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# Potential *In vivo* Immunomodulatory Effects of the most Active Lectin Isolated from Seeds of *Zizyphus oenoplia*

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## Abstract

**Objective:** Lectins are complex and heterogenous group of carbohydrate binding proteins, commonly found in all types of organisms with striking biological activities. Many anti-inflammatory synthetic medicines are available, but they show many side effects. In this context, anti-inflammatory compounds free from any adverse effects are the requirement of time. In the present study, the most active lectin isolated from seeds of *Zizyphus oenoplia* is tested for its anti-allergic and anti-inflammatory activities.

**Methods:** The most active lectin was isolated from seeds of *Zizyphus oenoplia*. The procedure of isolation involved extraction of lectins from dried seeds in sodium phosphate buffered saline (0.02 M, pH 7) followed by precipitation with ammonium sulphate (20%-60%) and dialysis. Later, affinity chromatography and ion-exchange chromatography were performed for further purification. The purified fraction was estimated for homogeneity and molecular weight determination on 10% SDS-PAGE. The isolated most active lectin was named as ZOSL (*Zizyphus oenoplia* seed lectin) and was tested for its anti-allergic and anti-inflammatory activity *in vivo* through Arthus reaction and anaphylactic shock on Wistar albino rats.

**Results:** ZOSL was found to be monomeric with molecular weight 25 kD. It was seen that regular oral administration of ZOSL to Wistar rats can prevent anaphylactic shock as well as Arthus reaction.

**Conclusion:** A novel monomeric lectin (ZOSL), with anti-allergic and anti-inflammatory activity, was isolated from the seeds of *Zizyphus oenoplia*.

**Keywords:** *Zizyphus oenopli*;, Affinity chromatography; Ion-Exchange chromatography; Arthus reaction; Anaphylactic shock

## Introduction

The immune system is remarkably versatile defense system that has evolved to protect animals from invading pathogenic microorganisms and cancer. It is able to generate an enormous variety of cells and molecules capable of specifically recognizing and eliminating an apparently limitless variety of foreign invaders. These cells and molecules act together in a dynamic network whose complexity rivals that of the nervous system [1]. Modulation of the immune system by various biomolecules may have effectiveness for the management of certain infections, autoimmune diseases, graft rejection as well as neoplastic diseases. Many proteins isolated from plant seeds, such as concanavalin A, phytohaemagglutinin (PHA), wheat germ agglutinin and pokeweed mitogen have been proven to be immunomodulatory in nature [2].

*Ziziphus oenoplia* belonging to family Rhamnaceae (vernacular name: Siakul) is a shrub, distributed in tropical and subtropical Asia (dry climates). It is useful for treating hyperacidity, ascaris infection, abdominal pain and healing of wounds [3]. Ethanolic extract of the plant has been studied for the anti-angiogenic potential on chorioallantoic membrane model [4]. Although locally and traditionally medicinal properties are known, the plant deserves systematic study of its each and every component. The present work is one step in the defined direction.

Lectins are complex and heterogenous group of carbohydrate binding proteins with diverse molecular structures, biochemical properties and carbohydrate binding specificities. In addition, plant lectins can be used to study glycoconjugates [5]. Lectins have attracted the interest of researchers because of their effects on various biological systems especially immune system [6-8]. In practical terms, lectins are excellent macromolecular tools for isolation and characterization of glycoconjugates [9]. Lectin affinity chromatography has been utilized in the assays of many different enzymes [10,11]. Though many types of lectins are isolated from plant seeds and are extensively studied, they are also found to be present in more or less quantities in other parts of plants such as root, stem, leaf and bark. Lectins from some of the plant species were reported to possess immunomodulatory activity [12-14], but studies on single, isolated and purified lectins are very few. Hence, in the present work the study is concentrated mainly on purified form of lectin (ZOSL).

Allergy is a hypersensitivity disorder of immune system which takes place when immune system reply to normally harmless substances and one of the main problems generated due to allergy is inflammation. Arthus reaction is a dermal inflammatory response (Type III hypersensitivity) caused by reaction of precipitating antibody with

antigen present in skin. Sometimes severe life-threatening reactions called anaphylaxis may also take place. Anaphylactic shock is a sudden immune response developed against the antigen for which the individual has IgE antibodies. Complement system mediators such as C3a, C4a etc. are called anaphylatoxins because of their ability to elevate anaphylactic shock and other allergic responses [15].

Here, the most active lectin, ZOSL isolated from *Zizyphus oenoplia* seeds by ammonium sulphate precipitation followed by affinity chromatography and ion-exchange chromatography was tested for its efficacy to prevent Arthus reaction and anaphylactic shock in Wistar albino rats.

## Methods

### Reagents and chemicals

Bovine serum albumin, phosphate buffer saline and ovalbumin were purchased from Himedia laboratories, Mumbai. Dialysis membrane and DEAE-Cellulose were purchased from Sigma Aldrich chemical company, St. Louis, USA. Guar-gum and all other reagents were purchased from authorized standard companies.

### Plant material

Mature seeds from a single growing plant were collected in January 2012. The plant was authenticated at University Department of Botany, Rashtrasant Tukdoji Maharaj Nagpur University, Nagpur and voucher specimen no. 9759 was deposited in the herbarium. The plant was identified to be *Zizyphus oenoplia* (Family Rhamnaceae).

### Erythrocytes for hemagglutination assay

Rabbit blood was collected in heparinized vials in the breeding house of University Department of Biochemistry, RTM Nagpur University, Nagpur (India). The RBCs were washed 2-3 times with normal saline and adjusted to 2% concentration with the same.

### Animals

Wistar albino rats, weighing between 180 g ± 20 g and age 2-3 months, were selected for in vivo studies. Animals were acclimatized to the standard laboratory conditions (Temp. 25°C ± 2°C) and maintained at 12 hr light, 12 hr dark cycle. Animals were fed with standard diet (Hindustan Lever) and water ad libitum and maintained as per the norms of Animal Ethics Committee. Experimental set ups were cleared by the Institutional Animal Ethics Committee.

## Experimental Design

### Acute toxicity study

A group of six rats was orally administered with ZOSL in graded doses of 50 mg/kg, 75 mg/kg, 100 mg/kg, 200 mg/kg, 250 mg/kg, 500 mg/kg, 1000 mg/kg, 2000 mg/kg and 4000 mg/kg body weight (b. w.). Rats were continuously observed for mortality and behavioral responses initially for 48 h and once daily thereafter till 14 days after initiation of experiment. LD50 (lethal dose 50), as obtained from this experiment was 2000 mg/kg b. w. Dose selection was performed by taking $1/10^{th}$ of the LD50. Therefore, the dose of the ZOSL selected for present experiments was 200 mg/kg b. w.

### Isolation of lectin

**Extraction:** Ten g dried seeds were powdered and extracted in a blender with 100 mL 0.02 M sodium phosphate buffer saline (PBS), pH 7. The mixture was kept on the shaker at 4°C for 1 hr, filtered through cheese cloth and centrifuged at 9668 g for 10 m on high speed centrifuge (Remi C24). The resultant supernatant was designated as crude extract and was used for further purification procedure.

**Precipitation by ammonium sulphate:** Fractionation of crude extract was performed by precipitation (salting out) using three different concentrations of ammonium sulphate (0%-20%, 20%-60% and 60%-100%) at 4°C. All the three precipitates were collected by centrifugation at 9668 g for 30 min and were used for measurement of protein content [16]. The precipitate obtained at 20%-60% concentration was found to be richer in activity and hence it was selected for further work. Further, it was dissolved in PBS and dialyzed at 4°C against the same to make it free from ammonium sulphate and carbohydrates [17].

**Affinity chromatography on cross linked guar gum:** Guar gum is a galactose based structure and is routinely used to separate galactose binding proteins (lectins) using affinity chromatography throughout the world. The mentioned fraction was subjected to affinity chromatography on cross-linked guar gum column (15 × 1.5 cm) previously equilibrated with PBS [17]. The unbound proteins in the column were removed by repeated washing with PBS till the optical density at 280 nm reached to 0.002 (Eppendorf AG 2331). Later the matrix bound proteins were eluted from the column by washing with extraction solution containing 0.1 M galactose. The flow rate was adjusted to 5 mL/10 min using fraction collector and peristaltic pump (L. K. B. Pharmacia). Such 25 fractions were collected with each fraction of 5 mL and checked for protein at 280 nm. All these fractions are subjected to dialysis against PBS at 4°C till galactose was removed. All fractions were checked for hemagglutination activity and protein content. The fraction number 4, 5 and 6 were found to be active amongst the 25 fractions. Hence these three fractions were mixed and were subjected to ion exchange chromatography.

**Ion-exchange chromatography on DEAE-cellulose:** Separation in ion exchange chromatography depends upon the reversible adsorption of charged solute molecules to immobilized ion exchange groups of opposite charges. The mentioned three fraction mixture of affinity chromatography was subjected to ion - exchange chromatography on DEAE-Cellulose which was equilibrated with 0.02 M sodium phosphate buffer after washing with 0.1 M HCl and 0.1 M NaOH, respectively [18]. The unbound portions are removed by washing with 0.02 M PBS. The bound proteins in the column were eluted with 0.05 M-0.25 M NaCl solution (gradient elution) and 20 different fractions were collected with each fraction of 5 mL. The absorbance of elutes was measured at 280 nm spectrophotometrically (EppendorfAG 2331). The fractions were then analyzed for protein estimation and hemagglutination activity and the most active fraction named as ZOSL was tested for homogeneity and molecular weight determination by SDS polyacrylamide gel electrophoresis.

### Polyacrylamide gel electrophoresis

Homogeneity of ZOSL was tested by simple PAGE and SDS PAGE as described by Weber and Osborn [19].

**Molecular weight:** Molecular weight of the purified lectin was determined by using 10% SDS-PAGE [19]. The standard proteins used

for comparison were Lysozyme-14 kD, Carbonic anhydrase-29 kD, Ovalbumin-44 kD, Bovine Albumin-67 kD, Phosphorylase-97 kD. After electrophoresis, the gels were stained with coomassie brilliant blue (R-250). The gels were destained with 7% acetic acid.

### Hemagglutination assay with rabbit erythrocytes

Method of Suseelan et al. was used to perform agglutination assay using 2% suspension of rabbit erythrocytes. Hemagglutination titer was determined by serial dilution in 96 round bottom well plate. Reciprocal of the last dilution showing detectable agglutination was taken as titer strength of lectin and expressed as hemagglutination units [6].

### Protein estimation

Protein content of ZOSL was measured by the method of Lowry et al. using BSA as standard protein [16].

### Carbohydrate estimation by phenol sulphuric acid test

Carbohydrate content of ZOSL was determined by phenol sulphuric acid method using D- glucose as standard [20].

### *In vivo* immunomodulatory activity

**Arthus reaction:** Twenty four animals in four groups containing 6 animals in each group were used for experiment. Animal groups were designated as positive control (PC), negative control (NC), experimental 1 (E1) and experimental 2 (E2). ZOSL dissolved in PBS was orally administered to E1 only. ZOSL was administered (200 mg/kg of b.w.) to E1 daily from 10 days prior to subcutaneous sensitization injection of 1 mg BSA in 0.2 mL PBS. Subcutaneous sensitization injection of 1 mg BSA in 0.2 mL PBS was given to all the four groups. ZOSL treatment was continued for next 15 days to E1 even after sensitization. Intradermal shocking injection of 0.5 mg BSA in 0.2 mL PBS was given to PC and E1 in right foot pad on day 1. In case of NC, as a shocking injection; an intradermal injection of ovalbumin (0.5 mg ovalbumin in 0.2 mL PBS) was injected in foot pad of animals. Immediate effectiveness of ZOSL on arthus reaction was tested in E2, where along with shocking injection of BSA, 1 mg lectin was injected in combination to animals on day 1. Thickness of footpad of each rat was recorded by vernier caliper after 2 hr, 4 hr, 6 hr, 1 day, 2 days, 3 days and daily after shocking injection until it came to normal [2].

**Anaphylactic shock:** Twenty four animals were divided in four groups before initiation of the experiment. Experimental groups were designated as Positive control (PC), negative control (NC), experimental 1 (E1) and experimental 2 (E2). ZOSL, solubilized in PBS, was administered at a dose of 200 mg/kg b.w. daily 10 days prior to intraperitoneal sensitization injection to animals in E1 group. Animals in E1 group were sensitized individually with sensitization injection administering 1 mg BSA in 0.2 mL PBS on day 1. Intraperitoneal injection (sensitization) of bovine serum albumin (BSA) (1 mg in 0.2 mL PBS) was administered to all the four groups. ZOSL treatment was continued for next 15 days to E1 after sensitization injection. After 15 days of sensitization injection, intravenous injection (shocking injection) of BSA (1 mg in 0.2 mL PBS) was given to PC and E1group while NC was given intravenous injection of ovalbumin (1 mg ovalbumin in 0.2 mL PBS). To E2, along with intravenous shocking injection of BSA, 1 mg ZOSL was injected in combination to check immediate effectiveness of ZOSL to prevent anaphylactic shock. Systemic anaphylactic reaction was observed within 10 min after shocking injection and rated as: *Positive reaction*; animals died or rendered stationary for at least 1 min, *Negative reaction*; no changes were observed in activity and movements of animals were normal [2].

## Results

### Isolation of lectin

The 20-60% ammonium sulphate precipitate, which was found to be rich with activity, was applied to cross linked guar-gum column at 4°C. Out of 25 fractions (5 mL each) collected (Figure 1), fraction number 4, 5 and 6 which were found to be active in hemagglutination activity were pooled and loaded on to the column of the ion-exchange chromatography (15cm × 1.5 cm).

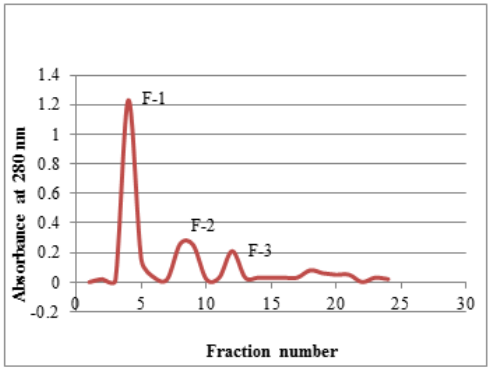


**Figure 1:** Elution profile of affinity chromatography of *Zizyphus oenoplia* seed lectins on cross linked guar gum. Fraction number 4, 9 and 12 are represented as F-1, F-2 and F-3.

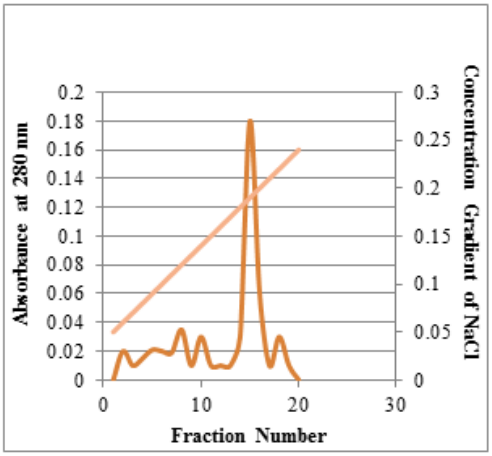


**Figure 2:** Elution profile of ion exchange chromatography of *Zizyphus oenoplia* seed lectins on DEAE-cellulose. Out of the 20 fractions, fraction number 15 was found to contain maximum proteins and named as ZOSL.

Here, 20 different fractions (5 mL each) were collected (Figure 2). Out of these, fraction number 15 was found to be most active and named as ZOSL. On further analysis on 10% SDS polyacrylamide gel electrophoresis, ZOSL was found to be monomeric with molecular weight 25 kD (Figure 3).

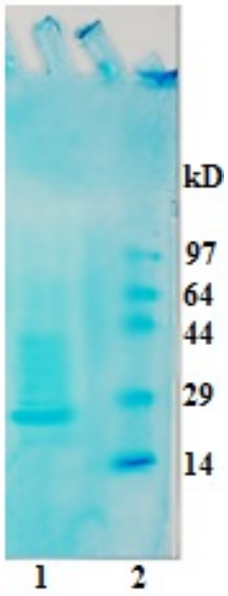


**Figure 3:** The 10% SDS-PAGE pattern of ZOSL reveals a single band, corresponding to the molecular weight to be 25 kD, Lane 1: Purified ZOSL, Lane 2: Molecular weight marker 14 to 97 kD. [Lane 1: Purified ZOSL, Lane 2: Molecular weight marker 14 kD to 97 kD (Merck, Eurolab GmbH Darmstadt, Germany)].

Results show that ZOSL was purified with good yield as shown in Table 1.

## *In vivo* immunomodulation

**Arthus reaction:** In PC, all animals were found to show positive footpad reaction within 2 hr of shocking injection (Figure 4A). While in NC, rats did not show Arthus reaction.

After administration of ZOSL to E1, no Arthus reaction was observed in the footpad (Figure 4B). In PC, the right footpad had shown an increase of approximately 7 mm within 2 hr-6 hr as compared to E1 where an increase of just 6 mm was recorded. Thus, severity of Arthus reaction was found to decrease greatly (Figure 5). However in E2, where ZOSL was administered along with shocking injection to check its immediate effect, ZOSL was found to be comparatively less effective in inhibiting Arthus reaction instantly but has decreased the severity of footpad reaction as it had taken more time to develop Arthus reaction as compared to PC.

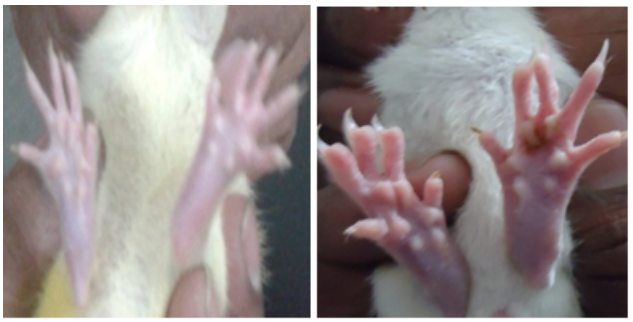


**Figure 4:** Photograph showing foot pad reaction in Wistar albino rats. Regular administration of ZOSL was found to be successful in preventing Arthus reaction. (Figure 4A: Positive Arthus reaction in PC, Figure 4B: Prevention of Arthus reaction due to ZOSL in E1).

| Purification step | Volume (mL) | Proteins (mg/mL) | HAU/mL a Rabbit erythrocytes | Total HAUa Rabbit erythrocytes | SAb Rabbit erythrocytes | Purification fold w.r.t. Rabbit erythrocytes | Yield percentage w.r.t. Rabbit erythrocytes |
|---|---|---|---|---|---|---|---|
| Crude extract | 100 | 9.5 | 2560 | 256000 | 269.47 | 1 | 100 |
| ASP Fraction | 22 | 6.0 | 5120 | 112640 | 853.33 | 3.1 | 40 |
| ACF Fraction | 15 | 1.84 | 10240 | 153600 | 5565.21 | 20.65 | 60 |
| IEC Fraction | 5 | 0.900 | 20480 | 102400 | 22755.55 | 84.44 | 40 |

**Table 1:** Purification chart of *Zizyphus oenoplia* seed lectin (ZOSL). Haemagglutination assays were conducted with a suspension of rabbit erythrocytes. It was observed that as the degree of purification increases, the haemagglutination activity also increases. (Haemagglutination assays were conducted with a suspension of rabbit erythrocytes. a) HAU- Hemagglutination Unit, b) SA- Specific activity, ASP fraction- Ammonium sulphate precipitated fraction, ACF fraction- Affinity chromatography fraction, IEC fraction- Ion-exchange chromatography fraction).

**Anaphylactic shock inhibition:** Results presented in Table 2 had shown that ZOSL was found to be effective in preventing anaphylactic shock in Wistar rats. All animals in PC displayed symptoms of systemic anaphylactic shock (animals remained stationary at least for 1 min) while NC did not show any anaphylactic reaction as they have been given shocking injection of ovalbumin. In E1, after oral supplementations of ZOSL, animals did not show systemic anaphylaxis. Immediate effect of ZOSL on anaphylactic shock was also observed, when injected in combination with BSA in shocking injection to E2.

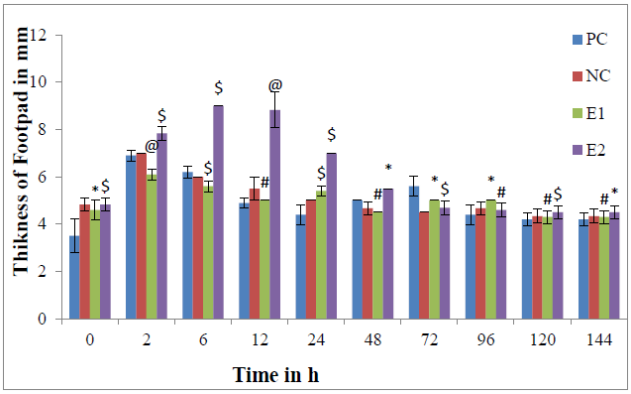


**Figure 5:** Effect of ZOSL in preventing Arthus reaction. It was seen that the footpad thickness came to normal after regular administration of ZOSL. (Values are mean thickness of footpad in mm measured by vernier caliper. The statistical significance was performed by the student T-test by using MedCalc software. Positive Control was compared with Experimental 1 and Experimental 2 where $^{*}P \leq 0.05$, $^{\$}P \leq 0.005$, $^{@}P \leq 0.0005$, $^{\#}P \leq 0.05$).

| | Sensitizing injection | Shocking injection | Oral ZOSL treatment | Results | |
|---|---|---|---|---|---|
| | | | | S/T | D/T |
| PC | BSA (i.p.) | BSA (i.v.) | - | 0/6 | 0/6 |
| NC | BSA (i.p.) | OVA (i.v.) | - | 0/6 | 0/6 |
| E1 | BSA (i.p.) | BSA (i.v.) | + | 0/6 | 0/6 |
| E2 | BSA (i.p.) | BSA (i.v.) + ZOSL (i.v.) | - | 0/6 | 0/6 |

PC: Positive Control; NC: Negative Control; E1: Experimental 1; E2: Experimental 2; OVA: Ovalbumin; i.p: intraperitonial; i.v.: intravenous; S/T: Number of anaphylactic symptoms/total no. of rats; D/T: Number of anaphylactic deaths/total no. of rats

**Table 2:** Effect of ZOSL in inhibiting systemic anaphylaxis in Wistar rats. It can be seen from the results that E1 is showing complete prevention of anaphylactic shock after regular administration of ZOSL.

## Discussion

Many synthetic anti-allergic and anti-inflammatory drugs are available today, but with some or more adverse effects. NSAIDs and DMARDs are effective anti-allergic as well as anti-inflammatory drugs, but demonstrate effects like gastrointestinal ulceration in many cases [21]. Hence, it is the real requirement of time to search for new, natural and adverse effect free compounds. That might be the reason why most of the researches have focused to work on studying natural compounds isolated from medicinal plants. This can be considered as the rebirth of *Ayurveda*. *Zizyphus* species is considered as the storehouse of medicinal compounds in *Ayurveda*. Many varieties of *Zizyphus* are explored by scientists and various medicinal properties are studied. *Zizyphus* plants are common in various medicinal preparations in India, Egypt and China for the treatment of various diseases [22]. Fruits of *Zizyphus oenoplia* are very common in India and are eaten by almost all age groups from decades, but no adverse effects have been observed.

Complement system mediators are also called anaphylatoxins, as they are the major one to amplify inflammatory reactions, which can lead to anaphylactic shock. The results of anaphylaxis might be very dangerous and can also cause death. Similarly, Arthus reaction is one of the hypersensitive reactions which can also take any dangerous form, if not controlled in time. ZOSL, a 25 kD monomeric lectin isolated from the seed cotyledons of *Zizyphus oenoplia* is found to be effective in preventing both Arthus reaction and anaphylactic shock in Wistar rats to a great extent. Though, ZOSL was not found to be very effective in immediate action to prevent Arthus reaction, but it has shown a comparatively better immediate action in preventing anaphylaxis. The results indicate that a regular oral dose of ZOSL is essential to prevent Arthus reaction as well as anaphylaxis completely. Thus, for ZOSL to act as an anti-allergic and anti-inflammatory drug, its regular administration shall be needed.

## Conclusion

According to experimental results, ZOSL isolated from *Zizyphus oenoplia* is found to possess excellent anti-allergic and anti-inflammatory activity as it has prevented both Arthus reaction and anaphylactic shock in Wistar rats. Being natural, ZOSL is free from any adverse effects. Thus, it has a potential to replace the synthetic anti-allergic and anti-inflammatory drugs.

## Conflict of interest

The authors declare no conflict of interest.

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