Bioactive Components and Antioxidant Properties of Stevia Beverage

Yohanes Martono⁸ and Hartati Soetjipto⁹

Abstract

Stevia rebaudiana (Bert.) is herb plant which is used to treat type 2 diabetes. It contains bioactive components, stevioside, phenolic and flavonoid as natural antioxidant. The objectives of this study were to develop a green process producing stevia beverage, to determine bioactive components and in vitro antioxidant activity of stevia beverage produced. Stevia beverage was produced by adjusting pH solution and formulated with curcumin and honey. Stevioside was determined by High Performance Liquid Chromatography (HPLC). Total phenolic compounds (TPC) and total flavonoid compounds (TFC) were determined by spectroscopy method. Total antioxidant capacity was determined by phosphomolybdenum method and antioxidant activity was measured by DPPH method. The results showed that pH adjustment from 4.0 – 10.0 influenced stevioside content in solutions ranged from 825.86 – 1349.45 µg/mL. Stevia beverage formulated with curcumin and honey has TPC and TFC, 0.62 mg/mL and 1.087 µg/mL, respectively. Total antioxidant capacity of formulated stevia beverage was 26.5 µg ascorbic acid equivalent/mL. Formulated stevia beverage exhibited DPPH scavenging activity with 57.81% inhibition and IC₅₀ 18.62 mg/mL. It can be concluded from the results that formulated stevia beverage is potentially developed as healthy natural drink with antioxidant activity.

Keywords: Stevia rebaudiana, beverages, stevioside, phenolics and antioxidant

Introduction

Reactive oxygen species (ROS) is unstable molecule that can attack health molecule in our body. Reactive oxygen species (ROS) produced from hyperglycaemia, hyperlipidemia, and inflammatory reactions by increased superoxide anions from mitochondrial. ROS can induce Diabetes Mellitus (DM) complications and vice versa (Sivitz and Yorek, 2010). DM patients were in high risk of complications. DM could damage liver and kidney and make new serious disease (Shivana et al., 2013). Therefore, it is important to prevent DM and its complications.

Stevia leaves have significant hypoglicemic activity and alleviating liver and kidney damage in the STZ-diabetic rats. It can reduce oxidative stress risk from streptozotocin induction in liver and kidney damage (Shivana et al., 2013). Stevia leaves also have antioxidant activity to scavenge DPPH free radical (Tadhanı et al., 2007). Stevia leaf extract contained phenolic compounds that can scavenge free radicals DPPH, hydroxyl, nitrogen oxide (NO), and superoxide anions (Sukhla et al., 2013; Kim et al., 2011). Curcumin is also bioactive compound from turmeric that can be used to help treat various diseases including DM. Healthy effects of curcumin raised from antioxidant and anti-inflammatory activity (Soetikno et al., 2013).

Curcumin has anti-hyperglycemic activity by increasing insulin sensitivity (El-Moselhy et al., 2011). Propolis-active substance from honeybees can prevent DNA damage and lipid peroxidation from liver and kidney in STZ-diabetic rats (Or’sol’c et al., 2013). Combination between stevia, curcumin and honey expected have effect in antioxidant activity to prevent DM and its complications. Chemical structure of stevioside and curcumin was shown in Figure 1.

Methodology

Samples and Chemicals

Stevia rebaudiana (Bert.) was obtained from Bandungan Hill, Semarang, Central Java, Indonesia. Turmeric was obtained from traditional market in Salatiga, Central Java, Indonesia. Honey was obtained from honey shop “MADU JAYA” at Salatiga, Central Java, Indonesia. For standard, stevioside (WAKO) and curcumin (Merck) were used. For reagent, DPPH (Merck), Follin-ciocalteou (Merck), and ammonium molybdate (Merck) were used.

Stevia Beverage production

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Stevia leaves were cleaned and dried less than 50 °C in cabinet drying for 24 hours. Leaves were grinded and kept in glass container. Thirty six gram dried stevia leaf were macerated with 1500 mL hot water gradually for 5 times, each maceration was 1 hour (5 x 300 mL). Solution was filtered and adjusted pH with phosphoric acid until pH 4.00. Solution was filtrated and adjusted pH with calcium carbonate until pH 10.00. Solution was filtered and adjusted pH until pH 7.00 and continued adjusted with citric acid 50% until pH 5.5. The solution was stevia beverage (STvB) and used for formulated stevia beverages (FSTvB).

Stevioside [1,7-bis-{(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione]. R1 = β-Glc; R2 = β-Glc-β-Glc(2→1) (source: Kawamori et al., 1999)
(13-[(2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] kaur-16-en-18-oic acid, β-D-glucopyranosyl ester) (source: FAO.org)

**CURCUMIN**

**Figure 1.** Chemical structure of stevioside and curcumin

**Curcumin Isolation**

Curcumin was isolated with column chromatography as procedure described in Revathy et al. (2011) with modification. Turmeric was cleaned, cutted, and dried in cabynet drying at 30 °C for 2 days. Dried turmeric was grinded and filtered with 40 mesh filter. Fifty gram powder was extracted with 500 mL acetonitrile by using a soxhlet for 6 hours. Solutions was concentrated and calculated percent yield. One gram acetonitrile extract was applied in coloum chromatography with silica gel as stationery phase. Column was eluted with dichlormethane. Curcumin was collected from second fraction. Fractions were plotted in Thin Layer Chromatography (TLC) with dichlormethane: methanol (95: 5, v/v) as eluent. Similar fractions were collected and concentrated. Concentrated extract was added methanol-dichlormethane and precipitated with petoleum ether. The crystals produced was allowed stand in refrigerator.

**Stevia Beverage Formulation With Curcumin and Honey**

Formulation was based on previous study. Formulation has tested with organoleptic assay. STvB was added with 5 ppm curcumin (C) and honey (Hy) 10%. The solution was homogenized and called Formulated Stevia Beverage (FSTvB).

**Stevioside Determination by HPLC analysis**

Stevioside determination was performed on reverse phase C18 column (150 x 4.6 mm) and the compound was monitored with UV detector (Knauer GMBh HPLC System). The solvent system used was H2O: Methanol (70:20) (76%) (Solvent A) and acetonitrile (24%) (solvent B). The solvent isocratic elution was used with flow rate 1.5 ml/min. 20 μl of the sample and standards was injected into the column and the phenolic acids were detected at 210 nm. Retention times and UV-Vis spectra of the peaks were compared with those of the standards.

**Curcumin Determination by HPLC Assay**

Curcumin determination was performed on reverse phase C18 column (150 x 4.6 mm) and the compound was monitored with UV detector (Knauer GMBh HPLC System). The solvent system used was acetonitrile: citrate buffer pH 3.0 (55: 45, v/v).The solvent isocratic elution was used with flow rate 1.0 ml/min. 20 μl of the sample and standards was injected into the column and the phenolic acids were detected at 428 nm. Retention times and UV-Vis spectra of the peaks were compared with those of the standards.

**Total Phenolic Compounds**

The procedure followed as describe Sukhla et al. (2009) with minor modification. 1.0 ml sample was added with 2 mL Folin Claro-Calteau 10% and followed by 2,5 ml Na2CO3 7.5%. Solutions allowed stand 30 minutes at room temperature. Solutions were measured absorbance at 765 nm by using spectrophotometer. Samples tested were WSTvE, STvB, Hy, STvB+Hy, STvB+C, FSTvB, WSTvE+Hy. Total phenolic compound (TPC) was expressed as µg equivalent gallic acid/mL according to equation obtained from gallic acid standard curve.

**Total Flavonoids Determination**

The procedure followed as describe by Chen et al. (2010). Four ml sample was added 0.4 mL NaNO3 5% for 6 min and mixed, into which 0.4 mL Al(NO3)3 10% was added and mixed. After 6 min, solution was added with 4 mL NaOH 4% and incubated for 15 min. Then, solution was measured absorbance at 400 nm by using spectrophotometer. Samples tested were WSTvE,
STvB, Hy, STvB+Hy, STvB+C, FSTvB, WSTvE+Hy. Total flavonoids compound (TFC) was expressed as µg quercetin equivalent/mL according to equation obtained from quercetin standard curve.

Antioxidant Activity Determination by DPPH Assay
The procedure followed as describe Sukhla et al. (2009) with minor modification. Total amount of 0.1 mL sample was added with 2.0 mL DPPH 2 mM and allowed to stand for 30 min in dark room. Then, solution was measure absorbance at 517 nm. Antioxidant activity was expressed as present inhibition as followed:

\[
\text{% inhibition} = \left( \frac{A_{517 \text{ DPPH}} - A_{517 \text{ sample}}} {A_{517 \text{ DPPH}}} \right) \times 100
\]

Samples tested were WSTvE, STvB, Hy, STvB+Hy, STvB+C, FSTvB, WSTvE+Hy, Md, and C. IC50 sample (WSTvE, STvB, FSTvB) to scavenge DPPH radical was determined by using linear regression method.

Antioxidant Capacity by Molybdate Reducing Method
The procedure followed as describe by Pal et al. (2012) with minor modification. Total amount of 0.3 mL sample was added 3 mL molybdate reagent (H2SO4,0.6 M, ammonium heptamolybdate 4 mM, and sodium phosphate 28 mM). Solution was incubated for 90 min at 95 °C. Solution was measured absorbance at 695 nm. Ascorbic acid was used as standard. Samples tested were WSTvE, STvB, Hy, STvB+Hy, STvB+C, FSTvB, WSTvE+Hy, Md, and C.

Data Analysis
Data were analysed by using ANOVA. Significant difference among data means were compare by using t test at 5% significant level. Statistical analysis was performed using software GNU Free Documentation License, Version 1.3.

Result and Discussion
Bioactive Stevioside Profile in Various Solutions
A Stevia beverage production process was based on improving solution visualization. Using water extract only without treatment will give a dark solution as chlorophyll extraction. Solution has low stability on solution colour caused by colour change as chlorophyll degradation. Chlorophyll was degrading rapidly by photo or light impact. Therefore, it needs to treat water extract of stevia for improving solution visualization. Based on our previous study, treatment by using pH adjustment can improve visualization by maintaining stevioside content (Martono and Hastuti, 2013). The result showed that pH adjustment can improve solution visualization. Stevioside is stable in pH range 2.00 – 10.00. Stevioside was influenced by pH alteration. Stevioside content tend to high level in pH 7.00. But, to improve stability and prevent protein precipitation, pH STvB was adjusted to 5.5. Stevioside content in various solutions was shown in Figure 2.

Figure 2. Stevioside content in various solution and pH

Curcumin Isolation
Curcumin is dominant as an active constituent in turmeric. Polarity of acetone is compatible to extract curcumin. According to like dissolve like principal, curcumin is semipolar compound because acetone is semipolar solvent. Curcumin extraction with acetone yielded 37.14% extract. Based on HPLC assay, curcumin isolation developed method yielded high purity curcumin crystal (94.73%) with yield crystal, 4.10%. The result showed that method developed can produce high purity curcumin from turmeric. HPLC chromatogram for curcumin determination was shown in Figure 3.

Figure 3. Curcumin chromatogram (tR = 12,417 min) in crystal produced from turmeric. Purity curcumin crystal is 94.73%. Mobile phase acetonitrile: citrate bufer pH 3,00 (55: 45, v/v).

Stevia Beverages Formulation With Curcumin and Honey and Bioactive component Determination
Based on our previous study, formula developed was water extract stevia that was added with 5 ppm curcumin and 10% honey. Stevia beverages developed expected to give significant effect between its combination.

Stevia beverages was standardized by determining its bioactive component, stevioside and curcumin. HPLC determination of stevioside showed that FSTvB developed contained 321.71 µg/mL of stevioside. Comparing with stevioside content in water extract, stevia solution pH 7.00 and 5.50, FSTvB has lower...
stevioside content than these solutions. It may be due to interaction occurred between bioactive component. Stevioside chromatogram analysis shown in Figure 4.

HPLC analysis of curcumin showed that FSTvB contained 0.023 µg/mL of curcumin. Curcumin content was much lower than concentration added, 5.00 ppm. It may be due to very low concentration of curcumin added, interaction occurred between the bioactive components in FSTvB and or compound degradation. Based on organoleptic test (data not shown), higher curcumin concentration will influence STvB taste and panelis acception.

Figure 4. Stevioside chromatogram, peak 11 (tR = 12.200 min) in FSTvB. Green chromatogram was stevioside standard (tR = 12.190 min).

Total Phenolics Compounds and Flavonoids

Phenolics compounds are commonly had linear correlation with antioxidant activity (Tadhani et al., 2007; Sukhla et al., 2009). Phenolic and Flavonoid compounds have hydroxyl groups that can donate its electron to free radicals. Therefore, phenolic and flavonoid are commonly assumed as antioxidant source. Table 1 show the total phenolic compounds and flavonoid content of samples.

WSTvE has highest both total phenolic compounds and flavonoids. Addition Hy in WSTvE reduced total phenolic compounds but increase flavonoids. There was no significant difference of total phenolic compounds and flavonoids between STvB and its combination with C and Hy both individually and together. Combination STvB with C and Hy found give no significant difference neither in phenolic compounds nor flavonoids content. According Shivana et al. (2013), some major identified phenolic compounds in Stevia leaf are dicaffeoylquinic acid, chlorogenic acid, Quercetin 3-O-xyllose, Apigenin-7-O-glucoside, 3,4-Dimethoxycinnamic acid, Luteolin 7-O-rutinoside, Caffeic acid etc.

DPPH assay

DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. When DPPH radical is scavenged, the colour of the reaction mixture changed from purple to yellow with decreasing of absorbance at wavelength 517 nm. Table 2 shows radical scavenging activity of samples expressed as percent inhibition.

Table 1. Total phenolic compounds (µg equivalent gallic acid/mL samples) and flavonoids (µg equivalent quercetin/mL)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total phenolic compounds (µg/gallic acid/mL)</th>
<th>F-value</th>
<th>Total flavonoids (µg/eq quercetin/mL)</th>
<th>F-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSTvE</td>
<td>1541.6 ± 13.97*</td>
<td>255.51*</td>
<td>255.51*</td>
<td>255.51*</td>
</tr>
<tr>
<td>STvB</td>
<td>499.23 ± 72.31bc</td>
<td></td>
<td>1.10 ± 0.03bc</td>
<td></td>
</tr>
<tr>
<td>Hy</td>
<td>140.13 ± 3.62a</td>
<td>0.39 ± 0.01a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STvB+Hy</td>
<td>678.48 ± 31.04a</td>
<td>1.19 ± 0.01c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STvB+C</td>
<td>475.64± 2.88b</td>
<td>1.03 ± 0.02b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSTvB</td>
<td>615.20 ± 3.71c</td>
<td>1.09 ± 0.02c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WSTvE + Hy</td>
<td>1308.57 ± 17.30d</td>
<td>2.58 ± 0.04e</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are a mean of four trials ± SE (n = 4).
Mean values with the same superscript within a column do not differ significantly (P>0.05).
NS: Non-significant. *Indicates significant difference (P<0.05).

Table 2. Percent inhibition (%) of DPPH radical to scavenge free radical

<table>
<thead>
<tr>
<th>Samples</th>
<th>WSTvE</th>
<th>STvB</th>
<th>STvB+C</th>
<th>STvB+Hy</th>
<th>FSTvB</th>
<th>C</th>
<th>Hy</th>
<th>C+Hy</th>
<th>Ascorbic acid (4 ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% inhibition</td>
<td>74.53±0.46</td>
<td>75.31±0.19</td>
<td>68.01±0.98</td>
<td>69.38±1.57</td>
<td>57.81±5.33</td>
<td>N.D</td>
<td>72.88±1.36</td>
<td>17.89±0.55</td>
<td>82.64±0.00</td>
</tr>
</tbody>
</table>

Values are a mean of four trials ± SE (n = 4).
Mean values with the same superscript within a row do not differ significantly (P>0.05).
N.D = not detected

Table 2 shows that all samples were found to be lower inhibition of DPPH than ascorbic acid (4.00 ppm). STvB were found to be no difference scavenging activity with WSTvE. Hy individually has high scavenging activity but
when combined with STvB and C was found to be lower. It may be due to antagonist interaction between Hy, C and STvB. High antioxidant activity to scavenge free radical of honey is agree with Khalil et al. (2012) study. There were no difference in Hy and C combination with STvB neither individually nor together. C individually was not detected. It may be due to low concentration of curcumin added. Curcumin in appropriate concentration should has antioxidant activity to scavenge free radical (Asouri et al., 2013). STvB alone was found to be higher antioxidant activity to scavenge free radical than FSTvB.

IC\textsubscript{50} between WSTv, STvB and FSTvB to scavenge 50% DPPH radical were found to be 15.83, 9.16, and 18.62 mg/mL, respectively. STvB was the strongest antioxidant solution among these solutions (Figure 5). This show that other constituent beside phenolic compounds may be involve in antioxidant activity. Some non-phenolic compounds that have antioxidant property are micronutrients such as selenium, zinc, manganese (Mandal and Madan, 2013), vitamin C, A, E, carotenoids (Sutradhar et al., 2013), or chlorophyll derivate such as pheophytin (Yun Hsu et al., 2013).

Figure 5. IC\textsubscript{50} between WSTv, STvB and FSTvB to scavenge 50% DPPH radical

Table 3. Antioxidant capacity (µg equivalent ascorbic acid/mL samples) samples

<table>
<thead>
<tr>
<th>Samples</th>
<th>WSTvE</th>
<th>STvB</th>
<th>STvB+C</th>
<th>STvB+Hy</th>
<th>STvB+C+Hy</th>
<th>C</th>
<th>C+Hy</th>
<th>WSTvE+Hy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reducing capacity</td>
<td>14.44±0.11</td>
<td>6.41±0.10</td>
<td>7.17±0.29</td>
<td>43.08±0.16</td>
<td>31.41±0.16</td>
<td>0.18±0.01</td>
<td>35.02±0.44</td>
<td>39.69±0.18</td>
</tr>
</tbody>
</table>

Values are a mean of four trials ± SEM (n = 4). Mean values with the same superscript within a row do not differ significantly (P>0.05).

**Conclusion**

It can be concluded that there was no difference in the effects considered possible by curcumin (low concentration) and honey combination with stevia beverages on the target of lowering blood glucose activity. Stevia beverage has potency as a natural antioxidant. Importantly, the combination of individual effects of stevia beverages involving honey or curcumin was more effective for enhancing antioxidant capacity.

**Antioxidant Capacity by Reducing Molybdate**

Natural sources can act as antioxidant by donating their electron to free radicals. Electron transfer from natural antioxidant will reduce substance gained electron. Therefore, antioxidant capacity is related with reducing power of natural antioxidant. Once substance that can be reduced is molybdate. Antioxidant capacity based on sample ability to reduce molybdate was shown in Table 3.

Hy has high antioxidant capacity. C enhanced antioxidant capacity of Hy. But, when both C and Hy combined with STvB, it was found to be lower in antioxidant capacity. Combination STvB with Hy individually could increase antioxidant capacity solution and found to be higher than FSTvB. This also occurred on combination STvB with C. Although, STvB+C has lower antioxidant capacity than STvB+Hy, C can enhance antioxidant capacity of STvB. It means that combination individually between STvB with C or Hy was more effective in increasing antioxidant capacity of solution. Combination WSTVE with Hy was the highest antioxidant capacity. Reducing power of natural antioxidant such as honey are commonly related with polyphenolic compounds (Khalil et al., 2012).

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**References**


