Materials Science Forum
ISSN: 1662-9752, Vol. 967, pp 38-44
doi:10.4028/www.scientific.net/MSF.967.38

Submitted: 2018-11-02
Revised: 2019-04-06
Accepted: 2019-06-22
Online: 2019-08-16

Antibacterial Activity of Secondary Metabolite Compounds in Ethyl Acetate Extract of Rumput Mutiara (*Hedyotis corymbosa* (L.) Lamk)

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Keywords: Isolation, *Hedyotis corymbosa*(L.) Lamk, steroid, antibacterial, *E.coli*.

Abstract. Plant Rumput Mutiara (*Hedyotis corymbosa* (L.) Lamk) is one of the family species of Rubiaceae which is used as a traditional medicine that is effective in healing boils, acne, anti-inflammatory, and anticancer. Research methods include maceration, fractionation, purification, class test and bioactivity test with Kirby-Bauer diffusion method using *Escherichia coli* (E.coli) bacteria. The results of this research are pure isolates with white crystal needle shape with melting point 137-138ºC. Pure isolates were analyzed using FTIR, and by the IR spectrum which showed the absorption band at wave numbers 3435.22 cm-1 indicated the presence of –OH, CH_3 and -CH_2- aliphatic groups (2956.87 cm^{-1}; 2935.66 cm^{-1}; 2893.22 cm^{-1}; and 2866.22 cm^{-1}), C = C (1641.42cm^{-1}), -CH_3 and -CH_2- bending (1462.04 cm^{-1} and 1377.17 cm^{-1}), CO (1056.99 cm^{-1}), and = CH (964.41 cm^{-1}). Based on the result, it showed that the isolate was a steroid group which has low antibacterial activity against *E.coli* with inhibition power of 10 mm.

Introduction

Indonesia is one of the countries that have rich in biodiversity. According to LIPI in 2010, there are 30.000 species of plants in Indonesia. Some of these natural resources have been used in daily life to meet the needs of the community as food, medicines, and others. Plants can be used as traditional medicines because they contain organic compounds that are bioactive. Some active compounds that are known to have physiological functions are carotenoids, phytosterols, saponins, glycosinolates, polyphenols, protease inhibitors, monoterpenes, phytoestrogens, flavonoids, sulfides, alkaloids, and phytic acid [1].

One of the plants that have the potential as a traditional medicinal commodity is Rumput Mutiara (*Hedyotis corymbosa* (L.) Lamk). This plant comes from the Rubiaceae family, which consists of 450 genera and 6500 species [2-4]. Rumput Mutiara Plants have been used as traditional medicines by the community to get rid of zits by pounding all parts of the plant, then rubbing them into the pimpled face.

Some research results show that this plant has the ability to cure ulcers (anti-carbuncular) [5], is useful as an antihepatotoxic [6], and has the potential as an anticancer [7, 8]. In addition, the herb *H. corymbosa* (L.) are efficacious as a reliever for fever (antipyretics), anti-inflammatory, diuretic, cooling fever and toxins (detox), activates blood circulation, drugs after childbirth, and ulcers and dysentery [3].

Plants of H. *corymbosa* (L.) Lamk is rich in known chemical constituents, including stigmasterol, ursolic acid, and oleanolic acid [2, 9]. Ursolic acid compounds which have several pharmacological effects, namely anti-tumor [7, 10], and oleanolic acid compounds that are thought to inhibit cancer [11]. Flavonoid compounds from the methylene chloride fraction are active against Shigelladysenteriae [2, 12].

Escherichia coli is one of the bacteria that are resistant to antibiotics harmful to health in excess doses. One of the efforts made to treat this bacterial disease is to use vegetable ingredients which are expected to be more effective, efficient and safe in an effort to inhibit bacterial growth. One alternative vegetable material that wants to know its potential in inhibiting bacterial activity is *H. corymbosa* (L.) Lamk. Based on the description above, further research is needed to examine the content of secondary metabolites in the ethyl acetate extract of the plant *H. corymbosa* (L.) Lamk

considering its potential is quite large as a medicinal plant and tests its bioactivity against *E.coli* bacteria.

Research Methods

Materials

The tools are used in this study are analytic balance, maceration vessel, Buchner funnel, rotary evaporator, oven, capillary pipe, Chamber, LC VL-4 LC 254-356 nm lamp, hot plate, KKCV column, KKT column, Erlenmeyer flask, measuring cup, ordinary funnel, separating funnel, beaker, dropper pipette, drop plate, vacuum pump, vial bottle, stirring rod, Stuart®SMP11 melting point, FT-IR spectrophotometer, petri dish, inoculum, incubators, autoclaves, tweezers, test tubes, and calipers.

Materials are used in this study are methanol, acetone, ethyl acetate, chloroform, n-hexane, aqua distilled, Liebermann-Burchard reagents, Wagner, Mayer, $FeCl_3$1%, Merck 60 silica gel, Merck silica gel 60 GF254, E-Merck plates TLC silica gel 60 F254, 10% $CeSO_4$, Whatman filter paper No.41, aluminum foil, tissue, label, nutrient agar, Nutrient Broth, paper discs, DMSO, spirits, tetracyclines, plastic wrap, cotton and *E.coli* bacteria.

Methods

1. Extraction

A total of 4.5 kg of fine powder *H. corymbosa* (L.) has been macerated with methanol for 3 x 24 hours. The extract obtained was concentrated using an evaporator to obtain a thick methanol extract, followed by a liquid-liquid (partition) extraction process with ethyl acetate solvent and the extract was concentrated to obtain thick ethyl acetate extract.

Class test for viscous ethyl acetate extract with Liebermann-Burchard reagent (terpenoids and steroids), 1% $FeCl_3$ (flavanoid), Meyer (alkaloids), and Wagner (alkaloids).

2. Fractionation

Condensed ethyl acetate extract was analyzed using thin layer chromatography (TLC) using n-hexane: ethyl acetate (8:2) eluent. The thick extract was fractionated by vacuum liquid column chromatography (KKCV) using Merck 60 GF_{254} silica gel.

The results of KKCV fractionation were analyzed by TLC using a mixture of eluent n-hexane: ethyl acetate (8:2) as elution. The fractions that have chromatograms with the same stain profile are combined and added so that solids are obtained to obtain the fraction which will be further purified through flash column chromatography (KKF). (8:2) as elution. The fractions which have the same stain profile are combined and evaporated so that solids are obtained for purification.

3. Purification

Solids (D4 fraction) obtained were recrystallized using methanol. The purity of the compound obtained was determined by conducting TLC of the three eluent system, said to be pure when showing a single stain pattern. As for the test melting point of the compound, if it shows a sharp melting point route, the compound is pure.

4. Identification

The isolates were tested with Liebermann-Burchard, $FeCl_3$ 1%, Wagner and Meyer reagents and further identified using FT-IR spectrophotometer.

5. Bioactivity Test

Pure compounds (isolate D4) obtained were tested for bioactivity against *E. coli* bacteria by Kirby-Bauer method, 1 ose of *E. coli* bacteria which had been planted in the medium of NB was diluted to a volume of ± 50 mL with sterile distilled water. petri dish contains NA media that has been made before. Disc paper that is 6 mm in size is taken using tweezers, saturated into negative control (DMSO), sample (isolate D4) and positive control (tetracycline). Then it is placed on the surface of the NA media which has been inoculated with the test bacteria. Petri dishes were incubated at 37°C for ± 24 hours. The response to the antibacterial activity was determined by

measuring the diameter of the bacterial inhibitory zone (clear zone) on the surface of the media by using a caliper.

Results and Discussion

Extraction Process

Rumput Mutiara samples (*H. corymbosa* (L.) extracted by ethyl acetate solvent, then obtained a brownish dark green thick extract with a weight of 123,6920 g. The thick ethyl acetate extract was tested using Meyer, $FeCl_3$ 1% reagent, Wagner and Lieberman-Burchard (LB).

Table 1.Group/Reagent Test Results Ethyl Acetate Plant Extracts *H. corymbosa* (L.)Lamk

No	Reagent	Observation	Information
1.	$FeCl_3$ 1%	Greenish yellow	Positive Flavonoid
2.	Liebermann-Burchard	Dark green	Positive Steroid
3.	Meyer	White sediment	Positive Alkaloid
4.	Wagner	Brown sediment	Positive Alkaloid

Fractionation

KKCV results obtained as many as 34 factions were identified by the TLC. The merger was carried out to obtain 8 main combined fractions (A-H).

Fraction D with a weight of 0.5396 g was selected to be further fractionated by the KKF method. The fractions obtained from the KKF results were 23 fractions, then merging was carried out to obtain 9 major combined fractions.

The D4 fraction forms a green needle crystal with a weight of 0.2075g. the choice of the D4 fraction because the weight of the D4 fraction is greater than the other fractions.

Purification

Crystal fraction D4 was purified by the recrystallization method, with methanol solvent producing isolates in the form of white needle crystals weighing 53.7 mg. Based on the TLC analysis, the three eluent system shows a single stain on the TLC plate. Furthermore, the D4 isolates were identified by the melting point test and obtained the observations of the isolates began to melt at 137ºC and melted completely at a temperature of 138ºC.

Identification

The obtained isolates were identified by testing the compound class using various kinds of reagents such as $FeCl_3$ 1%, Liebermann-Burchard, Meyer, and Wagner.

Table 2. Group Test Results on Pure Isolates of D4 Faction

No	Reagent	Observation	Information
1.	$FeCl_3$ 1%	Yellow	Negative Flavonoid
2.	Liebermann-Burchard	Green	Positive Steroid
3.	Meyer	Colorless	Negative Alkaloid
4.	Wagner	Brownish	Negative Alkaloid

Identify further using the FT-IR Prestige-21 SHIMADZU® spectrophotometer and obtain the IR spectrum results from the D4 isolate as follows:

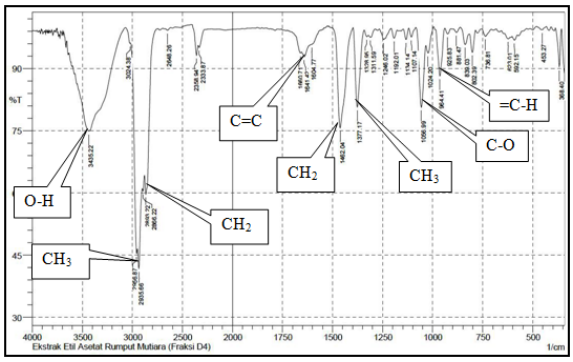

Figure 2. Isolate D4 Infrared Spectrum

Bioactivity Test

The D4 isolates obtained were tested for bioactivity against *E. coli* bacteria using Kirby-Bauer diffusion method. The results of the inhibition zone of D4 isolates against E. coli bacteria can be seen in Table 3.

Table 3. D4 Isolate Inhibiting Zone Areas Against *E.coli* Bacteria

Activity	Inhibition Zone Area
Isolate D_4	10 mm
Positive control (tetrasiklin)	32 mm
Negative control (DMSO)	0 mm

Discussion

Ethyl acetate Ekstract

The viscous ethyl acetate extract obtained from the extraction process was tested in groups which aimed to identify the class of secondary metabolite compounds contained in the extract ethyl acetate plant *H. corymbosa* (L.) Lamk. The results showed that there were flavonoid, steroid and alkaloid compounds in the ethyl acetate extract.

Fractionation was carried out by column chromatography method, namely KKCV and KKF. The selected fractions for further fractionation with the KKF method were considering several things to be achieved, namely, the stains that appear when KLT is not much and have a clear separation, have heavy mass, and there are signs that look like physical characteristics of crystals (solid and not greasy).

Crystal purification by recrystallization method, which aims to dissolve impurities from isolates to obtain pure compounds by using suitable solvents which are solvents that can dissolve impurities, but do not dissolve crystals or pure compounds. Needle crystal as much as 0.2075 g which is dark green recrystallized with methanol solvent to produce white needle crystals as much as 53.7 mg which indicates that the compound crystals obtained are pure.

Isolate

Isolates obtained were D4 isolates in the form of needle-shaped white crystals. Testing the purity of isolates with TLC three eluent systems using a variety of solvents and different comparisons. The identification results obtained showed a single stain in the solvent ratio, n-hexane: chloroform (5: 5), Rf: 0.42; n-hexane: ethyl acetate (9: 1), Rf: 0.65 and chloroform: ethyl acetate (8: 2), Rf: 0.85, detection of stain luminescence using UV VL-4 LC lamp 254-356 nm. The results of the stain that did not appear under the UV lamp sprayed with 10% $CeSO_4$ stain reagent, and heated on the hot plate, so that a single stain was obtained which was originally blue to reddish purple indicating that the D4 isolate was relatively pure. Melting point test results obtained the melting point of the isolate in the temperature of 137ºC-138ºC, the melting point route shows that the obtained isolate has been pure ie a sharp melting point route or not more than 2ºC [13].

Based on the results of identification, D4 isolates were secondary metabolites included in the steroid group. The identification results were supported by group test data and spectroscopy test.

Group test results with the addition of Liebermann-Burchard reagents gave a reaction of discoloration from colorless to green indicating positive steroid.

Spectroscopic tests were carried out using an IR spectrophotometer to determine the presence of functional groups contained in the pure isolate. FTIR test results are obtained by IR spectrum which shows the absorption band at wavenumber 3435.22 cm^{-1}, which indicates the presence of OH-group of isolates which are characterized by wideband and medium intensity based on the literature -OH group is in the area of 3550-3200 cm^{-1}. The vibration is supported by the C-O stretch vibration in 1056.99 absorption which according to the literature is in the range 1260-1000 cm-1. Vibration at wave numbers 2956.87; 2935.66; 2893.22 and 2866.22 cm-1 with strong absorption with strong intensity are aliphatic C-H stretching ¬-CH2- and -CH3 groups, based on literature in the range 3000-2800 cm^{-1}. This shows that the structure of the isolate compounds contains methylene and methyl groups, the presence of these groups reinforced by the presence of bending vibrations in the area of 1462.04 cm-1 indicates the presence of -CH2- and 1377.17 cm^{-1} groups for the -CH3 group based on literature found in area 1480-1430 cm^{-1} and 1395-1340 cm^{-1}. There is a wide absorption with a weak intensity in the area of 1641.42 cm^{-1} which shows the presence of stretching vibration C = C, which is reinforced by absorption at wavenumber 964.41 cm-1 which is a buckling vibration of C=H.

Based on the IR spectrum interpretation data that has been described shows that the isolates obtained contain several functional groups, including -OH (stretch), CO (stretch), aliphatic CH (stretch), aliphatic CH (bending), C = C (stretch) and = CH (buckling). These results indicate that isolate D4 is a steroid group compound. The steroid group with a melting point of 137.6-139.4ºC has also been successfully isolated by Siwe (2016) from the *Alchornea cordifolia* species, stigmasterol [14].

Bioactivity Test for *E.coli*

Antibacterial bioactivity test aims to determine the effectiveness of a compound in inhibiting the growth of a bacterium. Testing the growth inhibition of *E. coli* using Kirby-Bauer diffusion method. The choice of this method is due to the ease of the testing process and is relatively cheaper compared to other diffusion methods.

This test uses 6 mm disc paper. The disc paper serves as a place to accommodate antimicrobial substances. As for the sample used in this test is to isolate D4 ethyl acetate extract of *H. corymbose* plant which is dissolved with DMSO. The negative control, namely DMSO, was used to determine the effect of solvents on the activity of isolates on test bacteria. DMSO is an organic solvent that can dissolve almost all polar and non-polar compounds and is not bactericidal [15]. The positive controls used were tetracycline which was antibacterial, which was used as a reference for determining the activity of isolates as antibacterial.

The Nutrient agar (NA) that has been made and condensed on the surface of the petri dish serves as a place for *E. Coli* bacteria inoculation, the method of inoculation is by the spread method. The disc paper that had previously been saturated with D4 isolate, negative control (DMSO), and positive control (tetracycline) were placed on the surface of NA media. After incubation for ± 24 hours, the observation result was the existence of clear area formed around the disc paper which is an antibacterial inhibition zone area.

Based on the results of the isolation zone of isolate D4 against bacteria E. Data in Table 3 showed that isolate D4 with a 10 mm inhibition zone area had the potential as antibacterial, although the growth inhibition response was considered weak. As for negative control, it did not have an inhibitory zone area (0 mm). the effect of solvent (DMSO) on the activity of isolates on the test bacteria.

The mechanism of steroids as antibacterials is related to lipid membranes and sensitivity to steroid components that cause leaks in liposomes. Moreover, Steroids can interact with membrane phospholipid cells which are permeable to their respective lipophilic compounds, causing decreased membrane integrity and changes in cell membrane morphology. which causes brittle cells and lysis [16].

Conclusions and Suggestion

Conclusion

Based on the results of the study, it can be concluded that the Steroid group compound which has been isolated from the main fraction of Ethyl Acetate, namely the D4 fraction produces white needle crystals with a melting point of 137-138ºC and has weak antibacterial activity against *E.coli* with an inhibitory power of 10 mm.

Suggestion

The things suggested related to the improvement of this research are as follows:

1. Conducting further research on the factions which are not continued.
2. Continuing identification of the structure of compounds obtained using spectrophotometers GC-MS, 1H-NMR, 13C-NMR, and UV-Vis to ensure the actual structure of the compound.

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