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Original Article

Influence of ethanol concentration and temperature on antioxidant and antibacterial activity from *Artocarpus altilis* (Parkinson) Fosberg leaves

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ABSTRACT

Objective: *Artocarpus altilis* or Sukun is one kind of herbal medicine that is usually used by Indonesian people. It is widely known that the flavonoid derivative of *A.altilis*, one of its major bioactive constituent, has a broad range of pharmacological activity such as antioxidant, antibacterial, anti-inflamantory, antidiabetic and many others. In this study we investigated the bioactive constituent and influence of ethanol concentration, time and temperature on the antioxidant and antibacterial activity of *A.altilis* leaves in order to find appropriate and effective condition for its extraction. **Methods:** *A.altilis* leaves were extracted with different ethanol concentration (50, 60 and 70% v/v), time (1, 2, 3, 4 and 24 h) and temperature (70°C and room temperature). Influence of different extraction processes was analyzed on antioxidant capacity, antibacterial activity, total phenolic and total flavonoid content. Isolation of bioactive compound was performed by column chromatography method and structure was elucidated by 1H and 13C FT-NMR (Fourier Transform-Nuclear Magnetic Resonance) spectra. **Results:** Concentration of 70% v/v ethanol, 70°C and 1 h extraction process were found to be the most appropriate condition for extraction of *A.altilis* leaves in order to obtain bioactive components which gave both antioxidant and antibacterial activity against *E.coli* while yield of total flavonoid and phenolic content of extract should not less than 1.9 and 1.8% w/w, respectively. Structure elucidation of isolated compound identified that prenylated flavonoid, *i.e.* 1-(2,4-dihydroxyphenyl)-3-(8-hydroxy-2-methyl-2-(4-methylpent-3-en-1-yl)-2H-chromen-5-yl)propa-1-one is one of its important bioactive constituent. **Conclusion:** Use of ethanol concentration, temperature and time influenced the yield of total phenolic and flavonoid content and bioactivity of *A.altilis* extract. It is recommended to heat the extraction process to 70°C in order to obtain both optimal antioxidant and antibacterial activity from ethanol extract of *A.altilis* leaves.

KEY WORDS: Antibacterial, *Artocarpus altilis*, ethanol, temperature

INTRODUCTION

The use of alternative medicine has become popular in the past decade and the consumption of herbal medicines increased significantly [1-3]. WHO estimated that about 80% of world population use herbal medicine for primary health care. Alternative medicines were find as popular experience and nowadays research institutions are engaged for appraisal of effectiveness of traditional medicine [3]. In the World Health Assembly (WHA) resolution the need to ensure the quality of medicinal plant was emphasized.

There are multiple factors that affect the bioactivity of herbal medicine [4]. Concentration of active ingredients from extract of herbal medicine are usually influenced by time, temperature and type of solvent which were used during extraction process [5]. Those factors are important to be investigated because bioactive components of herbal medicine may become decomposed or inactive after wrong extraction process. Moreover, unappropriate temperature, solvent and extraction time also may cause difference in bioactivity.

Artocarpus altilis (Moraceae) had widely known as herbal medicine which is native from Malaysia, Philippines and Papua New Guinea but exotic from Indonesia [6]. Investigation of *Artocarpus* species had identified that every part of this plants were useful. There are several bioactivities for this plant which had been identified, *i.e.* antioxidant, antimalaria, antiviral, anti-platelet aggregation and cytotoxic activity for several cell lines [6-20]. The leaves were rich of phenolic compounds including flavonoids, stilbenoids and arylbenzofurans [7-13, 21-23]. More than fifty compounds had been identified and the group of flavonoid was known as the major constituent from the leaves [7, 9-11, 14]. Since flavonoid is a great polyphenol compound that have numerous biological activities, it is important to monitor the total flavonoid together with total phenolic content during extraction process for standardization of product. Herein, we report our investigation on structure elucidation of one bioactive component together with the influence of ethanol concentration (50, 60 and 70% v/v), time (1, 2, 3, 4 and 24 h) and temperature (70°C and room temperature) on total phenolic content, total flavonoid content together

with bioactivity (antioxidant and antibacterial) from *A.altilis* leaves.

MATERIALS AND METHODS

Plant material and chemicals

Artocarpus altilis leaves were collected from around campus of Indonesian Institute of Sciences located in Bandung, Indonesia and were identified at the Research Center of Biology, Indonesian Institute of Sciences, Bogor. The leaves were dried in a blower oven at 50°C then pulverized mechanically. The dry pulverized leaves were kept in dark and room temperature until used. Organic solvent, *i.e.* ethanol, was of technical grade which was distillated before use. Folin Ciocalteu, Na_2CO_3, $NaNO_2$, $AlCl_3$ and NaOH were bought from Merck with pro-analytical (p.a.) grade. Meanwhile DPPH• (2,2-diphenyl-1-picrylhydrazyl) free radical, gallic acid and rutin was from SIGMA Aldrich. Nutrient Agar was from Pronadisa. All pathogenic bacteria are obtained from the collection of School of Life Science and Technology, Bandung Institute of Technology (ITB).

Extraction

About 1 g of dry leaves of *A.altilis* were put into an Erlenmeyer of 250 ml, then added 100 ml ethanol solution. Concentration of ethanol solutions were varied as to be 50, 60 and 70% (v/v). Extraction process was conducted in a shaker incubator (New Brunswick Scientific, Edison, MJ, USA) for 1, 2, 3, 4 (70°C, 100 rpm) and 24 h (room temperature, 100 rpm). The filtrate of extract was separated by filtered paper (Whatman paper No. 1) and used for further analysis of total flavonoid and phenolic content, and assay of DPPH and antibacterial activity.

Isolation of bioactive compound

50 gr of crude concentrated ethanol extract was separated into 3 main fractions, *i.e.* n-hexane, ethyl acetate and water, by liquid-liquid partition method. Ethyl acetate fraction was used for further isolation. 20 g of ethyl acetate fraction was separated into 13 sub-fractions using liquid vacuum chromatography by stepwise gradient elution of n-hexane:ethyl acetate (100:0-0:100 v/v).

Fraction 6 (1.1 gr) was separated into 22 sub-fractions, *i.e.* Fr.6.1-Fr.6.22 on silica gel column choromatography using n-hexane:ethyl acetate (70:30) as eluent. Fr.6.4 was selected for isolation via preparative thin layer chromatography using n-hexane:ethyl acetate 7:3 as eluent and P-3 (20 mg) was selected for 1H and 13C NMR identification by JEOL 500 Mhz NMR.

DPPH assay (antioxidant activity)

About 50 μl of sample were put in well, then reacted with 200 μl of DPPH. The reaction was incubated for 15 min at room temperature (RT). Absorbance was recorded at λ 517 nm using the Varioskan™ Flash Multimode Reader (Thermo Scientific).

DPPH radical scavenging activity was estimated by following formula:

$$\% \text{ Activity} = [1 - (A_{sample} / A_{control})] \times 100$$

-A_{sample}; absorbance of DPPH radical solution mixed with the sample
-$A_{control}$; absorbance of DPPH radical solution mixed with methanol solution

Vitamin C was employed as positive control.

Antibacterial activity

About 20 μl of sample aliquots was filled in well of solid Nutrient Agar which were contaminated with pathogenic bacteria, *i.e. Escherichia coli*, *Staphylococcus aureus* and non-pathogenic bacteria *Bacillus subtilis*, aseptically. The bacteria were prepared as follows: a loop of fresh strain from slant Nutrient Agar was taken and placed in 3 ml of sterile saline water. The suspension of bacterial cells should be cloudy, similar to 0.5 of McFarland turbidity standard. The incubation was performed for 24 h at 37°C. Inhibition diameter was measured after incubation process and presented as antibacterial activity.

Total flavonoid content

Determination of total flavonoid content was performed using the aluminum chloride colorimetric method with slight modification. 20 μl of sample (aliquots of extract solutions) were put into a 96-well plate. Then each well was added with 80 μl of aquadest, 6 μl of $NaNO_2$, 6 μl of AlCl3 (2% w/v in 10% acetic acid solution), 40 μl of NaOH and 48 μl of aquadest were added sequentially. The test solutions were vigorously shaken. Absorbances at maximum wavelength standard (rutin) were recorded. A standard calibration plot was generated using known concentration of rutin. The concentration of flavonoids in the test samples were calculated from the calibration plot and expressed as rutin equivalent (RE) per g of sample (dry leaves).

Total phenolic content

Determination of total phenolic content was made using Folin Ciocalteu method. 20 μl of aliquots were put in well. Then 100 μl of Folin Ciocalteu (10% v/v) and 80 μl of Na_2CO_3 7.5% (w/v) were added sequentially. The test solutions were incubated (50°C, 15 min) and then vigorously shaken. Absorbance at maximum wavelength of standard (gallic acid) was recorded. Standard calibration plot was generated using known concentration of gallic acid. The concentration of total phenolic content in the test samples were calculated from the calibration plot and expressed as gallic acid equivalent (GAE) per g of sample (dry leaves).

Statistical Analysis

Statistical analysis was performed by using one way ANOVA test and differences were considered significant at the 95% confidence level ($P < 0.05$). Data presented as the value of mean ± standard deviation (SD).

RESULTS

Isolation of one bioactive constituent, namely **compound 1**, of *A.altilis* leaves extract was succesfully performed by the use of ethyl acetate fraction. Based on its ^{1}H NMR spectra, it could be determined that **compound 1** is a flavonoid derivative (downfield chemical shift at δH 12.82 (-OH) and aromatic protons at δH 6-8). ^{13}C NMR spectra indicated that **compound 1** had 25 carbons, including 3 methyl, 4 methylene, 8 sp^2 methine, 9 sp^2 quarternary carbons, 1 sp^3 quarternary carbon (δC 78.92 ppm) and 1 keton group (δC 204 ppm). The existence of two aromatic rings with AMX system was clearly revealed from ^{1}H NMR spectra which showed the presence of resonance at 6.34 (1H, dd, *J* 2.6 ;8.45 Hz), 6.37 (1H, d, *J* 2.6 Hz), 7.56 (1H, d, *J* 8.45 Hz) and 6.34 (1H, dd, *J* 2,6;8.45 Hz). Isoprenyl group was suggested as the presence of resonance at δH 5.08 (1H, *t*, H-21), 2.08 (2H, *m*, H-20), 1.7 (2H, *m*, H-19), 1.66 (3H, *s*, H-23), 1.57 (3H, *s*, H-24). There are ortho-coupled aromatic protons at δH 6.6 (H-11, *d*, *J* 8.45 Hz) and 6.72 (H-12, *d*, *J* 8.45 Hz). The presence of a methyl group in a singlet at δH 1.38 (CH3) and two cis-olefinic protons in doublets at δH 5.63 and 6.53 (each *J* 10.35 Hz) and a quaternary carbon at δC 78.92 implied the presence of a dimethylchromene group. The 1H NMR spectrum data also indicated the presence of isoprenyl group at δH 5.08 (1H, *t*, H-21), 2.09 (2H, *m*, H-20), 1.7 (2H, *m*, H-19), 1.66 (3H, *s*, H-23), 1.57 (3H, *s*, H-24) and 1.38 (3H, *s*, H-25).

By comparing the data with a previous study of Mclean *et al* [25] (Table 1), it could be determined that **compound 1** was 1-(2,4-dihydroxyphenyl)-3-(8-hydroxy-2-methyl-2-(4-methylpent-3-en-1-yl)-2H-chromen-5-yl)propa-1-one, also known as AC-31 [26]; its molecular formula was C25H28O5 (Figure 1).

The details for bioactivity of *A.altilis* leaves extract for different extraction conditions are presented in Table 2, and the total flavonoid and phenolic content of the extract is to see in Table 3.

Table 1. NMR spectral data for **compound 1** (in $CDCl_3$, 500 Mhz)

Position	Present experiment		McLean *et al* [25]	
	^{1}H (l, m, *J* in Hz)	^{13}C	^{1}H	^{13}C
1		165.29		165.15
2	6.37 (1H, d, 2.6)	103.65	6.37 (2)	103.51
3		163.28		163.14
4	6.34 (1H, dd, 2.6; 8.45)	108.14	6.34 (8.8; 2)	107.97
5	7.56 (1H, d, 8.45)	132.44	7.55 (8.8)	132.27
6		113.75		113.59
7		204.05		203.89
8	3.08 (2H, m)	39.82	3.08 (m)	39.65
9	2.98 (2H, m)	26.63	2.97 (m)	26.49
10		128.07		127.92
11	6.62 (1H, d, 8.45)	121.32	6.61 (8.2)	121.14
12	6.73 (1H, d, 8.45)	114.69	6.73 (8.2)	114.53
13		143.19		143.02
14		139.72		139.56
15		119.19		119.03
16	6.53 (1H, d, 10.35)	119.53	6.54 (10.2)	119.36
17	5.63 (1H, d, 10.35)	130.29	5.63 (10.2)	130.12
18		78.92		78.76
19	1.70 (2H, m)	40.92	1.7 (m)	40.77
20	2.08 (2H, m)	22.96	2.08 (m)	22.8
21	5.08 (1H, t)	123.99	5.08 (7)	123.84
22		132.13		131.95
23	1.66 (3H, s)	25.83	1.65 (bs)	25.66
24	1.57 (3H, s)	17.80	1.56 (bs)	17.64
25	1.39 (s)	26.24	1.38 (s)	26.07
1-OH	12.82		12.81 (s)	
3-OH			7.2 (bs)	
13-OH			5.58 (bs)	

Table 2. Bioactivity of *A. altilis* at different condition of extraction

Condition of extraction			Bioactivity			
Ethanol concentration (% v/v)	Temperature (°C)	Time of extraction (h)	% inhibition of DPPH (antioxidant)	Inhibition diameter (mm) (antibacterial)		
				SA	*BS*	*EC*
50	70	1	83 ± 2.25	9	12	12
		2	79 ± 0.31	10	12	12
		3	77 ± 2.93	11	13	14
		4	82 ± 1.35*	11	14	13
60	70	1	79 ± 0.51	9	12	12
		2	79 ± 0.19	10	12	11
		3	77 ± 0.69	10	11	11
		4	76 ± 0.83	11	12	11
70	70	1	79 ± 0.99	11	12	13
		2	78 ± 0.19	11	10	12
		3	79 ± 1.44	10	12	12
		4	77 ± 0.44	10	11	11
50	RT	24	78 ± 2.47*	10	6**	8**
60	RT	24	77 ± 0.52	10	6**	9**
70	RT	24	76 ± 0.22	6**	6**	10**
Ascorbic acid 3 ppm			81 ± 2.35			
Gentamycin sulphate 1%				30	32	20

RT: room temperature, ***SA:*** *Staphylococcus aureus,* ***BS:*** *Bacillus subtilis,* ***EC:*** *Escherichia coli*. Values are represented as mean ± SD (n=3). *****Significantly different when compared with 1 and 4 h extractions at 70 °C; ****** significantly different when compared with all respective extractions at 70 °C (95% confidence).

Table. 3. Total flavonoid and phenolic content of *A.altilis* at different conditions of extraction

Condition of extraction			Content	
Ethanol concentration (% v/v)	Time (h)	Temperature (°C)	Total flavonoid (RE, % w/w)	Total phenol (GAE, % w/w)
50	1	70	1.6 ± 0.24	1.4 ± 0.04
	2	70	1.6 ± 0.1	1.5 ± 0.02
	3	70	1.9 ± 0.04	1.8 ± 0.1
	4	70	1.9 ± 0.09	1.8 ± 0.14
	24	RT	1.5 ± 0.23	1.5 ± 0.23
60	1	70	1.6 ± 0.06	1.5 ± 0.04
	2	70	2.1 ± 0.19	1.9 ± 0.01
	3	70	2.4 ± 0.31	2 ± 0.11
	4	70	2.3 ± 0.09	2 ± 0.07
	24	RT	1.8 ± 0.05	1.4 ± 0.04
70	1	70	2.3 ± 0.4	1.8 ± 0.03
	2	70	1.9 ± 0.04	1.6 ± 0.15
	3	70	1.7 ± 0.11	1.9 ± 0.09
	4	70	2 ± 0.18	1.8 ± 0.05
	24	RT	1.5 ± 0.1	1.3 ± 0.04

RT: room temperature; **RE:** rutin equivalent; **GAE:** gallic acid equivalent. The values presented as mean ± SD (n=3).

DISCUSSION

Antioxidant action is one of the interesting bioactivity from *Artocarpus altilis*. Several studies reported examples of bioactive constituents from *Artocarpus* species that have antioxidant activity such as artelastin, prenylated flavonoid, cyclogeracommunin, artoflavone, artomuno-isoxanthone, artelastoheterol, cycloartobiloxanthone, artonol A and others [7, 24]. **Compound 1** was known as prenylated flavonoid and presented cytotoxic activity against P-388 leukemia cells [26], antioxidant activity [27], and also exhibited a strong antidiabetic activity [28]. Its antioxidant activity was expected as hydrogen donating ability. On the other hand, the antimicrobial activity **compound 1** was proposed following the mechanism of prenylated flavonoids; the lipophilic prenyl group could rapidly damage the membrane and cell wall function of bacteria [29].

Influence of different extraction processes on the antioxidant capacity of the isolated **compound 1** in the present study is shown in Table 2. The free radical scavenging activity of all filtrates at different ethanol solution are almost similar, although varied between 76 to 83% of inhibition. It was seen that the highest antioxidant activity was found at the extraction condition of 50% v/v ethanol solution (70°C, 1 h); however, the difference is not significant and elongation of extraction time did not increased antioxidant capacity of the extract. So, we suggested to choose ethanol 70% v/v as solvent, since it is easier to be concentrated than ethanol 50% v/v.

We also tested the filtrates of *A.altilis* extract on several pathogenic bacteria (Table 2). The experiments were conducted using test Gram-negative and Gram positive organisms, namely, *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. From tabulated data of inhibition diameter (Table 2), it is clearly found that extraction of *A.altilis* leaves at room temperature at different ethanol concentrations did not give a good results; at this condition, the inhibition diameter were less than 10 mm. However, antibacterial activity of *A.altilis* extract significantly increased after heating procedure: antibacterial activity of *A.altilis* leaves extract against *E.coli* was almost similar between 70% v/v ethanol, 1 h, 70°C and 50% v/v ethanol, 4 h, 70°C. Compared to other tested bacteria, *A.altilis* leaves were found to be more sensitive against *E.coli*. Based on the above mentioned condition, we suggest that, in an effort to obtain *A.altilis* leaves extract that needs to have antibacterial activity, the extraction process should be heated in solvent for 1 h, and ethanol 70% v/v could be an appropriate solvent.

Quantification of total flavonoid and phenolic content from *A.altilis* leaves extract were presented in Table 3. The results varied between 1.3 to 2.4% w/w (calculated based on dry leaves). From the results, it could be seen that temperature plays a significant role in the extraction process of flavonoid and phenolic compounds. Total flavonoid and phenolic content of extracts which were processed at room temperature for 24 h were lower than extracts which were processed at 70 °C. The highest flavonoid content was found at temperature of 70°C after 3 h processing time by use of 60% v/v ethanol solution. However, since being almost similar, 1 h extraction time and 70% v/v ethanol solution was chosen as to be the optimal processing condition; shorter extraction process will reduce the production cost. Similarly, for total phenolic content, although the highest value of phenolic content was found at 4 h extraction process by use of 60% v/v ethanol solution, it is preferable to perform extraction by using ethanol 70% v/v for 1 h, because the yield of total phenolic content did not significantly differ. It is recommended to set the default value of the total contents of flavonoids and phenols not to be less than 1.9 and 1.8 % w/w, respectively. Regarding the explanation above, the optimal extraction process of *A.altilis* leaves could be set as using 70% ethanol solution with 1 h extraction time. In conclusion, we suggest to use ethanol 70% v/v, 70°C temperature and 1 h extraction time as appropriate condition for extraction of A. altilis leaves to provide effective activity for both antibacterial and antioxidant action from.

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