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Optimization of concentration of stevia leaves (*Stevia rebaudiana* Bert.) and black tea: study on phytochemical composition and antioxidant activity of mixed stevia-black tea solution

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Abstract. Tea is used as an ingredient used every day in the diet of Indonesian people, with many health benefits of tea including one as an anti-diabetes compound; while stevia is a natural sweetener to replace sugar. Both ingredients are expected to produce a synergy function as antidiabetic and antioxidant compounds. This study aims to find out how to combine black tea and stevia which both have high activity. This mixture was prepared as a basic ingredient for making stevia-blacktea-cocoa candy. The experimental design for the optimization of phenol content and antioxidant activity (DPPH free radical scavenging) in the process of mixing stevia-black tea was carried out using the Response Surface Method (RSM), a central composite with 2 factors, 1 replication, a total run order of 13, axial point 4, α = 1.414421. Dry stevia tea mixture - black tea leaves are brewed with boiling water (93 ± 2°C, 3 ± 0.5 minutes). From the experiment, it is concluded that after optimization, the highest result acquired is from a mixture of stevia 0.4446% (w/v) and black tea 0.3427% (w/v) with the results of a total phenol value of 326.3626 mgGAE/L sample and a DPPH of 64.5157 mgGAE/L sample.

1. Introduction

Products that contain antioxidants and anti-diabetic properties are now sought after, due to health considerations, especially prevention of diabetes mellitus. Ingredients that are widely used include tea, cocoa, and natural sweetener stevia. Many benefits of tea have been studied and reported, among others, as immunomodulator, antigenotoxic, against cardiovascular diseases, cancer prevention, chemoprevention of prostate cancer, hepatoprotective properties, anti obesity, antibacterial and antiviral properties, antidiabetic *etc*, so that tea is classified as a product that has protective effects for human health [1]. In 2003 the antioxidant activity of green tea, oolong tea and black tea [2] was investigated with the results of the antioxidant properties of various types of tea not significantly different. A study on the differences in hypolipidemic properties and growth-suppressive effects of

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Oolong, Black, Pu-erh, and Green Tea Leaves in rats by Kuo *et. al.* [3] with the result that partially or completely fermented tea was found to be more effective on the effects of growth -suppressive and hypolipidemic compared to green tea. Cao stated that tea has antidiabetic, antiobesity, and anti-inflammatory activity in animal models, but the molecular mechanism of this effect is not fully understood [4]. Research on the effect of green tea extract and black tea on glucose control in adults with type 2 diabetes mellitus [5], there is no difference in the effect of both on glucose control. From the research of Nishiumi [6] found the ability of green tea and black tea in suppressing hyperglycemia and insulin resistance. Researchers from Sri Lanka in 2011 found hypoglycemic, antihyperglycemic, and antidiabetic activities of black tea in broken orange pekoe fanning grade (BOPF) black tea [7]. The effect of brewing time on catechin composition and antioxidant activity has been investigated by Koch [8]. In 2019 research has been carried out to look at the antioxidant activity and main chemical components of tea fermentation [9].

While stevia is used as a sweetener, plants or its derivatives are used in several countries including Japan, China, Russia, Korea, Paraguay, Ecuador, Argentina, Indonesia, Malaysia, Australia, New Zealand and Mexico as treatments for hypertension, obesity or skin disorders [10]. In this case, there are important biological properties of the Stevia rebaudiana plant, among which are its anti-cariogenic capacity, antineoplastic and antihyperglycemic [11], hypoglycaemic and hypolipidaemic [12]. Both ingredients, tea, and stevia have high antioxidants, and maybe have a synergy effect. Before utilizing the two mixtures for the manufacture of tea-stevia-cacao-based products, a study was conducted on the combination of tea and stevia both to obtain high antioxidant activity values. Both ingredients were expected to produce a synergy function as antidiabetic and antioxidant compounds. This study aims to find out how to combine black tea and stevia which both have high antioxidant activity.

2. Methods

The study was conducted with qualitative and quantitative tests. To determine the phytochemical composition of the mixture of the two materials qualitative phytochemical tests were used [13], based on 0,5% black tea, and stevia 0% up to 0.37% (w/v). For data optimization, RSM testing using the total phenol and antioxidant activity or the ability to counteract the DPPH free radical compound (diphenyl-2-picrylhydrazine) was carried out quantitatively. The experimental design for the optimization of phenol content and antioxidant activity (DPPH free radical scavenging) in the process of mixing stevia-black tea was carried out using the Response Surface Method (RSM), a central composite with 2 factors, 1 replication, a total run order of 13, axial point 4, $\alpha = 1.414421$. Dry stevia - black tea leaves mixture brewed with boiling water (93 ± 2 ° C, 3 ± 0.5 minute).

The total phenol from stevia and black tea leaf steeping was determined by the spectrometry method [14]. A sample (1,000 mg / L) of 1 mL was added with 4 mL of sodium carbonate solution (75 g / L) and then shaken. Folin-Ciocalteu Phenol reagent as much as 0.2 mL was added and shaken again. After homogeneous, distilled water up to a volume of 10 mL was added and shaken again. The mixture is left at room temperature for 1 hour. Then the mixture was filtered with Whatman paper No. 42 and the absorbance of the supernatant were measured at $\lambda = 760$ nm. Total phenol was determined using gallic acid solution. The results obtained are expressed as gallic acid equivalents (GAE). As for determining the antioxidant activity of counteracting DPPH free radical compounds was carried out according to the procedure of Astadi [15]. This method aims to determine the ability of extract material to capture DPPH radicals, in this case, the ability of the extract to donate hydrogen atoms to DPPH radicals so that it becomes non radical. This ability was characterized by a DPPH color change from purple to yellow. This change was measured in absorbance at λ 516 nm. To interpret the data, the calculation of the percentage of DPPH radical capture is obtained, this interpretation was called the percent radical scavenging capacity. Data for the phytochemical composition are shown in the table, while for the total phenol and DPPH tested are included in the Design of Experiment-Response Surface Method (RSM) using Minitab 16. The design of the study used was the Central Composite Response Surface Methods with two factors: stevia leaves (% w/v) and black tea concentration (% w/v). Design factors were processed using Minitab 16 software and generate 13 formulations

(treatments) which can be seen in Table 1. The parameters tested were total phenol and antioxidant activity by DPPH method.

Table 1. Formulation Design Factor for RSM.Minitab 16.

Run Order	Code	*Code*	Stveia % (w/v)	*Black Tea % (w/v)*
1	-1	1	0.1900	0.4445
2	0	1	0.1900	0.1900
3	-1	1	0.0000	0.1900
4	1	1	0.0100	0.3700
5	0	1	0.1900	0.1900
6	1	1	0.3700	0.3700
7	-1	1	0.1900	0.0000
8	1	1	0.0100	0.0100
9	-1	1	0.4445	0.1900
10	0	1	0.1900	0.1900
11	0	1	0.1900	0.1900
12	0	1	0.1900	0.1900
13	1	1	0.3700	0.0100

3. Result and discussions

From the experiments, with qualitative testing to see the phytochemical components of the stevia black tea mixture obtained the results as in Table 2.

Table 2. Phytochemical compound of brewed black tea-stevia.

Compound	0%	0.05%	0.13%	0.21%	0.29%	0.37%
Alkaloid	+9	+4	+5	+6	+6	+7
Flavonoid	+7	+8	+8	+8	+9	+10
Phenolic	+3	+4	+6	+6	+6	+7
Triterpenoid	-	-	-	-	-	-
Sterol	-	-	-	-	-	-
Saponin	+8	+6	+6	+8	+7	+8
Tannin	+1	+2	+1	+1	+1	+1
Cardiac glycoside	+5	+5	+6	+6	+7	+8

Note: + detected based on intensity - non detected based on intensity

The phytochemical compounds test results of black tea with the addition of stevia leaves contain alkaloids, flavonoids, phenolics, saponins, tannins, and cardiac glycosides. In the study, triterpenoid and sterol compounds were not identified because terpenoids and sterols are nonpolar. The higher the intensity value, the stronger the color.

The results of phytochemical testing were used as a basis for further analysis, including determining the nature of the antioxidant activity of the product. A positive (+) value on the test results indicates the product has antioxidant activity, so it is eligible to proceed with further analysis, namely optimization based on the nature of its antioxidant activity.

For optimization purposes, Table 1 is equipped with DPPH and Total Phenol test results, the complete contents of which are listed in the following Table3:

Table 3. Formulation Design Factor for RSM.Minitab 16.

Run Order	Code	*Code*	Stevia % (w/v)	*Black Tea* % (w/v)	*DPPH*	Total Phenol
1	-1	1	0.1900	0.4445	27.25	262.60
2	0	1	0.1900	0.1900	39.27	230.86
3	-1	1	0.0000	0.1900	23.42	208.86
4	1	1	0.0100	0.3700	15.12	250.67
5	0	1	0.1900	0.1900	44.26	221.50
6*	1	1	0.3700	0.3700	60.20*	299.65*
7	-1	1	0.1900	0.0000	0.00	22.37
8	1	1	0.0100	0.0100	23.15	176.87
9	-1	1	0.4445	0.1900	48.06	244.64
10	0	1	0.1900	0.1900	46.26	221.50
11	0	1	0.1900	0.1900	41.08	112.45
12	0	1	0.1900	0.1900	40.05	211.70
13	1	1	0.3700	0.0100	44.25	245.43

From the table above it is known that the sixth treatment produced the highest values for both DPPH and total phenol. Table 2 was used as input in the RSM program. For testing using RSM Minitab 16, the results depicted are shown in Figure 1A, Figure 1B, 1C, and 1D.

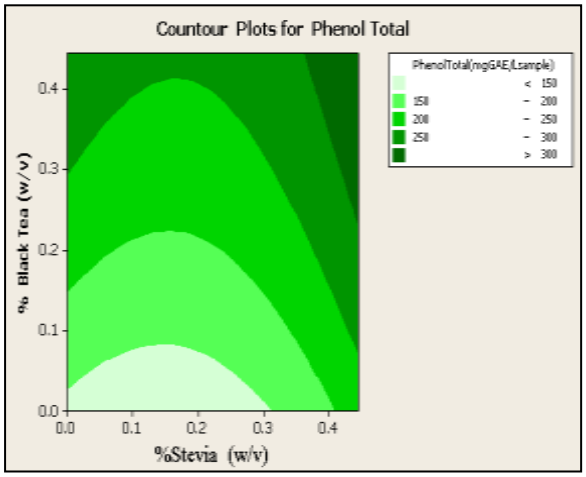

Figure 1A. Countour plot of total phenol.

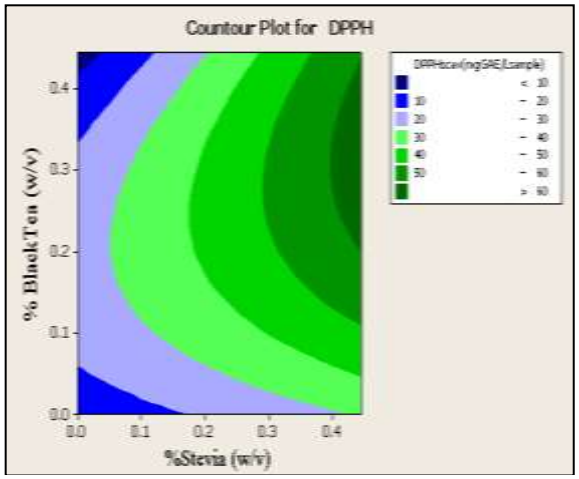

Figure 1B. Countour plot of DPPH.

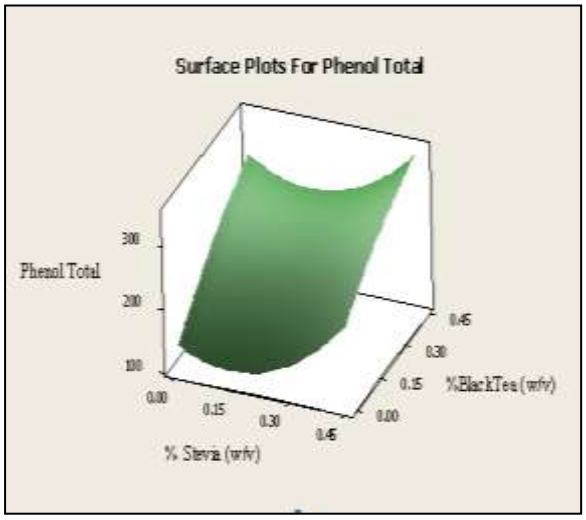

Figure 1C. Total phenol surface plot.

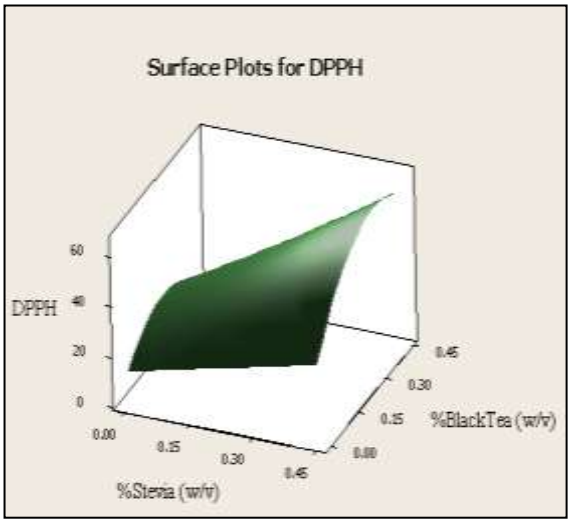

Figure 1D. DPPH surface plot.

The figure above shows the tendency that the increase in concentration between black tea and stevia, causing the same increase between DPPH and Phenol and it can be seen from Figure 1C and Figure 1D which have the same surface plot tendency. Apparently, the direction of the increase in DPPH that was different from the increase in phenol can be seen from Figure 1A and Figure 1B, so even though the effects of both were the same, the contour was different.

After optimization was done to find the maximum value, it was known that the maximum result of stevia mixing of 0.4446% with black tea of 0.3427% obtained a total phenol of 326.3626 mgGAE/L and a DPPH of 64.5157 mgGAE/L (Figure 2). These results indicate that the highest concentration of the treatment before being optimized was the 6th treatment (stevia concentration 0.37% (w/v) and black tea 0.37% (w/v)) which gives a total phenol value of 299.65 mgGAE/L and DPPH of 60.20 mgGAE/L, changing after optimization which gives the highest results was a mixture of stevia with a concentration of 0.4446% (w/v) and black tea with a concentration of 0.3427% (w/v) with the results of a total phenol value of 326.3626 mgGAE/L and a DPPH of 64.5157 mgGAE/L. From the results of the optimization, it appears that there has been a change in the treatment that gives maximum results after optimization. From the original treatment with stevia which was originally 0.37% increased to 0.4446%, and for black tea which was originally 0.37% decreased to 0.3427%.

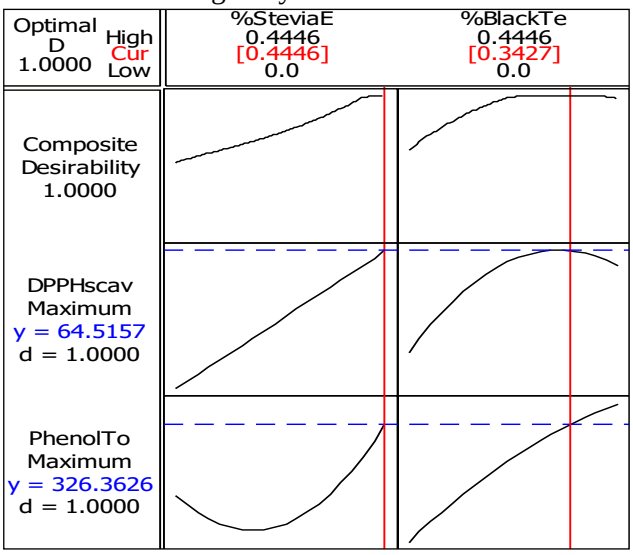

Figure 2. Optimization result.

The response surface regression result for this experiment as in Appendix. From the optimization process, the equation regression for DPPH was:

$[DPPH]=40.160+13.3375[S]+7.04248[B]+0.0921[S]^2 -10.8369[B]^2+ 5.9950\ [S][B]$

R-SQ: 70.81%

And the equation regression for Total Phenol was:

$[TP]=190.506+15.803[S]+59.318[B]+ 37.702\ [S]^2 – 8.806\ [B]^2 – 4.895\ [S][B]$

R-sq: 55.68%

Which,

[DPPH]: value of DPPH

[TP]: value of Total Phenol

[S]: concentration of Stevia (%w/v)

[B]: concentration of Black tea (%w/v)

For further use, it is still necessary to consider the sensory aspects of the mixture of the two ingredients, to what mix can be accepted by consumers. However, the use of tea and stevia for tea-stevia-cacao based products can still be continued, given their high antioxidant activity.

4. Conclusion

The highest value of the experimental results is obtained from a mixture of stevia with a concentration of 0.37% (w/v) and black tea with a concentration of 0.37% (w/v), which gives a total phenol value of 299.65 mgGAE/L and a DPPH of 60.20 mgGAE/L. After optimization which gives the highest results was a mixture of stevia with a concentration of 0.4446% (w/v) and black tea with a concentration of 0.3427% (w/v) with the results of a total phenol value of 326.3626 mgGAE/L and a DPPH of 64.5157 mgGAE/L, respectively.

5. Appendices

Response Surface Regression: DPPHscav(mgG versus %SteviaExtra, %BlackTea (w

The analysis was done using coded units.

Estimated Regression Coefficients for DPPH scav(mg GAE/L sample)

Term	Coef	SE Coef	T	P
Constant	40.1160	4.981	8.053	0.000
%SteviaExtract (w/v)	13.3375	4.421	3.017	0.019
%BlackTea (w/v)	7.0248	4.421	1.589	0.156
%SteviaExtract (w/v)* %SteviaExtract (w/v)	0.0921	5.097	0.018	0.986
%BlackTea (w/v)*%BlackTea (w/v)	-10.8369	5.097	-2.126	0.071
%SteviaExtract (w/v)*%BlackTea (w/v)	5.9950	5.720	1.048	0.329

S = 11.4400 PRESS = 6721.77

R-Sq = 70.81% R-Sq(pred) = 0.00% R-Sq(adj) = 49.96%

Analysis of Variance for DPPHscav(mg GAE/L sample)

Source	DF	Seq SS	Adj SS	Adj MS
Regression	5	2222.56	2222.56	444.51
Linear	2	1486.71	1541.81	770.91
%SteviaExtract (w/v)	1	1322.92	1191.28	1191.28
%BlackTea (w/v)	1	163.79	330.47	330.47
Square	2	592.09	592.09	296.04
%SteviaExtract (w/v)*%SteviaExtract (w/v)	1	0.48	0.04	0.04
%BlackTea (w/v)*%BlackTea (w/v)	1	591.60	591.60	591.60
Interaction	1	143.76	143.76	143.76
%SteviaExtract (w/v)*%BlackTea (w/v)	1	143.76	143.76	143.76
Residual Error	7	916.11	916.11	130.87
Lack-of-Fit	3	880.92	880.92	293.64

Pure Error	4	35.19	35.19	8.80
Total	12	3138.67		

Source	F	P
Regression	3.40	0.071
Linear	5.89	0.032
%SteviaExtract (w/v)	9.10	0.019
%BlackTea (w/v)	2.53	0.156
Square	2.26	0.175
%SteviaExtract (w/v)*%SteviaExtract (w/v)	0.00	0.986
%BlackTea (w/v)*%BlackTea (w/v)	4.52	0.071
Interaction	1.10	0.329
%SteviaExtract (w/v)*%BlackTea (w/v)	1.10	0.329
Residual Error		
Lack-of-Fit	33.38	0.003
Pure Error		
Total		

Unusual Observations for DPPHscav(mgGAE/Lsample)

Obs	StdOrder	DPPHscav(mgGAE/Lsample)	Fit	SE Fit	Residual	St Resid
7	7	0.000	20.626	7.561	-20.626	-2.40 R
13	2	44.250	29.689	9.643	14.561	2.37 R

R denotes an observation with a large standardized residual.

Estimated Regression Coefficients for DPPHscav(mgGAE/Lsample) using data in uncoded units

Term	Coef
Constant	13.3302
%SteviaExtract (w/v)	37.8612
%BlackTea (w/v)	130.970
%SteviaExtract (w/v)* %SteviaExtract (w/v)	2.84299
%BlackTea (w/v)*%BlackTea (w/v)	-334.472
%SteviaExtract (w/v)*%BlackTea (w/v)	185.031

Response Surface Regression: PhenolTotal(versus %SteviaExtra, %BlackTea (w

The analysis was done using coded units.

Estimated Regression Coefficients for PhenolTotal(mgGAE/Lsample)

Term	Coef	SE Coef	T	P
Constant	190.506	27.15	7.017	0.000
%SteviaExtract (w/v)	15.803	24.10	0.656	0.533
%BlackTea (w/v)	59.318	24.10	2.462	0.043
%SteviaExtract (w/v)* %SteviaExtract (w/v)	37.702	27.78	1.357	0.217
%BlackTea (w/v)*%BlackTea (w/v)	-8.806	27.78	-0.317	0.761
%SteviaExtract (w/v)*%BlackTea (w/v)	-4.895	31.18	-0.157	0.880

S = 62.3538 PRESS = 145850
R-Sq = 55.68% R-Sq(pred) = 0.00% R-Sq(adj) = 24.02%

Analysis of Variance for PhenolTotal(mgGAE/Lsample)

Source	DF	Seq SS	Adj SS	Adj MS
Regression	5	34186.0	34186.0	6837.2
Linear	2	26652.5	25439.3	12719.7
%SteviaExtract (w/v)	1	3987.4	1672.4	1672.4
%BlackTea (w/v)	1	22665.1	23563.7	23563.7
Square	2	7437.6	7437.6	3718.8
%SteviaExtract (w/v)*%SteviaExtract (w/v)	1	7047.0	7160.7	7160.7
%BlackTea (w/v)*%BlackTea (w/v)	1	390.6	390.6	390.6
Interaction	1	95.8	95.8	95.8
%SteviaExtract (w/v)*%BlackTea (w/v)	1	95.8	95.8	95.8
Residual Error	7	27216.0	27216.0	3888.0

```
  Lack-of-Fit                                        3  17538.0  17538.0   5846.0
  Pure Error                                         4   9677.9   9677.9   2419.5
Total                                               12  61402.0
Source                                                 F      P
Regression                                          1.76  0.240
  Linear                                            3.27  0.099
    %SteviaExtract (w/v)                            0.43  0.533
    %BlackTea (w/v)                                 6.06  0.043
  Square                                            0.96  0.429
    %SteviaExtract (w/v)*%SteviaExtract (w/v)       1.84  0.217
    %BlackTea (w/v)*%BlackTea (w/v)                 0.10  0.761
  Interaction                                       0.02  0.880
    %SteviaExtract (w/v)*%BlackTea (w/v)            0.02  0.880
Residual Error
  Lack-of-Fit                                       2.42  0.207
  Pure Error
Total
Unusual Observations for PhenolTotal(mgGAE/Lsample)
Obs  StdOrder  PhenolTotal(mgGAE/Lsample)      Fit  SE Fit  Residual  St Resid
  7         7                      22.370  118.081  41.214   -95.711     -2.05
Obs
  7  R
R denotes an observation with a large standardized residual.
Estimated Regression Coefficients for PhenolTotal(mgGAE/Lsample) using data in
     uncoded units
Term                                     Coef
Constant                               137.954
%SteviaExtract (w/v)                  -325.686
%BlackTea (w/v)                        461.528
%SteviaExtract (w/v)*                  1163.65
  %SteviaExtract (w/v)
%BlackTea (w/v)*%BlackTea (w/v)       -271.780
%SteviaExtract (w/v)*%BlackTea (w/v)  -151.080
```

Optimization Plot

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Response Optimization

```
Parameters
              Goal     Lower  Target  Upper  Weight  Import
DPPHscav(mgG  Maximum      0      61     61       1       1
PhenolTotal(  Maximum      0     300    300       1       1

Starting Point
%SteviaExtra   =   0
%BlackTea (w   =   0

Global Solution
%SteviaExtra   =   0.444558
%BlackTea (w   =   0.342656

Predicted Responses
DPPHscav(mgG   =    64.516  ,   desirability =   1.000000
PhenolTotal(   =   326.363  ,   desirability =   1.000000

Composite Desirability = 1.000000
```

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