In silico and biochemical studies on Ribosome-inactivating proteins (RIPs) from *Jatropha curcas* L. and other plant species

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BY

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UNDER THE SUPERVISION OF

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June 2022

CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the dissertation entitled, "***In silico* and biochemical studies on Ribosome-inactivating proteins (RIPs) from *Jatropha curcas* L. and other plant species**" in the partial fulfilment of the requirement for the award of degree of Master of Technology in 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.

Date: 28/06/2022

Place: TIET, Patiala

Kriti Sharma

(Kriti Sharma)

CERTIFICATE

TO WHOM IT MAY CONCERN

This is to certify that the dissertation entitled **"*In silico* and biochemical studies on Ribosome-inactivating proteins (RIPs) from *Jatropha curcas* L. and other plant species"** comprises research work carried out by Ms. Kriti Sharma (Regd. No. 602004011) under my supervision and guidance during the period between 2nd August, 2021 to 30th June, 2022 for the partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, submitted to Thapar Institute of Engineering & Technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

(Dr. N. Das) 27/06/2022

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LIST OF ABBREVIATIONS

Abbreviation	Name
µg	Microgram
µL	Microliter
aa	Amino acids
APS	ammonium persulfate
BLASTp	BLAST for proteins
BSA	Bovine serum albumin
BSAE g-1 FW	BSA equivalent per gram fresh weight
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
eEF-2	Eukaryotic elongation factor-2
EF-G	Elongation factor-G
FASTA	fast alignment
FBS	Fetal bovine serum
GAE g-1 FW	Gallic acid equivalent per gram fresh weight
GAP31	Gelonium Anti-HIV protein 31kDa
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
JIP60	Jasmonate-induced protein 60kDa
kDa	Kilo-Dalton
MAP30	Mirabilis antiviral protein 30kDa
MBP	Maltose-binding protein

mg	Milligram
mL	Millilitre
mRNA	Messenger RNA
MSA	Multiple sequence alignment
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Centrer for Biotechnology Information.
nm	Nanometer
NMR	Nuclear magnetic resonance
PAP	Pokeweed antiviral protein
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol
pH	Potential of Hydrogen
pI	isoelectric point
RIP	Ribosome-inactivating protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal RNA
RSP	radical scavenging potential
SDS	Sodium dodecyl-sulfate
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
TCS	Trichosanthin
TEMED	Tetramethylethylenediamine
ZOI	Zone of inhibition
α-MMC	Alpha-momorcharin

ABSTRACT

The biodiesel crop *Jatropha curcas* L. has gained worldwide importance during the last few decades. Apart from production of biodiesel, it has found its use in cosmetic, fertilizer and lubricant industries. It also has been used in traditional medicines for centuries. Various ribosome-inactivating proteins (RIPs) have been reported from different organs of *J. curcas.* One of the Type I RIP isolated from *J. curcas* seeds is Curcin 2A. RIPs irreversibly inhibit the protein synthesis by damaging the ribosome machinery of the cells. However, they possess various pharmacological properties. Therefore, they can be used as antibacterial, antiviral, antifungal, anticancerous as well as immunosuppressive agent. To use them as potent drug, it is important to study their structure and function in depth. Therefore, in present study we selected RIP sequences from various plant species to investigate structural and functional relationship using various bioinformatic tools. It revealed that the RIPs from same plant are significantly similar in amino acid length as well as other crucial regions. However, RIPs from other plant species of same type of RIP have a lot of variations. Furthermore, Ramachandran plot analysis revealed that most of the residues reside in the favored region. Thus, validating the structure. The extracts isolated from different organs of *J. curcas* at different stages of development were investigated for their therapeutic activity *in vitro*. Where, mature seed protein extracts showed the highest antibacterial potential. Whereas, for anticancerous activity immature seed extracts were found to be most potent when used in lower doses. Similarly polyphenol extracts of *J. curcas* were investigated for anti-oxidant activity and it was found that immature seeds had highest antioxidant potential. This comprehensive study would help in understanding biomedical, medicinal and traditional aspect about the RIPs.

Keywords: Jatropha (*Jatropha curcas*), Ribosome-inactivating proteins (RIPs), Polyphenols, Antibacterial potential, Anti-cancerous activity, Anti-oxidant activity

CHAPTER 1: INTRODUCTION

1.1 About RIPs

Ribosome-inactivating proteins or RIPs (EC 3.2.2.22) are a group of cytotoxic N-glycosidases (Lu et al. 2020). They form a family of well-characterized toxins, which specifically and irreversibly inhibit protein synthesis cells by enzymatically altering the 28S rRNA of the large 60S ribosomal subunit (**Fig. 1.1**). Over a hundred of RIPs have been isolated from various plants and bacteria with varying degrees of toxicity (Walsh et al. 2014).

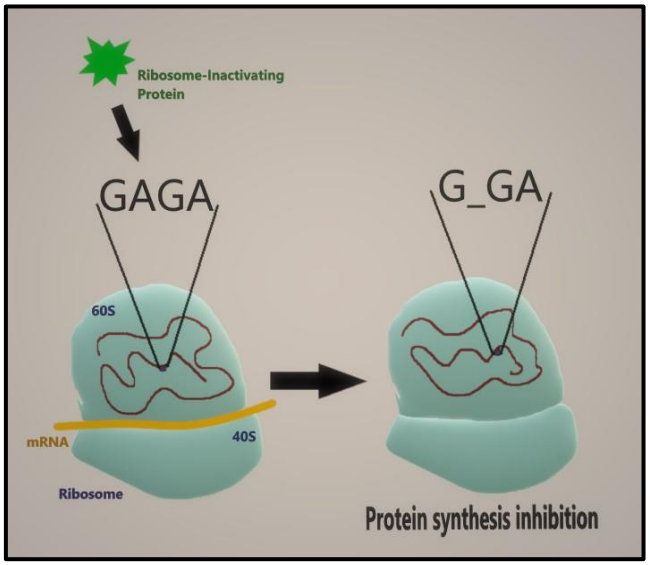

Fig. 1.1 RIP showing rRNA N-glycosidase activity by removing A-4324 in 28S rRNA

Types of RIPs

RIPs are generally classified into three main types based on their physical properties, i.e.

i) *Type I RIPs*: Type I RIPs are the most abundant RIPs. They generally consist of a protein with a single polypeptide domain of approximately 30kDa having N-glycosidase activity (**Fig. 1.2a**). Although type I RIPs share some of the highly conserved active site cleft residues, their overall sequence and post-translational modifications are significantly different. Various RIPs have been found in different plant species such as pokeweed antiviral protein (PAP) from American pokeweed, curcin from *Jatropha curcas*, trichosanthin (TCS) from *Trichosanthes kirilowii*, saporins from soapwort, moschatin from *Cucurbita moschata*, luffin from *Luffa cylindrica*, MAP30 from *Momordica charantia*, camphorin from *Cinnamomum camphora* (Schrot et al. 2015; Zhu et al. 2018).

ii) *Type II RIPs*: Type II RIPs are two-domain polypeptide proteins where enzymatically active domain (A-domain) of approximately 30 kDa and a slightly bigger binding domain (B-domain) of approximately 35 kDa are linked together by a disulphide bond (**Fig. 1.2b**). Here, A-domain is structurally similar to type I RIPs & B-domain possesses sugar-binding lectin properties. The absence of B-domain makes the type I RIPs relatively less toxic. The type II RIPs are further classified into 2 groups, i.e. toxic and non-toxic based on their cytotoxicity level. Modeccin, viscumin, volkensin, abrin and ricin are highly toxic whereas iris lectin, cinnamomin, nigrin and ebulin are non-toxic. However, the reason for this difference between toxicity levels of type II RIPs is still not known.

iii) *Type III RIPs*: Type III RIPs are composed of an enzymatically active A chain at N-terminal fused to another chain with an unknown function at C-terminal (**Fig. 1.2c**). They are generally synthesized as proenzymes and are transformed into active enzymes after the proteolytic removal of a short internal segment. Some of the examples include JIP60 from barley and b-32 from maize (Schrot et al. 2015; Chang et al. 2017; Zhu et al. 2018).

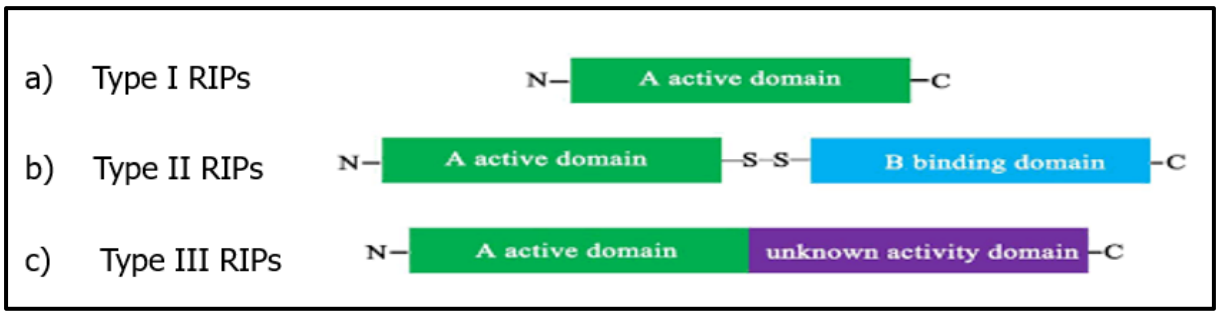

Fig. 1.2 Schematic depiction of Types of RIPs i.e. a) Type I RIPs b) Type II RIPs c) Type III RIPs.

Mode of Action

Type I RIPs have difficulty in entering the cells due to absence of sugar binding moieties. However, they can enter cell to some extent by interacting with phospholipids present on cell surface (**Fig. 1.3**). Investigations of their RNA N-glycosidase activity shows that they specifically act on 3' end of 28S rRNA present in large subunit of eukaryotic ribosome. They cleave the glycosidic bond of single adenine residue in the highly conserved α- sarcin-ricin loop (at A_{4324} position), leading to the loss of adenine base without directly cleaving the rRNA strand. This may leave the RNA strand susceptible to hydrolysis by cellular lyases. Thus, interfering with the binding of elongation factors EF-G & EF-2 subsequently inhibiting protein synthesis (Puri et al. 2012; Walsh et al. 2013; Citores et al. 2021).

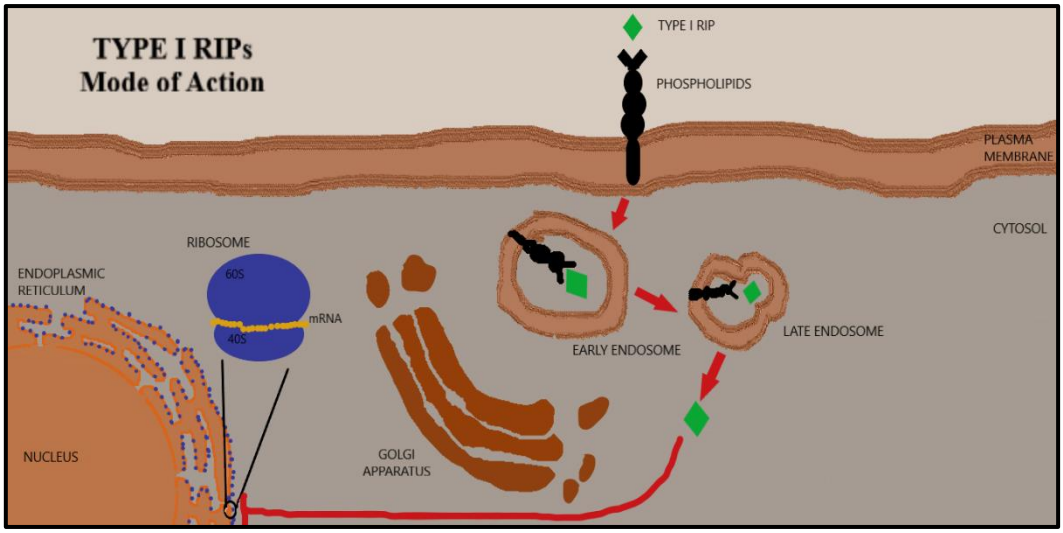

Fig. 1.3 Mode of action of Type I RIPs

In Type II RIPs the B-domain is cable of binding to glycoproteins or glycolipids present on eukaryotic cell surface and entering the cell via endocytosis. It also meditates retrograde transport of A-domain through golgi stack into endoplasmic reticulum. After reaching endoplasmic reticulum lumen, it has been suggested that the A chain is separated from B chain by reduction of the linking disulfide bond. A-domain is then further retrotransported to cytoplasm by a pathway usually reserved for disposal of misfolded proteins. Most of the RIPs that follow this route are ubiquitinated and degraded by proteosomes but a small fraction is able to escape degradation and bind to its target ribosome. Then, they depurinates A_{4324} at 3' end of 28S rRNA by a mechanism similar to the one used by type I RIPs with shared site of action known as α-sarcin-ricin loop (**Fig. 1.4**).

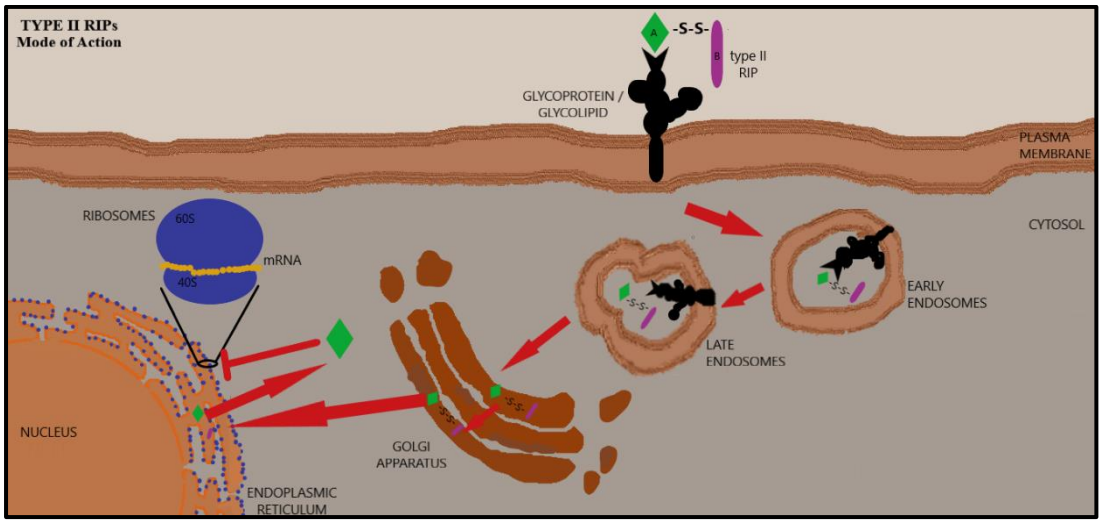

Fig. 1.4 Mode of Action of Type II RIPs

1.2 Some plant sources of RIPs used in the study

Ribosome-inactivating proteins are distributed broadly in different kingdoms of plants, fungi as well as bacteria. Although, they are found in more than one kingdom in nature but they are more abundant in plants (Domashevskiy and Goss 2015). Besides, some plants may have more than one RIP or more than one isoform of same RIP. So, it is important to know that some plant families contribute more to the RIP production than others.

Jatropha curcas

Jatropha curcas is a multipurpose, perennial, drought-resistant plant belonging to Euphorbiaceae family (**Fig. 1.5**). It is commonly known as barbados nut, termite plant, fig nut, black vomit nut, curcas bean, kukui haole, physic nut, purge nut, purging nut tree, bubble-bush, ratanjot etc (Abdelgadir & Staden 2013). It is widely distributed in tropical and sub-tropical regions like Central or South America, Africa, India etc (Prasad et al. 2012).

Sub-kingdom : Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Euphorbiales

Family : Euphorbiaceae

Genus : Jatropha.L.

Species : curcas

Fig. 1.5 *Jatropha curcas* plant

Primarily, it is used as a biodiesel crop but its seed oil can also be used to produce soaps, dyes, glue etc. Some farmers also grow it as a live fence around their crops to protect them from animals as parts of this plants are poisonous if ingested. This plant is also used for its medicinal properties in traditional folklore medicines. These medicinal properties are due to various secondary metabolites found in almost all parts of *J. curcas* (Islam et al. 2011).

Mirabilis jalapa

Mirabilis jalapa is a perennial, ornamental plant which reaches about 50-100 cm in height (**Fig. 1.6**). It is a native plant of tropical America belonging to Nyctaginaceae family.

Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Dicotyledonae
Order: Caryophyllales
Family: Nyctaginaceae
Genus: Mirabilis
Species: Mirabilis jalapa

Fig. 1.6 *Mirabilis jalapa* plant

It produces beautiful, colorful & fragrant flowers which open at around 4 o' clock in the afternoon. Therefore, it is also known as 4 o' clock plant in different countries. It is also known as Maravilha or bonina in Brazil, Marvel of peru in Peru, Gulambasa in Ayurveda, Gul-Abbas in hindi. It is used widely in traditional medicines in different parts of the world (Nath et al. 2010; Passari et al. 2020).

Trichosanthes kirilowii

Trichosanthes kirilowii is a perennial, unisexual, climbing herb belonging to Cucurbitaceae family (**Fig. 1.7**)

Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Dicotyledonae
Order: Violales
Family: Cucurbitaceae
Genus: Trichosanthes
Species: Trichosanthes kirilowii

Fig. 1.7 *Trichosanthes kirilowii* plant

It is widely distributed in South East Asia and its range is from India to East Asia and South East Australia. Different organs of *T. kirilowii* are used in traditional medicines in Liaoning, Anhui, Shandong, Henan and other places in China as well as different places in South Korea (Xu et al. 2015; Hu et al. 2020; Park et al. 2021).

Momordica charantia

Momordica Charantia is also commonly known as bitter gourd, bitter melon, kugua, balsam pear or karela (**Fig. 1.8**)

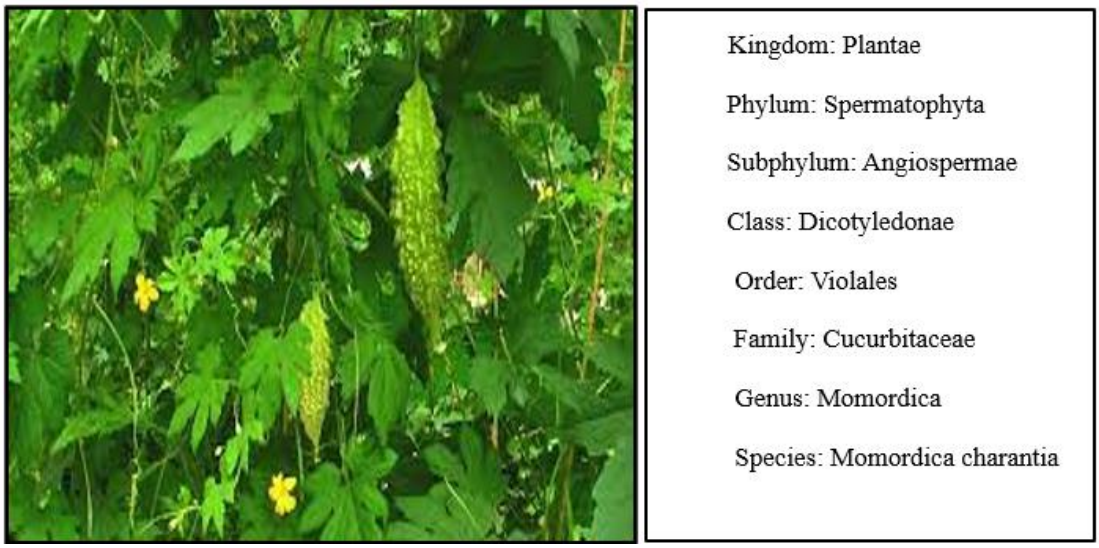

Fig. 1.8 *Momordica charantia* plant

It lives up to its name as it has special bitterness with good edible value as well as medicinal value. It belongs to 'Cucurbitaceae'. It is generally cultivated in Tropical and Sub-Tropical regions such as India, China, Japan, Thailand, Singapore, New Zealand, Mexico, Malaya, Amazon, Panama, etc (Jia et al. 2017; Chen et al. 2019; Fan et al. 2019).

Ricinus communis

Ricinus communis is a monotypic gene plant belonging to Euphorbiaceae family (**Fig. 1.9**). It originated from Africa. But now has been distributed worldwide and grows well in sub-tropical to tropical dry regions.

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Dicotyledonae

Order: Euphorbiales

Family: Euphorbiaceae

Genus: Ricinus

Species: Ricinus communis

Fig. 1.9 *Ricinus communis* plant

This perennial shrub has fruit bearing female flowers on the tips of the spikes whereas male flowers are present in lower regions that wither after yielding pollens. This plant can be used as an ornamental plant. However, its different parts have been used in different herbal medicines and cosmetics (Rehn et al. 2020; Franke et al. 2019; Worbs et al. 2011).

Hordeum vulgare

Hordeum vulgare also commonly known as Barley belongs to the family Poaceae (**Fig. 1.10**).

Kingdom: Plantae

Phylum: Spermatophyta

Subphylum: Angiospermae

Class: Monocotyledonae

Order: Cyperales

Family: Poaceae

Genus: Hordeum

Species: Hordeum vulgare

Fig. 1.10 *Hordeum vulgare* plant

This grass is grown all over the world as a cereal crop. Apart from being used as animal feed, it can also be used in human diets due to its health effects, such as lowering of Blood cholesterol, regulating glycemic activity and anti-oxidant activity (Punia 2020).

1.3 About Secondary metabolites found in *Jatropha curcas*

Plant secondary metabolites are small molecular products of different metabolic pathways that are non-essential for the growth and reproduction of the plant. They play a role in plant defense, response to the environmental stresses and meditating organismal interactions (Yang et al. 2018; Isah 2019). According to their biosynthetic pathways, they are classified into several large molecular families:

- Flavonoids: Flavonoids is a subclass of polyphenols. They have a characteristic C6-C3-C6 heterocyclic backbone structure. They are found abundantly in red-, blue- or purple-colored plants. It is further divided into different subclasses namely flavone, flavanones, flavanols and flavonols. These flavonoids play different roles such as anti-inflammatory, anti-allergic, anti-oxidative agents (Serafini et al. 2010; Yi 2018).
- Alkaloids: Alkaloids are plant-derived compounds, consisting of one or more Nitrogen atom (functional amine group) in a heterocyclic group. They are amino acid-derivative but are alkaline in nature. They play a role in germination and defense of plants against predators. Many alkaloids when ingested act as stimulants while some act as anesthetic in nature. They also have analgesic, anti-cancerous as well as anti-microbial activity (Teoh 2015).
- Saponins: Saponins are glycosides containing one or more sugar chains on a triterpene or a steroidal aglycone backbone. Due to the presence of a lipid-soluble aglycone and water-soluble sugar chain(s) in their structure (amphiphilic nature), saponins are surface active compounds with detergent, wetting, emulsifying, and foaming properties (Güçlü-Üstündağ and Mazza 2007).
- Phorbol Esters: Phorbol esters are polycyclic compounds in which two hydroxyl groups on neighbouring carbon atoms are esterified to fatty acids. They show anti-nutritional and toxicological effects to the animals who are fed phorbol ester containing plants. They also show toxic effect on pests feeding on them so they can be used as natural biopesticides. Besides this, studies have shown several phorbol esters have anti-tumor and anti-microbial activities (Goel et al. 2007).

CHAPTER 2: REVIEW OF LITERATURE

2.1 Ribosome-inactivating proteins (RIPs)

Ribosome-inactivating proteins (RIPs) are N-glycosidases, which depurinate non-mammalian ribosomes from insects (Zhou et al. 2000), plants (Iglesias et al. 1993), yeast (Roberts and Selitrennikoff 1986) and bacteria. It was also found that (i) from every ribosome, RIPs remove more than one adenine residues (Barbieri et al. 1994) and (ii) RIPs also remove adenine residues from polynucleotides and DNA.

Large rRNAs have the conserved alpha-sarcin loop which is important for anchoring elongation factor (EF-G for prokaryote or eEF2 for eukaryote) in mRNA translocation (Peumnas et al. 2001). RIPs with the help of various cofactors lead to inhibition activity on translation (Carnicelli et al. 1992).

RIPs were isolated, a century ago in Angiospermae, from both mono and dicotyledons plants and mushrooms (Yao et al. 1998; Lam and Ng, 2001). They are known to have catalytic toxins which create an irreversible toxic effect on protein synthesis by damaging ribosomal machinery (Joerg et al. 1997). The level of RIPs in plants varies from traces to hundreds of milligrams. They exist in almost every organ such as roots, stems, leaves, bark and flowers. (Park et al. 2004). The first Type I RIP to be isolated was pokeweed antiviral protein (PAP) by Dallal and Irvin (1978).

RIPs confer anti-cancerous, anti-viral and other therapeutic applications. The main obstacles of treatment with RIPs include short plasma half-life, non-selective cytotoxicity and antigenicity. Various strategies have been used to improve the pharmacological properties of RIPs on human immunodeficiency virus (HIV) and cancers. Coupling with other compounds such as polyethylene glycol (PEG) improves these properties such as increasing plasma time and reducing antigenicity. RIPs conjugated with antibodies to form immunotoxins also increases the selective toxicity to target cells (Lu et al. 2020).

Curcin is only present in seeds of *Jatropha curcas*. It does not express in leaves neither in stress nor under non-stress condition. However, curcin 2 gene expresses in leaves of seedlings growing under biotic or abiotic stress conditions (Qin et al. 2005).

Apart from this, curcin isolated from seeds of *J. curcas* was used against *Pheritima posthuma* to study its anthelmintic effects. In the test all earthworms died at 1600 mg/kg of curcin concentration. The adverse toxic effects were displayed at doses above 300mg/kg. However,

P. posthuma displayed physical changes with LD_{50} of 800 mg/kg per body weight. However, since toxicity of extracts is high in earthworms and cannot be extrapolated directly on humans. Therefore, caution should be exercised in its use especially at higher doses (Jummai and Okoli 2014).

RIPs are known to display antibacterial properties. For instance, MBP tagged MAP30 prepared using protein fusion and purification method in transformed *E.coli* has no toxicity in mice in doses lower than 1.25 mg/kg. It induced the production of anti-MAP30 IgG at both 0.42 & 0.14 mg/kg. Therefore, it showed bioactivity, synergistic anti-bacterial effect when used in combination with antibiotics such as chloramphenol or erythromycin. So, reduced dosages of these antibiotics lead to less side effects (Chang et al. 2017). Whereas, PD-L4 from *Phytolacca dioica* was investigated for its antimicrobial activity. It was found to be active on different bacterial strains in both native and denatured/alkylated forms. Its activity was found to be related to a cryptic peptide PD-$L4_{40-65}$. To date, PD-$L4_{40-65}$ is the first antimicrobial peptide identified in a type I RIP (Pizzo et al. 2015).

Curcin was found to be competent in suppressing the proliferation of six mammalian cell lines i.e. 3 normal cell lines (L929, HCN-1A, HuVEC) and 3 cancer cell lines (Glioma, MCF-7, MDA-MB-453). The cells exposed to 100 µg/mL of curcin for 72 hrs exhibited significant cellular metabolic arrest in various cancer cell lines. The viability assessment revealed highly restricted proliferation and near to complete dead cell proliferation. Incapacitation of vital cellular organelles such as nucleus, mitochondria and actin, with suppression of defence mechanism and elevated ROS levels were also studied. This ability of curcin to impact comprehensive shutdown of cells, complemented with its hemocompatibility, opening up the possibility of using it as ribotoxin in therapeutic industry (Mohamed et al. 2014).

RIPs such as TCS, MAP30, saporin, GAP31 and α-MMC have also been investigated for any anti-viral activities including anti-HIV activity. They have shown to affect the life cycle of HIV by inhibiting reverse transcriptase, integration, replication and assembly. They have adenosine glycosidase activity on DNA, genomic RNA or mRNA. MAP30 relax super-coiled DNA into linear form and clear double-stranded DNA whereas Saporin induce viral apoptosis and suppress HIV propagation (Lu et al. 2020).

Since, as studied extensively and discussed above that RIPs damage ribosomes of the host cells, hence, interfering with its protein synthesising machinery. As once synthesised, these toxic molecules are exported out of the cell and localized within the cell wall matrix (Domashevskiy

and Goss 2015). Henceforth, the cells synthesising RIPs should be capable of protecting their own RIPs during its biosynthesis. The cells follow 3 mechanisms for the safe synthesis of RIP:

(i) Relative resistance of autologous ribosomes to the actions of RIPs,

(ii) Synthesis of inactive pro-RIP forms

(iii) Synthesis of prepro-RIP form with N-terminal signal sequence that direct the nascent chain into lumen of reticulum, before it is finally folded in active configuration (Barbieri et al. 1993).

2.2 Polyphenols

Polyphenol is one of the secondary metabolites of the plants, consisting of an aromatic ring with one or more hydroxyl substituents. The weight of polyphenols ranges from 500-4000 Da (Beckman 2000). On the basis of their chemical constituents and structures, they are divided into different types likes flavonoids, phenolic acid, tannins, stilbenes, coumarins, and lignins (Lemos et al. 2015). Polyphenols affects various attributes of plant fruit organs such as colour, texture and taste (Akyol et al. 2016). It is found in both plants and animals. In plants, it has been reported in various organs such as leaves, fruits, flowers, and bark layers. They are known to play important role in various plant metabolism such as prevention against the microbial infection, suppression and release of growth hormones (Alasalvar et al. 2001). On the other hand, in animals, it is present in arthropods such as crustaceans and insects which results in epicuticle hardening (Reddivari et al. 2007). High levels of antioxidants present in fruits and vegetables are beneficial for human health (Kulen et al. 2013).

The leaf extracts of *J. curcas* showed high total polyphenolic content than stem extracts (5.79-48.95 mg GAE/g and 1.64-13.99 mg RE/g, respectively). However, leaf extracts show the most potent antioxidant capacity in term of free radical scavenging and reducing activity due to high polyphenolic content (Zengin et al. 2021).

2.3 Antimicrobial assay by disc diffusion method

Antimicrobial assay by disc diffusion method is easy to carry out. Small discs of filter paper are taken and placed on cultured petri-plate. The sample to be tested for antimicrobial assay is infused in the filter discs. After diffusion of the antimicrobial sample, a zone of inhibition is clear which can be measured to study the antimicrobial potential of the sample. Higher the ZOI, higher is the antimicrobial potential and vice-versa (Qadrie et al. 2009).

2.4 Antioxidant Capacity

Antioxidant capacity is defined as the ability to assess the antioxidant status of various biological samples and evaluate their antioxidant response against the free radicals produced in the particular disease (Rubio et al. 2016). It is measured based upon the abilities of different substances to scavenge free radicals compared with a standard antioxidant (Miller et al. 1996). *DPPH assay***:** α, α-diphenyl-β- picrylhydrazyl (DPPH) is a stable free radical that is used to determine the scavenging potential of antioxidant. The odd electron of Nitrogen atom in DPPH is reduced, by scavenging Hydrogen atom from antioxidants, to corresponding Hydrazine. This delocalization of free electrons turns the deep violet coloured DPPH solution to a yellow to a colorless solution. The absorbance is taken at 520 nm (Blois 1958; Contreras-Guzman and Srong 1982; Kedare and Singh 2011). The radical scavenging potential (RSP) of antioxidant compound is determined by using the following equation:

$$\% \text{ Antioxidant Capacity} = \frac{[A_{(radical)} - A_{(standard)}]}{A_{(radical)}} \times 100$$

2.5 MCF-7 breast cell line

MCF-7 is a breast cancer cell line. It shows that anti-cancerous extracts activity has ability to inhibit the growth of the cells and it also induce apoptosis in MCF-7 cancer cells through intrinsic and extrinsic pathways. Type I RIPs such as TCS, MAP30, gelonin, marmorin and α-MMC have displayed inhibitory effect on the growth of breast tumor cell lines such as MCF-7 & MDA-MB-231 both *in vitro* as well as *in-vivo*. TCS inhibits cell viability, cell mobility, causes cell cycle arrest and significantly reduces tumor volume and weight by inducing apoptosis through caspase-8 and caspase-9 (Zeng et al. 2015)

2.6 Origin of the problem

The plant derived products are of great importance in terms of traditional medicines. Modern concepts combined with biochemical studies unravel various attributes to aid the modern drug discovery process. Ribosome-inactivating protein (RIPs) are N-glycosidase enzymes that are synthesised in different parts of various plants. These RIPs are toxins that defend the plants from various pathogen attacks. However, they possess various therapeutic properties such as antibacterial, anticancerous and anti-helminthic. As a result, in terms of generating active and safer therapeutics with lesser side effects the full knowledge of RIPs is required. So, to understand the activity of RIPs, the comprehensive knowledge at both molecular and biochemical level is required. The plant *J. curcas* chosen for this study, has been used in traditional medicines for centuries. These medicinal properties can be contributed by the secondary metabolites present in the plant. These metabolites possess antioxidant activities in order to scavenge free radicals generated under stress conditions.

As, there is no comparable data available for RIPs in the database, present study focussed on studying different types of RIP at both molecular and biochemical levels using various bioinformatic tools in various plant species. Also, a comprehensive biochemical analysis was done using various organs of *J. curcas* plant. So, keeping all these crucial aspects in mind, following objectives have been proposed.

2.7 Objectives of the study

- Sequence analyses and comparison between the different types of RIPs from *J. curcas* and other plant species
- Prediction of the crucial motifs/domains and 3D-Structures of the RIPs
- Biochemical analysis of the crude extracts of the different organs for protein, RIP and antioxidants.

CHAPTER 3: MATERIALS AND METHODS

3.1 *In silico* analysis

For *In silico* analysis, curcas 2A sequence (sequence submitted by our lab) corresponding to type 1 RIP from *Jatropha curcas* L., was used as reference to find out homologous sequences in the NCBI database. In order to achieve this, BLASTp (http://www.ncbi.nlm.nih.gov) tool was used. RIPs from different plant species namely *Jatropha curcas* L., *Trichosanthes kirilowii, Mirabilis jalapa, Momordica charantia, Ricinus communis* and *Hordeum vulgare* were selected. The sequences were recovered and chosen for further analysis.

3.1.2 Identification of the conserved domains

Using MY HITS server (http://myhits.isb- sib.ch/cgi-bin/motif_scan) different motifs of RIPs were predicted by uploading amino acid sequence of RIPs from different plant species.

3.1.3 Biochemical characterization

Determination of different biochemical properties such as molecular weight (MW), aliphaticity, theoretical isoelectric point (pI), composition of amino acid (aa) and instability index of RIPs was done using EXPASY server (http://web.expasy.org/protparam/).

3.1.4 Three-dimensional model prediction

Tertiary structure prediction and protein folding identification were performed using the Phyre2 program (http://www.sbg.bio.ic.ac.uk/phyre/).

ProSA-web tool (https://prosa.services.came.sbg.ac.at/prosa.php*)* gives the protein structure analysis of the protein models. ProSA gave reliable protein models, which were selected on the basis of z-score and X-ray/NMR region. The 3-D structures were further validated for structural analysis by SAVES (https://services.mbi.ucla.edu/SAVES/) and PROCHECK in Rampage server (http://mordred.bioc.cam.ac.uk/ ~rapper/rampage.php).

3.2 Experimental

3.2.1 Harvesting of plant materials

Harvesting of different organs of *Jatropha curcas* was done at different stages of development. Various organs such as fruits, leaves and stems were collected and washed thoroughly to remove dirt. After drying under shade, different organs were frozen in liquid nitrogen to arrest them at their metabolic state and stored at -80 ºC for further experiments.

3.2.2 Protein isolation from various Jatropha organs

1 g of plant sample was taken and crushed into a fine paste using mortar pestle and 10 mL of 0.005 M Sodium phosphate buffer Saline solution (PBS) of pH 7.2 was added. Then, centrifugation at 12000 rpm for 20 minutes at 4℃ was done. After that sample was filtered and store at -20℃ for further use.

❖ *Total Protein estimation of extracts by Lowry method*

Lowry method was used for estimation of protein in different plant organs i.e. leaves, stems, fruits and seeds, according to Dunn et al. (1992). This method is sensitive at very low concentration of proteins. Amino acid derivatives such as certain buffers, drugs, lipids, sugars, nucleic acids, and sulfhydryl reagents interferes with Lowry procedures.

Reagents

- Solution A 2 % Na_2CO_3 in 0.1 M NaOH
- Solution B 0.5 % $CuSO_4.5H_2O$
- Solution C 1 % Sodium Potassium tartrate
- Reagent I (prepare fresh for every use): 0.5 mL of Solution B was mixed into 0.5mL of Solution C, and then 49 mL of Solution A was added.
- Reagent II: Folin-Ciocalteau reagent is diluted with water to 1 N.
- BSA (1 mg/mL)
- Sterile water
- Spectrophotometer

Methodology

100 µL of all the test samples (different stages of fruit, seeds, leaves and stems) was taken in different test tubes and 900 µL of distilled water was added to it. The test tube with 1 mL distilled water was used as blank. After that 5.0 mL reagent I was added and sample was

incubated for 10 minutes. Which was followed by the addition of 0.5 mL of reagent II. Then the samples were incubated for 30 minutes in darkness. The absorbance was measured at 750 nm. The BSA was used as standard and the values were plotted to prepare a standard curve.

❖ *Protein visualization by SDS PAGE*

Polyacrylamide gels are highly cross-linked gel matrix, formed by the reaction of acrylamide and bis-acrylamide (*N*,*N*'-methylenebisacrylamide), which act as a sieve for the movement of proteins in the presence of an electric field. In SDS-PAGE, the proteins are denatured and given a uniform overall negative charge. Thereby, making it possible for all the proteins to move towards the positive electrode on the basis of their size.

Reagents

- Vertical electrophoresis chamber with power supply, glass plates, spacers and combs
- Distilled Water
- 30 % acrylamide solution
- 1.5 M Tris-HCl (pH- 8.8)
- 0.5 M Tris-HCl (pH-6.8)
- 10 % Sodium dodecyl sulphate (SDS)
- 10 % Ammonium Persulphate (APS)
- TEMED
- n-Butanol
- Loading Buffer:

  Bromophenol blue 0.004 %
  2-mercaptoethanol 10 %
  Glycerol 20%
  SDS 4%
  Tris-HCl 0.125 M
- Running Buffer:

  Tris-HCl 25 mM
  Glycine 200 mM
  SDS 0.1% (w/v)
- Staining Solution

  CBB R-250
  Methanol
  Glacial acetic acid

- Destaining Solution

  Methanol
  Glacial acetic acid
  Distilled Water

Methodology

Gel Preparation: After cleaning the plates and spacer with distilled water and ethanol, the casting unit was assembled on a stable and even surface. The resolving gel solution was prepared using the following volume (for 10 mL) according to the percentage of gel required.

Gel %	**Distilled water (mL)**	**30% Acrylamide (mL)**	**1.5M Tris-HCl (pH-8.8) (mL)**	**10% SDS (µL)**	**10% APS (µL)**	**TEMED (µL)**
8%	4.6	2.6	2.5	100	200	20
10%	3.8	3.4	2.5	100	200	20
12%	3.2	4	2.5	100	200	20
15%	2.2	5	2.5	100	200	20

After preparing 12 % resolving gel solution, it was poured between the plates assembled with spacers. The surface of gel was overlayed with n-butanol to maintain an even and horizontal surface. Then, allowed it to set for about 20-30 min at room temperature. In the meantime, prepared 4 % stacking solution gel using the following volume (for 10 mL).

Gel %	**Distilled water (mL)**	**30% Acrylamide (mL)**	**1.5 M Tris-HCl (pH-8.8) (mL)**	**10% SDS (µL)**	**10% APS (µL)**	**TEMED (µL)**
4 %	5.9	1.3	2.5	100	200	20

After the solidification of resolving gel, overlayed n-butanol was discarded. The comb was inserted carefully between the plates over the resolving gel. Then, the stacking gel was added until it started to overflow. The gel was then further allowed to solidify for 20-30 min at room temperature. After the gel solidification, the comb was removed creating the wells in the gel. The running buffer was the filled in the SDS-PAGE unit.

Sample Preparation: An equal volume of both protein sample and loading buffer was added in an Eppendorf. it was then placed in boiling water for about 5 minutes. After loading the samples in the wells, electrophoresis was carried out.

Gel staining: After electrophoresis, placed the gel in a plastic tray and staining solution was added to it. Then, placed the tray on incubator shaker to stain the gel for 2-4 hrs.

Gel destaining: After staining, the gel was washed several times with distilled water to remove the excess stain. Then, the destaining solution was added and the tray was kept in incubator shaker for about 4 hrs until clear blue bands on clear background are visible.

❖ *Antibacterial assay*

Antibacterial assay of the extracts was determined by disc diffusion method.

Reagents

- Cultures of *E. coli and S. aureus*
- Filter paper discs
- Nutrient agar petri-plates
- Spreader
- Sterilized forceps
- Pipettes and sterilized tips
- Parafilms

Methodology

50 µl of freshly grown *E. coli* and S. *aureus* bacterial cultures were spread on LA plates. They were left to incubate for around 15 minutes. Then, some pre-sterilized filter paper discs were placed on the petri-plate with the help of a sterilized forceps. Then, with the help of pipette and tips around 15−20 µL of extracts were added to the discs. These plates were then incubated overnight at 37°C. Next day, the ZOI was measured.

❖ *Anticancerous activity determination*

Antitumor activity of the extracts was determined by MTT assay. MTT is a yellowish coloured solution which is reduced by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes to insoluble formazan, which has a purple color. Its absorbance is then taken at 593 nm

Reagents

- T-Flask, falcon tubes, Eppendorf
- Dulbecco's modified Eagle's medium (DMEM)
- Fetal Bovine Serum (FBS)
- Antibacterial solution (Penicillin + Streptomycin)
- Antifungal solution (Amphotericin)
- Phosphate Buffer Solution (PBS)
- Trypsin-EDTA solution
- MTT Reagent
- DMSO solution
- Cancer cell line (MCF-7)
- Plant Extracts
- CO_2 Incubator
- Haemocytometer
- Inverted Microscope
- Centrifuge
- Micro plate reader

Methodology

Maintenance of cell lines: MCF-7 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% antibacterial solution and 0.5% antifungal solution in an atmosphere of 5% CO_2 and 95% humidity at 37℃ until the confluency was achieved.

Cell counting: After achieving confluency, the used media was removed and the cells were washed carefully with PBS solution. Then, the trypsinization of the cells was done by adding about 1 mL of trypsin-EDTA solution to the cells. After the cells had detached from the flask surface, about 1mL of complete media was added to the flask to neutralize the trypsin and was left to incubate for about 1 minute. The media containing the cells was added to eppendorf and centrifuged at 1500 rpm for 5 min. The supernatant was removed and pellet contained cells. The pellet was dissolved in 1mL of complete media. Around 10 µL of culture was transferred

to another eppendorf and equal volume of trypan-blue was added to it and mixed gently. This solution was then checked in the haemocytometer to count the no. of cells present.

MTT assay: The colorimetric MTT assay was then carried out to check the inhibition of cells. Firstly, the 10^4 cells/ well were seeded in the PVC-coated 96 well plate and incubated overnight. Next day, the samples were added to the cells and again incubated overnight. On the third day, 10 µL of MTT Reagent was added to each well and incubated for 4 hrs. After incubation, approximately 150 µL of media was removed from each well and 150 µL of DMSO solution was added to the wells. The absorbance was taken in the plate reader at 593 nm.

Cell inhibition was calculated by following formula:

$$\text{Cell inhibition \%} = \left(\frac{A_{(control)} - A_{(sample)}}{A_{(control)}}\right) \times 100$$

3.2.3 Polyphenolic isolation from different Jatropha organs

*Seeds and Fruits***:** All the extracts from jatropha seeds and fruits were prepared according to Huang et al. (2020). Briefly, fruits and seeds were first collected, washed and dried and then freeze dried in liquid nitrogen and stored at -80 ºC. Then approx. 1.0 g of fresh weight of the sample was taken and crushed to fine powder with the help of liquid nitrogen in mortar pestle. Next, it was extracted with 20 mL of 100% methanol and was constantly agitated (100 rpm) at 4 ºC overnight followed by centrifugation at 6000 rpm at 4 ºC for 20 min to let the pellet settle down. The supernatant was further clarified by filtration and concentrated using rotary evaporator. Then, the concentrate was finally resuspended in 1.0 mL of 30% methanol and stored at -20 ºC for further use.

Leaf and stem: Stems and leaves were washed and freeze dried under liquid nitrogen at -80°C. One gram of the sample was crushed in mortar pestle and extracted with 50 mL of 80% methanol. After that extract was swirled for 1hr at room temperature using an orbital shaker and then above extract was filtered through filter paper and stored at -20℃ for further use.

- *Total Polyphenolic content estimation of different extracts*

  The Total phenolic content (TPC) was estimated spectrophotometrically as described Huang et al. 2020.

Reagents

- Reagent I: 10% Folin–Ciocalteu phenol reagent

- Reagent II: 7% Sodium Carbonate Solution
- Distilled Water
- Gallic Acid (1 mg/mL)
- Spectrophotometer

Methodology

200 µL of all extracts (different stages of fruit, seeds, leaves and stems) were taken in test tube and 800 µL of methanol was added to them and vortexed. The test tube with 1 mL of methanol was considered as blank. Immediately about 5 mL of 10 % Folin-Ciocalteu phenol Reagent was added followed by vortexing. Further, addition of 4 mL of 7% sodium carbonate solution was done. Then, the samples were incubated at 40℃ for 30 minutes. The absorbance was checked at 760 nm and values were plotted against the standard curve.

❖ *Antioxidant Capacity determination*

DPPH assay: The DPPH assay was carried out according to Ghasemzadeh et al. (2012).

Reagents

- Methanol
- DPPH
- Ascorbic acid
- Spectrophotometer

Methodology

Different concentrations of all polyphenol extracts (different stages of fruit, seeds, leaves and stems) were taken in test tube. Methanol was added to make up the volume up to 3 mL. 1 mL of DPPH solution was added to each test tube. Methanol was used as blank whereas methanol and DPPH was used as control. Further, incubation for 30 minutes under dark condition was carried out. The absorbance was checked at 570 nm and antioxidant capacity was calculated by the following equation:

$$\% \text{ Antioxidant Capacity} = \frac{[A_{(radical)} - A_{(standard)}]}{A_{(radical)}} \times 100$$

CHAPTER 4: RESULTS AND DISCUSSION

4.1 *In silico* analysis

RIPs are enzymes that play major role in defense of different plant and bacterial cells. There are three types of RIP i.e. (i) Type I RIPs which are made up of a single enzymatically active chain, (ii) Type II RIPs consisting of an enzymatically active chain linked to a lectin binding domain with the help of disulfide bond and (iii) Type III RIPs comprising of an enzymatically active chain linked to another peptide chain with an unknown function. The comprehensive report of their *in-silico* analysis is not available in database. Considerable efforts are made to study various structural and biochemical attributes using *In silico* approaches.

4.1.1 Sequence analysis of RIPs by BLASTp:

BLASTp was done using Type I RIP i.e. Curcin 2A (QWW18610.1). The results revealed significant similarity with other RIPs from same plant species i.e. *J. curcas* such as Curcin L-precursor (ABW17545.1) with 100% query coverage showed 98.38% identity, Curcin (ACO53803.1) with 94% query coverage showed 96.59% identity and Cucormosin (XP_012074346.2) with 100% query coverage showed 86.08% identity coverage. However, Type I RIPs from different plant species showed a lot less similarity. For instance, Trichosanthin (AAB31048.1) from *T. kirilowii* with 71% query coverage displayed 33.91% identity, Rip (AAS17014.1) from *M. charantia* with 75% query coverage revealed 35.56% identity, MAP precursor (BAA01079.1) from *M. jalapa* with 78% query coverage showed 26.67% identity.

When BLASTp was done using Type II RIP i.e. Ricin precursor (CAA26939.1) from *R. communis*; significant similarities were observed with other RIPs from the same plant species such as Preproricin (ADG29046.1) showed 91% query coverage with 99.81% identity & Agglutinin (XP_002534220.2) showed 100% query coverage with 89.58% identity.

Likewise, when BLASTp was carried out using Type III RIP i.e. JIP60 (AVK42932.1) from *H. vulgare*; significant similarities were observed with other RIPs such as 60 kDa JIP (KAE8787184.1) with 100% query coverage and showed 96.61% identity.
All the details are mentioned in the table 4.1 given below.

Table. 4.1 Some homologous sequences as available in the database.

Accession No.	RIP Name	Species	Amino acids	Maximum score	Query Coverage	% Identity
TYPE I RIPs Query sequence is 309-aa Curcin 2A (QWW18610.1) from *J. curcas*						
ABW17545.1	Curcin-L precursor	*Jatropha curcas*	309	628	100%	98.38%
ACO53803.1	Curcin	*Jatropha curcas*	293	580	94%	96.59%
XP_012074346.2	Cucurmosin	*Jatropha curcas*	309	556	100%	86.08%
AAB31048.1	Trichosanthin	*Trichosanthes kirilowii*	289	121	71%	33.91%
AAS17014.1	Rip	*Momordica charantia*	264	129	75%	35.56%
BAA01079.1	MAP precursor	*Mirabilis jalapa*	278	68.6	78%	26.67%
TYPE II RIPs Query sequence is 576-aa Ricin Precursor (CAA26939.1) from *R. communis*						
ADG29046.1	Preproricin	*Ricinus communis*	529	1087	91%	99.81%
XP_002534220.2	Agglutinin	*Ricinus communis*	605	1066	100%	89.58%
TYPE III RIPs Query sequence is 560-aa JIP60 (AVK42932.1) from *H. vulgare*						
KAE8787184.1	60kDa JIP	*Hordeum vulgare*	560	1114	100%	96.61%

4.1.2 Salient sequence features of RIPs

The polypeptide sequence of type I RIPs i.e., Curcin 2A (QWW18610.1) consisting of 309-aas was isolated earlier from developing seeds of *J. curcas.* The sequence was submitted to NCBI by our lab. The RIP region of this sequence ranges from 57−249 aa. Whereas, other type I RIPs from *J. curcas* namely Curcin L-precursor (ABW17545.1) consists of 309-aa with RIP region ranging from 57−249 aa, 209-aa long Curcin (ACO53803.1) has RIP region ranging from 57−249 aa, Curcin precursor (AAL86778.1) isolated from leaves consists of 293-aas also has

RIP region from 57−249 aa, Cucormosin (XP_012074346.2) isolated from mature leaves consists of 309-aas and has a RIP region ranging from 57−249 aa. MAP precursor (BAA01079.1) isolated from root tissue of *M. jalapa* consists of 278-aas, its signal peptide region ranges from 1−28 aa and the RIP region ranges from 45−235 aa. Rip (AAS17014.1) from *M. charantia* consists of 264-aas and it has a RIP region ranging from 10−199 aa. Trichosanthin (AAB31048.1) from *T. kirilowii* consists of 289-aas and has a RIP region ranging from 32−223 aa. All these results revealed that although, all the sequences of RIPs from *J.curcas* varied in number of amino acids but their RIP region is of same length. On the other hand, RIPs from other plants showed variation in RIP region

The polypeptide sequence of type II RIP i.e. Ricin precursor (CAA26939.1) from *R. communis* consists of 576-aas and has a signal peptide region ranging from 1−35 aa, RIP region ranging from 51−254 aa and 2 lectin domains from 324−446 aa and 454−572 aa, respectively.

The polypeptide sequence of type III RIP i.e. JIP60 (AVK42932.1) from Golden Promise cultivar of *H. vulgare* consists of 560-aas and has a RIP region ranging from 17−242 aa.

4.1.3 Protein motif search in various RIPs

Important protein motifs of various RIPs were identified using the MY HITS (http://myhits.isbsib.ch/cgibin/motif_scan) tool. These motifs are described in Table 4.2.

Table 4.2 Various protein motifs, their locations and their functions

RIPs	Motifs	Location	Function
A. TYPE I RIPs			
	ASN_GLYCOSYLATION *N-glycosylation site.*	7−10, 132−135, 266−269,274−277	The purpose of N-glycosylation sites is to alter suitable Asparagine residues in proteins with oligosaccharide structures in order to impact their bioactivities and characteristics.
Curcin 2A	PKC_PHOSPHO_SITE *Protein kinase C phosphorylation site.*	27−29, 57−59, 79−81, 227−229	Protein kinase C is a signal transduction cascade enzyme that controls other protein activities by phosphorylating the hydroxyl groups of amino acid residues -Serine and Threonine.

	CK2_PHOSPHO_SITE *Casein kinase II phosphorylation site.*	27−30, 136−139,158−161, 251−254, 294−297	Casein Kinase II catalyses the transfer of phosphate to the substrate peptide.
	MYRISTYL *N-myristoylation site.*	3−8, 44−49, 283−288	N-myristoylation site aids in lipid modification by attaching an amide bond to the alpha-amino group of glycine's N-terminal residues.
B. TYPE II RIPs			
Ricin	ASN_GLYCOSYLATION *N-glycosylation site.*	45−48, 271−274, 409−412, 449−452	The purpose of N-glycosylation sites is to alter suitable Asparagine residues in proteins with oligosaccharide structures in order to impact their bioactivities and characteristics.
	PKC_PHOSPHO_SITE *Protein kinase C phosphorylation site.*	364−366, 370−372, 480−482, 494−496, 555−557	Protein kinase C is a signal transduction cascade enzyme that controls other protein activities by phosphorylating the hydroxyl groups of amino acid residues -Serine and Threonine.
	CK2_PHOSPHO_SITE *Casein kinase II phosphorylation site.*	30−33, 69−72, 233−236, 276−279, 355−358	Casein Kinase II catalyses the transfer of phosphate to the substrate peptide.
	MYRISTYL *N-myristoylation site.*	22−27, 85−90, 272−277, 426−431, 536−541	N-myristoylation site aids in lipid modification by attaching an amide bond to the alpha-amino group of Glycine's N-terminal residues.
C. TYPE III RIPs			
JIP60	ASN_GLYCOSYLATION *N-glycosylation site.*	101-104	The purpose of N-glycosylation sites is to alter suitable Asparagine residues in proteins with oligosaccharide structures in order to impact their bioactivities and characteristics.
	PKC_PHOSPHO_SITE *Protein kinase C phosphorylation site.*	103−105, 243−245, 386−388,538−540	Protein kinase C is a signal transduction cascade enzyme that controls other protein activities by phosphorylating the hydroxyl groups of amino acid residues -Serine and Threonine.
	CK2_PHOSPHO_SITE *Casein kinase II phosphorylation site.*	21−24, 60−63, 224−227, 265−268, 290−293, 343−346, 353−356, 453−456, 518−521, 531−534	Casein Kinase II catalyses the transfer of phosphate to the substrate peptide.
	MYRISTYL *N-myristoylation site.*	130−135, 168−173, 261−266, 333−338, 400−405, 408−413, 461−466, 535−540, 543−548	N-myristoylation site aids in lipid modification by attaching an amide bond to the alpha-amino group of Glycine's N-terminal residues.

4.1.4 Comparison of various RIPs

Out of all the Type I homologous RIPs, 4 RIPs were chosen . three of these RIPs were from J. curcas i.e. Curcas 2A (QWW18610.1), Curcin (ACO53803.1) and Cucurmosin (XP_012074346.2); while the fourth RIP i.e. Trichosanthin (AAB31048.1) was isolated from *T. kirilowii.* They were schematically described in **Fig. 4.1** showing the crucial regions like underlined RIP region.

Similarly, **Fig. 4.2** shows Type II RIP i.e. ricin precursor (CAA26939.1) from *R. communis.* This figure schematically describes the RIP and their crucial regions such as signal peptide highlighted in Gray color, RIP region underlined and the 2-lectin binding region are marked by symbols "="and "~" respectively.

Similarly, **Fig. 4.3** schematically describes Type III RIP i.e. JIP60 (AVK42932.1) from *H. vulgare.* This Figure shows the crucial RIP region which is underlined.

A

MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKK 60

NYAQFIEDLREAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVAVGLNAITAHLV 120

AYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYADFESRAKLHREEVDLGVVAL 180

DNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240

WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKGLMGVLLNAVNYKVSMEEIIF 300

NDQKWLPWL 309

B

MKGGKMNLSIMVAAWFCRSCIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKK 60

NYAQFIEDLREAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLV 120

AYKVGSNSYFFNDSESLADAKKNLFTDTNQQTLAFTGSYADFESRAKLHREEVDLGVVAL 180

DNYVYTLEKSSQPADIAKPLVGFIEMVPEAARFKYIEKKISTQISKTFRPRGDIISLENN 240

WGDLSYQIQKSVDDVFLKPVQLQRENYTNILVSNVTQVKGLTGVLLNPVKYKV 293

C

MKGGKMNLSIMVAAWFCWSCIIFGWASAREIVCPFSSNQNYKAGSTPTLTITYDAAADKK 60

NYANFIRDLREAFGFSYSSHEIPVLRATVAANQKFIVAKVINVANLEVSLGLNVVNAYLV 120

AYKAGGTSYFFNDPESLADAKKYLFTDTKQQTLSFTGSYADFLSRANVHREDVDLGVLAL 180

DNYIYILHKSSQPADIAKPLVGFIEMVPEAARFKYIEKKVLTQISETFRPRGVIISLENN 240

WGDLSYQIQKSVNGIFLKPVQLQRENYTNILVNNVTQVTGLMGVLLNAVNYKVSMEEIIF 300

NYQKWLPWL 309

D

MIRFLVLSLLILTLFLTTPAVEGDVSFRLSGATSSSYGVFISNLRKALPNERKLYDIPLL 60

RSSLPGSQRYALVHLTNYADETISVAIDVTSVYIMGYRAGDTSYFFNEASATEAAKYVFK 120

DAMRKVTLPYSGNYERLQTAAGKIRENIPLGLPALDSAITTLFYYNANSAASALMVLIQS 180

TSEAARYKFIEQQIGKRVDKTFLPSLAIISLENSWSALSKQIQIASTNNGQFETPVVLIN 240

AQNQRVTITNVDAGVVTSNIALLLNRNDMAAMDDDVPMTQSFGCGSYAI 289

Fig. 4.1 Type I Ribosome-inactivating proteins(RIPs) from *Jatropha* and other plant species. **A** 309-aa Curcin 2A from *J. curcas* (QWW18610.1), RIP region (57-249); **B** 293-aa Curcin from *J. curcas* (ACO53803.1), RIP region (57-249); **C** 309-aa Cucurmosin from *J. curcas* (XP_012074346.2), RIP region (57-249); **D** 289-aa Trichosanthin from *T. kirilowii* (AAB31048.1), RIP region (32-223); the individual RIP regions are underlined.

```
MKPGGNTIVIWMYAVATWLCFGSTSGWSFTLEDNNIFPKQYPIINFTTAGATVQSYTNFI   60

RAVRGRLTTGADVRHEIPVLPNRVGLPINQRFILVELSNHAELSVTLALDVTNAYVVGYR  120

AGNSAYFFHPDNQEDAEAITHLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIELGNGPLE  180

EAISALYYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGEMRTRIRYNRRSAPDPSVI  240

TLENSWGRLSTAIQESNQGAFASPIQLQRRNGSKFSVYDVSILIPIIALMVYRCAPPPSS  300

QFSLLIRPVVPNFNADVCMDPEPIVRIVGRNGLCVDVRDGRFHNGNAIQLWPCKSNTDAN  360
                        ====================================

QLWTLKRDNTIRSNGKCLTTYGYSPGVYVMIYDCNTAATDATRWQIWDNGTIINPRSSLV  420
============================================================

LAATSGNSGTTLTVQTNIYAVSQGWLPTNNTQPFVTTIVGLYGLCLQANSGQVWIEDCSS  480
==========================          ~~~~~~~~~~~~~~~~~~~~~~~~

EKAEQQWALYADGSIRPQQNRDNCLTSDSNIRETVVKILSCGPASSGQRWMFKNDGTILN  540
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

LYSGLVLDVRASDPSLKQIILYPLHGDPNQIWLPLF                          576
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

Fig. 4.2 Ricin precursor, a 576-aa Type II Ribosome-inactivating protein (RIP), from *Ricinus communis* (CAA26939.1). Signal peptide (1–35) is highlighted grey; RIP region (51–254) is underlined; two Ricin-type beta-trefoil lectin domains i.e., 324–446 and 457–573 regions are marked by the symbols '=' and '~', respectively.

```
MALDKVAPIVIVTPFNVMTDTYDDFIEKVRAALAGKVPDSPTVVGPKSEVARPVMDKGTT   60

PVEQPPRWIHVELRGKTQGTTTPNPKVAIRSDDAYIMGFTNSTGRWFQLSKTGTKYKLVD  120

DKAVMAGFEGNYDTLVGGVKHLPDLNLNKFSMAQAAAALWNKASSISGGIGNDDVDDDGN  180

MLRANDPVKQAVATLAVAFCEAARFIPVSNVVKEGWSKDRVSVTPDEVNYIREWGDLSTA  240

LLSWKKKGYKDDATIFKIFNGIGITNGEQALAVVRLVKRVIRSNMADAPTTDEHLLAYAQ  300

LPKHGRYMAEVFAVRIPATAGGDPPSGTISLHGGHCSSDFIYSPEEEHTSQQTSCDSQGN  360

MVLTGPSVATSAYGPIVFNLDLHDGTRRQADEEEDEENTGTIVCDAIGGDFSNYNKAISE  420

TVLTRCGPAEVIYAVLSNGVQGRVDVKLAGLQSRDEVVLVGSIVARSKLFDIGCVLFYNE  480

AAGVRVRPGELVPLARHALAVPLHMPLTIELDIRHGGSGDEIVKGELEFKTAIDGLHTGR  540

LVGVNDAEFEVTILWSEYPW                                          560
```

Fig. 4.3 A 60 kDa jasmonate-induced protein i.e., a Type III Ribosome-inactivating protein (RIP) consisting of 560 amino acids from *Hordeum vulgare* cv. Golden Promise (AVK42932.1*)*. RIP region (17–242) is underlined.

- **Multiple Sequence Alignment**

Multiple Sequence Alignment of different sequences was carried out using MultAlin tool created by Florence Corpet in 1988 for the Multiple Sequence Alignment (MSA) of protein and nucleic acid sequences.

Six different sequences were taken from different plant species to perform multiple sequence alignment between Type I RIPs (**Fig. 4.4**). The sequences used for MSA were Curcin2A (QWW18610.1), Curcin-L Precursor (ABW17545.1), Cucurmosin (XP_012074346), Curcin (ACO53803.1) from *J. curcas;* Trichosanthin (AAB31048.1) from *T. kirilowii* and MAP Precursor (BAA01079.1) from *M. jalapa.* The amino acids highlighted with green color are highly conserved i.e. they are present in all the sequences. The amino acids highlighted with gray color are conserved in some sequences, but not in all sequences.

Multiple Sequence Alignment was also performed between Type I RIP and Type II RIP to find out any conserved domain between the two sequences (**Fig. 4.5**). The sequences used for MSA were Curcin2A (QWW18610.1) from *J. curcas,* a Type I RIP, and Ricin precursor (CAA26939.1) from *R. communis,* a Type II RIP.

Multiple Sequence Alignment was also performed between Type I RIP and Type III RIP to find out any conserved domain between the two sequences (**Fig. 4.6**). The sequences used for MSA were Curcin2A (QWW18610.1) from *J. curcas*, a Type I RIP, and JIP60 (AVK42932.1) from *H. vulgare,* a Type III RIP.

The amino acids highlighted with green color are highly conserved i.e. they are present in all the sequences. The amino acids highlighted with gray color are conserved in some sequences, but not in all sequences.

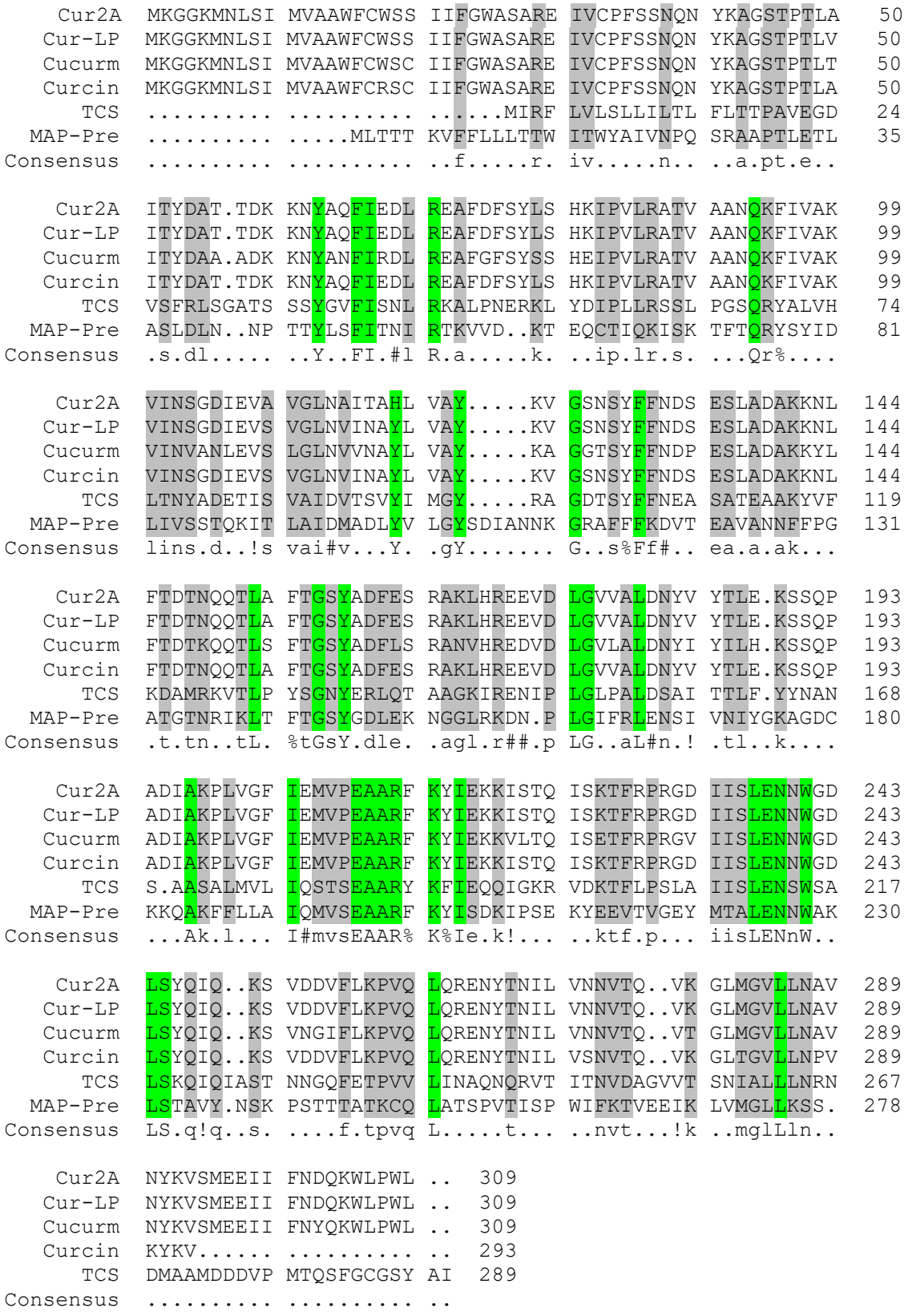

Fig. 4.4 Comparison between different Type I RIPs from different plant species, i.e. Curcin2A (QWW18610.1) from, Curcin-L Precursor (ABW17545.1), Cucurmosin (XP_012074346), Curcin (ACO53803.1) from *J. curcas;* Trichosanthin (AAB31048.1) from *T. kirilowii* and MAP Precursor (BAA01079.1) from *M. jalapa*. The capital

letters in consensus sequence indicate to the conserved amino acids (highlighted green) in all the sequences whereas the lowercase letters in consensus sequence indicate amino acids common in most of the sequences (highlighted gray); whereas ! is used for either I or V, $ is used for L or M , % is used for F or Y and # is used for N, D,Q,E,B or Z.

```
curcin2A   MKGGKMNLSI MVAAWFCWSS IIFGWASARE IVCPFSSNQN YKAGSTPTLA   50
   ricin   MKPGGNTIVI WMYAVATW.. LCFGSTSGWS FTL...EDNN IFPKQYPIIN   45
Consensus  MKgGgnnisI mmaAwacW.. icFGsaSare itc...e##N ikagqtPiia

curcin2A   ITYDATTDKK NYAQFIEDLR EAFDFSY.LS HKIPVL..RA TVAANQKFIV   97
   ricin   FTTAGAT.VQ SYTNFIRAVR GRLTTGADVR HEIPVLPNRV GLPINQRFIL   94
Consensus  iTtaaaT.kq nYa#FIralR ealdfga.lr HeIPVL..Ra glaaNQrFIl

curcin2A   AKVINSGDIE VAVGLNAITA HLVAYKVGSN SYFFN.DSES LADAKKNLFT  146
   ricin   VELSNHAELS VTLALDVTNA YVVGYRAGNS AYFFHPDNQE DAEAITHLFT  144
Consensus  aeliNha#ie ValaL#ainA hlVaYraGnn aYFFn.Dn#e dA#AiknLFT

curcin2A   DT.NQQTLAF TGSYADFESR AKLHREEVDL GVVALDNYV. ...YTLEKSS  191
   ricin   DVQNRYTFAF GGNYDRLEQL AGNLRENIEL GNGPLEEAIS ALYYYSTGGT  194
Consensus  Dt.NrqTlAF gGnYarlEqr AgnhRE#!#L GngaL##a!. ...Ytleggs

curcin2A   QPADIAKPLV GFIEMVPEAA RFKYIEKKIS TQISKTFR.. PRGDIISLEN  239
   ricin   QLPTLARSFI ICIQMISEAA RFQYIEGEMR TRIRYNRRSA PDPSVITLEN  244
Consensus  QladiArpl! gcI#M!pEAA RFqYIEgeir TrIrknrR.. Prgd!IsLEN

curcin2A   NWGDLSYQIQ KSVDDVFLKP VQLQRENYTN ILVNNVTQVK GLMGVLLNAV  289
   ricin   SWGRLSTAIQ ESNQGAFASP IQLQRRNGSK FSVYDVSILI PIIALMVYRC  294
Consensus  nWGrLStaIQ eSn#daFakP !QLQRrNgsn ilVn#Vsqli giial$lnac

curcin2A   NYKVSMEEII FNDQKWLPWL .......... .......... ..........  309
   ricin   APPPSSQFSL LIRPVVPNFN ADVCMDPEPI VRIVGRNGLC VDVRDGRFHN  344
Consensus  apkpSm#eii lnrqkwlnfn .......... .......... ..........

curcin2A   .......... .......... .......... .......... ..........
   ricin   GNAIQLWPCK SNTDANQLWT LKRDNTIRSN GKCLTTYGYS PGVYVMIYDC  394
Consensus  .......... .......... .......... .......... ..........

curcin2A   .......... .......... .......... .......... ..........
   ricin   NTAATDATRW QIWDNGTIIN PRSSLVLAAT SGNSGTTLTV QTNIYAVSQG  444
Consensus  .......... .......... .......... .......... ..........

curcin2A   .......... .......... .......... .......... ..........
   ricin   WLPTNNTQPF VTTIVGLYGL CLQANSGQVW IEDCSSEKAE QQWALYADGS  494
Consensus  .......... .......... .......... .......... ..........

curcin2A   .......... .......... .......... .......... ..........
   ricin   IRPQQNRDNC LTSDSNIRET VVKILSCGPA SSGQRWMFKN DGTILNLYSG  544
Consensus  .......... .......... .......... .......... ..........

curcin2A   .......... .......... .......... ..
   ricin   LVLDVRASDP SLKQIILYPL HGDPNQIWLP LF  576
Consensus  .......... .......... .......... ..
```

Fig. 4.5 Comparison between different Type I RIP i.e. Curcin2A (QWW18610.1) from *J. curcas* and Type II RIP i.e. Ricin precursor (CAA26939.1) from *R. communis*. The capital letters in consensus sequence indicate to the conserved amino acids (highlighted green) in all the sequences whereas the lowercase letters in consensus

sequence indicate amino acids common in most of the sequences (highlighted gray); whereas ! is used for either I or V, $ is used for L or M , % is used for F or Y and # is used for N, D,Q,E,B or Z.

```
Curcin2A  .......... .......... .......... .......... ..........
   JIP60  MALDKVAPIV IVTPFNVMTD TYDDFIEKVR AALAGKVPDS PTVVGPKSEV   50
Consensus .......... .......... .......... .......... ..........

 Curcin2A .......... .......... .......... .......... ..........
   JIP60  ARPVMDKGTT PVEQPPRWIH VELRGKTQGT TTPNPKVAIR SDDAYIMGFT  100
Consensus .......... .......... .......... .......... ..........

 Curcin2A .......... .......... .......... .......... ..........
   JIP60  NSTGRWFQLS KTGTKYKLVD DKAVMAGFEG NYDTLVGGVK HLPDLNLNKF  150
Consensus .......... .......... .......... .......... ..........

 Curcin2A .......... .......... .......... .........M KGGKMNLSIM   11
   JIP60  SMAQAAAALW NKASSISGGI GNDDVDDDGN MLRANDPVKQ AVATLAVAFC  200
Consensus .......... .......... .......... .........q agak$alaic

 Curcin2A VAAWFCWSSI IF..GWASAR EIVCPFSSNQ NYKAGSTPTL AITYDATTDK   59
   JIP60  EAARFIPVSN VVKEGWSKDR VSVTPDEVNY IREWGDLSTA LLSWKKKGYK  250
Consensus eAArFcpsSn !f..GWakaR eiVcPdesNq nreaGdlpTa aiswdakgdK

 Curcin2A KNYAQF.IED LREAFDFSYL SHKIPVLRAT VAANQKFIVA KVINSGDIEV  108
   JIP60  DDATIFKIFN GIGITNGEQA LAVVRLVKRV IRSNMADAPT TDEHLLAYAQ  300
Consensus d#aaqF.Ie# greaf#geqa lak!rllrat !aaNqadapa kdenlgaiaq

 Curcin2A AVGLNAITAH LVAYKVGSNS YFFNDSESLA DAKKNLFTD. TNQQTLAFTG  157
   JIP60  LPKHGRYMAE VFAVRIPATA GGDPPSGTIS LHGGHCSSDF IYSPEEEHTS  350
Consensus apghnaimAe lfAyr!gana ggdndSesia daggncfsD. inqqeeahTg

 Curcin2A SYADFESRAK LHREEVDLGV VALDNYVYTL E.....KSSQ PADIAKPLVG  202
   JIP60  QQTSCDSQGN MVLTGPSVAT SAYGPIVFNL DLHDGTRRQA DEEEDEENTG  400
Consensus qqadc#Sran $hreepdlat sAldniV%nL #.....rrqa da#eaeentG

 Curcin2A FIEMVPEAAR FKYIEKKIST QISKTFRPRG DIISLENNWG ....DLSYQI  248
   JIP60  TIVCDAIGGD FSNYNKAISE TVLTRCGPAE VIYAVLSNGV QGRVDVKLAG  450
Consensus fIecdaeaar Fkni#KaISe q!lkrcrPae dIialenNgg ....Dlklag

 Curcin2A QKSVDDVFLK PVQLQRENYT NI....LVNN VTQVKGLMGV LLNAVNYKVS  294
   JIP60  LQSRDEVVLV GSIVARSKLF DIGCVLFYNE AAGVRVRPGE LVPLARHALA  500
Consensus qqSrD#VfLk gsqlaRenlf #I....lyN# aaqVrgrmGe Llnaarhala

 Curcin2A ME.EIIFNDQ KWLPWL.... .......... .......... ..........  309
   JIP60  VPLHMPLTIE LDIRHGGSGD EIVKGELEFK TAIDGLHTGR LVGVNDAEFE  550
Consensus me.eiilnd# ldirhg.... .......... .......... ..........

 Curcin2A ..........
   JIP60  VTILWSEYPW  560
Consensus ..........
```

Fig. 4.6 Comparison between different Type I RIP i.e. Curcin2A (QWW18610.1) from *J. curcas* and Type III RIP i.e. JIP60 (AVK42932.1) from *H. vulgare*. The capital letters in consensus sequence indicate to the conserved amino acids (highlighted green) in all the sequences whereas the lowercase letters in consensus sequence indicate amino acids common in most of the sequences (highlighted gray); whereas ! is used for either I or V, $ is used for L or M , % is used for F or Y and # is used for N, D,Q,E,B or Z.

4.1.5 Biochemical attributes of different RIPs

Biochemical attributes play a very important role in the functioning of the proteins. Molecular weight of the different RIPs i.e. Curcin 2A, ricin precursor and JIP60 was found to be 34768.71 Da, 64090.57 Da and 60561.56 Da, respectively. While, the theoretical pI is a pH at which the particular molecule carries no net electric charge. For Curcin 2A pI is 6.34, for ricin precursor it is 6.34 and for JIP it is 5.19.The instability index gives the estimation of protein stability in test tube. A weight value comes from this technique which tells about the stability of the protein. Proteins with instability index less than 40 are considered to be stable and the proteins with instability index above 40 are unstable. Here, instability index of Curcin 2A is 29.27, for ricin precursor it is 39.03 and for JIP60 it is 34.08, indicating that these proteins are stable. The aliphatic index occupied by aliphatic side chains (A, V, I, L) is considered as positive factor and is defined as relative volume of a protein for the increase of thermal stability of globular proteins. Aliphatic index of curcin 2A, ricin precursor and JIP60 was found to be 91.84,88.89 and 87.55, respectively. The comparison between all other biochemical attributes of different RIPs is given in **Table 4.3**.

Table 4.3: Biochemical attributes of different Types of RIPs

Accession no.	Name	Molecular weight	Theoretical pI	Negatively charged residues	Positively charged residues	Instability index	Aliphatic index
QWW18610.1	Curcin2A	34768.71	6.34	33	32	29.27	91.84
CAA26939.1	Ricin precursor	64090.57	6.34	47	45	39.03	88.89
AVK42932.1	JIP60	60561.56	5.19	73	55	34.08	87.55

4.1.6 Predicted Secondary Structure

Secondary structure of the different RIPs was predicted using I-Tasser tool. The normalized B-factor (called B-factor profile, BFP) is predicted using a combination of both template-based assignment and profile-based prediction. Based on the distributions and predictions of the BFP, residues with BFP values higher than 0 are less stable in experimental structures. This can be used to predict the secondary structure of the RIPs as described in **Fig. 4.7**. The

purple color signify Helix structure, green color signify linear strand while black color signifies coiled structure.

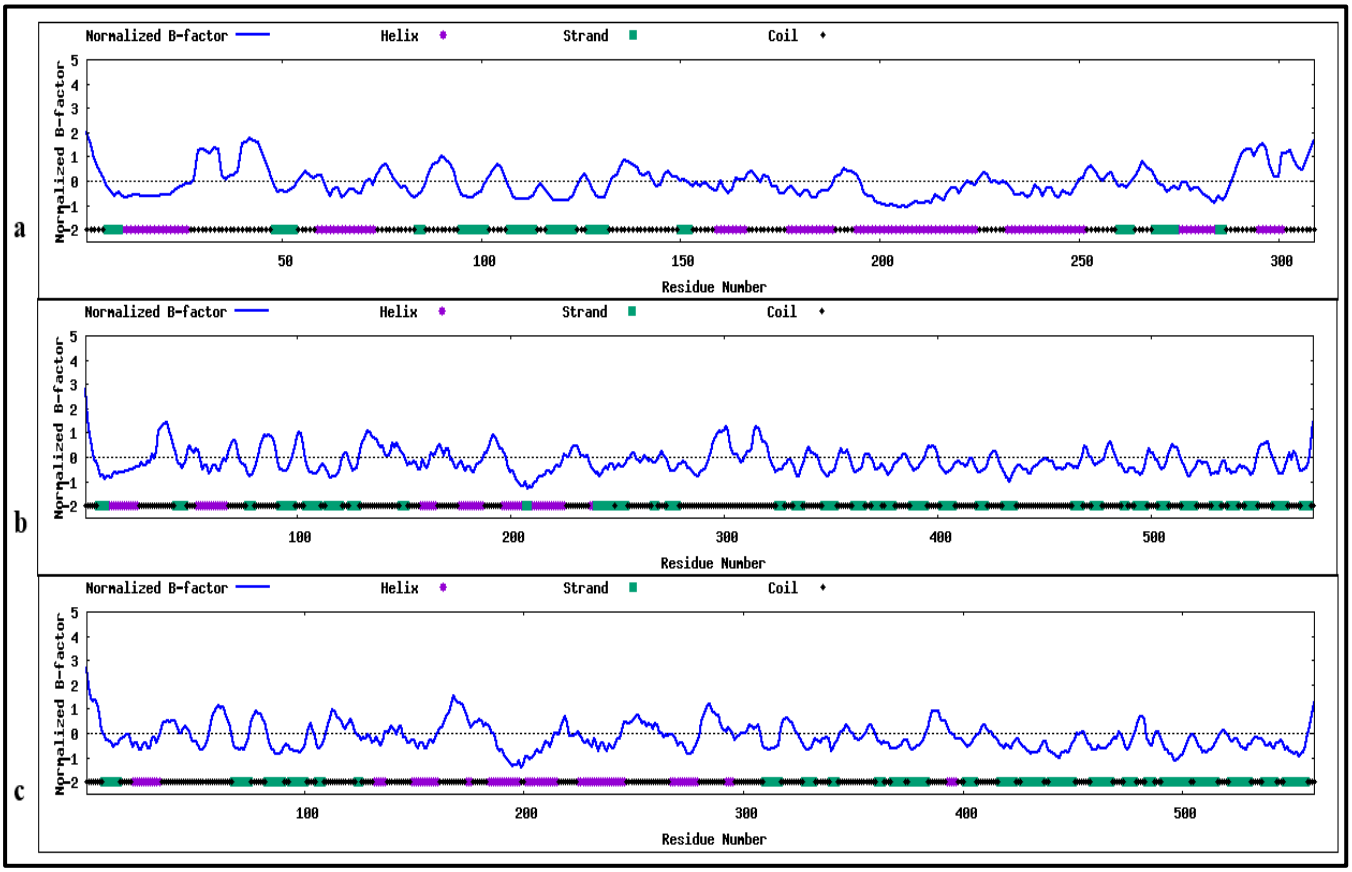

Fig. 4.7 Secondary structure of different RIPs using B-factor i.e. a) type I RIP i.e. Curcin2A; b) Type II RIP i.e. Ricin precursor and c) Type III RIP i.e. JIP60.

4.1.7 Three-Dimensional model studies

A detailed insight of a protein's 3D structure is required to annotate its biological activity. The whole RIP sequence was modelled using Swiss model tool. The amino acid FASTA sequences from NCBI were taken and uploaded in the tool, to find the best model (**Fig. 4.8**). The biological activity of a protein is determined by the arrangement of its atoms in three dimensions. A protein structure gives us a better knowledge of how a protein operates, allowing us to develop ideas about how to impact, regulate, or modify it. Knowing the structure of a protein, supports to create site-directed alterations with the goal of modifying function. Alternatively, anticipating molecules that bind to proteins.

❖ *Model structure*

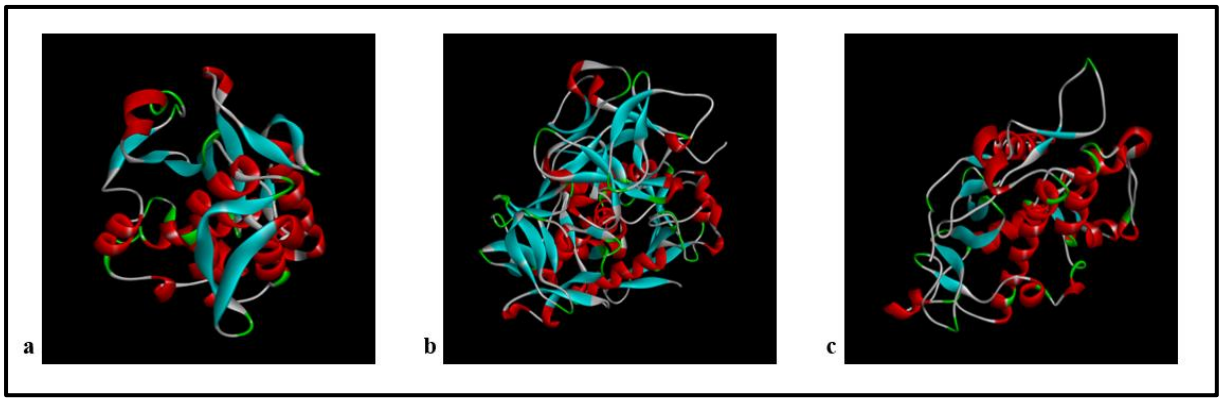

Fig. 4.8 Three-dimensional modelling of different RIPs. a) Type I RIP i.e. Curcin2A; b) Type II RIP i.e, Ricin precursor and c) Type III RIP i.e. JIP60.

❖ *X-ray/NMR structure*

Using ProSA-web tool the protein models were validated. For validation, the pdb files were uploaded on ProSA tool and the data obtained is shown in **Fig. 4.9** It depicts the position of protein in the X-ray/NMR region. The protein is shown by the black-coloured dot (.) if the protein falls in the region then it is considered as the best model.

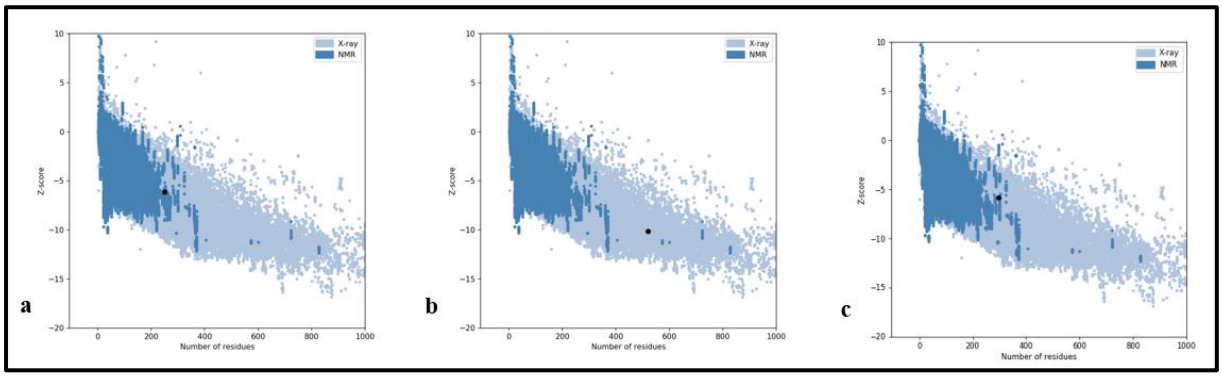

Fig.4.9 X-ray/NMR structure of different types of RIPs. a) type I RIP i.e. Curcin2A; b) Type II RIP i.e. Ricin precursor and c) Type III RIP i.e. JIP60.

❖ *Model quality*

An important way of validation of protein model is based on the energy level as shown in **Fig. 4.10**. If the energy level of the sequences is less than 0 then it is considered to be the best model.

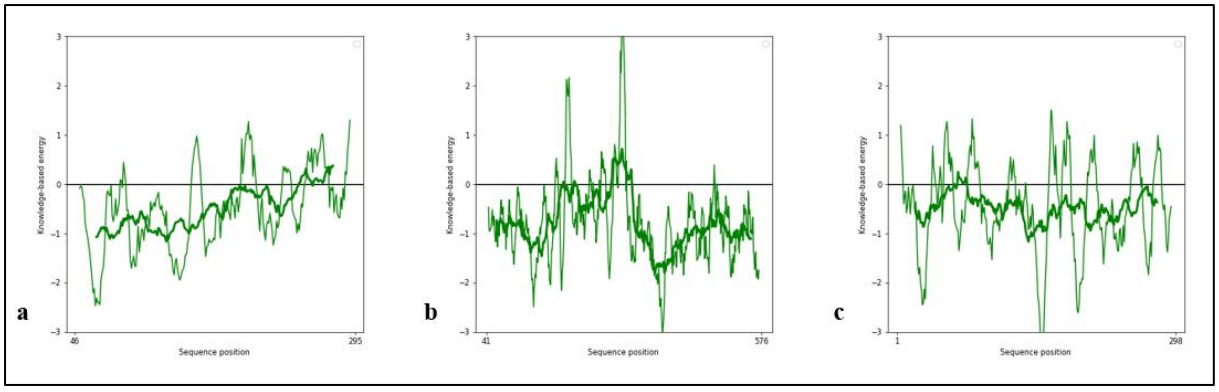

Fig. 4.10 Model quality of different types of RIPs. a) type I RIP i.e. Curcin2A; b) Type II RIP i.e. Ricin precursor and c) Type III RIP i.e. JIP60.

Another way of validation of the protein model is done by checking the z-score i.e. the difference between the raw and average scores in the unit of standard deviation. The model with minimum negative z-score is considered to be the best model (given in **Table 4.4**) So considering all the factors which help in prediction of best model contributes for its selection.

Table 4.4 Z-score and TM- score of different RIPs

Accession no.	Name	Z-score	TM-score
QWW18610.1	Curcin2A	34768.71	6.34
CAA26939.1	Ricin precursor	64090.57	6.34
AVK42932.1	JIP60	60561.56	5.19

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

Ramachandran plot is a way to visualize energetically allowed regions for backbone of amino acid residues in protein structure as indicated by dots in **Fig**. **4.11** The plots were obtained by uploading the pdb file to SAVES tool. In the Ramachandran plots of Curcin 2A, ricin precursor and JIP60 the dots indicate the residues of proteins lying in the favored, allowed and outlier region as described in **Table. 4.5** There is almost similarity in the values of the proteins lying in allowed region but a lot of differences in the proteins present in the negative region, this was identified by using Verify_3D. It determines the compatibility of an atomic model with its own amino acid sequence by comparing the results to good models.

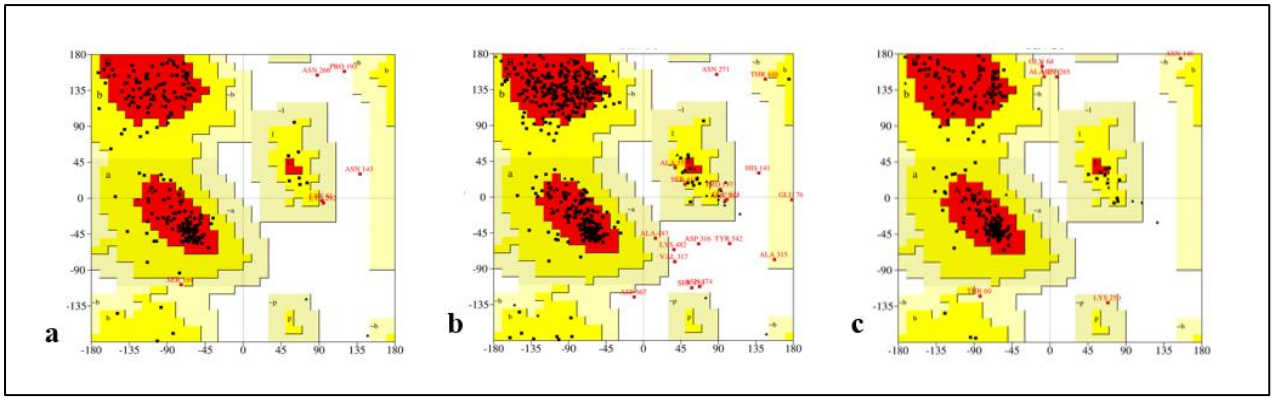

Fig. 4.11 Ramachandran plot of different types of RIPs. a) type I RIP i.e. Curcin2A; b) Type II RIP i.e. Ricin precursor and c) Type III RIP i.e. JIP60.

Table 4.5 Residual assessment of different RIPs

Species **RIP Name**	***Jatropha curcas*** **Curcas 2A** **(QWW18610.1)**	***Ricinus communis*** **Ricin Precursor** **(CAA26939.1)**	***Hordeum vulgare*** **JIP60** **(AVK42932.1)**
In most favoured regions [A,B,L]	191 (82.7%)	371 (81.7%)	222 (86%)
In additional allowed regions [a,b,l,p]	35 (15.2%)	66 (14.5%)	30 (11.6%)
In generously allowed regions [~a,~b,~l,~p]	3 (1.3%)	8 (1.8%)	5 (1.9%)
In disallowed regions	2 (0.9%)	9 (2%)	1 (0.4%)
Non-glycine and non-proline residues	231	454	258
End-residues	2	3	2
Glycine residues	10	37	22
Proline residues	7	26	16
Total residues	250	520	298

4.2 Experimental

Jatropha curcas is a multipurpose plant from Euphorbiaceae family. Various organs of the plant have been used in various folk and traditional medicines. They are used as raw material, in the form of decoction, dried powder or ingested as infusions to treat various diseases like malaria, rheumatism, dysentery, diarrhea, jaundice or to treat wounds, cough, fever, arthritis, etc. this means that this plant has some bioactive compounds with anti-bacterial, anti-viral, anti-fungal, anti-inflammatory properties. These compounds include secondary metabolites like polyphenols which have checked for antioxidant properties. One of the enzymes found in the seeds of this plant ribosome-inactivating protein (RIP) also has been checked for some of above-mentioned properties.

4.2.1 Harvesting of plant

Various organs of *J.curcas* like leaves stems, fruits and seeds were harvested and collected at different stages of development as shown in **Fig. 4.12**, **Fig. 4.13** and **Fig 4.14**.

Fig. 4.12 Seeds harvested at different stages i.e. a) Immature Seeds, b) Early growing Seeds, c) Growing Seeds, d) Premature Seeds e) Mature Seeds.

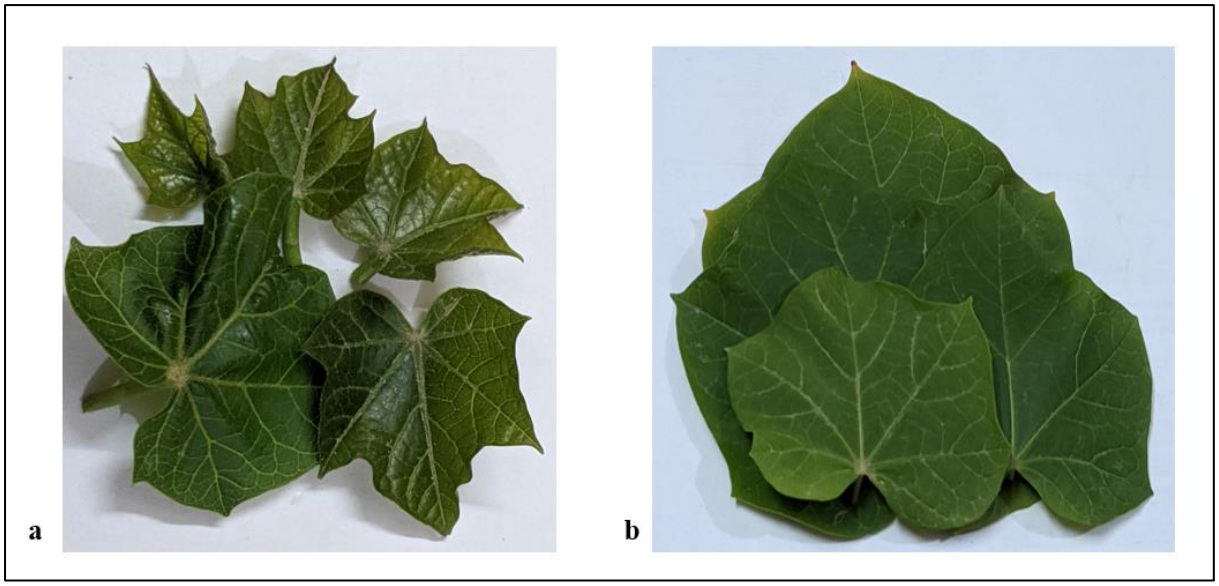

Fig. 4.13 Leaves harvested at different stages i.e. a) Immature Leaves b) Mature Leaves.

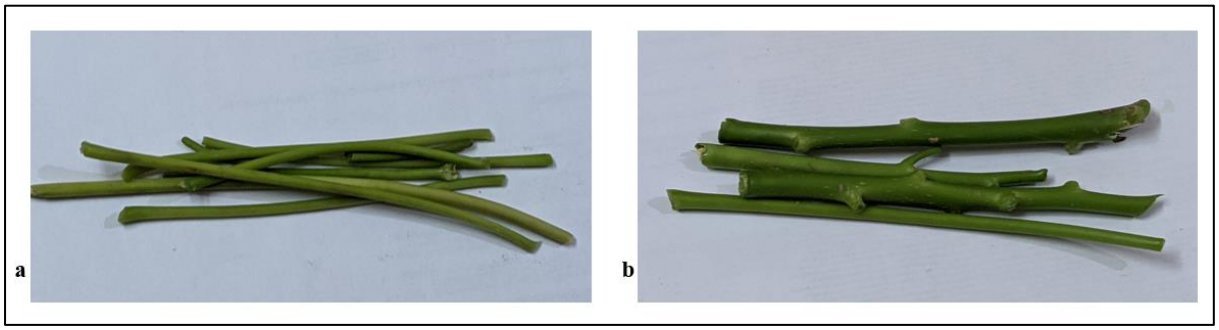

Fig. 4.14 Stems harvested at different stages i.e. a) Immature stems b) Mature stems.

4.2.2 Standard Curve for Protein and polyphenol estimation

I. *Standard curve for the estimation of total protein content:* BSA was used as standard and curve was made by using different concentration of 1 mg/mL stock of BSA solution as shown in **Table 4.6** and **Fig. 4.15**. The absorbance of the sample was taken 750 nm. It was estimated in µg of BSA equivalent per one gram of plant tissue fresh weight.

Table 4.6 Preparation of standard curve of BSA

Test Tube No.	Concentration (µg)	Absorbance (750nm)
1.	Blank	0
2.	100	0.279
3.	200	0.488
4.	300	0.66
5.	400	0.801
6.	500	0.962
7.	600	1.079
8.	700	1.237
9.	800	1.326

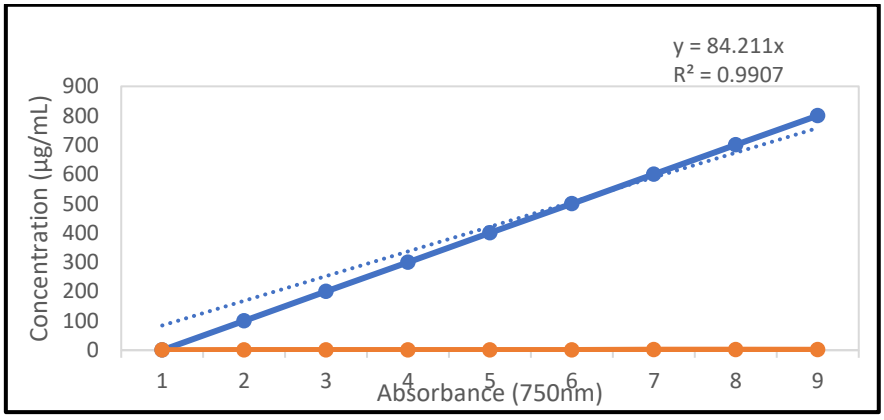

Fig. 4.15 Standard curve of BSA solution

II. *Standard curve for the estimation of total polyphenolic content*- For the estimation of total polyphenolic, Gallic acid (as reported in literature) was used as standard. The stock of 1 mg/ml was and different concentration from 20 to 100 µg was used to made standard curve according to Ghasemzadeh et al. (2012) and is shown in **Table 4.7** and **Fig. 4.16**.

Table 4.7 Preparation of the standard curve of Gallic acid (absorbance measured at 750 nm)

Test Tube No.	Concentration (µg)	Absorbance (750 nm)
1.	Blank	0
2.	20	0.037
3.	30	0.109
4.	40	0.221
5.	50	0.544
6.	60	0.575
7.	70	0.725
8.	80	0.8
9.	90	0.845
10.	100	0.869

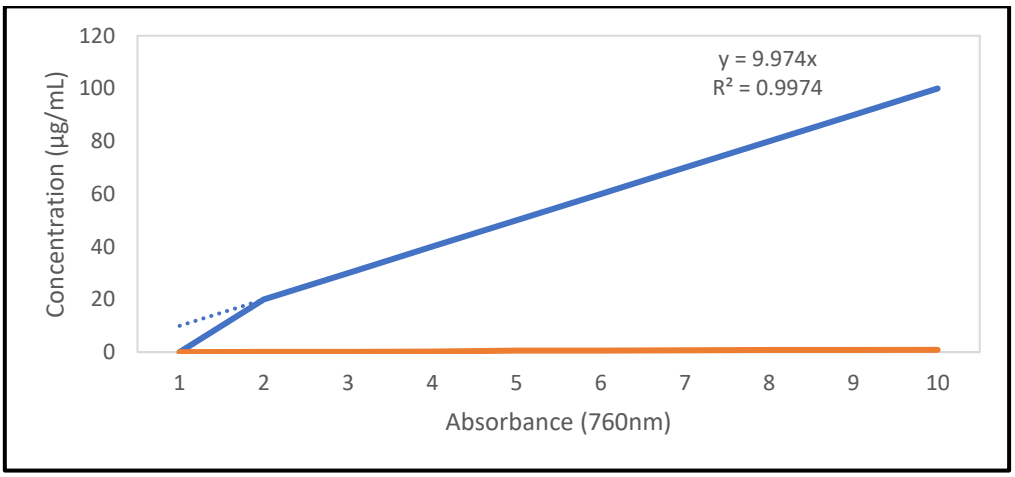

Fig. 4.16 standard curve of gallic acid

4.2.3 Estimation of protein content

- *Protein estimation:* Protein content in different organs of *J. curcas* i.e. leaves, stems, fruits and seeds at different stages was estimated using the Folin-Lowry method as shown in **Table 4.8**, **Table 4.9** and **Table 4.10**. Mature Seeds has the highest protein content (632.42 BSAE g^{-1} FW) whereas the immature fruit contains the lowest protein content (107.79 BSAE g^{-1} FW).

Table 4.8 Estimation of protein content in different organs of *J. curcas* (expressed in mg of BSA equivalent per gram of fresh weight of tissue)

Organ	**Stage**	**Concentration (BSAE g^{-1} FW)**
Leaves	Immature	309.89
	Mature	175.16
Stems	Immature	206.32
	Mature	175.16

Table 4.9 Estimation of protein content in different fruit stages of *J. curcas* (expressed in mg of BSA equivalent per gram of fresh weight of tissue)

Fruit Stage	**Concentration (BSAE g-1 FW)**
Immature	107.79
Early Growing	113.68
Growing	114.53
Premature	505.3
Mature	584.42

Table 4.10 Estimation of protein content in different seed stages of *J. curcas* (expressed in mg of BSA equivalent per gram of fresh weight of tissue)

Seed Stage	**Concentration (BSAE g^{-1} FW)**
Immature	344.42
Early Growing	291.37
Growing	351.16
Premature	253.48
Mature	632.42

❖ *Protein visualization*

The Protein bands were visualized using SDS-PAGE method. The bands were visible in white light and with the help of a ladder, the presence of the protein of our interest i.e. Curcin which has molecular weight of 28.2 kDa was observed. It is clear from the **Fig. 4.17** that highest intensity of curcin is present in mature stage of seeds.

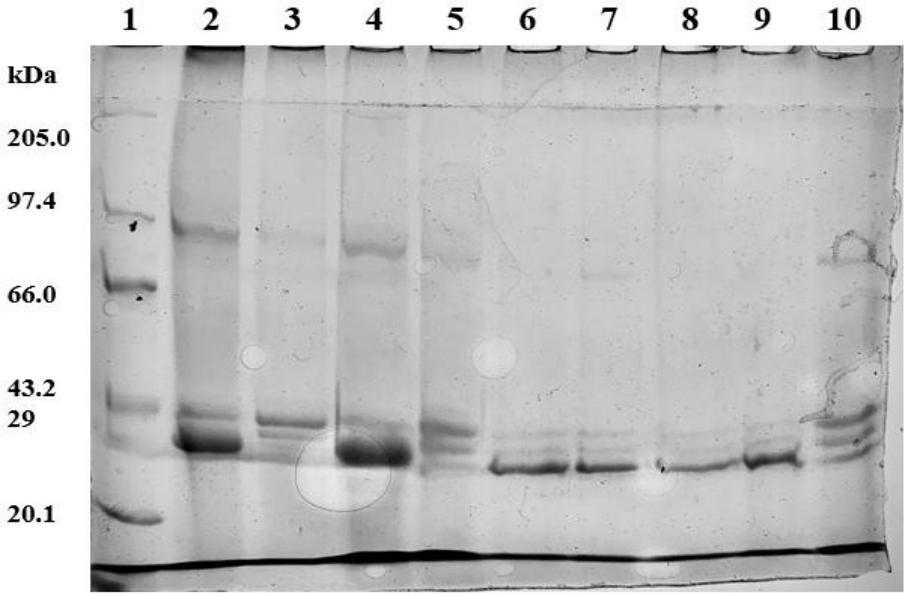

Fig. 4.17 SDS-PAGE of protein extracts i.e. 1) Ladder; 2) Mature seeds; 3) Premature seeds; 4) Growing seeds; 5) Early growing seeds 6) Immature seeds; 7) Mature stem; 8) Immature stem; 9) Mature leaves; 10) Immature leaves.

❖ *Antibacterial activity of protein extracts*

Antibacterial test was carried out on a gram- negative bacteria i.e. *E. coli* DH5α and a gram-positive bacterium i.e. *S. aureus*. The highest rate of inhibition was shown by the mature seed extract with about 9mm of zone of inhibition on *E.coli* as shown in **Table 4.11** and **Fig. 4.18**. However, seed extracts did not show any significant effect on *S. aureus.*

Table 4.11 Antibacterial activity of different seed stage extracts on E.coli and ampicillin as a positive control

Sample	**Zone of Inhibition (mm)**
Positive Control	35
Immature	6.5
Early growing	7
Growing	8
Premature	8.5
Mature	9

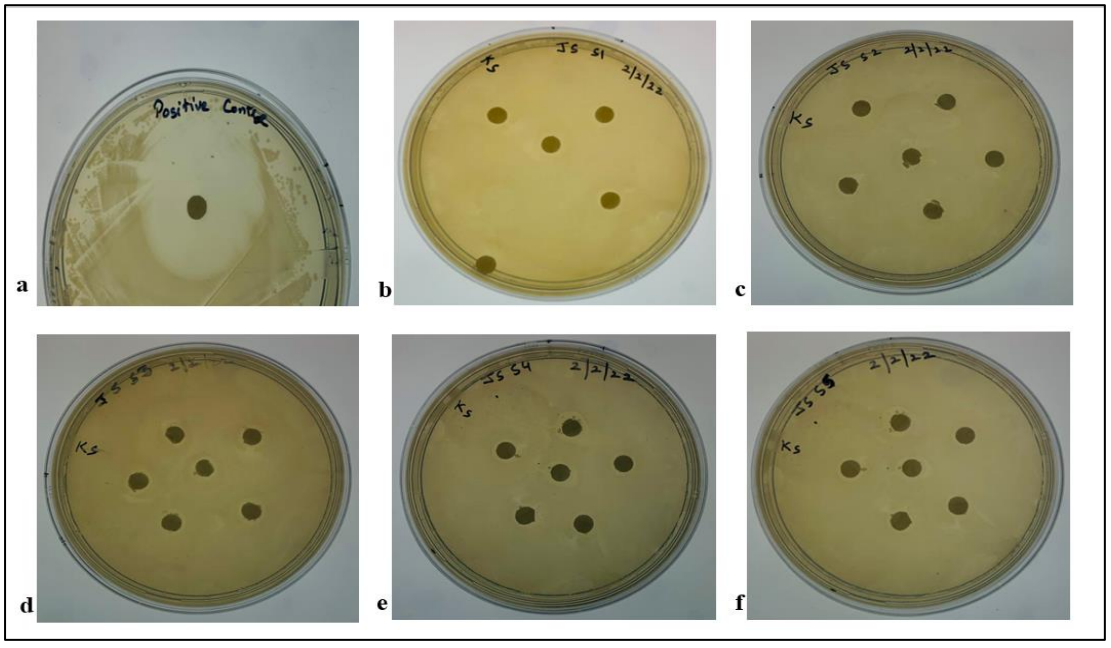

Fig. 4.18 Comparison between different stages of seeds for their antibacterial activity with ampicillin taken as a positive control. a) Ampicillin positive control; b) Immature seed extract; c) Early growing seed extract; d) Growing seed extract; e) Premature seed extract; f) Mature seed extract

❖ *Anticancerous activity of protein extracts*

The cytotoxic effect of different protein extracts was evaluated on MCF-7 cell lines. The cells were treated with different concentrations of (1, 2 and 4 eqiv./mL) for 24 hrs and then the cytotoxicity was measured by MTT assay. **Fig. 4.19** reveals the inhibition rate of MCF-7 cells treated with the extracts. The values are calculated between different measurement and reference measurement. The less the inhibition of the cells, the more purple crystals were formed. We took readings for blank, control and for each extract the analysis was done in triplicates. Blank reading was subtracted from measured reading and the result was divided by control reading. It was found that at 1 µg/µL all the samples showed some kind of inhibition with immature seed extracts showing maximum inhibition of 33.7%, growing seeds showed inhibition of 14.7% and mature seeds showed inhibition of 12.7%. However, at 2 µg/µL immature and growing seed extracts showed inhibition of 20.2% and 5.2% whereas mature seeds showed proliferation of cancer cell lines at further increase in concentration of extracts. Similarly, immature seeds showed inhibition of 14.3% at a concentration of 4 µg/µL whereas both growing seed extracts and mature seed extracts showed proliferation of cancer cell lines. These preliminary tests revealed that seed extracts showed some kind of inhibition at lower concentrations while at higher concentration they either show less inhibition or no inhibition.

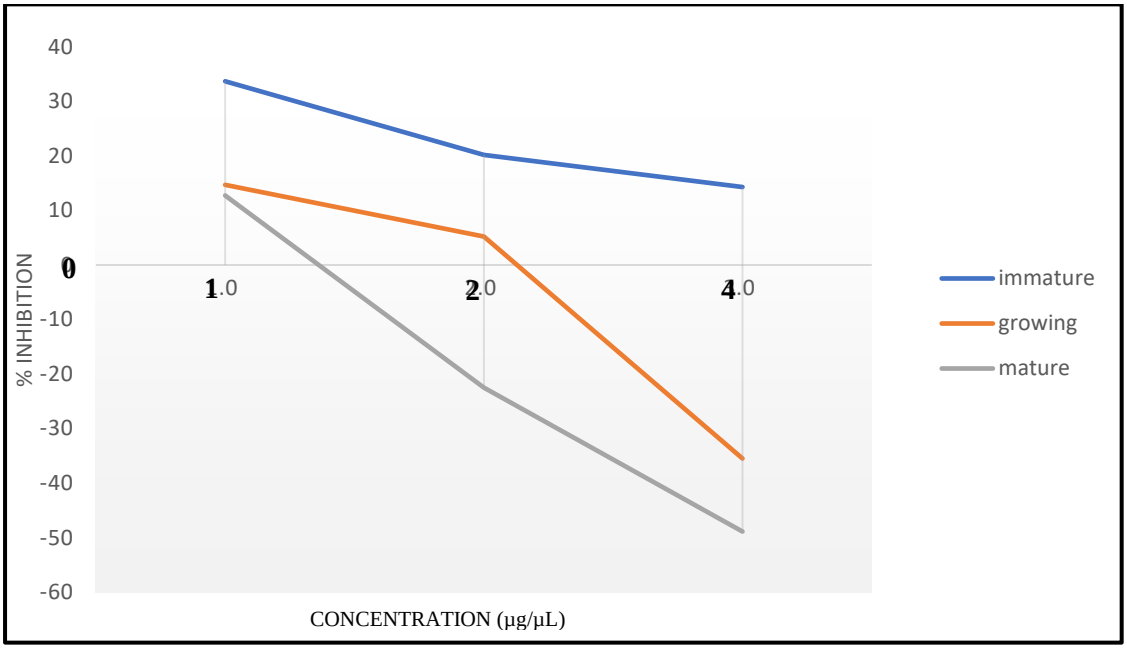

Fig. 4.19 Cytotoxic effects protein extracts of different stages of seeds on MCF-7.

4.2.4 Estimation of polyphenol content

❖ *Polyphenolic estimation*: Polyphenolic content in different organs of *J. curcas* i.e. leaves, stems, fruits and seeds at different stages was estimated as shown in **Table 4.12** and **Table 4.13** and **Table 4.14**. Mature fruit has the highest polyphenolic content (233.11 GAE g^{-1} FW) whereas the early growing fruit has the lowest polyphenolic content (94.03 GAE g^{-1} FW).

Table 4.12 Estimation of polyphenolic content in different organs of *J. curcas* (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Organ	Stage	Concentration (GAE g-1 FW)
Leaves	Immature	156.85
	Mature	196.81
Stems	Immature	160.92
	Mature	197.22

Table 4.13 Estimation of polyphenolic content in different fruit stages of *J. curcas* (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Fruit Stage	Concentration (GAE g-1 FW)
Immature	199.56
Early Growing	233.11
Growing	111.98
Premature	118.09
Mature	94.03

Table 4.14 Estimation of polyphenolic content in different seed stages of *J. curcas* (expressed in mg of Gallic acid equivalent per gram of fresh weight of tissue)

Seed Stage	Concentration (GAE g-1 FW)
Immature	191.92
Early Growing	148.28
Growing	107.08
Premature	108.31
Mature	108.31

❖ *Antioxidant Capacity of polyphenols*

Antioxidant capacity is the capacity of the samples to scavenge free radicals present in their surroundings. From the data in **Table 4.15** and **Table 4.16**, it was observed that antioxidant activity for leaf decreased as the plant matures while for the stem it increases. It also decreases as the seeds mature. And the highest anti-oxidant potential was found in immature seeds as 89.2%.

Table 4.15 Estimation of antioxidant potential in different organs of *J. curcas*

Organ	**Stage**	**% Antioxidant capacity**
Leaves	Immature	76.74%
	Mature	52%
Stems	Immature	65%
	Mature	80%

Table 4.16 Estimation of antioxidant potential in different seed stages of *J. curcas*

Seed Stage	**% Antioxidant capacity**
Immature	89.2%
Early Growing	81.7%
Growing	57.2%
Premature	53.9%
Mature	18.5%

4.3 Concluding remarks

- *J. curcas* is a multipurpose plant which has been used in traditional medicines for centuries.
- Multiple forms of different RIPs exist in nature. The individual RIP has some varying biochemical attributes which could reflect its overall functionality in different tissues.
- Apart from antibacterial, antiviral, anticancerous activities, these RIPs have many other therapeutic applications.
- In present study, the *In silico* analysis on different RIPs was done to study structural and functional relationship between them. It revealed that the RIPs from same plant are significantly similar in amino acid length as well as other crucial regions. However, RIPs from other plant species of same type of RIP have a lot of variations.
- Also, 3-D structure of these RIPs was predicted using different tools. Furthermore, Ramachandran plot analysis revealed that most of the residues reside in the favored region. Thus, validating the structure.
- Various biochemical attributes were studied using different parts of *J. curcas*. Antibacterial study of protein extracts of *J.curcas* revealed that mature seed protein extracts have the highest antibacterial activity on *E.coli* (ZOI= 9 mm). However, *S. aureus* showed no significant ZOI.
- Protein extracts at lower doses showed inhibitory effects on MCF-7 cancer cell line
- In antioxidant tests of polyphenol extracts of *J. curcas* highest antioxidant activity was found in immature seeds.
- To conclude, *In silico* analysis including structure-functional relationship and homologue modeling helped to investigate various physiochemical characterization. This will help us in optimization of different RIPs in drug designing. Also, biochemical characterization of different Jatropha plant organs has revealed some anticancerous and antibacterial properties. This study may further help to investigate the medicinal efficacy and potency of this plant.

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CHAPTER 1: INTRODUCTION 1.1 About RIPs
Ribosome-inactivating proteins or RIPs (EC 3.2.2.22) are a group of cytotoxic N-glycosidases (Lu et al. 2020). They form a family of well-characterized toxins, which specifically and irreversibly inhibit
protein synthesis cells by enzymatically altering the 28S rRNA of the large 60S ribosomal subunit (Fig. 1.1). Over a hundred of
RIPs have been isolated from various plants and bacteria with varying degrees of toxicity (Walsh et al. 2014).
Types of RIPs RIPs are generally classified into three main types based on their physical properties, i.e.
i) Type I RIPs: Type I RIPs are the most

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