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Original research article

Effects and Chemical Contents of Hydrolysis Modification of Aqueous Roselle Extract to Reflect the Antioxidant and Anti-Inflammatory Effects

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ABSTRACT

Hibiscus sabdariffa (roselle) has been widely used in Thai traditional medicine and food products. However, roselle effects may be unstable under the acidic conditions in the stomach. Thus, the objective of this research was to investigate the stability of bioactivity of roselle extract under acidic conditions. Roselle aqueous extract and acid hydrolysis of roselle extract were assessed for content of phenolic compounds by using Folin-Ciocalteu's colorimetric method, for antioxidant effect by DPPH radical scavenging assay, and for anti-inflammatory effect by using inhibition of nitric oxide production in RAW264.7 cell line. Five positive marker compounds in roselle extracts (chlorogenic acid, coumaric acid, ferulic acid, quercetin and cyanidin-3-o-sambubiosides) were determined by a modified HPLC method. The results showed that acid hydrolysis of roselle extract showed higher antioxidant activity with EC_{50} of 14.12±0.92 µg/ml and anti-inflammatory with IC_{50} of 16.84±3.18 µg/ml than the aqueous extract. Moreover, acid-hydrolyzed roselle extract showed higher total phenolic content than aqueous roselle extract. For HPLC analysis, the acid-hydrolyzed extract contained no chlorogenic acid and cyanidin-3-o-sambubiosides, but levels of the others were higher than for the aqueous extract. The results revealed roselle extract showed higher biological activity and active compounds content after acid hydrolysis.

Keywords: Acid hydrolysis; Roselle; *Hibiscus sabdariffa*.

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1. Introduction

Hibiscus sabdariffa L. is known as roselle, also called Kra-Jeab-Daeng in Thai. Its calyces have a sour taste that is used as food flavor and beverage production [1]. Moreover, it has been used for urinary tract infections and kidney stones, as a diuretic, mild laxative, and pyretic, and as a treatment for hypocholesterolemia and hypertension [2-4]. The roselle calyx mainly contains phenolic compounds such as anthocyanin, flavonoid and chlorogenic acid [5-7]. Phenolic compounds are antioxidants that protect against cell damage from free radicals and decrease LDL oxidation [8]. A previous study reported some phenolic compounds, such as naringenin and chlorogenic acid, were unstable under gastric digestion but some phenolic compounds were stable and increase after digestion in stomach [9]. Bioavailability of roselle under acidic conditions has not been reported. Therefore, this study investigated the stability of bio-accessibility and bioactivity in roselle extract under acidic conditions.

2. Materials and Methods

2.1 Extraction

An aqueous extract of *Hibiscus sabdariffa* calyces (voucher specimen No. SKP109081901) was obtained from Center of Excellence on Applied Thai Traditional Medicine Researches, Faculty of Medicine, Thammasat University, Thailand.

2.2 Acid hydrolysis of aqueous extract of *Hibiscus sabdariffa* L.

The acid hydrolysis was prepared with a modification method [10-13]. The aqueous extract of *Hibiscus sabdariffa* L. (20 g) was dissolved in 0.01 N hydrochloric acid for 15 minutes. After that, chloroform was added into solution with ratio 1:1. The chloroform part was collected, evaporated and stored at -20°C. All percentages of yield were calculated using the formulae below: (amount of hydrolysis extract/amount of original extract) x 100.

2.3 DPPH assay [14-15]

2.3.1 Preparation of sample solution

1 mg of roselle aqueous extract was dissolved in 1 ml of distilled water. 1 mg of Butylated hydroxytoluene (BHT) and acid hydrolysis extract of roselle were dissolved in 1 ml of absolute ethanol. The sample solutions were transferred to 1 mg/ml and diluted into 1, 10, 50 and 100 µg/ml.

2.3.2 Determination of DPPH radical scavenging assay

A DPPH solution concentration of $6x10^{-5}$ M was prepared in absolute ethanol. Then, 100 µl of sample solution and BHT were added into a 96-well microplate followed by 100 µl of DPPH solution. After that, the microplate was covered with foil for 30 minutes. Sample absorbance was measured at 520 nm. The percentage of inhibition was calculated and presented in 50% effective concentration (EC_{50}). The EC_{50} value and the standard error mean were calculated by the GraphPad Prism 5.

2.4 Nitric oxide assay [16]

2.4.1 Preparation of cell line

A RAW264.7 Murine macrophage cell line was purchased from (ATCC® Catalog No. TIB-71TM) (USA). The cell line was cultured in a DMEM medium containing 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were maintained at 37°C in an incubator with 5% CO_2 atmosphere and 95% humidity.

2.4.2 Preparation of sample solution

50 ml of roselle extract and acid-hydrolyzed roselle extract were prepared at a concentration of 50 mg/ml by dissolving in 1 ml of distilled water and sterile dimethyl sulfoxide (DMSO). After that, roselle aqueous extract was diluted to 50 and 100 µg/ml. Acid hydrolysis of the

roselle aqueous extract was diluted to 1, 10, 50 and 100 µg/ml in the DMEM medium. Prednisolone was used as a positive control and diluted to 0.01, 0.1, 1, 10 and 50 µg/ml in the DMEM medium.

2.4.3 Evaluation of nitric oxide production inhibition effect

RAW264.7 cells were seeded in a 96-well microplate with $1x10^5$ cells/well and incubated at 37°C for 24 hours. After incubation, 2 ng/ml of lipopolysaccharide (LPS) in DMEM was replaced in the upper part of 96-well microplate and DMEM medium without LPS being replaced in the lower part of 96-well microplate. Various sample concentrations were added into each well and incubated at 37°C for 24 hours. After that, 100 µl of supernatant was removed to another 96-well microplate and Griess reagent was added to identify nitric oxide production. The absorbance was measured by a microplate reader at a wavelength of 570 nm. The percentage of inhibition was calculated and presented in 50% inhibitory concentration (IC_{50}). The IC_{50} value and the standard error mean were calculated by the GraphPad Prism 5.

The MTT colorimetric method was used to determine cytotoxicity. MTT (5 mg/ml) was added and incubated for 2 hours. Then, supernatant was removed and replaced with isopropanol. Formazan production was measured at a wavelength of 570 nm by a microplate reader. The cytotoxicity was presented in percent survival.

2.5 Total Phenolic Content Assay

2.5.1 Preparation of sample solution

Stock sample solution was prepared at concentration 1 mg/ml. Roselle extract was dissolved by distilled water, but acid hydrolysis roselle extract was dissolved by absolute ethanol. All samples were diluted into 500, 1000 µg/ml.

Gallic acid was used as a positive control and the concentration was adjusted to 1 mg/ml. The stock solution was diluted to concentration 5, 10, 25, 50, 100, 250 and 500 µg/ml, respectively.

Sodium carbonate anhydrous was prepared at concentration 75% w/v in distilled water. Folin-Ciocalteu's reagent (5 ml) was added into a volumetric flask. Distilled water was added and the solution was kept in a dark room.

2.5.2 Evaluation of Total Phenolic Content

Sample and gallic acid solution 20 µl were added into a 96-well microplate. After that, 80 µl of sodium carbonate and 100 µl of Folin-Ciocalteu's reagent were added in each well. Then, the microplate was kept at room temperature for 30 minutes. Absorbance was measured at 765 nm and total phenolic content was calculated using the standard curve of gallic acid [17].

2.6 HPLC analysis [18]

For bioactive compound modified evaluation, there were 5 positive compounds in this study: chlorogenic acid, coumaric acid, ferulic acid, quercetin and cyanidine 3-sambubioside. Their structures are shown in Figure 1. Chlorogenic acid and ferulic acid were dissolved into 1 ml of 50% methanol and diluted into concentrations of 10, 20, 40, 60, 80 and 100 µg/ml. Coumaric acid and quercetin were dissolved into 1 ml of 50% methanol and adjusted concentration into 5, 10, 50, 100, 200 and 300 µg/ml. Cyanidin-3-o-sambubiosides was also dissolved in 1 ml of 50% methanol and diluted into 5, 10, 20, 30, 40 and 50 µg/ml. Moreover, 5 mg of roselle aqueous extract and acid hydrolysis were prepared in 50% methanol and adjusted in concentration to 500 and 1000 µg/ml in methanol.

The chromatographic system was performed in C18 100A analytic column (Phenomenax® Luna, 4.6 x 150 mm,10 µm) with G1322A solvent degasser, G1311A solvent pump, G1329A autosampler, G1316A column oven and G1315D

photodiode array detector. The data was presented by Chemstation software. Solvents were 0.1% phosphoric acid (A) and 100% acetonitrile (B). There were 3 solvent program elutions: isocratic elution with 6% B in 0 to 10 minutes, linear gradient to 20% B from 10 to 55 minutes and isocratic elution at 20% B from 50 to 60 minutes at flow rate 1 ml/min with injection volume 10 µl. The absorbance was detected at 325 (chlorogenic acid, coumaric acid and ferulic acid), 365 (quercetin) and 520 nm (cyanidin-3-o-sambubiosides).

3. Results and Discussion

The percentage of yield and biological activity are presented in Table 1. After being acid-hydrolyzed, the weight of the extract was 617 mg and the yield of the extract was 3.09 percent. For antioxidant activity, the results are presented in EC_{50} values. The acid hydrolysis roselle extract showed higher antioxidant activity than roselle aqueous extract and positive control (BHT). Besides, acid-hydrolyzed roselle extract still showed higher activity than roselle aqueous extract against nitric oxide production. For the MTT colorimetric results, prednisolone (positive control) at concentration 0.01, 0.1, 1, 10 and 50 µg/ml exhibited survival of cell lines with values of 89.46±0.68, 84.81±1.21, 75.80±0.58, 84.44±3.61 and 72.77±1.09%, respectively. Roselle aqueous extract at concentration 50 and 100 µg/ml showed survival of cell lines with values of 110.51±9.74 and 115.33±8.61%. Moreover, acid-hydrolyzed roselle aqueous extract at concentration 1, 10, 50 and 100 µg/ml displayed survival of cell lines with values of 107.40±8.59, 105.72±10.01, 86.42±5.41 and 81.64±6.95%, respectively. Therefore, roselle aqueous extract and acid-hydrolyzed extract did not exhibit cytotoxicity in RAW 264.7 cell in any concentration. In addition, total phenolic compound of roselle aqueous extract increased to 69.10 mg GAE/g after being acid-hydrolyzed as shown in Table 1. Roselle contains a bioactive compound for antioxidation and anti-inflammation [5]. The DPPH scavenging activity of chlorogenic acid, coumaric acid, ferulic acid and quercetin were exhibited with IC_{50} values at 22.8±1.50, >100, 61.90±0.01 and 9.70±0.80 µM, respectively [19] and cyanidin-3-o-sambubiosides showed against free radical with EC_{50} value at 7.29 µM [20]. For anti-inflammatory effect, chlorogenic acid and quercetin inhibited nitric oxide production at 1.8 and 1.7 µM [21]. Coumaric acid and ferulic acid also countered inflammation with EC_{50} values of 17 and 8.3 µM [22]. Cyanidin-3-o-sambubiosides were nitric oxide inhibitors with result values at 6.4-6.7 µM [23].

Bioactive compounds of roselle extracts were analyzed by the HPLC technique, as shown in Figure 3-5. Roselle extracts showed low ferulic acid, quercetin and cyanidin-3-o-sambubiosides content with 0.09, 0.57 and 0.56 mg/g, respectively. On the other hand, extracts contained high chlorogenic acid and coumaric acid content, as shown in Table 2. After the acid hydrolysable process, ferulic acid, coumaric acid and quercetin content in roselle extract were increased to 13.84, 10.84 and 2.03 mg/g, respectively, while chlorogenic acid and cyanidin-3-o-sambubiosides could not be detected. In the case of chlorogenic and cyanidin-3-o-sambubiosides, they were unstable during the thermal process [5]. According to previous studies, anthocyanin and phenolic compounds degrade increasingly with various high temperature and time combination [24-25]. The content of quercetin has been documented as decreasing as temperature and time increased [26-28]. Esterification of chlorogenic acid occurred after it was hydrolysed or reacted with water. This process transformed the chlorogenic acid isomer to its derivative compounds such as caffeoylquinic acid, p-coumaroylquinic acid and feruloylquinic acid [29]. The thermal process changed the isomer of cyanidin-3-o-

sambubiosides to cyanidin, protocatechuic acid, phloroglucinaldeyde and a few quercetin sambubioside as shown in Figure 2 [30]. These results showed that the biological activity and chemical content of roselle extract were increased after acid hydrolysis. Thus, acidic conditions in stomach may increase the anti-inflammatory and antioxidant activity of roselle aqueous extract and promote health benefits.

Table 1. Percent yield, total phenolic content and bioactivity of roselle extract.

	Aqueous extract	Acid-hydrolyzed
Percent yield	-	3.09
DPPH scavenging activity (EC_{50} µg/ml) Positive control (BHT) 18.78±0.47 µg/ml	50.40±1.41	14.81±2.39
Nitric oxide production inhibition (IC_{50} µg/ml) Positive control (Prednisolone) 0.14±0.03 µg/ml	more than 100	28.27±1.82
Percent survival at 100 µg/ml	115.33±8.61%	81.64±6.95%
Total phenolic compound mg GAE/g	46.51±2.58	69.10±0.60

EC_{50}: half maximal efficiency of extract concentration.
IC_{50} : half maximal inhibition of extract concentration.

Table 2. HPLC analysis of chlorogenic acid, coumaric acid, ferulic acid, quercetin and cyanidin-3-o-sambubiosides in extracts.

	Aqueous extract	Acid hydrolysis
Chlorogenic acid (mg/g)	5.76 ± 0.05	ND
Coumaric acid (mg/g)	2.23 ± 0.01	10.84 ± 0.03
Ferulic acid (mg/g)	0.09 ± 0.002	13.84 ± 0.20
Quercetin (mg/g)	0.57 ± 0.01	2.03 ± 0.11
Cyanidin-3-o-sambubiosides (mg/g)	0.56 ± 0.01	ND

ND: Not detection

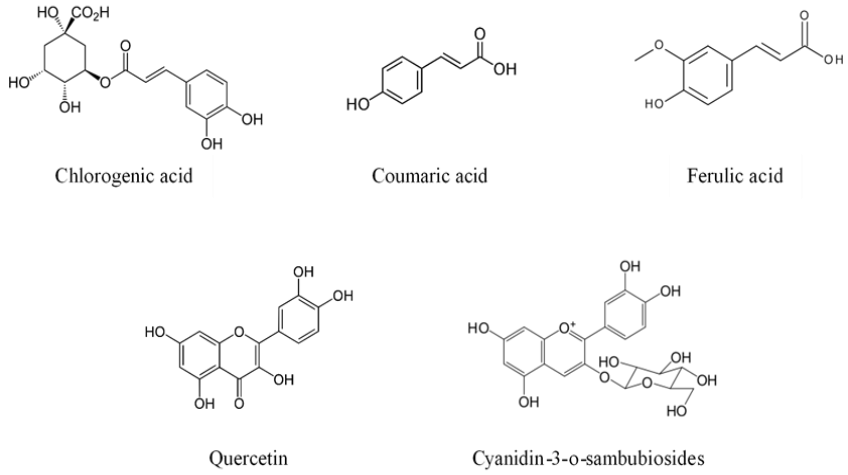


Fig. 1. Structure of standard compounds.

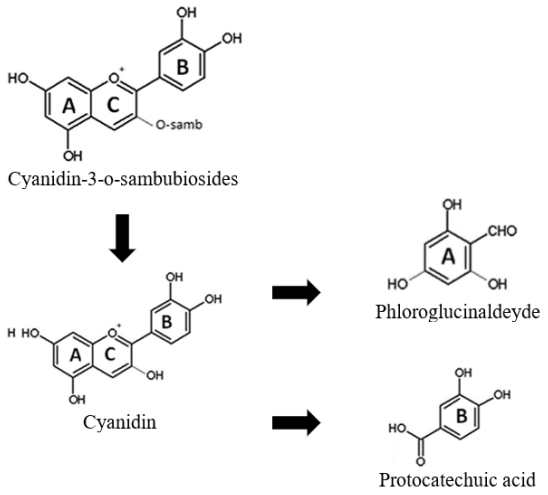
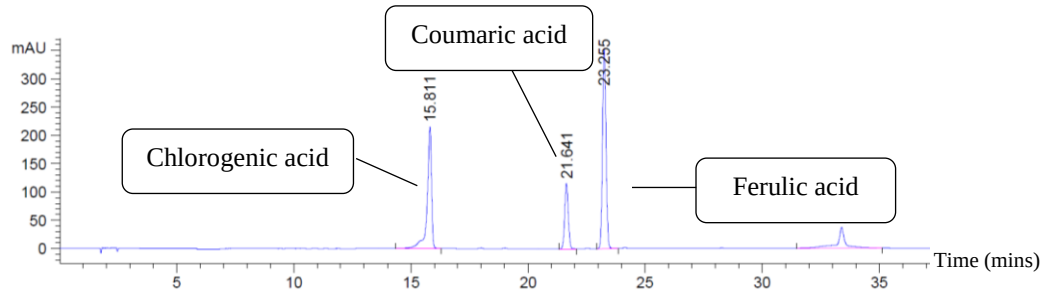
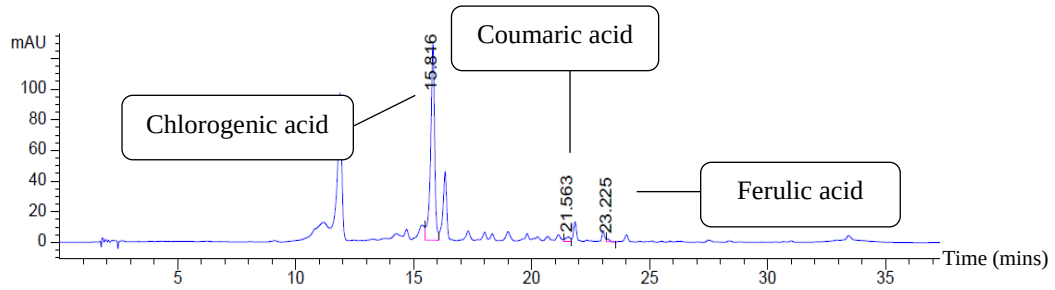


Fig. 2. Transformation of cyanidin-3-o-sambubiosides structure after thermal process [30].

a. Standard marker (chlorogenic acid, coumaric acid and ferulic acid)



b. Aqueous extract of roselle extract



c. Acid hydrolysis of roselle extract

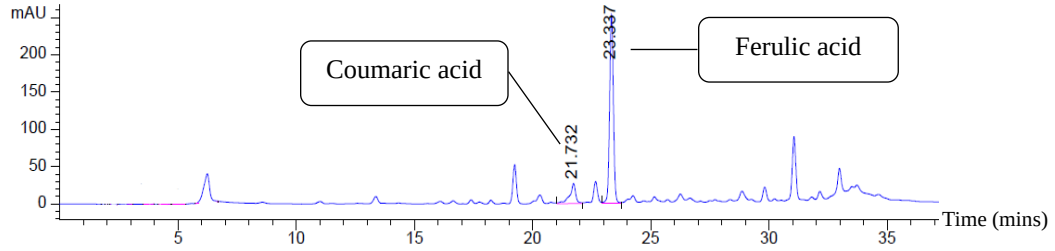
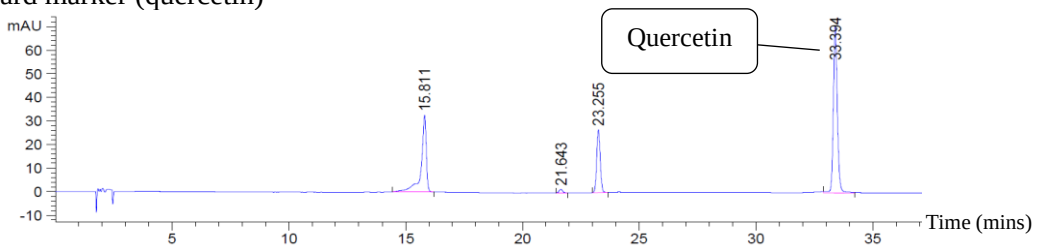
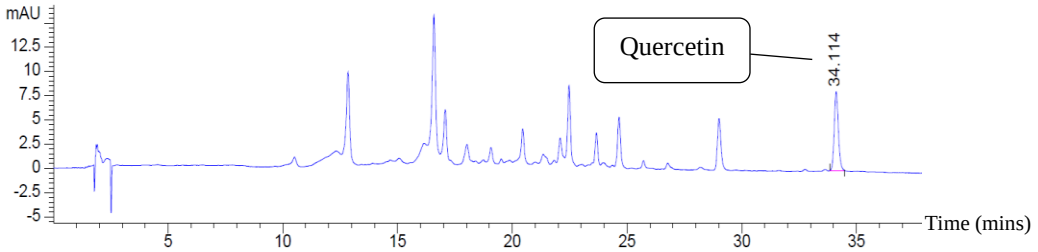


Fig. 3. Comparison of HPLC chromatograms of standard marker, aqueous extract and acid-hydrolyzed extract at wavelength 325 nm.

a. Standard marker (quercetin)



b. Aqueous extract of roselle extract



c. Acid hydrolysis of roselle extract

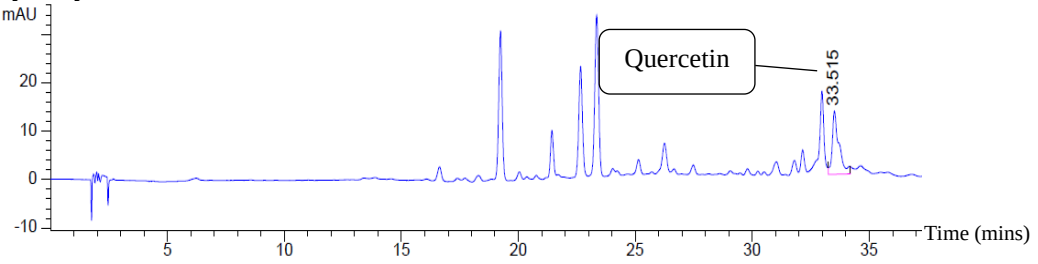
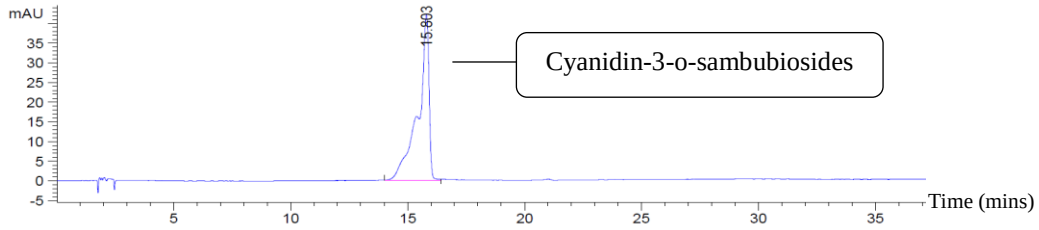
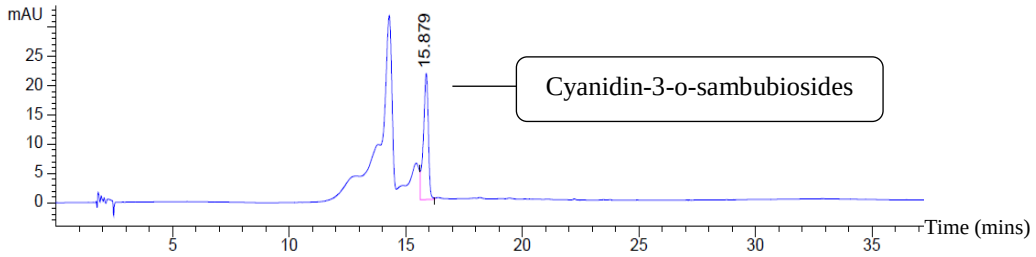


Fig. 4. Comparison of HPLC chromatograms of standard marker, aqueous extract and acid-hydrolyzed extract at wavelength 365 nm.

a. Standard marker (cyanidin-3-o-sambubiosides)



b. Aqueous extract of roselle extract



c. Acid hydrolysis of roselle extract

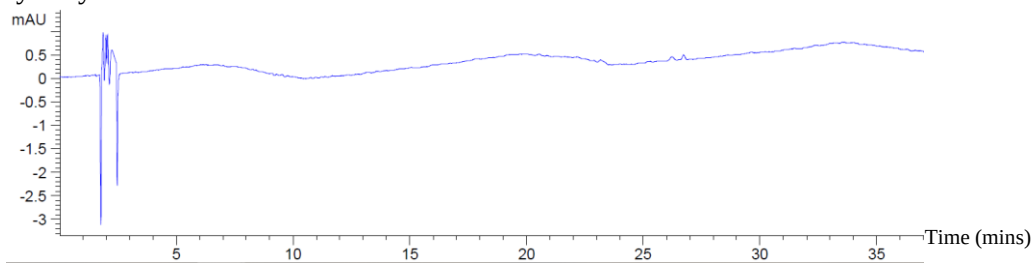


Fig. 5. HPLC chromatograms of standard marker cyanidin-3-o-sambubiosides, aqueous extract and acid-hydrolyzed extract at wavelength 520 nm.

4. Conclusion

After the acid hydrolysable process, roselle extract showed higher biological activity and more active compounds content than roselle aqueous extract before acid hydrolysis condition. Acidic conditions may affect the chemical structure of active compounds which increases biological activity. Therefore, the acid hydrolysable method of roselle should be developed to improve biological activity and increase some active compounds content to be higher than in a roselle aqueous extract. For the suggestion to the next research, it should be used gastric juice instead hydrochloric acid because when the health supplement pass to the stomach, there will digest by gastric juice in stomach. This is the first report for acid hydrolysis of aqueous extract and biological activity such as anti-inflammatory and antioxidant activity.

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