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Method development of quercitrin enrichment from asthma-plant (*Euphorbia hirta* L.) using aromatic macroporous resin

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ABSTRACT

Asthma-plant contains high amount of quercitrin which make it a potential new source for flavonoids. This study aims to develop a method of quercitrin enrichment by utilizing macroporous resin, which is known to be safer, more eco-friendly, economics, and efficient. Evaluations were conducted over the performance and separation characteristics of the macroporous resin in quercitrin enrichment as well as the adsorption and desorption of quercitrin by the macroporous resin. The results showed that the adsorption process of the macroporous resin in relation to the amount of quercitrin in the extract were in accordance with the second order model, which means that the process of adsorption is affected by other compounds. Furthermore, the examination of the isotherm adsorption fit the Freundlich's model (R^2 = 0.9850) rather than the Langmuir's one (R^2 = 0.4334). In the optimal condition, the enrichment of quercitrin by using macroporous resin increased the abundance of quercitrin by nearly five times, from 3.60% of quercitrin content in the extract to 17.02% in the quercitrin-rich fraction, with recovery yield of 50.39%.

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1. Introduction

Euphorbia hirta (*E. hirta*) L. belongs to family Euphorbiaceae and is commonly used as traditional medicine in tropical countries (Loh et al., 2009). Research showed that *E. hirta* exhibited various pharmacological activities, such as antibacterial, antioxidant, antiallergic, antitumor, and antidiabetes (Al-Snafi, 2017; Li et al., 2016; Basma et al., 2011; Cushnie and Lamb, 2005; Gálvez et al., 1993). In addition, the quercitrin content in this plant is high (0.047%/kg dry sample) (Mallavadhani and Narasimhan, 2009). Thus, this plant can be used as a potential new source of flavonoid.

That being said, conventional approaches used in the fractionation and isolation of chemical constituents still capitalize on the utilization of organic solvents, such as *n*-hexane, ethyl acetate, dichloromethane, and methanol. These solvents are known to be harmful to human health and are potential environmental hazards. Moreover, the use of the organic solvents has been known to result in low quercitrin yield. This problem has encouraged the development of quercitrin isolation methods from *E. hirta* which are safer, environmentally friendly, simpler, and more efficient to be applied in a large-scale isolation and purification. One of the methods that can potentially be used is the purification technique facilitated by the utilization of macroporous resin polymers.

Macroporous resin is a type of hydrophilic polymer possessing high level of stability, high adsorption capacity and selectivity, fast adsorption and desorption, easy regeneration, eco-friendly, and cost-efficient (Li et al., 2015). Adsorption principle is based on electrostatic force, hydrogen bonds, and the difference in molecular size of compounds is a sample. In the last few years, macroporous resin has been used as media in enriching and separating secondary metabolites from plants, such as flavonoids, coumarins, terpenoids, lignans, and phenolic compounds (Li et al., 2015). Furthermore, the popularity of the adsorption technology of macroporous resin has been increasing in pharmaceutical application (Jung et al., 2001; Zhang et al., 2007). One example of macroporous resin polymer that has been widely marketed is aromatic macroporous resin (polyvinyl benzene). The polyvinyl benzene resin demonstrates a higher attraction capability than gel type, has uniform pore size, and can be used in wider applications (Garcia diego and Cuellar, 2005). In light of that, in order to achieve large scale quercitrin separation which is safer, environmentally friendly, and cost-efficient, detailed research on macroporous resin is therefore required.

2. Materials and methods

2.1. Chemicals and reagents

Ethanol (EtOH) 96% food grade was used for preparation of herbal extracts and column chromatography. Quercitrin (Markherb,

Bandung, Indonesia), methanol (MeOH) HPLC grade (Merck), water, H_3PO_4 were used for HPLC analysis.

2.2. Adsorbent

Aromatic macroporous resin was purchased from Purosorb PAD900 (Purolite®). Physical properties of the resin are displayed in Table 1. Resin preparation was carried out by submerging resin in EtOH 96% for 24 h to remove monomers and porogenic agents trapped in the resin pores during the synthesis process. The resin was then washed with distilled water in a chromatography column before use.

Table 1. Physical properties of aromatic macroporous resin

Parameter	Score
Humidity	67 – 73%
Particle size	350 – 1200µm
Pore diameter	220Å
Pore volume	1.9 ml/g
Area	850 m^2/g
pH stability limit	0 – 14
Temperature stability limit	150°C (302.0°F)

2.3. Preparation of plant extract

E. hirta L. dry sample was procured from Borobudur®. The ground dried powder of *E. hirta* (2 kg) was macerated in 10 l of 70% MeOH for 1×24 h with three repetitions. The filtrates were combined and concentrated at 50°C into crude extract using rotary evaporator. For HPLC analysis purpose, a few milligrams of the crude extract was taken and diluted in 50% MeOH.

2.4. HPLC condition

HPLC-UV analysis was conducted using HPLC LC-20AD (Shimadzu) featured with CTO-20A pump (Shimadzu) and UV/Vis SPD-20A detector (Shimadzu). The separation system used was LiChrospher®100 RP-C18 5µm column (100 mm in length, 4 mm in diameter, 20 mm precolumn (Merck)) at 30°C. Separation of sample (20 µL) was run by using mobile phase A (water + 0.01% H_3PO_4) and mobile phase B (MeOH), with flow rate of 0.5 ml/min at UV wavelength of 210 and 360 nm. Solvent gradients were as follows: 40% B for the first 2.5 mins, continued with 60% B at minute 7.5. At minute 7.6-12.5, the solvent concentration was maintained at 70% B, and was maintained again at 40% B in the last 2.5 min for re-equilibration purpose before injecting the next sample.

Method validation requires several paramaters, including linearity, specifity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) (Ahuja, 2005). The validation of linearity was carried out by preparing six concentration series, i.e., 100, 75, 50, 25, 12.5, and 10µg/ml. The determination of accuracy and precision-repeatability was done by adding three different concentrations of quercitrin, i.e., 50, 75, and 100µg/ml, into the placebo solution. Each concentration was analyzed in triplication, and the quercitrin recovery percentages were calculated.

2.5. Static adsorption/desorption test

The adsorption capacity of the resin towards quercitrin was evaluated using the procedure by Hou et al., 2019; Du et al., 2012 with modification. Briefly, 25 ml of crude extract solution in water (0.2 mg/ml concentration) was poured into a 250 ml conical flask containing 1 g of wet resin. The mixture was shaken (120 rpm) in a shaker at constant temperature of 25°C for 60 min. Sample was taken from the solution every 5 min for HPLC quantitative analysis of quercitrin content.

The same resin used in the adsorption test above was added with 25 ml of EtOH 70% for desorption test. The mixture was shaken again (120 rpm) in a shaker at constant temperature of 25°C for 60 min. Sample was taken from the solution every 5 min for HPLC quantitative analysis of quercitrin content. The adsorption and desorption capacities, as well as the desorption ratio of the resin was calculated using the following equations:

$$q_t = \frac{(C_0 - C_t)V_i}{W}$$

$$A = \frac{(C_0 - C_t)}{C_0} \times 100\ \%$$

where,
A = adsorption ratio
C_0 = initial solution concentration
C_t = solution concentration at t min
q_t = adsorption capacity
V_i = initial solution volume
W = resin dry weight

$$D = C_d \times \frac{V_d}{(C_0 - C_e)V_i} \times 100\ \%$$

$$q_d = \frac{V_{d\ x\ C_d}}{W}$$

where,
D = desorption ratio
q_d = desorption capacity
C_d = solution concentration at desorption equilibrium
C_e = solution concentration at adsorption equilibrium
V_d = initial volume of desorption solution
V_i = initial volume of adsorption solution
M = resin water content
W = resin initial weight

2.6. Isothermic adsorption

Isothermic adsorption experiment on the aromatic macroporous resin (1g of wet resin) was carried out by connecting 4 (four) aliquots of 25 ml of sample solution at varying concentration (0.012, 0.024, 0.036, and 0.048 mg/ml) which were shaken (120 rpm) at constant temperature for 1 h. Each sample was analyzed using HPLC for quercitrin content. This procedure was conducted in three repetitions (Du et al., 2012). The isothermic adsorption analysis was done using the Langmuir and Freundlich equations below:

$$C_e/q_e = K_L/q_m + C_e/q_m$$

$$\log q_e = (1/n) \log C_e + \log K_F$$

$$R_L = \frac{1}{1 + C_{max}/K_L}$$

where,
C_e = solution concentration at equilibrium
q_e = adsorption ratio
K_L = Langmuir constant
Q_m = adsorbent maximum capacity
1/n = adsorption constant
K_F = Freundlich constant
R_L = dimensionless separation factor
C_{max} = highest concentration

2.7. Dynamic adsorption/desorption test

The dynamic adsorption test was conducted using a laboratory-scale column (21 cm in length, 3 cm in diameter), packed with 15 g of aromatic macroporous resin with 30 ml bed volume (BV). This test was done to observe the dynamic leakage curve. Extract solution at 12 mg/ml concentration was subjected to the resin

column with different flow rate (2, 3, 4 and 5 BV/h). The leakage curve was used to identify the leakage point (the condition in which the resin cannot adsorb more quercitrin from the extract solution, indicated by the detection of quercitrin in the eluate) to determine the optimal flow rate for the optimization the purification method. In this study, the leakage point was defined as the time recorded when the quercitrin total content in the eluate reaches 3µg/ml (Zhang et al., 2018). The gradient desorption test was carried out as follows: upon the completion of the adsorption process, the column was firstly washed with distilled water, and was subsequently eluted with EtOH-water solution (20, 30, 40, 50, 60, 70, 80, and 90%). The fractions collected were then analyzed using HPLC (Zhang et al., 2018).

3. Results and discussion

This study investigated the capacities and ratio of adsorption/desorption of aromatic macroporous resin towards quercitrin using static and dynamic approaches. Both the adsorption capacity and desorption ratio are important factors to consider in determining the suitable resin to be used for adsorbing the target compounds.

3.1. HPLC condition

3.1.1. Method validation

The validation of linearity gave determination coefficient (R^2) value of 0.9994, meeting the acceptability requirement of ≥ 0.999 (Ahuja, 2005). This indicates that the calibration curve can be used for the quantification of quercitrin.

According to Table 2, the average value of quercitrin recovery for each concentration was found to be in acceptable range of ±2% (Ahuja, 2005). Furthermore, the relative standard deviation percentage (% RSD) from the nine data was ≤ 2%, suggesting that the precision-repeatability met the requirement.

Tabel 2. Determination of accuracy and precision-repeatability with HPLC

Concentration (%)	Replication	Recovery (%)	Average	%RSD
100	1	100.503	99.922	1.208
	2	99.959		
	3	99.303		
75	1	101.197	99.487	
	2	98.980		
	3	98.284		
50	1	100.659	98.906	
	2	98.159		
	3	97.901		

3.2. Static adsorption/desorption test

3.2.1. Adsorption capacity and desorption ratio

According to the investigation on the resin PAD 900 adsorption capacity towards quercitrin, the adsorption towards quercitrin (Fig. 1) occurred in 3 stages. Firstly, the adsorption increase took place in rapid and linear fashion for the first 10 mins. In the second stage, the resin adsorption capacity improved slowly. The third stage was marked by the resin adsorption reaching the leakage point at the 35th min with the adsorption capacity of 6.050 mg/g dry resin and adsorption ratio of 97.43%. The EtOH desorption ratio towards quercitrin attracted to the macroporous resin was 85.39%, suggesting that approximately 85% (5.112 mg) of quercitrin was desorbed by the desorbent EtOH 70%. This indicates that the resin adsorption rate decreased as the amount of contact area that could attract the target compound went down.

Futhermore, Fig. 2 shows the presence of two peaks with high intensity that were also adsorbed along with quercitrin which affects the adsorption ability of the resin, which are predicted to be afzelin and myricitrin.

3.2.2. Adsorption and desorption kinetics

There are several kinetic approach models that can be used to understand the adsorption-desorption mechanism occurring, i.e., pseudo-first order reaction, pseudo-second order reaction, Elovich equation, and intraparticle diffusion kinetics. Table 3 shows that of the four models, the R^2 values of each the pseudo-second order reaction was the the closest to 1. This suggests that the process of quercitrin adsorption might be affected by the presence of other compounds, such as other polyphenols or flavonoid glycosides. Liu et al. (2007) reported that 3 compounds have been isolated from *E. hirta*, i.e., afzelin, quercitrin, and myricitrin. It is possible that the other two compounds had certain effects on the quercitrin adsorption by the resin.

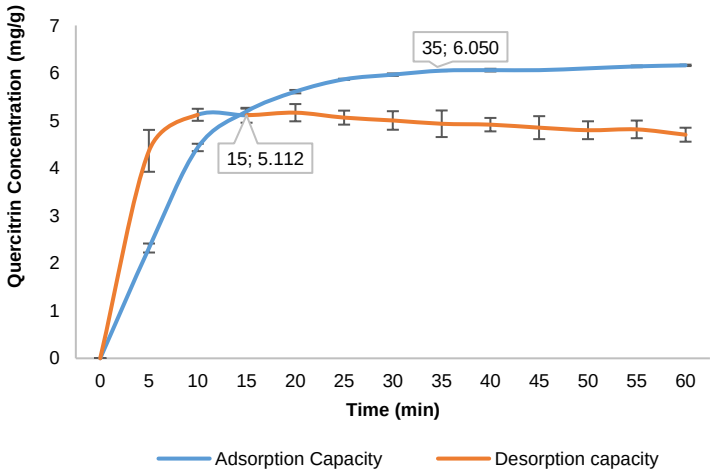

Fig. 1. Adsorption and desorption capacity curves for quercitrin on PAD 900 resin.

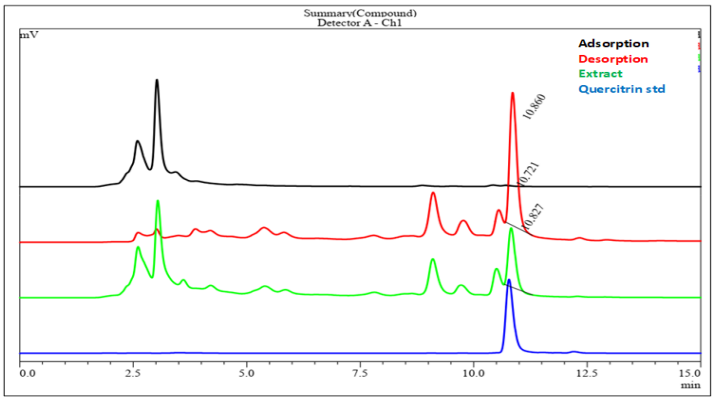

Fig. 2. Chromatogram overlay of extract, adsorption, desorption, and quercitrin standard

3.3. Isothermic adsorption

The Langmuir and Freundlich theoretical equations were used to describe the interaction between the adsorbent and the adsorbed materials. These equations are suitable to express the linearity suitability and to demonstrate how the solutes interact with the resin. The parameters obtained after data processing using Langmuir and Freundlich equations (Fig. 3) showed that the R^2 value that was closest to 1 was the Freundlich equation model (R^2 = 0.9879).

The acceptability requirement for Freundlich model is the Standard Deviation (SD) value of ≤13.21. The result showed that Freundlich isothermic model exhibited a lower SD value (11.11), indicating that the isothermic adsorption model of the aromatic

macroporous resin towards quercitrin in the sample extract was in agreement with Freundlich isothermic model.

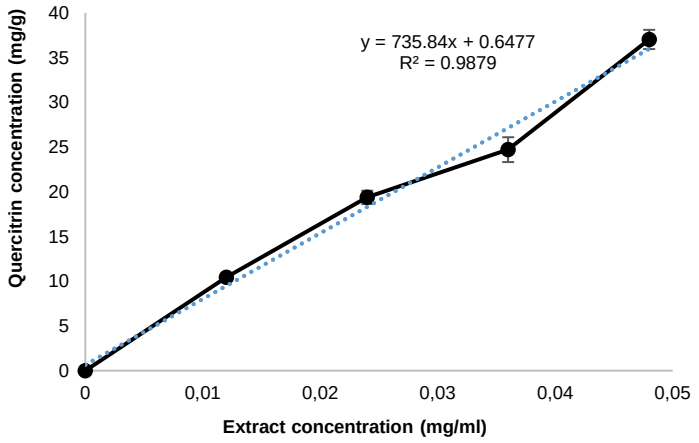

Fig. 3. Adsorption isotherms of quercitrin on PAD 900 resin at 25°C

Tabel 3. Adsorption kinetic paramaters

Adsorption kinetic parameter	Value
Pseudo first order	y = -0,00009x + 0,00006
k_1 (1/min)	-0,00009
R^2	0,99996
Pseudo second order	y = 0,00008x + 0,00009
k_2 [mg/(g min)]	0,00008
R^2	0,97443
Elovich	y = 525,21x + 8860,7
α [mg/(g min)]	525,21
R^2	0,7871

3.4. Dynamic adsorption/desorption

The dynamic adsorption and desorption studies were conducted by varying the flow rate of the sample solution, the concentration of desorbent solution, and the elution rate. The results of these studies are displayed in Fig. 4 and 5.

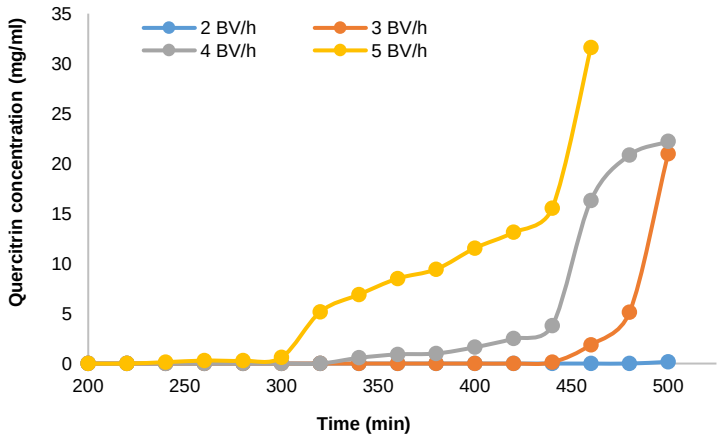

Fig. 4. Dynamic adsorption of quercitrin on PAD 900 resin

3.4.1. Optimum flow rate

The relationship between the flow rate, leakage point, and the quantity of adsorbed quercitrin is shown in Table 4. It is displayed that the increase in flow rate had caused difference in the duration to reach the leakage point. This might have promoted the dissimilarity in the capability of the resin to adsorb quercitrin. The more amount of quercitrin being adsorbed before the leakage point is reached, the better the adsorption capacity of the aromatic macroporous resin. The optimum flow rate that is potential to be applied in a large-scale purification was at 3 BV/h with the lowest adsorbed quercitrin of 237 mg.

Tabel 4. The relationship between the flow rate, leakage point, and the quantity of adsorbed quercitrin

Flow rate	Leakage point (min)	Adsorbed quercitrin (mg)
5 bv	240	216
4 bv	320	228
3 bv	440	237
2 bv	500	180

3.4.2. Optimum desorption

Dynamic desorption can be affected by several factors, such as eluent concentration, solvent flow rate, temperature, column length-to-diameter ratio, etc. In the dynamic desorption test, the optimum desorption was chosen based on the following methods: the column containing the sample was washed with 3 BV of distilled water, and was subsequently eluted with 1 BV each of EtOH 20-90% solutions. EtOH was chosen for its volatile and non-toxic properties. Fig. 5 shows that EtOH desorption capacity towards quercitrin increased at concentration range of 40-60% (v/v). EtOH 20-30% was used to remove the impurities, such as sugars and water-soluble pigments.

Fig. 5 demonstrates that lower elution flow rate resulted in longer contact duration between the desorbing solvent and the sample, resulting in a better desorption of quercitrin. Therefore, flow rate of 2 and 3 BV/h exhibited higher desorption results than did the 4 and 5 BV/h flow rate. At 2 BV/h, desorbed quercitrin was distributed in two desorbing solvent concentrations, i.e., 50 and 60%.

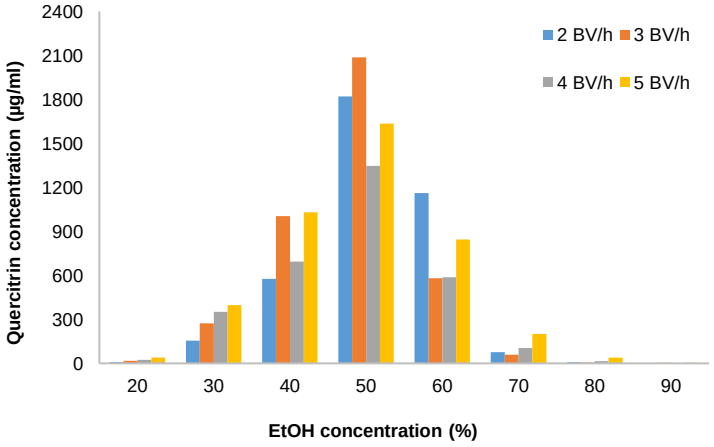

Fig. 5. Dynamic desorption of quercitrin on PAD 900 resin

On the other hand, the 3 BV/h flow rate render the quercitrin to be desorbed maximally at solvent concentration of 50%, hence it was considered the optimum flow rate. At the optimum condition, the enrichment process of quercitrin using macroporous resin managed to improve the quercitrin content by 4.72 times with 50.39% quercitrin recovery rate. These results were in line with the report published by Zhang et al. (2007) in which the adsorption/desorption of three flavonoid compounds by several types of resin with different building blocks were observed. The study revealed that aromatic macroporous resin constructed by styrene-divinylbenzene exhibited the most optimum adsorption/desorption capacity towards flavonoid compounds.

Compared to the static desorption process with 85% recovery rate, the dynamic desorption process was found to provide a lower recovery of quercitrin. This could be due to the contact duration between quercitrin and the resin being longer in the dynamic

process in comparison to that of the static one. In addition, the shaking procedure in the static desorption process might have improved the solvent's desorption capability, increasing the recovery rate.

4. Conclusion

The aromatic macroporous resin demonstrated a high adsorption capacity towards quercitrin. Kinetics study indicated that the adsorption process follows the pseudo-second model. The quercitrin content improved by nearly 5-fold with quercitrin recovery of 50.39%. The results gave a potential approach for large-scale quercitrin purification from *E. hirta* L. Moreover, the utilization of aromatic macroporous resin can be applied in the quality control for the results of quercitrin enrichment and purification processes.

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Conflict of interest

The authors declare there is no conflict of interest in this study.

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