Curcin proteins of biodiesel crop (*Jatropha curcas* L.): Cytotoxic effects of the crude curcin extract, and molecular approaches for isolation of curcin genes

A Dissertation

Submitted in partial fulfilment of the requirement for the award of degree of

Masters of Technology
in
Biotechnology

By

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CANDIDATE'S DECLARATION

I, the undersigned, hereby declare that the research work presented in the M.Tech project entitled "**Curcin proteins of biodiesel crop (*Jatropha curcas* L.): Cytotoxic effects of the crude curcin extract, and molecular approaches for isolation of curcin genes**" is an authentic record of the work carried out by me under the supervision and guidance of Dr. N. Das, Professor, Department of Biotechnology, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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CERTIFICATE

This is to certify that dissertation entitled, "**Curcin proteins of biodiesel crop(*Jatropha curcas* L.): Cytotoxic effects of the crude curcin extract, and molecular approaches for isolation of curcin genes.**" submitted by **Mr. Nitin Kumar** in partial fulfilment of the requirements for the award of Masters in Technology Degree in Biotechnology at Thapar University, Patiala is an authentic work carried out by him under our supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

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ABSTRACT

As of now, Jatropha (*Jatropha curcas* L.) stands as the best solution to the problems associated with production of biofuels, as it is well adapted to marginal soil with low nutrient content, and also suitable in different types of soil conditions, rainfall and climates. Disadvantages of Jatropha are the presence of toxic substances, particularly in the seeds, like curcin and phorbol esters which limits their applications. According to literature study, curcin protein has cytotoxic activity on cancer cell lines. Therefore, one of the objectives was to study the cytotoxic effects of curcin using crude curcin extracts. Here we chose four accessions of *Jatropha curcas* namely TJS17#03, TJS42#04, TJS35#01 and TJS01#03. First, quality and quantity of seed oils were checked; and oil content was found to be maximum in case of TJS42#05; whereas, free fatty acid content (FFA) content was found to be less in TJS17#03. Based on the available protocol, crude curcin extract was made from the seed kernel of TJS17#03; then its cytotoxic effect was assessed by MTT assay on RAW and HeLa cell lines. The crude curcin extract exhibited inhibitory effect on the cancer cell lines significantly. BLAST analyses revealed that the promoter region of the curcin gene showed considerable sequence divergence if compared with the other members of this family. Multiple sequence alignment was made using different forms of curcin namely curcin precursor, curcin2A, curcin-L precursor and ribosome inactivating protein (RIP). This exercise revealed both sequence identity and divergence between them. Good quality genomic DNA was isolated from field-grown tender leaves using a simple and efficient protocol as introduced in our laboratory. Quality and quantity of the genomic DNA preparations were checked by spectrophotometric analyses. Some forward and reverse primers were made based on the Curcin2A gene sequence as available in the database. Most of the forward primers correspond to the promoter region, and the reverse primers are from the coding regions. The purpose was to amplify the curcin genes with varying upstream (promoter) regions. PCR were carried out using the specific primer pairs. The amplicons corresponding to the individual PCR were noted in order to analyse and compare between the Jatropha accessions. Apart from the expected sizes, more amplicons of varying sizes were also found. All the promising amplicons i.e., PCR products as reported in the study need to be further studied at molecular level.

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ABBREVIATIONS

Abbreviations	**Name**
Bp	Base-pair
B20	20 % blend of biodiesel with fossil diesel
BSA	Bovine serum albumin
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
HeLa	Human cervical cancer cell line
IU/mL	International unit per mL
kb	Kilo base
kDa	Kilo-daltons
M	Molar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide
mM	Milli-molar
nm	nanometer
N	Normal
O.D	Optical Density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pmoles	picomoles
RIPs	Ribosome inactivating proteins
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
µL	Microlitre
µg	Microgram
U/mL	Unit per mL

1.1 *Jatropha curcas*: Green gold in a shrub

The genus Jatropha has 175 known plant species belonging to the family Euphorbiaceae (Dehgan 1984).The plants are drought resistant succulent shrubs and are recognized as potential biofuel crops (Jones and Miller 1991; Openshaw 2000). Among the several Jatropha species *Jatropha curcas, Jatropha integerrima, Jatropha glandulifera, Jatropha nana, Jatropha gossypifolia*, *Jatropha multifida* are cultivated in India. The centre of origin of *Jatropha curcas* is still questionable, but it is assumed to be Mexico and Central American region. It is cropped in many parts of the tropics and subtropics as a hedge crop and for conventional use (Heller 1996; Kumar and Sharma 2008). *Jatropha* occurs mainly occur at lower altitudes in areas with annual temperatures above 20°C but can grow at higher altitudes and bear little frost. The crops are highly toxic and can not be consumed by cattle. Jatropha is known by nearly 180 different names such as Chandrajot, Jungli arandi, danti, safed arand, physic nut etc. Jatropha is well known for its uses in various fields like biofuels, medicine, and environment. It is a boom for developing countries like India to fulfil their energy needs as they have surplus of wasteland.

1.2 Taxonomy of *Jatropha curcas*

Table 1 Taxonomic hierarchy of *Jatropha curcas*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malpighiales
Family	Euphorbiaceae
Subfamily	Crotonoideae
Tribe	Jatropheae
Genus	Jatropha
Species	Curcas

1.3 Botanical features of *Jatropha curcas*

Jatropha *curcas* is deciduous and diploid small tree or large shrub with 3 to 5 meters in height. It has smooth grey bark which secretes whitish coloured latex when cut. Under normal conditions it fruits once in a year. It can produce more than 1.5 to 2.0 kg seeds per plant per year. The life span of the plant is of about 40-50 years and it is well adapted to semi-arid climates.

Leaf of this plant is large, heart shaped, 4-6 lobed, initially yellowish green and at maturity it becomes dark green. The leaves are 10-15 cm in length. Flowering occurs during July to September.

Fig. 1 Leafs of Jatropha plant

Fruits are grey-brown capsule in shape, 3 cm long and generally tri-halved, each fruit comprises of three seeds. Depending upon the climatic conditions, maturation of fruits takes place from October-November and best time to harvest the fruits when they turned yellow to dark brown and seeds mature by three months after flowering.

Fig. 2 Mature fruits of Jatropha plant

Seeds are usually reaped after one year of plantation. Oil content in the seed of this plant is around 25-40 % by weight. Seeds contain about 22 % saturated fatty acids and 78 % unsaturated fatty acids. Oleic and linoleic acids are mainly present in Jatropha oil (Rehm et al.1991).The seeds also consist of some other chemical compounds, such as saccharose, raffinose, glucose, fructose, galactose, and toxic protein like curcin.

Fig. 3 Seeds of Jatropha

1.4 Cultivation of *Jatropha curcas*

Main states of India where Jatropha is cultivated are Gujarat, Madhya Pradesh, Rajasthan, Maharashtra and Tamil Nadu. Cultivation of Jatropha is simple. Jatropha Curcas can grow in tropical and subtropical areas. The plant can be grown in wastelands and it can be cultivated on any soil like gravel, sandy and saline .It can survive in poor and stony soils as well. It can be grown either by cuttings or seeds. Propagation of plant through seeds is faster than cuttings. The flowers develop at the tip of stem, so good ramification produces a large number of fruits. Another factor which favours productivity is the ratio of female and male flowers, more the number of female flowers results in more fruits. Seed bearing in Jatropha starts in first 9-12 months of its plantation but best seed yield is observed after 2-3 years of its plantation.

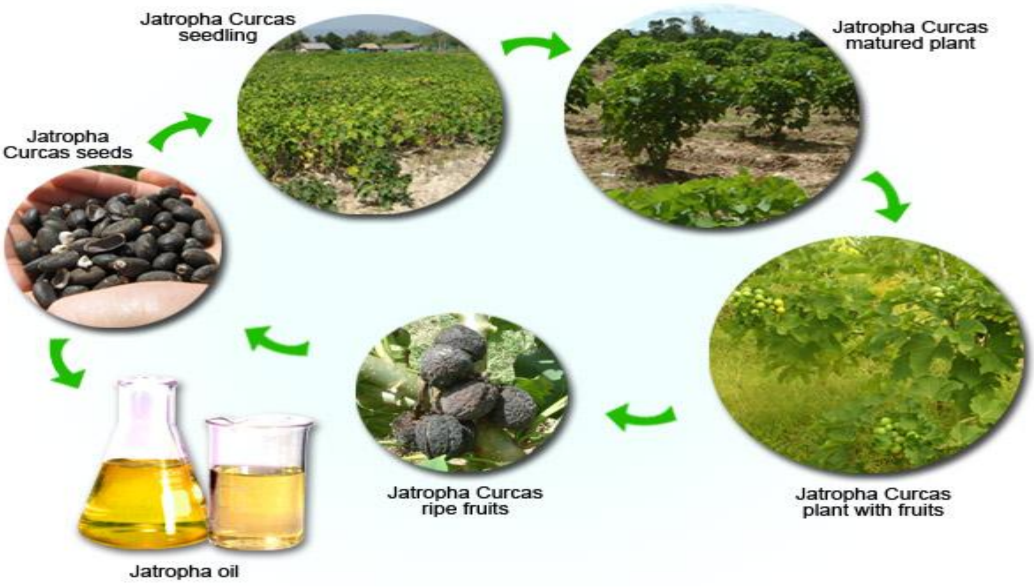

Fig.4 Jatropha life cycle (Source: www.neduet.edu.pk)

1.5 Uses of *Jatropha curcas*

Jatropha curcas is often called as green gold in a shrub. It has various benefits which are as follows:

Biodiesel production: Oil content in Jatropha seeds is relatively high ranging from 30-40 %. Biodiesel usually contains long chain alkyl-esters. These fatty-acids (triglycerides) in the oil extracted from the seed undergo transesterification reaction with alcohol to generate biodiesel and glycerol as shown in Fig. 5. The biodiesel so produced can be used in existing diesel engines up to 20 % mix generally termed as (B20). Biodiesel is biodegradable, non-polluting and environment friendly in nature as it does not produce Sulphur and Carbon monoxide (CO) on burning. It is believed as "carbon neutral" because the carbon dioxide produced is nearly equal to the carbon dioxide utilized for the growth of plant (Baranwal and Sharma, 2005). It has lesser mutagenic potency than commercial diesel. It reduces greenhouse gas emissions up to 50 % (Mukharjee et al. 2011).

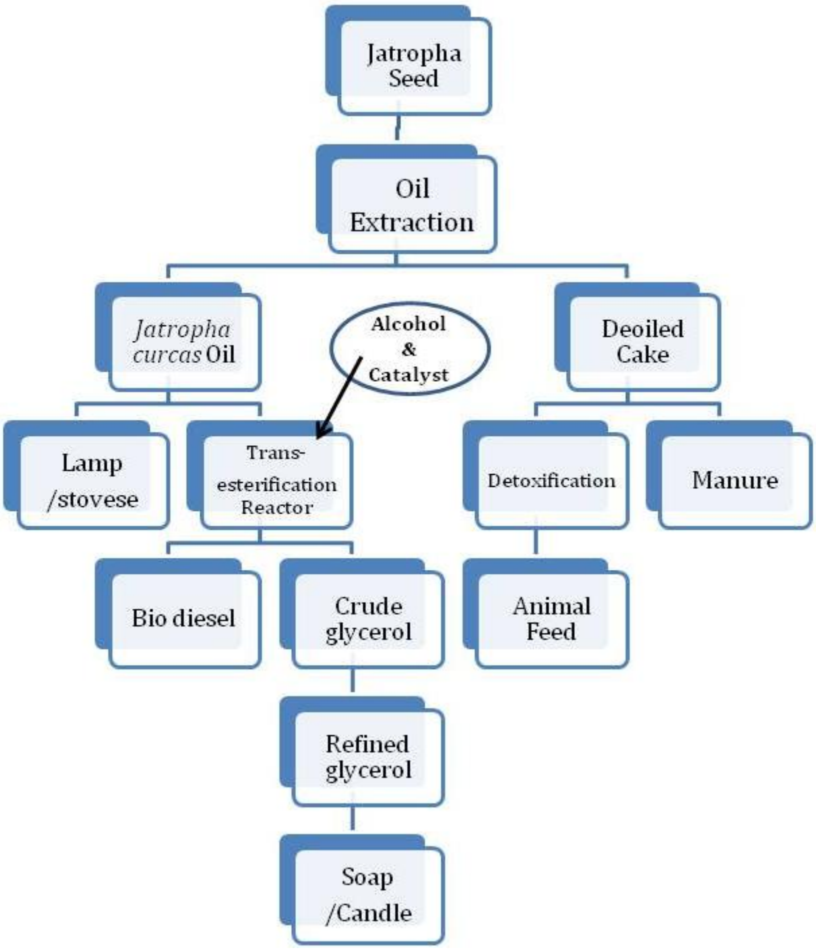

Fig. 5 Biodiesel production from Jatropha seeds

Medicinal Uses*:* Juice of the plant is useful in scabies, eczema and ringworm. The sap from stems can cure bleeding wounds. The latex contains several alkaloids with anti-cancer properties (Van den Berg et al. 1995; Thomas et al. 2008). The roots of Jatropha can act as antidote for snake-bites and have anti-inflammatory activity (Mujumdar and Misra 2004).Curcin protein in the seeds have anti-tumor property (Luo et al. 2007). The plant has pesticidal and fungicidal properties so the use of pesticide is not mandatory. In rural Bengal it is commonly used for treatment of *dhobhi itch* (common skin fungal infection).

Insecticidal property: Oil obtained from *J.curcas* contains insecticidal properties against various insects like cotton bollworm, and pests like pulses, corn and potato (Kaushik and kumar 2004). Extracted oil shows insecticidal effects against crop pests such as *Helicoverpa arimgera* and *Sitophilus zeamays* (Solsoloy 1995).

Industrial Uses: Glycerol obtained after the transesterification reaction can be used for the production of soap and candles. Dye obtained from bark and leaves is used for dying cloth.

Use of Seed cake, a by-product of extraction of curcas oil: Seed cake contains 3.2 -4.4% N, 1.4-2.09% P, 1.2 -1.6% K so it can be used as potential organic manure since it has high nitrogen content (Kumar and Sharma 2008).

Environmental benefits: It can be cultivated on marginal land and wasteland. Thus they don't put pressure on fertile land and control soil erosion by wind or water. It absorbs around 7 kg of CO_2 per tree per year (http://www.jatropha.pro/GHG%20and%20CO2.htm).

1.6 Biofuel from *Jatropha curcas*

In today's modern era, where the world's population is rising at a very fast rate, energy resources are depleting very fast. We are heavily dependent upon non-renewable sources of energy, so the whole world is facing an energy crisis. So, there is a need to search for alternatives to non-renewable resources of energy. Therefore biofuel from energy crops drew our attention because they are a renewable resource as compared to the fossil fuels and a suitable substitute to fulfil the ever increasing demand of petroleum. The biofuels originated from oil bearing plant species can fulfil around 13 % of the world's energy needs. Energy crops are the plants grown to make biofuels such as bioethanol, biogas, biodiesel etc. These plants are low cost and require low maintenance which makes them more suitable for production of energy. Before the production of biofuel various factors have to keep in mind such as food versus fuel needs, energy yield per unit area under farming, total cost of production and competitiveness with fossil fuels, reduction of greenhouse gas emissions, overall energy balance, and agricultural policy issue. The developed nations which have excess of edible oil seed crops can utilize them for biofuel purpose, but developing nations like India has to use non-edible oilseed crops like *Jatropha curcas* to nullify the competition of land for food crops.

Table 2 Oil producing crops

Oil producing Crop	**Liters of oil per hectare**
Oil palm	2,400
Jatropha*	1,300
Rapeseed (canola)	1,100
Sunflower	690
Soybean	400

*Indian Planning Commission (Sources: United Nations Development programme/world bank)

1.7 Scope and Prospects of Biodiesel in India

India imports crude petroleum to cover 65% of the energy requirement and by 2030 it is estimated to rise up to 90 %. Therefore biofuel is the only possible way out that can help us to cut down the country's dependence on foreign energy imports. Production of biofuel from edible crops is not an option for India. Therefore India had to shift its interest towards biofuels, generated from non-edible oil bearing crops. In the early 2000's former president Dr.A.P.J Abdul Kalam supported the contributions of Jatropha plant as a route to greening of waste land and production of biofuel. So on New Year's Eve 2002, the Shatabdi train from New Delhi to Amritsar was supplied in part with biodiesel for the first time, this shows Indian government's desire to wean India off imported petroleum (Fairless 2007). In September 2008, the Government of India announced the National Biofuels Policy. Main objective of this policy is to blend 20% biodiesel to conventional fuels by 2017. India has roughly 42.93 million hectares of wasteland which can be utilized to harvest *Jatropha curcas* plantation.

1.8 Toxic compounds in *Jatropha curcas*

Jatropha contains many toxic compounds such as lectins (curcin), saponins, phorbol esters and protease inhibitors. All these toxic substances are found in seeds of Jatropha (Sujatha 2009**).** Jatropha oil contains approximately 30% phorbol esters (Devappa 2010). Due to all these toxic substances found in *Jatropha curcas*, it cannot be used for cattle as a fodder and as cooking oil for animals. Adams in 1975 studied that when two goats were given Jatropha seeds 1g/day, they died within 2 to 9 days while on decreasing the amount of Jatropha seeds i.e. 0.25g/day and 0.5g/day, they both died within 11 to 21 days. This study revealed that seeds, press cake and oil of Jatropha cannot be utilized for human or animal feed.

1.9 Biotechnological aspects of *Jatropha curcas*

Although Jatropha can grow on marginal lands but for proper oil yield they require proper water and irrigation i.e. agricultural practices. There are many issues related to *Jatropha curcas* plant. The oil quantity produced by Jatropha is very low. Crude oil of Jatropha contains high amount of free fatty acids that leads to decrease the yield of biodiesel. The plant, seeds in particular, contains some toxic proteins/biomolecules like curcins and phorbol esters, which restrict their uses in many areas like as a fodder for animals and as cooking oil etc. Therefore, one of the major biotechnological aspects of *Jatropha curcas* is to increase or decrease the amount of toxic proteins like curcin particularly in the seeds. If the amount of toxic protein is more in Jatropha than its application will be increased because of some medicinal applications such as cytotoxic effects of curcin on cancerous cells. On the contrary, Jatropha varieties with less curcin content (which are nonhazardous) are more suitable for large scale cultivation in the marginal/semi arable lands for production of biodiesels. Moreover, various Jatropha tissues have other applications also.

2.1 Oil and fatty acid contents of *Jatropha curcas*

The term ‘Jatropha’ originated from the Greek word ‘iatros’(doctor) and ‘trophe’(food) which have medicinal uses. Crude oil extracted from Jatropha contains four main fatty acid i.e. Palmitic acid (9-22%), Linoleic acid (27-42%), Stearic acid (5-8%) and Oleic acid (35-51%) (Rehm et al.1992). Seed of *Jatropha curcas* contain 25-30% oil (Rug and Ruppel 2000). Biodiesel was obtained from crude oil of Jatropha after transesterification reaction of crude oil with alcohol. High amount of free fatty acid in crude oil have significant effects on the transesterification reaction (Goodrum 2002).Therefore the yield of biodiesel decreased and due to this cost of biodiesel increased. Biodiesel production cost can be reduced by using less expensive feedstock such as inedible oils, waste food oil and by-products of the refining vegetables oils (Veljkovic et al. 2006). Berchmans et al. in 2007 described a method to reduce the free fatty acid content in crude oil of Jatropha.

2.2 Toxins in Jatropha curcas

All parts of Jatropha are believed to be toxic in nature, especially the seeds of plant. Lectin, (curcin), phorbol esters and saponins are toxic substances in *Jatropha curcas* due to which seeds, press cake and oil cannot be utilized for human or animal feed (Sujatha 2009). Some study has reported that lectin is not the major toxic component in *Jatropha curcas* meal (Aderibigbe et al. 1997; Aregheore et al. 1998).

Lectins

Lectins are basically proteins that can bind with carbohydrate moiety with high affininty (Goldstein and Hayes 1978). They play a major role in plant defence against pathogen (Chrispeels and Raikhel 1991). Lectins are released into the water when a dry seeds absorbs water (Fountain et al. 1977). This will lead to the interaction of lectin with potential pathogen in germinating seed. Second method of Lectin defence system is when pathogen eat the seeds of plant, lectin present inside the seeds is released and binds with the glycoproteins present in the intestinal tract of pathogen and inhibit the absorption of nutrients, led to death of pathogen. Lectin and trypsin inhibiter in Jatropha can be reduced by heat treatment (Aderibigbe et al. 1997).

Phorbol esters

Phorbol esters found in both toxic and non-toxic varieties therefore this compound need to be studied further (Wink et al. 1997). The kernels of Jatropha seeds contain six different types of Phorbol esters (Haas et al. 2002). Many plants of family Euphorbiacaeae contain phorbol esters (Ito et al. 1983). Phorbol esters are structure specific with regards to their biological activities. Phorbol esters induces tumor by activating the protein kinase and important cellular target proteins. Phorbol esters have been extracted and studied in various human and animal cell lines for the tumor inducing property (Goel et al. 2007). Toxic effects of phorbol esters was studied on guinea pigs and results was positive (Goel et al. 2007). Phorbol esters showed bio pesticides property against fresh water snails and it was highly toxic to silkworm larvae (Goel et al. 2007). Toxic fraction of Jatropha oil was extracted and applied on the skin of rabbits and rats which showed irritant reaction followed by the necrosis (Gandhi et al. 1995). Heat treatment cannot inactivate phorbol esters which are main toxic agents in Jatropha (Aregheore et al. 1998). Phorbol esters can be detoxified by biochemical degradation process during the aerobic composting process. Toxicity of the seed cake exposed to air decreased the amount of phorbol esters after 30 days (Devappa 2010).

A non-toxic variety of *Jatropha curcas* has been found in Mexico, which can be consumable for human after roasting. Non-poisonous nature of this variety was checked using fish and rats as experimental models. Some molecular markers are used that can differentiate between toxic varieties of Jatropha and non toxic varieties. These Non-poisonous Mexican varieties of Jatropha have very low levels or nil levels of phorbol esters 0.27 mg g^{-1} in kernel meal as compared to the toxic varieties which contains around 2.49 mg g^{-1} phorbol esters (Makkar et al. 1997). But these non-toxic varieties still contain half the amount of toxic protein curcin as that of the toxic varieties, 30 % more trypsin inhibitors and 45-50% more saponins (Sujatha 2009).

2.3 Ribosome Inactivating Proteins (RIPs)

Generally Jatropha Plant growth and yield reduced due to various abiotic and biotic stresses. During cold and drought stress plant undergoes a number of physiological and developmental changes (Kazuo et al. 1996). Plants have formulated complex defence mechanism against pathogen attacks (Bowles 1990). The defence mechanism evolved by plants includes Ribosome inactivating proteins (RIPs). RIPs can be induced by abscisic acid, mechanical wounding, virus and fungi attacks on plant (Leah et al. 1991; Logemann et al. 1992;

Desvoyes et al. 1997; Joerg et al. 1997; Song et al. 2000). RIPs are proteins extracted from Plants that can conquer synthesis of protein in eukaryotic cells via catalytically damaging ribosomes (Barbieri et al. 1993). Ribosome inactivating proteins have rRNA N-glycoside activity, so the protein can enzymatically cleave the glycosidic bond of adenine of 28S rRNA due to this protein synthesis is inhibited as RIP prevent the binding of 28S rRNA to elongation factor factor(Peumnas et al. 2001). Plant RIPs which are tissue specific which are induced by environmental stresses. For example the expression of beetins in *Beta vulgaris* L was induced by salicylic acid and hydrogen peroxide in leaves (Iglesias et al. 2008). RIPs activity can be measured by different methods such as rapid quantitative determination of the choloroacetaldehyde-reactive material released by RIPs via HPLC and electrophoretic examination of any bases released from 28S rRNA (Luo et al. 2007). RIPs are categorized into two groups on the basis of their structure and functions. Molecular weight of type-1 RIP is 28-35 kDa consisting of a single polypeptide chain and isoelectric point (pI) value is 8 to 10. While type-2 RIP containing two chains, catalytically active chain A linked to cell binding B chain (Juan et al. 2003).

The A chain of type-2 RIP is functionally equivalent to type-1 RIP and B chain is kind of lectin (Barbieri et al. 1993). RIPs have some beneficial effect on plant as they involved in plant defence systems as they have anti-fungal and anti-viral activities (Krawetz and Boston 2000). So, now scientist realizes the importance of RIPs in agriculture as anti-viral and antifungal factors (Barbieri et al.1993; Chen et al. 2002b; Park et al. 2004; Stripe 2004). RIPs have some medicinal uses as they can be used as toxic components of Immunotoxins against malignant cells and antiviral agents in AIDS patients (Battelli 2004; Stripe and Battelli 2006).RIPs have anti-tumor activity (Lin et al. 2003).

2.4 Curcin

Toxicity of seeds is due to the protein component. This protein component was first extracted from the seeds of Jatropha by Felke (1914), and he named this protein as “Curcin”. It is mainly found in the endosperm of the seeds of Jatropha (Mourgue et al.1961). Felke in 1914 proposed that the curcin was a kind of toxalbumin. Barbieri et al. (1993) described that curcin is a type1 RIP. Cloning and characterization of Curcin, a toxin extracted from *Jatropha curcas* has revealed to encode the type-1 ribosome inactivating proteins (RIPs). Different forms of curcin gene are reported in NCBI database i.e. Curcin2A gene (Accession no. GQ925453), Curcin-L precursor (Accession no. EU195892), Curcin gene (Accession no.

EU395775), RIP partial *Jatropha curcas* gene (Accession no. AY435214) and Curcin precursor gene (Accession no. AF469003). Considerable differences could be noted between them at both nucleotide and amino acid sequence levels.

After extraction of oil from Jatropha seeds, the seed meal contains high amount of curcin (Stripe et al. 1976). Curcin is a highly toxic RIP similar to ricin, the phytotoxin found in the castor bean (Ricinis). Amino acid sequence of coding region of Curcin gene shows high homology with other RIPs such as ricin A-chain, gelonin and momorcharin (Juan et al. 2003). Comparison between amino acid sequences of curcin and ricin-A chain shows high similarities with 54 % homology. The minimum lethal dose of ricin is very small as 0.0000001% of body weight when administered by injection (Kingsbury 1964). It can lead to disease such as dehydration and cardiovascular collapse as a result of haemorrhagic gastro-enteritis, central nervous system depression. Although curcin was 1000 times less toxic than ricin (Juan et al 2003). With the help of protein-ligand docking approach the r-RNA binding site of curcin can be identified. Cloning of a peptide inhibitor to the r-RNA binding site of curcin can produce non-toxic varieties of *Jatropha curcas*.

2.5 Chemical structure and stability of Curcin

Curcin is a large complex protein molecule with high toxicity. It is a type of phytotoxin. Phytotoxins are heat labile and easily identified by precipitin reaction with known antibodies (Kingsbury 1964). They match bacterial toxins in physiological effects and in structure. Curcin remains in the seed after the oil has been extracted. Curcin is unable to penetrate cell walls as they lack a carrier moiety or a galactose binding group to bind to cell membrane like ricin. This was observed that in a cell free system the activity of curcin is not enhanced by treatment of 2-mercaptoethanol while in ricin the inhibitory effect is increased by treatment with 2-mercaptoethanol as it splits the molecules of ricin into two parts, one is effector and another is carrier moiety (Stripe et al. 1976)

Extraction and purification of curcin

Crude curcin can be extracted from seeds of *Jatropha curcas* by solvent extraction method using hexane as a solvent and Phosphate buffer saline as an extracting buffer (Jummai et al. 2014). The crude curcin can be purified by using CM-52 column and Sephadex G-100(Juan et al. 2003).

2.6 Medicinal potential of Curcin

Crude curcin can be used as components of ‘Immunotoxins’ which is a hybrid molecule consisting of a toxic peptide linked to an antibody (Frankel et al. 1986). Crude curcin have poor stability and immunogenicity so it can’t be used as components of Immunotoxins (Kreitman et al. 1999). Oil from *Jatropha curcas* seeds is used to cure skin ailments and in treatment of rheumatism (Heller 1996).

Anthelmintic Activity of Curcin: Although the majority of Helminth infections in humans are restricted to tropical regions only but it is worrying issue in today’s world. Helminth infection leads to various health hazardous problem such as anemia, eosinophilila and pneumonia (Bundy 1994). Jummai et al. in 2014 studied medicinal potentials of *J.curcas* on Helminth infection. They used Niclosamide as reference standard and normal saline as the control in the experiment. The result of the experiment shows that the crude curcin was more potent on Helminth infection than the standard (Niclosamide). *Jatropha curcas* consist of alkaloids and saponins in a huge amount (Ahirrao et al. 2011), which stop the supplies of glucose to helminthes which acts on CNS causing paralysis (Mute 2009; Sutar et al. 2010; Sharma et al. 2010; Mali et al. 2007).

Antitumor activity of Curcin: It was observed that curcin protein could inhibit the growth of tumor cells at very low concentration. Various observations proposed that RIPs can kill the tumor cells by apoptosis (Luo et al. 2007). The anti-tumor activity of curcin was checked by Luo et al. in 2007 on cellule pulmonary cancer cells (NCL-H446) and gastric cancer cells (SGC-7901).They inserted the fragment of DNA encoding the mature protein of curcin *E.coli* strain M15 and used 0.5mM isopropyl- β-D-thiogalactopyranoside as inducer. The recombinant protein was expressed in the form of the inclusion body. They purified this recombinant protein by Ni-NTA affinity chromatography and used different concentration of this purified protein to kill the tumor cells for different time. They observed that curcin protein inhibit the growth of cancer cells at a very low concentration.

Juan et al. in 2003 studied the anti-tumor activity of curcin by MTT assay (3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazoliumbromide) which includes testing the toxic effects of curcin on the viability of cells grown in culture medium via counting the number of viable cells and their proliferation. The reduction of Tetrazolium salts was used to check the cell proliferation. They observed that curcin have anti-tumor activity and the mechanism behind anti-tumor activity was N-glycosidase activity. They observed that curcin didn’t have harmful effects on normal cells.

The MTT assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS) (Mosmann 1983). MTT assay are the colorimetric assay in which the yellow colour Tetrazolium MTT is reduced to purple colour formazan by metabolically active cells. The MTT Reagent yields low absorbance values in the absence of cells.

2.7 Curcin cDNA sequences, and expression patterns of curcin in Jatropha

Juan et al. in 2003 studied the full length curcin cDNA sequence and its amino acid sequence which was obtained by the study of gene sequence (Genbank ID: AY069946).They found that first an immature protein with 293 amino acid was translated from amino acid sequence of curcin cDNA then after it’s processing, a mature protein with 251 amino acids residues was obtained. They revealed that Curcin contained one cysteine residues at 209th amino acid. Thus, curcin may directly form a disulphide linkage with an activated antibody. Therefore curcin can be used for the preparation of immunoconjugates which have great potential as chemotherapeutic agents for the cure of cancer or AIDS (Juan et al. 2003)**.**

Expression of Curcin2 gene in Jatropha curcas under stress conditions: Qin et al. in 2005 studied ‘Inducible expression of curcin2 gene under drought, temperature stress and fungal infection’. They used one month old seedlings of *Jatropha curcas* and treated with various concentration of PEG solution. After growing seedlings at 25°C, seedlings were transplanted to different temperature conditions maintained at 4° to 50°C. After temperature treatment, they inoculated leaves of one-month seedlings with fungus. Results of this experiment showed that the accumulation of curcin2 gene was expressed specifically in leaves of seedlings growing under stress conditions like temperature stress, drought and fungal infection and not expressed in normal conditions i.e. non-stressed conditions (Qin et al. 2005). Curcin gene is not expressed in leaves either in stress or non-stress conditions. Curcin gene expresses only in seeds and its molecular weight is 28.2 kDa (Lin et al. 2002), while curcin2 gene can expresses in leaves of seedlings growing under stress conditions. Molecular weight of curcin2 is 30.1 kDa (Qin et al. 2005).

3.1 Aim of the present study

Jatropha curcas has been a source of attraction for various researchers across the world because of its potential use as biodiesel, a renewable energy source. Oil from edible crops can be used only if they are in an excess. But in developing countries like India these edible oils cannot be used for biodiesel production as there is shortage of food. Therefore, non-edible crops like *Jatropha curcas* can be used for biodiesel production. Both Government and non-government agencies in India are promoting this biodiesel crop for the last two decades. In our country, considerable progress has been made on survey, selection, clonal propagation of elite Jatropha germplasm, large-scale cultivation in the field, and importantly biodiesel production through transesterification. However, relatively less progress has been made on studying the toxic proteins such as curcins, RIPs as found in the Jatropha seeds. One can develop a transgenic Jatropha with low curcin content gene silencing approaches. On the other hand, we can also develop a novel Jatropha plant with high curcin content in the seeds; since this toxic protein has some medicinal value in terms of antitumor effects. Keeping all the aforesaid points in view, one of the objectives is to see the antitumor property of crude seed extracts. The other objective is to standardize a suitable and easy-to-use protocol for the isolation of DNA from Jatropha plants so that structure and function of the curcin gene(s) can be studied through PCR approach using gene-specific primers. Earlier, in our laboratory the focus was on propagation of Jatropha plant through plant tissue culture technique. A total of 30 accessions of Jatropha plant from different regions of Punjab were cultivated in our university campus. Only four cultivars of Jatropha i.e. TJS 17#03, TJS 42#04, TJS 35#01, TJS 01#03 were chosen for both biochemical and molecular studies. The major objectives of the study are outlined below:

Objectives

- Isolation of crude protein extracts from Jatropha seeds, and studying their cytotoxic effects on different cancer cell lines (namely RAW and HeLa cell lines)
- Sequence analysis and comparison between different forms of curcin
- Isolation and purification of genomic DNA from some of the Jatropha accessions
- PCR amplifications using curcin gene-specific primers under different conditions, and analyses of the amplicons

4.1 Oil extraction from seeds of different Jatropha cultivars using Soxhlet apparatus

Soxhlet apparatus is designed by Franz von Soxhlet in 1879. A Soxhlet extraction technique is used when the desired compound has very low solubility in a solvent.

4.1.1 Materials

Procurement of *Jatropha curcas* L. plant material

Jatropha seed collection was carried out on the basis of genotypes having high seed yield and seed oil content. We have selected five accession for this purpose which are TJS17#03, TJS42#04, TJS06#01, TJS04#01 and TJS42#05.

Other materials

- Soxhlet apparatus
- Hexane
- Round bottom flask
- Water supply

4.1.2 Oil extraction method

Approx. 50 g kernel from seeds of Jatropha cultivar was taken inside a Soxhlet extractor made of glass. 200 mL of Hexane was taken into a round bottom flask and the Soxhlet extractor was placed over this flask. Over this Soxhlet apparatus a condenser was used for cooling. Solvent was heated at 80°C. The solvent vapors goes up and the condenser controls that solvent vapor cools and drop back down inside the chamber of Soxhlet extractor consisting of Jatropha seed samples. When the chamber of extractor completely filled with warm hexane, the chamber was automatically emptied by a siphon side arm as solvent goes back down to the distillation flask. After 8 hours of reaction, oil from seed was concentrated in the distillation flask. The flask was heated at 80°C so that all the hexane evaporates and only oil remains inside the flask.

Oil content can be calculated by using the below formula:-

$$\% \text{ oil} = \frac{W_B - W_A \times 100}{\text{Weight of seeds in gram}}$$

$W_B =$ Final weight of round bottom flask containing oil only

$W_A =$ Initial weight of empty round bottom flask

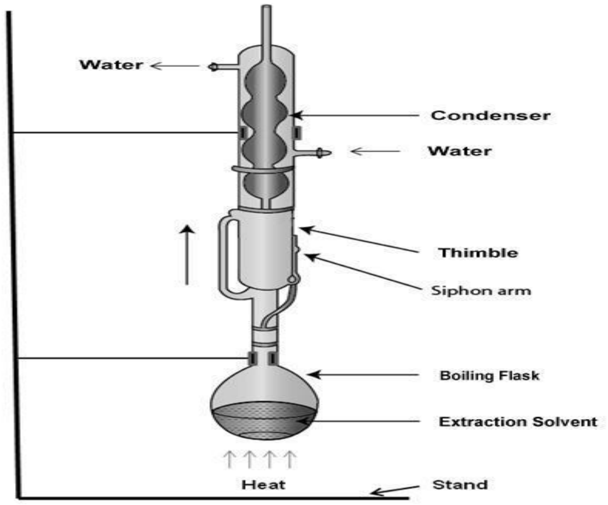

Fig. 6 Soxhlet apparatus operation

4.2 Free-Fatty acid content in oil from Jatropha

4.2.1 Materials

- Oil from *Jatropha curcas*
- 0.1 N KOH
- Neutral Ethanol
- Phenolphthalein

4.2.2 Method

Approx. 1 g of crude oil from seeds of Jatropha was weighed and mixed with 10 mL of neutral ethanol. The Solution was heated slowly for 2 minutes so that it became transparent. Five drops of Phenolphthalein was added into the above solution and titrated with 0.1 N KOH. The initial reading and final reading of KOH in burette was noted down.

Free Fatty acid content can be calculated by using the below formula:-

$$\% \text{ FFA} = \frac{28.2 \times N(\text{KOH}) \times \text{Volume of KOH used}}{\text{Weight of oil}}$$

4.3 Extraction of crude curcin extract from Jatropha seeds

4.3.2 Materials

- Seeds of Jatropha plant TJS17#03
- Mortar & Pestle
- Hexane
- Buchner funnel
- Rotary evaporator

- Sodium Phosphate buffer (0.005 M and pH 7.2 containing 0.2 Nacl)
- Ammonium Sulphate
- Centrifuge

4.3.3 Isolation of crude protein extract from Jatropha seeds

The seeds of Jatropha plant were dried properly so that there was no moisture remains inside the seeds. Then seeds were crushed into powder by grinder machine. 50 g of dried seeds powder was used to extract crude protein with 300 mL of hexane (Himedia, Mumbai). The solution was mixed properly and left overnight at 4°C. Buchner funnel connected to vacuum pump was used to filter solvent from the sample. The volume of the sample received after filtration was evaporated under reduces pressure in a rotary evaporator at 80°C to obtain crude protein extract. When all the hexane got evaporated, dried crude extract (20mL) was extracted with Sodium phosphate buffer (0.005M and pH 7.2 containing 0.2 Nacl) for 24h at 4°C. The sample was centrifuged at 10,000 rpm for 10 minutes and solid ammonium sulphate was added to the supernatant to about 60 % saturation. The solution was left overnight at 4°C. After being left overnight, the solution was centrifuged at 12,000 rpm for 15 minutes and the pellet was dissolved in 0.005 M sodium phosphate buffer and stored at 4°C. Dialysis of sample was done to remove the salt. The crude extract of protein was stored at 4°C.

4.4 Protein estimation by Folin-Ciocalteu Method

Folin-Ciocalteu method based on the principle of reduction of phosphomolybdate and phosphotungstate anions present in Folin-Ciocalteu phenol reagent by the tyrosine and tryptophan residues of protein to heteropolymolbdenum blue and tungsten blue respectively**.**

4.4.1 Materials

- Reagent-A

  2 % Na_2CO_3 in 0.1 NaOH
- Reagent-B

  2 % $CuSO_4.5H_2O$
- Reagent –C

  2 % Sodium Potassium tartarate
- Reagent –D (prepare fresh every day)

  0.25 mL of Reagent B was mixed into 0.25 mL of Reagent C, and then 49.5 mL of Reagent A was added.

- Reagent –E

  Folin-Ciocalteau reagent is diluted with water to 1 N.
- BSA (1mg/mL)
- Sterile water
- Spectrophotometer

4.4.2 Method

10 tubes were prepared containing increasing amounts of BSA (0.0 µg, 20 µg, 40µg, 60µg, 80 µg, 100 µg, 120µg, 140µg, 160 µg, 180 µg, 240 µg) in a total volume of 0.6 mL and six tubes were prepared with increasing volume of two unknown samples in a total volume of 0.6 mL . Then 3.0 mL of reagent D was added to each tube, mixed properly and incubated for 10 minutes at room temperature. Then 0.9 mL of reagent E (Folin's Ciocalteau reagent) was added and incubated for 60 minutes at room temperature in dark. After the incubation, absorbance was taken at 660 nm and graph was plotted between values of absorbance at 660 nm and concentration of BSA (µg). By using this graph, the value of unknown sample was calculated.

4.5 Effects of crude curcin extract on the different cell lines

4.5.1 Materials

- Crude curcin extract
- Cell lines(RAW & HeLa)
- Dulbecco's modified Eagle's medium (DMEM)
- MTT reagent (5 mg/mL)
- DMSO (Merck, Germany)
- Paclitaxel (positive control)
- Micro plate reader (Tecan infinite, Austria)

4.5.2 Methods

***Cell Culture maintenance*:** Two cell lines, RAW (mouse leukaemic monocyte macrophage cell line) and HeLa (Human cervical cancer cell line) were used to study anti-cancer activity. The cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. The Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS) , 100 IU/mL penicillin, 100 µg/mL streptomycin, 2.5 µg/mL amphotericin and 1mL/L tylosin. Cells were maintained in a humidified incubator with 5% CO2 for 37°C.

Cytotoxic assay: Response of crude protein extracts and standard drug was studied by means of a growth inhibition using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT assay). The cells were trypsinised and seeded in 96 well plate at a density of ~2×10^4 cells per well and incubated overnight. Sample extracts and positive control were added after 16 h in different concentration to the wells, making total volume 200 µL (cells + media + extract). The assay was carried out in triplicates. 20 µL of MTT reagent (5 mg/mL) was added after 48 hour of incubation to each well and again incubated for four hour. After 4h incubation, 180 µL of total volume was removed from each well and purple formazan crystals formed were dissolved in 100 µL DMSO (Merck, Germany) in each well. 4 µL of Paclitaxel was used as a positive control at the concentration 20 µg/mL .Using micro plate reader (Tecan infinite, Austria) absorbance was recorded at 570 nm and taking 630 nm as the reference wavelength; final O.D. was calculated by taking the difference between both O.D. Percentage of inhibition was calculated by following equation

$$\frac{\textbf{O.D Untreated cell – O.D test (extract)}}{\textbf{O.D Untreated cell}}\ \mathbf{x100}$$

4.6 Isolation and Purification of genomic DNA of *Jatropha curcas*

4.6.1 Materials

Procurement of *Jatropha curcas* L. plant material

For DNA isolation, leaf samples of Jatropha plant were collected from the different Jatropha cultivars such as TJS 17#03, TJS 42#04, TJS 35#01 and TJS 01#03 maintained at *Jatropha curcas* L. germplasm bank, COS-complex, Thapar University, Patiala.

Other materials

Various enzymes used were purchased from Bangalore Genei Pvt.Ltd., Bangalore. The chemicals required were bought from Siga-aldrich India Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai. Primers used were synthesized by Bangalore Genei Pvt.Ltd, Bangalore. The gel extraction Qiagen kit was purchased from Genetix.

Buffers and other chemical and Enzymes

- **Gel loading buffer (5X)**

    Sucrose - 35 % (w/v)
    EDTA - 50 mM (pH 8.0)
    Tris – 25 mM
    Bromophenol blue - 0.2 % (w/v)

- **TBE (5X) buffer**

  Tris Base - 54 g L^{-1}
  Boric acid - 28 g L^{-1}
  EDTA - 3.8 g L^{-1}
  The pH of the buffer was set at 8.0
- **TE buffer**

  Tris.HCl - 10 mM (pH 8.0)
  EDTA - 1 mM (pH 8.0)
- **Extraction buffer**

  50 mM Tris-HCL (pH8.0)
  50 mM EDTA
  250 mM Nacl
  15 % Sucrose
- Ethanol
- Isopropanol
- 5 M potassium acetate solution
- Sodium acetate
- RNase
- Alcohol
- Chloroform

4.6.2 Methods

Genomic DNA isolation from different varieties of Jatropha curcas

Genomic DNA was isolated from the Jatropha leaves by the protocol described by Kumari et al. (2012). Plant leaf samples were washed in tap water followed by sterile distilled water. Blotting filter paper was used to remove the excess water on the leaves. 0.7 g of plant material was weighed and grounded to fine powder in the presence of liquid nitrogen using mortar and pestle. The fine powder was then transferred to 20 mL tube containing 5 mL of extraction buffer and 0.5 % SDS (250 µL of SDS) maintained at 65°C. Contents were mixed properly with intermittent gentle shaking and incubated at 65°C for 15 min. The solution was centrifuged at 5500 rpm for 10 minutes. Then 170 µL of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and solution was centrifuged at 5500 rpm at 4°C for 15 min. The supernatant was filtered through a fine muslin cloth and equal volume of isopropanol was added, mixed gently and incubated at -20°C for overnight. DNA was extracted by centrifugation at 12,000 rpm at 4°C for 10 min. The crude DNA pellet was washed with ice cold 70% ethanol, air dried and suspended in 50 µL of TE buffer and stored at -20°C.

Purification of Jatropha DNA sample

Further purification of DNA was done by treatment with DNase–free RNase. 400 µL of sterile water was added into appendrof containing DNA sample. Then 3µL of DNase–free RNase enzyme was added into solution. The solution was incubated at 37°C for 45 minutes. Equal volume of phenol and chloroform (200 µL each) was added into the solution and mixed properly for 10-15 minutes by gently inversion mixing. Then the sample was centrifuged at 8000 rpm for 10 minutes. DNA was precipitated using 0.1 volume of 3.0 M sodium acetate (pH 5.5) and 2.0 volumes of ethanol. DNA was finally recovered by dissolving the pellet in 50 µL of TE buffer and stored at (-20°C). The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio.

4.7 Designing of Curcin2A gene-specific oligonucleotide primers

The following oligonucleotide primers were designed based on the available genome sequence corresponding to Curcin2A gene in GenBank database (GenBank ID: GQ925453). Curcin2A gene sequence of 3748 bps is comprised of the following structural features: It consist of 1790-bp 5′ flanking region (including promoter)and the transcriptional start site is located at the base 1791 corresponding to first exon. So, the above gene sequence provides an extended Curcin2A gene promoter. The translational start site is present at the base 2085. In curcin2A gene, the ORF of polypeptide spans from the base 2085 to 3014.

To ensure minimum chances of non-specific amplification, important factors kept in consideration while designing the primers were:

- PCR primers should be 15-20 nucleotides long.
- GC content should be 40-60%.
- The primer should not be self-complementary or complementary to any other primer to form primer-dimer or hairpin.
- Melting temperatures of primer pairs should not differ by more than 5º C, so the GC content and length must be chosen accordingly.
- The annealing temperature should be about 5°C lower than the melting temperature.
- Stretches of a particular nucleotide sequence is avoided.
- Primers with significant structures are avoided.

The features of four forward primers (each 20 mer) specific for Curcin2A genes are briefly discussed below:

Three forward primers were designed from different regions of upstream sequence of Curcin2A gene and one forward primer was designed from coding regions of curcin2A gene.

Forward Primers

- F1-CU126 is a forward primer that corresponds to bases 126-145 of the genome sequence i.e., 5′-AACTAGATTACAAGAGTAGC-3′
- F2-CU1318 is forward primer that corresponds to the bases 1318-1337 of the genome sequence i.e., 5′-TAAATGTTGGAATAGAAGAC-3′
- F3-CU1526 is forward primer that corresponds to the bases 1526-1545 of the genome sequence i.e., 5′-CTATGACGCGGCATTTATCA-3′

As the transcription start site is located at 2084 base, all three forward primers belong exclusively to extended promoter region of the gene.

- F4-CU2075 is a forward primer that corresponds to bases 2075-2094 of the genome sequence i.e., 5′-TGAAATCAATATGAAAGGTG-3′

Reverse Primers

- R1-CU2258 is a reverse primer that is complementary to the bases 2239-2258 of the genome sequence i.e., 5′-ATCAGTAGTAGCGTCATAAG-3′
- R2-CU3021 is a reverse primer that is complementary to the bases 3002-3021 of the genome sequence i.e., 5′-AGTAGGATTAAAGCCATGGC-3′

The gene sequence corresponding to the reverse primers lies in the coding region encoding the polypeptide of the Curcin2A gene i.e., 309-aa peptide. The purpose was to see whether the sequences are conserved in the coding region in curcin gene family members.

4.8 Polymerase Chain Reaction (PCR)

PCR was used to amplify a specific DNA sequence in a simple, rapid and automated manner using forward and reverse primer. PCR is repeated cycling of three steps: heat denaturation of template DNA (94°C); annealing of primers to the complementary sequences in template DNA (55°C); extension of annealed primers by a thermo stable DNA polymerase (72°C).

4.8.1 Materials

In PCR, genomic DNA preparations from the following four Jatropha cultivars were used:

- TJS 17#03
- TJS 42 #04
- TJS 35 # 01
- TJS 01 # 03

The following primer pairs were used to carry out individual PCR:

- F1-CU126 and R1-CU2258
- F2-CU1318 and R2-CU3021
- F3-CU1526 and R2-CU3021
- F3-CU1526 and R1-CU2258
- F4-CU2075 and R2-CU3021

4.8.2 Methods

Composition of PCR reaction: Total reaction volume: 50 µL

Template DNA	3 µL
Buffer 10X	5 µL
Forward primer	10 pmoles
Reverse primer	10 pmoles
dNTPs	2.5 mM
Sterile deionized water	volume made up to 50 µL
Taq DNA polymerase	3 U/µL

The thermal cycling parameters were as given below:

Step	*Temperature*	*Time*
Denaturation	94°C	1 min
Annealing	55°C	2 min
Polymerization	72°C	3 min

The reaction was carried out for 30 cycles with final extension at 72°C for 5 min.

4.9 Agarose gel electrophoresis

4.9.1 Materials

- Agarose (Himedia)
- 0.5 X TBE buffer
- Ethidium bromide dye (0.5 µg mL^{-1})
- Sterile water
- DNA samples and amplicons
- Bromophenol blue dye
- Gel Electrophoresis instrument
- UV Transilluminator
- Gel documentation system(BIO-RAD)

4.9.2 Method

Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 1.0 % agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5µg mL^{-1}) was added to it. Gel was then casted in the casting tray. The DNA samples were loaded in the wells after solidification of gel. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2 – 5 Volt per cm till the tracking dye covered two-third of the gel length. Finally, the DNA bands were visualized under UV light.

5.1 Extraction of oil from Jatropha seeds and quantitative estimation of free fatty acid (FFA) content in oil

Oil from seeds of different Jatropha cultivars was extracted using Soxhlet apparatus (see section 4.1.2 of 'Materials and Methods'). To get the pure biodiesel from crude oil, it was processed to remove the free fatty acid by the reaction known as 'Transesterification reaction' which takes place in the presence of alcohol. Free fatty acid percentage inside the crude oil was determined by titration method (see section 4.2.2 of 'Materials and Methods').

Table 3 Oil and free fatty acid content in Jatropha seeds

Variety of Jatropha	% Oil extracted	Free fatty acid % in oil
TJS 17#03	28	1.41
TJS 42#04	26	1.69
TJS 06#01	20	2.89
TJS 04#01	22	2.25
TJS 42#05	43.4	2.53

It was observed that seeds of TJS 42#05 have highest oil content (43.4%) as compared to other Jatropha cultivars (TJS 17#03, TJS 42#04, TJS 06#01, and TJS 04# 01). But the free fatty acid content is lowest in TJS 17#03 i.e. 1.41% as compared to other Jatropha cultivars studied. Transesterification reaction of oil with alcohol is effected with high content of free fatty acid and moisture (Goodrum 2002). The high free fatty acid content will led to the soap formation and separation of products will become difficult. So, as a result yield of Biodiesel is decreased from crude oil. Therefore, it is likely that the seeds corresponding to TJS 17#03 showing lowest free fatty acid (FFA) content could be a suitable resource for production of biodiesel effectively through transesterification.

5.2 Estimation of protein in the crude extract from Jatropha seeds by Folin-Ciocalteu method (see section 4.4.2 of ‘Materials and Methods’)

Table 4 Estimation of protein

BSA standard (µg)	Absorbance at 660 nm
Blank	0
20	0.037
40	0.067
60	0.123
80	0.146
100	0.184
120	0.223
140	0.249
160	0.303
180	0.353
200	0.378
Crude curcin (600 µL)	0.272
Residue of crude curcin extract (600 µL)	0.232

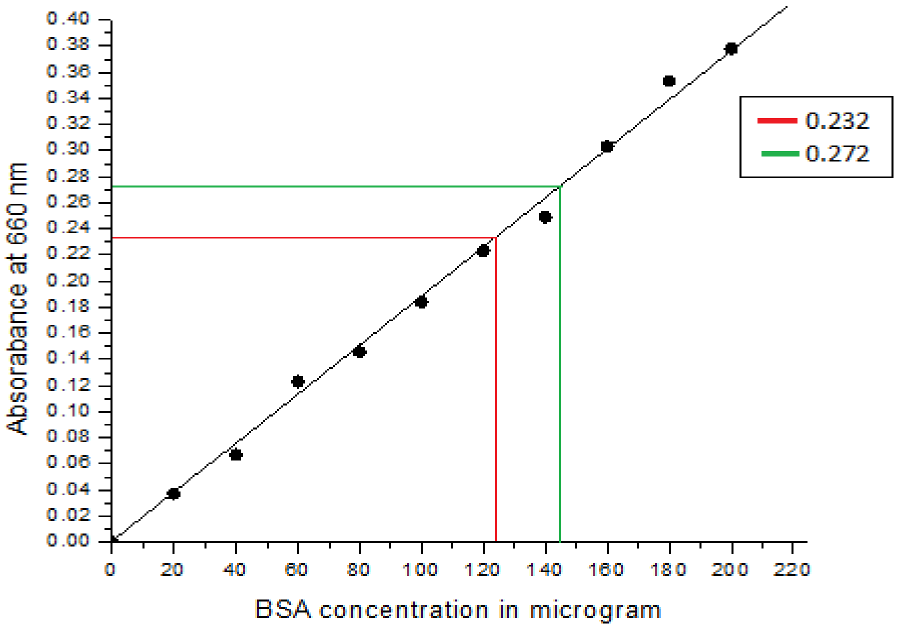

Fig. 7 Estimation of protein in the crude extracts of Jatropha seeds using BSA standard

Therefore from above graph we calculate that the amount of protein present in 600 µL of crude extract of curcin is 145 µg and the amount of protein present in 600 µL residue of crude extract is 122 µg.

5.3 Effect of crude extract of curcin on different cell lines

5.3.1 Effect of crude extract of Curcin on HeLa cell line

Crude extract of curcin was also assessed for its cytotoxic potential on the HeLa cells. 50 µL of crude curcin extract was added in the culture and incubated for 48 hrs at 37°C and 5% CO. MTT assay was done to evaluate the effect of extract on HeLa cell lines and then readings were taken at 570 nm, taking reference wavelength of 630 nm. Optical density, which corresponds to the number of viable cells, was analyzed for crude curcin extract as well as for untreated cells and positive control.

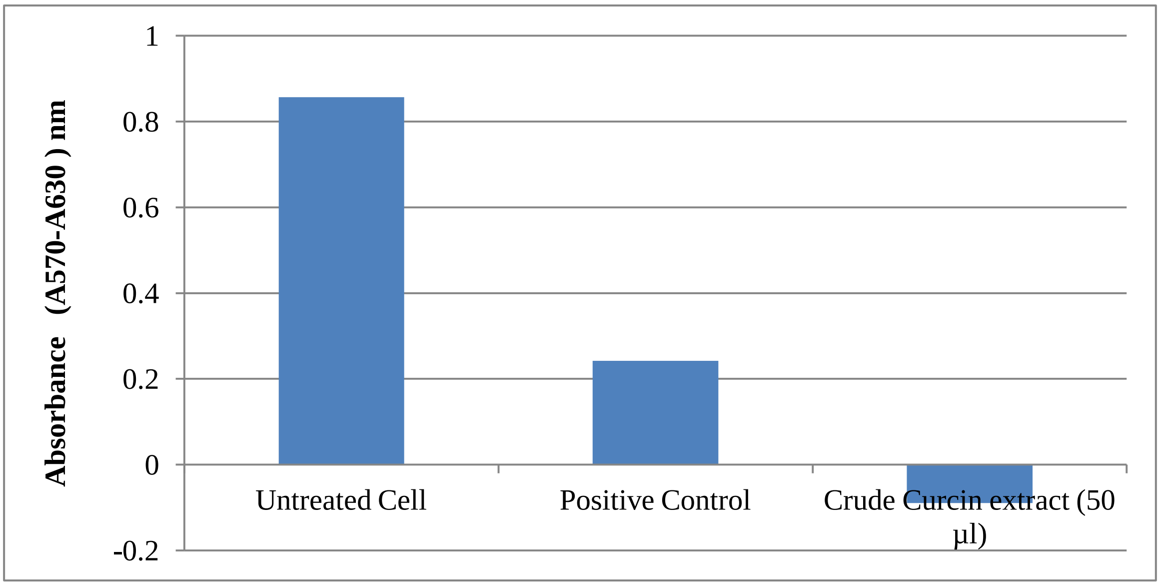

Fig. 8 Effect of crude protein extracts of Jatropha seeds on HeLa cell line

Percentage of inhibition was calculated by following equation (see section 4.5.2 of 'Material and Methods')

$$\frac{\text{O.D Untreated cell} - \text{O.D test (extract)}}{\text{O.D Untreated cell}} \times 100$$

It was observed from above readings that 50 µL of crude Curcin extract showed 100 percent inhibition to the proliferation of HeLa cell lines.

5.3.2 Effect of crude extract of Curcin on RAW cell line

Crude extract of curcin was assessed for its cytotoxic potential on the RAW cells (see section 4.5.2 of 'Materials and Methods'). Variable concentrations of the extract (10 µL, 20 µL, 30 µL and 50 µL) of crude curcin extract was added in the culture and incubated for 48 hrs at 37°C and 5% CO. MTT assay was done to evaluate the effect of these extracts on RAW cell lines.

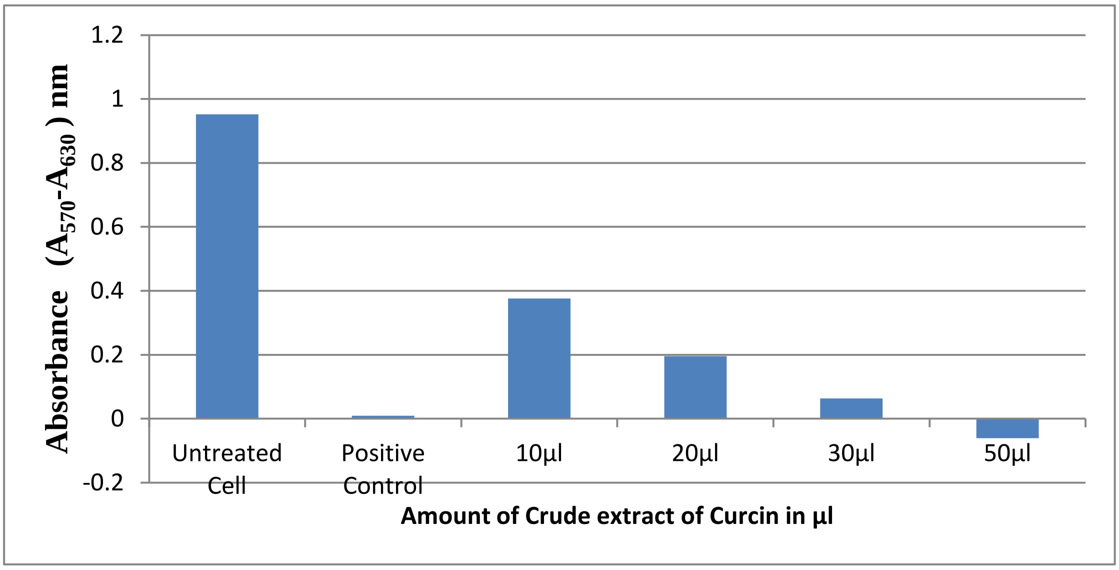

Fig. 9 Effect of different amount of crude curcin extracts of Jatropha seeds on RAW cell line

It was observed that 50 µL of crude extract has shown the maximum inhibition in proliferation as compared to the untreated cell. 10 µL of extract also show some inhibition in proliferation as compare to untreated cell.

Table 5 Percentage inhibition of RAW cell line by crude curcin extract

Amount of crude extract	Percent Inhibition
10 µL	60.50
20 µL	79.40
30 µL	93.36
50 µL	~100.00

The data as presented in Table 5 revealed that as the amount of crude extract was increased there is significant increased in the inhibition in the proliferation of RAW cell line. 50 µL of crude extract showed 100 percent inhibition in the proliferation of RAW cell line.

5.3.3 Effect of Residue of crude extract of Curcin on RAW cell line

Residue of crude extract of curcin was assessed for its cytotoxic potential on the RAW cells. Variable concentrations (10 µL, 20 µL, 30 µL and 50 µL) of the crude curcin residue was added in the culture and incubated for 48 hrs at 37°C and 5% CO. MTT assay was done to evaluate the effect of these extracts on RAW cell lines.

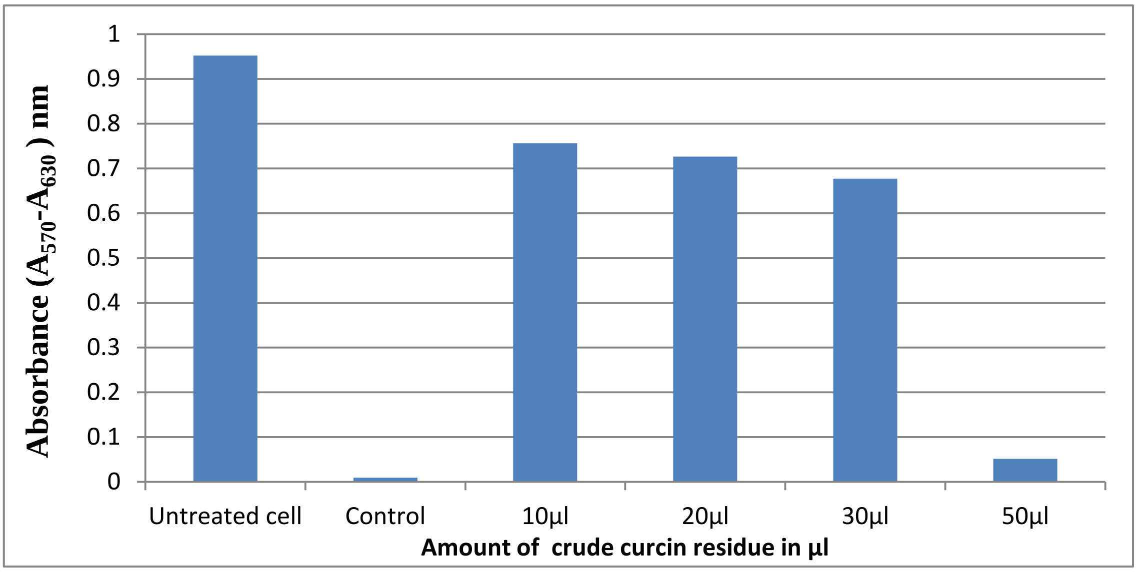

Fig. 10 Effect of different amounts of crude curcin extract residue on RAW cell line

It was observed that 50 µL of residue of crude extract has shown the maximum inhibition in proliferation as compared to the untreated cells .10 µL of extract also show some inhibition in proliferation as compare to untreated cell.

Table 6 Percentage inhibition by Residue of crude extract on RAW cell line

Amount of Residue of crude extract	Percent Inhibition on RAW cell line
10 µL	20.56
20 µL	23.56
30 µL	28.69
50 µL	51.72

The data as presented in Table 6 revealed that as the amount of residue of crude extract was increased there is significant increased in the inhibition in the proliferation on RAW cell line. 50 µL of the residue showed around 51 % inhibition in the proliferation of RAW cell line.

5.4 Some important biochemical attributes of Curcin2A protein (GenBank Protein ID: ADN39428)

The entire 309 amino acids sequence of curcin2A protein was analysed by using protparam tool of ExPASy resource portal under Swiss Institute of Bioinformatics (SIB) which revealed some of the important biochemical attributes. The calculated molecular weight of the curcin2A protein was found to be 34.8 kDa with a predicted isoelectric point (pI) of 6.20. Predicted formula of Curcin2A protein is $C_{1584}H_{2452}N_{404}O_{465}S_{8.}$ Out of its total 309 amino acids, 32 were strongly basic (+) (Arg + Lys), 33 were strongly acidic (-) (Asp + Glu), and 9 were polar (Asn, Cys, Gln, His, Met, Ser, Thr, Trp, Tyr), and 7 were hydrophobic (Ala, Gly, Ile, Leu, Phe, Pro, Val). The instability index (II) was computed as 29.55, which classified the protein as stable. The amino acid composition data revealed that some of the amino acids such as Asn (7.1 %), Ile (7.1%), Lys (7.4%), Phe (5.5%), Ser (8.1%), Tyr (4.5%) and Val (8.4%) occurred more frequently as compared to their average occurance; whereas the amino acids namely Arg (2.9%), cys (0.6%), Glu (5.2%), Gly (4.5%), Leu (8.1%), Met (1.9%), Pro (2.9%) and Thr (4.9%) occurred less frequently (Doolittle 1989). The estimated half life of curcin2A protein is 30 h as predicted in this analysis.

5.4.1 Hydropathy plot of Curcin2A protein

The hydropathy profile was generated for Curcin2A protein using the ProtScale tool based on the Kyte-Doolittle scale.

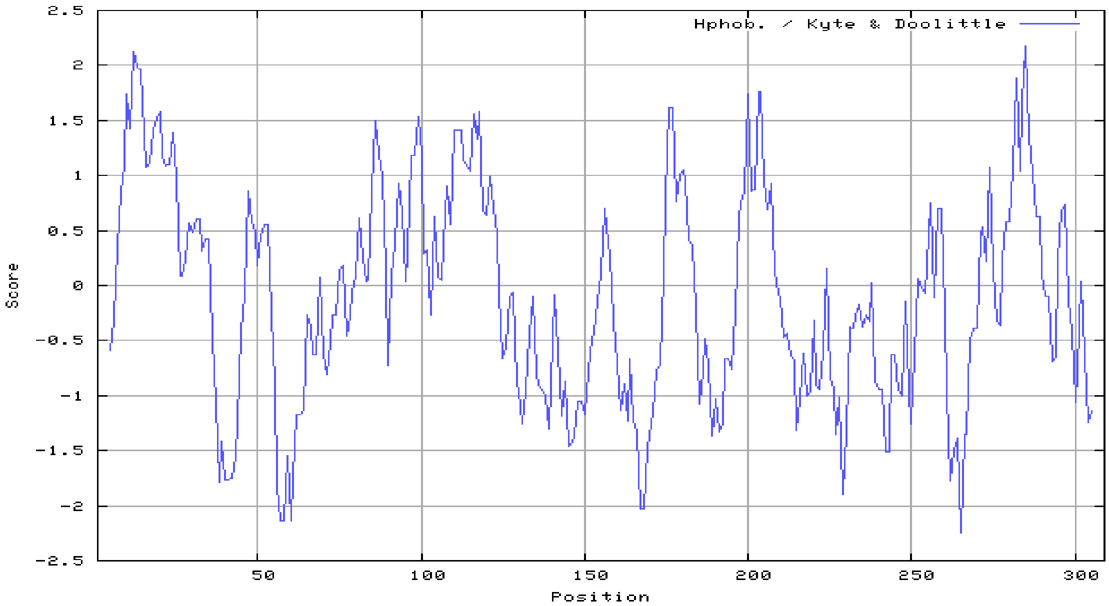

Fig. 11 Hydropathy plot of Curcin2A protein

Hydropathy plot of curcin2A protein revealed that N terminal of amino acid sequences of Curcin2A protein is Hydrophobic i.e. 5 to 30 position of amino acid sequence. Hydrophilic regions in the plots are at 30-40, 55-65, 120-150, 160-175, 220-255 and 265-275 positions of amino acid in curcin2A protein. Hydrophobic region in the plots are at 70-115, 165-170, 190-220 and 275-300 positions of amino acid in curcin2A protein.

5.4.2 BLASTp analysis of Curcin2A protein

When BLASTp was done for Curcin2A protein (GenBank protein ID: ADN39428); it shows more homology with other forms of Curcin proteins. The 309-amino acid sequence was found to be identical (100% sequence identity with 100% query coverage) to amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ABZ04128), 99 % sequence identity with 100 % query coverage to the amino acid sequence in *Jatropha curcas* curcin-L precursor gene (GenBank protein ID: ABW17545), 99 % sequence identity with 100% query coverage to the amino acid sequence in *Jatropha curcas* RIP gene (GenBank protein ID: AY435214), 98 % sequence identity with 94% query coverage to the amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ACO53803). The 309-amino acid sequence of Curcin2A gene showed C terminal variation with the amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ACO53803) as 16 amino acids are unique in Curcin2A gene at C-terminal.

5.4.3 Multiple sequence alignment (MSA) of different curcins from Jatropha

A total of four curcin protein sequence from Jatropha, i.e., Curcin precursor (Curcinp, AAL86778), Ribosome-inactivating protein, partial, *Jatropha curcas* (RIP-JAT, AAR08395), Curcin2A (curcin2A, ADN39428) and Curcin-L precursor (CurcinLP, ABW17545), were included in the multiple sequence alignment (Fig. 12). Minor manual adjustments were also made during alignment. This study was done to examine sequence similarities and divergence, the amino acid substitutions, insertions and deletions between the four different forms of curcin proteins.

```
CurcinP     MKGGKMNLSIMVAAWFCWSCIIFGWASAREIVCPFSSNQNYKAGSTPTLTITYDAAADKK   60
RIP-Jat     MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLVITYDATTDKK   60
Curcin2A    MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKK   60
CurcinLP    MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLVITYDATTDKK   60
            ******************* ***************************** *****  ***

CurcinP     NYANFIRDLREAFGFSYSSHEIPVLRATVAANQKFIVAKVINVANLEVSLGLNVVNAYLV   120
RIP-Jat     NYAQFIEDLREAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLV   120
Curcin2A    NYAQFIEDLREAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLV   120
CurcinLP    NYAQFIEDLREAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLV   120
            *** ** ****** *** ** *********************    *** **** *****

CurcinP     GYKVGGTSYFFNDPESLADAKTYLFTDTKQQTLSFTGSYADFLSRANVHREDVDLGVQAL   180
RIP-Jat     AYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYADFESRAKLHKEEVDLGVVAL   180
Curcin2A    AYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYADFESRAKLHREEVDLGVVAL   180
CurcinLP    AYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYADFESRAKLHREEVDLGVVAL   180
             ****  ****** *******  ***** **** ******** ***  * * ***** **

CurcinP     DNYIYTLEKSSKPADIAKPLVGFIEMVPEAARFEYIEKKISTQISKTFRPRGDIISLENN   240
RIP-Jat     DNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN   240
Curcin2A    DNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN   240
CurcinLP    DNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN   240
            *** ******* ********************* **************************

CurcinP     WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVKYKV-------   293
RIP-Jat     WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIF   300
Curcin2A    WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIF   300
CurcinLP    WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIF   300
            ************************************************* ***

CurcinP     ---------
RIP-Jat     NDQKWLPWL                                                      309
Curcin2A    NDQKWLPWL                                                      309
CurcinLP    NDQKWLPWL                                                      309
```

Fig. 12 Multiple sequence alignment of the deduced amino acid sequences of four different forms of Curcin protein from the Jatropha cultivars (see the text for GenBank protein ID).This alignment is based on ClustalW2 tool. The star symbols denote the conserved amino acid between all the four forms of curcin proteins studied. Alignment of Curcin P protein showed some divergence with respect to the other 3 forms of curcin protein which are highlighted grey

Segment wise comparison between the different forms of curcin protein revealed the following observations. In the N-terminal of the all the curcin forms the amino acids were nearly conserved with minor variations. The curcin P protein was found to be shorter by 16 amino acids at C-terminus if compared with the other forms of curcin as shown in Fig. 12. Considering Curcin2A protein as a reference, Curcin P was found to vary at 31 locations, mostly noncoservative substitutions such as T56A, T57A, K81E, D105N, N149K, K167N, Q192K, K214E, N290K and conservative substitutions such as S20C, Q64N, I106L, I115V, L168V, R170K, V183I with respect to other three forms of curcin protein studied.

5.5 Molecular approaches for isolation of curcin genes with upstream sequences from Jatropha

In-depth understanding of the structure and function of curcin/RIP genes and their expression patterns are important areas of research with regard to gene manipulation and other biotechnological aspects of the biodiesel crops. In this context, Jatropha is an important system to carry out molecular biology works. In this study, some efforts are made towards isolation of curcin genes from Jatropha mainly through PCR approach as presented in the following sections.

5.5.1 Salient sequence features of Curcin2A gene from Jatropha

For this thesis work we followed 3748-bp Curcin2A gene from *Jatropha curcas* (GenBankID: GQ925453) which encodes 309 amino acid of "Curcin2A" protein which is also regarded as ribosome inactivating protein. This 3748-bp Curcin2A gene consisting of 1790-bp 5′flanking region (including promoter) and two exons i.e. 1791 to 1845 and 2074 to 3194 bp. The entire transcriptional unit spans from 1791 to 3194. The length of first exon of this gene is 55-bp i.e. from 1791 to 1845 bp. The length of only one intron is 228-bp i.e. from 1846 to 2073 bp. The length of second exon is 1121-bp i.e. from 2074 to 3194 bps. Therefore the remaining portion i.e. from 3195 to 3748 bp refers to 3′ flanking region of this gene. Size of ORF in curcin2A gene is 930 bp (3014-2084) which is equal to 310 codons including stop codon. Therefore, the Curcin2A polypeptide consists of 309 amino acids. The length of 5′UTR is equal to 294 bp (including intron). Therefore, 3′ UTR of Curcin2A gene is equal to is equal to 180 bp.

5.5.2 Sequence analysis of the different regions of curcin2A gene by BLASTn

5′-flanking region as a query sequence**:** The nucleotide sequence of Curcin2A gene was analyzed by NCBI BLAST tools. When BLASTn was done for 5′ flanking region of Curcin2A gene, it shows largest promoter part in all *Jatropha curcas* gene available in database as query cover is very less. So, it shows divergence in distal part of promoter region of 5′ flanking region of Curcin2A gene. The 1790-bp 5′ flanking region of Curcin2A gene was found to be close (88 % sequence identity) to a gene copy in *Jatropha curcas* clone BAC 121e10 putative curcin 2b gene (GenBank ID: GQ925454). However sequence divergence was prominent if compared with some known curcin genes from Jatropha, since the sequence identity values were 99% (GenBank ID: EU195892), 99 %(GenBank ID: EF12740). Query coverage ranged from 74% to 22 % in the BLAST search data for these sequences. 5′flanking region of the Curcin2A gene was not found 100% identical with any other Curcin gene reported to date.

***Transcribed region as a query sequence*:** When BLASTn was done for transcribed region of Curcin2A gene, it shows more homology with other gene sequences of Curcin. The 1404-bp transcribed region was found to be close (99% sequence identity with 96 % query coverage) to a gene copy in *Jatropha curcas* curcin-L precursor gene (GenBank ID: EU195892), 100 % sequence identity with 87 % query coverage to a gene copy in *Jatropha curcas* curcin gene (GenBank ID: EU395775), 96 % sequence identity with 96% query coverage to a gene copy in Curcin precursor gene (GenBank ID AF469003).Although transcribed region of the Curcin2A gene showed some homology but it was not found 100% identical with any other Curcin gene reported to date.

***Coding region as a query sequence*:** When BLASTn was done for coding region of Curcin2A gene, it shows more homology with other gene sequences of Curcin. The 930-bp coding region was found to be identical (100% sequence identity with 100% query coverage) to a gene copy in *Jatropha curcas* curcin gene (GenBank ID: EU395775), 99 % sequence identity with 100 % query coverage to a gene copy in *Jatropha curcas* curcin-L precursor gene (GenBank ID: EU195892), 99 % sequence identity with 99% query coverage to a gene copy in *Jatropha curcas* RIP gene (GenBank ID: AY435214), 95 % sequence identity with 100% query coverage to a gene copy in *Jatropha curcas* curcin precursor gene (GenBank ID: JF357910).

5.5.3 Isolation of genomic DNA from Jatropha accessions

Total genomic DNA was isolated from Jatropha cultivars namely TJS 17#03, TJS 42 #04, TJS 35#01 and TJS 01#03 from the Jatropha leaf samples collected from the field grown plant (see section 4.6.2 of ‘Materials & Methods’). The quality of genomic DNA was checked by agarose gel electrophoresis as shown in Fig. 13.

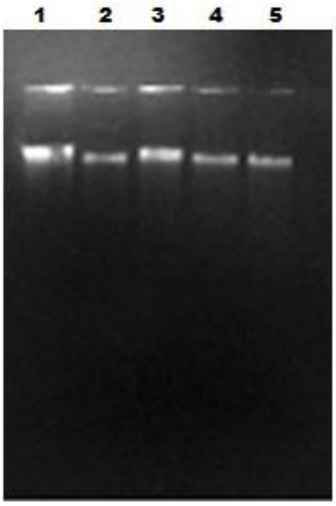

Fig. 13 Genomic DNA isolation from Jatropha accessions, TJS 17#03,TJS 42#04,TJS 35#01,TJS 01#03; Lane 1- λ DNA; Lane 2-TJS 17#03 genomic DNA; Lane 3- TJS 42#04 genomic DNA; Lane 4- TJS 35#01 genomic DNA; Lane 5- TJS 01#03 genomic DNA

The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio respectively.

Table 7 Quantification of Jatropha DNA using nanodrop spectrophotometer

Jatropha Cultivar	Amount of Material (g)	Volume of DNA soln. (µL)	Absorb. Ratio ($A_{260}/A_{2\,80}$)	Conc. (ng/µL)
TJS17#03	0.7	50	1.76	1081
TJS42#04	0.7	50	1.82	2212
TJS35#01	0.7	50	1.77	1156
TJS01#03	0.7	50	1.86	2009

5.5.4 Some details of Curcin2A gene specific primers as used in the study

- F1-CU126: BLASTn data clearly revealed that this primer sequence is unique to the Jatropha Curcin2A gene as expected. No substantial sequence identity could be seen with other available curcin gene sequences.
- F2-CU1318: This primer sequence appears to be specific to curcin2A gene of Jatropha. However some significant homology could be found with some unrelated gene sequences of different plant species.
- F3-CU1526: Apart from the source sequence, this primer sequence was found to be conserved in a no. of curcin related gene sequences such as Curcin gene (GenBank ID: EU39775), Curcin-L precursor (GenBank ID: EU195892), Curcin Ribosome inactivating protein of Jatropha (GenBank ID: EF612740).

- F4-CU2075: This primer sequence encompasses the coding region only and which appears to be conserved in a no. of curcin and curcin-related genes along with Cucurmosin like protein (Ribosome inactivating protein) as reported in the database.
- R1-CU2258: This primer sequence appears to be highly conserved if compared with other forms of curcin gene in Jatropha.
- R2-CU3021: This sequence also appears to be conserved with many other curcin gene sequences as reported from *Jatropha curcas*.

5.5.5 PCR using Curcin2A gene-specific primer pairs under different conditions

One of the major objective of the study is to amplify curcin gene(s) preferably with the upstream regions from a few Jatropha accessions through PCR approach under different conditions using specific primer pairs. For this purpose, genomic DNA preparations from the following Jatropha accessions namely TJS17#03, TJS42#04, TJS35#01 and TJS01#03 were used during PCR. For each specific primer pair, PCR was carried out under two different annealing temperatures i.e. 50°C and 55°C. Individual primer pair-specific amplicon profiles are shown in the following sections.

(i) Results of PCR using the primer pair: F4-CU2075, R2-CU3021

PCR was carried out under two different annealing conditions as shown Fig. 14A (annealing temp. 50°C), and Fig. 14B (annealing temp. 55°C)

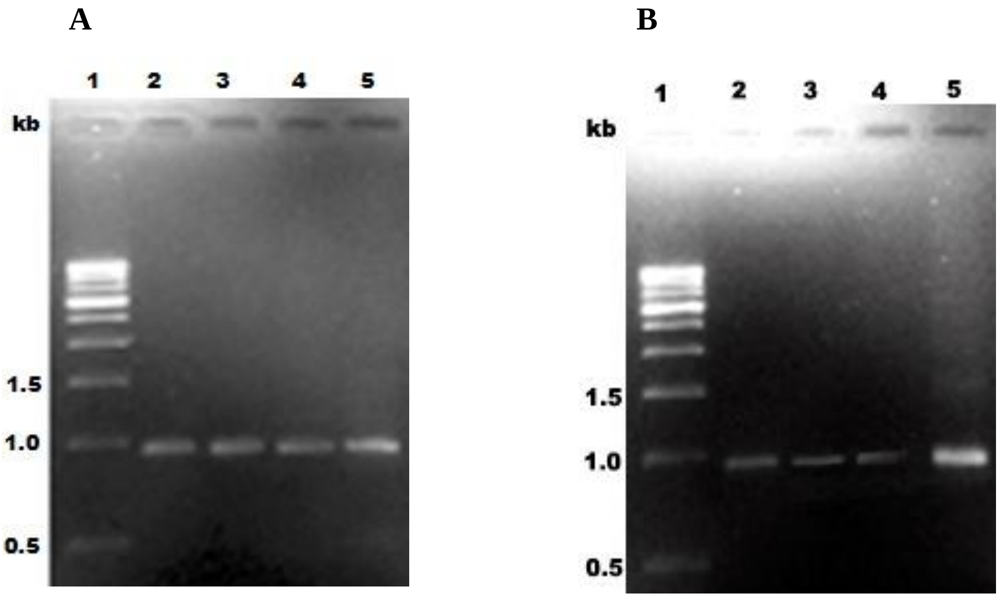

Fig. 14 PCR amplified products using gene specific primer pairs (F4-CU2075 & R2-CU3021)
A Annealing temp. 50°C and **B** Annealing temp. 55°C *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 42#04; *Lane* 4- PCR amplified band of TJS 35#01; *Lane* 5- PCR amplified band of TJS 01#03. Order of cultivars remains unchanged in both the figures

Table 8 F4-CU2075 & R2-CU3021 primer pair-specific amplicons

Jatropha accessions	Size of amplicons (annealing temp. 50°C)	Size of amplicons (annealing temp. 55°C)
TJS17 #03	~0.9 kb	~0.9 kb
TJS42#04	~0.9 kb	~0.9 kb
TJS35#01	~0.9 kb	~0.9 kb
TJS01#03	~0.9 kb	~0.9 kb

For F4-CU2075 & R2-CU3021 primer pairs, expected size is ~0.9 kb. At 50°C annealing temperature, similar banding pattern of PCR amplification products (~0.9 kb) was found in all the accessions of Jatropha. At higher annealing temperature i.e. 55°C amplicons of 0.9 kb size was found in all Jatropha accessions, suggesting that this region of curcin2A gene is almost conserved in different Jatropha germplasm; however there may be some sequence heterogeneity.

(ii) Results of PCR using the primer pair: F3-CU1526, R2-CU3021

PCR was carried out under two different annealing conditions as shown Fig. 15A (annealing temp. 50°C), and Fig. 15B (annealing temp. 55°C)

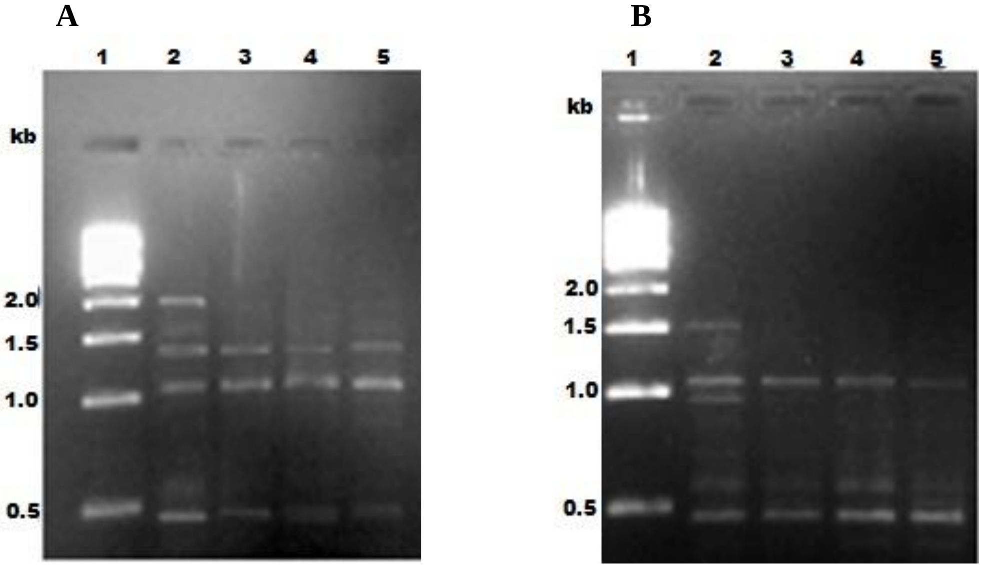

Fig. 15 PCR amplified products using gene specific primer pairs (F3-CU1526 & R2-CU3021)
A annealing temperature **50°C** and **B** annealing temperature **55°C** *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 42#04; *Lane* 4- PCR amplified band of TJS 35#01; *Lane* 5- PCR amplified band of TJS 01#03. Order of cultivars remains unchanged in both the figures

Table 9 F3-CU1526 & R2-CU3021 primer pairs-specific amplicons

Jatropha accessions	Size of amplicons (annealing temp. 50°C)	Size of amplicons (annealing temp. 55°C)
TJS17 #03	~ 1.1 kb, 1.4 kb, 2.0 kb	~0.9 kb, 1.1 kb, 1.5 kb
TJS42#04	~ 1.1 kb, 1.4 kb	~1.1 kb
TJS35#01	~ 1.1 kb, 1.4 kb	~ 1.1 kb
TJS01#03	~ 1.1 kb, 1.4 kb	~ 1.1 kb

For F3-CU1526 & R2-CU3021 primer pair, expected size of amplicon is ~1.5 kb. At 50°C, the amplicon profiles were found to be same in all the Jatropha accessions except TJS17#03 since a promising ~ 2.0 kb amplicon was only found in the latter. Interestingly, the amplicon profiles appear to be considerably different at 55°C annealing temperature. Instead of 1.4 kb DNA band as found in other conditions, 1.1 kb DNA band was found in this case; nearly 1.5kb amplicon appeared only for the accession TJS17#03. All these data clearly suggesting the presence of the allelic variants of curcin genes; however, all these amplicons need to be studied further.

(iii) Results of PCR using the primer pair: F2-CU1318, R2-CU3021

PCR was carried out under two different annealing conditions as shown Fig. 16A (annealing temp. 50°C), and Fig. 16B (annealing temp. 55°C)

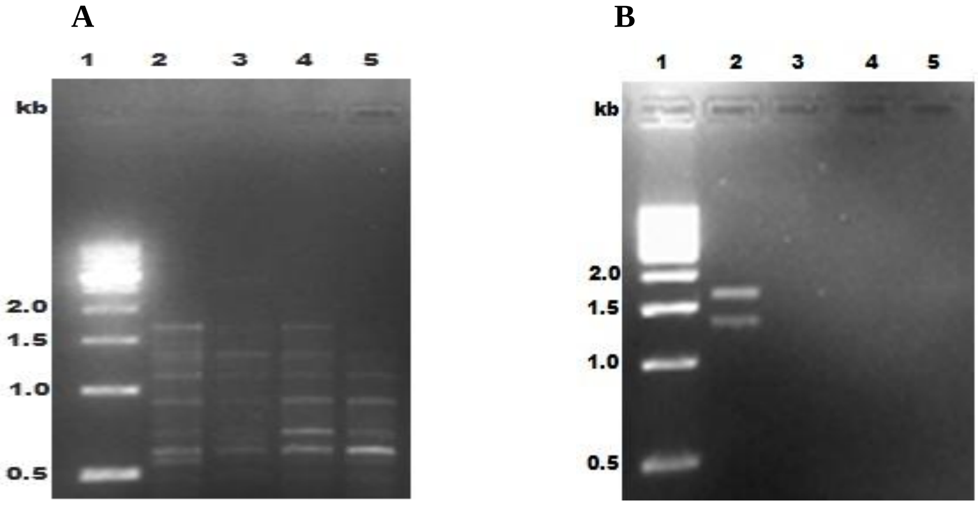

Fig. 16 PCR amplified products using gene specific primer pairs (F2-CU1318 & R2-CU3021)
A annealing temperature **50°C** and **B** annealing temperature **55°C** *Lane* 1- 500 bp ladde*r; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 42#04; *Lane* 4- PCR amplified band of TJS 35#01; *Lane* 5- PCR amplified band of TJS 01#03. Order of cultivars remains unchanged in both the figures

Table 10 F2-CU1318 & R2-CU3021 primer pair-specific amplicons

Jatropha accessions	Size of amplicons (annealing temp. 50°C)	Size of amplicons (annealing temp. 55°C)
TJS17#03	~0.6 kb, 0.9 kb,1.7 kb	~ 1.4 kb, 1.7 kb
TJS42#04	~0.9 kb, 1.4 kb	No bands
TJS35#01	~ 0.6 kb,0.9 kb,1.7 kb	No bands
TJS01#03	~0.6 kb, 0.7 kb, 0.9 kb	No bands

For F2-CU1318 & R2-CU3021 primer pair, expected size of amplicon is 1.7 kb. At 50°C, the expected size of amplicon was found only in TJS17#03 and TJS35#01. Apart from the expected size, shorter amplicons (~0.6 kb) was observed in all accessions of Jatropha which are less significant. Interestingly, the amplicon profiles appear to be considerably different at 55°C annealing temperature as 1.7kb amplicon appeared only for the accession TJS17#03. In all other accessions there was not a single amplicon. In TJS17#03, the second DNA band (~1.4 kb) was found to be slightly smaller than expected size of amplicon. All these data clearly suggesting the presence of the allelic variants of curcin genes; however, all these amplicons need to be studied further.

(iv) Results of PCR using the primer pairs: F3-CU1526 and R1-CU2258; F1-CU126 and R1-CU2258

PCR was carried out under two different primers at 55°C annealing condition as shown Fig. 17A (F3-CU1526 and R1-CU2258), and Fig. 14B (F1-CU126 and R1-CU2258)

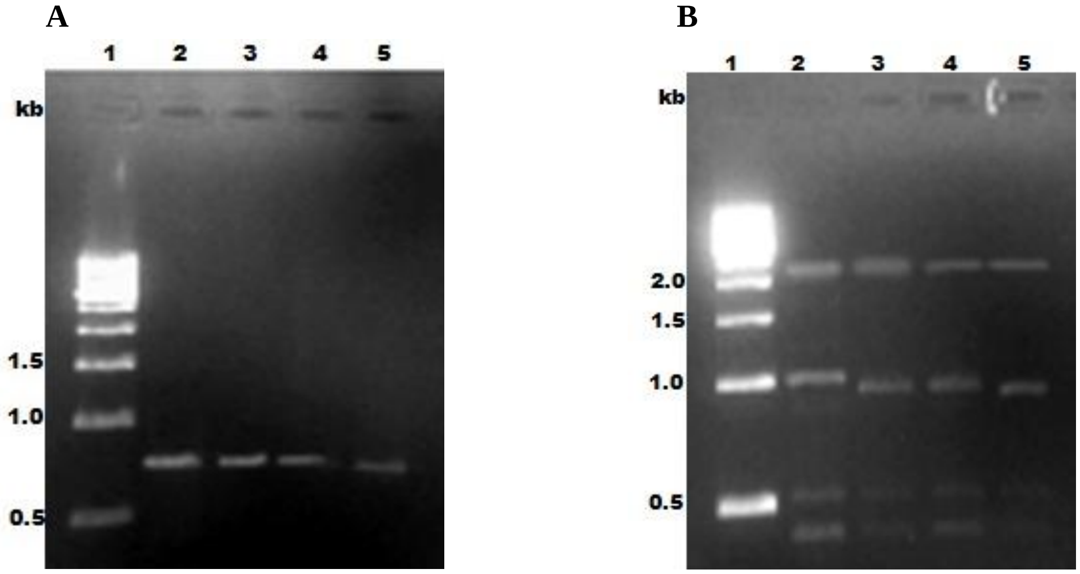

Fig. 17 PCR amplified products

⇨**Fig. 17contd. A** amplicons for primer pairs F3-CU1526 and R1-CU2258 and **B** amplicons for primer pairs F1-CU126 and R1-CU2258 *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 42#04; *Lane* 4- PCR amplified band of TJS 35#01; *Lane* 5- PCR amplified band of TJS 01#03. Order of cultivars remains unchanged in both the figures.

Table 11 F3-CU1526 & R1-CU2258; F1-CU126 & R1-CU2258 primer pair specific amplicons

Jatropha cultivars *curcas*	**Size of amplicons for F3-CU1526 and R1-CU2258**	**Size of amplicons for F1-CU126 and R1-CU2258**
TJS17 #03	~0.7 kb	~1.0 kb, 2.1 kb
TJS42#04	~0.7 kb	~ 0.9 kb, 2.1 kb
TJS35#01	~0.7 kb	~ 0.9 kb, 2.1 kb
TJS01#03	~0.6 kb	~ 0.8 kb, 2.1 kb

For F3-CU1526 & R1-CU2258 primer pairs, expected amplicon size is 0.7 kb. At 55°C, amplicons profiles was found to be same of 0.7 kb in size except TJS 01#03 in which 0.6 kb size amplicon was found. For F1-CU126 & R1-CU2258 primer pairs, expected amplicon size is 2.1 kb. At 55°C, intense DNA band of ~ 2.1 kb was found to be same in all Jatropha accessions. Apart from the expected amplicon, one more DNA band of ~1.0 kb was found in TJS 17#03; whereas in case of other three accessions of Jatropha, the second amplicon was slightly smaller than 1.0 kb.

Usually, the coding regions remain more or less conserved between the members of a gene family. Considerable divergence occurs in their upstream regions which in term control the overall functionality of the individual promoters. A number of forward primers were designed based on the upstream promoter sequence; whereas the reverse primers were based on the coding regions. PCR was carried out using different primer pairs. The purpose was to amplify the curcin gene along with the promoters of varying length. Most of the amplicons as found in the study are quite consistent in terms of the size. However, some amplicons were found which could be promising. Some of the amplicons may represent the allelic variants of curcin gene having distinct promoters. Therefore, it is apparent that different amplicons including the expected ones need to be cloned and sequenced for further molecular study.

CHAPTER 6 CONCLUSION

This report made a consolidated base for further studying the structure, functions of curcin genes in Jatropha. Moreover this study would be very helpful for transgenic work in order to improve Jatropha accessions. Results and their conclusions made in this study are discussed below:

- Seeds corresponding to Jatropha accession TJS17#03 having lowest free fatty acid (FFA) content could be a suitable resource for production of biodiesel effectively through transesterification reaction.
- Seed kernels of Jatropha accession TJS17#03 were used to prepare crude curcin extract. Folin- Ciocalteau method was used for the estimation of total protein content.
- Cytotoxic effect of crude curcin extract was tested on RAW and HeLa cell line by doing MTT assay. A very good percentage of inhibition was observed in the proliferation of these cancerous cells. Interestingly, on the other hand, residue of crude extract was found to have proliferation effect on RAW cell line.
- Multiple sequence alignment of the predicted polypeptides of different forms of curcin such as curcin, curcin-L precursor , curcin2A, RIP clearly revealed that some forms are highly conserved (nearly identical) whereas in some other forms of curcin considerable sequence variation could be found in terms of both conservative and non-conservative substitution of amino acids. Therefore it is likely that various curcin forms could differ with regard to their overall functionalities.
- Sequence comparison revealed that the promoter region of the curcin gene showed considerable variations if compared between members of this family; however their coding regions are mostly conserved.
- A number of forward and reverse primers were made based on curcin2A gene sequence. The aim was to amplify curcin genes with the upstream regions (i.e. promoters) of varying lengths. According to the aim of the study, PCR was carried out using different combinations of primers. A number of amplicons as obtained in the study appears to be quite promising. Further molecular cloning and sequencing of some of the amplicons could identify some new allelic variants of curcin gene from our Jatropha accessions.

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