Radical Scavenging Activity from Ethanolic Extract of Malvaceae Family’s Flowers

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Abstract Sea hibiscus flower (Hibiscus tiliaceus L.), shoe flower (Hibiscus rosa-sinensis L.), and turk’s cap flower (Malvaviscus arboreus Cav.) are a plant that belongs to the same family, Malvaceae. There are expected contain of anthocyanins as active compound. Several studied shows that some flowers could protect human body from free radical danger exposure. This study has been done to examine ethanolic extract from malvaceae family’s which has potency as radical scavenger. Antiradical activity assay was determined by DPPH method with IC50 value as parameter. Based on the study the malvaceae family’s flower was contain of tannins, polyphenols, saponin, and anthocyanine. The radical scavenging activity respectively from the lowest to the highest activity are vitamin c (4.05 ppm ± 0.094), Turk’s cap flower (6.80 ppm ± 0.22), shoe flower (14.62 ppm ± 0.104) and sea hibiscus flower (38.8 ppm ± 0.086). The three of the extract was having strong antioxidant activity.

1. Introduction
Free radicals in the body basically play a role in health maintenance because of its reactive nature to bind or react with foreign molecules that enter the body, for example to kill bacteria that enter the body. But if free radicals in large amounts, can cause disruption of the metabolic system, this is due to the nature of free radicals that can attack lipids, DNA, protein components of cells and tissues. Therefore, its existence must be controlled by the antioxidant system [1]. Hibiscus rosa-sinensis L. is widely grown as an ornamental plant in tropical and subtropical area. This plant has large flowers and is red. The leaves, flower and root contain flavonoids. The leaves contain saponins and polyphenols, as well as taraksyl acetate. Flowers contain polyphenols, cyanidin diglucosides, hibisetin, bitter and mucilage substances, vitamins, thiamin, riboflavin and ascorbic acid as well as alkaloids and saponins. While the root contains tannins and saponins [2]. The sea hibiscus flower (Hibiscus tiliaceus L.) is known to have many uses as a reliever of fever, hair grower, cough medicine and bloody or slimy diarrhea drug [3]. Malvaviscus arboreus Cav is a plant belonging to the family of Malvaceae. It is thought to have potential as an antioxidant agent, cytotoxic, and thrombolytic, with a total fenolic compound on leaves value of 20.06 ± 0.87, and an LC50 value of 3.82 ± 0.08 [4]. The success of this study is expected to provide an overview of the selection of plants that have antioxidant activity correlated with anticancer activity. Untill now, the result for cancer treatment is still unsatisfactory. Therefore, the study of cancer drug discovery is still intensively conducted. One of the medicinal plants that become the object of study is the flower of Malvaceae family.
2. Method

2.1 Chemicals and reagents

The sample of *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. was collected from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Tawangmangu, Karanganyar, Central Java, Indonesia. DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma), etanol p.a (Emure), technical etanol, aqua bidestilata (Merck), Thin Layer Chromatography (TLC) plates Silica GF$_{254}$ (Sigma).

2.2 Sample Preparation

The sample was extracted using 70% ethanol solvent. The maceration is carried out for 3 days to obtain the filtrate which extracts the active ingredient effectively. The obtained filtrate was then evaporated with a rotary evaporator at a temperature between 55-60 °C.

2.3 Identify phytochemicals

Chemical identification test in this research was done by using thin layer chromatography (TLC). The purpose of this identification test is to determine whether there are secondary metabolite compounds including tannins, polyphenols, saponins, and anthocyanins. For the stationary phase used is silica gel GF$_{254}$, while for the mobile phase, adjusted to the degree of polarity of the extract. The tannin test used the mobile phase of n-butanol: glacial acetic acid: water (14: 1: 5 v/v/v) [5], polyphenol test used glacial acetic acid: acetone: water (40: 20: 4 v/v/v) [6], while anthocyanin test used a mobile phase of n-butanol: glacial acetic acid: water (4: 5:15 v/v/v) [7].

2.4 Antioxidant Activity Assay (DPPH method)

The free radical scavenging method was performed based on Kikuzaki *et al* (2002) [8]. In this assay, sample from the stock solution of ethanolic extract from *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. were prepared in five different concentration (15, 30, 60, 90 and 120 µM). Each of sample added by 0.7 mL DPPH 0.4 mM and ethanol added up to 5.0 mL. This mixture was homogenized by mixing for 30 second and incubated for 30 minutes. The sample absorbance were measured by UV-Vis spectrophotometer (Shimadzu) with $\lambda_{\text{max}}$ 515 nm. The sample absorbance also compared with the control solution containing 0.7 mL DPPH 0.4 mM diluted in ethanol. The radical scavenging activity was calculated using the following formula:

$$\frac{\text{Ac}-\text{As}}{\text{Ac}} \times 100\%$$

Where Ac is absorbance of control (DPPH free radical without the addition of the sample solution), As is sample absorbance (absorbance of DPPH free radical after the addition of sample solution). The percentage (%) of antiradical activity were measured by linier regretron between concentration curve versus antiradical activity precentage were obtained. Then, the linier regression formula and sample concentration at 50% activity were determined.

2.5 Cytotoxic Activity

$1 \times 10^4$ T47D cells/well were grown in 96-well plate before being exposed to drug treatment. For cell viability assay, cells were treated for 24 hours with increasing concentration of extract. At 100 µg/ml of tetrazolium salt MTT [3-(4,5-dimetiltiazol-2-yl)-2.5-diphenyl terazolium bromide] solution (0.5 mg/ml in Phospfat Buffer Saline) was added to each well continued with incubation for 3 hours at 37°C. The reaction was stopped by dilution with 10% (w/v) Sodium Dodecyl Sulphate in 0.01 N HCl, and cells were incubated overnight. The absorbance was determined by using ELISA reader at $\lambda$ 595 nm.
3. Result and Discussion

3.1 TLC Analysis

The extraction with 800 grams of drying flowers of *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. were gave viscous extract of 35.23 gram (7.046%), 18.07 gram (3.614%) and 14.06 gram (2.812%) respectively. TLC analysis showed result was obtained based on Table 1.

**Table I. The comparison of the Rf value of tannin, plyphenol and antocyanin from the extract**

| Extract             | Tannin (Rf) | Polyphenol (Rf) | Antocyanin (Rf) |
|---------------------|-------------|-----------------|-----------------|
| *Hibiscus rosa-sinensis* | 0.5         | 0.74            | 0.25            |
| *Hibiscus tiliaceus*  | 0.66        | 0.83            | 0.16            |
| *Malvaviscus arboreus* Cav. | 0.63       | 0.82            | 0.47            |
| Standard            | 0.53-0.8    | 0.82            | 0.1-0.4         |

**Figure 1.** The result of Qualitative Phytochemistry Assay by TLC showed that *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. contain tannin (A) with Rf value are 0.5; 0.66; 0.63 respectively after eluated with mobile phase of n-butanol: glacial acetic acid: water (14: 1: 5 v/v/v), polyfenol (B) with Rf value are 0.74; 0.83 and 0.82 respectively after eluated with mobile phase glacial acetic acid: acetone: water (40: 20: 4 v/v/v) and antosianin (C) with Rf value are 0.25; 0.16; and 0.47 respectively after eluated with mobile phase n-butanol: glacial acetic acid: water (4: 5:15 v/v/v)

3.2 Radical scavenging activity

Antioxidant Activity Test of *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. determined by the ability of a compound contained in extract to reduce the purple color intensity of DPPH radical in its maximum wavelength. The reduction of purple colour intensity of DPPH radical is caused by the decrease of chromophore or conjugated double bond in DPPH compound. It caused by potency of extract compound which scavenging the radical by donating hydrogen atom to DPPH structure so that become reducted DPPH-H. DPPH-H is a compound which have yellow colour [9]. The principle reaction as bellow (Figure 2.) Antioxidant activity divide into 4 categories i.e active antioxidant if its IC$_{50}$ value is lower than 50 µg/ml, medium antioxidant if its IC$_{50}$ value between 50-100 µg/ml, less active in antioxidant activity if its IC$_{50}$ value between 100-200 µg/ml and if its IC$_{50}$ value is more than 200 µg/ml showed that the compound is not active as antioxidant [10].
Figure 2. The principle reaction of DPPH (Molyneux, 2004) [11]

Table II. The comparison of the antiradical activity of extract

| Extract                  | Concentration (µg/mL) | Antiradical activity (%) | IC<sub>50</sub> (µg/mL) |
|--------------------------|-----------------------|--------------------------|--------------------------|
| *Hibiscus rosa-sinensis* | 15                    | 50,72                    |                          |
|                          | 30                    | 53,77                    |                          |
|                          | 60                    | 64,98                    | 14,62                    |
|                          | 90                    | 73,84                    |                          |
|                          | 120                   | 83,25                    |                          |
| *Hibiscus tiliaceus*     | 15                    | 35,27                    |                          |
|                          | 30                    | 46,67                    |                          |
|                          | 60                    | 62,85                    | 38,89                    |
|                          | 90                    | 78,01                    |                          |
|                          | 120                   | 83,71                    |                          |
| *Malvaviscus arboreus*   | 15                    | 47,54                    |                          |
| Cav.                     | 30                    | 61,39                    | 10,45                    |
|                          | 60                    | 69,31                    |                          |
|                          | 90                    | 80,65                    |                          |
|                          | 120                   | 83,68                    |                          |

The parameter used in the interpretation of radical activity is the value of inhibitory concentration 50% (IC<sub>50</sub>) were obtained by linier regretion between the concentration versus the percentage of antiradical activity [12]. The radical scavenging activity respectively from the lowest to the highest activity are *Malvaviscus arboreus* Cav (10,45 µg/mL) ; *Hibiscus rosa-sinensis* L. (14,62 µg/mL) and *Hibiscus tiliaceus* L. (38,89 µg/mL) (Fig.2). With this IC50 value, all of extracts can be classified into active antioxidant [10] which states that crude extract with IC<sub>50</sub> less than 50 µg/mL.
Figure 3. The IC₅₀ curve of ethanolic extract of *Hibiscus rosa-sinensis* L. (A), *Hibiscus tiliaceus* L. (B), and *Malvaviscus arboreus* Cav. (C). The sample absorbance were measured by UV-Vis spectrophotometer with λ_max 515 nm.
3.3 Cytotoxic activity

Cytotoxic activity was tested by in vitro to determine the potential of a compound such as cytotoxic anticancer drugs. The parameter used in the interpretation of expressed in IC$_{50}$ value that can inhibit cell proliferation by 50% of the population. This study using MTT assay to determine the cytotoxic potential of *Hibiscus rosa-sinensis* L., *Hibiscus tiliaceus* L., and *Malvaviscus arboreus* Cav. The method is used to measuring the color intensity that occur as a result of reaction a substrate by living cells into colored product. The reaction of tetrazolium salt MTT [3-(4,5-dimetiltiazol-2-yl)-2,5-diphenyl tetrazolium bromide] reduced to formazan by the succinate tetrazolium reductase system, which is included in the mithocondria of living cells. Formazan intensity was measured using ELISA reader at a wavelength of 595 nm. Color intensity is proportional to the number of living cells. Sample extract was supplied with various concentration from 10 µg/ml to 150 µg/ml. Cell morphology was observed after treatment with extract for 24 hours, thus it being treated with MTT reagent. Treated cells showed cytotoxic effect with change of cell morphology and decreased cell viability. Viable cells had epithelial shape, but after being treated with certain concentration of samples they gave spherical-shape and shrunk cell wall. By using ELISA reader, absorbance value were obtained. IC$_{50}$ was calculated using the linier regression between extract concentration and percentage of living cell. The IC$_{50}$ value respectively are *Malvaviscus arboreus* Cav (152,45 µg/mL); *Hibiscus rosa-sinensis* L. (173,65 µg/mL) and *Hibiscus tiliaceus* L. (144,76 µg/mL) (Fig 3). With this IC$_{50}$ value, the extract can be classified into a potent chemotherapeutic agent which states that crude extract with IC$_{50}$ less than 100 µg/mL of highly potent as an anticancer agent [13].

| Extract                        | Concentration (µg/mL) | Cell viability (%) | IC$_{50}$ (µg/mL) |
|--------------------------------|-----------------------|--------------------|-------------------|
| *Hibiscus rosa-sinensis*       | 10                    | 91,753             |                   |
|                                | 50                    | 88,781             |                   |
|                                | 110                   | 64,706             | 173,65            |
|                                | 130                   | 63,008             |                   |
|                                | 150                   | 56,034             |                   |
| *Hibiscus tiliaceus*           | 10                    | 97,5               |                   |
|                                | 50                    | 75,2               |                   |
|                                | 110                   | 65,2               | 144,76            |
|                                | 130                   | 50,4               |                   |
|                                | 150                   | 46,2               |                   |
| *Malvaviscus arboreus* Cav.    | 10                    | 101,58             |                   |
|                                | 30                    | 95,88              |                   |
|                                | 50                    | 84,78              |                   |
|                                | 70                    | 84,72              | 152,45            |
|                                | 90                    | 90,05              |                   |
|                                | 110                   | 86,11              |                   |
|                                | 130                   | 75,99              |                   |
|                                | 150                   | 53,92              |                   |
Figure 4. The effect of ethanolic extract of *Hibiscus rosa-sinensis* L. (A), *Hibiscus tiliaceus* L. (B), and *Malvaviscus arboreus* Cav. (C) on the viability cells. Graph showed the treatment effect of various concentration of extract on T47d breast cancer cell viability.

Based on IC\textsubscript{50} value from antioxidant activity and IC\textsubscript{50} value from cytotoxic activity it indicated that the antioxidant might somehow contribute to growth inhibition in T47d cells. But further studies on the role of antioxidants growth inhibitory effect on T47d cell line is needed to be done. It also important to explore the mechanistic function between antioxidant and anticancer activity of flowers.

**Conclusion**
Antiradical activity of *Malvaviscus arboreus* Cav. is known to have highest activity than the other malvaceae family’s flowers. Its IC$_{50}$ value of *Malvaviscus arboreus* Cav. is 10.45 µg/mL. Also the cytotoxic activity of *Malvaviscus arboreus* Cav. is 152.45 µg/mL. Based on Husna et al (2013) [14] reported that antosianin compound in *Malvaviscus arboreus* Cav. is the compound responsible to the antiradical and cytotoxic activity, meanwhile the main compound from the extracts used in this study that is responsible in the antiradical and cytotoxic activity are not been known yet.

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