Antibacterial, Antioxidation, Antiproteolytic, and Cytotoxicity Activity of *Stevia rebaudiana* Bertoni Leaves

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บทคัดย่อ

การศึกษานี้ มีจุดมุ่งหมายเพื่อประเมินฤทธิ์ทางชีวภาพของสิ่งสกัดจากใบหญ้าหวาน ที่สกัดด้วยอัลตราซาวนด์ ค่าความถี่ระหว่าง 42 ถึง 45 กิโลเฮิรตซ์ โดยได้เก็บตัวอย่างพืชจากจังหวัดเชียงใหม่และสกัดพืชแห้งด้วยตัวทำละลายเฮกเซน ไดคลอโรมีเทน และเมทานอล ตามลำดับ นำสิ่งสกัดที่ได้มาทดสอบ ฤทธิ์ต้านออกซิเดชัน ฤทธิ์ยับยั้งเอนไซม์โปรตีโอไลติก ฤทธิ์ยับยั้งแบคทีเรียและการเป็นพิษต่อเซลล์ พบว่า สิ่งสกัดด้วยเฮกเซนความเข้มข้น 140 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์ต้านออกซิเดชันมากที่สุด (65.83%) สิ่งสกัดไดคลอโรมีเทนเข้มข้น 100 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์ยับยั้งเอนไซม์โปรตีโอไลติกมากที่สุด (39.27 เปอร์เซนต์) การทดสอบฤทธิ์ยับยั้งแบคทีเรียแกรมบวกและแกรมลบด้วยวิธี disc diffusion method พบว่า สิ่งสกัดด้วยตัวทำละลายไดคลอโรมีเทนความเข้มข้น 10 มิลลิกรัมต่อมิลลิลิตรมีฤทธิ์ยับยั้ง *Vibrio parahamalyticus* (4.33 มิลลิเมตร) ได้มากที่สุด สิ่งสกัดจากเมทานอลยับยั้งการเจริญของ *Escherichia coli* (4.00 มิลลิเมตร) และ *Micrococcus luteus* (4.00 มิลลิเมตร) ได้มากที่สุด สิ่งสกัดเฮกเซนยับยั้งการเจริญของ *Staphyllococcus aureus* (6.33 มิลลิเมตร) และ *Bacillus subtilis* (5.92 มิลลิเมตร) ได้มากที่สุด และผลการทดสอบฤทธิ์การเป็นพิษต่อเซลล์ของสิ่งสกัดความเข้ม 50 ไมโครกรัมต่อมิลลิลิตร มีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งปอด (NCI-H187) และ มีความเป็นพิษต่อเซลล์ไตของลิง (Vero cell) มีค่าการยับยั้งการเจริญของเซลล์มะเร็ง 98.45 เปอร์เซนต์ และค่าการเจริญของเซลล์ไตมีค่า 14.28 เปอร์เซนต์ ตามลำดับ การศึกษานี้พบว่า การสกัดสารสำคัญด้วยอัลตราซาวนด์มีประสิทธิภาพและมีค่าการยับยั้งการเจริญของเซลล์ปกติ Vero สูง ซึ่งควรพิจารณาจำกัดปริมาณการใช้สิ่งสกัดเมื่อใช้อัลตราซาวนด์

คำสำคัญ: หญ้าหวาน ฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ยับยั้งเอนไซม์โปรตีโอไลติก ฤทธิ์ยับยั้งแบคทีเรีย การเป็นพิษต่อเซลล์

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, CH_2Cl_2, and MeOH respectively. The obtained extracts were tested for antioxidation, antiproteolytic, antibacteria and cytotoxicity activity. The *n*-hexane extract concentration at 0.14 mg/mL was the highest antioxidation activity (65.83%). The CH_2Cl_2 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 CH_2Cl_2 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

10 mg/mL of *n*-hexane extract was the highest effect on *Staphyllococcus aureus* (6.33 mm) and was the highest inhibition on *Bacillus subtilis* (5.92 mm). At 50 µg/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 *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]

Sweetener	Approximate potency (times)
Acesulfame-K	200
Aspartam	200
Rebaudioside A	200-300
Neotame	8,000
Saccharin	300
Sucralose	600
Sucrose	1

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 irridation 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, CH_2Cl_2, 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.

$$\% \text{ DPPH Inibition} = (A_{control} - A_{sample})/A_{control} \times 100$$

Where $A_{control}$ = Absorbance of DPPH solution
A_{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 (1×10^8 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 187cell line was suspended in concentration at 6.6 x 10^4cell/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 CO_2 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;

$$\% \text{ Inhibition} = [1 - (FU_T / FU_C)] \times 100$$

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 sodiun bicarbonate, and 0.8 mg/mL of geneticin at 37°C. It was then incubated under 5% of CO_2. A cell suspension concentration of 3.3×10^4 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 CO_2. Ellipticine was the positive control. The absorbance was determined at 485 nm on the 1st day and 535 nm on the 4th day. Percent of inhibition was calculated according to the equation:

$$\% \text{ Cytotoxicity} = [1-(FU_T / FU_C)]\times 100$$

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 CH_2Cl_2 extract (Table 2).

Table 2 Antioxidation Activity of Crude Extract

Sample	Concentration (μg/mL)	% Inhibition
n-Hexane Extract	140	65.83±3.69
CH_2Cl_2 Extract		36.92±2.85
MeOH Extract		53.83±0.39
Ascorbic Acid	0.03	91.61±0.19

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 CH_2Cl_2 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

Crude Extract	Concentration (μg/mL)	% Inhibition
n-Hexane Extract	100	25.62±2.22
CH_2Cl_2 Extract		39.27±2.81
MeOH Extract		24.72±1.10
Ibuprofen		92.38±0.12

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 LaI 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 4 Clear Zone Inhibition of Bacteria of Crude Extract

Sample	*V. parahamalyticus*	*E. coli*	*S. aureus*	*M. luteus*	*B. subtilis*
	Clear zone Inhibition (mm) ± SE				
n-Hexane Extract	3.92±0.92	1.33±0.22	6.33±0.55	3.50±1.45	5.92±0.44
CH_2Cl_2 Extract	4.33±0.96	1.67±0.17	4.92±0.17	2.75±1.38	5.33±0.33
MeOH Extract	4.00±1.5	4.00±0.46	2.75±1.38	4.00±0.14	1.83±1.17

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_2Cl_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 5 Cytotoxicity on NCI-H187 cell line

Sample	Concentration (µg/ml)	Fluorescence Unit Mean ±SD	% Inhibition
Ellipticine	4.00	3,064±263	98.34
CH_2Cl_2-Extract	50.00	3,019±229	98.45

Eventhough 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_2Cl_2 extract of *S. rebaudiana* at 50 µg/mL had cytoxicity on Vero Cell and the viability cell was 14.28% (Table 6) which showed more activity than shaking extraction (150 rpm) with acetone and dulution to 1:8 of sample which was nontoxic on this cell according to Jayaraman *et al.* (2008).

Table 6 Cytotoxicity Activity on Vero Cell

Sample	**Concentration (µg/mL)**	**Fluorescence Unit Day0**	**Fluorescence Unit Day4**	**Growth (%)**
		Mean ±SD		
Vero cell + DMSO	DMSO 5 %	1,189±40	2,920±127	100.00
Ellipticine	0.13	1,207±37	2,736±174	88.32
CH_2Cl_2-Extract	50	1,260±29	1,507±24	14.28

Conclusion and Suggestion

S. rebaudiana extracts by ultrasonication with *n*-hexane, CH_2Cl_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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