Plant Archives Vol. 23, No. 1, 2023 pp. 425-429 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2023.v23.no1.069

ASSESSMENT OF ANTICANCER ACTIVITY AND GENE EXPRESSION IN P53 GENE OF *CISSUS QUADRANGULARIS* L. STEM MEDIATED SILVER NANOPARTICLES

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(Date of Receiving :18-02-2023; Date of Acceptance : 20-04-2023)

ABSTRACT

Present study was conducted to evaluate the anticancer activity of *Cissus quadrangularis* stem mediated silver nanoparticles and its effect on the expression of p53 gene. In the current study the anticancer activity was assessed using the MTT assay on the MCF-7 breast cancer cell lines. In addition, the Quantitative Real Time PCR analysis was carried out to study the expression of p53 genes. The analysis revealed that, Cisplatin exhibits an IC50 value of 5.51 µg/ml when used as a positive control in MCF-7 cell line, whereas the silver nanoparticle extract at the same concentration exhibit an IC 50 value of 56.74 µg/ml. The MCF-7 breast cancer cells that were treated with the *Cissus quadrangularis* stem mediated silver nanoparticles showed down regulation of the p53 gene, going from 1.0 fold in control cells to 0.43 fold in the treated cells. The relative gene expression of the control is usually set to 1 in comparison to the test sample.

In accordance with the MTT test results, the synthesized AgNPs have moderate anticancer efficacy. According to the findings of gene expression, MCF-7 cells treated with synthesized AgNPs from the stem of *Cissus quadrangularis* exhibit a 0.4564 fold decrease in the expression of p53 genes when compared to MCF-7 control cells.
Keywords: p53 gene, gene expression, MTT assays, anticancer, silver nanoparticles.

Introduction

With the constant increase in the rate of incidence of cancers worldwide, it has become the leading cause of death throughout the world. Such a scenario demands the discovery of novel and innovative drugs and therapies to put a check on the severity of this lethal disease. Herbal medicines have long been the sole and primary source of treatment of diseases in many developing and under-developed countries. The technological advancement has led to the detailed study and research of the specific phyto-compounds responsible for the curative properties of the plants.

The plant kingdom is recognized for producing abundant secondary metabolites that posess anticarcinogenic ability and is employed as a drug in curing cancer. However, the introduction of nanotechnology has set a new benchmark in the utilization of the therapeutically valuable phyto-constituents in the treatment of Cancer. The research has progressed to investigate the possible qualities and applications of terrestrial plant extracts for the development of prospective nanomaterial-based medicines for illnesses such as cancer (Greenwell & Rahman, 2015). Nanoparticles designed for targeted-drug delivery are of significant value in cancer treatment.

The anticancer activity of the silver nanoparticles synthesized from the stem of *Cissus quadrangularis* L. can be tested using the MTT assay on the MCF-7 breast cancer cell lines. This assay measures the cytotoxicity of the cancer cells taking Cisplatin as a standard. The MTT assay is applied to determine the sensitivity of the anticancer drug toward the malignancies. It is rapid and straightforward, and it enables a large number of tests to be performed in a single batch. This test is commonly used to assess the cytotoxic potential of medicines in vitro (Florento *et al*., 2012).

The synthesized silver nanoparticles can also be tested for expression of the P 53 gene of the MCF-7 cell line. The effect of the AgNPs from the stem of *Cissus quadrangularis* L. on the P 53 gene which is generally referred to as tumor-supressor gene can be indicative of its anticancer property. The most widely used method for measuring the amount of expression of individual genes in biological materials is real-time quantitative polymerase chain reaction (Tikellis *et al*., 2009). The use of PCR in conjunction with initial reverse transcription (RT-PCR) of the mRNA of interest allows for the measurement of gene expression with as little as one cell. The dependability of data generated by RT-PCR can be highly subjective due to the efficiency of both the RT and PCR stages (Riedy *et al*., 1995).

Materials and Methods

Synthesis of silver nanoparticles

Preparation of Plant extract

10gm of fresh *Cissus quadrangularis* L. stem was cut and rinsed well under running water to eliminate surface impurities and debris. Absolute alcohol was then used to sterilize it and disinfect it completely. The stem was cut into

small pieces and placed in a beaker with 100 ml of distilled water, which was then heated for about 20 minutes. After the temperature of the plant extract came to room temperature, a filtrate is extracted by filtering it using No. 1 Whatman filter paper. Refrigerate the filtrate at 4 degrees Celsius until the silver nanoparticles are synthesized.

Preparation of Silver Nanoparticles

0.1 Molar standard aqueous solution of Silver nitrate ($AgNO_3$) was prepared by dissolving 17 gm of silver nitrate in 1 litre of deionized water, which was utilized for the synthesis of nanoparticles. The next step involved the addition of 5 ml of plant extract of the *Cissus quadrangularis* to 45 ml of standard 0.1M $AgNO_3$ solution in a 100 ml Erlenmeyer flask. The entire process was conducted at room temperature to facilitate the formation of silver nanoparticles. The shift in color from white to dark brown could be considered as the positive indication for the formation of silver nanoparticles.

To collect the nanoparticles from the reaction medium, the solution is centrifuged at 10,000rpm and washed using 70% alcohol.

Anticancer Activity

Several in vitro studies evaluating a cell population's response to environmental stimuli are based on cell viability and proliferating measurements. The MTT Cell Proliferation Assay analyzes the rate of cell proliferation and, conversely, the decrease in cell viability triggered by metabolic processes such as apoptosis or necrosis.

Maintenance of Cell Line.

The MCF-7 breast cancer cell lines were procured from NCCS in Pune and were cultured in MEM supplemented with 10% FBS and the antibiotics penicillin/streptomycin (0.5 mL^{-1}) in an environment of 5% CO 2 /95% air at 37 0 C.

Preparation of Test Compound

The test extract was weighed and dissolved in DMSO one at a time for the MTT assay. The final concentration is made up to 1 mg/ ml with the media. The cells were treated with a range of doses ranging from 10 to 100 µg/ ml of medium.

MTT ASSAY

The MTT test, which is based on the mitochondrial succinate of metabolically active cells converting yellow tetrazolium salt-MTT (3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide) to purple-formazan crystals, gives a quantifiable measurement of viable cells (Chandra & Nagani, 2013).

The test is based on the number of cells present and the presumption that non-viable cells or their products do not reduce tetrazolium levels.

MTT penetrates the cells and reaches the mitochondria, where it is converted to insoluble dark purple formazan crystals. The cells are then solubilized in DMSO, and the solubilized formazan reagent is spectrophotometrically quantified at 570 nm.

Procedure

The MTT Assay was used to assess cell viability in three separate trials with six concentrations of chemicals in triplicate. To evaluate the viability of cells in cell suspension, cells were trypsinized, and the tryphan blue experiment was performed.

A hemocytometer is used to count the number of cells. Cells are plated in 96 well plates at a cell density of $5.0X10^3$ cells / well in 100 µl media of RPMI 1640 and grown in a CO_2 incubator for 24 hours (37 degree Celsius, 5% CO_2). After 48 hours, the medium is withdrawn and replaced with new media containing varying concentrations of the test sample. The cells are incubated for 24 to 48 hours (37 degrees Celsius, 5% CO_2). Cisplatin was utilized as a positive standard, and only PBS was added to the control wells. After 24 hours, remove the sample mixture and replace it in each well with a fresh medium supplemented with the MTT solution (0.5 mg/ml^{-1}). After that, the plates were incubated at 37°C for 3 hours. The formation of precipitates occurs at the completion of the incubation time as a result of the cells' metabolically active mitochondria reducing the MTT salt to chromophore formazan crystals. A microplate reader was used to measure the optical density of solubilized crystals in DMSO at 570 nm.

The following formula was used to determine the percentage growth inhibition.

$$\%\text{Inhibition} = \frac{100(\text{Control} - \text{Treatment})}{\text{Control}}$$

The IC_{50} value was calculated using the linear regression equation, y = mx+c.

The viability graph was used to get y = 50, m, and c values.

Quantitative Real Time-PCR Analysis of p53 Gene

Primer synthesis

A primer is a short nucleic acid sequence that serves as the initial point for DNA synthesis.

Table 1 : List of Primers

SNo	Gene	Primers
1	P^{53}	F-AGAGTCTATAGGCCCACCCC R-GCTCGACGCTAGGATCTGAC
2	GAPDH	F-ATGGCATTCCGTGTTCCTAC R-CCTTCAACTTGCCCTCTGAC

Following the selection of the sequences, the sequences were synthesized on a 50nm scale with HPLC purification. After the synthesis, the primers were adjusted to a 100 p molar stock and then to a 10 pmolar working stock for the Realtime-PCR.

RNA isolation

The MCF-7 cell lines were treated with the AgNPs extract for 24 hours, and then the cells were scraped, and total RNA was extracted using TRIzol reagent Takara Bio Inc. Cells were scraped with 1 ml Tri reagent, and any insoluble material was eliminated by centrifugation at 12,000 rpm for 20 minutes at 4°C. 0.2 ml chloroform was administered to the clear lysate and forcefully agitated. The phases were separated by centrifugation at 12,000g for 20 minutes at 4°C.

The top aqueous layer containing RNA was collected in a separate tube, and the RNA was precipitated by isopropanol (0.5ml) and centrifuged at 12,000g for 10 minutes at 4°C.

The pellet was rinsed in 75% ethanol, reconstituted in RNase-free water, and purified with the RNeasy Mini Kit (Qiagen). RNA samples were stored at -80 °C until they were needed.

cDNA Synthesis

To synthesize cDNA from extracted RNA, the Takara Prime Script 1st strand cDNA synthesis kit (Cat No 6110A) was utilized. The reverse transcription of 4 µg of RNA was carried out in a thermocycler using the 2 X reverse transcription (R.T.) which included 10X R.T. buffer, 25X dNTP mix, 10X R.T. random primers, RNase inhibitor and nuclease free water and Multiscribe reverse trancriptase.

The reaction conditions are as follows:

- Initial RT for 5 min at 65 °C
- 50 °C for 60 min
- 95 °C for 5 min (Inactivation of reverse transcriptase)

Real-time PCR

In the quantitative RT-PCR, 25 ng cDNA templates were tested using 50 pmol gene-specific primers. The Qiagen Roto-Gene PCR cycler and the SYBR green dye is employed in the study of gene expression. The detection of product formation was positioned in the center of the linear section of PCR amplification, and the cycle at which each reaction achieved the predetermined threshold (CT) was calculated.

The ΔΔCT values were used to determine the relative change in mRNA expression. Levels were adjusted relative to the housekeeping gene GAPDH mRNA and presented as fold change over controls. The PCR conditions were as follows:

- Initial denaturation was 2 minutes at 94 °C
- 35 cycles of denaturation 30 seconds at 94 °C
- Annealing at 60 °C
- Extension with 72 °C
- The final extension will be 72 °C with 5 minutes

All reactions were carried out in triplicate, including the no-template controls. The levels of relative gene expression were computed and summarized.

Results and Discussion

Anticancer activity

The colorimetric approach was used to detect anticancer activity in MCF-7 breast cancer cell lines using the tetrazolium MTT test. The MTT assay is a well-known in vitro method for analyzing cytotoxicity in cancer cell lines (Vijayalakshmi *et al*., 2013). The percentage viability of the cells is measured by the typhan blue assay, followed by the MTT assay. The percentage growth inhibition was calculated using the following formula.

% Inhibition = 100 (Control – Treatment) / (Control)

The percentage viability was calculated using the formula

% Viability = (Mean OD_{sample} / Mean $OD_{control}$) x 100

The IC_{50} value is calculated using the linear regression equation, y =mx+c, where y=50 and the values of m and c are taken from the viability graph plotted for each sample.

Table 2 : In vitro Anticancer activity of AgNPs of *Cissus quadrangularis* in MCF-7 cells

Concentration (µg)	Absorbance at 570nm	% Inhibition	% Viability	IC_{50} (µg)
5	0.574	20.16	79.84	56.74 ±0.718
10	0.497	30.87	69.13	
25	0.421	41.44	58.56	
50	0.339	52.85	47.17	
100	0.254	64.67	35.33	
Untreated	0.719	0	100	
Blank	0	0	0	

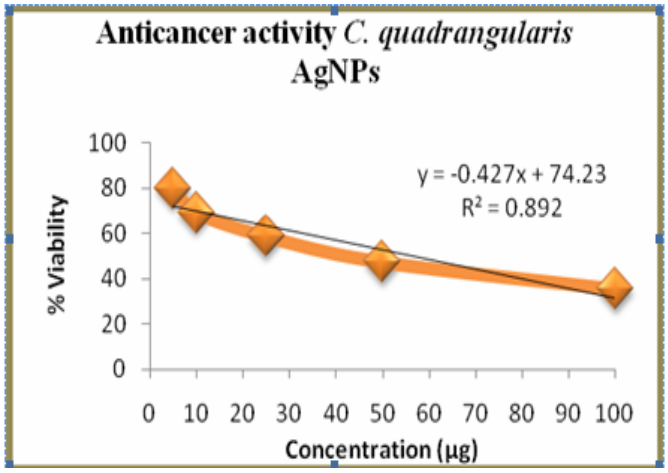

Fig. 1 : Determination of the IC_{50} value of AgNPs of *Cissus quadrangularis* in MCF-7 cells using the viability graph

The National Cancer Institute (NCI) of the United States defined a compound's cytotoxic activity as potent cytotoxic activity if the IC_{50} was less than 20 g/mL, moderate cytotoxic activity if the IC_{50} was between 21 and 200 g/mL, and weak cytotoxic activity if the IC_{50} is between 201-500 µg/mL. There was no cytotoxic activity if the IC_{50} was more significant than 500 g/mL (Damasuri AR *et al*., 2020).

The IC_{50} value of the synthesized AgNPs from the stem of *C.quadrangularis* was estimated to be 56.74 µg/ml. The results indicate that the AgNPs possess a good cytotoxic effect, and therefore, based on the criteria given by the NCI, it can be stated that the synthesized AgNPs possess moderate anticancer activity.

Table 3 : Comparision of the anticancer activity of the test samples and the positive control in the MCF-7 cells

S. No	Sample Name	IC_{50} (µg/ml) MCF-7
1	AgNps	56.74±0.718
2	Cisplatin (µM)	5.51± 0.139

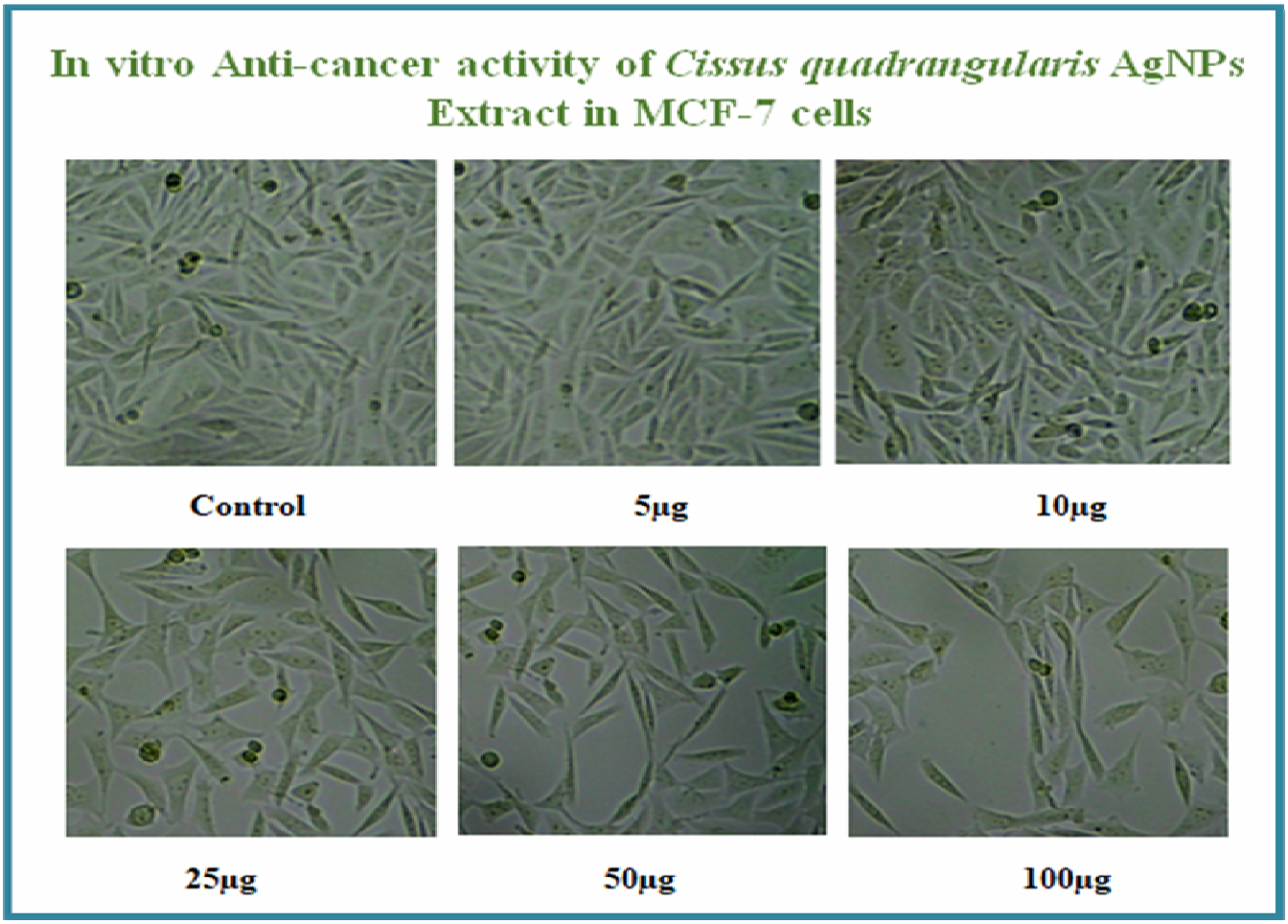

Fig. 2 : MCF-7 cells treated with the AgNPs extract

Quantitative Real Time-PCR Analysis of p53 gene

The effect of the AgNPs from the stem of *Cissus quadrangularis* on the expression of the p53 gene is out using the Quantitative Real Time-PCR Analysis. The GADPH (Glyceraldehyde-3-phosphate dehydrogenase) gene is chosen as the housekeeping gene, and the p53 gene is the gene of interest.

The primers for the specified sequences from the p53 gene and GADPH are synthesized at a scale of 50nm and purified using HPLC. RNA was then isolated from MCF-7 cells treated with AgNPs, constituting the basis for cDNA synthesis. In Q, real-time PCR, cDNA, and forward and reverse primers are used to investigate the expression of the p53 gene.

Table 4 : Concentration of cDNA

S. No	Sample Name	A260/280	cDNA Concentration (µg/ml)
1	AgNPs (Cissus)	1.78	427.2
2	MCF-7 Cell control	1.75	475.5

The A260/280 ratio measures the purity of the DNA, and the cDNA concentration is measured using the absorbance at 260nm.

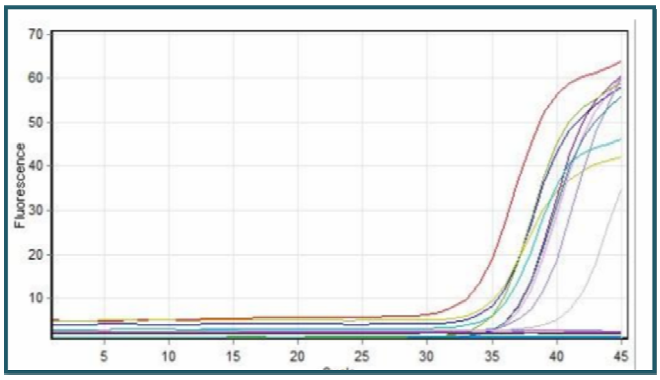

Fig. 3 : PCR efficacy graph

Table 5 : Ct Values of Samples

Samples	CT values		ΔCt	ΔΔCt	$2^{(-\Delta\Delta Ct)}$
	GOI	GADPH			
Control 1	31.22	28.92	2.3	0.97	0.51
Control 2	30.58	27.12	3.46	2.13	0.23
Control 3	29.62	31.39	-1.77	-3.1	0.12
Treated 1	30.69	29.13	1.56	0.23	0.85
Treated 2	31.11	29.21	1.9	0.57	0.67
Treated 3	31.29	27.41	3.88	2.55	0.17
			1.888333	**0.558333**	**0.43**

Here, the Ct values of the gene of interest and the reference gene are taken from the PCR efficacy graph. The ΔCt value is the difference between the Ct of the gene of interest and the Ct of the reference gene. The ΔΔCt value is the difference between the average ΔCt value of the control

and the individual ΔCt value of each sample. The $2^{(-\Delta\Delta Ct)}$ is the relative changes in gene expression. It is the difference in the gene expression of the target vs control gene in your test sample compared to variations in target vs control gene expression in your control sample.

Table 6 : Relative Gene Expression of P^{53}

	Control	Sample	Standard Error Mean(SEM)
Cissus quadrangularis AgNPs	1	0.43	0.63587

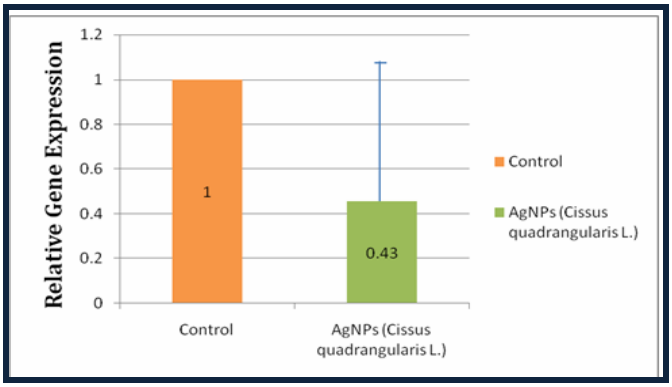

Fig. 4 : Relative Gene Expression of P^{53} gene in MCF-7 Cells

The relative gene expression in control is usually set to 1. If the ΔΔCt has a negative value, the gene of interest is upregulated because the fold change will be more significant than 1. On the other hand, if the ΔΔCt has a positive value, the gene is downregulated, and the fold change is <1. Down regulation of P^{53} gene expression was observed at 0.4564 folds in *Cissus quadrangularis* L. AgNPs 5 treated cells of MCF-7 compared to control cells of MCF 7.

Conclusion

The antioxidant activity and the presence of phenols and other bioactive compounds in the preliminary examination of the methanolic and AgNP extract of the *Cissus quadrangularis* stem to provide an insight into its possible anticancer potential. One of the most extensively used tests for assessing the preclinical anticancer activity of synthetic derivatives and natural products and natural product extracts is the MTT in vitro cell proliferation assay (Cauley *et al*., 2013). Cisplatin was selected as the positive control in the assessment. The MTT test findings showed that the synthesized AgNPs have moderate anticancer activity. This is confirmed by the IC50 values of 56.74 µg/ml in the AgNPs, which is considered moderate in cytotoxicity by the National Cancer Institute. Siddiqui *et al*. (2021) also established the anticancer potential of the ethanolic extract of *Cissus quadrangularis* against the human cervical HeLa cell lines.

Quantitative PCR (qPCR), also referred to as accurate PCR or quantitative real-time PCR, is a PCR-based method that amplifies a target DNA sequence to quantify the quantity of that DNA species in a reaction (Lorsch & Dymond, 2013). The comparative Ct approach is a mathematical model for measuring gene expression changes as a relative fold difference between an experimental and calibrator sample (Wong & Medrano, 2005). The process involved the isolation of RNA from the target cells and synthesizing c DNA from it, on which the expression studies are carried out. The AgNPs extract was used to evaluate its influence on the expression of the p53 gene in MCF-7 breast cancer cell lines as it demonstrated remarkable anticancer activity compared to the methanolic extract of *C. quadrangularis*. The results concluded that the MCF-7 cells treated with the synthesized AgNPs from the stem of *Cissus quadrangularis* bring about 0.4564 folds downregulation in the expression of p53 genes compared to the control cells MCF-7.

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