

## ***In Vitro* Studies on the Effect of Phototoxicity of A New Photosensitizer Extracted from Flowers and Aerial Parts of *Cichorium pumilum***

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**Abstract:** New photosensitizers have been extracted from the flowers and aerial parts of the *Cichorium Pumilum* Jacq (chicory). Chicory grows naturally in local wild areas and is known to cause quick death of only white sheep during its flowering season in autumn. Some of those isolated photosensitizers are yet to be further characterized. Preliminary investigations show a high degradation of isolated photosensitizers under direct sunlight (solar irradiance 450-950W m<sup>-2</sup>). This *in vitro* study also shows a very high photohemolysis rate at a concentration of 0.95 mg mL<sup>-1</sup>. The extracted compounds are also found to be temperature dependent. Multiple absorption peaks are also shown for tested extract both in ultraviolet region (at the wavelengths 314, 360nm) and in the visible region (at 445, 492 and 667nm). Results of the present investigation clearly show the potential of the light- and temperature-dependent extracted compounds as a new member of the photodynamic therapeutics. It is possible therefore that the claimed chicory-induced death of white sheep is simply a direct result of phototoxicity.

**Keywords:** *Cichorium pumilum*, photobiology, photohemolysis, chicory, photosensitizer

### **INTRODUCTION**

Natural pigments in some organisms absorb visible and near-ultraviolet light (UV). Natural photosensitizers are present in many organisms including bacteria, fungi, higher plants, protozoa, invertebrates and vertebrates. In northern parts of Jordan and during the flowering season, many incidences of sudden death of white sheeps have been reported immediately after grazing on chicory. Chicory flowers and aerial parts are found to be a source of photosensitizers and therefore their extract is used for this study for the first time. Similar extraction of red plant pigment hypericin (HY) from *Hypericum crispum* is known to cause severe damage to hemoglobin<sup>[1]</sup> and therefore developing a photodynamic disease "hypericism" of the grazing animals<sup>[2,3]</sup>. The transient intermediates held responsible for erythrocytes membrane damage include singlet oxygen, superoxide and free radicals.

Photosensitization implies that in the absence of an absorbing pigment, an organism does not react to light of certain wavelengths. Photohemolysis is the release of hemoglobin from erythrocytes induced by light-irradiation. This process is promoted by many photosensitizers such as Photofrin<sup>®</sup>, hypericin, Lutetium Texaphyrin (Lu-TEX) and protoporphyrin

IX,<sup>[1,4-6]</sup>. Photohemolysis of RBC *in vitro* has been studied for many drugs and chemical reagents including synthetic dyes (rose bengal, phloxine B<sup>[7,8]</sup>, hematoporphyrin, toluidine blue, methylene blue<sup>[9]</sup>, quinolinemethanol anti-malarial compounds<sup>[10]</sup>, butibufen and fluriprofen<sup>[11]</sup>, diflunisal<sup>[12]</sup>, chlorpromazine<sup>[13]</sup>, griseofulvin<sup>[14]</sup>, carprofen<sup>[12]</sup>, psoralen<sup>[15]</sup> and metallo-phthalocyanine sulfonates<sup>[16]</sup>. A comparative study for the quinolinemethanol anti-malarial compounds showed a good correlation between the *in vitro* photohemolysis rate and the minimum effective dose of erythema (an abnormal redness of the skin) in mice<sup>[10]</sup>. Some of these drugs are found to be sensitive to light. Root aqueous extracts of *Cichorium intybus* have been reported as a light-sensitive plant remedy for malaria with Lactucin and Lactucopicrin representing the anti-malarial compounds<sup>[17]</sup>. Light sensitivity of the root extract has been confirmed by other researchers<sup>[18,19]</sup>. In addition, an antibacterial activity has been reported for extracts of roots and aerial parts of *Cichorium pumilum*<sup>[20]</sup>. Photosensitization implies that, in the absence of an absorbing pigment, an organism does not react to light of certain wavelengths. Sensitization of RBC photohemolysis *in vitro* has also been used as an indicator of phototoxicity which is a major side-effect

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of most phototherapeutic drugs<sup>[21]</sup>. Hemolysis is defined as a released of hemoglobin from red blood cells (RBC) into the surrounding medium causing serious diseases such as babesiosis. In folk medicine herbal remedies have been and still are traditionally used<sup>[17,20]</sup>. In India chicory plant is cultivated and is commonly used to treat diarrhea, enlargement of the spleen, fever, vomiting and as a tonic reagent. Ancient Egyptians, Greeks and Romans used chicory to treat liver diseases. Chicory root is said to be a folk remedy for treating stomach ache<sup>[22]</sup>, an aperitif and an anti-inflammatory<sup>[23]</sup>, antioxidant activity<sup>[24]</sup>, healing kidney stone<sup>[25]</sup>, restoring or preventing immunotoxicity<sup>[26]</sup> and relief from gout<sup>[27]</sup>, Inhibition of Ehrlich ascites carcinoma and enlargement of spleen, gout and rheumatism<sup>[28]</sup>. Chicory root extracts are very rich in inulin and oligofructose. These extracts are found to be significantly inhibited the growth of various kinds of cancerous tumours in rats<sup>[27]</sup>. Chicory seeds are also known for its medicinal benefits. Chicory seeds are usually prescribed for week spleens and represent the main ingredient of "Jigrine", a commercial Indian product which is commonly used to treat of various liver diseases<sup>[29]</sup>. Aqueous root extract is said to heal breast and lung tumors<sup>[30]</sup>. Chicory leaves and roots also produce bitter sesquiterpene lactones which is used as an appetizer and to have diuretic properties<sup>[20,31]</sup>. Chicory extract is also known as an antioxidant and may also have anti-inflammatory properties<sup>[32]</sup>.

A comprehensive evaluation of the relationship between human phototoxicity and RBC photohemolysis has not yet been reported. *In vitro* studies of RBC photohemolysis are based on post-irradiation or delayed photohemolysis (DPH), where a suspension of RBCs along with aqueous extract is exposed to direct sunlight dose, followed by measurements of the photohemolysis rate in the dark. A typical photohemolysis curve has a sigmoidal shape.

A new kinetics model for photohemolysis was reported in which sensitized photohemolysis is attributed to cooperative photochemical and thermal processes<sup>[1,4-6]</sup>. The present investigation reports new DPH results fitted to Gompertz function module<sup>[5,6]</sup>. The Gompertz function is defined as:

$$H = H_0 e^{-ae^{-bt}} \quad (1)$$

where:

$H$  is the percentage of hemolysis during the lysis time  $t$  (the time measured from start of rupturing the RBCs at dark incubation).

$H_0$  is the initial maximum number of cells, normalized to one.

$a$  is a fractional hemolysis ratio and  $b$  is the rate of fractional hemolysis change.

To our knowledge, there are no prior reports discussed the phototoxicity or photo-allergy of the extract tested here. The objectives of this study are to identify and characterize the extract's effective compounds (which are sensitive to sunlight) and to examine their phototoxicity *in vitro*. In this study, the kinetics of red blood cell photohemolysis sensitized by tested compounds will also be investigated.

## MATERIALS AND METHODS

**Plant extraction:** Flowers and top parts of chicory were collected from local wild areas in northern Jordan during end of May 2006. Collected plant parts were washed with running water and were then air dried in a dark room.

Chicory flowers and aerial parts were extracted using a microwave-assisted extraction method as described in<sup>[33]</sup>. In this method, extraction is based on the selective heating of the matrix that contains the target extract when the matrix is immersed in a solvent such as ethanol and water, which is transparent to the microwaves. This solvent, allows for selective heating of particular components within the materials being treated, without using excessive energy. The dried flower and aerial parts of chicory were cut into small pieces and grinded for short periods of time to get powder mixtures. Hundred grams of dried powder were mixed in 1000 mL of 50% ethanol and 50% water. Every 10 gm of powder mixed with 100 mL of 50% ethanol in 250-mL conical flask. Mixtures were then irradiated with microwave for two minutes. The output power of the microwave oven is 300 W. The suspension was irradiated for 25 seconds with power on to give the desired temperature of about 80°C and then disconnect immediately and re-irradiate for 5 second with power on for heating and then for 10 minutes with power off for cooling. The process was repeated three times and the herb kept over night inside the refrigerator. The extract was finally filtered through gauze and evaporated under vacuum at 40°C using a rotary evaporator. The extraction was dissolved in phosphate buffered saline (PBS, P4417), purchased from Sigma/USA (P4417 Sigma, USA).

**Solar irradiance:** A Pyranometer instrument (LI-1000 LI-COR/USA) was used to measure the solar irradiance. The calibrated radiometer was mounted horizontally facing upwards.

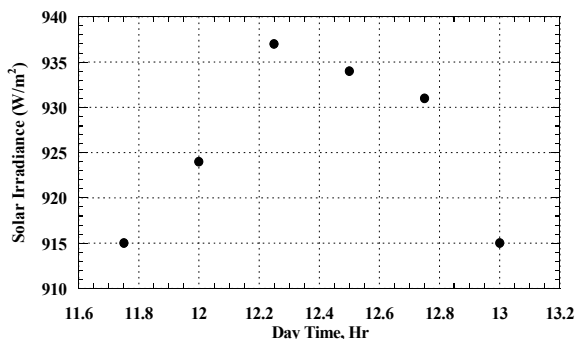


Fig. 1: Global solar irradiance measurements during the summer at UAE University (24°11'50.92"N and 55°41'03.04"E)

One of the measurements for the global solar irradiance during the summer is shown in Fig. 1 with an accuracy of about 3%. Present experiments were conducted at UAE University (24°11'50.92"N and 55°41'03.04"E).

**Sample preparation:** Fresh human blood samples were donated by healthy volunteers. RBCs were isolated by centrifugation at 3000xg for 10 min. at 4°C. RBCs were mixed with diluted extract in addition to other ingredients as detailed later. Blood samples were mixed with phosphate buffered saline (PBS), (pH= 7.4) and different dilutions of chicory's extract. All samples that contain extract are prepared in dark room. The kinetics of the fractional damage of the irradiated sample was followed spectrophotometrically by measuring the optical density as a function of time.

**Spectrophotometry:** Samples were incubated for 10 min at 37°C with or without light followed by the spectrophotometric measurements of the optical density at a fixed wavelength (650nm). The optical densities were measured as a function of lysis time. The measurements were taken in a dark room and at room temperature.

## RESULTS AND DISCUSSION

Figure 2 shows the spectrum of the extracted compounds from the flower and aerial part of *Cichorium Pumilum*. Strong absorption bands at 314 nm, 360 nm in UV region and 445 nm, 492 nm and 667 nm in the VIS region are observed. The extracted materials are found to be strongly dependent on temperature and very strongly dependent on solar intensity with a maximum effect around noon time (Fig. 1). Figure 3 shows the effect of light dose 950W m<sup>-2</sup> at 43°C with fixed extract concentration at 0.95mg mL<sup>-1</sup>. Typical hemolysis curves with higher steepness and higher  $t_{50}$  (the time required to rupture 50% of the total

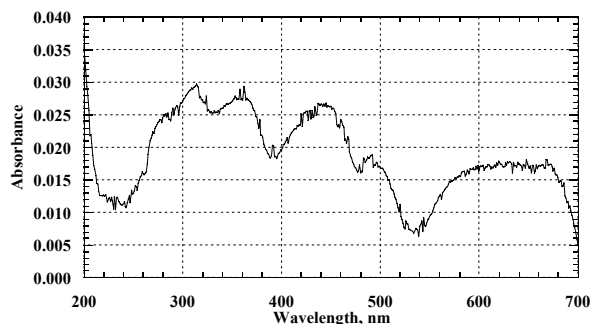


Fig. 2: Flower and aerial part spectrum with absorbance peaks at 314 nm, 360 nm in UV region and 445 nm, 492 nm and 667 nm in the VIS region

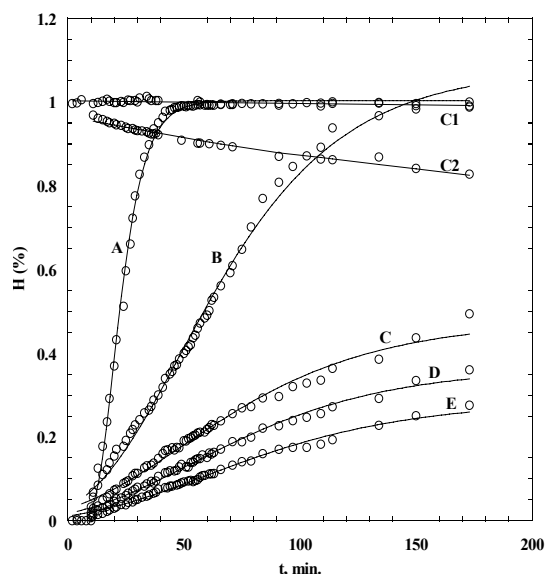


Fig. 3: Effect of light doses 950W m<sup>-2</sup> at 43°C with fixed extract's concentration 0.95 mg mL<sup>-1</sup>, the points are the experimental data points and the solid lines of A, B, C, D and E are the theoretical fitting to Eq. 1: C1, first control with RBC only; C2, second control RBC plus extraction (0.95mg mL<sup>-1</sup>) with no light; A) 2 min. Light irradiation B) 4 min. Light irradiation C) 6 min. Light irradiation D) 8 min. Light irradiation and E) 10 min. Light irradiation

RBCs when  $H=0.5H_0$ ) are noticed at lower concentration. All data points are normalized to one. The sample of control line C1 contained RBCs suspended in PBS showed no effect of temperature on RBC. Line C2 with extract concentration at 0.95mg mL<sup>-1</sup> served as control for the samples A, B, C, D and E. The slight decrease in C2 is due to temperature while the light effect can be seen clearly where higher irradiation time accelerates hemolysis much faster compared to control (C2). The solid lines of A, B, C, D and E are the theoretical fitting to Eq. 1 while the

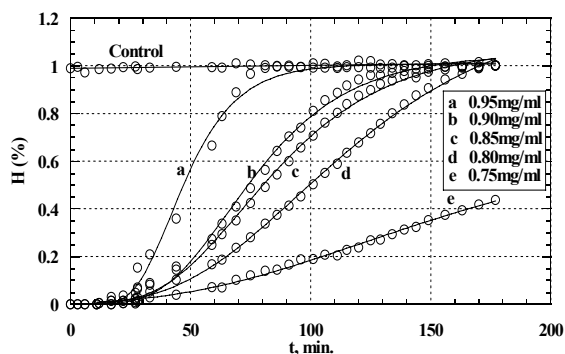


Fig. 4: Effect of room temperature on RBC sensitized by different concentrations of flower and aerial part extracts, where C is the control contained RBC only, a) 0.95 mg mL<sup>-1</sup>, b) 0.90 mg mL<sup>-1</sup>, c) 0.85 mg mL<sup>-1</sup>, d) 0.80 mg mL<sup>-1</sup> and e) 0.75 mg mL<sup>-1</sup>. The points are the experimental data points and the solid lines are the theoretical fitting to Eq. 1

circles are the experimental data points. The time required rupturing 50% of the cells  $t_{50}$  and the fitted parameters "a" and "b" are summarized in Table 1, Group I. The rate of fractional hemolysis "b" increases with the decrease of the hemolytic process and can be represented by  $t_{50}$ . The faster the hemolytic process goes, the greater the  $t_{50}$  gets and the slower the hemolysis becomes the lower  $t_{50}$ . The fractional hemolysis ratio "a" decreases at lower irradiation time and increases at higher irradiation time. Group II shows the increases in  $t_{50}$  with increasing "a" and "b" while the  $t_{50}$  is increasing with decreasing the concentration.

Figure 4 shows the effect of room temperature on RBC sensitized by different concentration of the extracted compounds. The line C is the control contained RBC only whereas the other lines represented by circles are the samples with different extract's concentrations (0.95, 0.90, 0.85, 0.80 and 0.75 mg mL<sup>-1</sup>). Solid lines are the theoretical fitting to Eq. 1. Samples with higher concentration have higher hemolysis rate in shorter time. The theoretical parameters are summarized in Table 2 (Group I). The data shown in Group II are for the samples exposed to 40°C. The ratio "a" and the  $t_{50}$  of group I are higher than that of Group II. The rate of fractional hemolysis "b" is very high at higher concentration 0.95 mg mL<sup>-1</sup> in both groups.

The present study reports a novel photohemolysis effect flowers and aerial parts extracts of *Cichorium Pumilum*. This extract is found to be viscous, dark pasty, soluble in water and is sensitive to both temperature and light. Unlike photo allergy, phototoxicity is rapidly developed after the initial light exposure, characterized by systemic responses and is accompanied by many histopathologic lesions. The

adverse reactions induced by phototoxicity such as photohemolysis (red cell membrane damage) and photoirritation (acute skin irritation and edema in guinea-pig and mice) are mainly oxygen-dependent reactions. Both *in vivo* and *in vitro* studies have related this phototoxicity to the generation of reactive oxygen species (ROS) such as hydrogen peroxides and hydroxyl radicals. Accumulation of ROS results in oxidative stress which induces oxidative modifications of biomolecules of membranes such as lipids, DNA and proteins<sup>[34,35]</sup>. However, photosensitivity reactions can have beneficial effects as in photodynamic therapy (PDT) to treat malignant tumors. PDT employs the interaction of a photosensitizer with light of the appropriate wavelength in the presence of molecular oxygen<sup>[36]</sup>.

The present results report a multi-step photohemolysis of RBCs and the associated membrane damage. First, the fractional hemolysis occurred in the presence of extracted compounds and temperature. Then, the fractional hemolysis occurred in the presence of extracted compounds plus temperature and light. The final step is rather complex process where both temperature and light are required in addition to the extracted compounds (Table 1). The theoretical fitting of all three steps predicts that the value of  $t_{50}$  is much lower on the light dose while the fractional photohemolysis rate is higher at higher temperature exposure (Table 1 and 2). These predictions were experimentally confirmed in prior studies for Sodium Hypericin (HY-Na) and Photofrin (PF)<sup>[1]</sup>, protoporphyrin IX<sup>[19]</sup> and for osmotic fragility<sup>[20]</sup>. The theoretical modeling is further supported by good fit of calculated lysis curves to the data in Table 1 and 2. Most of the prior studies were limited to roots and leaves extracts of the plant. Phototoxicity of the plant extracts received very little attention and is still poorly understood.

In general the values of  $t_{50}$ , "a" and "b" should depend on the effect of temperature and sensitizer concentration, the absorbed light dose and on the physical distribution of sensitizer molecules at membrane targets. The apparent anomaly was attributed in prior work to partitioning of the dye between the aqueous phase and RBCs.

The colloid osmotic kinetics model of Pooler proposes a specific mechanism for the thermal stage of hemolysis based on changes of membrane permeability to sodium and potassium ions<sup>[37]</sup>. Further investigation based on osmotic fragility measurements has led to an osmolarity-dependent fractional lysis that simulated lysis curves. The application of this model for

Table 1: Effect of light doses 915-950W m<sup>-2</sup> at 40-43°C with fixed extract's concentration (0.95 mg mL<sup>-1</sup>, Group I) and fixed irradiation time (5 min, Group II), the theoretical parameters "a" and "b" are the fitting data to Eq. 1 for the solid lines of A, B, C, D and E of Fig. 3

Sample	Irradiation time (min)	t <sub>50</sub> (min) <sup>*</sup>	a <sup>#</sup>	b <sup>#</sup> (min <sup>-1</sup> )	R <sup>§</sup>
Group I					
(0.95mg mL <sup>-1</sup> )	2	N.A	14.549±0.956	0.136±0.003	0.998
	4	N.A	3.530±0.098	0.026±0.001	0.997
	6	N.A	2.809±0.095	0.021±0.001	0.991
	8	59	3.035±0.083	0.021±0.001	0.995
	10	22	3.195±0.087	0.020±0.001	0.994
Group II					
0.95mg mL <sup>-1</sup>	5	12	3.226±0.149	0.269±0.008	0.998
0.75mg mL <sup>-1</sup>	5	35	2.838±0.073	0.066±0.003	0.997
0.65mg mL <sup>-1</sup>	5	N.A	2.552±0.052	0.048±0.002	0.997

Results are expressed as means ± SD

\*Experimental values obtained from data curves at H=0.5H<sub>0</sub>

#Theoretical values calculated from Eq. 1

§Correlation coefficient

Table 2: Effect of temperatures on RBCs with different extract's concentrations in dark. Group I was exposed to room temperature whereas Group II was exposed to 40°C for 10 min

Sample	C(mg/mL)	t <sub>50</sub> (min) <sup>*</sup>	a <sup>#</sup>	b <sup>#</sup> (min <sup>-1</sup> )	R <sup>§</sup>
Group I					
Group I	0.95	47	16.039±2.753	0.067±0.004	0.996
	0.90	74	10.849±1.146	0.036±0.002	0.998
	0.85	80	8.366±0.585	0.030±0.001	0.999
	0.80	99	6.691±0.269	0.020±0.001	0.999
	0.75	N.A	5.023±0.225	0.013±0.001	0.998
Group II					
Group II	0.95	4	2.875±0.419	0.342±0.026	0.990
	0.90	30	3.396±0.133	0.053±0.002	0.998
	0.85	43	3.472±0.153	0.034±0.001	0.997
	0.80	52	3.448±0.174	0.026±0.002	0.995
	0.75	60	3.381±0.076	0.019±0.001	0.998

Results are expressed as means ± SD

\*Experimental values obtained from data curves at H=0.5H<sub>0</sub>

#Theoretical values calculated from Eq. 1

§Correlation coefficient

photohemolysis assumes that the only effect of light is to increase the membrane cation-permeability and that the osmotic properties of the membranes are not altered from untreated (control) cells. The present findings and that of Pooler are complementary. Thus, *Cichorium Puntium* extract was much more photosensitizing than PF and Hy-Na owing to its strong absorption bands in the UV and VIS regions (Fig. 2).

These empirical parameters should be useful for comparing the relative photohemolysis efficiency of different compounds and for correlating *in vivo/in vitro* photohemolysis with photosensitization. The results might provide information applicable to the broader aspects of membrane photosensitization such as power dependency on drug concentration and irradiation time and temperature and the key kinetics parameters represented by mathematical modeling (Table 1 and 2). The present results may also lead to a new photodynamic drugs and/or a light-sensitive herb remedy for many diseases. Among the novel aspects of this work, is providing a qualitative and quantitative

modeling analysis of photohemolysis kinetics of an economically important plant. Several studies of chicory effects on sheep ended up with contradictory results. Guldberg et al., concluded that chicory had the largest impact on the establishment of the parasites in ram lambs due to gazing on *Cichorium Intybus* for six weeks<sup>[38]</sup>. On the other hand, chicory has been reported to reduce the number of infective parasite larvae available for ingestion by grazing sheep compared with ryegrass<sup>[39]</sup>. Other report has shown that when grazed by sheep and other ruminants, chicory may reduce internal parasite intensities<sup>[40]</sup>. In other *in vitro* study, it has been shown that chicory had no effect on hatching and development of nematode eggs and larvae, but did reduce the number of lungworm larvae developing to the L3 stage<sup>[41]</sup>.

The new results with chicory extraction fitted to Gompertz function show that the delayed photohemolysis rate parameters "b" is similar to previous finding<sup>[1,5,6]</sup>. This model extends the colloid-osmotic model<sup>[37]</sup> by attributing membrane rupture to

the combined effects of cell swelling induced by damage to the anion transport protein and thermally-activated photochemical damage to structural membrane proteins. The importance of the latter effect probably depends on the specific photosensitizer and the reaction conditions.

### CONCLUSION

For the first time the observations reported in this study suggest the hypothesis that the extractions from the flower and top part of *Cichorium Pumilum* are light and temperature dependent. Therefore, the chicory extracts pigments are could be the responsible for the photodynamic disease of grazing animals.

The most important conclusion derived from the present study is that photohemolysis requires thermal activation of photochemical damage. A similar cooperative process was proposed for psoralen by Potapenko *et al.*<sup>[42]</sup>. The rate of hemolysis photosensitized by hematoporphyrin derivative HPD was greatly accelerated by post-irradiation heating to 50°C, which is consistent with this mechanism<sup>[43]</sup>. Furthermore, these results showed that the mathematical analysis technique adapts to study the effects of light and thermal activations on the photohemolysis process at different conditions as a best-fit model with minimum parameters and minimum errors.

Further *in vivo/vitro* studies are needed to assess the activity of chicory extracts as potential tumor inhibitor.

### ACKNOWLEDGMENTS

This work was funded by the Scientific Research Council of UAE University, grant # 08-02-2-11/06 and partially by Higher Council for Science and Technology/Jordan, grant # 62/2000.

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