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In Vitro Evaluation of α-Glucosidase Inhibitory Activity of Fractions from *Stevia Rebaudiana* Collected in Lam Dong, Vietnam

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Abstract: *The α-glucosidase enzyme is involved in carbohydrate degradation and is a target for the treatment of type 2 diabetes. The search for α-glucosidase inhibitors from medicinal plants is of interest due to their safety and low side effects. Natural products from medicinal plants such as Stevia rebaudiana, a plant rich in diterpene glycosides – especially steviol glycosides, are a source of novel, safer metabolic inhibitors. This study aimed to investigate the in vitro α-glucosidase inhibitory potential of different solvent fractions from the ethanol extract of Stevia rebaudiana, and to isolate and identify the major active ingredient from the most potent fraction. The leaves and roots were extracted with ethanol and liquid-liquid fractionation to obtain n-hexane, ethyl acetate and aqueous fractions. All fractions were evaluated for α-glucosidase inhibitory activity by 96-well microplate spectroscopy. The highly active fraction was isolated by column chromatography, purified and its structure was determined by* H^1*-NMR and* C^{13}*-NMR spectroscopy. The ethyl acetate fraction showed the strongest inhibitory activity against 13 α-glucosidases (*IC_{50} *= 17.20 ± 1.16 µg/mL), higher than the positive control Acarbose (*IC_{50} *= 84.03 ± 4.74 µg/mL). The leaves and roots of Stevia rebaudiana are a source of potent α-glucosidase inhibitors. These findings provide a scientific basis for the research on the application of Stevia rebaudiana grown in Lam Dong, Vietnam, both as a natural sweetener and in the control of type 2 diabetes.*

Keywords: Stevia rebaudiana, α-glucosidase, bioassay-guided fractionation, type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus (T2DM) continues to be one of the significant global public health problems, with post-meal hyperglycemia identified to be a key cause of glucotoxicity, β-cell distress, and cardiometabolic risks. One accepted way of pharmacologically achieving a reduction of post-prandial glucose spikes is to inhibit intestinal α-glucosidase, and thus slowing complex carbohydrates hydrolysis and the rate of glucose absorption. This is the mechanism of clinical action of acarbose and its related analogs. Alongside and in addition to drug treatment, there is ongoing epidemiological research into dietary and herbal substances designed to aid in glycemic moderation which could be considered safe and acceptable for long-term use.

Stevia Rebaudiana Bertoni, also referred to as sweet grass, 'Co Ngot" in Vietnamese, is a Paraguay native and a perennial flowering shrub belonging to one of the 145 species of the Stevia genus. For long, its been utilized as a sweetener, herbal medicine, and in folk medicine in South America. Over the last few decades, the cultivation of S. Rebaudiana has spread to and is now found in countries such as Japan, Korea, Taiwan, Thailand, and Indonesia. Stevia was first introduced to Vietnam in 1988. Since then, cultivation has been promoted and developed in many regions across the country, from the northern provinces of Ha Giang, Cao Bang, Son La and Phu Tho to the southern provinces of Lam Dong and Dak Lak.

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Figure 1: Stevia rebaudiana

The most significant compound of S. rebaudiana is the diterpene glycoside known as stevioside; it is a member of the steviol glycoside family. These compounds are recognized as noncaloric sweeteners and are reputed to be 200 to 300 times sweeter than sucrose. Besides their functionality as a sugar substitute, many studies have reported biological activities of steviosides, including antibacterial properties, cardiovascular improvement, and blood pressure control. As a consequence, steviosides are used in the food and beverage sectors of developed countries such as Japan and the United States.

Although there are many international studies on the chemical composition, biological activities, and structural analysis of sweet-tasting compounds in *S. rebaudiana*, research in Vietnam is relatively limited. Previous studies have mainly focused on the agronomic aspects of plant growth and cultivation. There are very few studies related to the optimization of extraction and purification processes of valuable sweeteners from local plant sources and the evaluation of their bioactivities. Therefore, this study was conducted to find an efficient extraction and purification process for stevioside from S. rebaudiana Bertoni and to evaluate the glucosidase inhibitory potential of S. rebaudiana leaf and root extracts.

The results of this study were a meticulous and detailed extraction process. The analysis of each stage, together with the use of modern analytical standards, authenticated the final product through high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) spectroscopy.

2. Materials and Methods

2.1 Plant Material

The leaves and roots of Stevia rebaudiana were collected in the city of Da Lat, Lam Dong, Vietnam in the month of July, 2024. The raw material was subjected to pre-treatment, which in this case, was the cleaning of the material to get rid of foreign matter and parts of the material that were broken. The cleaned leaves were in the subsequently placed in an oven set to 60 degrees Celsius and dried for 4 hours.

2.2. Chemicals, Reagents, and Instrumentation

The reagents and chemicals employed in this study are all solvents of analytical purity (PA) of different polarities produced from the country of Vietnam, Chemsol. The adsorbents which were utilized in the methods of chromatography and Filtration were, for column chromatography, Silica gel 60 (Merck). For purification filtration, celite (Merck) was used, and for Thin Layer Chromatography (TLC), Silica Gel GF254 (Merck).

Can Tho University, Faculty of Natural Sciences, conducted High Performance Liquid Chromatography (HPLC) analysis. In Hanoi, Vietnam Academy of Science and Technology, Institute of Chemistry has Bruker Advance 500 MHz. Bruker has set up Advanced 500 MHz for NMR so the Bruker can record the NMR scans.

All extraction and chromothography solvents ranged from ethanol (96%) to n-hexane, ethyl, acetate, chloroform and methanol. All solvents are classified analytical grade and sourced from Chemsol, Vietnam. The inhibitory factor, acarbose, is also from Merck (Darmstadt, Germany) along with the α-glucosidase enzyme (Type I, Saccharomyces cerevisiae). The substrate for this reaction, p-nitrophenyl-α-D-glucopyranoside (p-NPG) is also provided. DMSO has been included and will serve as a co solvent. The grade salt solutions, disodium hydrogen phosphate (Na2 HPO4), potassium dihydrogen phosphate (KH2 PO4), sodium carbonate (Na2CO3), and sodium bicarbonate (NaHCO3), are also DMSO. Merck also provided the pre-coated TLC plates, Silica gel 60 F254, and Silica gel (0.040-0.063um) for Chromatography.

Other major instruments consisted of the ultraviolet- visible microplate for measuring solvent absorbance as well as the Yamato RP-18A rotary evaporator (Yamato Scientific Co. Ltd, Japan) for the rotary evaporator set, and the 600 MHz NMR spectrometer from Bruker, Germany to record the NMR. The spectra are electric and are recorded at the Vietnam Academy of Science and Technology, Hanoi.

2.3 Preparation of Extract and Fractions

This part of the study followed the standard protocol for bioassay-guided fractionation as depicted on figure 1. Per 500 g of dried and powdered stem bark, the powdered stem bark was soaked in 96% ethanol for extraction purposes, and the extraction was done at room temperature. The ethanol extracts were combined and concentrated in a rotary evaporator under reduced pressure at 40 ^{o}C. The combined extracts yielded a total of 45.05 g of a viscous dark crude extract which is referred to as total crude extract (TP).

The total crude extract (TP) was further partitioned in order to extract non-polar compounds. The remaining water in the flask was then partitioned and the n-hexane extracts were dried to obtain the n-hexane fraction (A). The ‘polar constituents’ (C) were obtained by concentrating the remaining aqueous layer. All the fractions obtained were stored in the refrigerator at 4 ^{o}C.

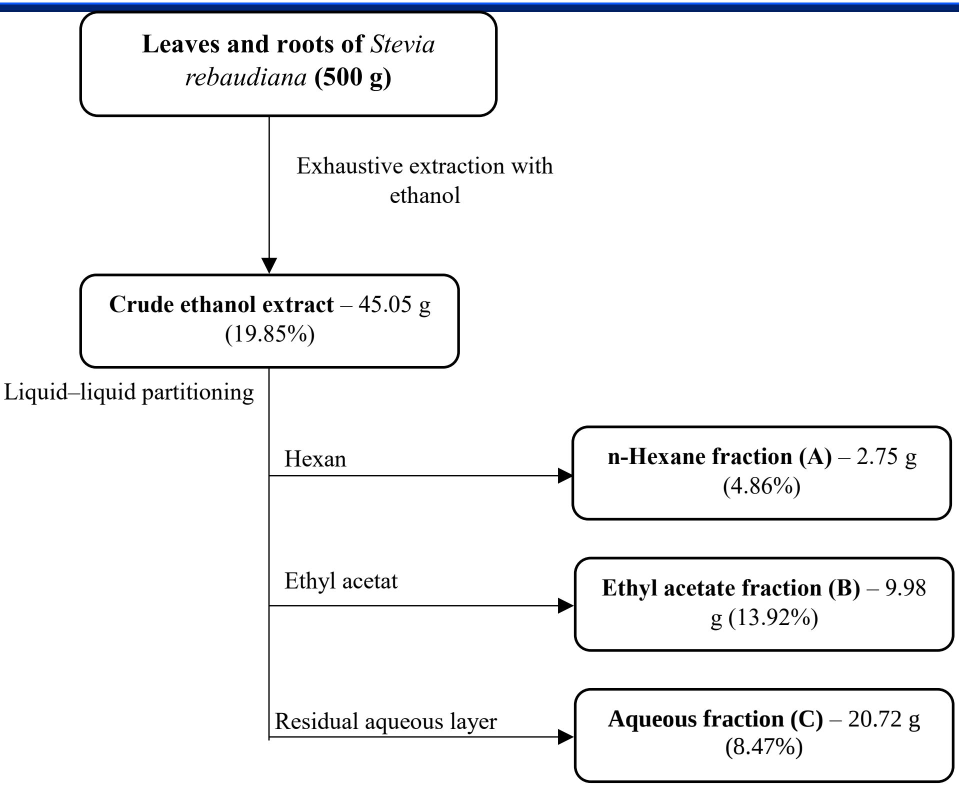

Figure 2: *Fractional extraction diagram of Stevia rebaudiana*

2.4. *In Vitro* α-Glucosidase Inhibition Assay

The inhibitory activity of the extracts, fractions, and the isolated compound against α-glucosidase was determined using a previously described 96-well microplate spectrophotometric method with minor modifications.

The assay is predicated upon the enzymatic hydrolysis of the substrate p-NPG by the enzyme α-glucosidase, which, in the course of the reaction, produces the colored product p-nitrophenol. The quantity of p-nitrophenol is a direct indication of the enzyme activity which is measured by the absorbance at 405 nm. Enzyme inhibition lowers the rate of the reaction.

The stock solutions of the test samples (extracts, fractions and pure compound) were prepared in DMSO and subsequently serially diluted with 5% DMSO to a range of concentrations. The assay was performed in a 96 well plate. Each well had a final reaction volume of 260 µL. The setup was comprised of wells for the test sample, test sample blank, control (enzyme activity without inhibitor), and control blank. The detailed pipetting procedure is given in Table 1.

In short, the test sample solution (or the control 5% DMSO solution) was incubated with 50 µL of DMSO, and, for alpha-glucosidase, 40 µL solution was added. For the alpha-glucosidase solution, buffering solution was used for the sample and 0.1 mL of 0.2 µN, then pH 6.8. The room temperature time was five minutes. After five minutes, 40 µL of the DMSO p-NBG solution was added. One 30-minute interval was taken for room temperature incubation. The next reaction was with 130 µL of the reaction solution (0.2 sodium carbonate). The detection of p-NF was measured with the microplate reader at the 405 nm wavelength. For Acabose, the same conditions and methods were used as a positive control. Each concentration was measured three times.

Table 1. *Pipetting Scheme for the 96-Well Plate α-Glucosidase Inhibition Assay*

Component	Test (µL)	Sample	Test (µL)	Sample	Blank	Control (µL)	Control (µL)	Blank

Test Sample / Acarbose	50	50	0	0
5% DMSO	0	0	50	50
0.1 M Phosphate Buffer (pH 6.8)	40	40	40	40
α-Glucosidase (0.2 µ/mL)	40	0	40	0
Incubate at room temperature for 5 minutes				
p-NPG (1 mM)	40	40	40	40
Incubate at room temperature for 30 minutes				
Na_2CO_3 (0.2 M)	130	130	130	130
Measure absorbance (OD) at 405 nm				

Calculation of Inhibition Percentage

IC_{50} Determination

- The α-glucosidase inhibitory activity was evaluated based on the inhibition percentage (I%). The inhibition percentage was calculated using the following formula:

$$I(\%) = \frac{A_0 - A_s}{A_0} \times 100$$

where:

A_0: Absorbance of the control (reaction mixture without inhibitor).

A_s: Absorbance of the sample containing the inhibitor.

- **IC_{50}** is defined as the concentration of the test sample that inhibits 50% of α-glucosidase activity. The IC_{50} value indicates the inhibitory strength of a sample—the lower the IC_{50} value, the stronger the inhibition. From the inhibition percentage (I%), the IC_{50} value was calculated.

2.5 Isolation and purification of fraction C

Use of column chromatography

Column preparation: The chromatographic column is packed with silica gel 60 as the stationary phase.

Desorption: The crude extract is loaded onto the column and desorbed with a series of chloroform-methanol mixtures, with increasing methanol ratios to create a polar gradient.

Fragment monitoring: The desorption process is monitored by thin layer chromatography (TLC) on silica gel GF_{254} plates. The mobile phase for TLC is a mixture of n-butanol, water and acetic acid in the ratio 4:5:1 (v/v/v). Spots are observed by spraying the plates with a 1:10 (v/v) solution of sulfuric acid in methanol, followed by drying at 110ºC for 2 min.

Isolation: The fractions identified to contain stevioside were pooled, concentrated and further purified by recrystallization in a suitable solvent to obtain the final product.

2.6 Structural Elucidation

The chemical structure of the isolated was determined using nuclear magnetic resonance (NMR) spectroscopy. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded in deuterated methanol (MeOD). The chemical shifts are reported in parts per million (ppm) relative to the residual solvent signal, and coupling constants (J) are given in Hertz (Hz). The obtained spectral data were meticulously analyzed and compared with data reported in the literature to confirm the compound's identity.

3. Results and Discussion

3.1 Extraction Yields and Screening of α-Glucosidase Inhibitory Activity

The initial extraction of 500 g of leaves and roots with 96% ethanol yielded 45.05 g of the total crude extract (TP), corresponding to a yield of 9.01%. Subsequent liquid-liquid partitioning of this crude extract resulted in three fractions with distinct yields, reflecting the distribution of phytochemicals based on polarity. The yields are summarized in Table 2. The ethyl acetate fraction (B) accounted for a significant portion of the crude extract, suggesting a high concentration of medium-polarity compounds.

Table 2. *Extraction and Fractionation Yields from Stevia rebaudiana*

Extract / Fraction	Code	Weight (g)	Yield (%)*
Total Ethanolic Extract	TP	45.05	9.01
n-Hexane Fraction	A	2.75	0.55
Ethyl Acetate Fraction	B	9.98	2.00
Aqueous Fraction	C	20.72	4.14

** Yield calculated based on the initial 500 g of dried plant material.*

The results of the *in vitro α*-glucosidase inhibition assay for the crude extract and all fractions are presented in Table 3. All tested samples demonstrated dose-dependent inhibitory activity. The most striking result was the exceptional potency of the ethyl acetate fraction (B), which displayed an IC_{50} value of 17.20 ± 1.16 µg/mL. This level of inhibition is remarkably potent, being nearly five times stronger than that of the standard clinical drug, acarbose (IC_{50} = 84.03 ± 4.74 µg/mL). The total ethanolic extract (TP) also showed significant activity (IC_{50}= 31.69 ± 0.63 µg/mL), indicating the presence of potent inhibitors in the raw plant material. The fractionation process successfully concentrated these active principles into the ethyl acetate fraction. The n-hexane fraction (A), containing non-polar compounds, exhibited the weakest activity among the organic fractions IC_{50}= 55.43 ± 7.45 µg/mL), suggesting that lipophilic constituents like sterols or fatty acids play a minor role in the observed *α*-glucosidase inhibition. The highly polar aqueous fraction (C) showed moderate activity IC_{50}= 68.62 ± 1.91 µg/mL which was comparable to that of acarbose.

Table 3. *α-Glucosidase inhibitory activity of Oro extracts and acarbose.*

Sample	Concentration (µg/mL)	Concentration in well (µg/mL)	Inhibition Rate (%)			IC_{50} (µg/mL)
			Trial 1	Trial 2	Trial 3	
TP	2000	384.62	85.19	88.45	88.24	31.69±0.63
	1500	288.46	85.66	84.50	84.24	
	1000	192.31	84.03	85.24	86.51	
	500	96.15	88.67	88.67	88.88	
	250	48.08	75.50	74.61	75.08	
	125	24.04	41.76	46.29	44.34	
	62.5	12.02	24.42	20.90	22.48	
A	2000	384.62	78.62	81.30	85.76	

	1500	288.46	71.59	75.27	78.90	55.43±7.45
	1000	192.31	66.30	79.73	77.45	
	500	96.15	63.40	81.24	69.81	
	250	48.08	37.13	48.17	57.99	
	125	24.04	34.68	27.37	23.97	
	62.5	12.02	9.64	7.63	7.08	
B	2000	384.62	89.66	85.48	93.28	17.20±1.16
	1500	288.46	85.03	82.80	85.76	
	1000	192.31	93.73	91.95	90.55	
	500	96.15	88.88	89.16	88.49	
	250	48.08	87.15	85.53	87.99	
	125	24.04	71.82	68.36	73.88	
	62.5	12.02	29.27	29.88	46.83	
C	2000	384.62	75.61	73.93	71.04	68.62±1.91
	1500	288.46	63.23	72.93	73.71	
	1000	192.31	72.54	76.50	75.44	
	500	96.15	67.13	67.91	65.12	
	250	48.08	30.50	26.70	35.63	
	125	24.04	10.37	12.04	6.30	
	62.5	12.02	3.73	5.57	2.67	
Acarbose	1000	192.31	80.66	80.55	80.76	84.03±4.74
	500	96.15	71.81	73.86	72.81	
	250	48.08	63.38	59.75	59.17	
	125	24.04	55.22	52.58	52.74	
	62.5	12.02	85.93	40.78	40.89	
	31.25	6.01	26.61	29.77	28.09	
	15.625	3.0	27.56	27.56	24.66	

3.2 Stevioside purification

Stevioside purification results: obtained 0.32 g. The purification efficiency by method 2 was 0.13%.

The purified product was examined by high-performance liquid chromatography (HPLC), with the HPLC spectrum shown in Figure 2, to check the purity of the product, before nuclear magnetic resonance spectroscopy.

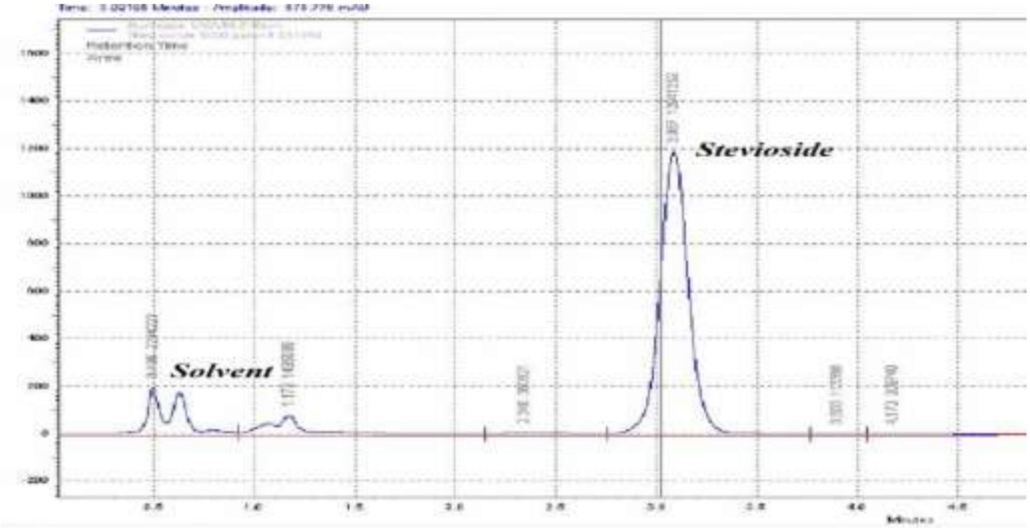

Figure 3: *HPLC chromatogram of Stevioside*

3.3 Isolation and Structural Elucidation of constituent C

Following the guidance of the bioassay results, the highly active water fraction (C) was subjected to silica gel column chromatography. This process led to the successful isolation of Compound 1 as a pure, yellow, crystalline solid. The structure of Compound 1 was elucidated by comprehensive analysis of its ^1H and ^{13}C-NMR spectra.

NMR spectral information was recorded from a Bruker Advance, 500 MHz, at the Institute of Chemistry, Vietnam Academy of Science and Technology. Based on the NMR spectral data of the compound, with pyridine solvent, the compound structure was interpreted, and then compared with the published standard compound data, specifically as follows: 1H-NMR spectrum (500 MHz, C5D5N): in the high magnetic

field region, two single peaks of methyl protons, δ (ppm): 1.27 (3H, s, H-18); 1.21 (3H, s, H-20); combined with the multi-peak signals of the protons in the 0.7 – 2.7 ppm region and the proton of the CH2=C< double bond at 5.67 ppm, representing the characteristic protons of the ent-Kaurene framework. In addition, there are 3 signals of anomeric protons with chemical shifts at 6.07 (1H, d, J = 8 Hz, H-1‴); 5.27 (1H, d, J = 7.5 Hz, H-1″); 5.12 (1H, d, J = 7.5 Hz, H-1′) showing that the compound carries 3 sugar groups.

^{13}C-NMR spectrum (125 MHz, C5D5N), δ (ppm): there are 38 signals of carbon atoms, in which, in the low magnetic field region, there is one signal with chemical shift at 177.1 ppm of carbonyl carbon (corresponding to C-19), two signals at 154.5 ppm and 104.6 ppm belong to two sp2 hybridized carbons carrying double bonds at C-16 and C-17 positions. In addition, in the 62.0 – 106.7 ppm region there are 19 carbon signals, including 18 carbons of the three sugar groups and one hydroxymethine carbon of the aglycon skeleton, at 86.0 ppm (corresponding to C-13); in the strong magnetic field region: 15.5 – 57.3 ppm there are 16 signals of the remaining carbon atoms of the entkaurene skeleton: with the characteristics of the skeleton, there is a one-carbon bridge (C-14: δ 28.2 ppm), between C-8 (42.6 ppm) and C-13 (86.0 ppm).

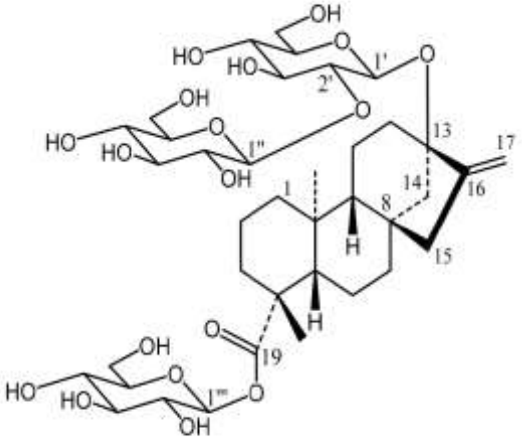

Figure 4: *Chemical Structure of stevioside*

4. Conclusion

The study showed that Stevia rebaudiana extract and its key compounds significantly inhibited α-glucosidase, demonstrating the potential to reduce postprandial hyperglycemia. The in vitro results were consistent across a range of concentrations tested and suggested a component-dependent mixed/competitive inhibition mechanism, while also demonstrating the role of the solvent matrix and extraction method on IC_{50}. However, the study had several limitations: (1) large differences in enzyme sources, pH/temperature conditions, and experimental matrices make cross-comparisons difficult; (2) no pharmacokinetic-pharmacodynamic (PK/PD) data linking enzyme inhibition to clinical response; and (3) insufficient sample size/design to allow generalization to the patient population. In future studies, we will prioritize improvements including: standardization of extraction procedures: external standards, quantification of key glycosides and polyphenols, establishment of unified assay protocols and enzyme kinetic models, expansion to in-vivo models and long-term safety evaluation, as well as implementation of randomized controlled trials focusing on postprandial glycemia, dose-response, and bioavailability of target components.

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