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**BIOACTIVE CONTENT IDENTIFICATION OF BULUBANGKAL LEAF
(NAUCLEA SUBDITA [KORTH.] STEUD.) AND IT'S ANALYSIS AS AEROMONAS
HYDROPHILA ANTIBACTERIAL BY IN VITRO AND IN SILICO METHODS**

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ABSTRACT

This research aimed at finding out a bioactive content of bulubangkal leaf and extractability of 3 polar solvents, semi-polar and non-polar as *Aeromonas hydrophila* antibacterial. The sample was extracted by maceration and fractionation methods using n-hexane solvent, ethyl acetate, and methanol. Antibacterial activity was carried out by disk-diffusion agar method. It was found that active agent contained within bulubangkal leaf are saponin, tannin, phenolic, and steroid as *A. hydrophila* bacterial based on the antibacterial test by in vitro and in silico methods.

KEY WORDS

Phytochemistry, antibacterial, *Nauclea subdita*, *Aeromonas hydrophila*.

Indonesia is the second largest country in the world after Brazil to have biological diversity. A plant is a home of various active agent content-rich chemical compounds, among others secondary metabolic products function to the process of metabolism. This compound group plays an important role in the process of interaction or competition, including defending self from pests and diseases attack. At a swampland is found more than 1000 kinds of a plant which can be used as a material of pest controller such as biopesticides material (Asikin, 2015). One of the swamp plants frequently utilized as an alternative medicine is the bulubangkal; it commonly results in a secondary metabolic compound such as tannin, phenolic, steroid, and alkaloids (Liew *et al.*, 2012). People commonly utilize bulubangkal to reverse injury; it is applied to the surface of ulcer and tumor, the water of the boiled leaf functions to reverse diarrhea and toothache. *N. subdita* also contains antioxidant and steroid working for cells growth so almost all of the Banjar utilizes it as the raw material of anti-acne powder and to soften skin face in which steroid compound has an ability to rejuvenate damaged cells.

Bioactive compound contained within *Nauclea subdita* is a type of secondary metabolic such as tannin, alkaloid, and phenolic which can be used as a natural bactericide. Leaf, flesh, and root of *N. Subdita* are found to contain alkaloid, tannin and phenolic and saponin (Amos *et al.*, 2005). Some phytochemistry such as tannin, alkaloid, and flavonoid has shown pathogen antibacterial within fish (Haniffa & Kavitha, 2012). This research made an identification of bioactive content and antibacterial test of bulubangkal leaf towards *Aeromonas hydrophila* bacterial which is pathogen bacterial contained within fish especially fresh water by in vitro and in silico methods.

MATERIALS AND METHODS OF RESEARCH

The materials used to conduct this research were bulubangkal leaf, methanol (polar), ethyl acetate (semi-polar), n-hexane (non-polar) all of them with PA grade, active extract of

bulubangkal leaf, TLC plate. A set of tools used are among others Miyako BL-152 GF blender made in Indonesia, a rotary evaporator (IKA RV 10 Digital V made in Germany), erlenmeyer flask, mixer, measuring cylinder, lint, silica gel, aluminum sheet, etc. While the equipment is TLC vessel, oven, splattering, UV lamp, glassware, UV-Vis.

N. subdita (Korth). Steud. leaf was cleaned, cut into small pieces and dried by being aerated. The dried leaves were then mashed with a blender until becoming soft powder. It was then given phytochemistry test towards the sample and conducted maceration. 50 grams of *N. subdita* (Korth). Steud. powder was soaked in 200 ml methanol, ethyl acetate and n-hexane for 2x24 hours in an erlenmeyer screw cap, the next step was being separated between extract and waste, repeated for 3x with the same solvent. The extract collected was then evaporated using vacuum rotary evaporator at the temperature of 40°C until there is no solvent condensation anymore at the condenser. It was then being baked for 3 hours under the temperature of 45°C aiming at relieving solvent still trapped in the bioactive extract, its waste was dried and then its final weight was weighed. A thick extract was the last one will use to conduct an antibacterial test.

This test is used to find out what solvent most effective to use as extracting of *N. subdita* (Korth). Steud. bioactive which is antibacterial in nature using disk method, that is an antimicrobial examination by measuring the diameter of obstacle area happens along the disk paper under the diameter of 6 mm, which has been given *N. subdita* (Korth). Steud. extract. Extract from the solvent giving the biggest drag zone was selected active extract.

The last extract that has been concentrated with gas N₂, taken only 0.1 ml and then diluted in 10 mL volumetric flask with applicable solvent. This solution was then entered into absorption cuvettes and its uptake reads using spectrophotometer UV which has been calibrated using proper solvent blanks in advance. The uptake is read at the wave length of 200-400 nm.

Pure isolates identification with spectrophotometer FTIR is used to take 2 gram solids of free-water KBr and the mashed and pressed at 2 torr pressure. KBr pellet was added by 1 drop of isolate, then analyzed using spectrophotometer FT-IR over the 4000-8000 cm⁻¹ range.

Active isolate analyzed using GC-MS, carrier gas used is helium with regulated stream below: Injector temperature is 320°C, initial temperature is 70°C. Increase rate of temperature is 10°C, and the last temperature in oven is 310°C.

In Silico Analysis - using PASS prediction method, that is GCMS result search using software Pass Online (WAY2DRUG).

RESULTS AND DISCUSSION

Identification result of bulubangkal plant used in this research was undertaken at Research Center for Biology in Cibinong, Bogor. Identity result revealed that this plant is categorized into the family of Rubiaceae and species (*Nauclea subdita* [Korth.] Steud.).

Phytochemistry Test Result

Table 1 – Phytochemistry test result of bulobangkal leaf

No.	Secondary Metabolic	Test Result
1	Phenolic	++
2	Flavonoid	
	• Reagen HCL concentrated + Mg	-
	• Reagen H ₂ SO ₄ 2N	+
	• Reagen NaOH 10%	-
3	Alkaloid:	
	• Reagen Mayer	-
	• Reagen Wagner	-
	• Reagen Hager	-
4	Steroid	+
5	Triterpenoid	+
6	Saponin	++
7	Tanin	++

According to the result of phytochemistry test above, bulubangkal leaf contains saponin, tannin, phenolic, flavonoid, triterpenoid, and steroid. Phenolic and flavonoid contained within the plant are known to have an important role in antioxidant activity test. The higher the phenolic content in the sample, the higher the activity of its antioxidant, (Trisharyanti, et al., 2011).

Tannin is a secondary metabolic compound contained within a plant. Tannin is polyphenol derivative with a characteristic which is able to form a complex compound with other macromolecules. Tannin is commonly soluble in the polar solvent. Chemically, there are two types of tannin, i.e. condensed tannin and hydrolyzed tannin. Condensed tannin is widely spread on woody plants and ferns, while the spread of hydrolyzed tannin is limited to dicotyledon (Auni, et al., 2013). According to Akiyama, et al., 2001, at the activity of antimicrobial, tannin has a positive effect on the different bacterial strain.

Tannin and saponin are proven to have anthelmintic activity (pal, et al., 2007). Tannin found functions to tie independent protein in the digestive tract of the host or glycoprotein in the parasitic cuticle and will cause death (Mali & wadekar, 2008). Benefit and effect potential of tannin, such as antimicrobial and antioxidant in nature. Tannin also has a physiological effect such as accelerating blood clots, and decreasing cholesterol content and lipid also blood as well as ability to act as anticancer (Chung, et al., 1998). An observation to utilize several sources containing tannin, food of several fish species show low lipid deposit in the digestive tract (Hossain, et al., 2001). Tannin has shown antibacterial (Banso & Adeyeme, 2007). Tannic acid can disturb microorganism growth by forming a complex with surface protein (Bhat, et al., 1998).

Saponin causes stimulus on a particular tissue, nasal epithelium, bronchus, kidney, etc. Stimulation on the kidney is expected to cause diuretics effect. Saponin can increase a respond of various substances from surface activity, saponin is also able to stretch insoluble particle and to assign that particle spread and divide softly into the solvent (Sartini, et al, 2012).

The test result of drag power of bulubangkal leaf towards *A. hydrophila* bacterial by disk method, drag zone resulted can be seen in Table 2.

Table 2 – Drag zone test result of bulubangkal leaf towards *A. hydrophila* bacterial growth after 24 hours

No	Sample	Drag Zone (mm)
1	Bulubangkal leaf - methanol	17.83 ± 0.28
2	Bulobangkal leaf-ethyl acetate	23.33 ± 0.57
3	N-hexane	-
4	Gentamicin control	21.66 ± 0.57

Antibacterial test result after 24 hour shows that the biggest drag zone of ethyl acetate fraction is 23.33 ± 0.57 mm, followed by methanol fraction with drag zone of 17.83 ± 0.28 mm. While n-hexane fraction has no drag zone from the initial observation until the last, gentamicin control of 21.66 ± 0.57 mm. Ethyl acetate fraction (semi-polar solvent) is able to dissolve active agent well contained within bulubangkal leaf compared to methanol (polar solvent) and n-hexane (non-polar solvent). Control actually has bigger drag zone than a treatment but at 72 hours bactericidal zone decreases and more decreases at 96 hours observation.

Drag zone formed shows an active agent ability contained within bulubangkal leaf using semipolar and polar solvent, it is expected that tannin contained within bulobangkal leaf has an antibacterial ability, in which tannin activity has been reported as bacteriostatic and bactericidal including *A. hydrophilla*, *E. coli*, *Listeria*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Streptococcus* (Chung et al., 1998). A complex capability of polyphenol and polymer as well as mineral have inhibition effect towards bacteria. Tannin contained within bulubangkal leaf also contains toxic characteristic and hamper enzyme in microbe (Bossi et al., 2007).

UV-VIS test Result. Analysis result of UV-VIS shows 6 spectrums of wavelength over the 200 until 800 nm, with maximum peak spectrum of 222 nm and 206nm. The occurrence of strong uptake at ultraviolet spectrum shows the existence of the number of double bonds C=C in the friction compound of bulubangkal leaf. This strong uptake indicates phenol compound and its derivative.

Figure 1 – Results of spectroscopy analysis

Spectroscopy analysis result of infrared shows the existence of –OH bending with widened bond at number absorption.

Table 3 – Extract components of bulubangkal leaf based on the result of UV-VIS

No	UV-VIS result	Literature Rojas & Ojeda, (2009&2013)	Component	PubChem CID
1	239.0	239.0	Citalopram ($C_{20}H_{21}FN_2O$)	2771
2	222.0	217.4 - 223.0	Fosinopril ($C_{30}H_{46}NO_7P$)	55891
3	215.0	215	Vincamine ($C_{21}H_{26}N_2O_3$)	15376
4	212	212	Tocopherol acetate ($C_{31}H_{52}O_3$)	86472
5	209.9	209.9	Piracetam ($C_6H_{10}N_2O_2$)	4843
6	206.9	200 – 210	Ramiopril ($C_{23}H_{32}N_2O_5$)	5362129

FTIR test result of ethyl acetate extract of bulubangkal leaf having 9 absorption bonds can be seen in Figure 2.

Figure 2 – HTIR test result towards bulubangkal leaf extract

According to figure 2 ethyl acetate fraction compound of bulubangkai leaf extract contains ionic fractions as listed in the following table.

Table 3 – Absorption band, wave number, vibration type and band form of FTIR result of bulubangkai leaf extract

Absorption band	Wave Number (cm ⁻¹)	Reference(Harborne, 2006)	Vibration type	Bond form
1	3467	3500 – 3200 alcohol	-OH bending	Widened
2	2926	3000 – 2700	-C-H (SP ³) stretching aliphatic	Weak
3	2856	3000 - 2700	-C-H (SP ³) stretching aliphatic	Weak
4	1710	2100 - 1700	aromatic C=H stretches	Weak
5	1638	1670 - 1475	-C=C- aromatic	average
6	1459	1470 – 1450	-CH ₂ , -CH ₃ scissoring	Weak
7	1383	~ 1050	-OH primer	Average
8	671	690 - 590	-C=CH out of plane	Weak
9	507	~ 570 – 540	monobranched alkanes (-CH ₃)	Weak

According to the result of GCMS test conducted to the ethyl acetate extract of bulubangkai leaf is obtained a graph as follows:

Figure 3 – GCMS analysis result of ethyl acetate extract of bulubangkai leaf

According to the figure above, it can be known that there is a different point, among other, there are 9 highest points. Of the point received, based on the literature review, is expected that the compound contained within bulubangkai leaf extract as follows.

Table 4 – Compound contained within bulubangkai leaf based on the library (PubChem)

n/n	Point	Name	Formula	Compound Structure	PubChem (CID)/BM
	1	2	3	4	5
1	18.925	Beta-caryophyllene	C ₁₅ H ₂₄		5281515/ 204.357 g/mol
2	19.283	Alpha guaiene	C ₁₅ H ₂₄		5317844/ 204.357 g/mol
3	19.400	Seychellene	C ₁₅ H ₂₄		519743/ 204.357 g/mol
4	20.125	Alpha panasinsen	C ₁₅ H ₂₄		578929/ 204.357 g/mol

n/n	1	2	3	4	5
5	20.558	Alpha-patchoulene	C ₁₅ H ₂₄		521710/ 204.357 g/mol
6	20.550 – 20.567	-	C ₁₅ H ₂₄		11095734/ 204.357 g/mol
7	20.443-20.450	Allo-aromadendrene	C ₁₅ H ₂₄		42608158/ 204.357 g/mol
8	20.442	Longifolene	C ₁₅ H ₂₄		204.357 g/mol
9	20.443-20.450	Alpha-Bulnesene	C ₁₅ H ₂₄		94275/ 204.357 g/mol

According to the result above, the compound contained within bulobangkai leaf has a formula and similar molecular weight of C₁₅H₂₄, with molecular weight of 204.357 g/mol, but having different compound structure.

According to the result of compound identification using GCMS is known 9 active compounds of Bulobungkal leaf which its potential is then analyzed using WAY2DRUG PASS prediction as antimicrobial and antifungal, etc, demonstrated in the following table as antibacterial, antibiotic, antiseptic, antiparasitic, and Peptidoglycan glycosyltransferase inhibitor.

Table 4 – Compound content in bulubangkai leaf based on in silico analysis

Compound	Antibacterial	Antibiotic	Antiseptic	Antiparasitic	Peptidoglycan glycosyltransferase inhibitor
Beta-caryophyllene	0.437			0.382	0.397
Alpha guaiene	0.306			0.414	0.482
Seychellene				0.401	0.589
Alpha panasinsen	0.33				0.566
Methandrostenolone					0.562
Alpha-patchoulene	0.356			0.477	0.457
Allo-aromadendrene	0.38				0.471
Longifolen					0.627
Alpha-Bulnesene	0.34			0.355	0.391

Assessment result of Pa value for a particular activity such as antibacterial, antibiotic, antiseptic, antiparasitic, and peptidoglycan glycosyltransferase inhibitor (playing role as cell cycle, cell form, and cell wall). Pa (probability To Be Active) value is a value reflecting a potential of a compound tested. An active agent of bulubangka leaf has various values. If Pa value above 0,7 so the compound is predicted to have a high potential as antimicrobial computationally and through laboratory test method. While if Pa value is more than 0.3 or less than 0.7 so the compound has computationally ability as antimicrobial and antifungal, but in a laboratory test way is not proven or its potential is small.

Figure 4 – Pa value of Bulubangka leaf

According to that Pa value above, an active agent of bulubangka leaf has a computational potential as antibacterial but its potential needs to be proven in a laboratory way since its score probability to be active is under 0.7. Trial result of in vitro method is proven that bulubangka leaf extract has a big drag zone towards *A. hydrophila* bacteria, ethyl acetate extract of 23.88 ± 0.74 mm and methanol of 17.03 ± 0.48 mm. Peptidoglycan (PG) is an important macromolecule like a tissue surrounding bacteria, giving them the form, and protecting them from its osmotic pressure. PG inhibition of synthesis causes bacterial cell lysis (Derouaux, *et al.*, 2013). This inhibition of synthesis of the cell wall is like a medicine containing β lactam, working procedure of inhibition was initiated by medicine binding of cell receptor-like transpeptidase enzyme among other. After adhesion at one or more receptor, reaction happened is a transpeptidase enzyme reaction and hampered peptidoglycan synthesis leading to the activity termination of autolysis on cell wall, and furthermore lysis enzyme becomes active (Jaweltz, *et al.*, 2001).

CONCLUSION

Bulubangka leaf has a computational potential as antibacterial under 0.7, it is proven by in vitro method of ethyl acetate fraction and effective methanol fraction hampering *A. hydrophila* bacteria while n-hexane fraction does not effectively hamper. It concludes that bulubangka leaf contains bioactive in form of saponin, tannin, phenolic, flavonoid, and steroid used as *A. hydrophila* antibacterial.

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