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Isolation and identification of secondary metabolites in ethyl acetate extract from the Maja bark (*Aegle marmelos* Linn.)

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ABSTRACT

This exploratory study resulted in the isolation and identification of secondary metabolites in ethyl acetate extracts from the Maja bark (*Aegle marmelos* Linn.) from Pattappa Village, Pujananting Subregency, Barru Regency, South Sulawesi. The stages of isolation included maceration, fractionation, and purification. Maceration was performed with methanol, fractionation with ethyl acetate, a particular ratio of acetone (9:1) was used, and purification was done by flowing nitrogen gaseous into the E₅ fraction. Furthermore, the classification test and FTIR spectrophotometer were carried out for identification. Pure isolates in the form of white crystals were obtained with a melting point of 242°C. Reagent tests indicated a brown precipitate on Wagner's test and a white precipitate on Mayer's test. Infrared-spectrophotometric identification provided typical absorption for such functional groups as NH, aliphatic CH, aromatic C=C, and amine CN. The reagent tests and FTIR-spectrophotometer identification confirmed that the secondary metabolites fit well into the class of alkaloids.

Keywords: *Aegle marmelos* (Linn.), alkaloids and secondary metabolites

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INTRODUCTION

Many natural compounds in Indonesia are widely used in therapeutic and health-enhancing properties. From traditional remedies until becoming the community's habits, natural ingredients, which are believed to cure various diseases, have been developed and used as pesticides, dyes, and medicines. Due to the uneven geographic and taxonomic distribution of plants, the success of a research program that aims to find new bioactive compounds with a unique and novel molecular structure still depends on biological resources (Achmad et al., 2001). One of the natural plant-based remedies is the Maja plant, commonly known as bael (*Aegle marmelos* linn.), a tribe of oranges or Rutaceae, which is native to Asian tropics and subtropics (Sastrohamidjojo, 1996); (Salempa, 2014).

Maja (*Aegle marmelos* linn) is a type of medicinal plant found in Indonesia's tropical forests. This tropical plant can be developed as a source of secondary metabolite compounds for medicinal substances. Commonly, Maja plants are known by various names such as Maja, Bila Gedang, Bilabila, Bilak, and Bila peak. The plant is widespread in Indonesia because its growth suits the climate of all parts of Indonesia (Sastrohamidjojo, 1996).

Several regions in Indonesia have acknowledged numerous benefits of the Maja plant and included it in traditional medicine. Maja is a traditional medicinal plant to overcome various diseases. Currently, many Maja medicinal plants have been used to develop traditional medicines. However, Maja's information to treat multiple diseases has not been fully collected. Hence, it is challenging to identify the Maja plant's primary uses by gathering all literature data from the internet (Mulyaningsih et al., 2020).

Maja fruit (*Aegle marmelos* (L.) correa) is one type of plant that is considered unique among Indonesians. The legendary Majapahit comes from Maja and Pahit (Indonesian phrase for bitter taste), which means Maja fruit that tastes bitter. It turns out that other parts of Maja plant hold a myriad of extraordinary benefits for health. It was used as traditional medicines such as for fever, constipation, dysentery, diarrhea, hepatitis, tuberculosis, inflammation of the nasal mucous membranes, brain disorders, liver disorders, itching (scabies, ulcers, boils, eczema), anti-inflammatory, hearing loss, urinary disorders, and abortive (Tasyrifah, 2010). The Maja fruit is also helpful as an anti-inflammatory and larvicidal (Harborne, 1987; Ng et al., 2018; Sari and Susilowati, 1981; Subhasini, 2014). Various phytopharmacological evaluations also have been reported for the important potential of the *Aegle marmelos* (Sanghi and Mushtaq, 2017).

The leaf extracts of *Aegle marmelos* (L.) Correa showed the presence of terpenoids, steroids, phytosterols, tannins, alkaloids, glycosides, saponins, reducing sugars, phenols, and flavonoids (Snehlata et al., 2018). Tannins may be used to cure urinary disorder, fever (antipyretic), swelling, vomiting, cuts, ulcers, thiamine deficiencies, diabetes mellitus, diarrhea, dysentery, and wound. It may also function as an anti-toxic substance, vegetable insecticides for stink bugs (*Leptocorisa acuta*). As a traditional medicine, its leaf may be boiled and then be drunk to reduce high blood pressure or hypertension (Mulyaningsih et al., 2020).

The result of phytochemical analysis has shown the presence of steroid compounds in the bark of *Aegle marmelos* linn. Some steroids act as an anti-inflammatory or to reduce inflammation. The skin of the *Aegle marmelos* Linn contains coumarin derivatives, and the structure appears as *R-(+)-marmine* based on the X-ray diffraction (Gupta et al., 2006). Previous work by Riyanto (2003) had subjected Maja leaves to extraction method using petroleum ether and chloroform, resulting in an aegeline compound, *N-2-hydroxy-2(4-methoxyphenyl ethyl cinnamamide)* were obtained. Such compounds were found to be cinnamic acid-derivative alkaloids.

According to previous research (Wilujeng et al., 2020), the antioxidant activity test result of the Maja fruit extract samples was profound, with the IC₅₀ values reaching the range of 50-100 µg/mL. Trials on mice were also carried out and proven to reduce their blood sugar levels. Interestingly, the study results had produced Maja fruit beverage that no longer tastes bitter but

1 changes the taste to be rather sweet, warmer when drinking it as its natural alcohol levels are reasonably safe for the body.

This study sought to show the potential secondary metabolites in the Maja bark similar to the prior work. The earlier mentioned descriptions showed that it is essential to find secondary metabolite in Maja bark to be used as an active ingredient in pharmacy by applying ethyl acetate solvent. The stem of the plant is one part that has a high possibility to be isolated since such part is expected to produce novel secondary metabolites and is often used by the community as traditional medicine.

MATERIALS AND METHOD

Material

The equipment used for the research includes FTIR Spectrophotometer Shimadzu Prestige-21. The identification of isolate was taken on a Shimadzu IR Prestige-21 using KBr pellets in the range of 4500–500 cm^{-1} wavenumber from which functional groups of the compound were obtained.

Test and procedure

As much as 4.5 kg of *Maja* bark was grounded and macerated with methanol solvent for 3x24 hours (Tobo et al., 2001), and the extract was harvested for 1x24 hours. Prior to weighing, the macerate was filtered through the Büchner funnel with Whatman filter and was evaporated using a rotary evaporator until the methanol extract thickened (Harborne, 1987). After weighing, the methanol extract was partitioned using liquid-liquid extraction with a separatory funnel and ethyl acetate solvent. The resulting filtrate was evaporated to be dried at room temperature and then weighed.

Ethyl acetate extract was fractionated using Vacuum Liquid Chromatography (VLC) method. The TLC's result was analyzed using Thin-Layer Chromatography (TLC) to obtain fractions of the same staining profiles. These fractions were combined and evaporated at room temperature. One of the fractions was selected and fractionated using Pressure Column Chromatography (PCC). The resulting fraction of PCC was, once again, analyzed using TLC, where fractions of the same staining profiles were combined. Fractions that indicated the presence of crystals at this point were recrystallized to obtain pure crystals. The purification of the fractions was subjected to three eluent systems. The compounds' isolation resulted from the qualitative test with suitable reagents, e.g., *Lieberman-Burchard*, FeCl_3 , *Wagner's*, and *Mayer's* reagents. The melting points were examined by using the Melting Point Meter, and the functional groups were tested using an FTIR spectrophotometer.

RESULT AND DISCUSSION

Classification test

The identification of ethyl acetate extract's chemical content was tested using such reagents as FeCl_3 , *Liebermann-Buchard*, *Mayer*, and *Wagner*. This test aimed to identify the class of secondary metabolites present in ethyl acetate extract. The result was presented in [Table 1](#)

Table 1. The classification test result of ethyl acetate extract

Reagents	Result	Description
<i>Wagner</i>	Brown precipitate	(+) <i>Alkaloids</i>
<i>Mayer</i>	Yellow	(-) <i>Alkaloids</i>
FeCl ₃	Green	(+) <i>Flavonoids</i>
<i>Lieberman-Burchard</i>	Green	(+) <i>Steroids</i>

Fractionation and purification

The fractionation process involved a stationary phase composed of 60 H silica gel and a mobile phase where eluents gradually increased in polarity. Separation was carried out based on color bands found in the stationary phase. It obtained 27 fractions, as shown in [Table 2](#).

Table 2. The results of vacuum liquid-column chromatography

Fraction	Eluent	Ratio (%)	Color
1-2	n-hexane	100	Clear
3-5	n-hexane:ethyl acetate	9:1	Clear
6-8	n-hexane:ethyl acetate	8:2	Green
9-11	n-hexane:ethyl acetate	7:3	Green
12	n-hexane:ethyl acetate	6:4	Green
13	n-hexane:ethyl acetate	5:5	Green
14	n-hexane:ethyl acetate	4:6	Yellow
15	n-hexane:ethyl acetate	2:8	Yellow
16	Ethyl acetate	100	Yellow
17	Ethyl acetate:acetone	9:1	Yellow
18-19	Ethyl acetate:acetone	7:3	Yellow
20	Ethyl acetate:acetone	6:4	Yellow
21	Ethyl acetate:acetone	4:6	Yellow
22	Ethyl acetate:acetone	2:8	Yellow
23-24	Ethyl acetate:acetone	1:9	Brown
25	Acetone	100	Brown
26-27	Methanol	100	Brown

Fractions that revealed the same staining profiles were combined and grouped into ten fractions. Fraction E that weighed 1.9526 g was further fractionated using PCC, from which 24 fractions were obtained ([Firdaus, 2011](#)), ([Iwan and Darminto, 2012](#)). Subsequent TLC was required for fraction combinations that consisted of 10 fractions according to the same staining profiles on TLC plates. The chosen fraction subjected to purification was fraction E₅ ([Table 3](#)) which weighed 0.5208 g. The resulting isolate was recrystallized with chloroform and methanol solvent that developed into a white crystal-forming isolate that weighed 43.1 mg.

Table 3. Combination of pressed-column chromatography fractions, fraction E.

Fraction	Combination	Weight (g)
1	E ₁	0.0128
2-3	E ₂	0.0085
4	E ₃	0.0047
5	E ₄	0.0180
6-7	E₅	0.5208
8-11	E ₆	0.2975
12-14	E ₇	0.1564
15-17	E ₈	0.0225
18-20	E ₉	0.0394
21-24	E ₁₀	0.0106

The purification of the E₅ isolate was carried out with repeated recrystallization. This technique aims to separate the isolates from the remaining impurities. The purification product was pure isolate (isolate E₅) in white crystals as much as 43.1 mg. The results of classification and spectroscopy tests that were previously reported corroborated this finding. Research conducted by (Nugroho et al., 2011) reported that aegeline (*N*-2-hydroxy-2-(4-methoxyphenyl)-ethylcinnamamide), as an alkaloid derived from *cinnamic acid*, was obtained in the petroleum ether extract and chloroform of *Maja* plant leaves. Anthraquinone (1-methyl -2-(3'-methyl-but-2'-enyloxy), which has anti-fungal activity against *Aspergillus fumigatus* and *Candida albicans*, was isolated in *Aegle marmelos correa* seeds (Mirsha et al., 2010). The results of *A. marmelos Correa* isolation found a Coumarin, *marmarin* 7-(6'-7' dihydroxygeranial-oxy), which actively inhibits leukemia cells (Nugroho et al., 2011). In another research, the highest concentration of *marmelosin* (108.65 µg / g) was observed in immature *Maja* fruit and the highest antibacterial activity of *marmelosin* against *E. coli* was shown at a concentration of 200 µg / ml by using the HPLC instrument (Svaefullah et al., 2020).

Purification test

The purity of the isolate was tested using three eluent systems where a pure isolate was indicated by a single spot's appearance when developed on a TLC plate. The result of the purity test with three eluent systems, i.e., n-hexane: acetone (3:7), chloroform: ethyl acetate (6:4), and n-hexane: ethyl acetate (7:3), appeared as a single spot, thus confirming the purity of the isolates. The exact temperature at which melting point was determined, using melting point apparatus, was 242°C (Firdaus, 2011). The classification of the resulting isolates was tested using these reagents, as shown in Table 4.

Table 4 The result of classification of pure isolated fraction E₅

Reagents	Color Reaction	Description
<i>Wagner</i>	Brown precipitate	(+) <i>Alkaloids</i>
<i>Meyer</i>	White precipitate	(+) <i>Alkaloids</i>
<i>FeCl₃</i>	Yellow	(-) <i>Flavonoids</i>
<i>Lieberman-Burchard</i>	Colorless	(-) <i>Steroids</i>

A pure isolate of fraction E₅ was tested for purity by TLC three eluent systems. The isolate was visible after the CeSO₄ stain was given, and the heating produced a brown color, as shown in Figure 1.

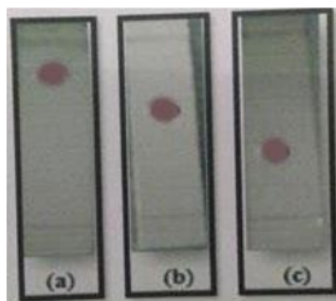


Figure 1. TLC Results of the isolate using Three Eluent Systems

- (a). Eluent n-hexane : acetone
- (b). Eluent chloroform : ethyl acetate
- (c). Eluent n-hexane : ethyl acetate

The isolate was further tested for purity using TLC with three types of eluents that displayed comparisons between different solvents. This aimed to confirm the isolate's purity as the developed TLC plates each showed a single spot. In the wake of the TLC development (Atun, 2010), a single spot appeared on these eluents, i.e., combinations of n-hexane and acetone; combinations of chloroform and ethyl acetate; combinations of n-hexane and ethyl acetate. As for the melting point, the results show that these isolates began to melt at 240 °C and melted completely at 242 °C. This supports the theory that a pure compound will have a sharp melting point, from solid-state to liquid-state, at no more than 2°C (Firdaus, 2011). The classification test led to positive alkaloid reactions to *Wagner's* reagent and *Mayer's* reagent, indicated by a brown-precipitate formation and a white-precipitate formation. This precipitate occurs because alkaloids are compounds from the nitrogenous base group. If the nitrogenous base is reacted with an acid, HCl (hydrochloric acid) will form an insoluble salt so that this salt will form a precipitate. In contrast, the difference in color that occurs is due to the original color of the used reagent. The three tests show that the Maja Bark (*Aegle marmelos*) extract was tested positive for alkaloids (Ibrahim and Rusdian, 2017).

FTIR spectroscopy test

Based on FTIR spectroscopy, the infrared spectrum of isolate E₅ was interpreted in terms of the wavenumber, band, intensity, and group, as shown in Figure 2.

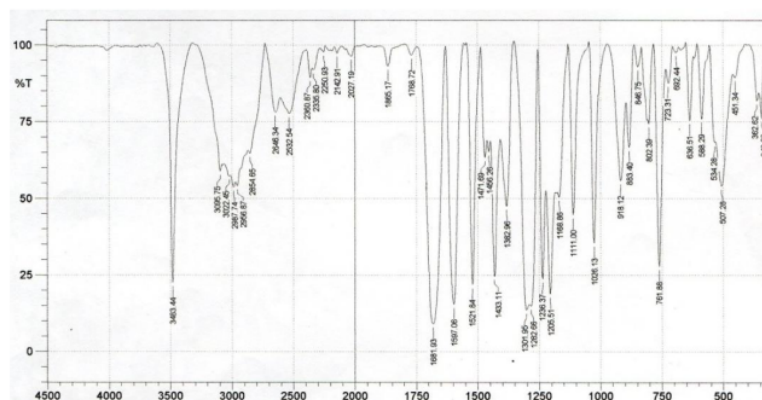


Figure 2. The infrared spectrum of pure isolate E₅

The IR spectrum analysis on isolated fraction E₅ revealed functional groups such as N-H stretching vibration, seen on a sharp band of strong intensity at 3483.44 cm⁻¹. This intense N-H vibration was confirmed by C-N stretch that exhibited absorption of 1236.37 and 1205.51 (ref.1250-1020 cm⁻¹). Sharp absorption that exhibited low intensity at 2987.74, 2956.87, and 2854.65 cm⁻¹ wavelengths was attributed to stretching vibration of -CH aliphatic (CH₂ and CH₃ groups) (ref. 3000-2850 cm⁻¹), thus indicating the presence of methyl and aliphatic methyl groups in isolated fraction E₅. Such indication was further confirmed by the presence of bending vibration in the region of 1433.11 and 1382.96 cm⁻¹, a typical absorption band for bending vibration of geminal dimethyl group - or CH(CH₃)₂ group (ref.1450-1375 cm⁻¹). Absorption at a wavenumber of 1681.93 accounted for the carbonyl group's (C=O) stretching vibration (ref.1715-1860 cm⁻¹), affirmed by the low-intensity absorption of C-O in the region of 1301.95 and 1282.66 cm⁻¹. The high-intensity absorption band at 1597.06 and 1521.84 wavelengths, which was aromatic C=C (ref.1650-1450 cm⁻¹) in the region of 1111.00 and 1026.13,35 cm⁻¹, was specific to a functional group of ether (C-O-C) (Sastrohamidjojo, 1996) (Mohrig et al., 2010).

Based on the IR spectrum interpretation, several functional groups were indicated: amine (N-H), aliphatic (C-H), carboxylate groups (HO-C = O), double-bond aromatic (C = C), CN, and COC. This indicates that isolate E₅ is one of the alkaloid compounds group. It was confirmed by the group test results showing that the pure isolates were positive for the alkaloid group.

CONCLUSION

The isolation of secondary metabolites from ethyl acetate extracts from Maja bark indicated the presence of pure, white crystalline isolates of alkaloid type, which melted at 242 °C.

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