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OPTIMIZING CONDITIONS FOR SAPONIN ENZYME-ASSISTED EXTRACTION FROM *Ficus auriculata* FRUIT

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ABSTRACT

This study aimed to find the optimal conditions for enzyme-assisted extraction (EAE) of saponin from *Ficus auriculata*. The investigated parameters included enzyme concentrations (0.2; 0.4; 0.6; 0.8; 1%), pH (4; 4.5; 5; 5.5; 6), and temperatures (40; 50; 60; 70; 80 ^{o}C). The extraction efficiency was shown by the saponin content. Optimized extraction conditions such as enzyme concentration, pH, and temperature were obtained by the response surface method (RSM). The experimental results at optimal conditions were 0.65%; pH 5.55; at 49.78 ^{o}C and the highest saponin content was 38.23 ± 0.98 mg/g dry matter. The finding indicated that EAE was an effective method to extract saponin from *F. auriculata*.

Keywords: Cellulase, enzyme, *Ficus auriculata*, RSM, saponins.

1. INTRODUCTION

Nowadays, the use of pharmaceutical products of natural origin, especially precious plants, to nourish and support the body's healing is increasingly being noticed. According to many remedies in traditional folk medicine, figs have many precious substances, like ginseng and ginseng, to help restore and improve health. Some studies on figs (*Ficus auriculata*) show that it is distributed mainly in temperate and tropical regions at 1800-2600 m altitudes. The dried fruit contains alkaloids, saponins 0.59 (g/100g), while in the bark extract of *F. auriculata* there are alkaloids, carbohydrates, phytosterols, diterpenes, proteins, etc., especially containing many saponins, so that *F. auriculata* has many great uses such as anti-oxidant, antibacterial, hepatoprotective, anti-cancer, anti-inflammatory [1, 2] to make medicines or valuable medicinal herbs to help improve health.

Saponins are secondary compounds found in many plants. Chemically. It is a large group of glycosides from one or more sugar units bound to a triterpene or a glycone steroid. Research indicates that their structural variety correlates with distinct physicochemical and biological characteristics, including foaming ability, cholesterol-lowering effects, immune system support, and anti-cancer activities [3, 4].

There are many studies on the genus *Ficus*, mainly about biological and antibacterial activities, but researchers have targeted extracts less [5]. The research on extracting saponins from plants is mainly done with solvents. In addition, some studies have shown that using enzymes can also aid in extracting compounds inside cells. The research results of Truong Hoang Duy *et al*. (2005) showed that using enzymes to support saponins from ginseng was 1.50 times more effective than the control sample [6]. Effect of factors (enzyme concentration, pH, temperature) on cell wall hydrolysis when using cellulase enzyme. Therefore, cellulase

enzyme improved the extraction efficiency of compounds in cells. Thus, in this study, the factors influencing saponins extraction from *F. auriculata* were carried out with the support of cellulase enzymes, including raw materials/ solvent ratio, pH, and extraction temperature. This research serves as a premise to explore further the extraction and determination of biological compounds from *F. auriculata*.

2. MATERIALS AND METHODS

2.1. Materials

Ficus auriculata was obtained from Tan Phat Limited Liability Company, 174/33/11 Nguyen Tu Gian St., P12, Go Vap district, Ho Chi Minh City. The raw materials received at the centre would be treated with the removal of impurities, soil, dust, and classification. The bright green fruits were washed with tap water and distilled water. The material was dried at 60 ℃ until under 10% moisture content, and then it was milled with a milling machine to obtain power form and stored in a PE bag under vacuum for experiments.

Cellulase enzyme - cellulast 1,5L (Novozymes, Denmark) provided by Vietnamese Brenntag Limited Company No. 202B, Hoang Van Thu St., Phu Nhuan District, Ho Chi Minh City.

Standard substances oleanoic acid (USA), vanillin (99.5%-China), acetic acid (99.5%-China), perchloric acid (99.5%-India), ethylacetate (99.5%-China), Ethanol (99.5%-China), distilled water. Other chemicals and solvents met analytical standards.

2.2. Methods

2.2.1. Effects of enzyme on saponin extraction

1 g of raw materials (based on the dry matter), enzyme concentrations (0.2; 0.4; 0.6; 0.8; 1%) at pH (4; 4.5; 5; 5.5; 6) with a selected enzyme/substrate ratio (E/S) of 1/30 (w/v). Then, the sample was incubated at temperatures (40, 50, 60, 70, and 80 ℃) for 60 min. Next, the sample was filtered to collect the clear solution, and spectral analysis was performed on the Model photolab 6100 Vis instrument of WTW-Germany.

2.2.2. Optimization of enzyme-assisted extraction conditions by RSM

The RSM method was used to determine the influence of factors in enzyme-assisted extraction on saponin content. To optimize the conditions for the extraction of saponins, the CCD complex centre model was chosen with 3 factors the ratio E/S (X_1, w/v), pH (X_2), temperature (X_3, °C), and the response is saponin content (Y, mg/g dry matter). The CCD experimental design is shown in Table 1.

Table 1. Optimal investigated experimental levels

Parameters	Low level (-1)	Center Level (0)	High level (+1)
Enzyme concentration (%)	0.4	0.6	0.8
pH	5	5	6
Temperature (℃)	40	50	60

The experiments were carried out with 17 experiments (8 at the edge, 6 at the centre, and three on the swingarm). The linear regression equation with quadratic form was determined by JMP 10 software:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_{12} + b_{22}X_{22} + b_{33}X_{32} + b_{12}(X_1X_2) + b_{13}(X_1X_3) + b_{23}(X_2X_3)$$

Where: b_0, b_1, b_2, b_3, b_{11}, b_{22}, b_{33}, b_{12}, b_{13}, b_{23} were the coefficients of the variables X_1, X_2, X_3, X_{11}, X_{22}, X_{33}, X_1X_2, X_1X_3, X_2X_3 respectively.

2.2.3. Total saponin content determination

Establishing a calibration curve for oleanolic acid: 1000 ppm oleanolic acid solution was added in different volumes, then 0.3 mL vanillin-acetic acid (5%), 1 mL perchloric acid, incubated at room temperature for 20 min. The mixtures were then cooled under cool water for 2-3 min, then volume up to 10 mL with ethyl acetate. The absorbance was measured using a spectrophotometer (UV-Vis) at 550 nm. The blank was performed similarly, replacing the sample with distilled water.

The saponin content was calculated according to the following formula [8]:

$$Saponin\ (mg/g) = \frac{C \times n \times V \times 100}{M \times (100 - h)}$$

C: Concentration of total saponins in the extract in terms of oleanolic acid (mg/mL); V: Volume of extract (mL), M: mass of the drug (g); n: Dilution factor; h: moisture content (%).

2.2.4. Data analysis

The experiments were repeated 3 times. Using the software SPSS 22 to analyze variance ANOVA ($P \leq 0.05$) to evaluate the difference between treatments. Experimental design and optimized results processing using JMP 10 software.

3. RESULTS AND DISCUSSION

3.1. Effects of EAE on saponin extract

Cellulase enzyme helps to bind and break down cell walls. The higher the enzyme concentration, the greater the yield extraction. In this study, 5 enzyme concentration levels (0.2; 0.4; 0.6; 0.8; 1%) were investigated to find the appropriate enzyme concentration to receive the saponin content. The results are shown in Fig. 1.

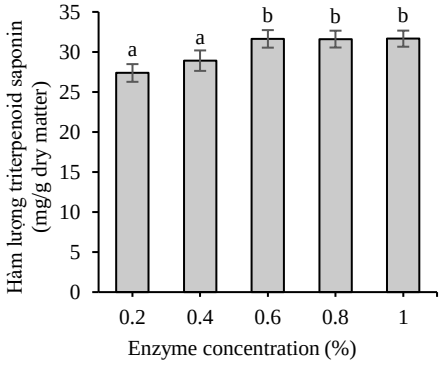

Fig.1. Effects of enzyme concentration on saponin extraction

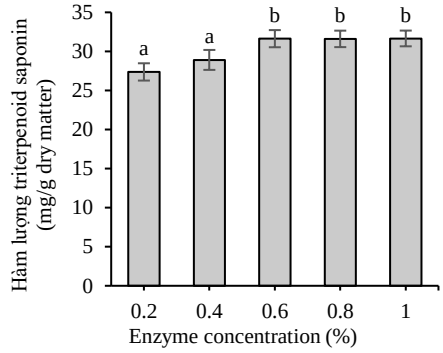

Fig. 2. Effects of pH on saponin extraction

(Note: [a–c] different letters on top of bars indicate significant differences, p < 0.05)

Fig. 1 indicated that the obtained saponin content was enzyme-dose-dependent. The saponin content increased from 27.37 ± 1.11 to 31.60 mg/g dry matter from enzyme 0.2% to 0.8%, an increase of 1.16 times. The extraction efficiency reached the maximum at enzyme 0.6%. The higher enzyme concentrations (0.8%, 1%) did not result in higher saponin contents

(31.60 ± 1.05 and 31.82 ± 1.06 mg/g, dry matter) with no significant differences according to ANOVA analysis. By the Michaelis-Menten equation, an increasing enzyme concentration would increase the efficiency of the enzyme reaction. Also, less enzyme concentration would not result in effective extraction because the substrate surpasses the enzyme, leading to a low reaction rate [9]. Therefore, an enzyme concentration of 0.6% was selected and fixed for the next survey steps.

Regarding pH, the effects of 5 pH levels (4.5; 5; 5.5; 6; 6.5) on saponin content were shown in Fig. 2. The saponin content increased sharply from 25.70 ± 1.02 mg/d dry matter to 31.96 ± 1.08 mg/g dry matter, an increase of 1.24 times and reached the highest value at pH 5.5 (31.96 ± 1.08 mg/g dry matter). The figure decreased to 30.29 ± 1.1 mg/g dry matter at pH 6 (Fig. 2). The ANOVA test showed no significant difference between the 3 pH levels of 5; 5.5 and 6. Each enzyme has an optimal pH range to work with the best performance. The different pH values from the optimal point would reduce the enzyme's catalytic ability and even denature the enzyme. So, the suitable pH range of the enzyme that works best for this experiment was 5.5.

The effects of 5 levels of temperature 40; 50; 60; 70 and 80 ℃ on saponin extraction were shown in Fig. 3.

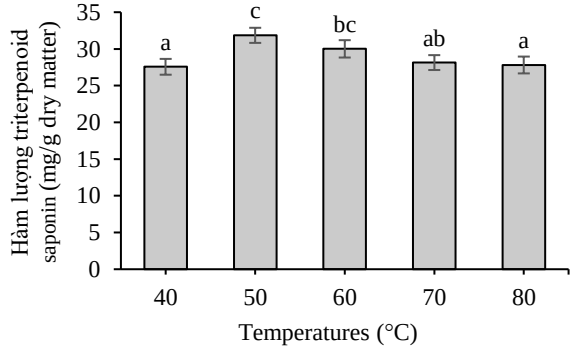

Fig. 3. Effects of temperatures on saponins extraction
(Note: $^{a-c}$ different letters on top of bars indicate significant differences, $p < 0.05$)

As results in Fig. 3, the saponin content increased from 27.57 ± 1.08 (40 ℃) to 31.85 ± 1.03 mg/g dry matter (50 ℃). Higher extraction temperature resulted in faster-moving components that enhance the solubility and diffusibility of saponins from the raw materials into the solvent. This also helps reduce the solvent's viscosity to penetrate easily into the material, making a larger surface contact area between the material and the solvent for increasing yield extraction [12]. However, the obtained saponin content decreased from 31.85 ± 1.03 to 27.81 ± 1.15 mg/g dry matter, accounting for 1.15 times at the temperature from 50 to 80 ℃. An increasing temperature accelerates the decomposition of compounds, but cellulase enzyme works best in the temperature range from 40 ℃ to 50 ℃. Thus, higher temperatures (50; 60 ℃) had no positive impact on saponin extraction, and the saponin content decreased at temperatures over 60 ℃.

3.2. Optimization of saponins extraction from *F. auriculata* by RSM

The purpose is to choose the optimal ranges of enzyme concentration (0.4; 0.6; 0.8%), pH (5; 5.5; 6), and temperature (40; 50; 60 ℃) to optimize. This is because after surveying the optimal region, it shows that after processing, there will be few optimal points, which makes the selection of the optimal point more evident than when choosing the optimal interval with

steps. Smaller or larger jumps lead to many optimal results, and it is difficult to determine the optimal condition.

The influence of a combination of factors (enzyme concentration, pH, temperature) on the extraction of saponins was carried out by the response surface method (RSM). The results of saponin content obtained at the experimental levels are presented in Table 2.

Table 2. Experimental planning matrix table and results

No.	Encoding variables			Real variables			Saponin content (mg/g dried matter)
	X_1	X_2	X_3	Enzyme concentration (%)	pH	Temperature (°C)	
1	-1	-1	-1	0.40	5.00	40.0	26.03 ± 1.044
2	-1	-1	1	0.40	5.00	60.0	25.37 ± 1.037
3	-1	1	-1	0.40	6.00	40.0	33.64 ± 1,125
4	-1	1	1	0.40	6.00	60.0	26.77 ± 1,076
5	1	-1	-1	0.80	5.00	40.0	29.00 ± 1,314
6	1	-1	1	0.80	5.00	60.0	31.47 ± 1,060
7	1	1	-1	0.80	6.00	40.0	34.68 ± 1,076
8	1	1	1	0.80	6.00	60.0	27.68 ± 1,097
9	-1.68	0	0	0.26	5.50	50.0	26.63 ± 1,072
10	1.68	0	0	0.94	5.50	50.0	31.82 ± 1,007
11	0	-1.68	0	0.60	4.66	50.0	29.76 ± 1,143
12	0	1.68	0	0.60	6.34	50.0	27.24 ± 1,295
13	0	0	-1.68	0.60	5.50	33.18	27.51 ± 1,076
14	0	0	1.68	0.60	5.50	66.82	28.89 ± 1,287
15	0	0	0	0.60	5.50	50.0	38.12 ± 1,033
16	0	0	0	0.60	5.50	50.0	38.88 ± 1,184
17	0	0	0	0.60	5.50	50.0	37.51 ± 1,119

Table 3. The results of the significance analysis of the coefficients of the regression equation

Regression coefficient	Coeff, SC	Std, Err,	P-value
b_0	38.09	1.28	<0.0001*
b_1	1.45	0.60	0.047*
b_2	0.49	0.60	0.445
b_3	-0.71	0.60	0.273
b_{12}	-0.89	0.78	0.294
b_{13}	0.37	0.78	0.648
b_{23}	-1.96	0.78	0.041*
b_{11}	-2.88	0.66	0.003
b_{22}	-3.14	0.66	0.002*
b_{33}	-3.25	0.66	0.002*

According to Joglekar and May, R^2 indicated a good fit of the model and should be at least between 0.80 and 0.97; In addition, the goodness of fit of the model was also evaluated by the F-value of lack of fit coefficient, P - value is used to test the significance level of each regression coefficient. Specifically, factors with a P-value ≤ 0.05 were considered to influence the objective function [14]. Among the 9 regression coefficients (except for b_o), 5 regression coefficients were not significant with the confidence P > 0.05, which were b_2, b_3, b_{12}, b_{13}, and b_{11}; this proved that the interaction between X_2, X_3, X_{12}, X_{13}, X_{11} has no significant effect on the response. The coefficient factors of b_{23}, b_{22}, and b_{33} were negative values on the response, reducing the obtained saponin content. Regression coefficient b_1 had the most significant and only positive value, showing that X_1 (enzyme concentrate) has a significant sizeable positive effect on the response.

Table 3 also showed that the obtained saponin content was impacted significantly by enzyme concentration (X_1) and treatment temperature (X_3) and less influenced considerably by pH (X_2). After analyzing ANOVA using SPSS software and setting up an experimental layout using JMP software, the following equation was given:

$$Y = 38.09 + 1.45X_1 - 1.96X_2X_3 - 3.14X_2^2 - 3.25X_3^2$$

The maximum saponin content was 38.32 mg/g dry matter at optimal extraction conditions: enzyme concentration 0.65%; pH 5.54; temperature 48.78 °C. The response surface model, showing the influence of the factors on the saponin content, and the predicted model are shown in Fig. 3 and Fig. 4. High enzyme content would break cell walls on the surface more effectively, creating conditions for better water penetration and making the solutes to escape easier. In addition, enzyme concentration had more impact on saponin extraction efficiency from *F. auriculata* than pH and temperature.

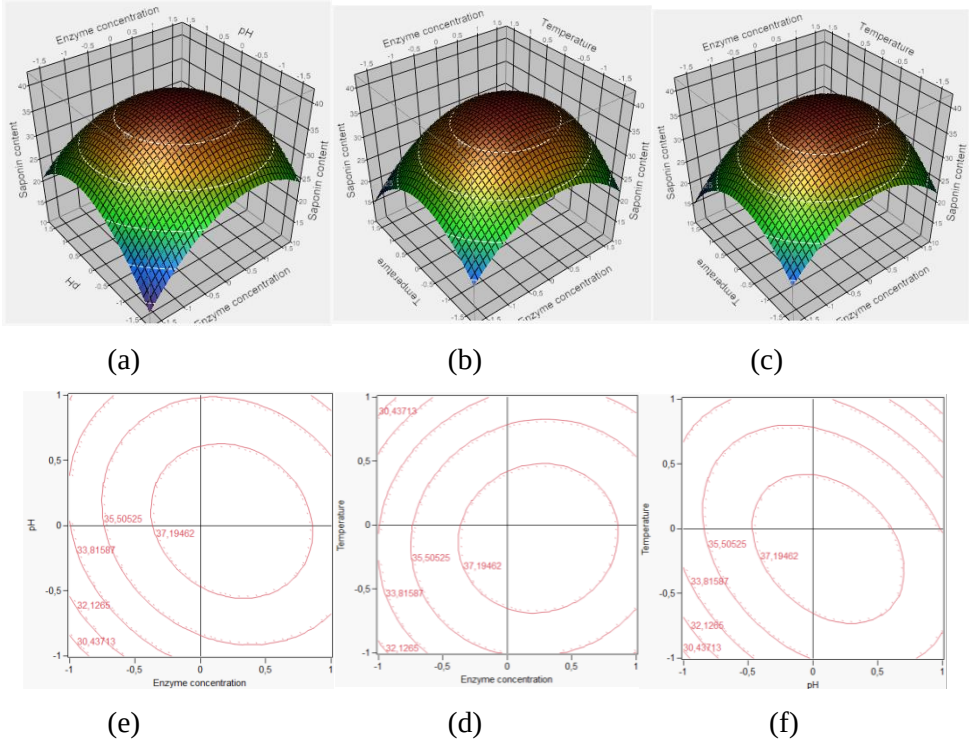

Fig. 3. The response surface (a, b, c) and contour (d, e, f) models represent the influence of 3 factors (enzyme concentration, X_1; enzyme pH, X_2; enzyme temperature, X_3) on saponin content; the effects between X_1 and X_2 (a, e); the impact between X_3 and X_1 (b, d); the effects between X_3 and X_2 (c, f)

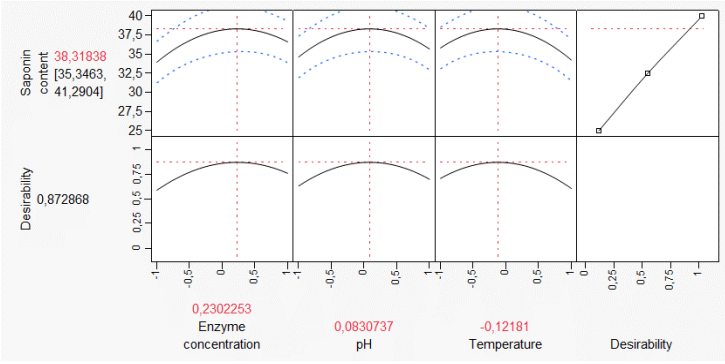

Fig. 4. Predictive model for saponin content

After obtaining optimal extraction conditions with a total saponin content equal to 38.32 mg/g CK at an enzyme concentration of 0.65%; pH 5.54, and temperature of 48.78 ºC, carrying out the verification experiment at optimal conditions, the total saponin content was 38.23 ± 0.98 mg/g CK, the difference was not more than 5% compared with the expected optimal result, demonstrating the optimal result was reliable superiority. Thus, the quadratic equation used is consistent with reality and has practical value.

4. CONCLUSION

This study indicated that *F. auriculata* contains a significant saponin content. The appropriate conditions to obtain saponin from *F. auriculata* with the support of enzyme at single-factor investigations were 0.6% enzyme cellulase, pH 5.5, and temperature of 50 °C. The optimal conditions from the RSM method of 0.65% enzyme, pH 5.54, 48.78 °C, and the obtained saponin content was 38.32 mg/g dry matter. These findings offer essential information for further studies on the isolation and bioactive properties of the obtained enriched-saponin extract in the food and pharmaceutical industries on a large scale.

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TÓM TẮT

TỐI ƯU HÓA ĐIỀU KIỆN TRÍCH LY THU NHẬN SAPONIN TỪ TRÁI VẢ (*Ficus auriculata*) CÓ SỰ HỖ TRỢ CỦA ENZYME CELLULASE

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Nghiên cứu này sử dụng enzyme cellulase nhằm hỗ trợ trích ly, khai thác hợp chất saponin từ trái vả *Ficus auriculata*. Các yếu tố nghiên cứu sàng lọc bao gồm nồng độ enzyme sử dụng (0,2; 0,4; 0,6; 0,8; 1%), pH (4; 4,5; 5; 5,5; 6), nhiệt độ (40; 50; 60; 70; 80 °C). Hiệu quả trích ly thể hiện qua hàm lượng saponin thu được đo bằng phương pháp quang phổ UV-Vis. Qua đó tiến hành tối ưu các điều kiện trích ly saponin như nồng độ enzyme, độ pH, nhiệt độ bằng phương pháp bề mặt đáp ứng (RSM). Kết quả thực nghiệm được tiến hành khảo sát gần với điều kiện tối ưu về nồng độ enzyme 0,65%; độ pH 5,55; nhiệt độ 49,78 °C thu được hàm lượng saponin cao nhất là 38,23 ± 0,98 mg/g chất khô.

Từ khóa: Cellulase, *Ficus auriculata*, mô hình bề mặt đáp ứng, trái vả, trích ly, saponin.