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Total phenolic content, cytotoxic and antioxidant activities of *Quisqualis indica* (Linn.) growing in Egypt

Ahmed A. A. Abd El-Rahman[1], Ibrahim M. Abd El-Aleem[1], Laila A. Refahy[2] and Maha A. El-Shazly[2*]

[1]*Agriculture Biochemistry Department, Faculty of Agriculture, Banha University, Moshtohor, El-Kalubya, Egypt*
[2]*Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt*

ABSTRACT

The aim of this study is to evaluate the antioxidant, cytotoxic activities and total phenolic content (TPC) of different fractions of Quisqualis indica(Linn.) leaves. The antioxidant activities were carried out quantitatively using 1,1'-diphenyl-2-picraylhydrazyl free radical (DPPH), phosphomolybdenum and reducing power assays, also qualitatively via dot-blot DPPH staining assay. Also, the cytotoxic activity was evaluated via brine shrimp lethality test (BSLT) and MTT assay against liver carcinoma cell line (HepG-2).The antioxidant activity results against DPPH radical (SC_{50}) were ranged from 24.38 to 72.10 µg/mlwith respect to ascorbic acid (SC_{50} = 7.45µg/ml), among all tested fractions EtOAc is the most active, furthermore the total antioxidant capacity results were ranged from 250.76 to 16.17 (mg AAE /g dry ext.),moreover at concentration 200 µg/ml the reducing power results (OD values) were arranged as; n-BuOH 0.680 > defatted 90% MeOH (0.465) > EtOAc (0.405) > 90% MeOH (0.225) > H_2O (0.90), in comparison with ascorbic acid (0.985). On the other hand, the cytotoxic activities were arranged as; n-BuOH (LC_{50}= 100), 90% MeOH (LC_{50}= 150), EtOAc (LC_{50}= 170), pet. ether (LC_{50}= 170) and defatted 90% MeOH (LC_{50}= 440) µg/ml.Finally, the cytotoxic results against (HepG-2) revealed that CH_2Cl_2 & n-BuOH are the most strong cytotoxic fractions (IC_{50}= 11.9, 17.9µg/ml respectively) against Doxorubicin (IC_{50} = 4 µg/ml). In conclusion, Quisqualis indica consider as a good source of naturally occurring antioxidant and cytotoxic agents.

Keywords: *Quisqualis indica*, cytotoxic, antioxidant, total phenolic content.

INTRODUCTION

Medicinal plants have a long history for treating various types of diseases all over the world, because they contain a variety of chemical substances that act upon the body to prevent, relieve and treat illnesses. Also, medicinal plants are important for pharmacological research and drug development **[1-3]**.The Combretaceae is a large family, it consists of 20 genera. The most commonly occurring genera are *Combretum*, *Terminalia* and *Quisqualis* each with about 250-300 species **[4, 5]**.Rangoon Creeper scientifically known as *Quisqualis indica* originated from South East Asia and occurs all over Africa, The flowers contain high quantity of poly- phenol that are believed to be strong antioxidants beneficial for human health **[6]**. The volatile oil of *Quisqualis indica* flowers consists of E- and Z-linalool oxides, 2,2,6-trimethyl-6-vinyl-3-keto-tetrahydro pyran, 2,2,6-trimethyl-6-vinyl-3-hydroxy- tetrahydropyran (linalool oxide pyranoid form), (E,E)-alpha farnesene, Z-3-hexenyl benzoate and benzyl benzoate **[7]**.Also, the phytochemical screening for *Quisqualis indica* showed the presence of, steroids, triterpenoids, phenols, tannins and flavonoid, glycosides in leaves and fol, 1-(3,4-dimethoxyphenyl) -2- (4-allyl-2,6 dimethoxyphenoxy) propan-1-ylacetate and 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(4-allyl-2,6 dimethoxyphenoxy) propan -1-ol **[8]**.

Free radicals or reactive oxygen species (ROS) including superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide ($NO^{\cdot}$) radical, and hydrogen peroxide (H_2O_2) are physiological metabolites. Free radicals have been

implicated in the causation of several diseases such as liver cirrhosis, inflammation, genotoxicity, atherosclerosis, cancer and diabetes **[9-12]**.In addition, the antioxidant can be defined as the chemical compounds which can delay the start or slow the rate of lipid oxidation reaction in food systems. The antioxidant free radicals can also react with lipid free radicals to form stable complex compounds and the resulting antioxidant free radical is not subject to rapid oxidation due to its stability **[13, 14]**. Natural antioxidants are found in many sources including foods, fruits and vegetables, such as vitamin C, vitamin E (tocopherols), vitamin A (carotenoids), various polyphenols including flavonoids, phenolic acids, tannins and anthocyanins as well as lycopene (carotenoids) **[15]**.

Furthermore, cancer is the second leading cause of death in the world, so scientists over the entire world do their best to discover safe cancer therapy. Cancer is considered as a major public health problem either in the developed and developing countries over the world**[16]**. It was estimated that 12.7 million recent cancer cases and about 7.6 million cancer deaths take place in the year 2008, which reflected the harmful effect of cancer on human by its various types. In fact, most of the artificial agents currently being used in cancer therapy are toxic and produce damage to normal cells. Therefore, chemoprevention or chemotherapy via nontoxic agents could be one solution for decreasing the harmful effects of cancer **[17-19]**. Therefore, in this study we will evaluate the antioxidant, cytotoxic activities and total phenolic contents of methanol extract of *Quisqualis indica* and its sub-fractions.

MATERIALS AND METHODS

1. Plant Material

The plant under investigation was collected from El-Zoharya Garden, Cairo, Egypt in May 2012. The identity of the plant was established by Eng. Tereez Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman Botanical Garden, Giza, Egypt. Voucher specimens (given number *QI*-2012) were kept in the Department of Medicinal Chemistry, Theodor Bilharz Research Institute (TBRI). The plant materials were air-dried in shade place at room temperature and then powdered by electric mill, finally kept in tightly closed container in a dark place till the extraction process.

2. Chemical, Reagents and Equipments

All solvents and reagents used were of analytical grade. 1,1'-diphenyl-2-picrylhydrazyl (DPPH) free radical and Folin–Ciocalteu's reagent (FCR) were purchased from (Sigma-Aldrich Co.). Trichloroacetic acid (TCA), pot. ferricyanide, ferric chloride, aluminum trichloride, sodium carbonate, disodium phosphate, ammonium molybdate, rutin, ascorbic acid and gallic acid were purchased from (Merck Chemical Co.), all solvents and acids (methanol, petroleum ether, chloroform, ethyl acetate, n-butanol), were purchased from (Sigma-Aldrich Co.). The absorbance measurements for antioxidant activity were recorded using the UV-Vis spectrophotometer Spectronic 601 (Milton Roy, USA).

3. Extraction and Fractionation

The dry powder of leaves of the plant (250 g) were soaked it in 90% MeOH (2.5 l), in room temperature with shaking day by day followed by filtration and again extraction for five times. Then each extract was filtered using Whatmann filter paper No.1 and concentrated by using a rotary evaporator (Buchi, Switzerland) at (40 ± 2°C) affording known weight of each crude methanol extract. The 90% methanolic crude extracts (31 g) was defatted by washing several times with petroleum ether (60-80°C), then 28 gram of the defatted 90% methanol extract was dissolved in distilled water then partitioned via; CH_2Cl_2, EtOAc and n-BuOH to afford 2.81g, 2.0 g, 3g , 10g and 11 g respectively for pet. ether, CH_2Cl_2, EtOAc, n-BuOH and H_2O extracts.

4. Total Phenolic Content

The total phenolic content was determined using Folin- Ciocalteu's reagent according to the method described by Kumar et al., 2008. In this method, the reaction mixture was composed of (100 µl) of plant extract (100 µg/ml), 500 µl of the Folin-Ciocalteu's reagent and 1.5 ml of sodium carbonate (20%). The mixture was shaken and made up to 10 ml using distilled water. The mixture was allowed to stand for 2 h, and then the absorbance was measured at 765 nm; gallic acid was used as standard. All determinations were carried out in triplicate. The total phenolic content was expressed as mg gallic acid equivalent (GAE) per g extract **[20]**.

5. Antioxidant Activity

5.1. DPPH free radical scavenging activity

The scavenging activity of the stable 1,1'-diphenyl-2-picrylhydrazyl free radical wasdetermined by the method described by Marwah et al., 2007. Briefly, the reaction medium contained 2 ml of 100 µM DPPH purple solution in methanol and 2 ml of plant extract, ascorbic acid was used as standard. The reaction mixture was incubated in the dark for 20 min and the absorbance was recorded at 517 nm. The assay was carried out in triplicate. The DPPH radical scavenging activity was calculated according to the equation: % DPPH radical scavenging activity [1-

($A_{sample}/A_{control}$) x 100], where $A_{control}$ and A_{sample} are the absorbencies of control and sample after 20 min, respectively. The SC_{50} (concentration of sample required to scavenge 50% of DPPH radicals) values were determined. The decrease of absorbance of DPPH solution indicates an increase of the DPPH radical scavenging activity **[21]**.

5.2. Total antioxidant capacity

The antioxidant activity of the plant extract was determined according to phosphomolybdenum method, using ascorbic acid as standard. In this method, 0.5 ml of each extract (200 µg /ml) in methanol was combined in dried vials with 5 ml of reagent solution (0.6 M sulfuric acid, 28 mM disodium phosphate and 4 mM ammonium molybdate).The vials containing the reaction mixture were capped and incubated in a thermal block at95°C for 90 min. After the samples had cooled at room temperature, the absorbance was measured at 695 nm against a blank. The blank consisted of all reagents and solvents without the sample and it was incubated under the same conditions. All experiments were carried out in triplicate. The antioxidant activity of the extracts was expressed as the number of equivalents of ascorbic acid (AAE) **[22]**.

5.3.Reducing power antioxidant assay (RPAA)

A Spectrophotometric method described by Ferreira *et al.*, 2007; was used for the measurement of reducing power. For this, 2.5 ml of each extract was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10 mg/ml). The mixture was incubated at 50ºC for 20 min, then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (2.5 ml) of the supernatant was diluted with distilled water (2.5 ml), and then ferric chloride (0.5 ml, 0.1%) was added and allowed to stand for 10 min. The absorbance was read spectrophotometrically at 700 nm, ascorbic acid was used as standard. Three replicates were made for each test sample **[23]**.

6. Cytotoxicity Activity

6.1.Brine shrimp lethality bioassay test

A solution of instant ocean sea salt (Aquarium System, Ohio) was made by dissolving 2.86 g in distilled water (75ml). 50 mg of *Artemia salina* Leach eggs (Artemia, Inc., California) was added in a hatching chamber. The hatching chamber was kept under an inflorescent bulb for 48 h for eggs to hatch into shrimp larvae. 20 mg of the tested extract was dissolved in 2 ml methanol or solvent in which it was soluble and from this, 500, 400, 300, 200, 100, 50, 5 µlof each solution was transferred into vials corresponding to 1000, 800, 600, 400, 200,100, and 10 µg/ml, respectively. Each dose was tested in triplicate. The vials and the control containing 500 µl of solvent were allowed to evaporate to dryness in about 48h at room temperature. 4.5 ml of instant ocean sea solution were added to each vial and 10 larvae of *Artemia salina* (taken 8-72 h after the initiation of hatching) were added to each vial. The final volume of solution in each vial was adjusted to 5 ml with sea salt solution immediately after adding the shrimp. 24 h later the number of surviving shrimp at each dosage was counted and recorded. LC_{50} values were determined with 95% confidence intervals by analyzing the data. The data were analyzed and LC_{50} values were calculated and carried according to Reed-Muench method. Potassium dichromate was used as standard **[24, 25]**.

6.2. MTT antitumor activity assay

The tested human carcinoma cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were maintained at 37ºC in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. For antitumor assays, the tumor cell lines were suspended in medium at concentration $5x10^4$ cell/well in Corning® 96-well tissue culture plates, then incubated for 24 hr. The tested compounds were then added into 96-well plates (six replicates) to achieve eight concentrations for each compound. Six vehicle controls with media or 0.5% DMSO were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO_2 for 4 hours. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration isplotted to get the survival curve of each tumor cell line after treatment with the specified compound.The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) [26-28].

6.3. Statistical Analysis

All data were presented as mean ± S.D. using SPSS 13.0 program. Correlation analysis of the antioxidant activity and free radical scavenging activity versus the total phenolic content of the different extracts of tested plant were carried out using the correlation and regression by Microsoft Excel program **[29]**. The Reed-Muench method assumes that an animal that survived a given dose would also have survived any lower dose, and conversely, that an animal that died with a certain dose would have also died at any other higher dose. Thus, the information from any one group can be added to that of the other groups in the range of dose tested **[24, 25]**.

RESULTS AND DISCUSSION

1. Total phenolic content (TPC)

The results in Table 1 revealed that the total phenolic contents of the 90% methanol extract and its sub-fractions are in the order; n-BuOH (380.78) > EtOAc (339.41) > defatted 90% MeOH (345.99)> 90% MeOH (309.0) > H_2O (78.59)(mg GAE /g dry ext.) as well as the remaining sub-fractions pet. Ether and CH_2Cl_2 which exhibit very small phenolic content 14.99 and 32.31 (mg GAE /g dry ext.) respectively. Many researchers are referring to the high positive correlation between TPC of the medicinal plant extracts/fractions and their antioxidant potential **[18, 19]**. The high phenolic content of the most tested fractions promote us to evaluate them as antioxidant agents.

2. Antioxidant activity

Due to the complex chemical profile of the medicinal plant extracts and to ensure high accuracy, therefore the antioxidant activity should be carried out via different antioxidant methods. According to the last mentioned concept, in our current study the antioxidant activity of different fractions of *Q. indica* was estimated quantitatively via 1,1'-diphenyl-2-picraylhydrazyl free radical, phosphomolybdenum and reducing power antioxidant assays, also qualitatively via dot-blot DPPH staining assays **[30]**.The results of the antioxidant activity of *Quisqualis indica* extracts against 1,1'-diphenyl-2-picrylhydrazyl free radical were listed in Table 1, their scavenging concentrations (SC_{50}) were ranged from 24.38 to 72.10µg/ml against ascorbic acid as standard (SC_{50} equal to 7.45), the high activity was recorded for EtOAc fraction and the low activity was recorded for water fraction and there is no activity was detected for pet. Ether and CH_2Cl_2.These results were reinforced by the total antioxidant capacity results which were ranged from 250.76 to 16.17 (mg AAE /g dry ext.)Table 1, finally the last mentioned results also were supported by the reducing power antioxidant results (OD values) according to the following order; n-BuOH 0.680>defatted 90% MeOH (0.465)> EtOAc (0.405)> 90% MeOH (0.225) >H_2O(0.90), in comparison with ascorbic acid as standard (0.985) at concentration 200 µg/ml. These results may be return to the presence of certain bioactive secondary metabolites in each test fraction like; phenolic acids, flavonoids, tannins and anthocyanins. The small variation in the results between each assay may be due to nature of chemical constituents and their modes of action in the individual sample**[8, 18, 19]**. Furthermore, the qualitative dot-blot antioxidant assayrevealed that most tested fractions are showing strong white and wider zones upon the purple background reflecting their potent potential as radical scavengers in comparison with ascorbic acid (Figure 1).

Table 1.Free radical scavenging potential (DPPH), total antioxidant capacity (TAC) and total phenolic content (TPC) of the 90% methanol extract of *Q. indica* as well as its derived sub-fractions

Sample	DPPH SC_{50} [µg/ml][a]	Total antioxidant capacity (mg AAE /g dry ext.)[b]	TPC (mg GAE /g dry ext.)[c]	RPAA (OD value)
90% MeOH	37.04 ± 0.18	235.0 ± 2.15	309.0 ± 1.58	0.225
Pet. ether	-ve	19.89 ± 1.06	14.99 ± 1.94	-ve
Defatted 90% MeOH	33.20 ± 0.94	250.76 ± 2.12	345.99 ± 1.45	0.465
CH_2Cl_2	-ve	16.17 ± 2.09	32.31 ± 1.27	-ve
EtOAc	36.59 ± 1.67	236.09 ± 2.80	339.41 ±1.30	0.405
n-BuOH	24.38 ± 0.53	229.37 ± 1.84	380.78 ± 1.46	0680
H_2O	72.10 ± 1.67	119.27 ±1.84	78.59 ± 2.48	0.90
Ascorbic acid	7.45 ± 1.25	------	-----	0.985

Results are expressed as mean values ± standard deviation (n = 3).

[a]DPPH values are expressed as µg dry extract/ml (µg/ml).

[b]Total antioxidant capacity values are expressed as mg ascorbic acid equivalent/g extract (mg AAE/g ext.).

cTPC (total phenolic content) values are expressed as mg gallic acid equivalent/g extract (mg GAE/g ext.).

Ascorbic acid

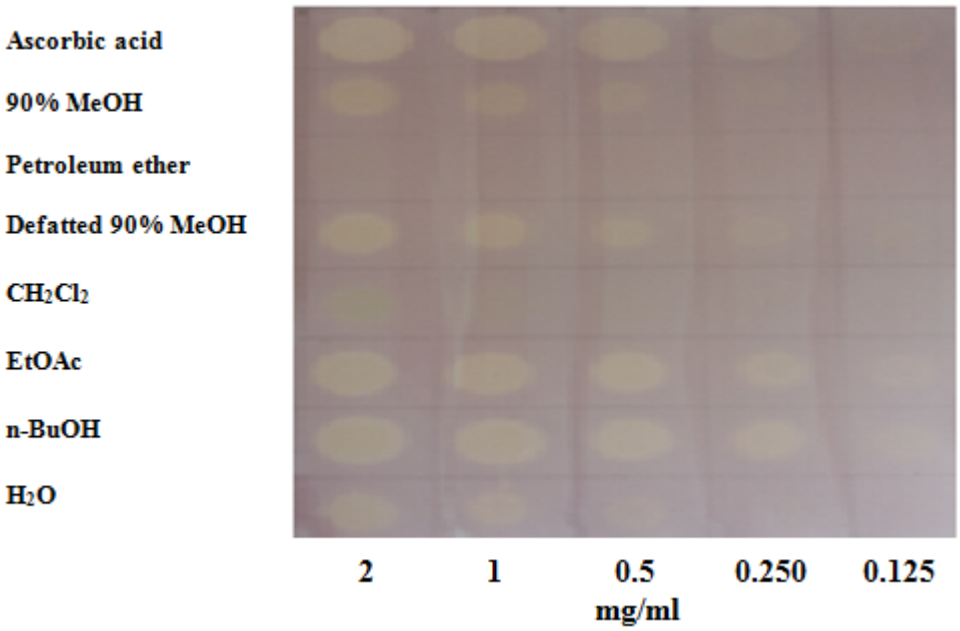

Fig. 1. Dot-blot qualitative antioxidant assay of different fractions of *Q. indica* on silica sheet stained with DPPH˙ solution in methanol against ascorbic acid

3. Cytotoxic activity

3.1. Cytotoxic activity via BSLT

The brine shrimp lethality test (BSLT) consider a rapid, cheap and simple test to evaluate the lethality of medicinal plant extracts which acts as a preliminary indicator to the cytotoxic and antitumor potentials **[16]**. In our current study the all fractions of *Q. indica* were undergo cytotoxic test using BSLT and their cytotoxic potentials were expressed by sublethal concentration LC_{50} which arranged in the following order; n-BuOH (LC_{50}= 100), 90% MeOH (LC_{50}= 150), EtOAc (LC_{50}= 170), pet. ether (LC_{50}= 170) and defatted 90% MeOH (LC_{50}= 440)µg/ml (Table 2).The previous reports revealed that the toxicity for plant extract or fraction as LC_{50} values above 1000 µg/ml are non-toxic, between 500&1000 µg/ml are weak toxic, and that below 500 µg/ml are toxic **[31]**. On the basis of such report all our tested samples are consider to be toxic against *Artemia salina* larva, and this promote us to evaluated them by more advanced and specific cytotoxic test (MTT) against liver carcinoma cell line HepG-2**[16]**.

3.2. Cytotoxic activity via MTT assay

The results of the cytotoxic activity of *Q. indica* fractions against liver carcinoma cell line (HepG-2) were presented in Table 3. The IC_{50} values in µg/ml were arranged as; CH_2Cl_2 (IC_{50} = 11.9), n-BuOH (IC_{50} = 17.9), defatted 90% MeOH (IC_{50} = 24.1), pet. ether(IC_{50} = 35.1) and EtOAc (IC_{50} = 65.1), against Doxorubicin (IC_{50} = 4) µg/ml. On the other hand according to the American Cancer Institute (ACI), the crude extract is considered to be strong cytotoxic with IC_{50} values ≤ 20 µg/ ml**[17, 18]**, therefore, both of CH_2Cl_2 & n-BuOH are strong cytotoxic followed by pet. ether and EtOAc.

Table 2.Cytotoxic activity using brine shrimp lethality Test (BSLT) of the 90% methanol extract of *Q. indica* as well as its derived sub-fractions

Plant extract	(LC_{50}±S.E.)[1]	(CL)[2]
90% MeOH	150 ± 6.67	2.17 ± 13.34
Pet.ether	175 ± 5.15	2.24 ± 10.3
Defatted 90% MeOH	440 ± 11.37	2.64 ± 22.74
CH_2Cl_2	inactive	
EtOAc	170± 5.2	2.2 ± 10.4
n-BuOH	100 ± 5.2	2 ± 10.4
H_2O	inactive	

[1] *Results are expressed as Means ±Standard Error Mean (SD/SEM) (n=3).*

[2] *95% confidence limits (CL) in parentheses.*

Table 3.Cytotoxic activity (HepG-2) of the 90% methanol extract of *Q. indica* as well as its derived sub-fractions

Sample conc. (µg/ml)	Tested Samples				
	Defatted 90% MeOH	Pet. ether	CH_2Cl_2	EtOAc	n-BuOH
	Viability %				
100	34.19	30.89	26.93	41.32	21.47
50	41.92	43.13	32.14	53.74	28.55
25	49.15	54.65	39.81	68.97	41.62
12.5	60.48	67.26	48.27	86.25	56.43
6.25	81.72	81.44	64.98	94.03	79.38
3.125	93.86	92.61	89.83	98.78	87.18
0	100.00	100.00	100.00	100.00	100.00
IC_{50} µg/ml	**24.1**	**35.1**	**11.9**	**65.1**	**17.9**

CONCLUSION

In the present study, the antioxidant and cytotoxic activities of *Q. indica* fractions were evaluated via different antioxidant and cytotoxic assays. Most tested fractions of *Q. indica* showed strong antioxidant activity by DPPH, phosphomolybdenum and reducing power activities, which may be return to the high amount of polyphenolic compounds. Furthermore, most of these fractions showed a significant cytotoxic effect via BSLT and MTT assays.

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