Research Article

Sequential extraction of quercetin-3-*O*-rhamnoside from *Piliostigma thonningii* Schum. leaves using microwave technology

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Abstract
Piliostigma thonningii (Schum.) Milne-Redh. is a plant rich in quercetin-3-*O*-rhamnoside (quercitrin), a flavonoid involved in the antioxidant and antimicrobial processes. Microwave assisted extraction (MAE) is a method which gives better extraction yield, enhance the quality of extracts while decreasing the extraction time. This effect, a sequential optimization by response surface methodology using a central composite design, help to determine the optimal conditions for obtaining more antioxidant compounds of *P. thonningii* leaves. The response surface curves showed that there was a positive interaction between the extraction time and the solvent concentration on the DPPH scavenging and iron chelating activities of the extracts. The optimal ethanolic extraction parameters for the highest yield of flavonoids were an extraction time of 69 s, an irradiation power of 380 W and a solid–liquid ratio of 1/10 (w/v). On the residue, the optimal extraction parameters for simultaneously obtaining the highest flavonoids yield and the highest antioxidant activity were an extraction time of 49 s, an irradiation power of 520 W and an ethanol concentration of 67% (v/v). HPLC analysis has shown the second optimization helped to further maximize the extraction of active compound quercetin-3-*O*-rhamnoside. Electron microscopy of the powders before and after extraction has shown that microwave heating causes cellular damage. Compared to the maceration extraction method, the combined extracts of sequential MAE provide higher antioxidant activities.

Keywords *Piliostigma thonningii* · Quercetin-3-*O*-rhamnoside · Microwave-assisted extraction · Antioxidant

Abbreviations

MAE	Microwave assisted extraction
HPLC	High pressure liquid chromatography
TLC	Thin layer chromatography
UV	Ultra violet
NMR	Nuclear magnetic resonance
HMQC	Heteronuclear multiple quantum correlation
TFC	Total flavonoids content
CCD	Central composite design
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ANOVA	Analysis of variance
MSR	Mean square of the regression
SEM	Scanning electron microscopy
DAD	Direct array detector
RSM	Response surface methodology

1 Introduction

Piliostigma thonningii (Schum.) Milne-Redh is a plant of the family of Caesalpiniaceae. The plant grows up to 8 m of height with branches. It has large two-lobed simple

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leaves without thorns or spines [1]. It is said that this plant has a vast economic importance [2] and possesses edible and chewable leaves which is believed to relieve thirst. It has been reported *P. thonningii* and other species in the genus *Piliostigma* possess a wide range of uses to mankind ranging from food for human and animals and also a wide range of medicinal benefits [3]. For medicinal uses, treating loose stool in teething children, wound healing, ulcers, stop bleeding, case of inflammations, bacterial infections, stomach pains, fevers [1, 4].

Some phytochemical investigations that had been reported on *P. thonningii*, demonstrated that the plant contains a various family of compounds, alkalloids, anthraquinones, flavonoids, glycosides, saponins, sterols and tannins. Some flavonoids have been isolated from this plant, this include quercitin-3-*O*-rhamnoside (quercitrin). Quercitrin is highly concentrated in *Piliostigma* leaves [3]. The antioxidant activity of quercetin-3-*O*-rhamnoside has been demonstrated by Agung et al. [5]. Mutalib et al. [6] has shown the antimicrobial activity of this flavonoid.

Conventional solvent extraction has been used in the last decades for extraction of bioactive compounds from *P. thonningii* leaves. However, many extraction methods with high efficiency developed for phenolic components extraction from plants, include pressurized liquid extraction [7], microwave assisted extraction (MAE) [8], ultrasound assisted extraction (UAE) [9], Soxhlet extraction and heat reflux extraction [10], and supercritical fluid extraction (SFE) [11]. MAE is an extraction method in which solvents containing solid samples material is heated with microwaves energy to facilitated distribution solutes between the solid material and the solvent, hence extraction time is reduced [12, 13]. From this process, higher extraction rates is observed as well as a better extraction yield [14]. In the mechanism of MAE, the solvents are heated by microwaves directly both in the surrender and inside the plant, it results in a rapid pressure increase within cells material, then the pressure-driven facilitated mass transfer of compounds of interest out from the plant material, causing disruption of the plant tissue with the release of the target compounds into the solvent [15, 16].

However, given the factors influencing the process of MAE, optimization of the extraction process parameters is necessary to extract the maximum amount of phenolic compounds [8]. Limited information has been published on the use of microwave technology for the sequential extraction of antioxidant compounds from plant materials. Two factors, irradiation power and extraction time influence each other to a great extent [17].

Although many flavonoids had been isolated and characterized from *P. thonningii* leaves, there is no work on the sequential MAE of these flavonoids. Therefore, the objective of this study was to realize successive optimization of sequential MAE of quercetin-3-*O*-rhamnoside from *P. thonningii* leaves by Response Surface Methodology (RSM). The optimum extraction parameters (extraction time, irradiation power, solvent concentration, and solid–liquid ratio) to maximize flavonoid yields and antioxidant activities are determined and the antioxidant activity of the combined extracts from the successive MAE had been evaluated.

2 Materials and methods

2.1 Materials

One batch of 5.0 kg of *P. thonningii* leaves was collected in Ngaoundere locality, North of Cameroon. The harvested plant was identified by Professor Mapongmetsem, botanist and lecturer in the Department of Biological Sciences, Faculty of Science at the University of Ngaoundere. The collected sample was saved to voucher number 32129/HNC.

These leaves were air dried for 24 h and milled. The powder obtained was stored in a sealed container for later use.

All other chemicals (analytical grade) and HPLC solvents (HPLC grade) used in the experiment were purchased from VWR International.

2.2 Isolation and identification of quercetin-3-*O*-rhamnoside

The extracts of *P. thonningii* leaves were obtained by maceration in solvents (n-hexane, ethyl acetate, acetone and methanol respectively) for 4 h and with mechanical stirring. For this, in an extractor of capacity of 8 L, 1.0 kg of powder of plant powder was mechanically macerated with 2 L of respective solvent, a metal rod driven in rotation by a motor (DEREIX S. A. PARIS). After 4 h of maceration, the mixture has been left to stand for 15 min for decantation and then filtered. The filtrate was concentrated using a rotary evaporator under reduced pressure (Laboratiriums-Technik AG CH-9230 Flawil/Schweiz, Switzerland). For the same solvent, the extraction was repeated three times.

The acetone extract (40 g) was separated by chromatography on a 60S silica gel column (240 g, 230–400 mesh) with the hexane–ethyl acetate and ethyl acetate–methanol systems by gradient of increasing polarity. Fifteen fractions (A-O) were collected on the basis of thin layer chromatography (TLC) analysis. The C fractions (91–104), eluted with hexane–ethyl acetate (70:30, v/v), has given a precipitate of yellow crystals (PA_4) (1.8 g) representing respectively 4.5% and 0.18% of the acetone extract mass and dry plant powder (w/w), respectively.

The Ultra Violet (UV) spectrum of the compound PA_4 resulting from the analysis by HPLC (High Pressure Liquid Chromatography) has maxima at 212; 256 and 350 nm, characteristics of a flavonol. Analysis of the ^{1}H-NMR (Nuclear Magnetic Resonance) spectrum, carried out in methanol indicates the presence of three signals of the chemical shift (ppm) of aromatic protons at 7.35 (d, J = 2.1 Hz, H-2′), 6.92 (d, J = 8.26 Hz, H-5′) and 7.32 (dd, J = 2.3 and 8.2 Hz, H-6′) in the form of an ABX spin system suggesting a flavonol with the 3′,4′-disubstituted positions of B nucleus. It is also observed a pair of meta-coupling proton signals at 6.21 (d, J = 2.1 Hz, H-6) and 6.38 (d, J = 2.1 Hz, H-8) corresponding to ring A (Table 1). There are also signals for the osidic fraction, with a signal at δ 5.37 ppm (d, J = 1.4 Hz, H-1″) indicating that the compound has bound sugar. The evaluation of the anomeric coupling constant and by comparison with the data in the literature the osidic part can be attributed to rhamnose.

The ^{13}C NMR spectrum supports this hypothesis and shows 21 signals including the carbonyl signal at δ 178.5 ppm (C-4). It revealed chemical shifts (ppm) at δ 134.8 (C-3), 161.8 (C-5), 164.6 (C-7), 148.50 (C-3′), 145.0 (C-4′) which suggests an oxygenated flavone nucleus in position 3, 5, 7, 3′ and 4′. This spectrum also shows significant signals of an osidic part at δ 102.5 (C-1″), 70.5 (C-2″), 70.72 (C-3″), 71.87 (C-4″), 70.65 (C-5′), 16.3 (C-6″). The chemical displacement of 16.3 (C-6″) compared to that of the literature is characteristic of CH_3 in rhamnose (Table 1).

Analysis of the HMQC (Heteronuclear Multiple Quantum Correlation) spectrum of the aglycone shows that the H protons of the carbons C6, C8, C5′, C2′, C6′, are not substituted since a correlation between C6–H6, C8–H8, C5′–H5′, C2′–H2′, and C6′–H6′ is observed. Therefore, the structure was determined to be quercetin-3-*O*-α-rhamnopyranoside (quercitrin) (Fig. 1) previously obtained from the leaves of the same plant by Ibewuike et al. [3].

2.3 Microwave assisted extraction

The process of MAE was performed with a microwave oven (Daewoo, KOG-360, Combi Grill, Ahyeon-Dong Mapo-Gu Seoul, Korea) with cavity dimensions (W × H×D) of 290 × 290 × 220 mm.

Successive extraction process of quercetin-3-O-rhamnoside compounds from *P. thonningii* leaves powders (previously defatted) was carried out in a sealed vessel of 150 mL of capacity using ethanol and aqueous ethanol solvents, respectively. First optimization was consisted to use ethanol as solvent to find first optimum conditions of extraction. For this purpose, a study was carried

Table 1 ^{1}H and ^{13}C NMR spectral data of PA_4

C/H	δ H (ppm), J(Hz)		δ C (ppm)		HMQC
1	PA_4	Quercetin-*O*-rhamnopyranoside	PA_4	Quercetin-*O*-rhamnopyranoside	
2	–	–	157.5	158.6	–
3	–	–	134.8	136.3	–
4	–	–	178.5	179.7	–
5	12.51 (s-OH)	–	161.8	163.3	–
6	6.21 (d, J = 2.1)	6.20 (d, J = 2.1)	98.8	99.9	C–H
7	–		164.6	165.9	–
8	6.38 (d, J = 2.1)	6.36 (d, J = 2.2)	93.31	94.8	C–H
9	–	–	158.1	159.4	–
10	–	–	104.5	106.0	–
1′	–	–	121.56	123.1	–
2′	7.35 (d, J = 2.1)	7.34 (d, J = 2.1)	115.54	117.1	C–H
3′	–	–	148.5	149.9	–
4′	–	–	145.0	146.5	–
5′	6.92 (d, J = 8.26)	6.92 (d, J = 8.3)	114.98	116.5	C–H
6′	7.32 (dd, J = 8.2 and 2.90)	7.31 (dd, J = 8.3 and 2.1)	121.4	122.0	C–H
1″	5.37 (d, J = 1.34)	5.35(d, J = 1.6)	102.5	103.6	C–H
2″	4.24 (dd, J = 3.2 and 1.73)	4.23 (dd, J = 3.3 and 1.7)	70.5	72.0	–
3″	3.77 (dd, J = 9.72 and 3.32)	3.77 (dd, J = 9.4 and 3.4)	70.72	72.3	–
4″	–	3.36 (t, J = 9.5)	71.87	73.4	–
5″	–	3.44 (dd, J = 9.6 and 6.1)	70.65	72.1	–
6″	0.96 (d, J = 6.07)	0.95 (d, J = 6.2)	16.3	17.7	C–H

out beforehand to choose and define the experimental domains of the three (3) factors (extraction time, irradiation power and solid–liquid ratio) (Table 2). Subsequently, a central composite design (CCD) on 18 experiments was used to determine the optimal levels of these three factors influencing this extraction as well as the study of interactions between these different factors. The response followed during the extraction was the total flavonoids content (TFC). The determination of the TFC was performed on the filtrates obtained after extraction at different extraction conditions. Four replicates were performed in each extraction.

Secondly, at the optimum condition previously found, the extraction was carried out and the residue resulting from this ethanolic extraction was air dried in laboratory. This residue was then used like our plant material for extraction using aqueous ethanol like solvent to optimize the yield of flavonoids and antioxidant activity. Modeling using the RSM approach was used. Thus, a second CCD was used to determine the optimal levels of three factors (extraction time, irradiation power, solvent concentration) influencing this extraction (Table 3). The responses followed during the extraction are the total flavonoids contents (TFC), the free-radical DPPH scavenging activity (% $DPPH_{scavenging}$) and ferrous ion chelating activity (% $Iron_{chelation}$). These analyzes were carried out on the filtrates obtained after extraction under different extraction conditions. In each extraction, four replicates had been performed.

2.4 Determination of total flavonoids content (TFC)

Total flavonoids content (TFC) were evaluated as described by Cornard and Merlin method, with slight modifications. This method is based on the oxidation of flavonoids by aluminum chloride. It results in the formation of a brownish complex that absorbs at 415 nm [18]. For experiment, 1 mL of methanolic solution of $AlCl_3$ (2%, w/v) was mixed with 20 µL of solution of the different extracts. After 20 min at room temperature in dark, the absorbance was read at 415 nm with the spectrophotometer (Spectrophotometer UV-6300PC, 634-0776, VWR International) against the blank (0.5 mL of methanolic solution of aluminum chloride (2% (w/v) and 1 mL of methanol). Using these absorbances of extracts, the standard curve of quercitrin (Absorbance = 20.325QE, $R^2 = 0.98$ with QE in mg) was used to determine the mass of quercitrin extracted. In this study, the results were expressed in mg of quercitrin equivalent (QE) per gram of material (mg QE/g).

2.5 Antioxidant activity

2.5.1 Determination of DPPH free-radical scavenging activity (%DPPHsc)

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity of the extracts (pre-diluted at a ratio of 1:100) was evaluated using Dahmoune et al. [13] method. For this study, aliquots of different extracts (10 µL) were added to 500 µL of methanolic solution fo DPPH (70 µM). The mixture obtained was incubated for 20 min at 37 °C in the dark. The decrease in absorbance of the mixture was measured at 517 nm. The DPPH free-radical scavenging activity (%) was calculated using the equation:

$$\%\mathrm{DPPH}_{\mathrm{scavenging}} = \frac{(A_O - A_F) \times 100}{A_O} \quad (1)$$

where A_O was defined as the absorbance before addition of extract, whereas A_F was defined as absorbance value after 20 min of incubation time.

Table 2 Experimental factors level table of central composite design (CCD) for ethanolic optimization

Factors (units)	Range and levels					
	Notation	−1.414	−1	0	1	1.414
Extraction time (s)	X_1	56	60	70	80	84
Irradiation power (W)	X_2	360	400	500	600	640
Solid–liquid ratio (g/20 mL)	X_3	0.8	1	1.5	2	2.2

Table 3 Experimental factors level table of central composite design (CCD) for hydro-ethanolic optimization

Factors (units)	Range and levels					
	Notation	−1.414	−1	0	1	1.414
Extraction time (s)	X_1	38	40	45	50	52
Irradiation power (W)	X_2	360	400	500	600	640
Solvent concentration (%)	X_4	16	20	30	40	44

2.5.2 Measurement of ferrous ion chelating activity

The iron-chelating abilities of the different extracts was estimated by the slightly modified method of Dinis et al. [19]. 0.05 mL of different extracts was added to a 2.7 mL phosphate buffer (pH = 7.2). Then, 0.05 mL of $FeCl_2$ (2 mM) were added. At 30 s, the reaction was initiated by the addition of 0.2 mL ferrozine (5 mM), the mixture was shaken vigourously at Vortex for 10 s. After 1 min beyond addition of $FeCl_2$ solution, absorbance of the solution was measured at 562 nm. The ability of extracts to chelate ferrous ion was calculated relative to the control (consisting of phosphate buffer, iron and ferrozine only) using equation:

$$\%\text{Iron}_{\text{chelation}} = \frac{\left(A_c - A_E\right) \times 100}{A_c} \quad (2)$$

where A_C is the absorbance of the control, and A_E is the absorbance of the extract.

2.6 Experimental design

The extraction parameters were optimized using response surface methodology. A central composite design (CCD) was employed in this regard. Irradiation time (X_1), irradiation power (X_2), solid–liquid ratio (X_3) and solvent concentration (X_4) were chosen for independent variables. The range and centre point values of four independent variables, presented in Tables 1 and 2 were based on the results of preliminary experiments. The experimental design in the two case of optimization consists of eight factorial points, six axial points at a distance of ± 1.414 from the centre and four replicates of the central point. TFC was selected as the responses for the combination of the independent variables given in Table 1, and TFC, $\%\text{DPPH}_{\text{scavenging}}$ and $\%\text{Iron}_{\text{chelation}}$ the responses for the combination of the independent variables given in Table 2. Four experiments were carried out at each experimental design point and the mean values were stated as observed responses. Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses.

The variables were coded according to the equation:

$$x = \frac{(Xi - Xo)}{X} \quad (3)$$

where X is the coded value, Xi is the corresponding actual value, Xo is the actual value in the centre of the domain and ΔX is the increment of Xi corresponding to a variation of 1 unit of X.

The mathematical model corresponding to the composite design is:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j + \varepsilon \quad (4)$$

where Y_i is the dependent variables (TFC, $\%\text{DPPH}_{\text{scavenging}}$ and $\%\text{Iron}_{\text{chelation}}$), β_0 is the model constant, β_i, β_{ii} and β_{ij} are the model coefficients, and ε is the error. They represent the linear, quadratic and interaction effects of the variables. Analysis of the experimental design data and calculation of predicted responses were carried out using Statgraphics centurion software (Version XVI.I). Additional confirmation experiments were subsequently conducted to verify the validity of the statistical experimental design.

2.7 Statistical analysis

Analysis of variance (ANOVA) was used to determine the influence of each factor and the significance of their effects. It then examines the statistical significance of each effect by comparing the squared average against an evaluation of the experimental error. The significance of each factor is determined by the Fisher test which is defined as the ratio of the mean square of the regression (MSR) to the experimental error (EE) (F = MSR/EE), representation of the significance of each variable controlled on the model examined. The regression equations were also subjected to the Fisher test to determine the coefficient of determination R^2.

The optimal extraction conditions were estimated through regression analysis and three-dimensional (3D) response surface plots and contour plots (obtained using Sigmaplot 12.0 software) of the independent variables and each dependent variable.

2.8 HPLC characterization of isolated quercetin-3-*O*-rhamnoside and extracts at optimum conditions

The HPLC method used for monitoring quercetin in the various optimized extracts was carried out as follows. Two mobile phases, solvent A (MeOH: H_2O, 80/20, v/v) with 1% phosphoric acid (v/v) and solvent B (MeOH) were used. The elution gradient of the mobile phases (A:B, v/v) was programmed as 60:40 to 0 min; 30:70 to 10 min (constant for 5 min); 20:80 to 15 min (constant for 4 min); 10:90 to 20 min (constant for 6 min); 0:100 to 25 min (constant for 6 min); 100:0 to 35 min (constant for 5 min). The flow rate and the temperature of the column were kept constant 1 mL/min and 40 °C, respectively. The analysis system consists of a DAD (Direct Array Detector) type detector. The detection wavelength was between 190 and 360 nm for an analysis time of 40 min per sample. The stock solution of the extracts (1.0 g/mL) was prepared by dissolving each extract in its respective solvent. The standard stock

solution (0.1 g/mL) was prepared by dissolving quercetin-3-*O*-rhamnoside in MeOH. Injection volume of the investigated samples was 20 µL.

2.9 Scanning electron microscopy (SEM) analyses

Powder of *P. thonningii* leaves was observed under SEM (Hitachi S4800, with voltage of 0.1–30 kV) for morphological characterization before and after the extraction processes. Three samples of the powders (untreated, dried residues of MAE and dried residues of successive MAE) were used for SEM analysis. All samples were dried at 70 °C during 1 h for preparing samples for SEM analysis. Dried sample particles were fixed on a specific support, then metallized by applying a layer of palladium on the sample surface, and their shape and surface characteristics were observed by using gaseous secondary electron detector.

3 Results and discussion

3.1 Optimization of MAE conditions of *P. thonningii* flavonoids with ethanol

3.1.1 Modeling and fitting the model with response surface methodology (RSM)

From a CCD of 18 experiments, the influence of the extraction time (X_1), the irradiation power (X_2) and the solid–liquid ratio (X_3) on the ethanolic extraction of flavonoids from *P. thonningii* by MAE was evaluated. The experimental design and corresponding response data for the total flavonoids content from *P. thonningii* leaves are presented in Table 4. As described by Zhang et al. [20], regression coefficients, linear, quadratic and interaction coefficients of the model were calculated using the least square technique (Table 5).

It was shown that all linear parameters extraction time (X_1), irradiation power (X_2) and solid–liquid ratio (X_3), two interactions (X_1X_3 and X_2X_3) and quadratic effect of solid–liquid ratio (X_3^2) were highly significant at the level of $p < 0.05$ (Table 5) on the ethanolic extraction of flavonoids assisted by microwaves. Considering the significant

Table 4 Central composite design (CCD) and responses of ethanolic MAE of flavonoids from *P. thonningii* leaves

Run	Actual values			Experimental responses	Calculated responses	Residual (%)
	Extraction time (s)	Irradiation power (w)	Solid–liquid ratio (g/20 mL)	Total flavonoids content (mg QE/g)	Total flavonoids content (mg QE/g)	
	X_1	X_2	X_3	Y_{TFC}	Y_{TFC}	
1	84	500	1.5	10.73	10.76	0.26
2	70	500	2.2	10.52	10.49	0.29
3	70	500	1.5	10.92	10.99	0.67
4	80	400	1	9.51	9.53	0.28
5	60	600	2	9.51	9.46	0.50
6	56	500	1.5	10.15	10.17	0.16
7	70	500	1.5	11.36	10.99	3.24
8	60	400	2	11.14	11.21	0.62
9	70	500	1.5	10.82	10.99	1.64
10	70	641	1.5	9.67	9.71	0.28
11	70	500	1.5	10.92	10.99	0.67
12	70	500	0.8	9.00	9.09	0.83
13	80	400	2	11.17	11.12	0.43
14	80	600	2	8.96	9.02	0.67
15	60	400	1	8.34	8.25	0.98
16	60	600	1	10.73	10.76	0.30
17	80	600	1	10.52	10.49	0.89
18	70	358	1.5	10.92	10.99	0.15

Table 5 Estimated regression coefficients for the quadratic polynomial model and the analysis of variance (ANOVA) for the experimental results of first extraction of flavonoids from *P. thonningii* leaves

Parameters	Estimated coefficients	Degree of freedom	Sum of squares	*F* value	*P* value
X_1	0.540	1	0.529	8.93	0.0582
X_2	0.063	1	1.297	22.00	**0.0183**
X_3	19.498	1	2.988	50.81	**0.0057**
X_1^2	−0.003	1	0.550	9.63	0.0532
X_1X_2	0.000	1	0.061	1.04	0.3833
X_1X_3	−0.069	1	0.938	16.01	**0.0280**
X_2^2	0.000	1	1.354	23.31	**0.0169**
X_2X_3	−0.013	1	3.226	54.54	**0.0051**
X_3^2	−2.451	1	2.923	50.21	**0.0058**
Lack of fit	–	5	0.045	0.13	0.9744
Pure error	–	3	0.175	–	–
R^2	0.985	–	–	–	–
Adjusted R^2	0.967	–	–	–	–
CV (%)	1.90				
Corr. total		17	14.147	–	–

Bold values indicate the corresponding independent variables are significant on the response

parameters only, the final predictive equation was as follows:

$$Y_{TFC} = -38.93 + 0.54X_1 + 0.063X_2 + 19.50X_3 - 0.069X_1X_3 - 0.013X_2X_3 - 2.45X_3^2 \quad (5)$$

The analysis of variance (ANOVA) of the results obtained given in Table 5 shows that the determination coefficient (R^2) was 0.98, this value implied that the variations of 98% for ethanolic MAE efficiency of flavonoids from *P. thonningii* leaves were attributed to the independent variables and could be explained by the defined model, and therefore only 2% of the total variations could not be explained by the model [21].

Table 5 shows that the values of R^2 and R^2 adjusted (0.98 and 0.97 respectively) for the model are not greatly different. Therefore, the model obtained is a good statistical model. The "Lack of fit *p* value" of 0.9744 implies that the Lack of fit is not significant relative to pure error ($p > 0.05$), this confirmed the model is validated. Zhang et al. [22] defined the coefficient of variation (CV) as the ratio of the standard error of estimate to the mean value of observed response. It is a measure of reproducibility of the models, expressed in percentage.

From Table 5, the coefficient of variation (CV %) obtained was 1.90% showing that the model was reliable and reproducible [23]. Karazhivan et al. [21] stated that a CV higher than 10% indicates that variation in the mean value is high and does not satisfactorily develop an adequate response model. From the value of CV obtained, the model could be validated in the prediction of ethanolic MAE of flavonoids from *P. thonningii* leaves.

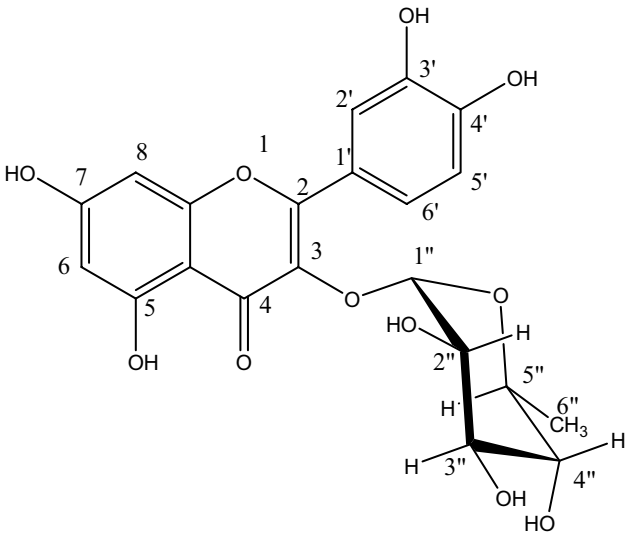

Fig. 1 Structure of isolated flavonoid quercetin-3-*O*-α-rhamnopyranoside

3.1.2 Analysis of percentage of factors' contributions diagram

The effects of the independent variables and their mutual interaction on the extraction yield of flavonoids are shown in Fig. 2.

This figure shows that the linear effects of extraction time as well as the load to extract (ratio) contribute to increase the extraction of flavonoids. This was probably due to the fact that more solvent could enter cells while more active compounds could permeate into the solvent under the higher solid–liquid ratio conditions [24, 25]. With further increase in liquid–solid ratio, a decline in flavonoids yield was observed by the negative effect of quadratic effect of liquid–solid ratio.

The Fig. 2 shows that the solid–liquid ratio has highest contribution (86.4%) in extraction of flavonoids (quercitrin) from *P. thonningii* leaves. This means that, for a given volume of extraction, the amount of flavonoids extracted increases with that of plant material in the medium due to mass transfer phenomenon. This figure also shows that there is a low contribution of linear effect of irradiation power as well as its quadratic effect (0.27 and 0.00%) on the ethanolic extraction of flavonoids. This phenomenon is considered to be caused by the low rate of mass transfer at low temperatures resulting to low irradiation power, which

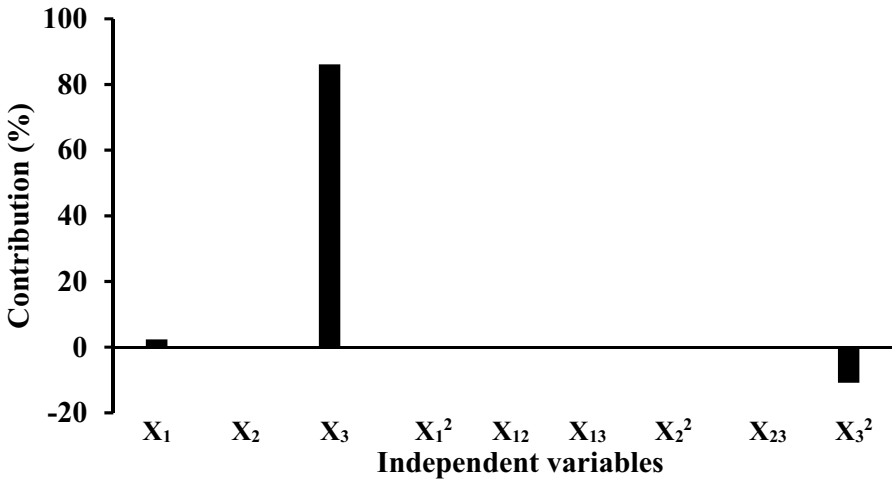

Fig. 2 Contribution percentage of independent variables on ethanolic MAE of flavonoids from *P. thonningii*

would require more time for the flavonoids to be dissolved from the raw materials into the solution. These results are similar to the research findings by Karabegovic´et al. [26]. In addition, the microwaves could accelerate cell damage and rupture by temperature rise and internal pressure increase inside the cells of plant material, which promotes the disruption of sample surface and in turns the exudation of the target substance within the cells into the surrounding solvent takes place [24, 27, 28]. At higher irradiation power however, dissolution of the compounds can reach the equilibrium in a shorter time then decreased by changes in the extraction time. This suggests that a higher irradiation power and a short extraction time are more effective in ethanolic extraction of flavonoids from *P. thonningii* using MAE. The negative effects observed at higher values of irradiation power (quadratic effect) and long period of extraction (quadratic effect) may be due to thermal degradation of the flavonoids [29]. Thus, for optimal extraction of flavonoids leaves of *P. thonningii* it would be advisable to work under moderate conditions of irradiation power in order to control this degradation of flavonoids.

3.1.3 Optimization of the ethanolic MAE and validation of model

The results of the optimization of the ethanolic MAE of *P. thonningii* leaves are shown in Table 6. The predicted extraction yield of TFC was 11.44 mg QE/g that was consistent with the experimental yield of 11.28 mg QE/g. The predicted values were in close agreement with experimental values and were found to be not significantly different ($p > 0.05$) using a paired t-test [13, 30]. It is noticed the predicted response values deviated slightly from the experimental values. The strong correlation between the real and predicted results confirmed that the response of regression model was adequate to reflect the expected optimization [20].

Table 6 Optimal conditions of ethanolic MAE of flavonoids from *P. thonningii* leaves

	Actual variables			Responses	
	Extraction time (s)	Irradiation power (W)	Solid–liquid ratio (g/20 mL)	Predicted	Experiment
TFC (mg QE/g)	63	380	2.01	11.44	11.28

3.2 Optimization of hydro-ethanolic MAE conditions of flavonoids and antioxidant activities of *P. thonningii*

3.2.1 Modeling and fitting the model with response surface methodology (RSM)

From a central composite design of 18 experiments, the influence of the extraction time (X_1), the irradiation power (X_2) and the variation of solvent concentration (X_4) on the flavonoids extraction and antioxidant activity by MAE was evaluated during this study. The three responses of interest were total flavonoids content (TFC), DPPH antiradical activity (%$DPPH_{scavenging}$) and iron chelating activity (%$Iron_{chelation}$). Table 7 shows the experimental design, the experimental responses, the calculated responses and the calculated residues. It appears from this table after the total flavonoid contents, the free DPPH antiradical activity (%$DPPH_{scavenging}$) and Iron chelating activity (%$Iron_{chelation}$) are between 7.80 and 13.15 mg QE/g; 72 and 88%; 70 and 92% respectively. The maximum values of TFC (13.15 mg QE/g) and %$DPPH_{scavenging}$ (88%) is obtained for experimental conditions of $X_1 = 45$ s, $X_2 = 500$ W, $X_4 = 30\%$; while

Table 7 Central composite design (CCD) and responses of MAE of flavonoids from *P. thonningii* leaves

Run	Actual variables			Experimental responses			Calculated responses		
	Extraction time (s)	Irradiation power (W)	Solvent concentration (%)	TFC (mg QE/g)	$\%DPPH_{scavenging}$ (%)	$\%Iron_{chelation}$ (%)	TFC (mg QE/g)	$\%DPPH_{scavenging}$ (%)	$\%Iron_{chelation}$ (%)
	X_1	X_2	X_3	Y_{TFC}	Y_{DPPH}	Y_{Iron}	Y_{TFC}	Y_{DPPH}	Y_{Iron}
1	50	400	20	11.68	75.53	80.60	11.61	75.31	80.57
2	38	500	30	10.29	73.50	78.65	10.44	73.95	78.71
3	40	400	20	7.88	72.61	76.70	7.89	72.39	76.67
4	45	360	30	10.31	79.00	79.84	10.44	79.45	79.90
5	40	400	40	10.59	79.09	78.18	10.80	78.86	78.15
6	50	400	40	11.34	82.91	82.98	11.35	82.68	82.95
7	45	640	30	11.02	81.75	82.58	11.03	82.20	82.65
8	40	600	20	9.57	71.75	70.85	9.49	71.53	70.82
9	50	600	20	10.96	83.27	83.34	11.07	83.05	83.31
10	52	500	30	11.95	84.35	90.87	11.94	84.79	90.93
11	45	500	30	12.45	85.64	85.57	12.56	86.00	87.80
12	45	500	30	13.12	86.95	88.65	12.56	86.00	87.80
13	40	400	40	11.19	75.23	79.32	10.80	75.01	79.29
14	50	600	40	10.67	87.65	92.72	10.59	87.43	92.69
15	45	500	30	12.55	88.11	87.56	12.56	86.00	87.80
16	45	500	44	12.65	85.76	86.60	12.80	86.21	86.66
17	45	500	16	11.12	78.09	78.92	11.11	78.53	78.98
18	45	500	30	12.25	84.21	89.56	12.56	86.00	87.80

the maximum value in $\%Iron_{chelation}$ is obtained for $X_1 = 50$ s, $X_2 = 600$ W and $X_4 = 40\%$ (Table 7). Thus, a combined optimization process to obtain desirable bioactive substances and antioxidant activity has been performed.

It was shown for the tree responses studied that all linear and quadratic parameters, extraction time (X_1), irradiation power (X_2) and solvent concentration (X_4) were highly significant at the level of $p < 0.05$. Two interactions in antioxidant activities responses ($\%DPPH_{scavenging}$ and $\%Iron_{chelation}$) are highly significant at the level of $p < 0.05$, excepting their interaction X_1X_4 (Table 8). For TFC, the interaction X_1X_3 was highly significative. Considering the significant parameters only, the final predictive equations obtained were given as below:

$$Y_{TFC} = -123.82 + 3.625X_1 + 0.145X_2 + 0.985X_3 - 0.028X_1^2 - 0.016X_1X_3 - 0.003X_3^2 \quad (6)$$

$$Y_{DPPH} = 86 + 3.83X_1 + 0.97X_2 + 2.71X_3 - 3.31X_1^2 + 2.15X_1X_2 - 2.58X_2^2 - 0.75X_2X_3 - 1.81X_3^2 \quad (7)$$

$$Y_{Iron} = 87.80 + 4.32X_1 + 0.97X_2 + 2.71X_3 - 1.48X_1^2 + 2.15X_1X_2 - 3.26X_2^2 + 1.74X_2X_3 - 2.48X_3^2 \quad (8)$$

3.2.2 Influence of extraction parameters on flavonoids content

The influence of three independent variables towards total flavonoids content was reported through the significant ($p < 0.05$) coefficient of the second-order polynomial regression equation. 3D response surfaces curves in Fig. 3 demonstrated the effects of the independent variables and their mutual interactions on the TFC values. They were obtained by keeping one another variable constant. The constant was equal to the corresponding true value of zero level.

The TFC increases with time until a time of 48 s, then remains stable (Fig. 3). It varies from 7.0 mg QE/g (time 38 s) to 12.0 mg QE/g (time 52 s). During the process, to release active compounds in the medium, the solvent of

Table 8 Variance analysis of regression equations of TFC, $\%DPPH_{scavenging}$ and $\%Iron_{chelation}$

Source	Sum of square	DF	Mean square	*F* value	*P* value
TFC (mg QE/g)[a]					
X_1	2.760	1	2.760	18.47	0.0127
X_2	0.405	1	0.405	2.71	0.1749
X_3	3.535	1	3.535	23.66	0.0083
X_1^2	3.646	1	3.646	24.40	0.0078
X_1X_2	1.615	1	1.615	10.81	0.0303
X_1X_3	3.712	1	3.712	24.84	0.0076
X_2^2	6.477	1	6.477	43.35	0.0028
X_2X_3	0.018	1	0.018	0.12	0.7451
X_3^2	0.707	1	0.707	4.73	0.0952
Pure error	0.60	3	0.15		
Lack of fit	0.11	5	0.03	0.20	1.0000
CV (%)	4.58				
Total	27.17	17			
$\%DPPH_{scavenging}$ (%)[b]					
X_1	176.46	1	176.46	62.42	0.0042
X_2	11.327	1	11.327	4.01	0.1391
X_3	88.350	1	88.350	31.25	0.0113
X_1^2	87.869	1	87.869	31.08	0.0114
X_1X_2	36.98	1	36.98	13.08	0.0363
X_1X_3	0.405	1	0.405	0.14	0.7303
X_2^2	53.629	1	53.629	18.97	0.0224
X_2X_3	4.5	1	4.5	1.59	0.2963
X_3^2	26.329	1	26.329	9.31	0.0554
Pure error	8.481	3	2.827		
Lack of fit	1.81	5	0.361	0.13	0.9753
CV (%)	2.05				
Total	496.14	17			
$\%Iron_{chelation}$ (%)[c]					
X_1	224.24	1	224.24	76.05	0.0032
X_2	11.327	1	11.327	3.84	0.1448
X_3	88.349	1	88.349	29.96	0.0120
X_1^2	17.741	1	17.741	6.02	0.0914
X_1X_2	36.98	1	36.98	12.54	0.0383
X_1X_3	0.405	1	0.405	0.14	0.7355
X_2^2	85.238	1	85.238	28.91	0.0126
X_2X_3	24.499	1	24.499	8.31	0.0634
X_3^2	49.567	1	49.567	16.81	0.0262
Pure error	8.8457	3	2.94		
Lack of fit	0.033	5	0.0067	0.00	1.0000
CV (%)	1.26				
Total	547.237	17			

[a]The coefficient of determination (R^2) and adjusted R^2 of the model was 97.34% and 94.40%

[b]The coefficient of determination (R^2) and adjusted R^2 of the model was 97.93% and 95.59%

[c]The coefficient of determination (R^2) and adjusted R^2 of the model was 98.37% and 96.55%

extraction (ethanol) takes a minimum time to enter the powder of the leaves, dissolves the active substances (flavonoids) which subsequently diffuse into the medium (solvent). The low values of extraction time indicate that the extraction of compounds could be facilitated by radiation, thus prolonged exposure leads to destruction of the structures of the compounds by heating, corresponding to the negative influence of the quadratic effect of the time observed on the extraction of TFC (Fig. 3). These results are in agreement with those of Hismath et al. [31] who observed that the quadratic effect of time has a negative influence on the extraction of phenolic compounds from the powder of *Azadirachta indica* leaves.

Concerning the effect of the irradiation power on the extraction of flavonoids, their content increases exponentially with the power then decreases. This content varies from 6.0 (at 360 W) to 11.0 (at 550 W) mg QE/g, then decreases to 10.0 mg QE/g for an irradiation power of 600 W (Fig. 3b). An increase in the irradiation power of microwave causes an increase in the heating temperature of the extraction system. As shown in Fig. 3b, the quadratic effects of irradiation power and interaction between irradiation power-extraction time tend to decrease flavonoids extraction. According to Gan and Latiff [32], high temperature could cause softening of plant tissue, disruption of phenolics compound interactions with proteins or polysaccharides, and increase their solubility and improve their diffusion rate. Once these compounds are extracted, a fairly long exposure to the oven waves would lead to a destruction of the latter under the effect of heat, hence the negative interaction between extraction time- irradiation power (Fig. 3).

Indeed, for prolonged exposure in heating waves, flavonoids are sensitive to degradation because of their hydroxyl groups and ketone, as well as their double unsaturated liaisons [33–35]. It is therefore important to find the optimal conditions for MAE of these flavonoids in order to avoid their possible degradation.

The TFC increases exponentially with the increase in the polarity of solvent (Fig. 3a). The addition of water in ethanol increases the polarity of the medium, the solubility of phenolic compounds and thus facilitates the extraction of these. The ethanol:water system obtained is therefore capable of extracting highly polar, less polar compounds, as well as those of moderate polarity [36].

3.2.3 Influence of extraction parameters on antioxidant activities ($\%DPPH_{scavenging}$ and $\%Iron_{chelation}$)

The influence of irradiation power and extraction time on the free DPPH antiradical activity is shown in Fig. 4. This activity evolves in a hyperbolic way with both the irradiation power and the extraction time.

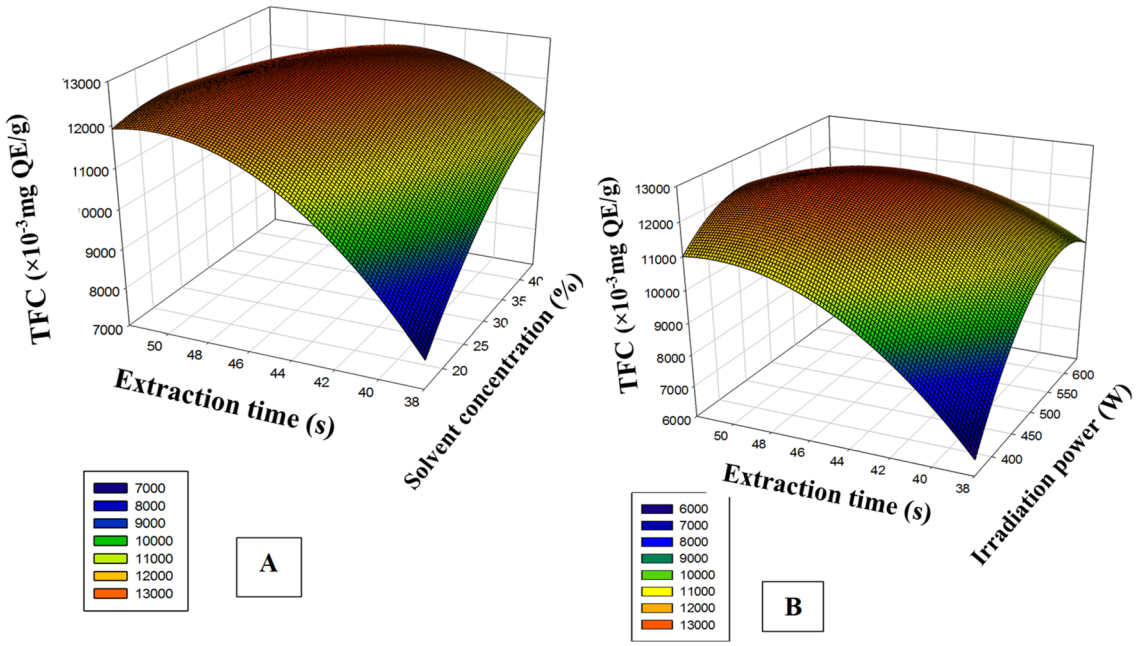

Fig. 3 Response surface analysis for the total flavonoids yield from *P. thonningii* leaves residues with MAE with respect to extraction time and solvent concentration (**a**) and extraction time and irradiation power (**b**)

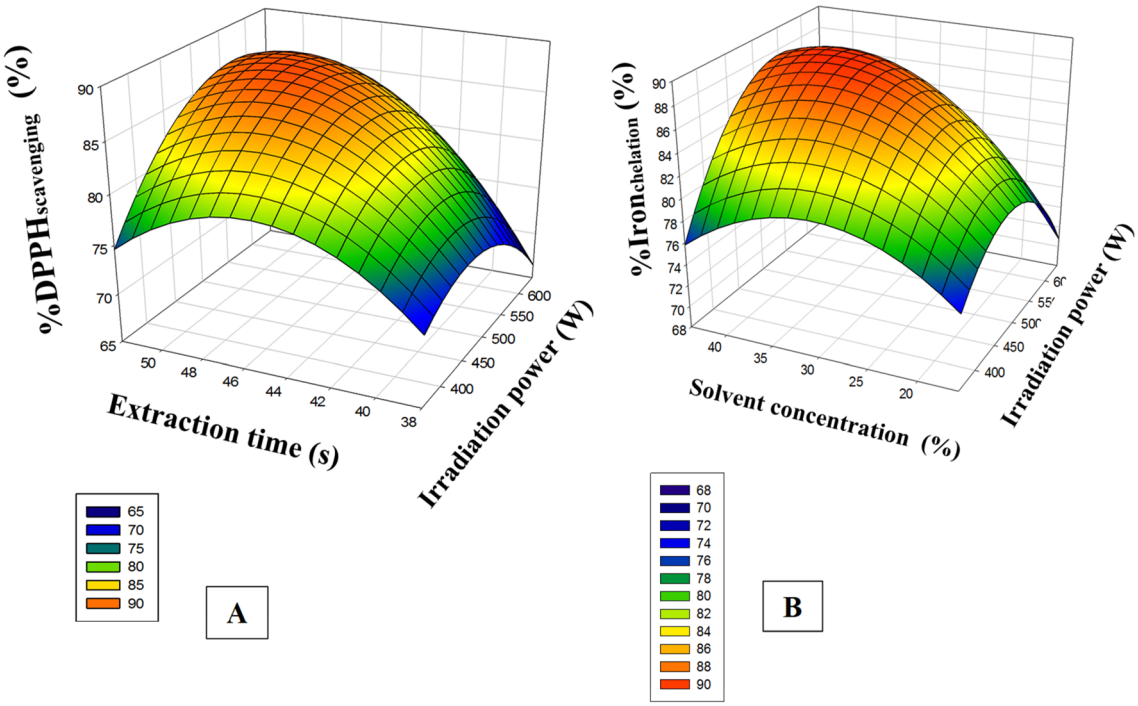

Fig. 4 Response surface analysis for the antioxidant activities from *P. thonningii* leaves with MAE with respect to extraction time and irradiation power (**a**); irradiation power and solvent concentration (**b**)

The antiradical activity increases with the extraction time and the irradiation power until reaching a maximum, then decreases. An increase in activity of 65–80% is observed when the time goes from 38 to 45 s. An increase of 65–75% is observed for the passage of the irradiation power from 360 to 500 W taken individually. However, a

combined effect of irradiation power and extraction time helps to maximize antiradical activity up to 87% for a power of 550 W and a time of 50 s (Fig. 4a).

The influence of the variation of the polarity of solvent and the irradiation power on the iron chelating activity is presented by the response surface (Fig. 4b). The chelating activity of iron increases with the concentration of water in the extraction medium. An increase in activity of 65–80% is observed when the water content in solvent increases from 20 to 35%. The extraction of the active substances is therefore favored in a hydroethanolic medium, the 100% (v/v) ethanol would not be effective enough for the extraction of phenolic compounds. Prasad et al. [36] show that 68% (v/v) ethanol is optimum for optimal total antioxidant activity. However, a reduction in activity when the water concentration is greater than 35% (v/v) (as solvent) is observed (Fig. 4b). This could be explained by the fact that the solubility of flavonoids decreases with the concentration of water in the medium, for high water content we would witness the extraction of other compounds with low chelating activity.

3.2.4 Optima of flavonoids MAE and antioxidant activity of *P. thonningii*

3.2.4.1 Graphical optimization: optimal zone Using the polynomial models presented above, the contour lines for each response were made as a function of the extraction time and the change in the polarity of the solvent, the irradiation power was kept constant. Before optimization, the limits were fixed according to the influence study of the factors (response surface curves). These are the maximum values obtained according to the effect of individual factors:

- Total flavonoids content ≥ 12.00 mg QE/g
- Free DPPH Antiradical activity ≥ 80%
- Iron complexing activity ≥ 80%.

Graphical optimization was made to have optimal conditions for the extraction of flavonoids, the free DPPH antiradical activity and the iron chelating activity. To do this, the contour curves obtained after modeling the different indices were superimposed, and the resulting graph shows the shaded area that respects the limits set for a better antioxidant activity of the microwave extracts of *P. thonningii* leaves (TFC ≥ 12.00 mg QE/g, $\%DPPH_{scavenging} \geq 80\%$ and $\%Iron_{chelation} \geq 80\%$). Any combination possible in this shaded area will result in extracts with high antioxidant activity (Fig. 5). To obtain an extract of optimal antioxidant activity, it is important to extract the leaves of *P. thonningii* under the following conditions: irradiation power of 500 W, extraction time between 43 and 52 s with an ethanol concentration from 55 to 75% (v/v) as solvent.

3.2.4.2 Multi-response optimization A multi-response optimization was performed for total flavonoids content, antioxidant activities (DPPH antiradical activity and iron chelating activity). The combination of the different factors is shown in Table 9. For this combination, the calculated optimal values are: TFC = 12.65 mg QE/g; $\%DPPH_{scavenging} = 88.55\%$ and $\%Iron_{chelation} = 94.04\%$.

The optimal conditions for the combined responses belong to the optimum domain predicted by the superposition of the contour plot. The combined optima were checked and the TFC values = 12.77 mg QE/g, the $\%DPPH_{scavenging} = 91.27\%$ and the $\%Iron_{chelation}$ of 88.11% were obtained compared to the theoretical values (TFC = 12.46 mg QE/g, $\%DPPH_{scavenging} = 91.4\%$ and $\%Iron_{chelation} = 88.11\%$).

3.3 HPLC chromatographic profile of extracts at different optimal conditions

The following figure (Fig. 6) shows the profile of extracts from leaves of *P. thonningii* optimized with ethanol and ethanol–water by MAE and by maceration. On this profile, the characteristic quercetin-3-*O*-rhamnoside (quercitrin) peak is identified at retention time of 15 min. The quantitation analysis was done by normalized area percentage methods, in which the area percentage of each peak is reported and the total area percentage equals 100%. The calculation of the percentages of characteristic quercitrin peak from the individual quercitrin peak areas and in relation to the total area is shown in the Table 10.

It is shown from Table 10 that for the same analysis conditions, the extract optimized with the ethanol–water solvent system has a greater total surface (69.35×10^6). Optimization of ethanolic extraction has helped to maximize quercetin-3-*O*-rhamnoside extraction. However, the second optimization (hydro-ethanolic extraction) has helped to further maximize the extraction of this active compound quercetin-3-*O*-rhamnoside. Adding water to ethanol is necessary to promote the solubilization of flavonoids and thus facilitate their extraction [37]. Furthermore, comparing the different total surfaces, it is noted the extract obtained by ethanolic maceration has the smallest total surface (3.87×10^6). In fact, heating due to the action of waves would help to maximize the extraction process of the active substances of *P. thonningii*.

3.4 Antioxidant activities of optimized extracts

The antioxidant activities of the various extracts obtained are recorded in Table 11. It can be seen from this table that

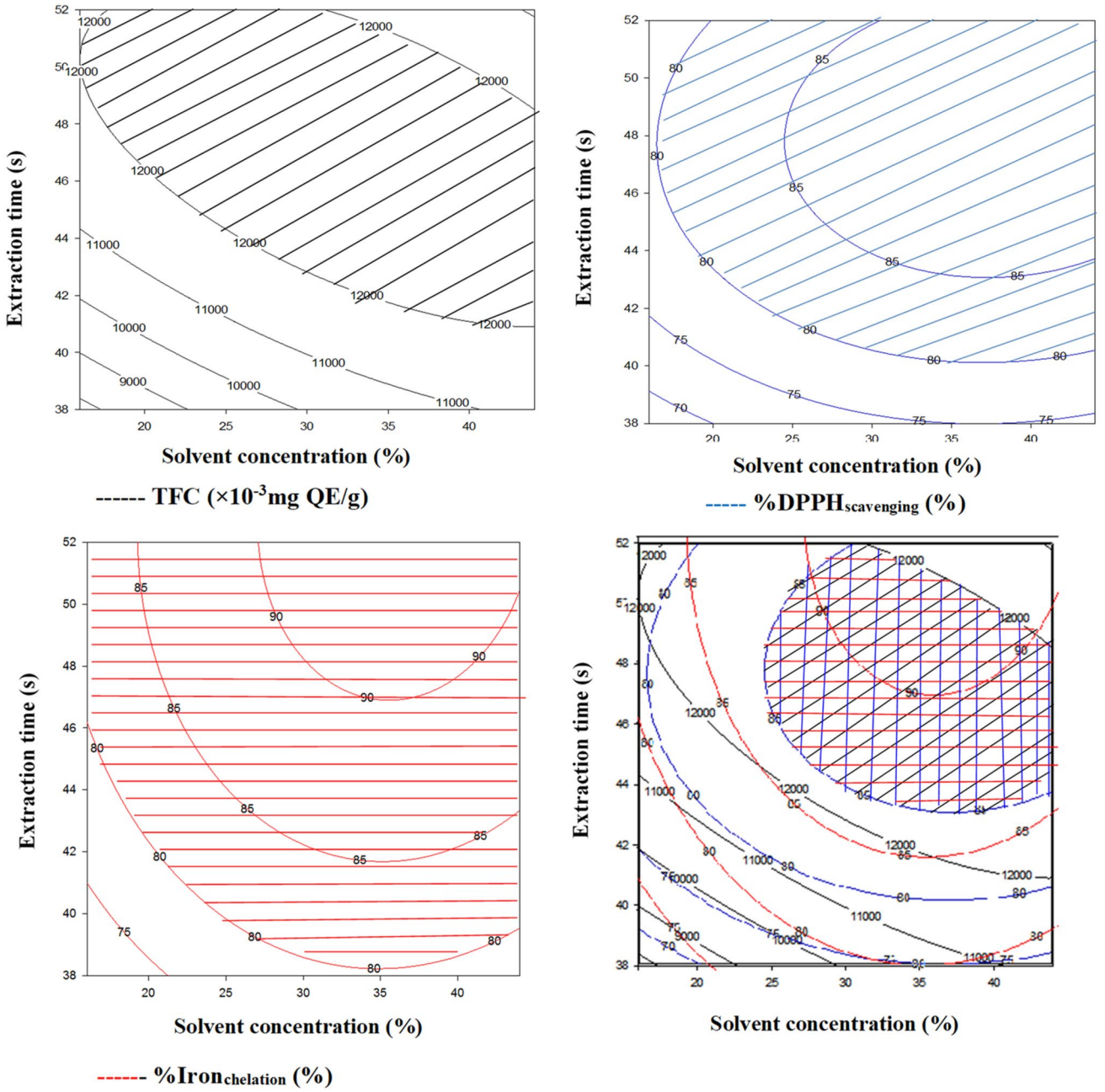

Fig. 5 Optimization by compromise area of flavonoids extraction and DPPH scavenging and iron chelating activities

Table 9 Optimal conditions of MAE of TFC, $\%DPPH_{scavenging}$ and $\%Iron_{chelation}$

	Actual variables		
	Extraction time (s)	Irradiation power (W)	Solvent concentration (%)
Combined optimum	49	520	33

the microwaves treatment contributes to increasing the extraction of the antioxidant compounds of *P. thonningii*. With regard to the iron chelating activity, it is found that the complexing activity of the optimized extracts of *P. thonningii* is twice the standard (ascorbic acid) on the one hand and at a dose 3 times lower than that of the extract obtained by maceration. This shows the interest of a sequential optimization by microwaves, because this process contributes to enrich the extracts in active compounds quercitrin.

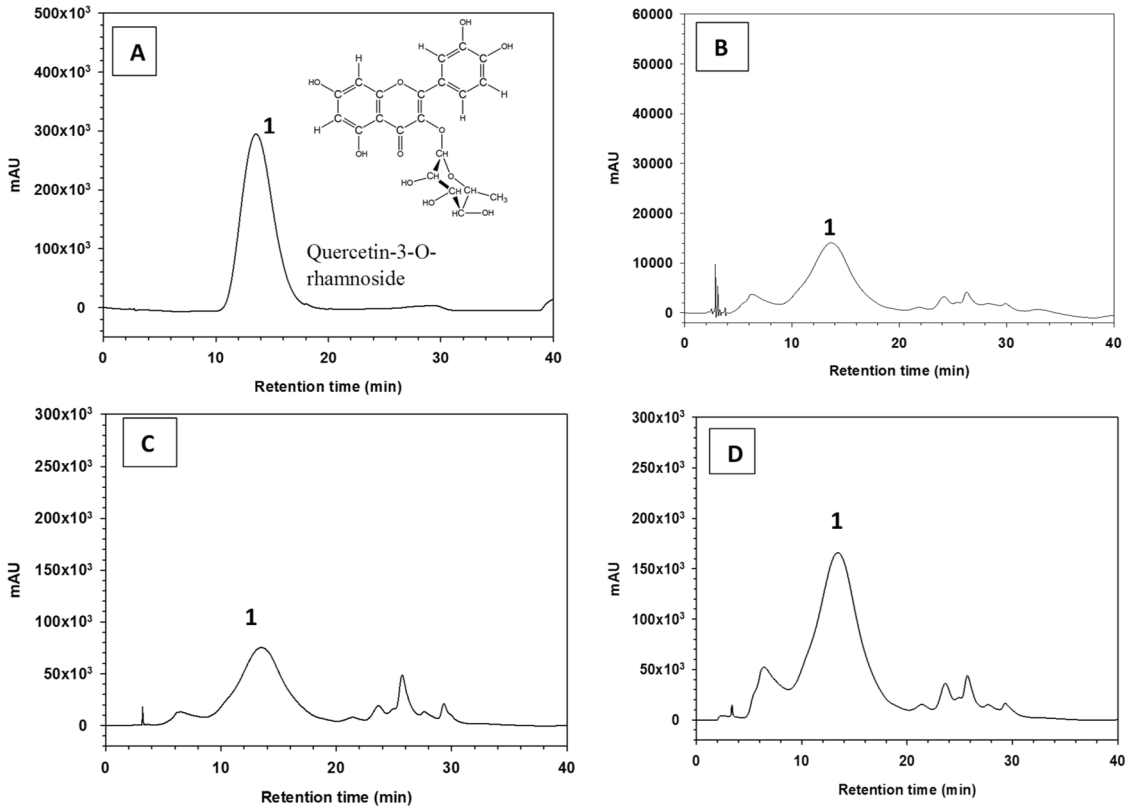

Fig. 6 HPLC Chromatograms at 254 nm of quercetin-3-*O*-rhamnoside (**a**) and extracts obtained by maceration (**b**), optimization with ethanol (**c**) and optimization with ethanol–water (**d**)

Table 10 Relative percentage of characteristic peaks and total areas for the different extracts obtained from *P. thonningii* by maceration and MAE

	Maceration	Ethanol optimization	Ethanol–water optimization
Retention time (min)	13.66	13.49	13.42
Surface of quercitrin peak ($\times 10^6$ mAU*min)	3.21	18.3	51.46
Relative percentage of quercitrin in extract (%)	82.83	71.06	74.17
Total peak area of extract ($\times 10^6$ mAU*min)	3.87	25.75	69.35

Table 11 IC_{50} (µg/mL) of the optimized extracts obtained by maceration and sequential optimization by microwaves

	Extracts				Standards
	Ethanolic maceration	Ethanolic MAE optimization	Optimization ethanol:water	Combination of MAE extracts	Quercitrin
$DPPH_{scavenging}$	98.9	77.0	74.07	63.49	25.7
$Iron_{chelation}$	35.5	21.1	17.08	15.05	11.3

3.5 Morphology of *P. thonningii* cake obtained by scanning electron microscopy (SEM)

The microscopic observation (Fig. 7.) of the cakes obtained by conventional method of extraction, MAE with ethanol and MAE with ethanol–water, showed that compared to the powder of *P. thonningii* leaves not treated with microwaves, the extraction maceration leads to changes in the cells morphology, but the damage is different depending on the extraction method applied.

The high pressure and temperature involved in the MAE process will destroy the cell walls of the plant matrix,

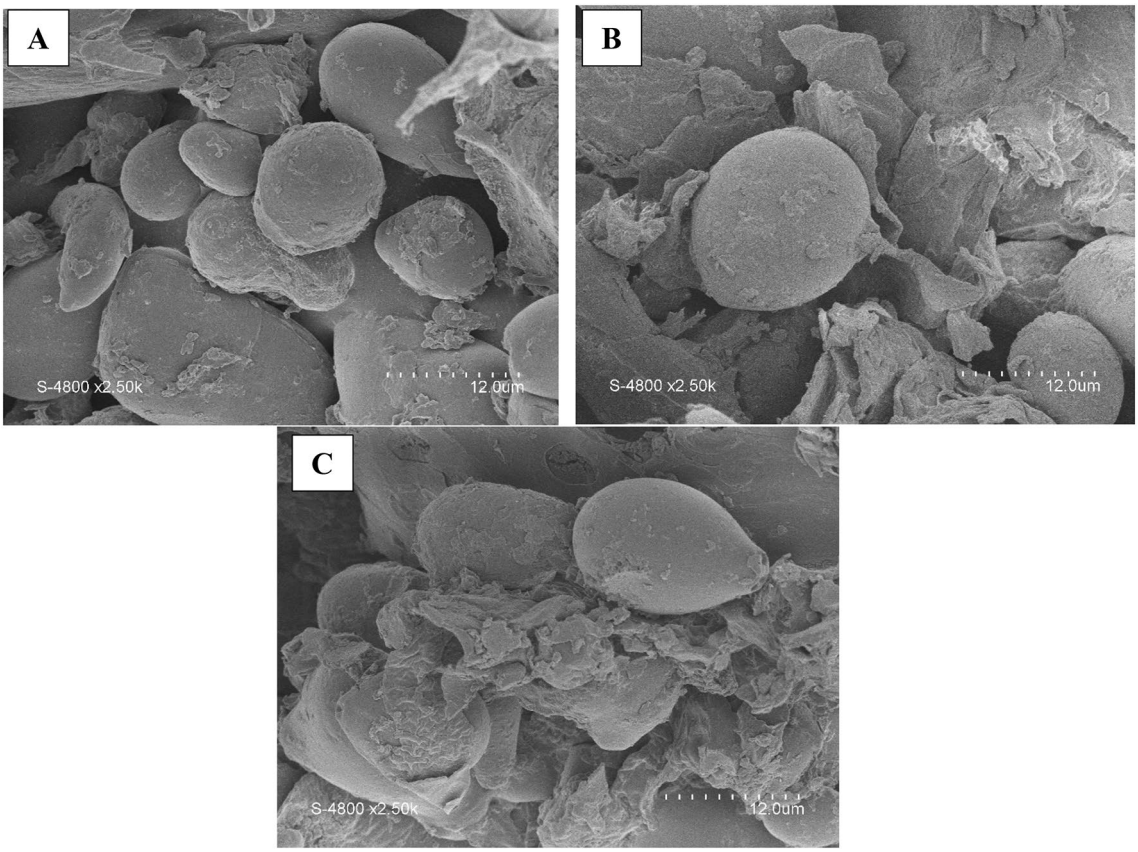

Fig. 7 Scanning electron microscopic images (×2500) of untreated *P. thonningi* leaves (**a**), residues in the extraction of conventional-solvent extracted leaves (**b**), and microwave-assisted extracted (MAE) leaves (**c**)

which facilitates the release of extractable compounds and improves mass transport by disrupting the cell walls of the product and its content can be released in the medium (C) [36]. Indeed, in MAE process, the microwaves dehydrate the cellulose and reduce its mechanical resistance, which allows easy penetration of the solvent into the cell channels [13]. The exposure of plant material to microwave has resulted in the increase of contact of solute with solvent through partial destruction of the solid phase and generation of cracks.

4 Conclusion

The objective of this study was to optimize the microwave assisted extraction (MAE) of quercetin-3-*O*-rhamnoside from *P. thonningii* leaves. Ethanol was used to a first optimization of this compound with antioxidant properties. Thereafter, on the residue obtained after the first optimization, the ethanol:water system was subsequently used for maximizing this extraction by optimization using the response surface methodology (RSM). The optimal conditions of extraction of active compound with maximum antioxidant activities are successively 63 s, 380 W and solid–liquid ratio of 1/10 (w/v) for the first extraction, and 49 s, 520 W and ethanol concentration of 67% as solvent for extraction from the residue. It is found that the antioxidant activity (complexing activity) of the optimized extracts of *P. thonningii* is twice the value of standard on the one hand and at a dose 3 times lower than that of the extract obtained by maceration.

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Availability of data and materials Research data have been provided in the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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