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Research Article
Chemical Constituents and Inhibition Xanthine Oxidase Activity of *Avicennia marina* Exudate

Khairul Anam, Dwi Susilo, Dewi Kusrini and L.N.A. Agustina

Department of Chemistry, Faculty of Science and Mathematics, Diponegoro University, Jalan, Prof. Soedarto, SH, Tembalang, 50275 Semarang, Indonesia

Abstract

Background and Objective: *Avicennia marina* is one of the mangrove species which have various properties in traditional medicine. Extracts of the leaves have anticancer and antiviral activity. Seeds have antimalarial activity. Stems and fruit used as a cure rheumatism, skin diseases and inflammatory. While, the tree exudates efficacious as contraceptives and gout medicine. Scientific support for the use of exudates in traditional medicine is relatively limited. The purpose of this study is the verification of the use of *A. marina* exudate as lowering uric acid levels. **Materials and Methods:** In this study, analyzed the content of chemical, anti-hiperurisemia activity test by inhibiting the enzyme xanthine oxidase method toward extract and fractions of *A. marina* exudate and identification of the primary chemical compounds in the active fraction. **Results:** The results showed that the *A. marina* exudate containing flavonoids, tannins, quinones, saponins and steroid/triterpenoid. Ethanol extract of *A. marina* exudate has anti-hiperurisemia activity (IC_{50} = 202.4 ppm) and their ethyl acetic fraction (IC_{50} = 0.42 ppm) had stronger activity than the total extract. **Conclusion:** The identification results of component in the active by UV-vis spectrometer and FTIR thought to contain 5,7,3',4' tetrahidroxyflavone (Luteolin).

Key words: Anti-hiperurisemia, flavonoid, uric acid, *A. marina*, antiviral activity

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Corresponding Author: Khairul Anam, Department of Chemistry, Faculty of Science and Mathematics, Diponegoro University, Jalan, Prof. Soedarto, SH, Tembalang, 50275 Semarang, Indonesia Tel: +62 81 325 555 513 Fax: +62 24 764 80824

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The mangrove ecosystem is a natural resource which has the various function and significant role in the country's development. Indonesian mangrove ecosystem is the largest in the world with an area of mangrove forests ±4 million hectares and consists of ±45 species[1]. One type of mangrove plants in Indonesia is fire tree (*Avicennia* sp). Utilization of *Avicennia* sp., as traditional medicine has long been used as anti-pregnancy drugs, drug box, accelerating the surgical wound drainage and gout medicine.

The content of bioactive compounds in *A. marina* has been reported the class of compounds tannins, saponins, terpenoids, alkaloids and steroids which have bioactivity as antimicrobial, antifungal, antiviral, antitumor, insecticides and antileukemia[2]. Shadariah *et al.*[3] reported that the *A. marina* leaves extract has potential as an anticancer (IC_{50} = 0.0002 ppm). The *A. marina* leaves extract also has potential as an antiviral (IC_{50} 66 ppm)[4]. Seeds of *A. marina* containing triterpenoids compounds and potential as an anti-malarial[5]. Stems and fruit of *A. marina* can be used as a cure rheumatism, skin diseases and inflammatory[6]. The *A. marina* has been used as a contraceptive and gout medicine, traditionally[7].

Uric acid is an end-product of purine metabolism of the compound in the body which are then excreted through urine, feces and sweat. Excess uric acid in the blood (hyperuricemia) cause uric acid settles in the joints and cause inflammation of the joints (gout)[8]. The prevalence of uric acid (gout) has increased in recent years. The prevalence of gout in America even reached 4% of the total population[8]. While in Indonesia, it is estimated to reach 1.6-13.6 per hundred thousand people. This prevalence increases with increasing age[9]. If this condition is left untreated would lead to the risk of diseases such as inflammation of the joints (gout), hypertension, diabetes mellitus and renal failure[8]. One of the conditions that can lower uric acid levels in the body is deficient as well as inhibition of the enzyme xanthine oxidase in the formation of uric acids, such as allopurinol administration of certain drugs that which has been used as an effective remedy for gout[10].

Allopurinol is a drug that is useful for treating gout, but if used for a long time and with a high dose has some adverse side effects, such as allergic reactions, gastrointestinal disorders, bone marrow depression and aplastic anemia[11]. Therefore, it is necessary to find compounds that have the inhibition of xanthine oxidase activity and moderate side effects derived from the natural product. One of them is *A. marina* that has traditionally been used as medicine by the people of uric acid. However, the scientific base of mangrove *A. marina* utilization as gout medication has not been reported. In this opportunity will be reported a compound in the active fraction of *A. marina* exudate. The aim of this study was to elucidate the chemical structure of active constituent fractions as xanthine oxidase inhibitor of *A. marina* exudate.

MATERIALS AND METHODS

General: Xanthine (Sigma), xanthine oxidase from bovine milk lyophilized powder (Sigma), silica gel 60 H (Merck EM 7734) column for vacuum liquid chromatography, silica gel 60 HF_{254} (Merck EM 5554) for thin layer chromatography analysis, UV light (254 and 365 nm) for visualized spots, sulfuric acids 10% or iron(III) chloride (10%) as spray reagent. Spectrophotometer UV-vis Hewlett Packard HP 8452 and spectrophotometer Shimadzu 8201PC.

Plant material: *Avicennia marina* exudates obtained from mangrove conservation Tapak- Mangunharjo area, Semarang, Central Java in February, 2015. The plant was determined by the Systematic and Ecology Laboratory, Departement of Biology, Faculty of Science and Mathematics, Diponegoro University, Indonesia.

Material characterization: Phytochemical screening conducted on biological material to determine the physicochemical properties of the *A. marina* exudate. The determination of simplicial characteristics including the determination of moisture content, ash content and the assay of extracts were performed according to developed methods by WHO[12].

Phytochemical screening: Phytochemical screening was carried out according to the developed methods by Harborne[13] and Farnsworth[14].

Extraction and fractionation: Exudate dried of *A. marina* maceration with ethanol 3×24 h. The filtrate obtained was concentrated using rotary vacuum evaporator to obtain viscous extract ethanol. Further fractionation ethanol extract was obtained by solvent n-hexane, ethyl acetate and methanol using a Vacuum Liquid Chromatography (VLC) stationary phase silica gel H 60. Then each eluent obtained was concentrated by rotary vacuum evaporator.

Inhibition activity test against xanthine *in vitro* oxidase activity: Test inhibitory activity against xanthine oxidase

activity *in vitro* was carried out by spectrophotometric method based on procedures[15,16]. One milliliter test solution was added to 2.9 mL of 0.05 M potassium phosphate buffer (pH 7.5). The mixture plus 0.1 mL xanthine oxidase (0.1 U mL^{-1} in phosphate buffer pH 7.5) and performed pre-incubation at 25°C for 15 min. The mixture was then added 2 mL of 0.15 mM xanthine and incubated at 25°C for 30 min. After incubation, the mixture immediately added 1 mL of 1 N HCl to stop the reaction. The UV absorbance measured the mixture at a wavelength of 290 nm. One unit activity of xanthine oxidase defined as the amount of enzyme required to produce 1 mmol of uric acid per minute at a temperature of 25°C. Xanthine oxidase activity expressed as the percent inhibition of xanthine oxidase which is calculated using the equation:

$$\text{Percent inhibition} = \left\{\frac{(A-B)-(C-D)}{(A-B)}\right\}\times 100$$

where, A is the application absorbance (without adding samples), B is the absorbance application control (without adding samples and enzymes), C is the absorbance samples and D is the absorbance sample control (without adding enzymes).

Identification of active compounds faction: Most active fraction to the inhibition of xanthine oxidase was identified using thin layer chromatography with spots developed by Wagner and Bladt[17] and purified preparative thin layer chromatography. Characterization of isolates using UV-vis and FTIR spectrophotometer.

RESULTS AND DISCUSSION

Characterising and screening of phytochemical *A. marina* exudates: *Avicennia marina* exudates are a sap which spontaneously out of the stem of the plant that has been dried up and stuck to the bark of plants. The *A. marina* exudates solid form transparent soft brown to dark brown. This sap is hot when exposed to skin and odorless.

The *A. marina* exudate moisture content amounted to 18.90%. The moisture content exceeds the provisions of WHO[12] at less than 10% so that the exudate of *A. marina* can not be stored for too long as the bulbs as easily damaged as easily damaged by enzymatic reactions and susceptible microbes in the water content of more than 10%. The *A. marina* exudate ash (4.69%) content indicates the amount of metal material in the exudate. This is possible because

Table 1: Physicochemical characteristics of *A. marina* exudate

Characteristics	Result (%)
Water content	18.90
Total ash	4.69
Ash insoluble acid level	2.03
Water soluble	16.95
Ethanol soluble	0.09

Table 2: IC_{50} value of the inhibition of xanthine oxidase activity

Extract	IC_{50} (ppm)
Allopurinol	0.01
Ethanol extract	202.03
n-hexana fraction	83.44
Etil asetat fraction	0.42
Methanol fraction	8.56

A. marina grows in marine environments that contain many metals or minerals. Acid insoluble ash (2.03%) content indicates silicate compound. Levels of water (16.95%) and alcohol soluble (0.09%) extract shows materials that can be extraction by water and alcohol (Table 1). Levels of soluble extract water and alcohol can qualitatively describe the quality of crude drugs, although not explicitly stated individual constituents in plants. Phytochemical screening results to show *A. marina* exudate contains flavonoid compounds, saponins, terpenoids, quinones and tannins.

Inhibition of xanthine oxidase activity: Inhibition of the enzyme xanthine oxidase test performed on extracts of ethanol, n-hexane fraction, ethyl acetate fraction and methanol fraction of *A. marina* exudates and allopurinol using a variant of concentration (Table 2). Test at various concentrations is aimed to determine the concentration effect of the extract to the increase in power of inhibition. It also made observations of enzyme activity without the addition of the sample (blank) to determine the effect of the sample on the enzyme inhibition activity and observed without the addition of the enzyme (control sample and blank control) to determine a correction factor for other compounds that give uptake.

The fraction of ethyl acetate and methanol fraction having IC_{50} values are smaller than ethanol extracts and fractions of n-hexane. This is because the compounds contained in these fractions are compounds that are polar or semipolar such as phenol compounds, tannins and flavonoids. Owen and Johns[15] reported a class of phenol compounds, tannins and flavonoids could potentially inhibit xanthine oxidase.

Phytochemical testing of the ethyl acetate fraction showed flavonoid-containing compounds, alkaloids and triterpenoids. Allegedly these compounds could potentially

inhibit the activity of xanthine oxidase. Flavonoid compounds known to play a role in inhibiting the activity of xanthine oxidase. Hydroxyl groups in flavonoids, especially at positions C5 and C7 plays a role in inhibiting xanthine oxidase. Flavonoids contribute to reducing oxidative stress by inhibiting the activation of the enzyme regulation (key regulating enzyme), such as the enzyme xanthine oxidase[18]. Flavonoid compounds inhibit xanthine oxidase activity by acting act as a competitive inhibitor[19]. Cos *et al.*[18] reported the presence of double bonds in flavonoids (mainly flavones and flavonols) in the C2 and C3 atoms in the ring C, resulted position ring B co-planar with the A ring because of the conjugate thereby increasing inhibition of xanthine oxidase activity. It is known that *A. marina* containing flavonoids luteolin 7-O-metileter-3'-O-β-D-glucoside and luteolin 7-O-metileter-3'-O-β-D-galactoside[20].

Identification of active fraction: The identification of chemical contents of ethyl acetate fraction which has most active on the inhibition of xanthine oxidase (IC_{50} = 0.42 ppm) by thin layer chromatography using n-hexane eluent: chloroform:ethyl acetate (2:2:1) shows the ethyl acetate fraction containing compounds alkaloids, triterpenoids and flavonoids. Separation of ethyl acetate fraction by preparative TLC (silica gel F_{254}) using the eluent n-hexane:chloroform:ethyl acetate (2:2:1) produced 12 isolates (A, B, C, D, E, F, G, H, I, J, K, L). Purity test showed only three isolates that are alleged to have pure isolates (A, C, F, G). Each of these isolates tested antioxidant activity against DPPH. This is due to the active compound on the inhibition of xanthine oxidase are compounds that are antioxidants to a compound that is a byproduct of the formation of uric acid by xanthine oxidase[18]. The test results of antioxidant activity produce isolates J as the only isolate active as an antioxidant. Therefore, it is separated by Thin Layer Chromatography (TLC) preparative thus obtained J isolate which is an antioxidant.

Purity test: Purity test done to J isolate using TLC with silica gel GF254 and developers are: n-hexane:chloroform:ethyl acetate [2:2:1] (R_f = 0.7), n-hexane:acetone [8:3] (R_f = 0.54) and benzene:acetone [3:1] (R_f = 0.59). The purity of the test results showed that J2 isolates is pure compounds.

Characterization and structure elucidation: The J isolates provides bright yellow color with spray reagent AlCl3 and gives blue color with ammonia vapor that can be seen under a light UV365. According to Markham[21] compound which gives a yellow light with spray reagent $AlCl_3$ and gives fluorescence blue color with ammonia vapor under the lights UV365 is a class of compounds flavones or flavonols.

Identification with the FTIR spectrophotometer against J isolates was conducted to determine the functional group of compounds found in J isolates. The FTIR analysis results showed that J isolates has an absorption band at wavenumber 3379.29 cm^{-1} indicate the presence of the -OH stretching that can form hydrogen bonds. While the absorption band at a wavelength of 2978.29 and 2893 cm^{-1} show aliphatic CH stretching asymmetry and symmetry reinforced their CH bend at 1450.03 and 1381.7 cm^{-1}. The absorption band at wavenumber 1652.07 cm^{-1} indicates the group C=O. The absorption band at wavenumber 1460.7 cm^{-1} indicates the group C=C aromatic. The absorption band at wavenumber 1153.4 cm^{-1} indicates the group COC (O bridge in the ring), an absorption band at wavenumber 1097.85 cm^{-1} shows CO alcohol groups. The absorption band at wavenumber 879.54 cm^{-1} indicates the substitution of benzene at the position. The absorption band at wavenumber 772.23 cm^{-1} indicates the substitution of benzene in the ortho position and an absorption band at wavenumber 671.23 cm^{-1} indicates the substitution of benzene at the meta position. Base on FTIR spectra analysis showed that J2 isolate has a functional group OH stretching, CH stretching (symmetry and asymmetry), CH buckling, C=O-carbonyl, C=C aromatic ether CO, CO substitution alcohol and benzene.

Identification using UV-vis spectrophotometer showed J isolate (Fig. 1) provides maximum absorption at a wavelength of 252 nm (Band II) and 344 nm (Band I). This wavelength showed the presence conjugated double bonds and their chromophore group that a characteristic absorption of the flavonoid class of flavonoids.

The addition a reagent shift to isolates aims to determine the location of the hydroxyl groups of flavonoids.

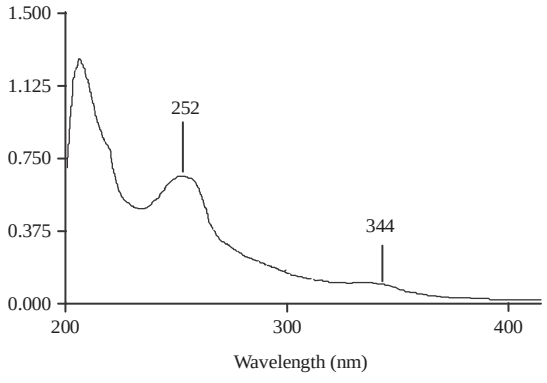

Fig. 1: UV-vis spectrum of J isolate

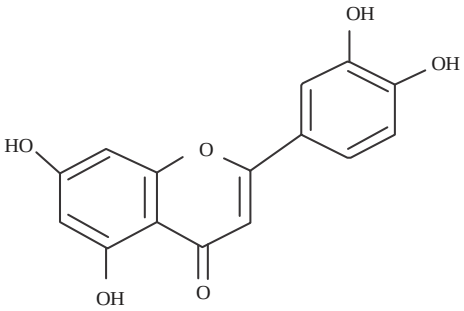

Fig. 2: Structure of the compound 5,7,3',4' tetrahidroxiflavone (Luteolin)

Table 3: Comparison of isolates J2 wavelength before and after the addition of NaOH

Band	λ isolate J2+methanol (nm)	+NaOAc (nm)	NaOAc/ H_3BO_3 (nm)
I	344 (A0,129)	372 (A0,134)	370 (A0,152)
II	252 (A0,664)	267 (A0,508)	263 (A0,559)

Table 4: Comparison of isolates J2 wavelength before and after the addition of $AlCl_3$ and $AlCl_3$/HCl

Band	λ isolate J2+methanol (nm)	+$AlCl_3$ (nm)	+$AlCl_3$/HCl (nm)
I	344 (A0,129)	390 (A0,204)	388 (A0,150)
II	252 (A0,664)	274 (A0,712)	270 (A0,700)

Table 5: Comparison of isolates J2 wavelength before and after the addition of NaOAc and NaOAc/H_3BO_3

Band	λ isolate J2+metanol (nm)	+NaOAc (nm)	+NaOAc/H_3BO_3 (nm)
I	344 (A0,129)	372 (A0,134)	370 (A0,152)
II	252 (A0,664)	267 (A0,508)	263 (A0,559)

Reagents shift used, among others, sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), hydrochloric acid (HCl), sodium acetic (CH_3COONa) and boric acid (H_3BO_3).

The addition NaOH solution to detect hydroxyl groups that more acidic or not substituted. The NaOH spectra explain their phenolic hydroxyl groups are ionized to a certain extent, so it is an indication spectral fingerprint of hydroxylation pattern. The addition of NaOH at flavones used to detect the presence 3-OH and 4'-OH group or a marked shift in the range of 45-65 nm in the band[21]. The UV spectra of J2 isolate with NaOH presented in Fig. 2.

The addition of NaOH in J isolates cause a bathochromic shift in the band I was 47 nm (344-391 nm) with a power that has not decreased for 5 min indicates the group is sensitive to acid 4'-OH groups in flavonoids (Table 3).

The addition $AlCl_3$ which aims to detect the presence of acid-resistant complex formation between hydroxyl and ketone neighboring as well as the compound is not an acid resistant o-dihydroxyl group in ring A and ring B[21]. The addition of $AlCl_3$/HCl is used to detect the 3-OH group and 5-OH which is detected by the shift of the absorption band I in the range of 0-60 nm as compared with the uptake in methanol. About 35-55 nm bathochromic absorption shift only indicates the presence of 5-OH[21].

Absorbance and wavelength of J isolate before and after the addition of reagents shift $AlCl_3$ and $AlCl_3$/HCl shown in Table 4. The addition $AlCl_3$ showed a bathochromic shift in the band I was 46 nm (344-390 nm) which showed o-dihydroxyl in ring B is in the C-3 'and C-4' as well as their hydroxyl group adjacent to the keto group. The addition of HCl after addition of reagents sliding $AlCl_3$ showed no change in wavelength but has the strength decreases. It indicates that flavonoids have a hydroxyl group at C-5.

The addition NaOAc solution which has aimed to detect the free 7-hydroxyl groups. It is demonstrated by the shift in absorption with a range of 0-20 nm in flavonoids[21]. The addition NaOAc and H_3BO_3 reagent are used to detect the presence of o-hydroxy group adjacent to the ring A and ring B. The presence of ortho-dihydroxy in ring B in flavonoids shown to a bathochromic absorption shift that occurred in the band I (ring B) is 12-36 nm[21]. The results of the analysis using UV spectrometer with the addition of NaOAc and NaOAc/H_3BO_3 against J isolate shown in Table 5.

The results of the analysis of UV spectra J isolate after the addition of reagent NaOAc slide show a bathochromic shift of 15 nm on the second tape that is of wavelength 252-267 nm and decreased strength after 5 min. It shows the -OH group in position C-7. The addition of reagents sliding NaOAc and H_3BO_3 resulted in a bathochromic shift of 24 nm (compared to absorption in methanol) in the ring I. This further reinforces the group o-dihydroxyl at position C-3' and C-4' in ring B at the time of adding reagent shift $AlCl_3$ and $AlCl_3$/HCl in flavonoids.

Based on the FTIR and UV-vis spectra analysis we propose that the primary component of ethyl acetate fraction is 5,7,3',4' tetrahydroxy flavone (luteolin). Flavonoid luteolin compounds are compounds that are known to have an inhibitory activity of xanthine oxidase high with IC_{50} of 0.55 ppm[18].

CONCLUSION

Avicennia marina exudate has been shown to inhibit the action of xanthine with ethyl acetate fraction had the highest activity (IC_{50} = 0.42 ppm) to extract ethanol, n-hexane fractions and fractions of methanol. The primary component of ethyl acetate fraction is 5,7,3',4' tetrahydroxy flavone (luteolin).

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