OPTIMIZING THE EXTRACTION CONDITIONS AND ANTIBACTERIAL ACTIVITIES OF SAPONIN FROM *Celastrus hindsii* Benth.

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SUMMARY

Saponins are natural compounds in plants which have been shown strong effects on health such as immune system stimulation, anti-cancer, analgesic, anti-vomiting, antioxidant, hypoglycemia, anti-fungal, bacteria and virus resistance. *Celastrus hindsii* Benth. is a medicinal plant species containing various bioactive compounds like flavonoids, saponins, tritrepenodid, diphenylpropanes, quinones that have been used as anticancer, anti-oxidant, antibacterial and anti-allergic drugs. The aim of this study is to optimize the extraction conditions of Saponin in *Celastrus hindsii* Benth. and identify its antibacterial activities. The results from experiments indicated that at the same conditions, the immersion method showed higher yeild of saponin content recovery (23.7 mg/g) than the ultrasound method (18.6 mg/g). Besides, the optimal parameters for the highest amount of saponins recovery by immersion method were also analyzed via surveying influenced factors and optimization by the Box-Behnken method. Accordingly, the optimal conditions for saponin extraction were identified: ethanol 74%, 3.32 hours, 80^{o}C, the ratio of solvent per gram of material 1:16.5. The antibacterial activites of saponin extracted from *Celastrus hindsii* Benth. against some test bacteria like *P. aeruginosa* HS, *S. areus* VS1, *E. coli* CA were also recored with the strong resistance at high inhibition zones from 1.6 to 2.2 cm. Thus, saponin extracted from *Celastrus hindsii* Benth. in the above conditions can be used to produce protect and care products for human health.

Keywords: Antibacterial, *Celastrus hindsii*, extract, Saponin.

1. INTRODUCTION

Saponins are steroid glycosides or triterpenoids, common in different plant species. Saponins have been demonstrated to have many effects on human health such as immune system stimulation, anti-cancer, pain relief, blood sugar reducing, anti-vomiting, antioxidant, anti-microoganisms (Aziz et al., 2019; Sapna et al., 2009).

Celastrus hindsii Benth. is a shrubs species growing wildly or planted in the provinces of Son La, Hoa Binh, Quang Ninh, Quang Binh in Vietnam (Trinh Thi Thuy et al., 2007). *Celastrus hindsii* Benth. has been considered as valuable medicinal ingredients containing flavonoids, saponins, triterpenoids, diphenylpropanes, quinone (Hu et al., 2013; Huang et al., 2000; Trinh Thi Thuy et al., 2007). The extracts from *Celastrus hindsii* Benth. have been studied to have strong cytotoxic activites against liver cancer, cervical cancer, colon carcinoma and nasal carcinoma cells as well as HIV replication prevention in cells (Jo et al., 2018; Kuo et al., 1997; Hu et al., 2013). Therefore, *Celastrus hindsii* Benth. extracts have been used as an anti-cancer agent in 108 Military Hospital (Le The Trung, 1999). Additionally, a number of other biological activities such as antioxidant, antibacterial, anti-allergic of *Celastrus hindsii* Benth. leaves extracts have been published recently (Ly Ngoc Tram, 2016).

In the medical and pharmaceutical industry, saponins are extracted from a variety of natural medicinal plants such as: *Celastrus hindsii* Benth., *Crinum latifolium*, *Ginseng*, *Codonopsis pilosula* by methods of immersion in hot and cold oganic solvent (Kuljanabhagavad et al., 2009; Semmar et al., 2010), microwave (Kwon et al., 2003; Vongsangnak et al., 2004), ultrasound (Wu et al., 2001; Navarro et al., 2018), supercritical fluid (Majinda, 2012). The purpose of this study is to identify the optimal extraction conditions of saponins from *Celastrus hindsii* Benth. leaves and determine the antibacterial activities of the extracts.

2. RESEARCH METHODOLOGY

2.1. Research materials

Celastrus hindsii Benth. leaves that were collected in Hoa Binh (July 2017 - August 2018), was morphologically determined by Vu

Quang Nam (Vietnam National University of Forestry). Leaves were washed, dried at low temperature (45^{o}C) to reach 8% moisture then grinded into powder and used as raw materials (Figure 1).

Pathogens strains: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* provided by Forestry Biotechnology Institute were used to measure the antibacterial activities of the extracts.

Figure 1. *Celastrus hindsii* Benth. leaves in fresh form (A) and dry powder form (B)

2.2. Research methods

2.2.1. Methods for saponin separation from Celastrus hindsii Benth. leaves

Saponins separated from C*elastrus hindsii* Benth. leaves were performed by method described previously by Cheng et al. (2017), Trinh Thi Thu Thuy et al. (2007). *Celastrus hindsii* leave powder is soaked in solvent or under the ultrasonic sound (the level of 40%, ultrasonic device Bandelin Sonopuls) in ethanol at concentrations of 50%, 60%, 70%, 80%, 90%, 100% and the ratio material per solvent of 1:5, 1:10, 1:15, 1:20, 1:25, 1:30 (g/ml) at temperature changing from 70 - 100^{o}C for 20 - 60 minutes (ultrasound method) and 1 - 5 hours (immersion method). The supernatant then was vacuumed at 50°C to become ethanol colloidal form (ethanol extracts). 100 g of ethanol extracts were dissolved into 500 ml of distilled water and continued extracting with butanol (3 times, 500 ml each time). This was vacuumed to remove butanol and collect total saponin.

2.2.2. Optimization of the saponins extraction conditions

In oder to optimize saponin content from *Celastrus hindsii* Benth. leaves, the software Design-Expert 7.1.5 (State-Ease, Inc., Minneapolis, USA) with Box-Benken planning (Liu et al., 2016; Kwon et al., 2003) was applied to analyze the effects of single and multiple factors on the objective function. The experiment matrix was establised consisting of 17 experiments with the running ranges of 3 surveyed elements. These elements were ethanol concentration (from 60% to 80%), the ratio of materials per solvent (from 1:10 g/ml to 1:20 g/ml) and immersion time (from 2 to 4 hours).

2.2.3. Methods of determining the antibacterial ability of saponins from Celastrus hindsii Benth. leaves

The antibacterial capacity of saponins extracted from *Celastrus hindsii* Benth. leaves was determined based on the inhibition zone diameter on agar medium (as described by Duong Thi Ly Huong et al., 2016). Accordingly, the test bacteria strains with the density of 10^{6} CFU/ml of each were supplemented and mixed well into agar nutrient medium at the rate of 1% innoculum. The plates containing both nutrient medium and test bacteria with wells (7 mm in diameter) were prepared. A volume 0.2 ml of saponins (at the concentration of 0.5 mg/ml) extracted from *Celastrus hindsii* Benth. leaves were added into wells and control well was filled with sterile water. The plates were placed in incubator at 28 - 30^{o}C for 24 hours. The antibacterial activity of saponin extracts was assessed based on diameter of inhibition zone around the agar wells. The activity was assessed as antibacterial properties when the diameter of inhibition zone was more than 15 mm.

2.2.4. Methods for determining total saponin contents

Total saponin content in saponin extracts from *Celastrus hindsii* Benth. leaves was determined by photometric method with diosgenin standard and vanillin reagent (Liu et al., 2016; Do Thi Ha et al., 2018; Nguyen Van Ban et al., 2018). Accordingly, the total saponin content is calculated by the linear equation between standard concentration and optical density at wavelength OD_{550} (y = ax + b) with the following formula:

Saponin content $S = \frac{y-b}{a} \times F$

F is the dilution factor, y is the OD index measured at 550 nm wavelength, x is the saponin concentration (mg/g).

2.2.5. ***Methods of data collection and processing***

The experiment was arranged with three iterations and the data was collected and processed by Excels, Design-Expert 7.1.5.

3. RESULTS AND DISCUSSION

3.1. Results of studying the conditions of saponin extraction from *Celastrus hindsii Benth. leaves*

3.1.1. The effects of ethanol concentration on saponin extracts

The composition of extraction solvent greatly affects the extraction efficiency of plant compounds (Ly Ngoc Tram, 2016). Variety of organic solvents have been used such as: methanol, ethanol, water... However, when saponin has been extracted by methanol that can lead to formation of methyl derivatives, this was not found in the plant (Oleszek and Bialy, 2006). Majinda (2012) suggested that the ethanol-water mixture is one of the effective solvents to extract saponins. Moreover, the use of nontoxic and safe as well as environmentally frendly is always encouraged in extracting natural compounds (Cheng et al., 2017). Therefore, the experiments were carried out with ethanol solvent at concentration from 50 to 100%. The results in figure 2 showed that the saponin content obtained was the lowest (3.96 mg/g) when the concentration of ethanol is 50%. When the concentration of ethanol increases, the saponin content also increases and at the 70% concentration of ethanol the saponin content is the highest (reached 12.58 mg/g). Otherwise, the concentration of ethanol is more than 70%, the content of saponin decreases gradually (6.22 - 10.52 mg/g). 70% ethanol is the most suitable concentration for extraction of *saponins from Celastrus hindsii* Benth.. Cheng et al. (2017) also reported that the highest content of saponin was obtained at 70% ethanol from *Cicer arietinum L.*. Zhao et al. (2012) studied the use of 10 - 90% ethanol for extracting *Codonopsis lanceolata* saponins and found that the concentration of 70% ethanol was suitable for obtaining the highest saponin content.

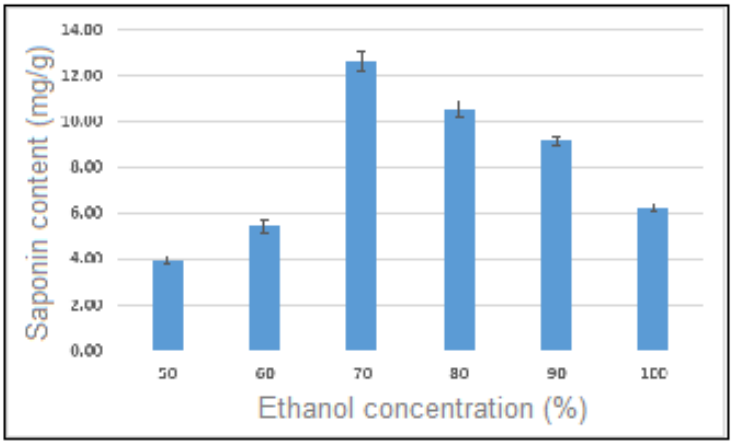

Figure 2. Effect of ethanol concentration on saponin content

3.1.2. Effect of the raw material per solvent ratio

On the same volume of material, the excess of solvent will lead to unnecessary waste, while the insufficience of it can lead to non-exhaustive extraction of substances in plants (Cheng et al., 2017). According to some studies, the ratio of materials per solvents is quite different when separating saponins from

different sources. For examples the ratio of 1:30 with *Codonopsis lanceolata*, ratio 1: 8 with *Cicer arietinum* L, ratio 1:15 with *Camellia oleifera*, ratio 1:10 with *Sapindus mukorossi* (Liu et al., 2016; Cheng et al., 2017; Zhao et al., 2012; Kose et al., 2016). Therefore, the effect of the ration of materials per solvents to the extraction efficiency of the target compound was evaluated at a ratio of 1:5 - 1:30 with a concentration of 70% ethanol. The result in figure 3 showed the proportion of materials: solvent has a strong effect on saponin extraction efficiency from *Celastrus hindsii* Benth. leaves. Accordingly, the highest saponin content reached 15.6 mg/g with the ratio of materials: solvent is 1:15. The efficiency of saponin extraction decreases gradually when the solvent content is ≥ 20 ml or ≤ 10 ml. The lowest content of saponin is 5.5 mg/g at the ratio of 1:30.

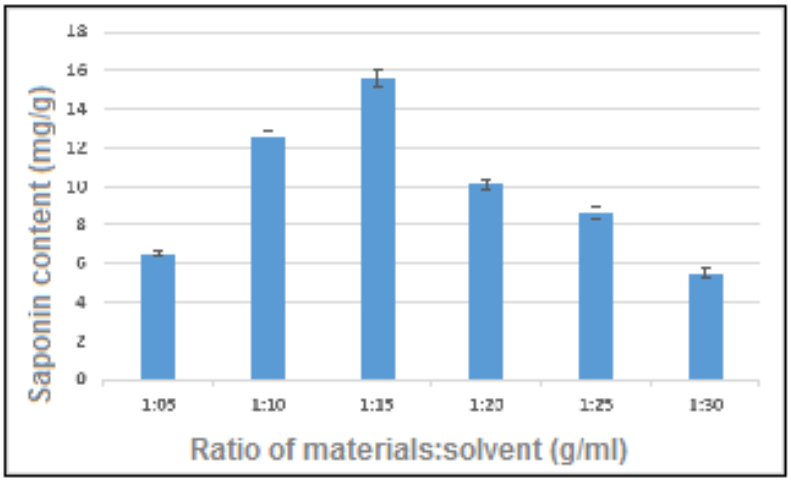

Figure 3. Effect of the ratio of materials : solvent on extracted saponin content

3.1.3. Effect of temperature

The saponin extraction efficiency changes when the extraction solvent temperature gradually increases. Liu et al. (2016) reported that when temperature changed from 60 - 80°C saponin extracted from *Camellia oleifera* leaves increased gradually. He also suggested that saponin was best dissolved at this temperature range, and at higher temperature saponin would be destroyed its structure. Figure 4 showed the lowest content of saponin extracted (9.4 mg/g) at 70°C and the highest (15.7 mg/g) at 80°C. When temperature was more than 90°C, the saponin extraction efficiency decreased (12.2 - 14.1 mg/g, 100°C and 90°C, respectively). This result may be due to high temperatures causing saponin loss (Cheng et al., 2017). Therefore, the temperature at 80^0C was chosen for the next study.

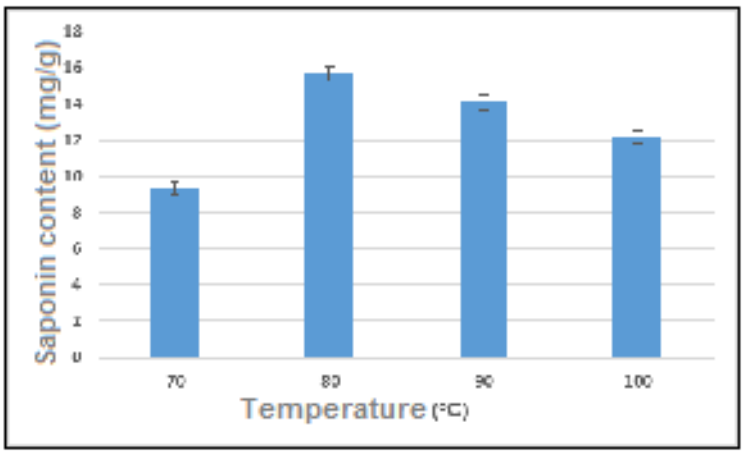

Figure 4. Effect of temperature on saponin extraction efficiency

3.1.4. Effects of method and time extraction

Saponins from *Celastrus hindsii* Benth. leaves were extracted based on two methods, immersion and ultrasound at a concentration of 70% ethanol, the ration of materials : solvents = 1:15, 80°C and 20 - 60 minutes (ultrasound method) or 1 - 5 hours (immersion method). The results in table 1 showed that, with ultrasound method, when the time increased from 20 to 30 minutes, the cell membrane was broken by the ultrasound wave and saponins were released at the content of 18.6 mg/g. Beside, with the immersion method, the extraction time of 2 hours obtained higher saponin content (15.4 - 23.7 mg/g) compared to the prolonged extraction time of 4 - 5 hours (7.7 - 11.4 mg/g). As the extraction of solute molecules, the long extraction time will increase the dissolved saponin content until the optimal content is reached; However, prolonging time at high temperatures will result in the decomposition of organic compounds, including decreasing saponin content. Research results of Majinda et al. (2012) revealed that organic matter had been changed by long-term high temperature and saponin content decreased sharply.

Ultrasound was one of the quick and effective extraction method for some oil seeds: quinoa, soybean, red lentils, pecan, lupine (Navarro et al., 2018) and ginseng (Wu et al., 2001). However, with *Celastrus hindsii* Benth. leaves, the immersion method gave higher saponin extraction efficiency than ultrasound (saponin contents are 23.7 mg/g and 18.6 mg/g, respectively).

Table 1. Effect of time and method on saponin content

Immersion method		Ultrasound method	
Time (hour)	**Saponin content (mg/g)**	**Time (minutes)**	**Saponin content (mg/g)**
1	8.9 ± 0.19	20	10.2 ± 0.26
2	15.4 ± 0.32	**30**	**18.6 ± 0.58**
3	**23.7 ± 0.85**	40	16.0 ± 0.41
4	11.4 ± 0.35	50	12.3 ± 0.31
5	7.7 ± 0.30	60	7.8 ± 0.24

3.1.5. Optimizing the saponin extraction conditions

Among various influent factors surveied, the concentration of ethanol (X_1), the ratio of materials : solvent (X_2) and the extraction time (X_3) showed strong influence on the extraction of saponins from *Celastrus hindsii* Benth. leaves. Then, the simultaneous effects of these three factors on expected function were determined by the second experimental planning method in order to optimize the saponin extraction conditions. The experimental conditions and saponin content are shown in Table 2.

Table 2. Experimental matrix of saponin extraction process

Test	X_1 (%)	X_2 (g/ml)	X_3 (hours)	Saponin content (mg/g)	Test	X_1 (%)	X_2 (g/ml)	X_3 (hours)	Saponin content (mg/g)
1	60	1:10	3	14.03	10	70	1:20	2	14.98
2	80	1:10	3	16.15	11	70	1:10	4	15.20
3	60	1:20	3	11.57	12	70	1:20	4	22.06
4	80	1:20	3	21.60	13	70	1:15	3	24.99
5	60	1:15	2	13.81	14	70	1:15	3	23.19
6	80	1:15	2	19.89	15	70	1:15	3	23.68
7	60	1:15	4	16.82	16	70	1:15	3	23.56
8	80	1:15	4	20.20	17	70	1:15	3	23.68
9	70	1:10	2	18.06					

The results from the table above showed that the saponin content was obtained from range 11.57 mg/g to 24.99 mg/g corresponding to experiment number 3 and experiment number 13. This also indicaded that, in the selected range, all variables affected the saponin extraction process. At the minimum and maximum levels of the factors, there was a difference in the amount of saponin obtained. Thus, all three factors were selected to have a strong link in influencing the extraction of saponins from *Celastrus hindsii* Benth. leaves.

The results of the analysis of the variance of the optimal model using DX7.1.5 software presented in Table 3 showed that all of 3 factors including time, ethanol concentration and the ration of materials: solvents have strong influence on the separation process. Extracting saponin (p value of X_1, X_2, X_3 < 0.05). The F value of the model was 72.74 with $p < 0.0001$ ($p < 0.05$), indicating that the model was correctly selected. The p value of "Incompatible" is 0.57 ($p > 0.05$), indicating that this model is compatible with the experiment. The p-value of X_1X_2 and X_2X_3<0.05, the co-effect of the ration of materials: solvents with ethanol concentration and time has a strong influence on the saponin extraction process from *Celastrus hindsii* Benth. leaves.

The regression equation shows that saponin content (Y - mg/g) describes the effect of the elements represented as follows:

$$Y = -1892.71 + 1.44*X_1 + 33.09*X_2 - 38.29*X_3 + 0.04*X_1*X_2 - 0.07*X_1*X_3 + 0.49*X_2*X_3 - 0.04X_1^2 - 0.16X_2^2 - 2.20*X_3^2.$$

Using the expected function method to optimize the content of saponin obtained after 43 experimental options of extraction. With the best solution, the concentration of ethanol is 73.91%, the ration of materials : solvents = 1: 16.5 and the immersion time is 3.32 hours, the calculated saponin content reached to 24.63 mg/g (Figure 5). Experimental at the condition of concentration of 74% ethanol, the ratio of materials : solvent = 1 : 16.5 and solvent temperature 80^0C after the immersion time of 3.32 hours, the amount of saponin obtained was 24.45 mg/g with the high compatibility compared with theory.

Table 3. Results of ANOVA variance analysis of the model

Parameter	Variance	Standard F	Significant level p	Parameter	Variance	Standard F	Significant level p
Model	31.59	74.72	< 0.0001	$X_2.X_3$	24.68	58.36	0.0001
Ethanol concentration (X_1)	58.38	138.08	< 0.0001	X_1^2	65.27	154.38	< 0.0001
Ration of raw materials: solvents (X_2)	5.73	13.55	0.0079	X_2^2	68.76	162.64	< 0.0001
Time (X_3)	7.09	16.77	0.0046	X_3^2	20.38	48.19	0.0002
$X_1.X_2$	15.60	36.89	0.0005	Incompatible	0.42	0.77	0.5675
$X_1.X_3$	1.80	4.26	0.0778				

Figure 5. Saponin content under the optimal conditions

3.2. Testing the antibacterial activities of saponin extract

Antimicrobial activity of saponin extract is carried out with 3 pathogenic bacteria strains: *Staphylococcus aureus* VS1*, Escherichia coli* CA*, Pseudomonas aeruginosa* HS. The results in Table 2 show that saponin extract from *Celastrus hindsii* Benth. leaves (10 mg/ml) is resistant to all 3 strains of bacteria tested with antibacterial ring diameter of 1.6 - 2.2 cm, however the ability antimicrobial action of extract is lower than ampicillin (0.5 mg/ml)

P. aeruginosa is known as the green pus *Bacillus* and is also a common bacterial pathogen, resistant to many common antibiotics such as penicillin, ampicillin, chloramphenicol, tetracyclin. *Staphylococcus aureus* causes many infections, pus and toxins in humans, usually occurs in areas of skin scratches such as pimples and ulcers. *S. aureus* is a bacterium with the strongest resistance to drugs. Some strains are resistant to all antibiotics except vancomycin and these lines appear more and more. The report of Jyothi et al. (2012) also confirmed high saponin extraction from *Celatrus paniculatus* with *Staphylococus aureus* antibacterial ability with 4 - 11 mm diameter ring at saponin concentration of 0.5 mg/ml. The resistance of *Staphylococus aureus* and *E. coli* of saponin from *Sapindus mukorossi* was also Kose et al. Report (2016), with a minimum concentration of 50% ethanol saponin extract is 12.5 - 25.0 mg/g. Therefore, the use of saponins from *Celastrus hindsii* Benth. leaves can kill these bacteria without causing resistance like antibiotics.

Table 2. Results of antibacterial activity test of saponins from *Celastrus hindsii* Benth. leaves

Microbial strains	Antibacterial ring diameter (cm)	
	Saponin extract	Ampicillin (0.5 mg/ml)
Pseudomonas aeruginosa HS	1.8 ± 0.05	11.6 ± 0.45
Staphytococus aureus VS1	1.6 ± 0.03	12.4 ± 0.31
E. coli CA	2.2 ± 0.06	10.2 ± 0.25

Besides, according to the researches of Avinash et al. (2014), Hu et al. (2013) *Celatrus paniculatus* and *Celastrus hindsii* contain variety of bioactive compounds with high antibacterial activity consitting of flavonoids, phenols, terpenoids, saponins, alkaloids, glycosides. Flavonoids bind to adhesin is the virulence factor of Gram-negative bacteria and an inhibitor of the release of acetylcholine - the phospholipid class component. In addition, alkaloid interferes with cell walls can break the structure and quinone inactivates enzymes associated with peptidoglycan synthesis of bacteria, especially transpeptidase. Thus, *Celastrus hindsii* Benth. is a precious medicinal herb that has effective antibacterial properties.

4. CONCLUSION

The concentration of ethanol strongly influenced the total saponin content (3.96 - 12.58 mg/ml, corresponding to 50 - 70% ethanol concentration) while the temperature did not significantly change saponin content (12.2 - 15.7 mg/g corresponds to 100^{o}C and 80^{o}C). The ratio of materials: solvents 1:15 obtained the highest total saponin (15.6 mg/g). The extraction of saponin by ultrasound method at 30 minutes with solvent extraction of 70% ethanol, temperature of 80^{o}C, the ration of materials: solvents is 1: 5, total saponin content of 18.6 mg/ml while also with these conditions, but using the immersion method, the total saponin content obtained was higher (23.7 mg/g). Optimize the extraction conditions with 3 factors of the survey elements: ethanol concentration (60 - 80%), the ration of materials: solvents (1:10 - 1:20 g/ml), immersion time (2 - 4 hours) maximum saponin content (24.45 mg/g) at 74% ethanol concentration, the ration of materials: solvents = 1: 16.5 and immersion time of 3.32 hours.

Saponins from *Celastrus hindsii* leaves have antibacterial activities, which have been tested with the saponin concentration of 30 mg/ml. The resistance ring diameter for each type of pathogenic bacteria type: *P. aeruginosa* HS (1.8 cm), *S. aureus* VS1 (1.6 cm), *E. coli* CA (2.2 cm).

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TỐI ƯU ĐIỀU KIỆN CHIẾT XUẤT VÀ HOẠT TÍNH KHÁNG KHUẨN CỦA SAPONIN TỪ LÁ XẠ ĐEN (*Celastrus hindsii* Benth.)

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TÓM TẮT

Saponin là một hợp chất tự nhiên từ thực vật có nhiều tác dụng: kích thích miễn dịch, chống ung thư, giảm đau, chống nôn, chống oxy hóa, làm suy yếu quá trình tiêu hóa protein, hạ đường huyết, chống nấm, vi khuẩn và kháng virút. Cây xạ đen – một loại dược liệu quý có chứa các chất như: flavonoid, saponin, triterpenoid, diphenylpropanes, quinon được sử dụng làm thuốc chống ung thư, chống oxi hóa, kháng khuẩn và chống dị ứng. Saponin chiết xuất từ lá xạ đen bằng phương pháp ngâm thu được hàm lượng cao hơn phương pháp siêu âm trong cùng điều kiện (23,7 mg/g và 18,6 mg/g). Các yếu tố nghiên cứu thay đổi bao gồm: nồng độ ethanol (50 – 100%), tỷ lệ nguyên liệu: dung môi (1:5 – 1:30), nhiệt độ (70 – 100^{o}C), phương pháp (ngâm và siêu âm), thời gian (20 phút – 5 giờ). Kết quả nghiên cứu cho thấy hàm lượng saponin thu được cao nhất bằng phương pháp ngâm trong ethanol 70% ở 80^{o}C trong 3 giờ với tỷ lệ nguyên liệu: dung môi = 1: 15 là 23,7 mg/g. Bài báo đã xác định được điều kiện tối ưu cho quá trình chiết saponin bằng quy hoạch thực nghiệm bậc 2 Box-Benken: nồng độ ethanol 74%, tỷ lệ nguyên liệu: dung môi = 1: 16,5 và nhiệt độ dung môi 80^{0}C, thời gian ngâm 3,32 giờ. Saponin từ lá xạ đen có khả năng kháng vi khuẩn kiểm định bao gồm: *P. aeruginosa* HS, *S. aureus* VS1, *E. coli* CA với đường kính vòng kháng 1,6 - 2,2 cm. Như vậy, saponin chiết xuất từ lá xạ đen theo các điều kiện trên có thể ứng dụng trong sản xuất các sản phẩm bảo vệ và chăm sóc sức khỏe con người.

Từ khóa: *Celastrus hindsii*, chiết xuất, kháng khuẩn, saponin.

Received : 08/10/2019
Revised : 10/8/2020
Accepted : 12/8/2020