ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF FLAX LECTIN

[1]Dr Suma TK, [2]Vasudha TK, [2]Raghavi G, [2]Rajalakshmi V, [2]Nisha S, [2]Priyadarshini M, [2]B Preethi, [2]M Vaishali Krishna and [3]Dr BK Manjunath
[1]Asst Professor, [2]Student, [3]Professor and Head
Department of Biotechnology
The Oxford College of Engineering, Bangalore, India
For correspondence – tksuma@gmail.com

Abstract: Lectins are a class of non-covalent carbohydrate-binding proteins of non-immune origins; possessing at least one non-catalytic domain, they can reversibly recognize and bind to monosaccharides or oligosaccharides. Legume lectins have been demonstrated to possess antifungal and antiproliferative potency on tumor cells. Seed lectin from *Linumusitassimum* (flax) was extracted and partially purified by acetone precipitation followed by DEAE-cellulose ion exchange chromatography and Sephadex G-100 gel filtration chromatography. Carbohydrate binding specificity of lectin was determined by the hemagglutination method and found to be specifically binding to N-acetyl galactosamine. Purified lectin was characterized for their biological activity like antiproliferative, antioxidant, anti-inflammatory and antimicrobial activity using *in vitro* assays and found to possess significant biological activities.

***(Indexterms :* flax, lectin, hemagglutination, carbohydrate binding, anti-oxidant)**

I. INTRODUCTION

Lectins are proteins/glycoproteins which bind reversibly to carbohydrates. Lectins with specific carbohydrate specificity have been isolated from distinct sources such as viruses, bacteria, fungi, algae, animals, and plants [Sharon and Lis2004]; they show specificity to distinct carbohydrates, such as mannose, sialic acid, fucose, N-acetylglucosamine, galactose/N-acetylgalactosamine, complex glycans, and glycoproteins [Wu A. M *et al* 2009].

Recent studies have demonstrated the potential of lectins from different origin and carbohydrate specificities as antifungal and antiparasitic agents [Hamed et al 2017]. Plant lectins investigated for antifungal potential, mainly against phytopathogenic species, have most reported antifungal effects binding to hyphae, causing inhibition of growth and prevention of spore germination.

Lectins from several origins exert cytotoxic effects such as inhibition of proliferation and activation of cell death pathways, on different types of cancer cells. In addition, many anticancer lectins usually possess low cytotoxicity on nontransformed cells. This fact is probably associated with the distinct expression of glycans on surface of cancer and normal cells, allowing lectins specifically to recognize malignant cells [PrzybyłoMet al 2002, Varki A et al 2009]. Since lectins have the property to bind carbohydrates their ability to antagonize, *in vivo*, neutrophil migration induced by inflammatory stimuli is well established (Alencar NM et al 2004). There is also data to suggest that some lectins down-regulate telomerase activity and hence inhibit angiogenesis (Sharon and Lis, 2004). A natural outcome of these studies has been the application of several lectins as therapeutic agents which favorably bind to cancer cell membranes or their receptors, thereby triggering cancer cell agglutination which translates into cytotoxicity, apoptosis, and inhibition of tumour growth (Sharon and Lis, 2004).

With the background of a wide variety of lectins isolated from diverse sources and studied for multiple biological activities, the current project was formulated to identify potential novel sources of lectins with useful properties. Flax seed or *Linumusitassimum* is important in the nutraceutical market, as an alternate source of fish oil being naturally high in polyunsaturated fatty acids (PUFA). Intake of flaxseed in daily diet may reduce the risk of cardiovascular diseases such as coronary heart disease and stroke. There is also evidence that flax has anticancer effects in breast, prostate and colon cancers. Information on lectins isolated from flax seeds and their properties is very little. The focus of the present study was to isolate, purify and characterize lectin from the seeds of *Linumusitassimum*(flax) and analyze*in vitro* antioxidant, anti-inflammatory and antiproliferative activities.

II. MATERIALS AND METHODS

a) Materials

Human blood of groups A, B and O were collected from healthy persons. Animal blood was collected from nearby veterinary hospitals. Flax (*Linumusitassimum*) seeds were obtained from local markets.

b) Extraction of lectin

Dry seeds were first powdered in a blender. Seed powders were then weighed and extracted using cold extraction buffer, 1X PBS overnight at 4ºC. The extract was then centrifuged for 15 minutes at 10,000 rpm at 4ºC and clear supernatant was collected. Protein was estimated by Bradford method using BSA as a standard protein (Bradford 1976).

c) Hemagglutination activity of lectins

Seed extracts were assayed for the presence of lectin with whole blood of different blood types (Jawade AA et al 2016). 10µl of whole blood was used as negative control. 10µl of human blood types A, B, AB and O are taken on a clean and dry slide. The whole blood was mixed with 20µl of seed extract and hemagglutination observed.

In order to confirm that hemagglutination is due to lectin interacting with RBC cell surface carbohydrates, agglutination test of lectin was done by using 2% suspension of erythrocytes (Deshpande&Patil 2003). 50µl of 2% RBC suspension was mixed with 20µl of seed extract and hemagglutination observed.

Hemagglutination assay was also performed in 96 well plates (John Shi et al., 2007). 50µl of PBS and 10µl each of whole blood of human blood types A, B, AB and O was added to wells. 20µl of sample was then added to the wells. 50µl of PBS and 10µl of whole blood of any blood type served as negative control. Hemagglutination was observed after one hour.

d) Carbohydrate inhibition assay

Agglutination inhibition assay was done by testing the ability of different carbohydrates like disaccharides, pentoses, hexoses, oligosaccharides to inhibit the agglutination (Kurokawa*et al.* 1976). To confirm sugar specificity of extracted lectins, 100 µl of 500 mM sugar solutions were incubated with 100 µllectin for 30 minutes at room temperature. 20µl of incubated mixture was mixed with 50µl of 2% RBC suspension. Hemagglutination or its absence was observed under a transilluminator.

e) Purification of lectin

Crude lectin extract was fractionated using ammonium sulfate salt (0 - 75%) (Devi *et al*., 2014). The salt precipitate was allowed to stand overnight in the cold for complete precipitation. After centrifugation, pellets were suspended in minimal volume of 1X PBS buffer and extensively dialysed against the extraction buffer for 24 hr in the cold for complete salt removal. Alternatively, crude extracts were treated with ice-cold acetone at 4ºC and allowed for complete precipitation overnight. The precipitate was then centrifuged, air dried and dissolved in PBS. Protein concentration and hemagglutination activity were determined for the precipitates.

f) Chromatographic separation of lectin(Devi et al., 2014)

Acetone precipitated flax seed extract was fractionated on a Sephadex G-100 gel filtation column equilibrated with 1X PBS, pH 7.4. Fractions were collected at a flow rate of 0.5ml/min and the protein was monitored by measuring absorbance at 280nm. Hemagglutination activities of the fractions were then assayed. The fractions containing hemagglutinating activities were pooled and again loaded on to a DEAE-cellulose ion exchange column pre-equilibrated with 1X PBS, pH 7.4. Fractions of 1 ml volume each were collected at a flow rate of 1 ml/min. The bound proteins were eluted with 1M KCl in 1X PBS, pH 7.4. All fractions were measured for absorbance at 280nm as well as for hemagglutination activity.

g) SDS-PAGE

Polyacrylamide gel electrophoresis of lectin samples was performed by the method of Laemmli (1970) with 15% polyacrylamide gel in the presence of sodium dodecyl sulfate (SDS) and 2- mercaptoethanol (SDS-PAGE). After electrophoresis the gel was stained with 0.2% Coomassie brilliant blue (R250) (Bunn et al 1987)and then destained.

h) pH and temperature stability studies

The effect of pH on activity of lectin was studied using different buffers in the pH range of 4-9 by the method described earlier (Devi et al., 2014). The thermal behavior of the partially purified lectin was also evaluated by incubating the lectin sample at temperatures of 17°C–77°C for 15min.

i) Antioxidant activity of lectin

Antioxidant activity of lectinwas assessed by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay (Janardhan*et al* 2014) and ABTS (2, 2'-azinobis, 3-ethylbenzothiazoline-6-sufonic acid di-ammonium salt) radical scavenging assay (Lee et al 2014). Standard used in assay was ascorbic acid.

j) Anti-inflammatory activity of lectin

To assess the anti-inflammatory activity of flax lectin, proteinase inhibition assay was performed according to the modified method of Oyedepo et al 1995 and Sakat et al 2010 and inhibition of albumin denaturation technique according to Mizushima et al 1968 and Sakat et al 2010 with minor modifications. The percentage inhibition of proteinase inhibitory and protein denaturation activity was calculated.

k) Anti-proliferative activity of lectins

MCF-7 and HEPG2cell line were cultured in DMEM and EMEM medium respectively supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in an humidified atmosphere of 5% CO2 at 37°C until confluent. The cells was dissociated with TPVG solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells / well of MCF-7 and HEPG2was seeded in a 96 well plate and incubated for 24 hrs at 37°C, 5 % CO2 incubator. The monolayer cell

culture was trypsinized and the cell count was adjusted to 1.0 x 105 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000cells/well) was added. After 24 h, when a partial monolayer was formed, 100 µl of different concentrations of test samples were added and plates incubated at 37°C for 24hrs in 5% CO2 atmosphere. Later, test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well and incubated for 4 h at 37°C. The supernatant was removed and 100 µl of DMSO was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line. % Inhibition = 100 – (OD of sample/OD of Control) x 100.

III. RESULTS AND DISCUSSION

Lectins were extracted from flax seeds into PBS and precipitated using salt or acetone. All extracts were tested for hemagglutination with human and animal blood. Table 1 shows hemagglutination of human blood types and animal blood by flax seed extracts. It showed hemagglutination reaction with only human blood group A (and AB) indicating its specificity to N-Acetylgalactosamine (Fig. 1).

Table 1 : Agglutination study of *Linum usitassimum* seed lectin with human and animal erythrocytes

Erythrocytes	Agglutination
Human 'O'	-
Human 'A'	+++
Human 'AB'	+++
Human 'B'	-
Rabbit	-
Sheep	-
Hen	-

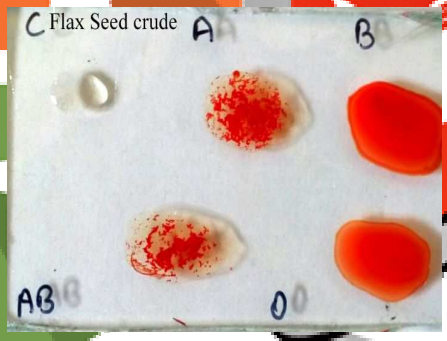

Figure 1 :Hemagglutionation of whole blood by flax extract

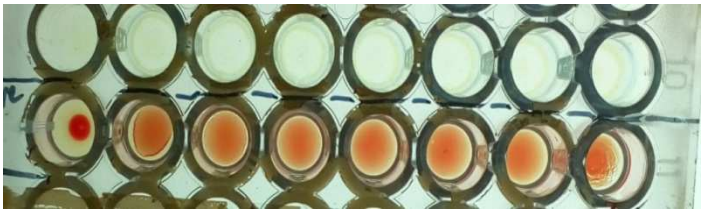

Figure 2: Hemagglutination assay in 96 well plate

Hemagglutination assay was also performed by using human erythrocyte suspension in microtitre plates. Formation of red button of RBCs at the bottom of the well indicated absence of hemagglutination. (Fig. 2).

In order to determine the absolute specificity of flax lectins towards various carbohydrates, carbohydrate inhibition assay was carried out. Table 2 shows the inhibition of lectin by different carbohydrates. Flax lectin was inhibited by N- Acetyl galactosamine (GalNac) alone and no other carbohydrate indicating the absolute specificity of this lectin for GalNac.

Table 2: Inhibition of lectin by different carbohydrates

Carbohydrates	Agglutination

Glucose	+++
Galactose	+++
Mannose	+++
Lactose	+++
Fucose	+++
N-acetyl galactosamine	-

- = inhibits lectin
+ = does not inhibit lectin

Flax lectin was purified from the acetone precipitated extract using Sephadex G-100 gel filtration column equilibrated with 1X PBS buffer, pH 7.4. Fig. 3 gives the purification profile of flax lectin by gel filtration chromatography. Hemagglutination activity was detected in the initial fractions (maximum in fraction 3) denoting the high molecular weight of the purified lectin. None of the later eluting protein peaks showed any trace of hemagglutination activity. Although gel filtration chromatography is commonly employed for lectin separations (Zhang et al 2014), such purification of *Linum usitatissimum* lectin using Sephadex G-100 has not been reported earlier.

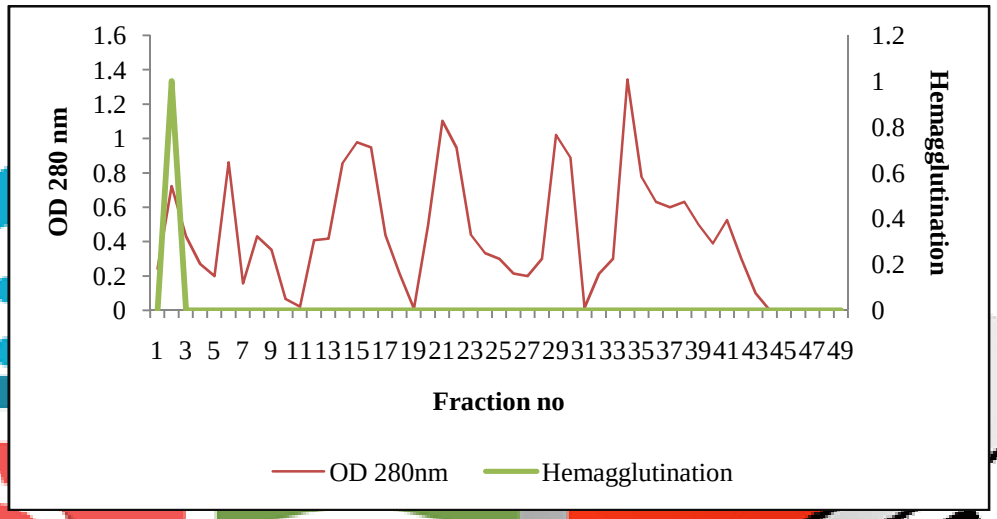

Figure 3: Sephadex G-100 gel filtration purification profile of flax lectin

Flax lectins were further purified on DEAE cellulose ion exchange column. The fractions were assayed for protein by measuring their absorbance at 280nm as well as for the hemagglutination activity. Among the fractions collected, activity was recovered in the unbound fractions and no activity was seen in bound (Fig. 4), indicating its anionic nature.

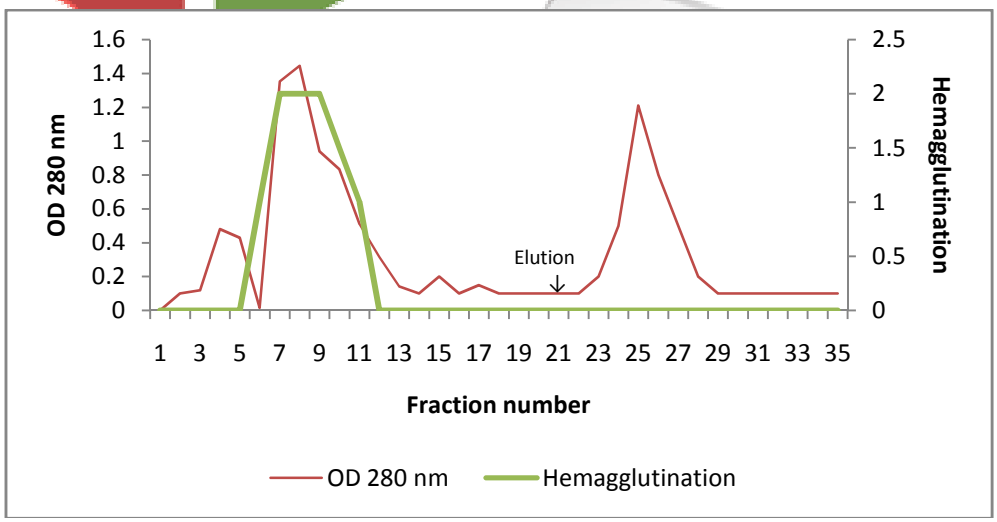

Figure 4: DEAE-cellulose ion exchange purification profile of flax lectin

SDS-PAGE analysis of lectin at each stage of purification was done to determine purity as well as molecular weight. Fig. 5 shows the gel profile of crude and partially purified flax lectin preparation on a 15% SDS-PAGE gel. In comparison with crude extract

and salt and acetone precipitates, different types of protein bands were seen in unbound and gel filtration fraction signifying a level of purification. The presence of protein bands in between that of BSA and lysozyme molecular markers shows that the molecular weight of the lectin or its subunits is between 14.3 kDa (lysozyme) and 66.5 kDa (Bovine serum albumin). This profile of unbound and gel filtration fractions was associated with hemagglutination activity while bound had no hemagglutination activity.

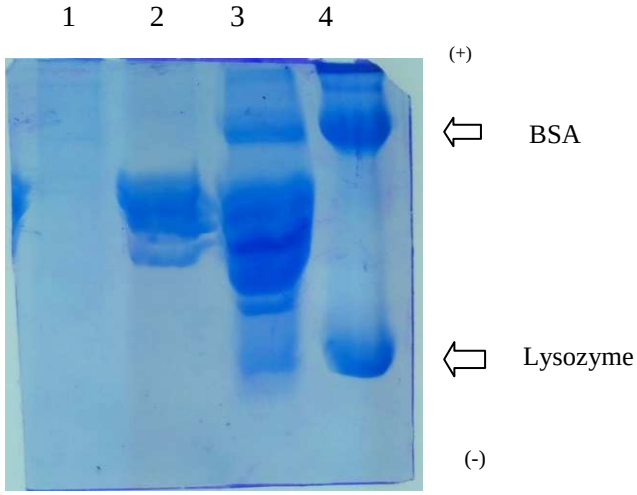

Figure 5: SDS-PAGE profile of flax crudeand partially purified samples. 15% polyacrylamide gel was run with following samples and stained with Coomassie Brilliant Blue for visualization. From left to right: Lane 1: Gel-filtration pooled fraction, Lane 2: Flax ion-exchange unbound fraction, Lane 3: acetone precipitate, Lane 4: BSA + Lysozyme molecular marker.

The antioxidant activity of lectins has been well-established (Sadananda *et al*., 2014). Anti-oxidant assays of DPPH and ABTS were performed for lectin extracts. The percentage scavenging activity of flax lectin was tested against that of ascorbic acid which is known to have potent antioxidant activity (Fig. 6 and 7). The IC50 value for flax lectin was found to be 3.008 mg/ml. The IC50 value for ascorbic acid at same concentration as lectin was found to be 3.018mg/ml.

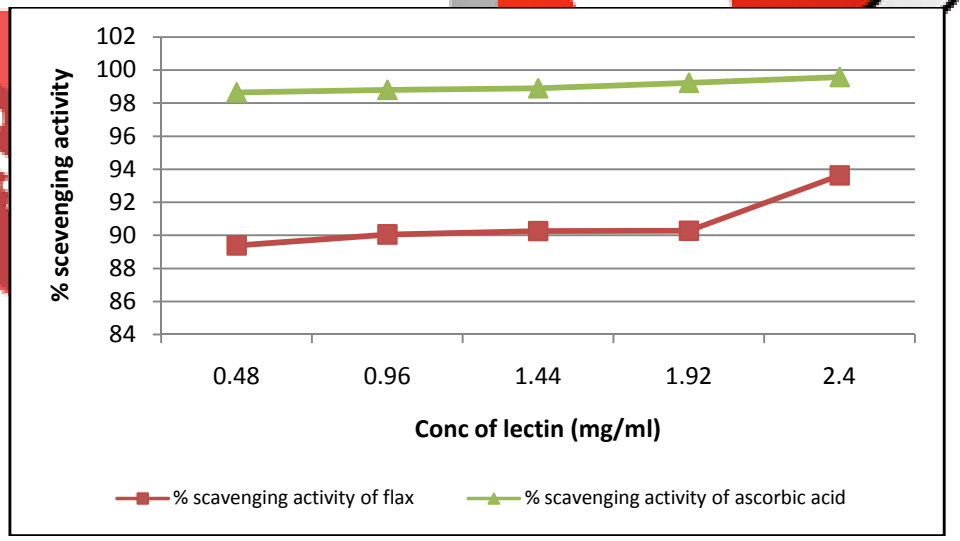

Figure 6: Antioxidant activity of flax lectin by DPPH assay. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517nm which is induced by different antioxidants.

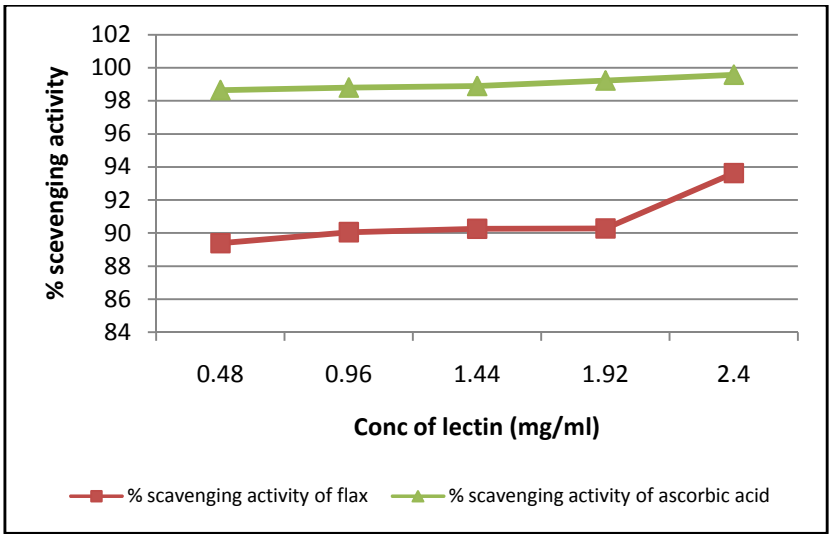

Figure 7: Antioxidant activity of flax lectin by ABTS assay.The decrease in absorbance due to scavenging of the proton radicals is monitored spectrophotometrically at 734nm

Lectins have been shown to have anti-inflammatory activity (Janaina K.L. Campos *et al*., 2016). Proteinase inhibition assay and albumin denaturation assays were performed to demonstrate anti-inflammatory activity. Fig. 8 shows the anti-inflammatory activity of flax lectin. Flax lectin exhibited significant antiproteinase activity at different concentrations and showed 100% inhibition at 3.2mg/ml of lectin.

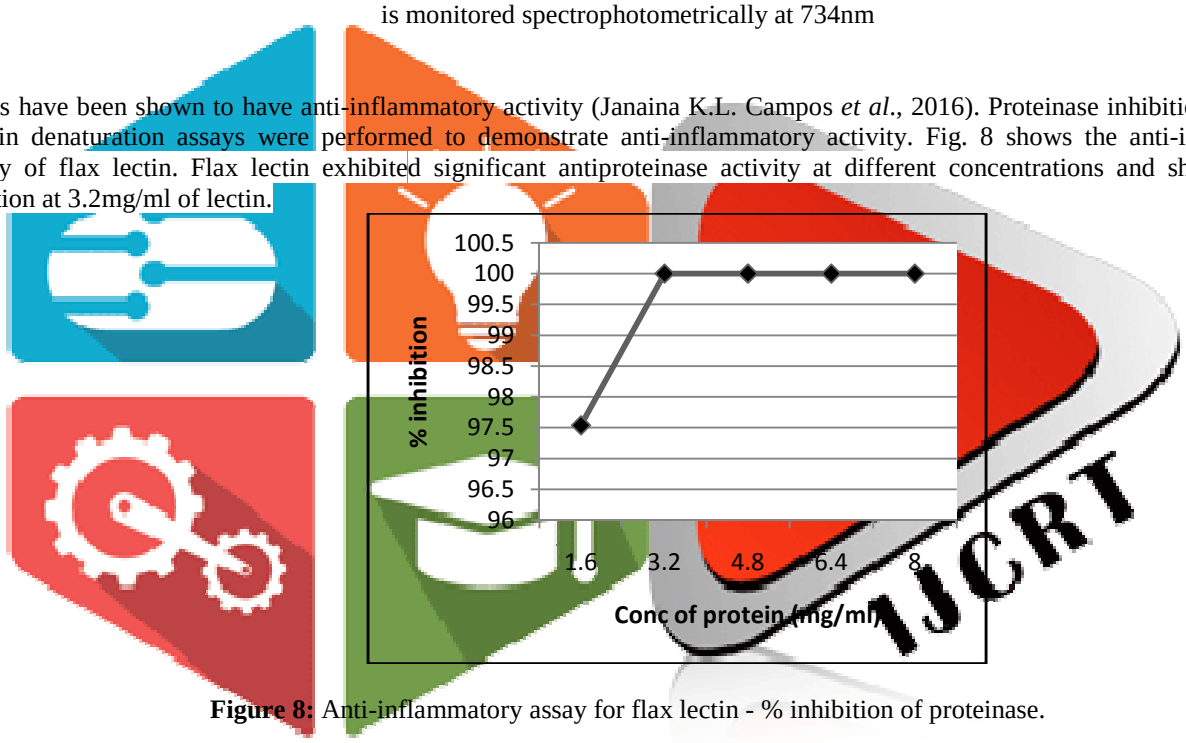

Figure 8: Anti-inflammatory assay for flax lectin - % inhibition of proteinase.

As part of the investigation on the mechanism of the anti-inflammation activity, ability of lectin to inhibit protein denaturation was studied (Fig. 9). It was effective in inhibiting heat induced albumin denaturation. Inhibition of 100% was observed at 8 mg/ml for flax lectin.

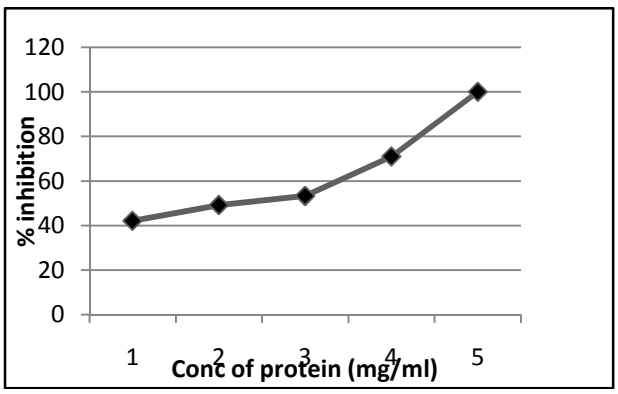

Figure 9: Anti-inflammatory assay for flax lectin - % inhibition of protein denaturation.

Flax lectins were tested for their pH stability by incubating the lectins in buffers of different pH. The lectins showed remarkable pH stability in the range 4-9 which was confirmed by hemagglutination of the lectins after incubation. When tested at a range of temperatures between 17-100°C, flax and lectins showed decreased activity beyond 57°C.

To evaluate the cytotoxic effect of flax lectin against cancer cells, human breast carcinoma cells (MCF-7) were incubated with different dosages of flax lectin samples. After 24 hours of incubation, cell viability was determined by MTT assay. Flax lectin extract was found to induce cell toxicity in a concentration dependant manner (Table 4). Dose response curves between the range 10µg/ml to 320µg/ml express decreased number of viable cells with increase in the concentration of compound. Results indicate that the cytotoxic effect steadily strengthens with increase in concentration.

Table 4: Cytotoxicity assays using flax lectin

MCF-7			
Sample	**Conc. µg/ml**	**OD at 590nm**	**% inhibition**
Linumusitatissimum	10	0.911	4.27
	20	0.875	7.95
	40	0.833	12.47
	80	0.811	14.78
	160	0.766	19.50
	320	0.721	24.19

IV. CONCLUSION

Very little information is currently available on flax seed lectins. In one study, hemagglutination activity and carbohydrate specificity of lectin seedlings revealed their role in plant adaptation to abiotic stresses (Levchuk*et al* 2013). A similar study on amaranth-like lectin genes in flax induced by defence hormones has also been reported (Kashfia Faruque*el al* 2015).

The current report sheds light on the hemagglutination activity, carbohydrate specificity, purification strategies and biological activity of flax seed lectins. *Linum usitatissimum* (Flax) lectin extracts were found to be specific to N-Acetylgalactosamine. Purification was achieved by concurrent use of gel filtration and ion exchange chromatography and it was found to be a high molecular weight cationic protein. Purified lectin was shown to possess anti-oxidant, anti-inflammatory and anti-proliferative properties and remarkable pH and temperature stability. These results support the general finding of lectins to be biologically active in different ways. Further work is warranted to establish possible utilization of flax lectin for specific applications.

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Acknowledgment

V. References

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