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SYNTHESIS AND CHARACTERIZATION OF CHITOSAN NANOPARTICLE CONJUGATED ASCLEPAIN c-II FROM THE PURIFIED LATEX

N. Karpagam, J.Kavina, Kumaresubitha.T, M. Sathiyanthan, M.Elamvaluthi, M. Gopalakrishnan*

PG & Research Department of Botany, Pachaiyappa's college Chennai-600 030.

***Corresponding author: ptcgopal@yahoo.com**

Keywords:

Asclepain c-II, Chitosan nano-particle, Cytotoxicity, Coagulation

ABSTRACT

Asclepias curassavica L., "Scarlet Milkweed" belongs to the family of Asclepiadaceae. It is an erect, evergreen perennial shrub with woody base, known for ages for its remarkable potential to be used for a variety of therapeutic purposes, which includes pro-coagulant (thrombin like), cardio-glycosidal, anti-haemorrhagic, activities and many more. The latex of this plant contains a specific cysteine protease named Asclepain c-II (Asp c-II) which is reported to have this pro-coagulant property which helps in clotting of blood, same as what thrombin does to our blood. The thesis reports the screening of Asp c-II from plant latex, purification and isolation of Asp c-II using ion exchange chromatography, its characterization using proteolytic assay and SDS PAGE. The synthesis, characterization and cytotoxicity studies of chitosan nano-particle conjugated Asp c-II on human blood was also carried out. The plant latex was collected and stored at -20o C. It was then subjected to SP Sepharose cation exchange column, to isolate the purified Asp c-II from crude latex. The isolated protein was characterized using SDS-PAGE. The molecular weight of the compound was found to be 23.59 kDa. Purified Asp c-II was then conjugated with chitosan nano-particle using ionic gelation method. Coagulation assays were performed for crude extract, purified Asp c-II and chitosan nano-particle conjugated Asp c-II. The clotting time reduced from 5 minutes for normal human blood to 2 minutes (on an average basis). Cytotoxicity studies were carried out using MTT assay on mammalian cell lines. The levels of cytotoxicity were found to be minimal, thus enabling nanoparticle conjugated Asclepain c-II to be further used as a pro-coagulant. Hence it could be fabricated as a therapeutic agent to aid in blood clotting.

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1. INTRODUCTION

Asclepias curassavica L., commonly known as Blood-flower, is a species of flowering plant in the milkweed family, Asclepiadaceae. It is a native plant of the American tropics but has become a naturalized weed in tropical and subtropical pastures and fields throughout the world. Typical plants are evergreen perennial sub-shrubs that grow up to 1 meter tall and have pale gray stems (Kartesz et al., 1994 & 2013). The leaves are arranged oppositely on the stems, narrowly elliptic and upon plucking the petiole, produce a white milky viscous sap. The flowers are arranged in umbels with refluxed, five parted corolla brilliant red-purple, exposing the crown of five orange horned hoods. It blooms continuously from spring until autumn. The scientific name, Asclepias curassavica L. refers to the Greek god of medicine, Asclepius, because of the long regarded medicinal qualities of the plants in this genus. However, many species in the family are poisonous and contain cardio-glycosides; the toxicity of which may cause death in livestock or humans. Root extracts of this plant is used as an emetic, expectorant and laxative. The crude extract is also employed for warts and fever. The roots are commonly known as "pleurisy root" and used as an expectorant for pneumonia, pleurisy and other lung problems. In Jamaica, a poultice of the root is used to treat ringworm and to stop bleeding. In Western Canada and the USA, milky sap of the stems has been used to treat warts and skin parasites. The roots are prepared in decoctions for constipation, venereal disease, kidney stones, asthma, and cancer. In the 1880s, Native Americans used the plant as a contraceptive and snakebite remedy. In Ayurvedic herbal medicine systems (India) the plant is considered diaphoretic, anthelmintic, purgative and emetic (Karpagam et al., 2013) The coagulation effect of the latex of A. curassavica is attributed to the presence of a specific cysteine protease. Proteases isolated from several plant latexes have been known to interfere in blood coagulation due to their thrombin like activity. Ficin, a cysteine protease isolated from Ficus carica was found to shorten the pro-thrombin time thereby accelerating clotting activity (Richter et al., 2002). Other plant cysteine proteases such as Ervatamin B from Ervatamia coronaria and a mixture of cysteine proteases isolated from Carica papaya show similar activity (Silva et al., 1997 & Suman et al., 2000). Asclepain c-II is a papain like endopeptidase isolated from the latex of A. curassavica L. It is reported to be the main proteolytic component in the latex of the plant. Asp c-II is found to show higher specificity of proteolytic activity when compared to Asp c-I. Both enzymes displayed quite distinct biochemical characteristics, confirming that they are different enzymes (Liggieri et al., 2008). Asp c-II has a molecular weight of 23.59 kDa. The activity of Asp c-II is inhibited by the inhibitor E-64 which is specific for cysteine proteases (Liggieri et al., 2009). Thus, cysteine proteases present in the latex exhibits thrombin like activity and helps in accelerating the blood clotting. Due to this potential application of the Asclepiadaceae plant latex, A. curassavica is chosen here. The isoelectric point of Asp c-II is greater than 9.3, with maximum proteolytic activity at pH 9.4-10.2. The CE can be purified using cation exchange chromatography to isolate Asp c-II. The IEX pre-packed column used was Hi-Screen SP Sepharose fast flow; purchased from GE Health Care, Chennai. Asp c-II is eluted using linear gradient of NaCl and can be stored at -20^{o} C for further studies (Nora Priolo et al., 2009).

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The Lowry‟s assay is the most common assay carried out to estimate the amount of protein present in a sample. It is a colorimetric method; which is based on absorbance at 660nm. The fractions obtained from the IEX column after elution of linear gradient NaCl eluent are to be estimated for protein concentration using Lowry‟s assay. The proteolytic activity of the fractions can be calculated using proteolytic assay with casein as the substrate. It is also a colorimetric assay; which is based on absorbance of product tyrosine at 280nm. The enzyme concentration is obtained as Units per mL of enzyme. Nanotechnology is being increasingly explored in science and industry for widely different applications. Nanotechnology and polymers have captivated a tremendous interest in many areas such as the pharmaceutical industry and therapeutic innovation among others. Natural and synthetic polymers have been used as a promising tool for nano scale drug carrier systems, especially in oral administration of poorly absorbed therapeutic drugs (Bravo Osuna I et al., 2008). The major advantages of nano-particles as a delivery system are in controlling particle size, surface properties, and release of pharmacologically active agents in order to achieve the site-specific action of the drug at the therapeutically optimal rate and dose regimen. The drug is usually dissolved, entrapped, adsorbed, attached, or encapsulated to the nano-particle matrix to favour drug delivery. The nano-particle matrix can be of biodegradable materials such as polymers or proteins. Depending on the method of preparation, nano-particles can be obtained with different properties and release characteristics for the encapsulated therapeutic agents.

In recent years, great developments have been made in the field of mucoadhesive polymer systems in formulations that increase the residence time of drugs on mucosal membranes and subsequently, enhance the bioavailability of drugs with poor oral absorption (Guggi D et al., 2004). Thus the polymer chitosan and its derivatives are potentially used as drug carriers. Chitosan is a natural, linear, aminopolysaccharide (pKa 6.5) copolymer of glucosamine and N-acetylglucosamine obtained by the alkylation and partial deacetylation of chitin. It is the second most abundant natural polysaccharide and originates from shells of crustaceans. Chitosan is a biodegradable, biocompatible, nontoxic mucoadhesive biopolymer. Since the main backbone of chitosan is made of amino group it can readily form nano-particles with substances like citrate, sulphate and tripolyphosphate. This method of chitosan nanoparticle synthesis is known as ionic gelation. Here the drug is trapped inside the nano-particle and releases the drug when in contact with skin or a mucosal layer, thus aiding in specific sustained release. The coagulation assays are a part of hematopathology analysis usually performed on human blood samples collected on Sodium Citrate (anti-coagulant) in the ratio 9:1; blood:anti-coagulant. Both blood and plasma samples are to be analysed for coagulation activity using crude latex, IEX fractions and nano-particle conjugated Asp c-II. This assay gives an idea of the clotting time and the efficiency of the Asp c-II. Cytotoxicity is the quality of being toxic to cells. Cells exposed to a drug can respond in a number of ways. The cells may undergo necrosis, in which they lose membrane integrity and die rapidly as a result of cell lyses; they can stop growing and dividing; or they can activate a genetic program of controlled cell death, termed apoptosis. Cytotoxicity assays are used widely in drug discovery research to help

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predict which lead compounds might have safety concerns in humans before significant time and expense are incurred in their development. The mechanisms of cytotoxicity is also viewed as a way to gain a better understanding of the normal and abnormal biological processes that control cell growth, division, and death. There are many ways to measure cytotoxicity, but most involve assessment of cell membrane integrity. Membrane integrity can be evaluated by using vital dyes (such as tryphan blue or propidium iodide), by protease biomarkers, with MTT or MTS redox potential assays, or by measuring ATP content. Many of these assays involve colorimetric, fluorescence, or luminescence detection. MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

2. MATERIALS AND METHODS

COLLECTION OF LATEX FROM A. curassavica L.

The plants were identified by their characteristic scarlet bright red flowers and woody stem. Nearly 10 new saplings were brought from the hilly areas of Salem, Tamil Nadu, India. They were subjected to hardening process in the college nursery. The latex was collected by plucking of the leaf petioles and by scraping at the stem nodes. The viscous milky white latex was then collected on 0.1 M Citrate Phosphate Buffer (pH 7.4) containing 5mM EDTA and 5mM Cysteine, in order to avoid phenoloxidase activity and oxidation, respectively (Nora Priolo et al., 2009).

PREPARATION OF CRUDE EXTRACT

Latex (2 ml), obtained by superficial incisions of petioles, was collected on 13 ml of 0.1M citrate phosphate buffer (pH 7.4) in four falcon tubes separately. This suspension was then allowed to stand overnight at 4^{o} C for the unwanted debris to settle down. The clear supernatant was then transferred into a fresh centrifuge tube the next day. The supernatant was subjected to centrifugation at 16,000 g for 30 minutes at 4^{o} C to discard the gums and other insoluble materials. The supernatant was again subjected to centrifugation at 16,000 g for 1 hour at 4^{o} C. The new supernatant containing soluble proteins (Crude Extract (CE), 12 ml), was stored at -20^{o} C for further studies (Liggieri et al., 2009).

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Fig. 1: Collection of plant latex

PROTEIN ESTIMATION BY LOWRY'S METHOD

ANALYSIS BY SDS PAGE
Procedure:

The SDS set up was cleaned and gels were casted as given in the above procedure and allowed to solidify. 20µL of CE were mixed with equal volume of SDS – sample buffer and heated on boiling water bath for 2 min and then cooled on ice immediately. Sample was then loaded into the gel and voltage was set to 50 V for stacking gel and 100 V for resolving gel. After complete running, the gel was stained for 2 hours using the staining solution. After staining the gel was placed in de-staining solution for overnight. The bands were observed the next day under gel illuminator and results were interpreted using DGelDAS software DGelDAS is computer based software which aids in predicting the molecular weight of proteins run through the gel (Lowry *et al.,* 1930; Schagger *el al.,* 2006)).

From right to left; Lane1: Low range protein molecular weight marker; 3.5 kDa Insulin, 6.5 kDa aprotinin, 14.3 kDa Lysozyme, 20.1 kDa Soyabean trypsin inhibitor and 43 kDa Ovalbumin.

Lane 2 & Lane 3 Crude Extract.

Lane 4 Lysozyme 14.3 kDa molecular weight marker.

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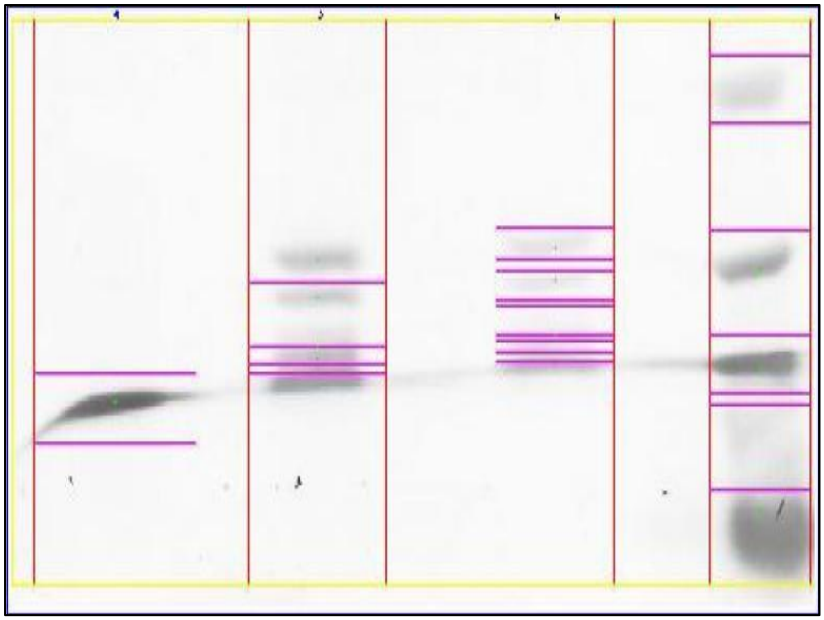

Fig. 2: DGelDAS Image

From right to left; Lane1: Low range protein molecular weight marker; 3.5 kDa Insulin, 6.5 kDa aprotinin, 14.3 kDa Lysozyme, 20.1 kDa Soyabean trypsin inhibitor and 43 kDa Ovalbumin.

Lane 2 & Lane 3: Crude Extract.

Lane 4: Lysozyme 14.3 kDa molecular weight marker.

PURIFICATION OF CRUDE EXTRACT AND ISOLATION OF ASCLEPAIN c-II USING IEX CHROMATOGRAPHY (Nora Priolo *et al.*, 2009)

PROTEOLYTIC ASSAY (Anson *et al.*, 1998)

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$$\text{Conversion from mg/mL to } \mu M/L = \frac{\text{Concentration of Tyrosine (mg/mL)} * 10^6}{\text{Mol. Wt. of Tyrosine (g/mol)}}$$

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Tubes containing proteolyzed samples, ready for measurement of absorbance at 280 nm.

From right to left; Lane1: Low range protein molecular weight marker; 3.5 kDa Insulin, 6.5 kDa aprotinin, 14.3 kDa Lysozyme, 20.1 kDa Soyabean trypsin inhibitor and 43 kDa Ovalbumin.

Lane 2: 0.4 M IEX fractions.

Lane 3: 0.6 M IEX fractions.

SYNTHESIS OF ASCLEPAIN c-II CONJUGATED CHITOSAN NANO-PARTICLES

(Anton Smith *et al.*, 2012)

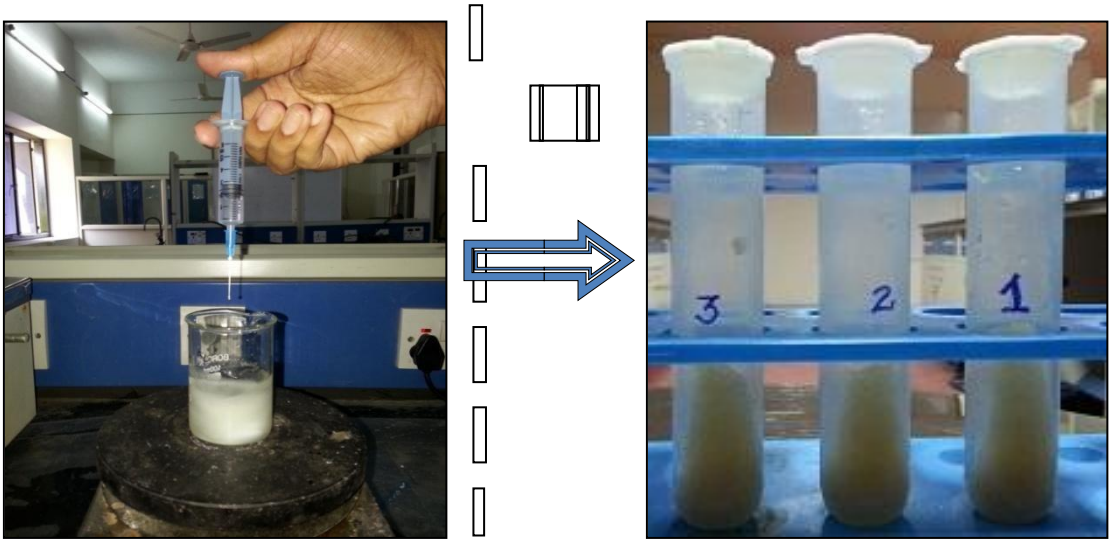

Fig. 3: Chitosan NPs conjugated Asp c-II synthesis

Sample 1 = Crude Extract Sample 2 = 0.4 M IEX Fractions
Sample 3 = 0.6 M IEX Fractions.

COAGULATION STUDIES

COAGULATION ASSAY USING PLASMA

5mL of blood was collected on 490 µL of sodium citrate, mixed thoroughly and subjected to centrifugation at 1600 g for 15 minutes at 4^{o} C to separate the plasma from the blood.50µL of plasma was added to an eppendorf tubes containing a pinch of chitosan NPs conjugated Asp c-II. 30µL of 0.01M Tris – HCl was added and mixed by slow inversion several times. 50µL of calcium chloride was added, mixed thoroughly and time for coagulation was found out. The process was repeated in

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similar fashion for IEX fractions. All assays were carried out in triplicates and the results were compared and studied (Sathish *et al.* 2012).

CYTOTOXICITY ASSAY (Mosmann *et al.*, 2005)

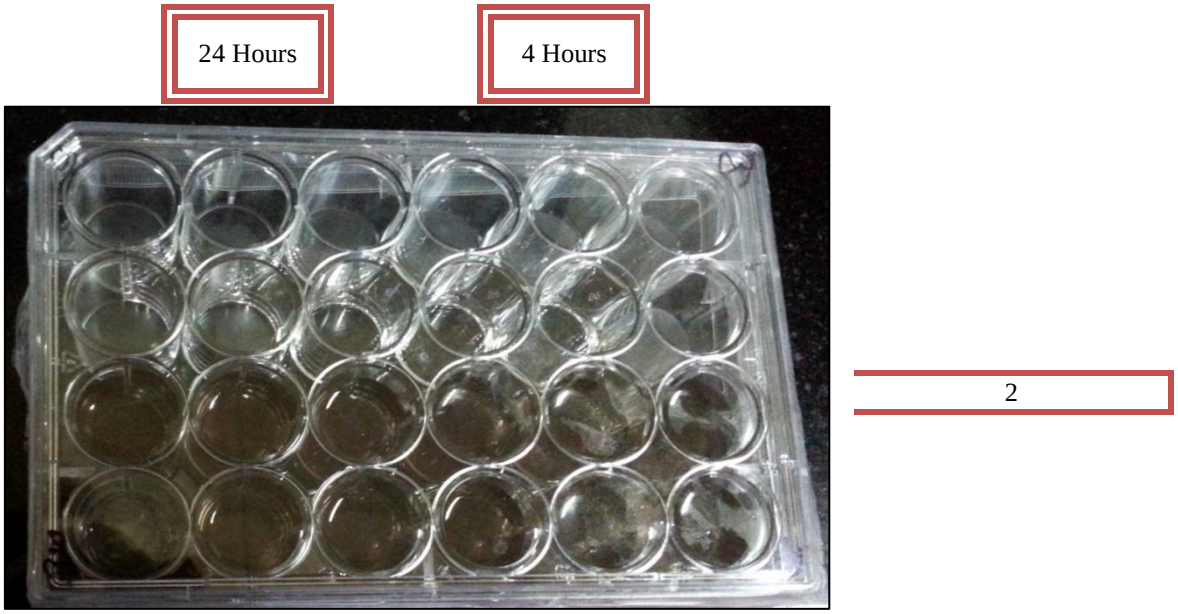

Fig. 4: 24 Well plate – MTT assay

Row No. 1: Control for MTT assay.

Row No. 1: MTT assay for Chitosan NPs conjugated Asp c-II.

RESULTS AND DISCUSSION

PREPARATION OF CRUDE EXTRACT

The latex sample after removal of all unwanted gums and other insoluble substances was stored on citrate phosphate buffer containing
EDTA and cysteine at -20^{o} C

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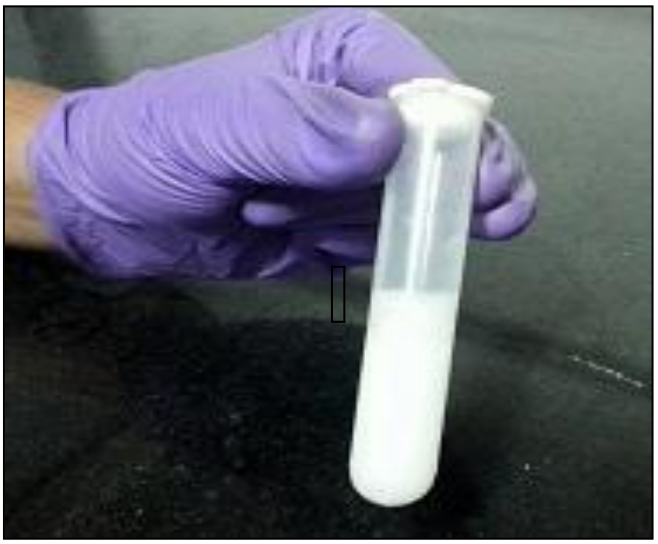

Fig. 5: Collection & storage of latex at – 20° C

This CE collected in four different tubes was then subjected to Lowry's Assay separately, in triplicates each and produced the following results;

PROTEIN ESTIMATION BY LOWRY'S METHOD

Table1: Absorbance values for Lowry's Assay

Sample No.	Concentration of BSA (mg/mL)	A660	A_{660}	A_{660}	Average of A_{660}	SD
Blank	0	0	0	0	0	-
S1	0.2	0.062	0.066	0.073	0.067	0.005568
S2	0.4	0.153	0.155	0.160	0.156	0.003606
S3	0.6	0.285	0.251	0.223	0.253	0.031048
S4	0.8	0.406	0.380	0.342	0.376	0.032187
S5	1	0.509	0.474	0.451	0.478	0.029206
U1	-	1.493	1.114	1.296	1.301	0.189549
U2	-	1.134	1.129	1.142	1.135	0.006557

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U3	-	1.116	1.098	1.095	1.103	0.011358
U4	-	1.401	1.382	1.375	**1.386**	0.013454

A_{660} – Absorbance at 660 nm.

SD – Standard Deviation.

S1...S5 – Standard BSA Solution.

U1...U4 – Unknown Crude Extract.

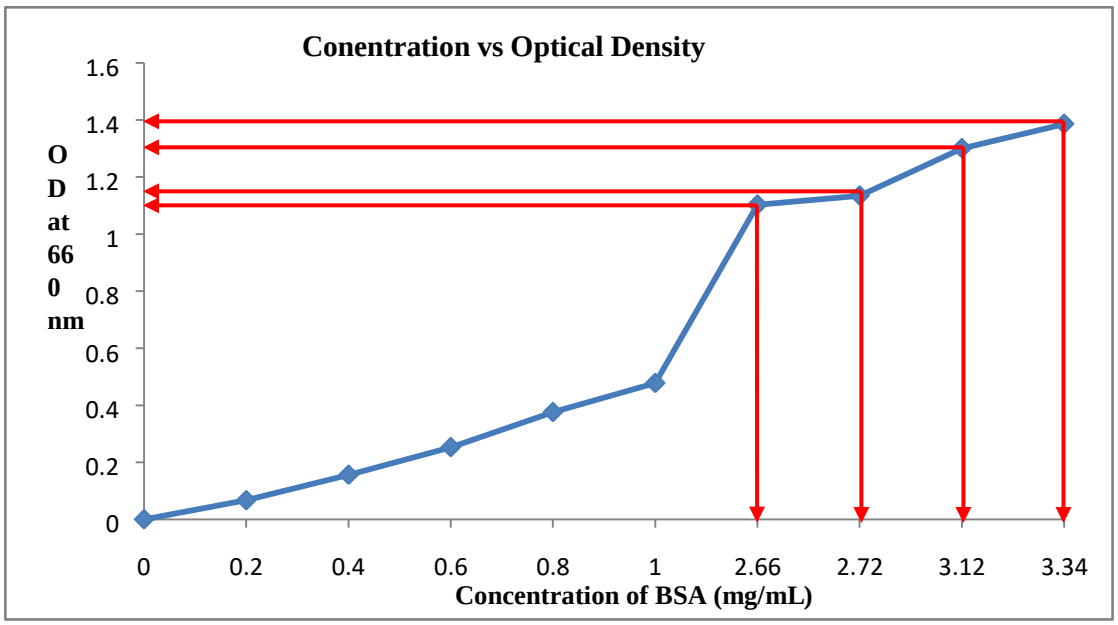

Fig. 6: Standard plot for Lowry's Assay along with unknown protein estimation

The concentration of proteins in the crude latex was estimated using the standard graph and found to be U1 = 3.12 mg/mL, U2 = 2.72 mg/mL, U3 = 2.66 mg/mL, **U4 = 3.34 mg/mL**.

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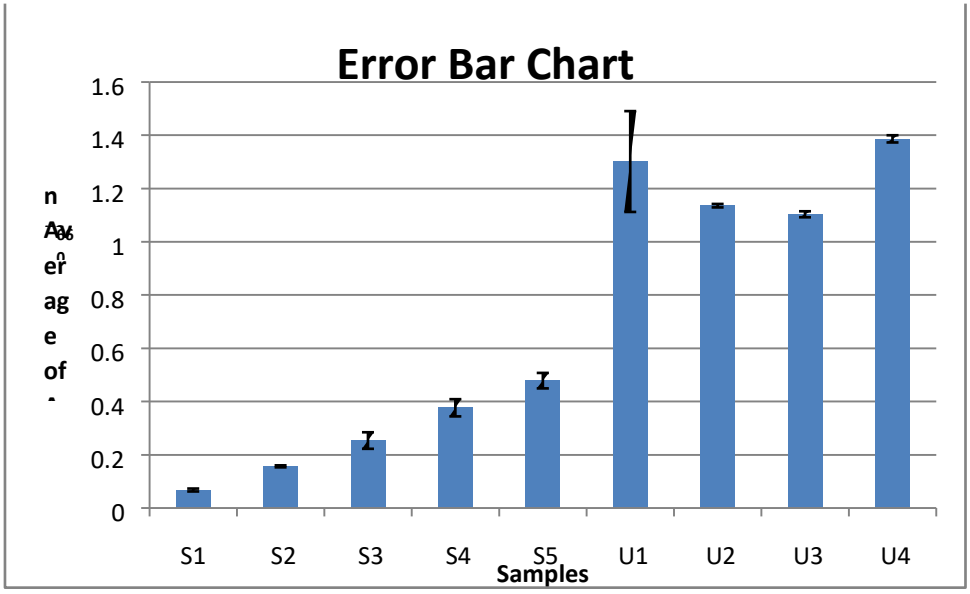

Fig. 7: Error bar chart for Lowry's assay

S1...S5 – Standard BSA Solution.

U1...U4 – Unknown Crude Extract.

ANALYSIS BY SDS PAGE

The CE after protein estimation was analysed by SDS PAGE in 10% polyacrylamide gels.

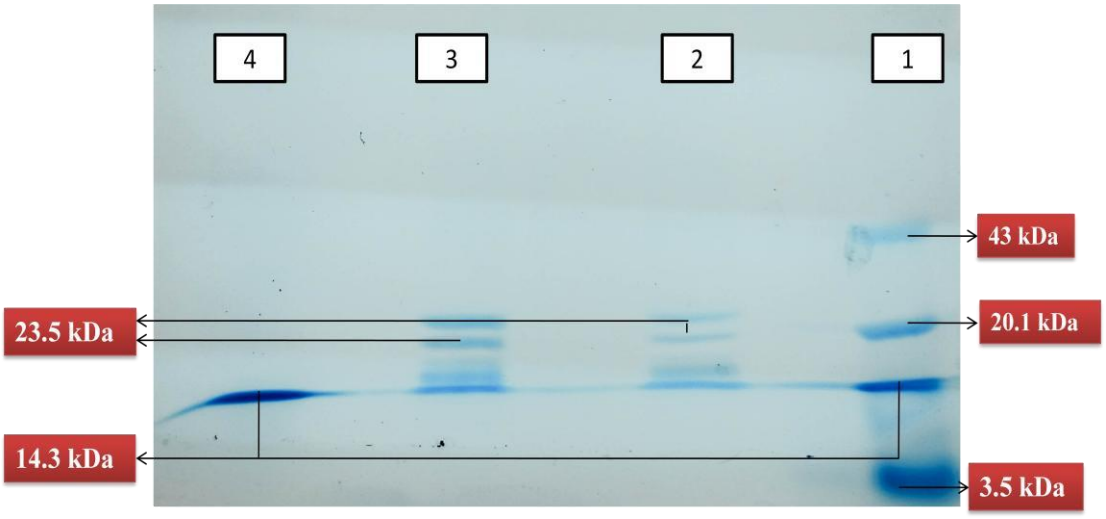

Fig.8: SDS PAGE gel loaded with CE

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From right to left; Lane1: Low range protein molecular weight marker; 3.5 kDa Insulin, 6.5 kDa aprotinin, 14.3 kDa Lysozyme, 20.1 kDa Soyabean trypsin inhibitor and 43 kDa Ovalbumin.

Lane 2 and Lane 3: Crude Extract.

Lane 4: Lysozyme 14.3 kDa molecular weight marker.

Table2: DGelDAS generated report

BAND	Molecular Weight (kDa)			
	LANE 1	LANE 2	LANE 3	LANE 4
1	43	33.54	30.61	14.39
2	20.1	**23.56**	**23.45**	
3	14.3	18.90	19.75	
4	6.5	16.92	17.85	
5	3.5	14.36	14.39	

Analysis by SDS PAGE confirmed the presence of Asp c-II with a molecular weight of 23.59 kDa. This was further confirmed by using DGelDAS software. ION EXCHANGE CHROMATOGRAPHY

The use of the SP Sepharose Fast Flow column allowed the separation of CE into three different fractions as shown in fig.14. Each fraction of was collected separately and stored at -20^{o} C.

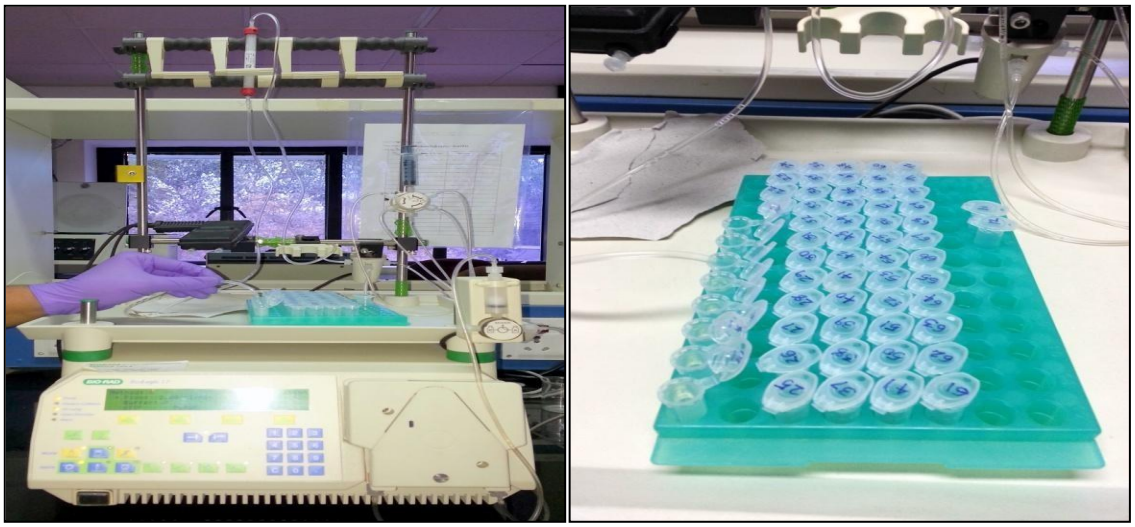

Fig. 9: Running of SP Sepharose column and fraction collection

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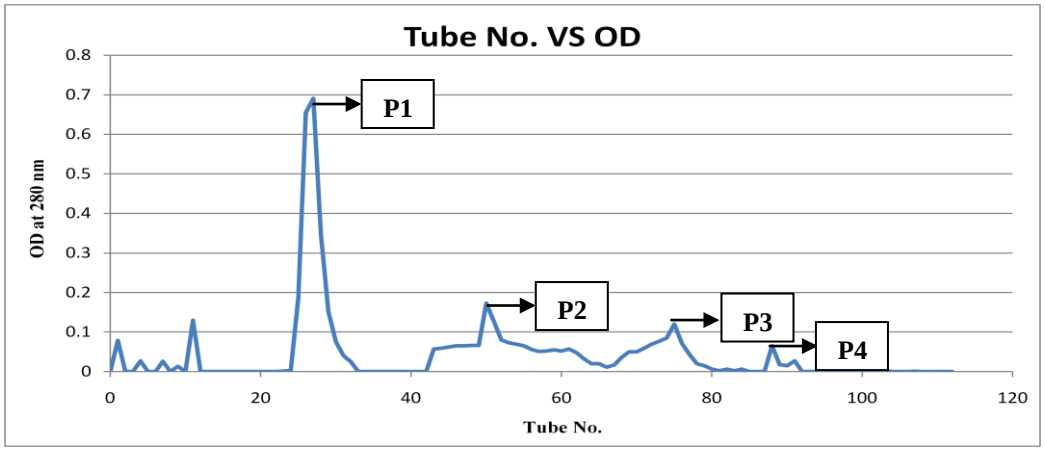

Fig. 10: Chromatogram for IEX chromatography

P1 – Sample injection peak.

P2 – 0.2 M NaCl eluent peak.

P3 – 0.4 M NaCl eluent peak.

P4 – 0.6 M NaCl eluent peak.

The presence of peaks (P2, P3 & P4) confirmed that Asp c-II was eluted along with linear gradient NaCl. These fractions were then subjected to proteolytic assay and other analysis.

PROTEOLYTIC ASSAY

The eluted IEX samples were analyzed for caseinolytic activity using proteolytic assay. The standard plot was obtained by observing the absorbance of tyrosine at different concentrations at 280 nm. The fractions exhibiting proteolytic activity were preserved at -20^{o} C and the rest of the fractions were discarded.

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STANDARD PLOT FOR PROTEOLYTIC ASSAY

Table 3: Absorbance of Tyrosine for standard plot

Sample No.	Concentration of Tyrosine standard (mg/mL)	A280	A_{280}	A_{280}	Average of A_{280}	SD
Blank	0	0	0	0	0	-
S1	0.01	0.045	0.043	0.050	0.046	0.003606
S2	0.02	0.085	0.094	0.085	0.088	0.005196
S3	0.03	0.093	0.155	0.112	0.120	0.031765
S4	0.04	0.145	0.161	0.186	0.164	0.020664
S5	0.05	0.194	0.168	0.226	0.196	0.029052

A_{280} – Absorbance at 280 nm.

SD – Standard Deviation.

S1...S5 – Standard Tyrosine Solution.

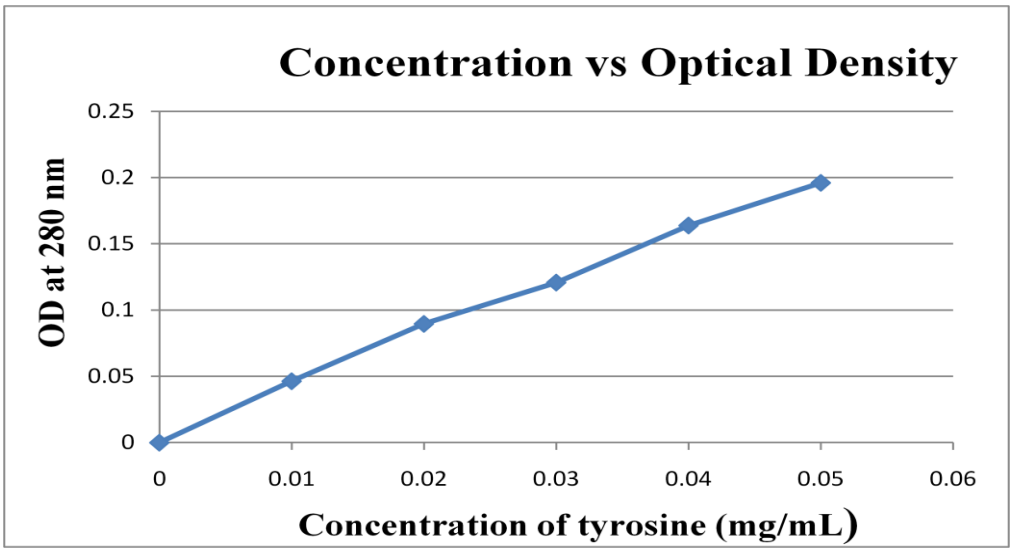

Fig. 11: Standard plot for proteolytic assay

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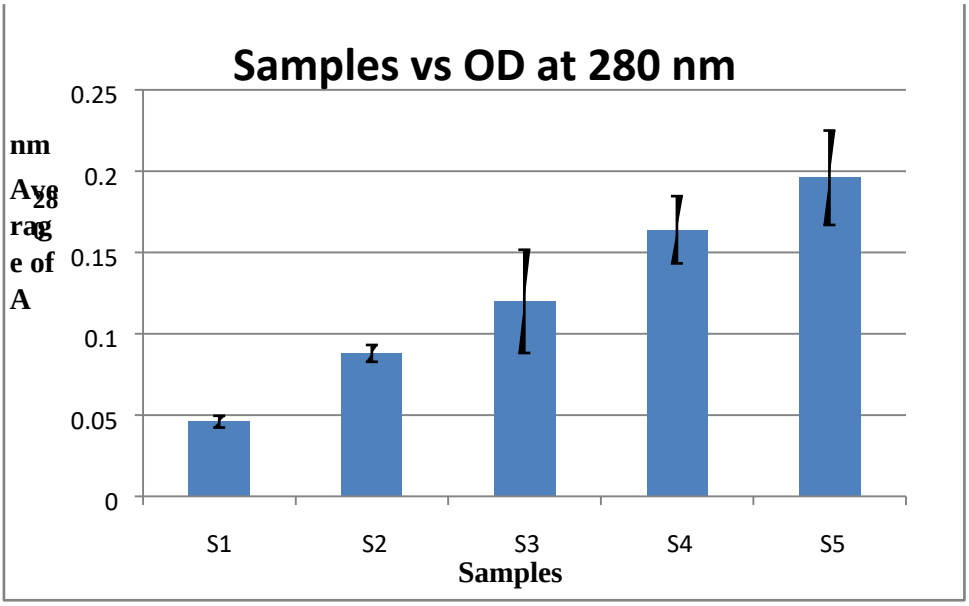

Fig. 12: Error bar chart for proteolytic assay

S1...S5 – Standard Tyrosine Solution.

A_{280} – Absorbance at 280 nm.

PROTEOLYTIC ACTIVITY OF IEX FRACTIONS

Table4: Proteolytic assay values

Sample No.	Average of A_{280}	mg/mL of tyrosine	µM/L equivalents of tyrosine	Units per mL of enzyme
S1	0.0010	0.0015	8.279	25
S2	0.0536	0.0100	82.786	166
S3	**0.0638**	**0.0150**	**82.786**	**248**

S1= 0.2 M IEX eluent.

S2 = 0.4 M IEX eluent.

S3 = 0.6 M IEX eluent.

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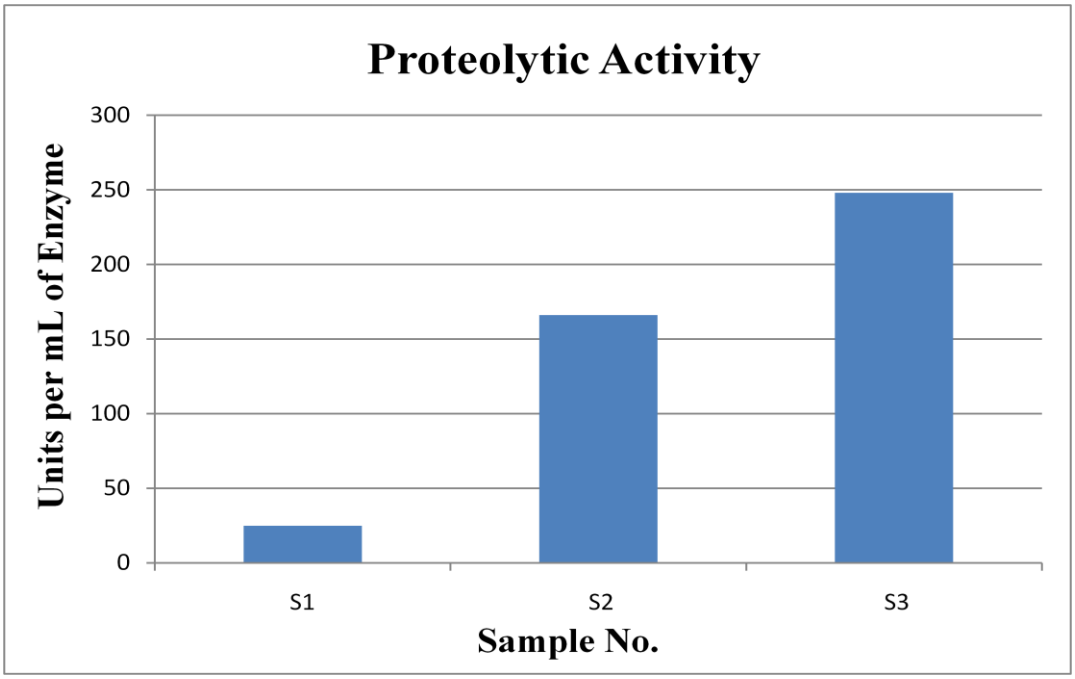

Fig.13: Proteolytic activity of different IEX fraction

S1= 0.2 M IEX eluent.

S2 = 0.4 M IEX eluent.

S3 = 0.6 M IEX eluent.

Since the 0.2 M IEX fraction contained very minimal amount of proteolytic enzyme (i.e., 25 units) it wasn‟t used for future analysis. Only
0.4 M and 0.6 M IEX fractions were further used.

TEMPERATURE STABILITY OF Asp c-II

The temperature stability of Asp c-II was found out by incubating 0.2 mL of 0.6 M IEX fraction of Asp c-II at 40^{o} to 70^{o} C for 30, 60, 90 and 120 minutes and then estimating the proteolytic activity.

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Table5: Proteolytic activity at different temperatures

Temp. ⇩ / ⇨ Time	Units per mL of enzyme			
	40° C	50 ° C	60 ° C	70 ° C
30 minutes	**240**	173	15	0
60 minutes	109	30	8	0
90 minutes	83	16	2	0
120 minutes	3	2	0	0

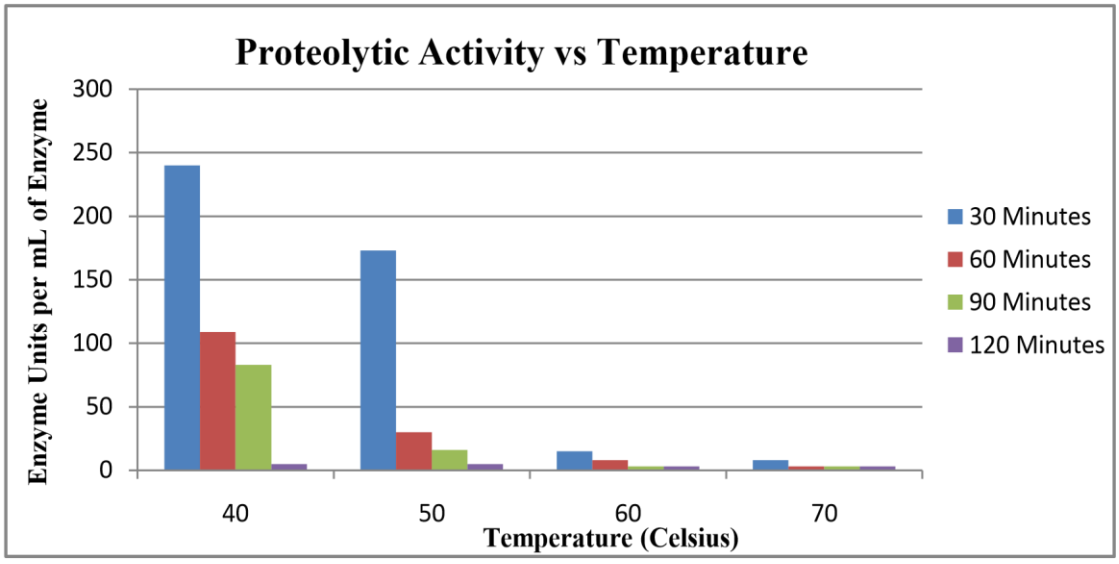

Fig. 24: Thermal stability of Asp c-II
Fig. 14: Thermal stability of Asp c-11

From the graph it is found that, Asp c-II has poor thermal stability properties and degrades at temperatures of above 50° C. pH STABILITY OF Asp c-II

The pH stability of Asp c-II was found out by incubating 0.2 mL of 0.6 M IEX fraction of Asp c-II in citrate phosphate buffer with different pH (5 to 10) for three hours and then estimating the proteolytic activity.

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Table 6: Proteolytic activity at different pH

S. No.	pH	Units per mL of enzyme
1	5	0
2	6	0
3	7	29
4	8	124
5	**9**	**223**
6	10	176

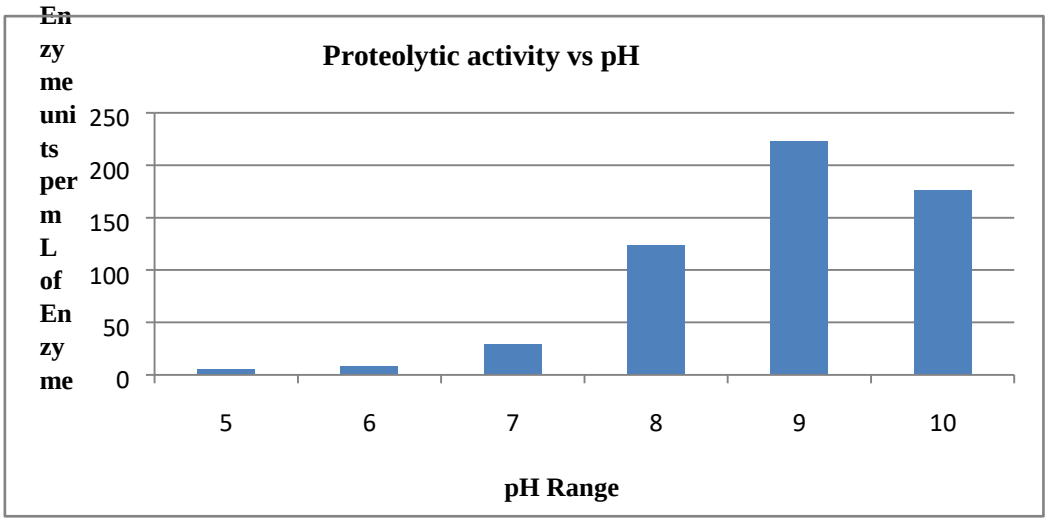

Fig. 15: pH stability of Asp c-II

From the graph it is found that, Asp c-II is stable in the pH range of 7 to 10 and has the maximum proteolytic activity at pH of 9.

ANALYSIS OF IEX FRACTIONS BY SDS PAGE

The IEX fractions were then subjected to SDS PAGE to confirm the presence of only Asp c-II in the eluted samples.

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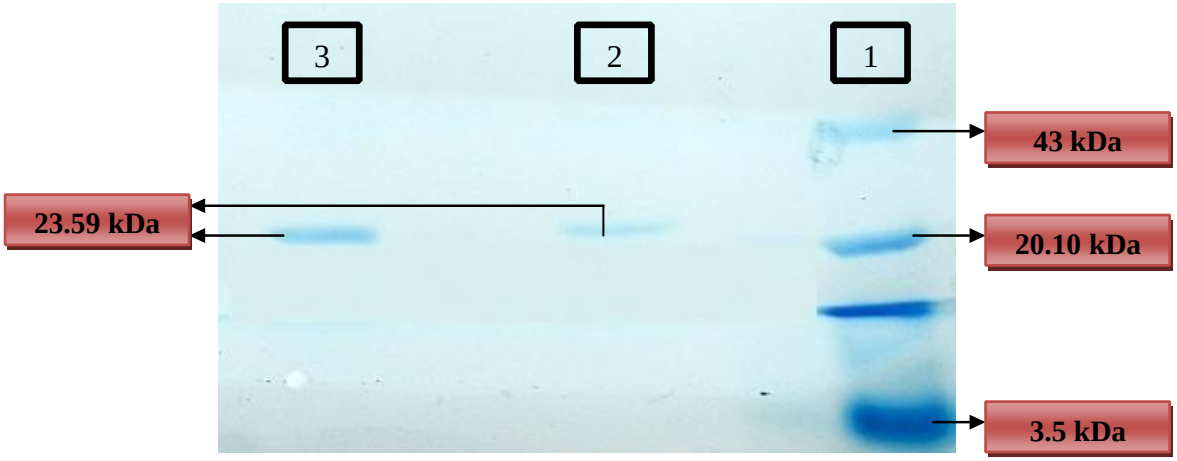

Fig. 16: SDS PAGE gel after IEX chromatography

From right to left; Lane1: Low range protein molecular weight marker; 3.5 kDa Insulin, 6.5 kDa aprotinin, 14.3 kDa Lysozyme, 20.1 kDa Soyabean trypsin inhibitor and 43 kDa Ovalbumin.

Lane 2: 0.4 M IEX fractions.

Lane 3: 0.6 M IEX fractions.

SDS PAGE confirmed the presence of only Asp c-II in both 0.4 M eluent and 0.6 M eluent. This confirmed the isolation of Asp c-II from the latex of *A. curassavica* L.

CHITOSAN NPs CONJUGATED Asp c-II SYNTHESIS

Chitosan NPs loaded Asp c-II were synthesized using ionic gelation method and the TEM results are as follows;

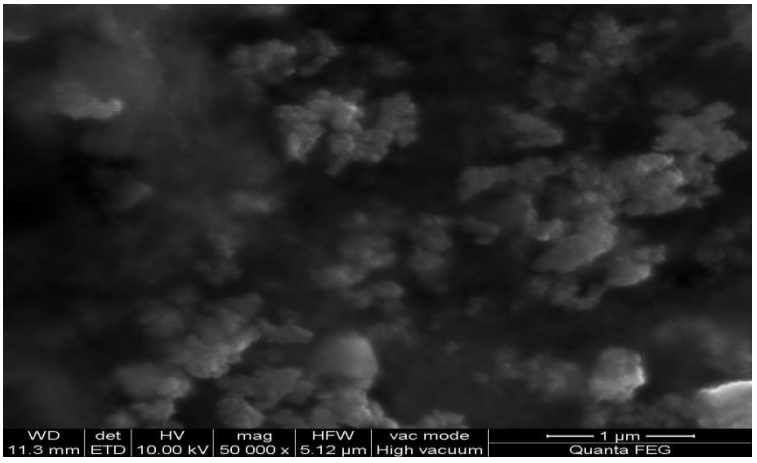

Fig. 17: TEM images of Chitosan NPs conjugated Asp c-II

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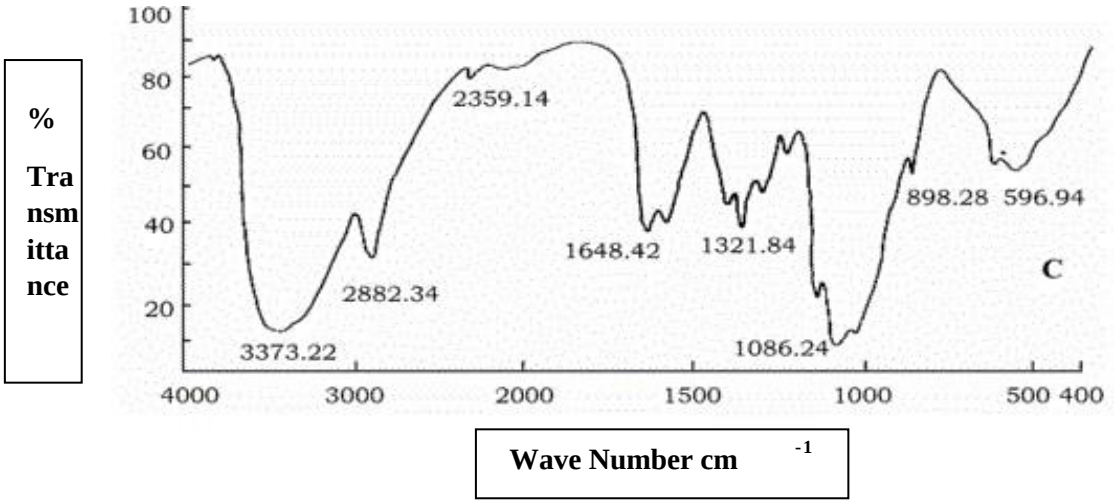
TEM images confirmed the formation of Chitosan NPs. The particle size of Chitosan nano-particle was around 1µm and the mean size distribution was found to be around 380 nm. Since the particle size is 1 µm, it can be used for drug delivery with relative ease.



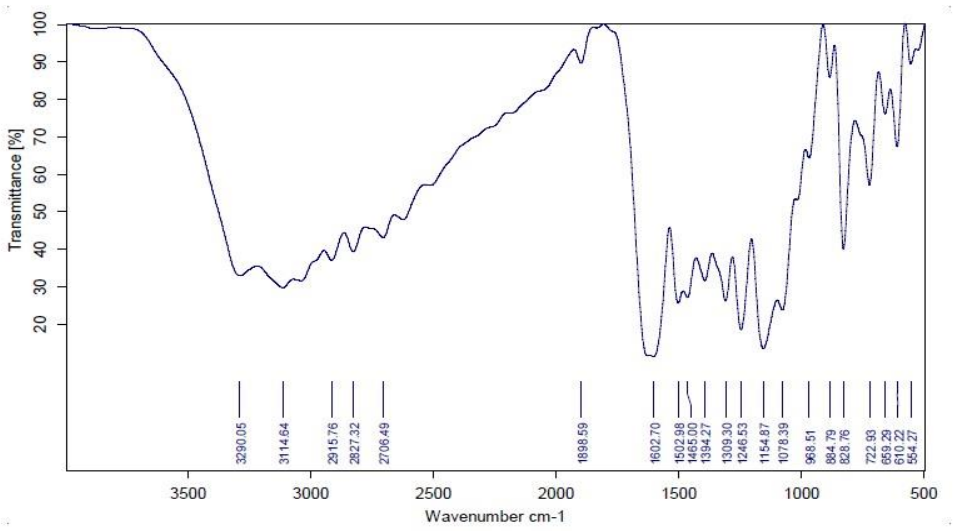


Fig. 19: FTIR for Chitosan NPs conjugated Asp c-II

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When the FTIR peaks of Chitosan NPs and Chitosan NPs conjugated Asp c-II were compared they showed significant difference, thus proving the entrapment of Asp c-II with Chitosan NPs. Fewer peaks were observed in Fig. 28 when compared to Fig. 29. However, the efficiency of binding of Asp c-II with Chitosan NPs is not significantly known.

COAGULATION STUDIES

Coagulation studies performed on human blood confirmed the role of Asp c-II on blood clotting and successful binding of chitosan NPs with Asp c-II.

COAGULATION ASSAY USING BLOOD

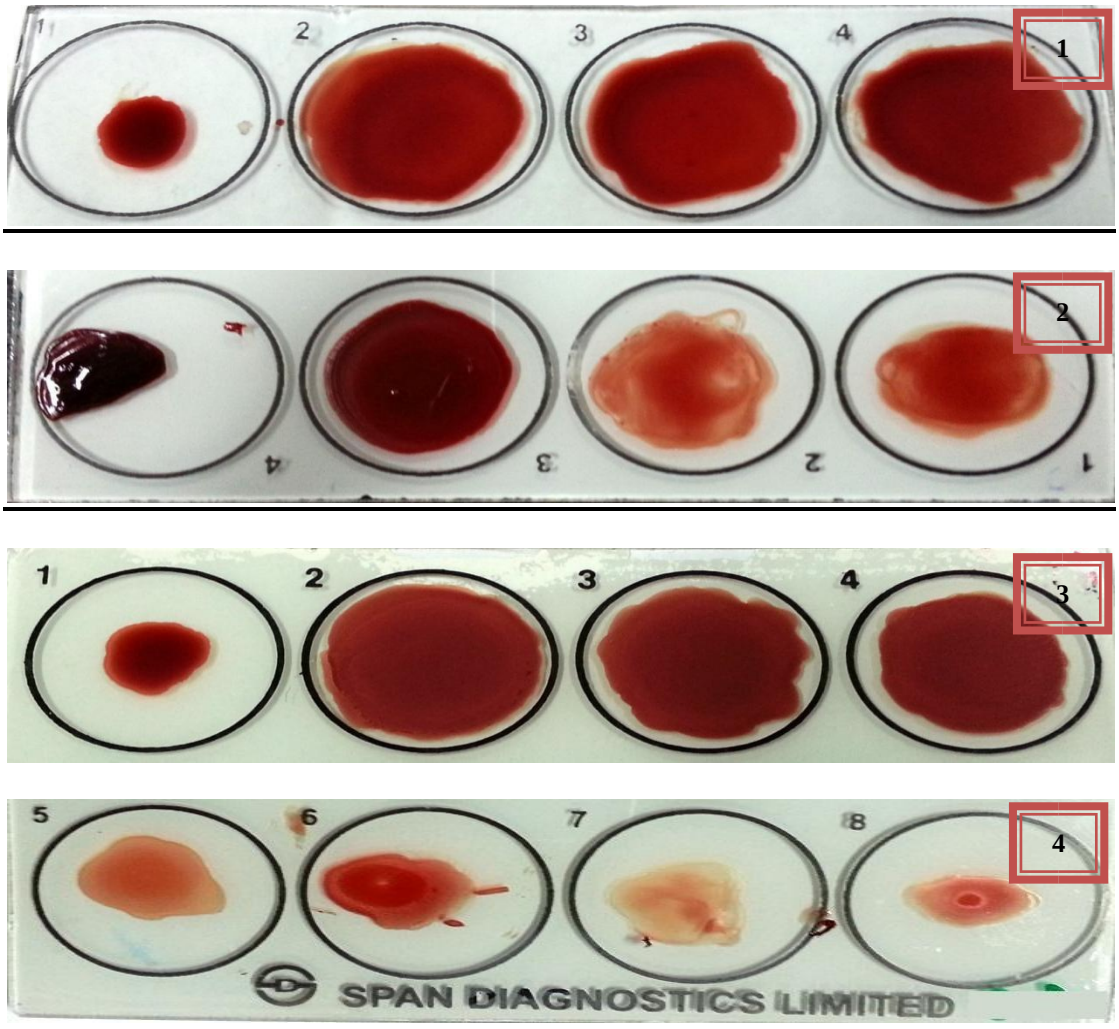

Fig. 20: Blood coagulation assay

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1 - Crude Extract.

2 - 0.4 M IEX fractions.

3 – 0.6 M IEX fractions.

4 – Asp c-II conjugated chitosan NPs.

The first circle in each glass slide is the control and rest three circles contain the test samples.

CE coagulates blood in 5 minutes, whereas 0.4 M and 0.6 M IEX fractions coagulate the blood in 1.5 minutes. Chitosan NPs conjugated Asp c-II coagulates blood in 2.1 minutes. Although the time of coagulation slightly increases for NPs conjugate, due to other beneficial properties of chitosan (aids in wound healing), it could be used as therapeutic product.

COAGULATION ASSAY USING PLASMA

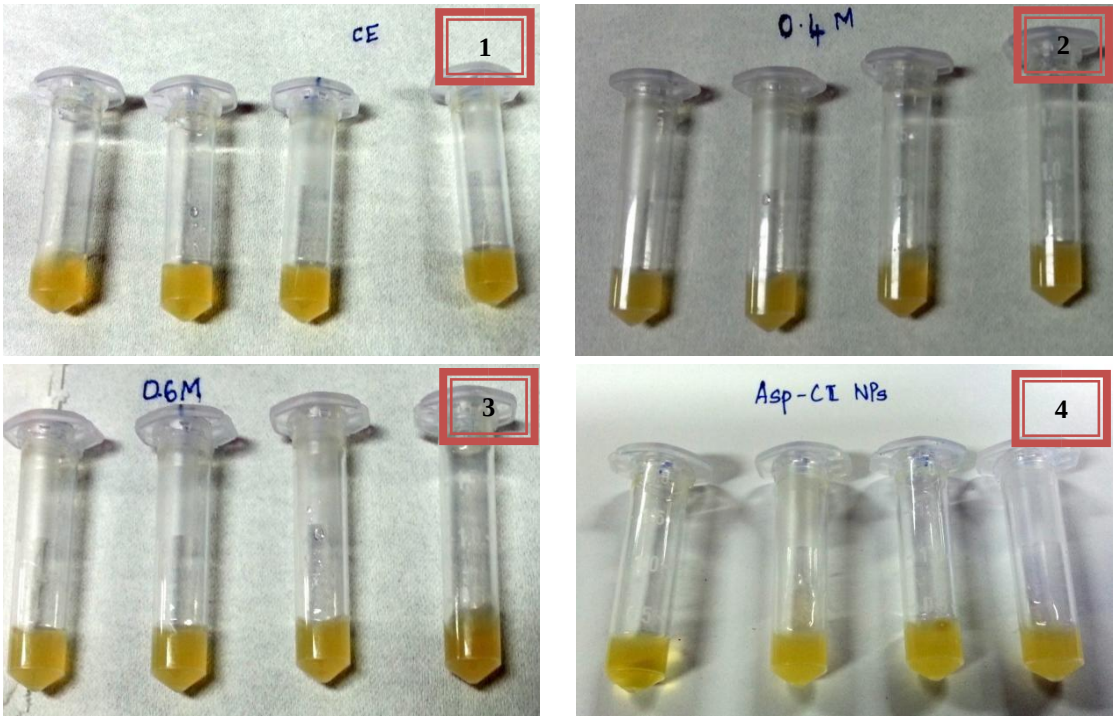

Fig. 21: Plasma coagulation assay

1 - Crude Extract.

2 - 0.4 M IEX fractions.

3 – 0.6 M IEX fractions.

4 – Asp c-II conjugated chitosan NPs.

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The first effendorf tube on the left is the control and the rest of the tubes are test effendorf tubes..

CE coagulates human plasma in 7 minutes, whereas 0.4 M and 0.6 M IEX fractions coagulate the plasma in 3 minutes. Chitosan NPs conjugated Asp c-II coagulates plasma in 3.5 minutes. Although the time of coagulation slightly increases for NPs conjugate, due to other beneficial properties of chitosan (aids in wound healing), it could be used as therapeutic product.

CYTOTOXICITY ASSAY

Table7: Absorbance at 570 nm by B and T lymphocytes – MTT assay

Sample No.	A570	A_{570}	A_{570}	Average of A_{570}	SD
A	1.854	1.769	1.813	1.812	0.0425
B	1.597	1.567	1.534	1.566	0.0315

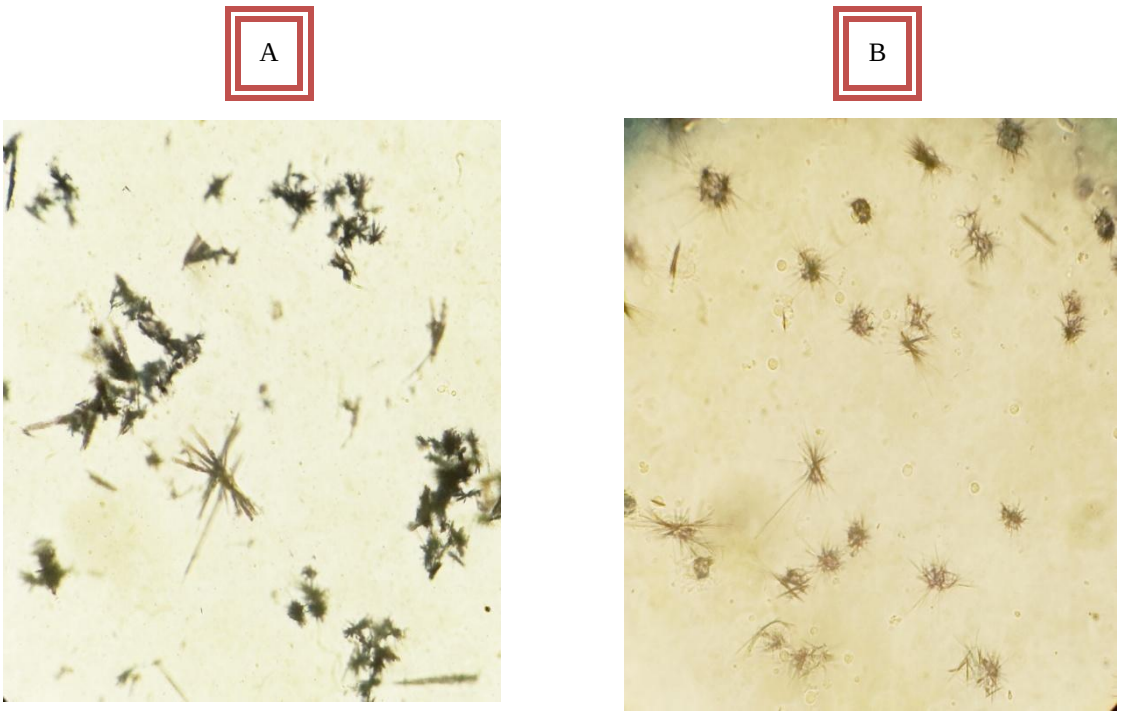

Fig. 22: Formation of formazan crystals – MTT assay

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A – Control.

B – Chitosan NPs conjugated Asp c-II.

A_{570} – Absorbance at 570 nm.

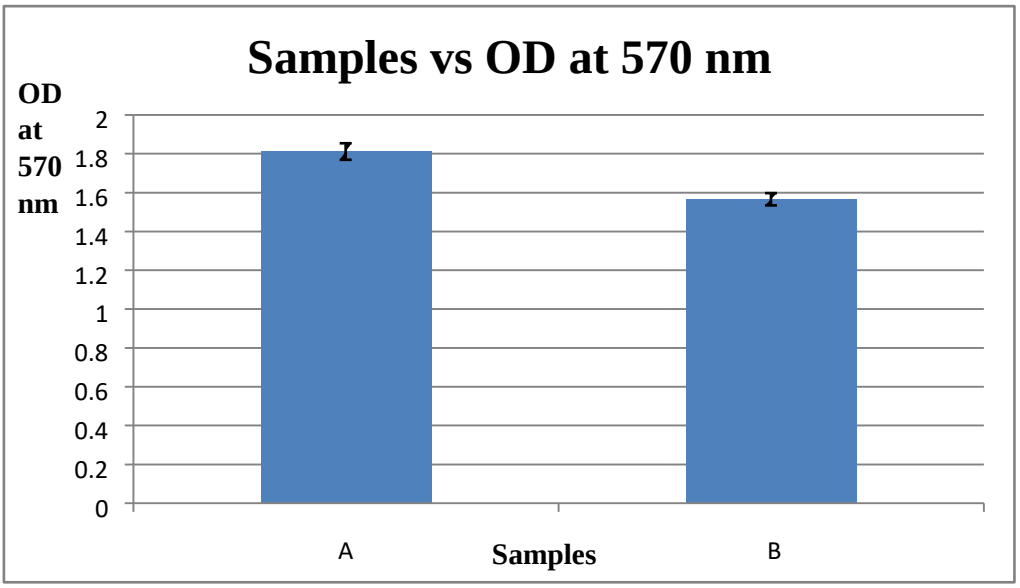

Fig. 23: Error bar chart for MTT assay

A – Control.

B – Chitosan NPs conjugated Asp c-II.

C A_{570} – Absorbance at 570 nm.

Table 8: Viability and Cytotoxicity values

Sample No.	Average of A_{570}	% Viability	% Cytotoxicity
A	1.812	100	0
B	1.566	86.42	13.58

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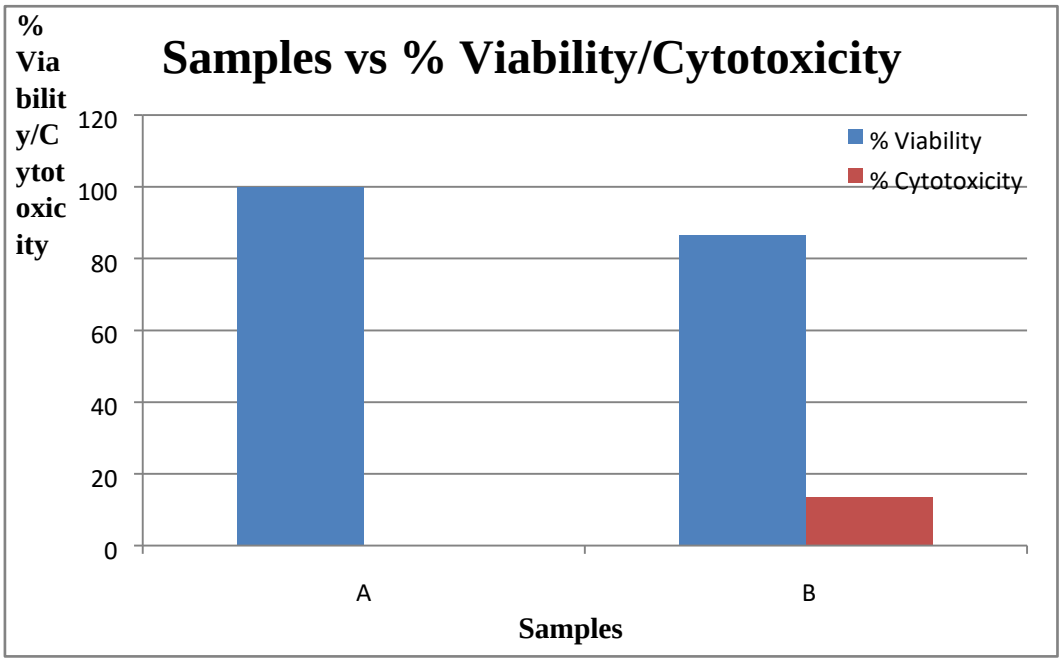

Fig. 24: Comparison of % Viability and %Cytotoxicity using MTT assay

A – Control.

B – Chitosan NPs conjugated Asp c-II.

The Chitosan NPs conjugated Asp c-II showed cytotoxicity of only 13.58% and hence it could be used as a therapeutic agent to assist in blood clotting without any harmful side effects.

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