9.ISOLATION, IDENTIFICATION, AND QUANTIFICATION OF MAJOR FLAVONOID IN LEAVES OF PERESKIA BLEO (KUNTH) DC

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Submission date: 22-Nov-2023 09:46AM (UTC+0700)
Submission ID: 2235755325
File name: 2022.IJAP_P.bleo.pdf (316.82K)
Word count: 3228
Character count: 17373

International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 14, Special Issue 4, 2022

Original Article

ISOLATION, IDENTIFICATION, AND QUANTIFICATION OF MAJOR FLAVONOID IN LEAVES OF *PERESKIA BLEO* (KUNTH) DC

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Received: 14 Jul 2022, Revised and Accepted: 20 Aug 2022

ABSTRACT

Objective: This study aimed to isolate, identify, and quantify major flavonoid in *P. bleo* leaves from West Java Regency, Indonesia.

Methods: Isolation began with maceration, followed by liquid-liquid extraction and various chromatographic separations. Identification of isolates was carried out using 2D TLC and shifting reagents. Quantification was determined with the colorimetric method.

Results: The results showed that extract, ethyl acetate, and distilled water fractions contain flavonoids. The flavonoid screening showed positive results for flavonols. The vacuum liquid chromatography separated 21 sub-fractions; the 12th and 13th sub-fractions were predicted to contain flavonols.

Conclusion: The novelty of this study was the isolation and identification of the major flavonoid of *P. bleo* leaves originating from West Java Regency, Indonesia, i.e. catechin. The content of the compound was 3.795±0.096 g QE/ml with a purity of 94.89%.

Keywords: Catechin, Chromatographic separations, Flavonol, Shifting reagent, Purity

DOI: https://dx.doi.org/10.22159/ijap.2022.v14s4.PP19 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

INTRODUCTION

Seven-star needle (*Pereskia bleo* (Kunth) DC) is a native plant of Brazil, Mexico, the United States and Central America. This plant has been cultivated in many tropical and subtropical countries, such as India, Malaysia, Singapore, and Indonesia. *P. bleo* contains alkaloids, flavonoids, phenolics, terpenoids, and steroids [1].

Plant phytoconstituents are currently used to prevent various diseases or as herbal medicines [2]. Flavonoids are widely distributed in nature. These compounds have antiviral, antibacterial, anti-inflammatory, cardioprotective, antidiabetic, anticancer, anti-aging, and anti-oxidant activities [3, 4]. This study is the first report of isolated flavonoid from *P. bleo* leaves. This study aimed to isolate, identify, and quantify the main flavonoids in *P. bleo* leaves. The structure of the isolated compound was important to determine the mechanism of action of the biological activity which produced by *P. bleo* leaves. Isolation began with maceration, followed by liquid-liquid extraction, vacuum liquid chromatography (VLC), and preparative thin layer chromatography (TLC) techniques. Identification of isolates was carried out using 2D TLC and shear reagents, then compared with a spectrum database. Quantification was determined based on Indonesian Herbal Pharmacopoeia with the colorimetric method using aluminum chloride.

MATERIALS AND METHODS

Materials

All chemicals were analytical grade and purchased from Merck (Germany), i.e. ethanol, methanol, acetic acid, hydrochloride acid, aluminum chloride, sodium hydroxide, boric acid, *n*-hexane, ethyl acetate, sodium acetate, ferric chloride, and magnesium powder. While quercetin was purchased from Sigma Aldrich (USA). Silica gel (G) 60F and silica gel F254 were purchased from Merck KGaA (Germany).

Plant collection and preparation

P. bleo leaves were collected from Manoko Experimental Garden, West Java, Indonesia (latitude-6.8087; longitude 107.6141). The plant was identified by Plant Taxonomy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Indonesia, with No. 32/HB/01/2021. *P. bleo* leaves were washed, then dried, and weighed. Water content of dried leaves was determined based on Indonesian Herbal Pharmacopoeia [5].

Extraction and fractionation

Dried leaves were powdered and macerated with 70% ethanol for 24 h, stirred occasionally. Every 24 h, ethanol was changed. All extract was collected and concentrated using a rotary evaporator, then the yield was calculated. Extract was dissolved in hot water, then extracted with *n*-hexane, with a ratio 1:1. Then, the *n*-hexane fraction was collected. The water fraction was extracted with ethyl acetate, with a ratio 1:1. All fractions were concentrated using a rotary evaporator, then the yield was calculated [6].

Flavonoid screening

Dried leaves, extract, and all fractions were dissolved in hot water and boiled for 5 min, then filtered. All filtrates were added with ferric chloride solution, Shinoda reagent, and acetic acid solution. The color changes were observed [7]. Extracts and fractions were monitored using TLC to select the further fraction for the isolation step. The mobile phase was *n*-butanol: acetic acid: water (2:2:6) with quercetin as standard. Plates were visualized directly after drying and under UV at 366 nm. The Rf value of all spots was observed and calculated [8].

Chromatographic separation

The selected fractions were separated using VLC with a gradient mobile phase using *n*-hexane, ethyl acetate, and methanol. Elution was carried out from lowest polarity to highest polarity. All fractions were monitored using TLC with the same procedure. The sub-fraction, which is predicted to contain flavonoids, used for the isolation step. Isolation was carried out by preparative TLC. The stationary phase was silica gel (G) 60F with an isocratic mobile phase, i.e. ethyl acetate: *n*-hexane (8:2). Fluorescent band was scratched and suspended in methanol for 24 h, then evaporated. The residue was collected and identified [8].

Isolate identification

The isolate was tested for purity using 2D TLC. The first mobile phase was ethyl acetate: *n*-hexane (2:8) and the second was ethyl

acetate: *n*-hexane (8:2). The isolate must produce a single spot [8]. The isolate was scraped off and dissolved in methanol to identify specific peaks for flavonoids. Identification was continued by adding shear reagent, i.e. 2 M sodium hydroxide, 5% aluminum chloride, sodium acetate, and sodium acetate/boric acid. Then the shifted absorptions were recorded to predict the flavonoid structure [8].

Total flavonoid content determination

About 0.2 ml of five different concentrations of quercetin as the standard and isolate were added to the volumetric flask containing 1 ml of 10% aluminum chloride and 1 ml of 1 M sodium acetate, then diluted to 10 ml with distilled water. The mixture was shaken and kept at room temperature for 30 min. All absorbances were measured at 431.6 nm against distilled water as the blank. The quercetin standard solution was prepared to generate a calibration curve. Total flavonoid content was express in term of µg/ml of quercetin in the isolate [5, 9].

RESULTS

A total of 10 kg of *P. bleo* leaves produced 1 kg of dried leaves with a water content of 4.8%. A total of 1 kg of dried leaves produced 174.79 g of concentrated extract, so the yield was 17.48%.

Table 1: Fractionation yield

Fraction	Weight (g)	Yield (%)
n-hexane	9.56	23.91
ethyl acetate	5.05	12.62
distilled water	25.01	62.52

Fig. 1: Flavonoids monitoring by TLC, i.e. extract (1), ethyl acetate fraction (2), distilled water fraction (3), and quercetin (4)

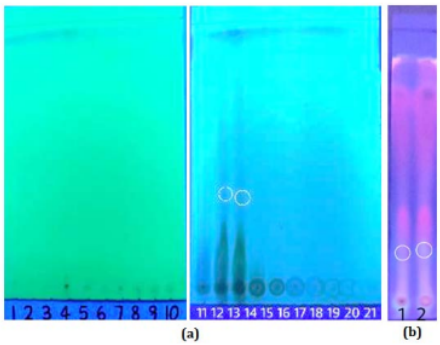

Fig. 2: Monitoring of 21 sub-fractions (a), sub-fractions 12 and 13 (b) with ethyl acetate: *n*-hexane (8:2) as mobile phase

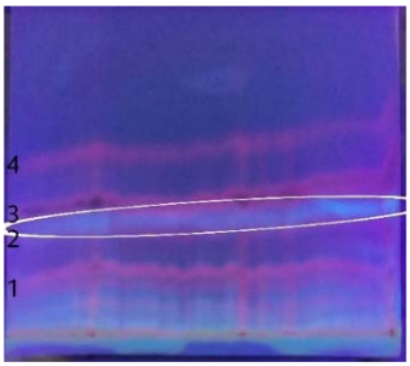

Fig. 3: Preparative TLC chromatogram showed 4 bands with ethyl acetate: *n*-hexane (8:2) as mobile phase

Table 2: Maximum wavelength of flavonol isolate with shear reagent

Shear reagent	Wavelength (nm)		Wavelength shift (nm)		Predicted substitution
	Band I	Band II	Band I	Band II	
MeOH	269.8	206.6			flavonol (free OH at carbon number 3 ring C)
2 M NaOH	212.8	201.8	-57.0	-4.8	C-4' group
2M NaOH, 5 min	273.8	215.4	4.0	8.8	4'-OH group
5% $AlCl_3$	270	208	0.2	1.4	no *o*-dihydroxyl in rings A and B
Sodium acetate	270.2	222.2	0.4	15.6	a hydroxyl group at C-7
Sodium acetate/boric acid	223.6	220.8	-46.2	14.2	*o*-dihydroxyl in rings A and B

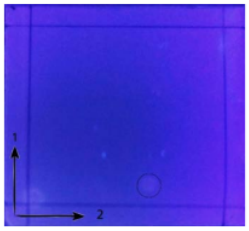

Fig. 4: Chromatogram of isolate identification with ethyl acetate: *n*-hexane (2:8) and ethyl acetate: *n*-hexane (8:2), respectively as mobile phase

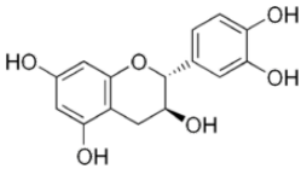

Fig. 5: Catechin structure [15]

Result of table 2 was used to identified the isolate, i.e. catechin (fig. 5). The schematic diagram for isolation of major flavonoid in *P. bleo* leaves was shown in fig. 6.

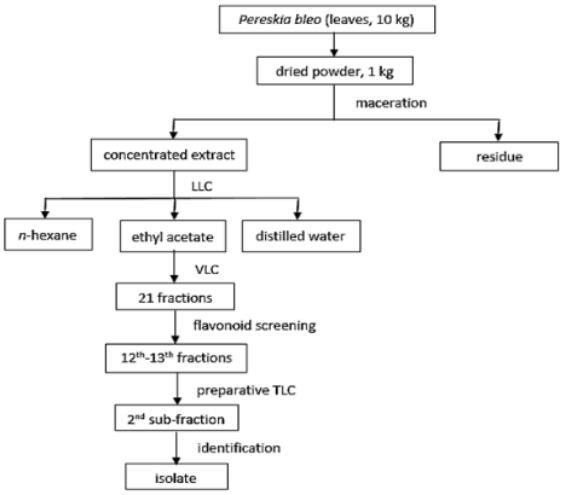

Fig. 6: The schematic diagram for isolation of major flavonoid in *P. bleo* leaves

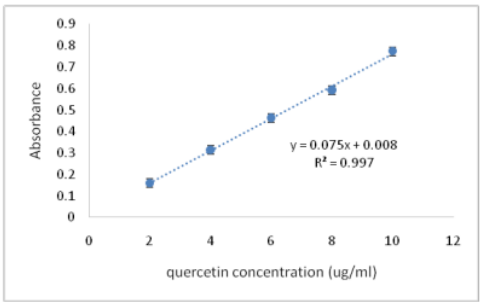

Fig. 7: Calibration curve of quercetin

DISCUSSION

Water content (4.8%) met the requirements, i.e. less than 10% [5]. Dried leaves were green and odorless. Maceration was chosen to prevent damage of thermolabile compounds and complies with Indonesian Herbal Pharmacopoeia. Ethanol was used as a solvent, as it can dissolve most secondary metabolites and is less toxic than methanol [5, 6]. The concentrated extract was dark green in color and odorless.

A total of 40 g of extract was dissolved in 80 ml of hot distilled water, then placed in a separating funnel for further extraction with *n*-hexane and ethyl-acetate, respectively. The result showed that secondary metabolites of *P. bleo* extract contain more polar compounds than no*n*-polar compounds because the distilled water fraction was the highest yield compare to ethyl acetate and *n*-hexane fractions (table 1).

Flavonoid screening based on color reaction. Flavones, flavonols, chalcones, and aurones give green or violet color when reacted with ferric chloride. Flavones give an orange color, anthocyanidins and flavonols give red color, flavanones give purplish color when reacted with Shinoda's reagent (cyanidin reaction). Flavonoids give yellow

color with acetic acid solution [7]. The results of flavonoid screening showed positive results for dried leaves, extract, distilled water fraction, and ethyl acetate fraction. This result was observed from dark green color when reacted with ferric chloride solution, red color with Shinoda reagent, and yellow color with acetate reagent. The flavonoids in *P. bleo* leaves were predicted to be flavonols. All reactions were negative for *n*-hexane fraction. This showed that the *n*-hexane fraction does not contain flavonoids.

The extract, ethyl acetate fraction, and distilled water fraction were monitored for flavonoids using TLC with quercetin as standard (fig. 1). Quercetin was chosen because it belongs to the flavonol group [10, 11]. The results showed that the extract and ethyl acetate fraction gave similar spots to quercetin (Rf = 0.3 cm). The ethyl acetate fraction contains fewer groups of secondary metabolites compared to the extract and has the same spot as the standard. So, the ethyl acetate fraction was used for further separation process.

A total of 1.5 g of ethyl acetate fraction was mixed with 5.5 g of silica gel 60 F254, then separated with gradient elution, from *n*-hexane and ethyl acetate (10:0) to ethyl acetate and methanol (0:10). VLC generated 21 sub-fractions, which were monitored using TLC (fig. 2a). The chromatogram showed that sub-fractions 12 and 13 gave the highest intensity spots and had Rf = 0.3. Sub-fractions 12 and 13 were sub-fractions with ethyl-acetate: methanol (7:3) and ethyl-acetate: methanol (8:2) as eluents, respectively. Both sub-fractions were re-monitored (fig. 2b). Both sub-fractions showed the same pattern and Rf, so both sub-fractions were mixed for further separation using preparative TLC.

The TLC preparative chromatogram showed 4 bands with Rf of 0.16, 0.30, 0.44, and 0.61 (fig. 3). The second band with Rf = 0.3 was predicted to be flavonol, then scraped and dissolved in methanol for isolate identification. Isolate identification was carried out using 2D TLC with two different mobile phases, i.e. ethyl acetate: *n*-hexane (2:8) and ethyl acetate: *n*-hexane (8:2), respectively. In this chromatogram, only 1 spot was observed, which indicated that the isolate was pure (fig. 4).

The UV spectrophotometer was identified the presence of conjugated double bond. The absorption was in conjugated diene absorption in the transtition $\pi \rightarrow \pi^*$ in the wavelength less than 270 nm. This shows the double bond in the conjugated flavonoid ring [8, 12]. Flavonols have a conjugated aromatic system and an C6-C3-C6 ring, so there is a characteristic UV spectrum, i.e., band I in the range 250-270 nm and band II in the range 200-280 nm [8]. These were corresponded to the spectrum of the isolate in methanol, i.e., band I at 269.8 nm and band II at 206.6 nm (table 2). Sodium hydroxide was identified the presence of hydroxyl groups on ring B [8, 13]. Table 2 showed that sodium hydroxide and sodium acetate showing a hypsochromic shift (blue shift), whereas aluminum chloride, sodium acetate, and NaOH incubated for 5 min showed a bathochromic shift (red shift). Wavelength shifting occurs due to differences in solvent polarity, conjugation or addition of groups that affect absorption [8].

UV-visible spectra from before and after the addition of shifting reagents were used to identify flavonoids. Substitution on the ortho-dihydroxyl position in rings A and B give the hypsochromic shift of band I in the range 30-40 nm range [8]. Aluminum chloride caused a hypochromic shift of 0.2 nm in the I band, so there was no ortho-dihydroxyl position in rings A and B. Sodium acetate ionized the hydroxyl group at C-7, produced a bathochromic shift of 15.6 nm in band II. Sodium acetate and boric acid was used to detect ortho-dihydroxyl at all locations in the flavonoid. These reagents produced a hypsochromic shift of 46.6 nm in band (table 2). The interpretation of wavelength shift of the UV spectrum were predicted to be flavonol, because the spectrum was similar to flavonol characteristic [8]. It concluded that the presence of a hydroxyl group at positions C3, C7, and without an ortho-hydroxyl group in ring B (table 2) identified the isolate was catechin (fig. 5). Our result was the same as Lakhanpal and Rai [11]. This was also because catechin provides the highest concentration of flavonoid quantification [14].

Flavonoids in *P. bleo* were the potential to donating a hydrogen atom to form a complex with aluminum ion. Flavonoid effectivity as metal chelating and radical scavenging activities was determined by the hydroxyl group of ortho position in catechol structure (B ring), double bond at C2-3 conjugated with a carbonyl group at C4 (C ring), and hydroxyl group at C5 (A ring) [16, 17].

Complex of quercetin and aluminum chloride solution forms a yellow solution. The solution gives the maximum wavelength at 431.6 nm. This wavelength is violet-blue wavelength (420-440 nm) as transmitted color [18]. This result was similar to Silva-Beltran *et al.* [19]. The correlation coefficient showed that the instrument response is proportional to the concentration and met the ICH criteria [20]. The linear equation of quercetin standard was $y = 0.0753x+0.0082$ with $R^2 = 0.9978$ (fig. 7). The isolate gave an absorbance of 0.304±0.005, so the total flavonoid content was 3.795±0.096 g QE/ml. The isolate concentration was 4 ug/ml, so the isolate purity was 94.89%.

CONCLUSION

The major flavonoid of *P. bleo* leaves was isolated and identified as catechin. The concentration of this compound was 3.795±0.096 g QE/ml with a purity of 94.89%.

ACKNOWLEDGMENT

The authors would like to thank Intan Puspasari for technical assistance.

FUNDING

Nil

AUTHORS CONTRIBUTION

All the authors contributed equally.

CONFLICT OF INTERESTS

Declared none

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