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In Vitro Mucolytic Activity Assay of The Acetone Extract of The Silver Fern (Pityrogramma calomelanos)

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Abstract **—The aim of The aims of this research is to determine mucolytic activity of methanol extract of silver fern (*P. calomelanos*). In this research extraction was carried out by maceration method. Qualitative test was done by ferric chloride reagent and Shinoda test to identify the existance of phenolic and flavonoid compounds, respectively. Mucolytic activity assay was performed in vitro based on the decreasing the viscosity of intestinal mucus cow. Viscosity values were analyzed statistically using one-way ANOVA test, followed by LSD test to determine significant differences between treatment groups. From the results of the study, it had been obtained the methanol extract in the form of brown solid. The qualitative test of the extract with the ferric chloride reagent produced a green color which showed the existence of phenolic compound, while the orange color in the Shinoda Test showed the existence of flavonoid compound. The result of mucolytic activity test showed that the extract had mucolytic activity. Extract with a concentration of 0.6% had mucolytic activity equivalent to acetylcysteine 0.1%.**

Keywords—Pityrogramma calomelanos, acetone extract, mucolyic activity

I. INTRODUCTION

Indonesia is a developing country, one of which is marked by the rapid development of industry. This has a positive impact on human life in the form of wider employment opportunities, so that it affects the socio-economic improvement of the community. In addition, there are negative impacts in the form of health problems due to air pollution from the factory fumes during industrial processes or byproducts of their production [1]. One of the diseases caused by air pollution is respiratory disease. Currently the disease is one of a major problem in Indonesia. The rates of morbidity and death from respiratory diseases such as acute respiratory infection, bronchitis, asthma, and tuberculosis are still in highest rank. According to WHO (2017), there are 1 - 4 million cases of respiratory problems (18.8 million - 12 million) globally, which are equivalent to 120 cases per 100,000 population. Most of them occur in the Southeast Asia region at 45% where Indonesia in second ranks among the five countries [2].

Patients with respiratory diseases such as bronchitis, asthma, and acute respiratory infections are usually characterized by cough with phlegm that persists for several weeks. Cough is a protective physiological mechanism that is useful for removing and clearing the respiratory tract of sputum, inhaled foreign stimulants, foreign particles, and elements of infection [3]. In these conditions, the mucus production increase. Mucus produced is thick so that it affects the breathing because it is difficult to remove. Physiologically cilia are unable to excrete mucus because it is too thick [4]. The mucus produced in the respiratory tract is a liquid complex of mucoprotein and mucopolysaccharide gel membrane membranes. The composition of mucus is 95% water and 5% glycoprotein. Meanwhile mammalian intestinal mucus consists of water (97.5%), protein (0.8%), other organic substances (0.73%), and organic salt (0.88%) [5]. The thick mucus can be removed through a dilution process.

The mucolytic active ingredients can help reduce the viscosity of sputum so that it is easily removed. The material is able to remodel and dissolve sputum so that the viscosity is reduced and makes it easier to removed. The active ingredients of synthetic mucolytic commonly used are bromhexin, ambroxol, and acetylcysteine. But the use of these active ingredients has side effects that are not good for health

The use of natural ingredients as traditional mucolytic medicine is safer and has the potential to be developed into medicine to overcome a variety of diseases. Some traditional medicinal plants had been known to the community for healing respiratory diseases such as bronchitis and chronic sore throat, including *Echinacea angustifolia*, bitter (*Andrographis paniculata*), and *Rhododendron dauricum*. Echinaceae plants contain active compounds, namely essential oils, alkamides, polyalcae, polyalcuna, and derivatives of caffeic acid. Sambiloto contains active compounds in the form of flavonoids and lactone terpenoids. Meanwhile, *Rhododendron dauricum* contains several bioactive compounds, namely flavonoid farrerol, scopoletin,

umbeliferone, hyperoside, kaempferol, and quercetin [6].

Studies on mucolytic activity of plants had been reported. The mucolytic activity of methanol extract of red betel leaf at a concentration of 0.3% was equivalent to 0.1% acetylcysteine. It contained saponin, flavonoid, and polyphenol compounds. The dichloromethane extract of the *Chingia sakayensis* fern with 0.6% concentration was reported to have mucolytic activity equivalent to 0.1% acetylcysteine and in the extract found the steroid compound β-sitosterol and flavonoid farrerol [7]. Ethanol extract of hibiscus leaves at a concentration of 0.60% has mucolytic activity equivalent to 0.1% acetylcysteine [4].

Silver fern (*P. calomelanos*) is one type of fern that grows in Indonesia [8]. The plant grows mostly in open areas, on rocky places on hill slopes, and on old wall marks, and is often found on the banks of open or rather protected rivers. It also thrives both in the lowlands and in the highlands with an altitude of 1200 m above sea level [9].

One of the secondary metabolite compounds contained in silver nail plants is the flavonoid compound. The types of flavonoids found in silver fern were 2 ', 6'-dihydroxy-4'-methoxy dihydrochalcone, kaempferol, and quercetin. Flavonoids have various activities as antiviral, antibacterial, antihistamine, and can increase respiratory activity, all of which are very supportive for healing respiratory inflammatory diseases [10].

The mucolytic activity of the silver fern acetone extract had never been reported. Therefore, in this paper we will report the results of mucolytic activity assay in vitro from the acetone extracts of silver fern s (*P. calomelanos*).

II. MATERIALS AND METHODS

A. Material

The dried powder of *P. calomelanos*'aerial part, n-hexane, dichloromethane, acetone p.a., $FeCl_3$ 5%, Mg ribbon, HCl p.a., mucus of intestinal cow, phosphate buffer solution (pH = 7), acetylcysteine , tween-80 (Sigma).

B. Instrument

The Ostwald viscometer, rotary vacuum evaporator (Buchi labortechnik B-491), analitical balance (Advanturer Ohaus), vacuum pump (Gast DOA-P-504-BN), stopwatch, waterbath (Memmert), picnometer (Pyrex), volumetric flask, beaker glass, test tube, drop pipette, spatula, maceration vessel, knife, vial bottle.

C. Reseach procedures

1. Collection and preparation of sample

Samples of the silver fern (*P. calomelanos*) were collected from the Kletak forest, Nongkojajar, Pasuruan, East Java. Before further investigation, the sample was identified at LIPI Kebun Raya Purwodadi. Furthermore, the sample is cleaned of attached dirt, then dried at room temperature. The dried sample was grinded into a fine powder that was ready for extraction

2. Extraction

The dried powder of silver fern (5 Kg) were macerated consecutively with n-hexane, dichloromethane, and acetone for 3 x 24 hours. The acetone extract was evaporated in vacuo using the rotary evaporator, yielded a reddish brown residue. Solid acetone extract was tested for phytochemistry by $FeCl_3$ reagent and Shinoda test, followed by mucolytic activity assay in vitro.

3. In vitro mucolytic activity assay

3.1. Preparation of mucus intentinal cow

The cow intestine is cleaned of attached dirt using running water, then the intestines are sorted and cut longitudinally. Furthermore, the mucous layer scraped slowly until clean. The collected mucus was used to test mucolytic activity.

3.2. Preparation of mucus-phosphate buffer solution of 20% (w/w)

The mucus-phosphate buffer solution of 20% (w /w) is made by mixing 20 parts of mucus (in weight) with 80 parts of phosphate buffer solution of 80 parts (in weight) so that a total of 100 parts (in weight). The mixture is stirred until homogeneous [11].

3.3. Preparation of negative control

The negative control solution was prepared by mixing 0.5% tween-80 (w/w) of a total weight or 0.15 gram with a phosphate buffer solution to obtain a total weight of 30 grams and stirring until homogeneous [11].

3.4. Preparation of positive control

The positive control solutions were made by mixing 0.1% acetylcysteine as much as 0.03 grams with tween-80 as much as 0.5% (w/ w) of the total weight or as much as 0.15 grams. Furthermore, mucus-phosphate buffer solution is added to obtain a total weight of 30 grams and stir until homogeneous [11].

3.5. Preparation of test solution

The test solution used was acetone extract from silver nail plants with a concentration of 0.2%; 0.4%, 0.6%, 0.8%, 1%, 1.2% and 1.4% (w/w). Each test solution was prepared by mixing as much extract as each concentration with tween-80 as much as 0.5% (w/ w) of the total weight or 0.15 gram. Subsequently a phosphate buffer mucus solution was added to obtain a total weight of 30 grams and stir until a homogeneous mixture [11].

3.6. In vitro mucolytic activity assay

The mucolytic activity test was carried out by measuring viscosity using the Ostwald viscometer. Negative control solution, positive control solution, and test solution were incubated in a water bath for 30 minutes at 37 ° C. Then the test solutions were put into the Ostwald viscometer. The density of test solutions were measured using a pycnometer. Finally, the viscosity of test solutions were calculated by multiplying the flow time data with the density value [11].

3.6. Data analysis tecniques

The mucolytic activity of silver fern acetone extract was analyzed descriptively by comparing the viscosity value of the extract with positive control of acetylcysteine 0.1%. Data of viscosity value of the extract were analyzed by one-way ANOVA, followed by LSD test in Post-Hoc analysis to determine the significance of the difference in the viscosity value of the solution between treatments. All statistical analyzes were carried out with the SPSS program.

III. RESULTS AND DISCUSSIONS

Samples in the form the dried powder of aerial parts of silver fern (*P. calomelanos*) as much as 5 kg were extracted by maceration successively using n-hexane, dichloromethane and acetone at room temperature for 24 hours and were repeated 3 times. The acetone extract obtained was evaporated by vacuum using a rotary evaporator to obtain a reddish brown residue of 76.0 grams. The solid acetone extract obtained was tested for phytochemistry with $FeCl_3$ reagent, yielding positive results indicated in green color and the Shinoda test in the extract showing orange color. Thus the acetone extract of silver fern contained phenolic compound called flavonoids.

Mucolytic activity assay was carried out with the aim to determine the mucolytic activity of the acetone extract obtained. The mucolytic activity assay was conducted on cow intestinal mucus because cow mucus has a mucus-like composition in the human respiratory tract. The mucus obtained was brownish and thick. Making mucus solution was done by diluting the mucus with phosphate buffer solution pH 7. The use of buffer solution was intended to maintain the mucus so that its composition does not change, other than that according to the degree of acidity of the blood and stomach or neutral conditions such as the degree of similarity of the body. The solution was incubated in a water bath at 37ºC so that the condition of the test solution was in accordance with the physiological conditions of human body temperature. Because if the temperature was less or more than 37ºC then the thickness of the solution would decrease and affected the viscosity value. The test was carried out using the Ostwald viscometer because mucus had a non-Newton flow type and was classified as pseudoplastic. The results of mucolytic activity tests were presented in Table 1.

Table 1. Viscosity value of test solutions

Solution	Viscosity (cps)			
	1	2	3	Average
Negative control	13.8850	13.8977	13.9104	13.8977
Positive control	9.1314	9.1314	9.1176	9.1268
Extract of 0.2%	9.9043	9.9043	9.9187	9.9091
Extract of 0.4%	9.3932	9.4071	9.4211	9.4071
Extract of 0.6%	9.1502	9.1641	9.1780	9.1641
Extract of 0.8%	8.7966	8.7966	8.8240	8.8057
Extract of 1.0%	8.6952	8.6952	8.7232	8.7045
Extract of 1.2%	7.9534	7.9670	7.9534	7.9579
Extract of 1.4%	6.9635	6.9635	6.9886	6.9719

After knowing the test results on mucolytic activity seen from the viscosity value in table 1 shows the value of change in each solution. The greater the concentration of a solution has a smaller viscosity value and the flow time is also small compared to the negative control. then the solution viscosity data were analyzed statistically using SPSS with normality test to find out whether the samples were normally distributed or not and the one-way ANAVA test was continued by LSD test in Post-Hoc analysis to determine the significance of the difference in viscosity between treatment groups. Based on the results of the normality test and homogeneity test there are significant differences with p values obtained of 0.640 and 0.401, respectively. Because the value is more than 0.05, it can be stated that the sample data is normally distributed and homogeneous. Then the analysis is continued with the LSD test to find out which groups have different meanings.

Table 2. The LSD test results in each treatment group

Treatment		Probability	Conclussion
Negative control	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Positive control	Negative control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.179	NSD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 0.2%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 0.4%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 0.6%	Negative control	0.000	SD
	Positive control	0.179	NSD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 0.8%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 1.0%	0.000	SD

	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 1.0%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.2%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 1.2%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.4%	0.000	SD
Extract of 1.4%	Negative control	0.000	SD
	Positive control	0.000	SD
	Extract of 0.2%	0.000	SD
	Extract of 0.4%	0.000	SD
	Extract of 0.6%	0.000	SD
	Extract of 0.8%	0.000	SD
	Extract of 1.0%	0.000	SD
	Extract of 1.2%	0.000	SD

Notes: SD = Significantly different,
NSD = Not significantly different

Based on the LSD test (Table 2) it could be seen that the comparison between negative control and positive control or extract compound had a significant difference with p value < 0.05 and a decrease in viscosity to negative control. This showed that both test solutions and positive controls had mucolytic effects. The LSD test showed that acetylcysteine could thin the mucus by breaking the disulfide bonds in the sputum mucoprotein structure. The LSD test showed that the extract had mucolytic activity. Furthermore, it was compared with positive control to find out the concentration that had mucolytic activity which was equivalent to 0.1% acetylcysteine. The result was an extract with a concentration of 0.6% did not show a significant difference (p > 0.05) to positive control (acetylcysteine 0.1%). From these results it could be concluded that the acetone extract with a concentration of 0.6% had mucolytic activity equivalent to 0.1% acetylcysteine.

The mucolytic activity of the silver nail plant acetone extract is caused by secondary metabolite compounds, specifically the flavonoids contained in the extract. These compounds can break the threads of mucoproteins and mucopolysaccharides from sputum (mucus). In mucus there are various types of bonds between molecules. The active group of compounds in the extract will break the disulfide bonds in the mucus so that it can reduce the viscosity of the mucus [3]

IV. CONCLUSIONS

Based on the discussion of the results of the study it can be concluded that the acetone extract of the aerial parts of the silver fern had mucolytic activity. The acetone extract with a concentration of 0.6% had mucolytic activity equivalent to 0.1% acetylcysteine as a positive control. Thus, acetone extract of silver fern had the potential to be developed as a natural mucolytic agent.

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