OPTIMIZATION OF ENZYME-ASSISTED EXTRACTION OF FLAVONOID FROM *Glinus oppositifolius*

Tran Thi Kim Nhan, Nguyen Thi Hai Hoa,
Hoang Thi Ngoc Nhon*
Ho Chi Minh City University of Food Industry
*Email: *nhonhtn@fst.edu.vn*
Received: 17 May 2022; Accepted: 15 June 2022

ABSTRACT

Glinus oppositifolius, a potential medicinal herb used in many countries around the world, contains lots of bioactive compounds. One of the essential ingredients was flavonoid, a group of natural compounds that have many beneficial effects on human health, such as antioxidant functions, antibacterial, anti-inflammatory, and anti-cancer. The independent variables, including enzyme concentration (10-50 UI/g), temperature (50-70 °C), and time (60-120 min), were investigated. The flavonoid extraction conditions were optimized with the CCD (Central Composite Design) design by response surface method (RSM). The results indicated that the optimal extraction conditions were found to be enzyme concentration (24.12 UI/g), temperature (68 °C), and time (99.8 min). Under such conditions, the highest content of flavonoid is 26.13 ± 0.05 mg/g of dry matter. These results suggest that enzyme treatment could help extract valuable components such as flavonoids that hold good potential for use in the food, cosmetic and pharmaceutical industries.

Keywords: Cellulase enzyme, extraction, flavonoids, *Glinus oppositifolius.*

1. INTRODUCTION

Glinus oppositifolius, an herbaceous plant with slender stem and branches, grows widely in Vietnam and tropical areas of Asia, Africa, and Australia [1]. It is distributed along with the coastal provinces, from the Hong River to the Mekong Delta in Vietnam. It is used as a vegetable and a precious medicine to treat some diseases. The extract has beneficial effects on digestion, aperitif, antibiotic, liver laxative, mouth sores, periodontitis, bleeding teeth, and diuretic [2]. Its extract has long been used as an antipyretic agent in traditional medicine for liver disease and jaundice. The active ingredients in this herbal medicine have been extracted and used in combination with other medicinal herbs to make soft capsules or tablets for modern medicine. It is known that *G. oppositifolius* has a prosperous chemical composition (alkaloids, saponins, steroids, anthocyanins, etc.) and especially contains large amounts of flavonoids with many important biological activities.

Flavonoid, a natural yellow pigment synthesized from phenylalanine [3], is a natural compound found in plants. More than 6000 flavonoids have been founded in vegetables, seeds, and fruits [4]. They reveal multiple positive effects because of their antioxidant and free radical scavenging action. So, it is beneficial for human health. This compound also has anti-inflammatory effects, antiviral or anti-allergic, and a protective role against cardiovascular disease, cancer, and various pathologies [5].

Figure 1. Glinus oppositifolius

In recent years, enzyme techniques have been increasingly interesting in studies on extracting bioactive compounds from plants. Enzyme-assisted extraction offers a safe, green, and novel approach to extracting bioactive compounds. This technique is also safe for targeted substances and users in both laboratory and industrial conditions [6]. However, their recovery from the plant matrix is generally limited by the presence of a physical barrier (cell wall). Thus, the use of novel extraction procedures to enhance their release is essential. Thus, the enzyme-assisted extraction method seems suitable for obtaining and applying bioactive substances such as flavonoids from plants such as *G. oppositifolius.* Therefore, this work aims to assess the potential use of cellulase to improve the extraction efficiency of bioactive compounds from *G. oppositifolius*, and to find out and optimize the flavonoid extraction conditions from the material to offer a foundation for further studies on applying this compound in practice.

2. MATERIALS AND METHODS

2.1. Materials

Fresh *G. oppositifolius* in green was collected in Chau Phu district, An Giang province, in July 2021. After being harvested, *G. oppositifolius* would be cleaned by washing to remove impurities. The leaves were dried at 60 ºC until under 10% moisture. The fine powder was obtained by grinding by a mechanical grinder (less than 80 mesh size) and stored in PE bags, protected from light and powder for the experiments.

Chemicals such as sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium hydroxide (NaOH), and methanol 99.5% were procured from Fisher Scientific (USA). Quercetin was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), and cellulase (10000U/g) from Antozyme Biotech Pvt.Ltd (India).

2.2. Methods

2.2.1. Effects of enzyme-assisted extraction

1g of raw materials (calculated by dry matter-dm), adding water as a solvent with the ratio of material/solvent 1/30 (w/v). The extraction process was conducted with the support of cellulase at the pH range investigated (3, 4, 5, 6, 7), and the concentrations of the studied enzyme (10, 20, 30, 40, 50 UI/g) at the temperature (40, 50, 60, 70, 80 ºC) in the period of (30, 60, 90, 120, 150 minutes). Then, the mixture was centrifuged at 5500 rpm/5 min. After centrifugation, the solution was filtered through Whatman No.1 filter (China) to collect the filtrate. Then, the total flavonoid content (TFC) content was determined by UV-Vis

spectrophotometer (Genesys 10s thermo, Made in the USA) to select the appropriate conditions for the flavonoid extraction.

2.2.2. Experimental design

RSM is a proper statistical and mathematical technique to evaluate multiple independent variables on the dependent variable and thus estimate the maximum yield of the process under a specific limited condition. The central composite design (CCD) is a common method to design experiments for building a quadratic model in RSM with response variables. CCD contains an embedded or fractional factorial design with a center point augmented with a group of new extreme values (low and high) for each factor in the design to allow curvature estimation, and the experimental matrix was built using JMP 10 software. Three independent variables include enzyme concentration (X_1), temperature (X_2), and time (X_3). The marginal values and experimental design with independent variables, their ranges, and 20 experiments (6 experiments at the central point) were carried out randomly to optimize the extraction process.

2.2.3. Total flavonoids content determination

Total flavonoid content was measured by the aluminum chloride colorimetric assay (Zhishen et al. 1999) using quercetin as a standard flavonoid. 1 mL of the extract was added to 4 mL of distilled water, and 0.3 mL of 5% $NaNO_2$, and the mixture was incubated at room temperature for 5 min. After incubation, the mixture was treated with 0.3 mL 10% $AlCl_3$ solution. After 1 min, 2 mL of 1 M NaOH was added, and 2.4 mL distilled water was added to the solution. The solution was mixed well, and the absorbance was measured at 415 nm against blank. The assay was performed based on the 6-point standard calibration curve of quercetin. The TFC was expressed as quercetin equivalents (QE) in milligrams per gram of dry material [7].

2.2.4. Experimental design and statistical analysis

The experiments were repeated three times. The results were presented as mean ± SD. Using IBM SPSS Statistics 20.0 software to analyze experimental data and evaluate the difference between samples ($p < 0.05$). JMP 10 software was used to analyze data in experimental optimization. The graph was drawn by Microsoft Excel 2016.

3. RESULTS AND DISCUSSION

3.1. Effects of enzyme and enzyme concentration on the flavonoids recovery yield

The effects of cellulase on TFC are shown in Table 1. There is a significant difference between the samples treated with cellulase (19.93 mgQE/gdm) and the control (12.24 mgQE/gdm). Thus, the cellulase positively supported the extraction efficiency of flavonoids from *G. oppositifolius*. The extraction process was carried out with water as a solvent, ratio 1/30 (g/mL), pH 5 at 60 in 60 min.

Table 1. Effects of cellulase on TFC

Samples	Flavonoid content (mgQE/gdm)
Control	12.24 ± 0.65^a
Cellulase	19.93 ± 1.20^b

The enzyme concentration also significantly affected the obtained flavonoid content. According to Puri *et al*., the enzyme disrupted the cell wall and membrane to release bioactive components into the solvent with high-yield recovery during enzyme-assisted extraction [8]. Plant cell walls are complex and heterogeneous, mainly composed of cellulose, hemicellulose, and lignin. These components were considered barriers, hindering some compounds' extraction [9]. Enzymes cause break plant cells to be fully exposed to the solvent and hydrolyze polysaccharides and lipids, promoting the release of intracellular components [10]. From Figure 2, the obtained flavonoid concentration gradually increased with the increase of enzyme concentration and reached 23.70 mgQE/gdm at 20 UI/g. Then, the flavonoid concentration decreased from 30 UI/g to 50 UI/g (11.34 mgQE/gdm). The effectivity of enzyme-assisted extraction was affected by its concentration and substrate concentration. While low enzyme concentrations resulted in a slow reaction rate and incomplete process, the high enzyme concentration caused fast and thorough speed until a certain percentage of enzymes. Thus, too much enzyme was unchanged in extracted targeted components and wasteful of the extraction process. With the appropriate enzyme concentration, an enzyme-assisted extraction method was an excellent approach to enhancing extraction efficiency [11].

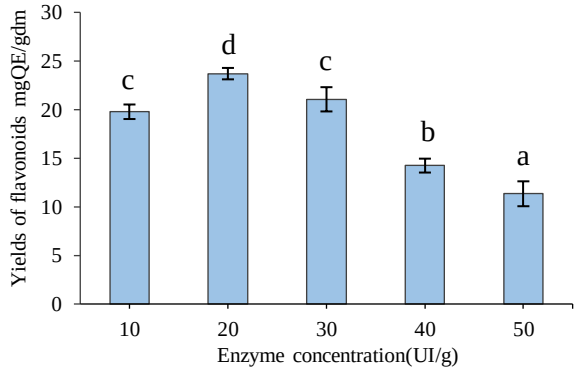

Figure 2. Effects of enzyme concentration on TFC
Note: Different letters a, b, c, and d in the same column represent statistically significant differences at p <0.05. This annotation applies to all charts.

3.2. Effects of times and temperatures on the flavonoids recovery yield

The TFC increased from 10.19 mgQE/gdm to 24.60 mgQE/gdm after an extraction time of 30 to 90 minutes (Figure 3). However, total TFC recovery tended to decrease to 23.52 mgQE/gdm up to 120 minutes. The extraction process was carried out with water as a solvent, the ratio of 1/30 (g/mL), the concentrations of the studied enzyme 20 UI/g (Fig.2), and pH 5 at 60 in 60 min.

A suitable period is essential for hydrolysis to occur entirely and thoroughly in the extraction stage. Long incubation time causes extract loss, the substrate is gradually decomposed, and produced substances during hydrolysis inhibit enzyme activity [12]. In addition, prolonged time will dissolve unwanted substances, affecting the extraction process [13]. On the other hand, the short incubation time is not enough for a thorough reaction, resulting in a low yield. In this study, 90 minutes of extraction was selected for further experiments. The result was in line with Nguyen Nhat Minh Phuong *et al.* [14]. The obtained TFC content peaked at 25.44 mgQE/gdm at 60 °C, but that figure did not increase at the higher temperature (Figure 4). Temperature reduces solvent viscosity and increases mass transfer and solvent penetration into cells. Thus, bioactive compounds are easily dissolved and diffused into the solvent. However, too high or too low temperature does not affect it well. For instance, an enzyme is a biological molecule with the nature of a protein, so it is quickly impacted by heat, especially at high temperatures. It would cut off the hydrogen bonds between the water surface and proteins and

amino acids [15]. On the other hand, the enzyme's active center would not be able to work well to break the cellulose chain in the plant cells at a low temperature. Therefore, the appropriate temperature for cellulase in this study was 60 °C.

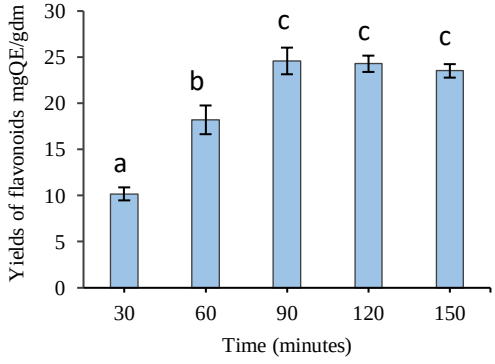

Figure 3. Effects of time extraction on TFC

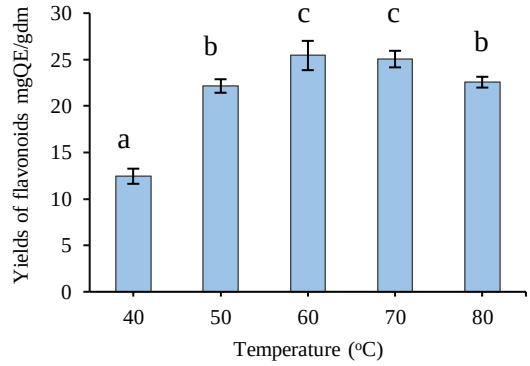

Figure 4. Effects of temperature extraction on TFC

3.3. Effects of different pH on extraction recovery yield of TFC

The effects of pH on flavonoid extraction from *G. oppositifolius* are shown in Figure 5. The shape of an enzyme would be changed in a too acidic or too alkaline medium, which impacted the extraction efficiency [16]. The TFC content increased to 23.80 mgQE/gdm at pH 5. This figure continued to rise at pH 6, but there are no significant differences from that at pH 5. The results were consistent with the study of Pan *et al.* (2014) [17]. Therefore, pH 5 is considered a suitable condition for the following experiments. Each enzyme has its own optimal active pH range; changing the pH value from the optimal pH point reduces the enzyme's ability to work and even denatures it. This result is similar to the study of Yan et al. (2012) [18], investigating the effect of pH on the activity of cellulase enzyme-produced strains of the fungus *Trichoderma reesei;* pH 5 is the optimal pH for the best cellulose hydrolysis for this enzyme.

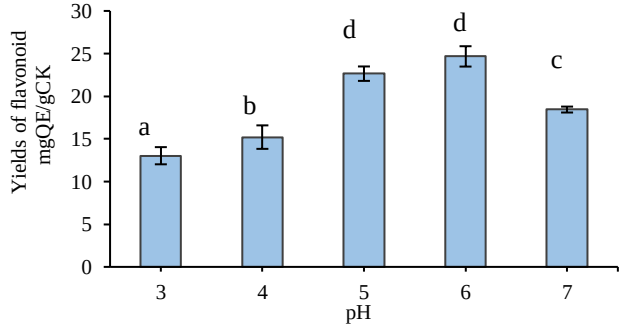

Figure 5. Effects of pH on recovery yield of TFC

3.4. The optimization of enzyme-assisted extraction of TFC

According to the CCD complex model, the total flavonoid content obtained from different optimal conditions is presented in the modeling table.

Based on suitable investigated conditions in the above single-factor experiments, the parameters such as enzyme concentration, temperature, and extraction time, were selected for the optimal study of extraction conditions to obtain the highest TFC content. The appropriate

ranges of these factors are presented in Table 2. The optimal experiment was designed in CCD style by the RSM method.

Table 2. Response surface central composite design and experimental flavonoids yield

No.	Independent variables			
	Concentration (UI/g)	Temperature (°C)	Time (min)	Y (Yield of flavonoids, mgQE/gdm)
1	10	40	60	18.96
2	30	40	60	18.83
3	10	60	60	17.74
4	30	60	60	22.51
5	10	40	120	22.98
6	30	40	120	19.72
7	10	60	120	21.89
8	30	60	120	22.79
9	3.18	50	90	17.19
10	36.82	50	90	22.75
11	20	33.20	90	18.35
12	20	66.80	90	22.73
13	20	50	39.54	20.53
14	20	50	140.46	22.71
15	20	50	90	24.42
16	20	50	90	25.04
17	20	50	90	26.35
18	20	50	90	24.93
19	20	50	90	24.95
20	20	50	90	24.43

The factors with $p < 0.05$ were considered to influence the objective function, and the influencing factors with regression coefficients were determined by the multivariable regression method, obtained as follows:

$$Y = 25.16 + 1.09X_1 + 1.15X_2 + 1.62X_3 + 0.76X_2X_3 - 1.13X_1^2 - 1.17X_2^2 - 1.11X_3^2$$

After conducting ANOVA analysis using JMP software, the following results were obtained: TFC obtained was 26.63 mgQE/gdm at optimal conditions with enzyme concentration (24.12 UI/g), temperature (68 °C), and time (99.8 minutes). The response surface model showed the influence of the investigated factors on the obtained total flavonoid content in the extract (Figure 6). The relationship between the repeat factors and flavonoids, while contour lines help visualize the shape of the response surface. Therefore, relying on surfaces helps assess the fit of the model [19].

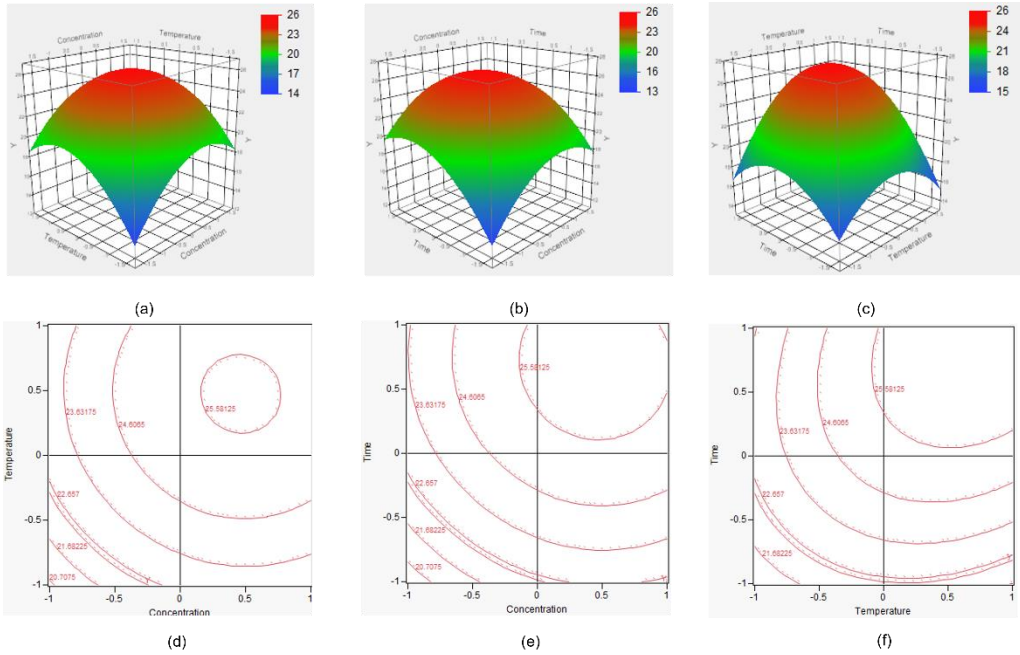

Figure 6. Response surface 3D (a, b, c) and 2D contour (d, e, f) plots showing the effect of different extraction parameters (X_1: concentration, UI/g; X_2: temperature, ^oC and X_3 time, min) added on the response Y.

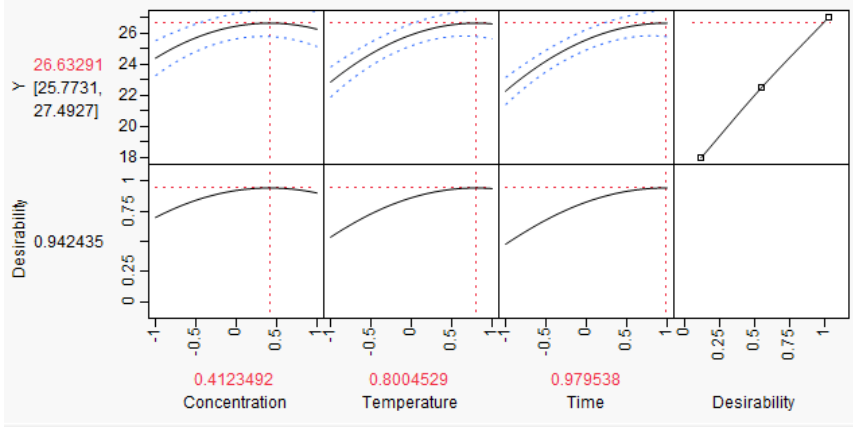

Figure 7. The predictive model of TFC extraction

For verification of the obtained parameters, experiments under optimized conditions were carried out (replicated three times). The obtained TFC of 26.13 mgQE/gdm, compared with the predicted TFC of 26.63 mgQE/gdm from the regression equation, accounting for 2.94% (<5%) in the difference. It showed that the obtained TFC content was completely consistent with the values predicted by the quadratic regression model. Thus, the quadratic equation to predict the TFC from *G. oppositifolius* under optimal conditions has practical value.

4. CONCLUSION

This study found the optimal conditions for flavonoid enzyme-assisted extraction from *G. oppositifolius* by cellulase enzyme. The RSM was used to find optimized conditions for flavonoid extraction, resulting in the optimal parameters of enzyme concentration (24.12

UI/g), temperature (68 ^{o}C), and time (99.8 min). At optimal conditions, the TFC was maximized at 26.13 ± 0.05 mg/gdm. The results showed that *G. oppositifolius* extract contained a significant amount of flavonoids. The obtained results are mainly to find the optimal conditions for flavonoid extraction by cellulase enzyme to the maximum TFC content. More studies need to be conducted to obtain comprehensive characteristics of flavonoids from *G. oppositifolius* to apply to functional foods and pharmaceuticals.

REFERENCES

1. Ridgway R. and Rowson J. - *Glinus oppositifolius* L. root - a substitute for senega, Journal of Pharmacy Pharmacology **8** (1) (1956) 915-926.
2. Mall T.P. and Tripathi S.C. - Exploitable vegetables for Food and health in Bahraich (UP) India, Agricultural Science Research Journal **6** (10) (2016) 241-246.
3. Harborne J.B. and Turner B.I. - Plant Chemosystematics, Academic Press, London, 1984.
4. Dixon R.A. and Pasinetti G.M. - Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience, Plant Physiology **154** (2) (2010) 453-457.
5. Chávez-González M.L., Sepúlveda L., Verma D.K., Luna-García H.A., Rodríguez-Durán L.V., Ilina A., and Aguilar C.N. - Conventional and emerging extraction processes of flavonoids, Processes **8** (4) (2020) 434.
6. Vergara-Barberán M., Lerma-García M., Herrero-Martínez J., and Simó-Alfonso E. - Use of an enzyme-assisted method to improve protein extraction from olive leaves, Food Chemistry **169** (2015) 28-33.
7. Simlai A., Chatterjee K., and Roy A. - A comparative study on antioxidant potentials of some leafy vegetables consumed widely in India, Journal of Food Biochemistry **38** (3) (2014) 365-373.
8. Puri M., Sharma D., and Barrow C.J. - Enzyme-assisted extraction of bioactives from plants, Trends in Biotechnology **30** (1) (2012) 37-44.
9. Khalil H., Lai T., Tye Y., Rizal S., Chong E., Yap S., Hamzah A., Fazita M., and Paridah M. - A review of extractions of seaweed hydrocolloids: Properties and applications, Express Polymer Letters **12** (4) (2018).
10. Nadar S.S., Rao P., and Rathod V.K. - Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review, Food Research International **108** (2018) 309-330.
11. Panja P. - Green extraction methods of food polyphenols from vegetable materials, Current Opinion in Food Science **23** (2018) 173-182.
12. Duong Thi Ngoc Hanh and Nguyen Minh Thuy - The use of α-amylase enzyme in starch hydrolysis from Red rice, Scientific Journal of Can Tho University (2014) 61-67.
13. Hahn T., Lang S., Ulber R., and Muffler K. - Novel procedures for the extraction of fucoidan from brown algae, Process Biochemistry **47** (12) (2012) 1691-1698.
14. Phuong Nguyen Minh Nhat, Hoang Che Van, Binh Ly Nguyen, and Ai Chau Tran Diem - Effect of pectinase enzyme treament to juice yield and fermentation conditions to the quality of mango wine (*Mangifera indica*), Can Tho University Journal of Science **20** (2011) 127-136.
15. Al-Farsi M.A. and Lee C.Y. - Optimization of phenolics and dietary fibre extraction from date seeds, Food Chemistry **108** (3) (2008) 977-985.

16. Nguyen Thi Ngoc Thuy, Nguyen Thi Thu Huyen, Truong Quang Duy, Phan Huynh Thuy Nga, and Tu Cao Thi Cam - Effect of solvent and pH value on extract of antioxidant activity compounds from perilla (*Perilla frutescens*) Journal of Science Techology and Food **14** (1) (2018) 66-74.
17. Pan S. and Wu S. - Cellulase-assisted extraction and antioxidant activity of the polysaccharides from garlic, Carbohydrate polymers **111** (2014) 606-609.
18. Zhong-Li Y., Xiao-Hong C., Qing-Dai L., Zhi-Yan Y., Yu-Ou T., and Juan Z. - A shortcut to the optimization of cellulase production using the mutant *Trichoderma reesei* YC-108, Indian Journal of Microbiology **52** (4) (2012) 670-675.
19. Baş D. and Boyacı I.H. - Modeling and optimization I: Usability of response surface methodology, Journal of Food Engineering **78** (3) (2007) 836-845.

TÓM TẮT

NGHIÊN CỨU TỐI ƯU HÓA CÁC ĐIỀU KIỆN TRÍCH LY FLAVONOIDS TỪ *Glinus oppositifolius* VỚI SỰ HỖ TRỢ CỦA ENZYME

Trần Thị Kim Nhân, Nguyễn Thị Hải Hòa, Hoàng Thị Ngọc Nhơn*
Trường Đại học Công nghiệp Thực phẩm TP.HCM
*Email: *nhonhtn@fst.edu.vn*

Rau đắng đất (*Glinus oppositifolius*) là một loại cây dược liệu tiềm năng được sử dụng phổ biến ở nhiều nước trên thế giới. Thành phần trong rau đắng đất chứa nhiều hợp chất hữu cơ mang hoạt tính sinh học, trong đó có flavonoid - nhóm hợp chất tự nhiên có nhiều tác dụng tốt cho sức khỏe con người, trong đó nổi bật nhất là các chức năng chống oxy hóa, kháng khuẩn, chống viêm nhiễm và ức chế tăng sinh của các tế bào ung thư. Để thu hồi được một lượng các hợp chất flavonoid ở mức cao nhất từ cây rau đắng đất flavonoids, nghiên cứu đã tiến hành trích ly kết hợp với sự hỗ trợ của enzyme cellulase trong quá trình trích ly flavonoids từ rau đắng đất và tối ưu hóa. Các thông số được khảo sát bao gồm: nồng độ enzyme (10-50 UI/g), nhiệt độ (50-70 ^{o}C), thời gian (60-120 phút). Điều kiện tối ưu trích ly flavonoids được thiết kế kiểu CCD (Central Composite Design) bằng phương pháp bề mặt đáp ứng (RSM), sử dụng phần mềm JMP 10. Kết quả nghiên cứu đã xác định được nồng độ enzyme, cùng nhiệt độ và thời gian trích ly tương ứng là: 24,12 UI/g, (68 °C) và 99,8 phút. Trong điều kiện tối ưu như thế có thể thu được 26,13 ± 0,05 mg/gck là điều kiện tối ưu để trích ly được hàm flavonoid cao nhất.

Từ khóa: Enzyme cellulase, flavonoid, *Glinus oppositifolius,* rau đắng đất, trích ly.