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Original Research

In Vitro Anticancer Activity of Ethanolic Extract of *Euphorbia hirta* (L.)

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Abstract

In the present study, *In vitro* anticancer effects of *Euphorbia hirta* were investigated. The objectives of this study are to find the presence of secondary metabolites by preliminary phytochemical investigation and FTIR analysis in the *Euphorbia hirta*. Ethanolic leaf extract of *Euphorbia hirta* was tested for its cytotoxicity against Dalton Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines using preliminary screening technique (Trypan Blue method and standard MTT colorimetric method). The *Euphorbia hirta* leaf extract was found to be more cytotoxic against Ehrlich Ascites Carcinoma cell lines when compared to cytotoxic activity against Dalton Lymphoma Ascites cell lines where as 59.67% cytotoxicity were noticed against Ehrlich Ascites Carcinoma cell lines. Extract of *Euphorbia hirta* showed a dose-dependent reduction of proliferation and induction of apoptosis in the Carcinoma cell lines cells. Ethanolic leaf extract of *Euphorbia hirta* showed potent cytotoxic activity against DLA and EAC cell lines and its IC_{50} value was found to be 560.83µg/ml and 384.7 µg/ml of extract respectively. The *in vitro* outcomes of *Euphorbia hirta* extract showed potent anticancer effect against both DLA and EAC cell lines.

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INTRODUCTION

Plants have been used as remedies and botanical literature has described the usage of plant extracts. Cancer is a dreadful disease and combating this disease is of great importance to public health. There is a necessity for search of new compounds with cytotoxic activity as the treatment of cancer with the available anticancer drugs is often unsatisfactory due to the problem cytotoxicity to the normal cells. Phytochemical examination has been making rapid progress and herbal products are becoming popular as sources of plausible anticancer compounds (Parag *et al.,* 2010).

Cancer is a class of diseases in which the body cells become abnormal and divide indiscriminately. Cancer cells may become invasive and transform normal adjacent cells into malignant cells. They may also spread through the blood stream and lymphatic system to other parts of the body to form metastatic tumors in distant organs. Cancer is caused by abnormalities in the genetic material of the transformed cells. Cancer may also be initiated by carcinogens, tobacco smoke, radiation, chemicals or infectious agents, especially some viruses. *Euphorbia hirta* of the family *Euphorbiaceae* is a medicinal, rhizomatous herb distributed in South Western Ghats of India and North East Coast of Tamil Nadu (Abdul Rahuman *et al.,* 2007). The plant is native to India but is a pan tropical weed and small, erect or ascending annual herb reaching up to 50 cm with hairy stem. The leaves are opposite, elliptical, oblong or oblong-lanceolate, with a faintly toothed margin and darker on the upper surface. The flowers are small, numerous and crowded together in dense cymes of about 1 cm in diameter. The fruits are yellow, three-celled, hairy, keeled capsules, 1-2 mm in diameter, containing three brown, four-sided, and angular, wrinkled seeds (Chika *et al.,* 2007). Leaves, stem and flowers are used for treating respiratory ailments especially cough, bronchitis and asthma. Worm infestations, dysentery, gonorrhoea, jaundice, pimples and digestive problems are also treated with *Euphorbia hirta* (Kirtikar and Basu, 1991). Extracts of *Euphorbia hirta* have been found to show selective cytotoxicity against several cancer cell lines. The plant is useful in the effective treatment of cancers, particularly malignant melanoma and squamous cell carcinoma (Abdul Rahuman *et al.,* 2007). The aim of the present study was to investigate the presence of secondary metabolites and cytotoxic activity of Ethanolic leaf extract of *Euphorbia hirta* against DLA an EAC cell lines.

MATERIALS AND METHODS

Extraction of Plant Material

Fresh disease free leaves of *Euphorbia hirta* was collected from in and around Tiruchirappalli District, Tamil Nadu, India and identified by Rev. Dr. John Britto, Botanist, St. Joseph's College, Tiruchirappalli. The shade dried leaves (*Euphorbia hirta*) were subjected to pulverization to get coarse powder. The coarse powder material was subjected to Soxhlet extraction separately and successively with ethanol. The extract was

concentrated to dryness in flash evaporator under reduced pressure and controlled temperature. The ethanol extract put in air tight containers stored in a refrigerator for experimentation purpose.

Phytochemical Analysis

Phytochemical analysis for major phyto-constituents of the plant extract was undertaken using standard methods. The plant extract was screened for the presence of biologically active compounds like sugars, amino acids, proteins, phenols, terpenoids etc. (Edeoga *et al.,* 2005; Trease and Evans, 1996; Smolenski *et al.,* 1974; Kapoor *et al.,* 1969; Harborne, 1984; Daniel, 2006; Prashith Kekuda *et al*., 2012).

Qualitative Phytochemical Analysis

Dragendoff's Test (Alkaloids): In a test tube containing 1 ml of extract, a few drops of dragendoff's reagent was added and the color developed was noticed. Appearance of orange color indicates the presence of Alkaloids.

Test for Terpenoids: In a test tube containing 1 ml of extract, a few drops of thionyl chloride were added. Appearance of pink color indicates the presence of terpenoids.

Leibermann-barchard Test (Steroids): The powder was dissolved in two ml of chloroform in a dry test tube. 10 droops of acetic anhydride and two drops of concentrated sulphuric acid were added. The solution becomes red then blue finely bluish indicates the presence of steroids.

Test for Coumarins: 1 ml of extract, 1 ml of 10% sodium hydroxide was added. The presence of coumarins is indicated by the formation of Yellow color.

Test for Tannins: To the few mg of powder, 10% alcoholic Ferric chloride was added; formation of dark blue or greenish black color shows the presence of Tannins.

Test for Flavanoids: To the few mg of powder, Magnesium and 1-2 drops of concentrated hydrochloric acid were added. Formation of the red or pink color shows the presence of flavanoids.

Test for Phenols: To 1 ml of extract, 2 ml of distilled water and few drops of 10% ferric chloride were added. Appearances of blue or green color indicate the presence of phenols.

Test for Volatile Oils: To 2 ml of extract, 0.1 ml dilute sodium hydroxide and small amount of dilute hydrochloric acid were added and the formation of white precipitate indicates volatile oils.

Test for Quinones: To 1 ml of methanolic extract, 2 drops of concentrated hydrochloric acid was added. Formation of red color indicates presence of quinines.

Test for Sugar: To the 1 ml of extract, Fehling's solution was added. Appearance of red color indicates the presence of sugar.

Borntrager's Test (Anthroquinones): The extract was macerated with ether and after filtration, aqueous ammonia or caustic soda was added. Pink red or violet color in the aqueous layer after shaking indicates the presence of anthroquinones.

Test for Saponins: To 1 ml of the extract, 5 ml of water was added and the tube was shaken vigorously. Copious lather formation indicates the presence of Saponins.

Fourier Transform Infrared Spectrophotometer (FTIR)

Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. The powdered sample of plant specimen (*Euphorbia hirta*) was loaded in FTIR spectroscope (Shimadzu, IR Affinity, Japan), with a Scan range from 400 to 4000 cm^{-1} with a resolution of 4 cm^{1}.

Source and Maintenance of Cell Lines

Tumor Cell Lines

1. Dalton's Lymphoma Ascites (DLA) cells
2. Ehrlich ascites tumor (EAC) cells

The cell lines were maintained at Amala Cancer Research Centre, Amala Nagar, Thrissur, India. They were chosen for following reasons

(i) Adaptability to growth in RPMI-1640 or DMEM plus FBS with glutamine,
(ii) Suitability for use in micro culture, and
(iii) Mass doubling time that allows for harvesting of approximately $1.5x10^5$ cells/ml every week.

The two cell lines were cultured in 200 ml flasks with the complete culture medium. The cell was maintained at 37^oC in a 5% CO_2 humidified incubator. The culture medium was replaced three times weekly and the cells were trypsinised when nearly confluent.

Preparation of Cell Suspension

All the cells were detached from the culture flasks by the addition of 1 ml of 0.25% trypsin 0.1% EDTA. Trypsin was then inactivated by the addition of 10 ml of experimental medium. Cells were separated in to a single cell suspension by a gentle pipetting action and 1 ml of the cell suspension was returned to the culture flask containing 20 ml of the culture medium. The remaining cell suspension was centrifuged at 1000 rpm for 3 minutes and the supernatant was discarded.

***In Vitro* Cytotoxicity Assay**

The cytotoxicity was determined by Trypan blue exclusion method (Babu *et al.,* 1995). It is based on the principle that live cells possess intact cell membranes that exclude the dye while the dead cells do not and have blue colored cytoplasam under light microscope. For the assay DLA and EAC cells, to about 0.8 ml of PBS add 0.1 ml of cell suspension containing 1 x 10^6 cells and different concentration of extract ranging from 50 µg – 500 µg/ml. These were incubated for 3 hours at 37^oC. After incubation, 0.1 ml trypan blue dyes was added and apply a drop of tryphan blue – cell mixture to a hemocytometer and count the stained (non-viable) and unstained (viable) cells separately under a microscopic field.

MTT Assay

Sensitivity of Dalton Lymphoma Ascites and Ehrlich Ascites Carcinoma cells to *Euphorbia hirta* was determined individually by the MTT colorimetric assay (Jack, 2005). Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 h at 37°C and in 5% CO_2. Both cell lines were exposed to plant species mentioned above.

The solvent DMSO treated cells served as control. Cells were then treated with MTT reagent (20µl/well) for 4 h at 37°C and then DMSO (200 µl) was added to each well to dissolve the formazan crystals. The optical density (OD) was recorded at 492 nm in a microplate reader. Percentage of residual cell viability was determined as [1−(OD of treated cells/OD of control cells)]×100.

RESULTS AND DISCUSSION

Phytochemical Analysis of *Euphorbia hirta*

The class of phytochemical compounds were present in the shade dried leaf of *Euphorbia hirta* sample are presented in Table 1. Ethanol was used as extractant. The results of phytochemical analysis showed that the leaf of *Euphorbia hirta* have different classes of bioactive constituents. Steroids, coumarin, tannins, saponin, flavonoids, anthraquinones, total phenol, total proteins, total carbohydrates and fixed oil were found to be present in the extractant. The ethanolic extract showed negative results for quinines and volatile oils.

Table 1: Preliminary phytochemical investigation in the *Euphorbia hirta*.

S.No	Phytochemical compound	Ethanol
1	Anthroquinone	+
2	Terpenoids	+
3	Alkaolids	+
4	Phenolic Compounds	+
5	Tannins	+
6	Flavonoids	+
7	Volatille oil	-
8	Quinones	-
9	Steroids	+
10	Coumarins	+
11	Saponin	+

(+) Present and (-) Absent

In the present investigation the phytochemical analysis of the powdered leaves of *Euphorbia hirta* ethanolic extract showed the presence of a lot of secondary plant metabolites which are responsible for its numerous medicinal effects. Most phytochemical analysis investigated the properties of *Euphorbia spp*. Several coumarins were isolated from several different *Euphorbia spp*. And multiple flavonoids have been identified from *Euphorbia spp*. Stems, leaves, and roots. Also prominent were triterpenoids from the roots, leaves (Vijaya *et al.,* 1995).

The Aliphatic Compounds Analysis

Fourier transform infrared (FTIR) spectral analysis showed the presence of aliphatic compounds and they were identified as Oximes, alkenes, aminoacids and aromatic compound (Figure 1). FTIR spectroscopy was useful for the compound identification and when run under IR region in the range of 663-4000 cm^{-1} there was a variation in the peaks in the plant samples. The peaks showed that the plant samples have the compounds like poly, Amide II band (hioamide), Ketones-dialkyl ketones group, Amide salts, oximes and primary alcohols (Figure 1).

Carbohydrates in the leaves were the major constituents in these absorption bands. The very strong peak at 1032 cm^{-1} and 1039 cm^{-1} in the spectrum also indicates the starch content in the sample. The stronger the relative intensity of the band was indications of the higher the chemical constituents. The secondary peaks at 770-922 cm^{-1} are assigned as characteristic absorption of the carbohydrate. The absorbance bands at 837-721 cm^{-1} represent C-H in plane and out of plane bending for the benzene ring and bands at 533-633 cm^{-1} represent C-O-O and P-O-C bending of aromatic compounds (phosphates).

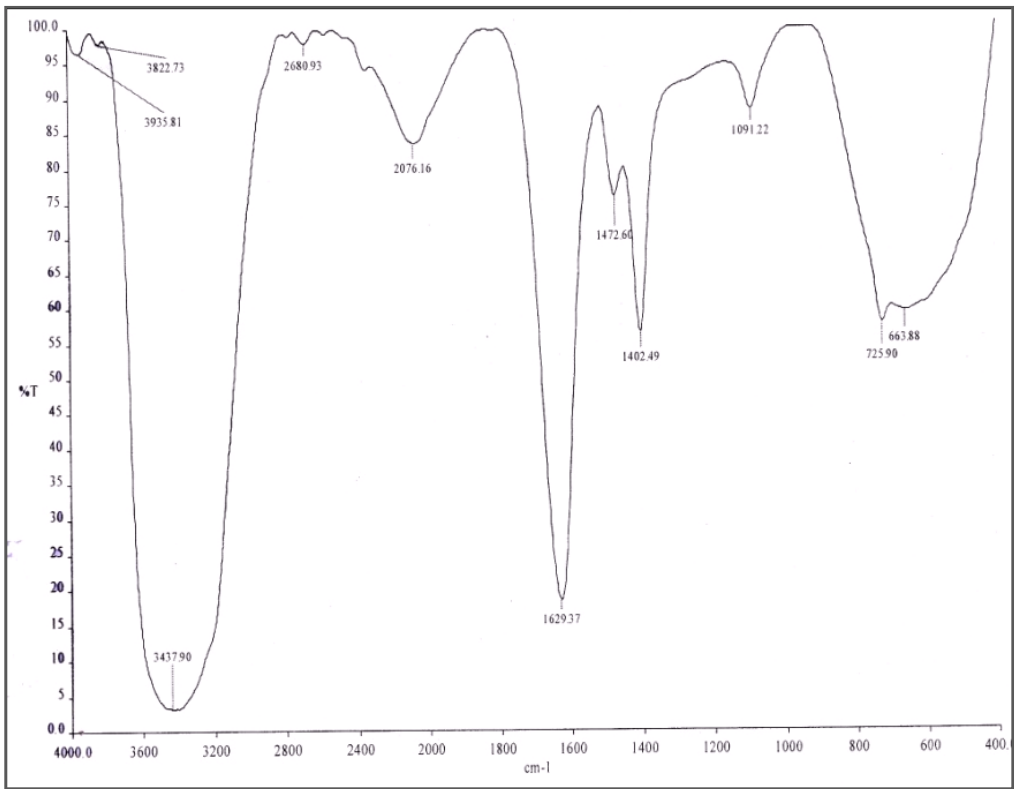

Figure 1: FTIR spectra for ethanolic extract of *Euphorbia hirta*.

Table 2: Identification of compound from extract of *Euphorbia hirta* using FTIR technique.

No	Frequency Range	Wave length Range	Type and Group
1	3920.52	2.43-2.57	Oximes
2	3412.36	2.82-2.90	R-OH, Ar-OH dimeric group
3	2342.32	3.79-4.24	All aminoacids
4	2084.26	4.67-4.81	Aminoacids
5	1638.20	6.06-6.33	Pyrimidines, Quinolines
6	682.12	14.49-16.26	Cis form nitrites
7	760.38	12.98-13.61	Benzene ring with four adjacent Free H Atoms
8	611.46	15.90	Alkenes
9	1440.86	6.90	Aromatic multiple band

Kareru *et al.* (2008) carried the spectral analysis for saponins in the crude dry powder of 11 plants and detected that *Albizia anthelmintica, Senna singueana, Maytenus senegalensis, Senna didymomotrya, Terminalia brownii*, and *Prunus africana* were likely to be bidesmosidic, oleanane-type triterpenoids, while those detected in Entada leptostachya and Rapanea rhododendroides might be monodesmosidic saponins.

Muruganantham *et al.* (2009) carried out the FTIR and EDS spectral analysis of plant parts like leaf, stem, and root of the medicinal plants, *Eclipta alba* and *Eclipta prostrate* and reported the presence of characteristic functional groups of carboxylic acids, amines, amides, sulphur derivatives, polysaccharides, nitrates, chlorates, and carbohydrate that are responsible for various medicinal properties of both herbal plants.

Trypan Blue Method and MTT Assay

Short term *in vitro* cytotoxic effect (Trypan Blue method) of ethanolic leaf extract of *Euphorbia hirta* against Dalton Lymphoma Ascites cell lines were tabulated in Table 3. The cytotoixcity increased with increase in concentration of extract, 50 µg/ml concentration showed 16.2% cell death where as in high concentration (500 µg/ml) 76.93% of cell death was noticed.

Short term *in vitro* cytotoxic effect of the ethanolic extract of *Euphorbia hirta* leaf against Ehrlich Ascites Carcinoma cell lines were tabulated in a Table 4. The cytotoxicity increased with increase in concentration of extract. 50 µg/ml concentrations showed 11.8% cell death where as in high concentration (500 µg/ml) 61.8% of cell death was noticed.

Table 3: Effect of ethalonic leaf extract of *Euphorbia hirta* of cytoxicity of DLA cells (Trypan Blue Method).

Concentration of Plant extract (µg/ml)	Number of visible cells	Visible Cells (%)	Number of Dead cells	Dead cells (%)
Control	113	96.59	4	3.41
50	119	83.8	23	16.2
100	88	61.5	55	38.5
250	67	41.8	93	58.2
500	33	23.07	110	76.93

Table 4: Effect of ethalonic leaf extract of *Euphorbia hirta* of cytoxicity of EAC cells (Trypan Blue Method).

Concentration of Plant extract (µg/ml)	Number of visible cells	Visible Cells (%)	Number of Dead cells	Dead cells (%)
Control	113	96.59	4	3.41
50	88	82.2	19	11.8
100	131	71.19	54	28.81
250	100	50.76	97	49.24
500	68	38.2	110	61.8

Cytotoxic effect (MTT Assay) of ethanolic extract of the leaf was studied against both Dalton Lymphoma Ascites cell lines and Ehrlich Ascites Carcinoma cell lines and the results were tabulated in Table 5 and Table 6 respectively. The *Euphorbia hirta* leaf extract was found to be more cytotoxic against Ehrlich Ascites Carcinoma cell lines (59.67%) when compared to cytotoxicity against Dalton Lymphoma Ascites cell lines (43.93).

Table 5: Effect of ethalonic leaf extract of *Euphorbia hirta* of cytoxicity of DLA cells (MTT assay).

Concentration (µg/ml)	Cytotoxicity (%)	IC_{50} (µg/ml)
Control	---	
50	8.52	
100	19.13	560.83
250	30.49	
500	43.93	

Table 6: Effect of ethalonic leaf extract of *Euphorbia hirta* of cytoxicity of EAC cells (MTT assay).

Concentration (µg/ml)	Cytotoxicity (%)	IC_{50} (µg/ml)
Control	---	
50	6.55	
100	31.14	384.7
250	39.6	
500	59.67	

The extracts found to be cytotoxic against Dalton Lymphoma Ascites cell line and Ehrlich Ascites Carcinoma cell line. At 500 µg/ml leaf extract showed 76.93% cytotoxicity against Dalton Lymphoma Ascites cell line whereas 61.8% of cytotoxicity Ehrlich Ascites Carcinoma cell line at the same dose (Table 3 and Table 4). As a part of the apotosis precedes the loss membrane integrity thereby the cells were permeable to trypan blue.

Further, in depth cytotoxic activity of the plant extract under study were evaluated against Dalton Lymphoma Ascites cell line and Ehrlich Ascites Carcinoma cell lines (MTT assay). 24hrs treatment with plant extracts showed growth inhibition of Dalton Lymphoma Ascites cell line and Ehrlich Ascites Carcinoma cells. The death of the cells caused by the extract under study was due to the loss of mitochondria which is one of the hallmarks of the apoptosis pathway. From the results, it clearly evident that at minimum concentration of the extract activities and resulted in death of Dalton Lymphoma Ascites cell line and Ehrlich Ascites Carcinoma cell lines.

The global burden of cancer more than doubled during the past 30 years with 2008 estimates of over 12 million new cases and 25 million persons alive with the diagnosis of cancer. Medicinal plants constitute a common alternative for cancer treatment in many countries around the world. At this time, more than 3000 plants worldwide have been reported to have anti-cancer properties. Globally, the incidence of use of plant-derived products for cancer treatment is from 10% to 40% with this rate reaching 50% in Asiatic patients. Though therapeutic benefits can be traced to specific plant molecule, many herbs contain many active principles which together synergies to give the desired anticancer effect (Boyle *et al.,* 2013).

Chemotherapy and radiotherapy, the conventional cancer treatments used nowadays, are expensive and cause many side effects, including such minor ones as vomiting, alopecia, diarrhea, constipation, and major ones such as myelosuppression, neurological, cardiac, pulmonary and renal toxicity. All such side effects reduce the quality of life and discourage patients to observe mediation protocols which then lead to the progression of cancer and associated complications. In addition, many of these treatments present limited anti-cancer activity. Therefore, there is a need to discover alternative anticancer drugs, hopefully more potent, as well as more selective and toxic than those currently in use.

Early examples of cytotoxic agents developed from higher plants are the antileukemic alkaloids (vonblastine and vincristine), which were both obtained from the Madagascar periwinkle (*Catharanthus reseus*), paclitaxel isolated from *Taxus baccata* used in treatment of lung, overian, breast, neek cancers and advanced form of Kaposi's sarcoma as well as camptothecin from *Camptotheca acuminata* which synthetic derivatives such as topotecan and irinotecan are used in cancer.

The initial screenings for plants used for cancer treatment are cell-based assays using established cell lines, in which the toxic effects of plant extracts or isolated compounds can be measured. According to the National Cancer Institute (NCI), plant extracts and pure compounds with cytotoxic ED50 (Effective Dose 50) values ≤30 µg/ml and ≤4 µg/ml, respectively, are considered active. Cytotoxic screening models supply important preliminary data to select plant extracts or compounds with potential antineoplastic properties (Mosmann, 1983).

In addition, cytotoxic assays do not provide false negative results since they consider plant extracts or compounds which affect cell viability. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. In most cases, the cytotoxic effects of Mexican plant extracts and their isolated compounds have been studied employing established cancer cell lines, and few reports have used primary cultures derived from human or animal tumors or drug-resistant-induced cell lines.

Initial scientific investigations of *Ficus carica* latex against cancer were performed in the 1940s. Injection of an extract of *Ficus carica* latex was found to inhibit of a benz-[a]-pyrene-induced sarcoma and resulted in the disappearance of small tumors in albino rats. This work inspired the isolation and structure elucidation of a mixture of 6-O-acyl-β-d-glucosyl-β-sitosterol iosforma from the latex of *Ficus carica* that demonstrated anti-proliferative activity in several tumor cell lines. Some of the most promising cytotoxic compounds in Ficus species include a series of triterpenoids with C-28 carboxylic acid functional groups and phenanthroindolizidine alkaloids (Borenfreunds *et al.,* 1998). Thus, triterpenoids, isolated from the aerial roots of *Euphorbia hirta* demonstrated cytotoxicity in three human cancer cell lines with IC_{50} values from 4.0 to 9.4 µM, including HONE-1 nasopharyngeal carcinoma cells, KB oral epidermoid carcinoma cells, and HT29 colorectal carcinoma cells.

CONCLUSIONS

The presence of secondary metabolites by preliminary phytochemical investigation and FT IR analysis supports the claims made by the tradition healers about *Euphorbia hirt*. Thus the data of the results of the *in virto* studies clearly depicted that even minimum concentration of the *Euphorbia hirta* leaf extract showed potent anticancer activity against both Dalton Lymphoma Ascites cell lines and Ehrlich Ascites Carcinoma cell line by activating the apoptotic pathway. Further studies are warranted for the isolation and identification of individual compounds and also *in vivo* studies are needed for understanding their mechanism of action.

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