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OPTIMIZATION OF PHENOLS EXTRACTION FROM ROSELLE (*Hibiscus sabdariffa*) BY MICROWAVE ASSISTED EXTRACTION AS ANTIBACTERIAL AND ANTIOXIDANT AGENTS

OPTIMASI EKSTRAKSI FENOLIK DARI ROSELA (Hibiscus sabdariffa) BERBANTU GELOMBANG MIKRO SEBAGAI BAHAN ANTIBAKTERI DAN ANTIOKSIDAN

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ABSTRAK

Kelopak bunga rosela merupakan sumber potensial senyawa phenolik. Untuk mendapatkan senyawa bioaktif tersebut digunakan metode ekstraksi berbantu gelombang mikro yaitu ekstraksi menggunakan alat microwave skala rumah tangga. Response Surface Method digunakan untuk menentukan kondisi optimum variabel ekstraksi, yaitu daya gelombang mikro, konsentrasi etanol dan lama waktu ekstraksi. Kondisi optimum proses ekstraksi yaitu pada daya gelombang mikro 250 Watt, konsentrasi etanol 78,36% dengan lama waktu 4,91 menit. Hasil verifikasi penelitian total phenol yang diperoleh pada kondisi optimum adalah 23,77 ± 0,25 mg/g, lebih rendah dari estimasi optimasi proses, yaitu 24,61 mg/g. Kandungan anthosianin, vitamin C dan rendemen MAE berturut turut 14,80 ± 0,08; 10,74 ± 0,14 mg/g dan 22,09 ± 3,3%. Aktivitas antioksidan ditunjukkan dengan zona hambat yang terbentuk 12,6 ± 0,6 mm terhadap E. coli dan 11,6 ± 0,3 mm terhadap S. aureus. Aktivitas antioksidan ditunjukkan dengan nilai IC_{50} 202,47 µL/mL

Keywords: microwave assisted extraction, Phenols, rosella, response surface method

ABSTRACT

Roselle calyx is rich with phenolic substances. In this study, microwave-assisted extraction was used to extract the phenolic compounds from Roselle calyx. By using response surface methodology, the effects of microwave output power, ethanol concentration and extraction time on total phenols yield were investigated and the optimal conditions were determined as follows: microwave output power 250 W, ethanol concentration 78.36% and extraction time 4.91 min. The estimated values for total phenols yield was 24.61 mg/g obtained at these conditions. The yield of a verification experiment at the optimum condition was 23.77 ± 0.25 mg/g total phenols higher than those of conventional method 19.84 ± 0.46 mg/g was obtained. The detailed yield consist of anthocyanin, vitamin C and yield of microwave assisted extraction were 14.80 ± 0.08; 10.74 ± 0.14 mg/g and 22.09 ± 3.3% which were higher and significantly different than conventional extraction , which were 9.28 ± 0.04; 9.99 ± 0.16 mg/g and 16.18 ± 1.9%, respectively. Inhibitory zone was 12.6 ± 0.6 mm against *E. coli* and 11.6 ± 0.3 mm against *S. aureus.* IC_{50} value of the extract was 202.47 µL/mL. Compared with conventional method, with inhibitory zone 10. 2 ± 0.4 mm against *E. coli* and 9.8 ± 0.6 mm against *S. aureus.* Antioxidant activity expressed with IC_{50} value 293.09 µL/mL.

Keywords: microwave assisted extraction, Phenols, roselle calyx, response surface method

INTRODUCTION

Roselle (*Hibiscus sabdariffa)* is also known as rosella in Indonesia. Initially, it is cultivated in this region for its fiber, but now also for leaf, fleshly calyx and seed according to their respective properties. The thick red and fleshly cup shaped calyx of the flower are consumed worldwide as a cold beverage and as warm tea. This plant is also used in traditional medicine against many complaints that including high blood pressure, liver diseases and fever (Tsai *et al.*, 2004).

Roselle as medicinal plant are known to produce bioactive compounds which react with other organism in environment, inhibiting bacterial growth. These substances that inhibit pathogen and have little toxicity to cell are considered prospective candidates for developing new antimicrobial drug. According to Pietta (2000), dried calyces contain the flavonoid gossypetin, sabdaretin, hibiscetin and anthocyanin.

Mourtzinos *et al*. (2008) stated that the calyx of Roselle was rich in phenolic compounds including anthocyanin. It was reported that those compounds could be considered as a great source of natural antioxidants. As anthocyanin are derivatives of the basic flavylium cation structure, which has an electron deficient nucleus, they generally are highly reactive. This reactivity shows the capabilities of anthocyanin as antioxidant. In the other hand, this

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reaction usually involves decolorization on the pigment. The rate of anthocyanin destruction depends on many factors such as pH, temperature, intermolecular copigmentation, ascorbic acid, oxygen, etc.

Usually, a conventional techniques as heating, boiling or refluxing can be used to extract the phenols. However the disadvantages are the loss of phenols due to oxidation, hydrolysis and ionization during extraction as long as the long extraction time. Other techniques such as Supercritical Carbondioxide Extraction, Subcritical Water Extraction, Ultrasonic Assisted Extraction and Microwave Assisted Extraction (MAE) have become interesting alternatives for the conventional methods. Among these, MAE is the most economical and simplest technique for extraction of many plant derived compound (Li *et al.*, 2009; Wang *et al*., 2010). Product recovery by microwave is generally attributed to its heating effect, which occurs due to the dipole rotation of the solvent in the microwave field (Zhang *et al*., 2005; Hemwimon *et al*., 2007). Nevertheless, no reports on MAE of phenol compounds from roselle have been published, thus this experiemen using MAE technique should be found.

Based on the dependent characteristic of phenols, and the need to find the most efficienct extraction have emphasized the need of optimizing the extraction process. The Response Surface Method (RSM) is a collection of mathematical and statistical technique for the investigation and modeling of complex problem processes whose response of interest is influenced by several variables and objective to optimize this response (Montgomery, 2001). RSM takes interactions into consideration and optimizes the process parameters to reasonable range, with the advantage of less the number of replicates and the total time required to perform the experiments (Lee *et al*., 2006). RSM uses an experimental design such as the central composite design (CCD) to fit a model by least squares technique. There has been no report on optimizing MAE of phenols from roselle using RSM technique. The objectives of the work were to establish an optimised condition of MAE for roselle calyx phenols, and to characterize of the extract of the optimum condition.

MATERIAL AND METHODS

Material

Roselle calyxes were bought from Beringharjo Market, Yogyakarta Indonesia. Solvent used in this experience was ethanol (p.a, 101986, Merckmillipore, Germany).

Extraction

The dried roselle calyxes were ground for 1 minute using grinder and sieved in 60 mesh. The extraction with various concentrations of ethanol (50%, 60%, 70%, 80%, and 90%), microwave power extraction (100, 175, 250, 325, and 400 W) and time of extraction (1, 3,5,7, 9, and 11 minutes) were conducted for each parameter using non factorial design. Microwave used for extraction was Electrolux EMM 2007X. The extraction was done in pH 2, the ratio dried roselle and solvent 1:10 w/v (wet weight), 10 g dried roselle powder, in 100 mL solvent. The slurry was radiated in microwave oven at regular intervals (one minute radiation and two minute off) to keep the temperature not rise above the boiling point (Li *et al*., 2009). Roselle extract was filtered and concentrated with vacuum evaporator at 70^{o}C, 44 cmHg and blowing with N_2 to ensure the solvent totally evaporate. The main response parameters that analyzed were total phenols. The best combination was also analyzed vitamin C, (AOAC, 2000) and total anthocyanin content (Fuleki and Francis, 1968).

Determination of Total Phenols

Total phenols were determined by the Folin-Ciocalteu method of Chew *et al.* (2009). Briefly, 1.5 mL Folin-Ciocalteu reagent (10% v/v) was mixed with 1.5 mL 7.5% (w/v) Na_2CO_3 solution, then 0.4 mL sample solution was added. After a 90-min incubation at room temperature in dark, the absorbance was measured at 765 nm using Spectrofotometer UV Vis 1800 Shimadzu. Gallic acid was used as a standard compound for the standard curves. The results were presented in mg Gallic acid equivalent (GAE)/g extract. All the experiments were carried out in four replicates.

Screening of Antibacterial Activity (Doughari, 2006).

Screening of antibacterial activity of the plant extract was performed by disc diffusion technique which is highly effective for rapidly growing microorganism. The 20 µL test microorganism were inoculated on to the respective NA medium by pour plate method with 24 hours incubation. After solidification the filter paper disc (6 mm diameter) impregnated with 10 µL crude extract sample, standard antibiotic (Kloramfenicol) and a blank disc impregnated with 10 µL respective solvent had included. This screening was tested against gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Escherichia coli.* The inoculated plates were incubated for 24 hours at 30^{o}C. The antibacterial activities of the extracts were determined by measuring the clearance zone surrounding the disc.

Minimum Inhibitory Concentration and Minimum Killed Concentration (Doughari, 2006).

Ethanol extract with varying concentration were added to 1 mL NB containing of test bacteria. Each of the solution was incubated at 37^{o}C, 24 hrs. The lowest concentration of an antimicrobial that will inhibit the visible growth of microoaganism is

expressed as the MIC value. This clearance solution was mixed with NA medium and poured to the plate incubated at 37°C for 24 hours. The Minimum Killed Concentration was determined by the least concentration extract without any bacteria growth.

Antioxidant Activity (Al Hashimi, 2012)

0,6 mL of sample was dissolved in 0.12 mL of 98% ethanol and 2.88 mL of a 2.51% linoleic acid and 9 mL of a 40 mM phosphate buffer (pH 7.0) were added. The mixture was incubating at 40°C in a test tube in the dark for 3 days (72 hours). After incubation, a 0.1 mL was taken from the mixture and diluted with 9.7 mL of 75% ethanol, followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Precisely three minutes after adding the 0.1 mL of 20 mM ferrous chloride in 3.5% hydrocloride acid, the absorbance of the red color was measured at 500 nm, using Spectrofotometer UV Vis 1800 Shimadzu. The level of lipid peroxidation inhibition by each fraction was calculated from the absorbance ratio to that of a blank without any sample. A half lipid peroxidation inhibition expressed with $IC_{50.}$

Antioxidant activity (%) =

$$\frac{\text{Control absorbance - sample absorbance}}{\text{control absorbance}} \times 100\%$$

Experimental Design

One response was measured: Total phenolic yield (Y), defined as the ratio of total phenols in the extract to total amount of raw material expressed as GAE milligrams per gram of raw material (wet weight). Each of variables to be optimized was coded at 3 levels: -1, 0, and 1. A Central Composite Design (CCD), was arranged to allow for fitting of a second-order model. The model proposed for the response (Y) was:

$$Y = \beta_0 + \beta_i x_i + \beta_{ii} x_i^2 + \beta_{ij} x_i x_j + €$$

Where $_0$ was the value of the fitted response at the center point of the design, which is point (0, 0, 0). $_0$, $_i$, $_{ii}$, and $_{ij}$ were the constant, linear, quadratic and cross-product regression terms, respectively.

RESULT AND DISCUSSION

The Effect of Ethanol Concentration on Total Phenol Extraction

The influencing factors on MAE were ethanol concentration, microwave power and time of extraction. To determine the range for each variable, and degree polynomial should be used, the influencing factors must be investigated separately by doing the single experiment for every variable (Li *et al*., 2009; Wang *et al.*, 2010).

Solvent selection, which is fitted with the method of extraction used, was the first step to develop the method of extraction included MAE. Ethanol was used in this research because it nontoxic and relative commonly used. Methanol was not used in this investigation although having higher dissipation factor. It could absorb the microwave power and change to heat energy better, but the toxicity was higher made it not food grade (Hemwimon *et al.*, 2007). For this reason, additions ethanol with water have to be done. Water was added to reach the concentration as follows: 40, 50, 60, 70, 80 and 90%. In these experiment, variables were used 250 Watt of microwave power and 5 minutes extraction time.

The result of the research is shown in Figure 1. Ethanol concentration influenced the yield, of phenols. The yield of phenol was increasing with the increasing of ethanol concentration. However the increase of ethanol concentration above 80% made the yield of phenol decreased. In extraction process, the polarity of solvent and the substances play an important role

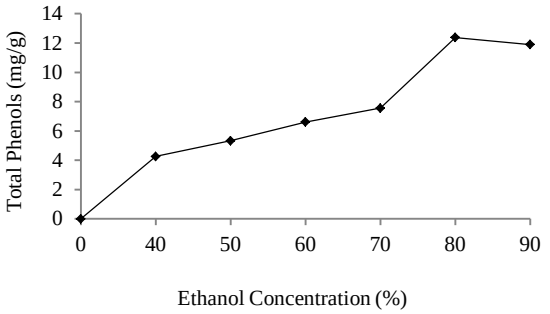


Figure 1. The effect of ethanol concentration to the yield of total phenols

The polarity of solution is determined by its dielectric constant. Markom (2007) stated that dielectric constant of water and ethanol respectively were 80.20; 24.30 and according to Durst and Worldstad (2005) in roselle almost 80-90% phenols content was anthocyanin. The polarity of anthocyanin was classified as semi polar which is around 30-40 (Richter *et al.*, 2006), mean that the polarity of phenols as a result of secondary metabolism on plant was fitted with the polarity of ethanol 80%. It was decided to use the ethanol 80% for the next experiment.

The Effect of Extraction Time to The Yield of Phenols

Extraction time is the factor which must have to be investigated to increase the effectiveness of extraction. The research was done in varying time: 1, 3, 5, 7, 9 and 11 minutes and 250 Watt of microwave power. Figure 2 shows that the yield of total phenols is increasing until 5 minutes of extraction time. The extraction time longer than 5 minutes decreases the yield of phenols. The reason of this condition is that the deterioration of phenols is depending on many factors including temperature.

The longer time this substance irradiated with microwave power, it would increase the contact duration between the bioactive compound and heat, causing the loss of phenols, although the temperature was stable at 50°C. Chumsri *et al*. (2008) stated that the rate of anthocyanin destruction depend on many factors such as pH, temperature, intermolecular copigmentation ascorbic acid, and oxygen. The purpose of determining the extraction time was to know the time needed for the absorption process on extraction with minimum loss of total phenols. In this case no need to do the extraction experiment more than 5 minutes.

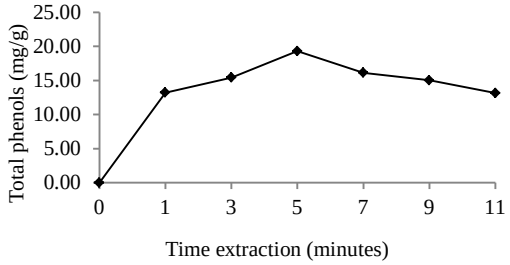


Figure 2. The effect of extraction time to the yield of total phenols

The Effect of Microwave Power to The Yield of Phenols

Microwave power is the important parameter in microwave assisted extraction. The varying microwave power used were 100, 175, 250, 325 and 400 W, 80% ethanol for 5 minutes extraction time. Figure 3 shows that microwave power of 250 W was enough to the next experiment.

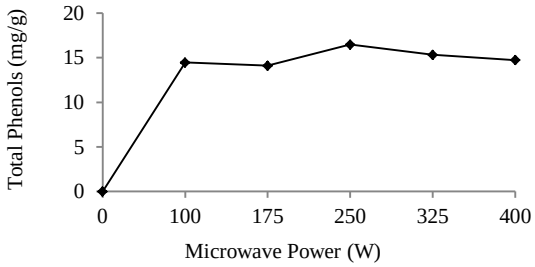


Figure 3. The effect of microwave power to the yield of total phenols

The extraction effectiveness improved when the microwave power increased from 100 to 250 W. This condition can be explained that the higher microwave power resulted the higher effect of microwave energy on biomolecules by ionic conduction and dipole rotation which results in power dissipated inside the solvent and plant material and then generate molecular movement and heating (Chen *et al.*, 2008). Microwave power above 250 W decreased the yield of phenols. Phenols are very sensitive to the heat according to Gao *et al.* (2006) in higher microwave power (400-1200W).

Optimization of MAE Condition of Phenol

This research evaluated 3 parameters, which are: (A) microwave power, (B) ethanol concentration, and (C) extraction time. Based on the previous study, there were three level for each parameter and second order model used, shown in Table 1.

Table 1. Code and variable of experimental design

Variable	Code	-1	0	1
Microwave power (Watt)	A	175	250	325
Ethanol Concentration %)	B	70%	80%	90%
Time of extraction (min)	C	3	5	7

In order to reduce the number of experiment, a Central Composite Design was used (Table 2). In this way, only 20 experiment were necessary. Multiple regression analysis is following second-order polynomial stepwise equation:

$$Y = 24.50 + 1.76A - 2.32B - 0.63C - 0.89AB + 0.48BC - 2.05A^2 - 3.03B^2 - 1.39C^2 + 2.00A^2B + 0.93A^2C$$

The result of ANOVA is shown in Table 3. The model F-value of 145.86 implies that the model is significant. The coefficient of determination (R-Squared) is the proportion of variability in the data explained or accounted for by the model. The "R-Squared" of 0.9979 is desirable, mean that the model was fitted for this case.

It is shown from Table 3 that the calculated of probability value (p) is smaller than 0.01. Consequently, the model is statistically significant for microwave power and ethanol concentration factor. The interaction between microwave power and ethanol concentration also significantly different within 99% of confidence level but not for the interaction of all factors. A quadratic change of factor would significantly affect the response (Y).

Figures 4 shows three dimensional response surface was presented for the independent variables (microwave output power, and ethanol concentration) which were obtained by keeping another variable (extraction time) constant. The figure indicate the changes in total phenols yield under different MAE conditions. Phenol substances just like anthocyanins could solute in polar solution ethanol, aceton and water. But from polarity level, between phenol as salute and ethanol as solvent are disbalance.

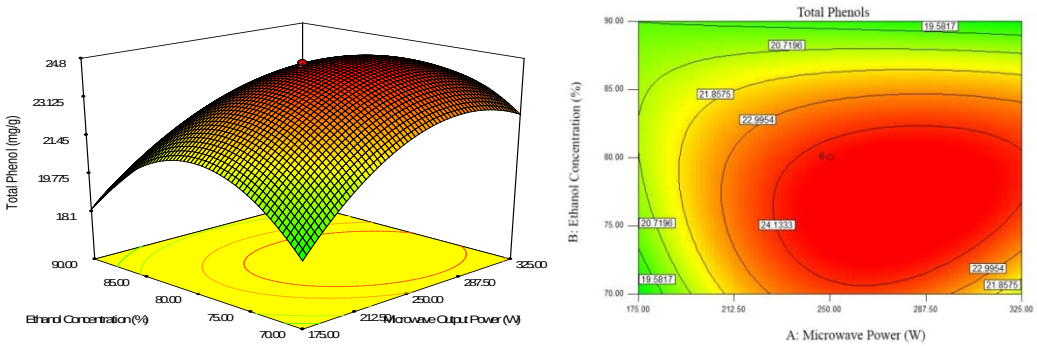
Table 2. Matrix of central composite design

Unit	Type	A (Watt)	B (%)	C (Minute)	Total phenol (mg/g)
1	center	250	80	5	24.49
2	center	250	80	5	24.49
3	Fact	175	70	3	17.59
4	Fact	175	90	7	18.98
5	Fact	325	90	3	16.46
6	Fact	325	70	7	19.84
7	Fact	325	90	7	17.59
8	center	250	80	5	24.61
9	Fact	325	70	3	19.04
10	Fact	175	70	7	16.11
11	Fact	175	90	3	16.98
12	center	250	80	5	24.61
13	Axial	250	80	1.64	22.00
14	Axial	376.13	80	5	22.02
15	center	250	80	5	24.38
16	Axial	123.87	80	5	16.11
17	center	250	80	5	24.38
18	Axial	250	63.18	5	20.20
19	Axial	250	80	8.36	19.89
20	Axial	250	96.82	5	12.38

Table 3. ANOVA for the complete codified mode

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob>F
Block	0.76	2	0.38		
Model	251.39	13	19.34	145.86	0.0001**
A-Power	17.46	1	17.46	131.72	0.0003**
B-Concent	30.58	1	30.58	230.62	0.0001**
C-Time	2.23	1	2.23	16.79	0.0149*
AB	6.28	1	6.28	47.39	0.0023**
AC	0.25	1	0.25	1.87	0.2428
BC	1.81	1	1.81	13.69	0.0208*
A^2	60.56	1	60.56	456.79	<0.0001
B^2	132.37	1	132.37	998.40	<0.0001
C^2	27.67	1	27.67	208.67	0.0001
ABC	0.55	1	0.55	4.14	0.1116
A^2B	13.30	1	13.30	100.34	0.0006
A^2C	2.89	1	2.89	21.78	0.0095
AB^2	6.02	1	6.02	45.44	0.0025

Ket: **significantly difference in 1% confidence level range
* significantly difference in 5% confidence level range



(A) (B)

Figure 4. The Interaction between microwave power and ethanol concentration to the yield of total phenols (A) Contour 3D, (B) Plotting Contour

The polarity of ethanol according Richter *et al*. (2006) is 24, and the polarity of the phenol substances and anthocyanins around 30-40. So, it needs addition of water to increase the extraction efficiency. In the other side King *et al*. (2009) stated that the increasing of temperature will decrease water polarity. Based from these reasons made the influenced cause by the interaction between microwave power and ethanol concentration is significant different.

Optimization of Microwave-Assisted Extraction

The optimum conditions were obtained by running the program of central composite design. The optimum conditions for independent variables and the predicted values of the responses also are presented as follows: 250 W microwave output power, 78.36% ethanol concentration and extraction time 4.91 min. The estimated values for total phenols yield, 24.61 mg/g was obtained at those conditions. A verification experiment at the optimum condition, consisting of 4 runs, was performed and the experimental yield of 23.77 $\pm$ 0.25 mg/g total phenols higher than using conventional method 19.84 $\pm$ 0.46 mg/g was obtained.

In this research, microwave assisted extraction was compared with conventional extraction techique using electricity boiling at 50°C (temperature of MAE), 5 minutes extraction time, and 78.36% of alcohol concentration. Those variables are the optimum variables using MAE. In this research, the heat generated by the microwave at the optimum condition are equal to 50°C based from preliminary research.

From the Figure 5, compared with conventional method, total phenol, anthocyanin, and vitamin C and yield of microwave assisted extraction were 23.77 $\pm$ 0.25, 14.80 $\pm$ 0.08, 10.74 $\pm$ 0,14 mg/g and 22.09 $\pm$ 3.3% respectively which are higher and significantly different within 99% confidence level than conventional extraction, which were 19.84 $\pm$ 0.46; 9.28 $\pm$ 0.04; 9.99 $\pm$ 0.16 mg/g and 16.18 $\pm$ 1.9% respectively.

Compared to the conventional method of extraction, MAE extraction produces a higher total phenols, anthocyanin, total solid and vitamin C. The fact that the increased extraction of phenols by the microwave power is related to the direct effects of microwave energy on biomolecules by ionic conduction and dipole rotation which result in power dissipated inside the plant material and the solvent which generate the molecules movement and heating (Chen *et al*., 2008).

The different of total phenol, anthocyanin and vitamin C also made the different of antioxidant activity between the two extract, shows in Figure 6. Extract using MAE has IC_{50} value 202.47 µL/mL lower than conventional method which has IC_{50} 293.09 µL/mL.

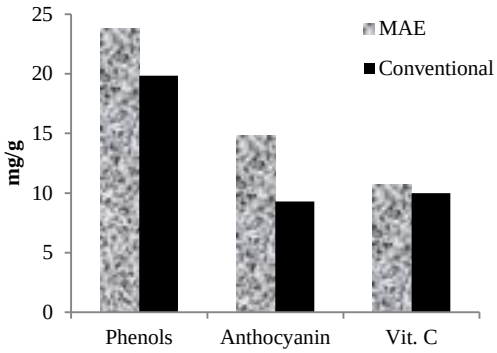


Figure 5. Phytochemical characterization

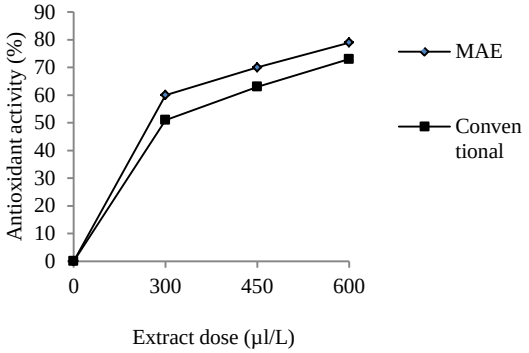


Figure 6. Antioxidant activity

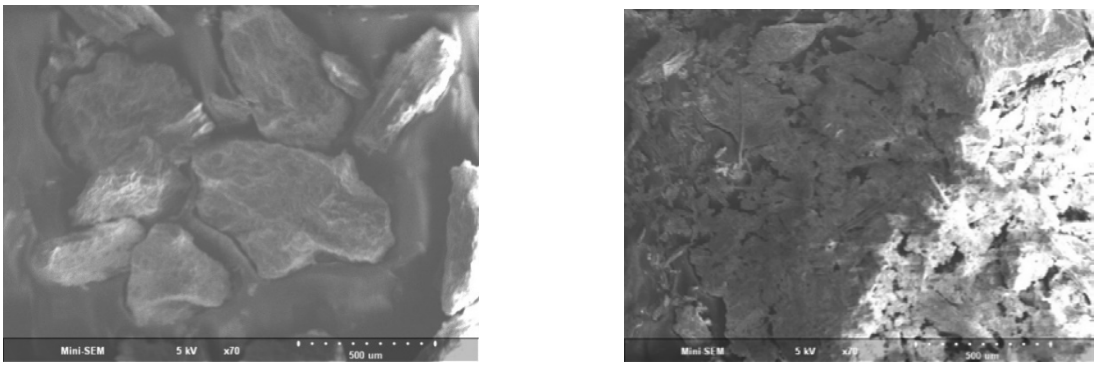
So that, the MIC and MKC from optimum extraction, lower than the conventional ones (Table 4). The results of this study clearly indicated that the roselle extract inhibit the growth of tested microorganism, however the effectiveness are varied. The antibacterial activity can be attributed to the action of phytochemical. Phenols substance known to be synthesized by plants in response to microbial infection and it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against microorganism. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacteria cell walls. This results agree with the previous (Al-Hashimi, 2012; Alonso *et al.,* 2006) who found that plant polyphenols have been demonstrated as potential antibacterial.

Microscopic Characterization

In order to study cell damages during the convensional extraction (boiling in 50^{o}C at the same time and the same ethanol concentration) and MAE, roselle calyx samples were examined by mini SEM (Scanning Electron Microscopy) with 7000x of magnification. The result shows that there are structural difference between MAE and convensional extraction. Figure 7 shows that the surface of the sample is found to be greatly destroyed after MAE.

Table 4. Antibacterial activity of roselle extract

Method	*E. coli*			*S. aureus*		
	Clear zone (mm)	**MIC (ppm)**	**MKC (ppm)**	**Clear zone (mm)**	**MIC (ppm)**	**MKC (ppm)**
MAE	12.6 + 0,6	5500	6000	11.6 + 0.3	5500	6000
Conventional	10. 2 + 0.4	7000	7500	9.8 + 0.6	7000	7500



(a) Conventional extraction (b) MAE extraction

Figure 7. Cell of roselle powder after the extraction in 7000x of magnification

This observation suggests that the microwave treatment affects the structure of the cell wall due to the sudden temperature rise and internal pressure increase. A rapid exudation of the bioactive substance within the cell into the surrounding solvent takes place during the rupture process (Wang *et al*., 2010). The effect of microwave energy is also strongly dependent on the dielectric susceptibility of both the solvent and solid plant matrix.

CONCLUSIONS AND RECOMMENDATION

Conclucions

Optimum condition of microwave-assisted extraction of total phenolic compounds from roselle calyx could be achieved by extracting at the microwave output power of 250 W and 78.36% ethanol concentration for 4.91 min. These conditions resulted in estimate the total phenolic yield of 24.61 mg/g. The average experimental phenolic yield under the optimum conditions was found to be 23.77 $\pm$ 0.25 mg/g. Anthocyanin, vitamin C and yield of microwave assisted extraction were, 14.80 $\pm$ 0.08; 10.74 $\pm$ 0.14 mg/g and 22.09 $\pm$ 3.3% respectively. Inhibitory zone was 12.6 $\pm$ 0.6 mm against *E coli* and 11.6 $\pm$ 0.3 mm against *S aureus.* EC_{50} value 202.47 µL/mL.

Recomendation

These research need further investigation about another response to find the optimum microwave assisted extraction condition.

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