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In Vitro Antioxidant and Anti-Mycobacterial Activities of Roselle (*Hibiscus sabdariffa* L.) Calyx Extract against Clinical Isolate of Multidrug Resistant *Mycobacterium tuberculosis*

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ABSTRACT
Hibiscus sabdariffa L. or roselle calyx has been known to have biological activities including antioxidant and antibacterial. In this research, we examine the antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as scavenging agent and the anti-mycobacterial activity towards clinical isolate of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB). Roselle calyces were dried and extracted through infusion methods using distilled water, and maceration methods using 50% ethanol, within the ratio of sample (1): solvent (10), followed by spray-drying using maltodextrin as diluting agent. The total phenolic content (TPC) was analyzed using Folin-Ciocalteu method calculated as gallic acid equivalent (GAE), antioxidant activity was analyzed using DPPH method, and anti-mycobacterial activity was assay using Microscopic Observation Drug Susceptibility (MODS) method. As the results, TPC of infusion extract and ethanolic extract were 17.70±0.7 mg GAE/g and 13.15±0.3 mg GAE/g, respectively and IC_{50} toward DPPH were 0.88 mg/ml for infusion extract and 3.78 mg/ml for ethanolic extract, respectively. Anti-mycobacterial activity of both extracts expressed as Minimum Inhibitory Concentration (MIC) were 10 mg/ml toward clinical isolate of MDR-TB.

Keywords: roselle (*H.sabdariffa* L.) calyx, MDR *Mycobacterium tuberculosis*, Microscopic Observation Drug Susceptibility, 1,1-diphenyl-2-picrylhydrazyl

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INTRODUCTION
Tuberculosis (TB) become one of the leading causes of death in the developing country. In 2018, TB caused an estimated 1.2 – 1.4 million death from 10.0 million people developed TB diseases, globally. From 64 % of the estimated 10.0 million news caes that occured in 2018, The top three being Indonesia represented India 27%, Cina 9 % , and Indonesia 8 % (1).

Considering the standard treatment for tuberculosis, it has been suggested for the use of fixed-dose regimen including isoniazid (INH), rifampicin (RIF), and pyrazinamide for 2 months, followed by 4 months of INH and RIF. In fact, according to some studies, those combinations have shortened the duration of therapy, and also may treat multidrug-resistant tuberculosis. In many developing countries, ethambutol has been usually added to this regimen, and streptomycin is recommended by the World Health Organization (WHO) in retreatment cases (2,3).

In addition, anti-tuberculosis drug resistance issues and the risk of hepatotoxic effects, using natural ingredients is an important option. This natural materials may be safer and have been empirically and scientifically proved as potent antibacteria (4,5). For example, several Malaysian plants had been studied for this purposes, of which the resulting methanolic extract had MICs in the range of 0.4– 1.6 mg/ml (6).

Roselle (*Hibiscus sabdariffa* L.), from Malvaceae family, contains anthocyanin group, such as delphinidin-3-O-sambubioside and cyanidin-3-O-sambubioside, responsible for the color of calyces (7,8). The calyces also generate some secondary metabolites, for instance, protocatechuic acid; flavonoids such as quercetin, luteolin, and their glycosides; also a major organic acid such as citric acid, maleic acid, hibiscus acid, tartaric acid, ascorbic acid; dietary fiber; compounds like vitamin, mineral, and phytosterol (9,10). According to the previous research, roselle calyx extract potentially showed effect as antibacterial and antioxidant. The antibacterial activity of the roselle water and ethanolic extracts was tested on *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC6538, and *Escherichia coli* ATCC 8739 (9). Another researcher has also concluded that roselle extract elucidated antibacterial activity against *Mycobacterium tuberculosis* (11). In addition, the seed of roselle was also reported protein hydrolysate (12) which also had antibacterial and antioxidant activity (13,14). Thus, this research aimed to scientifically determine the antioxidant capacities of roselle calyx extracts toward DPPH along with the antibacterial activity against MDR *M. tuberculosis*.

MATERIAL AND METHODS
Material
Roselle calyces were collected from the garden of the Faculty of Pharmacy, clinical isolate of MDR *M. tuberculosis* was provided by Hasanuddin University Microbiology Research Center, Indonesia

Preparation of *Roselle calyx* Extract
The dried calyces were ground into coarse powder using an electric grinder. About 100 g was extracted through maceration methods, using 1 L of 50% ethanol (sample: solvent ratio = 1:10) for 5 days, and by infusion methods using distilled water for 15 minutes (sample: solvent ratio = 1:10), followed by filtration through Whatmann no.1 filter membrane. The obtained liquid extract was added with 10 g maltodextrin until dissolved and subsequently processed with spray-drying to have roselle calyces extract.

Determination of Total Phenolic Content

The total phenolic content (TPC) of the extract was determined using the Folin–Ciocalteu method, based on Indonesia Herbal Pharmacopeias (Kemenkes, 2011). Briefly, about 200 µL of crude extract (10 mg/mL), mixed thoroughly with 2.5 mL of 7.5 % Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 1% (w/v) sodium hydroxide and demineralized water up to 5 mL. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 650 nm. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent (GAE) per gram extract.

Determination of Antioxidant Activity by DPPH Method

Determination of antioxidant activity by DPPH was conducted according to Indonesia Herbal Pharmacopeias (Kemenkes, 2011). Briefly, crude extract samples were prepared at 100 µg/ml. A total of 2 ml samples were placed in test tubes, in which was added 3 ml of a 0.3 methanolic solution of DPPH, mixed well and allowed to incubate at 30 °C for 20 min. The absorbance value of the sample was measured at 517 nm. The percentage of DPPH radical scavenging activity was theoretically calculated by the following formula, according to Chen et al. (2013).

Determination Antibacterial Activity by Microscopy Observation Direct Susceptibility (MODS) Method

Determination antimycobacterial activity wasconducted according to Isrul et al (17) with slightly modification. Briefly, the roselle calyx extract solutions were added 50 µl to give final concentration 0.125, 0.5, 1.0 and 2.0 % in sterile 24 well plates containing 950 µl Middlebrook 7H9 medium with 10% oxalic acid, albumin dextrose and catalase (OADC) enrichment and 10^6 colony/ml culture MDR *M.tuberculosis*, respectively. The well plate was incubated at 35°C for 7 - 14 days. Cord-like form of MDR M.tuberculosis was observed by inverted light microscopy. The MIC was defined as the lowest concentration of extract where the absence of *Mycobacterium* growth (unobservable cord-like structure) after 7 – 14 days incubation. Each antibacterial assay for MIC determination was carried out in triplicate.

RESULTS AND DISCUSSION

The Extraction yield, total phenolic content, antioxidant activity of Roselle Calyx Extract

The results of extraction yield, total phenolic content (TPC), and antioxidant activity expressed as IC_{50} against DPPH for both of sample extracts shown in table 1.

In this research, both of roselle calyx extracts were diluted/encapsuleted in maltodextrin and dried using spray drying method. Anthocyanin and other phenolic compounds in roselle calyx are unstable, one alternative for improving anthocyanin is encapsulation technique which entraps the sensitive ingredient inside a coating material like maltodextrin. Encapsulation techniques have been widely used to reduce interactions of food components with environmental factors, such as temperature, light, moisture, and oxygen (18).

Figure 1 and table 1 showed that ethanolic extract slightly more active than water extract, contrary the total phenolic of aqueous extract is higher than ethanolic extract which correlated with their antioxidant activity. Previously reported that water extract indicated higher levels of polyphenols (especially anthocyanins) than methanol, ethyl acetate, and hexane extracts, respectively (19). According to Borrás-Linares *et al.* (2015), the total phenolic compounds (TPC) vary in the different roselle extracts, ranging from 24.0 ± 3.0 to 100.0 ± 4.0 mg GAE / g dry calyx. In fact, this study obtained a lower total phenolic level than the previous study, because the extracts contained maltodextrin as diluent or encapsulating material. Antioxidant activity of water extract showed higher level than the other, because water extract provided the highest concentrations of anthocyanin (cyanidin 3-sambubioside and delphinidin 3-sambubioside). According to Jafarian *et al.* (2014) the presence of phenolic compounds could account for the reasonably strong antioxidant activity in the roselle extracts.

The results of antimycobacterial activity of extract after 14 days incubation in Middlebrook 7H9 showed in fig.2 .

In this study, the MODs method was used to determine MIC of the extracts. Providing both, accurate and inexpensive, the method also has been used to test the sensitivity of tuberculosis drugs in a shorter incubation period compared to conventional methods. The presence of bacterial growth is characterized by the formation of a *cord* shape when observed under microscope after 7-14 days of incubation (Park *et al.,* 2002). As a result, it showed that in 0.5% extract, the cord shape was still observable under the microscope, but 1% extract was undetectable. This implied that roselle extract obviously provide the antibacterial ability. Furthermore, as reported from the previous study, the plant phenolic compounds have the ability to inhibit *M. tuberculosis* growth (Brown, *et al.*, 2007; Garcia *et al*, 2012). Sasikhumar *et al*. (2018) in line with extract yielding quercetin and rutin flavonoids could inhibit *M. tuberculosis* growth with MIC values around 6.25 µg/ml until 25 µg/ml. In fact, research about the ability of anthocyanins in inhibiting *M. tuberculosis* has not been found yet. In this study, the MIC value of 10 mg/ml was obtained because they were in the form of a crude extract coated with maltodextrin. The chemical substances of roselle should be considered in addition to anthocyanins including protocatechuic acid; flavonoids such as quercetin, luteolin, and their glycosides; a major organic acid such as citric acid, maleic acid, hibiscus acid, tartaric acid and ascorbic acid; dietary fiber; compounds such as vitamins, minerals and sterols.

CONCLUSION

Both Roselle extracts had antibacterial activity expressed as Minimum Inhibitory Concentration (MIC) at 10 mg/ml against MDR *M. tuberculosis* and antioxidant activity against DPPH of water extract (0.88 mg/ml) stronger than ethanolic extract (3.78 mg/ml). Further study regarding the chemical analysis of compounds in Roselle and the potential effect as antioxidant must be elaborate. The mechanism

involve and specific substance contributed to antimicrobial activity against MDR Tuberculosis are still unclear. However, basic study of calyx extract from Roselle illustrate a potential candidate as new natural resources in infection treatment.

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CONFLICT OF INTEREST

All authors confirmed that there is no conflict of interest.

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TABLE AND FIGURES

Table 1: Extraction yield, Total Phenolic Concentration, IC_{50} against DPPH of roselle calyx extract

extraction methods	extraction yield (%)	TPC (mg GAE/g)	IC_{50} against DPPH (mg/ml)
Infusion (water)	8.5	17.70 ± 0.7	0.88
Maceration (50% ethanol)	10.25	13.15 ± 0.3	3.78

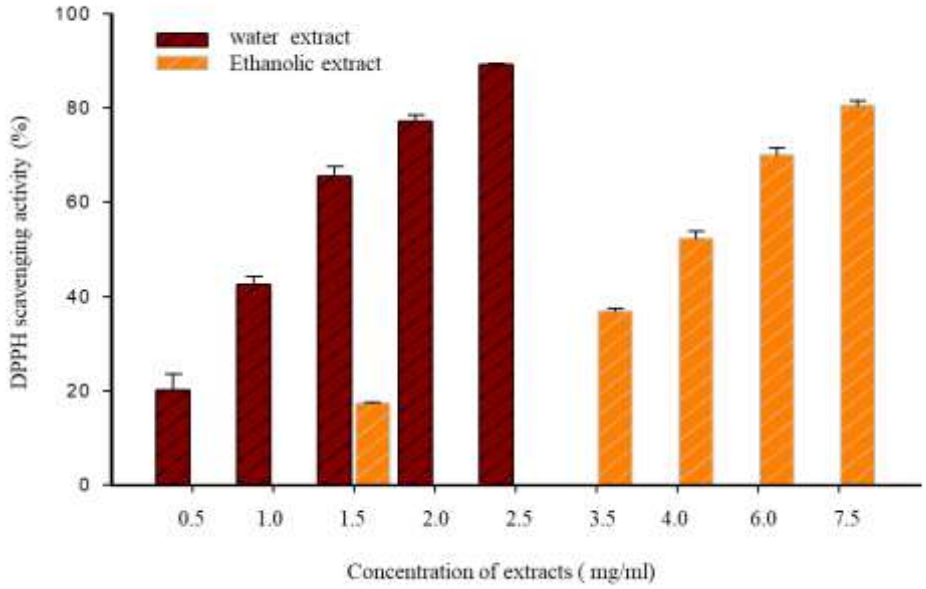

Figure 1: DPPH scavenging extracts in several concentration activity of roselle calyx

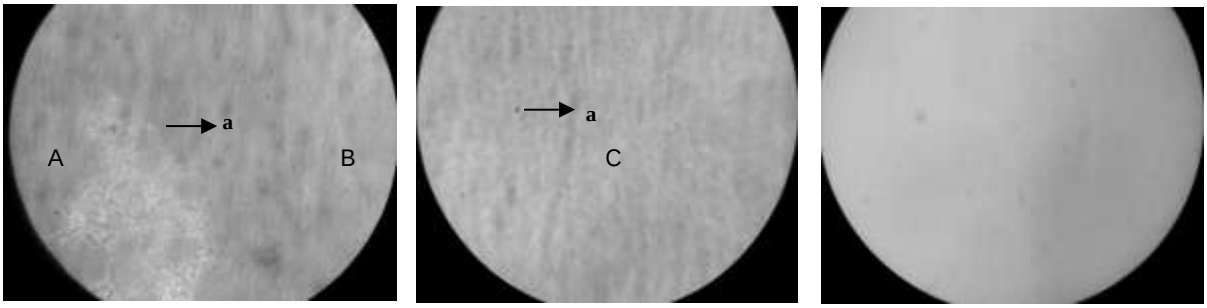

Figure 2: Microscopic of MDR *M. tuberculosis* after exposured with 50% ethanolic extract of roselle calyx in media Middlebrook 7H9 with 14 days incubation. (A) without roselle extract; (B) 0.5 % roselle extract; (C) 1 % roselle extract; (a) Cord shape of M. *tuberculosis* in Middlebrook 7H9 medium with 10% OADC enrichment media