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Optimization of ultrasound-assisted extraction and the antioxidant activities of Sidaguri (*Sida rhombifolia*)

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ABSTRACT

Sidaguri (*Sida rhombifolia*) is widely distributed in tropical and subtropical regions and has been reported to possess many bioactive compounds that are beneficial for human health, including polyphenolics having antioxidant activities. The objective of this study was (1) to optimize ultrasound-assisted extraction (UAE) of Sidaguri and (2) to evaluate antioxidant activities of extracts resulting from the optimized condition using radical scavenging assay of 2,2′-diphenil-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid. The optimum UAE conditions obtained were solvent to solid ratio of 26:1, temperature of extraction of 45.45°C, methanol concentration of 42%, power of sonication of 86%, and time of extraction of 5 minutes. Sidaguri obtained from locations of Kretek and Ngemplak had the highest radical scavenging activity accounting for 83.69% ± 0.30% and 82.95% ± 0.49%, respectively.

INTRODUCTION

Sidaguri (*Sida rhombifolia*) is one of the famous *Sida* species among 200 others, belonging to the Malvaceae family. This species is widely distributed in tropical and subtropical regions and has been used to treat many diseases such as headaches, tuberculosis, diabetes, malaria, hemorrhoids, wounds, rheumatic, cardiac disease, diarrhea, and skin diseases for thousands of years (Ferro *et al*., 2019; Rohman *et al*., 2020). The studies of Sidaguri have been conducted for the past 50 years. Many studies reported that Sidaguri extract provides potent free radical scavenging activity toward 2,2′-diphenil-1-picrylhydrazyl

(DPPH) radical, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS•+) radical, reducing power, superoxide scavenging activity, antibacterial activity, and anti-inflammatory activities (Ferro *et al*., 2019; Mah *et al*., 2017). These biological activities are manifestation of the secondary metabolites which are bioactive compounds produced by Sidaguri. The production of the bioactive compound was significantly affected by extrinsic factors such as climate, temperature, sun exposure, rainfall, and soil type (Kumar *et al*., 2017). The fact that Sidaguri produced various metabolites which are beneficial to human health makes it necessary to analyze those metabolites and bioactive compounds expressed by antioxidant activities for controlling their quality comprehensively.

Extraction is a critical analytical step to evaluate those bioactive compounds from solid samples prior to their analytical determination. However, the extraction using conventional method is time-consuming and needs more solvent leading to the massive

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production of the waste of organic solvents which are harmful to human health and environment (Khoddami *et al.*, 2013). The combination of these facts with the recent trends of increasing awareness in environmental, economic, and safety considerations make the extraction techniques move to use green extraction techniques to be used in food, cosmetics, and pharmaceutical industries (Irnawati *et al.*, 2021).

Several novel extraction techniques have been developed, such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and pressured liquid extraction, in order to obtain a higher concentration of phenolic compounds from their sources in a shorter time with a lower volume of solvent. UAE is more considered over other novel extraction methods due to its lower cost and comfort to operate (Tao *et al.*, 2014). The use of UAE is not limited by a solvent, contrary to MAE that should use solvents that are transparent to microwaves. Therefore, UAE could be run for a wide range of targeted compounds. In addition, the application of UAE also inhibits the degradation of the bioactive compounds (Wang *et al.*, 2016).

The mechanism of UAE is based on the cavitation effect, which leads to cell wall rupture generating the solvent to penetrate into the plant material to wash out the desired compounds efficiently (Wu *et al.*, 2019). The formation of the cavitation bubble is related to the power of ultrasound employed. Aside from the power of ultrasound, there are some other factors affecting the extraction efficiency, including the concentration of solvent, extraction temperature, and ratio of solvent to solid (Setyaningsih *et al.*, 2019). Therefore, those factors require optimization to obtain extract with the optimum antioxidant activity.

The optimization of extraction could be conducted using Box–Behnken design (BBD) to describe the optimum condition of those selected factors. The BBD is selected due to the fact that BBD can predict the parameters of the quadratic model, build sequential designs, and detect lack of fit, thus making it suitable for response surface methodology (RSM) (Ferreira *et al.*, 2007). From literature studies, the extraction of Sidaguri was carried out using one variable at one time which neglected the interaction among factors contributing to the extraction efficiency; therefore, experimental design-based extraction is needed to overcome this problem. In addition, there are no reports regarding the antioxidant activities and phenolics contents from Indonesian Sidaguri; therefore, the objectives of this study were (1) to optimize the extraction of Sidaguri having antioxidant activities, (2) to evaluate the antioxidant activities of extracts resulting from the optimized condition using DPPH radical scavenging assay and Trolox equivalent antioxidant capacity (TEAC) assay, and (3) to evaluate the total phenolic contents (TPCs) using Folin–Ciocalteu method.

MATERIALS AND METHODS

Materials

The leaves of Sidaguri were harvested from several regions in Daerah Istimewa Yogyakarta (Depok, Imogiri, Kretek, Cangkringan, and Ngempak) and Central Java (Manisrenggo, Borobudur, and Mungkid) in the morning, during the stages of flower appearance. Distilled water and purified water were purchased from PT, Ikapharmindo Putramas, Indonesia. The identification of plant material was carried out in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, under the supervision of botanist Dr. Djoko Santosa. Methanol for analysis, Na_2Co_3, and Folin–Ciocalteu reagent were purchased from E. Merck (Darmstadt, Germany); DPPH, ABTS, potassium persulfate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and gallic acid 97.5% were purchased from Sigma (Aldrich, USA).

Sample preparation

The preparation of powder from leaves was carried out according to Rafi *et al.* (2020) with slight modification. The leaves of Sidaguri from different regions were sorted from the herb and then dried using the heating method using an oven at a temperature of 60°C for 24 hours. The size reduction of the dried leaves is carried out using a coffee grinder that is available on the market and the homogenization is carried out using a household sieve. The dried leaves of Sidaguri were then pulverized into *powder using* a disk mill and the obtained powder was used for further analytical steps.

Optimization of UAE

The Sidaguri leave powder is weighed as much as 0.5 g, placed in a 30 ml vial, and extracted using UAE. The UAE used in this study is ultrasound probe Hielcher UP200ST with ultrasonic frequency of 24 kHz. The optimum condition of UAE was obtained by evaluating four factors, namely (1) solvent to solid ratio, (2) temperature of extraction, (3) MeOH concentration (in distilled water), and (4) sonication power using BBD based on response to radical DPPH activity (Table 1). The extraction time was evaluated subsequently of 5, 10, 15, 20, and 25 minutes. The optimum UAE condition was subsequently used for extraction of Sidaguri leave powder and the extracts obtained were subjected to its antioxidant activities and total phenolic contents.

Experimental design

Box–Behnken randomized block design is employed to obtain the optimum extraction condition by evaluating the four aforementioned factors. Twenty-seven experiments with three repetitions of center points along with responses were statistically analyzed using Statgraphics Centurion XVII (Statpoint Technique, Inc., Warrenton, VA). The experimental design used along with responses obtained was compiled in Table 2.

Antioxidant activity using DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to Muritala *et al.* (2018) with slight modification. A 0.5 g of dried Sidaguri powder is extracted with 5, 10, and 15 ml of 0%, 50%, and 100% methanol/distilled water mixture solvent (according

Table 1. Codes of independent variables and their actual levels.

Factors	Code	−1	0	+1
Solvent to solid ratio	X_1	10:1	20:1	30:1
Temperature of extraction (°C)	X_2	30	45	60
MeOH concentration in water (%)	X_3	0	50	100
Power of ultrasound (%)	X_4	20	60	100

Table 2. Experimental design of Box–Behnken for four factors along with responses.

Run	Block	X_1	X_2	X_3	X_4	Observed value (% RSA)	Fitted value (% RSA)
1	1	0	0	1	−1	72.5352	76.8341
2	1	1	−1	0	0	57.3944	57.0575
3	1	−1	1	0	0	84.507	79.8279
4	1	0	0	1	1	50.5869	49.4062
5	1	0	0	−1	−1	81.338	77.9561
6	1	1	1	0	0	27.3474	32.9192
7	1	−1	−1	0	0	14.2019	19.2727
8	1	0	0	−1	1	31.4554	19.4641
9	1	0	0	0	0	64.4366	71.0651
10	2	0	1	−1	0	33.0927	34.9884
11	2	0	−1	−1	0	84.8375	74.7438
12	2	1	0	0	−1	83.2732	84.1814
13	2	0	0	0	0	35.0181	31.0561
14	2	−1	0	0	−1	58.0024	69.7492
15	2	−1	0	0	1	83.0325	83.7992
16	2	0	1	1	0	64.0193	62.1262
17	2	1	0	0	1	30.0842	32.7611
18	2	0	−1	1	0	51.6245	49.579
19	3	0	0	0	0	78.2097	83.6239
20	3	1	0	−1	0	48.6455	50.9375
21	3	0	1	0	−1	31.4488	31.7991
22	3	0	1	0	1	29.3286	26.613
23	3	−1	0	−1	0	37.2203	29.4559
24	3	−1	0	1	0	81.5077	84.182
25	3	0	−1	0	1	86.3369	86.5484
26	3	1	0	1	0	87.7503	81.7275
27	3	0	−1	0	−1	63.8398	69.4001
28	4	0	0	1	−1	76.9536	84.3127
29	4	1	−1	0	0	75.5644	64.5361
30	4	−1	1	0	0	83.7981	87.3065
31	4	0	0	1	1	71.3147	56.8848
32	4	0	0	−1	−1	82.8685	85.4347
33	4	1	1	0	0	30.0662	40.3978
34	4	−1	−1	0	0	14.741	26.7513
35	4	0	0	−1	1	31.7397	26.9427
36	4	0	0	0	0	84.0637	78.5437
37	5	0	1	−1	0	28.6195	34.7167
38	5	0	−1	−1	0	85.4097	74.472
39	5	1	0	0	−1	86.7565	83.9097
40	5	0	0	0	0	27.7217	30.7843
41	5	−1	0	0	−1	59.7082	69.4774
42	5	−1	0	0	1	85.1852	83.5275
43	5	0	1	1	0	58.5859	61.8545
44	5	1	0	0	1	33.7823	32.4894
45	5	0	−1	1	0	54.7699	49.3072
46	6	0	0	0	0	84.9873	87.2506
47	6	1	0	−1	0	48.6005	54.5642
48	6	0	1	0	−1	32.9517	35.4257
49	6	0	1	0	1	31.1258	30.2397
50	6	−1	0	−1	0	34.3511	33.0825
51	6	−1	0	1	0	86.3868	87.8087
52	6	0	−1	0	1	87.2774	90.1751
53	6	1	0	1	0	87.7863	85.3541
54	6	0	−1	0	−1	83.4606	73.0267

to experimental design) and 100 µl was taken and was added to 2.9 ml DPPH (91.296 µmol dissolved in methanol) in a test tube. The solution is incubated for 50 minutes in a dark place at room temperature. The absorbance of the solution is measured using spectrophotometer (Genesys 10S UV-Vis, Japan) at a maximum wavelength of 516 nm. The percentage of radical scavenging activity radical scavenging activities (% RSA) is calculated as

$$\%RSA = \frac{\text{Absorbance of blank} - \text{absorbance of sample}}{\text{absorbance of blank}} \times 100\%$$

The blank used was 2.9 ml DPPH 100 µl of methanol/water mixture and treated the same as the sampling procedure and recorded at 516 nm.

ABTS radical cation decolorization assay

ABTS$^{•+}$ assay was performed according to Prommuak *et al.* (2008) with slight modification. This assay was carried out by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution and allowing the mixture to stand in dark at room temperature for 12–16 hours before use. The procedure is carried out by adding 30 µl of extracted dried Sidaguri powder that the extraction condition obtained from the optimization of extraction and 3 ml of the ABTS$^{•+}$ radical cation solution which was diluted with methanol at a ratio of 1:10 and the absorbance was recorded at 731 nm against blank after 6 minutes. The standard curve is prepared using seven different concentrations of Trolox (2, 5, 7, 10, 12, 15, and 17 mg/l) and treated the same as the sampling procedure. The results were expressed as mg Trolox Equivalent per gram Sidaguri powder (mg TE/g sample).

Total phenolic content assay

Determination of phenolics content was carried out according to Chun *et al.* (2003) with slight modification. A 0.1 ml of sample (extracted dried Sidaguri powder that the extraction condition obtained from optimization of extraction) is placed to 10 ml volumetric flask and added to 0.4 ml of Folin–Ciocalteu reagent, 4 ml of Na_2CO_3 7% (*w/v*), and 5.5 ml of purified water. The solution is incubated for 2 hours and the absorbance of the solution is recorded at 765 nm. The standard curve is prepared using different concentrations of gallic acid (2–8 mg/l dissolved in purified water), treated the same as the sampling procedure, and recorded at 765 nm. The equation obtained from the standard curve

regression ($R^2 \geq 0.99$) was employed to calculate the concentration of TPC in extracted Sidaguri powder. The levels of TPC were expressed as mg gallic acid equivalent/kg samples.

Data analysis

BBD used as the design of experiment for the optimization of UAE was created and analyzed using Statgraphics Centurion XVII (Statpoint Technique, Inc., Warrenton, VA) with RSA as the response. The confidence interval was set at 95%.

RESULTS AND DISCUSSION

The preliminary study of extraction optimization is carried out by screening the suitable solvent mixture to be one of the optimization variables. Three types of solvents that have been examined in this study were methanol, ethanol, and water. The antioxidant activity was evaluated using RSA toward DPPH radical. The result in Figure 1 shows that the best solvent for extracting the active compounds as antioxidants is water, followed by methanol and ethanol. The significance of effects was determined by analysis of variance (ANOVA) single factor, followed by least significant difference (LSD) since they showed a significantly different result. Therefore, in this study, the mixture of methanol/water was employed as a variable in optimizing extraction condition to provide high antioxidant activity.

Optimization of UAE

Several factors affecting the efficiency of antioxidant extraction from Sidaguri such as solvent to solid ratio (X_1), temperature of extraction (X_2), methanol concentration (X_3), and power of sonication (X_4) were evaluated. The optimization was facilitated by BBD. Fifty-four extractions were finished based on the experimental design, including three center points and one repetition (Table 2).

Standardized Pareto charts were employed to obtain the significant variables for antioxidant activity. The length of each bar of the Pareto chart is proportional to the value of a *t*-statistic calculated by dividing the corresponding estimated effect by its standard error. The bars that exceed the vertical line demonstrate the factors or combination of factors that possess a significant
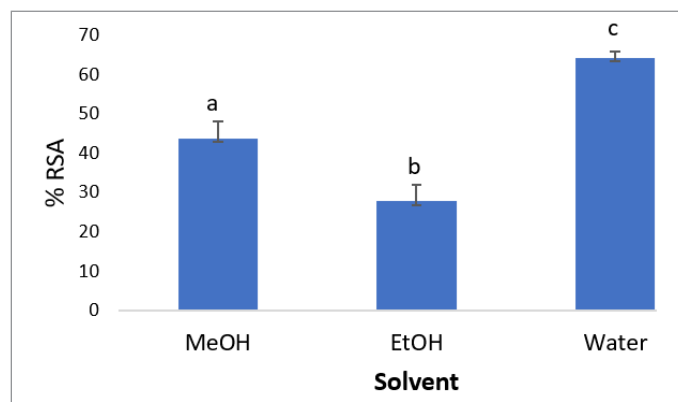
effect on the response (*p*-values < 0.05) sorted from the highest to the least contribution.

Based on the Pareto chart, the factors provide significant effects to the response, namely sample to solvent ratio, extraction time, methanol concentration, power, and other combination factors (Fig. 2).

RSM results were calculated based on the BBD as the experimental design in order to create a regression model for response. The obtained mathematical quadratic model was used to estimate a predictive equation for the RSA by only considering the significant factors. Only the significant factors (*p* < 0.05) were considered for the optimization to avoid variability if the number of factors for an optimized design is higher. The second-order polynomial equation for the model obtained for RSA is as follows:

$$RSA\ (\%) = 85.5947 + 6.71709x_1 + 3.26771x_2 - 4.08682x_3 + 4.23048x_4 - 6.93621x_1{}^2 - 45.0591x_3{}^2 - 10.9595x_3x_4$$

The predictive capability of the regression model was 0.9362 which was obtained from evaluating the observed versus predicted values (Fig. 3). The high slope values demonstrate that the model equations are convenient to represent the response surface. Table 3 describes the optimum point that is in the range of the studied level of each factor with RSM results that are shown in Figure 4.

The optimum solvent to solid ratio is located at a coordinate of 0.5466, which is 26:1. The positive value indicates that the higher the solvent to solid ratio, the higher the obtained response. This is complying with the statement that gradient of
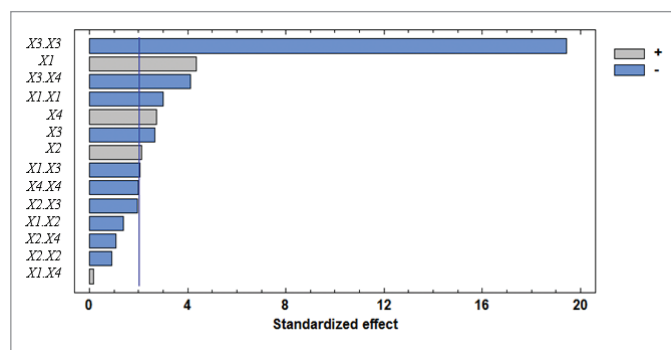

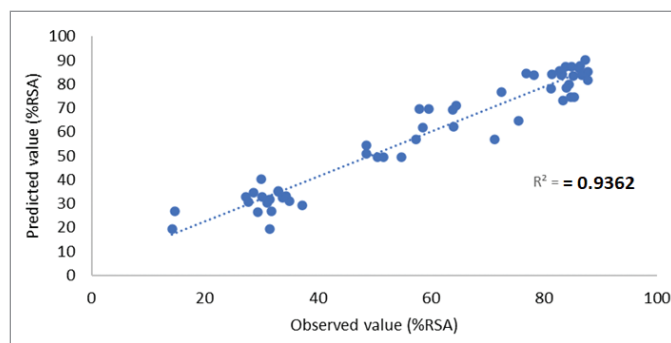**Figure 2.** Standardized Pareto chart for the studied variables.

Figure 3. Prediction capability of the regression model (the correlation between observed and predicted values of RSA) of Sidaguri.

Figure 1. Comparison of three types of solvent for extracting Sidaguri using response of antioxidant activity. RSA: radical scavenging activities using DPPH radicals.

concentration is considered to be the driving force of mass transfer, which was greater when a higher solvent to solid ratio was used, resulting in an increase in the diffusion rate until the equilibrium has reached (Nour *et al.*, 2016). Even though too much solvent usage could extract the majority of bioactive compounds, it can make the extract too dilute which requires additional concentration step and not enough solvent can lead to an insufficient extraction (Jakobek *et al.*, 2015). From those statements, it is confirmed that optimum solvent to solid ratio (26:1) is the equilibrium point; thus, it could wash out the compounds responsible for antioxidant activity effectively.

Table 3. Optimum coordinate points of each factor.

Factor	Low	High	Coordinate	Optimum
X_1	−1	1	0.546644	26:1
X_2	−1	1	0.067194	45.45°C
X_3	−1	1	−0.16169	42%
X_4	−1	1	0.653628	86%

Extraction temperature leads to softening the plant tissues, decreasing the viscosity of the solvent, increasing the diffusion coefficient of the substance, breaking down certain chemical bonds, and increasing the solubility of biochemical compounds; thus, it can help to release subjected compounds from the evaluated samples (Al-Farsi and Lee, 2008; Dai and Mumper, 2010; Setford *et al.*, 2017). Nevertheless, cavitation is reduced at higher extraction temperatures because voids are filled with solvent vapors, leading to less violent collapse. It can be concluded that the optimized extraction temperature (45.45°C) could extract the subjected compounds in Sidaguri and it also produces optimum cavitation.

The optimum methanol concentration of 42% is located at the coordinate of −0.1615 which negatively affects the response. The negative coordinate indicates that, before reaching the optimum methanol concentration, the lower the methanol concentration is, the more the compounds responsible for antioxidant activities are extracted. At this optimum methanol concentration, the optimum antioxidant level produces the highest antioxidant activities. Thus, in accordance with the principle of like dissolve like, the result
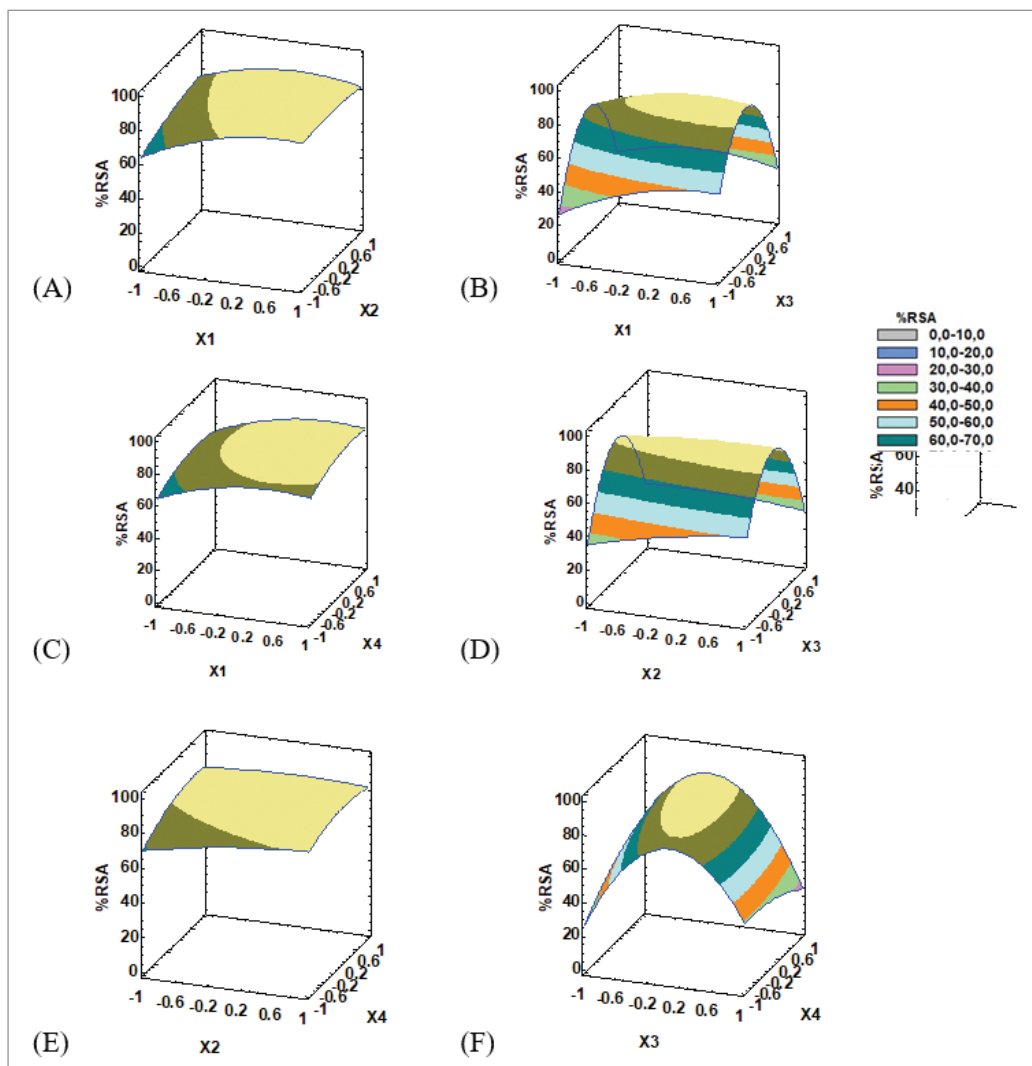

Figure 4. RSM plot of the antioxidant optimization: (A) X_1 versus X_2, (B) X_1 versus X_3, (C) X_1 versus X_4, (D) X_2 versus X_3, (E) X_2 versus X_4, and (F) X_3 versus X_4.

shows that the polarity of 42% methanol in water is similar to the polarity of the majority of compounds that possess antioxidant

The optimum sonication power is located at the coordinates of 0.6596 which is 86% and has a positive effect. The use of high sonication power would create a great cavitation effect. At this level, the cavitation effect could rupture the plant wall entirely but not break the subjected compounds. This result agrees with other studies conducted by Saifullah *et al.* (2020) reporting that higher power is more suitable for the extraction of bioactive compound.

The interaction between variables of methanol concentration and power of ultrasound (X_3X_4) was found significant ($p < 0.05$), with a negative effect. Meanwhile, quadratic effects, which are methanol concentration (X_3X_3) and solvent to solid ratio (X_1X_1), are also found significant ($p < 0.05$) and provide a negative effect on the response.

Extraction time is considered one of the influencing factors to extraction efficiency; thus, the optimization of extraction time is necessary to be conducted. A kinetic study was used to obtain the optimum extraction time by applying five different extraction times, which are 5, 10, 15, 20, and 25 minutes, under the optimized condition. The longer the extraction time is, the longer the sample and solvent exposed to high temperature would be; it would cause a loss of solvent due to vaporization, which affects the solvent to solid ratio and leads to antioxidant losses through oxidation. Based on this result, the optimum UAE conditions obtained were solvent to solid ratio of 26:1, temperature of extraction of 45.45°C, methanol concentration of 42%, power of sonication of 86%, and time of extraction of 5 minutes.

Antioxidant activities of Sidaguri

The optimum extraction condition was applied to extract antioxidant compounds contained in Sidaguri leaves from several different regions, including the Special Region of Yogyakarta (Depok, Imogiri, Kretek, Cangkringan, and Ngemplak) and Central Java (Manisrenggo, Borobudur, and Mungkid). Another antioxidant activity assay that was used to evaluate antioxidant compounds in Sidaguri aside from RSA toward DPPH radical was the TEAC method known as ABTS radical cation decolorization assay. The mechanism of those two assays is pretty similar; the antioxidant compound in Sidaguri would donate the hydrogen atom to the DPPH and ABTS radicals; therefore, the radicals become stable and the oxidation chain reaction would be stopped.

The result of RSA toward DPPH radical and TEAC of Sidaguri from eight regions evaluated its significance by ANOVA single factor, followed by LSD. The RSA values of Sidaguri from eight regions are 65.58% ± 1.38% to 83.698% ± 0.30%. Imogiri possesses the lowest RSA value; meanwhile, Kretek and Ngemplak are having the highest RSA value accounting for 83.69% ± 0.30% and 82.95% ± 0.49%, as shown in Figure 5. The different annotation of the bar indicates that the RSA is significantly different. The correlation between RSA using DPPH radical and TPC yielded coefficient of determination (R^2) of 0.3805, which means that TPC does not really contribute to the RSA toward DPPH radical. The antioxidant activity using ABTS assay as expressed by TEAC of Sidaguri from eight regions is shown in Figure 6. The highest TEAC value was obtained from regions of Cangkringan and Ngemplak, accounting for 285.04 ± 59.35 and 320.81 ± 15.09 mg
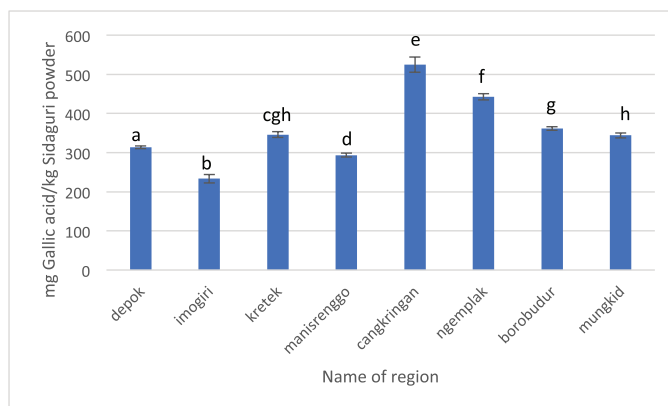

Figure 5. RSA using DPPH radicals of Sidaguri extract extracted using the optimized condition of UAE. Different letters indicate significant difference ($p < 0.05$).
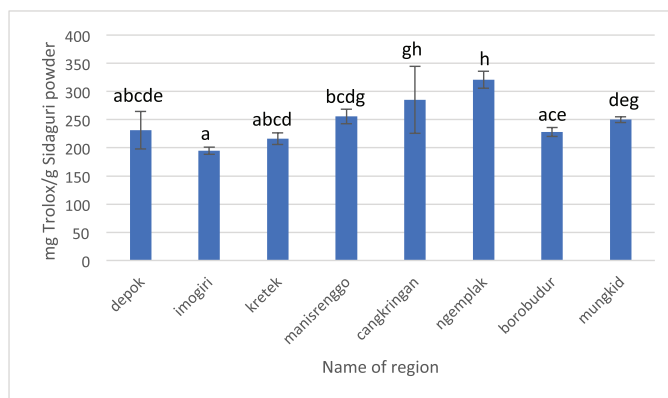

Figure 6. RSA using ABTS radicals of Sidaguri extract extracted using the optimized condition of UAE. Different letters indicate significant difference ($p < 0.05$).
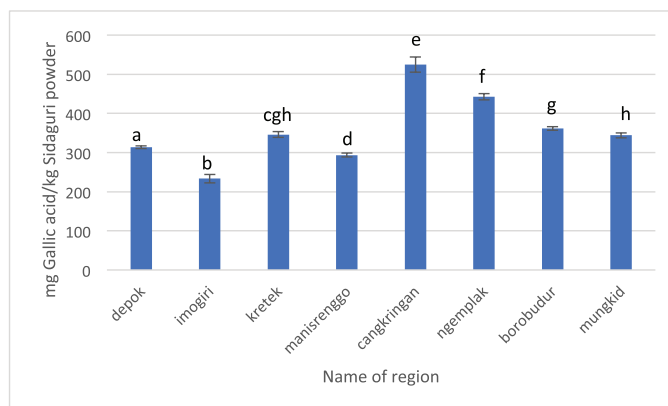

Figure 7. TPC of Sidaguri extract extracted using the optimized condition of UAE. Different letters indicate significant difference ($p < 0.05$).

TEAC/g Sidaguri powder, respectively. The correlation between TEAC and TPC, as expressed by R^2-value, is 0.5923.

Total phenolic content

The TPC result of Sidaguri obtained from eight regions is demonstrated in Figure 7. It can be seen that the TPC value

ranges from 233.34 ± 10.76 to 525.06 ± 19.61 mg gallic acid/kg Sidaguri powder. The lowest TPC value belongs to Imogiri, while the highest TPC value is possessed by Cangkringan. It is clear that the TPC value of Sidaguri from eight regions has a wide range, shown by the TPC value of Cangkringan which is more than twice higher than Imogiri.

CONCLUSION

The factors affecting the efficiency of UAE were solvent to solid ratio, temperature of extraction, amplitude extraction, and methanol concentration. Solvent to solid ratio, temperature of extraction, and amplitude extraction gave the positive effect on the response, while methanol concentration exhibited gave the negative effect. The optimum conditions were solvent to solid ratio of 26:1, temperature of extraction of 45.45°C, methanol concentration of 42%, amplitude of extraction of 86%, and extraction time of 5 minutes. The RSA of Sidaguri extract extracted from the optimum condition of UAE condition were 65.5 ± 1.38% to 82 ± 0.47% (using DPPH radicals) and 194.60 ± 6.51 to 320.81 ± 15.09 mg Trolox/g Sidaguri powder (ABTS assay).

ACKNOWLEDGMENT

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

PUBLISHER'S NOTE

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