In silico and molecular cloning studies on the curcin isoforms in *Jatropha* (*Jatropha curcas* L.)

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Dissertation
Submitted in the partial fulfillment of the requirement for the award of degree of
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In
Biotechnology

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CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled, **"*In silico* and molecular cloning studies on the curcin isoforms in *Jatropha* (*Jatropha curcas* L.)"** in the partial fulfillment of the requirement for the award of degree of Master of Science in Biotechnology, Thapar University, Patiala, is an original record of my own research work carried out under the guidance and supervision of **Dr. N. Das,** Professor, Department of Biotechnology, Thapar university, Patiala, India. The content in the dissertation has not been submitted to any other university or institute for award of any other degree.

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CERTIFICATE

This is to certify that the dissertation entitled "***In silico* and molecular cloning studies on the curcin isoforms in *Jatropha* (*Jatropha curcas* L.)"** submitted by **Neha Bhardwaj** (Regd. No. 301501012) in partial fulfillment of the requirement for the award of the degree of Master in Science in Biotechnology, to Thapar University is a record of student's own work carried out by her under my guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

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(Neha Bhardwaj)

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LIST OF ABBREVIATIONS

Abbreviation	Name
BLAST	Basic Local Alignment Search Tool
BLASTp	BLAST for Proteins
Bp	Base-Pair
$CaCl_2$	Calcium chloride
DEPC	Diethyl pyrocarbonate
DNA	De-oxy Ribo nucleic acid
dNTP	2'-deoxynucleoside-5,-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
HCl	Hydro chloric acid
IgE	Immunoglobin E
IU/ml	International unit per mL
Kb	Kilo Base
kDa	Kilo-Daltons
LiCl	Lithium chloride
M	Molar
mM	Mili-molar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
Nm	Nanometer
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
Pmoles	Picomoles
RIPs	Ribosome inactivating proteins
RNA	Ribo nucleic acid

rRNA	Ribosomal RNA
Rpm	Revolutions per minute
S (60S)	Svedberg unit
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
µL	Microlitre
µg	Microgram

ABSTRACT

The biodiesel crop *Jatropha* (*Jatropha curcas* L.) has gained world-wide importance during the last few decades. Its non-edible seed oil could be converted to biofuel for blending with fossil fuels. A number of important metabolites of Jatropha have significant commercial importance as the contents could be used in making cosmetics, soaps, fertilizers and lubricants. Curcins, a group of toxic proteins are present in *Jatropha* seeds and other tissues. They are basically Ribosome inactivating proteins (RIP) which render cytotoxic effects. Interestingly, curcin proteins have some pharmacological importance. Since, curcin proteins could be used as anti-tumor, anti-HIV, antiviral and immunosuppressive agents. The present study focused mainly on two curcin isoforms namely Curcin 2A (GenBank protein id: ABZ04128.1) and Curcin precursor (GenBank protein id: AAL86778.1). Several facile modern bioinformatics tools have been employed to generate data comprising of searching protein motifs, 3-D modeling, phylogenetic tree, Ramachandran plot and some other important attributes. Many of these features as obtained in this study were not reported earlier. Good quality RNA was isolated from different *Jatropha* tissues namely leaf, seed kernel and seed pericarp. RT-PCR approach was adopted using total RNA and oligo (dT) primer. The size of each amplicon was ~1.0 kb. A number of putative Curcin 2A specific cDNA clones were obtained using total RNA from different *Jatropha* tissues. The putative cDNA clones in pMD 20-T vector were partially characterized by restriction analysis and PCR. These cDNA clones will be sequenced for further use in recombinant protein expression. sequenced prior to undertake molecular approaches for recombinant expression of the individual curcin isoforms.

Key words- *Jatropha curcas* L., Curcin isoforms, Protein motifs, 3-D modeling, Polymerase chain reaction, Molecular cloning

CHAPTER-1 INTRODUCTION

1.1 *Jatropha curcas* L.

Jatropha curcas L. (physic nut or purging nut) an all-purpose plant is a drought repellent succulent shrub and a potential biofuel crop (Kazuo et al. 1996). The crop is known by 180 other names across the world such as Chandrajot, Jungli Arandi, Danti, etc. *Jatropha* oil meets the American standards due to its property of getting transformed to liquid bio-fuel. Most of the *Jatropha* plants are poisonous; therefore, the seed oil is non-edible by humans. Moreover, the other parts of this plant are not consumed by animals. It occurs mainly at lower altitudes with temperature above 20°C but can grow at higher altitudes also. *Jatropha* is derived from the Greek word *jatr'os* (doctor) and *troph'e* (nutrition), reflecting medicinal aspects of the crop.

Features of *Jatropha:* The *Jatropha* plant is perennial, small woody shrub with 3 to 5 m average height. Under favorable conditions, the plants can grow up to a height of 8 to 10 m. The bark of the tree is grey in color but when cut secretes white colored latex (Fairless 2007). Leaves are heart-shaped 4-6 lobed and turn from yellow to dark green on maturity. Fruits are grey brown in color and have 3 diploid seeds. Harvesting of seeds is done once in a year. *Jatropha* seeds are rich source of unsaturated fatty acids thus used for extracting oil.

Cultivation of *Jatropha:* *Jatropha* can grow in tropical and subtropical areas where the soil quality is very poor, saline, eroded and stony (Rehm et al. 1992). This crop can be grown in wastelands due to its properties like easy and rapid propagation, wide adaptation and short gestation period. The crop is cultivated in many states of India namely, Gujarat, Madhya Pradesh, Rajasthan, Maharashtra and Tamil Nadu. The flowers develop at the tip of stem and the best seed yield is observed after 2-3 years of its plantation (Janick et al. 2008). Different parts of *Jatropha curcas* are shown in **Fig.1**.

Fig. 1: Flower, Leaf, Fruits and seeds of *Jatropha* (Ref: http://www.flickr.com/photos/naufal/)

1.2 Multifarious uses of *Jatropha*

- *As food*: Some non-toxic varieties found in Mexico and Central America do not contain phorbol esters(toxin present in *Jatropha),* hence, leaves after steaming and seeds after roasting can be consumed as food (Makkar et al. 1998).
- *Industrial application:* Glycerine, a byproduct of *Jatropha* seed oil during transesterification, is used in formation of soaps and candles. Another widely used application is the production of biodiesel from seed oil (Nahar et al. 2011). Typically, biodiesel refers to methyl or ethyl esters of long and medium chain fatty acids.
- *Insecticidal property:* The aqueous extract has the property to control pests of pulses and potato crop.
- *Medicinal uses:* All tissues of *Jatropha* plant have medicinal importance be it leaf, stem, seed, root or bark. Traditionally, they have been used for veterinary purpose (Dalziel 1955; Duke 1985; Duke, 1988).
- *Environmental benefits:* The plants can be cultivated on wasteland and thus controls soil erosion.

The above aspects are presented in **Fig. 2**

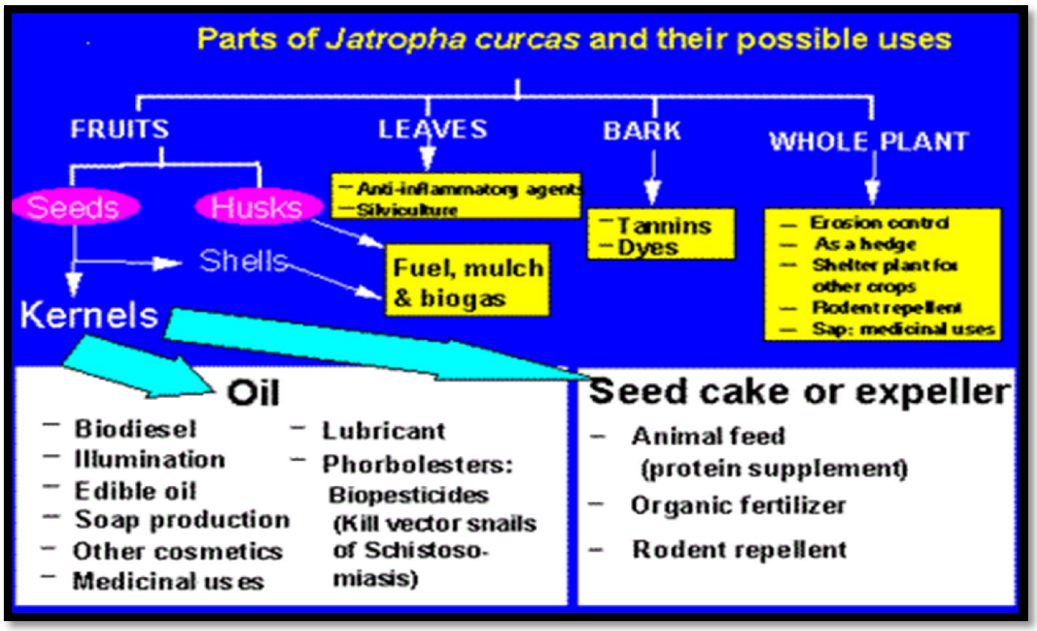

Fig. 2: Various parts of *Jatropha* and their uses (Ref: http://www.asapempowers.org)

The medicinal values of *Jatropha* are well-recognized as shown in **Fig. 3**. Leaf extract of this plant is used for treating scabies, eczema and ringworm. The stem sap is used to cure toothache, gum bleeding and other wounds. It also cures dermatological diseases. Antidote for snake bite and other inflammation

can be cured by the root extracts. Oil of the seeds has the property to treat gout, arthritis, jaundice and skin infections.

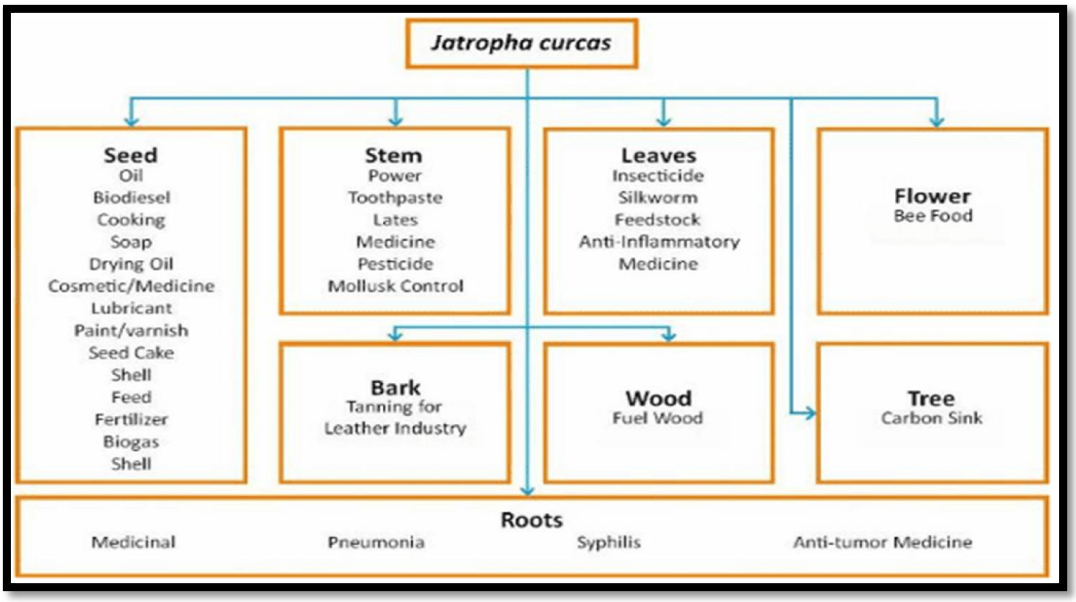

Fig. 3: Medicinal uses of *Jatroph*a (Ref: http://www.thecropsite.com)

Curcin, a toxic protein found earlier in the seeds, shows *anthelmintic property*. Anthelminthics are group of antiparasitic drugs that expel parasitic worms from the body. Helminths cause life threatening diseases like anaemia, eosinophilia and pneumonia (Bundy 1994). Ahirrao et al. (2011) reported that *Jatropha* has saponins and alkaloids in huge amount. Jummai et al. (2014) also carried out experiments on helminths and concluded that curcin causes breakdown of central nervous system (CNS) of helminthes by blocking the glucose supply thus paralyzing the parasite (Mute 2009; Sutar et al. 2010; Sharma et al. 2010; Mali et al. 2007). Curcin also shows antitumor activity. It was shown that curcin protein could inhibit the growth of tumor cells at very low concentration. El-Baz et al. (2014) reported anntihyperglycemic activity of methanolic extracts of *Jatropha* leaves. Leaves also have antiulcer activity.

1.3 Toxicity of *Jatropha*

Jatropha contains curcins, lectins, saponins, phytate, phorbol esters which are briefly described below. Toxic components have made *Jatropha* non-edible for both humans and animals (Sujatha 2009). Seeds are the most toxic part of the plant.

Curcin: The first toxic protein extracted was curcin (Felke 1914), a toxalbumin, which is a Type I Ribosomal Inactivating Protein (RIP). Crude curcin is found in the seed endosperm. It is a hybrid molecule consisting of toxic peptide chain linked to an antibody, and act as immunotoxins.

Lectin: Lectin is not the major toxic component in *Jatropha* (Aderibigbe et al. 1997; Aregheore et al. 1998). However, the activity of lectins in the seeds can be reduced by heat treatment.

Phorbol esters: These are the major toxic constituents of *Jatropha* seeds. The toxicity of phorbol esters affects humans and animals by causing tumor promotion, cell proliferation, erythema of skin, lymphocyte mitogenesis, prostaglandins production.

CHAPTER 2: REVIEW OF LITERATURE

2.1 *Jatropha curcas:* A biodiesel plant

During the last few decades, *Jatropha* is of great interest due to its ability to produce biofuel. *Jatropha* seeds contain proteins, fibers and four most important fatty acids namely palmitic acid, linoleic acid, oleic acid and stearic acid esterified to glycerol i.e., in the form of triacylglycerol (Rehm and Espig 1991). Oil content in the seeds is very high ranging from 30-50%. The seed oil is allowed to undergo transesterification reaction with alcohol, namely methyl alcohol and ethyl alcohol to produce the esters of the fatty acids i.e., biodiesel along with glycerol. The biodiesel thus produced can be used in existing diesel engines (Achtena et al. 2008). Biodiesel is non-polluting, biodegradable and environmental friendly as it does not produce sulphur and carbon. Process of production of biodiesel is presented in **Fig. 4**.

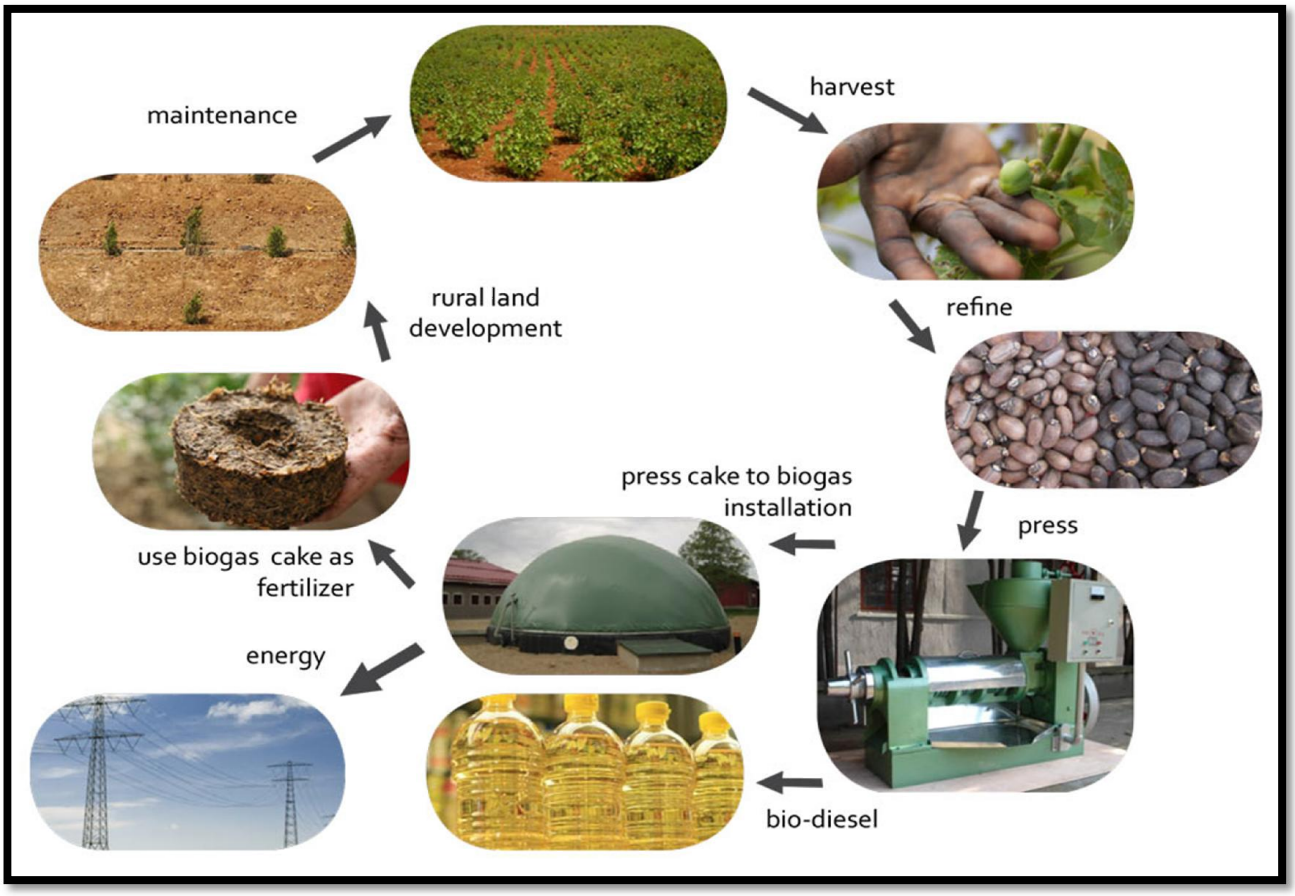

Fig. 4: Process of Biodiesel formation (Ref: http://www.nstda.or.th)

Jatropha grows fast and begins yielding oil in the second year and for the next forty to fifty years. Optimal yield is obtained from the sixth year of plantation, and spaced at a distance of 2 meter. It is

observed that in an area of 1 hectare 2500 plants can be cultivated. It is environmental friendly as it absorbs large amount of carbon dioxide from the atmosphere. Detoxified oil of *Jatropha* can be used as a rich protein supplement in feed for animals.

2.2 Ribosome Inactivating Proteins (RIPs)

Toxins present in *Jatropha* are Phorbol esters, Saponins and Lectins (curcin). Phorbol esters are structure specific and induces tumor by initiating the activation of protein kinase pathway and other cellular proteins (Ahmed et al. 2009). Lectins have high affinity for getting bound to the carbohydrate moiety (Goldstein and Hayes 1978), and its major role is in plant defense system against pathogen. The toxicity of curcin is due to presence of Ribosome Inactivating Proteins (RIP).

Plants have various defense mechanisms against pathogen attacks (Chrispeels et al. 1991). The defense mechanism evolved by plants includes RIPs, which are capable of inactivating ribosomal proteins. Isolation of RIPs were done a century ago in *Angiopermae*, in both mono and di-cotyledons and in mushrooms (Yao et al. 1998; Lam and Ng, 2001), and are known to have catalytic toxins which create an irreversible toxic effect on protein synthesis by damaging ribosomal machinery (Joerg et al. 1997). The level of RIPs in plants varies from traces to hundreds of milligrams. They exist in almost every tissue like roots, stems, leaves, bark, flowers etc. (Park et al.).

RIPs possess antifungal and anti-tumor activity (Luo et al. 2007). They have been used as a mid-term aborting agent and for the treatment of hydration moles (Stirpe et al. 2006). RIPs are attractive molecules because immunotoxins could be constructed from them, namely conjugates with antibodies capable of selectively directing them towards specific targets. Pharmacological attributes associated with RIPs are diverse; they include Protein synthesis-inhibitory, anti-tumor, antiviral immunosuppressive and anti-HIV.

RIPs need various cofactors for maximal inhibitory activity on translation (Carnicelli et al. 1992). They depurinate non-mammalian ribosomes from insects (Zhou et al. 2000), plants (Iglesias et al. 1993), yeast (Roberts and Selitrennikoff, 1986) and bacteria. It was also found that (i) from every ribosome, RIPs remove more than one adenine residues (Barbieri et al. 1994) and (ii) RIPs also remove adenine residues from polynucleotides and DNA.

Large rRNAs have the conserved alpha-sarcin loop which are depurinated by the toxic N-glycosidases of Ribosome-inactivating proteins (RIPs) (Peumnas et al. 2001). Participation in protein synthesis is blocked by the inactivation of depurination.

A well-characterized toxin enzymatically alters the 28S rRNA of the large 60S ribosomal subunit which specifically and irreversibly inhibits protein synthesis in eukaryotic cells (Stirpe et al. 1976). Depending on structure and function, RIPs are usually divided into two subgroups: type 1 RIP and type 2 RIP as given in **Fig. 5**. Type 1 RIPs are monomeric proteins of approximately 30 kDa which possess RNA *N*-glycosidase enzymatic activity. In contrast, type 2 RIPs are composed of an A-chain with RNA *N*-glycosidase activity associated to one or several B-chain(s) of approximately 35 kDa. The A chain is function equivalent of a Type 1 RIP, and the B chain is a lectin (Stirpe et al. 1986).

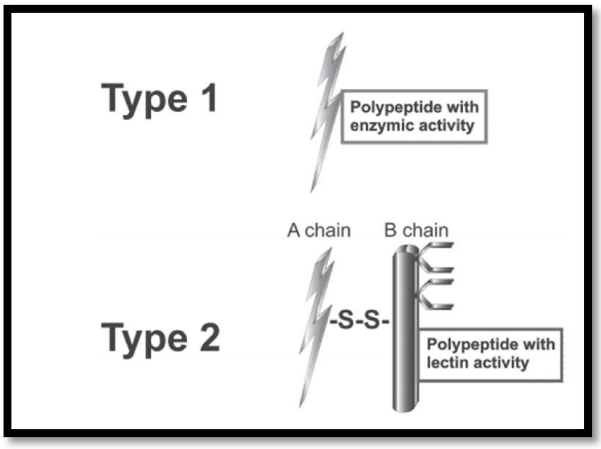

Fig. 5: Types of RIPs (Ref: F.Stirpe/ Toxicon 44 (2004))

Type I RIPs are made up of the toxic subunit (A-chain) alone, while type II RIPs consist of a toxic A-chain and a lectin-like subunit (B-chain) linked together by a disulfide bond (Stirpe 2004). The toxicity of A-chain is due to its RNA-N-glycosidase activity (Peumnas et al. 2001), by which it brings about depurination of adenine at position 4324 in the 28S rRNA. This activity prevents the formation of a critical stem–loop configuration, to which the elongation factor is known to bind during the translocation step of translation (Qin et al. 2005). The end result of this activity leads to complete inhibition of cellular translation. The role of the B-chain is to help in the transport of RIPs into the cells by binding to specific sugar residues of glycoproteins or glycolipids on the plasma membrane and internalization by endocytosis. The B chain, once binds to the cell, allows and facilitates the entry of toxins in cell (Walsh et al. 2013). The A chain can then exert its enzymatic activity leading to damage of ribosomes and other structures resulting in cell damage and cell death. A schematic representation of biochemical action of RIPs is given in **Fig. 6.**

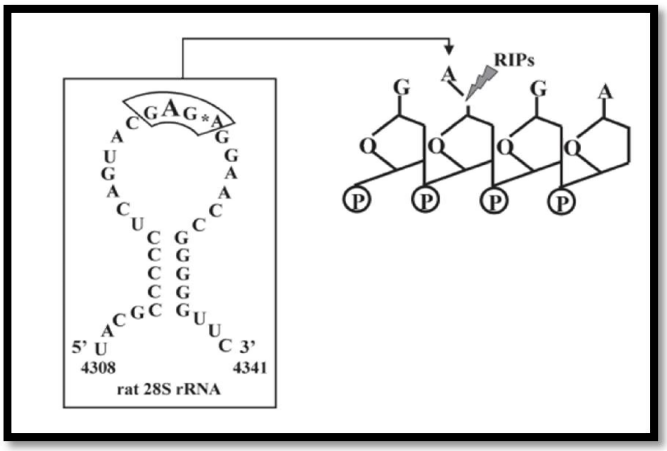

Fig. 6: Biochemical action of ribosome-inactivating proteins (RIPs)
(Ref: http://www.jnsci.org/files**)**

N-glycosylation activity of curcin was checked by incubating ribosome with various amounts of curcin (Lin J et al.). The extracted rRNA was then checked by Agarose gel electrophoresis. RNA fragments were observed when rRNA from ribosome which was curcin-treated was treated with aniline at acidic pH. When ribosome was treated with curcin only these fragments did not appear. The fragment got released due to action of curcin with acid-alinine treatment depicting *N*-glycosylation activity.

2.3 Entry of RIPs inside cell

During entry into cell, part of a RIP first enters into golgi apparatus followed by to cytoplasm, and finally to lysosomes where they get degraded and are expelled from the cells. At every destination, difference in cytotoxicity inhibits the protein synthesis and results in cell damage.

2.4 Anti-tumor activity of Curcin

Earlier, it was studied that cell death was induced by inhibiting cell-free translation and protein synthesis. Also, Type I RIPs were known to be less toxic than Type II as they lack binding moieties which hinder them. Recent works by Mohamed et al. (2014) demonstrated that cells exposed to type I RIP curcin showed cytological and subcellular response. It was done by exposing normal and cancerous cell lines to curcin. Experiments were performed to explore the toxicity of normal cell line to cancer cell lines. Curcins are known to affect Nuclear factor κB (NF- κB), Focal adhesion Kinases and Reactive Oxygen Species (ROS). Destruction of cancer colonies were also observed along with suppression of anti-apoptotic survivin. It was concluded that the level of cellular activity depends on proper functioning of cell-organelles and dysfunction leads to impaired cyto-metabolism.

Currently, pharmaceutical agents available are of natural origin, which are of great advantage and also there is a need to find new and potent versions. Curcin is a cysteine-containing RIP, which might be ideal for preparation of immunoconjugates, which can be used as a promising chemotherapeutic agent in future.

2.5 Immunological effects of RIP

Immunogenic: Paul Ehrlich gave the knowledge about immunogenicity of RIPs. When animals consume them, antibodies formation occurs because they are strongly immunogenic.

Allergenic: Administration of Type I RIPs induces an IgE response and enhances the response against other antigens.

Immunosuppressive: Administration of RIPs create an immunosuppressive effect on both cell mediated and humoral response, i.e. prevent formation of antibodies.

2.6 Work done so far on *Jatropha* in the laboratory

Jatropha accessions have been collected from different agro-climatic zones in the state of Punjab, a North-Western state of India. Kumar (2015) worked on the four *Jatropha* accessions namelyTJS17#03, TJS42#04, TJS35#01 and TJS01#03. Crude curcin extract was prepared from the seed kernel of TJS17#03. The cytotoxic effect of crude curcin was assessed by MTT assay on RAW and HeLa cell lines. The crude curcin extract exhibited inhibitory effect on the cancer cell lines significantly. There are different forms of curcin namely curcin precursor, curcin2A, curcin-L precursor and ribosome inactivating protein (RIP). Some studies were also done at molecular level. For example, BLAST analyses revealed that the promoter region of the curcin gene showed considerable sequence divergence if compared with the other members of this family. In addition, efforts were made to isolate good quality genomic DNA from field-grown tender leaves using a simple and efficient protocol as introduced earlier in the laboratory. PCR was carried out to amplify the 5′-flanking regions (i.e. promoter regions) of various curcin genes and the amplicons of various sizes were reported. Kaur (2016) carried out the sequence analysis of curcin, and predicted some motifs. Multiple sequence alignment was made using different forms of curcin namely, curcin precursor, curcin2A, curcin-L precursor, ribosome inactivating protein (RIP) and ribosome-inactivating protein cucurmosin-like, *Jatropha curcas*. Moreover, Hydropathy plots of curcin2A protein revealed that the N terminal part of this protein is hydrophobic. In

addition, efforts were made to isolate RNA from different tissues of *Jatropha*. RT-PCR approach was adopted to see the expression patterns of curcin2A and curcin precursor in *Jatropha.*

2.8 Objectives of the study

Molecular cloning and thorough sequence analysis are prerequisites for recombinant expression of curcin. Since curcin protein has considerable pharmaceutical importance. Keeping in view the overall progress so far in the laboratory, this thesis work focused on the following objectives:

- To carry out sequence analysis, and prediction of various protein motifs and 3-dimensional structural features of curcin isoforms

- Molecular cloning of cDNAs encoding curcin isoforms through RT-PCR approaches, and the preliminary characterization of the cDNA clones

CHAPTER- 3 MATERIALS & METHODS

3.1 *In-silico* analysis

A flowchart of strategies for the identification and study of curcin isoforms of *Jatropha*:

Collection of the GenBank accession numbers of the curcin isoforms of *Jatropha*

↓

BLASTp at NCBI (http://www.ncbi.nlm.nih.gov)

↓

The sequences were retrieved and selected for further analysis

↓

Sequence analysis, predicting motifs and 3-dimensional structure of curcin protein

Biochemical characterization: The theoretical pI, amino acid composition, aliphaticity and molecular weight were determined by using EXPASY server (http://web.expasy.org/protparam/).

Identification of conserved domains: The curcin protein sequences were then uploaded to MY HITS (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and different sites were predicted.

In silico characterization**:** Phyre2 tool (http://www.sbg.bio.ic.ac.uk/~phyre/) was used for secondary structure prediction and protein fold recognition. Many pdb files of the curcin protein having homology were generated out of which one best model was selected and uploaded on ProSA.

*3-D modelling***:** ProSA-web tool (https://prosa.services.came.sbg.ac.at/prosa.php) gives the protein structure analysis of the protein models. ProSA gave reliable protein models, which were selected on the basis of z-score and X-ray/NMR region. The models which had lowest negative z-score and fall in the x-ray/NMR region were selected. Further energy minimization of selected models was done by NOMAD Ref (http://lorentz.immstr.pasteur.fr/gromacs/minimization_submission.php).

Validation of model: The minimized pdb models obtained from NOMAD-Ref were validated for structural analysis and verification by SAVES (https://services.mbi.ucla.edu/SAVES/) and stereochemical quality and accuracy by PROCHECK in Rampage server (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

3.2 Molecular cloning studies

3.2.1 *Jatropha* plant samples

For RNA isolation, stem and seed samples were collected from *Jatropha* cultivar TJS 04 #42 maintained at *Jatropha curcas* L. germplasm bank, COS complex, Thapar university, Patiala.

3.2.2 Other requirements

Enzymes were purchased from Bangalore Genei Pvt. Ltd., Bengaluru. Chemicals required were bought from Sigma-aldrich India Pvt. Ltd., and Himedia Pvt. Ltd., Mumbai. Primers used were synthesized by Eurofilms Genomic Pvt. Ltd., Bengaluru. Other chemicals were prepared in laboratory given in **Table 1**.

Table 1. Different chemicals and their composition

Chemicals	Composition	Volume
Gel Loading Buffer (5X)	Sucrose EDTA Tris Bromophenol	35% (w/v) 50 mM (pH 8.0) 25 mM 0.2% (w/v)
TBE (5X) Buffer (pH 8.0)	Tris Base Boric Acid EDTA	54 g L^{-1} 28 g L^{-1} 3.8 g L^{-1}
TE Buffer	Tris HCl EDTA	10 mM (pH 8.0) 1 mM (pH 8.0)
Extraction Buffer	Tris HCl EDTA NaCl Sucrose	50 mM (pH 8.0) 50 mM 250 mM 15%

Ethanol, Isopropanol, Potassium acetate solution (5.0 M), Sodium acetate, RNase, Alcohol, Chloroform

3.1.6 Enzymes used in molecular techniques

Restriction enzymes

Restriction enzymes such as *Eco*RI, *Hind*III, *Bam*HI and *Sma*I were used in this study. Restriction digestion was carried out in buffer supplied by manufacturer. Depending on specific enzyme, reaction was carried out at appropriate temperature and BSA was added as required (for e.g. BSA is required during *Bam*HI restriction digestion reaction).

Other enzymes used in the study

Ribonuclease A

Stock solution - 10 mg mL^{-1}

Working solution - 10-15 $mgmL^{-1}$

DNAse free Ribonuclease A was prepared in a buffer containing 10 mM Tris (pH 8.0) and 15 mM NaCl. The solution was boiled for 10 minutes, followed by the slow cooling by placing at room temperature for 2 hours, and then stored at - 20 °C for subsequent use.

Chemicals/Biochemicals

X- gal (5-Bromo- 4-chloro- 3-indolyl-β-D galactoside)

Stock conc. - 20 mg mL^{-1}

Working conc. - 20 μgmL^{-1}

X-gal was prepared by dissolved in N, N- dimethyl formamide.

IPTG (Isopropyl thio ß- D- galactoside)

Stock conc. - 100 mg mL^{-1}

Working conc. - 100 μgmL^{-1}

It was prepared in fresh and sterile (autoclaved) water.

3.3 Methods

3.3.1 Isolation of total RNA from different tissue samples of *Jatropha curcas*

Isolation of total RNA was done from various tissues like seed, seed kernel and seed pericarp. *Jatropha* plants were harvested from candidate plus trees (CPT's) of *Jatropha* maintained at COS complex, Thapar University. From every CPT, fruits were harvested and seeds were separated manually. The seeds were then cleaned and stored under appropriate conditions in muslin bags. The seeds get their constant weight when dry so they were dried under sun. Materials required for isolation of RNA are given in **Table 2**.

Table 2. Stock and working solution concentrations for Isolation of total RNA

Solution	Stock Solution	Working Solution
Tris buffer	0.5 M (pH 8.0)	100 mM
LiCl	8 M	100 mM
EDTA	0.5 M (pH 8.0)	10 mM
SDS	10%	1%
B-mercaptoethanol	0.2%	0.1 ml

Procedure:

Plant tissues contain polysaccharides, nucleases, phenolics and proteins. Thus, isolation of RNA from plant is a difficult process in term of quality. Literature has stated many methods for isolation. Here, SDS-Phenol method by Gilman (1987) was used for isolation.

Liquid nitrogen was used to freeze plant tissues which were further grounded into fine powder by mortar and pestle. The powder was mixed in buffer containing lithium chloride and SDS and further extraction was done with phenol chloroform (1:1). 1/3rd volume of 8 M LiCl was added for the precipitation of RNA.

3.3.2 Purification of total RNA

Procedure:

The crude RNA was purified by treatment with RNase free DNase followed by solvent extraction and ethanol precipitation. RNA was dissolved in RNase-free deionized water and aliquots were stored at -70°C for further use.

3.3.3 Designing of Oligonucleotide primers

Oligonucleotide primers were designed based on genome sequence of curcin 2A (GenBank Acc. No.: GQ925453) and curcin precursor (GenBank Acc. No.: AF469003) available in GenBank database.

Curcin 2A gene sequence (3748 bps) consists of 1790-bp 5'flanking region and the transcriptional start site is located at the base 1791 corresponding to first exon. The translational site is present at the base 2085 and ORF spans from the base 2085 to 3014.

Curcin precursor gene sequence (1802 bps) consists of 694-bp 5'flanking region and the transcriptional site is located at the base 1576. The ORF spans from the base 695 to 1576.

3.3.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Requirements:

DEPC water, Oligo dT primer, 10mM dNTP, RNase inhibitor, Reaction buffer, RNA sample

Procedure:

Reverse transcription*:* Revert Aid H Minus M-MuLV reverse transcriptase enzyme was used to synthesize first strand cDNA. Ribonuclease H activity specific to RNA in RNA-DNA hybrids is not present in this enzyme; therefore RNA degradation does not occur. Lack of RNA degradation leads to higher yields of full-length cDNA from long templates upto 13kb.

RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas Life Sciences) containing M-MuLV reverse transcriptase and the gene-specific reverse primers, JCuPR-R1590, R2CU3021 was used for reverse transcription. Template used for each RT reaction was sample of stem or leaf (approx. 2.0µg) from *Jatropha* cultivars. According to manufacturer's instruction the steps for RT were performed. Isolation of full length cDNA was done by performing PCR. PCR mixture was made by using RT product as template, and adding 6 µl DEPC water, RNA sample and oligo dT primer. The mixture was properly mixed and eppendorfs were kept at 65°C for 5 minutes followed by incubation at room temperature for 5 minutes. Reaction buffer (4 µl), RNase inhibitor (1 µl), 10 mM dNTPs were added to the mixture and the eppendorfs were incubated at 42°C for 1hour and 65°C for 5-7 minutes.

RT-PCR: For RT-PCR, cDNA was used as template and following thermal cycling parameters were followed. The thermal cycling parameters followed during PCR are given in **Table 3**.

Table 3: Thermal cycling parameters for PCR

Stage	Temperature	Time
Initial denaturation	94°C	1 min 30 s
Denaturation	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	2 min
Final extension	72°C	5 min

3.3.5 Polymerase chain reaction (PCR)

Requirements: For PCR reaction the requirements are given in **Table 4**.

Table 4: Composition for PCR

Material	Volume taken	Concentration
Template DNA	1µL	0.5 µg
Buffer 10X	5 µL	10X
Forward primer	2 µL	10 pmoles
Reverse primer	2 µL	10 pmoles
DNTPs	2.8 µL	2.5 mM
Sterile deionized water	Volume made upto 50 µL	Volume made upto 50 µL
Taq DNA polymerase	0.7 µL	3 U/ µL

Procedure:

Polymerase chain reaction amplifies the DNA sequence using reverse and forward primers. PCR occurs in following steps: heat denaturation of template DNA, annealing of oligonucleotide primers to complementary sequences in single strand template DNA, and extension of annealed primers by a thermo stable DNA polymerase. Thermal cycling parameters for 30 cycles are as follows:

3.3.6 Agarose gel electrophoresis

Requirements:

Agarose (Sigma), 0.5X TBE buffer, Ethidium bromide dye (5 mg/mL), Sterile water, DNA samples, Bromophenol blue dye, Gel electrophoresis apparatus, UV transilluminator, Gel-Documentation system

Procedure:

Standard method (Sambrook- a laboratory manual) was followed to perform Agarose gel electrophoresis. 0.8% agarose gel was weighed and mixed in 0.5X TBE buffer and ethidium bromide dye was added to it. Gel was then casted in tray and allowed to polymerize. The DNA samples were loaded in the wells by mixing them with Bromophenol blue. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2-5 volt till the tracking dye covered 3/4th length in agarose gel. The DNA bands were then visualized under UV light.

3.3.7. Elution of Purified RT-PCR Product from Agarose gel

Requirements: Gel elution kit, sterile blade, eppendorfs, water bath, vortex

Procedure:

Silica bead DNA gel extraction kit was used to elute the DNA bands of curcin 2A isoform for cloning purposes. Silica bead DNA agarose gel extraction protocol was followed for elution of DNA bands. DNA samples were run in 0.8% agarose in 1X TAE buffer. In one lane, DNA sample was loaded as control. This control lane was excised with a clean scalpel and visualized under UV-trans illuminator to mark the position of DNA bands. The corresponding DNA bands were excised from the gel (without UV exposure) by matching the position of bands in the control lane. The gel slices containing the DNA bands were weighed in 1.5 mL microfuge tubes. As per the manufacturer's instructions, three volumes of binding buffer was added to one volume of gel and was incubated at 55°C, till the gel completely solubilize. Following this, 7µL of silica gel suspension beads was added and mixed properly by vortexing for 30 seconds. The tubes were then incubated at 55°C in water bath for 5 minutes with intermittent vortexing after every 1 min for 10 sec in order to allow adsorption of DNA on the beads. The samples were then centrifuged at 6,000 rpm for 30 sec and supernatant was removed very carefully with the help of a

pipette. The pellet was washed with 500 µL of washing buffer and then centrifuging at high speed for 30 sec followed by removal of supernatant. In the same manner, pellet was washed two times with 500 µL of washing buffer. Pellet was properly air dried in the laminar air flow until it appeared in a white powdery form. 7 µL of DEPC treated water and 7 µL of TE buffer was added to the dried pellet. Pellet was resuspended in TE buffer by vortexing for 15-20 sec. It was incubated at 55°C water bath for 5 minutes. Then centrifugation was done at 6,000 rpm for 30 sec. The supernatant containing the purified DNA was carefully transferred to a fresh sterile microfuge tube. The last two steps were again performed to increase the yield of DNA. 1.5 µL of eluted DNA was loaded in 0.8% agarose gel to check the yield.

3.3.8 Ligation reaction

Requirements: Mighty TA-cloning kit (TAKARA) for ligation was used for the reaction and pMD 20-T vestor was used. The vector map is given in **Fig. 7** and the requirements for ligation reaction are given in **Table 5**.

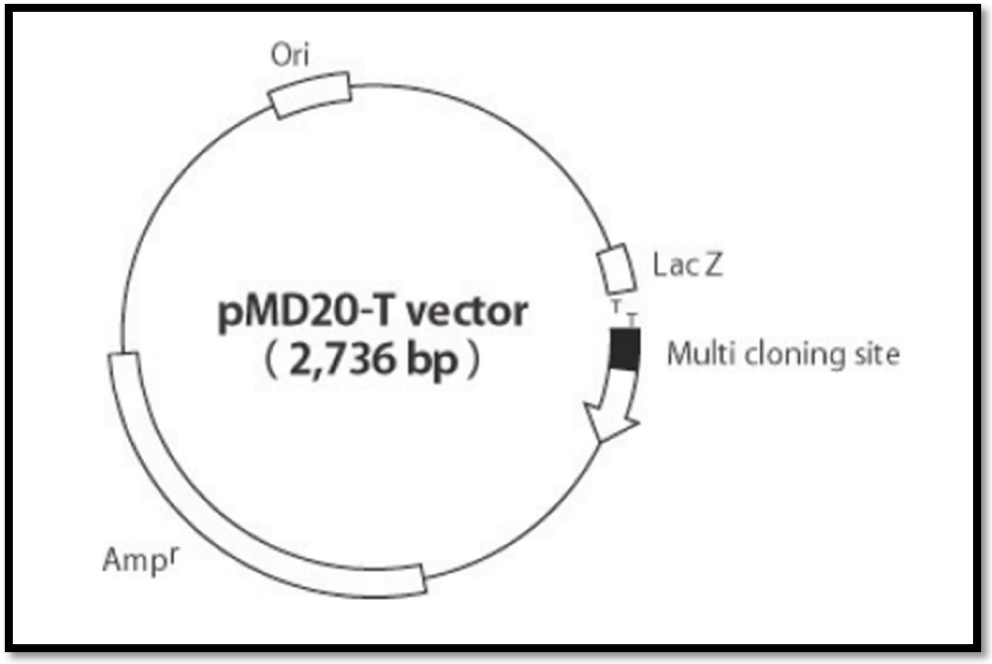

Fig. 7: Vector map of pMD 20-T

Table 5: Components for Ligation Reaction

Component	Volume
Linearized vector (pMD 20-T)	1 µL
PCR product (insert)	1 µL
Sterilized water	3 µL
Ligation Mighty Mix	5 µL

Procedure:

Added 1 µL of the PCR product, 1 µL pMD 20-T vector and 3µLsteriled distilled water to a microtube and mixed properly. Then to it, added 5 µL of Ligation Mighty Mix and gently mixed. Incubated the reaction mixture at 16°C for 1hour.

3.3.9 Genetic transformation of *E.coli* DH5α with recombinant plasmid

Requirements: E.coli. fresh plate, Luria broth, $CaCl_2$, X-Gal, IPTG

Media: **Luria Bertani Medium**

Yeast extract – 0.5% (w/v), Tryptone – 1.0% (w/v), NaCl – 1.0% (w/v), Agar – 1.5% (w/v)

For preparing LA–Ampicilin medium, ampicillin was added to the LA medium at working concentration of 50 µg m L^{-1}. Autoclaved media was used.

Procedure:

Preparation of competent cells*:* A single colony of *E.coli.* DH5α was picked from freshly grown plate and inoculated in freshly made autoclaved Luria broth. The culture was then incubated overnight at 37°C with shaking at 120 rpm. Next morning, 200 µL of the grown culture was inoculated in fresh Luria broth and incubated at 37°C for 2-3 hours at shaker to obtain an O.D. around 0.4 to 0.5 at 590 nm. Then the culture was transferred to centrifuge tube and kept on ice for 5 minutes to slow down the metabolism. Culture was then centrifuged at 8000 rpm for 10 minutes at 4°C. Cell pellet was then resuspended properly in 20 mL of chilled 50 mM $CaCl_2$ and kept in ice for 10 minutes. The cell-$CaCl_2$ suspension was then centrifuged at 8000 rpm for 10 minutes. To the pellet, 1 mL chilled $CaCl_2$ was added and kept in ice for 3 hours. Transferred the competent cells (100 µL) to eppendorf and added 10 µL ligation mixture, then kept the tube ice for 30-40 minutes and then heat shock was given 42°C for 90 seconds. Transferred the tube to ice for 1-2 minutes, added 1 ml media and kept at 37°C for 90 minutes. In the meantime, spreaded X gal-IPTG on LA-ampicilin plates ampicillin (50 µg mL^{-1}), then spreaded the ligated-competent cells on them (after 1 hour). Let the plates dry for few minutes and kept in incubator at 37°C for 12-16 hours. The transformants were selected by blue-white screening. Each pure white colony containing the recombinant (pMD 20-T vector) was picked up with a sterile tooth pick and patches were made on LA- ampicillin plates ampicillin (50 µg mL^{-1}), with X-Gal and IPTG.

3.3.10. Isolation of plasmid DNA by alkalysis method

Solutions for plasmid isolation:

Solution I – 50 mM glucose, 10 mM EDTA, 25 mM Tris HCl (pH 8.0)

Solution II – 0.2 M NaOH, 1.0% SDS (freshly prepared)

Solution III – 3.0 M with respect to potassium and 5.0 M with respect to acetate

Procedure:

Alkali lysis method*:* As described by Brinboim and Doly (1979), mini scale plasmid isolation was carried out by alkali lysis method. Transformed *E.coli* colony was inoculated in 25 ml of Luria broth containing ampicillin (50 μgmL^{-1}). The culture was inoculated at 37°C for overnight with vigorous shaking. 1.5mL of the above grown culture was poured in microfuge tubes and centrifuged at 8000rpm for 5 min. Supernatant was decanted and pellet dried , resuspended in 200 µL of ice cold Solution I with vigorous vortexing and incubated at ice for 10 minutes. Followed by addition of 300 µL of freshly prepared solution II and gently mixed. The tubes were then stored on ice for some time. Then 400 µL of ice cold Solution III was added and mixed by gentle inversion of tubes till curdy white precipitate was formed. The tubes were stored on ice for 15 to 20 min. the tubes were centrifuged at 12,000 rpm for 15 min at 4°C. Supernatant was carefully transferred to fresh microfuge tubes. DNA was precipitated with equal volume of isopropanol and was kept at 4°C for 30 min. Then centrifugation was done at 10,000 rpm for 15 min at 4°C. Supernatant was decanted and pellet was air dried and dissolved in 50 µL of TE buffer. Further purification of crude plasmid was done by adding 370 µL of sterile water to crude plasmid dissolved in TE buffer, followed by addition of 2 µL DNase free RNase to remove RNA contamination. The tubes were then incubated at 37°C for 60 min. Extraction of DNA was done with equal volume of phenol and chloroform. Then centrifugation was done at 8000 rpm for 10 min and upper aqueous layer was transferred to fresh microfuge tube and $1/10^{th}$ volume of 3 M sodium acetate was added and mixed well for 5 min. Double volume of dehydrated alcohol was added and mixed properly. It was kept at -20°C for overnight. Centrifugation was done at high speed for 10 min and supernatant was decanted. Pellet was washed with 70% ethanol and air dried and then dissolved in 30 µL TE buffer.

CHAPTER-4 RESULTS AND DISCUSSIONS

4.1. *In silico* analyses

Curcin is a toxic compound but has some pharmaceutical importance. It is found that very less work has been done in the literature for the *in silico* analysis. So, efforts have been made to find the structure and other biochemical attributes of curcin isoforms.

Sequence analysis, comparison and motif search in different curcin isoforms of *Jatropha*

This study focused on the two isoforms of curcin namely Curcin 2A and Curcin precursor from *Jatropha.*

4.1.1 Salient sequence features

Curcin 2A gene: Curcin 2A protein consisting of 309 amino acids encoded by a 3748-bp gene sequence from *Jatropha* (GenBank Acc. No.: GQ925453). This gene consists of 1790-bp 5′-flanking region, and the entire transcriptional unit spans from 1791 to 3194 bp. Size of ORF in Curcin 2A gene is 930 bp including stop codon.

Curcin precursor: Curcin precursor is an isoform of curcin consisting of 293 amino acids. It is encoded by 1802-bp gene from *Jatropha* (GenBank Acc. No.: AF469003). This gene consists of 694-bp 5′-flanking region and the transcriptional unit spans from 695 to 1576 bp. Size of ORF is 882 bp including stop codon.

The Curcin 2A (GenBank protein id: ADN39428.1) and Curcin precursor (GenBank protein id: AAL86778.1) sequences were used as reference sequences for protein modeling. Amino acids were retrieved in FASTA format from NCBI and submitted in BLASTp server of NCBI. BLASTp gave the homologous sequences with maximum identity and query coverage. The homologous sequences were used for comparison, characterization and validation of protein models.

4.1.2 BLASTp analysis using the sequences of Curcin isoforms

(a) Curcin 2A

BLASTp analysis was carried out using Curcin 2A sequence (GenBank protein id: GQ925453) as a query. The 309 amino acids of Curcin 2A showed 90-99% sequence identity with other curcin isoforms. In most of the cases query coverage was 100% except Curcin isoform i.e., 94%. The data generated by the BLASTp analysis are presented in **Fig. 8** and **Table 6**.

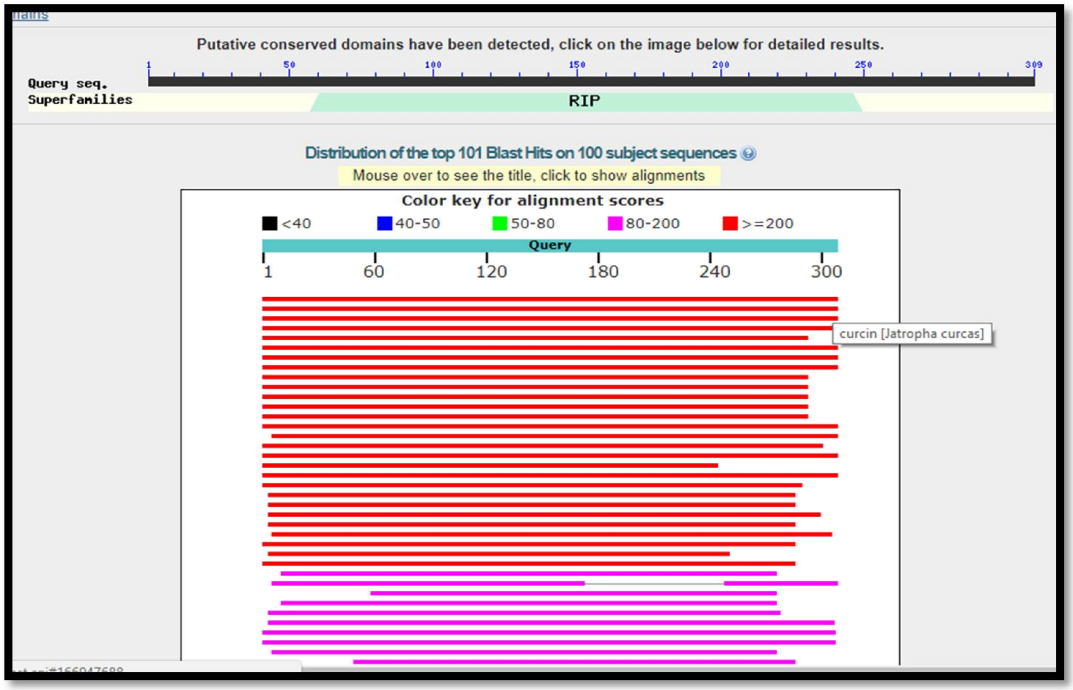

Fig. 8: BLASTp analysis of Curcin 2A

Table 6: Details of homologous sequences of Curcin 2A

Protein name	Accession number	ORF length	Max Score	Identity %	Query coverage %	Amino acid
Curcin	ABZ04128	930	636	100	100	309
Curcin-L-precursor	ABW17545	930	635	99	100	309
RIP	AAR08395	930	633	99	100	309
Curcin	ACO53803	882	587	98	94	293
Curcin precursor	AEA72440	930	580	90	100	309

(b) Curcin precursor

Likewise, BLASTp analysis was carried out using Curcin precursor sequence (GenBank protein id: AF469003) as a query. The 293 amino acids of Curcin precursor showed 89-95% sequence identity with other curcin isoforms. The query coverage was 100% for each case. The results of the BLASTp analysis are given in **Fig. 9** and **Table 7.**

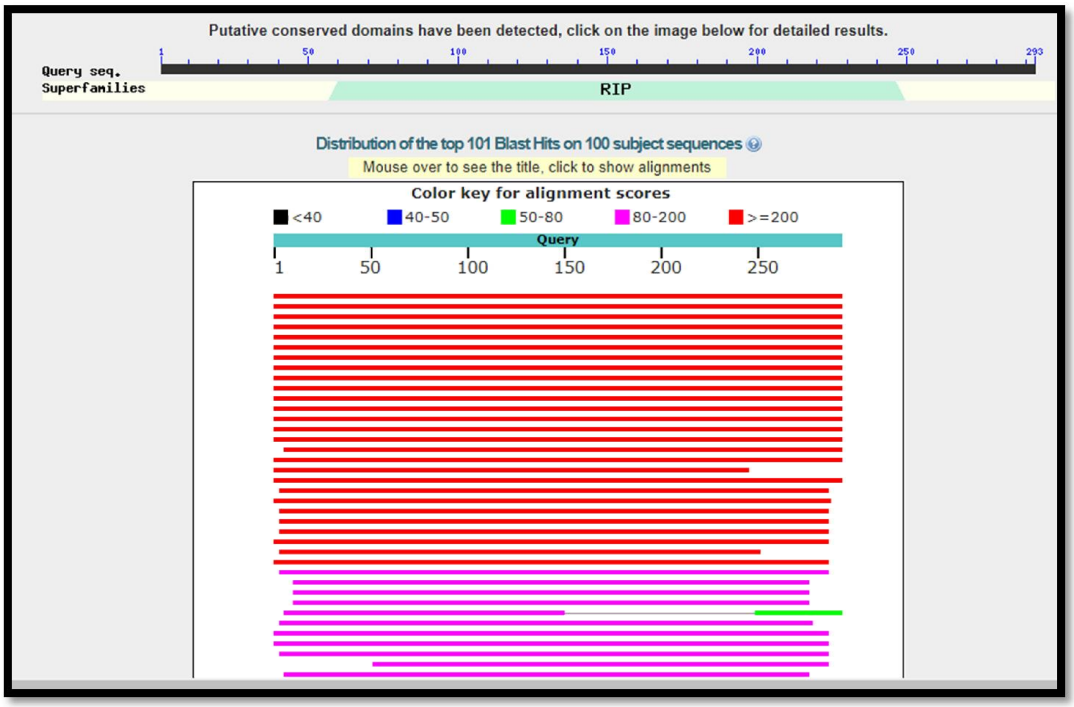

Fig. 9: BLASTp analysis of Curcin precursor

Table 7: Details of homologous sequences of Curcin precursor

Protein name	Accession number	ORF length	Max Score	Identity %	Query coverage %	Amino acid
Curcin precursor	AAL86778	882	605	100	100	293
Curcin precursor	AEA72440	930	575	95	100	309
Curcin 2b	AJW31110	882	571	94	100	293
RIP	AAR08395	930	539	89	100	309
Curcin-L precursor	ABW17545	930	540	89	100	309

4.1.3 Multiple sequence alignment using Constraint-based Multiple Alignment Tool (COBALT)

For multiple sequence alignment (MSA), the amino acid sequences of five curcin isoforms were taken which included Curcin 2A, Curcin-L-precursor, RIP (Ribosome-inactivating-protein), Curcin, and Curcin precursor using multiple sequence alignment tool COBALT as shown in **Fig. 10**. MSA reveals that most of the domains are highly conserved including the N-glycosylation sites. Comparison between the

sequences shows variation at approximately 33 sites because of mutations, deletions or insertions as highlighted in the figure. The N-terminal sequences of the curcin isoforms appear to be highly conserved as compared to their variable C-terminal sequences.

```
Cur2A    MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKKNYAQFIEDLREAFDFSYLSH  80
Cur-L    MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLVITYDATTDKKNYAQFIEDLREAFDFSYLSH  80
RIP      MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLVITYDATTDKKNYAQFIEDLREAFDFSYLSH  80
Cur      MKGGKMNLSIMVAAWFCRSCIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKKNYAQFIEDLREAFDFSYLSH  80
Cur P    MKGGKMNLSIMVAAWFCWSCIIFGWASAREIVCPFSSNQNYKAGSTPTLTITYDATTDKKNYAQFIKDLREAFGFSYSSH  80
         *****************.*******************************.****************.******.***.**

Cur2A    KIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLVAYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYA 160
Cur-L    KIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLVAYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYA 160
RIP      KIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLVAYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYA 160
Cur      KIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLVAYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYA 160
Cur P    EIPVLRATVAANQKFIVAKVINVANLEVSLGLNVVNAYLVAYKVGGTSYFFNDPESLADAKKYLFTDTKQQTLSFTGSYA 160
         .*********************....***.****.**********.*******.********.**********.******

Cur2A    DFESRAKLHREEVDLGVVALDNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240
Cur-L    DFESRAKLHREEVDLGVVALDNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240
RIP      DFESRAKLHKEEVDLGVVALDNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240
Cur      DFESRAKLHREEVDLGVVALDNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240
Cur P    DFLSRANVHREDVDLGVLALDNYIYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKVLTQISKTFRPRGDIISLENN 240
         **.***..*.*.*****.*****.***********************************..*******************

Cur2A    WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIFNDQKWLPWL  309
Cur-L    WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIFNDQKWLPWL  309
RIP      WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIFNDQKWLPWL  309
Cur      WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVSNVTQVKGLTGVLLNPVKYKV----------------  293
Cur P    WGDLSYQIQKSVNGVFLKPVQLQRENYTNILVNNVTQVTGLMGVLLNAVNYKVSMEEIIFNYQKWLPWL  309
         ************..******************.*****.**.*****.*.***
```

Fig. 10: Multiple sequence alignment of amino acid sequences of five different forms of curcin protein. The alignment is based on COBALT tool. The asterisk (*) symbol denotes the conserved amino acids between all forms; full-stop (.) denotes the amino acids which have divergence from other sequence.

4.1.4 Amino acids composition analysis in the curcin isoforms

All the standard amino acids are present in the curcin isoforms in varied amounts. The amino acid composition of Curcin 2A and Curcin precursor was closely inspected. The composition of some of the amino acids is presented in **Table 8** which revealed deviation if compared with their average occurrence in the natural proteins. The increase or decrease in the amount of amino acid in a protein changes its structure and functionality. For example, Histidine and Proline occur relatively at less frequency in both Curcin 2A and Curcin precursor; and some hydrophobic amino acids such as Valine, Isoleucine and Phenylalanine occur more in these isoforms. All these significant changes in the amino acid composition could have profound effects on the structure and function of the curcin isoforms.

Table 8: Analysis of some amino acid composition (in %)

Amino Acid	Average occurrence	Curcin 2A	Curcin precursor
Valine	6.6	8.9	8.4
Isoleucine	5.3	6.5	7.1
Phenylalanine	3.9	5.5	5.5
Tyrosine	3.2	5.1	4.5
Serine	6.8	7.5	8.1
Asparagine	4.3	6.8	7.1
Lysine	5.9	7.2	7.4
Proline	5.2	3.1	2.9
Cysteine	1.9	1.0	0.6
Histidine	2.3	0.7	0.6
Arginine	5.1	3.4	2.9

4.1.5 Searching protein motifs

The amino acid sequence of Curcin 2A was analysed for prediction of some protein motifs using the tool MY HITS as shown in **Fig.** 11

```
MKGGKMNLSI MVAAWFCWSS IIFGWASARE IVCPFSSNQN YKAGSTPTLA ITYDATTDKK   60
NYAQFIEDLR EAFDFSYLSH KIPVLRATVA ANQKFIVAKV INSGDIEVSV GLNVINAYLV  120
AYKVGSNSYF FNDSESLADA KKNLFTDTNQ QTLAFTGSYA DFESRAKLHR EEVDLGVVAL  180
DNYVYTLEKS SQPADIAKPL VGFIEMVPEA ARFKYIEKKI STQISKTFRP RGDIISLENN  240
WGDLSYQIQK SVDDVFLKPV QLQRENYTNI LVNNVTQVKG LMGVLLNAVN YKVSMEEIIF  300
NDQKWLPWL                                                          309
```

Fig. 11 Amino acid sequence of Curcin 2A (GenBank protein id: ADN39428). Some protein motifs are highlighted using different colors

The position and amino acid sequence of the individual protein motifs are presented in **Table 9.**

Table 9: Motif sites and position of Curcin 2A

S. no.	Site	Sequence	Amino acid position	Color
1.	N-glycosylation site	NLSI NDSE NYTN NVTQ	7-10 132-135 266-269 274-277	Skyblue
2.	*cAMP- and cGMP-dependent protein kinase phosphorylation site*	KKIS	218-221	Purple
3.	*Casein kinase II phosphorylation site*	SARE SLAD SYAD SVDD SMEE	27-30 136-139 158-161 251-254 294-297	Olive green
4.	*N-myristoylation site*	GGKMNL GSTPTL GVLLNA	3-8 44-49 283-288	Orange
5.	*Protein kinase C phosphorylation site*	SAR TDK SHK TFR	27-29 57-59 79-81 227-229	Black
6.	*Cell attachment sequence*	RGD	231-233	Red
7.	*Tyrosine kinase phosphorylation site*	REAFDFSY	70-77	Light orange
8.	*Shiga/ricin ribosomal inactivating toxins active site signature*	IEMVPEAARFKYIEKKI	204-220	Grey

Likewise, the amino acid sequence of Curcin precursor was analysed for prediction of some protein motifs using the tool MY HITS as shown in **Fig. 12**

```
MKGGKMNLSI MVAAWFCWSC IIFGWASARE IVCPFSSNQN YKAGSTPTLT ITYDAAADKK   60
NYANFIRDLR EAFGFSYSSH EIPVLRATVA ANQKFIVAKV INVANLEVSL GLNVVNAYLV  120
GYKVGGTSYF FNDPESLADA KTYLFTDTKQ QTLSFTGSYA DFLSRANVHR EDVDLGVQAL  180
DNYIYTLEKS SKPADIAKPL VGFIEMVPEA ARFEYIEKKI STQISKTFRP RGDIISLENN  240
WGDLSYQIQK SVDDVFLKPV QLQRENYTNI LVNNVTQVKG LMGVLLNAVK YKV         293
```

Fig.12 Amino acid sequence of Curcin precursor gene (GenBank protein Id: AAL86778)**.** Some protein motifs are highlighted using different colors

Table 10: Motif sites and position of Curcin precursor

S. no.	Site	Sequence	Amino acid position	Color code
1.	N-glycosylation site	NLSI NYTN NVTQ	7-10 266-269 274-277	Skyblue
2.	*cAMP- and cGMP-dependent protein kinase phosphorylation site*	KKIS	218-221	Purple
3.	*Casein kinase II phosphorylation site*	SARE SSHE SLAD SYAD SVDD	27-30 78-81 136-139 158-161 251-254	Olive green
4.	*N-myristoylation site*	GGKMNL GSTPTL GVLLNA	3-8 44-49 283-288	Orange
5.	*Protein kinase C phosphorylation site*	SAR SSK TFR	27-29 79-81 227-229	Black
6.	*Cell attachment sequence*	RGD	231-233	Red

N-glycosylation sites are present in both the curcin isoforms where sugar molecules could be attached at the specific sites. The types of N-glycans synthesized depend on the accessibility of different enzymes present in the cellular components. It occurs in the selective Asparagine residues. Casein kinase II catalyses the transfer of phosphate to peptide substrate. N-myristoylation site helps in the lipidation modification i.e., in attachment of amide bond to alpha-amino group of N-terminal glycine residue. It plays role in membrane targeting and signal transduction in plant responsible to environmental stress. Proteinase kinase C enzymes are signal transduction cascade and are involved in controlling function of other proteins through phosphorylation of hydroxyl groups of serine and threonine amino acid residues. Cell attachment sequence present in both the isoforms acts as receptor for cell adhesion molecules and cell-cell interaction. Further, biochemical approaches are required to validate the functionality of the individual motifs as predicted in this study.

4.1.6 3-D modeling studies

3-dimensional protein structures for Curcin 2A and Curcin precursor were predicted by using Phyre2 tool. The amino acid FASTA sequences from NCBI were taken and uploaded in the tool, many pdb files were generated which were further used to find the best model. The protein models are given in **Fig. 13.**

- **Model structure**

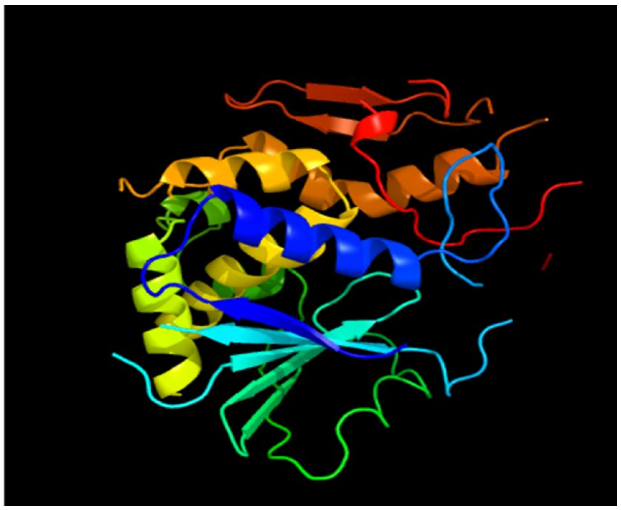

Fig. 13 (a): Model Structure of Curcin 2A

Fig. 13 (b): Model Structure of Curcin Precursor

- **X-ray/NMR structure**

Using ProSA-web tool the protein models were validated. For validation, the pdb files were uploaded on ProSA tool and the following data was obtained as given in **Fig. 14 and Fig. 15**. **Fig.14** depicts the position of protein in the X-ray/NMR region. The protein is shown by the black colored dot (.) if the protein falls in the region then it is considered as the best model.

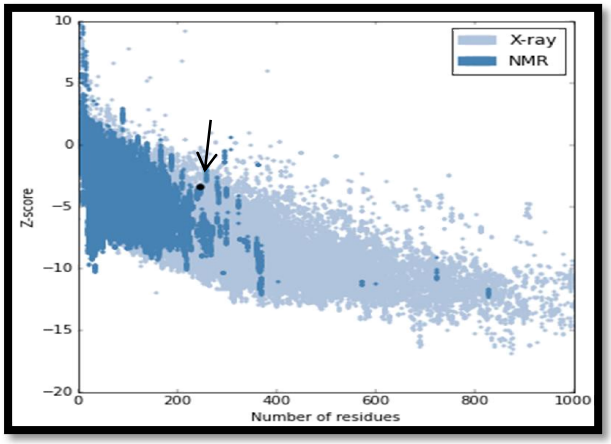

Fig. 14(a): X-ray/NMR structure of Curcin 2A

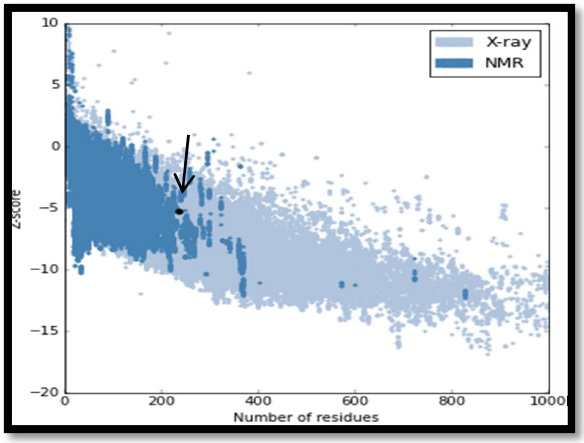

Fig. 14(b): X-ray/NMR structure of Curcin precursor

- **Model quality**

An important way of validation of protein model is based on the energy level as shown in **Fig.15.** If the energy level of the sequences is less than 0 then it is considered to be the best model.

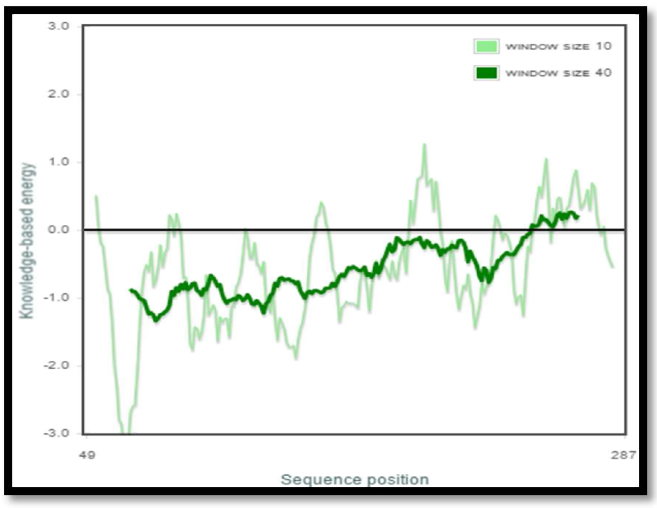

Fig. 15 (a): Model quality of Curcin 2A

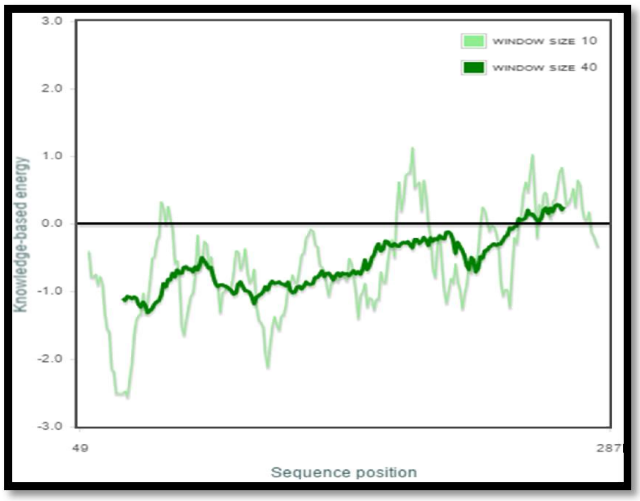

Fig. 15 (b): Model Quality of Curcin precursor

Another way of validation of the protein model is done by checking the z-score. The model with minimum negative z-score is considered to be the best model. So considering all the factors which help in prediction of best model contributes for its selection.

- **Minimized pdb file by energy minimization in Nomad Ref**

The selected models as presented above were uploaded on the Nomad Ref tool which gave the minimized protein model as given in **Fig. 16**.

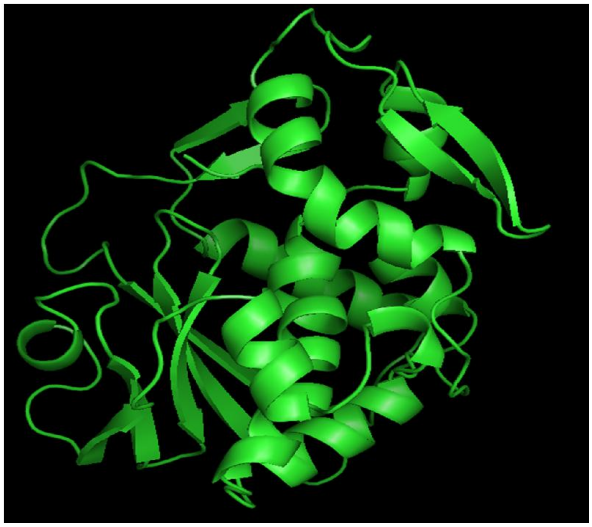

Fig.16 (a): Minimized pdb model of Curcin 2A

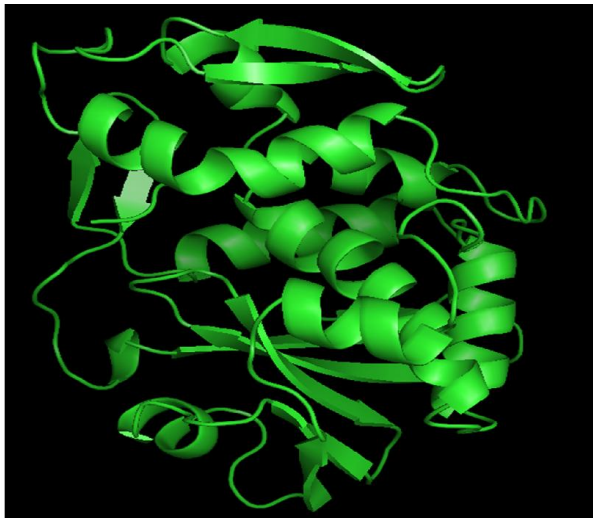

Fig. 16 (b): Minimized model of Curcin precursor

- **Ramachandran analysis of curcin protein as visualized by SAVES software**

Ramachandran plot is a way to visualize energetically allowed regions for backbone of amino acid residues in protein structure as indicated by dots in **Fig.17 and 18**. The plots were obtained by uploading the minimized pdb file to SAVES tool. In the Ramachandran plots of Curcin 2A and Curcin precursor, the dots indicate the residues of proteins lying in the favored, allowed and outlier region as described in **Table 11**. There is almost similarity in the values of the proteins lying in allowed region but a lot of differences in the proteins present in the negative region, this was identified by using Verify_3D. It determines the compatibility of an atomic model with its own amino acid sequence by comparing the results to good models

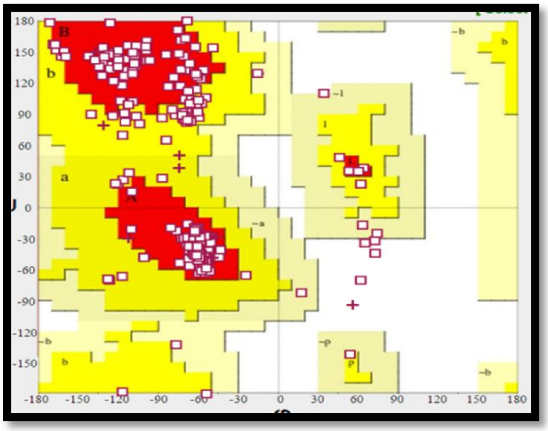

Fig. 17 (a): Ramachandran Analysis of curcin 2A

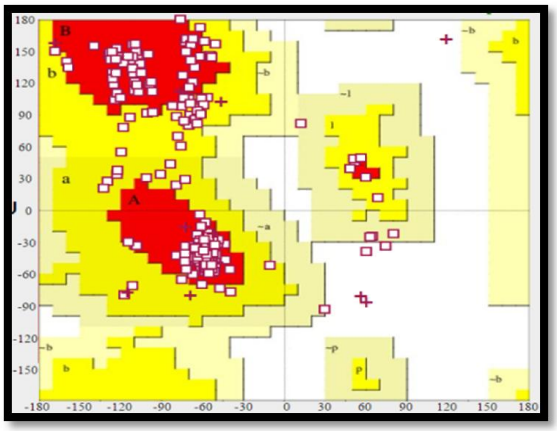

Fig. 17 (b): Ramachandran Analysis of Curcin Precursor

- **Assesment of residues plot**

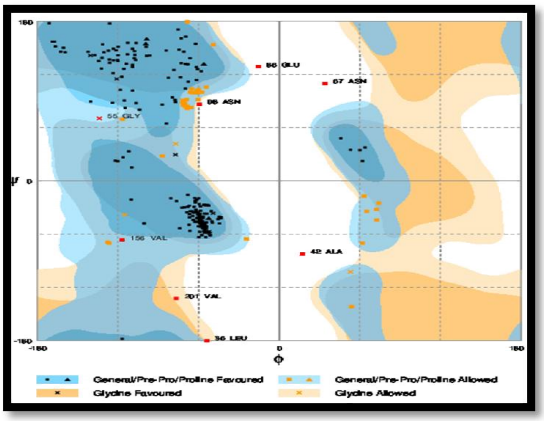

Fig. 18 (a): Residual proteins in Curcin 2A

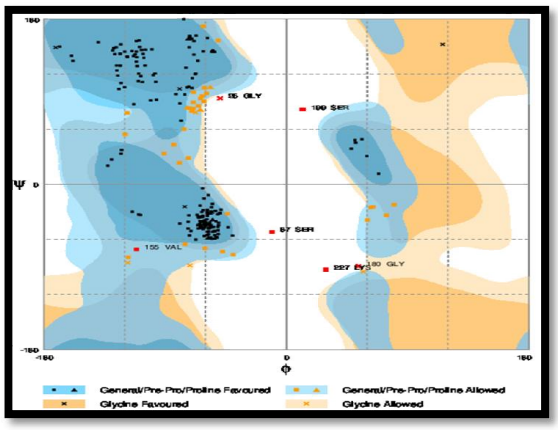

Fig. 18 (b): Residual proteins in Curcin precursor

- **No. of residues**

Table 11: Ramachandran plot values of Curcin 2A and Curcin Precursor

Ramachandran plot values	Curcin 2A	Curcin precursor
Favoured	194	192
Allowed	35	38
Outlier	8	6
VERIFY 3D AVERAGED SCORE(%)	72	88.28
NEGATIVE LYING RESIDUE No.	220	58
Name	Leucine	Glutamine
Score	-0.03	-0.01

- **Transmembrane structure:**

Transmembrane helices play an important role in study of membrane associated proteins specially with respect to cell signaling and energy transport. The transmembrane structures were predicted by uploading the pdb files protein models to Phyre2 tool. In both the transmembrane structure of Curcin 2A and Curcin precursor peptide signals entering from the extracellular membrane for cell signaling is different for both the curcin isoforms, i.e., 1-38 and 1-32 respectively as shown in **Fig. 19 (a&b)**. Maximum number of proteins is present towards the N-terminal and very less towards the C-terminal in both the curcin isoforms.

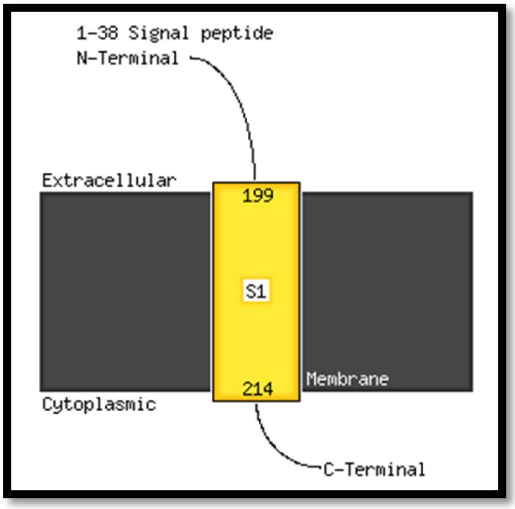

Fig. 19(a): Transmembrane structure of Curcin 2A

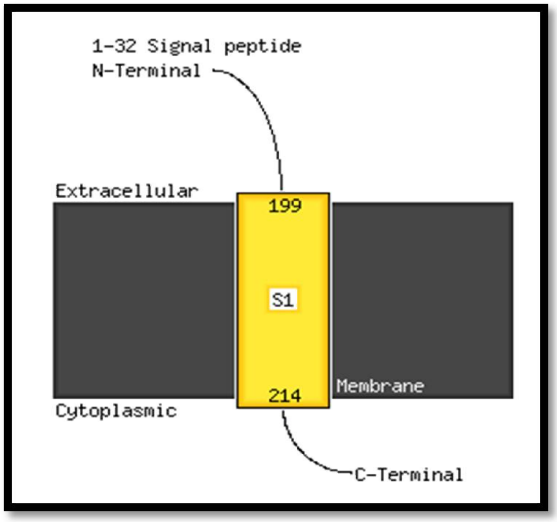

Fig. 19(b): Transmembrane structure of Curcin Precursor

4.1.8 Comparison between different isoforms of curcin

Curcin isoforms have some biochemical attributes which play a very important role in the functioning of the proteins. The comparison between different isoforms of curcin with Curcin 2A and Curcin precursor as reference is given in **Table 12**. The instability index gives the estimation of protein stability in test tube. A weight value comes from this technique which tells about the stability of the protein. Proteins with instability index less than 40 are considered to be stable and the proteins with instability index above 40 are unstable. Here, Curcin 2A ranges from 27.33-30.31 and Curcin precursor ranges from 23.31-30.31, telling all the curcin proteins to be stable.

The aliphatic index occupied by aliphatic side chains (A, V, I, L) is considered as positive factor and is defined as relative volume of a protein for the increase of thermal stability of globular proteins. In curcin it was found to be in the range 91.40 – 96.57.

Table 12: Biochemical attributes of different isoforms of Curcin

S. no.	Accession no.	Name	Molecular weight	Theoretical pI	Negatively charged residues	Positively charged residues	Instability index	Aliphatic index
Ref 1	ADN39428	curcin2A	34851.80	6.20	33	32	29.55	92.14
1.	ABZ04128	Curcin	34851.80	6.20	33	32	29.55	92.14
2.	ABW17545	Curcin-L-Precursor	34879.86	6.20	33	32	29.55	92.75
3.	AAR08395	RIP	34851.84	6.20	33	32	30.31	92.75
4.	ACO53803	Curcin	32789.48	8.54	30	33	28.76	91.50
5.	AEA72440	Curcin Precursor	34829.05	8.74	27	31	27.33	94.01
Ref 2	AAL86778	curcin precursor	32766.50	8.55	28	31	23.31	91.84
1.	AAL86778	Curcin Precursor	32766.50	8.55	28	31	23.31	91.84
2.	AEA72440	Curcin Precursor	34829.05	8.74	27	31	27.33	94.01
3.	AJW31110	Curcin 2B	34774.02	8.75	26	30	29.65	96.57
4.	AAR08395	RIP	34851.84	6.20	33	32	30.31	92.75
5.	ABW17545	Curcin-L-precursor	34879.86	6.20	33	32	29.55	92.75

4.1.9 Phylogenetic analysis of curcin by mega 7

Phylogenetic tree also known as evolutionary tree is a branched diagram which is used to find the evolutionary relationships between homologous sequences. The sequences joined together imply similarities and common ancestors, whereas, the sequences which have evolved from different root imply divergence. The length of the branches depicts the evolutionary time. There are numerous ways to construct the phylogenetic tree. Here, neighbor joining method by Mega 7 has been used to construct the phylogenetic tree for different isoforms of curcin protein of *Jatropha* as shown in **Fig. 20**. A different color in the phylogenetic tree depicts different species of the plant which show the evolutionary history.

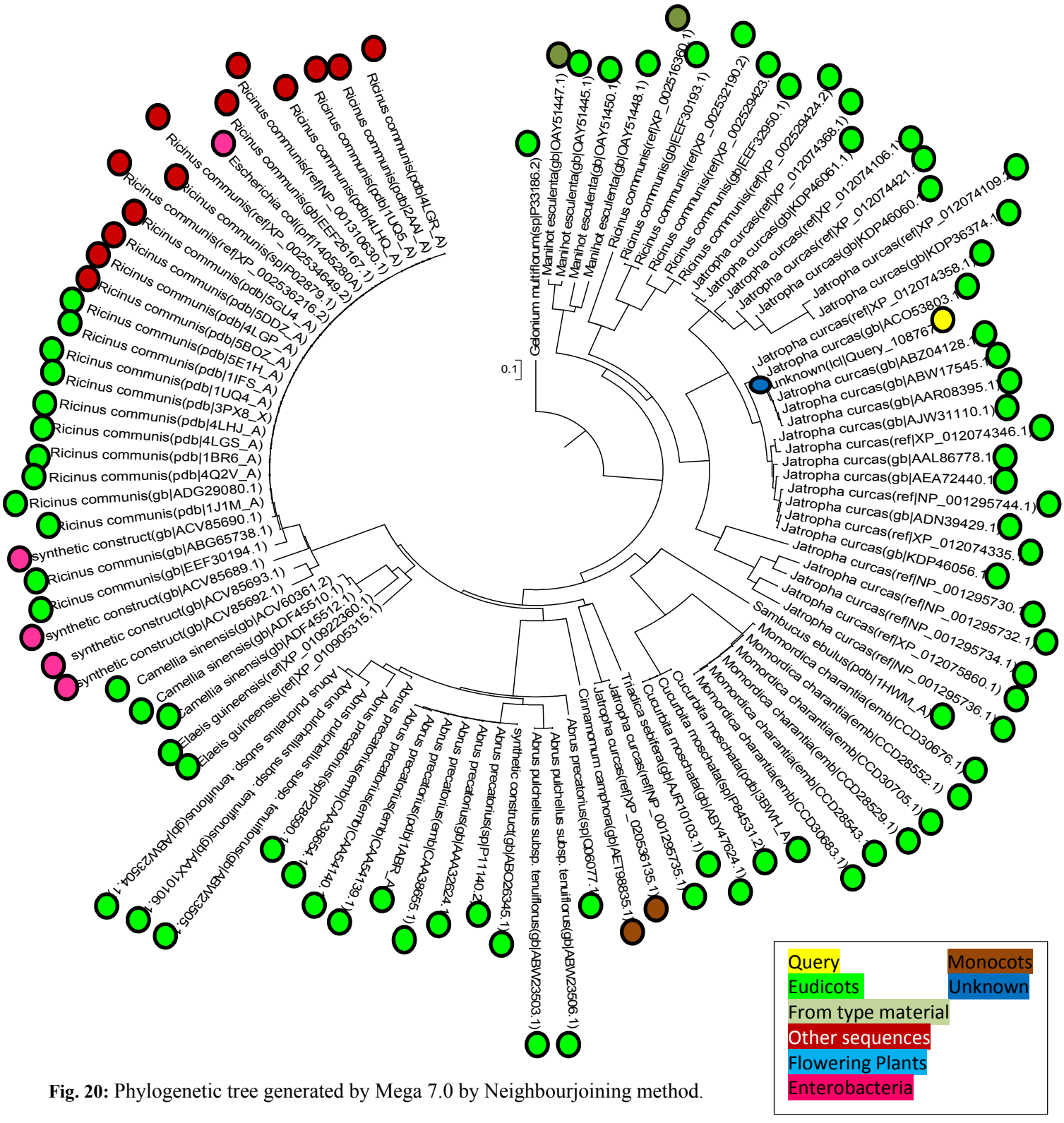

Fig. 20: Phylogenetic tree generated by Mega 7.0 by Neighbourjoining method.

All these data on curcin proteins as generated by various bioinformatics tools would be quite useful for further in-depth biochemical and molecular studies; particularly during recombinant expression.

4.2 Molecular cloning and partial characterization of curcin isoforms

Recombinant protein expression has become an important aspect of biotechnology during the last few decades. Although the curcin proteins in *Jatropha* are inherently cytotoxic in nature but they have various pharmaceutical importance. Therefore, molecular cloning of different curcin cDNAs is a prerequisite to carry out recombinant expression of such proteins for production at commercial scale. Keeping in view, this study focused on curcin cDNA cloning studies as described precisely in the following sections.

4.2.1 Isolation of total RNA from different tissue of Jatropha: Total RNA was isolated from different tissues of *Jatropha,* i.e., leaf, seed kernel, seed pericarp and stem. The crude RNA samples were checked by agarose gel electrophoresis as shown in **Fig.21**. Ribosomal RNA bands were distinct indicating the intactness of total RNA preparations. Some of the RNA samples had genomic DNA impurities.

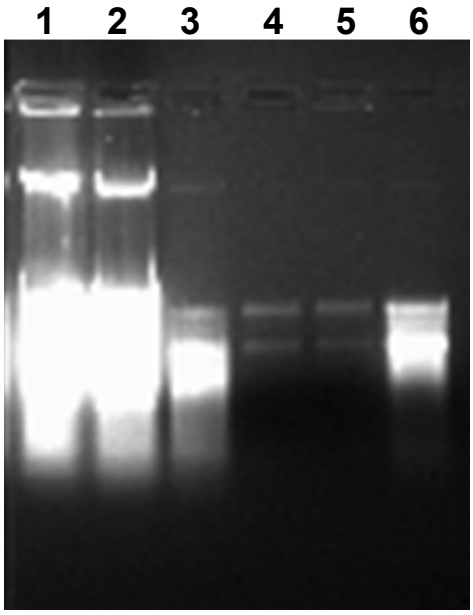

Fig. 21: Total RNA from different *Jatropha* tissues. Lane 1-leaf, Lane 2-seed kernel 1, Lane 3-seed kernel 2, Lane 4-seed pericarp 1, Lane 5-seed pericarp 2, Lane 6-seed pericarp 3

4.2.2. Purification of total RNA*:* Total RNA was purified by RNase-free DNase treatment followed by solvent extraction. Purified RNA samples were checked by Agarose gel electrophoresis as shown in **Fig. 22.** The distinct bands shows the quality of the purified RNA present. Nanodrop spectrophotometer was used to find A_{260}/A_{280} ratio to access the quality and quantity of the prepared RNA samples.

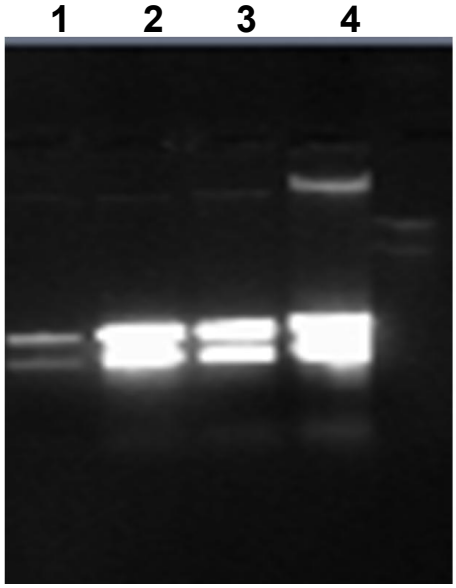

Fig. 22: Purified RNA from different *Jatropha* tissues. Lane 1- leaf, Lane 2-seed kernel 1, Lane 3-seed kernel 2, Lane 4-seed pericarp 2, Lane 5-seed pericarp 2

4.2.3 RT-PCR: Reverse transcription was carried out using the purified total RNA. The RT products were used to carry out PCR using Curcin 2A specific primers as described in Material and Methods. The PCR products were analyzed by agarose gel electrophoresis as shown in **Fig. 23.** Intact bands were observed in seed kernel and seed pericarps. As expected, the amplicon size in each case was around 1.0 kb. The amplicons were used for cloning in the plasmid vector. In case of leaf RNA there was no RT-PCR product. The data suggest that expression of Curcin 2A varies between different tissues in *Jatropha.*

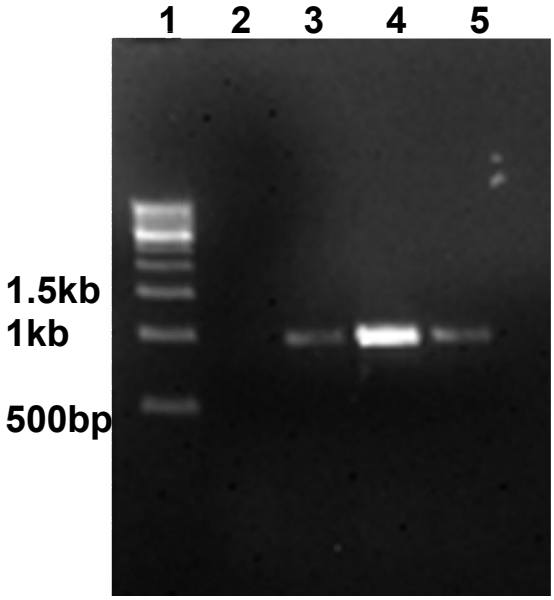

Fig. 23: Agarose gel electrophoresis of RT-PCR products. Lane 1- 500 bp ladder, Lane 2-leaf, Lane 3-seed kernel, Lane 4-seed pericarp 1, Lane 5-seed pericarp 2

4.2.4 Purification of Curcin 2A specific RT-PCR product: First, the RT-PCR products (~1.0 kb) were resolved in agarose gel followed by elution of the DNA bands using silica bead gel extraction kit and confirmed further by agarose gel electrophoresis as shown in **Fig. 24 (a) and Fig. 24 (b).**

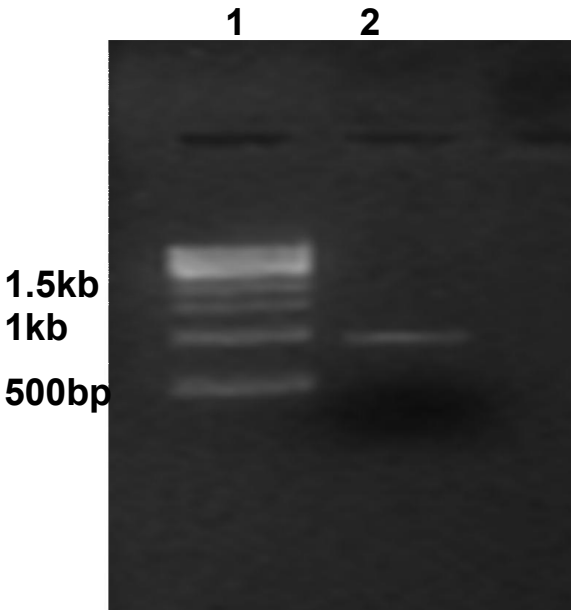

Fig. 24(a): Elution of RT-PCR product. Lane 1-500 bp ladder, Lane 2-eluted DNA specific to seed kernel

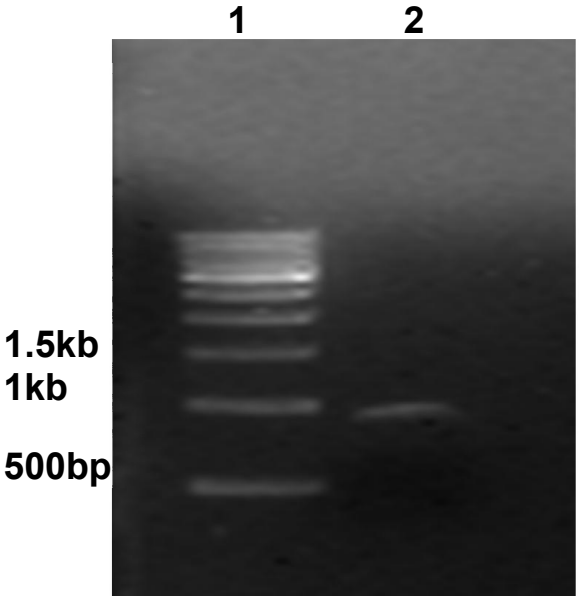

Fig. 24(b): Elution of RT-PCR product. Lane 1-500 bp ladder, Lane 2-eluted DNA specific to seed pericarp

4.2.5 Cloning of Curcin 2A specific cDNA in the pMD 20-T vector: The Curcin 2A specific eluted RT-PCR products corresponding to *Jatropha* seed kernel and pericarp as shown in **Fig. 24 (a) and Fig. 24 (b)** were ligated to the pMD 20-T vector. The ligated products were used to transform *E.coli* DH5α for further blue-white screening of the recombinant plasmid clones. The procedure in detail is provided in Materials and Methods. A number of white colonies (i.e., putative clones) were clonally purified by streaking. The individual white colonies were picked up for isolation of plasmid DNA in mini-scale by alkali lysis method. The crude plasmid DNA samples were analyzed by agarose gel electrophoresis as shown in **Fig. 25A and Fig. 25B.** Mobility clearly suggests that 2 out of 2 (specific to seed kernel) and 6 out of 7 (specific to seed pericarp) plasmid DNA samples appeared to be recombinant clones.

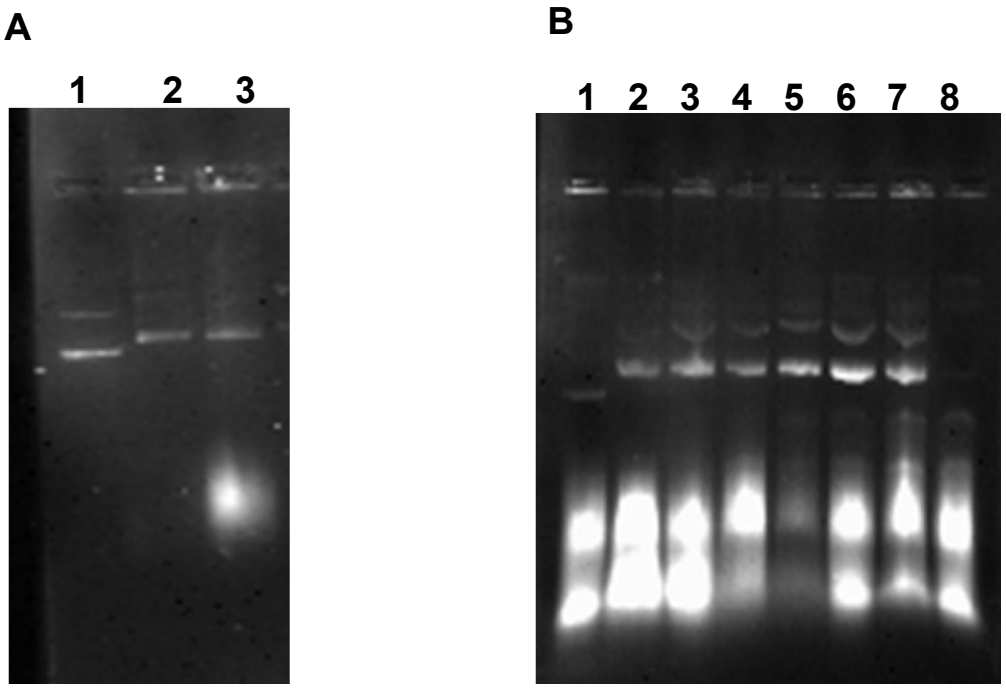

Fig. 25: Gel electrophoresis of Curcin 2A specific putative clones. (**A)** Specific to seed kernel, Lane 1-plasmid vector, Lanes 2 & 3-putative clones.

(**B)** Specific to seed pericarp, Lane 1- plasmid vector, Lanes 2 to 7-putative clones

4.2.6. Partial characterization of the putative Curcin 2A clones:

- *RNase treatment***:** Three Curcin 2A putative clones corresponding to both seed kernel and seed pericarp were treated with RNase and resolved by agarose gel electrophoresis, as shown in **Fig. 24**.

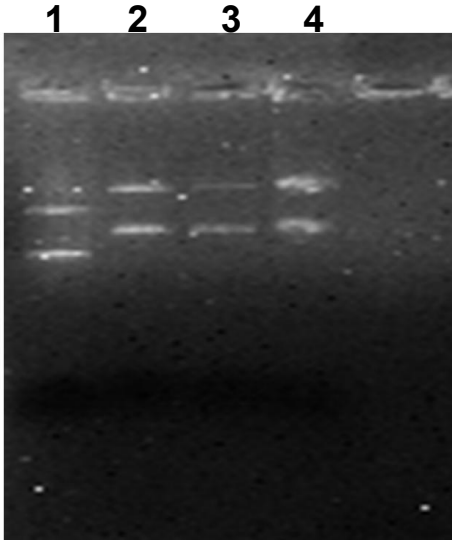

Fig. 26: RNase treatment of the Curcin 2A putative clones.
Lane 1- pMD 20-T, Lane 2-specific to seed kernel, Lanes 3& 4- specific to seed pericarp

- **Restriction analysis by *Eco*R1**

Restriction digestion of the putative clones was carried out and the digested DNA samples were resolved in agarose gel electrophoresis as shown in **Fig. 27**.

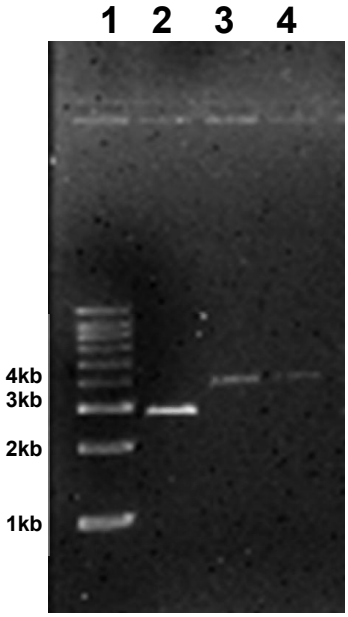

Fig. 27: Restriction analysis of the putative clones of Curcin 2A. Lane 1-1.0 kb ladder, Lane 2- pMD 20-T vector, Lane 3-putative clone specific to seed kernel, Lane 4- putative clone specific to seed pericarp 1

- *Polymerase Chain Reaction (PCR):* PCR approach was adopted for initial characterization of the Curcin 2A specific putative clones using the same set of primer pair as employed in RT-PCR earlier (**Fig. 28)**. As expected, the size of each amplicon is ~1.0 kb.

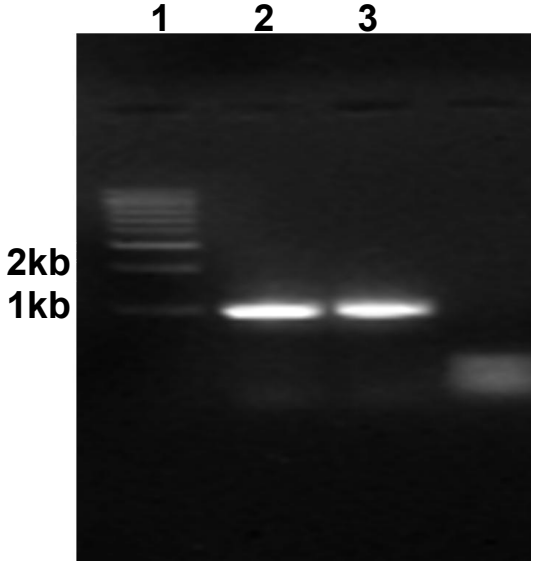

Fig. 28: Characterization of the putative Curcin 2A clones through PCR. Lane 1-1.0 kb ladder, Lane 2- amplified product corresponding to seed kernel specific clone, Lane 3- amplified product corresponding to seed pericarp 1 specific clone

The putative Curcin 2A cDNA clones derived from the total RNA samples of different *Jatropha* tissues need to be further characterized by sequencing. Likewise efforts are to be made to obtain the Curcin precursor cDNA clones through RT-PCR approach as adopted in this study.

Concluding remarks:

Multiple forms of curcin exist in *Jatropha.* The individual forms have some varying biochemical attributes which could reflect their overall functionality in different tissues. Preliminary studies indicate that they are expressed differentially in the *Jatropha* tissues. Apart from cytotoxic effects, curcins have become attractive because of their pharmacological importance. Keeping all these points in view, several facile modern bioinformatics tools have been employed in this study to generate data comprising of searching protein motifs, 3-D modeling, phylogenetic tree and some other important attributes. Many of these features as obtained on the study were not reported earlier. All these information would be quite useful to carry out various aspects of protein engineering using curcin protein as molecular tools.

Apart from *in silico* approaches, RT-PCR approach was adopted to get a number of putative Curcin 2A specific cDNA clones. For this purpose total RNA preparations from different *Jatropha* tissues were employed. This would help to see any tissue specific variation of a particular curcin isoform. The cDNA as obtained in this study were partially characterized by restriction analysis and PCR, and appeared to be promising ones. However, these cDNA clones needs to be thoroughly sequenced prior to undertake molecular approaches for recombinant expression of the individual curcin isoforms.

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