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Antioxidant activity of fulvic acid: A living matter-derived bioactive compound

Noemí Cárdenas Rodríguez [1]*, Elvia Coballase Urrutia [1], Bernardino Huerta Gertrudis [1], José Pedraza Chaverri [2] and Gerardo Barragán Mejía [1]

*[1] Laboratorio de Neuroquímica, Instituto Nacional de Pediatría, Insurgentes Sur 1300, Letra C, Col. Insurgentes-Cuicuilco, Del. Coyoacán, 04530, Mexico D.F. [2] Departamento de Biología, Facultad de Química, Universidad Nacional Autónoma de México. *e-mail: noemicr2001@yahoo.com.mx*

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Abstract

Fulvic acid (FA) is a class of compound including humic substances together with humic acid and humin. It is formed through the degradation of organic substances by chemical and biological process. FA consists of a mixture of closely related complex aromatic polymers with the presence of aromatic rings, phenolic hydroxyl, ketone carbonyl, quinone carbonyl, carboxyl and alkoxyl groups. The possible application of coal-derived FA as an antimicrobial and anti-inflammatory property has been reported. Actually, it is used as a soil supplement in agriculture and as a human nutritional supplement. In this work, we examined, for the first time, the scavenging activity of biosynthesized fulvic acid in comparison with reference compounds. It was evaluated the *in vitro* superoxide ($O_2^{\bullet-}$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$) and singlet oxygen ($^1O_2$) scavenging capacity of the fulvic acid synthesized from a compost elaborated with poultry manure by spectrophotometric methods. The IC_{50} (mg/ml) values were as follows: 4.97±0.02, 1.56±0.06, 1.39±0.03, 2.5±0.04, 5.73±0.05 and 3.54±0.03 for $O_2^{\bullet-}$, HOCl, H_2O_2, $OH^{\bullet}$, $ONOO^-$ and $^1O_2$, respectively. FA displays a scavenging activity compared with the reference compounds although it was less efficient than nordihydroguaiaretic acid (NDGA), ascorbic acid, pyruvate, dimethylthiourea (DMTU), penicillamine and glutathione (GSH) for $O_2^{\bullet-}$, HOCl, H_2O_2, $OH^{\bullet}$, $ONOO^-$ and $^1O_2$, respectively. The antioxidant properties of the FA partially support the health beneficial properties of this compound; and therefore, the FA is a good candidate to be used in pharmaceutical or food industries as an accessible source of natural antioxidants.

Key words: Fulvic acid, scavenging capacity, antioxidant properties.

Introduction

Fulvic acid (FA) is a class of compounds including humic substances together with humic acid and humin. It is formed through the degradation of such organic substances as dead plants, microbes and animals by chemical and biological process. FA is also found abundantly in peat, weathered coal and other humified substances [1].

Chemical and spectroscopic analyses have revealed the presence of aromatic rings and phenolic hydroxyls, ketone carbonyl, quinone carbonyl, carboxyl and alkoxyl groups [1-3]. Humic substances are used in medicine and antimicrobial, anti-inflammatory and antitumor agents, as liver stimulants, remedies for gastric ulcer to stop bleeding and for the treatment for skin burns [4].

FA has various useful effects due to its functional groups. Studies on the physiological actions of FA exerted on the living body are gradually being carried out. The possible application of coal-derived FA as an antimicrobial and antioxidant substance has been described and the inflammatory property of coal-derived FA has been also reported [5-7]. It has been used externally to treat haematoma, phlebitis, desmorrhexis, myogelosis, arthrosis, polyarthritis, osteoarthritis and osteochondrosis. Likewise, FA has been taken orally as a therapy for gastritis, diarrhoea, stomach ulcers, dysentery, colitis and diabetes mellitus [8]. FA and humic substances isolated from soil and water reservoirs have been reported to stimulate neutrophil and lymphocyte immune function [1]. It is especially reactive with metals, forming strong complexes with Fe^{3+}, Al^{3+} and Cu^{2+} [9, 10]. FA and its related compounds have no toxic compounds [11]. Humic and FA are commonly used as a soil supplement in agriculture and as a human nutritional supplement.

ROS contribute to the development of various diseases such as atherosclerosis, diabetes, cancer, neurodegenerative diseases, liver cirrhosis and ageing process [12]. To prevent the damage caused by ROS, tissues had developed an antioxidant defence system that includes nonezymatic antioxidants (*e.g.*, glutathione, uric acid, bilirubin and vitamins C and E) and enzymatic activities such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [13]. A second level of prevention against ROS-induced damage is constituted by scavenging compounds, which are able to reduce the incidence of free radical-mediated diseases [13].

The use of antioxidants, both natural and synthetic, in the prevention and cure of various diseases is expanding. There is a considerable interest in the antioxidant activities of molecules such as vitamins E and C and plant polyphenolic and carotenoid components [14]. In this sense, FA displays activity against superoxide and hydroxyl radicals [15].

Despite the broad spectrum use of FA for a variety of medical conditions, far less is known regarding the mechanisms of action

of FA. Therefore, the objective of this work was to evaluate, for the first time, the *in vitro* superoxide ($O_2^{\bullet-}$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), peroxynitrite ($ONOO^-$) and singlet oxygen ($^1O_2$) scavenging capacity of the FA derived from a compost elaborated with poultry manure (Miyamonte, Mexico) by established spectrophotometric methods.

Materials and Methods

Chemicals: Sodium pyruvate, dimethyl thiourea (DMTU), nordihydroguaiaretic acid (NDGA), ascorbic acid, histidine, xylenol orange, butylated hydroxytoluene, ammonium iron (II), sulphate hexahydrate, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), bovine serum albumin (BSA), dimethyl sulphoxide (DMSO), NN-dimethyl-4-nitrosoaniline (DMNA), catalase, xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), potassium nitrite (KNO_2), manganese dioxide (MnO_2), diethylene triamine pentaacetic acid (DTPA), butylated hydroxytoluene (BHT) and DL-penicillamine were purchased to Sigma Aldrich (St. Louis, MO, USA), and 2,7-dichlorodihydrofluorescein diacetate (DCF) and dihydrorhodamine 123 (DHR-123) were obtained from Cayman Chemical (Ann Arbor, MI, USA). Potassium persulphate and sodium carbonate (Na_2CO_3) were from Mallinckrodt (Paris, KY, USA). Absolute ethanol, hydrogen peroxide (H_2O_2), sulphuric acid (H_2SO_4), methanol, ethylenediamine-tetraacetic acid disodium salt (EDTA), sodium hypochlorite (NaOCl) and sodium nitrite ($NaNO_2$) were purchased to JT Baker (Mexico City, Mexico). All other chemicals were reagent grade and commercially available.

Preparation of fulvic acid: FA was obtained from a compost elaborated with poultry manure, its characteristics are described in Table 1. It was prepared by Miyamonte México S.A. de C.V.

FA was extracted from the compost following this process in brief: a suspension was elaborated with 1 M NaOH, followed a precipitation with 10% H_2SO_4. The obtained solution was centrifuged to precipitate impurities and eliminate them. The solution was placed on NH_4OH (pH 2 to 7) in a roto-evaporator to solidify the FA (greyish powder). Finally, it was dried in an oven at 60ºC [16].

Table 1. Characteristics of a compost elaborated with poultry manure by Miyamonte México S.A. de C.V.

Element	Contents (%)
Organic matter	58.13
Nitrogen (NH_3)	23.57
Amoniacal nitrogen	0.2836
Nitric oxide	0.062
Phosphates	2.66
Calcium	8.08
Potassium	2.61
Magnesium	0.59
Zinc	0.024
Iron	0.36
Aluminium	0.059
pH	6.5-7.5
Humidity	15-20

Determination of $O_2^{\bullet-}$ scavenging capacity: Xanthine-xanthine oxidase system was used to determine the $O_2^{\bullet-}$ scavenging capacity of the FA. $O_2^{\bullet-}$ in the assay system and xanthine oxidase activity were measured as NBT reduction using a DU-64O series Beckman spectrophotometer. This system is useful to test the $O_2^{\bullet-}$ scavenging capacity only when the samples used do not interfere with the xanthine oxidase activity. A compound with $O_2^{\bullet-}$ scavenging capacity should decrease NBT reduction without interfering with the xanthine oxidase activity measured as uric acid production. Eight hundred µl of the following reaction mixture: 90 µM xanthine, 16 mM Na_2CO_3, 22.8 µM NBT and 18 mM phosphate buffer (pH 7.0) were mixed with 100 µl of different concentrations of FA. The reaction was started by the addition of 100 µl of xanthine oxidase (168 U/litre). Optical density was registered both at 295 nm (for uric acid production) and 560 nm (for $O_2^{\bullet-}$ in the assay system) [17]. Scavenging percent was obtained from the optical densities at 560 nm. NDGA was used as standard for $O_2^{\bullet-}$ scavenging in this assay.

HOCl scavenging assay: The catalase assay involves a spectral analysis of the enzyme. A spectrum (200-500 nm) of catalase, catalase treated with HOCl and catalase containing varied mixtures of HOCl treated with increasing concentrations of FA or the reference compound was obtained. The HOCl scavenging capacity of FA or the reference antioxidant was evident by the inability of HOCl to eliminate/decrease the peak in a concentration-dependent way. Experiments were carried out essentially as described before [18]. A solution of 49.8 µM bovine liver catalase (16.6 µM, final concentration) was mixed with 18 mM HOCl (6 mM, final concentration) in the presence of increasing concentrations of FA or the reference compound.

Spectra (370-450 nm) of catalase alone, catalase plus HOCl, catalase plus HOCl and the FA or the reference compound were registered and the optical densities (OD) at 404 nm were obtained. The value of the OD of catalase alone minus the OD of catalase plus HOCl was considered as 100% of degradation of catalase (or 0% of scavenging activity), and the difference of the catalase alone minus the OD of the catalase plus HOCl in presence of either FA or reference compound was compared against this value[18]. The ability of FA to scavenge HOCl was compared with that of ascorbic acid.

Determination of H_2O_2 by the ferrous ion oxidation-xylenol orange (FOX) assay: A solution of 75 mM H_2O_2 was mixed (1:1v/v) with water (0% scavenging tube) or with different concentrations of FA and incubated for 30 min at room temperature. After this, H_2O_2 was measured by the following method: briefly, 9 volumes of 4.4 mM BHT in HPLC-grade methanol were mixed with one volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 0.25 M H_2SO_4 to give the working FOX reagent.Of the extract solutions 45 µl and 45 µl of 75 µM H_2O_2 were dispensed in 1.5 ml Eppendorf tubes and mixed with 10 µl of HPLC-grade methanol immediately followed by the addition of 0.9 ml of FOX reagent. Solution was mixed on a Vortex mixer for 5 s and incubated at room temperature for 10 min. The tubes were centrifuged for 15,000×g for 10 min and absorbance at 560 nm was read against a methanol blank. The concentration of H_2O_2 was calculated from a standard curve prepared with increasing H_2O_2 concentrations. Pyruvate was used as standard for H_2O_2 scavenging activity [18].

Determination of OH• scavenging capacity: The ability of FA to scavenge OH• was conducted in the Fe^{3+}-EDTA-H_2O_2-deoxyribose system [19]. The reaction mixture containing deoxyribose (0.056 mM), H_2O_2 (1 mM), potassium phosphate buffer (10 mM, pH 7.4), $FeCl_3$ (0.2 mM), EDTA (0.2 mM) and ascorbic acid (0.2 mM) was incubated in a water bath at 37±0.5°C for 1h. The extent of the deoxyribose degradation by the OH• formed was measured directly in the aqueous FA phase by the thiobarbituric acid test at 532 nm. The ability of FA to scavenge OH• was compared with that of DMTU.

Synthesis of $ONOO^-$: $ONOO^-$ was synthesized as previously described [17]. Five ml of an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with 5 ml of 0.6 M KNO_2 on an ice bath for 1 s and the reaction was quenched with 5 ml of ice-cold 1.2 M NaOH. Residual H_2O_2 was removed using prewashed granular MnO_2 and the reaction mixture was then left overnight at -20°C. The resulting yellow liquid layer on the top of the frozen mixture was collected for the experiment.

$ONOO^-$ scavenging assay: The $ONOO^-$ mediated oxidation of DHR-123 was performed as described before [18]. A 50 mM solution of DHR123 was prepared from a 28 mM stock solution in DMSO. The solution was maintained protected from light at 4°C during the assay. All reaction mixtures contained 5 mM DHR-123, 0.1 mM DTPA, different concentrations of extracts and 25 mM $ONOO^-$. Optical density was registered at 500 nm, the optical density of a mixture without sample was considered as 100% and the optical densities of the mixtures containing the FA were compared against it. The ability of the tested FA to scavenge $ONOO^-$ was compared with that of penicillamine.

$^1O_2$ assay: The production of $^1O_2$ by NaOCl and H_2O_2 was determined using DMNA as selective acceptor of $^1O_2$ as reported elsewhere with minor modifications [18]. The bleaching of DMNA was monitored spectrophotometrically at 440 nm. The assay mixture contained 45 mM Na-phosphate buffer (pH 7.1), 10 mM histidine, 10 mM NaOCl, 10 mM H_2O_2, 50 μM DMNA and 0.1 ml of FA. The total volume of reaction (2.0 ml) was incubated at 30°C for 40 min. The extent of $^1O_2$ production was determined by measuring the decrease in the absorbance of DMNA at 440 nm. The relative scavenging efficiency (percentage of inhibition of $^1O_2$ production) of FA was estimated from the difference in absorbance of DMNA with and without the addition of FA, being tested or reference compound. Glutathione was used as standard for $^1O_2$ scavenging.

Statistical analysis: Data are expressed as mean±SD. The data were compared against the blank tube without FA or the reference compounds using student *t* test. (GraphPad Prism 4.0 Software, San Diego, CA, USA). $P<0.05$ was considered statistically significant. The scavenging capacity was expressed as the 50% inhibitory concentration value (IC_{50}), which denotes the concentration of FA or the reference compounds required to give a 50% reduction in oxidating effect relative to the blank tube.

Results and Discussion

This is the first time that IC_{50} values of FA for HOCl, $ONOO^-$, H_2O_2, $^1O_2$ and OH• are described. The FA, as well as the reference compounds, scavenged $O_2^{\bullet-}$, HOCl, H_2O_2, $ONOO^-$, $^1O_2$, and OH• in a concentration-dependent way (Figs. 1-6). The IC_{50} values, calculated from the linear portion of the dose-response curve, are shown in Table 2. The analysis of the IC_{50} values indicated that FA displays a scavenging activity compared to the reference compounds; although it was less efficient than NDGA, ascorbic acid, pyruvate, DMTU, penicillamine and GSH for $O_2^{\bullet-}$, HOCl, H_2O_2, OH•, $ONOO^-$ and $^1O_2$, respectively ($P<0.0001$).

FA is a class of compounds consisting of complex polymeric aromatic structures. It is formed through environmental degradation of animal, plant, fungal and bacterial biopolymers[20-22]. The FA act as an antioxidant like other high molecular weight plant phenolics such as tannins [7]. In the present paper, we have shown that FA *in vitro* scavenged of $O_2^{\bullet-}$, HOCl, H_2O_2, OH•, $ONOO^-$ and $^1O_2$ in a concentration-dependent way. These specific scavenging properties of FA contribute to explain their antioxidant properties [7]. The ability of FA to scavenge the above mentioned reactive species was compared with reference compounds with the purpose to know the relative efficacy of FA to scavenge these species.

To our knowledge this is the first time that IC_{50} values of FA for $O_2^{\bullet-}$, HOCl, H_2O_2, OH•, $ONOO^-$ and $^1O_2$ are described. Based on these comparisons, FA is less effective than the reference compounds to cope with all species studied. In this context, it has been demonstrated the scavenging activity of four FA (named XWCS-1, XWCS-4, XWCS-8 and XWCFA), obtained by ozonolysis of humic acid extracted from Xinjiang (China) weathered coal, for $O_2^{\bullet-}$ and OH• radicals investigated with an electron spin resonance (ESR)-spin trapping method [7] and quenching $^1O_2$ generated through visible light irradiation of Rose Bengal [23]. FA also reduced OH• radical formation, rate and time dependent, in aqueous iron-hydrogen peroxide reaction [24].

The hydrophobicity of the antioxidants plays a role in the efficacy of inhibition. The presence of structural units O-functionalized, including aromatic domains in FA, could explain their tendency to form molecular aggregates (hydrogen bridges, metal bridges and hydrophobic interactions) in solution [2, 3, 7]. Moreover, it has been known that phenolic hydroxyl group is the main active group which scavenges OH• (this effect can be primarily attributed to the hydrogen donation and electron transfer capacities of OH group) and favours the encapsulation of the pro-oxidant iron species, which generates OH• through the Fenton reaction [25-27]. This suggests that the phenolic hydroxyl group and metal-chelating ability by FA could explain the ROS scavenging activity observed.

Table 2. Scavenging capacity of FA and reference compounds.

Species	Fulvic acid IC_{50} (mg/ml)	Reference compound	IC_{50} (mg/ml)
$O_2^{\bullet-}$	3.87±0.19	NDGA	0.003 ± 0.0003 *
HOCl	14.93±0.47	Ascorbic acid	0.0016 ± 0.0009 *
H_2O_2	49.79±7.86	Pyruvate	1.3 ± 0.0708 *
OH•	48.59±2.12	DMTU	0.166 ± 0.0634 *
$ONOO^-$	57.2±5.78	Penicillamine	0.0017 ± 0.00013 *
$^1O_2$	32.56±2.22	GSH	0.75 ± 0.0732 *

Data are presented as mean ± SD of six independent assays.
*$P<0.0001$ *vs* FA.

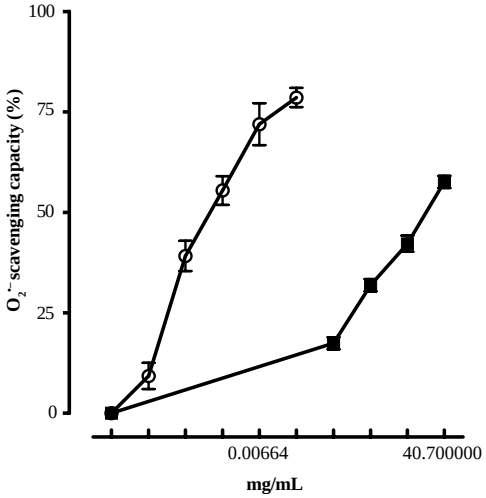

Figure 1. Fulvic acid scavenges $O_2^{\cdot-}$ in a concentration-dependent way; (○) reference compound: NDGA and (■) FA. Data are mean±SD, n = 3 assays. P<0.001 *vs* 0 g/ml.

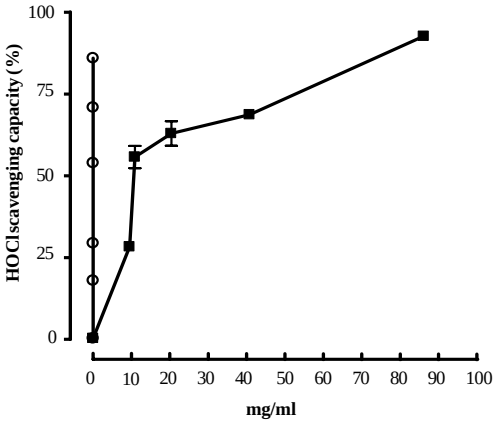

Figure 2. Fulvic acid scavenges HOCl in a concentration-dependent way; (○) reference compound: ascorbic acid and (■) FA. Data are mean±SD, n = 6 assays. P<0.0001 *vs* reference compound.

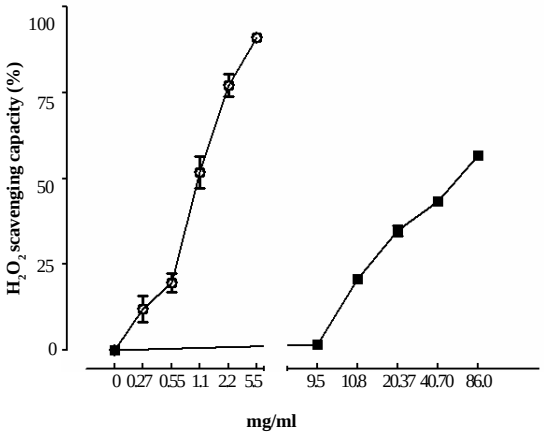

Figure 3. Fulvic acid scavenges H_2O_2 in a concentration-dependent way; (○) reference compound: pyruvate and (■) FA. Data are mean±SD, n = 6 assays. P<0.0001 *vs* reference compound.

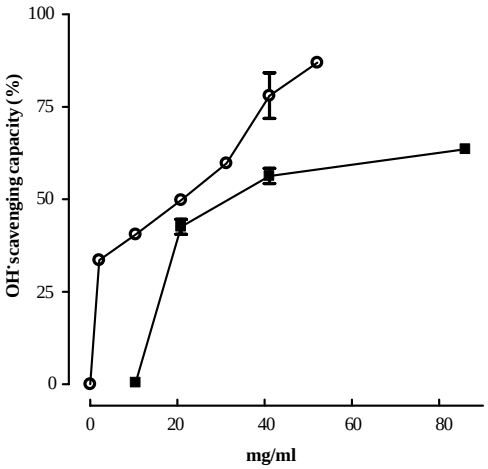

Figure 4. Fulvic acid scavenges $OH^{\cdot}$ in a concentration-dependent way; (○) reference compound: DMTU and (■) FA. Data are mean±SD, n = 6 assays. P<0.0001 *vs* reference compound.

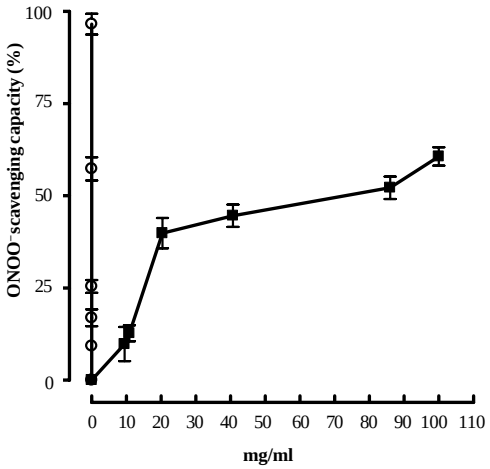

Figure 5. Fulvic acid scavenges $ONOO^-$ in a concentration-dependent way; (○) reference compound: penicillamine and (■) FA. Data are mean±SD, n = 6 assays. P<0.0001 *vs* reference compound.

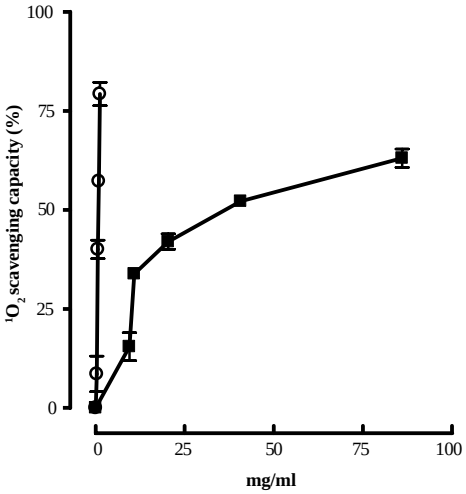

Figure 6. Fulvic acid scavenges $^1O_2$ in a concentration-dependent way; (○) reference compound: GSH and (■) FA. Data are mean±SD, n = 6 assays. P<0.0001 *vs* reference compound.

Conclusions

The antioxidant properties of FA described in this work could explain some of the health beneficial effects of this compound since the excessive production of $O_2^{\bullet-}$, HOCl, H_2O_2, $OH^{\bullet}$, $ONOO^-$ and $^1O_2$ are involved in several pathologies. Moreover, they could be a good candidate for use in pharmaceutical or food industries as an accessible source of natural antioxidants and for the improvement of food quality by retarding lipid oxidation.

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