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TLC and UV-Visible Spectrophotometry Validation for Identification of Sildenafil Citrate in Aphrodisiac Herbal Medicine

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Abstract

Traditional medicines, also known as jamu, have sometimes been found to contain medicinal chemicals to enhance the effectiveness of these products. Sildenafil citrate, which is clinically effective in improving erectile function, can cause harmful side effects when included in herbal products. This study, therefore, aims to validate the identification of sildenafil citrate in aphrodisiac herbal medicine using Thin Layer Chromatography (TLC) and UV-visible spectrophotometry. The samples were prepared by macerating 100 mg of the jamu in 96% ethanol for 24 hours. The samples were then evaluated using TLC and a UV-visible spectrophotometer, with validation parameters including linearity, precision, limit of detection (LOD), and limit of quantitation (LOQ). This study uncovered that one of the three samples tested positive for sildenafil citrate, as evidenced by similar Retention factor (Rf) values in two TLC systems. Additionally, analysis using UV-visible spectrophotometry revealed that the average content of sildenafil citrate in the sample was 23.96%, with a relative standard deviation (RSD) of 0.74% and LOD and LOQ values of 10.28 and 34.27 µg/mL, respectively. The methods of analysis, including TLC and UV-visible spectrophotometry, for sildenafil citrate identification are expected to be valuable for regulatory and supervisory agencies in monitoring the distribution of such herbal medicines.

Keywords: traditional medicine; *jamu*; sildenafil citrate; validation; TLC; UV-vis spectrophotometry

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INTRODUCTION

Herbal medicine, also known as *jamu,* is an herbal concoction made from natural ingredients, a traditional knowledge or cultural heritage of Indonesia intended for preservation, improvement, recovery, or treatment of health, as well as disease prevention.[1] Nevertheless, an issue encountered in *jamu* products is the presence of Medicinal Substance (MS). According to the Indonesian Ministry of Health Regulation Number 7 of 2012 on Traditional Medicines Registration, it is stated that traditional medicines are prohibited from containing MS with medicinal properties or isolated products.[2] The consumption of MS in traditional

medicines such as *jamu* is very harmful to health, especially with long-term use, which can lead to side effects.[3]

One type of *jamu* that is well distributed in a community is an herbal aphrodisiac. *Panax Ginseng* (Korean Red Ginseng), *Epimedium* (Horny Goat Weed), and *Eurycoma longifolia* (Tongkat Ali) are commonly used herbs for treating erectile dysfunction. The key secondary metabolites responsible for their effects include ginsenosides in Panax Ginseng, particularly various steroid glycosides and triterpene saponins;[4,5] icarisid II, a flavonoid compound, in Epimedium; and quassinoids such as canthin-6-one and eurycomalides in *Eurycoma longifolia*.[6] These metabolites have been identified as the active constituents contributing to the therapeutic potential of these herbs in improving erectile function. To improve product marketing, producers, however, often misuse MS to enhance the effectiveness of their products.

Several studies have indicated that certain dietary supplements, herbal medicines, and alcoholic beverages illegally contain sildenafil.[7] The trend of adding MS is predominantly marked by the presence of sildenafil citrate and tadalafil in herbal aphrodisiac products claiming to enhance male stamina. Out of 61 identified herbal products containing MS, sildenafil citrate was identified in 35 of them.[8] Herbal aphrodisiacs are herbal products that should not include active MS such as Sildenafil Citrate (SC) in their formulations. Clinically, SC has been shown to enhance erectile function, including aspects of orgasm, sexual desire, and overall sexual satisfaction.[9] The presence of SC in herbal aphrodisiacs can lead to side effects such as headaches, flushing, stroke, chest pain, heart attack, loss of hearing and vision, dizziness, facial swelling, and even death.[9,10] Thin-Layer Chromatography (TLC) and densitometric analysis by Setiawan et al. identified SC in 14 out of 22 samples collected from Surabaya, while UV-Visible spectrophotometric analysis by Elsan et al. found SC in 3 out of 5 samples collected from Ungaran.[11,12]

Therefore, this study aims to provide data validation for methods used to detect SC in *jamu* products from Magelang Regency, utilizing a combination of TLC and UV-visible spectrophotometric techniques.

METHODS

Tools and materials

Glassware (Pyrex®), TLC silica gel plate 60 GF_{254} (Merck®), TLC chamber, micropipette (Dragonlab®), UV-Visible spectrophotometer (Cecil®), quartz cuvette (Starna®), filter paper, UV lamp analyzer 254 nm, analytical balance (Ohaus®), orbital shaker (DLAB®), water bath (PT Rizky Scientific Techno), aluminum foil (Klinpak®), stretch warp, chloroform (SmartLab®), methanol (Merck®), ethanol 96% (Surya Arta®), Liquid Sildenafil Citrate (LSC) (Sigma®), Sildenafil Citrate Tablet (SCT) (Novell®), and three samples of herbal aphrodisiac were used. Sampling was conducted in Magelang Regency, covering two main shopping areas: Jambu Market in Tempuran Sub-district with two samples and Chinatown Area in Central Magelang Sub-district with one sample. The samples obtained were coded A, B, and C.

Preparation of TLC samples

Sample A of *jamu* was weighed to 100 mg and placed into a 50 mL volumetric flask. The sample was dissolved in ethanol 96% and shaken for 15 minutes at 300 rpm, followed by maceration for 24 hours. The sample was then shaken again for 15 minutes at 300 rpm and filtered. The filtered macerate was then evaporated using a water bath until a concentrated extract of sample A remained. Samples B and C were subjected to the same treatment as sample A.

Preparation of UV-Visible spectrophotometry samples

Sample A of *jamu* was weighed to 100 mg and placed into a 100 mL volumetric flask. The sample was dissolved in ethanol 96% and shaken for 15 minutes at 300 rpm using an orbital shaker, followed by maceration for 24 hours. The sample was then shaken again for 15 minutes at 300 rpm, after which the liquid extract was filtered and stored in a glass bottle. Samples B and C were subjected to the same treatment as sample A.

Preparation of TLC standard

The SCT outer coating was peeled off to obtain its key components, then grinded, weighed 300 mg, and dissolved in a 10 mL volumetric flask using methanol and homogenized. The SCT standard solution was obtained at a concentration of 3000 ppm, stored in a dark amber vial, and covered with aluminum foil.

Preparation of a UV-Visible spectrophotometry standard

LSC was taken 100 µL, dissolved in a 10 mL volumetric flask using methanol solvent, and homogenized. The LSC standard solution was obtained at a concentration of 10 ppm, stored in a dark amber vial, and covered with aluminum foil.

Organoleptic evaluation

The primary packaging of the sample was opened, and then the sample underwent an organoleptic evaluation. For samples in capsule form, the capsules were opened to observe the contents. Through direct sensory observation, the organoleptic evaluation was conducted to obtain a general description of the sample, including its color, taste, aroma, and dosage form.[13]

TLC evaluation

The SCT and the concentrated extracts of samples A, B, and C were spotted onto TLC plates with systems 1 and 2 as listed in Table 1. Qualitative analysis was performed by comparing the Retention factor (Rf) values from the elution spots of the standard, and the samples were scanned under a UV lamp analyzer at 254 nm wavelength. The sample was declared to contain the standard if it had an Rf difference from the reference within the range of 0.02.[14]

Determination of maximum wavelength using UV-Visible spectrophotometry

A sufficient amount of LSC standard solution with a concentration of 10 ppm was used to measure its maximum absorbance within the wavelength range of 200 - 400 nm using a UV-Visible spectrophotometer.[16]

Table 1. Mobile Phase System for TLC Evaluation

Standard	System 1	System 2
Sildenafil citrate	Methanol: chloroform (1:1)	Methanol: chloroform (4:1)
Stationary phase	Silica gel 60 GF_{254}	Silica gel 60 GF_{254}
Spotting volume	10 µL	10 µL [15]

Determination of standard calibration curve using UV-Visible spectrophotometry

The LSC standard with a concentration of 10 ppm was subjected to serial dilutions. The absorbance of the LSC standard at concentrations of 2, 4, 6, 8, and 10 ppm was measured using a UV-Visible spectrophotometer. The absorbance and concentration data were then input into a regression equation in Microsoft® Excel 2021 to obtain the SC standard calibration curve.

Determination of medicinal substances content in *jamu*

Samples A, B, and C absorbance were measured using a UV-Visible spectrophotometer. The absorbance data of the samples were input into the y-variable of the linear regression equation obtained from the standard calibration curve, i.e., y = a + bx, to obtain the x-value (µg/mL). The x-value was then substituted into Equation 1:

Equation 1. Determination of Sildenafil Citrate Content

$$\text{content of SC in sample (\%)} = \frac{\text{x} \times \text{solvent volume (L)} \times \text{Dilution factor}}{\text{sample mass (mg)}} \times 100\%$$

Validation of analytical method

Method validation is an essential element in quality control to guarantee reliable measurements. According to ISO 17025, validation is the verification through examination and the provision of objective documentation to fulfill specific requirements for a particular purpose.[17] The following parameters were evaluated, including linearity, precision, limit of detection (LOD), and limit of quantification (LOQ).[17,18]

Linearity

The proportional response of the analytical method to the analyte concentration in the sample is referred to as linearity, which is tested using a series of standard solutions ranging from at least 50 - 150% in four different concentrations of the sample analyte. The linearity variable uses the correlation coefficient (r) with the coefficient of determination (r^2) in the linear regression equation y = bx + a.[17] The standard solution concentrations used in this analysis were 2, 4, 6, 8, and 10 ppm. The r^2 value should approach 1 to satisfy the linearity parameter requirement.[17]

Precision

Testing of homogeneous samples under normal conditions was performed consecutively and repeatedly at least six times using the same equipment. If the

standard deviation (SD) calculation yields a relative standard deviation (RSD) of 2% or less, the method can be considered precise.[17] The SD and RSD values were calculated using Equations 2 and 3, respectively.[17]

Equation 2. Determination of Standard Deviation

$$SD = \sqrt{\sum_{i-1}^{n} \frac{(x_1 - \bar{x})^2}{n-1}}$$

*n: total number of observations or data points; x_i: each individual data point in the dataset; $\bar{x}$: mean

Equation 3. Determination of Relative Standard Deviation

$$\%RSD = \frac{SD}{\text{average value}} \times 100\%$$

LOD and LOQ

The LOD is the smallest amount of analyte in a sample that can be detected and provides a significant response compared to the blank, whereas the LOQ is the smallest quantity of analyte that meets the criteria for precision and accuracy.[17] On a linear calibration curve, the instrument response y is assumed linearly related to concentration x within a limited concentration range and is expressed in the model y = bx + a. To calculate the sensitivity b as well as the LOD and LOQ, the following formulas were used.[17]

Equation 4. Determination of LOD

$$LOD = \frac{3Sa}{b}$$

Equation 5. Determination of LOQ

$$LOQ = \frac{10Sa}{b}$$

*Sa: standard deviation; b: slope

RESULT AND DISCUSSION

Organoleptic evaluation

The organoleptic evaluation includes evaluating the sample's color, taste, aroma, and dosage form.[13] Most of the organoleptic evaluation results of the *jamu* samples revealed a characteristic *jamu* aroma and a bitter taste.[19] The samples in this study are herbal aphrodisiacs in capsule form, consisting of fine powder and having a distinctive aroma of ginseng and vanilla-like coffee. The three samples exhibited bitter, coffee bitter, and slightly spicy tastes. The organoleptic evaluation results for samples A, B, and C are shown in Table 2. Based on these evaluations, sample A appeared white in color and is suspected to contain SC, as it lacked the typical characteristics of *jamu*.[19]

Table 2. Organoleptic Evaluation Results of Herbal Aphrodisiac Samples

Sample	Aroma	Taste	Color	Form	Dosage Form	Figure
A	Vanilla-like coffee	Bitter	White	Powder	Capsule	
B	Ginseng	Bitter	Cream	Powder	Capsule	
C	Ginseng	Slightly spicy	Yellowish brown	Powder	Capsule	

TLC analysis

Three concentrated *jamu* extracts obtained from maceration and the standard stock solution were spotted onto different systems TLC plates, respectively. The resulting elution spots were compared. The TLC system used a mobile phase comprising a mixture of two solvents for optimized separation and silica gel 60 GF_{254} as the polar stationary phase. The results of the TLC evaluation for two mobile phase systems with samples A, B, and C are shown in Figure 1.

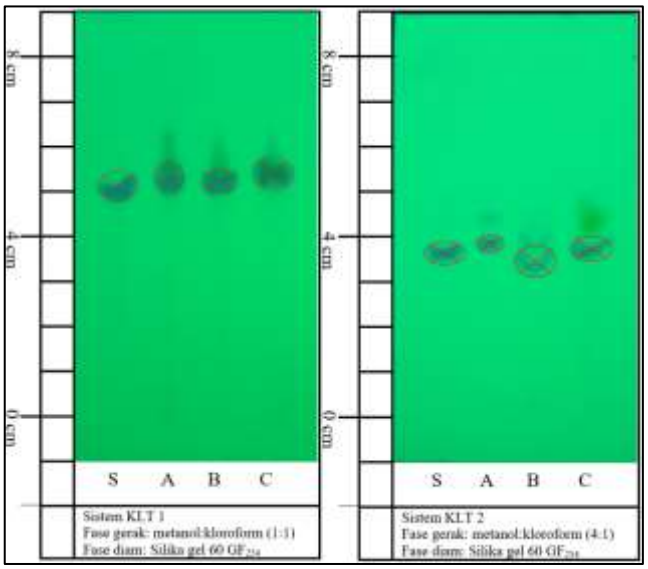

Figure 1. TLC Analysis of Standard (S) and Samples (A, B, and C)

Chromatography is a simple and rapid analytical technique for selectively separating compounds with similar structures using different absorbent media.[20] Research by Setiawan *et al.* (2020) found that 14 of 22 herbal

aphrodisiac samples evaluated using TLC were positive for SC based on similar Rf values.[11] The elution results were observed under UV light at 254 nm. TLC systems 1 and 2 showed Rf value differences between sample B and the standard (S) of 0.01 and 0.00, respectively. These nearly identical values strongly suggest that sample B contained a compound with the same polarity and migration behavior as the SC standard. Additionally, both systems exhibited a similar purple spot color. The Rf values from the TLC elution results are presented in Table 3. These Rf differences were used as the basis for suggesting that the sample might be positive for SC, with an Rf value difference within 0.02.[14] Research by Lawal *et al.* confirmed that the similarity in Rf values was strongly indicative of product adulteration with sildenafil, which was further validated using Fourier transform infrared analysis.[21] These varied data were used to support the conclusion that sample B contained SC based on the TLC results from two systems. Sample A was not suspected of containing SC because the similar Rf value to the standard in only one TLC system was not sufficiently convincing.

Table 3. Rf Values from TLC Analysis of Samples and Standard

Standard (S)	Sample	System 1 (Rf)	Rf value difference from S	Spot Color	System 2 (Rf)	Rf value difference from S	Color	Result
Sildenafil citrate	S	0.64	0	Purple	0.43	0	Purple	
	A	0.66	0.02	Purple, brown	0.48	0.05	Purple	-
	B	0.65	0.01	Purple, dark yellow	0.43	0.00	Purple	+
	C	0.68	0.04	Purple, brown	0.48	0.05	Purple. yellow	-

Determination of MS contents in *jamu* samples

Samples suspected of containing SC based on qualitative TLC analysis were then further quantitatively analyzed using UV-visible spectrophotometry.[22] UV-visible spectrophotometric analysis was chosen because SC contains chromophore groups that can absorb UV-visible light.[7,23] The sample subjected to further analysis was sample B. The maximum absorbance wavelength of SC was determined using a 10 ppm LSC standard solution. The maximum absorbance of SC was detected at a wavelength of 292.5 nm with an absorbance value of 0.384. The maximum wavelength scan result for the LSC standard is depicted in Figure 2.

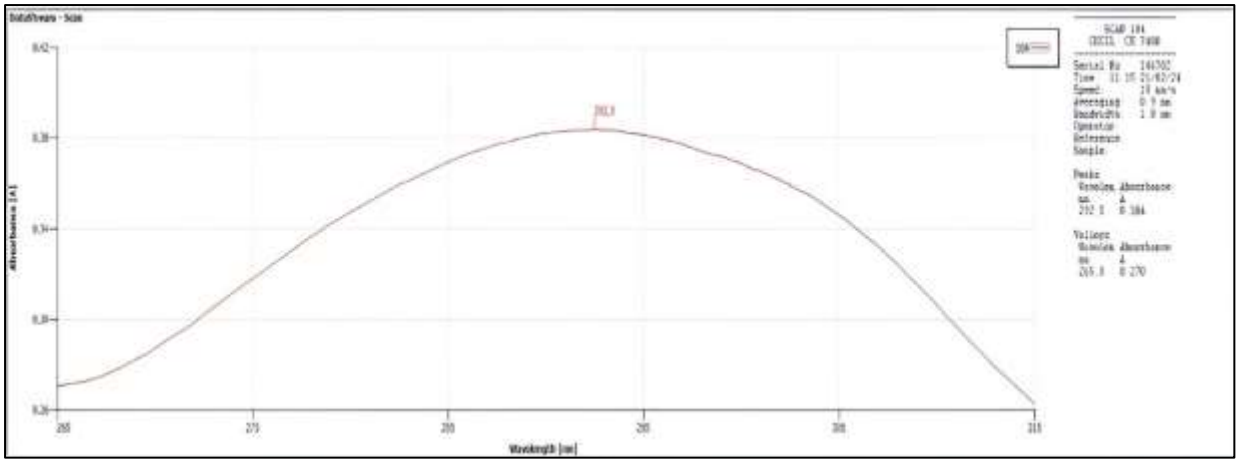

Figure 2. Maximum absorbance of the LSC standard at 10 ppm

The obtained maximum absorbance wavelength was used to establish the standard calibration curve and determine the SC content in the sample. The standard curve for the LSC standard was determined using concentrations of 2, 4, 6, 8, and 10 ppm at a wavelength of 292.5 nm. The absorbance measurement results for the LSC standard are shown in Table 4.

Table 4. Measurement Results of LSC Standard Absorbance

	ppm	Absorbance
C_1	2	0.127
C_2	4	0.181
C_3	6	0.241
C_4	8	0.291
C_5	10	0.332

Based on the absorbance measurement results for the SC standard, a linear relationship was observed between increasing concentration and increasing absorbance. From the measurement data in Table 3, the standard curve for the SC standard was established. The regression equation for SC was determined as $y = 0.026x + 0.0784$ with $r = 0.9978$ and $r^2 = 0.9956$. The obtained linear standard curve for SC is ideal, with $b = 0$ and $r = +1$ in a rightward direction.[17] The standard curve for the SC is shown in Figure 3.

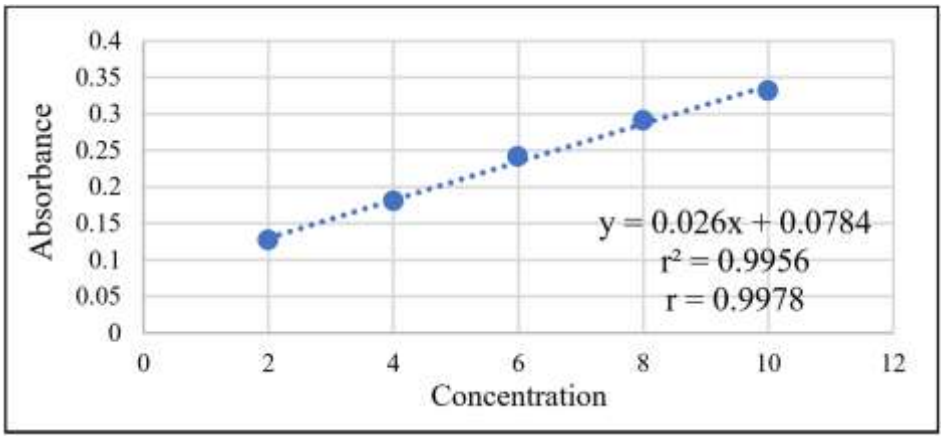

Figure 3. Calibration Curve for SC

Absorbance measurements of six replicates of *jamu* sample B, with a maceration time of 24 hours, using spectrophotometry resulted in SC contents of 23.89%, 23.97%, 23.74%, 23.97%, 24.28%, and 23.89%, with an average of 23.96%. Research by[16] reported that herbal aphrodisiac samples with a maceration time of 120-150 minutes showed SC levels of 8.00 - 21.00%, while Elsan *et al*. reported that 3 samples showed SC levels of 0.16 – 0.53%.[12,16] The SC content for sample B is shown in Table 5.

Table 5. SC Contents for Sample B

Replication	Absorbance	X (µg/mL)	Content of SC (%)
1	0.393	11.95	23.89
2	0.390	11.98	23.97
3	0.387	11.87	23.74
4	0.390	11.98	23.97
5	0.394	12.14	24.28
6	0.389	11.95	23.89

Validation of analytical method

Linearity refers to an analytical method's adequate response to the analyte concentration in the sample. The range refers to the interval between the lowest and highest concentrations of the analyte. The linearity evaluation was conducted using at least four standard solutions with serial concentrations ranging from 50% to 150% of the analyte concentration in the sample.[17] The concentration range used and the linearity for the liquid SC standard are shown in Table 6.

Linearity was measured using the obtained r^2 value. A correlation coefficient (r) is considered acceptable if the r^2 value approaches 1.[24] The linear regression equation for sildenafil citrate was y = 0.026x + 0.0784 with r^2 = 0.9956 and r = 0.9978 within the range of 2, 4, 6, 8, and 10 ppm. The obtained r^2 value being close to

1 indicates a strong linear relationship between concentration and absorbance.

The precision variable includes reproducibility and repeatability. The relative standard deviation (RSD) value of a good study or one that can be considered precise is less than 2%.[17] The results of this study are considered good because the RSD value obtained was 0.74%. The RSD value for the sample is shown in Table 6.

The detection limit (LOD) refers to the smallest amount of analyte in the sample that gives a significant response compared to the blank, while the quantitation limit (LOQ) refers to the smallest amount of analyte in the sample that can be measured with accuracy and precision.

Research conducted by Setiawan et al. determined the LOD of SC using a densitometer to be 2.503 mg/600 mg capsule.[11] Based on absorbance measurements from six replicates of sample B, the LOD and LOQ were determined to be 10.28 and 34.27 µg/mL, respectively. LOD and LOQ data are displayed in Table 7.

On the basis of the identification results of the three herbal aphrodisiac samples available in the market, sample B was found to contain sildenafil citrate, as indicated by its Rf value and spectrum comparison with the sildenafil citrate standard. This research could be further extended using more sensitive instruments, such as liquid chromatography–mass spectrometry (LC-MS) or high-performance liquid chromatography (HPLC). This finding is expected to be useful for regulatory agencies in monitoring the distribution of herbal medicines containing MS.

Table 6. Linearity Analysis of SC Standard for Standard Curve

Standard	Concentration (ppm)	Absorbance	Equation
Sildenafil citrate	2	0.217	y = 0.026x + 0.0784 r = 0.9978 r^2 = 0.9956
	4	0.181	
	6	0.241	
	8	0.291	
	10	0.332	

Table 7. Relative Standard Deviation (RSD) of Herbal Aphrodisiac Sample B

Sample	Replication	X (µg/mL)	SD	RSD (%)
B	1	11.95	0.0891	0.74
	2	11.98		
	3	11.87		
	4	11.98		
	5	12.14		
	6	11.95		

Table 8. LOD and LOQ of Herbal Aphrodisiac Sample B

Sample	Replication	Concentration (µg/mL)	Slope	LOD (µg/mL)	LOQ (µg/mL)
B	1	11.95	0.026	10.28	34.27
	2	11.98			
	3	11.87			
	4	11.98			
	5	12.14			
	6	11.95			

CONCLUSION

Based on the research conducted on three samples of herbal aphrodisiac, it was found that 1 out of 3 samples marketed in the main market area of Magelang Regency contained medicinal chemicals with an average content of 11.98 µg/mL, a relative standard deviation (RSD) of 0.74%, and LOD and LOQ values of 10.28 and 34.27 µg/mL, respectively. The thin-layer chromatography and UV-Visible spectrophotometric method for analyzing sildenafil citrate demonstrated linearity, precision, detection limit, and quantitation. This method can be easily and affordably performed, providing a valuable tool for regulatory agencies in monitoring the distribution of herbal aphrodisiacs containing medicinal chemicals in their respective areas.

CONFLICT OF INTEREST

The authors declare no conflict of interest in the preparation of this manuscript.

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