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Solvent concentration effect on total flavonoid and total phenolic contents of *Averrhoa bilimbi* leaf extract

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ABSTRACT

Averrhoa bilimbi is one of the Indonesian indigenous plants containing phenolic and flavonoids which exhibit several ethnopharmacological effects i.e. antidiabetic, anti-microbial, anti-inflammatory, cytotoxic, anti-oxidant, antifertility, and antibacterial activities. However, the optimum solvent for extracting these compounds using percolation have not been reported, yet. This research was aimed to evaluate the various concentrations of ethanol solvents towards total phenolic (TPC) and total flavonoid content (TFC). Extraction was carried out using percolation technique with ethanol solvent ratio of 50%, 70%, and 96%. The phytochemical screening, thin layer chromatography, total flavonoids, and total phenolic levels were obtained from the concentrated extract. The TPC and TFC were determined using visible spectrophotometry method. Phytochemical screening study indicated that ethanolic extract contains classes of flavonoids, phenolic, alkaloid, saponins, and steroids. The TFC of ethanolic extract with 50%, 70%, and 96% solvent concentrations were investigated at 62.74, 64.81 and 59.1 mg RTE / g extract, respectively while the TPC were recorded as follows: 103.79; 119.47; 110.10 mg GAE / g extract. Hence, 70% ethanol were higher among others, and therefore remains as optimum solvent for the extraction of *A. bilimbi* leaves.

Keywords: *Averrhoa bilimbi* L., total flavonoid content, total phenolic content

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INTRODUCTION

Traditional medicine from natural product has been known for a long time by the people of Indonesia. Although modern medicine from synthesis drugs remains as the main choice for medical treatment, natural ingredients is also taken into account as its important contribution to health. Various extracts of fruit and leaves of *Averrhoa bilimbi*, L. shows beneficial pharmacological effects viz. anti-microbial, anti-oxidant, antidiabetic, antifertility, anti-inflammatory, and cytotoxic activities. Phytochemical contents of *A. bilimbi* fruit extracts include flavonoids, saponins, tannins, and triterpenoid. Instead, the bark extracts contains saponins, flavonoids (Kumar *et al*., 2013) while the fruit and leaf parts contains procyanidins (Ramsay and Mueller-Harvey, 2016). Water extract of *A. bilimbi*'s fruit was reported to contain carbohydrates, proteins, amino acids, flavonoids, tannins, and hydrolysable tannins (Patil *et al*., 2013). In general, pharmacological profile from various fruit and leaves extracts of *A. bilimbi* have antidiabetic, antimicrobial, anti-inflammatory, cytotoxic, antioxidant, and antifertility effects (Kumar *et al*., 2013). antihypertensive, antithrombotic, hypolipidemic, wound healing, and anthelminthic (Alhassan and Ahmed, 2016).

Flavonoids is well-known as one of the largest groups of phenolic compounds (Harborne, 1998). Phenolic compounds tend to be polar because they are generally in the form of bonded sugar as glycosides (Harborne, 1998). Flavonoids have activity as anti-cancer, anti-inflammatory, hepatitis and ischemic stroke. Polyphenol have activity as antihistamine and liver disease (Lipinski, 2011). Phenols have the ability as anti-inflammatory, anticoagulant, antioxidant and immune system enhancers (Arukwe *et al*., 2012). Interestingly, a research showed the toxicity of *A. bilimbi* in which the compound of caramboxin has been isolated, explaining the mechanisms of its neurotoxicity (Caetano *et al*., 2017).

Evaluation of total phenolic content (TPC) and total flavonoids content (TFC) of *A. bilimbi* fruits was already conducted by Yan and co-workers (Yan *et al*. (2013). However, the investigation on the ratio of ethanol solvent concentration has not been studied, yet. This research is aimed at evaluating (rather *compare*) the various concentrations of ethanol (50%, 70 and 96%) towards the TPC and TFC of *A. bilimbi* leaf extract. The TFC and TPC were investigated using visible spectrophotometry methods.

MATERIALS AND METHOD

Materials

Averrhoa bilimbi leaf, ethanol 50% v/v, ethanol 70% v/v, ethanol 96% v/v, Mg, HCl, amyl alcohol, $FeCl_3$, ammoniac, $CHCl_3$, dragendorff's reagent, ether, H_2SO_4, silica gel 254 F, butanol, acetic acid, hexane, ethyl acetic, methanol, $AlCl_3$, rutin, Folin-Ciocalteu, Na_2CO_3, galic acid, rotary evaporator (Shimadzu®), water bath, and spectrophotometer UV-Visible (Shimadzu®) type 1240.

Methods

Plant and extraction

The leaves of *A. bilimbi* were collected from Semarang, Central Java on August 2017. The plants were then identified by a taxonomic botanist in the Botanical Pharmacy Laboratory STIFAR "Yayasan Pharmasi Semarang". The two kg of fresh *A. bilimbi* leaf were dried in a drying cabinet, then sprayed with a mesh sieve 60. A total of 60 g of dry leaf powder added by 200 mL of ethanol for each solvent concentration (50%, 70%, and 96%). The ethanolic extract were subsequently moved to the percolator and soaked for 24 hours. Afterwards, the percolator faucet was opened and allowed to drop until the percolate is obtained. A new solvent was continuously added to the percolator. The percolate was evaporated using a rotary evaporator at 50°C on 100 RPM in order to obtain the viscous extract. The rendemen of *A. bilimbi* leaf extract was calculated using following formula.

$$Rendemen = \frac{\text{Weight of the extract}}{\text{Weight of } A.\ bilimbi\ dry \text{ leaves}} x\ 100\%$$

Phytochemical screening

Preliminary screening of secondary metabolites such as flavonoid, phenolic, alkaloid, and steroid were performed according to the methodology developed by Aiyegoro and Okoh (Aiyegoro and Okoh (2010), while the screening of saponin was carried out according to Harborne (Harborne (1998) as the common phytochemical methods.

Thin Layer Chromatography

Thin layer chromatography (TLC) was performed using standard methods (Harborne, 1998) with modification.

Total Flavonoid Content

The TFC were measured by colorimetric assay (Chang *et al*., 2002) with modification. Rutin was used to produce calibration curve. Ten mg of Rutin was dissolved in methanol and subsequently diluted to 20, 40, 60, 80, and 100 µg/mL. Rutin as standard solution (0.5 mL) were separately mixed with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 413 nm wavelength with a spectrophotometer UV-Visible Shimadzu type 1240. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. The TFC of the extracts were expressed as mg Rutin equivalents (RTE) per-gram of sample (mg/g).

Total Phenolic Content

The method used for determination of TPC using Follin-Ciocalteu reagent was adapted from Mc Donald and co-workers (McDonald *et al*., 2001) with modification. Herein, gallic acid was used to make the calibration curve. The 25 mg of gallic acid was dissolved in methanol and then diluted to 100, 200, 300, 400, and 500 µg/mL. Gallic acid as standard solution (0.5 mL) were separately mixed with 0.4 mL Folin Ciocalteu reagent, incubated at room temperature 4-8 min, then added with 4 mL $NaCO_3$ 7% and 10 mL of distilled water. The TPC were determined at 770 nm colorimetric wavelength. The TPC were expressed as mg gallic acid equivalents (GAE) per gram of sample (mg/g).

Data Analysis

Data obtained from the absorbance value of each sample was then plotted into the standard Rutin (TFC) or the gallic acid (TPC) and then calculated by the formula,

$$\text{TFC or TPC} = \frac{\text{c x V}}{\text{m}}$$

Where c is concentration from calibration curve, V is volume of the extract, and m is the mass of the extract. The data analysis was carried out using IBM Statistic SPSS 23 methods.

Result and Discussion

Extraction yield

Extract of *A. bilimbi* leaf obtained from the extraction process by percolation method using percolator. The principle of the percolation method is the release of the compounds by flowing the liquid through the wetted of *A. bilimbi* powder, penetration the cell wall by liquid, the dissolved of active substance due to the difference of concentration between the active substance solution inside

and outside the cell, and lastly the solution with high concentration is pushed out of the cell. The extract was concentrated using a rotary evaporator at 50° C in order to obtain the viscous extract. The results of yielded ethanol leaf extract of *A. bilimbi* are described in Table I.

Table I. The result of yielded *A. bilimbi* extract

Solvent (%)	Weight Extract (g)	Weight of dry leaf (g)	Rendemen (%)
Ethanol 50	60	14.4	24
Ethanol 70	60	25.2	42
Ethanol 96	60	13.1	21.83

Based on the above results, the solvent which has the highest effectiveness in extracting the compound is 70% ethanol. The effectiveness of extraction may be influenced by the type and amount of the solvent, powder size, extraction method, extraction time, and solvent concentration.

The rendemen of extraction by various aqueous ethanol solvents is indicated as follows: 70% > 50% > 96%. It shows that the randemen using 70% ethanol (42% yield) remains higher than that of 50 % (24% yield) and 96% (21.83% yield). The water and organic solvents may facilitate the extraction of desired compounds which are soluble in aqueous and/or organic solvent. These results are in a well agreement with the previous similar study (Abdillah *et al*., 2015) confirming the use of 70% ethanol. The use of this solvent allows to produce high-level yields of the extraction owing to its similar polarity with most of the components in the plant.

The 70% ethanol solvent can remarkably dilute phytochemical compounds since the solvent contained optimum water content (30%) that could help in the extraction process. In this way, most of the compounds would be attracted in the ethanol and some in the water.

Phytochemical screening

Phytochemical screening is an early tool to identify the chemical content in the extract. In this study, phytochemical screening includes identification of flavonoid, phenolic compounds, alkaloid, saponin, and steroid contents. The results are presented in Table II.

Table II. Phytochemical screening result of *A. bilimbi* extract

Compounds	Solvent concentrations			Observations
	50%	70%	96%	
Flavonoids	+	+	+	The occurrence of red or orange colorations
Phenolic (tannin)	+	+	+	A blue coloration resulting in the addition of ferric chloride
Alkaloids	+	+	+	Orange-brown spots on a yellow background with Dragendroff reagent
Saponins	+	+	+	A persistent foam is formed above the liquid surface
Steroids	+	+	+	A reddish-brown color at the interface

Thin Layer Chromatography

TLC is the modest among other chromatography methods. TLC method uses the silica gel 254 F as the stationary phase and mixture of several organic solvents with various comparisons as the mobile phase. The plate is then inserted into a vessel containing a predetermined solvent which will be absorbed inside the plate and separate the compounds mixture based on their polarity. The results are shown in Table III below.

Table III. The results of TLC identification

Compounds	Solvent Conc. (Ethanol)	Mobile Phase	Retardation Factor		Observations
Alkaloids	50%	Methanol-Ammonium hydroxide (200:3)	(-)		(+) Orange-brown spots on a yellow background with Dragendroff reagent (Harborne, 1998)
	70%		(+) (Rf = 0.84)		
	96%		(-)		
Flavonoids	50%	n-Butanol-Acetic acid-water (4:1:5)	(+) (Rf_1 = 0.16) (Rf_2 = 0.88)		(+) Bright yellow (Harborne, 1998)
	70%		(+) (Rf_1 = 0.15) (Rf_2 = 0.88)		
	96%		(+) (Rf = 0.17)		
Saponins	50%	Chloroform-methanol-water (13:7 :2)	(+) (Rf_1 = 0.50) (Rf_2 = 0.86)		(+) Pink to purple spots (Harborne, 1998)
	70%		(+) (Rf = 0.88)		
	96%		(+) (Rf = 0.89)		
Phenolic	50%	Acetic acid-chloroform (1:9)	(+) (Rf_1 = 0.16) (Rf_2 = 0.95)		(+) Blue spots (Harborne, 1998)
	70%		(+) (Rf = 0.11) (Rf = 0.89)		
	96%		(+) (Rf_1 = 0.11) (Rf_2 = 0.93)		
Steroids	50%	hexane-ether (97:3)	(-)		(+) Orange color with a green fluorescence in UV (Harborne, 1998)
	70%		(+) (Rf_1 = 0.72) (Rf_2 = 0.92)		
	96%		(+) (Rf = 0.57) (Rf_2 = 0.82) (Rf_3 = 0.88) (Rf_4 = 0.89) (Rf_5 = 0.93) (Rf_6 = 0.96)		

Total Flavonoid Content (TFC)

TFC and TPC of *A. bilimbi* extract were measured using visible spectrophotometric method. The results are presented in Table IV.

Table IV. TFC and TPC of *A. bilimbi* extract

Ethanol extract	TFC (mg RTE/g) ± SD	TPC (mg GAE/g) ± SD
50%	62.74 ± 1.16 [a]	103.79 ± 3.19 [c, d]
70%	64.81 ± 1.85 [b]	119.47 ± 1.76 [c, e]
96%	59.10 ± 0.33 [a, b]	110.10 ± 1.73 [d, e]

GAE = gallic acid equivalent; RTE = rutin equivalent; TFC = total flavonoid content; TPC = total phenol content

Values were expressed as mean ± standard deviation (n = 3)

[(a-e)] indicate significant difference from one another ($p > 0.05$).

Flavonoids are probably the most important phenolic compounds. All of these compounds possess a broad spectrum of chemical and biological activities (Al-Matani *et al*., 2015) including hepatoprotective (Nurkhasanah and Rahardhian, 2015), free radical scavenging capacity, anti-inflammatory, coronary heart disease prevention, and cytotoxic activities (Kumar and Pandey, 2013), inflammatory bowel disease (Vezza *et al*., 2016), upper respiratory tract infections and immune function (Somerville *et al*., 2016). TFC was determined by $AlCl_3$ colorimetric method (Rohman *et al*., 2010) based on the formation of color complex quantified by visible spectrophotometry. The formation of complex is between $AlCl_3$ and keto groups at C-4 and hydroxyl groups at C-3 or C-4 neighboring from flavones and flavonols. The total flavonoid content is expressed with RTE value comprising the number of mg equivalent of Rutin in gram of sample.

The operating time of Rutin was found at 35 min with the maximum wavelength (λ max) of 413 nm. Generally, the standard curve shows the linear relationship between absorbance and standard concentrations. Here, the linear regression equation was obtained as $y = 0.0028x + 0.0352$ with $R^2 = 0.9846$.

The result show that extract with 70% ethanol yielded the highest TFC (64,8 mg RTE/g extract whereas the lowest obtained from 96% ethanol extract (59,1 mg RTE/g extract). The yield of TFC presented in the following order: 70% > 50% > 96%. The TFC in the 75% ethanol extract is higher than 100% and 50% ethanol. This may be attributable to the higher solubility of proteins and carbohydrates in 70% ethanol in which contains 30% water. The combined use of aqueous and organic solvent may facilitate the extraction of chemicals that are soluble in water and/or organic solvent. The results of this study are in agreement with the TFC of *Limnophila aromatica* (Do *et al*., 2014), *Ananas comosus* and *Musa paradasiaca* (Alothman *et al*., 2009).

Total phenolic content

Phenolic have multiple biological properties such as anti-inflammatory, antioxidant, chemopreventive (Servili *et al*., 2013), neuroprotective (Szwajgier *et al*., 2017), and antimicrobial (Marcucci *et al*., 2001). Determination of TPC is commonly performed by using Folin-Ciocalteu reagent. This method is based on the reducing strength of the phenolic hydroxyl group. The presence of aromatic nuclei in phenol compounds (phenolic hydroxyl groups) can reduce phosphorus phosphotungstate into molybdenum blue (Sudjadi and Rohman, 2004). The total phenolic content in the plant is expressed in GAE corresponding the number of mg equivalent of gallic acid in gram of sample (McDonald *et al*., 2001).

The results obtained the standard operating time at 2 hours with the λ max of 770 nm. The linear regression equation was acquired at $y = 0.0006x + 0.0043$ with $R^2 = 0,9862$. Table III show

that 70% ethanol extract yielded the highest TPC (119,5 mg GAE/100 g extract) whereas the lowest amount obtained from 50% ethanol extract (103,8 mg RTE/100 g extract). The yield of TPC was found to be 70%> 96% > 50%. Instead, it may also be triggered by the possible complex formation of some phenolic compounds in the extract that are soluble in 70% ethanol. These phenolic compounds may possess more phenol groups or have higher molecular weights than the phenolics in the water extract (Do *et al*., 2014). According to this result, the best extraction solvent was 70% ethanol. Here, we note that level of phenolic content of this study remains higher than the previous study reported by (Hasanuzzaman *et al*., 2013). However, the results are in agreement with the TFC of pineapple *Ananas comosus* (Alothman *et al*., 2009). Polyphenols recovery from plant materials is affected by the solubility of the phenolic compounds in the solvent during extraction process. Further, polarity of solvent will important role in increasing phenolic solubility (Naczk and Shahidi, 2006). Commonly, the less polar solvents are considered to be appropiate for the extraction of lipophilic phenols unless very high pressure is used. This is due to the wide range of phenols which the aqueous ethanol mixtures can dissolve (Alothman *et al*., 2009).

Industrially, the economical feasibility of the extraction process includes the search for the optimal extraction in order to amplify the the process of efficiency. In this study, 70% ethanol exhibit the highest TPC and TFC towards extracts. These results could be the first step for the implementation of large scale process, being a satisfactory starting point for further studies in regards of the optimization of continuous process, as of it is the major interest from industrial point of view.

CONCLUSION

The 70% of ethanolic solvent was the optimum solvent concentration based on total flavonoid and total phenolic content for the extraction of *A. bilimbi* leaves.

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