Estimation and stability study of oleuropein from olive leaves extract (Olea europaea L.)

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Received 15 October 2023; revised 24 October 2023; accepted 24 October 2023

Abstract

This study included extraction and estimation of oleuropein concentration in different types of olive leaves, It is the most important medicinal plants which is widely distribution in Libya and used in floclore medicine , effect of different parameters (solvent, temperature, pH, extraction method and time) during and after extraction was studied. The obtained results showed that chemlel and Corantina olive leaves have the highest Oleuropein amount. in addition, the ideal conditions for extraction and maintaining the stability of the compound when using dioxane as a solvent were: 60% dioxane at 25° C and pH3, and the soxhlet method is better than maceratiom method.

Keywords: Oleuropein, dioxane, olive leaves, UV-Spectrophotometer, Soxhlet

1. Introduction

The olive leaves contain significant amounts of phytochemicals, which are correlated with an array of health effects [1], the extract of olive leaves help to lower blood pressure in cases of hypertension, reduces levels of bad cholesterol(LDL), stimulates immune system and fights cold and the flue, it has antifungal, antibacterial, the natural antioxidants present help to fight free radicals and detoxification of carcinogens and harmful chemicals [2].

polyphenols are the most known group of bioactive compounds in olive leaf extracts, The oleuropein and its derivatives as oleuropein aglycone, oleuropein-aglycone di-aldehyde, ligstroside, and hydoxytyrosol are the most characteristic phenolic compounds in olive leaves [1]. oleuropein concentration can reach up to 140mg/g on dry matter basis in young olives [3], and 60-90mg/g of dry matter in the leaves [4].

There are several factors affecting on Oleuropein extraction such as enzyme effect, solvent, effect of pH and temperature. in addition, the qualitative and quantitative composition of olive leaves depends on several conditions such as climatic and storage conditions, geographical origin, the drying and extraction methods [1]. There are several studies have been showed that Oleuropein contents are dependent on varieties of olive leaves, Geographic origin, time of cultivation, type of extraction method, temperature, pH and solvent type and. Himours

et al,2017. Studied oleuropein content on olive leaves extract from two varieties of Olea Europea L (Chemlel and Dathier) [5], Hadeel Homssi, Muberra Kosar* 2019. Determined oleuropein amounts of olive leaves from different regions of Northern Cyprus [6]. Chaleb TayouB,Huda Sulaima et al 2012.Studied determination of Oleuropein in leaves and fruits of some Syrian Olive varieties[7]. Ibraheem AFaneh et al, 2015.studied the effect of olive leaves drying on the content of oleuropein, and found that highest Oleuropein content with olive leaves dried at ambient temperature and extraction performed with 20% acetonitrile [8]. Yateem, H et al,2014. Studied optimum conditions for oleuropein extraction from olive leaves, and found that the highest Oleuropein yield with 80% ethanol, in addition there was increase on Oleuropein content with increase of temperature, ideal temperature was 60c◦, while pH 3 was ideal pH, where soxhlet extraction was given highest Oleuropein content [9]. Nasirs.S.A.Malik and Joe M Bradford,2008. Studied recovery and stability of oleuropein and other phenolic compounds, and showed that oleuropein and other polyphenols in methanol extracts were quite stable for 30 days [10]. Mohammad Shah Faisal et al 2015. Studied development and validation of UV Spectrophotometer method for the quick estimation of olive leaf extract in pharmaceutical formulation. Study showed that methods were accurate and precise as indicated by good recoveries of the drugs and low % RSD values. The proposed methods could be applied for routine analysis for quantitative determination of the olive leaf extract in the both pure and dosage form [11]. Therefore, there was no literature review to estimate Oleuropein content by UV Spectrophotometer method, also there was no scientific literature to estimate the Oleuropein content from olive leaves in Libya, there was no studies dealing with dioxane solvent for Oleuropein extraction. The object of current work to estimate Oleuropein content for different types of olive leaves in Misurata city Libya. Study also aims to Find the best conditions to extract the highest amount of Oleuropein, and study stability of different extracts.

2.Materials and methods:

2.1. Sample collection:

different types of green fresh Olive leaves (chemlel, Arbequina, Corantina) were randomly collected from different area from Misurata city, Libya, in middle November, And random collection from different positions from olive trees.

2.2.sample drying:

Fresh green olive leaves were dried at ambient temperature (25c°) for 7-8 days. The dried samples were then ground to obtain powder which was stored in the dark container at room temperature until extraction.

2.3. chemicales:

Oleuropein standard 75%(Pro Health, USA), Dioxane (MRS SCIENTIFIC UK), Ethanol (CARLOERBA.France).

2.4. preparation of Standard stock solution:

Accurately amount of 10 mg oleuropein standard was weight and dissolved in 10ml (80% Ethanol) and the diluted to 100ml.

2.5. Preparation of calibration curve for oleuropein for UV-spectroscopy:

Oleuropein standards were prepared at 1mg/ml concentration for stock solution and the five dilutions (0.005,0.01,0.02,0.03,0.05mg /ml) were prepared for calibration curve. The absorbance of solution was measured at 280 nm with UV- spectrophotometer (ALigent).

2.6. Extraction of olive leaves with 80%Ethanol and 60% Dioxane:

15 gram of Arbequina olive leaves powder were placed in thimble of a soxhelt apparatus and then extracted with 300ml of solvent mixture for 4 hours, with 80% Ethanol at 60c◦ and/or with 60% Dioxane at 75c◦. The extract was cooled at room temperature, and then filtered off, the filtrate extracts were then evaporate in rotatory evaporator at room temperature under vacuum for 2 hours. The concentrated extracts were stored in a refrigerator at 2c◦ to 8c◦ until analyzed.

2.7. Extraction of chemlel olive leaves with maceration with different temperature:

5 grams of olive leaves powder were macerated with 50ml 60% Dioxane at different temperature 25c◦, 40c°,75c◦ for four hours, the extract was filtrated off, then the filtrate was evaporated to get concentrated extract which was stored in refrigerator at 2-8c◦ until analyzed.

2.8. Extraction of chemlel olive leaves with different PH (60% Dioxane):

5 grams of chemlel olive leaves were macerated with 50ml (60% Dioxane) at PH (3, 5,7,9) for four hours at room temperature, pH was adjusted with 0.1N HCL and/or 0.1N NaOH, the extract was filtrated off, then the filtrate was evaporated to get concentrated extractwhich was stored in refrigerator at 2-8c◦ until analyzed.

2.9. Extraction of chemlel olive leaves with different PH (60% Dioxane) at 60c°:

5 grams of chemlel olive leaves were macerated with 50ml (60% Dioxane), at PH (3,9) for four hours at 60c◦, the extract was filtrated, the filtrate was evaporated to get concentrated extract which was stored in refrigerator at 2-8c◦ until analyzed.

2.10. Preparation of sample solution for UV- spectrophotometer analysis:

0.1ml of each extract was diluted to 10ml with 80%ethanol and/or 60% Dioxane. the absorbance of the solution was measured by UV- spe-ctrophotometer at 280nm against blank.

2.11. Stability study of the analyte:

Known concentrations of oleuropein for Ethanol, Dioxane, and Dioxane extract with pH (3,5,7,9) were prepared and in order to minimize it´s possible degradation of analyte, they had stored in dark colored vials and kept refrigerated at 2-8c°, and at room temperature, samples were drawn on days 1,7,14,21,30 days, and assayed for their oleuropein concentration.

2.12. statistical analysis:

Data were subjected to statistical analysis using Package for social science-21(spss). Data were presented as the mean± standard deviation. Difference were considered significant at P<0.05. comparing the mean concentration for different solvent and pH at different days by using one-way analysis of variance(ANOVA), followed by Fishers least significant Difference (LSD) test.

3.Results and discussion:

3.1. Calibration curve for Oleuropein standard:

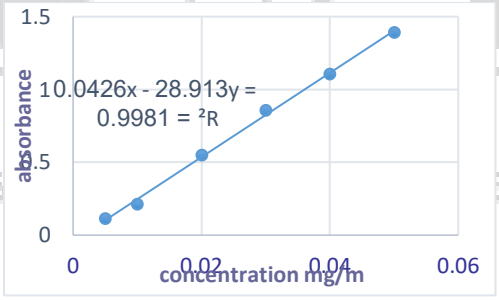

Figure3.1: calibration curve at 280nm by UV-Visible spectrophotometer

The regression equation was found to be y=28.913x-0.0426. the correlation coefficient(r^2) of the standard curve was found to be greater than 0.998 (figure 3.1).

3.2. Extraction of olive leaves with 80%Ethanol and 60% Dioxane:

The results showed effect of extracting solvent on oleuropein content by two types of solvent with soxhlet extraction, Oleuropein content for Arbequina olive leaves with 80% ethanol (35.41mg/g) (3.5%) was significantly ($p<0.005$) higher than 60%dioxane (31.01mg/g) (3.1%) (table 3.1).

this may be due to 80% has high polarity and it has ability to form intermolecular hydrogen bonds with polar components, and 60% dioxane was good solvent due to ability to form hydrogen bond with Oleuropein, 80% ethanol, 60% dioxane increases solubility of polyphenols because by addition of water to organic solvents weakening hydrogen bods between polyphenol and protein in cells [12].

Table 3.1: Oleuropein content of Arbequina olive leaves by soxhelt extraction**:**

Solvent type	Oleuropein content (mg/g) 280nm	percentage
80% ethanol	35.41 ±0.687[ab]	3.5
60% Dioxane	31.01 ±0.317[ab]	3.1

[ab]…significant difference, n=3 (P=0.001) t-test=0.001 at 280nm

3.3. Extaction of chemlel and Corantina olive leaves with 60% Dioxane:

Determination of oleuropein from different types of olive leaves were studied by extraction with soxhlet method with the same solvent. The results showed that Chemlel and Corantina olive leaves have higher oleuropein content (54.66mg/g) (5.5%) for chemlel olive leaves and (54.55mg/g)(5.5%) for Corantina olive leaves than Arbequina olive leaves (31.01mg/g)(3.1%) at 280nm (table 3.2).

This difference was probably releated to genetic feature of each variety, science their growth in the same climatic and geographical conditions. When genetic difference is great, which resides in gene, the morophology and biochemical diversity for each species are different. They can bring difference in amount or the type of chemical conistuents produced. Whenever such biochemical variation occure, each particular type is known as physiological variety[13].

Table 3.2: Oleuropein content of olive leaves by soxhelt:

Type of olive leaves	Solvent type	Oleuropein content (mg/g) 280nm	Percentage
Arbequina olive leaves	60% dioxane	31.01 ±0.317	3.1
Chemlel olive leaves	60% dioxane	54.66±0.306	5.5
Corantina olive leaves	60% dioxane	54.55 ±0.234	5.5

t-test=92.474 p=0.000, for Arbequina and chemlel olive leaves, t-test=8.594 p=0.000 for Arbequina and Corantina olive leaves

t-test=1.873 p=0.134, for chemlel and Corantina olive leaves.

3.4. Extraction of chemlel olive leaves with maceration with different temperature:

Effect of temperature on Oleuropein content was studied by maceration of olive leave powder with 60% dioxane at different temperature. there was decreasing in oleuropein yield with increase in temperature by maceration method, the highest oleuropein amount at 25c◦(21.68mg/g) (2.2%), followed by (16.50mg/g) (1.7%) at 40c◦ and (13.06mg/g) (1.3%) at 75c◦ (table3.3).

This is due to use 60% dioxan as solvent with high temperature causes rapid oxidation of hydroxyl group resulted from basic characteristic for solvent, temperture above 50c◦,70c◦ causes rapid polyphenol degradation[14] and activtion of enzymes responsible for Oleuropein degradation.

Table3.3: Oleuropein content of chemlel olive leaves by maceration with different temperature:

Temperature	Solvent type	Oleuropein content (mg/g)	Percentage
25c°	60% dioxane	21.68 ±0.201[ab]	2.2
40c°	60% dioxane	16.50 ±0.234[ab]	1.7
75c°	60% dioxane	13.06 ±0.099[ab]	1.3

[ab]…significant difference

(p=0.000) T-test=29.062 at 25c° and 40c°, (p=0.000) T-test=66.644 at 25c° and 75c°, (p=0.000) T-test 23.459 at40c°and 75c°

3.5:Oleuropein content of chemlel olive leaves by soxhelt and maceration method:

soxhelt method given significant ($p<0.05$) higher oleuropein content (54.66mg/g) than Maceration method at different temperature 25c◦(21.68mg/g), 40c◦(16.50mg/g), 75c◦(13.06mg/g) figure (3.2).
this is because soxhelt extraction is more efficient for short time than maceration, maceration method for extraction is need longer time for extraction at 25c°, because soluble phenolics are present within the plant cell vaculoes[15], it need longer time to diffuse through cell wall.

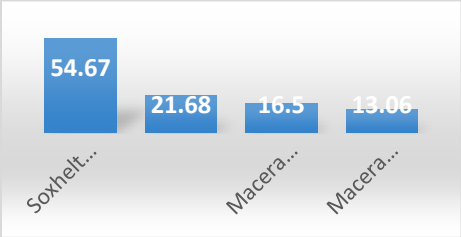

Figure3.2: Oleuropein content with soxhlet and maceration method

3.6. Extraction of chemlel olive leaves with different pH (60% Dioxane):

Extraction of oleuropein was performed with 60% dioxane maceration with different pH. higher oleuropein content at pH 3 (38.87mg/g) (3.9%) and pH 9 (38.79mg/g) (3.9%), followed by pH 5 (28.32mg/g) (2.8%), pH 7 (20.30mg/g) (2%) table (3.4).

The highest amount of Oleuropein at pH 3 because polyphenols are weakly acidic and generally more stable at low pH, and acidic condion helps polyphenol to stay neutral, thus readilly extracted into organic solvent[16] and because effect of acid on glycoside bonds helps to breaking down glycoside bonds. dioxane is basic solvent, so extraction occurs more efficient at alkaline pH, , Extraction with 60%dioxane at pH 5 (2.8%) is relatively quite, due to Oleuropein is weakly acidic, so it is quite soluble at pH 5, least amount of Oleuropein extraction at pH 7 (2%), this is may be unsuitable PH for Oleuropein extraction, science Oleuropein Polyphenol (weak acid) is not suitable PH to maintaining stability of polyphenols.

Table 3.4: Oleuropein content of chemlel olive leaves by maceration method with different PH (60% dioxane):

pH	Oleuropein content (mg/g)	Percentage
pH 3	38.87 ±0.208^{ab}	3.9
pH 5	28.32 ±0.416^{ab}	2.8
pH 7	20.30 ±0.244^{ab}	2.0
pH 9	38.79 ±0.238^{ab}	3.9

(P=0.817)T-test=0.248 at pH 3 and pH 9, (p=0.000) T-test=39.266 at pH 3 and pH 5

(p=0.000)T-test=100.152 at pH 3 and pH 7, (p=0.000) T-Test=28.776 at pH7and pH 5

(p=0.000)T- test=37.790 at pH 5 and pH 9, (p=0.000) T-test=93.678 at pH 7 and pH 9

3.7. Oleuropein content of chemlel olive leaves by maceration (60% dioxane) with different pH at 60c◦:

extraction was performed by maceration of olive leaves powder with 60% dioxane (pH 3, pH 9) at 60c◦. the results showed that there was 70% decreasing in Oleuropein content at PH 3 (11.76mg/g) (1.2%) and 60% at PH 9 (16.20mg/g) (1.6%) at 60c◦, compared to extraction by maceration at 25c◦ (table 3.5).

this is due to high temperture with acidic and alkaline pH facilitate Oleuropein degradation, first catalyst hydrolysis of glycoside bonds to Oleuropein aglycon and glucose by acid, and then catalyst ester bond hydrolysis on Oleuropein aglycon to produce hydroxytyrosol and elonic acid, science temperature at 60c◦ acts as catalyst for this reaction more than assist for extraction.

Table3.5: Oleuropein content of chemlel olive leaves by maceration (60% dioxane) at 25c◦; 60c◦:

pH	Oleuropein content(mg/g) 25c◦	Percentage	Oleuropein content (mg/g) 60c◦	percentage
pH 3	38.87 ±0.208[ab]	3.9	11.76 ±0.111[ab]	1.2
pH 9	38.79 ±0.238[ab]	3.9	16.20±0.043[ab]	1.6

[ab]:non-significant T-test=199.608 p=0.000 for pH 3 at 25c◦ and 60c◦

T-test=1.55.545 p=0.000 for pH 9 at 25c◦ and 60c◦

3.8. Stability of oleuropein for ethanolic extract:

Stability of oleuropein with same initial concentration with 80% ethanol solvent at 25c◦ and 2-8c◦ for 30 days was studied. the results showed that initial Oleuropein concentration(0.061mg/ml) was stable for ethanolic extract at room temperature and 2-8c° for 30 days table (3.6).

This is due to ability of 80% ethanol to form hydrogen bonds with polyphenols, and acts as preservative to prevent microbial growth, microbial growth produce enzymes responsible for oleuropein degradion.

Table 3.6: Oleuropein concentration of 80% ethanol extract for 30 days:

time	concentration (mg/ml) 25c◦	Concentration (mg/ml) 2-8c°
1st day	0.0614±0.0004	0.0614±0.0004
7th day	0.0621±0.0006	0.0666±0.0004
14th day	0.0636±0.0003	0.0605±0.0005
21st day	0.0633±0.0003	0.0633±0.0003
30th day	0.0636±0.0003	0.0696±0.0003

3.9. Stability of oleuropein for dioxane extract:

Stability of oleuropein with same initial concentration with 60% dioxane solvent at 25c◦ and 2-8c◦ for 30 days was studied. The results showed that there was non-significant difference for Oleuropein concentration (0.0617-0.0589mg/ml) for 60% dioxane extract from first to seventh day at refrigerated and room temperature figure(3.3). There was (72.6%) decreasing from initial Oleuropein concentration(0.0159mg/ml) at room temperature, (0.0122mg/ml) at 2-8c◦ after 14 days, there was slightly increasing in Oleuropein concentration to (0.0227mg/ml) (0.0239mg/ml) at 21st day at room temperature and 2-8c◦ (Table 3.7).

60% Dioxane mixture was stable for 7days, due to formation of hydrogen bonds science 60% dioxane is heterocyclic diether that contains two oxygen atoms with two pair of electrons that it has ability to form two hydrogen bonds with polar components, after 7 days hydrolysis occure with lose of 74.3% from initial Oleuropein concentration at 14 days at RT and 2-8c°, this due to dioxane solvent has basic characteristic and acts on hydrolysis of ester bond and ionizion of hydroxyl group for Oleuropein.

Table 3.7: Oleuropein concentration of 60% Dioxane extract for 30 days:

Time	Oleuropein concentration (mg/ml) 25c◦	Oleuropein concentration (mg/ml) 2-8c◦
1st day	0.0617±0.0003	0.0617±0.0003
7th day	0.0589±0.003	0.0589±0.0006
14th day	0.0159±0.0292	0.0122±0.0087
21st day	0.02270±0.003	0.0239±0.0003
30th day	0.02497±0.0006	0.0229±0.0007

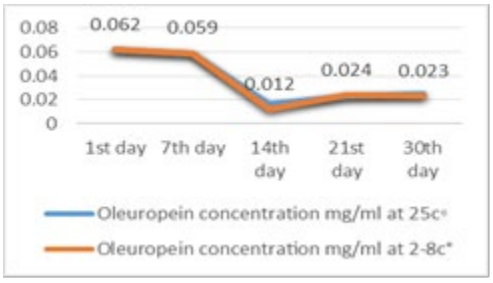

Figure 3.3: Oleuropein concentration for 60% dioxane with time

3.10. Stability of Oleuropein at different pH with 60% dioxane:

Stability of different extract with different pH at the same initial concentration was studied for 30 days. Oleuropein concentration is more stable at pH 5 (0.058mg/ml), pH 3(0.048mg/ml) than pH 9(0.034mg/ml), pH 7(0.025mg/ml) at 30th day figure (3.5). Oleuropein concentration was highly unstable at pH 3 (0.014mg/ml) after 7 days followed by pH 9 (0.037mg/ml), pH5(0.041mg/ml), pH 7 (0.044mg/ml), there was gradual increasing in concentration at pH 3(0.046mg/ml), pH 5 (0.050mg/ml) at 14th day, pH 3 (0.048mg/ml), pH 5 (0.057,0.058mg/ml) at 21st ,30th day. Oleuropein concentration was nearly stable (P>0.005) at pH 9(0.037-0.034mg/ml) from 7th to 30th day, there was significantly gradual decreasing in Oleuropein concentration at pH 7(0.035-0.025mg/ml) from 7th to 30th day table (3.8).

Oleuropein concentration was highly unstable at pH3(0.014mg/ml) after 7 days, this is explained acid –catalyzed ester hydrolysis with dilute aqueouse HCL, with less effect by pH5(0.041mg/ml) this is explained that reaction proceed very slowly in absence of strong acids, there was graduall increase in concentration at pH3(0.046mg/ml) and pH5(0.050) at 14 days this Is explain Fisher esterfication, that reach equlibrium by reversible reaction [17]. Oleuropein concentration was more stable at acidic pH 5, pH 3 for 30 days, this due to acidic nature of Oleuropein. science Oleuropein contains number of OH group on Benzene ring give acidity for Oleuropein, polyphenols is weakly acidic, so it stays stable on weak acidic pH 5(0.058mg/ml) than pH 3(0.048mg/ml), After 7 days Oleuropein concentratin at pH 9 (0.037mg/ml) this reflux Base-promoted hydrolysis called saponfication (figure3.4). and the concentration was stable from 7 to 30 days, this is reflux base promoted hydolysis of an ester, as result is essentially irreversible reaction due to the carboxylate ion is very unreactive toward nuclophile substitution because it is negativelly charged[17]. and ionization of hydroxyl group on polyphenol by alkaline pH.

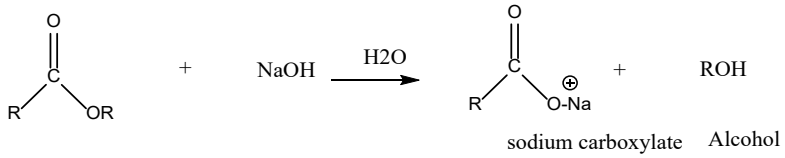

Figure 3.4:hydrolyis of ester bond by base

Table 3.8: Effect of pH on oleuropein concentration after extraction with 60% dioxane**:**

Sample pH	1st day	7th day	14th day	21st day	30th day

pH3	0.0503±0.0002	0.014±0.0003	0.046±0.0016	0.048±0.0002	0.048±0.0002
pH5	0.050±0.0002	0.041±0.001	0.050±0.0002	0.057±0.0003	0.058±0.0001
pH7	0.050±0.0002	0.044±0.0002	0.035±0.0001	0.038±0.0003	0.025±0.0001
pH9	0.050±0.0002	0.037±0.0046	0.039±0.0005	0.036±0.0001	0.034±0.0001

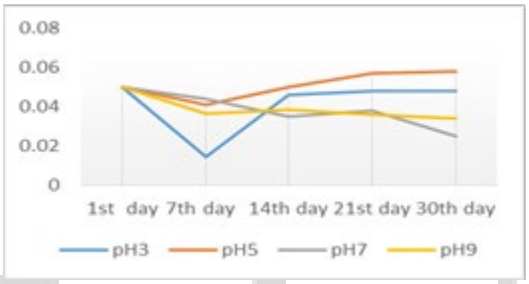

Figure 3.5: Oleuropein concentration at different pH with Time

4. Conclusion:

80% ethanol were the best solvent for oleuropein extraction and maintaining Oleuropein stability, chemlel and Corantina olive leaves have significantly higher oleuropein content than Arbequina olive leaves.

Maceration with 60% dioxane at 25c° was given the highest oleuropein content, there was (23.9%) decrease for oleuropein concentration at 40c°, and 39.85% at 75c°.

Soxhlet method extraction was given higher oleuropein concentration compared to maceration at different temperature.

Extraction at acidic pH (pH 3) and alkaline pH (pH 9) were given significantly higher oleuropein content followed by pH 5, while the lowest oleuropein content at neutral pH (pH 7).

There was 67.85% decrease on oleuropein content and 57.1% at pH 3 and pH 9 respectively when extraction performed at temperature 60c°.

Initial oleuropein concentration for 80% ethanolic extract was quite stable for 30 days, when stored at room temperature and 2-8c°.

Initial oleuropein concentration for 60% dioxane was quite stable for 7 days, there was sharply decreasing on oleuropein concentration at 14th day when stored at room temperature and 2-8c◦.

Oleuropein concentration is highly stable at acidic pH (pH 3, pH 5), highly decreasing on oleuropein concentration for alkaline and neutral pH (pH 7, pH 9).

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