Original Article

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Factorial Design Applied to Subcritical Water Extraction for the Investigation of Flavonoids and Antioxidant Capacity of *Gynura calciphila* Kerr

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Abstact

The aim of this work was to study of antioxidant capacity and the flavonoid content of *Gynura calciphila* using different extraction procedures. An environmental kindly technique, subcritical water extraction (SWE), was compared to the traditional extraction (ethanolic extract). The results revealed that quercetin, a plant-derived flavonoid, was found as a major component in both extraction procedures. The use of SWE provided higher quercetin content and antioxidant activity. Quercetin content by SWE and traditional extraction were 44.6 μg/ml and 35.1 μg/ml, respectively. The EC_{50} (effective concentration, 50%) of SWE and traditional extraction were 508 and 518 μg/ml, respectively. Additionally, potential factors affecting SWE (temperature, extraction time and material ratio) were investigated their influence and interaction on the antioxidant activity and the flavonoid content using full factorial design. Results from factorial design provided good model equations for the prediction of SWE condition on the antioxidant activity and the flavonoid content. The equations indicate that temperature is the most significant factor of SWE technique on the antioxidant activity and the flavonoid content. This work demonstrated the traditional time-consuming techniques for 12 hours of the extraction of flavonoids could be substituted for the SWE technique within 1 hour. Most of all, SWE is an alternative procedure for consumer safety.

Key words: Subcritical water, Antioxidant activity, *Gynura calciphila* Kerr, Factorial design

INTRODUCTION

Traditional medicine is widely used throughout Thailand. Many Thai medicinal plants have provided the foundation for modern pharmaceuticals and drug leads. For *Gynura*, could lead for the development of the future anti-inflammatory drugs such as *G. pseudochina*[1]. However, there is no such report for pharmaceutical activities of *G.calciphila*.

Gynura calciphila is an endemic species to peninsular Thailand[2]. The species is a subsucculent plant arising from small, tapering tubers 1–2 cm diameter up to 20–60 cm high, sparsely pubescent with ovate or elliptic leaves. Flowering and fruiting throughout the year. The plant grows in open rocky places of limestone hills, 0–500 m^2.

The main components in *Gynura* were identified as flavonoids[3]. Flavonoids are secondary plant metabolites in various medicinal plants, which occurs in free state (*i.e.*, aglycone) and glycosides. The basic structure of flavonoids contains a phenolic ring with 2-phenylbenzopyrone (flavone). Their derivatives differ in the substitutions, the number and position of hydroxyl and

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methoxy groups, and the number of sugar moieties in the molecules[4]. Moreover, flavonoids show important pharmacological activities, such as anti-allergy, anti-inflammatory, anti-viral activities[5], anti-cancer[6,7,8], antimicrobial activities[9] and antioxidant activity[10]. However, the traditional extraction method of flavonoids from plant tissues has usually been accomplished using conventional extraction processes such as solid–liquid extraction employing methanol, ethanol, acetonitrile and acetone and also through steam distillation.

Extraction is a critical step for the isolation and recovery of high added valued compound, in particular flavonoids. Especially, the methods for the determination of trace analysis for medicinal plants are usually various procedures[11]. These procedures are disadvantaged by the consumption of organic solvents which cause the environmental problems, whereas, SWE can play a significant role to overcome the drawbacks. SWE is an environmental kindly technique for the future based on using water as extraction solvent at temperatures between 100 ^{o}C and 374 ^{o}C (critical point of water, 374 ^{o}C and 22 MPa at high pressure which is high enough to keep the water as the liquid solvent)[12]. Increasing the temperature at moderate pressure also reduces the surface tension and viscosity of water causes the subcritical water is comparable to organic solvents[13, 14]. Nevertheless, to the best of our knowledge, potential factors affecting SWE (temperature, extraction time and material ratio (subcritical water : dry raw material (ml: g)) were investigated their influence and interaction on the responses using full factorial design.

The objective of this work was to compare the efficacy of SWE with that of conventional extraction (ethanolic 95% v/v) regarding the flavonoid content and antioxidant activity from *G.calciphila*. Moreover, this current work investigated the potential factors affecting SWE and their interaction on flavonoid content and antioxidant activity of the *G.calciphila* extract.

MATERIALS AND METHODS

Plant material

G.calciphila was collected from Krabi province, Thailand in September in which the plant grows widely under natural condition. Voucher specimens of all accessions are deposited in the Phranakhon Rajabhat University Herbarium, Bangkok Thailand. *G.calciphila* was dried and ground into powder before the extraction.

Apparatus

The HPLC-UV/DAD analyses were carried out on a ^{3D}HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector, an automatic injector, an autosampler, and a power supply. Liquid chromatograph connected to a model 1100 (DAD).

Chemicals and standards

Investigated standards (catechin, rutin, quercetin, apigenin, hesperitin) were purchased from Sigma (St. Louis Missouri, U.S.A.). 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) was purchased from Sigma (St. Louis Missouri, U.S.A.). Ethanol (EtOH), acetonitrile (ACN) of HPLC grade were purchased from Labscan Asia (Bangkok, Thailand) and glacial acetic acid was purchased from Sigma (St. Louis Missouri, U.S.A.).

Extraction method

Subcritical extraction 100 g of powdered *G. calciphila* sample was soaked in water (1:50 w/v) and conducted at the temperature of 160 ^{o}C, under sufficient pressure (enough to maintain water in the liquid state) in a homebuilt apparatus for 60 min. The apparatus was slightly modified with the report from Cacace and Mazza (2006)[15]. Nitrogen was used to purge for drying the extracts. The collected extracts were kept protected from light, at 5^{o}C for the further studies.

Soxhlet extraction 100 g of powdered *G.calciphila* sample was extracted with 95% ethanol for 12 h. at temperatures of 60 °C in a Soxhlet apparatus. The extract was filtered and alcoholic content in extract was evaporated using Rotavapor® at 70 °C and the dried extract was stored at 5 °C for the further studies.

Identification and Quantitation of flavonoids The separation was performed by Reversed Phase High Performance Liquid Chromatography (RP-HPLC) using an ACE-129-2546® C18 column (25 cm x 4.6 mm i.d; 5 µm). The extracts were automatically injected (10.0 µL). The column was thermostatically controlled at 25 °C and a 1.0 ml/min flow rate was applied. The chromatogram was monitored at 270 nm, and UV spectra of individual peak was recorded in the range of 200–400 nm. A simple and gradient elution-based RP-HPLC method was developed for the analysis of typical flavonoids (*i.e.*, catechin (1), rutin (2), quercetin (3), apigenin (4), hesperitin (5)) in the extracts. For the development of an effective mobile phase, various solvent systems, including 0.1% glacial acetic acid (solvent A) and acetonitrile (ACN) of HPLC grade (solvent B) were investigated. For the preparation of the calibration curve, standard stock solutions of the both extracts at the concentration of 1,000 µg/ ml were prepared in ethanol, filtered through 0.22 µm filters (Millipore), and appropriately diluted (10–100 µg/ml). The linearity of analytical signals of the target compounds was tested in five different concentrations and each concentration was injected in triplicate. The calibration curves were plotted of the peak areas versus concentrations to obtain the desired concentrations in the quantification range.

Determination of antioxidant activity For 2, 2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) assay, an aliquot of samples will be mixed with 4.5 mL DPPH reagent (0.006 mM of DPPH redical in methanol) and the final volume was adjusted to 5.0 ml with water. After 10 min incubation, the disappearance of DPPH radical colour upon radical reduction was monitored by measuring the absorbance at 517 nm using UV/VIS spectrophotometer with deionized water in reagent as a control blank. The percentage of the remaining DPPH radical was calculated and plotted against sample and represented the results as the effective concentration at 50 % (EC_{50}).

Experimental design The factors affecting SWE including temperature, extraction time and material ratio (subcritical water : dry raw material (ml: g)) were investigated under full factorial design. The response surface methodology (RSM) combined with a 3^2 full factorial design was used to determine the optimum operating conditions for the extraction of flavonoids of the *G.calciphila*. The Design Expert (6.0.5) program was used to conduct the regression and the numerical optimization analysis, respectively. The models of flavonoid content and antioxidant activity were presented in forms of actual variables. For the modeling purposes, pre-defined ranges of dependent variables were considered regarding their statistical significance. A two-level full factorial design was employed using the following upper and lower limits of each factor : temperature (X_1, 120 and 160 °C), extraction time (X_2, 10 and 60 min) and material ratio (X_3, 25:1 and 50:1, subcritical water, ml: dry raw material, g).

Statistical analysis Each analysis was done three times for each extraction procedure in order to determine their reproducibility. Results are expressed as mean + SD. Statistical comparisons were made by one-way analysis of variance (ANOVA). Differences were considered to be significant when the p values were <0.05.

RESULTS

Plant Identification[2] For morpho-logical identification, Description: *Gynura calciphila* Kerr in Bull. Misc. Inf. Kew. 330. 1935. Type: Thailand, Chumpawn, Siepzuan, *Put 953* (holotype, K!; isotype, BM!). Plants 20–60 cm high, stems decumbent or erect from small tapering tubers of approximately

1–2 cm diameter, subsucculent, sparsely pubescent. Petioles 3–7 cmlong, with small auricles, 0.5–1×0.5–2 cm, or auricles absent, sparsely pubescent. Blades cordate, ovate or elliptic, 5–11 × 1.5–6 cm, sparsely pubescent, base cordate, apex acute, margin sinuate–dentate. Capitula 1–5 in lax cymose panicles; peduncles 4–10 cm long, sparsely pubescent, bracts 3–7, 2–4mmlong; involucres 7–10 mm long, 3–8 mm in diameter; calycular bracts 4–7, 3–5 mm long, pubescent; phyllaries 14, 1–2 mm broad, sparsely pubescent. Florets 15–25; corollas orange to yellow, 10–13 mm long, exserted part 2–3 mm. Anthers 2mm long, anther collars elongated. Style arms 3 mm long. Cypselas 4 mm long, brown, glabrous; carpopodium cylindrical, yellowish; pappus 7–10 mm long.

Identification and Quantitation of flavonoids

The optimized gradients of Reversed Phase High performance Liquid Chromatography (RP-HPLC) employed in *G. calciphila* extracts was: 0–2 min, 50% B (ACN) in A (0.1% glacial acetic acid); 2–10 min, 50-20% B in A and 10-20 min, 50% B in A. Linearity data from the optimized RP-HPLC conditions shows good correlation coefficient with acceptable %RSDs of slope and intercept for all tested compounds. Table 1 summarized the repeatability (express as %RSD). Method identification of compounds was performed on the retention time, coinjections, and spectral matched with 100 µg/mL investigated standards (catechin (1), rutin (2), quercetin (3), apigenin (4), hesperitin (5)). A simple RP-HPLC method was obtained for the identification and quantification of the five flavonoids and the result was well separated as Fig.1. As results, quercetin was found in both extracts from the different extraction procedure (Fig.2-3) under spiking technique and comparing with the spectrum of quercetin standard. It is evident that, quercetin is a major component in the both extracts. The results were shown that amount of quercetin in *G.calciphila* extract by SWE (44.6 + 5.2 µg/g, of dry raw material) was higher than soxhlet extraction (35.1+ 1.3 µg/g, of dry raw material) (Table 2).

Table 1. Repeatability and linearity data

	catechin	rutin	quercetin	apigenin	hesperitin
% RSDs	0.05-0.21	0.06-0.26	0.04-0.11	0.02-0.15	0.03-0.18
(calculated from peak area)					
Slope (%RSDs, n = 3)	0.2023 (1.42)	0.1153 (1.94)	0.3541 (1.13)	0.3813 (1.23)	0.2681(2.03)
Intercept (%RSDs, n =3)	1.493 (2.12)	1.135 (3.56)	1.093 (3.12)	0.9883 (1.89	1.343(1.78)
r^2	0.9997	0.9996	0.9999	0.9998	0.9997

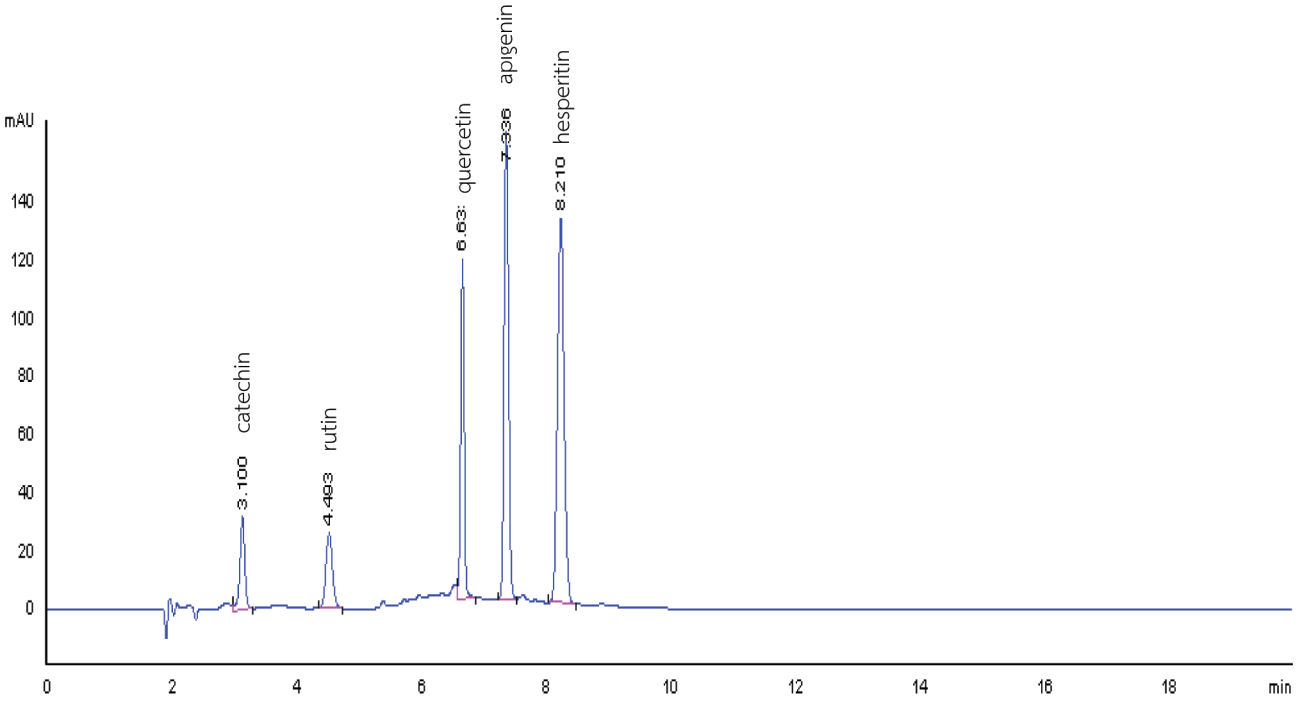

Figure 1. RP-HPLC chromatogram of the investigated flavonoids (100 µg/mL) for each standard under the optimized RP-HPLC conditions

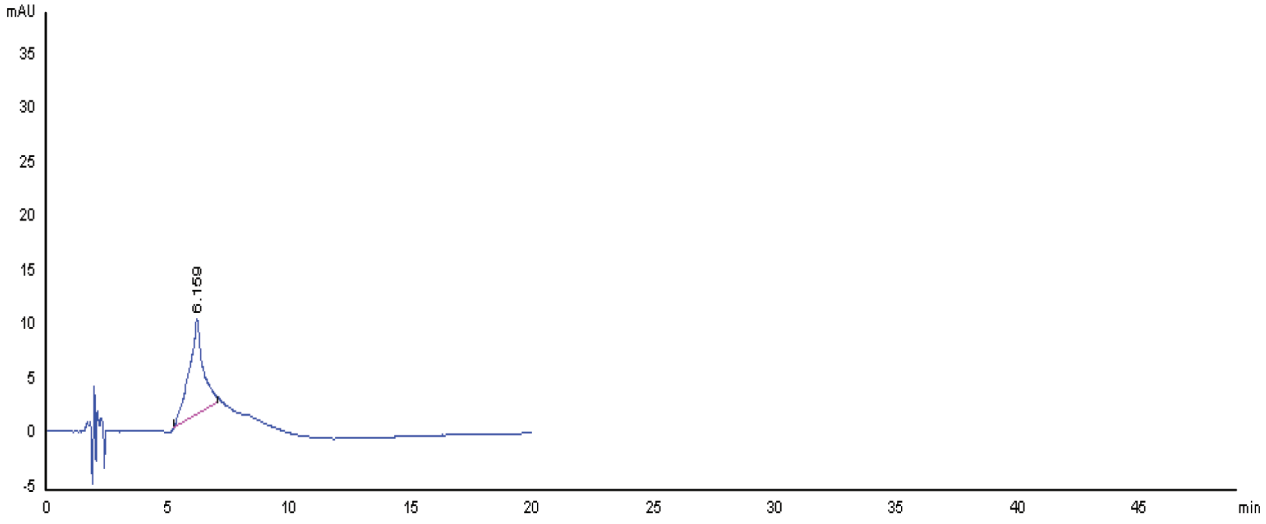

Figure 2. RP-HPLC chromatogram of subcritical water extract of *G. calciphila* Kerr. (1,000 µg/mL).

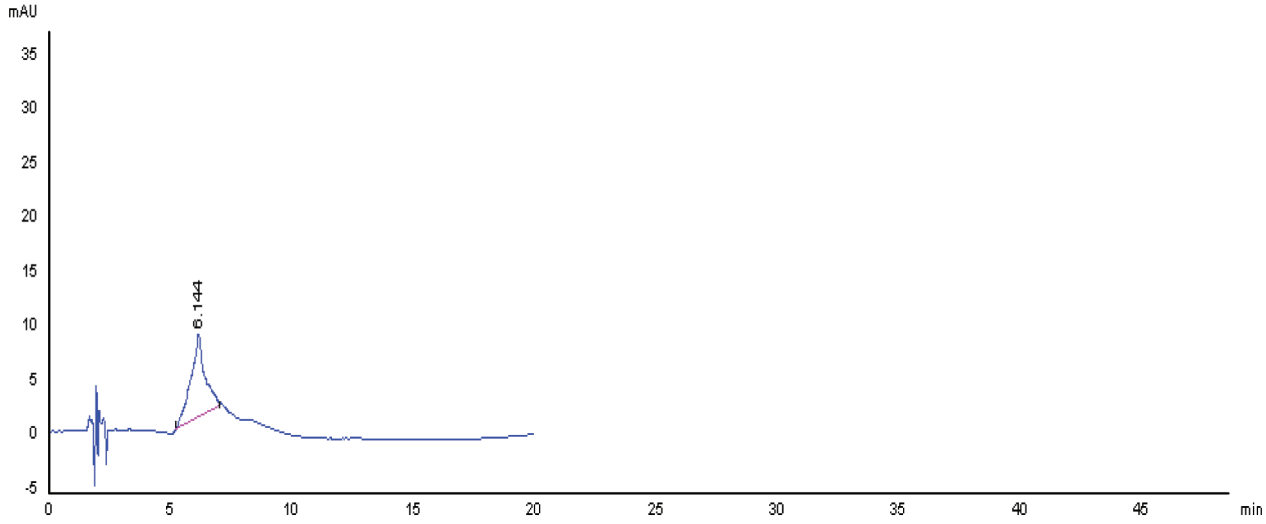

Figure 3. RP-HPLC chromatogram of soxhlet extract of *G.calciphila* Kerr. (1,000 µg/mL).

Table 2. Contents of quercetin in *G.calciphila* extracts from the different extraction

No.	Quercetin (µg/g, of dry raw material)	
	Subcritical water extraction	Ethanol extraction
1	41.4	36.5
2	45.2	34.7
3	47.1	34.0
Average	44.6	35.1
SD	2.9	1.3

Determination of antioxidant activity

Quercetin has been found as a major component in the extracts of *G.calciphila* and may acts as an antioxidant. Quercetin has a higher reduction potential compared with curcumin and comparable to trolox[16]. The addition of *G.calciphila* extract infusions the DPPH solution induced a decrease in the optical density at 517 nm. The effective concentration for being an antioxidant at 50 percent (EC_{50}) of the subcritical water extract and an ethanolic extract were at 508 and 518 mg/ml, respectively. Obviously, subcritical extraction was comparable to ethanolic extraction.

Experimental design

Experimental design was appied to investigate the factors affecting SWE, which performed better results than the soxhlet extraction in term of quercetin content and antioxidant. Quercetin content by subcritical water and traditional extraction were 44.6 µg/ml and 35.1 µg/ml, respectively. The EC_{50} of subcritical water extract and traditional extract were 508 and 518 µg/ml, respectively. The content of quercetin and EC_{50} of the extracts at the different SWE conditions are represented in Table 3. From the experimental design, results of each response were analyzed in randomized order to transform systematic

error into random error. Applying a full factorial design provided the goodness fitting for model equations to predict the effect and their interaction of temperature, extraction time and material ratio on quercetin content and antioxidant activity. Multiway factorial ANOVA showed that the measured and predicted values were in good agreement and no outlier was identified. In a model with significant p-value (p<0.05), a high regression coefficient demonstrates significant effect of independent variables on the corresponding responses. The goodness of fittings for models was expressed by coefficients of determination (R^2) (Table 4). The model equations derived from the experimental data that gave the most fit for each response are shown as the following.

Quercetin content (µg/g, dry raw material) = $35.36 + 9.43\ X_1 + 4.24\ X_2 + 0.24X_3 + 0.71\ X_2\ X_3$ ..... (1)

EC_{50} (µg/mL) = $627.8 - 129.94\ X_1 - 63.44\ X_2 - 30.88\ X_3 - 22.69\ X_1\ X_2$ ..... (2)

Table 3. Quercetin content and EC_{50} from SWE under the different conditions from 3^2 full factorial design.

No.	Temperature (°C)	Time (min)	Material ratio (subcritical water: dry raw material, ml:g)	Quercetin content (µg/g, dry raw material)	[a]EC_{50} (µg/ml)
1	120	10	25	20.1+1.2	805
2	120	10	50	22.6+3.1	792
3	120	60	25	30.8+4.4	775
4	120	60	50	30.2+2.5	659
5	160	10	25	36.8+3.6	709
6	160	10	50	32.4+2.6	602
7	160	60	25	40.2+1.3	519
8	160	60	50	44.6+2.9	508

[a]EC_{50} (effective concentration, 50%)

Table 4. Results of the model from ANOVA of quercetin content and antioxidant activity (EC_{50})

Response	Sum of squares	p-value	R^2
Quercetin content (µg/g, dry raw material)	438.43	0.0158	0.9068
EC_{50} (µg/ml)	95493.50	0.0139	0.9604

According to the effect estimated values (coefficients of the equation), the most significant factor for quercetin content and EC_{50} were the temperature (X_1) and extraction time (X_2), respectively.

The vary SWE conditions from 3^2 full factorial design were investigated using response surface analysis. The results of response surface analysis of quercetin content on the effect of factors affecting SWE including

temperature, extraction time and material ratio on quercetin content were shown in Fig.4. In this study, increasing extraction temperature from 120 °C to 160 °C, significantly enhanced quercetin content from 20.1 to 44.6 µg/g of dry raw material. Temperature has more crucial effect on the quercetin content compared to extraction time and material ratio. The results are in agreement with previous studies when subcritical water was used for the extraction[17, 18]. Cheigh and Chung (2012)[19] reported the maximum yields of flavanones including hesperidin and narirutin from *Citrus unshicu* were achieved by SWE at the temperature of 160 °C. However, degradation of compounds could be occurred with increased temperature. The 3D response surface of EC_{50} as a function on the effect of factors affecting SWE was shown in Fig. 5.

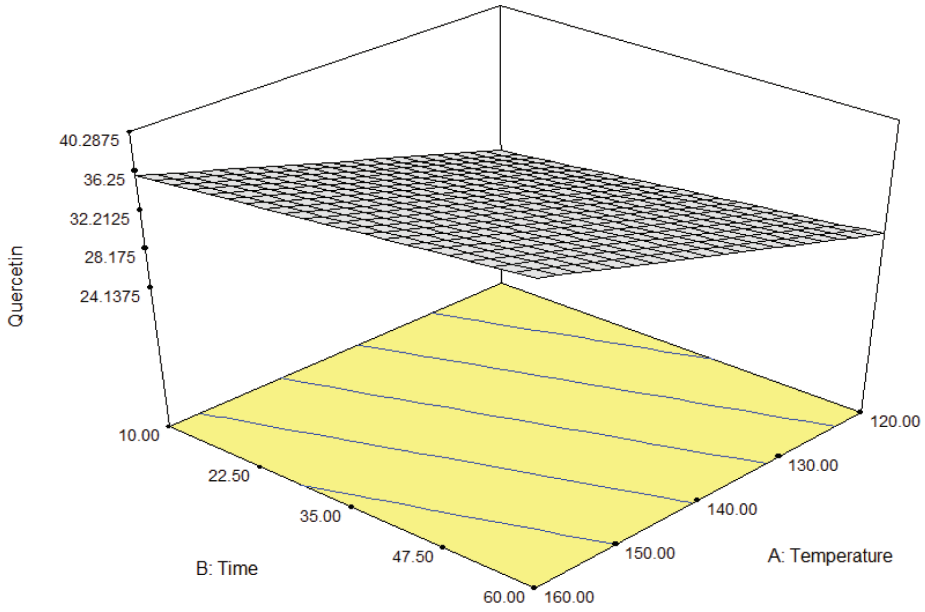

Figure 4. Response surface of quercetin content (µg/g, dry raw material) as simultaneous functions of SWE temperature (X_1) (°C), extraction time (X_2) (min) and material ratio (X_3) (subcritical water, ml: dry raw material, g).

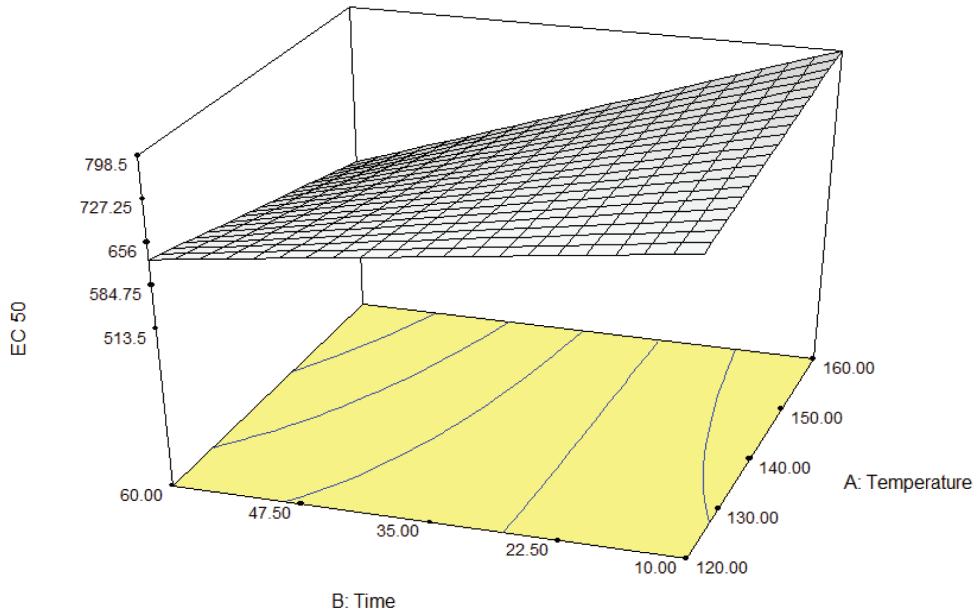

Figure 5. Response surface of EC_{50} (µg/mL) as simultaneous functions of SWE temperature (X_1) (°C), extraction time (X_2) (min) and material ratio (X_3) (subcritical water, ml: dry raw material, g).

DISCUSSION

This result demonstrated that the extraction time and temperature are predominant factors on the EC_{50} while, material ratio have minimal effect on EC_{50} or antioxidant activity. This effect might be due to reducing the polarity of subcritical water that allows acting as an organic solvent and dissolving less polar compound, which causes higher antioxidant activity. This current study is one of Thai National Researches with emphasis on the standardization and quality control of Natural products. In addition, this is the first report on the analysis of quercetin content and antioxidant activity of *G. calciphila*. SWE and factorial design were employed to carry out the results. The results of this work demonstrated that subcritical water extraction of *G. calciphila* was comparable to soxhlet with ethanol. The use of subcritical water provided higher quercetin content and antioxidant activity. The amounts of quercetin by subcritical water and traditional extraction were 44.6 µg/ml and 35.1 µg/ml, respectively. The EC_{50} of subcritical water extract and traditional extract were 508 and 518 µg/ml, respectively. Additionally, results from factorial design indicated that temperature and extraction time are the main affecting factors in SWE technique on the responses. This work confirmed the capability of safety SWE as a substitute for the traditional time-consuming techniques for the extraction of flavonoids as antioxidative compounds.

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