Antibacterial, Antioxidation, Antiproteolytic, and Cytotoxicity Activity of 

_Stevia rebaudiana_ Bertoni Leaves

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Abstract

This study aimed to evaluate the biological activity of _Stevia rebaudiana_ Bertoni leaves extract using ultrasound assisted extraction (42-45 kHz). The plant material was collected from Chiangmai province and dried plant was extracted with n-hexane, CH₂Cl₂, and MeOH respectively. The obtained extracts were tested for antioxidant, antiproteolytic, antibacteria and cytotoxicity activity. The n-hexane extract concentration at 0.14 mg/mL was the highest antioxidation activity (65.83%). The CH₂Cl₂ extract concentration at 0.1 mg/mL had the highest antiproteolytic activity (39.27%). The anti-bacteria static activity on gram positive and gram negative bacteria was determined by the disc diffusion method. Extract concentration at 10 mg/mL of CH₂Cl₂ inhibited _Vibrio parahamalyticus_ growth (4.33 mm) best. At a concentration of 10 mg/mL, MeOH extract exhibited the highest inhibitory activity against _Escherichia coli_ (4.00 mm) and was found to inhibit Micrococcus luteus best (4.00 mm). The extract concentration at

บทคัดย่อ

การศึกษานี้ มีจุดมุ่งหมายเพื่อประเมินฤทธิ์ทางชีวภาพของสิ่งสกัดจากใบหญ้าหวานที่สกัดด้วยอัลตราซาวนด์ ค่าความถี่ระหว่าง 42 ถึง 45 กิโลเฮิรตซ์ โดยได้เก็บตัวอย่างพืชจากจังหวัดเชียงใหม่และสกัดพืชแห้งด้วยตัวทำละลาย เนต คลอโรเมทANE และเมทแอนอล ตามลำดับ จากสิ่งสกัดได้มาทดสอบ ฤทธิ์ต้านออกซิเดชัน พฤทธิ์ยับยั้งเอนไซม์โปรตีโอไลติก พฤทธิ์ยับยั้งแบคทีเรีย และการเป็นพิษต่อเซลล์ พบว่า สิ่งสกัดด้วยเนตความเข้มข้น 140 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์ต้านออกซิเดชันมากที่สุด (65.83%) สิ่งสกัดโดยคลอโรเมทANE ความเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์ยับยั้งэнไซม์โปรตีโอไลติกมากที่สุด (39.27 เปอร์เซนต์) การทดสอบฤทธิ์ยับยั้งแบคทีเรียแกรมบวกและแกรมลบด้วยวิธี disc diffusion method พบว่า สิ่งสกัดจากเนตความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตรมีฤทธิ์ยับยั้ง _Vibrio parahamalyticus_ (4.33 มิลลิเมตร) ได้มากที่สุด สิ่งสกัดจากเนตความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตรมีฤทธิ์ยับยั้ง _Escherichia coli_ (4.00 มิลลิเมตร) และ _Micrococcus luteus_ (4.00 มิลลิเมตร) ได้มากที่สุด สิ่งสกัดจากเนตความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตรมีฤทธิ์ยับยั้ง _Staphylococcus aureus_ (6.33 มิลลิเมตร) และ _Bacillus subtilis_ (5.92 มิลลิเมตร) ได้มากที่สุด และผลกระทบต่อแบคทีเรียเป็นพิษต่อเซลล์ของสิ่งสกัดความเข้มข้น 50 ในโครงการต่อ มีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็ง (NCI-H187) และ มีความเป็นพิษต่อเซลล์ไตของลิง (Vero cell) มีค่าการยับยั้งการเจริญของเซลล์มะเร็ง 98.45 เปรียบชนะ และมีค่าการเจริญของเซลล์ไต มีค่า 14.28 เปรียบชนะ ตามลำดับ การศึกษานี้พบว่า การสกัดสารสกัดจากหญ้าหวานมีประสิทธิภาพและมีค่าการยับยั้งการเจริญของเซลล์บัคทีร์ Vero สูง ซึ่งควรพิจารณาการใช้สิ่งสกัดเมื่อใช้สกัดเนื้อเยื่อกระดูกชาร์น
10 mg/mL of \( n \)-hexane extract was the highest effect on \( Staphylococcus aureus \) (6.33 mm) and was the highest inhibition on \( Bacillus subtilis \) (5.92 mm). At 50 \( \mu \)g/mL of concentration, this extract had cytotoxicity effect on lung cancer cell (NCI-H187) and kidney cell of green monkeys (Vero cell). The growth inhibition of lung cancer cell line was 98.45% and the cell growth of Vero cell was 14.28%. This study supported the efficient ultrasound to extract the active ingredients. Normal cell of Vero cell was toxic with high level, that should be considered for dose limiting with this application.

**Keywords:** Stevia rebaudiana: antioxidation activity: antiproteolytic activity: antibacterial activity: cytotoxicity

**Introduction**

*Stevia rebaudiana* Bertoni is a natural sweetener and the plant species is in the genus *Stevia* of the sunflower family (Asteraceae) (Figure 1). The phytochemicals in this plant are glycosides, alkaloid, saponins, tannin, and sugar [1] including soluble vitamin of folic acid (52.18 mg/100 g), vitamin C (14.98 mg/100 g), vitamin B12 (0.43 mg/100 g), vitamin B6, niacin, and thiamine [2].

![Figure 1](image1.png)

*Figure 1 Stevia rebaudiana* Bertoni

The glycosides present in *S. rebaudiana* are stevioside (9.1%), rebaudioside A (3.8%), rebaudioside C (0.6%), and dulcoside (0.3%) [3]. Rebaudioside The sweetness in this plant is 200 to 300 times of sucrose (Table 1).

**Table 1 Sweetness potency of sweetener [4]**

<table>
<thead>
<tr>
<th>Sweetener</th>
<th>Approximate potency (times)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acesulfame-K</td>
<td>200</td>
</tr>
<tr>
<td>Aspartam</td>
<td>200</td>
</tr>
<tr>
<td>Rebaudioside A</td>
<td>200-300</td>
</tr>
<tr>
<td>Neotame</td>
<td>8,000</td>
</tr>
<tr>
<td>Saccharin</td>
<td>300</td>
</tr>
<tr>
<td>Sucralose</td>
<td>600</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1</td>
</tr>
</tbody>
</table>

This plant consists of phytochemicals having biological effects, for example, antioxidation activity [5-8] that includes antibacterial activity and antifungal activity [9,10], antiretrovirus activity [11], and antidiabetic activity [12,13]. Extraction is one of bioprocess technology to separate constitutes from matrix. That the conventional extraction can improve yield by ultrasonication application [14-16]. Ultrasonication process is liquid irradiation by ultrasonic waves agitation. Sound waves propagate into the liquid causing alternating high-pressure (compression) and low-pressure (rarefaction) cycles. During rarefaction, high-intensity sonic waves create small vacuum bubbles in the liquid that can collapse violently during compression.
As a development of previous results, this study aimed to evaluate some biological activities by ultrasonication application from *S. rebaudiana*. That antibacterial activity, antioxidation activity, antiproteolytic activity, and cytotoxicity are our interests. Supporting by previous studies have found the phytochemicals from this plant has antibacterial, antioxidation, and cytotoxicity activity in the conventional extraction.

**Materials and Methods**

1. **Plant preparation and Extraction**

*S. rebaudiana* Bert. leaves were collected from Chiangmai province in November 2013. The plant sample was dried and ground before partition extraction of 300 g by ultrasonication bath at 50°C for 30 min. with *n*-hexane, CH₂Cl₂, and MeOH respectively. The sample was extracted in duplicate. The solvent was evaporated under a vacuum to obtain a crude extract. This extract was left until the remaining solvent evaporated at room temperature before assay.

2. **Antioxidation activity assay [17]**

Crude extracts at 0.5, 1, 5 and 10 mg/mL were diluted in ethanol 95%. 0.1 mM of DPPH solution was prepared in ethanol 95%. Antioxidation activity was determined by 100 uL of crude extract suspension and 700 uL of DPPH solution was mixed before incubation under the dark for 30 min. The sample was assayed in triplicate. The absorbance was then determined at 517 nm. DPPH inhibition was calculated according to an equation by ascorbic acid as the positive control and ethanol 95% as the negative control.

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

Where \( A_{\text{control}} = \) Absorbance of DPPH solution \\
\( A_{\text{sample}} = \) Absorbance of sample

3. **Antiproteolytic activity assay [18,19]**

Crude extract at 10 mg/mL was suspended in 50 mM Tris buffer saline (pH 6.74-8.53). Sample 5 µL was mixed with 500 µL of 0.7 – 1.0% (w/v) of Bovine Serum Albumin in this buffer. It was then incubated at 72°C for 5 minutes and cooled at room temperature for 20 min before absorbance determination at 660 nm compared to Tris- buffer saline (negative control) and ibuprofen (positive control).

4. **Anti-bacterial activity assay by agar disc diffusion**

A single colony of bacteria of *M. luteus*, *S. aureus* and *B. subtilis* (gram positive) and *E. coli* and *V. parahaemolyticus* (gram negative) was isolated by cross-streaking on nutrient agar and incubation at 37°C for 24 hr. It was suspended in 0.85% of NaCl and the turbidity was adjusted with McFarland No. 0.5 (1×10⁸ CFU/mL). This suspension was spread-plated on nutrient agar and crude suspension concentrations at 10 and 20 mg/mL in 95% ethanol at 20 µL were pipetted on AA disc. Standard levofloxacin was a positive control, concentration at 5 mg/mL (2 µL) was a positive control. It was then incubated at 37°C for 24-48 hr and clear zone inhibition was evaluated in comparison to the control.

5. **Cytotoxicity on NCI-H187 by Resazurin microplate [20]**

NCI-H 187 cell line was suspended in concentration at 6.6 x 10⁶ cell/mL and the sample at 5 µL was diluted in 45 µL of 5% DMSO in microplate before incubation at 37°C under 5% of CO₂ atmosphere for 5 days. Resazurin
concentration at 62.5 µg/mL was 12.5 µL was added and then incubated at 37°C for 4 hr before measurement the absorbance at 530 nm and 590 nm. Ellipticine was a positive control and 0.5% DMSO was a negative control. Percent Inhibition was calculated according to the equation:

\[ \% \text{ Inhibition} = \left( 1 - \frac{FU_T}{ FU_C} \right) \times 100 \]

where

- \(FU_T\) Absorbance of sample
- \(FU_C\) Absorbance of control

6. Cytotoxicity on Vero Cell [21]

Cell line was suspended in 10% of fetal bovine serum containing 2 mM of L-glutamine, 1 mM sodium pyruvate, 1.5 g/L of sodium bicarbonate, and 0.8 mg/mL of geneticin at 37°C. It was then incubated under 5% of CO₂. A cell suspension concentration of 3.3×10⁴ cell/mL at 5 µL was diluted with 45 µL of 5% DMSO in microplate incubation for 4 days at 37°C under 5% of CO₂. Ellipticine was the positive control. The absorbance was determined at 631 nm on the 1st day and 691 nm on the 4th day. Percent of inhibition was calculated according to the equation:

\[ \% \text{ Cytotoxicity} = \left( 1 - \frac{FU_T}{FU_C} \right) \times 100 \]

where

- \(FU_T\) Absorbance of sample
- \(FU_C\) Absorbance of control

### Results and Discussion

#### Antioxidation activity

The antioxidation activity of \(n\)-hexane extract yielded the highest activity compared to MeOH and CH₂Cl₂ extract (Table 2).

#### Table 2 Antioxidation Activity of Crude Extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/mL)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane Extract</td>
<td>140</td>
<td>65.83±3.69</td>
</tr>
<tr>
<td>CH₂Cl₂ Extract</td>
<td>140</td>
<td>36.92±2.85</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>100</td>
<td>53.83±0.39</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>0.03</td>
<td>91.61±0.19</td>
</tr>
</tbody>
</table>

This was similar to Ahmad et al. (2010) the reports using shaking extraction and Shukla et al. (2009) using soxhlet extraction that observed from EtOH extract activity. Kim et al. (2011) also observed that activity from callus yields less than leaves using high polarity solvent of water.

#### Antiproteolytic activity

The antiproteolytic activity of \(S. rebaudiana\) leaves extract with CH₂Cl₂ at 39.27% yield had higher activity than \(n\)-hexane (25.62%) and MeOH extract (24.72%) respectively (Table 3).

#### Table 3 Antiproteolytic Activity of Crude Extract

<table>
<thead>
<tr>
<th>Crude Extract</th>
<th>Concentration (µg/mL)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-Hexane Extract</td>
<td>25.62±2.22</td>
<td></td>
</tr>
<tr>
<td>CH₂Cl₂ Extract</td>
<td>39.27±2.81</td>
<td></td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>24.72±1.10</td>
<td></td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>92.38±0.12</td>
<td></td>
</tr>
</tbody>
</table>
The antiproteolytic assay was the first reported in this study, and this property was postulated in relation to antiinflammatory activity. During inflammation, inter alpha inhibitor (IAI) reacted with inflammation association protein (TSG-6), pentraxin (PTX3) and vitronectin (VN), TSG-6 reacted with hyaluronic acid (HA) and heavy Chain Lal bonding to extracellular matrix (EMC) caused antiproteolytic activity. Bonding to EMC may cause NF-kappaB and Erg-1 transcription factor inhibition [22].

**Antibacterial activity**

The crude extracts of *S. rebaudiana* leaves had antibacterial activity against the sample of gram positive and gram negative bacteria (Table 4).

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>V. parahamalyticus</em></th>
<th><em>E. coli</em></th>
<th><em>S. aureus</em></th>
<th><em>M. luteus</em></th>
<th><em>B. subtilis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear zone Inhibition (mm) ± SE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>n</em>-Hexane Extract</td>
<td>3.92±0.92</td>
<td>1.33±0.22</td>
<td>6.33±0.55</td>
<td>3.50±1.45</td>
<td>5.92±0.44</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$ Extract</td>
<td>4.33±0.96</td>
<td>1.67±0.17</td>
<td>4.92±0.17</td>
<td>2.75±1.38</td>
<td>5.33±0.33</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>4.00±1.5</td>
<td>4.00±0.46</td>
<td>2.75±1.38</td>
<td>4.00±0.14</td>
<td>1.83±1.17</td>
</tr>
</tbody>
</table>

That was the first ultrasonication application testing in *M. luteus*. Clear zone inhibition was related to Jayaraman *et al.* (2008) using shaking extraction and Ghosh *et al.* (2008) using soxlet extraction of *E. coli, B. subtilis* and *S. aureus*. Tomita *et al.* (1997) [23] found that ferment aqueous extract inhibited *V. parahaemalyticus* VP 78 more than *S. aureus* ST86.

**Cytotoxicity**

CH$_2$Cl$_2$ extract at 50 µg/ml of *S. rebaudiana* was toxic on NCI-H187 cell similar to Ellipticine (4 µg/ml) effect (Table 5).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Fluorescence Unit Mean ±SD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellipticine</td>
<td>4.00</td>
<td>3,064±263</td>
<td>98.34</td>
</tr>
<tr>
<td>CH$_2$Cl$_2$-Extract</td>
<td>50.00</td>
<td>3,019±229</td>
<td>98.45</td>
</tr>
</tbody>
</table>
Even though the cytotoxicity on NCI-H187 of this plant was never reported but it was observed this activity in 2’-O-acetyl cerleaside A isolated from seed of Cerbera odollam [24]. Glycosides toxic on cell by it may inhibit fibroblast growth factor-2 (FGF-2) exporting through the membrane interaction with the Na1. K1-ATPase pump of cancer [25-27]. Supporting by glycosides are important of phytochemicals in S. rebaudiana [28].

CH\textsubscript{2}Cl\textsubscript{2} extract of S. rebaudiana at 50 µg/mL had cytotoxicity on Vero Cell and the viability cell was 14.28% (Table 6) which showed more activity than shaking extraction (150 rpm) with acetone and dilution to 1:8 of sample which was nontoxic on this cell according to Jayaraman et al. (2008).

**Table 6 Cytotoxicity Activity on Vero Cell**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/mL)</th>
<th>Fluorescence Unit Day0</th>
<th>Fluorescence Unit Day4</th>
<th>Growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero cell + DMSO</td>
<td>DMSO 5 %</td>
<td>1,189±40</td>
<td>2,920±127</td>
<td>100.00</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>0.13</td>
<td>1,207±37</td>
<td>2,736±174</td>
<td>88.32</td>
</tr>
<tr>
<td>CH\textsubscript{2}Cl\textsubscript{2}-Extract</td>
<td>50</td>
<td>1,260±29</td>
<td>1,507±24</td>
<td>14.28</td>
</tr>
</tbody>
</table>

**Conclusion and Suggestion**

S. rebaudiana extracts by ultrasonication with n-hexane, CH\textsubscript{2}Cl\textsubscript{2} and MeOH had biological activity, including antibacterial, antioxidation, antiproteolytic, and cytotoxicity activity. Vero cell toxicity was observed with higher activity by ultrasonication application compared to conventional extraction.

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**References**


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