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The immune modulatory activity of lectin extracted from the Algerian *Castanea crenata*

Imen Torche*[1], Youcef Necib, Ahlem Bahi, Sana Boufker

[1]*Department of Biochemistry & Molecular and Cellular Biology, Faculty of Natural and Life Sciences, University Mentouri Brothers Constantine, Algeria*

Laboratory of Microbiological Engineering and Applications, Faculty of Natural and Life Sciences, University Mentouri Brothers Constantine, Algeria

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Abstract

A lectin has been purified from the Algerian chestnut seeds (*Castanea crenata*), one of the natural biosourced plants in Algeria. This lectin extracted from the inner shell of the seeds exhibited a high hemagglutinating activity toward rabbit and human erythrocytes, and remained stable under a wide range of pH levels 2-12 and temperatures up to 120 °C. The agglutinating activity was inhibited by glucose, N-acétyl-glusamine, Saccharine sodique but more by mannose also by BSA, fetuin, Ovalbumin, mucin. The extracted lectin from *Castanea crenata* found to be non-metal binding lectin when tested against several metals. In the last part of this study, The immunomodulatory potential effect of *Castanea crenata'* s inner shell lectin on the phagocytic activity was measured by the carbon clearance rate test, at different doses (30, 50, and100mg/kg), where the administration of the extracted lectin intravenously in mice increases significantly the phagocytic index K comparing to control, especially with high doses, which enhances the clearance rate of carbon in blood indicating the stimulation of the reticulo-endothelial system. Thus, the present study reveals that *Castanea crenata*'s inner shell lectin holds promise as immunomodulatory agent, which acts by stimulating dose dependent phagocytic function.

* **Corresponding Author:** Imen Torche ✉ torche.imen@gmail.com

Introduction

Lectins are a highly spread group of sugar-binding proteins, being present in all types of organisms including animals, plants, bacteria, fungi and even viruses Lis and Sharon (1980). Lectins considered being proteins or glycoproteins of non-immune origin, which bind reversibly to carbohydrates and usually agglutinate cells or precipitate polysaccharides and glycoconjugates (Goldstein, 1980). According to Peumans and Van Damme (1995) lectins possess at least one non-catalytic domain, which binds reversibly to a specific mono or oligosaccharide. However lectins cannot have enzymatic activity (Cummings, 1997).

These bioactive molecules play many roles such as defense, growth, regulation of membrane glycoprotein synthesis, recognition and intercellular communication,due to their protein-carbohydrate interactions, and the ability to specifically recognize glyco-conjugates carried on cell membranes Klafke *et al.* (2013); Kamiya *et al.* (2012), these properties allowed lectins to become a useful tool in several fields of biological research (cell biology, membrane structure, cancer research, genetic engineering and immunology). The Immunomodulation means any immune response changes, including the induction, expression, amplification, and even the inhibition of any part of the response. Especially that in certain cases the stimulation of immune response is required, whereas the suppression of the immune response in other conditions is needed Saroj *et al.* (2012), most immunomodulatory drugs have oftenely a slite side effects, including a less potential for creating microbial resistance diseases. Novel immunomodulating agents are used for the treatment of various conditions, such as infections, organ transplantation, cancer, rheumatoid arthritis etc. Masihi (2001). Thus, the screening for better immunomodulating agents and evaluating their immunomodulatory potential increases and become an important field of research.

Plant resources providing and enhancing the health care system by providing new natural bioactive substances such as lectins. The reason why, many plant lectins have been purified in the last century, and their bioactivities and sugar-binding specificities have been characterized. Liu *et al.* (2010).

In the present work we describe the extraction of a lectin from *Castanea crenata* collected from Skikda east of Algeria and investigated the immunomodulatory effect by using phagocytic activity by carbon clearance test in vivo experimental model mice.

Materials and methods

The lectins used in this study were extracted from the Algerian Castanea crenata's seeds

Human blood group erythrocytes A, B and O were collected from healthy donors, and rabbit red blood cells was obtained by venous puncture of a healthy animals.

Preparation of Extracts

The seeds of *Castanea crenata* used in this study were collected and truthfully cleaned, then their inside shell was dried and finely grounded using a high speed blender. The obtained fine powder was soaked in a phosphate buffer (0.1M, pH 7.3) and incubated for 24 h at 4°C. The sample was collected after passing the previous mixture in centrifugation at 9000 rpm for 30 min, and removing the remaining debris by filtering the supernatant through filter paper Hamshou *et al.* (2010). The partial purification of the proteins was performed by successive precipitation in ammonium sulphate under 90% saturation. Suspension was centrifuged at 11000 rpm/ min for 20 min at 4 °C, then the precipitate was dissolved in phosphate buffer (0.1M, pH 7.3) at the ratio of 1:10 (w/v) and centrifuged at 9000 rpm/ min for 15 min at 4 °C. The supernatant was filtered through syringe filter 0.22µm then dialysed in dialyse tube (12kD-14 kD) toward phosphate buffer (0.1M, pH 7.3) at 4°C. The crud extract was partially purified by gel filtration chromatography using dextran G-75, the fractions containing lectins were tested against rabbit erythrocytes, then freez-dried. The lyophilized partial purified extract was dissolved in 0. 9% NaCl and injected intravenously into mice at doses of : 30, 50 and 100mg/Kg body weight for evaluating of phagocytic activity.

Preparation of Sephadex G-75

4g of sephadex G-75 were soaked in 100mL of phosphate buffer (0.1M, PH: 7.3). The suspended gel was then incubated for 48h at room temperature. Finally it was packed into a 30 x1cm column to use for the extracted *Castanea crenata* lectin**.**

Extraction of lectin from Castanea crenata by sephadex G75

The partial Purification of lectin, was carried out using gel filtration chromatography. The sample was loaded to (10 x 1.2cm) column of sephadex G75.then it was eluted from the column by using a phosphate buffer (0.1M, PH7.3), fractions of 5ml were collected in each tube, where the absorbance was measured at 280 nm in 1cm path length cell using spectrophotometer UV to estimate proteins concentration, where the fraction containing lectin was freez-dried and stored at –20°C**.**

Hemagglutination Assay

In the aim of estimating the Agglutination activity, a serial of two fold dilutions of lectins was applied in 96-well microtiter U-shaped plate. Each well of the micro-titer plate contained 50µl of extracted lectins and 50µl of rabbit red blood cells (3%), the results were obtained after one hour of incubation at room temperature. according to Correia and Coelho (1995). The hemagglutination titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination (Pando *et al*., 2004).

Blood group specificity

The blood group specificity of the lectin was established using human erythrocytes (A^+, B^+, AB^+ and O^{+ve}) by using the same method as before.

Inhibition Tests

The agglutination activity was tested against several glycoproteins, sugars and metal ions. Each solution containing 400mm of each substance in PBS was submitted to serial two-fold dilution in micro -titer plates, then mixed 1:1 volume with the extracted lectin, after incubating the mixture for 30 min at room temperature, rabbit red blood cells (3%) were added, the results were read after one hour, where the hemagglutination inhibition activity was estimated as the highest substance dilution which inhibited the agglutinating activity.

Heat Stability Test

The heat stability of the agglutinating activity of lectin was determined by measuring the remaining agglutinating activity. After incubating aliquots of the lectin extract solution at different temperatures (40, 60, 80 and 100°C) for 1h.

pH Test

To estimate *Castanea crenata* lectin stability under different conditions of pH. A range of PH (1 to 12) was applied using phosphate buffer (0.1M) with different pH values.

Phagocytic Activity

Albinos Wistar mice male were housed under hygienic conditions in the animal house, they were kept under standard conditions of temperature (21±1°C), and fed with standard pellet diet with a free access to water for 12h of light daily. All the experiments were performed in accordance with the institutional animal ethics committee. Phagocytic activity index was determined as per the method reported by Cheng *et al.* (2005).

Phagocytic activity of reticulo-endothelial system was determined by carbon clearance test. Phagocytic index was calculated as a rate of carbon elimination of reticulo -endothelial system by clearance test.

In this experiment animals were equally divided to four groups, of seven animals each. Animals of Group I were kept as a Control, while animals of the treated groups: II, III and VI were an intravenously injected by the extracted lectins from *Castanea crenata* at dose of: 30, 50and 100mg/kg body weight respectively. 48 h later mice were received an injection of French Carbon ink suspension at a dose of 0.1mL/100g through tail vein, which contained black carbon ink 3mL, 0.9% NaCl 4ml and 3% gelatine solution 4ml. The blood samples were taken from the retro orbital vein using glass capillaries, at 5 and 15 min. Blood sample (30µL) were mixed with 4mL of 0.1% sodium carbonate solution to occur the

lysis of erythrocytes then the absorbance measured using a spectrophotometer JENWAY 7305 at 675 nm Knight (1997). The liver and spleen of each mouse were removed and weighed immediately. The phagocytic activity is expressed by the phagocytic index K which measures all the reticulo-endothelial system function in the contact with the circulating blood, and by the corrected phagocytic index α which expresses this activity by unit of weight of active organs: liver and spleen. The clearance rate is expressed as the half-life period of the carbon in the blood (t1/2, min). These are calculated by means of the following equations (Satnam *et al.,* 2012; Biozzi *et al.*, 1953).

$$K = \frac{(lnOD1 - lnOD2)}{(t2 - t1)},$$

$$t1/2 = 0.693/K,$$

$$\propto = \sqrt[3]{K}\ \frac{Body\ weight\ of\ animal}{Liver + Spleen\ weight}$$

Where OD1 and OD2 are the optical densities at times t1 and t2 respectively.

Statistical Analysis

Statistics were applied by using Graph PAD prism 8.0.1(244) for Windows. The statistical significance was assessed using one-way analysis of variance (ANOVA). Followed by Dunnett's comparison test. The values are expressed as Mean ± SEM and $P<0.05$ was considered significant.

Results

Extracted lectin from Castanea crenata by Sephadex G-75

The second peak correspond to *Castanea crenata'* s inner shell lectin.

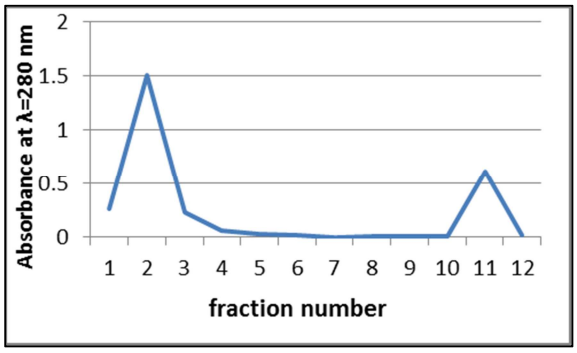

Fig. 1. The partial purification of *Castanea crenata'* s inner shell lectin by chromatography using sephadex G-75.

Hemagglutination Assay

Castanea crenata inner shell lectin exhibited a very high agglutinating activity when tested against rabbit red blood cells 18th well (Fig. 2).

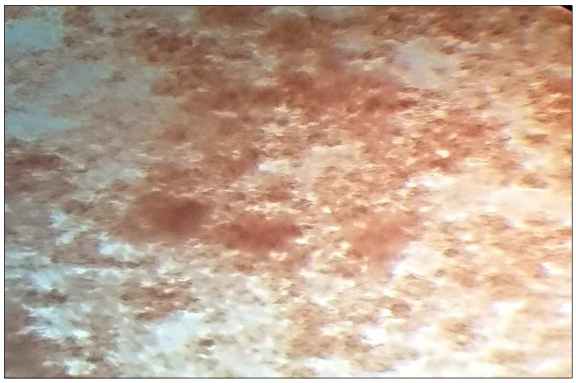

Fig. 2. Hemagglutinating activity of the lectin extracted from of *Castanea crenata* toward rabbit erythrocytes GX40.

Inhibition Tests

The results of inhibition tests using a large number of simple sugars, glycoproteins and minerals for *Castanea crenata* lectin are presented in table1 and 2.

Table 1. Inhibition of the hemagglutinating activity of the lectin extracted from the *Castanea crenata* by Sugars.

Carbohydrates	Minimum inhibitory concentration (mM)
Glucose	+ (MIC 400mm)
Saccharose	NI
Maltose	NI
Galactose	NI
Mannose	+ (MIC 50mm)
Cellulose	NI
Fucose	+ (MIC 100mm)
Arabinose	NI
N-acétyl-glusamine	+ (MIC 100mm)
Xylose	NI
Manitol	NI
Sorbitol	NI
Fructose	NI
Ribose	NI
Saccharine sodique	+ (MIC 100mm)
HCl Glucose-amine	NI
Raffnose	NI

+: Inhibition of the heamagglutinating activity.

NI: non inhibitory.

From the tested molecules: Six simple sugars (Glucose, fucose, N-acetyl Glucosamine, saccharine sodique and Mannose, shown an inhibition against the extracted lectin of *Castanea crenata*.

For the tested glycoproteins only BSA, fetuin, mucin and Ovalbumine were inhibitors. For the tested minerals no one had an inhibitory effect against *castanea crenata*'s lectin.

Table 2. Inhibition of the heamagglutinating activity of the lectin extracted from the *Castanea crenata* by glycoproteins.

Glycoproteins	Hemagglutinating activity
Insuline	-
BSA	+
Ovalbumine	+
fetuin	+
mucin	+

+: Inhibition of the heamagglutinating activity.
-: non inhibitory.

Table 3. Inhibition of the heamagglutinating activity of the lectin extracted from the inner shell of *Castanea crenata's* seeds by metals.

Metals	Hemagglutinating activity
Copper Dichloride $CuCl_2$	-
Aluminium Trichloride $AlCl_3$	-
Manganese Dichloride MnCl2	-
Stannous Dichloride $SnCl_2$	-
Cobalt Dichloride CoCl2	-
Barium Dichloride BaCl2	-
Magnesium DichloridemgCl2	-
Calcium Dichloride CaCl2	-
Zinc Chloride ZnCl2	-

+: Inhibition of the heamagglutinating activity.
-: non inhibitory.

Effect of Heat on Heamagglutinating Activity of Extracted lectin from castanea crenata

The hemagglutinating activity of extracted lectin from *Castanea crenata* when it submitted to heat treatment was stable until 120°C during 1h (Table 4).

Table 4. Effect of Heat on heamagglutinating activity of extracted lectin from *Castanea crenata*.

Temperature	40°	60°	80°	100°	120°
Hemagglutinating activity	+++	+++	+++	++	++

+++: highest heamagglutinating activity.

Effect of PH on Heamagglutinating Activity of Extracted lectin from Castanea crenata

The extracted lectin retains 60% of its hemagglutinating activity at PH 2-3, and it was stable in the range of pH (4 to12)

Table 5. effect of pH on heamagglutinating activity of extracted lectin from *Castanea crenata*.

pH	1	2	3	4	5	6	7	8	9	10	11	12
Hemagglutinating activity	-	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

-: no heamagglutinating activity
++: medium heamagglutinating activity
+++: highest heamagglutinating activity.

Blood Human Test (ABO)

The Extracted lectin from *Castanea crenata* did not present any specify for human blood Groups (Table 6).

Table 6. effect of human erythrocytes suspension on the agglutinating activity of the extracted lectin from *Castanea crenata*.

Human erythrocyte groups	A	B	AB	O
Hemagglutinating activity	+++	++	++	++

+++: highest heamagglutinating activity.
++: moderated heamagglutinating activity.

Effects of lectin extracted from Castanea crenata on Phagocytic Activity

The *Castanea crenata'* s inner shell lectin enhances Significantly the phagocytic activity in the treated groups: 50mg/kg and 100mg/kg comparing to control group (Fig. 4). And decreases remarkably in half-time of carbon in blood (Fig. 5). In addition it exhibits increasingly a positive effect on the reticuloendothelial system of mice in the treated groups :30mg/kg, 50mg/kg and 100mg/kg. (Fig. 6).

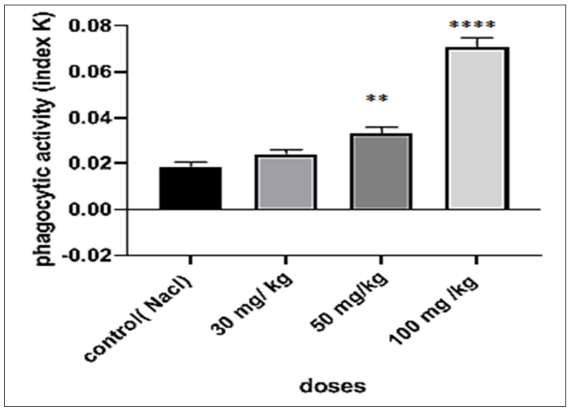

Fig. 3. Effect of *Castanea crenata'* s inner shell lectin on phagocytic activity.
(All values are mean± SEM, n= 7, *P<0.05, with*: significant (P<0. 033), **: highly significant (P<0. 002), ****: very highly significant, (P<0.001) comparing to control group).

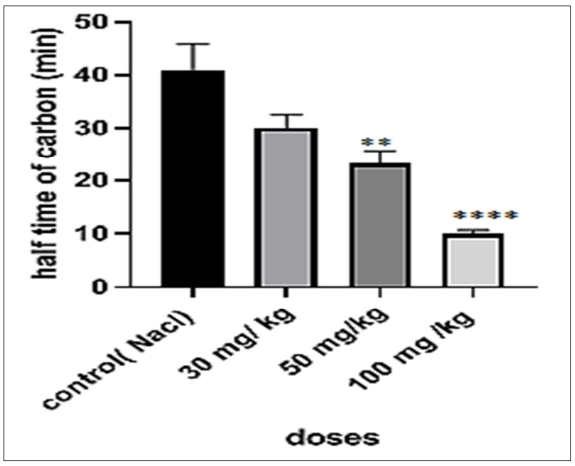

Fig. 4. Effect of *Castanea crenata'* s inner shell lectin on half -life ($t_{1/2}$) of carbon in blood.
(All values are mean±SEM, n=7,*P<0.05, with: *: significant(P<0. 033), **:highly significant (P<0. 002),****: very highly significant (P<0.001) comparing to control group).

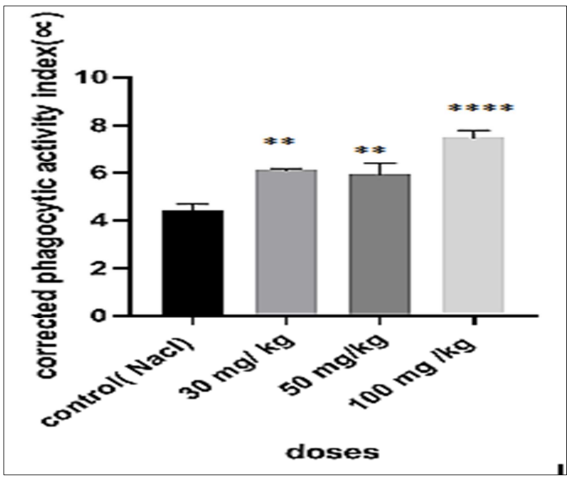

Fig. 5. Effect of *Castanea crenata's* inner shell lectin on corrected phagocytic index.
(All values are mean± SEM, n=7,*P<0.05, with: *: significant (P<0. 033),
: highly significant (P<0. 002), **: very highly significant (P<0.001) (comparing to control group)

Discussion

The results of this study shows that the extracted lectin of *Castanea crenata* seeds presents a very high hemagglutinating activity when tested against rabbit red blood cells, the same was found by Yao *et al.,* 2010 for *Setcreasea purpurea* lectin where it exhibited a strong hemagglutinating activity towards rabbit erythrocytes (Yao *et al.*, 2010).

Other lectins have relatively activity toward red blood cells. For example, a lectin from extralong autumn purple beans another cultivar of *Phaseolus Vulgaris cv*, with multiple anti-pathogenic properties, can agglutinate human, mouse, rabbit, and rat red blood cells (Fang *et al.*, 2010).

Moreover *Castanea crenata* lectin agglutinants all human erythrocytes with no specificity but it exhibits more activity toward the A blood group (as shown in Table 6) this results are the opposite of those found by J. W. Han *et al.,* 2010, where *Bryopsis plumose* lectin doesn't agglutinate any human erythrocytes type (Han *et al.*, 2010).

The Stability under highly acidic and basic conditions is a common property for lectins. The lectin from *Castanea crenata* seeds showed stability and kept its activity at a high level when submitted to pH levels from 2 to 12, Bahi *et al.* (2022) obtained similar results when studying lectin of *Ephedra alata*, finding a considerable stability in the pH range of 2-12.

The heat stability is also an interesting property for lectins. The results obtained for *Castanea crenata* lectin are similar to the chitin binding lectin that purified from *Setcreasea purpurea* wich shows an exelent heat resestency and retains 50% of their activity in 100°C Yao *et al.* (2010), *Castanea crenata* lectin is fairly thermostable due to the stability of the hemagglutinating activity between 40°C and 120°C, although it demonstrated a considerable decreasing of the agglutinating activity level as temperatures increases up to 120°C, whereas Jack Wong *et al.* (2008) found that *Castanopsis chinensis* lectin retains its agglutinating activity just at temperatures below 60°C. Lectins are proteins with a well-known quality for Carbohydrate binding specificity which was determined by inhibition of hemagglutinating activity. this studies demonstrated that *Castanea crenata* lectin was specifically inhibited by glucose (MIC 400mm), fucose, N-acetyl Glucosamine, saccharine sodique (MIC 100mm) but more strongly by monnose (MIC 50mm), which is similar to those of other lectins in the same family such as *Castanea mollissima* mannose binding lectin isolated by NGg *et al.* (2002).

It is known from other researchers on *Castanea* genus lectins that they tend to be mannose/glucose specific, as it was found for lectins from the species *crenata* by Nakamura *et al.* in (1998). Where several studies like the study realised by Pando *et al.* (2004) indicates that the agglutinating activity of *Crotalaria paulina* lectin has been inhibited by D-Galactose (MIC 50mm) and N-Acetyl-D-galactosamine with a Minimum inhibitory concentration of 25mM.Where as other studies on plants and alga lectins reveals their specificity for other sugars like green algae Spirogyra spp lectin inhibited by N-acetyl-glucosamine and N-acetyl-beta-D-mannose, and D-galactose Oliveira *et al.* (2017), while *Brassica napus* L. lectin exibeted a specificity for glucose, mannose and galactose Bahi *et al.* (2022). The hem agglutination inhibition studies carried out with the purified lectin from *Castanea crenata* seeds, revealed that the inner shell seeds lectin is also inhibited by glycoproteins such us: BSA, Ovalbumine and mucin while l other lectin like those of *Helianthus tuberosus* inhibited by Fetuin, Bovine fibrinogen Human and Bovine lactotransferrinthise (Van Damme *et al.*, 1999).

The primary role of phagocytosis is the removal of microorganisms, foreign particles and also the elimination of dead cells this is why phagocytosis considered being the first line of defence of the host against infectious agents. The reason why various pathological conditions in human are related to phagocytic defects. Singhal and Ratra (2013). Since mannose is widespread in animals, insects and microorganisms, mannose-binding lectins are considered biologically important proteins, (Wong *et al.* (2008), thus, the investigation of the potential immune modulatory effect of *Castanea crenata* lectin was an object of this study. The present data shows that administration of the extracted lectin at concentrations of 50 and 100mg/kg, increase very significantly and very high significantly Respectively the clearance of carbon rate by the reticulo-endothelial system cells as compared with the control group which is expressed by phagocytic index K (Fig. 3).

This indicates that *Castanea crenata* inner shell lectin stimulates the reticulo-endothelial system which enhances the phagocytic activity, furthermore it decreases remarkably the half-time of carbon in blood circulation in treated groups :50mg/kg and 100mg/kg comparing to control group ($P<0.005$).

This result indicates that the administration of the extract stimulates the elimination of carbon particles by reticulo-endothelial system cells, where the rate of elimination was proportional to the dose administered to mice. As it appears in the last part of this study (Fig. 5) organs such as the liver and spleen were involved to get rid of carbon particles from the blood, due to their stimulation by *Castanea crenata* lectin in the treated groups when compared to the control group, especially in the group that received a dose of 100mg/Kg which was the highest ratio of corrected phagocytic index (alpha), this results are similar to those found by f. Merouan *et al.,* in 2015 on the intracellular lectin of *Micromonospora aurantiaca* Merouan *et al.* (2015). Other studies demonstrated the lectin from *Viscum album L*, which is isolated as immunomodulatory activity of macrophage-mediated immune responses and enhanced also the term of various cytokines (IL-3, IL-23 and TNF-a) (Lee *et al.* (2007).

Conclusion

Mannose is an important cell surface sugar present in glycoconjugates that are implicated in a wide variety of important receptor mediated process, *Castanea crenata* inner shell lectin specificity towards mannose has provided an interesting line further researches with some therapeutic applications.

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