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# Investigation of solvent effect on antioxidant property of some flavonoids in water- methanol mixture

**Hamidreza Moallem[1], Iman Nasrollahi[2]*, Ebrahim Talebi[2]**

**[1]Damghan university, Semnan, (IRAN)**

**[2]Young Researchers and Elite Club, Darab Branch, Islamic Azad University, Darab, Fars, (IRAN)**

**E-mail: iman67nasr@yahoo.com**

## ABSTRACT

(IC50), were analyzed by employing the Kamlet-Abbot-Taft (KAT) multiple-Flavonoids are considered useful for human health because they have antioxidant properties. In were studied in the two-component solvent water-methanol (50-90% v/v)at physiological pH and room temperature using the DPPH method. In this this work, the antioxidant properties of flavonoids such as chrysin, quercetin, and naringenin method, the ascorbic acid, which has extremely high antioxidant power, was used as the standard reference material. The effects of the solvent on antioxidant property, which is expressed as the concentration of flavonoids with 50% radical inhibition parameter equetion and Dimroth-Reichardt's normalized polarity parameter using the multivariate linear regression (MLR) method. The results showed that by reducing the polarity of the solvent (increasing the organic component) increase the antioxidant properties of all used compounds.



## KEYWORDS

Solvent;
Antioxidant;
DPPH;
Quercetin;
Flavonoid;
Polarity.

## INTRODUCTION

Antioxidants are health beneficial compounds thatfight against reactive oxygen and nitrogen species and free radicals that may eventually give rise to various diseases such as neurodegenerative diseases, cancer, arteriosclerosis, malaria, rheumatoid arthritis, some forms of anemia, auto-immune diseases, ageing, and diabetes[1,3].

Most of the natural antioxidants are found in wood, bark, stems, leaves, fruits, roots, flowers and seeds of the plants[4]. The most important natural antioxidants are: vitamin antioxidants, flavonoids etc[4,5]. Flavonoids are a diverse group of polyphenolic compounds that are widely found in plants and over lot number unique structural distributions have been identified for flavonoids in plant sources. Flavonoids are primarily recognized as pigments and they are the cause of most of yellow, orange and red colors in flowers, fruits and leaves of plants[6,7]. Flavonoids are compounds with 15 carbon atoms skeleton and dual nucleus structure in which there is a carbon triple bond between two phenyl groups[8-11]. However, most interest has been devoted to the antioxidantactivities and radical scavenging activities of flavonoids, whichare due to their ability to reduce radical formation and to scavenge radicals[12]. These interesting properties

have been studiedby using cyclic voltammetry[13,17], and spectroscopic methodsincluding UV–visible technique[18-21]. and electron spin resonance (ESR) technique[22,24]. One of the direct methods for clearing the free radicals by flavonoids is oxidizing the flavonoids in the presence of free radicals to the radicals with less reactivity and more stability. in addition, the studyon the structure-activity relationship of flavonoids has received a great success, for example, the presence of the ortho-diphenolgroup in the ring-B and a 3-hydroxyl group in the ring-C, the number of free hydroxyl groups in flavonoids, and a C2–C3 double bondin the ring-C, are closely related to the antioxidant activities andradical scavenging activities of flavonoids[13,25,26]. Antioxidant characteristics of compounds vary depending on different circumstances. In fact, the antioxidant capacity of compounds varies based on the factors such as the type of solvent, temperature, pH of the environment, ionic strength, etc. Solvent usually plays an important role in the reactions of antioxidants. Many researchers have studied the effect of solvents on the antioxidant properties of compounds[27,28]. However, there is limited information on the effect of various two-component solvents on antioxidant characteristics. Type of a solvent and its properties may affect a single electron transfer (SET) and a hydrogen atom transfer (HAT), which are key aspects in the measurements of antioxidant capacity. Flavonoids are practically insoluble in water, but they are often soluble in organic solvents. This is a frequent problem today since the new molecules in drug research are less water-soluble and more lipophilic. The mixed-solvent procedure mainly using organic solvent–water mixtures provide a good alternative for sparingly or nonsoluble compounds.

This study examined the effect of solvent on the antioxidant properties of a number of flavonoids such as:naringenin, quercetin and chrysine in different percentages of methanol -water mixture (50-90% v/v). There are various technique for the evaluation of antioxidant activity of flavonoids such as: ABTS, FRAP, Cu, ESR, DPPH method, etc. Among these methods, the one which iscurrently popular is based upon the use of the stable free radical 1,1-diphenyl-2-picryhydrazyl (DPPH)[27,28]. DPPH method was used in this project. DPPH method is fast, simple, repeatable and inexpensive for measuring the antioxidant capacity of the compounds and shows how the considered antioxidant can give hydrogen or electrons to DPPH radical to stabilize the compound. In this method, ascorbic acid solution (vitamin C) was used as the standard material. In other words, the values obtained for the antioxidant activity of the samples are compared with its amount for ascorbic acid and therefore, it is comparative.

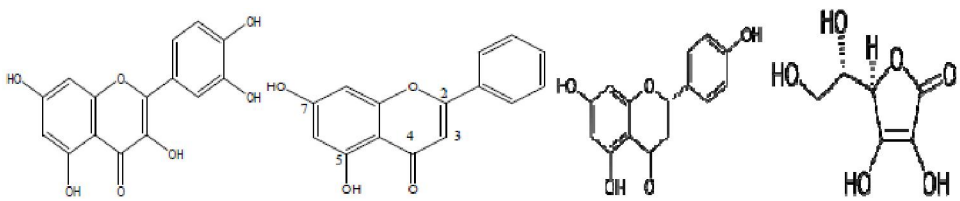
## EXPERIMENTAL SECTION

### Chemicals

All chemicals and solvents used were of the highest quality available. methanol, water, tris (2-amino-2-hydroxymethyl-propane-1,3-diol), and hydrochloric acid (for preparation of buffer and adjustedto physiological pH) were obtained from Merck. The stable radicalDPPH, ascorbic acid, naringenin [4,5,7-trihydroxyflavanone], nar, chrysin[5,7-dihydroxy flavone], chry, and quercetin [3',4',3,5,7-pentahydroxy flavone], quer, (Scheme 1) were obtained from Sigma as analytical reagent grade materials and were used without further purification.

### DPPH method

DPPH method was used in this project. DPPHmethod was carried out with little modifications to the procedure reported by Etcheverry*et al*[28]. and it constituted the following



querchrynarasc. acid

**Scheme 1 : The structures of nar, chry, quer and asc. acid**

steps:

Step 1: Preparation of the stock solution of samples: Samples were weighed accurately and were dissolved using various percentages of methanol-water mixture (50-90% v/v) and 1 mM solution was prepared for them.

Step 2: preparation of DPPH: The amount of 2.2 mgof DPPH were accurately weighed in 250 mL volumetric flasks and were dissolved using various percentages of methanol-water mixture (50-90% v/v) and its 40 ppm solution was prepared. Solutions werestored in the dark and in a dry environment.

Step 3: Preparation of buffer solution: The amount of 0.1211 g of Tris- HCl solid buffer was weighed and using the solvent, the volume reached to 10 mL to prepare 0.1 M solution.

Step 4:Reaction system: In sixcontainers, 4 ml of DPPH solution was added and 0.500, 0.375, 0.250, 0.125, 0.050 mLof the antioxidant sample was added to the first five containers, respectively. Then, 0.500, 0.625, 0.750, 0.875, 0.950 and 1 ml buffer solution was added to the six containers, respectively. The sixth container is the blank. The samples were placed in the dark for an hour at 25, so that the flavonoid could react with the free radicals. After an hour, it was observed that the blue color of the solutions within the containers that was associated with DPPH changed into yellow. This showed that the reaction between DPPH radical and flavonoid was done successfully. Finally, the UV - visspectroscopy device was adjusted for the wavelength of 520 nm (according toDPPH maximum absorption in water–methanol mixture) and the absorbance of each sample was measured, then according to the following equations[30]. the $IC_{50}$ of compounds were calculated.

$$IC(\%) = [(A_0 - A_t)/A_0] \times 100$$

Where$A_0$and$A_t$ are the absorbance values of the blank sample andthe test sample

Each measurement was repeated 3 times on average and the calculated average of the results was reported as the final data.

## RESULTS AND DISCUSSION

### Measurement of $IC_{50}$

$IC_{50}$ or the concentration of antioxidant that neutralizes half of the free radicals in the testing environment is an accurate and a good measure for the performance of antioxidants. The lower value of $IC_{50}$ indicates the better antioxidant performance of the compound[30,31]. The results showed that measuring $IC_{50}$ in combination with different percentages of methanol- water mixtures using DPPH method is very useful for measuring the antioxidant activity offlavonoids. Figure 1 shows the amount of $IC_{50}$ for each combination in methanol-water mixture. As can be seen, the amount of $IC_{50}$ for different flavonoids is: chrysine>naringenin>quercetin> ascorbic acid. The above results indicate that quercetin exhibits the lowest amount of $IC_{50}$ and the highest antioxidant activity among threeflavonoids This is due to the structural properties of the compounds. For example, by increasing the hydroxy groups in the structure of flavonoids, the antioxidant property of these compounds also increases. However, the amount of $IC_{50}$of quercetin is lower

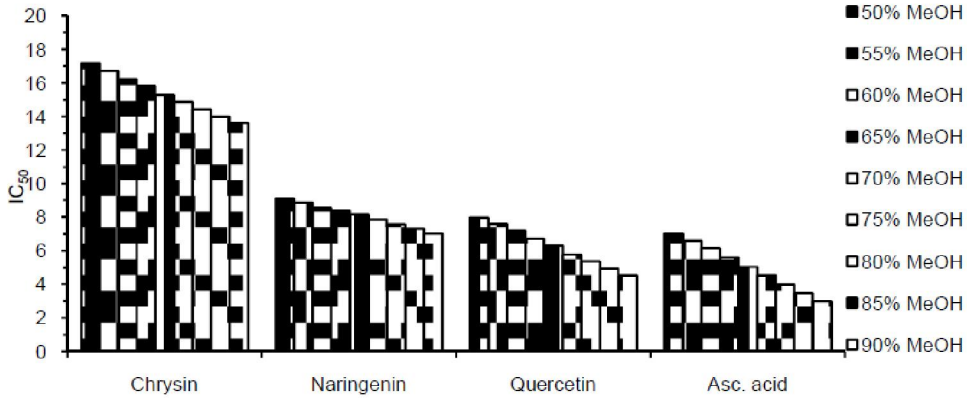



**Figure 1 : The $IC_{50}$ plots of the flavonides and asc. acid. (Standard deviations are based on three replicates)**

**TABLE 1 : Regression coefficient of the KAT equation (dual parameter) in different aqueous solutions of methanol (50–90% v/v)**

| Samples | Regression coefficient[a] | ose[b] | Rss | f-test | $r^{2}$ [c] |
|---|---|---|---|---|---|
| Asc.acid | $IC_{50}$= 6.05 (±1.23) – 12.45(±1.22) β + 8.82 (±0.45) $\pi^{*}$ | 0.03 | $^{-3}$×104.23 | 11316.35 | 1.00 |
| Quer | $IC_{50}$= 8.55 (±1.23) – 12.00 (±1.22) β + 6.98(±0.45)$\pi^{*}$ | 0.03 | $^{-3}$×104.23 | 8170.61 | 1.00 |
| Nar | $IC_{50}$= 6.27(±1.78) –3.90 (±1.76) β + 5.24 (±0.65) $\pi^{*}$ | 0.04 | $^{-3}$×108.79 | 1352.40 | 1.00 |
| Chry | $IC_{50}$= 22.91 (±1.62) –17.42 (±1.61) β+5.24 (±0.59) $\pi^{*}$ | 0.03 | $^{-3}$×107.33 | 4926.86 | 1.00 |

**(a) Values in the parentheses are the standard error for that coefficient; (b) the overall standard error; (c) the regression coefficient.**

**TABLE 2 : The calculated values of the $IC_{50}$ using the dual parameter of the KAT equation in different aqueous solutions of methanol (50–90% v/v)**

| MeOH%(v/v) | Asc.acid | quercetin | naringenin | chrysine |
|---|---|---|---|---|
| 50 | 7.04 | 7.98 | 9.07 | 17.16 |
| 55 | 6.59 | 7.59 | 8.86 | 16.73 |
| 60 | 6.11 | 7.17 | 8.63 | 16.28 |
| 65 | 5.60 | 6.73 | 8.38 | 15.81 |
| 70 | 5.07 | 6.28 | 8.13 | 15.34 |
| 75 | 4.53 | 5.82 | 7.86 | 14.86 |
| 80 | 3.99 | 5.37 | 7.59 | 14.41 |
| 85 | 3.47 | 4.94 | 7.31 | 14.00 |
| 90 | 2.97 | 4.53 | 7.03 | 13.66 |

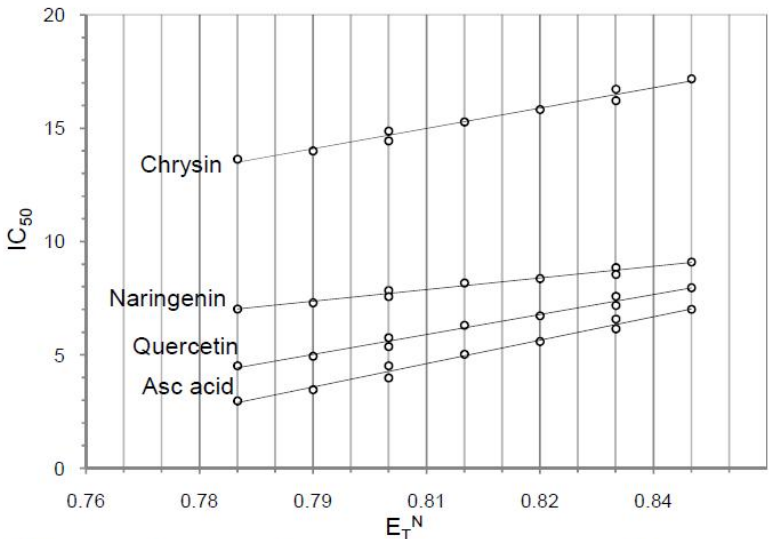



**Figure 2 : Plot of the $IC_{50}$ versus $E_T^N$ for nar, chry, quer and asc.acid in 50–90% methanol**

than that of chrysine andnaringenin; this is due to the presence of the 3-OH group in the ringC and a C2–C3 double bond in the ring-C of Quercetin[29]. The formeris favorable for increasing the electron-donating capacity [32]. the latter leads to conjugation with 4-oxo function in the ring-C that plays an important role in the electronic delocalization involving the ring-A and the ring-B, through the ring-C, and for spreadingconjugation over the entire molecule[33]. Another reason for the increased antioxidant power of Quercetin is the presence of orthohydroxy groups in the catechol part of the structure of these compound. Therefore the lower $IC_{50}$ (the higher antioxidant power) of quercetin compared to that of chrysine and naringin is reasonable. These results indicate that both

antioxidant activity andradical scavenging activity of naringenin are higher than thatof chrysine;this is due to the presence of the 4-OH group in the ringC.

**Solvent effect**

It is understood that a solvent could play a major role on thechemical behavior of antioxidant compounds[34]. The natural polyphenols likeflavonoids can usually exert their antioxidant action bythree mechanisms including hydrogen atom transfer, single electrontransfer to free radicals, and finally metal chelation. These mechanisms are affected by antioxidant structure and properties, solubilityand partition coefficient, and solvent system. In any analysis of solvent effects on antioxidant properties, it is customary to seek a linear relationship between some empirical solventparameter and antioxidant activity for the antioxidant compounds that in many cases is based on linear free-energy relationships. There are several empirical ways to measure the effects of solvent inorganic-water binary mixtures[35]. one of the most ambitious and successful method is the quantitative treatment using a multiparameters' equation that is known as linear solvation energy relationship (LSER) introduced by Kamlet, Abboud, and Taft (KAT)[36,37]. Thismethod explains any solute property varying with solvent compositionas a linear combination of the solvatochromic parameters of the solvent,$\pi^*$ [an index of solvent dipolarity/ polarizability accounting the ability ofthe solvent to stabilize a charge or a dipole by virtue of its dielectric effect (non-specific interaction)], $\alpha$(solvent hydrogen-bond donating(HBD) acidity), and$\beta$(solvent hydrogen-bond accepting (HBA) basicity(specific interactions). The appropriate form of the KAT equation in thiscase is:

$$IC_{50} = A_0 + a\alpha + b\beta + p\pi^* \quad (2)$$

Where$IC_{50}$ represents the concentration of flavonoids with 50% radical inhibition in different aqueous organic solvent mixtures, and$A_0$is the regression value ofthe solute property in cyclohexane as the reference solvent. The regression coefficientsa, bandpmeasure the relative susceptibilities of thesolvent dependence on the $IC_{50}$ to the indicated solvent parameters.

In order to explain the obtained $IC_{50}$ values through the KAT parameters, the $IC_{50}$ were correlated with solvent properties by means of single-, dual- and multi-parameter regressionanalysis by a suitable computer program (Microsoft Excel SOLVER andLINEST)[38]. We used the Gauss–Newton non-linear least-squares method in the computer program to refine the $IC_{50}$ by minimizing the error squares sum from Eq.(3)

$$S = \sum[(IC_{50})_{exp} - (IC_{50})_{cal}]^2 \quad (3)$$

The procedure used in the regression analysis involves a rigorousstatistical treatment tofind out which parameter in Eq.(2)is bestsuited to the water-organic mixed solvents. The obtained resultsshowed that the dual-parameter model using $\beta$ and$\pi^*$ parametersrepresents a significant improvement in the regression analysis withrespect to the single- or multi-parameter models. In order by a dual-parameter correlation of the $IC_{50}$ versus $\beta$ and$\pi^*$ was obtainedand is summarized in TABLE 1.

From TABLE 1, it is evident that compounds of asc. acid, quer and chry the regression coefficients of$\beta$and$\pi^*$ in Eq.(2)are in the order of$\pi^* > \beta$. This suggests that the hydrogen-bond acceptingparameter of the media is the most important and the polarity-polarizability parameter plays a relatively small role on $IC_{50}$.. But for naringenin the polarity-polarizability parameter will have a greater impact on antioxidant capacity of the compound. This is due to its structural characteristics. The negative sign of $\beta$ coefficient shows that the increase of basicity of solvent mixture reduces $IC_{50}$. Also the positive sign of the coefficient of $\pi^*$ indicates that the decrease in polarization of solvent mixture decreases the $IC_{50}$. According to the results, the more the amount of organic component in the solvent mixture increases, the less the value of $IC_{50}$ becomes. The calculated values of the $IC_{50}$ using$\beta$and$\pi^*$, via Eq.(2), in comparison with the experimental ones, showthat the interpretations are accurate (TABLE 2). However, the singleparameter correlation of the $IC_{50}$ versus$\beta$or$\pi^*$ gives poor results($r^2$=0.96 and 0.94, respectively).

Since normalized polarity parameter is a blend of dipolarity/polarizability and hydrogen-bond donating acidity, we also used the polarityscale proposed by Dimoroth and Reichardts,$E_T$, based on

**TABLE 3 : The KAT, $E_T^N$solvatochromic parameters of different methanol–$H_2O$ mixtures**

| MeOH%(v/v) | □ | □ | □ | $E_T^N$ |
|---|---|---|---|---|
| 50 | 1.01 | 0.63 | 1.01 | 0.84 |
| 55 | 1.01 | 0.65 | 0.98 | 0.83 |
| 60 | 1.01 | 0.67 | 0.95 | 0.83 |
| 65 | 1.01 | 0.68 | 0.91 | 0.82 |
| 70 | 1.02 | 0.70 | 0.87 | 0.81 |
| 75 | 1.03 | 0.71 | 0.83 | 0.80 |
| 80 | 1.05 | 0.73 | 0.79 | 0.80 |
| 85 | 1.06 | 0.74 | 0.75 | 0.79 |
| 90 | 1.08 | 0.75 | 0.70 | 0.78 |

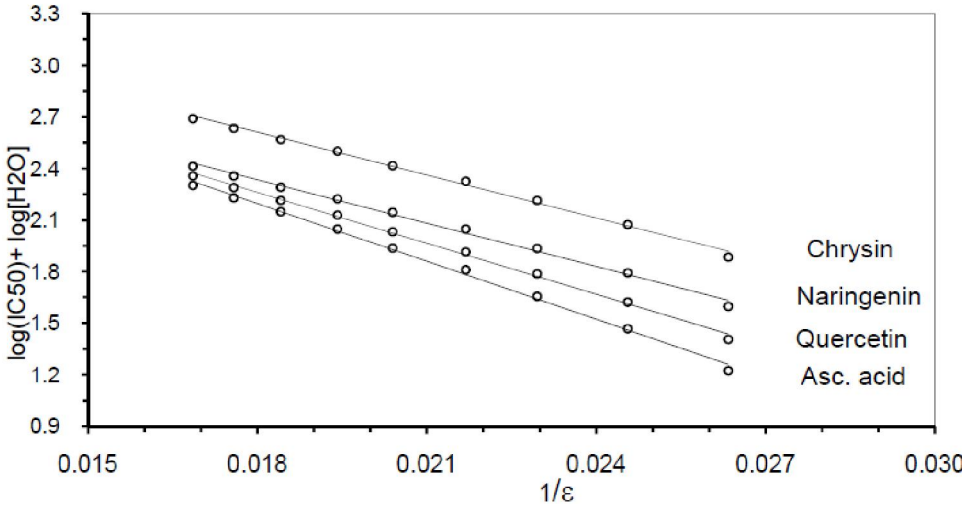



**Figure 3 : The Yasuda–Shedlovsky plots of the $IC_{50}$values of theflavonoids and asc. acid in different aqueous solutions of methanol**

the solvatochromic behavior of pyridinium N-phenoxidebetaine dye. This dye isthe most solvatochromic compound reported to date[39]. This scalehas now been revised and normalized to$E_T^N$, known as the normalizedpolarity parameter, due to the introduction of SI units. $E_T^N$is relatedwith the ability of a solvent to stabilize charge separation in the dye. According to this approach, the $IC_{50}$ values were correlated with $E_T^N$as a single linear regression analysis. The KAT and$E_T^N$parameter values for all the water–methanolmixtures used in this work were obtained from the plot of eachproperty versus the mole fraction of the organic solvent of thevalues that were reported in the literature for some other percentages of aqueous solutions of methanol[40,42]. Those are given in TABLE 3.

A very good linear correlation of the $IC_{50}$ values versus$E_T^N$wasobtained in the aqueous methanol mixtures (50–90% methanol v/v), Figure 2. The $IC_{50}$ values of theflavonoids and ascorbic acidincreasewith increasing solvent polarity parameter. The results clearly indicate that a solvent system with higher polarity ability decreased transferring of a hydrogen atom from theflavonoids to free radical and therefore has an increasing role in theantioxidant activity.

## Yasuda–shedlovsky extrapolation method

The $IC_{50}$ values of theflavonoids in pure water have been determined by extrapolation ofYasuda–Shedlovsky approach in different aqueous solution of methanolmixtures[43,44]. This approach was successfully applied before todetermine protonation constants of many weak acids or bases inpure water from the protonation constant values in different water–methanol mixtures[45]. However, in this work we used this methodto evaluate the $IC_{50}$ values of theflavonoids and asc. acid in pure water. It is

**TABLE 4 : The values of the $IC_{50}$ by extrapolation by the Yasuda–Shedlovsky approach to pure water in the range 50–90% (v/v) methanol**

| $IC_{50}$ | Component |
|---|---|
| 11.20 | Asc. acid |
| 11.59 | Quercetin |
| 12.23 | Naringenin |
| 21.54 | Chrysine |

claimed that Yasuda–Shedlovskyextrapolation procedure is generally more accurate than conventional method(the proposed property versus weight percent of an organic solvent)and can be applied for broad ranges of insoluble or sparingly watersoluble drug compounds[45]. On the basis of Yasuda–Shedlovsky approach[43,44]. the plot of log($IC_{50}$) +log[H2O] versus 1/εproduces astraight line (Eq.(4)), where $IC_{50}$ represents the concentration of flavonoids with 50% radical inhibition value in different aqueous organic solvent mixtures, [H2O] isthe molar concentration of water, andεshows the dielectric constantof the medium, aandbare two constants that should be determined for the various methanol–water mixtures used in this work. Figure 3 shows theYasuda–Shedlovsky plots of the systems studied are linear withcorrelation coefficients 0.99 or more. The $IC_{50}$ values determinedby extrapolation to zero percent methanol have been summarized inTABLE 4, which shows a lesser antioxidant activity of the flavonoid andasc. acid in a poor water.

$$\log [IC_{50}] + \log[H_2O] = \frac{a}{\varepsilon} + b \quad \textbf{(4)}$$

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