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Phytochemical and Analytical Characterization of Constituents from *Fagopyrum esculentum*

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ABSTRACT

Fagopyrum esculentum (buckwheat) is an annual herb that is commonly cultivated in Vietnam for ornamental and food purposes. Previous studies also indicated the medicinal potential of the plant due to its high content of bioactive compounds. However, every year, more than 300 ha of buckwheat cultivation has been discarded after flower festival in North Vietnam. The present study was conducted to clarify the main chemical constituents of *F. esculentum* aerial parts collected in Ha Giang, Vietnam. The investigation has led to the isolation and identification of nine compounds, specifically flavonoids (rutin, quercetin, quercetin 3-*O*-rhamnopyranoside, quercetin 3-*O*-galactopyranoside), lignan (isolariciresinol), phenylpropanoids linking with sucrose (lapathoside A, lapathoside D) and phytosterols (*β*-sitosterol, and daucosterol). An HPLC quantification method of the major component of *F. esculentum* leaves, rutin, was also developed. The optimized condition was performed on a Shimadzu LC-20D HPLC system; Shim-pack GIST C18 column (4.6 mm × 250 mm × 5 µm); eluting solvent system (0-20 min:16-26% acetonitrile in water containing 0.1% formic acid); injection volume 20 µL; flow rate 1.2 mL/ min; column temperature 35°C; UV wavelength 254 nm. The method was also validated by various parameters, including linearity (R^2= 0.9995), precision (6 samples; RSD= 1.58%), accuracy (recover rates from 94.4 to 103.9%), limit of detection (LOD= 0.049 µg/mL), limit of quantification (LOQ= 0.147 µg/mL). The analytical results showed that the content of rutin in leaf and flower samples were 1.794 ± 0.002% and 1.137 ± 0.002% , respectively.

Keywords: *Fagopyrum esculentum*, Chemical constituents, HPLC quantification, Buckwheat residue

Introduction

F. esculentum Moench. (Polygonaceae family) is an annual herb which can be up to 90 cm tall. The plant is commonly cultivated in Asian (e.g China, Vietnam, Korea), European, and North American countries.[1] Studies relating to the phytochemical composition and the pharmacological activities of the plant have been reported in literature. Phytochemical studies indicated that *F. esculentum* contained numerous secondary metabolites. To date, more than 20 compounds have been isolated and structurally elucidated from the hull, seed, and sprout parts, including flavonoids with rutin as the main composition,[2-5] catechins,[6] phenolics,[2] D-chiro-inositol derivatives (fagopyritols),[7,8] and monoterpenoids.[9] Rutin was identified as a compound of interest of *F. esculentum*. Rutin was found in different parts of buckwheat, including hull, seed, sprout, leaf, and flower with different content. The highest content of rutin was found in the leaf (0.34%) and flower (0.82%)[10] while hulls and seeds were found to contain 0.04% and 0.02% rutin, respectively.[2,11] Buckwheat sprouts were also studied for their phenolic accumulation during growth period with the highest rutin content under optimal condition of 1.6%.[12] It was also observed that rutin content changed variably according to places of cultivation, harvest seasons, and plant varieties.[11]

There have been a number of pharmacological studies conducted on different parts of the plant. Recent studies have shown that the flower and leaf extracts of *F. esculentum* exhibited potential antioxidant effects, such as hydrogen peroxide (H_2O_2) scavenging, hypochlorous acid (HClO) scavenging, superoxide anion scavenging, and DPPH assay.[13,14] With respect to anti-inflammatory effects, the ethanol extract of *F. esculentum* sprouts possessed both *in vitro* and *in vivo* anti-inflammatory activity through down-regulation of IL-6 and TNF-α levels in LPS-stimulated mice. In addition, the extract also has a direct impact on the expression of the regulatory genes IL-6 and IL-8 in HeLa cells.[15] The ethanol extract of the germinated seeds also showed hepatoprotective effect by reducing the amount of triglycerides and cholesterol in rats fed with a high-fat diet. The mechanism was identified to be the inhibition of the expression of adipogenic transcription factors, such as PPARγ and C/EBPα in hepatocytes.[16] The water extract of buckwheat concentrate (standardized by D-chiro-inositol) was also shown to decrease blood sugar levels in streptozotocin (STZ)-induced diabetic rats.[17]

In Vietnam, *F. esculentum* has been cultivated mainly for ornamental purposes, especially the annual 'Tam giac mach' (Vietnamese common name of *F. esculentum*) flower festival celebrated in Ha Giang province. When the festival ends, the whole plant will be uprooted and discarded; farmers will plant the new ones for next year's festival. Although buckwheat is one of major sources of natural rutin in Western countries, the species in Vietnam has almost no pharmaceutical application and every year, *F. esculentum* residues from 300 ha cultivation in Ha Giang province is discarded or used to feed cattle. Because of these reasons, the current research was designed to identify the chemical composition and rutin content of *F. esculentum* collected in Vietnam, which hopefully supports the medicinal potential of the dry residues of buckwheat after the flowering season.

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Materials and Methods

General procedures

NMR experiments were performed on a Bruker Avance 500 NMR spectrometer (500 MHz, BrukerSpin, Germany) using TMS (tetramethylsilane) as an internal reference. High performance liquid chromatography (HPLC) analysis was conducted on a Shimadzu LC-20D system (Japan) equipped with SPD-M20A diode array detector (DAD), DGU-20A5R degassing unit, and CTO-10ASVP column oven. Column chromatography for isolation was carried out using normal-phase silica gel (40-63 μm; Merck, Germany), reverse-phase silica gel RP-C18 (Merck, Germany), and Sephadex LH-20 (Merck, Germany) as adsorbents. Thin-layer chromatography for fraction profiling was of Merck 60 F_{254} aluminum plates (Merck, Germany). Organic solvents used for isolation procedure were from Samchun Pure Chemicals (Korea). HPLC solvents for analytical procedures were from Macron Fine Chemicals (Avantor, USA). Formic acid as acidic additive was from VWR Chemicals (Avantor, USA). Standard rutin was purchased from Chengdu Biopurify Phytochemicals Ltd. (China).

Plant material

The aerial parts of *F. esculentum* were collected at Quan Ba district, Ha Giang province, Vietnam in September 2020. The sample was dried in the oven at 60°C and kept in the freezer at -20°C for preservation. The sample was taxonomically authenticated by Dr. Pham Ha Thanh Tung, Department of Botany, Hanoi University of Pharmacy, Vietnam. A voucher specimen (PU/TGM/09-20) was deposited in the Laboratory of Pharmacognosy and Traditional Pharmacy, Faculty of Pharmacy, Phenikaa University.

Extraction and isolation of compounds

The ground dried plant materials (1.6 kg) were extracted with ethanol 96% (4 times × 1.5 L) in an ultrasonic bath. The extracts were then filtered, combined, and evaporated under reduced pressure to obtain 350.7 g of green-brown crude extract. The crude extract was then suspended in distilled water and partitioned with *n*-hexane, EtOAc, and *n*-BuOH successively. Each organic layer was evaporated by a rotary evaporator to obtain respective portions.

The non-polar *n*-hexane fraction (147.2 g) was first fractionated by a normal-phase silica gel (Φ =10 cm × l.55 cm), eluting with a gradient solvent system consisting of *n*-hexane and ethyl acetate (from 9:1 to 0:1) to obtain 8 sub-fractions (HX1-8). Based on TLC profiles, two fractions (HX2 and HX5) were chosen for further fractionation. Specifically, fraction HX2 (1.6 g) was separated using a gradient of *n*-hexane and dichloromethane (from 2:1-0:1) as eluent to yield compounds **8** (5 mg) and **9** (10 mg). Fraction HX5 was fractionated by a solvent system of dichloromethane and methanol (from 99:1 to 9:1) to isolate compound **5** (4 mg). The medium-polar EtOAc fraction (12.1 g) was also subjected into a normal-phase silica gel (Φ =8 cm × l=50 cm), eluting with a three-component solvent system (EtOAc, methanol, and water; gradient from 9:1:0.1 to 1:1:0.1) to yield 9 sub-fractions (EA1-9). The fraction screening by TLC indicated further exploration of fractions EA3 and EA5-7. Fraction EA3 (2.8 g) was separated on a reverse-phase silica gel column (2 cm × 60 cm) with a gradient of methanol and water (30-100%) to obtain compound **2** (5 mg). The same procedure was applied to isolate compound **6** (4 mg) from fraction EA5 (1.4 g). By using methanol 50% in water as an eluting solvent system, compounds **3** (4 mg), **4** (5 mg), and **7** (8 mg) were isolated from fractions EA6 (0.9g) and EA7 (1.2 g).

The *n*-BuOH fraction (40.3 g) was dissolved in 100 mL methanol and the solution was kept at the temperature of 4°C in the fridge. After a week, yellowish crystals were formed at the bottom of the flask. The crystals were then filtered and washed with cold methanol to remove impurities, which resulted in 20 mg of compound **1**.

HPLC analytical conditions

The separation of rutin (**1**) from *F. esculentum* leaves was optimized on a Shim-pack GIST C18 column (4.6 mm × 250 mm × 5 μm). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B). The gradient ratio started from 16B:84A and gradually changed to 26B:74A in 20 minutes. The column was then washed with pure acetonitrile in 10 minutes and re-equilibrated with the initial solvent system (16B:84A) for 10 minutes before the next analysis. The sample was injected with a volume of 20 μL (manual injector with a fixed 20-μL loop). The flow rate was set at 1.2 mL/ min, the column temperature was 35°C. The detected UV wavelength was chosen as 254 nm.

HPLC sample preparation

The finely ground plant materials (approximately 130 mg) were extracted with methanol (3.0 mL × 15 min × 3 times) by sonication. After centrifugation (1500g, 10 min), the supernatants were combined in a 10 mL volumetric flask and the flask was then filled with pure methanol to the volume. Before analysis, each sample solution was filtered by 0.45 μm membrane (PureTech Syringe Filter, Finetech 13 mm, 0.45 μm) and placed in the sample tray of the HPLC system.

Method validation

System suitability was confirmed by injecting a standard solution (approximately 200 μg/mL) six times. As a result, the relative standard deviation of retention time and peak area of reference compound were 0.11 and 0.87%, respectively, which indicated the chosen HPLC condition was suitable for quantification (Figure S10.2, Supplementary Materials). The reference compound (**1**) was then used to construct calibration curve. A stock solution was prepared by dissolving 4.3 mg of compound **1** into 5.0 mL pure methanol in a volumetric flask. Five other concentrations of calibration curve were prepared by serial dilution of stock solution with methanol in the ratio of 1:1. Respective solutions were then used to calculate linearity of calibration curve, and determine limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were determined by visual evaluation with signal-to-noise ratio of 3 and 10, respectively. Selectivity of HPLC peak was confirmed by Peak Purity function in the LabSolutions software (Shimadzu, Japan). Precision was evaluated by consecutive analysis of independent six samples. Accuracy was evaluated by respectively spiking leaf samples with three concentrations (high spike: 2.00 mg, medium spike: 1.00 mg, low spike: 0.50 mg) of compound **1** prior to extraction.

Results and Discussion

Identification of Isolated Compounds

Phytochemical investigation on *F. esculentum* has led to the isolation and identification of total nine compounds (**1**-**9**, Figure 1). The structures of isolates were elucidated by extensive spectroscopic and spectrometric analysis, including NMR and MS, along with comparison with published literature. The isolated compounds were identified to be rutin (**1**),[18] quercetin (**2**),[19] quercetin 3-*O*-rhamnopyranoside (**3**),[20] quercetin 3-*O*-galactopyranoside (**4**),[21] isolariciresinol (**5**),[22] lapathoside A (**6**),[23] lapathoside D (**7**),[23] β-sitosterol (**8**),[24] and daucosterol (**9**).[25]

Compounds **5**-**7** (**5**: $[\alpha]_D^{20}$ +41.0 (*c* 0.2, MeOH); **6**: $[\alpha]_D^{20}$ +24.3 (*c* 0.2, MeOH); **7**: $[\alpha]_D^{20}$ +17.2 (*c* 0.2, MeOH) were isolated and structurally elucidated the first time from *F. esculentum*. Previous publications indicated the vast majority of chemical constituents were flavonoids, catechins, cyclitol (fagopyritols), common phytosterols, and fatty acids.[26] Therefore, the presence of lignan and phenylpropanoid glycoside provided new information on secondary metabolites of the plant. Tartary buckwheat (*F. tataricum*) was found to contain phenylpropanoid glycosides (tatarisides) and these compounds were found to exhibit cytotoxic activity against several cancer cell lines.[27] Our study indicated that *F. esculentum* also contained similar compounds (**6** and **7**), which might exhibit interesting bioactivities in pharmacological assays.

HPLC Method Development

Our TLC initial screening showed rutin (**1**) as major composition in methanol extract of *F. esculentum* leaves and flowers (Figure S10.1, Supplementary Materials), which led to the method development of rutin quantification by high performance liquid chromatography. The optimized HPLC chromatogram was shown in Figure 2.

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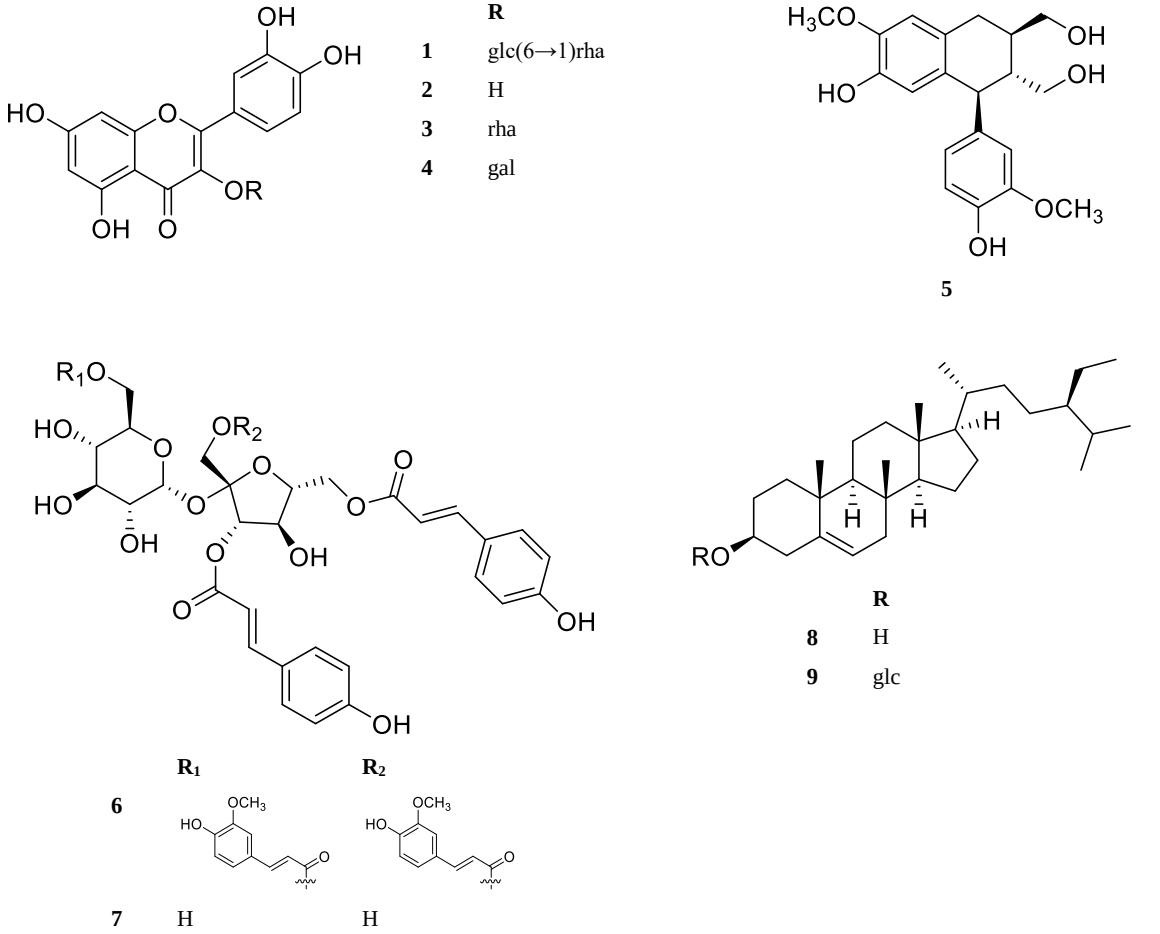

(glc= glucopyranosyl; gal= galactopyranosyl; rha= rhamnopyranosyl)

Figure 1: Structures of isolated compounds from *F. esculentum* aerial parts

The purpose of optimization is to produce good peak shape with high peak purity (≥ 95% in Peak Purity function of LabSolutions software) and narrow peak width, and good tailing factor. For mobile phase composition, acetonitrile was shown to be superior to methanol in terms of peak symmetry and peak width (Figure S10.3, Supplementary Materials). Moreover, the use of acetonitrile as organic solvent also reduced analytical time and column back pressure. It is also observed that the presence of low concentration of formic acid in water (0.1%, v/v) improved resolution between target peak and minor adjacent signals (Figure S10.4, Supplementary Materials). The variable of flow rate was also evaluated; the flow rate of 1.2 mL/min considerably improved peak width when compared with those of 0.8 and 1.0 mL/min (Table S1, Supplementary Materials). The change of column temperature also contributed to the sharpness of peak without affecting peak purity. The optimal column temperature was determined at 35°C (Table S2, Supplementary Materials). The detected UV wavelength was 254 nm due to the UV maxima of rutin under optimized HPLC condition.

Method validation

Following the guideline of ICH (International Conference on Harmonisation),[28] the optimized HPLC method was validated for selectivity, linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ). Selectivity was confirmed by Peak Purity function of LabSolutions software (Shimadzu, Japan) (≥ 95%). In terms of linearity, the coefficient of determination (R^2) of calibration curve was 0.9995 in the concentration range of 25.8-860 µg/mL. By visual evaluation method, LOD and LOQ were determined 0.049 and 0.147 µg/mL, respectively. In terms of precision, the parameter was determined by quantifying six independent samples in triplicate with relative standard deviation of 1.58%. Accuracy experiments were conducted by spiking plant samples with compound **1** at three different concentrations (2.0, 1.0, and 0.5 mg). As a result, the recovery rates of **1** were 94.4% (high spike), 96.1% (medium spike), and 103.9% (low spike). The validation results were summarized in Table 1.

Analysis of samples

The validated method was then applied to quantify rutin (**1**) content in leaf and flower samples of *F. esculentum*. As a result, the contents of 1 in leaf and flower samples were determined 1.794 ± 0.002% and 1.137 ± 0.002%, respectively (in triplicate).

Previous quantification studies indicated the variability of rutin content in leaf and flower parts of *F. esculentum*. Buckwheat samples collected in Slovenia were found to contain rutin with content ranging 0.014-0.065% (for leaves) and 3.62-5.89% (for flowers) by capillary electrophoresis.[29] Another HPLC-based quantification study in United Kingdom resulted in rutin content of 3.42% (for leaves) and 0.82% (for flowers). It is known that the accumulation of rutin reaches maximum during inflorescence and starts declining in the end of harvest season.[30] Our study indicated that dry residues of *F. esculentum* after flowering season still contained a considerable amount of rutin, especially in leaf and flower parts (approximately 1.8 and 1.1%, respectively). Since the aerial parts of *F. esculentum* will be discarded after flower festival, the significantly high rutin content of residues indicated the potential of natural resources for rutin extraction in pharmaceutical industry, especially in the context of Vietnam.

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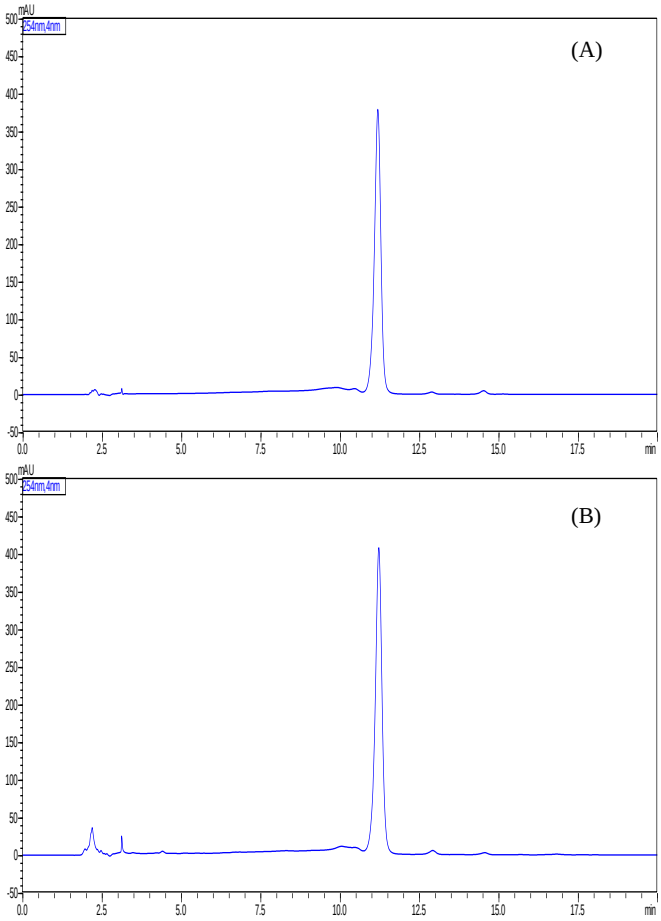

Figure 2: (A) HPLC chromatogram of rutin under optimized condition; (B) HPLC chromatogram of *F. esculentum* leaf extract under optimized condition.

Table 1: Results of method validation

	1
Regression equation	y = 27510x - 265904
R^2	0.9995
Range (µg/mL)	25.8-860
LOD (µg/mL)	0.049
LOQ (µg/mL)	0.147
Accuracy[1]	
high spike	94.4
medium spike	96.1
low spike	103.9
Precision[2]	
intra-day	1.58

[1] expressed as recovery rates in percent.
[2] relative standard deviation (n = 6).
Abbreviations: y = peak area, x = concentration (µg/mL), R^2 = coefficient of determination, LOD = limit of detection, LOQ = limit of quantification.

Conclusion

The dry residues of *F. esculentum* after flowering season collected in Quan Ba district, Ha Giang province were found to contain a wide range of phytochemicals, including rutin (**1**), quercetin (**2**), quercetin 3-*O*-rhamnopyranoside (**3**), quercetin 3-*O*-galactopyranoside (**4**), isolariciresinol (**5**), lapathoside A (**6**), lapathoside D (**7**), β-sitosterol (**8**), and daucosterol (**9**). Among them, rutin (**1**) was identified as major compound of total extract with contents of 1.794 ± 0.002% and 1.137 ± 0.002% in leaf and flower samples, respectively. The above mentioned results indicated that *F. esculentum* can be a promising natural source of bioactive compounds, especially rutin. Further pharmacological studies should be carried out to support industrial scale extraction of dry residue and encourage its uses as dietary supplement.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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