

# Optimization of Microwave-Assisted Extraction to Obtain Optimum Antioxidant Activity and Anthocyanin Concentration from *Myrmecodia pendens* Tubers Using Response Surface Methodology

Nurul Fajry Maulida, Bernita Yanuaritamala, Arry Yanuar<sup>1</sup>, Fadlina Chany Saputri<sup>2</sup>, Abdul Mun'im

Departments of Pharmacognosy-Phytochemistry, <sup>1</sup>Biomedical Computation and <sup>2</sup>Pharmacology-Toxicology, Faculty of Pharmacy, Universitas Indonesia, Depok 16424, West Java, Indonesia

## ABSTRACT

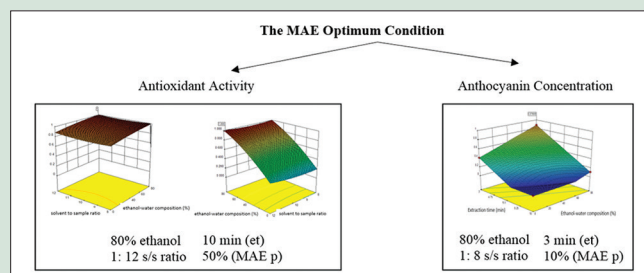
**Background:** One of the major antioxidants in *Myrmecodia pendens* (sarang semut) tubers is anthocyanin. **Objective:** To obtain antioxidant activity and anthocyanin concentration from sarang semut optimally through an appropriate method. **Materials and Methods:** Microwave-assisted extraction (MAE) was chosen because of its brief extraction time (ET), saving solvents, and being inexpensive compared to conventional extraction methods. Experiment design was prepared using response surface methodology. 1,1-diphenyl-1-2-picrylhydrazyl and reducing power method were used for the determination of the antioxidant activity, while the Association of Analytical Communities official method 2005.02 was used for the anthocyanin concentration calculation. **Results:** It was found that the optimum antioxidant activity was obtained at 80% ethanol, sample-to-solvent ratio (S/S ratio) 1:12, ET 10 min, and MAE power 50%. Meanwhile, the optimum anthocyanin extraction was obtained at 80% ethanol, S/S ratio 1:8, ET 3 min, and MAE 10%. **Conclusion:** The optimum condition of antioxidant activity and anthocyanin concentration was the same at the solvent used, yet different at the S/S ratio, ET, and power level which open further research to support this study.

**Key words:** Anthocyanin, antioxidant, microwave-assisted extraction, *Myrmecodia pendens*, response surface methodology

## SUMMARY

- The microwave-assisted extraction (MAE) optimum condition of antioxidant activity from *Myrmecodia pendens* tubers using response surface methodology was obtained at 80% ethanol, sample-to-solvent ratio (S/S ratio) 1:12, extraction time (ET) 10 min, and MAE power 50%
- The optimum condition of anthocyanin concentration was obtained at 80% ethanol, S/S ratio 1:8, ET 3 min, and MAE power 10%
- The optimum condition for antioxidant activity and anthocyanin concentration was the same at ethanol-water (8:2)

- Based on Hildebrand theory, ethanol-water (8:2) can be the solvents which have the most similar polarity level to the target compound, so it can give its maximum dissolution
- While the optimum condition for antioxidant activity and anthocyanin concentration were different at S/S ratio, ET, and power level
- It may occur since the extract does not only contain anthocyanin but also other major antioxidant compounds (rosmarinic acid and the polymer of procyanidin B<sub>1</sub>).



**Abbreviations Used:** S/S ratio: Sample-to-solvent ratio, ET: Extraction time, MAE power: Microwave-assisted extraction power, RSM: Response surface methodology.

## Correspondence:

Dr. Abdul Mun'im,  
Department of Pharmacognosy-Phytochemistry,  
Faculty of Pharmacy, Universitas Indonesia,  
Depok 16424, West Java, Indonesia.  
E-mail: abdul.munim61@ui.ac.id  
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## INTRODUCTION

*Myrmecodia pendens* was classified as the member of Rubiaceae family, locally known as sarang semut. Tuber of the plant has antitumor activity in human oral tongue squamous cell carcinoma cell line through induction of cyclin-dependent kinase inhibitor p27Kipl and suppression of cyclin E,<sup>[1]</sup> immunomodulatory effect,<sup>[2]</sup> and antidiabetic and antidiarrheal activity.<sup>[3]</sup> Flavonoid is the active component of the tuber.<sup>[4]</sup> Its major antioxidant has been analyzed, then it was found that anthocyanin contributes as one of its major antioxidants.<sup>[5]</sup> It was found that anthocyanin from red rosella tea (*Hibiscus sabdariffa* Linn.) has a role in lysosomal system by counteracting free radicals 1,1-diphenyl-1-2-picrylhydrazyl (DPPH), lowering lactate dehydrogenase bond, decreasing malondialdehyde formation, and reducing oxidative.<sup>[6]</sup> An appropriate method is required to obtain antioxidant activity and anthocyanin concentration optimally from sarang semut tubers extract. Extraction is a process to separate active compounds from plant or animal tissues using selective solvents through a standard procedure.<sup>[7]</sup> There are

numerous extraction methods from conventional methods (maceration, percolation, reflux, soxhletation, digestion, infusion, and decoction) to modern methods (microwave-assisted extraction [MAE], ultrasound extraction, and pressurized liquid extraction [PLE]).<sup>[7]</sup> MAE is more excellent compared to conventional methods because of its brief extraction and saving solvents.<sup>[8]</sup> Other alternatives are ultrasound extraction and PLE. However, free radicals can be produced when using

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ultrasound wave, so an active compound susceptible to free radicals is not appropriate using these methods.<sup>[7]</sup> Furthermore, MAE is more inexpensive than these methods.<sup>[9]</sup>

Optimum condition of MAE is influenced by the solvent concentration, sample-to-solvent ratio (S/S ratio), extraction time (ET), and power.<sup>[9]</sup> Experiment design is required to determine the optimum condition. Classical experiment designs, mostly applied by researchers to investigate the impact of a single factor or multifactor toward a result, are completely randomized design, randomized block design, and factorial design.<sup>[10]</sup> A large number of experiments, spending a lot of time, labor, and cost, must be done if multifactorial classical experiment design is used. In the past few years, the design of experiment (DoE) was developed to reduce the number of experiments.<sup>[11]</sup> DoE kept developing until lately was referred as response surface methodology (RSM).<sup>[12]</sup> Therefore, in this study, RSM was used to determine the optimum condition of MAE in obtaining antioxidant activity and anthocyanin concentration from sarang semut tubers.

## MATERIALS AND METHODS

The plant materials were obtained from Wamena, Papua, Indonesia, was determined by Lembaga Ilmu Pengetahuan Indonesia as *M. pendens* Merr. and L. M. Perry. The voucher specimen was deposited in Pharmacognosy Herbarium, Faculty of Pharmacy, Universitas Indonesia.

Software Design Expert® version 10 (Stat-Ease, Inc, Minneapolis, USA) was used for setting the experiment design for optimization using RSM. A total of 30 runs were resulted as shown in Table 1. Four factors and three levels with coded values as minimum (-1), center (0), and maximum (1): Solvent of ethanol-water composition (0%, 40%, 80%), S/S ratio (1:8, 1:10, 1:12), ET (3, 6.5, 10 min), and MAE power (10%, 30%, 50%).

Sarang-semut tubers were cleaned from contaminants, dried, ground into powder, and collected for extraction. The powdered sample (60 g per run) was extracted using MAE (modified Modena). The extraction yield was filtered with Kiriyaama-Rohto S-60 filter. The solvent was removed using rotary vacuum evaporator (Janke and Kunkel IKA®-Labortechnik, Germany). The extraction yield was determined as follows:

$$R_{\text{extract}} (\%) = \frac{m_{\text{extract}}}{m_{\text{sample}}} \times 100$$

where  $R_{\text{extract}}$  is the extraction yield,  $m_{\text{extract}}$  is the crude extract mass (g), and  $m_{\text{sample}}$  is the sample mass (g).

This was performed according to a method by Blois<sup>[13]</sup> with a small modification. Briefly, extract (25 mg) was diluted in 25 ml methanol (Merck, Germany). Serial solutions in the concentration range between 1 and 75 ppm were prepared. Quercetin (1–5 ppm) (Sigma-Aldrich, Singapore), the positive control, was prepared by diluting in methanol. Radical activity percentage of DPPH was obtained as follows:

$$\frac{A_{\text{blank}} - A_{\text{test}}}{A_{\text{blank}}} \times 100$$

where  $A_{\text{blank}}$  is the absorbance of blank solution (AU) and  $A_{\text{test}}$  is the absorbance of test solution (AU).  $IC_{50}$  value was calculated using equation  $y = a + bX$ , by inserting  $y = 50$  and  $x$  as the test solvent concentration which can reduce radical of DPPH in the amount of 50%.

Extract (25 mg) was diluted in 25 ml demineralized distilled water. The serial solution in the concentration range between 3 and 500 ppm was prepared. Ascorbic acid (10, 20, 30, 40, and 50 ppm) (Sigma-Aldrich), the positive control, was prepared by diluting in demineralized distilled water. Each test solution (1 ml) and positive control (1 ml) was transferred into a test tube. Then, 2.5 ml buffer phosphate (0.2 M, pH 6.6) and 2.5 ml hexacyanoferrate (1%) (Merck, Germany) were added, respectively.

The solution was incubated at 50° for 20 min, added with 2.5 ml trichloroacetate (10%) (Merck, Germany), and centrifugated in 3000 rpm for 10 min. Next, 2.5 ml of the reaction mixture was pipetted and added, respectively, with 2.5-ml demineralized distilled water and 0.5 ml  $FeCl_3$  (0, 1%). The absorbance was measured by spectrophotometer UV/Vis (Thermo Scientific Evolution 201) at 700 nm.

Anthocyanin concentration was determined using the Association of Analytical Communities (AOAC) official method 2005.02.<sup>[14]</sup> The extract was diluted in 10-ml ethanol-water (80%). Two test tubes were prepared and added 1 ml sample solution. Each test tubes were diluted with 4 ml different buffer, one with pH 1.00, while the other one with pH 4.50. The ratio of the sample solution to buffer should be 1:4, so the buffer capacity of reagents would not be exceeded. Absorbance measurement was done within 20–50 min from the preparation. Absorbance was determined at both 520 and 700 nm and read versus a blank cell filled with distilled water. The absorbance should be between 0.2 and 1.4 AU (within the linear range of spectrophotometer UV/Vis) by determining appropriate dilution factor. Anthocyanin concentration was calculated using an equation as follows:

$$C = \frac{A \times MW \times DF \times 10^3}{\epsilon \times l}$$

where C is the anthocyanin concentration (cyanidin-3-glucoside equivalents, mg/l), A is (A520 nm-A700 nm) pH 1.0–(A520 nm-A700 nm) pH 4.5, MW is the molecular weight (449.2 g/mol for cyanidin-3-glucoside), DF is the dilution factor, l is the path length in cm,  $\epsilon$  is 26900 molar extinction coefficient in  $L \times mol^{-1} \times cm^{-1}$  (for cyanidin-3-glucoside), and  $10^3$  is the factor for conversion from g to mg.

**Table 1:** Experiment design obtained by response surface methodology

Number	Run	Factor 1	Factor 2	Factor 3	Factor 4
1	3	0	1:12	10	10
2	6	0	1:12	3	10
3	12	0	1:8	3	50
4	13	0	1:8	10	10
5	19	0	1:8	3	10
6	24	0	1:10	6.5	30
7	26	0	1:12	3	50
8	28	0	1:8	10	50
9	30	0	1:12	10	50
10	2	40	1:10	6.5	30
11	4	40	1:10	6.5	30
12	5	40	1:12	6.5	30
13	7	40	1:10	6.5	30
14	10	40	1:10	6.5	30
15	14	40	1:10	6.5	30
16	15	40	1:10	10	30
17	20	40	1:10	6.5	10
18	21	40	1:10	3	30
19	23	40	1:10	6.5	30
20	25	40	1:8	6.5	30
21	27	40	1:10	6.5	50
22	1	80	1:8	3	10
23	8	80	1:8	10	50
24	9	80	1:12	3	50
25	11	80	1:8	3	50
26	16	80	1:10	6.5	30
27	17	80	1:12	3	10
28	18	80	1:12	10	50
29	22	80	1:8	10	10
30	29	80	1:12	10	10

## RESULTS AND DISCUSSIONS

Data obtained from all measurements were used to set mathematical equation using software Design Expert<sup>®</sup> version 10. The equation obtained would be used to determine the optimum condition of MAE.

Based on Engida *et al.*,<sup>[15]</sup> five flavonoids: kaempferol (13.767 mg/g), luteolin (0.005 mg/g), rutin (0.003 mg/g), quercetin (0.030 mg/g), and apigenin (4.700 mg/g) were identified from sarang semut tuber extract. An advanced research was followed, three major antioxidants: rosmarinic acid (20.688 mg/g), procyanidin B<sub>1</sub> (3.236 mg/g), and the polymer of procyanidin B<sub>1</sub> were analyzed. The polymer of procyanidin B<sub>1</sub> was not quantified due to a lack of commercial standard. The study was conducted using heat reflux extraction with ethanol–water (80%) as the solvent.<sup>[5]</sup>

MAE, chosen as the appropriate extraction method in this study, is an extraction method assisted by microwave at 2.45 GHz in the form of electromagnetic unionization.<sup>[16]</sup> This energy causes molecules movement with ion migration and rotation from two poles, but the molecule structure will not be changed. The extraction process mechanism of MAE is as follows; the heat of microwave radiation will heat up and evaporate the water from material cell, cause the cell wall pressure to increase, as a result, the cell is swelling and the pressure will push the cell wall from inside, tense, and break the cell.<sup>[17]</sup> In a short time, cellulose will turn into a dissolved fraction. The heat energy in the material cell wall will also increase the cellulose dehydration and reduce the cellulose mechanical force and hence the cell wall permeability will be disturbed. The material matrix damage will allow the target compound to escape and dissolve in the solvent. Furthermore, the increase of the temperature will increase the solvent penetration into the material matrix and the active compound will be extracted by the heated solvent.<sup>[18]</sup>

In general, MAE system is divided into two, the multimode system (closed type MAE system) and the monomode system (open type MAE system). MAE with the multimode system has microwave radiation with a random dispersion or random wave direction in an unlimited area. While MAE with monomode system has focused microwave radiation or one wave direction in a limited area.<sup>[19]</sup> In this research, the material was extracted using opened type MAE system because of thermolabile anthocyanin and volatile solvent.<sup>[20]</sup> In open type MAE system, the extraction was done under atmospheric pressure, so the temperature can be lower and safer for a thermolabile target compound. Moreover, this system is connected with a condenser to condense the evaporated solvent.<sup>[19]</sup>

Optimization is a normative approach to identify the best solution in decision-making toward an issue. The most important element in the optimization issues is the function of a purpose influenced by some factors.<sup>[21]</sup> In this study, four factors were identified which are solvent concentration, S/S ratio, ET, and power.

Ethanol and water, a solvent combination used in this study, are the examples of appropriate solvents used in MAE because of its high dielectric constant and dielectric loss that can absorb microwave energy. Higher contact surface area will increase the extraction efficiency by improving solvent penetration. Meanwhile, ET and power correlate to the temperature reached. The longer ET and the higher power, the higher temperature produced.<sup>[9]</sup> Thus, ET and power in this study were set, respectively, within the range 3–10 min and 10%–50% because of the thermolabile anthocyanin. As shown in Figure 1, Run 17 was the highest extract rendement. Run 17 was a condition at 80% ethanol (the highest solvent composition level), S/S ratio 1:12 (the highest ratio level), ET 3 min (the lowest time level), and power 10% (the lowest power level). In general, the higher ratio, the higher extraction yield.<sup>[22]</sup>

That DPPH solution losing its specific purple color is the principle of antioxidant activity determination by DPPH since DPPH is a stable free radical.<sup>[13,23]</sup> The antioxidant activity can be calculated by observing

reduction in absorbance. While the principle of antioxidant activity determination by reducing power method is that antioxidant will reduce ferri to ferro then form a Per's Prussian color complex as the indicator of color change from yellow to green until blue which depends on the capacity to reduce the antioxidant.<sup>[24]</sup> Either antioxidant activity test using DPPH or reducing power method result to have run 18 as the optimum condition. The response surfaces for antioxidant activity are shown in Figure 2. Run 18 was a condition at ethanol–water 80%, S/S ratio 1:12, ET 10 min (the highest time level), and power 50% (the highest power level). By DPPH, the smallest IC<sub>50</sub> was obtained, 0.98 ppm classified to have a very active antioxidant activity, was smaller than IC<sub>50</sub> obtained from *Antrodia amphorata* red (2.00 ppm) and white *mycelia* (1.56 ppm).<sup>[25]</sup> It occurred because of the time difference of ferric chloride solution addition. Compared to the positive control (ascorbic acid), IC<sub>50</sub> obtained was also lower. Meanwhile, the lowest IC<sub>50</sub>, 0.78 ppm classified to have a very active antioxidant activity, was also obtained by reducing power method and lower than positive control (quercetine) because stronger antioxidant activity from flavonoid may be contained in the extract. IC<sub>50</sub> from the same plant obtained by Engida *et al.*,<sup>[15]</sup> using reflux extraction method, 96.21 ppm, was higher than IC<sub>50</sub> obtained from this study. Different extraction method, time difference in incubating, sample solution to DPPH ratio, and time difference after incubating may be the reasons for this different IC<sub>50</sub> obtained.

Anthocyanin concentration determination through the AOAC 2005.02 method was based on color changes caused by pH changes reversibly. Anthocyanin in pH 1.00 will be in the colored oxonium form while in pH 4.50 will be in colorless hemiketal form. The absorbance difference at 520 nm will show its concentration proportionally. If the absorbance is high, the concentration will be high too and vice versa. Degraded anthocyanin in polymer form is not able to undergo color changes, so its determination could not use this method.<sup>[14]</sup> The highest anthocyanin concentration, 3807.31 mg/l higher than 3236 mg/g of dry sample found by Engida *et al.*<sup>[5]</sup> was obtained in run 1. The surface response of anthocyanin concentration is shown in Figure 3. Run 1 was a condition at ethanol–water 80%, s/S ratio 1:8, ET 3 min, and power 10%. Some runs (3, 13, 20, 23, 25, 26, 27, 28, and 30) had negative absorbances as shown in Table 2 and flat blue surface in Figure 3. It occurred because of blank correction error when a blank solution used was able to absorb more UV/Vis light than the sample solution.<sup>[26]</sup> The highest anthocyanin concentration was obtained at the lowest S/S ratio, exactly different with the optimum ratio level in obtaining highest extraction yield and antioxidant activity. Even though the higher ratio, the higher extraction yield, the higher ratio can also cause the mixing process not sufficient on solvents by the microwave and excessive swelling may occur on the material extracted. Excessive solvents also can cause dissolution of unwanted compounds which may reduce the target compound selectivity.<sup>[22]</sup> The lowest level of ET and power showed the best

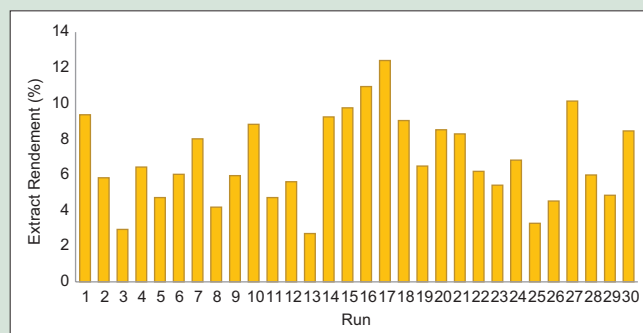


Figure 1: Variation of extraction yield run 17 as the highest and run 13 as the lowest

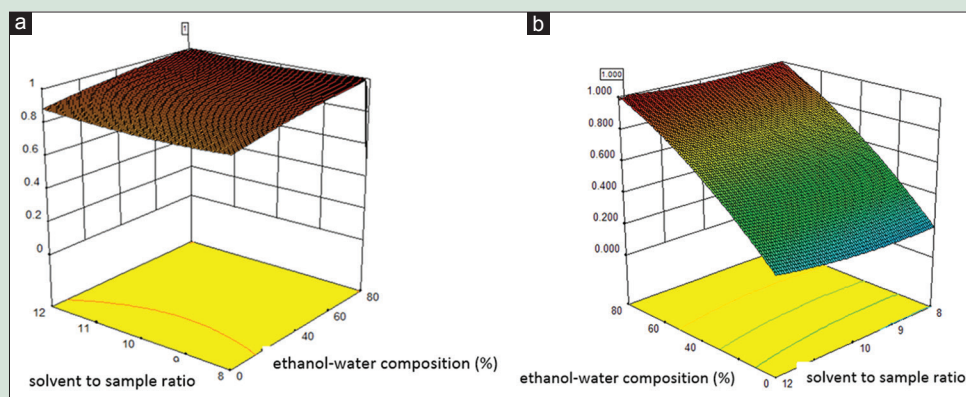


Figure 2: Response surfaces for antioxidant activity test. (a) 1,1-diphenyl-1-2-picrylhydrazyl assay and (b) reducing power method

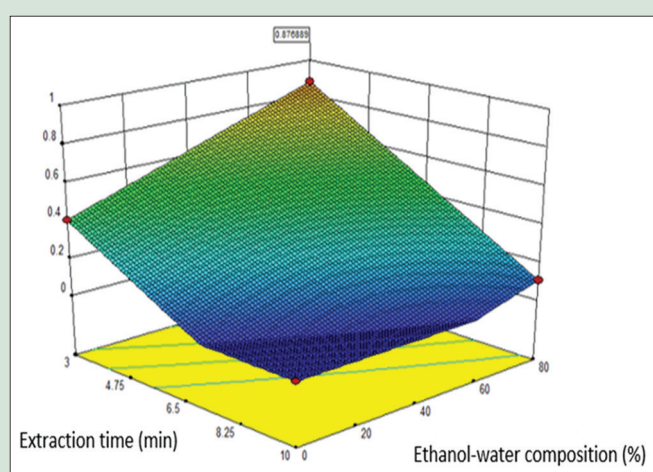


Figure 3: Response surface for anthocyanin concentrations

Table 2: Anthocyanin concentration obtained Association of Analytical Communities 2005.02 method

Number	Run	Response: concentration (mg/l)
1	3	-144.15
2	6	574.63
3	12	330.35
4	13	-97.98
5	19	821.3
6	24	531.57
7	26	-117.42
8	28	-831.87
9	30	-355.3
10	2	713.31
11	4	0
12	5	1156.72
13	7	676.97
14	10	280.82
15	14	314.1
16	15	62.39
17	20	-199.87
18	21	880.83
19	23	-147.61
20	25	-40.47
21	27	-63.39
22	1	3807.31
23	8	0
24	9	0
25	11	2293.83
26	16	931.79
27	17	564.98
28	18	1270.69
29	22	0
30	29	1137.91

combination to produce an appropriate extraction temperature since anthocyanin is unstable at high temperature. Anthocyanin can be degraded at the temperature above 65°–95° merely in a short time.<sup>[20]</sup>

Therefore, the optimum condition for antioxidant activity and anthocyanin concentration were the same at ethanol–water 80%. In general, anthocyanin is extracted using polar solvents such as methanol or ethanol.<sup>[27]</sup> However, some researchers found that a combination of two polar solvents can obtain extraction yield by MAE better, such as extraction of gymnemagenin from *Gymnema sylvestre* R. Br. (using methanol–water [85:15]) and extraction of paclitaxel from *Taxus baccata* (using methanol–water [90:10]).<sup>[9]</sup> The combination of two polar solvents will tend to have similar polarity level to the target compound. Based on Hildebrand theory that the maximum dissolution will be obtained by the solvents which have similar polarity level to the target compound.<sup>[28]</sup> While the optimum condition for antioxidant activity and anthocyanin concentration were different at S/S ratio, ET, and power level. It may occur since the extract does not only contain anthocyanin but also other major antioxidant compounds (rosmarinic acid and the polymer of procyanidin B<sub>1</sub>). Another research to obtain the optimum condition of those compounds may be conducted in the future to support this study.

### CONCLUSION

The optimum condition of antioxidant activity and anthocyanin concentration was the same at solvent used, yet different at the S/S ratio, ET, and power level which open further research to support this study.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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