

Original Article

# Identification of the Major Fractioned Compounds and Bioactivity Testing from

# Crude Stevia Ethanolic Extracts.

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Abstract: A medicinal plant Stevia rebaudiana bertoni is considered as a natural non-caloric 17 sweetener. The plant leaf has sweetness potency 200-350 times of sucrose. Diterpenoid 18 steviol glycosides are abundant in stevia leaf which composed of various sweet com-19 pounds mostly stevioside and rebaudioside A and also other compounds. The aim of this 20 study is to evaluate the number of other major bioactive compounds and to prove whether 21 the crude stevia ethanolic extracts (CSEE) have biological activities. The first objective was 22 to identify the major phytochemical profiles after separating the fraction from CSEE. Com-23 pound identities would lead to the information about possible biological and pharmaco-24 logical properties of the compounds. So, the second objective was to test for biological 25 activities of CSEE which would be toxicity, antioxidant, anti-inflammatory, and other re-26 ported properties in harmony with steviol glycosides. From crude stevia ethanolic ex-27 tracts, we have determined major peaks by HPLC. We were able to collect 7 fractions ac-28 cording to their retention time as well as high base intensity peak chromatogram. Then 29 those fractions were analyzed individually by LC-MS/MS. Those compounds were iden-30 tified as oleamide, caffeic acid, apigenin-7-O-glucoside, quiercetin-3-O-rhamnoside, 3,4-31 dicaffeolquinic acid, pinocembrin-7-O-glucoside, protocatechuic acid, chlorogenic acid, 32 rutin, and palmitamide. The CSEE was found to have high antioxidant property by DPPH 33 assay. The extract also had low cytotoxicity and exhibited fastest cell migration in wound 34 healing assay with human primary fibroblast cell at level up to 100  $\mu$ g/ml. Form the data, 35 stevia ethanolic extract is safe to be used for food supplement or cosmetic ingredient 36 which would help to enhance some therapeutic properties for diseases caused by oxida-37 tive stresses and inflammation. Further investigations will use these compounds as key 38 active ingredients for safety, quality control or evaluation of other biological efficacies of 39 using stevia extracts. 40

### Graphical abstract:

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Keywords: Keywords: Stevia rebaudiana, Crude stevia ethanolic extracts (CSEE), HPLC, ESI-LC-44MS/MS, Antioxidant, Anti-inflammation45

#### 1. Introduction

Inflammation is the complex mechanisms response to cell injury by toxic entities, infec-47 tious agents and, noxious stimuli which provoke to initialize healing process. Acute in-48 flammation is an interaction of molecular and cellular events that lead to control infection 49 or injury, reform tissue integrity and restore its function is actively coordinated by this 50 process [1]. Perpetuation of inflammation may develop form repetitive stimulation, poor 51 regulation of inflammatory responses, possible damage normal healthy tissue and subse-52 quently resulting the chronic inflammation [2]. Stevia rebaudiana Bertoni. has been shown 53 potential anti-inflammatory properties against some chronic inflammatory disease, two 54 of the major compounds stevioside and rebaudioside A from *Stevia rebaudiana* already has 55 been reported to exhibit high therapeutic properties and effectively attenuated Chronic 56 liver inflammation and human colon carcinoma [3,4,5]. However very limited information 57 is available regarding other major bioactive compounds of this plant besides stevioside 58 and rebaudioside A. Traditional treatment relies on regimens of Phyto-medicine which 59 has come from wild plants that has been guided researchers for investigating novel med-60 ication strategies to apply for humans instead of depending on single synthetic product. 61 In this regards, medicinal plants are always considering as a tremendous valuable re-62 source from nature and considerably contributed to human survival at this era [6]. A 63 widely known medicinal plant Stevia rebaudiana, a plant belongs to the family Asteraceae 64 is well known for its natural high potency non-caloric sweetener. More than 200 species 65 of plant in the genus Stevia present around the world but only the Stevia rebaudiana has 66 natural sweet taste. Traditionally, the plant leaves are commonly used to combine with 67 other herbal products to treat several chronic diseases in South America such as Paraguay 68 as folk medicine. Steviol glycosides are abundant in the leaves of stevia plant which are 69 responsible for sweetener components. Many reports suggested that stevia plant consists 70 of eight glycosides which are major active sweet compounds- stevioside, steviol bioside, 71

rebaudiosides (A-E) and dulcoside. Most abundant steviol glycosides (stevioside and re-72 baudioside A) are high potency sweetener which has 300 times sweeter more than sucrose 73 by weigh equivalent [7,8] and has been used worldwide. However, apart from the sweet-74 ener part of this plant, it has contained some countable health benefits for example, the 75 leaves of stevia are used as green tea or herbal remedies for sweet taste, keeping naturally 76 refresh mind and body, treating hurt burn. The most reported steviol glycosides, stevio-77 side, has been claimed for antihyperglycemic and antihypertensive [9]. But some reports 78 showed that using leaf or leaf extract was more effective in such properties than using 79 stevioside alone [10]. It has long been known that plant produces many essential phyto-80 chemicals with significant biological activity from the secondary metabolites, such as fla-81 vonoids, phenolic compounds, complex lipids (phenolipids) and vitamins which are nat-82 urally produced during the plant growth. Many studies showed that Stevia leaf extracts 83 has contained a lot of phytochemicals which are a group of phenolic compounds, flavones, 84 flavonoids, terpenes etc. [11, 12]. To clarify what are the actual phytochemical compounds 85 in stevia plant would help to understand synergistic effects on using stevia as a functional 86 food with some therapeutic properties. 87

Stevia ethanolic extracts can produce a significant number of essential bioactive secondary88metabolites those are significantly valuable in pharmaceutical and nutraceutical industry,89cosmetic industry, chemical industry, and food industry as well. In this present study90aims to perform a more in-depth investigation about the major phytochemical composi-91tion of stevia ethanolic extracts besides steviol glycosides in order to evaluate the com-92pounds responsible for various biological activity such as high antioxidant, anti-inflam-93matory and other properties.94

2. Materials and Methods	95
<b>2.1</b> Extraction and purification from crude stevia extracts.	96
Stevia plant was collected in large amounts from cultivated field grown of Sugavia Co. Ltd. at Pakchong, Nakhon Ratchasima.	97 98

The *S. rebaudiana* leaves were dried and crushed into small pieces which was then heated 99 80°C for 1 hr. with water. The infused water and liquid squeezed from boiled plant were 100 removed for steviol glycosides purification. Then the plant residual was dried again, 101 weighted and infused in 80% ethanol 1:10 w/v overnight. Finally, ethanolic extract was 102 filtered and concentrated by rotary evaporator. The concentrated extract was stored in 103 dark at room temperature 25°C. Different solvent extracts can be prepared from dried 104 materials of stevia in the same way. 105

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**2.2.** Sample preparation for HPLC analysis.

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An aliquot of Crude stevia ethanolic extracts (CSEE) was diluted to 0.05g/mL, with 80% 109 ethanol. Then filtration was taken by filter paper, 2~4 µm and syringe 0.22 µm filter before 110 applying to a HPLC vial and injected into the HPLC system. The Peak identification was 111 achieved by comparison of both the retention time and UV absorption spectrum. 112

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The identification of the individual fraction compounds was carried out by a HPLC-MS 115 method. The HPLC quantitation was performed using a Waters HPLC system equipped 116 with a model e2695 separation module system, 2487 dual wavelength detector (Waters 117 Corporation, Milford, MA) and Acclaim 120 C18 column (Thermo Fisher Scientific, 4.6 118 mm × 100 mm, 3  $\mu$ m). The temperature of the column oven was 25 °C. The mobile phase 119 was composed by solution A H2O 100% and ACN 100% solution B. The gradient elution 120 was modified as follows: 0-1min 95% A, 1-1.5 min 90% A, 1.5-2 min 85% A, 2.5-3.5 min 121 75% A, 4-5.5 min 60%A, 5.5-7.5 min 70% B, 7.5-9.5 min 90% B, 10 min 100% B. The post-122 running time was 5 min. Injection volume was 6  $\mu$ L at a flow rate of 1 mL/min. The DAD 123 acquisitions were performed and the chromatograms were detected by monitoring the 124 UV absorbance in the range from 210 to 280nm wavelength. Total seven fractions from 125 chromatographically separated compounds were collected by retention time individu-126 ally.Screening the fraction compounds through HPLC system were operated inde-127 pendently. 128

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#### 2.4. HPLC-HRESI-MS and MS/MS conditions.

The MS/MS data was obtained to a high-resolution mass spectrometer fitted with an elec-131 trospray ionization in positive mode (ESI+) a mass spectrometer equipped with a quadru-132 pole. Cone voltage and capillary voltage were set at 20V. ESI-MS/MS was done for isola-133 tion and fragmentation of target compounds by direct infusion. The multiple reaction 134 monitoring (MRM) and manual quantitative method was developed for each target com-135 pounds. A mass spectrum plots the relative ion intensities against the m/z values, and a 136 series of mass spectra are generated at each time point. 137

## 2.5. Mass spectra analysis.

The MS and MS/MS data were collected for molecular masses and mass fractions of 140 cleaved functional groups. The mass spectra were searched using (1) Pubchem databases 141 (https://pubchem.ncbi.nlm.nih.gov) (2) Massbank databases (https://massbank.eu/Mass-142 Bank/Search) and (3) Chemspider (http://www.chemspider.com/). The compound identi-143 ties were searched from mass spectra with the highest similarity hit to the same compound from more than one database. 145

2.6. Antioxidant activity: DPPH Radical scavenging activity.

/DPPH radical scavenging assay were performed using slight modification of Brand-Wil-147 liams et al method [13]. The crude Stevia rebaudiana ethanolic extracts were tested for their 148 radical scavenging capacity by in vitro DPPH (2,2-diphenyl-1-picrylhydrayl-hydrate) as-149 say. The CSEE (1mg/mL) were diluted in 80% ethanol at different concentrations to pre-150 pare working concentration (1.25, 2.5, 5, and 10 µg/mL). Then, 3mL of 0.1mM DPPH so-151 lution were added with 1mL of different working concentration of CSEE. The reaction 152 mixtures incubated at room temperature for 30 min at a dark place and the reaction be-153 tween different sample concentrations able to reduce the radical DPPH to the yellow col-154 ored diphenylpicrylhydrazine was monitored by spectrophotometer at 517 nm after 30 155 min and quantified the percentage of scavenging activity. 156

Absorbance of Control – Absorbance of Sample	157
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% scavenging = -

 $\times 100$ 

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2.7. Cell Culture. The human primary fibroblast cell (HPF) was cultured in low glucose
containing Dulbecco's modified eagle's (DMEM) media with 10% fetal bovine serum
(FBS). The cells were maintained at a controlled condition followed by 5% CO<sub>2</sub> and 37°C
temperature in an incubator. The cells were required to passaged (1:5 ratio) when the confluency of cells around 80-90%. Hemocytometer were used to normalize cell number before conducting assays.

**2.8.** Trypan Blue Exclusion Assay. It's an effective method to distinguish between living 166 and dead cells [14]. The human primary fibroblast cell was incubated for 24 hours at 5% 167  $CO_2$  and 37°C temperature in a humidified incubator. After getting attached the cell, different working concentration (5.5 µg/mL – 800 µg/mL) of CSEE (1mg/mL) were treated 169 for 24 hours. After the completion of treatment period, trypan blue (0.4%) dye were used 170 to stain the death cells and count the percentage of viable cells. 171

	Total Number of Viable cells		172
% of Viable cells =		—— × 100	
	Total Number of cells (Live + Death)		173
			174

2.9. MTT assay for Cytotoxicity analysis. It's a colorimetric assay to evaluate cytotoxicity 175 of cells [15,16], CSEE (1mg/mL) were subjected to determine adverse effect on human pri-176 mary fibroblast cell. The dose was selected by considering higher percentages of cell via-177 bility (5.5  $\mu$ g/mL to 100  $\mu$ g/mL) for treating human primary fibroblast cell. The human 178 primary fibroblast cells were seeded in 96 well plate at density 1 × 10<sup>4</sup> cells/well/100 µL 179 DMEM and incubate for 24 until cells attach to the bottom of wells. Cells were treated 180 with selected concentration of crude stevia ethanolic extract (5.5, 12.5, 25, 50, 100 µg/mL) 181 for 24 and 48 hours at an incubator considering 5% CO<sub>2</sub> and 37°C. After the treatment 182 ended, medium was removed from each well and  $10 \,\mu\text{L}$  of 5mg/mL MTT solution with 90 183 µL of complete medium (DMEM/10% FBS/Low glucose) was added into each well and 184 incubated for 3 hours at 5% CO2 and 37°C. Finally, 100 µL of DMSO was added into each 185 well to solubilized formazan crystal. Absorbance at 540 nm was measured using micro-186 plate reader. Here, 80% ethanol were used for positive solvent control and untreated cells 187 were served as negative control. 188

	Test Sample Mean Absorbance		189
% of cell viability = —		× 100	190

Negative Control Mean Absorbance

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This calculation was formulated to determine percentages of viable cells.

Percentage of cell viability was plotted against selected concentrations of test sample 193 CSEE. Experiments were performed triplicates and the data were presented as mean +/- 194 SD. 195

**2.10.** Wound Scratch Assay. This assay was used to analyse migration rate of human primary fibroblast cell [17]. Cells were seeded first in 6 well plate at  $2 \times 10^4$  cells/well density, incubated 24 hours under 5% CO<sub>2</sub> and 37°C condition in incubator. After 24 hours of incubated. old medium was removed and scratched horizontally with 200 µL pipette tips. Put 1X Phosphate-buffered saline was added and aspirated carefully to get rid of existing cell debris. The cells were treated with CSEE at different concentrations (50, 100 µg/mL) 201 with serum free DMEM/low glucose media. Scratched untreated cell were used as control.
Photographs were taken by invert microscope 10X magnification at 0, 24, 48 hours. The
wound area and migration rate of human primary fibroblast cells at scratched site were
determined by using ImageJ software. Percentage of wound closure were calculated by
following equation and the statistical analysis were performed using GraphPad Prism 9.

0/ 0337 1 1	Wound area at 0 h – Wound area at 24 and 48 h		207
% of wound clouser =	Wound area at 0 h	× 100	208
			209
			210
3. Results			211
3.1. Identification and qu	antification of major phytochemicals from cru	de ethanolic ex-	212

tracts of Stevia rebaudiana by HPLC-HRESI-MS:

In order to evaluate the major fractionated compounds which were responsible for the 214 various biological activities, the extracts have been subjected to HPLC-DAD. By observing 215 HPLC analysis, we were able to collect 7 fractions according to their retention time as well 216 as high base peak intensity chromatogram (Figure 1). Each fraction has individually de-217 tected by analysis both liquid chromatography- mass spectrometry (LC/MS-MS), (ESI-218 MS/MS) and Orbitrap. Moreover, LC-MS/MS-ESI analysis showed the major compounds 219 according to m/z (table1). After investigating high peak intensities molecular weight of 220 compounds, target those compounds for isolation and fragmentation as well as predict 221 the compound structure and properties. After that, identifying compounds were again 222 examined by using Orbitrap mass analyzer in order to search the exact mass and the 223 MS/MS fragmentation pattern. To compare our data, both orbitrap and ESI-MS/MS were 224 used to screen, the exact mass of the parent ion was calculated. 225

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2.00 6 1.80 1.60 1 1.40 1.20 1.00 AU 0.80 0.60 2 0.40 0.20 0.00 1.00 2.00 3.00 4.00 5.00 6.00 7.00 9.00 0.00 8.00 10.00 Time (Min)

Peak	RT (min)	[M+H] +	[M+H] + (m/z)	Molecular	MS/MS	<b>Tentative Identity</b>
No.		Observed	Calculated	Formula		
1	1.101	281.5	282.2791	C18H35NO	265,149,247,109,93,69	Oleamide
						derivatives
2	2.070	180.2	181.0836	C9H8O4	163,149,121,111	Caffeic acid
						derivatives
3	3.147	432.4	433.3256	$C_{21}H_{20}O_{10}$	271,149,85,71	Apigenin-7-O-
						glucoside
4	4.454	448.2	449.1063	$C_{21}H_{20}O_{11}$	287,303,85	Quercetin-3-O-
						rhamnoside
5	5.189	516.2	517.134	C25H24O12	163,145	3,4-Dicaffeolquinic
						acid derivatives
6	6.734	418.2	419.3080	C21H22O9	149,167,185,287,71,85	Pinocembrin-7-O-
						glucoside
7	7.220	154.1	154.9885	C7H6O4	113,131,72	Protocatechuic acid
8	7.93	354.3	355.0975	C16H18O9	163,145,107,93,79,	Chlorogenic acid
					61,355	
9	8.25	610.5	611.1530	C27H30O16	303,287,85,71	Rutin derivatives
10	9.60	255.2	256.2599	C16H33NO	256,239,102,88	Palmitamide

**Figure 1:** Stevia ethanolic extract fractions base peak intensity (BPI) chromatogram was examined by HPLC (High performance liquid chromatography). 229

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**Table 1:** Identification of these compounds (1-10) according to standards retention time, m/z, and fragmentation mass232pattern.233

This is the first report of major phytochemical profile of fractionated compounds from 234 crude stevia ethanolic extracts (table 1). More than 10 compounds were identified through 235 accurate mass and fragmentation patterns which aided by the existing databases and lit-236 erature reviews. These compounds are fatty amide derivatives (oleamide derivatives (1), 237 palmitamide (10)), phenolic acids and derivatives (caffeic acid (2), 3,4-dicaffeolquinic acid 238 (5), protocatechuic acid (7), chlorogenic acid (8)), flavones and flavonols (apigenin-7-O-239 glucoside (3), quiercetin-3-O-rhamnoside (4), flavanones (pinocembrin-7-O-glucoside (6)) 240 , flavonoid derivatives rutin (9). 241

This study exhibited that, Ethanol was a good solvents for extraction of polyphenols and242fatty acid derivatives from dried stevia plant materials. The solvent also showed minimal243effect on cell assays (Figure 4-5). Such polyphenolic and flavonoid compounds were de-244termined the highest content in those fractions. \*In addition, this study aims to analyze245the phytochemical profile of the stevia ethanolic extracts as well as its fractionated com-246pounds. Moreover, major fractionated compounds identified, those are individually in-247volved in treating several chronic diseases.248

**Oleamide**, a primary fatty amide which was firstly identified in human luteal phase 249 plasma [18]. It's an endogenous brain lipid act as a neuromodulator. The interesting fact, 250

Oleamide in experimental mice model could induce sleep, However the molecular mech-251 anisms of Oleamide on sleep was not well illustrated [19]. Oleamides were categorized as 252 an endocannabinoid compound [20]. It's mostly found in human cerebrospinal fluid un-253 der sleep ablation condition [21]. One recent study demonstrated, oleamide has neuropro-254 tective activity, it can activate or stimulate endocannabinoid system of Central and pe-255 ripheral CB1 or CB2 receptor that can initiate to provide protection against neurotoxic 256 agents [22]. Caffeic acid derivatives has multi-purpose biological properties, Caffeic acid 257 derivatives has potential role against inflammation and can inhibit NF-k $\beta$  pathway which 258 is responsible for expressing pro-inflammatory genes [23]. On the other hand, STAT 3 259 (Signal transducers and activation of transcription 3) is responsible for its crucial role on 260 carcinogenesis, inflammation and proliferation but JAK-STAT3 were considered as the 261 main factor for UV-mediated skin carcinoma, interestingly caffeic acid derivatives can se-262 lectively suppresses JAK-STAT3 expression and induce apoptotic genes expression [24]. 263 This compound has exhibited potential neuroprotective performance against Parkinson 264 disease in cellular model and also showed anti-aging properties as well [25,26]. Besides 265 that, Apigenin-7-O-glucoside, a flavonoid compound that can inhibit the growth of par-266 asite Toxoplasma gondii a zoonotic protozoan disease [27]. Nowadays researchers are look-267 ing for novel plant-based antifungal drugs by considering fungi resistant to many syn-268 thetic drugs. One recent study suggested this compound has ability to combat against 269 colon cancer and also has potent anti-fungal activity against Candida spp., moreover these 270 compounds were used for treatment against some inflammatory disease [28]. Not only 271 anti-parasitic, anti-fungal and anti-inflammatory activity but also this compound has 272 gained considerable attention because of its anti-viral, antioxidant, anti-bacterial, anti-can-273 cer activities [29,30,31]. In vitro study suggested that hemorrhagic and hemolytic activities 274 of snake venom were completely demolished using quercetin-3-O-rhamnoside, so this 275 compound might take place as alternate for anti-venom and could serves as complemen-276 tary treatment for snake bite [32]. Like Caffeic acid, 3,4-dicaffeoylquinic acid were iden-277 tified as a potent anti-inflammatory and anti-fungal constituents [33,34]. On the other 278 hand, pinocembrin which is well used for natural flavonoid drug and can capable enough 279 to cross BBB (Blood-Brain-Barrier), carrying neuroinflammatory activity and also having 280 ameliorative activities against ovarian cancer and can induce ER (endoplasmic reticulum) 281 mediated apoptosis [35,36,37]. A phenolic acid compound protocatechuic acid has diverse 282 biological properties, recently a group of scientists provide information regarding in vitro 283 and in vivo experiments of protocatechuic acid to evaluate anti-wrinkles and anti-ageing 284 potency, protocatechuic acid is now subjected to test in human as clinical trial [38]. Besides 285 that, this compound has other functional properties such as provide protection against 286 nephrotoxicity, hepatotoxicity and prevent inflammatory bone loss [39,40,41]. Chloro-287 genic acid an intermediate of lignin biosynthesis and belongs of polyphenolic compound 288 family. This compound has distinct biological activity. People use this compound as an 289 effective treatment for obesity or weight loss. Additionally, it has been found that, some 290 devastating metabolic disorder such as diabetic, cardiovascular disease, liver disease was 291 ameliorated by using chlorogenic acid in vitro and in vivo [42,43,44]. This compound has 292 been reported to exhibit lethality against some certain types of cancer. It can completely 293 suppress the growth and proliferation of breast cancer, lung cancer and glioblastoma. 294 Chlorogenic acid also shows Anti-inflammatory properties with an absolute efficacy 295 [45,46,47,48]. Rutin derivatives, essential flavonoids which has different biological activ-296 ities. These metabolites had been shown an increasing level of antioxidant and zero cyto-297 toxicity for mammalian cell [49]. Rheumatoid arthritis a joint inflammatory disorder is a 298 common problem for human over several century but some flavonoids and rutin deriva-299 tives had been shown a promising anti-immunomodulatory activity in mice model [50]. 300  $\alpha$ -glycosylated rutin, a water soluble rutin derivatives were used to evaluate its potency 301 for reducing abdominal visceral fat (AVF) and its under clinical trial now additionally 302 some studies have documented rutin as a lipid metabolism mediator [51,52]. Moreover, 303 this metabolite has been reported to have many other significant bioactivities such as anti-304



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**Figure 2:** Molecular structure of identified compounds, screened through HRESI-MS/MS from Crude Stevia ethanolic 310 extract (CSEE). 311

# 3.2 Antioxidant profiling through DPPH scavenging

DPPH scavenging assay is a model attempt for antioxidant profiling, at the room temperature (25°C) it's a stable free radicle and has capability to accept an electron and developed 314 into strong diamagnetic molecules [57]. 517 nm absorbance were used to determine for 315 DPPH reduction induced by antioxidants. To monitoring positive antioxidant activity of 316 crude stevia ethanolic extract (1 mg/mL), different working concentration (1.25, 2.5, 5 and 317 10  $\mu$ g/mL) were prepared. Ascorbic acid was used as positive full scavenging percentage. 318

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Figure 3: DPPH scavenging capacity of Crude *Stevia* ethanolic extract (CSEE).

Crude stevia ethanolic extract showed potent antioxidant activity by scavenging DPPH. 321 Increasing concentration of CSEE 1.25-10  $\mu$ g/mL demonstrated significant DPPH scavenging. 10  $\mu$ g/mL of Crude *Stevia* ethanolic extract and ascorbic acid exhibited 60% and 88% 323 of DPPH scavenging efficacy respectively. Additionally, IC50 value were calculated, IC50 324 value for CSEE and ascorbic acid found to be 7.2  $\mu$ g/mL and 1.4  $\mu$ g/mL. The lowest IC50 325 value represents and associates with the highest DPPH scavenging strength. 326

# 3.3 Effect of Crude Stevia ethanolic extract (CSEE) on Cell Survivability

In the beginning, we analyse *in vitro* cytotoxicity of CSEE and determine non-cytotoxic 328 concentration of CSEE for further in vitro assays. HPF cells were treated with various con-329 centration of CSEE (5.5 µg/mL to 800 µg/mL) for 24 h. In accordance with our findings, 330 cells survivability was significantly lessened, when the concentration of CSEE goes above 331 200 µg/mL. Cell survival below of 80% consider as toxic. In this regard, the nontoxic CSEE 332 concentration range 5.5 to 100 µg/mL were considered which had determined 85-100% 333 cell viability. This nontoxic CSEE concentration (5.5 to 100 µg/mL) were used for following 334 up experiment in order to investigate wound healing potentiality. 335



**Figure 4:** Effect of CSEE (5.5 to 800 µg/mL) on HPF cell survivability after 24 h exposure. 337

MTT assay was employed to determine cytotoxic effect of CSEE on HPF cell viability by 339 reducing MTT. Nontoxic concentration of 5.5 to 100 µg/mL dose were selected according 340 to HPF cell survival that was confirmed from trypan blue exclusion assay. Selected CSEE 341 concentration (5.5 µg/mL to 100 µg/mL) were treated to HPF cell for 24 and 48 hours. 342 However, CSEE did not exhibit any cytotoxic effect on HPF cell both 24 h and 48 h. MTT 343 reduction capacity of CSEE (5.5 to 100  $\mu$ g/mL) were comparable higher than control in 344 both treatment period. 345



Figure 5: MTT reduction percentage performed in HPF. Various working concentration 347 of CSEE were treated for 24h and 48h. Each value represent mean +/- standard error for 348 triplicate. 349

The increasing concentrations of treatment demonstrated significant proliferative effect of 350 these cells (Figure 4) with negative cytotoxic influence.

# 3.5 Wound healing activity of crude stevia ethanolic extract (CSEE)

To observe the wound migration activity on HPF cell, the best concentrations (50 and 353 100µg/ml) of CSEE were selected, that were confirmed from the cell cytotoxicity assay. To 354 determine the wound repair acceleration of CSEE, the wound on the HPF cell was created 355 and treated with 50 and 100 µg/ml of CSEE for 24 to 48 hours. Cell migration were cap-356 tured at 0, 24 and 48 hours and the wound healing distance were measured by ImageJ 357 software (Figure 6). 358

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# **Wound Healing Activity of CSEE**

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Figure 6: Migration of HPF cell in the presence and absence of CSEE. Cell migration ac-360tivity of CSEE through wound closure at 24 and 48h.361

This result demonstrated that, CSEE at different concentrations 50  $\mu$ g/ml and 100  $\mu$ g/ml 362 significantly ameliorated by closing the wound area. 50  $\mu$ g/ml and 100  $\mu$ g/ml treatment 363 of CSEE exhibited 79% and 98% wound closure activity at 48 hours. Our findings demonstrated that, CSEE has a potential cell migration stimulating effect on HPF cell. The figure 365 7 shows the microscopic images of untreated and stevia extract treated (50 and 100 $\mu$ g/ml) 366 HPF cell. 367



**Figure 7:** Microscopic representation of cell migration at 0, 24 and 48h in untreated and CSEE treated HPF cells (50 and  $\mu$ g/ml). The microscopic views were captured under 10X-magnification. (A) represent control, (B) represent 50 $\mu$ g/ml 371 CSEE treatment and (C) represent 100 $\mu$ g/ml treatment. The dash line represents wound scratch boundaries. 372

#### 4. Discussion

The medicinal plant Stevia rebaudiana has shown interesting health benefits and evoked 374 attenuating characteristics against several metabolic disorders. Some previous reports 375 have been proposed on antidiabetic, anti-hypertensive, anti-microbial and renal protec-376 tive properties of this plant [58,59]. Most of the author emphasizes to reveal biological 377 importance of stevioside and rebaudioside, however, those are the most abundant com-378 pounds of this plant [60,61,62,63]. Here, we investigated the major bioactive compounds 379 from the plant beside stevioside and rebaudioside. Employing HPLC technique, we were 380 able to identify 7 major peaks and collect those 7 fractions according to their retention time 381 as well as high base intensity peak chromatogram. Furthermore, we analyzed individual 382 fraction using ESI-LC-MS/MS and identified major compounds according to their mass to 383 charge ratio (m/z) and tandem mass fragmentation pattern (MS/MS) [64]. From the crude 384 stevia ethanolic extract (CSEE), we were able to detect the 10 major bioactive compounds 385 such as oleamide derivatives, caffeic acid derivatives, apigenin-7-O-glucoside, quercetin-386 3-O-rhamnoside, 3,4-dicaffeolquinic acid derivatives, protocatechuic acid, chlorogenic 387 acid, rutin derivatives, and palmitamide beside stevioside and rebaudioside from the 7 388 fractions, respectively. In the bioassay experiments, we preliminary prioritized in vitro 389 antioxidant screening assay through DPPH scavenging. The rationale behind that assay 390 was to evaluate antioxidant capacity of CSEE. The CSEE presented a strong antioxidant 391 activity with the IC50 of 7.2 µg/mL. 392

So, our next aim was to evaluate the cytotoxicity effect of CSEE on human primary dermal 393 fibroblast cell line. We employed trypan blue exclusion method to determine HPF cell 394 survivability upon different courses of CSEE treatment (5.5  $\mu$ g/mL – 800  $\mu$ g/mL). We in-395 vestigated non-toxic concentration of CSEE doses from 5.5 to 100µg/ml, where >80% HPF 396 cell were survived. Cell survivability <80% was determined as cytotoxic and >100µg/ml 397 CSEE treatment exhibited significant cyto-toxicity effect on the HPF cells. Furthermore, 398 the selected treatment doses 5.5-100µg/ml of CSEE were utilized to evaluate cell viability 399 using MTT reducing assay. We performed this assay for 24 and 48h. The HPF cells were 400 treated with selected treatment doses of CSEE (5.5 to 100µg/ml) from trypan exclusion 401 assay. We treated the HPF cells with selected dose manner (5.5 to 100µg/ml) for 24 h and 402 48h. At the 24h, MTT reduction percentage for untreated cell (control) were 80% and for 403 the 48h it was declined at 70%. One the other hand, MTT were reduced by CSEE dose 404 dependent manner For 24h, 5.5 µg/ml exhibited 85% MTT reduction and after 48h it was 405 increased 95%, subsequently, 12.5 µg/ml exhibited 95% and for 48h were 107%, 25 µg/ml 406 at 24h 100% and for 48h 116%, 50 µg/ml at 24h 109% and 48h 120%, and 100 µg/ml at 24h 407 117% and 48h 126% respectively. So, our present study demonstrated that, the increasing 408 of the treatment doses of CSEE (5.5-100µg/ml) significantly accelerated MTT reduction by 409 promoting cells growth. 410

Employing antioxidant screening test in-vitro and wound healing test in cell line or in-411 vivo is the simplest way to evaluate the natural products anti-inflammatory activities 412 [65,66]. Our present study mainly focused on anti-inflammation activity of CSEE on HPF 413 cell. Here we selected HPF cell as a model of dermal subcutaneous cell, because skin is the 414 largest part of human body and acts as primary defense system. But different kinds of 415 adverse stimuli e.g. injury or damage of tissue, UV irradiation can induce inflammation. 416 Here our present study was conducted to investigate the potential anti-inflammatory ac-417 tivity of CSEE. From the previous experiment we determine, 50 and 100µg/ml CSEE treat-418 ment could reduce MTT significantly and proportionally could accelerate the cell growth. 419

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So, we choose 50 and 100µg/ml CSEE dose for wound scratch assay. At the 24 and 48h 420 untreated (Control) cells migrated 11 and 30%. However, the CSEE treated cells, 50 µg/ml 421 at the 24 and 48h migrated towards to repair the wound about 50% and 81%, on the other 422 hand, the 100µg/ml CSEE treated cells for 24 h repair the wound 65%, and the wound 423 were completely repaired at the 48h of CSEE treatment. In this regard, we hypothesized 424 that, the CSEE treatment might induce upregulated fibronectin, Wound heal associated 425 heat shock protein 47 (HSP47) and collagen type 1 gene which promote cell migration by 426 healing the wound [67,68]. This hypothesis still needs further investigation to confirm. 427 Our present study shows the biological importance of CSEE and unveiled the major com-428 pounds from this extract. Our next goal is to standardize fractionated compounds and 429 address some of the biological importance of those compounds individually. 430

#### 5. Conclusions

Beside of steviol glycosides, stevia ethanolic exatract was found to have many phytochem-432 ical compounds. The major compounds which were identified in this study are olemide, 433 caffeic acid, apigenin-7-O-glucoside, quercetin-3-O-rhamnoside, dicaffeolquinic acid, pi-434 nocembrin-5-O-glucoside, protocatechuic acid, chlorogenic acid, rutin and palmitamide. 435 All of these compounds have been reported for many biological activities with low cyto-436 toxicity. The crude ethanolic extract had high antioxidant properties about 20% of ascorbic 437 acid. The extract also had low cytotoxicity which can applied up to 100  $\mu$ g/ml and at this 438 level it exhibited fastest cell migration in wound healing assay. Form these data, they can 439 prove that stevia ethanolic extract is safe to use as a food supplement or a cosmetic ingre-440 dient which would help to enhance therapeutic properties of steviol glycosides or other 441 drugs especially for diseases caused by oxidative stresses and inflammation such as 442 wound, diabetes, hypertension and aging. The vast applications may be claimed in the 443 future after further scientific proves can be tested. 444

Author Contributions: Conceptualization, SR and JM.; methodology, SR, MH, and JM.; software, 446 SR, MH, JM.; validation, SR, MH, PW, MK, and JM.; formal analysis, SR, JM; investigation, SR, MH, 447 JM.; resources, JM.; data curation, SR, JM.; writing-original draft preparation, SR; and JM. writ-448 ing-review and editing, SR, MH, PW, MK, JM.; visualization, SR, MH, JM.; supervision, JM.; pro-449 ject administration, JM. All authors have read and agreed to the published version of the manu-450 script. 451

Funding: This research received no external funding

Acknowledgments: This research was supported by the Department of Biochemistry and Faculty 453 of Science, Mahidol University for facilities and funding. We thank current members of the Depart-454 ment of Biochemistry for support and encouragement. We also show our gratitude to the Center of 455 Neuroscience, Faculty of Science, Mahidol University. 456

Conflicts of Interest: The authors declare no conflict of interest.

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