Original Article

The colorimetric method for determination of total Alkaloids and Flavonoids content in Indonesian black nightshade (*Solanum nigrum* L.)

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ABSTRACT

Plant standardization requires determination of the total content of various classes of secondary metabolites, due to dosage determination in the preparation. Black nightshade (*Solanum nigrum* L.) is one of the ethomedicines in Indonesian society. The purpose of this study was to determine the total alkaloids and flavonoids content in leaf, fruit, and stem of black nightshade and to determine the effect of acidified solvent in the alkaloids and flavonoids extraction. The novelty of this study was black nightshade from West Java, Indonesia. The colorimetric method was conducted to determinate total alkaloids content using thiourea and aluminum chloride to determinate total flavonoids content. The total alkaloids content was 0.256-0.389 mg/g in fruit, 0.066-0.159 mg/g in leaf, and 0.144-0.283 mg/g in stem. Total flavonoids content was 0.420-0.582 mg/g in fruit, 0.492-0.556 mg/g in leaf, and 0.240-0.372 mg/g in stem. Fruit has highest total alkaloids content and leaf has highest total flavonoids content. Acidified solvents were increasing the alkaloid and flavonoid extraction by 120.7-240.9% and 108.9-155.0%, respectively.

Keywords: acidified solvent, colored complex, thiourea, aluminum chloride

Introduction

Plant standardization requires determination of the total content of various classes of secondary metabolites. Alkaloids and flavonoids have biochemical properties and therapeutic efficacy. Determination of total alkaloids and flavonoids contents in plants is important to determine the dosage of plants in the preparation. [1] Colorimetric methods for alkaloids and flavonoids are reliable, accurate, and time-saving compared to chromatographic methods, which require advanced instrument and expertise, and are time-consuming, and expensive. [2] Colorimetric methods are simple, rapid, and efficient methods for the routine determination of total alkaloids and flavonoids content. The amount of bismuth is estimated after alkaloids precipitation with Dragendorff reagent; bismuth forms a yellow complex with thiuourea. [3, 4] Flavonoids form yellow-green complex with aluminum chloride. [2]

Black nightshade (*Solanum nigrum* L.) is ethomedicine for Indonesian society. The fruit is antiseptic, anti-inflammatory, antidisentric, antimalarial, and ulcerogenic. The seed is used for the gonorrhea and dysuria treatment. The fruit is used as antipyretic and stomach ailment treatment, while the bud is used as skin treatment. Flower and leaf are used as antipyretic and alcohol antidote. The leaf is used as anthelmintic, joint and ear treatment. [5] The purpose of this study was to determine the total alkaloids and flavonoids content in leaf, fruit, and stem of black nightshade and to determine the effect of acidified solvent

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in the alkaloids and flavonoids extraction. The novelty of this study was black nightshade from West Java, Indonesia.

Materials and Methods

Materials

Black nightshade (3-4 months old) plants were collected from Sindang Village, Garut Regency, West Java, Indonesia. The plant was identified in Plant Taxonomy Laboratory, Biology Department, Universitas Padjadjaran, with No. 769/HB/06/2014.

All chemicals were of analytical grade (Merck), i.e. thiourea, bismuth nitrate pentahydrate, potassium iodide, methanol, hydrochloric acid, disodium sulfide, mercury chloride, and nitric acid. Papaverine was purchased from Sigma Aldrich.

Determination of Loss on Drying

A 5 g simplicia was dried on 105°C at atmospheric pressure for 5 h, then weighed. Drying and weighing were continued until a constant weight. [6]

Extract Preparation

5 g of simplicia was extracted with 10 mL of various solvents, i.e. 96% ethanol and acidified ethanol (2, 4, and 6%) at 40° C for 60 min in reflux apparatus. Extracts were filtered, then re-extracted with the same solvent. All extracts were transferred to a 25 mL of volumetric flask and diluted with the same solvent.

Determination of Total Alkaloids Content

Preparation of bismuth nitrate calibration curve

1 mL of 2 mg/mL bismuth nitrate solution was added to 5 mL of 3% thiourea solution. The absorbance value was measured at 440-460 nm. Each of five various bismuth nitrate concentrations were added to 5 mL of 3% thiourea solution in a 10 mL of volumetric flask. The absorbance was measured at the maximum wavelength against thiourea solution as blank. [1]

Validation of analytical method

Each of 5 mL of various concentrations of papaverine solution (pH 2-2.5) was reacted with 2 mL of Dragendorff reagent. The precipitate was centrifuged and washed with 96% ethanol. The precipitate was reacted with 2 mL of disodium disulfide to form blackish brown compound, then centrifuged. The precipitate was dissolved in 2 mL of concentrated nitric acid and diluted with aquadest in a 10 mL of volumetric flask, then 1 mL of solution was pipetted and added to 5 mL of 3% thiourea solution. Absorbance was measured at the maximum wavelength against blank containing nitric acid and thiourea. The amount of bismuth was calculated by multiplying the absorbance with the factor, taking a suitable dilution factor into consideration. The factor is obtained from the calibration curve, which is a constant for different concentrations. The results were used to determine recovery, quantification limits, and detection limits. [1]

Determination of total alkaloids content

Determination of total alkaloids content of 5 mL extract was carried out with the same procedure as papaverine. [1]

Determination of Total Flavonoids Content

1 mL of 1% rutin solution was added to 2 mL of 5% $AlCl_3$ solution in a 10 mL of volumetric flask. Absorbance was measured 300-450 nm against $AlCl_3$ solution as blank. Determination of total flavonoids content of 1 mL extract was carried out with the same procedure as rutin at maximum wavelength. Total flavonoids content was calculated with the below formula: [2]

$$\text{TFC} = \frac{A \; x \; DF}{A_{1cm}^{1\%} \; x \; (w - ld)}$$

Where, TFC = total flavonoids content, A = absorbance, DF = dilution factor, $A_{1cm}^{1\%}$ = specific absorption for rutin-$AlCl_3$ complex (259.4), w = mass of sample (g), and ld = loss on drying.

Statistical Analysis

Data were shown in mean ± standard deviation (SD). Data were analyzed statistically using the one-way ANOVA to determine the significancy effect of acidified solvent in extraction alkaloids and flavonoids.

Results and Discussion

Determination of Loss on Drying

Loss on drying was 94.88% for fruits, 93.97% for leaves, and 91.24% for stem. The values were high, due to simplicia was the fresh part of the plant. Loss on drying must be determined, due to the effect of the simplicia weight and calculation on the total flavonoids content.

Extract Preparation

Methanol is better solvent to dissolve secondary metabolites, but ethanol was chosen as an extraction solvent, because methanol is more toxic than ethanol. [7] Heating in reflux extraction increase solvent diffusion, that will increase the alkaloids and flavonoids extraction from simplicia. [8] All extracts were light green color with the characteristic odor of black nightshade.

Determination of Total Alkaloids Content

The nitrogen atoms in alkaloid bind to heavy metal atoms in Dragendorff reagents ($KBiI_4$) to form ionic bonds in complex compounds (BiI_3) (Alk•HI) which precipitate. [3] The nitric acid medium causes tiourea $\{(H_2N)_2C{=}S\}$ and bismuth form a yellow bismuth complex $\{Bi[CS(NH_2)_3]\}(NO_3)_3$. The maximum wavelength of bismuth nitrate-thiourea complex was 460 nm, i.e. the blue wavelength (450-495 nm) as complementary or transmitted color. [9] The medium pH must be maintained; high acidic medium will re-dissolve the precipitated complex. Disodium sulfide will release bismuth from alkaloid complexes. [10] 3% thiourea solution was used, due to stability of solution, which forms a stable complex for 24

h. Higher concentration of thiourea solution forms thiourea precipitates. [1]

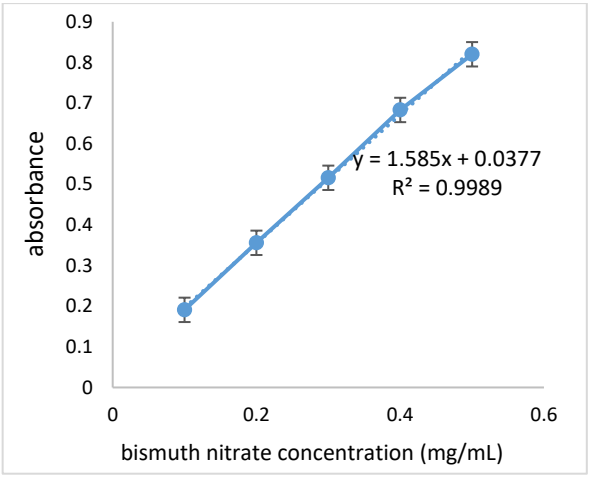

Fig. 1. Calibration curve of bismuth nitrate-thiourea complex (n = 3)

The calibration curve of bismuth nitrate concentration and absorbance obeyed Lambert-Beer's law in the concentration range of 0,1-0,5 mg/mL and linear, with coefficient correlation of 0.9989 (Fig. 1). The bismuth nitrate-thiourea complex was 1:1, so the amount of bismuth nitrate corresponds to the amount of alkaloids, which was calculated by multiplying absorbance with factor. The accuracy was determined from recovery, i.e. 99.60 ± 0.25%, which met the requirements. [11] Limit quantification was 0.102 mg/mL and limit detection was 0.034 mg/mL.

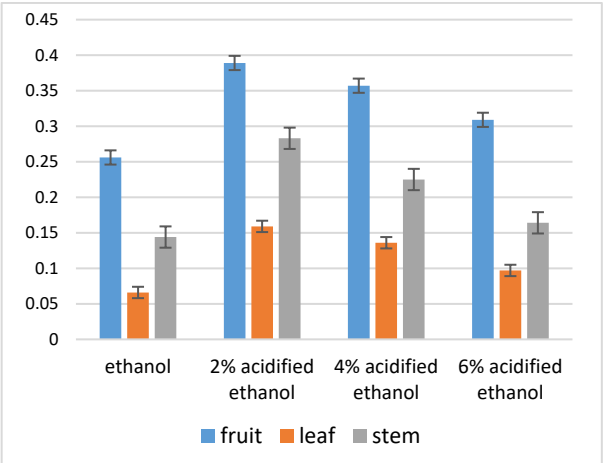

Fig. 2. Graph of total alkaloids content (mg/g) in black nightshade (n = 3).

Table 1: Total alkaloids content (mg/g) in black nightshade in various solvents.

Plant part	Total alkaloid content (mg/g) in mixed solvent (96% ethanol:HCl)			
	100:0	98:2	96:4	94:6
Fruit	0.256 ± 0.019	0.389 ± 0.012	0.357 ± 0.021	0.309 ± 0.012
Leaf	0.066 ± 0.005	0.159 ± 0.002	0.136 ± 0.004	0.097 ± 0.005
Stem	0.144 ± 0.005	0.283 ± 0.004	0.225 ± 0.007	0.164 ± 0.009

The total alkaloids contents were varied in the various part of black nightshade and the highest content was in the fruit (Table 1). Alkaloids of *Solanum* species are in the form of water-soluble salts of sugar, i.e. glycoalkaloids, rather than as free bases that are not water soluble. [12] Acidified ethanols increased the total alkaloids content, i.e. 120.7-151.9% for fruit, 147.0-240.9% for leaf, and 138.9-196.5% for stem (Fig. 2). The highest total alkaloids content was achieved with 2% acidified ethanol. Our prediction was that, acid increases the ethanol polarity, and glycoalkaloids reach the highest solubility in 2% acidified ethanol. Statistical analysis showed a significant difference due to various solvents (p value = 0.00129).

Glycoalkaloids synthesis is affected by temperature, daylight exposure, physical wounding, [13] climate, growth conditions, and storage conditions. [14] Fruits' total alkaloids content in this study (0.256 ± 0.019 mg/g) was lower than other study (0.3 mg/g), [15] due to differences in growth location and the analytical method. Fruits of black nightshade were collected from two different locations in Indonesia (in this study from Garut, while in other study from Bogor). Garut has a tropical climate (Am), while Bogor has a tropical rainforest climate (Af) according to Köppen climate classification. Bogor is more humid and rainy than Garut. [16] Bogor and Garut have an average temperature of 23.1 °C and 26.3 °C, and relative humidity of 80.3% and 83.8%, respectively. [17] The analytical method in this study was colorimetric method, while in the other study was gravimetric method of ethyl acetate fraction. Colorimetric method is more accurate, because Dragendorff reagent reacts with alkaloids specifically, while ethyl acetate fraction contains alkaloids and secondary metabolites which have the same polarity as ethyl acetate.

Determination of Total Flavonoids Content

The maximum wavelength of the flavonoid-$AlCl_3$ complex was 412 nm, i.e. the violet wavelength (380-450 nm) as complementary or transmitted color. [9] Total flavonoids content was varied in the various part of black nightshade and the highest content was in the leaf (Table 2). Acidified ethanols increased the total flavonoids content, i.e. 136.2-138.1% for fruit, 108.9-113.0% for leaf, and 153.3-155.0% for stem (Fig. 3). It was the flavonoids O-glycosides form, which can be hydrolyzed by acid. The flavonoids contented in the sample, the polyphenol structure, the concentration of $AlCl_3$ solutions, and the reaction time affected the flavonoid-$AlCl_3$ complex formation. [18] The highest total flavonoids content was achieved with 4% acidified ethanol. Our prediction was that, the acid increases the ethanol polarity, and flavonoids reach the highest solubility in 4% acidified ethanol. Statistical analysis showed a significant difference due to the acidified solvent (p value = 0.00185).

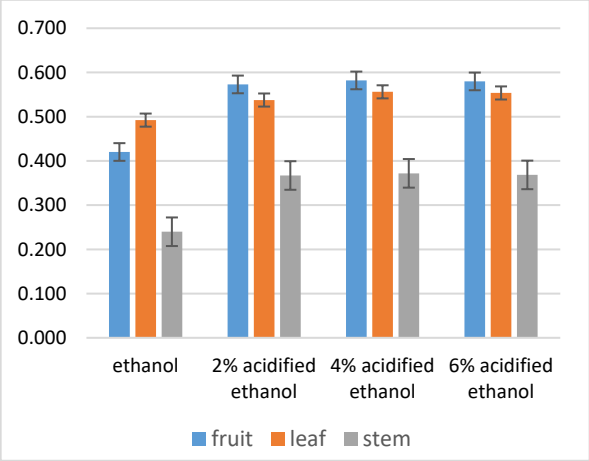

Fig. 3. Graph of total flavonoid content in black nightshade (n = 3).

Table 2. Total flavonoids content (mg/g) in black nightshade in various solvents.

Plant part	Total flavonoids content (mg/g) in mixed solvent (96% ethanol:HCl)			
	100:0	98:2	96:4	94:6
Fruit	0.420 ± 0.010	0.573 ± 0.014	0.582 ± 0.014	0.580 ± 0.014
Leaf	0.493 ± 0.016	0.538 ± 0.018	0.556 ± 0.018	0.554 ± 0.018
Stem	0.240 ± 0.010	0.367 ± 0.016	0.372 ± 0.016	0.368 ± 0.016

Total leaf flavonoids content (0.493 ± 0.016 mg/g) was lower than study by Kwinana-Mandindi (13.30 ± 0.08 mg/g), [19] due to the difference in the growth location and the used simplicia. This study used the wet simplicia which was collected from Garut District (Indonesia) with ethanol as a solvent, while the Kwinana-Mandindi's study used dry simplicia collected from various sites of the Amathole District in South Africa with methanol as a solvent. Flavonoids were phenolic compounds; their content depends on the cultivation techniques, growing conditions, plant types, ripening processes, and storage conditions. South Africa is a more suitable growth location for black nightshade. Dry simplicia gave higher flavonoids content, due to low water content, which increased flavonoids hydrolysis by the solvent. Extraction methods and the solvent polarity affect the phenolic content. [20] The dielectric constant of methanol (1.66) is lower than ethanol (1.69), [21], so more flavonoids are extracted in methanol.

Conclusion

Fruit has highest total alkaloids content and leaf has highest total flavonoids content. Acidified solvents increased the alkaloid and flavonoid extraction by 120.7-240.9% and 108.9-155.0%, respectively.

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Conflict of Interests

There are no conflicts of interest.

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