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Original Article

Isolation and characterization of a bioactive lectin from *Zizyphus oenoplia*

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ABSTRACT

A lectin isolated from *Zizyphus oenoplia* leaves, agglutinating rabbit and human 'B' group erythrocytes, was purified to homogeneity by affinity chromatography on Guar gum column. The purified lectin showed single band, both in non-denaturing PAGE and SDS-PAGE. The molecular weight determined by SDS-PAGE was found to be 23000 Da. Treatment of lectin with 10 mM EDTA diminished the haemagglutinating activity to 50% of the original level. Addition of divalent cations, Mg^{2+} and Mn^{2+} at 1mM totally restored hemagglutinating activity. The protein shows maximum activity over the pH range and was stable at higher temperature. It was identified to be a glycoprotein. The lectin has shown to promote proliferation of human lymphocytes after 72 h of culture under standard conditions. The purified lectin was also found to be pH and temperature stable.

Keywords: Zizyphus, lectin, Hemagglutinin, Phytoagglutinin, Cell Proliferating protein

INTRODUCTION

Zizyphus oenoplia belonging to family Rhamnaceae (vernacular name: Siakul) is a shrub, distributed in tropical and subtropical Asia in dry climates. The roots are astringent, bitter, antihelmintic, digestive, and antiseptic [1-4]. They are useful for treating hyperacidity, ascaris infection, abdominal pain, and healing of wounds [5-8]. This plant is used traditionally as a folk medicine for its antiinfectious, antidiabetic, antidiuretic activities. [9-11].

Lectins are carbohydrate binding proteins widely distributed in plant kingdom. This class of proteins has been isolated from many plants and animals. Their characteristics have also been extensively exploited in many aspects of biochemistry and biomedicine [12]. In plants, leguminous seed are particularly the rich source of lectins, while only a few have been isolated from other families [13]. The main properties of lectin are based on their ability to interact with carbohydrates and thus bind with glycocomponents on the cell surface [14]. Lectins are able to tightly bind with and cause the precipitation of specific polysaccharides and glycoproteins because they are polyvalent (i.e. each lectin molecule has at least two carbohydrate binding sites to allow cross-linking between cells or between the sugar containing macromolecules). Lectins vary, however, in molecular size, amino acid composition, metal ion requirement and their three dimensional structures [15].

Most of the lectins are shown to depend on metal ions such as Mn^{2+} and Ca^{2+} activity. In some cases, evidences have been presented for the requirement of metal ions for activity. Interaction of molecules with carbohydrates requires tightly bound Ca^{2+} and Mn^{2+} (or any other transitional metal ion). These metal ions are located close to the carbohydrate binding site and are identically positioned. Thus metal ions serve to maintain the integrity of the subunits of the lectins and in addition, help to position amino acid residue for carbohydrate binding [16].

Furthermore many plant lectins are reported to induce cell proliferation and also act as a immuno-modulating agent *"in vitro"* and *"in vivo"*. Lectin from *Viscum album coloratum* modulates proliferation and cytokine expressions in Murine splenocytes [17]. Lectins isolated from *Volvariella volvacea* (straw mushroom) exhibit stronger immunomodulatory activity than other known lectins such as concanavalin A [18], and a lectins isolated from a mushroom [19], etc. The diatary lectins isolated from legumes (peanut, groundnut, kidney bean, jackbean) modulate immune function in rheumatoid arthritis [20], Mistletoe lectin is

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having cytotoxic and immunomodulatory effect in breast cancer cells [21], dietary garlic (*Allium sativum*) lectins, ASA I and ASA II, are highly stable and are shown to be immunogenic [22].

Isolation and characterization of lectin, with property of human lymphocyte proliferation, from the leaves of *Zizyphus oenoplia* (ZOLL) has been described here. Isolation and characterization of non-leguminous lectin is important in order to study their minor structural differences that can lead to dramatic differences in biological properties and also to investigate the ubiquitous nature of lectins.

MATERIALS AND METHODS

Materials: Leaves were collected from a single identified and authenticated tree *Zizyphus oenoplia*. (Voucher No. 9759). Human blood of ABO system were obtained from healthy donors from clinical laboratory and rabbit blood was withdrawn from the animals maintained in animal house of Department of Biochemistry, RTM Nagpur University, Nagpur. Molecular weight markers were obtained from Genei (Bangluru, India), Acrylamide, guar-gum, various carbohydrates, methylene bisacrylamide and all of the other reagents were analytical grade purchased from SRL (Mumbai, India).

Cell lines culture media: Lymphocyte separation medium (HiSep LSM), fetal calf serum, RPMI-1640, Antibiotic antimycotic solution, XTT cell assay kit (EZ Count, CCK015) were purchased from Himedia (Mumbai, India). Concanavalin-A was purchased from SRL (Mumbai, India).

Methods

Extraction: Ten g of dried leaves were powdered and extracted in a blender with 100 ml of 0.02 M sodium phosphate buffer saline, pH 7 (PBS), kept on the shaker at 4º for 1 h, filtered through cheese cloth, centrifuged at 10,000 rpm for 10 m (Remi C24). The resultant supernatant designated as crude extract was used for further purification of lectins.

Ammonium sulphate precipitation: Lectins were precipitated from the crude extract by adding ammonium sulphate at 4 º. The precipitate obtained between 20 to 60% saturation was collected by centrifugation at 12000 rpm for 30 m (Remi C24). Precipitate was dissolved in PBS and dialyzed against the same till the solution was free from carbohydrates and designated as ammonium sulphate fraction (ASF) [23].

Affinity Chromatography: The ASF was subjected to affinity chromatography on cross-linked guar-gum column previously equilibrated with PBS [24]. The unbound proteins were washed thoroughly with PBS till elute showed no readings at 280 nm on spectrophotometer (Eppendorf AG 2331). The bound proteins were eluted from the column by extraction solution containing 0.1 M galactose. The flow rate was adjusted to 5 ml/10 m using fraction collector and peristaltic pump (L. K. B. Pharmacia). Fractions of 5 ml each were collected and checked for protein at 280 nm and subjected to dialysis against PBS till galactose was removed. All fractions were checked for agglutination activity [25]. Proteins in the fractions showing maximum agglutination were tested for homogeneity.

Polyacrylamide Gel Electrophoresis: Homogeneity of the affinity purified fractions was tested by simple PAGE and SDS PAGE as described by Patil and Shastri (1982). Homogeneous fractions were designated as ZOLL (*Zizyphys oenoplia* leaf lectin).

Molecular weight: Molecular weight of the purified lectin was determined by the method of Weber and Osborn, (1969) [26] using 10 % SDS-PAGE. The standard proteins used for comparison were Lysozyme – 14 kD, Carbonic anhydrase – 29 kD, Ovalbumin – 44 kD, Bovine Albumin – 67 kD, Phosphorylase-b – 97 kD. After electrophoresis the gels were stained with coomassie brilliant blue (R-250). The gels were destained with 7% acetic acid [27].

Protein concentration: Protein concentration of ZOLL was measured by the method of Lowry *et al*., (1951) using BSA as standard protein [28].

Estimation of Carbohydrate content:
Carbohydrate content of ZOLL was determined by phenol sulphuric acid method using -D- glucose as standard [29].

Agglutination assay: Method of Deshpande and Patil, (2002) [30] was used to perform agglutination assay using 2% suspension of erythrocytes of rabbit and human blood group ‘A’, ‘B’, ‘O’. Hemagglutination titre was determined by serial dilution in 96 µl well plate. Reciprocal of the last dilution showing detectable agglutination was taken as titer strength of lectin and expressed as hemagglutination units [31].

Agglutination inhibition assay: Carbohydrate specificity of ZOLL was investigated by the ability of various carbohydrates to inhibit the agglutination. Method of Kurokawa *et al*., (1976) [32] was used to test the agglutination inhibition assay. Various pentoses, hexoses, monosaccharides, disaccharides and

oligosaccharides were used to study the agglutination inhibition assay. 100 µl of 0.1 M carbohydrate solution was incubated with 100 µl of ZOLL solution at 37 º for 1 h. Agglutination assay was performed by the method described earlier. The minimum inhibitory concentration was taken as the one which did not agglutinate erythrocytes.

pH Stability: Effect of pH on the hemagglutinating activity was determined by carrying out the haemagglutination assay of the lectin using following buffers of different pH values ; for pH 1- 0.1 N HCL, for pH 2 and 3 - 0.2 N glycine-HCL buffer, for pH 4 and 5 - 0.2 M sodium acetate buffer, for pH 6 and 7 - 0.2 M sodium phosphate buffer, for pH 8 - 0.2 M - tris-HCL buffer, for pH 9 - glycine-NaOH buffer, for pH 10 to 13 - carbonate bicarbonate buffer. 100 µl ZOLL and 100 µl buffer solution was incubated for 1 h at 37 º. Aliquots were withdrawn and assayed for agglutination as described earlier [33].

***Effect of temperature and thermal inactivation on haemagglutination activity*:** To study the effect of temperature 30 µl ZOLL solution was added to 1 ml 0.006 M PBS pH-7 and incubated at 20, 40, 60, 80 º for 1 h and agglutination assay was carried out at 37 º after cooling. The effect of thermal inactivation was tested by heating 250 µl ZOLL with 250 µl of 25 mM PBS pH -7 at 37 º in multiblock heater. Aliquots were withdrawn and estimated for agglutination after 20, 40, 60 80 and 100 min [34].

Effect of metal ion on agglutination activity: Method of Kawagishi *et al*., (1990) [35] was used to check the effect of metal ion on agglutination activity of ZOLL by adding 100 µl EDTA treated ZOLL with 100 µl 0.1 M metal ion solution and incubating at 37 º for 1 h, agglutination assay was carried out as described earlier.

α - and β – galactosidase activity: Substrates used in this study were 3 mM α – pNPGal and 3 mM β – oNPGal. α and β galactosidase assay was carried out by the method of Murray *et al*.,(1983) [36]. The assay mixture contained 20 to 100 µl of ZOLL and 3 mM substrate prepared in 0.1 M sodium acetate buffer pH 4.7. Reaction mixture was incubated at 37 º for 40 m and the reaction was stopped by adding 2 ml, 0.2 M sodium carbonate. The liberated p-nitrophenol was measured at 400 nm. Lectin was also inhibited by α – D – galactose and checked for galactosidase activity.

Cell proliferation assay: Human peripheral blood lymphocytes (PBLs) were separated by the method of Boyum [37], using HiSep LSM, and density gradient centrifugation. The cell count was adjusted to 1×10^6 cells/ml in RPMI-1640 complete medium. PBLs were stimulated with each of the four concentration (2, 4, 6, 8, 10 µg) of ZOLL. After 72 h of incubation, XTT assay was done by the instructions given by the manufacturer of XTT cell assay kit (EZ Count, CCK015) then the plates were read after 4 h, at a test wavelength of 450 nm to obtain specific absorbance. Mean background values were obtained by scanning blank wells containing medium only. Proliferative effect of ZOLL on human PBLs was expressed in terms of proliferation index. The index for control is taken as 1.0, and for others it is calculated as a ratio of the absorbance at 450 nm of test sample to that of the control.

RESULTS AND DISCUSION

Results show that ZOLL was able to agglutinate human erythrocytes of group ‘A’, ‘B’, ‘O’ and rabbit erythrocytes also. The haemagglutination titre was more with ‘B’ group of human erythrocytes as that with rabbit erythrocytes hence rabbit erythrocytes were used for further studies (Table 2). Agglutinating property of ZOLL was inhibited by α-D-galactose suggesting the lectin to be galactose specific (Table 3). The ZOLL could be purified in good yield on cross linked guar-gum by affinity chromatography (Table 1). ZOLL was found to be homogeneous as exhibited a single band on SDS-PAGE, suggesting the lectin to be monomeric and glycoprotein in nature as tested by phenol sulphuric acid method. Similar type of results were also observed with *Vigna mungo* lectins [38].

Estimation of Carbohydrate content: The purified lectin (ZOLL) tested positive with phenol sulphuric acid method indicating ZOLL to be glycoprotein in nature.

SDS Polyacrylamide Gel Electrophoresis: The ZOLL exhibited little low molecular weight of 23 kD, on SDS-PAGE as shown in Figure 1. *Tricosanthus anguina* lectin was also found to have *Mr* of 23 kD in the presence of β- mercaptoethanol [39].

Agglutination inhibition assay: The purified ZOLL was inhibited in the presence of galactose and galactose derivatives (Table 3) indicating the lectin to be galactose specific. Agglutinins of *Vigna mungo* and *Vigna radiate* were also found to be galactose specific [40, 41]. The minimum inhibitory concentration of D-galactose and lactose were 100 mM, for α – pNPGal and β – oNPGal was 3 mM and for raffinose was 250 mM. On the other hand sugars like α – D – glucose, D – mannose, D

– fructose, D – maltose, D – sorbitol, D – ribose, and sucrose and glucosamine derivatives did not inhibit agglutination by ZOLL.

pH Stability: The optimum pH for agglutination activity was found to be pH 7. The activity of ZOLL was lost at pH below 4 and above 12. The activity of lectin was stable between pH 4 to 12 (Figure 2). Similarly *Erytherina velentina* and *Fonna aurantica* lectins were stable at neutral pH with optimum activity pH at 7.5 [41].

***Effect of temperature and thermal inactivation on haemagglutination activity*:** The ZOLL when heated at several temperatures ranging from 37 to 80 ^{o} for 1 h showed 100% activity indicating it to be thermostable. The ZOLL did not lose its agglutination activity when incubated at 37 ^{o} from 20 to 80 m (Figure 3). *Vigna mungo, Erytherina veluntina* and *Fonna aurantica lectins* also showed similar properties of thermostability [38, 42].

Effect of metal ion on agglutination activity: Result presented in figure 4 show the effect of metal ions on agglutination of ZOLL. Demetalized ZOLL was used for agglutination assay. ZOLL was unable to agglutinate erythrocytes in the presence of Hg^{++},Cd^{++} ions (*P<0.05).

α - and β – galactosidase activity: The ZOLL exhibited α and β – galactosidase activities with substrate α-pNGal and β- oNGal, respectively. The ZOLL did not lose enzyme activity in the presence of galactose suggesting it to possess an enzyme as well as an agglutination activity. Likewise, *Vigna mungo* lectin also exhibited similar properties [38]. ZOLL shows both the types of galactosidase activities, wherein α – galactosidase activity was minor than β – galactosidase, thus it can be stated that ZOLL exhibited bifunctional property. Lectin from *mung beans*, [41], and *Vicia faba* seed lectin [43], also exhibited both α and β galactosidase activities. *Black gram* lectin [44], also possessed both α and β galactosidase activities.

Effect of lectin on proliferation of PBL: To estimate the mitogenic activity of ZOLL in PBLs, 1 x 10^{6} cells were added to 96-well micro titer plates, and the cells were then stimulated with 2, 4, 6, 8 and 10 µg/ml of ZOLL. At the end of 72 h incubation, the proliferation indices were 13%, 52%, 89%, 246% and 297% of control, respectively (Figure 5). After simultaneous treatment with Con A (5 µg/ml), the cell proliferation was significantly increased to 311% (*P<0.05). ZOLL significantly enhanced the cell proliferation in normal human PBLs, with highest at 10 µg/ml shows that ZOLL can act as a mitogen for PBL cells. Effect of ZOLL on viability of PBL cells has shown that it has no adverse effect on cell viability when exposed for 72 h.

CONCLUSION

The galactosidase activity and agglutination activity exhibited by ZOLL indicated the lectin to be bifunctional in nature. Studies with respect to pH, temperature stability and cell proliferative nature make the ZOLL to be unique in properties.

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Table (I): Summary of purification procedures for *Z. oenoplia* leaf lectin

Purification step	Volume (ml)	Proteins (mg/ml)	HAU[a] w.r.t. Rabbit erythrocytes	SA[b] w.r.t. Rabbit erythrocytes	Purification fold w.r.t. Rabbit erythrocytes	Yield % w.r.t. Rabbit erythrocytes
Crude extract	100	7.6	5120	673.68	1	100
Ammonium sulphate fraction	27	1.14	1280	1122.8	1.66	67.5
Affinity chromatography fraction	10	0.04	800	20000	29.71	16

a) HAU- Hemagglutination Unit b) SA- Specific Activity

Table (II): Agglutination of human and rabbit erythrocytes by *Z. oenoplia* leaf lectin

Erythrocytes	No. of Sample	Agglutination of erythrocytes Haemagglutination by ZOLL	
		Trypsin treated	Non treated
Human 'O'	100	+	+
Human 'A'	100	+	+
Human 'B'	100	++++	+++
Rabbit	100	++++	++++

Agglutination (+), No agglutination(-)

Table (III): Inhibition of agglutination BY *Z. oenoplia* leaf lectin IN PRESENCE OF various carbohydrates

Sr. No.	Carbohydrates	Minimum concentration required to Inhibit the haemagglutination (mM) by ZOLL
1	D-Arabinose	NI
2	N acetyl – D – glucosamine	NI
3	D-Fructose	NI
4	D-Galactose	100
5	α-D-Glucose	NI
6	Lactose	100
7	D-Maltose	NI
8	D-Mannitol	NI
9	P-nitrophenyl α-D galactopyranoside	3
10	o-nitrophenyl β-D galactopyranoside	3
11	D- Raffinose	250
12	D-Ribose	NI
13	D- Raffinose	250
14	Sucrose	NI
15	D-Sorbitol	NI
16	D-Xylose	NI

NI- No Inhibition

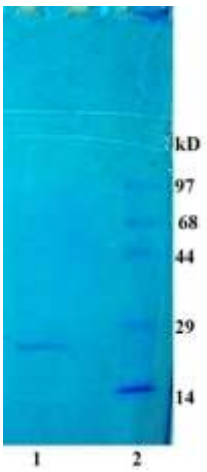


Figure (I:. The SDS-PAGE electrophoretic pattern of *Z. oenoplia* leaf lectin

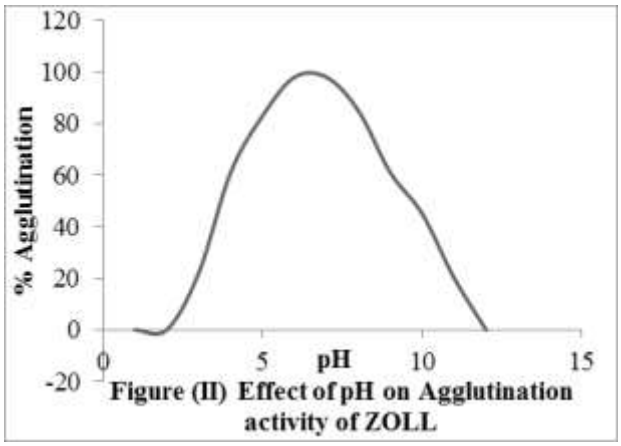
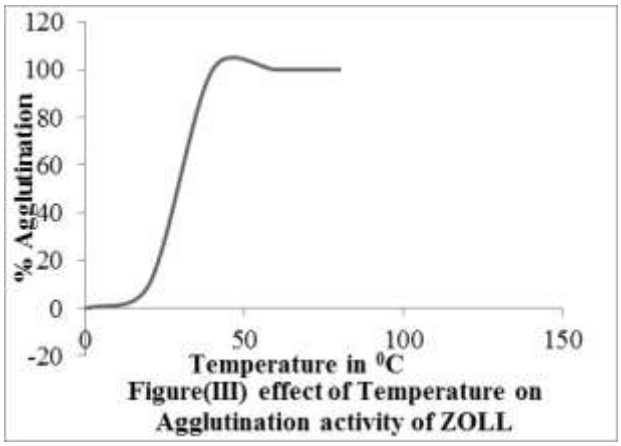


Figure (II) Effect of pH on Agglutination activity of ZOLL



Figure(III) effect of Temperature on Agglutination activity of ZOLL

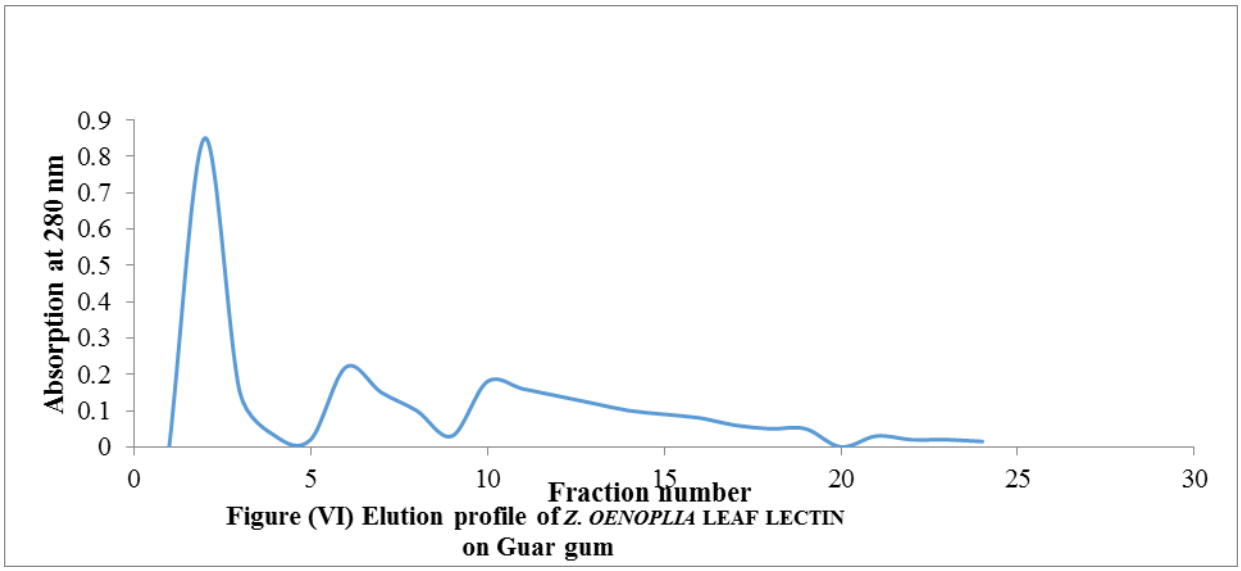


Figure (VI) Elution profile of *Z. OENOPLIA* LEAF LECTIN on Guar gum

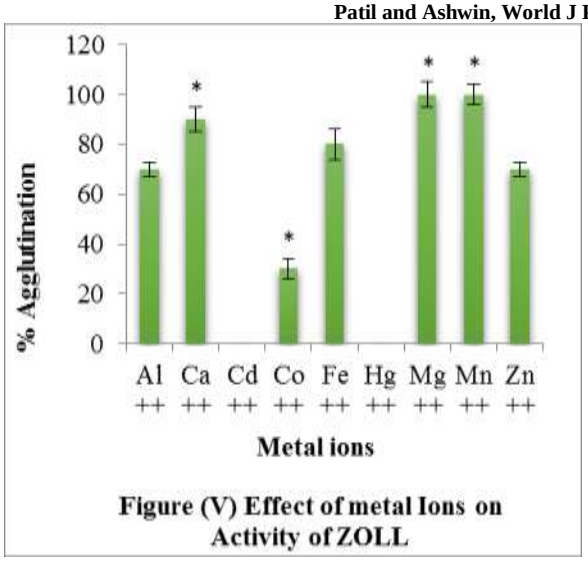


Figure (V) Effect of metal Ions on Activity of ZOLL

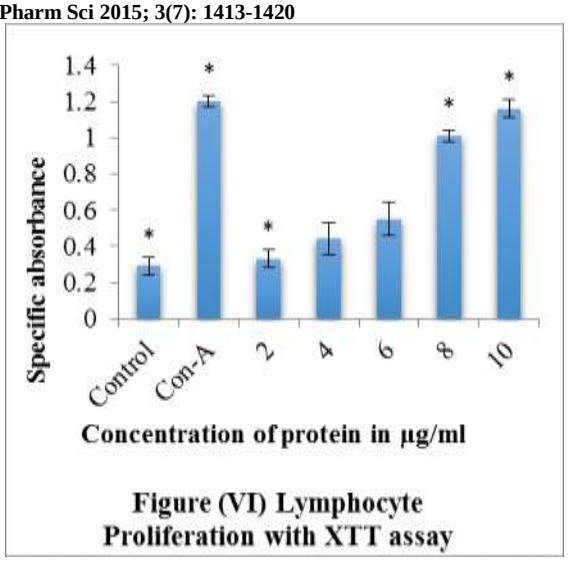


Figure (VI) Lymphocyte Proliferation with XTT assay

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