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Total Phytosterols Content In Roselle (*Hibiscus sabdariffa*) Calyx From Subang And Bandung.

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ABSTRACT

The roselle (*Hibiscus sabdariffa*) calyx is used as an anti-cholesterol empirically. This is suggested because of the phytosterols content which can decrease cholesterol levels in the body. This study aimed to determine the total phytosterols content in roselle calyx from Subang and Bandung. Colorimetric method was used to measure the colored compounds from sterols and Liebermann Buchard reagents. The results showed that total phytosterols content in 100 g of roselle calyx from Bandung and Subang was 152.667 ± 2.456 mg and 171.667 ± 3.126 mg, respectively. We conclude that the total phytosterols content of roselle calyx influenced by the growth location.

Keywords: anti-cholesterol, colorimetric, Liebermann Buchard

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INTRODUCTION

Roselle (*Hibiscus sabdariffa*) calyx contains dietary fiber, minerals, vitamins, and bioactive compounds, such as organic acids, phytosterols, and polyphenols [1]. Roselle calyx (5% or 10%) was a cholesterol-lowering agent to hypercholesterolaemia rats. It was hypothesized that bioactive compounds activate the hormonal secretions, such as adrenocortical hormones, which stimulate the cholesterol metabolic pathway [2]. The anticholesterol action of roselle (0.5% to 1%) was confirmed in rabbits. This treatment was reduced the serum concentrations of triglycerides, total cholesterol and low-density lipoprotein cholesterol, and atherosclerosis mitigation in the aorta [3].

Bandung and Subang are roselle calyx-producing areas in Indonesia. This study aimed to determine the total phytosterols content in roselle calyx from Bandung and Subang. The synthesis of secondary metabolites was stimulated by different parameters, such as environmental factors, precursors of the targeted molecules, elicitors, and genetic transformation of the plants [4]. Determination of total phytosterols content was conducted like our previous study about phytosterols, i.e. colorimetric method from the colored compounds from sterols and Liebermann Buchard reagents [5].

MATERIALS AND METHODS

Materials

Roselle calyces were collected from Bandung and Subang sub-district, Indonesia, in July 2016. Calyces were identified by School of Biological Sciences and Technology, Bandung Institute of Technology, Indonesia with No. 1123/II.CO2.2/PL/2016. All chemical reagents are analytical grade (Merck), including the 95% phytosterols standard (Jiatian Biotechnology, Xi'an, China).

Moisture content determination

A total of 5 g of roselle calyx was dried on 105°C at atmospheric pressure for 5 h, then weighed. Drying and weighing continued, every 1 h, until a constant weight [6].

Steroid identification

A total of 5 g of roselle calyx was grinded in chloroform and 3 drops of concentrated sulfuric acid were added to it followed by 3 drops of acetic anhydride. The color was turned to violet blue and finally green [7].

Phytosterols Extraction

A total of 100 g of roselle calyx was extracted with 200 mL of *n*-hexane:ethanol (82:18) for 24 hours at 25 °C. The extract was filtered, and the residue was re-extracted twice, using 200 mL of the same solvent for 24 hours. The extract was concentrated by rotary vaporator at 40 °C, then saponified with 26.73 M KOH solution. The unsaponified phase was separated with *n*-hexane, then the crude sterols extract was concentrated by rotary vaporator [5].

Quantitative Analysis of the Total Phytosterols Content

Solution preparation. (i) Liebermann-Buchard reagent (LB): The acetic anhydride is cooled for 30 min, then added concentrated sulfuric acid in the ratio 10: 1. The reagent should be fresh. (ii) Standard solution: The phytosterols standard (50 mg) was dissolved with chloroform in a 100 mL volumetric flask [5].

Optimization of analysis conditions. (i) Wavelength selection: Standard solution (1 mL) was added to 4 mL of LB, and chloroform in 10 mL of volumetric flask. The mixture was incubated for 5 min, then measured the absorbance at 400-900 nm with spectrophotometer. (ii) Determination of the time reaction: Standard solution (1 mL) was added to 4 mL of LB, and chloroform in a 10 mL volumetric flask. The absorbance was measured at the maximum wavelength every 5 min for 60 min [5].

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Validation Methods. (i) Linearity was obtained by plotting the five standard concentrations against absorbance. Each concentration was measured three times. The results are averaged, then made the equation and the correlation coefficient (r) with linear regression. (ii) Limits of detection (LOD) and limits of quantitation (LOQ) were calculated by the following equations, i.e. LOD = 3 SD/slope and LOQ = 10 SD/slope, where SD is the standard deviation from linear curve. (iii) Accuracy was determined by the recovery values which expressed the percentages of the ratio of the total phytosterol contents experimentally and their theoretically. Three standard concentrations, each concentration was measured three times, and the recovery value was calculated. (iv) Precision was expressed by the coefficient of variation (CV) = SD/average from six individual standard concentrations which measured for repeatability [8].

Quantification of total phytosterol content. A total of 50 mg crude extract was dissolved in 25 mL of chloroform, then 2 mL of LB reagent was added to 1 mL of this solution, followed by chloroform in 5 mL volumetric flask. The mixture was incubated for 5 min, then measured the absorbance. Total phytosterols content was calculated from the linear regression in the calibration curve [5].

Statistical analysis

The results were presented as the mean ± standard deviation (SD). Statistical analysis was conducted by one way ANOVA followed by t-test with statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

The roselle calyces from Bandung and Subang were purple-red color with slightly berrylike aroma. The moisture content of roselle calyx from Bandung and Subang was 4.8 ± 0.23% and 4.6 ± 0.12%, respectively. The moisture content met the criteria, i.e. 12% [9]. The moisture content showed the water and volatile compound inside the dried plant material. Qualitative test showed that roselle calyx of Bandung and Subang contain steroids. It was observed from the formation of a green solution from phytosterols and LB reagent [7]. The known sterols in roselle were β-sitosterol and ergosterol [1].

Phytosterols extraction using a maceration method because of phytosterols are thermolabile. The solvent is a mixture of *n*-hexane and 70% ethanol (82:18), which is non polar, to maximize the sterols extraction. Extracts were saponified with KOH to separate the unsaponified and saponified components. Roselle calyx of Bandung and Subang require 5 mL and 3 mL KOH, respectively. It was shown that the roselle calyx of Bandung contains more saponified compounds than Subang. The unsaponified phase was evaporated to obtain concentrated extract of Bandung and Subang, i.e. 1.397 ± 0.271% and 1.686 ± 0.231%, respectively. The results showed that the extracted compounds in roselle calyx from Bandung was higher than Subang.

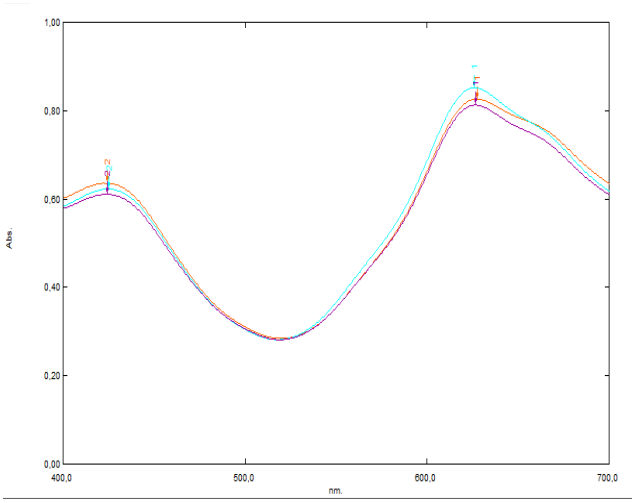

Fig 1: Spectrum of phytosterols-LB reagent product

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The reaction between phytosterol with LB reagent which contain the concentrated acids form the oxidated blue product with a maximum wavelength at 626.7 nm (Fig 1). This result was consistent with the literature [10] and our previous study [5]. One additional maximum was observed at 416 nm from rearranged aromatic sulfonic acids [11]. Analysis was using 626.7 nm because more specific for the phytosterol-LB reagent product [10].

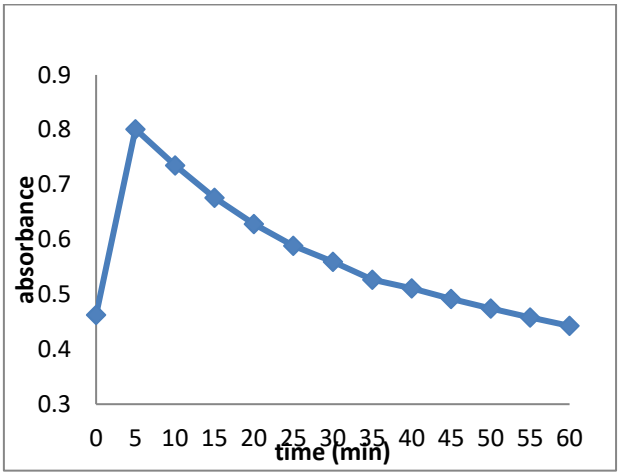

Fig 2: The graph of reaction time of phytosterol and LB reagent

Time reaction determination was conducted to optimize the reaction time of the LB reagent with phytosterols. The maximum absorbance was occurring at 5 min after the LB reagent addition which indicate the complete reaction (Fig 2). This graph was corfirmed the accelerated reaction [12] because of the conversion of acetate derivatives of the steroids after reacting with LB reagent [13] and the instability product produces absorbance reduction.

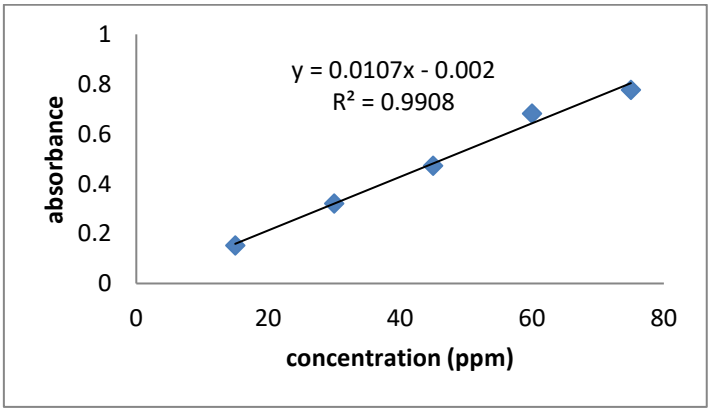

Fig 3: Calibration curve of standard phytosterols

Table 1. Validation result

Parameter	Results	Reference [8]
Linearity (correlation coefficient)	0.9992	0.990
Accuracy (% recovery)	96.7-106.9%	80-110%
Precision (coefficient of variance)	1.553%	less than 2%
Limits of detection (LOD)	0.915 µg/mL	-
Limits of quantitation (LOQ)	3.051 µg/mL	-

The validation results showed that all value were met the criteria (Fig 3 and Table 1) [8]. It means the instrument response was comparable to the analyte concentrations with good accuracy and precision. The LOD and LOQ were good to analysis the total phytosterol content in sampels. Total phytosterols content in 100

g of roselle calyx from Bandung and Subang was 152.667 ± 2.456 mg and 171.667 ± 3.126 mg, respectively. The total phytosterols content of roselle calyx from Subang was higher than Bandung. There was statistically significant on total phytosterols content (P = 0.0014). It was because the extract yield from Subang was higher, so the possibility of phytosterol content in yield was more higher.

The elevation of Bandung and Subang is 800 m (2624 ft) and 95 m (312 ft), respectively. The altitude affected the temperature, i.e. from 23.3 to 28.7 °C for Bandung, and from 27.7 to 32.2 °C for Subang [14]. The temperature of both place meets the criteria for the roselle growth, i.e. between 18 and 35ºC, with an optimum of 25ºC [15]. Rainfall in Bandung is high (301-400 mm), whereas in Subang relatively low (51-100 mm) [14]. Phytosterols in roselle calyx were responsive to the climate, high temperature and low rainfall, caused a increase in total phytosterols content. So, growth location has a significant impact on the total phytosterol content in roselle calyx.

CONCLUSION

The total phytosterols content of roselle calyx influenced by the growth location.

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