Sequencing and *in silico* analysis of the curcin isoforms in Jatropha (*Jatropha curcas* L.), and cloning in the expression vector

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

Submitted by:
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June 2018

CANDIDATE'S DECLARATION

I, hereby declare that the work which being presented in the thesis entitled, "**Sequencing and *in silico* analysis of the curcin isoforms in Jatropha(*Jatropha curcas* L.), and cloning in the expression vector**" in the partial fulfillment of the requirement for the award of degree of Master science of Biotechnology, Thapar Institute of Engineering & Technology, 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 Institute of Engineering & Technology, 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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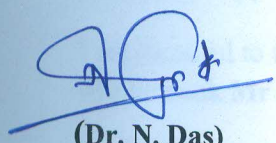
Place: T.I.ET, Patiala

CERTIFICATE

This is to certify that the dissertation entitled "**Sequencing and *in silico* analysis of the curcin isoforms in Jatropha(*Jatropha curcas* L.), and cloning in the expression vector**" submitted by Shreya Arora (Regd. No 301601021) in the partial fulfillment of the requirement for the award of the degree of Master Science of Biotechnology, to Thapar Institute of Engineering & Technology 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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(Shreya Arora)

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LIST OF ABBEREVATIONS

Abbreviation	Name
BLAST	Basic Local Alignment Search Tool
BLASTp	BLAST for Proteins
Bp	Base-Pair
$CaCl_2$	Calcium chloride
DEPC	Diethyl pyrocarbonate
DNA	Diethyl pyrocarbonate
EDTA	Ethylenediamine-tetra acetic acid
HCl	Hydro chloric acid
IU/ml	International unit per mL
Kb	Kilo Base
KDa	Kilo-Daltons
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
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
RIPs	Ribosome inactivating proteins
RNA	Ribo nucleic acid
Rrna	Ribosomal RNA
Rpm	Revolutions per minute
S (60S)	Svedberg unit
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
µL	Microlitre
µg	Microgram
mins	Minutes
O.D	Optical density
nm	Nanometer

ABSTRACT

Nowadays Jatropha(*Jatropha curcas* L.) is gaining world-wide importance due to its seed oil which is becoming a promising source of biodiesel. It can be grown on degraded and marginal land areas. It is also suitable in different types of soil conditions, rainfall and climates. It contains number of significant commercial metabolites which could be used in making cosmetics, soaps, fertilizers and lubricants. Presence of toxic substances in seeds like curcin and phorbol esters, limit their applications. Curcin, a major toxin protein, is present in seeds of Jatropha and other tissues. This is primarily a ribosome inactivating proteins (RIPs) which produces cytotoxic effects. Significantly, curcin protein has some pharmacological importance i.e. since its protein can be used as anti-tumor, anti-HIV, anti-viral and immune-suppressive agents. The present study focused on a curcin cDNA clone in pMD 20-Tvector namely Curcin 2A-17 having 99% sequence identity with a curcin isoform i.e Curcin2A (GenBank protein id: ADN39428). Various facile bioinformatics tools were used to study different attributes generating 3-D models, protein motifs, phylogenetic tree, Ramchandran plot, Hydropathy characters of the curcin isoform encoded by Curcin 2A-17, apart from studying its relatedness and divergence from other curcin isoforms. In order to clone the above Curcin cDNA in a suitable expression vector, the following efforts were made: Good quality of plasmid DNA i.e., protein expression vector, pET28a DNA was isolated. Curcin cDNA clone, approx. ~1.0 kb *Bam*HI-*Hind*III fragment was eluted by gel extraction technique. Then it was cloned in to pET28avector digested with *Bam*HI-*Hind*III. A few putative recombinant clones were isolated which will be duly characterized and used for recombinant protein expression.

Key words-Jatropha curcas L., curcin isoforms, protein motifs, 3-D modeling, Protein expression vector.

Chapter1: Introduction

1.1 Jatropha *(Jatropha curcas*L.)- a promising biodiesel crop

Speedy depletion of fossil fuels has caused an alarming situation as well as the negative effect of their use on environment has made scientist to broaden area of clean technology. Nowadays, *J.curcas* a multi-purpose plant is attracting attention as an oilseed crop for biofuel (Maghuly and Laimer,2013). Its seed oil is gaining world-wide extensive significance and is considered as best alternative form of fossil fuels as it could possiblybeat the conventional petroleum products. It can actually grow under climate and soil conditions which are not suitable for plants. Its seed contain 30% of oil which is used for biodiesel production. The oil produced meets American and European requirements.
J.curcas compounds are poisonous and thus not consumed by both animals and human beings.Seeds are the most toxic parts of *J.curcas.*

1.1.1 Taxonomy of Jatropha curcas

J. curcas L. also known asphysic-nut or purging-nut.Itis a perennial plant belonging to family(*Euphorbiaceae)*. The name of the genus Jatrophais derived from the Greek word i.e.,*iatros* (doctor) and *trophe* (food). It includes approximately 180 species of drought repellent succulent plant shrub.

Table 1 *Taxonomic hierarchy of J.curcas*(https://www.cabi.org/isc/datasheet/28393)

Domain	Eukaryota
Kingdom	*Plantae*
Phylum	*Spermatophyta*
Subphylum	*Angiospermae*
Class	*Dicotyledonae*
Order	*Euphorbiales*
Family	*Euphorbiaceae*
Genus	*Jatropha*
Species	*Jatropha curcas*

1.2 General description of *J.curcas*plants

1.2.1 Botanical features of Jatropha

Jatrophais usually a small shrub. It may be grown in low to excessive rainfall areas and can be utilized to reclaim land, as a hedge or industrial crop (Openshaw, 2000). Jatrophacan grow up to about 3 to 5 meters and for the duration of negative conditions they are able to attain heights of 8 to 10 meters and have a life span of 4 to 5 years. The plant has a gray bark and when chopped it forms white watery latex (Fairless, 2007).

From seedling phase generally five roots are formed: one is the central and four are peripheral. It fruits annually and per plant more than 1.5 to 2.0kg seeds areproduced. The plant has a history in medicinal uses from all over the world.Jatropha fruit, seeds, flower, leaf as shown in **Fig. 1**.The different parts of theJatrophaplant are used for different medicinal purposes asdescribed below:

- *Leaves*: The leaves of theJatrophaplant are 10 to 15 cm in size, 4 to 6 lobes and are heart shaped. They show green to pale coloration and are used in the treatment of inflammation.

- *Flowers*: The flora is formed terminally and individually. The plant produces male and female flower separately.The lengthof the petiole ranges about 6-23mm and shows inflorescence at leaf axis. Most female flowers are higherthen male flowers and occur during hot seasons (July to September).

- *Fruits*: The fruits are produced in autumn. The fruit is generally grey brown ovoid capsule of approximately 3cm and matures during October-November. If the conditions are suitable such as soil moisture and temperature then the crop can producefruits throughout the year.

- *Seeds*: The seeds are the most poisonouspart of theJatrophaplant due to thetoxic protein Curcin.From the date of fertilization the capsule changes from green to yellow in color. This suggests that the seeds are at the maturing stage. Seeds include about 22% of saturated fatty acids and 78% of unsaturated fatty acids. In Jatrophaoil mainly oleic and linoleic acid are present. It also comprises some sugar compounds such as phorbol esters, saponinsetc (Fairless, 2007).

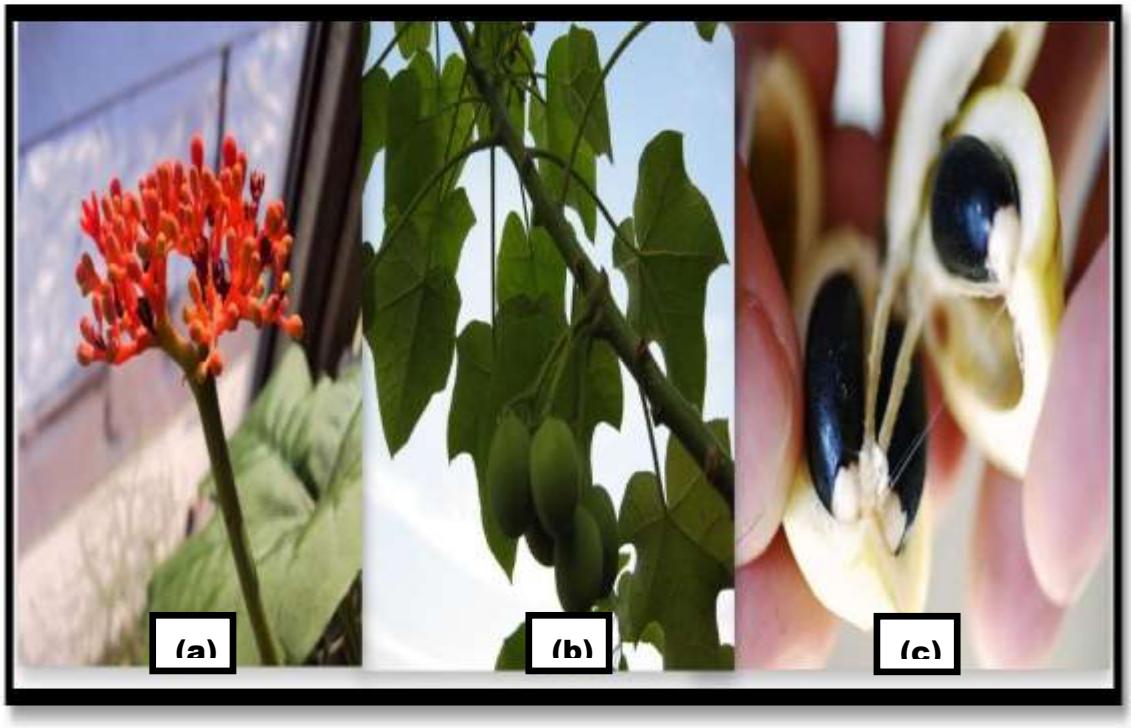

Fig. 1 Jatropha(**a**)Flowers, (**b**) Fruit &Leaf, (**c**)Seeds
(https://www.shutterstock.com/search/jatropha)

1.2.2 Distribution and cultivation of Jatropha

The Portuguese seafarers dispensed Jatropha seedsby way of the Cape Verde islands and guinea Bissau to other nations in Asia and Africa (Heller, 1996).Jatrophaplant can grow on wasteland due to its characteristics like hardiness,rapid, easy propagation, short gestation period, wide adaptation and optimum plant size which are suitable for cultivation. In India the plant is cultivated in Gujarat, Madhya Pradesh, Rajasthan, Maharashtra and Tamil Nadu.Jatropha plant cultivation is in general executed via two approaches vegetative propagation (cuttings) or generative propagation (seeds). Propagation through cutting is slower than from generative propagation.

1.3 Various applications of J.curcas

1.3.1 *Industrial use***:**Glycerin, which is a byproduct of Jatropha has high saponification value and is largelyused for making soap in India and world-wide. Another widely used application is production of biofuel from plant seed oil (Naharand Ozores-Hampton,2011).The protein content of Jatrophaoil cake is used as raw materials for plastics and synthetics fibers.

1.3.2 *Medicinal use***:**Almost all parts of Jatropha are used for medicinal purposes i.e., leaves, bark, stems etc. The latex of Jatropha contains an alkaloid which comprises anti-cancerous properties. The leaves are used as a remedy for malaria and high fever. *Curcas*oil also contains purgative properties additionally used for external problems such as skin diseases and Rheumatism. Diseases cured with Jatropha plant parts are shown in **Table 2**.

Table 2 Jatropha plant parts for medicinal uses (Kumar and Sharma, 2008)

Plant parts	Diseases
Seeds	To treat jaundice, gout, arthritis
Steam	Pyorrhea, toothache, gum bleeding
Plant sap	Dermatomucosal diseases
Water extract of branches	Tumor, HIV
Plant extract	Burns, wound healing

1.3.3 Source of biodiesel:Jatropha has a byproduct which is known as seed cake it contains excessive quantity of toxic protein called curcinhas similarities as of ricin protein present in castor oil which makes it unsuitable for the use in cattle feed. However, it has a potential as fertilizer or for biogas production (Staubmann*et al.*, 1999).Seed cake used as straight fertilizer rich in potassium, phosphorus, potassium the properties of seed cake are compared given below in **Table 3**.

Table 3 Nutritionalsurvey of oil seed cake and manure(Delgado and Parado, 1989)

Property	*J. curcas*oil cake	Neem oil cake	Cow dung manure
Nitrogen	3.2-4.44	5.0	0.97
Potassium	1.2-1.68	1.5	1.66
Phosphorus	1.4-2.90	1.0	0.96

1.3.4 Insecticidal property:Aqueous extract made from leaves contain insecticidal property. It is used for farming to control pest in pulses, potatoes, corns and reduce nematodes in soil and controls insects pest of cotton including cotton bollworm (Kaushik and Kumar, 2005).

1.3.5 *Environmental benefits*: To control soil erosion, Jatropha plants can be cultivated on wasteland.

1.4 Seed toxicity of J.curcas

Jatropha contain toxic compunds such as lectins, phorbal esters, saponins and phytate. These compounds are described below:

Jatropha contains toxic compound which are non-edible for both humans and animals major toxins are present in seeds.

1.4.1 *Curcin*:Atoxalbumin which is a type 1 RIP. It is a toxic protein isolated from Jatropha seeds, which has been found to inhibit protein synthesis.

1.4.2*Phorbalester*s:The main toxic protein i.e. phorbalesters present in Jatropha in very high concentration. It is a main toxic agent responsible for Jatropha toxicity (Aderibigbe*et al*,. 1997; Aregheore*et al*,.1998).

1.4.3 Lectins:Lectins are essentially protein that belong to plant origin that binds exceptionally to carbohydrate moiety. It has been proven that lectin will not be the predominant poisonous factor in Jatropha meal. Lectin are heat labile and their activity can be reduced by heat treatment.

1.5 Medicinal potential of Jatropha

All elements of Jatropha (seeds, leaves and bark) have additionally been used in natural treatment for veterinary purposes for a couple of centuries. Curcin is a toxic protein present in seeds of Jatropha. Antihelminthics are group of antiparasitic. Helminthes causes frightening diseases like pneumonia, anemia, eosinophilia (Bundy, 1994).It was reported by (Ahirrao*et al*.,2011) that Jatropha contains huge amount of saponins and alkaloids. Experiment was also carried out byJummaiand Okoli(2014)on helminthes and it was finalized that due to curcin which causes breakdown in central nervous system (CNS) block the glucose supply thus paralyzing the parasite. Curcin also shows antitumor activity. It was proven that curcin protein could inhibit the progress of tumor cells at very low concentrations. Leaves also show antiulcer activity (El-Baz*et al*.,2014).Medicinal uses of Jatropha as shown in **Fig. 2.**

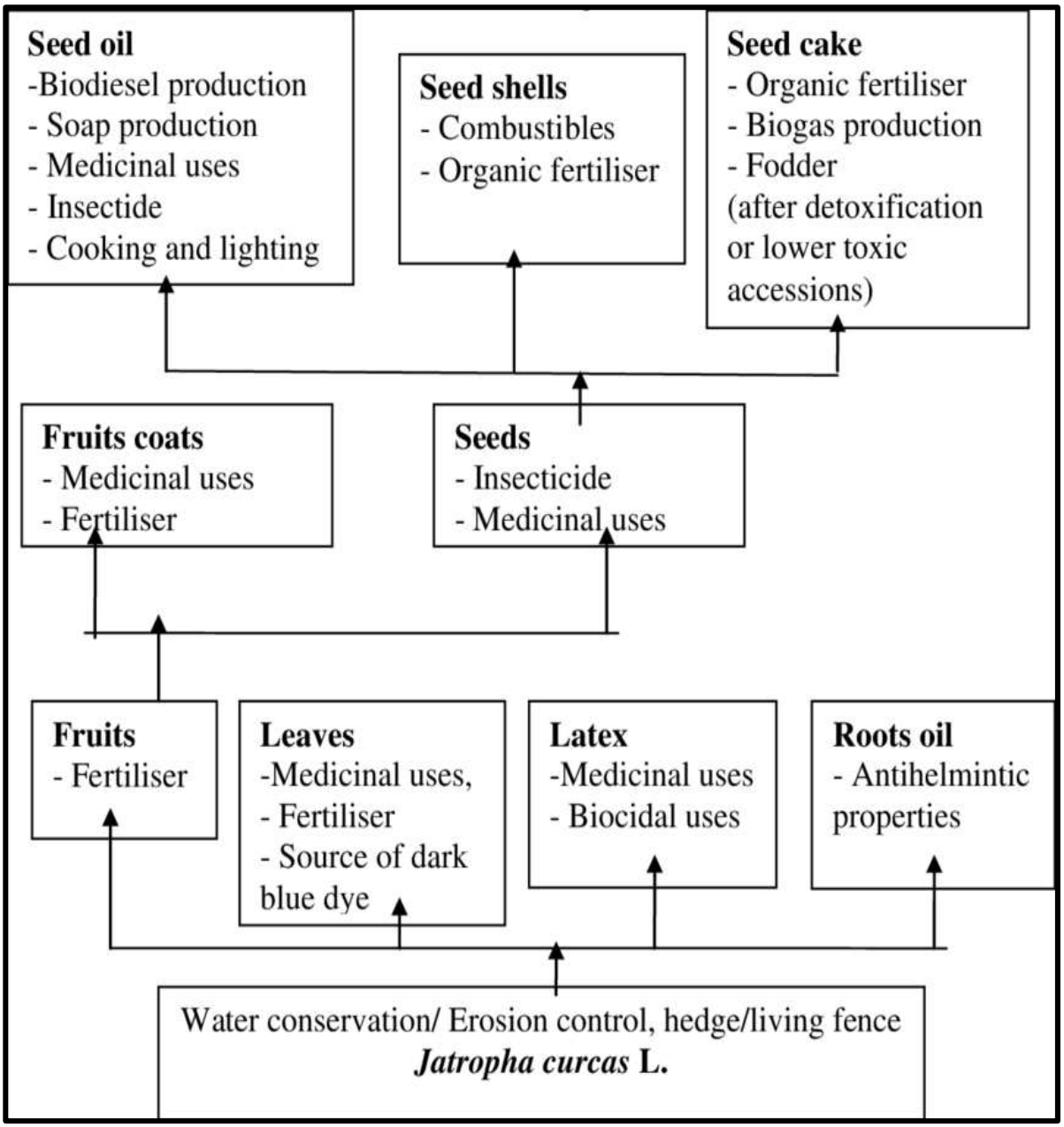

Fig.2 Medicinal uses of *J.curcas*(ttps://whww.researchgate.net/publication/258279438)

Chapter 2: Review of Literature

2.1 Extraction of biofuel from Jatropha

There's a steady seek for renewable resources of fuels as a result of the cost of depletion of fossils (Folaranmi, 2013).The oil extracted from this plant is further transesterified to receive biodiesel in usable for. Steps involved in the extraction of biofuel from Jatropha seeds are shown in **Fig. 3**. Jatropha seeds contain number of protein, fibers and four major fatty acid namely palmitic, linoleic, oleic and steric acid i.e., in the form of triacylglycerol.The biodiesel so obtained could be utilized in current diesel engines (Achten*et al*., 2008). On burning biodiesel does not produce carbon and sulphur monoxide. Hence reduces green-house gas emission up-to 50% and it also possess mutagenic potency. Biofuel from Jatropha seeds as shown in **Fig. 3**

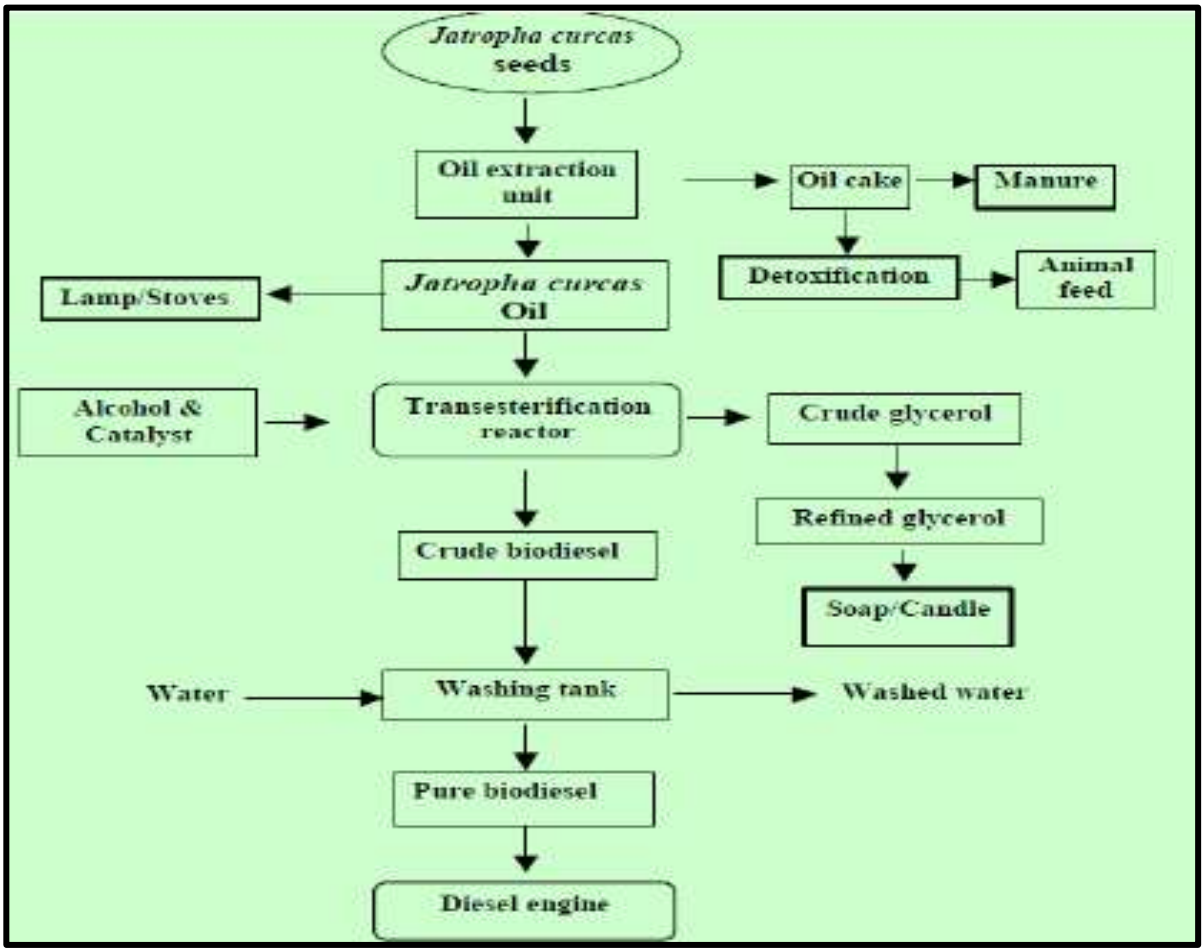

Fig. 3 Extraction of biofuel from Jatropha seeds (Arakerimath and Rachayya, 2012)

2.2 Curcin

Jatrophacontains a toxic protein curcin which was isolated from the *J.curcas* seeds by Felke(1914).It is a toxalbumin which is mainly found in the endosperm of the seeds ofJatropha(Mourgue*et al*., 1961).Curcin is a type 1 RIP (Ribosome inactivating protein) which irreversibly inhibit protein synthesis in both prokaryotic and eukaryotic cells by enzymatically altering 28SrRNA of the large 60S ribosomal subunit(Walsh *et al.,* 2013). In NCBI database exclusive types of curcin genes are pronounced which are, i) curcin2A gene (Accession no. GQ925453), ii) curcin L precursor (Accession no. EU195892), iii) curcin gene (Accession no. EU395775), iv) RIP partial *J. curcas* gene (Accession no. AY435214), v) curcin precursor gene (Accession no. AF469003). According to in vitro studies, it was found that curcin is 1000 times less toxic then ricin (phytotoxin found in castor bean).Crude curcin used tobe extracted from seeds of *J.curcas* with aid of solvent extraction approach usingsaline phosphate buffer as an extracting buffer and hexane as a solvent (JummaiandOkali, 2014).

2.3 Pharmacological importance of Curcin

Anthelmintic activity: Helminths infection generally spread in humans, which affects huge population. In tropical region helminthes infections are limited. It causes life-threatening diseases such as undernourishment, anemia, eosinophilia, and pneumonia (Bundy, 1994).

Antitumor activity: Research has been conducted by (Mohamed *et al*.,2014).They demonstrated that the cells which are exposed to type I RIP showed cytological and subcellular response. Normal and cancerous cell line was exposed to curcin. Many experiments were conducted to observe normal cell line to cancer cell lines. Curcin is known to affect reactive oxygen species, focal adhesion kinases and nuclear factor Kb(NF-Kb). Eradication of cancer colonies were observed with suppression of anti-apoptotic surviving. It was accomplished that proper functioning of cell-organelles and dysfunction leads to impaired cyto-metabolism which depends on the level of cellular activity.

Curcin as Immunotoxin: Hybrid molecules are type of immunotoxins and their molecule consist of toxic peptide chain which is linked to an antibody. According to crude curcin activity are intense and can be attributed its use as a component of immunotoxin. At

209^{th}position one cysteine residue is present in curcin. Thus with an activated antibody curcin may directly form a disulphide linkage.

2.4 Mode of action of RIP (Ribosome inactivating protein)

Toxin molecules present in Jatropha are lectins (Curcin), phorbal esters, and saponins and because of their high affinity lectins bound to carbohydrate moiety (Goldstein and Hayes, 1978), Abiotic and biotic stress reduces the growth and yield of Jatrophaplant. During drought stress and cold plant undergoes a number of developmental and physiological changes.Plant have evolved complex defense mechanism against viral or pathogen attacks Bowles, 1990).It includes ribosome inactivating protein (RIP), which are specifically capable of inactivating ribosomal proteins. Ribosome inactivating protein are mostly found in plants, mushrooms and bacteria (Stirpe, 2013). They are known to have catalytic toxins which cause an irreversible toxic effects on protein synthesis by damaging its ribosomal machinery (Joerg*et al*., 1997). The elevation of RIP, in plants varies from traces hundreds of milligram and they are present in most of the tissues like flowers, stem, leaves, bark etc. (Park *et al.,* 2004*)*.For the effective translation RIP require various cofactors (Carnicelli*et al*., 1992). They depurinate non-mammalian ribosomes from plants,insects(Zhou *et al*., 2000),bacteria and yeast (Roberts and Selitrennikoff, 1986).As it was also found that RIP remove more than one adenine residue from every single ribosome (Barbieri *et al*., 1994),RIPs also remove adenine residues from polynucleotides and DNA. RIP belongs to family of well-characterized toxins, which irreversibly inhibit protein synthesis in eukaryotic cells by enzymatically altering 28S rRNA of the large 60S ribosomal subunit.

RIPs are usually divided in to two subgroups on the basis of their structure and function(i) Type I RIP and (ii) Type II RIP as shown in **Fig. 4**. Type I RIP 30kDa single polypeptide chainwhich possessRNA N-glycosidase enzymatic activity and pH 8 to 10 of alkaline isoelectric point with or without carbohydrates. Similarly Type II RIP is a heterodimer of 35kDa chain A (active domain) linked to B chain (binding domain)through disulphide bond chain Apossess RNA N-glycosidase activity and B chainwith lectin specific properties binding with of sugar with galactosestructure(Stirpe, 2013).

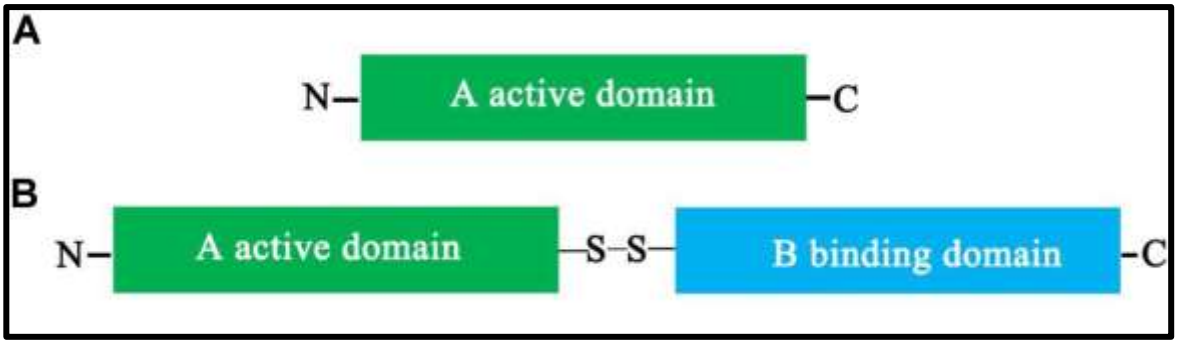

Fig. 4 Types of RIP: (**A**) Type1 RIP (**B**) Type II RIP (https://doi.org/10.3389/fpls.2018.00146)

Type IRIP (A chain) made up of toxic subunit whereas type II RIP with A chain along with B-chain which is lectin specific and are joined together by disulphide bond (Stirpe, 2004). Due to its RNA-N-glycosidase activity which causes toxicity in A-chain (Peumnas*et al*., 2001), at position 4324 in the 28S rRNAit brings about depurination of adenine. Critical stem loop configuration is prevented by this activity and the elongation factor is known to bind during the translocation step of translation types (Qin *et al*., 2005). At the end resulting cellular translation leads to complete inhibition. The role of B-chain is to help in the transport of RIPs into the cells by binding to specific sugar residues of glycoprotein or glycolipids on the plasma membrane and the internalization by endocytosis. Once B chain binds to the cell, it facilitates and allow entry of toxins into the cell (Walsh *et al*., 2013). Due to exertion of enzymatic activity which leads to damage of ribosome and other structures resulting cell death and cell damage. A diagrammatical representation of biochemical action of RIP is shown in **Fig. 5.**

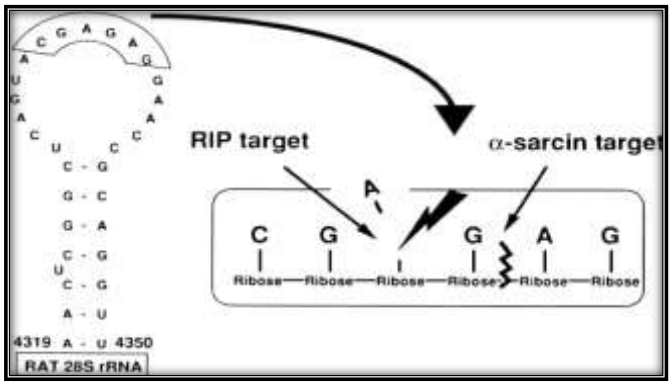

Fig. 5 Biochemical action of ribosome-inactivating proteins (RIPs)(http://www.jnsci.org/files)

2.5 Salient features of an expression vector

An expression vector is used to introduce a specific gene in to a target cell, and control cell mechanism for protein synthesis to produce protein encoded by gene. The vector is engineered to contain regulatory sequences that act as enhancer and promoter region and leads to efficient transcription of the expression vector carried by the gene.Expression vector produces efficient amount of protein with the help of stable messenger RNA which can then translated in to protein.Expression vector have promoter for DNA insert that are induciblei.e., they are only expressed with certain polymerases under certain conditions (Clark and Pazdernik, 2013).Promoters initiates transcription and is therefore control the expression of cloned genes. Protein synthesis is only initiated by the introduction of an inducer such as IPTG. Additionally expression vector contains tags, produce a peptide sequence which is linked to the protein of interest, when expressed,that is then used to isolate the protein (Clark and Pazdernik, 2013). Features of expression vector shown in **Fig 6**.

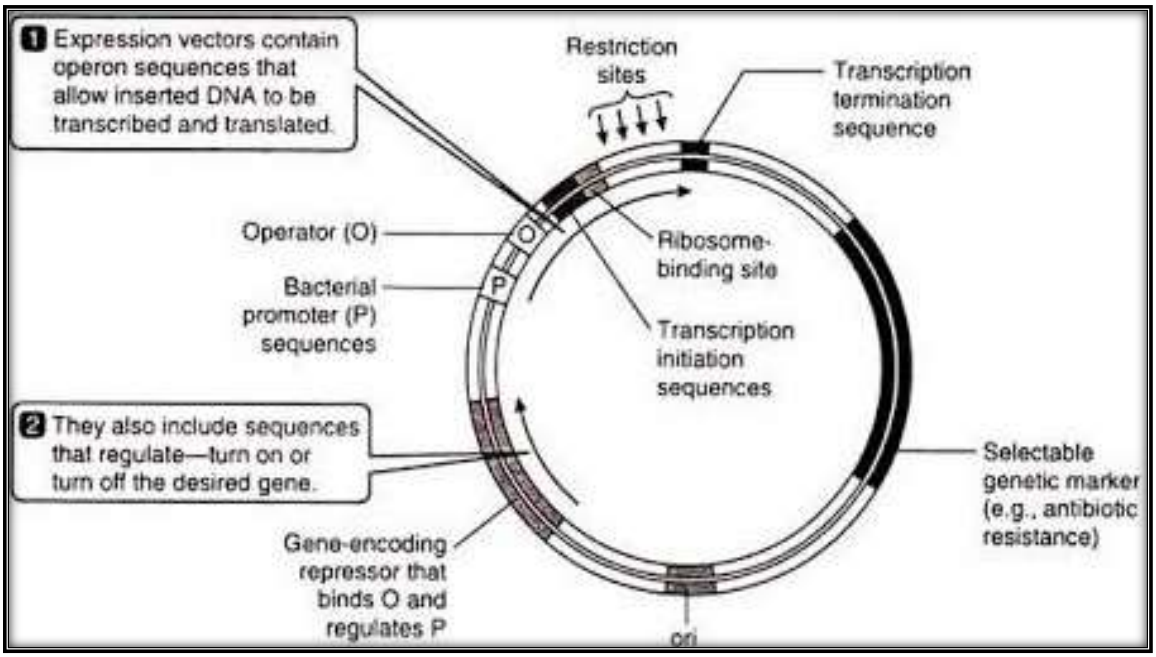

Fig. 6 Features of an expression vector(http://www.biotechnologynotes.com/recombinant-dna-technology/3-main-classification-of-vectors-with-diagram/395)

Following features of expression vector are explained below:

Promoter: This is recognized by sigma subunit of RNA polymerase and gene of interest of transcription which is required for initiation.

Termination: It is an element of DNA which is present at the end of gene where transcription ends. It's a short nucleotide sequence which itself can base pair with to form hair pin loop

Ribosome Binding site: It is a short nucleotide sequence recognized by the ribosome as the point at which it should attach to the messenger molecule. The initiation codon of the gene is always a few nucleotide downstream of this.

2.6Work done so far in the laboratory on Jatropha

In north-western state of India, from the state of Punjab different agro-climatic zones *Jatropha* accessions have been collected. Earlier work on the four *Jatropha* accession namely TJS01#03, TJS17#03, TJS35#01, TJS42#04 was done in 2015. From accession no. TJS17#03 of seed kernel crude curcin extract was prepared. By MTT assay on RAW and Hella cell lines crude curcin cytotoxic effect was assessed. Significantly on cancer cell line extract of crude curcin exhibit inhibitory effect. There are different form of curcin namely ribosome inactivating protein (RIP), curcin2A, curcin precursor, curcin-L precursor. At molecular level some studies were also done for example BLAST analysis predicted that curcin gene promoter region showed considerable sequence divergence when compared with the other member of this family. In inclusion, efforts were made to isolate good quality of genomic DNA from field grown tender leaves with applying simple and efficient protocol introduced in the laboratory. 5-flanking regions of various curcin gene was used to amplify and PCR was carried out and various sizes of amplicons were reported. Curcin sequence analysis was carried out by and predicted some protein 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, *J.curcas*. Moreover, Hydropathy plots of curcin2A protein revealed that the N terminal part of this protein is hydrophobic and 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 at molecular level curcincDNA clone was carried out. Sequence analysis of curcin isoforms was done which predicted 3-dimensional structural of curcin. Molecular cloning of cDNAs encoding curcin isoforms through RT-PCR approaches, and the preliminary characterization of the cDNA clones.

2.7Objectives of the study

Molecular cloning of a cDNA, sequencing, *insilico* sequence analysis, and prediction of important protein motifs along with related features are prerequisites prior to any recombinant protein expression using an expression vector.Curcins are toxic proteins in Jatropha as they are ribosome inactivating proteins (RIPs); however, curcins have some pharmacological importance. Keeping in view the following objectives were framed for this study:

- Sequencing, and sequence analysis of a curcincDNA from *J. curcas*
- *In silico*analysis for predicting structural and functional aspects of the different protein motifs in curcin
- Strategies for molecular cloning ofcurcincDNA in an expression vector

Chapter 3: Materials & Methods

3.1 *In silico*analysis

Procedure for the identification, structure prediction and study of curcinisoforms in *Jatropha*. The sequence was retrived fromBlastpNCBI http://www.ncbi.nlm.nih.gov. For further analysis sequences were retrieved3-dimensional structure of curcin protein, prediction motifs and sequence analysis was done.

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

Identification of conserved domain: MY HITS tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan) uploaded curcin TJs-17 protein sequence and different sites were predicted.

3-D modeling: ProSA-web tool gives the analysis of protein structure of the protein model. This tool gives reliable protein models and according to Z-score and X-ray/NMR region. The model which fall in the X-ray/NMR region and which contain lowest Z-score were selected. Further NOMAD is used for energy minimization (http://lorentz.immstr.pasteur.fr/gromacs/minimization_submission.php).

Sequence retrieval and 3D structure prediction: Amino acid sequence of Curcin 2A-17 deduced and was retrieved from DNA dynamo tool and it consist of 309 amino acid residues. Similarity, search of curcin2A-17 sequence retrieved, was done using protein blast tool of NCBI. It was observed that lower e-value with no single template was able to satisfyand 100% query coverage. Therefore combination of multiple templates were opted to enhance query coverage, hence for full length protein structure prediction, implemented I-TASSER server (http://zhang.bioin-formatics.ku.edu/I-TASSER).

3.1.1 Reagents and chemicals

Enzymes where obtain from Bangalore Genei Pvt. Ltd., and chemicals were taken from Sigma-Aldrich India Pvt. Ltd., and HI media Pvt. Ltd.,. Vector strains were purchased from were maintained in the laboratory. For gel extraction silica gel extraction bead was used. Other chemicals were prepared in laboratory given in **Table 4**.

Table 4 Different buffers, chemical and their composition

Chemicals	Composition	Volume
Gel loading buffer (5X)	Sucrose EDTA Tris Bromophenol	35% (v/w) 50mM (pH 8.0) 25mM 0.2%(w/v)
TBE (5X) buffer (pH 8.0)	Tris Base Boric Acid EDTA	54 g L^{-1} 54 g L^{-1} 3.8 g L^{-1}
TE Buffer	Tris HCL EDTA	10mM (pH8.0) 1mM (pH8.0)
Extraction buffer	Tris HCL EDTA Sucrose NaCl	50mM(pH8.0) 50mM 15% 250Mm

- Ethanol (Merck)
- Isopropanol (Sisco Research Lab PVT. LTD.)
- 5M Potassium acetatepH (5.5) (SRL)
- 3M Sodium acetatepH (5.5) (SRL)
- RNase (2mg/ml) (Thermoscientific)
- Alcohol (SRL)
- Chloroform (SRL)

3.1.2 Enzymes used during experimental work

Restriction enzymes such as *Sma*I, *Eco*RI, *Hind*III, *Bam*HI (Bangalore Genei)were used during laboratory work. The restriction was carried out in buffer and were maintained according to temperature requirements recommended by manufacturer.

3.2 Methods

3.2.1 Agarose gel electrophoresis

Requirements:

0.5X TBE buffer

Ethidium bromide dye (5mg/mL)

Agarose (Sigma)

Sterile water

DNA samples

Bromophenol blue dye

Gel electrophoresis apparatus

Gel-Documentation system.

Procedure:

To perform agarose gel electrophoresis standard method was followed. 0.8% agrose gel was weighed and mixed in 0.5 X TBE buffer and 8ulethidium bromide dye was added to it. Then gel was casted in a tray and then allowed to polymerize. The DNA samples was mixed with bromophenolblue and loaded in the wells. 0.5X TBE buffer was used in electrophoresis at 5-6 volt till the tracking dye covered $3/4^{th}$ length in agarose gel. Under the Bio Rad Gel Doc instrumentDNA bands were then visualized.

3.*2.2 Isolation of plasmid DNA by Alkali lysis method*

*Solutions for plasmid isolation**:***

Solution I – 50mM glucose, 10 mM EDTA, 25 mMTrisHCl (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:

Alkalilysis method**:** At mini scale level plasmid isolation was carried out by alkali lysis method as described by Bimboim and Doly (1979), transformed *E.coli* colony was inoculated in 25ml of Luria broth containing ampicillin (50µg/mL) the culture was inoculated overnight at 37°Cwith vigorous shaking. In microfuge tube 1.5ml of above grown cultured was taken and centrifuged at 8000rpm for 5mins. Supernatant was decanted and pellet was dried and resuspended in 200 µL of ice cold solution I and incubated at ice for 10mins. Followed by addition of 300 µL solution II freshly and mixed gently. For 5 minsthe tubes were stored on ice. Then addition of ice-cold 400µL solution III was added mix solution by gently inversion of tubes so that white curdy precipitate is formed. Then , for 15 to 20 mins tubes were stored on ice.After the incubation, at 12000rpm tubes were centrifuged for 15minsat 4°C. In fresh microfuge tubes supernatant was carefully transferred. For precipitation of DNA equal volume of isopropanol was add and was kept at 4°C for 30mins. Followed by centrifugation at 10,000rpm for 15mins at 4°C. Decanted supernatant pellet was air dried and dissolve in 50 µL of TE buffer. For further purification 370 µL of sterile water was added to crude plasmid and 2 µL of DNAase free RNAase was added. The tubes were then incubated at 37°C for 60mins.Extraction of DNA was done with equal volume of phenol and chloroform then centrifugation was doneat 8000rpm for 10mins and upper aqueous layer was shifted in fresh tube and 1/10 volume 3M sodium acetate was added and followed mixing for 5mins. Double volume of dehydrated alcohol and was kept at -20°C overnight. At high speed for 10 min centrifugation was done and supernatant was decanted. The pellet was washed with 70% ethanol and air dried and finally dissolved in 30µL TE buffer.

3.2.3 Gel elution of Purified RT-PCR product from agarose gel

*Requirements:*Vortex, Gel elution kit, sterile blade, eppendrofs, water bath,UV-transilluminator

Procedure:

For cloning purpose silica bead DNA gel extraction kit was used to elute curcinisoforms DNA bands. Silica bead DNA agarose gel extraction protocol was followed for elution of DNA bands. In 1X TAE buffer DNA samples were run in 0.8% agarose. As control DNA samples were loaded in one lane and with clean scalpel this control lane was excised and to mark the position of DNA bands it was then visualized under UV-trans illuminator. In the control lane the position of bands were matched and the corresponding DNA bandswere excised from the gel (without UV exposure). In 1.5mL microfuge tubes the gel slices containing DNA bands were weighed. As per the kit instructions, three volume 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 sec. The tubes were then incubated at 55°C in water bath for 5 mins with intermittent vortexing after every 1 min for 10 sec in order to allow adsorption of DNA on the beads. Samples was then centrifuged for 30 sec at 6000rpm and with the help of pipette very carefully the supernatant was removed. At high speed centrifugation was done for 30 sec to wash the pellet with 500 µLof washing buffer. Similarly with 500 µL washing buffer pellet was washed for two times. In laminar air flow the pellet was dried properly until it looks white powdery form. 7µL DEPC and 7µL TE buffer was added to dried pellet. Pellet was resuspended in TE buffer by vortexing for 15-20 sec. And for 5mins it was incubated in water-bath at 55°C. For 30 sec at 6000rpm centrifugation was done. The purified DNA containing supernatant was transferred in a fresh sterile microfuge tubes carefully. To increase the yield of DNA the last two steps were performed again. To check the yield 1.5 µL of eluted DNA was loaded in 0.8%agarose gel.

3.2.4 Ligation reaction

Requirements: For ligation reaction T4 DNA Ligase purchased from Bangalore Genei and pET28a vector maintained in laboratory was used. In **Fig. 7** vector map is shown. In **Table 5**ligation reaction requirements were given.

Table5 Components for Ligation Reaction

Components	Volume
Water	5 µL
Linearized Vector	3 µL
PCR product (Insert)	3 µL
Buffer	3 µL
Enzyme	1 µL

Procedure: In a sterile micro tube insert, pET28a vector and ligation buffer sterile distilled water was added and mixed properly. Then T4 DNA ligase was added to it and was mixed gently. The reaction was incubated for 5 hours at 25°C.

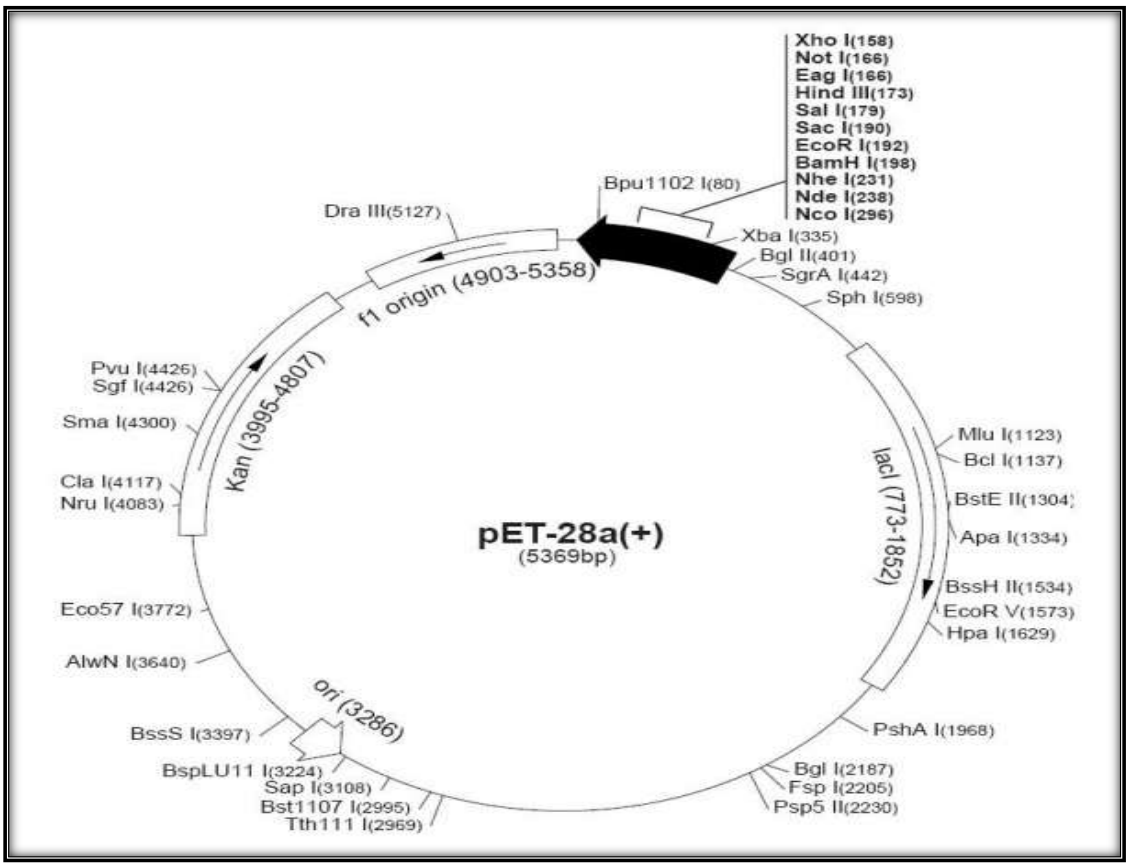

Fig. 7 pET28a expression vector map (http://ecoliwiki.net/colipedia/index.php/pET-28a(%2B))

3.2.5 Genetic transformation of E.coli DH5α with recombinant plasmid

Requirement: *E.coli,* fresh plate, Luria broth, $CaCl_2$, X-gal, IPTG

Media: LuriaBertani medium

For preparing LA –Kanamycin, at working concentration 50µgm L^{-1}.kanamycin was added to the LA medium. Freshly, autoclaved media was used.

Procedure:

Preparation of competent cells: A single colony of *E.coli* was picked from freshly grown plate and inoculated in autoclavedfreshly made media Luria broth. The culture was then incubated at 37°C with continuous shaking. Next morning 200µL of the grown culture was inoculated and incubated at 37°C for 2-3hours at shaker to obtain an O.D around 0.4 to 0.5 at 590nm. To slow down the metabolism the culture was transferred to centrifuge tube and kept on ice for 5mins. At 8000rpm for 10mins at 4°C culture was then centrifuged. In 20 mL of chilled 50 mM $CaCl_2$ cell pellet was then suspended properly and kept in ice for 10mins. For 10mins the cell-$CaCl_2$ suspension was kept for centrifuged, 1mL chilled $CaCl_2$ was added to the pellet and kept for 3hours in ice. In a fresh eppendorf (100µL) competent cells were transferred and 10ul ligation reaction mixture was added, for 30-40 mins the tube were kept on ice then for 90 sec. At 42°C heat shock was given. Place the tubes in ice, added 1ml of media and kept the tubes for 90 mins at 37°C. Further spread on LA-kanamycin plates (50 µg mL^{-1}), ligated-competent cells are then spreaded on the plates (after 1 hour). Kept the plates to dry and place them in incubator at 37 °C for 12-16 hours. The transformed white colonies were selected. Each white colony contain pET28a vector andpET28avector + insert. The colonies were picked up with sterile tooth pick and patches were made on LA-kanamycin plates and further DNA was isolated.

Chapter 4: Results & Discussion

4.1 *In silico* analysis

- Curcin is the most toxic compound present in the seeds of Jatropha plant and has a significant pharmacological importance. Considerable reports on the structure and isoforms of Jatropha are not available in the literature. So, the study was focused on the *in silico*analysis to understand the curcin isoforms its biochemical attributes, structure sequence analysis, comparison and protein motifs
- This study is mainly focused on the isoform of Curcin 2A-17 from Jatropha

4.1.1 Sequencing of Curcin2A cDNA clone

The cDNA specific to Curcin-2A (GenBank id: GQ925453.1) was cloned in from the seed pericarp of TJS # 01 previously in the laboratory and was sequenced.

4.1.2 Salient sequence features

The 947-bp CurcinTJS-17 consisted of 10 –bp 5´- UTR, 927 –bp ORF (bases 11-937), and 10 –bp 3´ UTR. The predicted protein designated as Curcin 2A-17 consists of 309 amino acids as shown in **Fig 8**. The G/C content was 10%, 38% and 30% in 5´- UTR, ORF and 3´ UTR respectively. NCBI search revealed that Curcin 2A-17 shows 99% sequence identity with full length *Jatropha curcas* Curcin 2A gene complete cds (GQ925453.1) and *Jatropha curcas*Curcin gene complete cds (EU395775). The coding region is significantly similar with other Curcin isoforms cDNAi.e., 93% with *Jatropha curcas*, RIP, cucurmosin like transcript variant X1, mRNA (XM _012218945) and 88% with *Jatropha curcas*RIP cucurmosinmRNA (XM_012219301).

```
5´tgaaatcaatATGAAAGGTGGAAAGATGAACCTCTCCATTATGGTGGCTGCCTGGTTTTGCTGGAGTAGT 70
M  K  G  G  K  M  N  L  S  I  M  V  A  A  W  F  C  W  S  S  20

ATTATATTCGGATGGGCATCGGCTAGGGAAATAGTTTGTCCATTCTCATCAAACCAAAACTACAAAGCTG  140
I  I  F  G  W  A  S  A  R  E  I  V  C  P  F  S  S  N  Q  N  Y  K  A  G 44

GTTCCACTCCAACTTTAGCCATTACTTATGACGCTACTACTGATAAGAAAAACTACGCCCAGTTCATTGA  210
  S  T  P  T  L  A  I  T  Y  D  A  T  T  D  K  K  N  Y  A  Q  F  I  E    67

AGATCTAAGAGAAGCATTTGACTTCAGTTATTTAAGCCATAAAATACCAGTCTTACGGGCCACGGTTGCT  280
 D  L  R  E  A  F  D  F  S  Y  L  S  H  K  I  P  V  L  R  A  T  V  A     90

GCAAATCAGAAATTTATTGTAGCCAAAGTCATAAATTCTGGGGACATAGAAGTAGCAGTAGGATTAAACG  350
A  N  Q  K  F  I  V  A  K  V  I  N  S  G  D  I  E  V  A  V  G  L  N  A  114

CCATTACTGCACATCTAGTGGCTTATAAGGTAGGAAGTAATTCCTATTTCTTTAACGATTCGGAATCTTT  420
  I  T  A  H  L  V  A  Y  K  V  G  S  N  S  Y  F  F  N  D  S  E  S  L  137

GGCTGATGCAAAAAAAAATCTTTTCACAGACACAAACCAACAAACACTAGCATTTACTGGTAGCTATGCA  490
 A  D  A  K  K  N  L  F  T  D  T  N  Q  Q  T  L  A  F  T  G  S  Y  A    160

GATTTTGAATCTAGGGCAAAGTTACATAGAGAGGAAGTGGATTTAGGAGTGGTGGCATTGGATAATTACG  560
D  F  E  S  R  A  K  L  H  R  E  E  V  D  L  G  V  V  A  L  D  N  Y  V  184

TATATACACTTGAAAAAAGTTCTCAGCCAGCAGACATTGCTAAACCTCTAGTTGGTTTTATCGAAATGGT  630
  Y  T  L  E  K  S  S  Q  P  A  D  I  A  K  P  L  V  G  F  I  E  M  V   207

TCCAGAGGCAGCAAGATTCAAATATATTGAGAAAAAAAATATCAACTCAAATTAGCAAAACCTTTAGGCCG  700
 P  E  A  A  R  F  K  Y  I  E  K  K  I  S  T  Q  I  S  K  T  F  R  P    230

CGTGGTGACATAATTAGCCTTGAGAACAACTGGGGAGACCTCTCTTATCAAATACAGAAATCTGTTGATG  770
R  G  D  I  I  S  L  E  N  N  W  G  D  L  S  Y  Q  I  Q  K  S  V  D  D  254

ATGTATTTCTGAAGCCAGTTCAATTGCAACGTGAAAACTATACCAATATCCTAGTGAACAATGTCACCCA  840
  V  F  L  K  P  V  Q  L  Q  R  E  N  Y  T  N  I  L  V  N  N  V  T  Q 277

AGTAAAAGGTCTCATGGGAGTCTTGTTGAATGCAGTCAATTACAAAGTCTCAATGGAAGAAATTATTTTC  910
 V  K  G  L  M  G  V  L  L  N  A  V  N  Y  K  V  S  M  E  E  I  I  F    300

AACGACCAAAAGTGGCTGCCATGGCTTtaatcctact3´947
N  D  Q  K  W  L  P  W  L  *        309
```

Fig. 8 947- ntcurcincDNA(Curcin2A-17) sequence encoding Curcin- 2A specific isoform from the Jatropha TJs #01 variety showing deduced amino acid sequence (309-aa). The 5´- UTR and 3´- UTR are shown in lower cases.

4.1.3 Analysis of BLASTp using the sequence of Curcin isoform

(a) Curcin 2A-17

BLASTp analysis was carried out using sequence Curcin 2A-17(to be submitted to NCBI) as a query. Curcin 2A-17 shows 309 amino acids and 99-86% sequence identity with other curcin isoforms. In most of the cases query coverage was 100% Curcin (*J. curcas*) i.e., 94%. The data generated by the BLASTp is given below in **Fig.9** and **Table6**

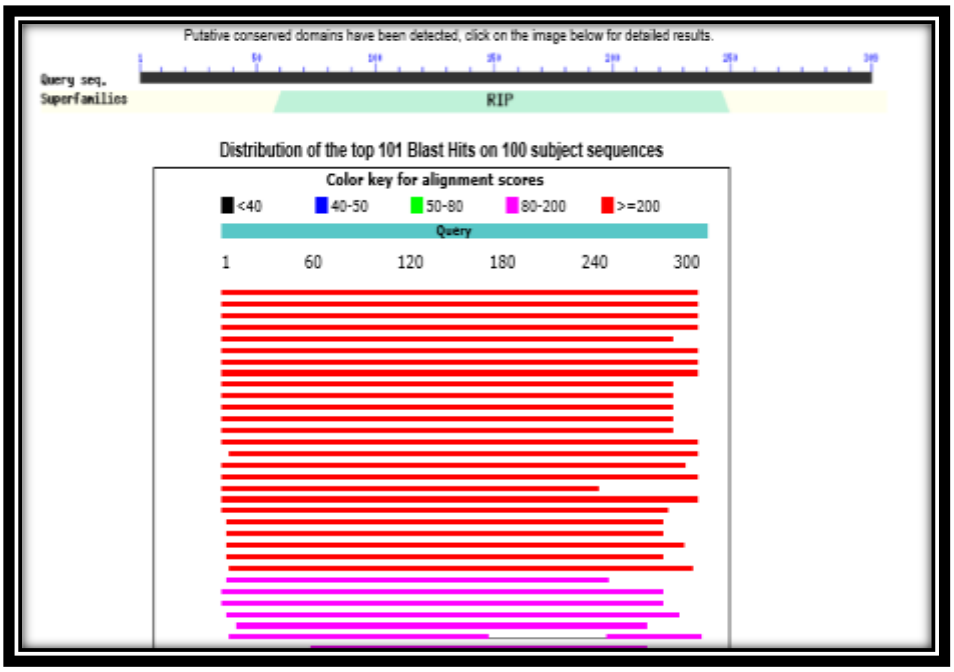

Fig. 9 Blastp analysis of Curcin precursor

Table 6 Blastp analysis for sequence identity between the curcin isoforms

Protein name	Accession number	ORF length	Max score	Identity %	Query	Amino acid
Curcin 2A-17	To be submitted to NCBI	940	636	99	100	309
Curcin	ABZ04128	930	636	99	100	309
Curcin-l-precursor	ABW17545	930	635	98	100	309
RIP	AAR08395	930	633	98	100	309
Curcin	ACO53803	882	587	97	94	293
Curcin precursor	AEA72440	930	580	89	100	309

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

Six curcin isoforms were taken for multiple sequence alignment (MSA) such as Curcin 2A-17, Curcin L precursor, RIP (Ribosome inactivating protein), Curcin precursor, Curcin (Jatropha curcas), putative Curcin 2b (Jatropha curcas) using multiple sequence alignment tool COBALT as presented in **Fig.10**MSA reveals the highly conserved domain including N-glycosylation sites. Because of mutation comparison between sequences shows approximately 33% variations. Variations such as deletion insertion are highlighted in the **Fig. 10** given below

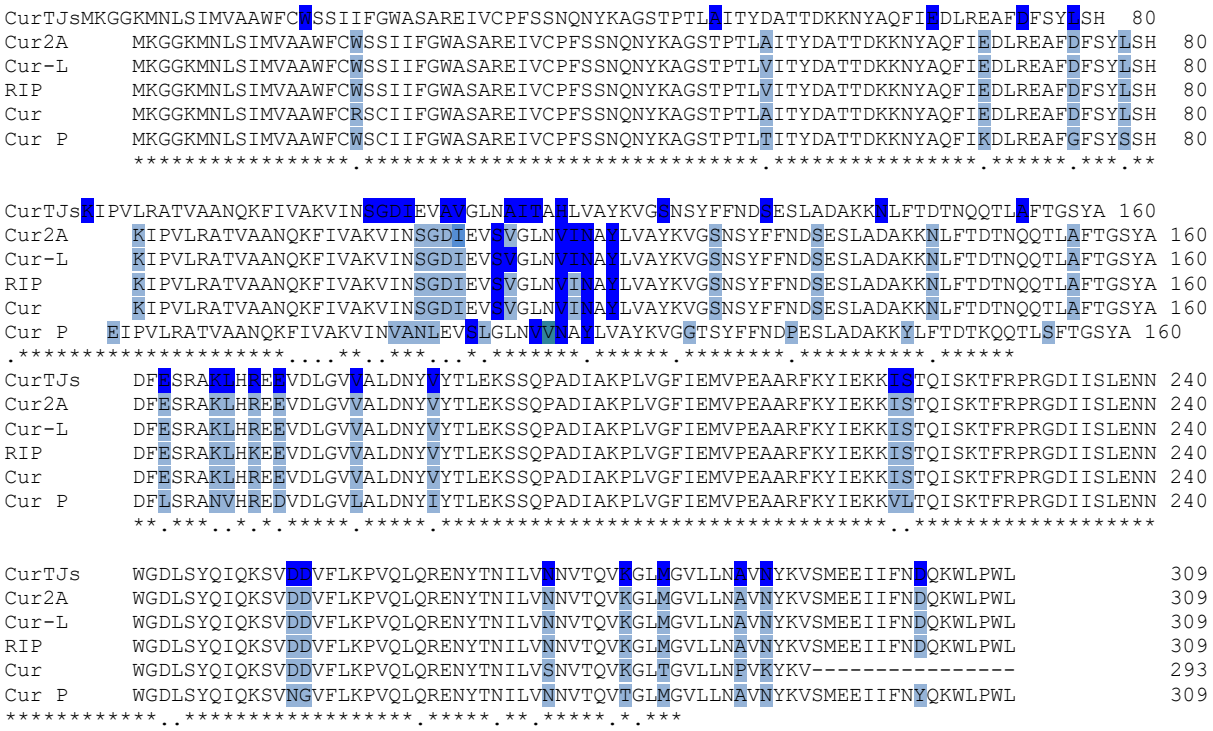

Fig. 10 Multiple sequence alignment of amino acid sequences of six different forms of curcin protein. The alignment is based on COBALT tool. The asterisk (*) symbol denotes the conserved amino acids between all forms; full-stop (.) denotes the amino acids which have divergence from other sequence

4.1.5 Hydropathy plot of Curcin TJS-17 protein

Hydropathy plot was trigger for Curcin 2A-17 using the ProtScale tool based on the Kyte-Doolittle given in the **Fig. 11**

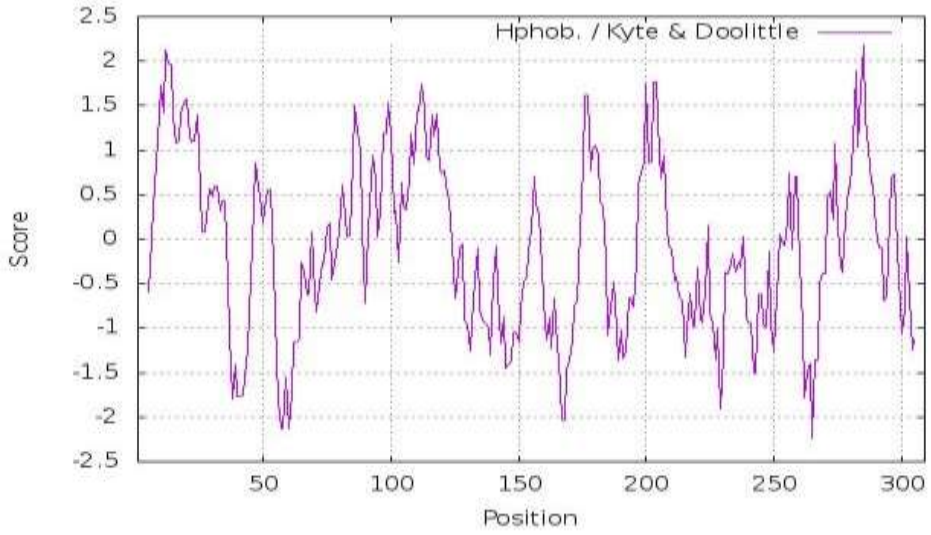

Fig. 11 Hydropathy plot of Curcin TJS-17

Hydropathy plot of Curcin 2A-17 protein revealed that N-terminal amino acid sequences of Curcin 2A-17 is Hydrophobici.e 7 to 35 positions of amino acid sequences. Hydrophilic regions in the plot are 36-35, 55-72, 129-139, 143-155, 166-175, 184-196, 209-223 positions of amino acid protein, whereas Hydrophobic region in the plot include 47-54, 91-124, 197-207 and 275-300 positions of amino acid in Curcin 2A-17 protein.

4.1.6 Amino acid composition analysis in the Curcin isoforms

In varied amount all the standard amino acids are present in Curcin isoforms. The amino acid composition of Curcin 2A-17was closely inspected. In **Table 7** the composition of some of the amino acids are presented which states deviation if comparison occur between their average occurrence in the natural points. Structural and functionality changes in protein due to increase and decrease in the amount of amino acid for example Proline and Histidine occur relatively at less frequency in both Curcin 2A and Curcin 2A-17; and some hydrophobic amino acids such as Isoleucine, Valine and Phenylalanine occur more in these isoforms. All the significant composition changes in amino acids could have profound effects on the structure and function of the curcin isoforms.

Table 7 Analysis of some amino acid composition (in%)

Amino acid	Average occurrence	Curcin 2A	Curcin 2A-17
Valine	6.6	8.9	8.1%
Isoleucine	5.3	6.5	7.1%
Phenylalanine	3.9	5.5	5.5%
Tyrosine	3.2	5.1	4.2%
Serine	6.8	7.5	7.8%
Asparagine	4.3	6.8	5.5%
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	1.0%
Arginine	5.1	3.4	2.9%

4.1.7 Searching protein motif

For prediction of some protein motifs the amino acid sequence of Curcin 2A-17 was analysed with the tool **MY HITS** as shown in **Fig. 12**

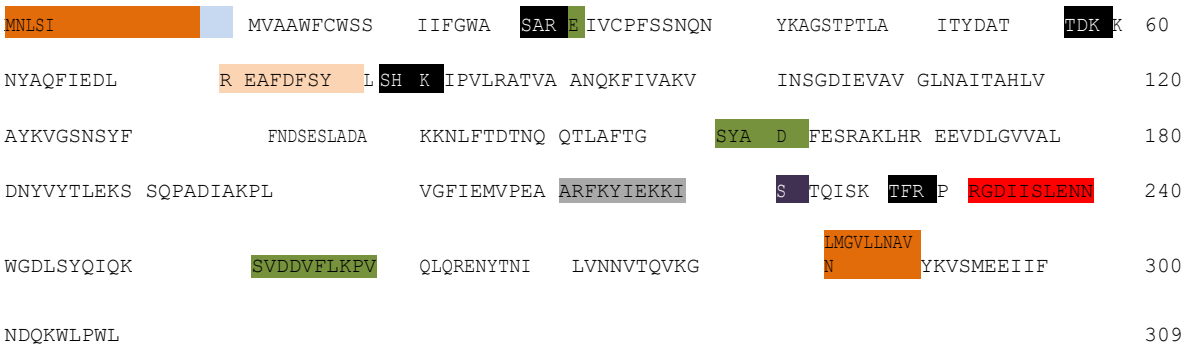

Fig. 12 Using different colors showingsome highlighted protein motifs of amino acid sequence of Curcin 2A-17.

The position of individual protein motifs of amino acid sequence are presented in the **Table 8** given below:

Table 8 Motif sites and position of Curcin 2A-17

S. No.	Site	Sequence	Amino acid position	Color
1.	N-glycosylation	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-myristolat.ion 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 toxin active signature*	IEMVPEAARFKYIEKKI	204-220	Grey

In curcin N-glycosylation sites are present where sugar molecules could be attached at specific sites. The accessibility of different enzymes which are present in cellular component are synthesized from type of N-glycans. It occurs in selective Asparagine residue. Casein kinaseIIcatalyses the transfer of phosphate to peptide substrate. N-myristoylation site help in the lipidationi.e, in attachment of amide bond to alpha amino group of N-terminal glycine residue. It plays important role in signal transduction and membrane targeting in plants which is responsible to environmental stress. Signal transduction cascade are protein kinase C enzyme which are involved in controlling function of other proteins through phosphorylation of hydroxyl groups of serine and threonine amino acid residue. Both the isoforms of cell attachment sequence are present which act as receptor for cell adhesion molecule and cell-cell interaction. Further, biochemical approaches are required to validate the functionality of the individual motifs as predicted in this study

4.1.8 3-D modeling studies

Phyre2 tool is used to predict 3-dimesional protein structure of Curcin 2A-17. From NCBI amino acid FASTA sequence were taken and was uploaded in the tool, many PDB files were generated and which were further used to find best model. Protein model asshown in **Fig. 13**.

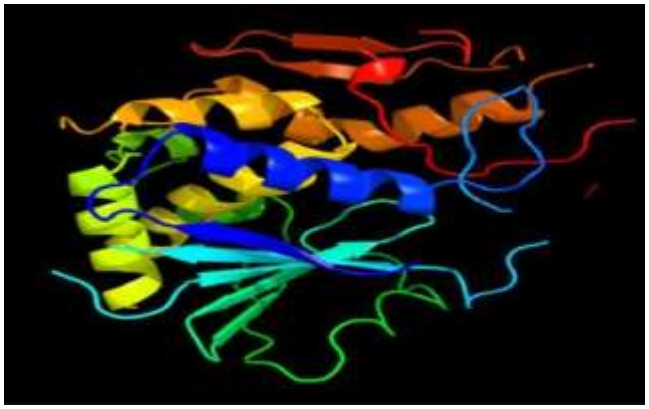

Fig. 13 Model structure of Curcin 2A-17

▪ *X-ray/NMR structure*

For validated protein models ProSA-web tool is used, many pdb files were generated for validation on ProSA-web tool in fig the following data were obtained the figure depicts the position of protein in the X-ray/NMR region. Black colored dot showing protein region. This considered as the best model if protein falls in this region as shown in **Fig. 14**

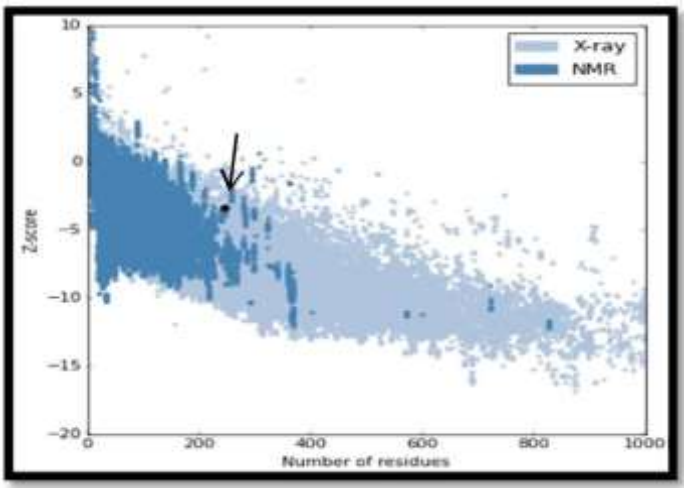

Fig. 14 X-ray/NMR structure of Curcin 2A-17(Arrow indicates the protein region)

- *Model quality*

Validation is an important way for protein models which is based on energy levels as shown below in **Fig. 15** the best model is considered when it shows energy level of the sequence less than 0

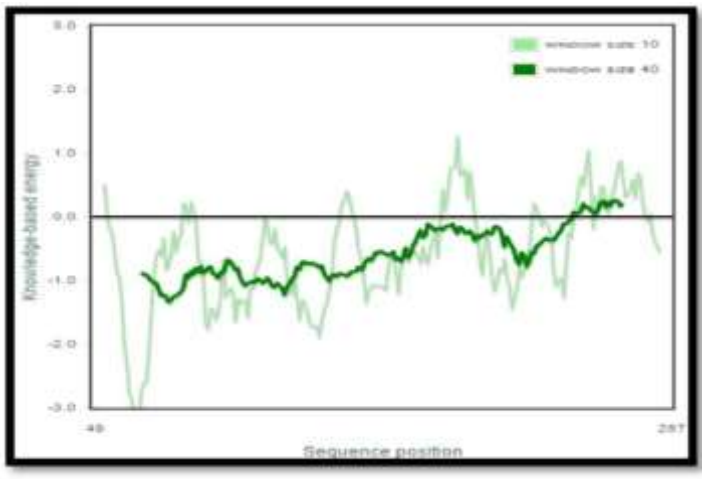

Fig. 15 Model quality of Curcin 2A-17(Dark green line indicates the energy level which is less than 0)

Another way of validation of protein model is done by checking the z-score. Minimum negative z-score in the model considered as the best model. So, keeping all the factors the best model was selected.

- *Minimized pdb files by energy minimization in NOMAD Ref*

Selected models were uploaded on Nomad Ref tool in **Fig. 16** which gave the protein minimized models.

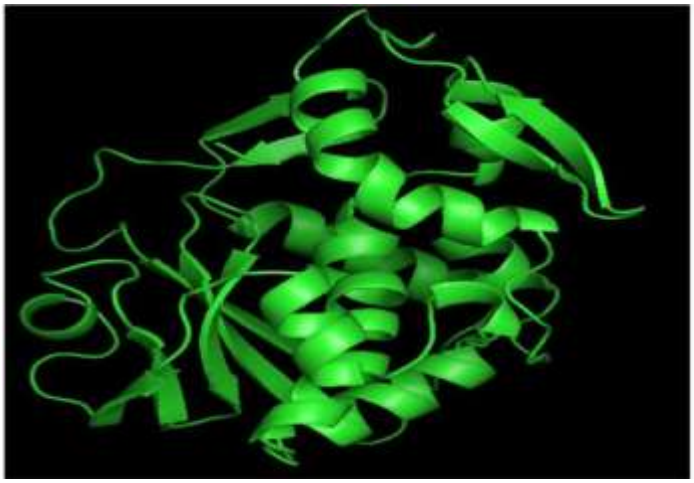

Fig. 16 Minimized pdb model of Curcin 2A-17

- *Ramachandran analysis of curcin protein as visualized by*

Ramchandran plot is a way to predict and visualize allowed regions backbone of amino acid in protein structure as shown in **Fig. 17** by uploading the files on tool plot were obtained. In Ramchandran plot Curcin 2A-17 it indicates that dots of protein residue were lying in favored, allowed outlier region as represent in the table. Mostly protein value similarities occurred in the allowed region and major differences in the proteins are present in negative region. This was identified by using Verify_3D. Its purpose is to determine the atomic model compatibility with its own amino acid sequence by comparing the results to good models.

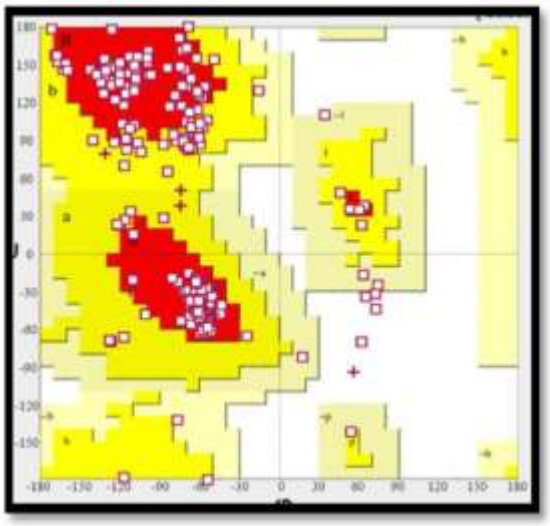

Fig.17 (a) Ramchandran analysis of Curcin 2A

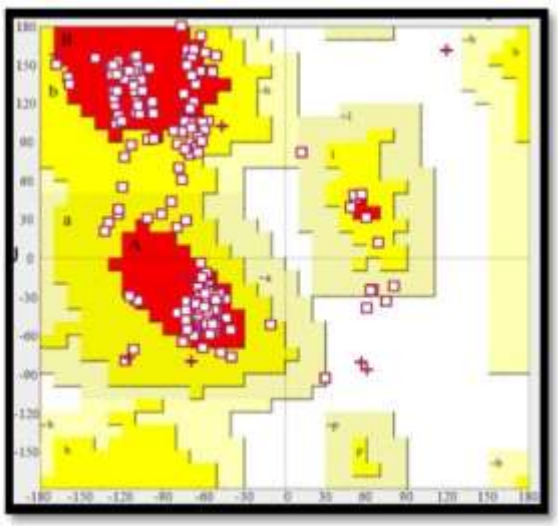

Fig. 17 (b)Ramchandran analysis of Curcin 2A-17

- Assesment of residues plot

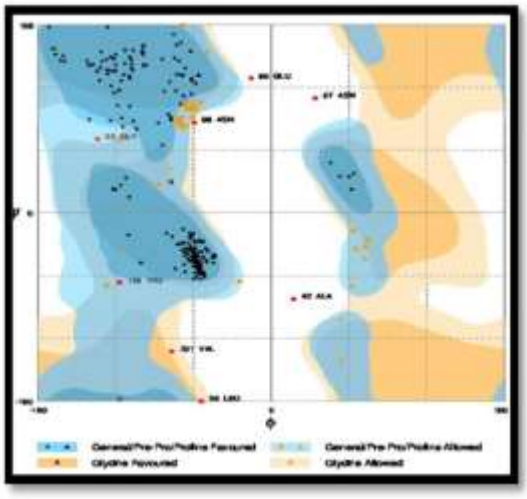

Fig. 18(a) Residual proteins in Curcin 2A

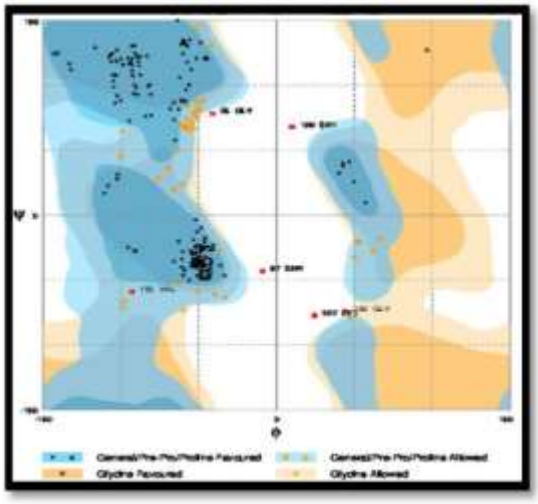

Fig. 18(b) Residual proteins in Curcin 2A-17

- No of residue

Table 9 Ramachandran plot values of Curcin 2A and Curcin 2A-17

Ramchandran plot values	Curcin 2A	Curcin TJS-17
Favoured	194	192
Allowed	35	38
Outlier	8	6
VERIFY 3D AVERAGE SCORE	72	88.2
NEGATIVE LYING RESIDUE NO.	220	58
NAME	Leuicne	Glutamine
SCORE	-0.03	-0.01t

- *Transmembrane structure*

In cell signaling and energy transport transmembrane play an important role in study of membrane associated with protein specially. It was analysed by uploading the PDB files protein models to Phyre2 tool. In transmembrane structure of Curcin 2A-17 entry of peptide signals from the extracellular membrane for cell signaling is different i.e., respectively as shown in **Fig. 19** towards N-terminal maximum number of protein are present and less towards the C-terminal in curcin.

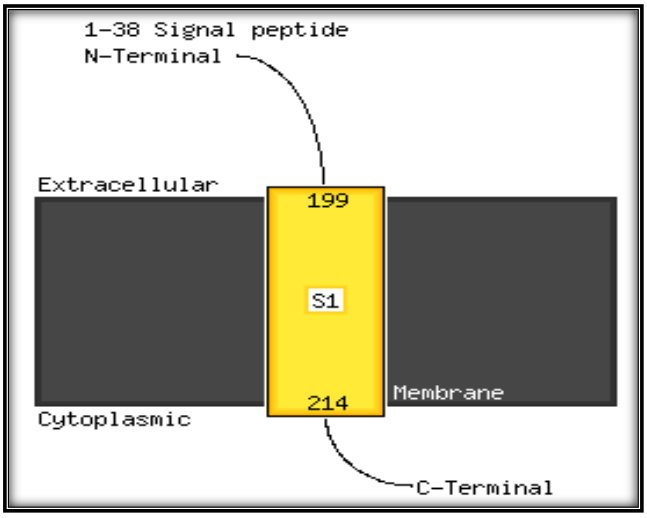

Fig. 19 Transmembrane structure of Curcin 2A-17

4.1.9 I-TASSER

The quality estimation of the threading alignment is usually based on the Z-score, since the top ten templates are generated by different threading algorithms which is generated by I-TASSER along with normalized Z-score. The alignment of Curcin 2A-17 with threading templates is given below in **Table 10**

Rank	PDB hit	Normalized Z-score
1.	3ku0A	2.22
2.	4z8Sa	3.55
3.	1br6A	2.43
4.	3ku0	2.55
5.	3ku0	1.85
6.	1br6A	2.94
7.	3ku0	2.79
8.	3px8X	4.13
9.	3ku0A	2.33
10.	3mvgA	3.52

Table 10 Threading templates along with normalized Z-score of the threading alignment.

In comparision TM-score counts all residues pairs using the Levitt-Gerstein weight whereas short distance weighted is stronger than the long distance. Hence, the TM-score stays in the global topology than local variation. TM-score stays in (0-1) where higher value indicates stronger similarity between structures. The top ten PDB structures of similar topology are shown in table given below in **Table 11**.

Rank	PDB hit	TM-score
1.	1br6A	0.813
2.	2vlcA	0.774
3.	1abrA	0.770
4.	1pumA	0.768
5.	3ku0A	0.767
6.	1onkA	0.766
7.	1d6Aa	0.766
8.	2zr1A	0.765
9.	1hwnA	0.764
10.	2z53A	0.764

Table 11PDB structures structurally closest to model along with TM-score of the structural alignment

4.1.10 Phylogenetic analysis of Curcin by mega 7

Phylogenetic tree also called as evolutionary tree which represents evolutionary relationship between homologous sequences. The sequences joined together imply similarities and common ancestors whereas from different root the sequence which have evolved imply divergence. Evolutionary time depict the length of the branches. There are different ways to construct phylogenetic tree, here neighbor joining method by Mega 7 has been used to construct the phylogenetic tree for Curcin protein of *Jatropha* as shown in **Fig. 20** In the phylogenetic tree depicts different species of the plant which represent 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 whole and/or segment-wise recombinantcurcin expression. It is likely that different curcin segments could vary with regard to pharmacological importance


Escherichia coli(1405280A)
Ricinus communis(5GU4_A)
Ricinus communis(ADG29080
Ricinus communis(EEF26167
Ricinus communis(P02879.1)
Ricinus communis(1IFS_A)
Ricinus communis(4LHJ_A)
Ricinus communis(3PX8_X)
Ricinus communis(4LGR_A)
Ricinus communis(1UQ5_A)
Ricinus communis(5E1H_A)
Ricinus communis(1UQ4_A)
Ricinus communis(5BOZ_A)
Ricinus communis(4LHQ_A)
Ricinus communis(4LGP_A)
Ricinus communis(1J1M_A)
Ricinus communis(4LGS_A)
Ricinus communis(2AAI_A)
Ricinus communis(1BR6_A)
Ricinus communis(5U4L_A)
Ricinus communis(5DDZ_A)
Ricinus communis(ABG65738.1)
synthetic construct(ACV85693.1)
synthetic construct(ACV85692.1)
Ricinus communis(XP_002529062.1)
synthetic construct(ACV85687.1)
Elaeis guineensis(XP_010922360.1)
Elaeis guineensis(XP_010905315.1)
Camellia sinensis(ADF45512.1)
Camellia sinensis(ADF45510.1)
Cucurbita moschata(XP_022959960.1)
Cucurbita maxima(XP_023005049.1)
Cucurbita moschata(P84531.2)
Cucurbita moschata(ABY47624.1)
Cinnamomum camphora(AET98835.1)
Sambucus ebulus(1HWM_A)
Abrus precatorius(CAA38654.1)
synthetic construct(ABQ26345.1)
Abrus precatorius(CAA38655.1)
Abrus precatorius(1ABR_A)
Abrus precatorius(CAA54139.1)
Abrus precatorius(CAA54140.1)
Triadica sebifera(AJR10103.1)
Manihot esculenta(XP_021611042.1)
Manihot esculenta(XP_021610530.1)
Manihot esculenta(XP_021610006.1)
Manihot esculenta(XP_021609231.1)
Jatropha curcas(KDP46056.1)
Jatropha curcas(XP_012074335.1)
Jatropha curcas(ADN39429.1)
Jatropha curcas(NP_001295744.1)
Jatropha curcas(AAL86778.1)
Jatropha curcas(XP_012074346.1)
Jatropha curcas(AJW31110.1)
Jatropha curcas(AEA72440.1)
Jatropha curcas(XP_012074358.1)
Jatropha curcas(ABZ04128.1)
Ricinus communis(XP_002532190.2)
Momordica charantia(XP_022158193.1)
Momordica charantia(XP_022156715.1)
Ricinus communis(EEF30193.1)
synthetic construct(AFR78255.1)
synthetic construct(AFR78253.1)

4.2 Molecular cloning and expression of Curcin in pET28a vector

Recombinant protein expression has gain world-wide importance in the field of biotechnology during the last years. Curcin protein of Jatropha also has a significant pharmaceutical value although being potentially toxic.Therefore, molecular cloning of different curcincDNAs is a prerequisite to carry out recombinant expression of such proteins for production at commercial scale. Keeping the foresaid, in view this study was focused on cloning of Curcin cDNA in protein expression vector as described in the following sections.

4.2.1 Plasmid DNA isolation of pET28a

Plasmid DNA of XLpET28awas isolated as described in material and method sections. Agarose gel was run to check the quality of crude DNA sample and good quality of plasmid DNA shown in **Fig.21**. Plasmid DNA bands were distinct indicating the quality of total DNA isolated.

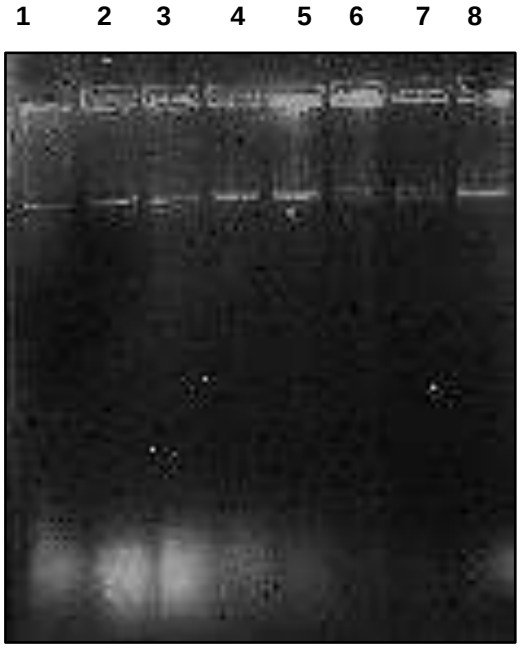

Fig. 21 Plasmid DNA isolation from pET28a

4.2.2 *Purification of DNA from pET28a*

DNA was purified by RNase-free DNase treatment followed by solvent extraction. Purified DNA samples were checked by 0.8% Agarose gel electrophoresis in 0.5X TBE buffer as shown in **Fig. 22**.

Fig. 22 Purified DNA of pET28a

4.2.3 Restriction digestion of pET28a with *Bam*HI *and Hind*III:

Restriction digestion of pET28a was carried out and digested DNA samples were resolved in agarose gel electrophoresis as shown in **Fig. 23**.

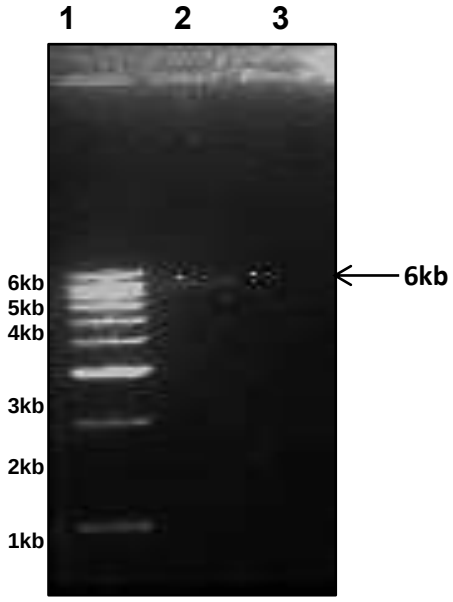

Fig. 23 Restriction analysis of pET28a.Lane1 -1.0kb ladder, Lane 2-Linearised pET28a, Lane 3-Control pET28a

4.2.4 Plasmid DNA isolation of Curcin cloned in pMD 20-T vector:

Plasmid DNA was isolated from Curcin cloned in pMD 20-T vector and 0.8% agarose gel was run to resolve and check the crude plasmid DNA as shown in **Fig. 24**.

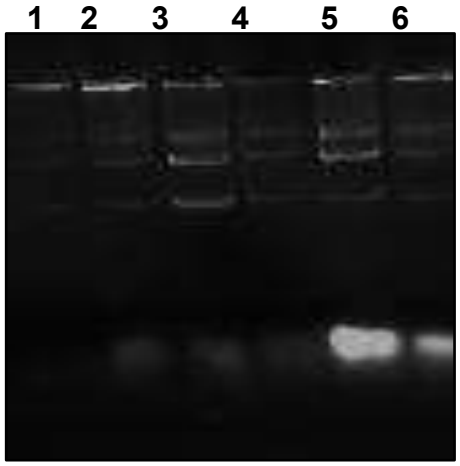

Fig. 24 Plasmid isolation curcin cloned in pMD 20-T vector

*4.2.5 Purification of Curcin cloned in*pMD 20-T:

DNA was purified by RNase-free DNase treatment followed by solvent extraction. Purified DNA samples were checked by Agarose gel electrophoresis as shown in **Fig. 25**.

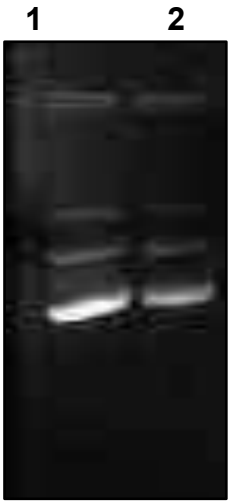

Fig. 25 Purification of Curcin clone cloned in pMD 20-T vector

4.2.6 Restriction digestion of pMD20-T

Restriction digestion out of curcin cloned in pMD 20-T was carried out usingBamHI and HindIIIenzymes compatible in buffer D and the digested DNA samples were checked in agarose gel electrophoresis as shown in **Fig. 26**.

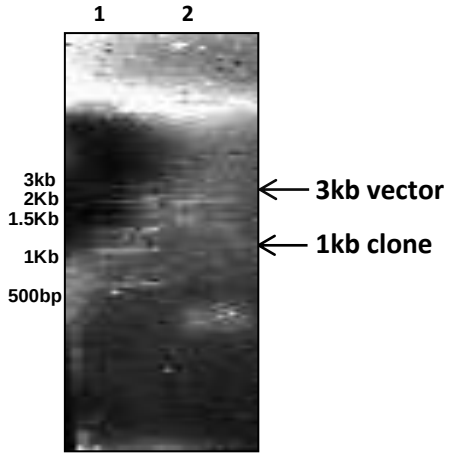

Fig. 26 Restriction analysis of rpMD20-T.
Lane1- 1.0 kb ladder, Lane 2-showing vector along with ~1.0 kb curcincDNAinsert.

4.2.7 Purification of curcincDNA insert using gel extraction kit

Curcin insert from the clone was further eluted out by using silica gel beads extraction kit and confirmed further by agarose gel electrophoresis. A sharp band of ~1.0kb was observed as shown in **Fig. 27**.

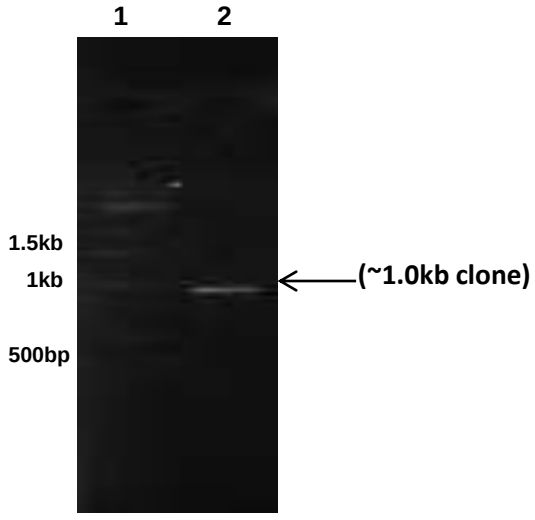

Fig. 27 Elution of curcin clone insert.Lane 1- 500 bp ladder, Lane 2- ~1.0kb clone.

4.2.8 Cloning of Curcin 2A-17 cDNA in pET28a vector

The Curcin 2A-17 Cdnawas ligated in *pET28a* vector as described in material and methods section. The ligated product was then used to transform *E.coli* DH5α for further growth on Luria Agar Kanamycin plates. A number of white colonies (i.e., putative clones)was observed and were purified by streaking.Plasmid DNA isolation was done by alkali lysis method of different colonies. Agrose gel electrophoresis was resolved to check the crude plasmid as shown in **Fig. 28**. Putative recombinant clones were identified on the basis of their mobility in the gel.

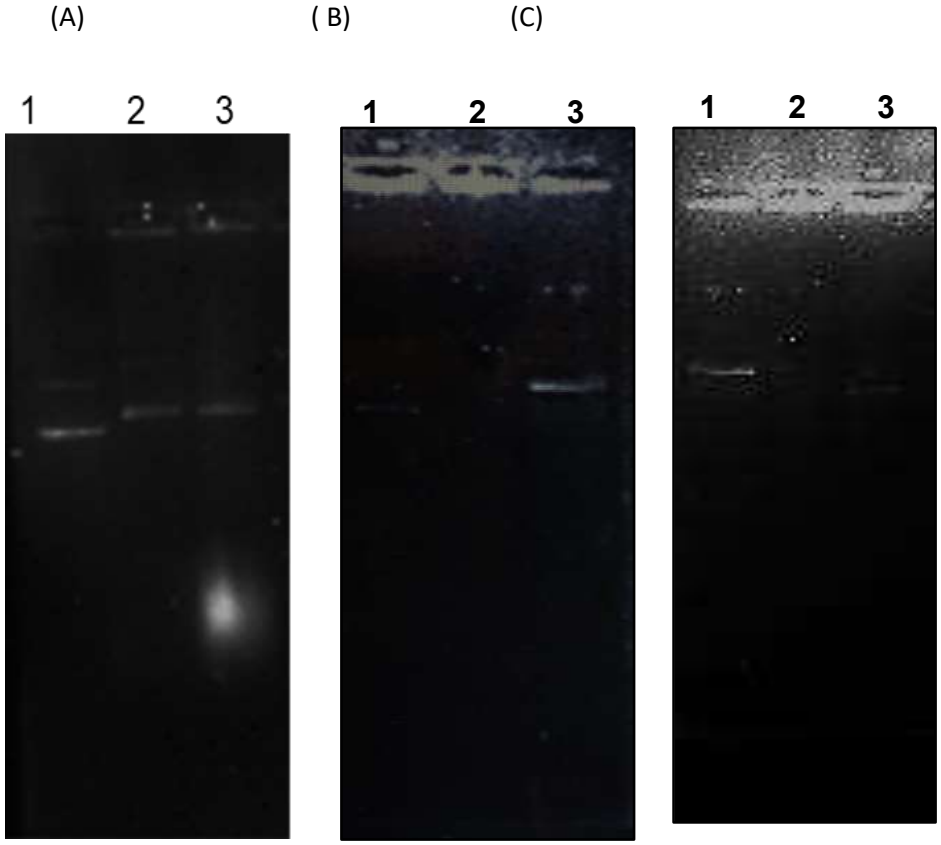

Fig.28 Gel electrophoresis of the plasmid clones. Lane 1- control *pET28a*, Lane 2-putative clone #14, Lane 3- putative clone #08

4.2.9 Purification of the putative clones:

RNase-free DNase*treatment*:Two putative clones were purified by RNase-free DNasetreatment followed by solvent extraction and DNA precipitation.To check the result further resolved by agarose gel electrophoresis, as shown in**Fig. 29**.

Fig. 29 Purification of putative clones.Lane 1-*pET28a*, Lane 2- Putative clone 14

4.2.10 Restriction analysis of putative clone

Restriction digestion of putative clone was carried out and the digested DNA samples were resolved on gel electrophoresis as shown below in **Fig. 30**.

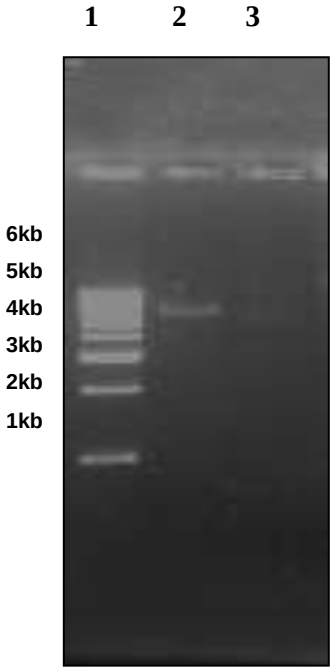

Fig. 30 Restriction digestion of the putative clone.

Lane 1-1.0 kb ladder, Lane 2- clone #14 digested with *BamHI* , Lane 3-control (clone #14)

The cDNA clone in recombinant pET28a vector was partially characterized by restriction analysis. The slow mobility of digested putative clones as compared with the digested pET28a indicates the presence of the insert, which needs to be further characterized prior to recombinant expression in a suitable *E. coli* host.

4.3 Discussion

Curcin is a toxic compound but has a significant pharmaceutical value. Despite of its importance in pharma and other industries,less reports are available on the isoforms of curcin and their structures. Genomics along with *in silico* studies are key steps for further studies on genes and their expression and function. Here, we provide a comprehensive report on the structure of Curcin 2A specific clone Curcin 2A-17 and its cloning in the expression vector pET28a:

- In order to know about relatedness of the clone, multiple sequence alignment (MSA) was carried out using COBALT. It was found to be 99% similar to the reference sequence. MSA revealed that most of the significant domains and the signature sequences of the Curcin isoforms were highly conserved. We speculate that N-terminus of protein was highly conserved with low and conserved mutations. The similarity between the Curcin protein sequence suggested that their biochemical activity would likely be very similar. Indeed Curcin 2A (GenBank id: GQ925453.1) and the clone Curcin 2A-17 (to be submitted to NCBI) showed the variation in the percentage of positive amino acids (Histidine, Arginine, and Lysine) and also in other amino acids like Asparagine and Isoleucine, depicting their different conformation and activity. I-TASSER is known for its reliability in the protein modeling. The model obtained after threading process was depicted in rainbow color starting from N to C as publication format. All the reliable models were checked by ProSA tool. An significant validation of the protein models is based on the energy level, lower the energy level more reliable is the model. The reliable model lying in NMR region and with low energy and z score was further carried for energy minimization. The negative value indicates best models. Modeled structure of the Curcin protein contained coiled structure. The stereochemical quality and accuracy of the new modeled structure (min.4 PDB) wasanalysed by PROCHECK and verify 3D. The Ramachandran plot displays the phi-psi torosion angles for all the residues in the structure. Glycine residues donot restrict to any regions so they were separately identified by triangles. Each residue based on its location and environment is assigned a structural class. Analysis of phylogenetic tree reveals its similarity with other Curcin isoforms from Jatrophacurcas and Jatropha .

  To understand the vast amount of genomics information, proteomics has become a major focus. Studying proteins in global way is a challenge, which is driving new technologies for systematic analysis of structure and function of proteins. So apart from *in silico* analysis this study is also focused on the molecular clonig studies. The Curcin 2A specific DNA was cloned in the protein expression vectorpET28a. The pET28a was isolated in good quality and was purified by RNase-free-DNase followed by phenol-chloroform treatmemt; a conventional method for isolation. The Curcin insert was cut from rpMD20-T vector, purified and ligated in pET28(a). The transformed colonies were further processed for DNA isolation. This process could provide a start point for engineering the protein expression studies.

Conclusions

- Earlier, RT-PCR approach was adopted to get Curcin 2A specific cDNA clone from Jatropha. The clone was sequenced, and the amino acid sequence was deduced, designated as Curcin 2A-17. Sequence analysis was done at both nucleotide and protein level.

- Multiple sequence alignment between different Curcin isoforms that are highly conserved with some divergence. This study also generated data comprising 3D-modelling, searching protein motifs, phylogenetic tree and some other important attributes. Many features as found in this study were not reported earlier.

- Apart from *in-silic*o approach, efforts were made to clone the curcincDNA in an expression vector for the purpose of recombinant protein expression.

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