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ISOLATION AND PURIFICATION OF A LECTIN FROM SEEDS OF A DROUGHT RESISTANT PLANT, *CARAGANA GERARDIANA* AND EVALUATION OF ITS ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES

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ABSTRACT

A galactose-specific lectin was purified from seeds of a drought resistant leguminous plant, *Caragana gerardiana*, by affinity chromatography on lactose–agarose. Protein extract agglutinated human erythrocytes (treated with proteolytic enzyme). Among various carbohydrates tested, the lectin induced agglutination was inhibited by D-galactose. SDS-PAGE showed that the lectin, named CGL produce a single band establishing that the lectin is composed of similar type of subunits. The lectin was reasonably thermostable showing full activity within a temperature range of 20°C to 90°C. pH stability of the lectin falls in the range of 2-9. The antimicrobial activity of the purified lectin was carried out by agar ditch diffusion method at different concentrations using appropriate standards. CGL demonstrated a remarkable antibacterial activity against the pathogenic bacteria *Pseudomonas aeroginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. Antioxidant activity of the purified lectins *were* carried out by DPPH- free radical scavenging activity (DPPH assay) and Ferric (Fe^{3+}) reducing power assay.

KEYWORDS: Lectin, DPPH, *Caragana gerardiana*.

1. INTRODUCTION

Caragana gerardiana is a species of flowering plant in the family Leguminosae. Found at elevation of 3700- 4100 metres in Xizang provience in China, has also been reported from

Pakistan and Kashmir. Caragana gerardiana is a decidious shrub growing to 1 meter. It flowers in may. The flowers are hermaphrodite and are polinated by insects. It can fix nitrogen. *Caragana* is an important genus of drought resistant leguminous shrubs. Sixty species have been recorded in China; *Caragana* can overwinter safely where temperatures go down to - 40^0 C and withstand summer heat above 45^0 C. They are excellent shrubs for sand fixing, erosion control, animal feed, wildlife-cover, fuel and fibre. *Caragana* has been widely planted in watershed management and soil conservation projects in northern China; mainly *C. korshinskii* and *C. microphylla*, are used (Niu and Gao., 2001). Lectins are carbohydrate-binding proteins that interact with specific sugars or glycoconjugates and mediate several biological activities such as cell–cell interactions, fungi and bacteria adhesion to host cells and immune responses, among others (Patra *et al*., 2011; Michele *et al*., 2011). Due to their properties, these proteins have been used in the isolation and structural characterization of glyco-conjugates, blood typing and studies of the architecture of the cellular surface (Diaz *et al*., 2008; Parillo and Supplizi., 2008; Kapoor *et al*.,2008). Because of their effects in processes such as immunosupression, mitogenicity and cytotoxicity, they have been used as tools in immunology, cellular biology and cancer research (Luciano *et al*., 2008). In spite of their wide distribution, the best studied lectins are those obtained from plants, especially from the Leguminosae family. In fact, these proteins have been used for decades as model systems for the study of protein–sugar interactions, because of their wide range of specificities (Luciano *et al*., 2008).

2. Experimental Details

2.1 Material

2.1.1 Collection of plant material: All experiments were carried out with *C.gerardiana* seeds collected from north Kashmir region of India. The collected plant was authenticated from the Department of Botany, ICSC College, Srinagar.

2.2 Methods

2.2.1 Isolation of lectin

Fifty grams of the seed were de-skinned & soaked overnight in 7.2 pH phosphate buffer. It was then homogeneted in phosphate buffer and the homogenate was filtered through four layers of cheese cloth and the filtrate thus obtained was centrifuged at 10,000 rpm for 30 minutes. The supernatant was then collected and ammonium sulphate was added to the sample with constant stiring to a concentrant of 100% saturation and kept overnight at 4°c.

The precipitate thus formed was collected by centrifugation as above, the obtained precipitate was than dissolved in minimum amount of phosphate buffer and then dialysed extensively against three changes of PBS (pH 7.2), and then checked for heamagglutination activity (Gabriel *et al*., 2013). This was labeled as crude extract.

2.2.2 Haemagglutination and haemagglutination inhibition tests

Haemagglutination tests were performed using 3% trypsin-treated erythrocytes of human (Sadanand *et al*., 2013a). Assays of haemagglutination inhibition studies by a range of simple sugars were performed using 4 haemagglutinating units of lectin as described (Sadanand *et al*., 2013b). In each tube, 50 μl of a two-fold serial dilution of simple sugars in buffer was added to an equal volume of lectin solution, which had been carefully diluted to contain four minimum agglutination doses. After 1 h at room temperature, 50 μl of the erythrocyte suspension was added. The mixture was incubated for 1 h at room temperature and then examined for agglutination. Results were expressed as the minimum concentration (millimolar) of simple sugar required to completely inhibit the 4 haemagglutinating units.

2.2.3 Determination of protein concentration

Protein concentration was determined by Lowry method (Lowry *et al*.,1951), using bovine serum albumin as standard. Readings at 280 nm were used to determine protein content of the column elute.

2.2.4 Lectin purification

Crude extract of seeds of *C. gerardiana* was homogenized with 100 mM Tris- Hcl, pH 7.6, containing 0.15 M NaCl (1:20 w/v) for 3 h at room temperature. This crude protein fraction was loaded onto a lactose–agarose affinity column equilibrated and eluted with extraction buffer at a flow rate of 30ml.min^{-1} until the column effluent showed absorbance at 280 nm of less than 0.05. Bound proteins were eluted with 100 mM lactose in equilibration buffer. The elution was monitored at 280 nm and 3 ml fractions were collected manually and tested for haemagglutinating activity on human trypsinized erythrocytes. Active fractions were pooled, dialysed extensively against Tris-Hcl buffer pH 7.6, freeze-dried and stored at 4°C until use.

2.2.5 SDS-PAGE

SDS-PAGE was carried out in a 12.5% gel and run at 30 mA for 5 h (Manpreet *et al* ., 2013). Proteins with known molecular mass were used as markers: bovine serum albumin (66 kDa),

ovalbumin (45 kDa), soybean trypsin inhibitor (20 kDa), bovine gammalactalbumin (14.4 kDa). Protein bands were stained with 0.05% Coomassie brilliant blue R-250.

2.2.6 Effect of Temperature and pH on the Stability of the Lectin

Thermal stability of of lectin isolated from selected leguminous plant (*C. gerardiana*) was monitered in the range of 20-100°C by incubating the lectins for 30 minutes at respective temperatures, followed by cooling on ice and determination of agglutination activity under standard conditions. The pH dependance of the lectins was determined by incubating 50µg of lectin in ech case with buffers in different pH: .1 M glycine/ HCl (pH2-3), .05M sodium acetate/ acetic acid (pH 4-5),.05M potassium phosphate (pH 6-7), .05M tris- HCl (pH 8-9) and .01M glycine/ NaOH (pH 10-12).

2.2.7 Antimicrobial Activity

The purified lectin was screened for their antimicrobial activity by using agar ditch diffusion method (Barry., 1980) by measuring the diameter of the inhibitory zones in mm using different concentration of purified lectin in methanol. The diameters of the zones of inhibitions of the samples were than compared with the diameter of the zone of inhibition produced by the standard antibiotic such as ciprofloxacin (antibacterial). Nutrients agar medium was used for determining antibacterial activity.

2.2.8 Antioxidant activity

2.2.8.1 Assay for *in vitro* DPPH- free radical scavenging activity (DPPH assay)

DPPH free radicle scavenging activity is one of the basic reproducible method for antioxidant screening of numerous compounds. Free radicle scavenging potentalities were tested against the methonolic solution of DPPH. DPPH accepts an electron or hydrogen radicle to become a stable, diamegnetic molecule. It can be oxidised only with difficulty and reversibily. Because of its odd electron, 1,1- diphenyl-2-picryl hydrazyl shows a strong absorption band at 517nm its solution appearing in a deep violet colour. As the electrons becomes paired off, the absorption vanishes and the resulting decolourization is stiochimetric with respect to the electrons taken up. Antioxidant reacts with DPPH and converts into 1,1-diphenyl-2- picryl hydrazine. The purple colour of DPPH changes to yellow (decolorization), indicating the scavenging efficacy of added substance. The change in absorbance at 517nm has been used to measure antioxidant property. The assay was standardized using ascorbic acid as standard. The DPPH assay was performed as described by (Bozin *et al.*, 2001). The reaction mixture 4ml contained, 0.1ml of (50, 100, 150, 200, 400µg/ml) various

concentration of lectin samples in 0.1M Phosphate buffer (pH 7.2), 3.9 ml of DPPH (.025gm/l). solution was addedto all above test tubes. Incubated at room temperature in the dark for 30 minutes. A blank determination with 0.1 ml of methanol solution instead of standard treated similary was maintained. For control 0.1 ml of methanol with 3.9 ml of DPPH solution was used. The optical density was measured at 517 nm using a spectrophotometer.

Scavenging activity of DPPH free radicle in percent was calculated according to equation.

DPPH radical scavenging activity (%) = 1-A/B×100

Where: A= Absorbance of blank(control)

B= Absorbance of test sample.

IC_{50} values, which represented the minimum concentration of extracts required to neutralization 50% of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

2.2.8.2 Ferric (Fe^{3+}) reducing power assay

Ferric reducing power was determined by the method of (Yildirim *et al*.,2000). Fe^{3+} reducing power as an indicator of antioxidant activity is widely accepted. In this method antioxidant compound forms a coloured complex with potassium ferricyanide in the presence of trichloro acetic acid and ferric chloride. Purified lectins were dissolved separately in methanol and solutions of different concentrations (100–400 µg ml^{-1}) were prepared. 1 ml of this solution was mixed with 2.5 ml, 0.2 M phosphate buffer (pH 7.2) and 2.5 ml, 1% potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. After this, 2.5 ml, 10% trichloroacetic acid was added to the reaction mixture and was centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant liquid was mixed with 2.5 ml distilled water and 0.5 ml, 0.1% $FeCl_3$ solution. A blank was prepared with 1ml methanol instead of sample, treated similarly was maintained. The absorbance was measured at 700 nm using UV–visible spectrophotometer. The higher the absorbance of the reaction mixture at 700 nm, the higher is the reducing power of the extract. The intensity or absorbance at this λmax was used as the measurement of the ferric reducing antioxidant activity of these extract.

The relative percentage reducing power of the sample as compared to the maximum absorbance tested which appeared in Ascorbic acid at 10µg/ml was calculated by using the formulla: **(A- Amin)/(Amax-Amin)× 100**

Here,

$\mathbf{A_{max}}$ = absorbance of maximum absorbance tested

$\mathbf{A_{min}}$ = absorbance of minimum absorbance tested and

A = absorbance of a sample

2.3 Statistical Analysis

All the measurements were done in triplicate and statistical analysis was performed by statistical software. All the data were expressed as±S.E.M. Statistical analysis were determined using one way analysis of variance (ANNOVA).

3. RESULTS AND DISCUSSION

3.1 Isolation and Purification

Legume lectins represent the largest and most thoroughly studied family of the simple lectins. The members of this protein family consists of two or four subunits (protomers), either identical or slightly different each with a single small carbohydrate combining site with the same specificity. A lectin was isolated and purified from the seeds of a drought resistant plant *Caragana gerardiana* by salt fractionation followed by affinity chromtography on lactose-agarose. The crude extracts strongly agglutinated native human red blood cells **(Table 1)**. Similar results were found with *I. heterantha* (Qadir *et al*., 2013). However, the crude extracts of *B. purpurea* and *B. vahlii* exhibited low haemagglutinating activity when in contact with human blood of the ABO system (Luciano *et al.*, 2008). The protocol employed to purify the seed lectin from *C. gerardiana* by affinity chromatography on lactose–agarose column (**Figure 1**) was simple and very efficient. Data on the purification of *Caragana gerardiana* lectin is summarized in **Table 2**. The yield of the lectin was about 0.46% and about a 5.2 fold purification of the lectin was achieved.

Table. 1: Specific haemagglutination activity (H.U mg/g) and protein concentration in crude extract of *C. gerardiana.*

Enzyme treated	H.U/mg			P. conc. mg/ ml
	A	B	O	
Trypsin	6.24	9.04	6.24	70.0

Table. 2: Purification profile of *Caragana gerardiana* seed lectin.

Purification step	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg) #	Activity (H.U)*	Total activity@	Specific activity (H.U/mg)	Fold purification$
Crude extract	200	70	14000	125	980000	1.78	1
Affinity chromatography	20	10.5	210	95	2205	9.04	5.07

#Total protein= volume × concentration…….@Total activity= total concentration × total protein.

*One hemagglutinating unit (HU) is defined as the minimum amount of the lectin per ml required to give positive agglutination of 1 ml of a 3% erythrocyte suspension. HU is expressed in microgram of lectin/ml of the protein solution.

$Purification fold: The purification per step can be obtained by dividing the specific activity after that step by specific activity of the material before that step.or dividing specific activity at any stage by the specific activity of the original homogenate.

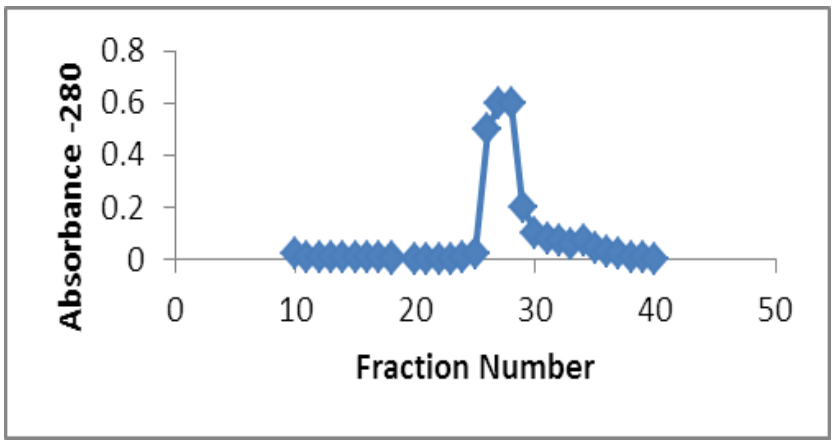

Figure. 1: Purification of the lectin from *C. gerardiana* by affinity chromatography on an agarose–lactose column. The column was equilibrated and washed with Tris-HCl 100 mM pH 7.6 containing 150 mM NaCl to remove unbound proteins. The lectin was recovered with 100 mM lactose in equilibration buffer (Absorbance at 280 nm).

3.2 Electrophoretic Analysis

In 12.5% SDS-PAGE, the lectin moved as a single band, establishing that the lectin is composed of similar type of subunits (**Figure 2**) with molecular weight of approximately 30 KDa.

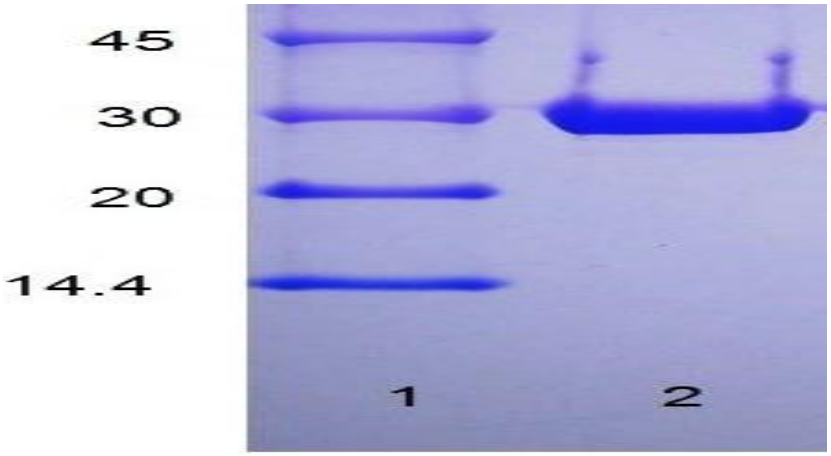

Figure. 2: SDS-PAGE (12.5%). Lane 1, molecular mass standards– ovalbumin (45 kDa), carbonic anhydrase (30 kDa) soybean trypsin inhibitor (20 kDa), bovine gammalactalbumin (14.4 kDa). Lane 2, CGL.

3.3 Haemagglutination assay

The Caragana gerardiana lectin (CGL) does not show any marked blood group specificity (**Table 1**). As is evident, the lectin agglutinated human erythrocytes of all blood groups, being somewhat more specific towards blood group B erythrocytes. Extent of hemagglutination was found to be same, when human erythrocytes of blood group A, B and O were incubated with purified CGL for overnight at 7°C . CGL showed no specificity in its ability to hemagglutinate human (A, B and O) erythrocytes as the lectin from Egyptian *Pisum sativum* seeds and *Erythrina variegata* lectin (Datta and Basu., 1983).

3.4 Carbohydrate Specificity (Inhibition of haemagglutination)

In order to determine the sugar specificity of the lectin, inhibition and reversal of inhibition by a number of sugars and sugar derivatives was studied. In each case the ability of the sugar to inhibit agglutination was measured. Results on such specificity studies are shown in (**Table 3)**. It is clear from the table that D-Gal is the most potent inhibitor of the *Caragana gerardiana* lectin mediated hemagglutination. The carbohydrate specificity was similar to the lectin from *B-monandra* (Penate *et al*.,1988), *Eupharbia tirucalli* (Shanzio *et al*., 2014)

Table. 3: Inhibition of agglutination of human red blood cells induced by *C.gerardiana* lectin by mono or disacharide by using 2% human trypsin treated erythrocytes.

S.No	Sacharides	Minimium Inhibitory Concentration (mM)
1	D. Gal	0.250
2	D. Gal Nac	NI
3	D. Glucose	NI
4	D. Lactose	NI
5	D. Mannose	NI
6	N. Acetylglucoseamine	NI

NI- No inhibition at 100 mM concentration.

3.5 Effect of pH and Temperature

The examination of CGL activity in different pH (pH 2 - 12) values showed that the lectin was stable in the pH range of 2 - 9 (**Figure 3**) indicating that the amino acid residues involved in carbohydrate binding are not affected by changes in pH in this range. The purified lectin sample was found to be heat stable up to 80°C, at incubation temperatures of 20 to 90°C (**Figure 4**). Even heating at 90°C for 30 minutes caused a loss of 50% of its original activity. However the hemagglutinating activity of the lectin was completely lost when exposed to 100°C.

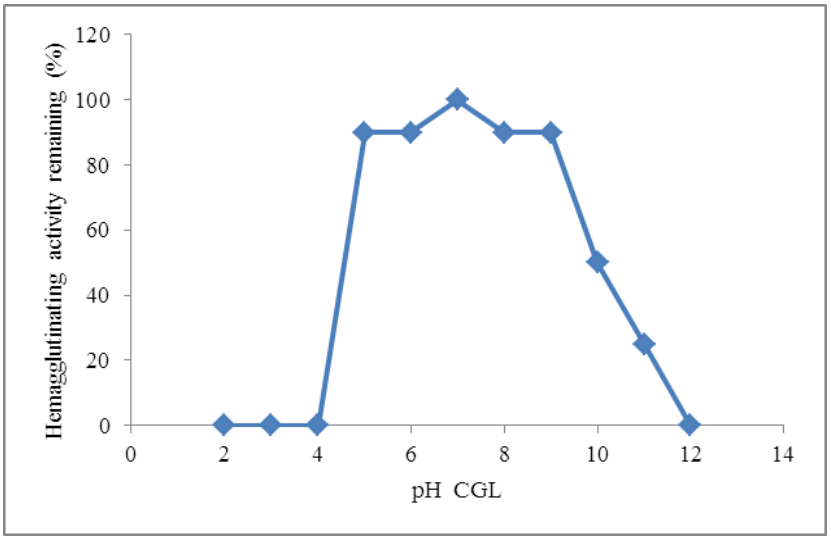

Figure. 3: Effect of pH variations on CGL activity. The pH dependence of the lectin was determined by incubating 50 µg of CGL with buffers in different pH.

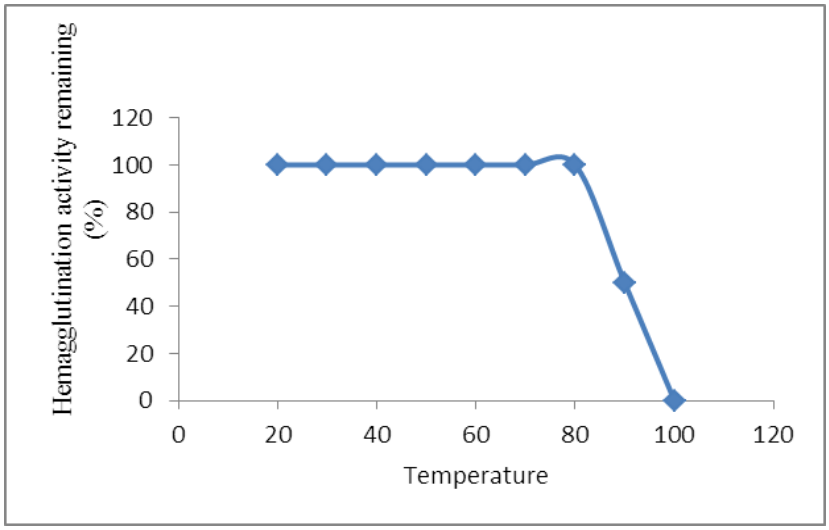

Figure. 4: Effect of temperature on lectin activity. The lectins were incubated for 30 minutes at respective temperatures followed by cooling on ice and determination of activity under standard conditions.

3.6 Antioxidant activity

3.6.1 DPPH scavenging

DPPH assay is one of the most widely used method for screening antioxidant activity of plant extracts. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radicle to become stable diamegnetic molecule. Tha reduction capability of DPPH radicle was determined by decrease in its absorbance at 517 nm, which is induced by different antioxidants. The decrease in absorbance of DPPH radicle caused by antioxidants because of the reaction between antioxidant molecule and radical progress which results in the scavenging of the radical by hydrogen donation.It is visually noticeable as a change in colour from purple to yellow.

Table. 5: % DPPH Radical Scavenging Activity of lectin extracts isolated and purified from a drought resistant leguminous plants (*Caragana gerardiana*) and standard (Ascorbic acid).

Conc. µg/ml	50	100	150	200	400	IC_{50}
Caragana Gerardiana lectin	41.37± 0.1	46.15± 0.1	47.52± 0.1	50.0± 0.1	51.0± 0.1	72.96
Ascorbic acid	51.0 ± 0.2	63.6± 0.2	70.2± 0.2	78.9± 0.2	93.8± 0.2	17.86

The lectin extracts of selected leguminous plant (*Caragana gerardiana*) showed a promising free radicle scavenging effect of DPPH in a concentration dependent manner (**Table 5**) and compared with ascorbic acid. The highest percentage of inhibition values of ascorbic acid is

93.8% with IC_{50} value of 17.86 µg/ml while as *Caragana gerardiana* lectin shows highest percentage of inhibition values of 51.0% with IC_{50} value of 72.96 . Lower the IC_{50} value greater the antioxidant activity. The significant DPPH scavenging potential of selected plant lectin extract may be due to hydroxyl group present in the lectin.

3.6.2 Ferric ion reducing assay

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. In this assay the ability of extracts to reduce Fe^{3+} to Fe $^{2+}$ is determined. The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to form ferrous cyanide form(Fe^{2+}). In the reducing power assay, antioxidants cause the reduction of the Fe^{3+} to Fe^{2+} thereby changing the solution into various shades from green to blue, depending on the reducing power of the compound. Strong reducing agents formed Perls Prussian blue colour heaving a sharp absorbance at 700 nm. The relative reducing percentage of lectin isolated from selected plant in comparison with ascorbic acid as standard is depicted in **Table 6.**

Table. 6: Relative ferric reducing power assay of lectins from a drought resistant leguminous plants (*Caragana gerardiana*) and standard (Ascorbic acid).

Conc. µg/ml	100	200	300	400	IC_{50}
Caragana Gerardiana lectin	11.97±0.01	24.64±0.01	74.64±0.01	83.80±0.01	248.79
Ascorbic acid	15.49±0.01	28.87±0.01	85.91±0.01	85.91±0.01	230.31

Value represents in the results are mean ± SD of three replicates.

Relative reducing percentage of ferric (Fe^{3+}) reducing power assay showed reducing power percentage of lectin from selected leguminous plant(*Caragana gerardiana* lectin) and positive control were in concentration related and increase with the increase of sample concentration on the range of (100 to 400 µg/ml). Their relative reducing power on Fe^{3+} were as follows ascorbic acid (92.95), (83.80) in *Caraana gerardiana* lectin at (400 µg/ml). Similarly in the glycoprotein extracts from *C.borg, C. amenda, C. casne* has abundent ferric reducing power in the concentration dependent manner (Angel *et al*., 2013; Ebrahimzadeh *et al*., 2010). The IC_{50} values was calculated and listed in the (**Table 6**).

4. CONCLUSION

Caragana gerardiana is an drought resistant leguminous plant. Before this study no bioactive component has been isolated from it. CGL represents the first isolated proteinaceous

constituent of the plant and being active against several pathogenic microorganisms could be used as an antimicrobial agent for animal and plant infections. The results of the present study have also shown that CGL *in vitro* possess significant antioxidant property. It has also has a significant haemagglutination activity. In future the protein isolate could be used for blood typing, bacterial typing and may have the potential to play role as biotechnological tools. Hence, further work can be continued for exploring its medicinal value as well as its other biomedical uses.

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