# Purification and partial characterization of a Fructose-binding lectin from the leaves of *Euphorbia helioscopia*

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**Abstract**: A lectin was purified from leaves of *Euphorbia helioscopia*, by a combination of ion-exchange and gel filtration chromatography. On ion exchange using a DEAE- cellulose column in 0.2 M phosphate buffer, pH 7.2, the bound protein was eluted with a linear sodium chloride gradient of 0.1 M to 0.5 M. Further purification of the lectin was achieved by gel filtration on Sephadex G-100. *Euphorbia helioscopia* lectin (EHL) agglutinates only chick erythrocytes, showing no agglutination of all human blood group erythrocytes. The EHL induced hemagglutination is inhibited by fructose. The purified protein showed one band, both in non-denaturing PAGE and SDS-PAGE establishing the charge and size homogeneities of the lectin preparation. The molecular mass of the lectin as indicated by SDS-PAGE was approximately 31 kDa and that estimated from G-100 gel filtration chromatography was about 65 kDa establishing that the lectin is a homodimer. The lectin was stable within a temperature range of 0°C-40°C and exhibited a narrow range of pH stability, being optimally active at around pH 7. EHL also possesses antimicrobial activity and is an inhibitor of bacterial growth particularly *Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Escherichia coli.*

**Keywords**: *Euphorbia helioscopia*, hemagglutination, lectin, antimicrobial.

## INTRODUCTION

Lectins are carbohydrate binding glycoproteins of non-immune origin capable of specific recognition of and reversible binding to carbohydrates (Peumans and Van Damme 1998). Lectins are found in wide range of organisms like viruses, bacteria, fungi, plants and animals (Lanno and Van Damme 2010). It has been established in mouse models that blocking lectins present in microbes, which help in recognition, can prevent infection. However, success of the treatment in humans has not been achieved yet (Sharon 2006).

Lectins from both plant and animal origin are being evaluated for potential antimicrobial activity. A novel galactoside binding lectin from *Bothrops leucurus* snake venom was purified and exhibited antibacterial effect against the pathogenic gram positive bacteria *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis* (Nunes *et al*., 2011). *Archidendron jiringa* seed lectin showed inhibitory activity against *Bacillus subtilis* and *Staphylococcus aureus* (Charungchitrak *et al*., 2011). Lately with the emerging problem of multiple drug resistance, research in characterization of newer lectins to combat infections is gaining momentum.

*Euphorbia helioscopia* belongs to Euphorbiaceae family. It is cosmopolitan in nature, and has great medicinal importance. It has been traditionally used for the prevention, improvement or cure of various diseases like ascites, edema and pulmonary tuberculosis (Feng *et al.*, 2009, Feng *et al*., 2010). The roots are used as anthelminthic and seeds mixed with roasted pepper have been used in the treatment of cholera (Uzair *et al.,* 2009). However, *Euphorbia helioscopia* has not been investigated for lectins till date. In this communication we have reported the purification and biological characterization of new lectin from the leaves of this plant. Antibacterial activity of EHL is also reported here.

## MATERIAL AND METHODS

### *Plant material*

The plant (*Euphorbia helioscopia*) was collected from the premises of the Department of Botany, University of Kashmir, India and authenticated from the Centre of Plant Taxonomy of the same department. DEAE- cellulose and Sephadex G-100 were purchased from Sigma Aldrich Company, USA. Other chemicals were of highest purity grade.

### *Isolation and purification*

The lectin was isolated by a slight variation of the method devised by (Kuku *et al*., 2003), Twenty grams of freshly cut leaves were taken, washed and homogenised in a Remi auto mix blender for 10 minutes in 0.2M NaCl containing 1g/L of ascorbic acid at pH 7.The homogenate so obtained was filtered through 4 layers of cheese cloth. The filtrate was then centrifuged on Remi C24 cooling centrifuge at 3000g for 10 min. The pellet was discarded and the supernatant retained to which $CaCl_2$ (20mM) was added and the pH was adjusted to 9. The supernatant was kept in refrigerator overnight and then centrifuged next

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day at 3000g for 10 min. The pellet was again discarded, the supernatant retained and its pH adjusted to 7 and kept in ice overnight. The filtrate was next centrifuged at 9000g for 20 min and the supernatant filtered through Whattman 3mm filter paper. The filtrate so obtained was dialysed against 0.2M phosphate buffer, pH 7.2, concentrated by evaporation using dialysis tubing and checked for the hemagglutination activity. This was labeled as crude extract and stored at 4ºC.

***Ion- exchange chromatography***

To purify the lectin, the crude extract was subjected to ion-exchange chromatography on DEAE- cellulose column in 0.2M phosphate buffer, pH 7.2. The adsorbed lectin was eluted using linear sodium chloride gradient from 0.1 to 0.5M.

***Gel- exclusion chromatography***

The size homogeneity was established by pooling the active fractions eluted from the ion-exchange column and subjecting these to chromatography on Sephadex G-100 column in 0.2M phosphate buffer, pH 7.2. The standard markers employed were: cytochrome c (12.4kDa), carbonic anhydrase (29kDa) and bovine albumin (66kDa).

***Protein estimation***

Protein concentration was determined by the method of Lowry *et al.* 1951, using BSA as the standard protein.

***Native Polyacrylamide gel electrophoresis (PAGE)***

Polyacrylamide gel electrophoresis in presence and absence of SDS was performed at pH 8.3 on 10% gel with a discontinuous buffer system. The gels were stained with Coomassie Brilliant Blue R-250.

***Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)***

The subunit molecular mass of the lectin was estimated by discontinuous SDS-PAGE according to method of (Laemmli, 1970).

***Hemagglutination assay and inhibition of hemagglutination***

The hemagglutination activity of the lectin was determined by a slight variation of the method devised by Peumans *et al*., 2000, using human erythrocytes bearing blood groups A, B, O, AB and chick erythrocytes. Human blood was obtained courtesy Hematology department, SKIMS, Srinagar and chick blood was procured from a local veterinary outlet. The erythrocytes were collected by centrifugation of the blood at 2500g (5000 rpm) for 10 min, and washed thrice with normal saline. Finally the erythrocytes were suspended in normal saline to obtain a final concentration of 3% erythrocyte suspension.

Assay of agglutination was carried out on microtitre slides by mixing the erythrocytes with the test solution. Fifty microlitres each of erythrocyte suspensions (A, B, O, AB and Chick erythrocytes) were taken on a slide. To this, 50µl of the test solution was added. After an incubation period of 15 min at room temperature, agglutination was monitored unaided on the slides. Also the control slide, using the buffer instead of the lectin solution was run simultaneously. The lectin activity has been expressed as H.U (hemagglutinating Unit). One hemagglutinating unit (H.U) 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 as µg of lectin per ml of the protein solution.

The carbohydrate specificity was investigated by observing the inhibition of the lectin induced hem-agglutination by various sugars namely D-glucose, D-galactose, D-mannose, D-fructose, lactose, maltose, sucrose, D-ribose and sugar derivatives like N-acetyl galactosamine and N-acetyl glucosamine. The inhibition assay was performed on microtitre slides. Different dilutions of the above sugars (final volume 20µl) were added to microtitre slides on which agglutination was performed. To each dilution, 20µl of purified lectin was added. The mixture was incubated at room temperature for 1 hr after which 80µl of 3% suspension of erythrocytes was added to each microtitre slide. The minimum concentrations of each sugar capable of fully inhibiting agglutination after 1 hr at room temperature were noted.

***Effect of pH***

The pH dependence of the lectin was determined by incubating 50 µg of EHL with buffers in different pH: 0.1 M glycine/HCl (pH 2-3), 0.05 sodium acetate/acetic acid (pH 4-5), 0.05 M potassium phosphate (pH 6-7), 0.05 M Tris-HCl (pH 8-9) and 0.1 M glycine-NaOH (pH 10-11) for 5 hrs at 25ºC and in each case pH was adjusted to 7.2 just prior to hemagglutination assay.

**Table 1**: Summary of Purification Steps for *Euphorbia helioscopa* leaf lection

| Purification step | Volume (ml) | Protein concentration (mg/ml) | Total protein (mg) | Activity (H.U)* | Total activity | Specific activity (H.U/mg) | Fold purification | Yield % | Activity Yield % |
|---|---|---|---|---|---|---|---|---|---|
| Crude extract | 300 | 490 | 147000 | 100 | 30000 | 0.204 | 1 | 100 | 100 |
| Ion exchange chromatography | 20 | 97 | 1940 | 30 | 600 | 0.309 | 1.514 | 1.319 | 2 |
| Sephadex gel chromatography | 5 | 6.97 | 20.91 | 15 | 75 | 3.58 | 17.54 | 0.014 | 0.25 |

*One Hemagglutination Unit (H.U) is defined as the minimum amount of the protein required to give positive agglutination of a 3% suspension of Chick erythrocytes. H.U is expressed in µg of lectin/ml of erythrocyte suspension.

***Thermal stability***

Thermal stability of EHL was monitored in the range of 10-100ºC by incubating the lectin for 60 min at the respective temperatures, followed by cooling on ice and determination of agglutination activity under standard conditions.

***Evaluation of antimicrobial activity***

For the evaluation of antibacterial activity of *Euphorbia helioscopia* lectin, firstly all the glassware was sterilized in an autoclave. After sterilization, the subsequent steps were the preparation of media, selection of the test organisms and sensitivity tests of antibacterial activity.

***Purified EHL was used for analysis. Ceftrixone was used as standard.***

Three pathogenic bacterial strains were tested against purified *Euphorbia helioscopia* lectin. Certified pure culture strains of bacteria viz. *Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa*, were obtained from the Microbiology Lab, SKIMS and stored according to the prescribed instructions. Whenever required these bacterial strains were used for determination of antibacterial activity.

The Agar well diffusion method was used to determine antibacterial activity of the lectin preparation. This test was done according to method of Perez *et al*. 1990. The bacterial colony was picked by the inoculating loop and was passed into the normal saline tube. The turbidity of this tube was compared with the McFarland`s opacity tube, and was diluted or concentrated accordingly. The media plates were subsequently inoculated with specific bacterial strains and labeled accordingly. The inoculated plates were left to dry for at least 5-10 minutes, after which a well was formed on plates using sterile borer which were loaded by samples and the antibiotic ceftrixone. Volume accommodated in one well was 100µl. Loaded plates were kept as such for some time under the laminar hood. The plates were then incubated at $37^0$C for 24 hrs in an incubator. Zone diameters (mm) around each of the discs were measured to the nearest mm and recorded.

## RESULTS

***Isolation and purification***

The isolated and purification of the lectin from crude extract of the *Euphorbia helioscopia* leaves was obtained by a two-step procedure. This involved an initial ion-exchange chromatography of the crude extract on DEAE-cellulose, using a linear 0.1-0.5M NaCl gradient, the bound protein eluted in two peaks (I and II). The chromatographic profile is shown in fig. 1. The fractions under peak II showed lectin activity and were then chromatographer onto a gel filtration column. The single peak that was obtained from the gel filtration column contained all the hemagglutinating activity, establishing the size homogeneity of the purified lectin. The chromatographic profile is shown in fig. 2 and table 1 summarizes the overall purification scheme for EHL.

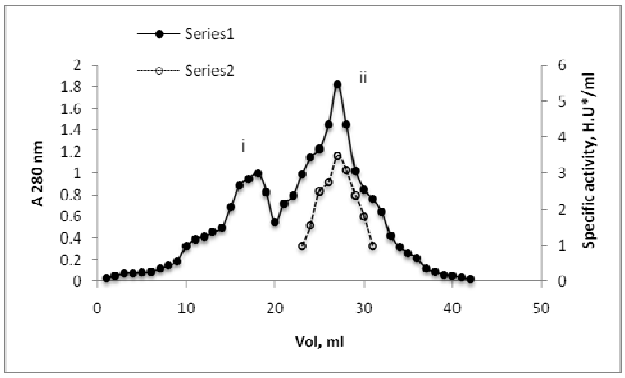


**Fig. 1**: Elution profile of the lectin extracted from leaves of *Euphorbia helioscopia* on DEAE cellulose column. Series 1 represents absorbance at 280nm and series 2 represents the hemagglutinating activity About 65 milligram of protein was applied on the column (20×2cm) equilibrated with 0.2M phosphate buffer pH 7.2. The protein was eluted using a linear sodium chloride gradient of 0.1 to 0.5M in 5ml fractions at a flow rate of 30ml/hr. Peak I had no lectin activity while peak II showed agglutination against chick erythrocytes.

*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 as µg of lectin per ml of the protein solution

***Electrophoretic analysis***

The purified Lectin preparation was homogeneous with respect to charge as revealed by the single band when subjected to polyacrylamide gel electrophoresis (fig. 3).

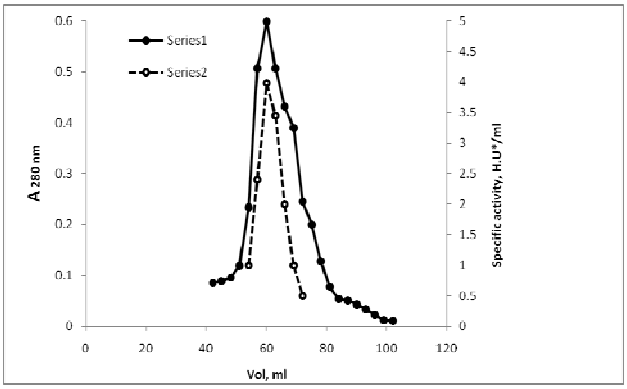


**Fig. 2**: Elution profile of peak II obtained from DEAE cellulose column on Sephadex G-100. Series 1 represents absorbance at 280nm and series 2 represents hem-agglutinating activity. About thirty milligram of protein was applied on the column (20×2cm) equilibrated with 0.2M phosphate buffer pH 7.2. The protein was eluted at a rate of 30ml/hr in 3ml fractions. The void volume of column was 39 ml.

*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 as µg of lectin per ml of the protein solution.

***Characterization***

*Molecular weight determination*

The molecular weight of lectin as determined by gel filtration chromatography was 31kDa (fig. 2). Based on SDS polyacrylamide gel electrophoresis data, the molecular weight was found to be 65kDa (fig. 4). The results of SDS-PAGE together with gel filtration data revealed that EHL (*Euphorbia helioscopia* lectin) is a homodimer.

***Biological characterization***

*Hemagglutination*

The hemagglutinating activity of *Euphorbia helioscopia* lectin (EHL) is specie specific. EHL agglutinates only chick erythrocytes at a minimal concentration of 15µg/ml, while exhibiting no agglutination towards any human blood group (A, AB, B and O) erythrocytes. The agglutination profile of the purified EHL is shown in table 2.

***Carbohydrate specificity***

Among the variety of sugars tested, Fructose at a concentration of 10mM was able to inhibit 15 hem-agglutinating units of the lectin, while other sugars could not inhibit the hemagglutinating activity even at a concentration of 200mM. Results on such specificity studies are shown in table 3.

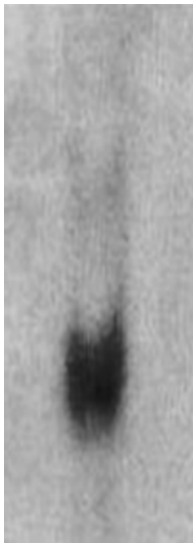

**Fig. 3**: Polyacrylamide Gel Electrophoresis (PAGE) pattern of *Euphorbia helioscopia* lectin (EHL) under native conditions. About 40µg of the ion exchange purified EHL was applied on 8% polyacrylamide gel. Tris-glycine buffer pH 8.3 was used. Current was 8mA per well. The staining reagent used was Coomassie brilliant blue G-250.

***Thermal stability of the lectin***

The lectin activity was stable up to a temperature of 40ºC. Thereafter the lectin showed a complete absence of activity (fig. 5).

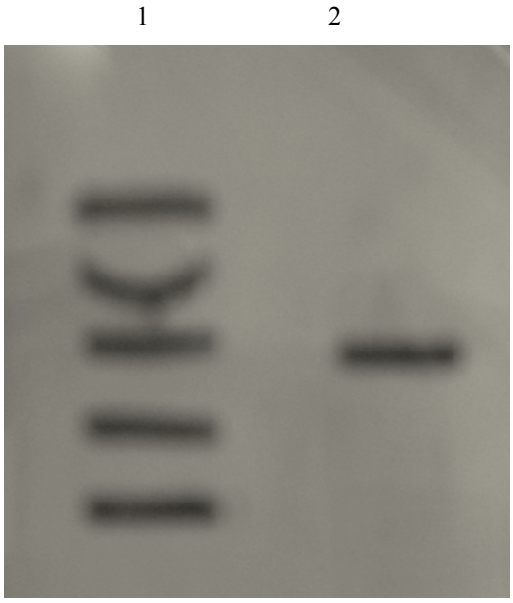


**Fig. 4**: SDS-Polyacrylamide Gel Electrophoresis pattern of *Euphorbia helioscopia* lectin (EHL). About 40µg of EHL was electrophorised on 8% polyacrylamide gel in presence of 0.1% SDS. Tris-glycine buffer pH 8.3 was used. Current was 8mA per well. The staining reagent used was Coomassie brilliant blue G-250.The standard molecular mass markers from top to bottom are: Bovine serum albumin (67kDa), Ovalbumin (45kDa), Carbonic anhydrase (30kDa), Trypsin inhibitor (20.1), α-lactalbumin (14.4kDa). Lane 1 represents Standard markers; lane 2 represents EHL

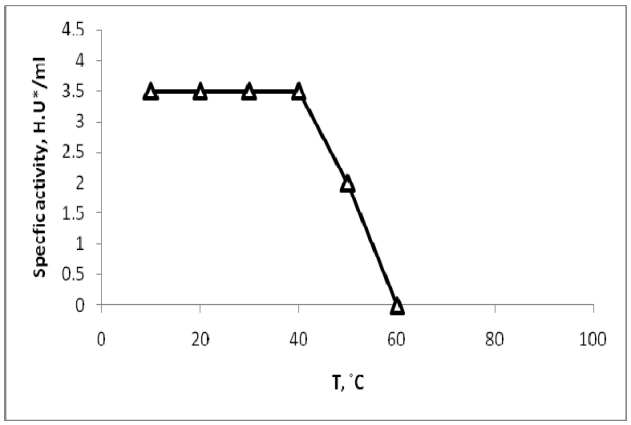


**Fig. 5**: Effect of Temperature on EHL activity. The lectin was incubated for 60 min at the respective temperatures, followed by cooling on ice and determination of activity under standard conditions.

*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 as µg of lectin per ml of the protein solution.

***Effect of pH on lectin activity***

The lectin is stable within a narrow pH range of 6-8 but shows optimum activity at pH 7 (fig. 6).

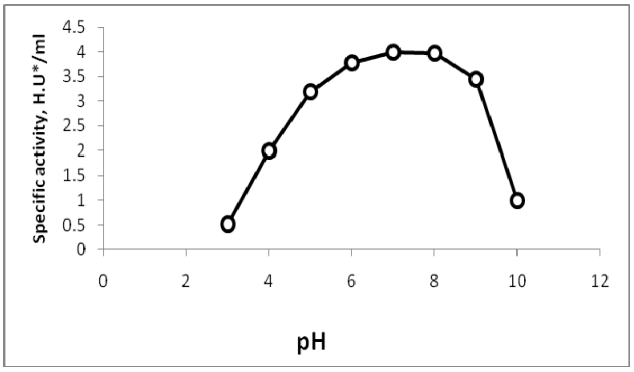


**Fig. 6**: Effect of pH variations on EHL activity.
*****One hemagglutinating unit (HU) is defined as the minimum amount of the lectin per ml required to give positive agglutination of 1 ml of a 3% erythrocyte suspension. HU is expressed as µg of lectin per ml of the protein solution.

**Table 2**: Agglutination profile of purified *Euphorbia helioscopia*

| Blood Group | | Specific activity HU*/mg |
|---|---|---|
| Human Blood Group | O | NH[#] |
| | A | NH |
| | B | NH |
| | AB | NH |
| Chick erythrocytes | | 3.58 |

*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 as µg of lectin per ml of the protein solution.

[#]No hemagglutination

***Antimicrobial activity***

The lectin was tested against three different bacterial strains and compared to that of antibacterial antibiotic, ceftrixone. The results of the sensitivity test are presented in table 4. Purified EHL (15 µg/disc) exhibited a strong antibacterial effect on *Klebsiella pnuemoniae, Escherichia coli* and *Pseudomonas aeruginosa.* The diameter of zone inhibition by the addition of EHL being significantly effective to be 16mm, 12mm and 09 mm respectively (table 4).

## DISCUSSION

A lectin was purified from leaves of *Euphorbia helioscopia*. The *Euphorbia helioscopia* lectin agglutinates only chick erythrocytes and shows no activity towards any human blood group erythrocytes. This is inconsistent with all other reported lectins of the Euphorbiaceae family. Almost all of the lectins from Euphorbaceae family agglutinate human blood group erythrocytes non-specifically, (Rojas *et al*., 2001, Souza *et al.*, 2005). Lectins can be classified according to their carbohydrate binding specificity, such as galactose binding, glucose binding, mannose binding, etc. EHL is a fructose specific lectin, which is in accordance with a lectin isolated from *Musa accuminata* (Allen *et al*., 2009).

**Table 3**: Carbohydrate Inhibition of *Euphorbia helioscopia* Lectin induced Hemagglutination

| Sugar | Minimum Concentration (mM)* |
|---|---|
| D-Glucose | NI[**] |
| D-galactose | NI |
| Mannose | NI |
| Fructose | 10 |
| Lactose | NI |
| N-acetyl galactosamine | NI |

*Minimal sugar concentration necessary for complete inhibition of agglutination of a 3% chick erythrocyte suspension by *Euphorbia helioscopia* lectin.
**NI-no inhibition of hemagglutinating activity of the crude extract even at 200mM concentration.

**Table 4**: Anti-bacterial activities of *Euphorbia helioscopia* lectin

| S. No | Strains of microorganisms | Diameter of zone of inhibition (mm) | |
|---|---|---|---|
| | | EHL (15µg/disc) (Peak II) | Standard ceftrixone (5µg/disc). |
| 1 | *Pseudomonas aeruginosa* | 16 | 21 |
| 2 | *Klebsiella pnuemoniae* | 12 | 20 |
| 3 | *Escherichia coli* | 09 | 20 |

The gel filtration chromatography gave a single peak which corresponded to a molecular weight of 65 kDa. The results are comparable to other reported lectins (Pereira *et al.*, 2012, Chan *et al*., 2012). EHL moved as a single band of 31 kDa on SDS- PAGE under reducing conditions. Based on the calibration curve of the gel filtration column, EHL has a molecular size of around 65kDa.This showed that the lectin is a dimer with a subunit molecular weight of about 31 kDa. Almost all the plant lectins with a few exceptions are multimeric proteins. The EHL was stable up to 40ºC after which the lectin activity dropped with a complete loss of the hemagglutination activity at 60ºC. These results are in agreement with those obtained from *Synadenium carinatum* lectin and *Arachis hypogaea* lectin (Souza *et al*., 2005, Jie *et al*., 2011). The pH activity profile of the lectin shows that EHL induced hem-agglutination is markedly affected by variation of the pH and its maximum activity is seen at pH 7. The activity falls off rapidly thereafter, with essentially all activity lost

after pH 10. This indicates that the saccharide binding site on the lectin is reasonably sensitive to the conformational changes induced on the lectin molecule by pH changes. EHL showed a significant antibacterial activity against different bacterial strains namely *Klebsiella pnuemoniae*, *Escherichia coli* and *Pseudomonas aeruginosa.* A number of lectins have been reported to possess antibacterial activity (Gomes *et al*., 2012, Sindh *et al*., 2013).

In conclusion, a lectin was purified from *Euphorbia helioscopia*, which agglutinates only chick erythrocytes and thus is different from other lectins belonging to this family. EHL shows a remarkable antibacterial activity that can be of great importance for clinical microbiology and possible therapeutic applications.

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