
Morphological Characteristics of Bioactive Compounds on Api-Api Mangrove Leaves Extract (*Avicennia marina*) Based on Leaves Age

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Abstract Api-api leaves *A. marina* has a variety of active compounds that have been confirmed to too wordy be used as antibacterial agents, where harvesting of mangrove leaves is carried out at the location of Betooguci Gresik and differentiated by age, this age differentiation is based on morphological characteristics because it has color differences significant. Where age is one of the factors that affect the level of active compounds found in leaves. Extraction results of mangrove api-api gave different yield levels in each age level. The order of yields from the largest to the smallest is the old leaves 3%, fall 2.86% and young leaves 2.04%. Whereas based on phytochemical test the best extracts were obtained, namely old leaves, fall leaves and young leaves, FT-IR results with 10 detected absorption files which indicate the presence of functional group characteristics that can be used as an illustration of the presence of antibacterial compounds. In the LC-MS test with identification of several molecular weights which indicated a signal derivative of flavonoid compounds in the form of dihydroquercetin, quercetin-3-O- β -D-xylopyranoside, quercetin-3-O- β -D-galactopyranoside, isohametin-7-O-pentoside and routine, and derivatives of detected alkaloid compounds in the form of berberine and papaverine.

Introduction

The control of bacterial diseases mostly still use antibiotics until now. The use of antibiotics has a negative impact on consumer health in the form of antibiotic residues and causes contamination of the aquatic environment (Haditomo *et al.*, 2016). Continuous use of synthetic antibiotics can cause pathogenic bacteria to become resistant. Besides, it is very possible for the emergence of residues or accumulation of antibiotics in the fish body (Setyowati *et al.*, 2014).

The alternative that can be taken from the above problems is by using parts of mangrove plants that have antibacterial compounds to avoid the negative effects of these antibiotics. One medicine that can be used to treat *A.*

salmonicida infection is by using natural ingredients in the form of api-api mangrove leaves were used as an extract. In Gresik, *A. marina* is a type of mangrove that dominates mangrove populations in the area of ponds. These mangrove leaves contain various chemicals with potential medicinal properties that can be used in healing. Organic compounds can act as antioxidants and immunostimulants (Kumar *et al.*, 2018). While other studies have reported that *A. marina* can be used as an antibacterial *A. salmonicida* in stems, leaves and fruit. In general, *A. marina* has been reported to function as an antibacterial, anti-fungal, antiviral, anti-plasmodial, anti-tumor and anti-ulcer (Dhayanithi *et al.*, 2015). Based on research conducted by Karimuna (2015), the

levels of bioactive leaves will increase with age even the content of each leaves position on the availability of nutrients in the area.

While based on research conducted by Schimel and Hattenschwiler (2007), leaves that fall at ground level will undergo decomposition process that can cause a reduction in the content of the leaf, whereas leaves that contain N will transfer the N of the result of the movement of nitrogen on microsite which is an important control in N cycle control. Where Karimuna (2015) states that nutrient and leaf age differences will correlate straight to the amount of bioactivity contained in the leaf, where old leaves have higher bioactive levels than young leaves. In this study we hope to provide information related to how much the division of *A. marina* leaf bioactive content based on age differences.

Materials and Methods

Materials



Figure 1. Morphology of young (A), old (B) and fall (C) leaves.

The test material in this study was presented as follows: *A. marina* mangrove leaves taken from Betoayokauman village, Manyar, Gresik Regency, East Java, Indonesia with coordinates S 7°5'59.7876 "E 112°33'47.412", label paper, 70% alcohol, 96% ethanol, tissue, distilled water, aluminum foil, plastic wrap, gloves and masks. While the tools

used are containers, blenders, glass jars, bottles, vacuum evaporators, refrigerators, spatulas and scales. Where leaf morphology is shown in Figure 1.

Methods

Extraction

The *A. marina* mangrove leaves are carried out in several stages, the initial stage is the leaves taken from the mangrove ecosystem around the Sembayat pond area, Gresik, East Java, Indonesia on S 7°5'59.7876" E 112°33'47.412". Samples of leaves (young leaves, old leaves and leaf fall) then collected as much as 2 kg of each sample and dried, then dried powdered crude drugs into a powder using a powder maker. The maceration process by soaking 200gr of leaf powder into 1 liter ethanol solvent. After soaking for at least 24 hours, the sample is separated from the pulp by filtering using filter paper. If it has been separated, the filtrate is evaporated using a rotary evaporator to obtain a mangrove leaf extract and extract of *A. marina* can be stored in the refrigerator. According to Ulmursida *et al.* (2017) and Ekawaty *et al.* (2015) state that the ratio of solvent and sample at least 1:3 and then soaked for 24 hours. According to Kumar *et al.* (2018), the mangrove leaves after being taken then washed and dried at room temperature. After drying, the leaves are mashed using a blender. The leaf powder from the grinding process is withdrawn by the active compound using ethanol. Where the active compound in the form of the extract can be stored at a temperature of 4°C. According to Ekawaty *et al.* (2015), the evaporation process was carried out at a temperature of 40°C, while the withdrawal of active ingredients from several solvents in the antibacterial test was in the form of inhibition using discs starting from the best sequentially namely ethanol, n-hexane and ethyl acetate.

Phytochemical Test

Phytochemical test in Matera Medica, Batu City. This test is useful for describing

compounds in extracts which include examination of flavonoids, alkaloids, tannins, steroids/triterpenoids and phenolics (Syafitri *et al.*, 2014). The steps of the phytochemical test are as follows:

a. Flavonoids Test

Samples were added 0.1 mg magnesium powder and 0.4 mL amyl alcohol (37% hydrochloric acid mixture and 95% ethanol with the same volume) and 4 mL alcohol then the mixture was shaken. The formation of red, yellow or orange in the amyl alcohol layer shows the results of flavonoids.

b. Alkaloids Test

0.1 gram extract was added with 10 mL chloroform and a few drops of ammonia were added. The chloroform fraction is separated and acidified with a few drops of concentrated H₂SO₄. Acid fraction is taken and divided into 3 tubes, then Dragendorf, Meyer, and Wagner reagents are added. The presence of alkaloids is characterized by the formation of white deposits in Meyer reagents, red deposits in Dragendorf reagents, and brown deposits in Wagner reagents.

c. Tannins Test

Extracts of 1 gr is added with 10 mL of distilled water and then boiled. After cooling the filtrate was added 5 mL FeCl₃ 1% (w/v). If the color changes to dark blue, it means that the sample contains tannin

d. Steroids / Triterpenoids Test

A total of 1 gr of sample was dissolved with 25 mL of 50°C hot ethanol, then filtered into a porcelain dish and evaporated to dryness. The residue is dissolved with ether and transferred into a test tube, then added 3 drops of anhydrous acetic acid and 1 drop of concentrated H₂SO₄ (Lieberman Burchard Test). Red or purple indicates triterpenoids and green or blue indicates steroids.

f. Phenolic Test

A total of 1 gram of sample was extracted with 20 mL of 70% ethanol. Extract as much as 1

mL then add 2 drops of 5% FeCl₃ solution. The formation of green or green color determines the phenol compounds in the ingredients.

Fourier Transform Infra-Red (FT-IR)

FT-IR test in Malik Ibrahim Islamic State University Malang. In another reference, the process of compound analysis using FT-IR is by taking as much as 0.5 mg of the sample then mixed with 180 mg KBr and homogenized to form a pellet. Then the measurements were carried out with the FT-IR spectrum with a wavelength of 4000-400 cm⁻¹ and analyzed the results (Rohaeti *et al.*, 2011). Samples before testing FT-IR were centrifuged at 10,000 rpm for 15 minutes and dry samples were mixed with KBr pellets used in FT-IR measurements. The spectrum was recorded in the 4000-400 cm⁻¹ range using the Thermo Nicolet Nexus 670 spectrometer in diffuse reflectance mode which operates at a resolution of 4 cm⁻¹ (Mallikarjuna *et al.*, 2011).

Liquid Chromatography Mass Spectrophotometry (LC-MS)

The sample test is done with LC-MS / MS equipment (Thermo) in Politeknik Negeri Malang. The column used with the Hypersil Gold specification (50mm x 2.1mm x 1.9µm). UHPLC The ACCELLA type 1250 brand made by Thermo Scientific consists of a vacuum degasser, quarter pump, thermostatic autosampler controlled by a personal computer through the x-calibur 2.1 program. Solvent A=0.1% formic acid in Water and B=0.1% formic acid in acetonitrile. A mobile phase gradient with a speed of 300 µl / minute with a setting of 0.0-0.6 minutes 10% B, 0.6-5.0 minutes 55% B, 5.0-5.5 minutes 55% B, 5.5-5.75 minutes 10% B, 5.75-7.5 minutes 10% B. The injection volume in LC is 2 µL at 16°C. The column is controlled at 30°C, and the autosampler compartment is set to 16°C. Use of MS / MS Triple Q (quadrupole) mass spectrometer TSQ quantum access max from thermo finnigan with ESI ionization source (electrospray ionization) controlled by TSQ tune

software which is operated in negative mode. Qualitative determination by the SRM method (selected reaction monitoring) is arranged in Table 1. Where the ESI ionization conditions are as follows: 2.5 kV spray stress; Evaporation temperature of 250°C; Capillary temperature, 300°C; nitrogen as sheath gas pressure 40 psi, and Aux gas pressure 10 psi with argon gas. In another reference by (Rachmawati dan Widiyanti, 2013) the LC-MS testing system is using the stationary and mobile phases, the separation technique of the LC pump gradient system and the flow rate vacuum pump with units ml / min. In this test also uses the column dimensions contained in the tool. The column temperature can be adjusted at 40°C, the injection volume is 10 ul, and it uses a gas generator, where the gas temperature is set at 250°C then enters the mass spectrometry detector (SPD-10 AVP) and Electrospray Ionization (ESI) ionization technique of positive ions. Table 1 presents ion ranges based on Politeknik Negeri Malang standard.

Table 1. Optimization of Mass Parameters of Analytes

Molecular Ion	Ion Precursor	Ion Product
	Q1 (m/z)	Q2 (m/z)
Quercetin	301	179
Dihydroquercetin	303	286
Berberine	334	288
Papaverine	338	292
Quercetin-3-O- β -D-xylopyranoside	433	300
Quercetin-3-O- β -D-galactopyranoside	463	301
Isohametin-7-O-pentoside	477	301
Rutin	609	300

Result and Discussions

Phytochemical Test

The results of phytochemical screening of 6 compounds in 3 leaf ages (young, old and fall) showed different results and provided information that the old leaves had bioactive levels that were more diverse than the falling leaves and the last young leaves. Where the results of the phytochemical screening are presented in Table 2.

Table 2. Phytochemical Screening Results of Mangrove Leaf Extract (*A. marina*)

Sample	Identification of Compounds					
	Flavonoids	Alkaloids	Tannins	Triterpenoids	Steroids	Phenols
Young	-	-	+	-	-	+
Old	+	+	+	+	-	+
Fall	+	+	+	-	-	+

Permata and Asben (2017), the number of Bioactive leaves are affected by age differences which can be characterized by differences in leaf color. Based on research conducted by Karimuna (2015) that the levels of bioactive leaves will increase with age even in each leaf stalk position differ greatly influenced by the availability of nutrients in the area. Whereas based on research conducted by Schimel and Hattenschwiler (2007), states that falling leaves located on the ground surface will undergo a decomposition process that can cause reduced content of the leaves, where leaves that

have a high N content will transfer N due to the movement of nitrogen on microsities which are important controls in N cycle control. This transfer movement will cause uneven decomposition of litter. This is the result of N mineralization by microbes that have first access to N to control how much N is transferred to other microsities in the system. Karimuna (2015) states that the nutrients and the age difference leave straight correlated to the amount of bioactive contained in the leaves, where the older leaves have higher bioactive level than young leaves. So that from the above statement indicates that the loss / reduction of triterpenoids in fallen leaves is the effect of reduced N elements on leaves during the N transport process from high concentrations to low concentrations that affect the reduced

bioactive levels of leaves indicated by the correlation between nutrient levels and the amount of bioactive contained.

Fourier Transform Infrared (FT-IR)

The results shown in the FTIR testing showed a difference in every age of the leaf, where the older leaves have the highest and lowest absorption file which is then followed by the young leaves and leaf fall. The infrared spectrum shows absorption bands derived from the double bond hydroxyl, aliphatic, isolated into the ether (Harizon *et al.*, 2014). Where naming functional groups based on the absorption of Bruno *et al.* (2010), Harizon *et al.* (2014), Sopiah (2014), Jain *et al.* (2016), and Ningrum *et al.* (2017) are presented in Tables 3, 4, and 5.

Table 3. FTIR Test of Mangrove Leaf Extract *A. marina* (Young)

No.	Absorption File	Functional groups
1.	520.960	C-H Bending
2.	668.765	C-H Aliphatic
3.	1339.901	C-F Alkyl Halide
4.	1399.954	C-H Alkana
5.	1457.087	C-NO ₂ Nitro Aromatic
6.	1633.807	C = C Alkene
7.	2926.012	C-H Alkana, Alifatik
8.	3444.084	O-H Phenol
9.	3749.085	O-H Hydroxyl
10.	3855.227	O-H Alcohol

Table 4. FTIR Test of Mangrove Leaf Extract *A. marina* (Old)

No.	Absorption File	Functional groups
1.	519.915	C-H Bending, Aromatic
2.	668.227	C-H Aliphatic
3.	1073.557	C-O Alcohol
4.	1261.301	C-N Amina
5.	1400.831	C-H Alkana
6.	1456.993	C-H Alkana
7.	1635.247	C = C Aroinatik, O-Quinone, Polyquercetin
8.	2927.956	-C-H Ulur aliphatic
9.	3425.717	O-H Alcohol, Phenol
10.	3855.617	O-H Alcohol

Table 5. FTIR Test of Mangrove Leaf Extract *A. marina* (Fall)

No.	Absorption File	Functional groups
1.	667.500	C-H Aliphatic
2.	1038.672	C-O Alcohol
3.	1085.182	C-O Alcohol
4.	1188.455	C-O Ether
5.	1382.662	C-H Alkana
6.	1458.170	C-H Alkana
7.	1650.850	C = C Aroinatic, Alkene, O-Quinone, Polyquercetin
8.	1688.824	C = C Aroinatik, Alkene
9.	2926.666	C-H Ulur Alifatik, Alkana
10.	3424.264	O-H Alcohol, Phenol

Liquid Chromatography (LC-MS)

Liquid chromatography in combination with mass spectrometry aims to detect the presence or absence of the target compound at the test extracts characterized by the information signal, which is based on the form of peak time required (time) and strong low signal received on the relative abundance. In this test, the focus is on flavonoid and alkaloid derivatives due to limitations of data-based compounds, but it can mark the difference in content or derivative of the target compound which can be seen in Table 6.

Table 6. Results of Detection of LC-MS of Mangrove Leaves of *A. marina*

Age	Compound Signals Detected							
	Quercetin	Dihydroquercetin	Berberine	Papaverine	Quercetin-3-O- β -D-xylopyranoside	Quercetin-3-O- β -D-galactopyranoside	Isohametin-7-O-pentoside	Rutin
Young	--	+	+	+	+	+	+	+
Old	--	+	+	+	+	+	+	+
Fall	--	+	+	--	+	+	+	--

The derivatives of flavonoids detected in the form of dihydroquercetin, quercetin-3-O- β -D-xylopyranoside, quercetin-3-O- β -D-galactopyranoside, isohametin-7-O-pentoside and can be used as antibacterial agents where their activity will increase because of the presence of hydrophobic substituents. Xie *et al.* (2015) stated that flavonoids are antibacterial agents that can be used in various pathogenic microorganisms. Flavonoids attract attention because they have the potential to be a substitute for antibiotics. It was concluded that hydroxyl in a specific place on the aromatic flavonoid ring can increase activity. However, methylation active hydroxyl groups can reduce the activity. besides, ring A lipophilicity is very important in supporting chalcone activity. Hydrophobic substituents such as phenyl groups, alkyl chains, alkylamino chains, and oxygen or nitrogen

containing heterocyclic groups generally can increase the activity of all flavonoid derivatives. The antibacterial mechanism of flavonoids can be in the form of inhibition of nucleic acid synthesis, inhibition of energy metabolism, inhibition of cytoplasmic membrane function, inhibition of biofilm attachment and formation, inhibition of porin (cell membrane), changes in membrane permeability to attenuate the degree of pathogenicity.

A derivative of flavonoids such as quercetin, Jaisinghani (2017) has said quercetin is a potential polyphenolic flavonoid that is chemoprotective and can be used as an antibacterial agent in relatively small doses. Quercetin is a flavonoid flavanol group that is safe and has antioxidant properties, antiatherogenic, anti-inflammatory, neuroprotective, anti-carcinogenic, antibacterial and antiviral. According to Maalik, *et al.* (2014), chitosan is generally used by quercetin in antimicrobial activity against bacterial species such as *E. coli*, *Salmonella enterica*, and *Listeria monocytogenes*. Also, the bacteriostatic properties of quercetin can inhibit the ligation of D-Ala-D-Ala in bacterial cells, by inhibiting D-alanine: Dalanin ligase enzymes and preventing bacterial growth.

While according to Dabas *et al.* (2019), isohametin is a polyphenolate from plant extracts that act as antioxidants and has bactericidal and fungicidal characteristics in the study where he is still bound to other elements. Meanwhile according to Arima *et al.* (2002), routine mixing with quercetin will increase antibacterial activity by inhibiting DNA synthesis. Similarly, the mixture of quercetin and morin will also increase with the routine. Where an antibacterial activity is based on MIC values, the use of kaempherol decreases sharply with routine additions.

After exposure function of the derived flavonoid, if passed by seeing derivative of

alkaloid compounds were detected in the form of berberine and papaverine was also to be used as an antibacterial agent, but cannot be used as a substitute for antibiotics modification of the structure. Based on Ling *et al.* (2018), Berberine (BBR) is a derivative of the isoquinoline alkaloid known have a significant effect that acts like a drug in gastroenteritis and dysentery in the Chinese and Ayurvedic medicine system. Other pharmacological effects of BBR are antimicrobial, hepatoprotective, anti-hyperlipidemic, anticancer, anti-diabetic and anti-inflammatory. Even according to Bandyopadhyay *et al.* (2015), BBR can provide therapeutic efficacy to multidrug resistant environments. Although BBR has a capacity in the treatment of microbial infections, Battu *et al.* (2010) stated that antimicrobial activity tested in vivo was not effective enough to replace antibiotics commonly found in drug stores.

Conclusions and Suggestion

Several bioactive content tests, it was stated that old api-api mangrove extract has the highest amount of yield of 3% quantitatively and the phytochemical test also had the most complete compound because older leaves had a tendency to have more bioactive content than young leaves. The bioactive compounds are the result of a process plant secondary metabolites to maintain the life of an extreme environment. The antibacterial properties evidenced by the group commonly used as an indicator of an antibacterial compound and when seen qualitatively using LC-MS that target flavonoid and alkaloids, young leaves and old leaves only have one difference in the compound, papaverine. So, further research is needed to determine how effective the extract is when used as an antibacterial to reduce the use of antibiotics.

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