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Hydro-alcoholic Extraction by an Ultrasound Assisted Leaching Method of *Crotalaria assamica* Benth Seed Powder for Pharmaceutical Purposes: Optimization using RSM and Basic Properties

Long Hoang Nguyen[a,b], Viet Tien Vo[c], Minh Thong Le Tuan[a,b], Tan Phat Vo[a,b], Nhan Tu Le Tan[a,b], Dinh Quan Nguyen[a,b,*]

[a] Laboratory of Biofuel and Biomass Research, Faculty of Chemical Engineering, Ho Chi Minh City University of Technology (HCMUT), 268 Ly Thuong Kiet, District 10, Ho Chi Minh City, Vietnam.
[b] Vietnam National University Ho Chi Minh City, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam.
[c] Faculty of Pharmacy, Ton Duc Thang University, Vietnam
ndquan@hcmut.edu.vn

Many components in *Crotalaria assamica* benth composition make this plant a potential antioxidant and antibacterial herbal material, which can find many applications in medicine and biochemistry. In this study, *Crotalaria assamica* benth seed powder was extracted with aqueous alcohol solutions by an ultrasound-assisted leaching method. The ultrasound wave was 37 kHz, and the extraction was carried out at 30 °C for 10 mins. An alcohol content of 54.78 vol% and a mass ratio of solid to liquid (RSL) of 1/55 are suitable conditions according to the reaction surface method (RSM) developed by the Box–Beknen approach to design matrix experiments. With the above conditions, the actual result was that the TPC was 12.02 ± 0.24 mg GAE/g DW and the TFC was 7.68 ± 0.34 mg RE/g DW). *Crotalaria assamica* confers antioxidant activity of the extract with an IC50 value of 644 μg/mL, and antibacterial activity against *P. aeruginosa* strain, with an average antibacterial ring diameter of 13.5 mm.

1. Introduction

Crotalaria assamica Benth is a Fabaceae family plant commonly used as a herb in Asian nations such as China, Vietnam, and India (Figure 1) (Krishnaraj et al., 2011). *Crotalaria assamica* was used to cure bladder stones, genetic wounds, leprosy, bone discomfort, inflammation, and bleeding. Its seeds have been used to prepare beverages, such as herbal teas, which are supposed to help people sleep deeper. There has not been substantial scientific investigation to discover the remarkable content and applications of this plant, so these were essentially folk cures. Studies on natural chemicals and medicine have been developed in recent decades, and various species of the genus *Crotalaria* have been included in the list. In 1992, Edgar with his colleagues discovered pyrrolizidine alkaloid compounds in extracts of *Crotalaria assamica* (*C. assamica*) (Edgar et al., 1992). In another study in 2004, new isoflavones of 5,7,4-trihydroxy-2-methoxyisoflavones were discovered when analyzing the fluid composition of *Crotalaria pallida* and *C. assamica* Benth (Ko et al., 2004). The antioxidant and anti-inflammatory capabilities of these substances are well-known. In a study of Islam and his collaborators on *Crotalaria pallida* in 2018, the results also showed that emphasized plants' antioxidant activities (Islam et al., 2018). Alkaloids have a wide range of biological effects, including anti-inflammatory, demulcent, ganglionic blocking, anti-plasmodic, and hepatoprotective properties (Debnath et al., 2018). Flavonoids have various biological activities such as: anti-inflammatory, anti-cancer, antiviral, antioxidant, enzyme inhibitor, hormone, and immune modulators activities (Lee et al., 2007). As a result, this study employed an ultrasound-assisted leaching approach to enhance the efficiency of extracting the essential compounds from *Crotalaria assamica* Benth.

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Ethanolic maceration is commonly applied to extract the antioxidant active components in vegetables and plants because of the low temperature and solvent's capacity to dissolve various compounds (Jusoh et al., 2017). This method does not give high extraction efficiency, they can take a long time, possibly from a few days to a few weeks. Ultrasonic-assisted extraction has been suggested to increase efficiency. Ultrasonic irradiation can rupture cell structures, release chemicals and enhance the solubility of solutes in solvents (Kumoro et al. 2021). This method is considered green technology and has been developed to extract heat-sensitive compounds.
An investigation of the conditions for soaking alcohol combined with ultrasonic extraction for *Crotalaria assamica* Benth was carried out in this study, including aqueous alcohol concentration and the ratio of solid to liquid. The antibacterial and antioxidant activities of the extracts were also assessed. These results are expected to serve fundamental knowledge for establishing the medicinal plant's usage and potential application.

Figure 1: Crotalaria assamica Benth plant and flower

2. Materials and methods

2.1 *Crotalaria assamica* Benth seed and chemicals

C. assamica Benth seeds from Duc Trong, Lam Dong Province, Vietnam, were provided by the Department of Plant Resources – Tay Nguyen Scientific Research Institute (Vietnam). The dried seeds were treated under roasting for 3 h and land cooling process. The seeds were crushed and filtered so that its had evenly sized and less than 1 mm.

2.2 Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH, 95 wt%) and Dimethyl sulfoxide (DMSO, analytical standard) were purchased from Aldrich Sigma Co. Ltd. Ethanol (alcohol) (90 vol%) was purchased from Vina Chemsol (Vietnam). Standard compounds gallic acid (> 99.8 %) and rutin (> 99.5 %) were purchased from S&P Global.

2.3 Experiment design

C. assamica Benth seeds powder was mixed with alcohol and the mixture was subjected to ultrasound wave at 37 kHz for 10 min at 30 °C and then filtered. The vacuum rotary evaporator was used to empty all solvents from the extraction.
Response Surface Methodology (RSM) was used to investigate the influence of two independent on total phenolic content (TPC) and total flavonoid content (TFC).
One-factor experiments were carried out to determine the range of the factors of the RSM model. The mass ratio of solid to liquid was investigated with the following values: 1/10, 1/20, 1/30, 1/40, 1/50, 1/60, 1/70, and 1/80, and alcohol concentration was surveyed with values: 0, 30, 50, 70, and 90 vol%.
A two-level factorial design was used to build the model for the extraction process of solvent concentration and the mass ratio of solid and liquid. Design Expert v.11 software was used to develop the design matrix using the Box-Behnken approach and to analyse the results statistically. Based on findings during the implementation of this method research, the solvent concentrations (x_1, 30-50-70 vol%), mass ratio of solid and liquid (x_2, 1/70-1/60-1/50), were considered as the major influential variables. The effects of the two independent variables on two responses: total phenolic content (TPC) and total flavonoid content (TFC). An empirical model was obtained by correlating the measured responses with independent variables by multiple regression analysis. In the RSM experiments, the second-order response function was predicted by the following Eq(1):

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (1)$$

where y is the responses, x_1 and x_2 are independent variables, b_1, b_2, b_{11}, b_{22}, and b_{12} are estimate of the theoretical coefficient.

A Box-Behnken design in the software was used to develop the experimental matrix. The low value and the high value of two factors were supplied in the Box - Behnken Design. Center points per block were two. The number of experiments is determined according to the Eq(2):

$$N = 2^k + 2k + n_0 = 2^2 + 2.2 + 2 = 10 \quad (2)$$

With k = 2 is the number of factors and n_0= 2 is the number of repeated experiments at center. Ten trials were arranged as shown in Table 1.

Table 1: Experimental design and results

Exp.	Independent variables		Responses	
	x_1: Solid/liquid	x_2: Alcohol Concentration (%)	y_1, TPC (mg GAE/g DW)	y_2, TFC (mg RE/g DW)
1	1/50 (+1)	30 (-1)	6.49	1.86
2	1/60 (0)	30 (-1)	9.26	2.72
3	1/70 (-1)	30 (-1)	7.39	1.47
4	1/50 (+1)	50 (0)	8.22	4.28
5	1/60 (0)	50 (0)	12.11	7.68
6	1/60 (0)	50 (0)	12.97	7.81
7	1/70 (-1)	50 (0)	10.50	6.28
8	1/50 (+1)	70 (+1)	6.28	1.76
9	1/60 (0)	70 (+1)	10.37	5.14
10	1/70 (-1)	70 (+1)	10.10	6.56

where (-1) is the low value, (+1) is the high value, and (0) is the median value.

2.4 Determination of total phenolic content (TPC)

The total polyphenol content was analysed using the Folin-Ciocalteu modified (Wang et al., 2018). In Brief, 1 mL of the extract was mixed with 1 mL of Folin-Ciocalteu reagent, and then incubated at 30 °C for 5 mins. Afterward, 2 mL of saturated Na_2CO_3 was added to each well for another 20 mins of incubation at 30 °C. The absorbance was recorded at λ = 765 nm by spectrophotometer model (NiR V770, Japan). Blank samples were carried out in the same way but without the extract. A calibration curve was drawn with gallic acid at different concentrations (5–100 µg/mL, R^2 = 0.9998). The results were expressed as milligram gallic acid equivalents (GAE) per gram of dried weight of the sample (mg GAE/g DW). The experiment for each sample was repeated 3 times and averaged.

2.5 Determination of total flavonoid content (TFC)

TFC was measured using the previously described method, with some modifications (Wang et al., 2017). In Eppendorf tube, 100 µL of sample or standard was mixed with 400 µL and 30 µL $NaNO_2$ 10 %. After being kept at room temperature for 5 min, 30 µL of the 10 % $AlCl_3$ solution was added, then incubated for another 6 min, followed by the addition of 200 µL of 1 M NaOH, and diluted with methanol solution to a constant volume of 1 mL. After that, the solution was thoroughly mixed again and incubated for 30 min at 30 °C. The absorbance was recorded at 510 nm by spectrophotometer (NiR V770, Japan). Blank samples were carried out in the same way but without the extract. A calibration curve was drawn with rutin at different concentrations (10–100 µg/mL, R^2 = 0.9997). Results were expressed as mg rutin equivalents (RE) per gram of sample in dried weight (mg RE/g DW). The experiment for each sample was repeated 3 times and averaged.

2.6 Antioxidant activity

The antioxidant capacity of the extract is evaluated by DPPH scavenging activity. The volume of sample or alcohol (blank) was 0.4 mL mixed with 0.8 mL of 0.1 mM DPPH solution in alcohol solvent (Carmona-Jiménez et al., 2014). Concentrations of extraction in mixed solution were from 400 to 60 µg/mL (ppm). The positive control was aqueous ascorbic acid (vitamin C) diluted in alcohol with different concentrations. The negative control was alcohol. The reaction solutions were incubated at room temperature for 30 min before identifying the light absorbance at 517 nm wavelength. The tests were carried out in triplicate.
The free radical scavenging activity was calculated as following Eq(3):

$$\text{DPPH radical scavenging activity} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100\ (\%) \quad (3)$$

where $Abs_{control}$ is the absorbance of DPPH radical + alcohol; Abs_{sample} is the absorbance of DPPH radical + sample extract/standard.

2.7 Antibacterial activity

Pseudomonas aeruginosa strains were implanted onto agar surface. Holes with a diameter of 5.5 mm were created using sterile tool. A compound extract was ịnjected 70 µL into the holes created above. The control sample was DMSO. DMSO is used as a tool in in vivo studies of test compounds, and it is used as a flavonoid adsorption aid. The petri dish was incubated at 37 °C for 16 – 18 h before measuring the inhibition zone diameter (Sen and Batra, 2012).

3. Results and discussion

3.1 RSM results and optimization of hydro-alcoholic extraction by an ultrasound assisted leaching method

One-factor experiments were conducted to enhance hydro-alcoholic extraction by ultrasound-assisted leaching method. The local maximum point of the responses (TPC and TFC) was considered the center point (0), the upper and lower conditions of the center point were expressed +1, -1. The conditional range for solid and liquid ratios is [1/50 - 1/70], as shown in Figure 2. The concentration of alcohol is [30-70] vol% (Figure 3). At 0 vol% alcohol, water is used to extract using ultrasound. The vacuum rotational process of the extract solution in this case takes a longer time since the temperature was not higher than 60 °C.

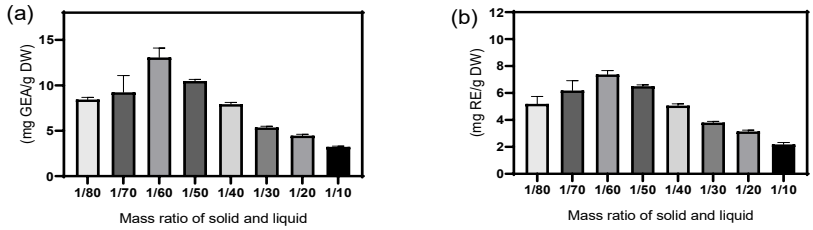

Figure 2: (a) TPC and (b) TFC of extracts with different mass ratios of solid and liquid

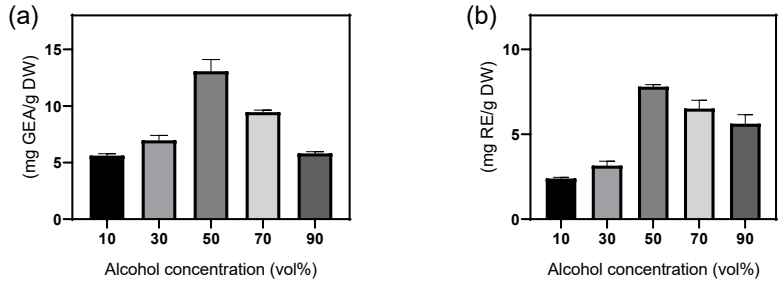

Figure 3: Alcohol concentration effect on (a) TPC and (b) TFC

Graph 3D TPC and TFC responses were presented in Figure 4. TPC and TFC values increased as alcohol levels increased, with TPC increasing from 6.27 to 12.97 mg GAE/g DW and TFC increasing from 1.47 to 7.81 mg RE/g DW. With solid and liquid ratios, the trend reactions to value changes increased the rate from 1/70 to 1/60, then declined somewhat in the region [1/60 - 1/50]. It could be explained that the decrease in viscosity of the medium enhanced the cavitation leading to an intensive sponge effect and erosion toward the material's surface. Further decreasing solid and liquid ratios, the TPC and TFC decreased probably due to the more substantial cavitation that caused the disintegration of these compounds (Kumar et al., 2021). When increasing alcohol concentration to 50 vol%, the polarity of systems could decrease improving the solubility of TPC and TFC. Further increasing alcohol, the protein in materials could be denatured that hindered the diffusion of alcohol solution into cell matrix, leading to a decrease in the alcohol's ability to wash out the compounds in the cells and reduce the extraction yield (Kumar et al., 2021). These results indicated that both solid and liquid ratios, as well as alcohol concentration, had significant effects on TPC and TFC. The correlation between the TPC (y_1) and TFC (y_2) responses values and the real value of the factors is represented by quadratic polynomials, expressed as Eq(4) and Eq(5):

$$y_1 = -88.25 + 9788.01x_1 + 0.37x_2 + 12.12x_1x_2 - 2.94.10^5x_1^2 - 0.01x_2^2 \text{ , } R^2 = 0.9812 \quad (4)$$

y_2 = -54.66 + 5456.38x_1 + 0.44x_2 + 21.63x_1x_2 – 1.82.10$^5x_1^2$ – 0.01x_2^2 , R^2 = 0.9780 (5)

The significant of the model was also analysed by ANOVA in Design Expert. With the result for the TPC model, there was a p-value of 0.0015 and the TFC had a p-value of 0.0021. The R^2 values of both models expressed in Eq(4) and Eq(5) with values greater than 0.95 indicate the reliability of the model and the experimental process equation (Le Tan et al., 2021). Besides, the p-value of the lack of fit of TPC was 0.7663 and that of TFC was 0.095. This indicated that it is negligible compared to the pure error.

Eq(4) and Eq(5) equations were used to optimize hydro-alcoholic extraction by an ultrasound-assisted leaching process. The appropriate value for the process to be carried out with a solid and liquid ratio is 1/55 with an alcohol concentration of 54.78 vol%, the predicted TPC value was as high as 12.45 mg GAE/g DW and the TFC was 7.728 mg RE/g DW. The margin of error is 0.955 from the actual result (TPC was 12.02 $\pm$ 0.24 mg GAE/g DW and TFC was 7.68 $\pm$ 0.34 mg RE/g DW).

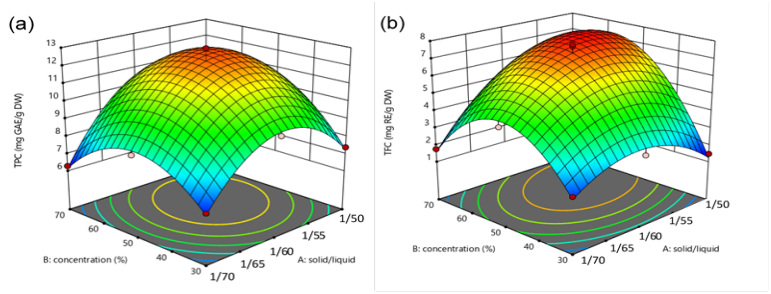

Figure 4: 3D graphs of the response surface of (a) total phenoic content (TPC) (b) total flavonoid content (TFC)

3.2 Antioxidant activity

Antioxidant activity is a beneficial feature of foods and medications that help resist and protect DNA and lipid oxidation in the human body. The DPPH free radical neutralization capacity of each extract was evaluated based on the IC_{50} value. The IC_{50} value is the extract concentration at which 50 % of DPPH free radicals are inhibited. The smaller the value, the stronger the antioxidant capacity. Figures 5a and 5b show that the IC_{50} value of the vitamin was 8.84 ppm (at a log(x) value of 0.947), and the *C. assamica* Benth seed extract value was 644 ppm (log(x) is 2.809). The above results show that *C. assamica* contains active ingredients that are less capable of neutralizing DPPH free radicals than vitamin C. Compared with some other plants such as *Solanum hainanense* Hance (IC_{50} value of 1734 ppm) and *Streptocaulon juventas* Merr (IC_{50} value of 2586 ppm) (Nguyen and Eun, 2011), *C. assamica* extract has much higher antioxidant activity, proved that *C. assamica* is a plant with effective antioxidant capacity.

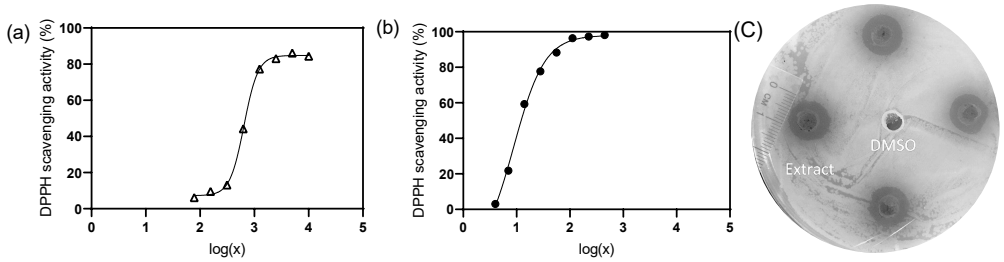

Figure 5: DPPH scavenging activity (%) of (a) extraction and (b) vitamin C according to the log concentration – log(x) and (c) antibacterial activity with P. aeruginosa

3.3 Antibacterial activity

P. aeruginosa strain is a Gram-negative bacteria that is hazardous to human health, causing pneumonia and sepsis syndromes via a hospital ventilator. Figure 5c shows *C. assamica*'s antibacterial activity against the *P. aeruginosa* strain of bacteria, with an average inhibition zone diameter of 13.5 mm. The diameter of the DMSO control sample was 5.5 mm. With antibacterial activity against *P. aeruginosa, C. assamica* also resistant to many other negative bacteria such as *Escherichia coli* and *Klebsilla pneumonieae*, as some reports have shown that compound extracts of the genus *Crotalaria* are strongly resistant to bacterial strains Gram-negative above. (Kiruthiga et al. 2014).

4. Conclusions

Crotalaria assamica Benth is originally used as a local drink and for medical purposes in folklore. In this study, the alcohol extracts of *Crotalaria assamica* Benth seeds were first time analysed and found that contains potentially rich medicinal medication with antibacterial and antioxidant components. With RSL of 1/55 and an alcohol concentration of 54.78 vol%, hydro-alcoholic extraction by an ultrasound-assisted leaching yielded an extract with significant TPC (12.24 mg GAE/g DW) and TFC content (7.728 mg RE/g DW). The extract's antibacterial and antioxidant characteristics were also proven when the IC_{50} value was 644 ppm in the DPPH scavenging activity experiment and antibacterial ability, with an antibacterial ring diameter of 13.5 mm with *P. aeruginosa* strain. These results were the initial stage in identifying a possible plant's pharmacological capabilities. More research was required to identify other compounds in the plant. Apart from the study's limitations, the study results help improve the usefulness and economic valuable of *Crotalaria assamica* Benth.

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