Extraction methods effect on antioxidant activity of ethanol extract of ‘pasak bumi’ (Eurycoma longifolia Jack.) root

K Anwar, A Lutpi, A Melinda and S Hadi

Department of Pharmacy, Universitas Lambung Mangkurat, Banjarbaru, Indonesia 70714
*Corresponding author e-mail: samsul.hadi@ulm.ac.id

Abstract. Pasak bumi (Eurycoma longifolia Jack.) has various benefits, such as the root of E. longifolia is often used as an anti-microbial, anti-malarial, anti-inflammatory, dysentery and aphrodisiac. The antioxidant activity is closely related to the ability of E. longifolia root to maintain a fit condition. The benefits of the medicinal plants can be felt by the general public after going through both traditional and modern processing processes. One of the modern processing methods is to isolate secondary metabolites in plants or known as extraction. This study aims to determine the antioxidant activity of the roots of extracted E. longifolia using 96% ethanol solvent with different extraction methods. Extraction was performed by maceration, percolation, reflux, and soxhletation methods. The antioxidant activity test was carried out using the DPPH(1,1-diphenyl-2-picrylhydrazyl) method. The results shows that the antioxidant activity of E. longifolia extract using various extraction methods in the order soxhletation > reflux > percolation > maceration. From this research, it can be concluded that the percolation can be developed as extraction method of E. longifolia roots.

Keywords: extraction, methods, antioxidant, Eurycoma longifolia, root

1. Introduction

Eurycoma longifolia is a plant that is often used as traditional herbal medicine, especially in Southeast Asia such as Indonesia, Malaysia, Vietnam, and Thailand [1,2]. This plant has a local name in Indonesia, namely pasak bumi or bedara pahit, while in Malaysia it is often called tongkat Ali. This plant extract has many properties including as an aphrodisiac, antimalarial, antimicrobial, and antidiabetic [2]. One part of the plant that is most often used as traditional medicine is the root. Eurycoma longifolia root extract has provided many benefits to humans such as restoring energy and vitality, increasing blood flow, and as an herbal ingredient for women after childbirth [1].

Eurycoma longifolia root extract has several secondary metabolites such as phenolic compounds, terpenoids, and flavonoids, while the stem extract shows cardiac glycosides, proteins, phenolic compounds, flavonoids, terpenoids, and alkaloids. Flavonoids are compounds that have antioxidant, anti-inflammatory, anticancer, and antimicrobial activities. Antioxidant activity in plants is usually due to the presence of phenolic compounds and flavonoids [3].

Antioxidants play a role in fighting free radicals in the body, usually obtained from the body's metabolism, air pollution in the environment, sunlight, and so on. Free radicals can cause various kinds of disturbances such as the production of DNA, the effect on blood vessels, the production of other proteins such as enzymes and prostaglandins [4]. Flavonoids present in plants can prevent injury from free radicals by directly capturing free radicals. Flavonoids will produce radicals that are less reactive and more stable by reacting with reactive compounds of radicals [5].
The antioxidant activity test was often carried out using the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. The activity of this method is based on the ability of the sample to capture the DPPH radical which is indicated by the reduction in the purple colour of the DPPH solution. The reduced colour intensity indicates the potential of the sample to capture DPPH radicals [6]. The antioxidant and anti-inflammatory activity of *E. longifolia* will increase if the concentration is increased. The study showed that the extract from this plant had an IC50 value of 169.56 ppm [7]. *Eurycoma longifolia* root has activity against free radical scavenging with the ES50 value of the ethanol extract (15.64 g/mL) greater than the ethyl acetate fraction (13.948 g/mL) [6].

*Eurycoma longifolia* has various bioactive compounds, one of which is eurycomanone. This quassinoids compound is the main compound of *E. longifolia*. Concentration of this compound plays an important role in the biological activity of *E. longifolia* plants [8]. Eurycomanone is one of the compounds that play an important role in plant activity as an aphrodisiac and anticancer [9].

Phenolic compounds and flavonoids are compounds that play a role in plant activity as antioxidants. The extraction method is considered to have an influence on the success of obtaining certain compounds related to the pharmacological activity of a plant. Therefore, in this study optimization of various methods of extracting the roots of *E. longifolia* to produce optimal antioxidant activity.

2. Materials and Methods
2.1. Sample preparation
The roots of *E. longifolia* were collected from Sabuai Village, Pangkalan Bun. Wet sortation was carried out to separate foreign materials or unnecessary impurities. The washing process with water flow is carried out to remove impurities and adhering soil. The roots of *E. longifolia* firstly shaved to get smaller particles. Drying process of the simplicial was done in the oven at a temperature of 60°C. The dried simplicia were sorted, and then blended to get a smaller particle [10].

2.2. Extraction of *E. longifolia* roots
There are 4 extractions carried out in this study, namely maceration, percolation, soxhletation and reflux. The maceration method was carried out by mixing 250 g of dry powder of the roots of *E. longifolia* mixed with 96% ethanol solvent in the macerator. The mixture was stirred every 6 hours. After 24 hours, it was filtered with filter paper and filtrate was collected. Maceration process is repeated 2 times for residue from filtration result. The collected filtrate was evaporated with a water bath to obtain a thick extract [11].

The percolation process carried out in this study used two solvents, namely 96% ethanol, and hot water. Dry powder of *E. longifolia* root (75 g) wrapped like candy by filter paper and put into the percolator. As a solvent, 750 mL ethanol 96% was put into the percolator and allowed to stand for 3 hours. Then the solvent was dripped at a rate of 20 drops per minute and followed by a new solvent. Percolation was carried out by changing the solvent 6 times. After that, it was filtered with filter paper and filtrate was collected. Maceration process is repeated 2 times for residue from filtration result. The collected filtrate was evaporated with a water bath to obtain a thick extract with a constant weight. Hot water percolation method was carried out the same way as ethanol 96% percolation [12].

The soxhletation process was carried out using 50 g of dry *E. longifolia* powder. The powder is wrapped in filter paper like candy. The ethanol solvent was put into a 250 ml round bottom flask. Boiling stones are also added to the round bottom flask. The temperature of the bath is adjusted according to the boiling point of the solvent to obtain ± 12 cycles. Filtering is done with filter paper. The filtrate was evaporated with a water bath to obtain a thick extract with a constant weight [13].

The reflux method was done by adding 100 g of *E. longifolia* powder into a round bottom flask, and adding it with 900 ml of ethanol 96% solvent. The reflux apparatus is assembled, then extracted at the boiling point temperature of the solvent. The reflux process was carried out for 3-4 hours with 3 repetitions. The extracted filtrate was filtered using filter paper and evaporated with a water bath to obtain a thick extract with a constant weight [14].
2.3. DPPH antioxidants assay
The first step of DPPH antioxidants assay is determination of maximum wavelength. The 0.4M DPPH reagent was prepared using 8 mg in a 50 ml volumetric flask, dissolved in 96% ethanol to mark the limit. 0.5 ml of DPPH solution was taken with 2 ml of 96% ethanol. The mixture was then vortexed, and incubated for 22-30 minutes at room temperature and dark conditions. Wavelength measurements were carried out at 450-550 nm [15]. A blank solution was prepared by inserting 2 ml of ethanol into a test tube. The positive control solution used quercetin with concentrations of 1, 2, 3, 4, 5 and 6 ppm [16].

A total of 25 mg of each extraction method was made of 1000 ppm stock solution with 96% ethanol as solvent. The concentration series solutions were made with concentrations of 50, 100, 150, 200, 250 and 300 ppm. Each concentration was taken as much as 2 ml and added 0.5 ml of DPPH reagent, then vortexed. The mixture was incubated for 22-30 minutes at room temperature and dark conditions. The absorbance of each sample was read at a wavelength of 515 nm [17].

2.4. Determination of IC50 value
The percentage inhibition value can be represented by the IC50 value which is calculated by the following formula [18].

\[
\text{Percentage of Inhibition} = \frac{(A_k - A_s)}{A_k} \times 100\%
\]

\(A_k\) = Absorbance control or Absorbance that does not contain a sample

\(A_s\) = Absorbance of sample or Absorbance of extract

The results of the calculation of the percentage of inhibition were entered into the regression equation with the concentration of the extract (μg/mL) as the abscissa (X axis) and the value of % antioxidant inhibition as the ordinate (Y axis). The value of \(x\) as IC50 can be calculated by the equation [19]

\[y = bx + a\]

\(y\) = percentage of inhibition

\(x\) = sample concentration (ppm)

\(a\) = intercept or regression constant

\(b\) = slope or regression coefficient

3. Results and Discussion
3.1. Extraction of E. longifolia roots
Eurycoma longifolia samples were extracted using the cold method and the hot method extractionn. The cold extraction method is maceration and percolation, while the hot method is soxhletation and reflux. The final result of the extraction process is a thick extract, where the rendement of the extract will be calculated. Rendement is the ratio of the final weight of the resulting product to the initial weight of the product before treatment and is expressed as a percentage. The higher the yield of the extract, the higher the chemical compounds that are interested in the extract [20].

Based on Table 1, rendement of hot water percolation > maceration > soxhletation > reflux > ethanol 96% percolation. Rendement is influenced by several factors including the extraction method, the number of solvents and the type of solvent used [21-23]. The maceration method will produce more yields than the percolation method, because in maceration stirring occurs to accelerate the dissolution process by the analyte, compared to percolation which is not stirred and only immersed in the process [23]. The rendement of the maceration and percolation methods using 96% ethanol as a solvent showed the appropriate results, where the maceration was higher than the percolation rendement.
Table 1. Rendement of *E. longifolia* extract with different extraction methods.

| Method      | Solvent       | Simplicia Weight (g) | Thick Extract Weight (g) | Rendement (%) |
|-------------|---------------|-----------------------|--------------------------|---------------|
| Maceration  | Ethanol 96%   | 250                   | 7.62                     | 3.048         |
| Percolation | Ethanol 96%   | 75                    | 1.16                     | 1.547         |
|             | Hot water     | 75                    | 6.43                     | 8.573         |
| Soxhletation| Ethanol 96%   | 50                    | 1.25                     | 2.5           |
| Reflux      | Ethanol 96%   | 100                   | 2.49                     | 2.49          |

The rendement of percolation with hot water solvent was higher than the yield of all other extraction methods using ethanol 96% as solvent. Water is a very good solvent for ionic compounds and the –OH group in water is polar. Ethanol 96% is semi-polar solvent which can form hydrogen bonds between molecules. Ethanol as solvent will evaporate faster than the water solvent and make faster extraction process [24].

Generally, the hot extraction method will produce higher yields than the cold method. Higher extraction temperature will result in faster molecular and solvent movement, so the rate of transfer of compounds from plant cells into the solvent will increases. However, this study showed different results where the rendement from the cold extraction method was higher than the hot method. This possibility occurs because the cold method is extracted at room temperature and is relatively safe so that most of the compounds can be extracted [22]. In addition, maceration and percolation extraction (with hot water) uses a larger number of solvents than soxhletation and reflux extraction. The transfer of active compounds from plants into the solvent can run more optimally and solvent saturation can also be avoided [21].

3.2. Antioxidant activity of *E. longifolia* roots extract

The antioxidant activity test in this study used the DPPH (1,1-diphenyl-2-picrylhydrazil) method. Samples are considered to have antioxidant abilities if they can capture or reduce DPPH radicals, indicated by the fading of the purple color of the DPPH solution. The sample solution that is able to capture the DPPH radical will turn yellow because of the reaction between the hydrogen atom donor from the sample and the DPPH radical molecule [6].

The capacity of antioxidant activity of the sample can be calculated by IC$_{50}$ (Inhibition Concentration 50%). The IC$_{50}$ value is the concentration at which the sample is able to capture or reduce 50% of the DPPH radical activity. The higher the concentration, the lower the absorbance of the sample, because the absorbance measured is the absorbance of the remaining DPPH which is not reduced by the compounds in the sample. The lower the absorbance, the higher the antioxidant activity of the sample [6]. The maximum wavelength and absorbance of the solution were read using a UV-Vis spectrophotometer at 450-550 nm. The maximum wavelength obtained from the 0.4 mM DPPH solution is 515 nm [25].

The concentration of quercetin which is able to inhibit 50% DPPH radicals is 1.2755 ppm (figure 1). Quercetin was used as a comparison or positive control because it has high free radical reduction activity. OH- groups in quercetin can stabilize free radicals by means of hydrogen atom donation or electron transfer [26-27].
Figure 1. Graph of the relationship between concentration of quercetin solution and free radical inhibition (%)

The result of DPPH radical inhibition of *E. longifolia* root extract from different extraction methods was various (figure 2). The IC$_{50}$ values of soxhletation < reflux < percolation (ethanol 96%) < maceration < percolation (hot water). The DPPH radical inhibition activity (IC$_{50}$) in soxhletation extraction was 96.2941 ppm (strong antioxidant), reflux extraction was 126.3039 ppm (moderate antioxidant), percolation extraction (ethanol 96%) was 220.8113 ppm (moderate antioxidant), maceration extraction was 254.1297 ppm (weak antioxidant), and percolation extraction with hot water was 417.4432 ppm (weak antioxidant). When compared with quercetin, *E. longifolia* root extract had lower antioxidant activity. Extraction with soxhletation showed strong antioxidant activity but not as strong as quercetin.

Figure 2. Free radical inhibition (%) of *E. longifolia* extract from different extraction methods.

The antioxidant activity of percolation extraction with hot water solvent is much lower than the antioxidant activity of various other extraction methods with 96% ethanol solvent. This is because phenol compounds that act as antioxidants cannot be extracted completely in water solvents. The amount of phenol content is usually influenced by the use of solvent in the extraction. Effective solvents used to extract these compounds are methanol and ethanol, because of their universal use and able to extract almost all metabolites present in plants [28].

The best antioxidant activity was found in cold extraction methods such as maceration and percolation [29-30]. Phenolic compounds such as flavonoids, that believed to have antioxidant properties
and are able to prevent reactive free radicals from damaging cells and their cellular components, is stable to heating with a certain temperature. An increase in temperature will result in increased levels of flavonoids and then will decrease if the temperature is increased continuously. Flavonoid content increases with increasing extraction temperature, but too high temperature can cause denaturation of heat-sensitive antioxidants and will be more stable at low temperatures.

However, the results of this study are inversely related to the research of conducted by Verawati et al. [29] and Wathan et al. [30]. In this study, hot extraction such as soxhletation and reflux showed better antioxidant activity than cold extraction. This may be due to high flavonoid levels and the presence of other chemical compounds such as phenolic groups or tannins which also have antioxidant activity, as well as the absence of damage to antioxidant activity due to excessive heat. Comparison of the extraction temperature was not carried out in this study, so that it could not estimate the optimal extraction temperature for antioxidant capacity.

4. Conclusion
From this study it can be concluded that rendement of hot water percolation > maceration > soxhletation > reflux > ethanol 96% percolation. The soxhletation method has the best antioxidant activity with an IC50 value of 96.2941 ppm, followed by reflux, percolation, maceration, and percolation methods with hot water solvent. Percolation can be developed as extraction method of E. longifolia roots.

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