# Characterization of Ethanol Extract from Agarwood (*Aquilaria microcarpa* Baill.) Leaf

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## ABSTRACT

The aim of this research is to determine the specific and non-specific parameter from ethanolic extract of A. microcarpa. Specific parameters such as the organoleptic properties, content of water and ethanol soluble compound, and chhromatogram profile of the extract. In contrast, the non-specific parameter includes drying losses, ash levels, microbiological contamination levels, and heavy metal contamination levels. The results showed that the organoleptic properties of ethanolic extract of A. microcarpa was thick, blackish brown color, bitter taste and have distinctive odor. The average content of water-soluble compounds of 66.93%, ethanol-soluble of 47.97%, and chromatogram profile with Rf 0.636. The non-specific parameters results were drying losses of 5.50%, total ash content of 3.73%, acid-insoluble ash of 2.13%, microbial contamination testing results of 1.2x102-5.5x103 colonies/g, and total mold/ yeast contaminants of 10-100 colonies/g. Heavy metal contamination Pb levels and Cd levels were 5.47 mg/kg and 0.19 mg/kg respectively. These studies suggested that the observed specific and non-specific parameters may be helpful for establishing pharmacopoeia standards of A. microcarpa and to ensure uniformity of extract effect.

## INTRODUCTION

Agarwood is one of non-timber forest products (NTFPs) which has high economic value. Agarwood-producing plants are widely known as members of the Thymelaeaceae family, Aquilaria and Gyrinops genus. They can grow in the temperature range of 24-32$^{o}$ C, humidity of 80-90%, with rainfall of 1,000-1,500 mm/year (Jensen and Meilby 2008). The land condition for growing of agarwood producing trees are mostly classified as podsolic soil with sandy clay or marginal land with altitude of 10-400 m a.s.l. One type of them have been widely known cultivated by agarwood producers is *Aquilaria microcarpa* (Paoli *et al.* 2001).

Agarwood has many benefits such as anti-asthmatic, stomach pain medication, hepatitis, and cirrhosis (Saidu *et al*. 2014; Fatmawati and Hidayat 2016). Ethanol extract

of agarwood leaf has reported contains flavonoids, alkaloids, terpenoids, and glycosides of secondary metabolite compounds (Khalil *et al*. 2013). *Aquilaria microcarpa* leaf empirically used by the Tamiyang Layang community in Central Kalimantan for diabetes treatment. Antihyperglycemic activity of agarwood leaf most likely to be associated with increasing of glucose uptake mechanism (Pranakhon *et al*. 2011; Manoka *et al*. 2016)

The content of active compounds and the quality of extracts from medicinal plants cannot be guaranteed to always be in constant amounts. Variations in the content of active compounds in the extract products can be due to the following aspects: genetic (seed), environment (place to grow, climate), agronomic (fertilizer, treatment during growth), harvest (time and post-harvest) (Salminen *et al*. 2001; Osadebe and Ukwueze 2004; Feng *et al*. 2014). Therefore, the standardization process of extracts is necessary to produce good quality extracts before they are produced on an industrial scale. Standardization of raw materials of medicines from natural ingredients such as medicinal plant extracts is a set of parameters, procedures and methods of measurement whose results are elements related to the pharmaceutical quality paradigm. Standardization is necessary to ensure the efficacy quality uniformity and safety of *A. microcarpa* leaf extract (Jeong *et al*. 2016). Standardized raw materials and controlled process, they will produce quality products (MoH RI 1995).

The agarwood (Aquilaria microcarpa Baill.) leaf empirically used to reduce blood glucose levels. Previous research has shown an extremely strong antioxidant activity in its ethanolic extract. This study aims to standardize ethanol extracts of *A. microcarpa* leaf based on specific and non-specific parameters. Specific parameters include organoleptic properties, the average content of water-soluble and ethanol-soluble compounds, and chromatogram profile using TLC technique. Meanwhile, non-specific parameters observed were drying losses, total ash content, acid-insoluble ash, microbial contamination, total mold/ yeast contaminants, and heavy metal contamination (Pb and Cd).

## METHODS

### Chemicals and Reagents

Potato Dextrose Agar, Nutrient Agar, acetic acid, sulfuric acid, ethanol, Folin–Ciocalteau's reagent, NaOH, and silica gel 60 $F_{254}$ plate were obtained from E. Merck, Darmstadt, Germany. Pb acetate and Liebermann-Burchard reagent were purchased from Sigma-Aldrich. The other chemical used were analytical grade.

### Preparation of Ethanolic Extract

*Aquilaria microcarpa* leaves were collected from Barabai, South Kalimantan in November 2016. Authentication of the plant was conducted in Biology Laboratory, Faculty of Mathematic and Sciences, Lambung Mangkurat University. Dry powder of *A. microcarpa* leaf (500 g) was macerated using ethanol 70% in the macerator with ratio of 1:10 for 24 hours while stirring occasionally. Filtrate was separated by filter paper. The residual was remacerated 2 times. All the obtained filtrate was collected and evaporated by rotary evaporator to obtain 1/10 parts, followed by evaporation in water bath until obtained thick extract (Wigati *et al*. 2017).

### Determination of Specific Parameters

*Organoleptic properties of extracts*

The organoleptic parameters of ethanolic extract of *A. microcarpa* leaf are determined using the five senses in describing the shape, color, smell, and taste.

*Water-soluble compound*

The powdered leaves of 5 grams saturated for 24 hours using 100 mL water-chloroform (40: 1) in the maceration vessel. The liquid extract obtained is then filtered. The 20 mL filtrate was evaporated to dry in a shallow, flat-bottomed bowl that had previously been tied. The remainder of the sample was heated at a temperature of 105° C to a fixed weight (MoH RI 1995).

*Ethanol-soluble compound*

Dry powdered leaves of 5 grams macerated for 24 hours using 100 mL ethanol. The liquid extract obtained is filtered. The 20 mL filtrate was evaporated to dry in a shallow, flat-bottomed bowl that had previously been tied. The remainder of the sample was heated at 105° C until fixed weight (MoH RI 1995).

*Phytochemical contents*

Determination of phytochemical contents conducted by color reaction (tube) test for detected alkaloids, saponins, phenolics, tannins, flavonoids, steroids, terpenoids, and diterpenes contents.

*Chromatogram (TLC) profile*

TLC profile analysis was used silica gel F254 as stationary phase and mobile phase used chloroform: methanol (9:1) (MoH RI 2009). Otimization of mobile phase have conducted with several solvent.

**Determination Of Specific Parameters**

*Drying losses*

Extract (1 gram) in porcelain cruces heated at 105 ° C for 30 minutes and weighed. The extract is flattened to form a thin layer and dried to a fixed weight. The fixed weights obtained were recorded to calculate the percentage of drying shrinkage (MoH RI 2000).

*Total ash content*

Determination of ash content is done by weighing 3 grams of ethanolic extract of *A. microcarpa* leaf, placed on asbestos, flattened and heated until ash. Then the ash was weighed (MoH RI 1995).

*Acid-insoluble ash*

The ash obtained dissolved 25 mL chloride acid 10% v/v for 5 min in porcelain cup then boiled. The obtained solution is filtered using a filter paper. The rest of the ash on filter paper is then washed with hot water. Filter paper and residual ash were heated until the weight is fixed (MoH 1995).

*Total bacterial contaminants*

Extract from $10^{-4}$ dilution pippetted with 1 mL sterile pipette, implanted in Nutriene Agar medium. then incubated at 37 °C for 24 hours. The number of colonies that grow then observed and counted and multiplied by dilution factor (MoH 2000).

*Total mold contaminants*

Extract from $10^{-4}$ dilution pippetted with 1 mL sterile pipette, implanted in Potato Dextrose Agar medium, then incubated at 25 °C for 3 days. The number of colonies that grow then observed and counted and multiplied by dilution factor (MoH 2000).

*Metal contaminants (Pb and Cd) using ICP-OES*

One gram of extract was deconstructed with $HNO_3$ and then fed into a 100 mL measuring flask and aquadest is added to the boundary marker. Standar solution made with 1; 2; 3; 4; and 5 ppm series consentration. Serial samples and series solutions were analyzed by ICP-OES. The metal content in the extract was shown in units of mg/ kg (MoH 2000).

**RESULT AND DISCUSSION**

**Specific Parameters**

*Organoleptic properties*

Organoleptic test of ethanol extract of *A. microcarpa* leaf was aimed for initial identification of extract by describing shape, color, smell and taste directly with senses (MoH RI 2000). *A. microcarpa* leaf ethanol extract was thick extract with blackish brown color, distinctive odor and bitter taste. The thick extract was obtained by the separation process and the solvent had evaporated completely. The compound content of the extract gave distinctive odor. Bitter taste was caused by secondary metabolite compounds containing by the extract. (Harborne 2006). Most flavonoids have a bitter or astringent taste or a bitter taste with sweet aftertaste (Hounsome *et al*. 2008).

*Determination of Extract Compound*

Determination of extract compound is aimed to estimate roughly the amount of polar (water-soluble) compounds and the semi-polar or non-polar (soluble-ethanol) compounds (Saifudin *et al.* 2011). Determination of this parameter was carried out gravimetrically used two solvents, i.e. water and ethanol solvents. Water is a polar solvent that will dissolve polar compound. Ethanol has the ability to dissolve with a wide polarity ranging from non-polar compounds to polar compounds (Arifin *et al*. 2006). The level of active compounds in an extract is influenced by age of the plant, time of harvest, climate and place of growth. The results showed that the content of ethanolic extract of *A. microcarpa* leaf in aqueous solution was 66.93% and ethanol solution was 47.97%. These results simply indicate that the active compound in the *A. microcarpa* leaf was easily extracted by both of these solvents (Devaraj *et al*. 2010). So the extraction process will be most favorable using polar solvent. Ethanol 70% is used as a solvent on extraction because of its polarity and solubility of the extract.

*Phytochemical content*

Phytochemical screening was aimed to provide an overview of the constituent compounds in the extract (Kristanti *et al*. 2008). The result showed that the extract compounds contained saponins, phenolics,

tannins, and flavonoids (Table 1). Saponins were glycosides form of sapogenin, so it will be polar. It was surface-active compounds and can cause foam if shaken in water, because it has micelles form, so the polar group faces out while the non-polar group faces inwards. This condition caused foam formed (Kristanti *et al*. 2008; Simaremare 2014). Phenolics were widespread in higher plants especially on leaf. Phenol testing was performed with the $FeCl_3$ which was characterized by blackish green or dark blue color after $FeCl_3$ was added. This condition caused by the $Fe^{3+}$ complex formed (Sa'adah 2010). The tannins test use a gelatinous solution resulting in a precipitate showing the presence of a complex bond between tannins and proteins. The characteristic of tannin is bitter taste especially on the leaf. Flavonoids had diverse types and present in free form (aglycones) or bonded as glycosides. The polymethoxy aglycone was non-polar, the polyhydroxy aglycone was semi-polar, while flavonoid glycosides was polar because it contains hydroxyl and sugar groups (Harborne 2006). Therefore, the flavonoid group can be extracted by universal ethanol solvent. Flavonoids and phenolic have antioxidant effect by capture and neutralize free radicals (Anwar *et al*. 2017).

**Table 1.** The phytochemical content test results of ethanolic extract of *A. microcarpa*

| No | Test | Result |
|---|---|---|
| 1. | Alkaloids | - |
| 2. | Saponins | + |
| 3. | Phenolics | + |
| 4. | Tannins | + |
| 5. | Flavonoids | + |
| 6. | Steroids | - |
| 7. | Terpenoids | - |
| 8. | Diterpenes | - |

*Chromatogram Profile*

TLC profile analysis was used silica gel F254 as stationary phase and mobile phase used chloroform: methanol (9:1). The spraying reagent used 10% $H_2SO_4$. The sulphuric acid ability as reducer agent can damage the chromophore group of the active compound, the wavelength shifted towards longer wavelength and spot will be seen more clearly (Gandjar & Rohman 2008). This Rf value can be used as a marker or to indicate the counterfeit compound on quantitative and qualitative examination. Based on TLC result (Fig 1) obtained Rf value was 0.109, 0.709, and 0.909. It showed the compounds tended to be semi-polar. The polar stationary phase with non-polar mobile phase interacted with the compounds contained in the extract. If the spot was seen in the lower position with small Rf value, the compounds tended to had similar the stationary phase. This study shows that Rf value in the middle ang higher position, so the dominant compound in the extract was semipolar and polar, which leads to flavonoid or phenolic compounds.

**Non-specific Parameters**

The non-specific parameter included drying losses, total ash content, acid insoluble ash contents, bacterial and mold contamination, and metal contamination (Table 2). Drying losses is aimed to provide a maximum range about the compounds lost in the drying process. The drying losses parameter measured residual substance after drying at 105 °C to constant weight, as percent value (MoH RI 2000). The results showed that *A. microcarpa* leaf extract drying was 5.50%. The shrinkage rate of drying simplicia based on the raw material drug requirement maximum of 10% (NADFC RI 2014). Sample drying and extract evaporation process by the non-optimal solvent would leave water residue in the resulting thick extract and it can be bacterial and fungus growth medium. The storage of thick extract in

**Table 2.** Non-specific parameter results of ethanolic extract of *A. microcarpa*

| No. | Parameters | Result |
|---|---|---|
| 1. | Drying losses | 5.50 % |
| 2. | Total ash content | 3.73 % |
| 3. | Acid-insoluble ash | 2.13 % |
| 4. | Total bacterial contamination | $1.2x10^2$–$5.5x10^3$ colonies/g |
| 5. | Total mold contamination | 10 – 100 colony/g |
| 6. | Pb contamination | 5.47 mg/kg |
| 7. | Cd contamination | 0.19 mg/kg |

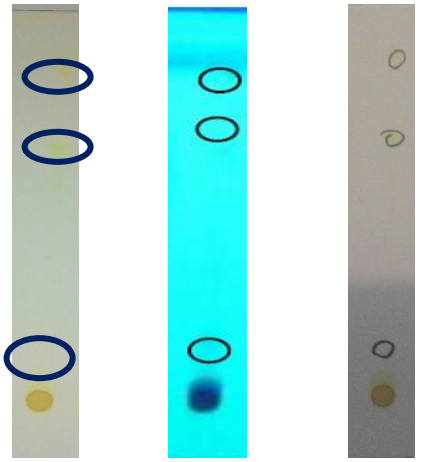


**Figure 1.** TLC profile analysis of ethanolic extract of *A. microcarpa*. Stationary phase Silica Gel F254, mobile phase chloroform: methanol 9:1

moist places would cause the absorbed water to the extract (Saifudin *et al.* 2011). Other affecting factors to water content was biological factors such as species, growth area and plant part used for making extract and chemical factors such as extraction method, equiptments, extracted material dryness, and solvent used in the extraction process (MoH RI 2000).

Inorganic material contained in the extract can be determine by total ash content. Ash is inorganic residual of combustion from an organic material. The ash content depends on material and spying manner. Determination of ash content extract was conducted to determine whether or not processing to extract is good, know the material used type, and as parameter value before being used as food or pharmaceutical preparation. Ash content should have small value because this parameter indicates the presence of heavy metal contaminant that is resistant to high temperature. The total ash content of ethanolic extract of *A. microcarpa* leaf was 3.73%. According to Herbal Pharmacopoeia Indonesia, the requirement of extract total ash content maximum at value of 13.3% (MoH RI 2009).

Determination of acid-insoluble ash content was aimed to evaluate the extract on contamination of silicate-containing materials, such as soil and sand. The hydrochloric acid solvent was used to dissolve the organic metals, while the acid-insoluble usually contain silicates. The acid-isoluble ash content of *A. microcarpa* leaf ethanol extract was 2.13%. This showed the acid-insoluble ash content obtained on *A. microcarpa* leaf ethanol extract fulfilled the standard. The acid soluble ash content is not more than 2.6% according to FHI requirement (MoH RI 2009). This indicates that the acid soluble ash content obtained on ethanol extract of *A. microcarpa* leaves meets the standard.

Total bacteria and mold test of *A. microcarpa* leaf ethanol extract were conducted to calculate microbe contaminating the extract. This test included the determination of the number of microorganism and to indicate the presence or absence of certain bacteria in the extract. Excessive microbe can alter organoleptic characteristics and nutritional or nutritional status changes in the extract (NADFC RI 2008). The maximum limit of microbes or bacterial in food is $1.0x10^4$ colonies/g and maximum limit of molds is $1.0x10^3$ colonies/g. Total bacteria and mold test on *A. microcarpa* leaf ethanol extract was $1.2x10^2$-$5.5x10^3$ colonies/g and 10-100 colonies/g respectively.

The determination of heavy metal contamination was conducted to ensure that the extract didn't contained heavy metals exceeding limits that can be toxic to the body. Based on the study results, ethanol extract of *A. microcarpa* leaf contained Pb of 5.47 mg/kg extract. This level was in accordance with the required maximum limit of <10.00 mg/kg (NADFC RI 2014). The Cd content obtained of 0.19 mg/kg, and fulfilled the required maximum limit of <0.3 mg/kg (NADFC RI 2014). High levels of metal in the soil caused by fertilizers, pesticides, industrial or household waste and mining (Charlena 2004). The impact of high Pb concentration to the body results in poisoning symptoms such as acute gastrointestinal irritation, vomiting, abdominal pain, and diarrhea. Pb affect the intelligent nervous system and growth, so it can cause brain damage, convulsion, behavioral disorders, and death. The impact of high Cd concentration to the body in the long term can accumulate the kidney and liver. Cd can cause abnormal uric acid calcium and phosphorus in urine. It can also cause lung respiration damage, bone fragility, and affect the reproductive system (Widaningrum *et al*. 2007).

With the manufacture of standardized extracts, it is expected to produce extracts that meet the requirements of NADFC RI. The results of this study will be useful to complete information related to the

identification and standardization of ethanol extract quality of *A.microcarpa* leaf.

## CONCLUSION

From this study, it can be concluded that the specific parameter testing result shows ethanolic extract of *A. microcarpa* leaf takes the form of thick extract with blackish brown color, distinctive odor and bitter taste, water-soluble content of 66.93% and ethanol 47.97%, phytochemical contents of saponins, phenolics, flavonoids, and tannins, with TLC profile shows some spots on Rf 0.636. Non-specific parameter testing results shows drying losses of 5.5%, total ash content of 3.73%, acid-insoluble ash content of 2.13%, bacterial contamination of $1.2x10^2$-$5.5x10^3$ colonies/g, mold contamination of 10-100 colonies/g, Pb levels of 5.47 mg/kg, and Cd levels of 0.19 mg/kg.

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