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Research article

Influence of variation extraction methods (classical procedure) for antibacterial activity of Rarugadong (*Dioscorea pyrifolia* Kunth.) tuber

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Keywords: Secondary metabolites, antibacterial, *Dioscorea pyrifolia* Kunth., screening phytochemistry, maceration, reflux, soxhlet.

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Abstract

Drug resistance due to widespread abuse and excessive use of antibiotics has become an increasingly serious problem, making the development of alternative antibacterial a very urgent problem. The aim of this study was to define whether suitable extraction methods, such as maceration, reflux and soxhlet method. In this study, rarugadong (*Dioscorea pyrifolia* Kunth.) tuber extract, was extracted by maceration, reflux and soxhlet method respectively using methanol as solvent. Phytochemical screening carried out on rarugadong (*Dioscorea pyrifolia* Kunth.) tuber in simplicia powder and each extract. Antibacterial activity was determined using agar well diffusion method. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were used as test bacterial in this study. Phytochemistry screening results of simplicia and extract gave the same result, it was showed positive results against the class of chemical compounds alkaloids, flavonoids, glycosides, saponins, triterpenoids and tannins, anthraquinone glycosides and cyanogenic glycosides. Antibacterial activity of methanol extract from maceration, reflux and soxhlet methods showed inhibition for both *bacteria* (*Staphylococcus aureus* and *Escherichia coli)* from the measurement of inhibition diameter in each extract concentration.

Introduction

Infectious diseases cause a death rate of 13 million people in worldwide each year, especially in developing countries such as Indonesia [1]. The use of antibiotics is a necessity in overcoming various infectious diseases, but the rate of resistance produced continues to increase in line with the use of antibiotics. In 2005 more than 19,000 cases of death in America and England were caused by *Staphylococcus aureus* resistant to methicilin. The case also caused the death rate to rise sharply from 51 cases in 1993 to 1.652 cases in 2006 in the United Kingdom [2]. About 70% of nosocomial infections are caused by *Staphylococcus aureus*. Various infections of the skin and soft tissues, such as pneumonia, osteomyelitis, meningitis and endocarditis caused by *Staphylococcus aureus* [3]. *Escherichia coli* is one of the bacteria that causes infection in the digestive tract. These microorganisms become pathogens only when they reach tissues outside the digestive tract, especially the urinary tract, bile ducts, lungs, peritoneum or lining of the brain, thus causing inflammation in these places [4]. Research carried out on plants (*Dioscorea pyrifolia* Kunth.), especially for antibacterial activity, has not been carried out mainly on antibacterial activity. Research conducted in 2012 stated that the methanol extract of *Dioscorea dumetorum* (Kunth) Pax and *Dioscorea hirtiflora* (Linn.) [5] tuber had antibacterial activity against gram-positive bacteria and gram-negative bacteria, also antioxidant activity. In addition, the antimicrobial effects of tuber *Dioscorea deltoidea* for several microbes [6] and for traditional treatment of eye infections [7] was carried out. From the existing studies it can be concluded that the Dioscoreaceae family has the potential as an antimicrobial and antioxidant. This research is the initial part of the whole research in obtaining antibacterial active compounds from rarugadong tubers to gram positive and gram negative bacteria represented by each bacterium, namely *Staphylococcus aureus* and *Escherichia coli*. The research phase is to optimize the most suitable extraction method in terms of its antibacterial activity as a first step in isolating its chemical active compounds.

Experimental

Preparation of plants sample

The plants were tuber rarugadong (*Dioscorea pyrifolia* Kunth.), taken from the Urug Besan Forest in Buah Nabar Village, Sibolangit District, Deli Serdang Regency, Medan, North Sumatera Province, Indonesia. As much as

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5 kg of fresh rarugadong tuber (*Dioscorea pyrifolia* Kunth.) were cleaned from dirt by washing one by one with water then drained, then thinly sliced, weighed, then dried by aerating in the open air protected from light. The slices of the rarugadong tuber were put into the drying cupboard at a temperature of 40 ± 5ºC. Simplicia powder was stored in a well-closed container protected by sunlight, hot and humid.

Identification of plant

Identification of plant was carried out in "Herbarium Bogoriense" Field of Botanical Biology Research Center in Indonesian Institute of Sciences (LIPI) - Bogor in Indonesia was a species of plant *Dioscorea pyrifolia* Kunth. and Dioscoreaceae as family (No. 737/IPH.1.01/If.07/III/2016).

Phytochemical screening for simplicia powder and extract

Phytochemical screening is carried out based on standard procedures with slightly modification of the alkaloid chemical compounds [8-9], flavonoids [9], glycosides [9,10], anthraquinone glycosides [8], cyanogenic glycosides [11], saponins [10], tannins [12], triterpenoids [12] and steroids [9,13].

Extraction procedure

Maceration method

Making extract using the maceration method with methanol solvent was as followed. As much as 500 g of simplicia powder rarugadong tubers were macerated with methanol solvents until submerged in tightly closed containers for 3 days protected from sunlight while stirring several times, then filtered so that they were obtained macerate. The obtained macerate was transferred into another container which was tightly closed and protected from sunlight while the simplicia was added to the re-maceration with liquid then macerated to obtain a clear final macerate. The macerate obtained was collected and evaporated by using a rotary evaporator at a temperature of no more than 40ºC until a viscous extract was obtained, then dried over a water bath with the low heat [14].

Soxhletation method

Making extract using the soxhlet extraction method with methanol solvent was as followed. As much as 30 grams of simplicia of rarugadong tubers were wrapped in filter paper that had been formed according to a thimble with a height not exceeding the siphon tube boundary and inserted into a thimble. Then as much as 300 ml of methanol was put into a round flask. Subsequently, the soxhlet was assembled and it was ensured that the water entering and exiting water connected to the cooler was stable and not leak. Then turned on the heater, the soxhletation process was running. This process was terminated if the solvent in the round flask was colorless. Then the solvent in the round flask was concentrated with the help of rotary evapotaror for 30-40 minutes at a temperature no more than 50ºC until a viscous extract was obtained, then dried over a water bath at the lowest temperature until the dried extract was obtained and weighed [15].

Reflux method

Making extract using reflux extraction method using methanol as solvent was as followed with some modification. A total of 100 g of simplicia of rarugadong tubers were put into a round flask added with methanol as much as 300 ml. Then reflux was assembled, the heater was turned on and counted for 5 hours starting from the boiling mass. Then the reflux results in a cooled rounded flask, filtered with Whatmann paper. The filtrate was concentrated with the rotary evaporator for 30-60 minutes at a temperature no more than 50ºC until a viscous extract was obtained, then dried over a water bath at the lowest temperature to obtain a dry extract and weighed [16].

Antibacterial activity assay

Antibacterial activity assay was carried out using the agar well diffusion assay method. The test bacteria cultures used were gram-positive bacteria (*Staphylococcus aureus* ATCC 25923) and gram-negative bacteria (*Escherichia coli* ATCC 25922) which were obtained from the Microbiology Laboratory, Faculty of Pharmacy, Gadjah Mada University – Yogyakarta, Indonesia. Antibacterial activity assay was performed of methanol extract obtained by the agar well plate diffusion assay method using a sterile cork borer [17].

Preparation of test extract solution

Each extract was weighed as much as 1 g then dissolved in dimethylsulfoxide until dissolved and the volume was satisfied with ethanol to the mark line on the flask measuring 10 ml to obtain a concentration of 100mg/ml.

Agar well plate diffusion assay method

The base layer of the media was made by pouring 10 ml of mueller hinton agar (MHA) into a sterile petri dish, then left to solidify. After solidifying, on the surface of the layer, poured 0.1 ml of suspension of test bacterial inoculum (the inoculum solution was measured to achieve an optical density solution (OD) = 0.5 using a uv-vis spectrophotometer at a wavelength of 600 nm) [18-20] and 20 ml of mueller hinton agar (MHA) media as the second layer, then homogenized. Sterile cylinder stainless steel were immediately placed and arranged on the surface of the media and arranged in such a way that the observation area was not overlap. Furthermore, the cylinder stainless steel was lifted slowly using sterile tweezers from the surface of the agar media that had solidified, so that wells (holes) were formed which would

be inserted into each of the test extract solutions with various concentrations. A solution of the test extract with various concentrations and blanks (mixtures of dimethylsulfoxide and ethanol) was included in the available wells as much as 0.1 ml. Petri dishes were immediately closed and left for 30 minutes, then the petri dishes were incubated in an incubator at a temperature of 35±2ºC for 24 hours. Observations were made by measuring the clear area in the form of a circle around the well using a calliper, so that the growth inhibition diameter was known in units of millimeters (mm) [18,19].

Results and discussion

Simplicia preparation and extraction

Simplicia powder obtained from 5 kg of fresh rarugadong tuber as much as 910 g. Extraction results by maceration method using methanol solvent from 200 g of simplicia powder obtained a dry extract of 35.3467 g. Extraction results by reflux method using methanol solvent from 50 g of simplicia powder obtained a dry extract of 5.1512 g. Extraction results by soxhlet method using methanol solvent from 87.5 g of simplicia powder obtained a dry extract of 10.0275 g.

Chemical compound analysis by phytochemistry screening

Phytochemistry screening results of simplicia of Rarugadong (*Dioscorea Pyrifolia* Kunth.) tuber was showed positive results against the class of chemical compounds alkaloids, antraquinone glycosides, flavonoids, glycosides, saponins, cyanogenic glycosides, tannins and triterpenoids. Phytochemical screening was the first step in determining the content of plant chemical compounds (secondary metabolites) qualitatively. Results of phytochemistry screening can be seen in Table 1 below. Results of simplicia phytochemistry screening on Rarugadong (*Dioscorea Pyrifolia* Kunth.) tuber indicated the profile of chemical compounds existenced in methanol extract from maceration, reflux and soxhlet method using methanol as solvent, this showed methanol as extraction solvent was one of the organic solvents which can dissolved and attracted chemical compounds from plant of various types polarity, namely nonpolar, semipolar and polar.

Table 1. Results of Phytochemistry Screening of Simplicia and Extract of Variation Extraction Methods (Maceration, Reflux and Soxhlet) of Rarugadong (*Dioscorea Pyrifolia* Kunth.) Tuber.

No.	Chemical Compound	Simplicia	Extract of Maceration Method	Extract of Reflux Method	Extract of Soxhlet Method
1.	Alkaloids	(+)	(+)	(+)	(+)
2.	Antraquinone glycosides	(+)	(+)	(+)	(+)
3.	Flavonoids	(+)	(+)	(+)	(+)
4.	Glycosides	(+)	(+)	(+)	(+)
5.	Saponins	(+)	(+)	(+)	(+)
6.	Cyanogenic glycosides	(+)	(+)	(+)	(+)
7.	Tanins	(+)	(+)	(+)	(+)
8.	Triterpenoids / Steroid	(+) triterpenoids	(+) triterpenoids	(+) triterpenoids	(+) triterpenoids

Note: (+) = containing the compound

It has been reported that solvents which were generally used to search for compounds that have antimicrobial activity are methanol, ethanol and water. It has also been reported previously that acetone and methanol solvents were better solvents in the process of extracting antimicrobial chemical compounds than hexane and dichloromethane. The advantage of using methanol solvents is their ability to dissolve antimicrobial substances, rate of extraction, ease of removal and toxicity in bioassay [17]. In the data it appears there was no difference in the composition of the chemical compound content of each extract obtained based on differences in extraction methods. This illustrates that the chemical compounds found in rarugadong tubers were not affected by heat extraction or cold extraction. It has been reported in previous studies that the content of phenol compounds in *Dioscorea alata* was not completely lost after vacuum frying. This can be seen in the data they presented that after vacuum frying, the content of phenol, anthocyanin, sinapic acid and ferulic acid were reduced by 57.32%, 74.45%, 58.89% and 69.71% respectively. In the journal it was also reported that there were no negative effects of temperature changes on the content of phenol compounds and their antioxidant activities [21].

Antibacterial activity

Antibacterial activity of variation extraction methods of rarugadong (*Dioscorea pyrifolia* Kunth.) tuber for *Staphylococcus aureus* (SA) ATCC 25923 at concentration 100mg/ml can be seen in figure 1.

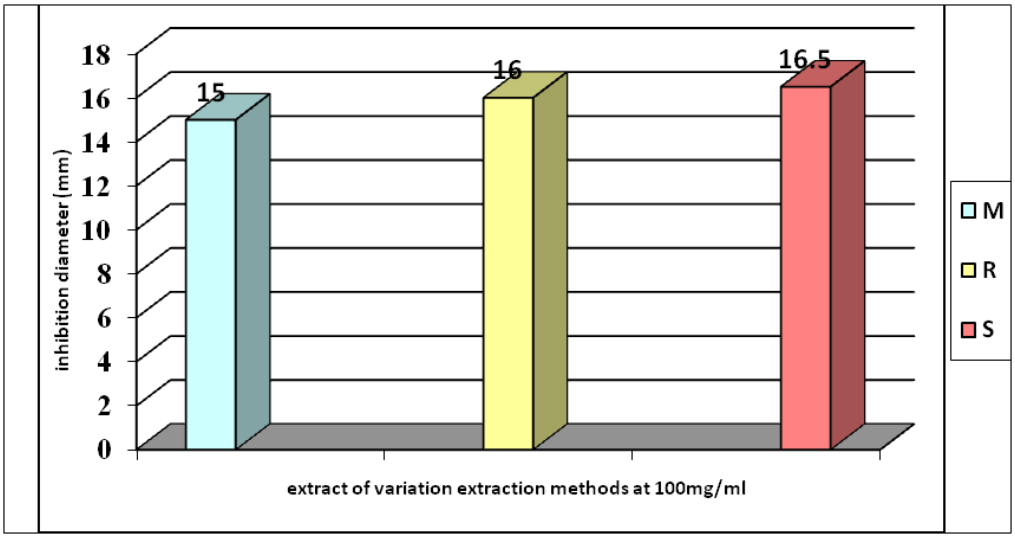

Figure 1. Antibacterial activity of variation extraction methods of Rarugadong (*Dioscorea pyrifolia* Kunth.) Tuber for *Staphylococcus aureus* ATCC 25923 at concentration 100mg/ml.
Note: M= extract from maceration method using methanol as solvent, R= extract from reflux method using methanol as solvent, S= extract from soxhlet method using methanol as solvent.

The antibacterial activity of rarugadong tuber extract from various extraction methods against SA bacteria showed varying results. The strongest antibacterial activity against SA bacteria was shown in extract S (d = 16.50 mm). From this overall data showed the inhibition diameter of one another had a difference that was not too large for the growth of SA bacteria. Antibacterial activity of variation extraction methods of rarugadong (*Dioscorea pyrifolia* Kunth.) tuber for *Escherichia coli* ATCC 25922 at concentration 100mg/ml can be seen in Figure 2. The antibacterial activity of rarugadong tuber extract from various variations of extraction methods against bacterial EC showed varied results. The strongest antibacterial activity against bacteria EC was shown in extract S (d = 14.10 mm). In testing the antibacterial activity carried out at a concentration of 100 mg / ml, this concentration was obtained from the results of the orientation carried out previously by looking for the same concentration of each extract showing the inhibitory diameter. In this study there was no testing of positive control antibacterial activity using antibiotics and the same was done by several previous researchers [20,22-24]. This is because in this study it was not aimed at comparing the antibacterial activity of extracts with antibiotic agents. In addition, agar well plate diffusion assay method was not need to prove its validity because this method as testing antibacterial activity have been carried out by previous researchers referring to standardized procedures.

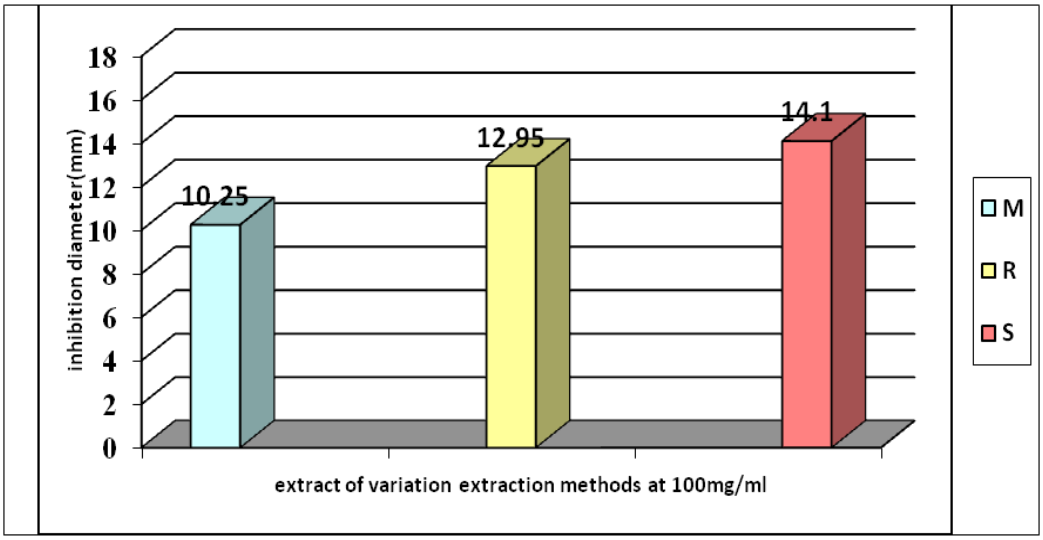

Figure 2. Antibacterial activity of variation extraction methods of Rarugadong (*Dioscorea pyrifolia* Kunth.) Tuber for *Escherichia coli* ATCC 25922 at concentration 100mg/ml.
Note: M= extract from maceration method using methanol as solvent, R= extract from reflux method using methanol as solvent, S= extract from soxhlet method using methanol as solvent.

The soxhlet extraction method can be used well against chemicals that are resistant to the boiling point of the solvent. For plants and plant chemical compounds that are not durable it will cause degradation of chemical components in plants [17]. From the results obtained, the effect of heat extraction methods (reflux and socletation) did not have a negative effect on antibacterial activity in *Staphylococcus aureus* and *Escherichia coli* bacteria. In contrast, the antibacterial activity of the extract (reflux and soxhletation) was greater than the methanol extract from the maceration method. From the results of phytochemical screening showed no differences in the composition of the chemicals contained in each extract. According to a previously published study, using heat to get the extract adds to the antimicrobial activity and increases the acidity of the extract, increasing the total phenol and individual phenolic compounds [25]. Overall rarugadong tuber extracts contain greater antibacterial activity against *Staphylococcus aureus* than *Escherichia coli*. In previous studies, gram negative bacteria were more resistant to gram-positive bacteria, this was also approved by previous researchers [22, 25]. This can be explained further that the antimicrobial potential and mechanism of action are influenced by the environmental conditions at which antimicrobial substances. The environment that tends to be hydrophilic, nutritious substances, temperature and pH, are important influences on the nature of secondary metabolites. This is the tendency of gram-positive bacteria and fungi to be more affected by secondary metabolites, where the main work target is the cell wall, whose disintegration or changes in permeability are followed by efflux of the intracellular compounds and damage to the cytoplasm [26]. In another study, it was also added that in general gram-positive bacteria were more affected by natural chemical compounds of plant extracts than gram-negative bacteria due to differences in the structure of cell walls by both. Gram negative has a phospholipid outer membrane that carries a structural component of lipopolysaccharide, which makes the cell wall impermeable to antibacterial chemicals. Another case with gram-positive bacteria, which consists of the peptidoglycan layer which is not effective as a layer of permeability [27].

Based on the results of the analysis of the chemical compounds carried out on the powder simplicia and extract containing variety chemical compound (secondary metabolites), namely alkaloids, antraquinone glycosides, flavonoids, glycosides, saponins, cyanogenic glycosides, tannins and triterpenoids (table 1). The biological activity of a plant was determined by the chemical compounds in it. There had been a lot of preliminary research and further research investigating the biological properties of plants, especially their antibacterial properties. In previous studies it had been found that the class of indolizidine compounds (belonging to the alkaoid compound) had antibacterial activity by inhibiting the formation of nucleic acids [28]. Anthraquinone compounds, namely chrysophanol, isolated from plant root extracts of *Colubrina greggii*, had been tested to have antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* [29]. Extracts containing saponin compounds had been tested for antimicrobial activity, but only have effects on gram-positive bacteria, whereas for gram-negative bacteria and fungi have no effect [30]. In this study, it is seen that methanol extract has a greater effect on SA bacteria, this is possible because the saponin compounds have no effect on the EC bacteria which is a gram negative bacteria. Flavonoid compounds (such as galangin and pinocembrine) have also been reported to have antimicrobial properties [31]. Cyanogenic groups of glycosides [32] and tannins [32-33] have antimicrobial properties that have been demonstrated through research on *Ximenia americana* [33]. The antibacterial activity of the triterpenoid group which showed the mechanism of action of bacterial membrane cells has also been reported [34].

Conclusion

Phytochemistry screening results of simplicia and extract were showed positive results against the class of chemical compounds alkaloids, flavonoids, glycosides, saponins, triterpenoids and tannins, anthraquinone glycosides and cyanogenic glycosides. Antibacterial activity of methanol extract from maceration, reflux and soxhlet methods showed inhibition for both *bacteria* (*Staphylococcus aureus* and *Escherichia coli)* from the measurement of inhibition diameter in each extract concentration.

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