Journal of Research in Pharmacy

Research Article

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Antioxidant evaluation and HPLC analysis of *Buchanania lanzan* and *Buchanania siamensis* leaf extracts

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Received: 10 November 2022 / Revised: 13 March 2023 / Accepted: 20 March 2023

ABSTRACT: *Buchanania lanzan* Spreng and *Buchanania siamensis* Miq. (Anacardiaceae) are traditional medicinal plants that are rich in phenolic compounds and are used for the treatment of inflammatory diseases and pain. In the present study, the antioxidant potential of the distilled water and 80% ethanol extracts of *B. lanzan* and *B. siamensis* leaves was evaluated by various antioxidant assays, including DPPH, ABTS, FRAP, and ROS assays. The simultaneous HPLC determination of three major compounds was developed and validated. In addition, the total phenolic contents of each extract were also measured. From HPLC parameter validation, good linearity was achieved, with a correlation coefficient greater than 0.997. The method provided acceptable accuracy, specificity, and precision, suggesting that it could be suitably used for the quantification of the major compounds (myricitrin, isoquercitrin, and quercitrin) in the crude extracts of *B. lanzan* and *B. siamensis*. The results of the antioxidant assays showed that the ethanol extracts exhibited higher antioxidant activity than the aqueous extracts. The ethanol extract of *B. lanzan* leaves expressed the most antioxidant activity and could serve as a potential antioxidant source. Moreover, our validated technique would be beneficial for the quality control of the extract.

KEYWORDS: Antioxidant; *Buchanania*; HPLC; ROS; validated.

1. INTRODUCTION

Free radicals are derived from both endogenous (e.g., cell metabolism) and exogenous (e.g., pollution, tobacco smoke, and pesticides) sources. They are a substance that can be a cause of health problems. Free radicals have been associated with various diseases, including cancer, heart disease, Parkinson's disease, and Alzheimer's disease, as well as health problems such as aging. The human body produces antioxidants to protect cells from free radicals, but sometimes it can be imbalance between free radicals and antioxidants due to the overproduction of free radicals. Therefore, intake of exogenous antioxidants is a good option to prevent health problems [1, 2].

Buchanania lanzan Spreng (*B. Lanzan*) and *Buchanania siamensis* Miq. (*B. siamensis*), are members of the family Anacardiaceae and are traditionally used as food and medicine. Leaves of *B. lanzan* are used as traditional medicine such as expectorant, aphrodisiac, purgative, blood purifier, and to treat digestive diseases [3]. The ethanol extracts inhibited genotoxicity and oxidative stress, which were induced by cyclophosphamide in mice, by reducing chromosomal damage and lipid peroxidation [4]. For *B. siamensis*, the young shoots and leaves are locally consumed as fresh vegetables and appetizers and are used to relieve food poisoning and fever. The stems and leaves of *B. siamensis* have been shown to have a high total phenolic content, as well as strong antioxidant and anticancer properties [5]. The phytochemical investigation of genus *Buchanania* leaves revealed the presence of phenolics and flavonoids such as gallic acid, ellagic acid, myricitrin, isoquercitrin, and quercitrin [6, 7].

These bioactive constituents, myricitrin (myricetin-3-*O*-α-L-rhamnoside), isoquercitrin (quercetin-3-*O*-β-D-glucoside) and quercitrin (quercetin-3-*O*-α-L-rhamnoside), exhibited strong antioxidant, anti-inflammatory, and cytoprotective properties [8-10]. To the best of our knowledge, there is no study on validation protocols for HPLC method to ensure that there was no interference from others. Thus, this study aimed to evaluate the antioxidant activities of *B. lanzan* and *B. siamensis* leaf extracts as well as validate the HPLC method for analyzing these plants' main phytochemicals.

How to cite this article: Prompanya C, Petchsomrit A, Vongsak B. Antioxidant evaluation and HPLC analysis of *Buchanania lanzan* and *Buchanania siamensis* leaf extracts. J Res Pharm. 2023; 27(6): 2480-2486.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.535

2. RESULTS AND DISCUSSION

Flavonoids and phenolic compounds display the role of antioxidants which are essential parts that can protect cells from oxidative stress and aging caused by free radicals [2, 9, 11]. The ultrasonic-assisted extraction (UAE) is a suitable method to give the extract with high amount of total phenolic content [12-14]. In the present investigation, *B. lanzan* and *B. siamensis* leaf extracts were prepared by UAE with 80% ethanol and decoction with distilled water. The results of total phenolic content (TPC) and antioxidant activities were shown in Table 1.

Table 1. The total phenolic contents, FRAP value, 50% inhibition concentration (IC_{50}) of DPPH, ABTS and % ROS product in Hs68 cells from *B. lanzan* and *B. siamensis* leaf extracts

The extracts / positive control	Total phenolic content [mg GAE/g extract]	FRAP assay [mg Fe^{2+}/g extract]	DPPH assay IC_{50} [µg/mL]	ABTS assay IC_{50} [µg/mL]	%ROS Product in Human fibroblast cells (Hs68)
ELL	111.66±0.13^a	35.35±0.51^a	4.13±0.08^a	5.43±0.08^a	44.58±6.28^a
ESL	91.54±0.17^b	36.11±0.78^a	4.48±0.09^b	9.85±0.52^b	55.43±3.20^b
ALL	109.80±0.05^c	35.11±0.20^a	5.07±0.25^c	5.93±0.04^c	56.10±3.66^b
ASL	105.24±0.75^d	33.49±0.41^b	5.32±0.28^d	5.37±0.21^a	56.14±4.61^b
Ascorbic acid	-	-	2.46±0.17^e	2.51±0.13^d	50.44±4.67^b

Expressed as means ± SD (n = 3). A statistically significant difference exists between different alphabets. ELL: Ethanolic *B. lanzan* leaf extract; ESL: Ethanolic *B. siamensis* leaf extract; ALL: Aqueous *B. lanzan* leaf extract; ASL: Aqueous *B. siamensis* leaf extract.

Ethanol extract of *B. lanzan* leaves (ELL) exhibited TPC at 111.66±0.13 mg GAE/g extract, which was higher amount than other extracts. The antioxidant activities were evaluated using ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and reactive oxygen species (ROS) production assays. In a previous study, *B. siamensis* leaves were extracted by the UAE using a mixture of water and propylene glycol as a solvent. The results exhibited TPC and anti-radicals of ABTS lower than UAE using 80% ethanol and distilled water this study [14]. ELL showed the most activity in DPPH (IC_{50} 4.13±0.08 µg/mL) and ROS production (44.58±6.28%) assays. ESL exhibited the greatest activity in the FRAP assay with 36.11±0.78 mg Fe^{2+}/g extract, followed by ELL with 35.35±0.51 mg Fe^{2+}/g extract. In the ABTS assay, the most powerful antioxidant was ASL, followed by ELL, with IC_{50} values of 5.37± 0.21 and 5.43 ±0.08 µg/mL, respectively. Overall, the results revealed that ELL exhibited the most antioxidant capacity with the highest level of TPC.

Phytochemical analysis revealed the presence of myricitrin, isoquercitrin, and quercitrin in *Buchanania* spp. in prior research [6, 7]. Thus, the HPLC method was developed and validated in the current study. Mobile phase with 0.2% formic acid in water and methanol (55:45, v/v) was good for separating myricitrin, isoquercitrin and quercitrin. Validation parameters for the developed HPLC technique were found to be acceptable (Table 2).

Table 2. Method validation parameters by the proposed HPLC method

Parameters	Results		
	Myricitrin	Isoquercitrin	Quercitrin
Regression equation[a]	Y = 585.7X - 11398	Y = 162.52X - 7956	Y = 466.06X - 11111
Correlation coefficient [r^2]	0.9979	0.9983	0.9981
Linear range [µg/mL]	15.63-500	62.5-1000	31.25-500
Average recovery [%]	108.27±4.76	107.82±7.20	108.14±8.52
Intraday, %RSD	1.74	2.17	1.66
Interday, %RSD	2.99	3.71	2.69
LOD, [µg/mL]	0.98	0.98	7.81
LOQ, [µg/mL]	2.97	2.97	23.67

[a]x is the concentration of the compounds; y is the peak area at 254 nm.

Myricitrin, isoquercitrin, and quercitrin had linear calibration curves as 15.63-500, 62.5-1000, and 31.25-500 µg/mL, respectively. The correlation coefficient value was ≥ 0.9979, confirming the linearity of the method. The %RSD values of intra- and inter-day precision were < 3.8%. The LOD of myricitrin, isoquercitrin, and quercitrin were 0.98, 0.98, and 7.81 µg/mL, respectively, and the LOQ of these compounds were 2.97, 2.97, and 23.67 µg/mL, respectively. The developed HPLC method was found to be specific and accurate using standard addition techniques. The myricitrin, isoquercitrin, and quercitrin were added directly to the aliquots of

analyzed extract, and the extract and average recovery were 108.27±4.76, 107.82±7.20, and 108.14±8.52%, respectively. For suitability of the method, the chromatographic properties such as resolution and peak asymmetry satisfied the criteria for these compounds (Table 3). The resolution values measured for each peak pair were more than 1.06. The tailing factors of myricitrin, isoquercitrin, and quercitrin were 1.21, 1.17, and 1.16, and the numbers of theoretical plates of these compounds were 3789.89, 4350.00, and 5922.22, respectively. Ideal system suitability parameters are a tailing factor of less than 2 and theoretical plates greater than 2000. Hence, the validation parameters indicated that the analytical procedure has a reasonable degree of precision and accuracy [15]. The content of myricitrin, isoquercitrin, and quercitrin in the extracts ranged from 5.62-15.01, 1.18-3.54, and 3.00-19.68 mg/g extract, respectively (Table 4).

Table 3. System-suitability report of myricitrin, isoquercitrin and quercitrin

Compound (*n*=9)	*Rt*	*Rs*	*T*	*N*
Myricitrin	8.66	11.19	1.21	3789.89
Isoquercitrin	10.266	1.82	1.17	4350.00
Quercitrin	13.214	5.35	1.16	5911.22

n, Number of determinations; *Rt*, retention time; *Rs*, USP resolution; *T*, USP tailing factor; *N*, number of theoretical plates

Table 4. The content of myricitrin, isoquercitrin, and quercitrin in *B. lanzan* and *B. siamensis* leaf extracts from different extraction methods

Extract	Content of major compounds [mg/g extract]		
	Myricitrin	**Isoquercitrin**	**Quercitrin**
ELL	11.14±0.03[a]	2.59±0.01[a]	19.68±0.05[a]
ESL	15.01±0.03[b]	3.54±0.07[b]	10.48±0.01[b]
ALL	10.10±0.01[c]	2.86±0.02[c]	16.86±0.05[c]
ASL	5.62±1.41[d]	1.18±0.32[d]	3.00±0.81[d]

Expressed as means ± SD (*n* = 3). Different letters of the extraction method in the same column indicate significant differences. ELL: Ethanolic *B. lanzan* leaf extract; ESL: Ethanolic *B. siamensis* leaf extract; ALL: Aqueous *B. lanzan* leaf extract; ASL: Aqueous *B. siamensis* leaf extract.

Anacardiaceae plants, including mango, cashew, and marula, are shown to have potent antioxidant properties and are employed in the manufacture of cosmeceutical and food products. The plant flavonoids have been identified as active chemicals [6]. In this study, *B. lanzan* and *B. siamensis* which are distributed in the east of Thailand were investigated and the ethanolic *B. lanzan* leaf extract exhibited the strongest antioxidant activity. In addition, our HPLC validation method was also effective to analyze the major compounds, myricitrin, isoquercitrin, and quercitrin contents in these extracts (Figure 1). Ethanolic leaf extract had the highest content of these compounds, and *B. lanzan* also provided the highest quercitrin content. This information regarding the concentration of these pharmacologically active chemicals in the leaf extracts of *B. lanzan* and *B. siamensis* could aid in the identification of their active constituents, which is of great value to the pharmaceutical sector. The ethanolic *B. lanzan* leaf extract might be a source of antioxidant substances that illustrated the strongest activity for the development of cosmetics and nutraceuticals. For further research, these phenolic compounds in the leaf extracts may need to be fully characterized using LC-MS/MS, and their biological mechanisms may need to be studied.

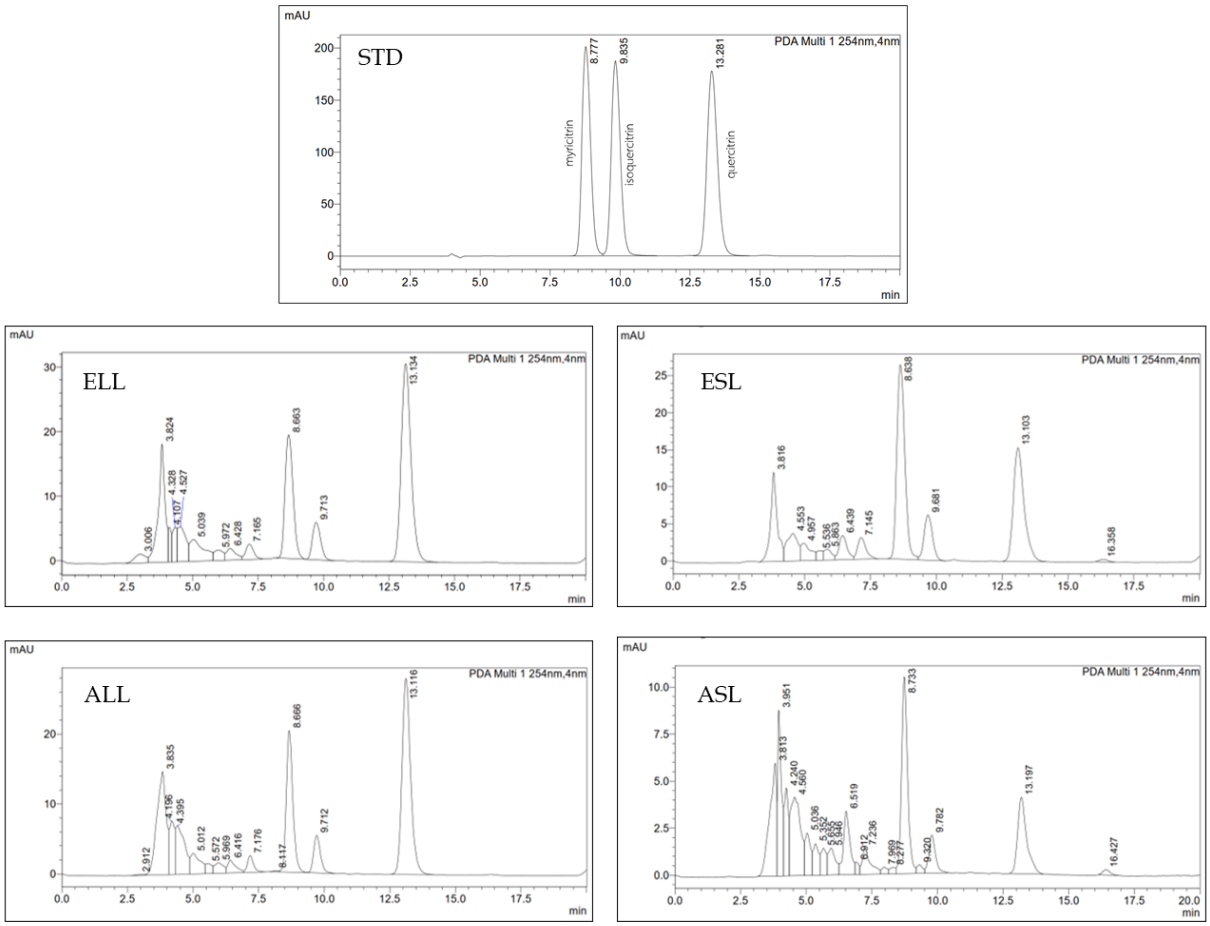

Figure 1. HPLC chromatograms of (STD) myricitrin, isoquercitrin, and quercitrin standards, (ELL) Ethanolic *B. lanzan* leaf extract; (ESL) Ethanolic *B. siamensis* leaf extract; (ALL) Aqueous *B. lanzan* leaf extract; (ASL) Aqueous *B. siamensis* leaf extract

3. CONCLUSION

According to the findings of the antioxidant assessments, the ethanolic *B. lanzan* leaf extract had better antioxidant activity than the other extracts. For the simultaneous measurement of three bioactive substances (myricitrin, isoquercitrin, and quercitrin) in extracts of *B. lanzan* and *B. siamensis* leaves, a practical and accurate HPLC-DAD method has been developed and would be advantageous for the quality inspection of the extracts.

4. MATERIALS AND METHODS

4.1. Materials

B. lanzan and *B. siamensis* samples from Sa Kaew province, Thailand, were collected and identified by plant taxonomist, Dr. Chakkrapong Rattamanee in October 2020. The leaves of these plants were cleaned with tap water, and dried in a hot air oven at 50 °C for 24 h. Then, the dried leaves were cut into small pieces and separately ground to a fine powder.

The ethanolic extract was prepared by using the ultrasonic-assisted extraction (UAE) method. The dried leaf powder was combined with 80 %ethanol in an Erlenmeyer flask at a solid-to-solvent ratio of 1:20 (w/v). The sample was extracted at 35 °C and 35 kHz ultrasonic frequency for 30 min.

The decoction was prepared by adding dried leaf powder to an Erlenmeyer flask and boiling for 15 min with distilled water (1:20, w/v).

Each method's mixture was filtered with Whatman® filter no. 1 paper, then the residue was re-extracted. The pooled filtrates of each extraction were dried in a vacuum evaporator at 50 °C under reduced pressure, and the extracts were maintained in sealed containers at 4 °C. Analytical grade chemicals, solvents, and reagents were used.

4.2. Total phenolic content

The Folin-Ciocalteu colorimetric method was used to determine the total phenolic content of the extracts, as described before [2]. In brief, 200 µL of each sample were thoroughly mixed with 800 µL of 7.5% sodium bicarbonate solution and 500 µL of Folin-Ciocalteu reagent. The absorbance of the mixture was measured at 765 nm after 30 min in the dark. The total phenolic content per gram of extract was determined in milligrams of gallic acid equivalents (GAE). The average and standard deviation of triplicate analyses were used to illustrate the results.

4.3. Free radical scavenging assay

The DPPH radical scavenging activity and ABTS radical cation-scavenging activity of the extracts were tested using Vongsak et al. method [2]. The IC_{50} value was evaluated as the sample concentration required to inhibit 50% of scavenged DPPH.

Ferric Reducing Antioxidant Power (FRAP) assay was also carried out according to the Vongsak et al. [2] method. The amount of $FeSO_4$ equivalents in 100 g of extract was calculated using a standard curve comparison.

4.4. Cell culture

Human fibroblast cell strain Hs68 (ATCC CRL-1635) was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium with a low glucose concentration (DMEM-LG; Cytiva, United States) containing 100 U/mL penicillin, 100 µg/mL streptomycin (AppliChem, Germany), and 10% fetal bovine serum (Cytiva) and maintained at 37 °C with 5% CO_2 in a humidified chamber. The medium was changed every 3 days until it reached 80% confluence.

4.5. Determination of inhibition effect on intracellular ROS production

The final concentrations of the extracts (20 µg/mL) and positive control (5 µg/mL) that provided more than 90% survival of Hs68 cells evaluated by the MTT assay were selected for evaluation of intracellular ROS production. The improved dichloro-dihydro-fluorescein diacetate (DCFH-DA) procedure of Pattananandecha et al. [16] was used to assess the inhibitory effect of the chosen substances on intracellular ROS production. Hs68 cells (1×10^6 cells/mL) were plated for 12 h on a 96-well plate, then pre-treated with 20 µg/mL of tested samples for 12 h. To promote ROS generation, cells were treated with 5 mM hydrogen peroxide (H_2O_2) for 30 min. After that, each well-plate was filled with 40 µM of DCFH-DA solution and incubated for 30 min at 37 °C with 5% CO_2. Excitation wavelengths of 480 nm and emission wavelengths of 525 nm were used to measure the green fluorescence intensity. Positive control was L-ascorbic acid (5 µg/mL). The equation 1 was used to calculate the percentage of ROS production.

(Eq. 1) Percent ROS production = 1 – [Fluorescence intensity in the presence of the extract or ascorbic acid / Fluorescence intensity of the control (cells without the extract)] × 100

4.6. Quantitative analysis of major active compounds by HPLC

Standard stock solutions (1000 µg/mL) were made by accurately weighing myricitrin, isoquercitrin, and quercitrin standards (purity > 98.0%) and dissolving in methanol in a volumetric flask. All stock solutions were serially diluted with methanol to provide six different concentrations (500-15.6 µg/mL) for a calibration curve construction.

4.7. Chromatographic conditions

The system consisted of a quaternary pump (LC-20AD), a diode array detector (SPD-M20A), a thermostated column compartment (CT0-10ASvp), and an autosampler (SIL-20A HT) from Shimadzu (Kyoto, Japan). Shimadzu LabSolutions software was used to collect the data. At 25 °C, a Luna® C18 column (250×4.6 mm, i.d., 5 µm) with a C18 guard column (Phenomenex, California, USA) was used to accomplish chromatographic separation. The mobile phase for isocratic separations was made up of 0.2 %formic acid and methanol (55:45, v/v) and flowed at 1.2 mL/min at 25 °C with a 10 µL injection volume and detected at 254 nm.

4.8. Validation

The International Conference on Harmonization (ICH, 1996/2005) requirements were followed for the validation of accuracy, precision, linearity, limit of quantitation (LOQ), and limit of detection (LOD) [17].

The accuracy of the method was measured by spike recovery experiments. The determined UAE was spiked with three different concentrations of myricitrin, isoquercitrin, and quercitrin (500, 1000, and 1500 µg/mL). The recoveries were obtained by subtracting the values from the control (unspiked matrix) from the spiked standard samples and dividing by the quantities added. For each concentration, recovery was determined in triplicate, and recovery (%) was calculated as Equation 2.

(Eq. 2) Percent recovery = [(Amount found - Original amount)/Amount spiked] x 100

To investigate measurement precision and repeatability, the peak area of six duplicates of sample solutions with maximum concentrations of myricitrin, isoquercitrin, and quercitrin from the UAE was analyzed. To determine repeatability (intra-day precision), the sample was examined six times on the same day, while the intermediate precision (inter-day precision) was recorded for three days. The relative standard deviation (%RSD) percentages were determined.

HPLC was used to assess the linearity of myricitrin, isoquercitrin, and quercitrin working standard solutions (500 to 15.6 g/mL) in triplicate. The peak area vs the known concentration of each standard was plotted to construct the calibration curve.

Serial dilutions of sample stock solutions with methanol were used to determine the LOD and LOQ tests. The LOD and LOQ had a signal to noise ratio (s:n) of 3:1 and 10:1, respectively.

4.9. Statistical Analysis

The data were presented as means ± SD (n = 3). In SPSS for Windows (v21.0), one-way ANOVA testing with the least significant difference statistical tests was run. Statistical significance was indicated by a *p*-value less than 0.05.

Acknowledgements: This research was financially supported by a grant from the Faculty of Pharmaceutical Sciences, Burapha University (Grant no. Rx05/2564) and the Pharmaceutical Innovations of Natural Products Unit (PhInNat), the Research and Development Fund, Burapha University (Grant no.11/2565).

Author contributions: Concept – B.V., A.P.; Design – B.V., C.P.; Supervision – B.V.; Resources – B.V., A.P.; Materials – B.V.; Data Collection and/or Processing – B.V., A.P., C.P.; Analysis and/or Interpretation – B.V., A.P., C.P.; Literature Search – B.V., A.P., C.P.; Writing – B.V., A.P., C.P.; Critical Reviews – B.V., A.P., C.P.

Conflict of interest statement: The authors declared no conflict of interest.

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