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Formula of *Piper crocatum, Cinnamomum burmanii,* and *Zingiber officinale* extracts as a functional beverage for diabetics

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Abstract

Red betel (*Piper crocatum* Ruiz & Pav.), cinnamon (*Cinnamomum burmanii* Blume), and red ginger (*Zingiber officinale* var. Rubrum) were shown to have antidiabetic properties. However, there has been no studies on the antidiabetic activity analysis of that three plants formula in a functional beverage. This study aimed to determine the formula of those plants that had the best sensory quality. This research also analyzed the antioxidant and antidiabetic activity of the favored formula. Plants extracts were made by boiling method, while sensory evaluation was analyzed using acceptance test. Antioxidative activity was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and antidiabetic activity was obtained by *in vitro* method. The results showed that the highest acceptance score by the panelists was a formula with the composition of 42%(v/v) red betel leaves, 28%(v/v) cinnamon bark, 15%(v/v) red ginger, and 15% (v/v) lime. The chosen formula had antioxidant activity and α-glucosidase inhibitory activity as much as 873.2 µg/mL and 88.7%, respectively. This antidiabetic activity was higher than 0.01%(w/v) acarbose which had value of 31.1%. Investigation on its water extract showed that red betel contained flavonoids, tannins, and alkaloids. Therefore, it can be concluded that there was a functional beverage possessed high antioxidant and antidiabetic properties which acceptable by the consumers.

Introduction

Diabetes mellitus (DM) is a group of metabolic diseases characterized by increased blood glucose levels due to decreased insulin secretion, the inability of the body to use insulin effectively, or both (American Diabetes Association, 2014). According to Danaei *et al*. (2011), there are 347 million people suffering from diabetes. More than 80% of death from DM occurs in low- and middle-income countries (Mathers and Loncar, 2006). People with DM may undergo complications such as nephropathy, neuropathy, retinopathy, cardiovascular disorders, and hypertension. Those complications occur due to free radicals generated during conditions of hyperglycemia. Actually, free radicals can be reduced by enzymes in human body such as superoxide dismutase (SOD), catalase, and NADPH oxidase (Ceriello, 2003). However, a strong radical attack would lead to more severe DM and its complications.

Diabetic complications can be prevented by taking hypoglycemic drug. They also can be precluded by functional beverage made from herbs and spices that have antioxidant and antidiabetic activities (Rates, 2001). Safithri (2012) reported that the mixture formula of red betel leaves and cinnamon bark extracts could activate SOD and catalase, but inhibit alpha-glucosidase activity. This formula showed antihyperglycemic activity as much as 61% and contained 1067.65 µg/mL phenol in total. Sub-acute toxicity tests showed that consumption of the formula of red betel leaves and cinnamon bark extracts (3:2) at a dose of 1890 mg (BW) for 28 days did not cause toxic effects on mice (Safithri and Fahma, 2008).

Based on the previous results, further research is needed to apply the mixture extracts into a functional beverage products that meet the standards of functional food protocol. A functional food should have raw materials that meet quality standards, give health benefits based on scientific studies, have acceptable sensory characteristics by the consumers, and safe to be consumed (Shimizu, 2002; BPOM RI, 2005). According to the established criteria, the research focused on the sensory characteristics (especially: appearance, color, consumer acceptability) and the determination of functional components which possess antioxidant and antidiabetic activities.

Materials and Methods

Source of materials

Plant materials such as red betel leaves (plant age:

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>4 months, leaves: 4th-8th from the tip), cinnamon bark (plant age: >8 years), and red ginger (plant age: 8-10 months) were obtained from Biopharmaca Research Center, Bogor Agricultural University. The materials were cutted into small pieces and sun-dried from 10:00 to 13:00 o'clock (for 3 days with a total drying time of 9 hours). After that, the material was crushed into a powder by the size of 40 mesh. All chemicals used were analytical grade reagents.

Determination of moisture content

The moisture content was determined according to the AOAC method No. 925.10 (AOAC 2006). The measurements were carried out in triplicate.

Red betel leaves, cinnamon bark, and red ginger extractions

The extracts of red betel leaves, cinnamon bark, and red ginger were made refer to Safithri and Fahma (2008). A total of 10 g of red betel powder was put into 200 mL of distilled water (1:20), then boiled in a closed-container for 15 minutes. The sample was filtered by using cloth, and the volume of resulted-filtrate was measured. The distilled water was added to filtrate until the volume was 100 mL in total. Cinnamon bark and red ginger extractions stage were similar with the extraction of red betel leaves, but the comparison of cinnamon and red ginger with distilled water was 1:10. The solutions obtained were called the stock solution and then stored at 8°C before used.

Formulation of functional beverage

From the stock solutions, red betel leaves and cinnamon bark extracts were mixed by the volume ratio 60% and 40%, respectively. Stevia was added as a sweetener to the mixture with the amount of 0.67%(w/v). The result was referred as a basic formula of functional beverage. Then, the basic formula was optimized by red ginger extract from stock solution and lime juice, with same concentration proportion of both (in %(v/v)): 5, 10, 15, 20, and 25. The mixtures were stirred until homogen and stored in bottles at 8°C for the next stage of research.

Sensory evaluation

Sensory evaluation of beverage formulas was done by acceptance test (Meilgaard *et al.*, 2006). The panelists were 30 untrained panelists consist of undergraduate students, graduate students and staffs of the Dept. Food Science and Technology (FST) and staffs of SEAFAST Center, Bogor Agricultural University. The test was conducted in the Laboratory of Sensory Analysis SEAFAST Center. Five samples with three digit random code were served monadic. Panelists in individual booths were asked to taste the samples (30 mL/panelist) and drink water as neutralizer. Panelists had to give acceptance level of the color and flavor (aroma and taste) of samples by using a 5-point hedonic scale (1 = dislike very much, 2 = dislike, 3 = slightly like, 4 = like, and 5 = like very much).

pH value

The pH value of 30-50 mL of sample was measured using a pH meter based on AOAC method No. 970.21 (AOAC 2006). The pH meter was calibrated with buffer solutions of pH 4.0 and pH 7.0 before used.

Total dissolved solid

Total dissolved solids (TDS) of samples were measured using a refractometer by AOAC method No. 932.14C (AOAC 2000). The filtrate of sample was dripped on the prism of refractometer that has been stabilized, and then the total solid was measured. Before and after being used, the prism of refractometer was cleaned by alcohol (Merck, Germany). TDS was represented in °Brix.

Degree of color

The analysis was performed by the method of Hunter blue (Hutching, 1999) using Minolta Chroma Meters (USA). The sample was placed in a uniform-sized sample container (petri dish) and the values of L, a, and b were measured. L is the brightness parameter (lightness) which has a value of 0 (black) to 100 (white). The a value represents reflected light which produces chromatic colors of red-green mixture, with the value +a (positive) of 0-80 for the red color and the value -a (negative) from 0 - (-80) for the green color. The b value represents chromatic colors of blue and yellow mixture, with the value +b (positive) of 0-79 for yellow color and -b value (negative) of 0 - (-70) for blue color.

Analysis of α-glucosidase inhibition

Analysis of α-glucosidase inhibition referred to method of Sutedja (2003). A 20 µL of sample was added by 980 µL of 0.1 M phosphate buffer pH 7 (Merck, Germany) and 500 µL of p-nitrophenyl-α-D-glucopyranoside substrate 20 mM (Sigma Aldrich, Germany). After incubated at 37°C for 5 minutes, the solution was added by 500 µL of α-glucosidase 2.5 units/mL, and incubated at 37°C for 15 minutes. The reaction was stopped by addition of 2 mL of Na_2CO_3 200 mM (Merck, Germany), and the absorbance was measured at a wavelength of 400 nm with a reference solution using 0.01%(w/v) acarbose. Distilled water

was used as a negative control.

Standard curve was prepared by diluting the p-nitrophenol (pNP) (Cat. 1041, Sigma Aldrich, Germany) into a series of concentrations, i.e 0, 1, 5, 10, 15, and 20 µM. Furthermore, the treatment of the standard curve and negative controls were similar with enzyme activity test of the sample. Extract inhibitions were calculated using the following formula (Sutedja 2003).

$$\%\,\text{inhibition} = \frac{[\text{pNP}]\,\text{negative control} - [\text{pNP}]\,\text{extract}}{[\text{pNP}]\,\text{negative control}} \times 100\%$$

Antioxidant activity assay by DPPH (2,2-diphenyl-1-picrylhydrazyl)

The antioxidant activity was measured by the method developed by Sharma and Bhat (2009). The extract was dissolved in absolute methanol at various concentrations (25, 50, 75, 100, and 200 µg/mL). A 2 mL of sample was added by 2 mL of DPPH 0.1 mM (in methanol) and incubated at room temperature (25°C) for 30 minutes. Absorbance measurements were done at a wavelength of 517 nm. Methanol was used as a correction factor and α-tocopherol compounds with various concentrations (1, 2.5, 5, 7.5, and 10 µg/mL) were used as controls. All materials were purchased from Sigma Aldrich (Germany). Free radical inhibition (%) was measured with the following formula (Alfarabi, 2010).

$$\%\ \text{inhibition} = \frac{\text{absorbance of control} - \text{absorbance of extract} \times 100\%}{\text{absorbance of control}}$$

Total phenolic of the chosen beverage formula

1 mL sample of the formula beverages which had been chosen on the sensory evaluation was added to a test tube containing 1 mL of 95% (v/v) ethanol and 5 mL of deionized water. The mixture was added by 0.5 mL of Folin-Ciocalteu reagent (Sigma Aldrich, Germany), and then incubated at 25°C for 5 minutes. Subsequently, the sample solution was added by 1 mL of Na_2CO_3 5%, and then homogenized and incubated at 25°C for 1 hour in the dark room. Absorbance of the solution was measured at a wavelength of 725 nm. The standard used was tannic acid with the concentration 0, 6.5, 13, 32.5, 65, and 130 µg/mL (Pourmorad *et al*., 2006). Calculation of total phenolic content was done by plotting the sample absorbance value on the line equation obtained from a standard curve.

Secondary metabolites of red betel leaves extract

Secondary metabolites those being analyzed were flavonoid, tannin, and alkaloids. The selection was based on the active compounds which allegedly important in the process of antioxidants and inhibition of α-glucosidase. The first stage was extraction of red betel leaves, which was done by reflux method. A 200 g of dry powder of red betel leaves were extracted with 1 1000 mL of distilled water for 2 hours at 70°C. Ethanol extract was obtained by adding 200 g dry powder of red betel leaves in ethanol 30%. The extract yielded then filtered and freeze dried.

Flavonoids fractionation

Fractionation of flavonoids was done by using the method developed by Mabry *et al.* (1970). Concentrated ethanol-extract and concentrated water-extract were dissolved separately in the water, then put in separating funnels. After that, the extracts were fractionated respectively by n-hexane, chloroform, diethyl ether, ethyl acetate, and n-butanol solvents (Sigma Aldrich, Germany). The initial step, fractionation by 40 mL of n-hexane solvent, resulted n-hexane fraction and water fraction. N-hexane fraction was separated. The water fraction was fractionated by 40 mL of chloroform, resulted chloroform fraction and water fraction. Chloroform fraction was separated. The water fraction was fractionated by diethyl ether, resulted diethyl ether fraction and water fraction. Water fraction was fractionated further by ethyl acetate, resulted ethyl acetate fraction and water fraction. Ethyl acetate fraction was separated. The water fraction was added by n-butanol and concentrated. Fractionation was performed three times using 40 mL of solvent for each fractionation.

Tannins fractionation

Tannin fractionation was done referred to the research method of Makkar and Becker (1994). Concentrated ethanol-extract and concentrated water-extract were dissolved in hot water separately, stirred until homogeneous then filtered and put in separating funnels. Subsequently, the solution was fractionated by n-hexane, acetone: water (70:30) with 0.1% ascorbic acid, chloroform, and ethyl acetate solvents (Sigma Aldrich, Germany). After that, water fraction obtained was concentrated and resulted tannin extract. Fractionation was performed three times using 40 mL of solvent for each fractionation.

Alkaloids fractionation

According to the method of Martono (1983), ethanol extract and water extract were soaked with 10%(v/v) acetic acid in ethanol for minimum 4 hours. Then, the solution was filtered and the filtrate was concentrated up to ¼ of the initial volume.

After being concentrated, NH_4OH was added drop wise until a precipitate formed. The precipitate was separated by centrifugation and washed with 1% NH_4OH. The precipitate was dissolved in chloroform with 10% HCl and then shaken vigorously. There were two phases obtained, namely water phase and chloroform phase. The chloroform phase was added by 10% NH_4OH until the solution becomes alkaline, then added by chloroform. The water phase was discarded and chloroform phase was concentrated to obtain the alkaloid fraction.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

The fraction obtained from the previous step was injected into UPLC-QToF-MS/MS system (Waters, Milford, USA). The LC system was equipped with a binary solvent delivery system, an auto sampler, and a photoiode-array detection system. The chromatography was performed using acquity UPLC BEH C18 (1.7 µm, 2.1×50 mm) column. Two mobile phases composed of (A) consisted of H_2O+0.1%(v/v) formic acid, and (B) consisted of acetonitrile+0.1%(v/v) formic acid. The gradient elution was 0–9 min, 5–90% A. About 5 µL of the fraction was injected with the flow rate kept at 0.3 mL/min and the temperature of the column was maintained at 40°C.

The mass spectrometry was equipped with an electrospray ionization source (ESI). High purity nitrogen was used as the nebulizer and auxiliary gas and argon was used as the collision gas. The Q-ToF mass spectrometer was operated in positive ion mode with a capillary voltage of 3 kV, a sampling cone voltage of 38 V, a cone gas flow of 18 L/h, a desolvation gas flow of 500 L/h, a desolvation temperature of 300°C, a source temperature of 110°C, a collision energy of 6 V, and the full scan spectra from 100 to 1000 Da. The data was analyzed by MassLynx version 4.1 software (Waters Corp., Milford, USA).

Data analysis

Data were analyzed using analysis of variance (ANOVA) of completely randomized design at the 95% of confidence level and $\alpha = 0.05$. The post-hoc test was Duncan test. All data were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

The results showed that sun-drying process was able to reduce the moisture content of simplicia below 12%(w/w). The moisture content of red betel leaves, cinnamon bark, and red ginger were 8.22±0.41%(w/w), 8.72±0.39%(w/w), and 10.53±0.15%(w/w); respectively. The moisture content determined the percentage of water in a sample extract. These values were already in accordance with the criteria of Indonesian National Standard for spice powder category (BSN, 1995). Heat of the sun can maintain the number of active compounds in the simplicia leaves which vulnerable to heat (Igual *et al*., 2010). Low water content also prevent enzymatic process and damage of simplicia by microbes. In addition, proper drying will extend shelf life without changing the active compounds in simplicia (Manoi, 2006).

Table 1. Sensory evaluation of functional beverage formulas

Formula	Composition (Red betel leaves:Cinnamon bark:Red ginger:Lime) in %	Average of hedonic score			Total score
		color	aroma	taste	
1	54:36:5:5	2.8±1.2	2.6±1.2	2.3±1.2	2.8
2	48:32:10:10	3.1±0.9	2.9±1.0	2.8±1.1	2.9
3	42:28:15:15	3.1±0.8	3.1±1.0	2.7±1.1	3.0
4	36:24:20:20	3.0±1.0	3.0±0.9	2.6±1.1	2.9
5	30:20:25:25	3.0±1.3	3.0±1.1	2.6±1.3	2.9

n=30

Sensory evaluation of five beverage formulas showed that formula 3 was the most accepted beverage by the panelists (Table 1). The mean score of color, aroma, and taste of the beverage formula was 3.0 (slightly like). The least accepted beverage by the panelists was formula 1, with mean score of color, aroma, and flavor was 2.8. Three other formulas showed similar acceptance score, with mean total score of 2.9. These results indicated that the addition of red ginger and lime (both each: 15%(v/v)) could improve the sensory quality of functional beverages by Safithri (2012) which only consisted of red betel and cinnamon.

Result of the physical and biochemical function analyses of formula 3 were shown in Table 2. The brightness level of formula 3 was L=31.30. The beverage could be classified to low pH beverage, because the pH value was below 4.6. Safithri (2012) reported that functional beverage from red betel and cinnamon had pH value 5.59. Another research showed that the red ginger functional beverage had a pH value 6.38 (Ibrahim *et al.*, 2015). This result showed that the addition of 15%(v/v) red ginger extract and 15%(v/v) lime extract could reduce the pH value. Low acidity caused pathogenic bacteria difficult to grow so that their germinations were hampered.

The total dissolved solid of formula 3 was as

Table 2. Physical and biochemical functions of the functional beverage (formula 3)

Parameters	Value
pH	2.8±0.06
Color	L= 31.30±0.04; a=9.18±0.03; b= 11.22±0.02
Total dissolved solid (°Brix)	2.60±0.00
Phenolic content (µg/mL)	1385.25±0.96
Antioxidant activity (µg/mL)	873.21±9.16
α-Glucosidase inhibition (%)	88.66±1.25

low as 2.60 °Brix. Other researchers reported that TDS in red betel-based beverage was 15.62 °Brix (Sarah, 2013), whereas in fruit juice-based beverage was in range of 10.2-14.2 °Brix (Pratiwi, 2009). TDS value was directly proportional to the level of beverage viscosity. The increase of the TDS would raise the viscosity (Juszczak and Fortuna, 2004). TDS expressed the amount of dissolved solids and suspended solids, both organic and inorganic compounds, contained in the solution. The difference was caused by the variety of other ingredients in a functional beverage.

Selected formula had total phenol of 1385.25±0.96 µg/mL formula. Compared to Safithri (2012), the addition of 15%(v/v) red ginger extract and 15%(v/v) lime increased the levels of total phenol in functional beverage up to 317.6 µg/mL formula. Another research reported by Sarah (2013) showed that beverage made of emprit ginger (*Zingiber officinale* var. Amarum) had a total phenol value 424.75 µg/mL formula. The addition of honeybee to red ginger beverage reduced total phenolic compounds (Ibrahim *et al*., 2015). Bioactive compounds which measured as total phenolic compounds of the formula of red betel leaves extract and cinnamon bark allegedly belong to flavonoids, tannins, and alkaloids (Shihabudeen *et al.*, 2011; Safithri and Fahma, 2008). The high level of phenolic compounds in formula 3 indicated its potency as an antioxidant beverage (Javanmardi *et al.*, 2003).

The antioxidant activity assay showed that formula 3 could inhibit free radical DPPH compounds with the inhibitory rate of 873.21 µg/mL. This value was higher than functional beverage which only consisted of the red betel and cinnamon, with the inhibitory rate of 631.6 µg/mL (Safithri, 2012). Another research reported that the functional food with cinnamon bark and emprit ginger had antioxidant capacity 442.09 µg/mL (Sarah, 2013). These antioxidants compounds might be from the plants extracts composed the functional beverage.

Table 3. Allegations of molecular weight and molecular formula of secondary metabolites in the water extract of red betel leaves

Type of fraction	Molecular weight (m/z)	Molecular formula
Flavonoids	486.2250	$C_{27}H_{34}O_8$
	418.1765	$C_{26}H_{26}O_5$
	450.2034	$C_{27}H_{30}O_6$
	452.2197	$C_{27}H_{32}O_6$
	470.2294	$C_{27}H_{34}O_7$
	510.2247	$C_{29}H_{34}O_8$
	552.2734	$C_{32}H_{40}O_8$
	570.2461	$C_{31}H_{38}O_{10}$
	610.2780	$C_{34}H_{42}O_{10}$
Tannins	148.0152	$C_8H_4O_3$
	222.0571	$C_5H_{11}N_3O_6$
	283.3210	$C_{19}H_{41}N$
	287.2803	$C_{13}H_{33}N_7$
	578.1620	$C_{24}H_{22}N_{10}O_8$
	594.1556	$C_{23}H_{26}N_6O_{13}$
	539.5256	$C_{34}H_{69}NO_3$
Alkaloids	239.2230	$C_{15}H_{29}NO$
	341.2525	$C_{15}H_{31}N_7O$
	387.2268	$C_{20}H_{29}N_5O_3$
	421.2021	$C_{29}H_{27}NO_2$
	421.2030	$C_{29}H_{27}NO_2$
	459.2233	$C_{25}H_{33}NO_7$
	599.4562	$C_{37}H_{61}NO_5$

According to Alfarabi (2010), Piper crocatum leaves extract could inhibit free radical DPPH with IC_{50} value of 58.82 µg/mL. Prasad *et al*. (2009) reported that *Cinnamomum burmanii* had antioxidant activity, slightly above *Cinnamomum tamala.* Study on the red ginger showed that its rhizome extracts could inhibit free radical DPPH 8.9% (Wan-Ibrahim *et al*., 2010). Compounds that could counteract free radical attack can be used to treat DM and its complications (Maritim *et al.*, 2003; Ceriello, 2003; Baynes and Thorpe, 1999; Baynes, 1991).

Antidiabetic potency of the formula was also showed by the result of α-glucosidase inhibitory analysis. Functional beverage formula 3 could inhibit that enzyme by 88.66±1.25%. This value was higher than 0.01%(w/v) acarbose which could inhibit α-glucosidase only 31.13±1.31%. Another study reported that the functional beverage made of red betel leaves and cinnamon had an inhibitory activity of α-glucosidase by 61±2.55% (Safithri, 2012). The difference of value indicated that the addition of 15%(v/v) red ginger and 15%(v/v) lime improved the inhibition of α-glucosidase by 27%. Safithri and Fahma (2008) reported that red betel decoction could reduce blood glucose level. In addition, antidiabetic activity of ginger extract was higher than cinnamon based on Mccue *et al.* (2005) research. Another research reported that red ginger could enhance insulin synthesis in alloxan-induced rats (Iranloye

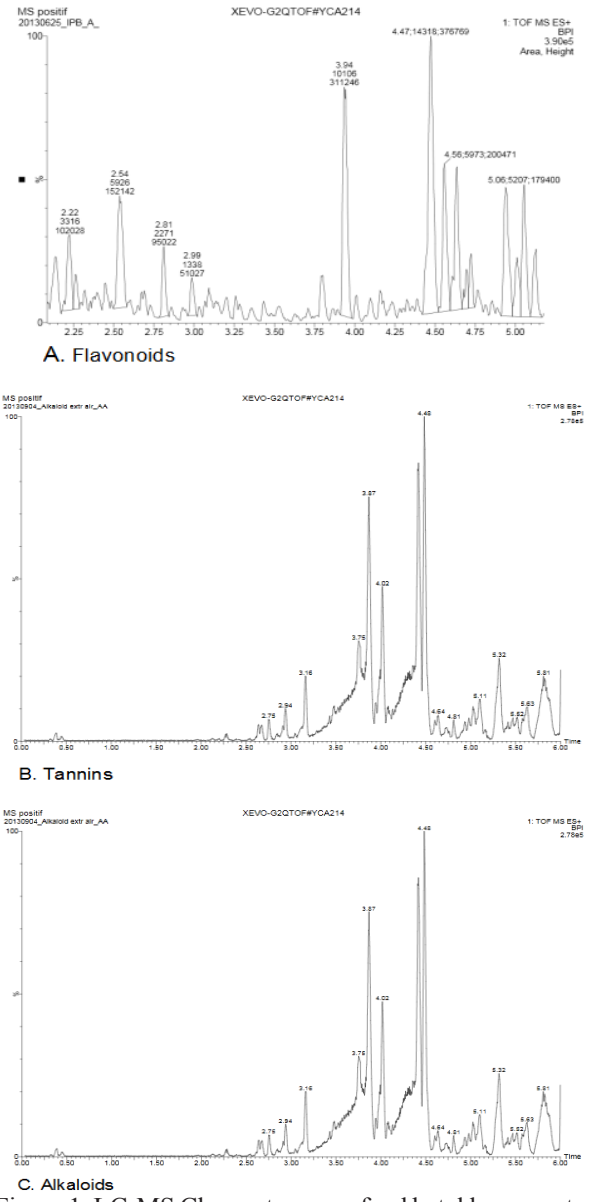

Figure 1. LC-MS Chromatogram of red betel leaves extract

et al., 2011). Alpha-glucosidase inhibitory activity decreased blood glucose levels (Ross *et al*., 2004). Toxicity study of functional drink made of red betel leaves and cinnamon bark revealed that there was no significant bad effect to Sprague dawley albino rats at dose of 1890 mg/kg BW (Safithri *et al.*, 2012).

Analysis of secondary metabolites showed that red betel leaves extract contained flavonoids, tannins, and alkaloids with their derivates (Table 3). LC-MS analysis resulted that the highest peak of flavonoids had a retention time of 4.47 with a molecular weight of 450.2034 (m/z), and the molecular formula estimation was $C_{27}H_{30}O_6$ (Figure 1a). A molecular weight of 450.2034 (m/z) estimated had a formula $C_{27}H_{30}O_6$. This molecule was alleged as flemiphilippinin B or glabrescione B, a class of isoflavonoids (Andersen and Markham, 2005). Tannin compounds with retention time 4.21 and molecular weight 148.0152 (m/z) was estimated to catechin or gallocatechin (Harborne, 1984) (Figure 1b). Chromatogram of alkaloids produced the highest peak with a retention time of 4.48 and the molecular weight of 400.1879 (m/z) (Figure 1c). Estimation of molecular formula of the compound was $C_{23}H_{29}O_6$. However, the least compound was not included in the results on Table 3 because most of the alkaloid compounds contain one or more N atoms (Hopkins and Huner, 2004).

Conclusions

The most preferred functional beverage by the panelists was the formula 3 with the composition of 42%(v/v) red betel leaves, 28%(v/v) cinnamon, 15%(v/v) red ginger, and 15%(v/v) lime. Functional beverage formula 3 had inhibitory activity against α-glucosidase by 88.7% and antioxidant activity by 873.2 µg/mL. The result of the secondary metabolites analysis showed that the flavonoid fraction had nine types of molecular weight, tannin fraction had seven types of molecular weight, and the fraction of alkaloids had seven types of molecular weights.

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