Optimizing The Protocol For Extraction Of Bioactive Components From *Hibiscus Sabdariffa* With Potent Antioxidant Activity

Nur Royhaila Mohamad*[1], Farrah Payyadhah Borhan[2], Mohd Shahrizi Razali[3], Roswanira Abdul Wahab[4]

Abstract

The extract of *Hibiscus sabdariffa* (HS) also known as roselle from the Malvaceae family is prized for its exceptionally high contents of polyphenols and anthocyanins, the calyces of HS being one of the many sources of natural antioxidants. Considering the numerous health benefits associated with the consumption of such compounds as well its broad application in the food industry, the development of an extraction protocol for such compounds from HS at high yields and antioxidant activity merits scientific relevance. In this study, the optimum conditions to achieve such goals were established by the method of response surface methodology (RSM) using three independent variables: time (30, 165, and 300 min), temperature (50, 70, and 90°C) and ethanol concentration (60, 75 and 90%). A Box-Behnken design (BBD) was utilized to determine the optimum condition that yielded the highest extraction yield and antioxidant activity measured by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical inhibition activity test. The results showed that under optimum conditions [300 min, 70°C in 90% ethanol concentration and 30 min, 70°C in 90% ethanol concentration], a corresponding 48.44% and 87.93% were obtained for the extraction yield and DPPH activity of HS, respectively; hence verifying the suitability of RSM for optimizing the extraction of HS.

Keywords: *Hibiscus Sabdariffa*; DPPH; extraction yield; antioxidant; response surface methodology

Significance | Utilize statistical methods to determine which different variables impact the response to the extraction of roselle.

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Introduction

Hibiscus sabdariffa (HS) also known as roselle from the Malvaceae family is a bushy plant that may reach a height of up to 2.5 m, producing numerous single flowers that change color from yellow or buff with a rose or maroon eye, gradually turning pink during maturation (Mohamed et al., 2007). Being high in polyphenols (Mourtzinos et al., 2008) and anthocyanins that confer their prized red color (Mohamed et al., 2007), the calyces of HS constitute one of the many great sources of natural antioxidants. Owing to their high antioxidant content and protective effects against degenerative diseases, infusions of both dried and fresh HS calyces have been prevalently used in local herbal medicines for their effects of diuretic, choleretic, febrifugal and hypotensive, thinning of the blood as well as stimulating intestinal peristalsis (Da-Costa-Rocha et al., 2014). In addition to their medicinal properties, the

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Table 1: Experimental design and results of response surface design

Std	Time (min)	Temp (°C)	[EtOH] (%)	Variable			
				Extraction Yield (%)	Pred	DPPH (%)	Pred
1	30	50	75	41.88	41.79	85.65	85.99
2	300	50	75	46.65	46.99	83.56	83.85
3	30	90	75	40.08	39.74	86.42	86.13
4	300	90	75	45.61	45.70	85.83	85.49
5	30	70	60	40.46	40.44	85.87	85.66
6	300	70	60	44.12	43.66	86.42	86.26
7	30	70	90	40.08	40.54	87.92	88.08
8	30	70	90	48.44	48.47	84.47	84.68
9	165	50	60	39.89	40.00	83.78	83.65
10	165	70	60	35.65	36.02	84.08	84.58
11	165	50	90	40.51	40.14	84.61	84.11
12	165	90	90	40.89	40.78	84.84	84.97
13	165	70	75	39.47	39.90	82.56	83.10
14	165	70	75	40.28	39.90	83.56	83.10
15	165	70	75	38.78	39.90	83.08	83.10
16	165	70	75	41.09	39.90	83.16	83.10
17	165	70	75	39.87	39.90	83.13	83.10

Table 2: The ANOVA for the extraction of HS. ^{ns}not significant; *significant (α = 0.05)

Response/ Variable	Extraction Yield (%)		DPPH	
	F-value	P-value	F-value	P-value
Model	30.15	< 0.0001	40.46	< 0.0001
A-Time	110.04	0.0001	40.32	0.0007
B-Temperature	9.92	0.0162	26.78	0.0021
C-Ethanol Concentration	21.21	0.0025	0.083	0.7830
AB	0.26	0.6290^{ns}	5.83	0.0523^{ns}
AC	9.76	0.0168*	41.44	0.0007*
BC	9.43	0.0180*	4.82	0.0705^{ns}
A^2	110.20	0.0001*	183.44	0.0001*
B^2	0.27	0.6175^{ns}	0.049	0.8318^{ns}
C^2	1.65	0.2393^{ns}	20.33	0.0041*
Lack of fit	0.43	0.7449	0.28	0.7696^{ns}

HS concentrate is also used as a food coloring and flavoring agent in fermented drinks, jellied confectionaries, wine, jam, chocolates, ice cream, puddings, and cakes (Bako et al., 2009; Da-Costa-Rocha et al., 2014).

In view of serious concerns over the carcinogenic effect that synthetic antioxidants may have on humans, the use of natural plant-based antioxidants in food preparations is gaining popularity among consumers (Lourenço et al., 2019). Consumption of foods rich in antioxidants confers a myriad of health benefits which include protection against degenerative diseases (Liyana-Parthirana and Shahidi, 2005) as well as overcoming the damaging effects of free radicals on the body owing to oxidative stress. Incorporation of antioxidants into foods also retards lipid oxidative rancidity (Salminen et al., 2012; Zhang et al., 2022) allowing for a longer shelf life. As a matter of fact, functional foods have found a very important place in modern times, seeing the ever-increasing diseases such as diabetes, cardiovascular diseases, and various types of cancers.

Considering the potential health benefits of plant polyphenols (Liyana-Parthirana and Shahidi, 2005; Salminen et al., 2012; Zhang et al., 2022), the development of an efficient extraction method for isolating high concentrations of such phytochemicals deserves scientific consideration. Moreover, in vitro and animal studies have shown that the actions of such chemicals were more effective at much higher doses (Liyana-Parthirana and Shahidi, 2005). Because of the increasing consumer demands (Lourenço et al., 2019) and susceptibility of such compounds to process treatments (Wani et al., 2017), extraction protocols from natural sources that allow for complete extraction as well as retention of their full nutritional benefits and prophylactic or therapeutic potency, may be proven necessary. It has been indicated that the composition of the natural sources of plant polyphenols as well as their physicochemical and structural properties are diverse (Kaur et al., 2022; Nekkaa et al., 2021), hence utilization of a universal extraction protocol may not be conceivable.

While the conventional method of extraction can be laborious and time-consuming (Abd Manan et al., 2016; Prasad et al., 2011) as well as unable to explicitly illustrate the complete interactive effects of various parameters on the response (Isah et al., 2017), resorting to a statistical optimization procedure in the form of response surface methodology (RSM) i.e. Box-Behnken design (BBD) may prove useful. RSM is a statistical experimental protocol used in mathematical modeling (Abt et al., 2018; Gong et al., 2012) that uses multiple variables to establish optimum conditions with a reduced number of experimental runs (Marzuki et al., 2015; Mohamad et al., 2015; Yim et al., 2012). Pertinently, the method is more expedient and less costly (Abdul Wahab et al., 2014) and has been successfully employed to optimize extraction conditions for phenolic compounds from several other food products viz. wheat (Liyana-Parthirana and Shahidi, 2005), onion (Pandey et al., 2018), pink guava (Prasad et al., 2011), and stink beans (Campone et al., 2018). Herein, this present study was aimed at establishing the optimized conditions for extracting antioxidant-rich compounds from the extract of HS calyces. The parameters investigated included time, temperature, and ethanol concentration in achieving the maximum extraction yield and antioxidant activity.

Materials and Methods

Roselle (*Hibiscus sabdariffa)* was purchased from the Federal Agricultural Marketing Authorities (FAMA) in Rengit, Malaysia in February 2017. The plant was authenticated by an expert and deposited at the Herbarium Unit of the Institute of Bioscience, Universiti Putra Malaysia. The reagents used were all analytical grade and used as received without further purification. Distilled water was used for preparing solutions. Reagents 2,2-Diphenyl-2-picrylhydrazyl (DPPH) and solvents, ethanol, methanol, and chloroform were purchased from Sigma Aldrich (Milwaukee, WI, USA).

Preparation of HS extracts

The calyces were washed with tap water three times and air-dried for 7 days prior to grinding into coarse powder. HS extracts containing high quantities of antioxidant-rich compounds was prepared according to the method of Yang et al. (2013) with some minor modifications. HS powder (1.5 g) was transferred into a 150 mL round-bottom flask containing 50 mL of ethanol concentration (60, 75, 90%) and refluxed for (30–300 min) at temperature (50–90 °C). The mixture was filtered through a Whatmann filter paper No. 4 and the residues were further extracted with a fresh batch of ethanol (300 mL) and filtered once again. The supernatants of the extracts were combined and concentrated in a rotary evaporator (IKA RV 10 Digital V, German) at 55 °C and lyophilized. The extraction yield was determined using Eq. 1:

$$EY\ (\%) = \frac{\text{Extracted weight (g)} \times 100}{\text{Total sample weight (g)}} \quad (1)$$

Measurement of DPPH radical scavenging activity

Free radical scavenging activity of the HS extract was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical analysis according to the method described in Mishra et al. (2012) with some modifications. Freshly prepared DPPH (1 mL, 0.135 mM) in ethanol was added to a test tube containing the extract of HS (1 mg/mL). A negative control was prepared by adding DPPH solution (1 mL) into another test tube containing methanol (1 mL, 80%). Both mixtures were incubated in darkness for 30 mins prior to quantification by UV-visible spectrophotometer (Tecan Infinite 200 Pro, Switzerland) at 517

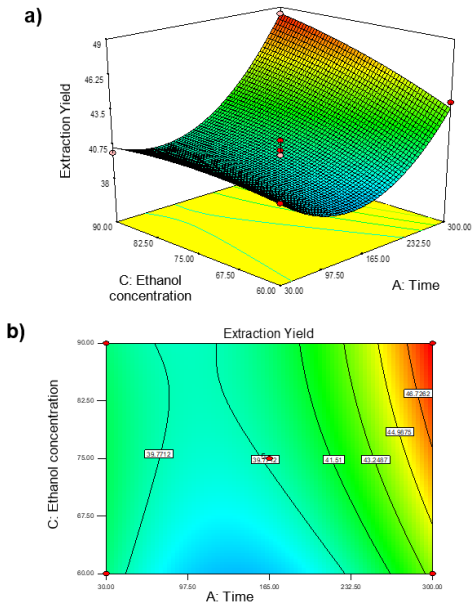

Figure 1. Response surface plot (a) and contour plot (b) showing the effect of reaction time (A) and ethanol concentration (C) and their mutual interaction in the extraction yield of HS extracts.

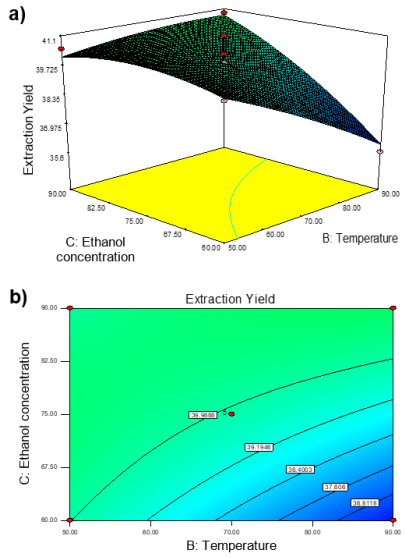

Figure 2: Response surface plot (a) and contour plot (b) showing the effect of temperature (B) and ethanol concentration (C) and their mutual interaction in the extraction yield of HS extracts.

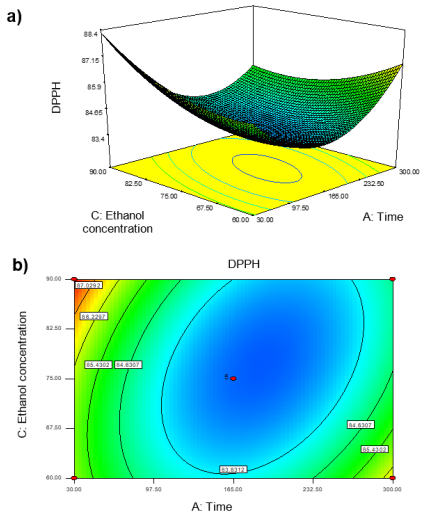

Figure 3. Response surface plot (a) and contour plot (b) showing the effect of reaction time (A) and ethanol concentration (C) and their mutual interaction in antioxidant capacity (DPPH) of HS extracts.

nm wavelength. DPPH is a commercially available radicle, and the method is simple and rapid for the evaluation of antioxidant activity of extracts based on the reduction of DPPH radical and the results are presented as percentage inhibition (Eq. 2):

$$\text{SCA (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (2)$$

Whereby $A_{control}$ and A_{sample} is the absorbance of the control sample and sample obtained from the crude extract, respectively.

Experimental design and optimization for HS extraction and antioxidant

A three-factor, three-level BBD that required 17 experiments following a fractional factorial design that consisted of 12 factorial points and 5 center points were employed to evaluate the reaction variables (time, temperature, and ethanol concentration). A software package, Design Expert Version 7.1.6 (Stat-Ease, Statistical Made Easy, Minneapolis, MN, USA) was used to fit the second-order model to the independent variables using the following Eq. 3:

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j \chi_j + \sum_{j=1}^{k} \beta_{jj} \chi_{j^2} + \sum_{i=1}^{j-1} \sum_{j=2}^{k} \beta_{ij} \chi_i \chi_j + \varepsilon \quad (3)$$

Where Y is the response (extraction yield and DPPH), i and j are the linear and quadratic coefficient, respectively, X_i and X_j are encoded independent variables (time, temperature, and ethanol concentration) and regression coefficient, k is the number of studied optimized variables in the experiment. β_0 is a constant coefficient, β_j, β_{jj} and β_{ij} are interaction coefficient of linear, quadratic, and second order terms, respectively, and the ε is the error. The variables and the levels selected for responses extraction yield and antioxidant capacity of HS were time (30–300 min), temperature (50–90 °C), and ethanol concentration (60–90%). The experiments were randomized for statistical reasons and the measurements of the extraction yield and antioxidant activity of the HS extract in each experiment were run in triplicate. The value of the correlation coefficient (R^2) was used to express the fitness of the polynomial model and a P-value < 0.05 represents the significance of the model. Analysis of variance (ANOVA) was also carried out to determine the adequacy of the constructed model to describe the observed data. Pertinently, the optimum combination of variables can be established based on ridge analysis as well as maximum analysis.

Results and Discussion

Model fitting and analysis of variance (ANOVA)

RSM is a widely accepted statistical technique capable of solving simultaneous multivariate equations from analyses of multivariate data as it entails a minimum number of experiments to predict the optimum conditions (Abdul Wahab et al., 2014; Liu et al., 2014). The graphical response surface generated in this study is the representation of the relative equation that describes the individual and cumulative effect of the experimental variables and their subsequent effect on the response (Alara et al., 2018).

In this study, the percentage of extraction yield and antioxidant properties of HS was modeled using the method of RSM, utilizing three reaction variables: reaction time (min), reaction temperature (°C), and ethanol concentration (%). The experimental designed conditions and their employed responses are tabulated in Table 1.

It is generally accepted that a good-fitting model should attain an R^2 value of at least 0.80 (El-Boulifi et al., 2014). Multiple regression analysis on the experimental data afforded the response Y_{EY} for the percentage of extraction yield and Y_{DPPH} for DPPH activity for the extraction of HS, obtainable through the second-order polynomial model Eq. 4 and Eq. 5:

$$Y_{EY}(\%) = (+39.90 + 2.79A - 0.84B + 1.23C + 0.19AB + 1.18A + 1.16BC + 3.85A2 - 0.19B2 - 0.47C2)$$

$$Y_{DPPH} = (+83.10 - 0.70A + 0.70B - 0.039C + 0.38AB - 1.00AC + 0.48BC + 2.30A2 - 0.038B2 + 0.77C2)$$

where A: time, B: temperature, C: ethanol concentration. The positive and negative signs of the terms implied the synergistic and the antagonistic effects, respectively. Hence inferring the influence of the independent variables on the extraction yield and antioxidant activity of HS. In the models, the terms AB, AC, BC and A2 were associated with synergistic effects while B2 and C2 were found to be antagonistically related for the extraction yield of HS (Eq. 4). For the DPPH activity, terms AB, BC, A2 and C2 showed synergistic association whereas AC and B2 revealed antagonistic effects (Eq. 5). While the terms AB (P-value = 0.6290) for extraction yield as well as AB (P-value = 0.0523) and BC (P-value = 0.0705) for the response of DPPH activity were found insignificant, all coefficients were retained to minimize any possible error (Table 2).

Interactive effect of factors on the percentage of extraction yield of HS

Effect of time and ethanol concentration

Figure 1 shows the effect of the reaction time and ethanol concentration and their mutual interaction on the extraction yield of HS with the temperature held constant at 70°C. The considerably higher F-value for time (110.04) over ethanol concentration (21.21) showed that the effect of the former was more impacting than ethanol concentration in achieving high percentage yields of the HS extract. The small P-value (0.0168) for AC, indicated that their interaction was significant. It was clear that the percent extraction yield of HS was significantly increased with the increasing ethanol concentration from 75% to 90%, and the highest extraction yield (46.7%) was attained at ethanol concentration between 82.50% to 90% at 300 min of extraction time. In contrast, minimum yield of the HS extract was obtained at the lowest ethanol concentration and extraction time. Pertinently,

the trend of higher extraction yields of HS observed here when the extraction time and ethanol concentration were at their highest suggested that their interaction was synergistic, consistent with the obtained positive term (+ 1.18AC) in the generated model equation (Eq. 4).

The attainment of higher yields of the HS extracts when longer extraction time and higher ethanol concentration were used was consistent with previous reports that indicated that increasing the polarity of the solvents (Prasad et al., 2011) and lengthening the contact time would promote the efficacy of the process. This is because by increasing the ethanol concentration, the solubility of the phenolic compounds in the mixture of water and ethanol will also increase. In fact, several studies have shown an excellent linear correlation between solvent polarity and extraction yield (Liyana-Parthirana and Shahidi, 2005; Majeed et al., 2016; Mohamad et al., 2015). Likewise, increasing extraction time tends to promote longer contact time between the plant cells with the solvents. Under such conditions, more solvent molecules are allowed access into the cells, thereby permitting progressive extraction of phenolic compounds into the solvents (Prasad et al., 2011; Michiels et al., 2012), consequently improving the efficacy of the extraction process.

Effect of temperature and ethanol concentration

The effect of temperature and ethanol concentration on the extraction yield of HS at a constant time of 165 min (Figure 2) clearly indicated that the effect of ethanol concentration (21.21) was more significant than that of temperature (9.92), hence implying that the former being the more impacting variable. According to the ANOVA of factors (Table 3), their mutual interaction was significant, indicated by a small P-value (0.0180). As seen in Figure 2, only a slight increase in the extraction yield of HS was observed with the increase in ethanol concentration and temperature. The highest extraction yield of HS (approximately 40 %) could be achieved under conditions of both moderate temperature and ethanol concentration, corresponding to a temperature of 65°C while the ethanol concentration was set at 70%. The synergistic interaction between both variables seen in this study was consistent with the positive coefficient (+ 1.16BC) in the model equation (Eq. 4).

According to the review of literature, a rise in the reaction temperature could disrupt the cellular constituent of plant materials (Quispe-Fuentes et al., 2022), thus enhancing the release of bounded and cell wall bioactive compounds. Moreover, higher extraction temperatures may also influence the membrane structure of plant cells by coagulating the lipoproteins, hence making them less selective (Corrales et al., 2008; Prasad et al., 2011). Likewise, the higher yields of HS seen here is also consistent with reports that described better extraction of phenolic compounds at elevated temperatures were attributable to reduction in the dielectric constant of water and solvent property, resulting in a more efficient extraction of phenolics (Prasad et al., 2011). In addition, an elevation in temperature could improve extraction of such compounds by improving their solubility, extraction and diffusion rate as well as reducing solvent viscosity and surface tension (Saldaña et al., 2021). However, the study found that raising the temperature beyond 65°C was counterproductive presumably due to increased degradation of the phenolic compounds. Such unwanted reactions would have been brought about by chemical or enzymatic degradation, or reaction with other plant components (Pompeau et al., 2009); these have been proposed as the main mechanisms that reduce extraction efficacy. Aside from incurring unnecessary increase in cost of the process, increasing the reaction temperature too high may prove futile when extracting most plant phenolic compounds, likely due to such compounds being relatively heat labile.

Interactive effect of factors on the antioxidant activity, DPPH of HS

Effect of time and ethanol concentration

For this evaluation, the temperature was held at its midpoint (50°C), the mutual effects of time and ethanol concentration on the antioxidant capacity of HS extract were observed (Figure 3). According to the ANOVA of factors, the mutual interaction of time and concentration of HS was very significant because of the small P-value (0.0007). The F-value for the effect of time (40.32) was more significant in comparison to the ethanol concentration (0.083) (Table 3) to affect the antioxidant activity in the HS extracts.

While it had been indicated in the literature that increasing the extraction time would cause a favorable increase in the concentrations of the extracted bioactive compounds (Kamaludin et al., 2016), the results of this study indicated otherwise. The antioxidant activity obtained in this work showed an increasing trend up to 38 mins of extraction time before a decline was observed. This outcome can be explained by Fick's second law of diffusion which states that after a certain period, the solute concentration in the plant matrix would achieve final equilibrium with the bulk solution (Pompeau et al., 2009). Hence, the highest antioxidant capacity in the HS extract was achieved at low extraction time signifies that the equilibrium between the two phases was reached rather quickly. In contrast, the highest antioxidant activity of the HS extract was attained when a relatively high percentage of aqueous ethanol was used as the solvent. This observation agrees well with several reports describing that ethanol has significant influence on the antioxidant activity in many plant extracts (Lee et al., 2013; Liyana-Parthirana and Shahidi, 2005; Prasad et al., 2011; Pompeau et al., 2009).

Generally, plant extracts containing high yields of phenolic contents demonstrate better antioxidant activity (Majeed et al., 2016) and using high concentrations of ethanol promotes high responses for both the extraction yield and antioxidant activity. Thus, the findings in this study support such correlations. The obtained results strongly indicate the phenolic compounds in the HS extracts were good scavengers of free radicals. Similar observations were also described by earlier studies (Alara et al., 2018; Majeed et al., 2016).

Comparison on the efficacy between RSM and other conventional methods for high extraction yield and antioxidant

Among the key considerations when extracting antioxidants from plant materials is the appropriate selection of the extraction parameters. Optimization by the one-variable-at-a-time approach (OVAT) is not recommended. In any case, the use of OVAT for optimizing processes is less accurate as the method overlooks the interactive component between each factor. Optimization by RSM circumvents this problem and offers a better prediction of optimal experimental conditions (Anastácio and Carvalho, 2013; Andrade et al., 2021). Several studies that successfully employed RSM to extract bioactive plant components have been reported (Andrade et al., 2021; Lee et al., 2016). Amado et al. (2014) employed the RSM technique to optimize extraction of antioxidants from potato peel wastes which optimum conditions that gave high concentrations of the phenolic compounds and flavonoids were reached at 89.9°C and ethanol concentrations of 71.2% and 38.6% respectively using a relatively short extraction time of 34 mins. In another study, RSM predicted the optimum condition for extracting bioactive extracts from Korean Red Ginseng (KRG) with maximum antioxidant activity (43.7%) and extraction yield (48.8%) used an ethanol concentration of 48.8%, an extraction time of 73.3 min, and an extraction temperature of 90 °C (Lee et al., 2016). Extraction from apricot powder (*Prunus armeniaca L.*) carried out by Wani et al. (2017) assisted by the RSM showed the optimum conditions for a favorable yield of polyphenols and the corresponding antioxidant potential can vary from 76.15% to 96.68% and 8.77 to 12.11 mg GAE/g, respectively.

Conclusion

The method of RSM was successfully applied to optimize the extraction of phenolic compounds from calyces of HS. The second-order polynomial models were found to give satisfactory descriptions of the experimental data for both the extraction yield as well as antioxidant activity via DPPH analysis. The results revealed that the highest percentage of extraction yield of HS at 48.44% was attained under an optimized condition of 70°C at 300 min using ethanol concentration of 90%. Similarly, the highest DPPH activity corresponding to 87.47% was attained under 70°C after 30 mins of extraction using 90 % ethanol concentration. Pertinently, the results of this study would be useful in developing large-scale extraction process for obtaining high yield of the HS extract showing high antioxidant activity suitable for use in cosmeceutical products.

Author Contributions

Royhaila conceived, designed the experiments, wrote the paper, and performed the experiments; Farrah analyzed and interpreted the data; Shahrizi revised the manuscript; Roswanira supervised the study.

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Competing financial interests

The authors have no conflict of interest.

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