



Effect of Different Solvent on Total Phenolic, Total Flavonoid, and Sun Protection Factor of Belimbing Wuluh (*Averrhoa Bilimbi linn.*) Fruits Fraction

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Abstract

Ultraviolet (UV) radiation is related with a variety of damaging effects. *Averrhoa bilimbi* Linn (AB) known as Belimbing Wuluh in Indonesia, it contains phenolic compounds such as flavonoids, where the compound has the potential as a sunscreen. Objective: the study was to determine the Total Phenolic Content (TPC), Total Flavonoid Content (TFC) and investigate Sun Protection Factor (SPF) activity from different solvent for fractionation of AB among N Hexane (NH), Ethyl Acetate (EA), and Water. Methods: TPC, TFC, and Sun SPF were investigated using in vitro assay with spectrophotometric UV-Vis coupled chemometric using principal component analysis (PCA). In this study, TPC was determined using Folin-Ciocalteu reagent, the outcomes were expressed in (mg of gallic acid equivalent (GAE)/g sample), TFC content was determined by colorimetric using AlCl₃ methods, the results were expressed in (mg of rutin equivalent (RE)/g sample), and SPF using Dutra calculation. Result: EA showed the highest SPF (15, 38±1, 3). The similar fraction having the highest TPC (56, 87±1, 5212) mg (RE)/g of EA) and the highest TFC (24, 99±3, 2895) mg (GAE)/g of EA). When principal component analysis (PCA) was applied, high correlation among TFC and SPF activities was found. Conclusion: Hence, AB potential as sun screens.

Keywords: *Averrhoa bilimbi*, Total Flavonoid Content, Total Phenolic Content, Sun Protection Factor, Chemometric.

Introduction

Excessive sun exposure can cause skin like sun burn, ageing, pigmentation, wrinkles, dermatitis, immune suppression and skin cancer [1]. UVB in wavelength (280-320 nm) associated with erythema, however UVA in wavelength (320-400 nm) is makes

photoaging, tanning, and DNA damage [2]. Since 1928 and present, sunscreens play a key mechanism in sun protection and prevention of skin cancer [2]. Sunscreen commerce firstly focused on growing the sun protection factor (SPF) [2].

Used of sunscreen in the market is expanding, not only for the skin but also developing for the lips, eyes and even oral preparations are also available to protect the skin. The most sunscreen comes from chemicals, such as octocrylene, cinnamates, benzophenones, and PABA derivatives notice to chronic or acute allergic symptoms [1]. Therefore it is necessary to develop sunscreens that are safe from natural product such as *Averrhoa bilimbi* Linn (AB).

AB also called *Belimbing Wuluh* in Indonesia, It is also common in other Southeast Asian countries [3]. AB contains phenolic like flavonoids compounds, natural chemical like polyphenols as flavonoids, are more actual than synthetic chemicals which is owing to their long-term useful effects mainly counter to free radical produced skin harmful along with UV-rays blocking [4]. Chemometric methods using Principal component analysis (PCA) make overview of the inter-relationships between TPC, TFC, and antioxidant [5]. Previous research on AB with total flavonoids and total phenolic content has been investigated by [4].

Therefore, no reports are available to effect of different solvent for fractionation on TPC, TFC, SPF and combination with chemometric using Principal component analysis (PCA). The aims of this research to determine of TPC, TFC, and SPF activity from AB Fruits Fraction. PCA gave the impression of the inter-relationships between TPC, TFC and SPF activity of AB.

Materials and Methods

Materials and chemicals

The materials were used Ethanol, Ethyl acetate, N Hexane, distillate water, Methanol, Folin-Ciocalteu's, Natrium

Carbonate, aluminium chloride, Natrium Acetate, Gallic acid and Rutin by Sigma-Aldrich (St. Louis, MO, USA). UV- Vis spectrophotometer (Shimadzu UV-1280, Japan), Rotary evaporator (Heidolph, Germany).

Extraction and Fractionation of *Averrhoa Bilimbi*

Fresh of AB fruits were collected from Ketileng, Semarang, Indonesia. Determination by a botanist at laboratory of pharmaceutical biology at Semarang Pharmaceutical Collage (Stifar). 300 g dried/powdered fruits of AB, remaserated by 96% ethanol solvent for 5 day, evaporated at 60°C at 100 rpm using rotary evaporator (Heidolph, Germany) until it is obtained viscous extract. For fractionation, as much as 10 g viscous extract of AB dissolve with water and n hexane (NH) each 50 mL on separating funnel, separate NH phase and then add ethyl acetate (EA) 50 mL in the water phase, separate the EA phase with the water phase, evaporate fraction of NH, EA and water until obtained viscous fractions.

Determination of Total Flavonoid Content (TFC)

TFC was determined according to [6] [7] with modification, rutin as standard reference with concentration 50-400 µg/mL and sample of AB fraction made concentration 1000 µg/mL. 0,5 ml standard reference was included in 5,0 mL volumetric flask. Basic of method is correlated formation complex between AlCl₃ and flavonoids make a yellow solution. Added 1,5 mL methanol, 0,1 mL AlCl₃ 10 %, 0,1 mL NaNO₂, and 2,5 mL distillate water, incubated for 30 minutes (Operating Time) in room temperature and measured λ max 415 nm using a UV- Vis spectrophotometer.

Do it with same method with sample (triplet). TFC was Expressed in gram rutin equivalent (RE) an mg rutin equivalent each g of sample.

Determination of Total Phenolic Content (TPC)

TPC was determined according to [6] with modification. Gallic acid as standard reference with concentration 20-140 µg/mL and sample of AB fraction made concentration 1000 µg/mL. 0,5 ml standard reference were into 10 mL volumetric flask, than added with 0,4 mL Folin-Ciocalteu incubated for 4-8 minutes, 4.0 mL 7 % Natrium Carbonate. Then, added with distilled water, incubated at room temperature (Operating Time) 120 minute and measure at λ max 718 nm using Uv Vis

Spectrophotometry. Do it with same method with sample (triplet). TPC was expressed in (GAE) Gallic acid equivalent [8] a mg Gallic acid equivalent each g of sample. Calculate to TPC and TFC, following formula

$$\text{TPC or TFC} = c \times V / m$$

Where c= concentration from calibration curve

V= Volume of the fraction used

m= Mass of the fraction used

Determination of Sun Protection Factor (SPF)

SPF was determined according [9] methods with modification. 300 µg/mL of fraction NH, EA, and water diluted with ethanol. With interval 5 nm increment using 1 cm quartz cell, and ethanol as a blank. Measure at 290-320 nm. Calculate average of three determinations and calculate SPF by [9] equation. EE* I values are constant [10] and given in Table 1.

$$\text{SPF (Spectrophotometric)} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where: EE (λ) = erythemal effect spectrum; I (λ) = solar intensity spectrum; Abs (λ) = absorbance of sunscreen product; CF= correction factor (=10).

Chemo Metrics

The chemometrics analyses were performed using the software Minitab® 18.1 © 2017 Minitab, Inc. Classification of relationships among TPC, TFC, and SPF were carried out using principal component analysis (PCA).

Result and Discussion

Determination of Total Flavonoid Content (TFC)

Total Flavonoid Content (TFC) was analyzed using ALCl₃ colorimetric assay. Flavonoids the most compound of plant phenolic. The absorbances of series concentrations to linear calibration curve of rutin Y=0.0026x + 0.018.

TFC of AB equivalents were found to be

highest in EA (56,87) followed by NH (29,87) and water fraction (16,60). According to [11] high (>500 mg GAE/100 g), medium (100–500 mg GAE/100 g) and low (<100 mg GAE/100 g). If converted in mg/g shown high (>5 mg GAE/g), medium (1–5 mg GAE/g) and low (<1 mg GAE/g), All tested samples in this research was classified as a high category. Effect of solvent type on the fraction was studied by fixing the n hexane, ethyl acetate and water fraction.

Results in Table 2 show, semi polar solvent like ethyl acetate is preferable for flavonoid from A. Bilimbi. Semi polar solvents can absorb flavonoids of poly hydroxy and poly methoxy types, so that in this study the largest total flavonoid content was found in

ethyl acetate solvents. Then followed by lower polarity such as n-hexane can absorb flavonoid poly methoxy types, and the lowest total flavonoid is water fraction. Further research is needed to find out what types of flavonoids are found in ethyl acetate, n hexane and water fractions. Ethyl acetate may facilitate the fraction of chemicals that are soluble in polar and/or non-polar solvent. This may be the reason why yields of TFC higher than another fraction.

The spectrophotometric assay based on $AlCl_3$ formation is one of the record generally used procedure for the total flavonoid determination, as the content of these compounds is measured as an significant parameter for estimating food or medicinal plant samples [12]. The process which involves the measurement at 410-430 nm after adding of $AlCl_3$ solution is careful only for flavones, flavanols and luteolin.

The process in the attendance of $NaNO_2$ in alkaline medium seems to be specific for rutin, catechins and luteolin, but also phenolic acids exhibition extensive absorbance at 510 nm. Application of both events to natural samples provided different order in terms of flavonoid content. Hence, the expression TFC is not satisfactory as the results of both methods are reliant on the structure of the specific flavonoids present [12]. The frequent uses and belongings of flavonoids can be used to develop the field of topically useful sun protection.

The polyphenols and flavonoids studied are respectable candidates for use in photoprotective products [13] Determination of Total Phenolic Content (TPC) - TPC was determinate using Folin-Ciocalteu method with gallic acid as standard. The absorbances of series concentrations to

linear calibration curve of gallic acid $Y=0.0054x+0.0325$. Plant products a great number of phenolic compounds with some biological actions [12]. Phenolics such as phenolic acids, flavonoids and tannins are considered to be the major sponsor to the sunscreen ability of plants [1]. Phenolic compounds contribute obviously to sunscreen action and they establish the major class of natural sunscreen present in plants.

Consequently, it is needed to determinate total phenolic content in plant species. In current study, using Folin-Ciocalteu method we create different response from three different samples in various solvent for each which were quantitatively. expressed as mg GAE/g of sample. Here, $NaCO_3$ products blue colour of phosphormolybdicphosphor tungstic phenol complex. Hence, most concentrated blue colour contains highest total phenolic content [14].

Current studies indicate the presence of polyphenolic compound in n hexane, ethyl acetate and water fraction. Phenolic compounds are generally found in equally inedible and edible plants and have been noted to have broader biological effects, including sunscreens activity. It has been reported that compounds such as phenolics, flavonoids, which contain hydroxyls, are responsible for the sunscreen activity. The highest TPC was obtained in the ethyl acetate fraction, followed by the water fraction, and the least is n hexane fraction.

This may be attributable to the containing of extra nonphenolic compounds such as terpene and carbohydrate in water fraction than in ethyl acetate fraction. It may also be caused by the likely complex formation of approximately phenolic compounds in the fraction that are soluble in ethyl acetate, n

hexane, and water. These phenolic compounds may possess more phenol groups or have higher molecular weights than the phenolics in the water solvent [14]. Based on the results of TPC, the best fraction solvent was ethyl acetate. Determination of Sun Protection Factor (SPF) - In the present research, SPF was determined according the methods of [9] with modification. SPF were investigated using *in vitro* assay with spectrophotometric UV-Vis, with interval 5 nm increment using 1 cm quartz cell, and ethanol as a blank.

Result of SPF can be seen in Table 2. (AB) contain many secondary metabolite compounds such as flavonoids and other phenolics that are thought to function as sunscreen active ingredients. The determination of the effectiveness of sunscreen is done by determining SPF values *in vitro* with spectrophotometer [9].

The absorption of the sample solution shows the effect of the active ingredient absorbing or reflecting UV light in the solution. Ethyl acetate fraction, with concentration was similar to the commercial sunscreen (SPF-15). Followed by n hexane fraction and the last water fraction. The SPF value linear with Total Flavonoid Content.

Topical application of flavonoids from caper shows effective inhibition of erythema on the healthy human [15]. The significant secondary metabolites act as UV blockers with phenolic acids and flavonoid. and three flavonoids (kaempferol, quercetin, and galanin), which are identified to be effective have a high rate of UV absorption [15]. According to [16] plant extracts rich in flavonoids are capable of absorbing ultraviolet light in the UV-B and UV-A regions.

Flavonoids included organic chemical, organic chemicals absorb UV energy through their conjugated aromatic ring structures. Upon UV exposure, electrons of these compounds are energized and jump to an excited and unstable state. In time, the electrons back to their ground and stable state and release the energy in the form of heat[15].

Evaluation of a sunscreen is usually expressed by the sun protection factor (SPF) [17], which is definite as the Ultraviolet (UV) energy required to produce a minimal erythema dose (MED) on protected skin, divided by the UV energy required to produce a MED on unprotected skin [18].

The MED is definite as the lowest time interval or dosage of UV light irradiation adequate to produce a minimal, observable erythema on unprotected the skin [19]. The higher of SPF, the more effective in preventing sunburn. Photoprotection can be determined *in vivo* or *in vitro*.

The *in vitro* methods divided into two types. The first methods is the transmission of UV energy through sunscreen product films in bio membranes or quartz plates, and the second methods in which the absorption appearances of the sunscreens are determined based on spectrophotometric analysis [1].

The UV spectrophotometric method is rapid, simple, employs low cost reagents and can be used in the *in vitro* determination of SPF values in many samples and can give important information before *in vivo* tests. The effect that different solvents have upon the wavelength of maximum absorbance and upon the UV absorbance of some sunscreen chemical, single or in mixture are well

known and known different solvent can also produce UV absorption groups, therefore interfering with those of UVA and UVB sunscreen. This outcome is reflected exclusively for with an SPF greater than 15. The higher SPF values are more problematic to measure. A high SPF generally leads to a greater uncertainty also in the final in vivo effect, due to the biological differences of the volunteers [18]. Chemometric - principal component analysis (PCA) are well known projection methods for analysis of multivariate data.

PCA can be used in the analysis to determine the relationship of activity to natural product [5]. In the results of the study (Table 3), it appears that the eigenvalue is = 3, equal to the number of variables used in PCA analysis. Here are the values that contain variable contributions for each component, the greater the coefficient value, the greater the contribution of the variable to the PCA value. Eigenvalue shows the number of variations related to a factor.

Table 1: Calculation of SPF used normalized product function

Wavelengths λ in nm	EE x I (Normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
	Total 1

Table 2: Result Total Phenolic Content (TPC); Total Flavonoid Content (TFC); Gallic Acid Equivalent (GAE); Rutin Equivalent (RE); Value is given as mean \pm SD of three independent experiment in triplicate

Fraction	TFC mg (GAE)/g of sample	TPC mg (RE)/g of sample	SPF
NH	29,87 \pm 0,59	7,55 \pm 0,70	4,51 \pm 0,80
EA	56,87 \pm 1,52	24,99 \pm 3,29	15,39 \pm 1,33
Water	16,6 \pm 0,60	20,24 \pm 1,08	1,97 \pm 0,08

Table 3: Eigenvector of TFC, TPC, and SPF

Variable	PC1	PC2	PC3
TFC	0,613	-0,420	-0,670
TPC	0,466	0,876	-0,122
SPF	0,638	-0,238	0,732

Each variable has an Eigen value of ≥ 1 so that the factor whose Eigenvalue < 1 is not used. from the data obtained, the value of eigen value used is PC1. With Scree plots

(Fig.1), it is possible to do visual assessment of the importance of each component. a scree plot is a graph that shows the relation between a factor and its Eigen value. The

form of a scree plot is used to determine the number of factors taken. In general, the limit of the number of factors taken is marked by a very sharp slope between factors one with the next factor. The loading plot (Fig.2) indicates the similarities and correlations between elements.

The elements with small loadings located near the origin have only little influence on data structure, whereas the elements with high loadings represent those elements with the greatest influence on the grouping and separation of plant samples [20]. A loading plot shows how strongly each characteristic influences a principal component. Angles between vectors show how these variables correlate with each other. If two vectors are close to one another which form a narrow

angle, it shows a positive correlation between the two variables.

For example, TFC with SPF, TPC with SPF, and TPC with TFC are positively correlated, but the biggest correlation is TFC with SPF because the angle between the two is the smallest. If between variables form an angle close to 90 degrees, then they are not correlated.

If the variable vector is scattered and forms an angle close to 180 degrees, it shows that both are negatively correlated. Scores of plots (Fig. 3) are also called latent variables because the collected samples that have the value of the plot score given have the same physical chemical properties. Samples that have high similarities will have PC1 and PC2 score values that are similar.

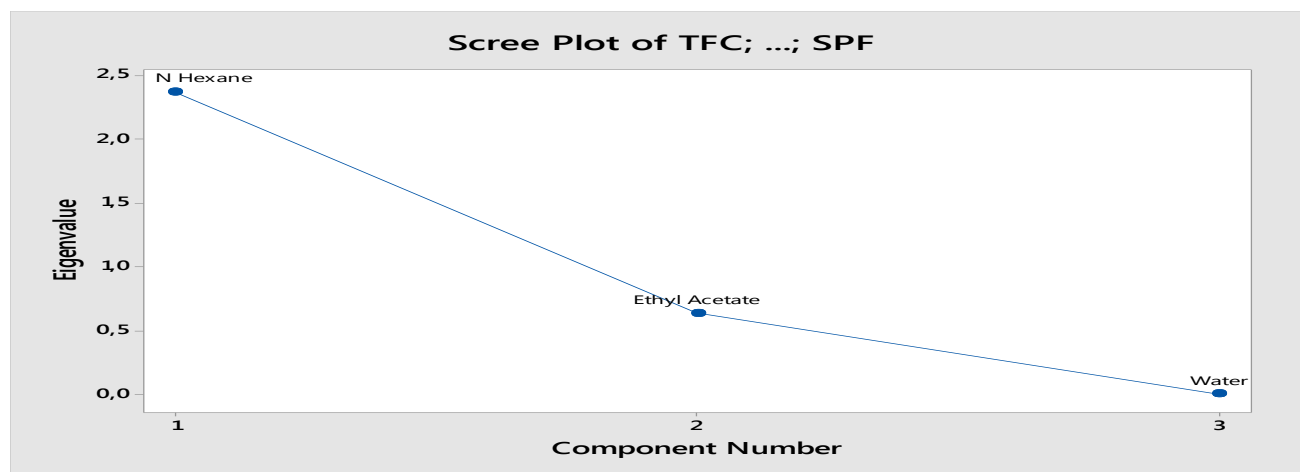


Fig. 1: A Scree plots of TFC, TPC, and SPF

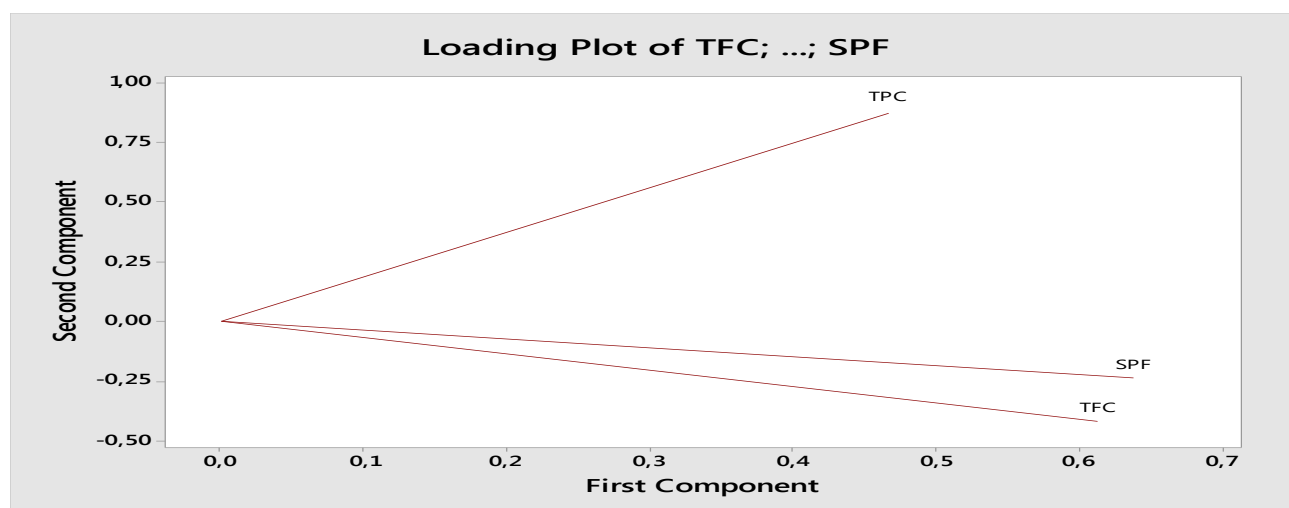


Fig. 2: A Loading plots of TFC, TPC, and SPF

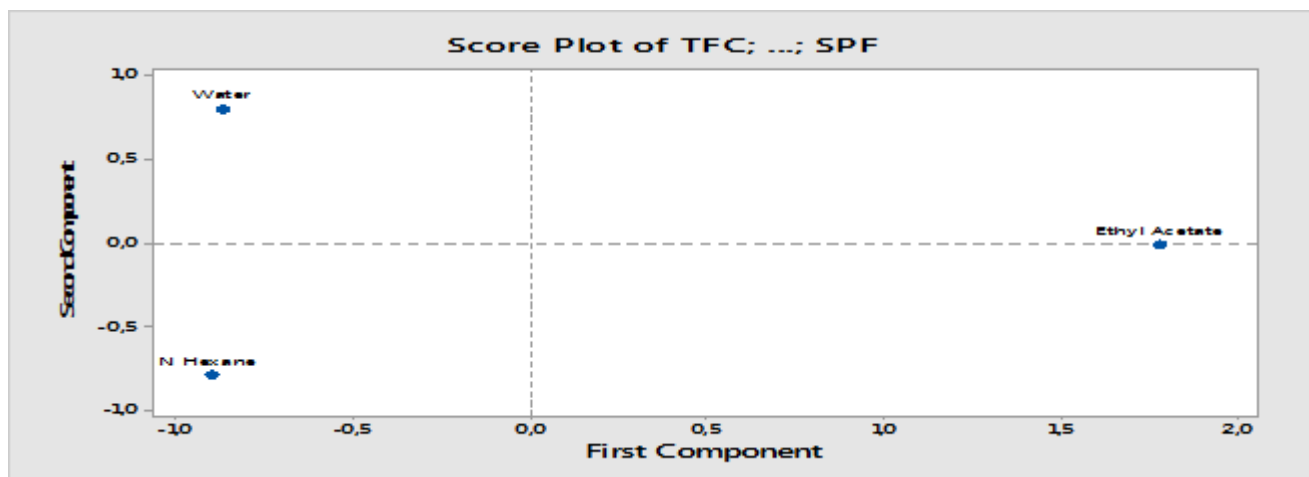


Fig. 3: A Score plots of TFC, TPC, and SPF

Conclusion

The significant linear association was established between the values for the total phenolic content, total flavonoid content and SPF activity of ethyl acetate fraction. The high contents of phenolic and flavonoids compounds showed that these compounds contribute to the sunscreen activity. Chemometric analysis such as PCA clearly showed impression of the inter-relationships

between TPC, TFC and SPF activity of AB. The ethyl acetate fraction of AB can be observed as talented candidates for natural product sources of sunscreens.

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