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BIOACTIVITIES OF SoRIP1, A TYPE 1 RIBOSOME INACTIVATING PROTEIN FROM *SPINACIA OLERACEA*

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Abstract

A single-stranded ribosome inactivating protein SoRIP1 from *Spinacia oleracea* (Spinach) is a 30 kDa protein. It is a multipurpose protein with versatile biological activities. This paper defines cloning and expression of SoRIP1 in bacteria to study its versatile biological properties and cytotoxic potential. The recombinant plasmid pET29a-SoRIP1 was synthesized and then transferred into top10 and BL21 (DE3) cells of *Escherichia coli*. The protein SoRIP1 was expressed after giving induction of 0.5 mM IPTG for 16 h at 37 ℃. The purification was achieved by Ni–NTA affinity chromatography. The protein SoRIP1 reserved its inherent biological activities like topological inactivation and N glycosidase activity when tested and exhibited hemaglutination activity as well. SoRIP1 demonstrated cytotoxicity against a liver carcinoma cell line (HepG-2) and human breast adenocarcinoma cell line (MCF-7). SoRIP1 protein when tested against pathogenic bacterial (*Ralstonia solanaceum* and *Acinetobacter lwoffii*) and fungal (*Alternaria solani* and *Alternaria Alternata*) strains demonstrated growth inhibiting effect. These results suggested that SoRIP1 may be a potential anticancer and antimicrobial agent to be used in medicinal and agricultural field.

Keywords: Antibacterial, Antifungal properties, cytotoxic protein**,** Glycosidase activity, Ribosome inactivating protein.

1. INTRODUCTION

Ribosome inactivating proteins known as RIPs have glycosidase activity and are specific in nature. These proteins depurinates eukaryotic as well as prokaryotic rRNAs and hence arrest synthesis of protein during translation. Ribosome inactivating proteins prevent binding of elongation factor 2 by deglycosylating a certain base in the 28S rRNA. The two major types include type 1 with a single polypeptide chain whereas type 2 with an A chain having inhibition related roles and B chain with lectin like properties (Zhu et al., 2018). Type 3 Ribosome inactivating proteins are less common type of RIPs. These had been synthesized in form of inactive precursor molecules and need proteolytic cleavage to be active (Domashevskiy & Goss, 2015). Type 3 RIPs consist of an amino acid domain at their terminal region that share similarities with type 1 RIPs (Fabbrini et al., 2017). RIPs are majorly produced by many plant species. Certain fungi and bacteria produces such kind of proteins as well. Mostly RIPs were found in *Phytolaccaceae, Caryophyllaceae* and *Cucurbitaceae* (Girbés et al., 2004). Under stressful conditions expression of RIPs had been increased. Important bioactive properties of RIPs include their antibacterial, antiviral, antifungal and insecticidal activity (Zhu et al., 2018). In case of plants, enhanced

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expression of RIPs, increased the resistance to Viruses, bacteria, Fungi, Insects, Drought and Salinity (Stirpe, 2013). RIPs are one of the most effective tool against viruses in transgenic plants (Citores et al., 2021). Ribosome inactivating proteins are potential antifungal agents. Disruption of fungal cell wall is caused by antifungal proteins and hence the cell wall became unable to perform its function and finally results in the death of fungus (Morais et al., 2010). Most of the Ribosome Inactivating Proteins are cytotoxic in nature and have anti-tumor properties (Maqsood et al., 2021). Clinical trials, for gelonin in myeloid malignancies, TCS, ricin in leukemia and pokeweed antiviral protein in HIV (Uckun et al., 1999) have been carried out. Currently, a RIP based drug named as denileukin diftitox (Ontak) approved by FDA has been used for T-cell lymphoma treatment (Lu et al., 2020).

SoRIP1 (Ishizaki et al., 2002) that is a type-1 RIP was isolated from *Spinacia oleracea* (Spinach). SoRIP1 share the shiga/ricin toxic domain (Kawade et al., 2008). The type I RIPs have difficulty in penetrating into the cells and this is probably because of absence of lectin domain, so the RIP1 have relatively low cytotoxic activity (Puri et al., 2012). Type I RIPs are of considerable interest as they can be used to construct targeted drugs with desirable properties by coupling them to cell penetrating peptides and antibodies and in this way make them potential candidate for targeted delivery into cells and hence their bioactivity and specificity can be increased. Here, we are interested in studying the bioactivities of SoRIP1.The construct named pET29a -SoRIP1 was made to express recombinant protein and was recovered by Ni–NTA affinity chromatography. The topological inactivation and N-glycosidase activity was determined. Antimicrobial activities along with cytotoxic properties of the recombinant protein was evaluated as well.

2. MATERIALS AND METHODS

Materials and reagents

The codon optimized SoRIP1 gene was synthesized by Molecular Biology (https://mbps.pk/). The restriction endonucleases, DNA ladder (1kb: thermo: cat no: SM0311 and 100bp plus: thermo: cat: SM0323), DNA polymerase, PCR kit and PAGE ladder (26616) were purchased from thermoscientific. PAGE ladder (Zokeyo: 025A1) was also bought from Takara Company (Dalian, China). Primers were synthesized by THE WORLDWIDE SCIENTIFIC (Lahore, Pakistan).

The Anti-His Tag Mouse Monoclonal Antibody, was purchased from thermo scientific. Resin for affinity chromatography (Ni–NTA) was from Invitrogen (Carlsbad, CA, USA). Rabbit reticulocyte lysate assay system was bought from Promega Corporation (Madison, WI, USA). Cell lines MCF-7 and HepG-2 were gifted by Institute of Micro Biology and Molecular Genetics (MMG) University of the Punjab, Quid e Azam Campus. Microbial and fungal strains were purchased from FCBP, Faculty of Agricultural Sciences, University of the Punjab, Quid e Azam Campus.

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Insilco analysis and Molecular Characterization of SoRIP1

Nucleotide sequences of BP31/SoRIP1 protein (accession number AB435548.1) was regained from GenBank NCBI (Ishizaki et al., 2002). The gene sequence of the BP31/SoRIP1 protein was analyzed for complete open reading frames by Expert Protein Analysis System (ExPASy) (Gasteiger et al., 2003). The amino acid sequence of the SoRIP1 gene contains various important information. The physicochemical properties and amino acid composition was analyzed by ProtParam server (Gasteiger et al. 2005) whereas the secondary structural features of SoRIP1 were predicted by the PSIPRED server (McGuffin et al., 2000). The amino acid sequence of SoRIP1 was submitted in bioinformatics structure prediction tools including C I Tasser and alpha fold (Pearce and Zhang, 2021). The structure produced was visualized in Pymol for identification of sequence and structure refinement. Then (BLASTp) basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence homology and meg11 software was used for Phylogenetic tree building (https://doi.org/10.1093/molbev/msab120). Molecular and biological function of the protein SoRIP1 was predicted through COFACTOR server (Roy et al., 2012).

2.1 Cloning and Expression of SoRIP1 gene

The codon optimized SoRIP1 gene synthesized by Molecular Biology Products (https://mbps.pk/) was cloned in pET-29a (+) vector by using Nde1 and XhoI restriction sites by amplification (PCR) with gene specific primers described in Table 2.1. The PCR temperature profile was as follows; initial denaturation was at 94 ℃ for 4 min and there was 30 cycles of amplification, adjusted for 40 sec of denaturation at about temperature 95 ℃, annealing temperature was set at 60 ℃ for 35 sec.

The extension temperature was set at 72 ℃ and the final extension was for 10 min at 72 ℃. Hold was set at 4 ℃. The product taken was (100 ng) and 10 pmol of forward and reverse primers were used; 200 µM dNTPs, 20 mM $MgSO_4$, 10X taq Buffer and Taq DNA polymerase enzyme used in a 20 µL PCR master mixture was 1 unit . This PCR product (SoRIP1) was digested with Xho1 and Nde1 and gel purified. After that it was inserted into pET-29a (+) vector (digested with same enzyme). The recombinant plasmid pET29a-SoRIP1 was checked by PCR and restriction digestion (with Xho1 and Nde1 enzymes).

Table 2.1: Primers used for amplification of SoRIP1 gene. The respective restriction sites were highlighted (bold)

Primer name	Sequence	Source
SoRIP1 (forward primer)	5'_**CATATG**GTGGTTGAGGACAGGA_3'	Self-design
SoRIP1 (reverse primer)	5'_**CTCGAG**AATAAGGGCTGACTCTAGGAACTCG_3'	Self-design

Recombinant plasmid pET29a-SoRIP1 transformed into *Escherichia coli* BL21 (DE3) cells and transformation was confirmed by colony PCR. After confirmation, it was induced by isopropylb-D-1-thiogalactopyranoside (IPTG). About 0.5 mM conc., at 37 ℃ for time period of 16 h of IPTG was used. Centrifuged the culture to obtain the pellet.

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Washing of pellet was done twice with 1 X PBS and finally resuspended the pellet in 1 X PBS. Before giving heat shock to the samples, SDS loading dye was added in them. After that, centrifugation was done for 6 min at 13000 rpm. Debris was removed and supernatant was picked and loaded onto 12% SDS-PAGE for analyzing protein bands.

For expression at large scale, 100 mL of overnight grown culture was inoculated in one-liter terrific broth containing 50 µg/mL kanamycin and incubated for 4 h until the OD_{600} reached 0.6 – 0.8, at 37 ℃ in a shaker and then induced with 0.5mM IPTG for 16 h. Cell culture was then centrifuged at 3000 g for 15 min at 4 ℃. The obtained biomass was weighed and suspended in Lysis buffer (100 mM Tris-NaCl, 0.1 M PMSF and 0 1mg/mL lysozyme). After incubation for 1 h at 37 ℃, cells experience Lysis by sonication at 17 Hz followed by centrifugation for 25 min at 12,000 g to get the supernatant (Lv et al., 2015). All the protein fractions were investigated by SDS PAGE analysis.

2.2 Protein Purification

Affinity Chromatography was used for purification purpose. The protein was passed through his tag spin column. For that purpose, first removed the bottom cap of column, placed column in a falcon and centrifuged for 2 min to remove all storage buffer. After that added 10 mL of equilibrium buffer (20 mM sodium phosphate, 10 mM imidazole and 300 mM sodium chloride,) and allowed it to pass through the column. Then placed the column closed from bottom in new falcon tube and added filtered protein in it.

Placed this on orbital shaker for 30 min. After that removed the bottom cap and added wash buffer (20 mM sodium phosphate, 25 mM imidazole and 300 mM sodium chloride) about 7 mL. Repeated washing step. After that added Elution buffer (20 mM sodium phosphate, 250 mM imidazole and 300 mM sodium chloride) and collected protein fractions (Rezaei-Moshaei et al., 2021). The eluted fractions of protein were run on 12% SDS-PAGE for analyzing single desired protein band.

2.3 Western Blot Analysis

About 15 µg of protein from *E. coli* expressing SoRIP1 gene was resolved on 12 % SDS polyacrylamide gels. Protein was then shifted from gel to the membranes in a Trans-Blot (Semi-Dry) Transfer Cell (Bio-Rad) (Towbin et al., 1979). Whatman blotting paper was used to make a sandwich of membrane and gel. The whole assembly was placed in a transblotter at 17 V. After transfer, blocking of membrane was done by skim milk 3% in 1xPBST having 0.05%Tween-20 for time period of 1 h.

After that it was incubated with anti-His primary antibody (1: 10,000 dilutions, Santa Cruz) with shaking at 4 ℃ for overnight. Membrane washing was performed with 1X PBST having 0.05% Tween-20 in 1X PBS and incubated with AP-conjugated secondary antibody (1: 5,000 dilutions, Santa Cruz) for about 1 hour. After washing thrice, the membrane was flooded with substrate BCIP/NBT (1-bromo-3-chloro-3- indolyl phosphate/nitro blue tetrazolium) in dark.

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2.4 Activity assessment assays of SoRIP1 protein

RNA glycosidase activity

In order to check RNA N-glycosidase activity, Rabbit reticulocyte lysate (Promega, REF; L4540, USA) was used as rRNA substrate. In a 100 µL reaction buffer (5 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.65, and 25 mM KCl), the (test) samples were mixed with rabbit reticulocyte lysate. It was then incubated for 10 min at 37 °C. About 10 µL of 10% SDS was used to stop the reaction. Phenol/chloroform (1:1 v/v) was used for extraction of reaction mixture and ethanol precipitation was done for RNA recovery that was followed by dissolving of RNA pellet and treatment with 1 M aniline/ 0.8 M acetic acid at 60 °C for 10 min. It was then analyzed by 6% polyacrylamide gel having 7 M urea. Ethidium bromide was used for staining of gel and UV transilluminator for visualization purpose (Lin et al., 2003).

Topological inactivation activity

The plasmid DNA (1 µg) was incubated with 2 µg of SoRIP1 protein in a 20 µL reaction mixture (20 mM Tris–HCl having pH 8.5, 10 mM $MgCl_2$ and 100 mM KCl) for 1 h at 37 ℃. After that 1% agarose gel electrophoresis was used for analyzing DNA samples (Lv et al., 2015).

Hemagglutination assay

Different blood (human) samples that were A+, B+ and O+ were taken form students from different labs of Center for Applied Molecular Biology (Punjab University, Pakistan). About 2 mL blood sample was centrifuged and the erythrocyte cells pellet was dissolved in PBS buffer with 0.1% trypsin (pH 7.4) to get 2% erythrocyte suspension. This suspension was then used for studying Hemagglutination activity of recombinant protein. Different conc., of the protein (5, 10, 20, 30, 40, 50, 70, 90, 110, 130, and 150 µg/µL) were made in a 50 µL of PBS buffer (pH 7.5). Incubation for 1 h at 21 ℃ was done after mixing these concentrations of recombinant protein with 50 µL of erythrocyte suspension inside a 96-well microtitre plate. In negative control no protein was added. When red blood cells in negative control sample were fully precipitated, results were read (Yagi et al., 2002).

Antimicrobial Susceptibility Testing

Agar well diffusion method was used for evaluation of the antimicrobial activity of purified SoRIP1 protein (Valgas et al., 2007). For that purpose, inoculated the agar plate by evenly spreading the microbial inoculum. A hole of size 6 to 7 mm in diameter was created aseptically with the help of a tip and different concentration of SoRIP1 protein (20, 40, 60 and 80µg) were introduced to the wells (holes). Incubated plates at 37 ℃ for overnight. The purified SoRIP1 protein was diffused and inhibited the growth of the *Ralstonia solanaceum* (acc. No: FCBP-BP0407) and *Acinetobacter lwoffii* (acc. No: FCBP-BP0435).

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Antifungal Susceptibility Testing

Agar well diffusion method was used for evaluation of antifungal activity of purified protein (Kirubakaran and Sakthivel 2007). For that, fungal inoculums (*Alternaria solani* (acc. No: FCBP-PTF831) and *Alternaria Alternata* (acc. No: FCBP-PTF1174) obtained from Fungal Bank Punjab University) were prepared and a PDA plate was inoculated by spreading this in the middle of plates.

Kept these plates at 28 ℃ for three days. A hole of size 6 to 7 mm in diameter was created at growing margins of fungus aseptically with the help of a tip and different concentration of SoRIP1 protein (10, 20, 30 µg) were introduced to the wells (holes).

Incubated plates at 28 ℃ for 2 more days. The purified SoRIP1 protein was diffused and inhibited the growth of the *Alternaria solani* and *Alternaria Alternata* (fungal strains) tested.

Cytotoxicity assay

The SoRIP1 protein cytotoxicity was checked by (inhabitation of proliferation) the cell lines MCF-7 and HepG-2 by a 3-2, 5-diphenyltetrazolium bromide (MTT)-based colorimetric assay kit (Millipore, USA) as per manufacturer's instructions. Dulbecco’s modified Eagle medium (DMEM) (with 10% fetal bovine serum, 100 IU/mL penicillin and 100 µg/mL streptomycin) was used to culture one hundred µL (1×105) of MCF-7 and HepG-2 cells in 96 wells plate separately. CO_2 incubator was used for culturing these cells for 24 h at 37 ˚C.

SoRIP1protein was differentially diluted and added into the plate followed by incubation in a CO_2 incubator at 37 ˚C for 24 h. Three replicates were analyzed for each dilution. The media was decanted after 24 h and 100 µL of freshly prepared media was added. According to manufacturer’s instructions, 20 µL MTT solutions (5 mg/mL in PBS) was supplemented. Again incubated the plate for 4 hours at 37˚C in a CO_2 incubator.

After 4 h 0.1 mL DMSO was added for dissolving formazen crystals formed in the wells. In order to find out the optical density of the MTT formazan product Enzyme-linked immunosorbent assay (ELISA) plate reader was used and recorded the test wavelength of 570 nm and reference wavelength of 620 nm.

3. RESULTS

3.1 Insilco Analysis of SoRIP1

Analysis of the Primary Sequence of SoRIP1

Some of the important properties of the protein SoRIP1 are number of amino acids, isoelectric point (PI), aliphatic index (AI), grand average hydropathicity (GRAVY) value, molecular weight (MV) and instability index (II). All these important properties of the protein SoRIP1 was listed in table 3.1 and amino acid composition was presented in Fig. 3.1.

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Table 3.1 Shows Physico Chemical Properties of SoRIP1 by Protparam

Physico-chemical properties	SoRIP1
No. of amino acids	289
Molecular weight (kDa)	32.5
Theoretical pI	5.44
Instability index	30.96
GRAVY	-0.340
Total no. of atoms	4546
Total no. of negative charged residues (Asp + Glu)	36
Total no. of positive charged residues (Arg + Lys)	32
Formula:	$C_{1433}H_{2258}N_{400}O_{446}S_9$

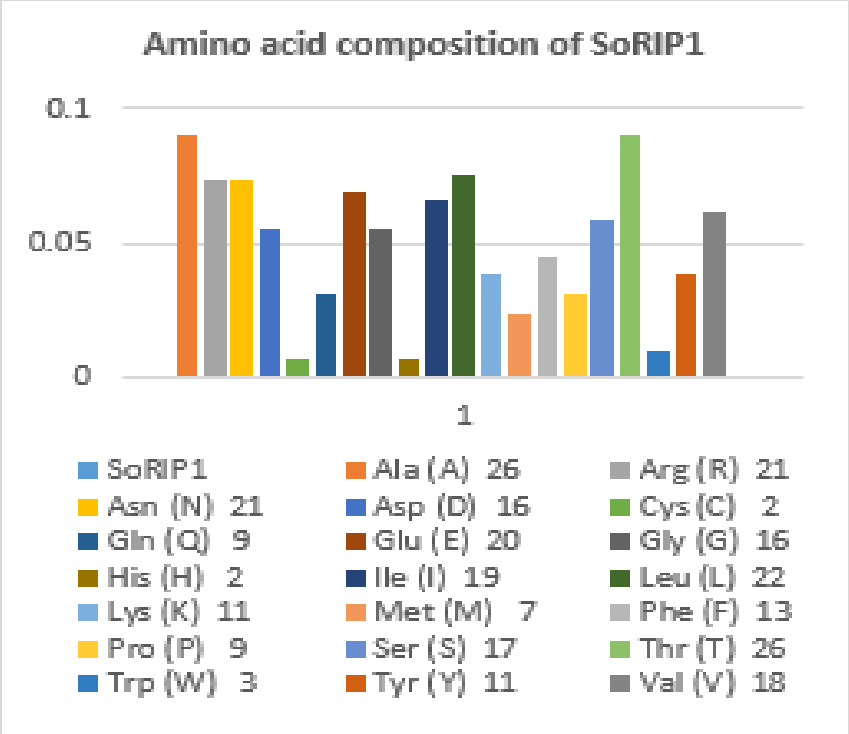

Fig 3.1: Shows amino acid composition of SoRIP1

3D-structure prediction

Structure prediction by I Tasser, Alpha fold and trRosetta is achieved by providing the FASTA sequence of SoRIP1. The best model from I Teaser was model 1 with c score value 96.1 and from alphafold was model 3 with c score value 79.00, whereas from trRosetta was model 3 having c score value 89.62.

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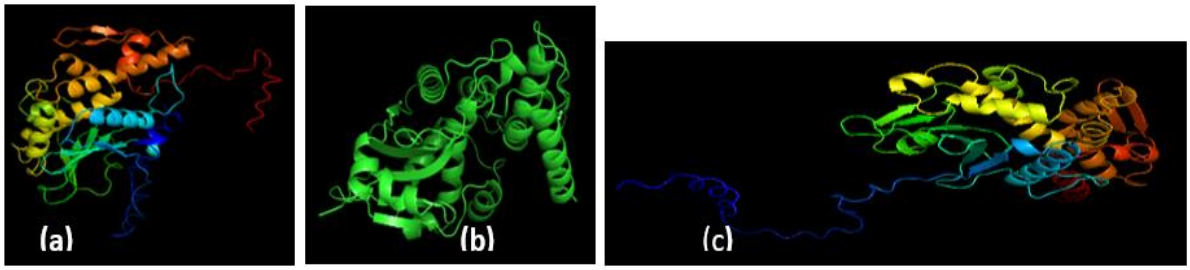

Fig 3.2: Shows 3D structure of SoRIP1. (a) Shows best model of SoRIP1 by I Tasser (b) Shows best model of SoRIP1 by alpha fold (c) shows best model of SoRIP1 produced by trRosetta

Based upon saves server and Ramachandran plot the best among these three models was the one obtained from I Tasser. Each model of the SoRIP1 given in Fig. 3.2 from the 3 servers are the one with best score and stable conformation.

Secondary Structure Predication

The features related to secondary structure of SoRIP1 were predicted by using server named PSIPRED which shows that the SoRIP1 protein contains 38.41% (111 residues) alpha helix, 14.2% (41 residues) extended strands and 47.40 % (137 residues) random coils (Fig. 3.3). These result suggests that random coils are dominated in SoRIP1 protein.

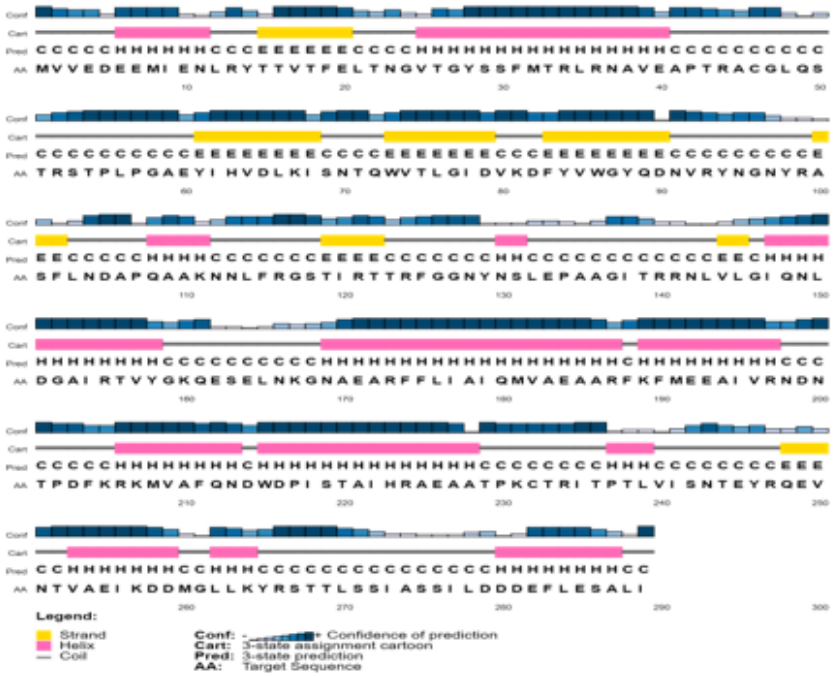

Fig 3.3: Shows secondary structure present in SoRIP1

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Phylogenetic Tree

Phylogenetic tree is a diagram which helps in understanding evolutionary relationship amongst the biological species. The drawn phylogenetic tree showed that the SoRIP1 is closed to ribosome-inactivating protein from *Beta vulgaris* and ribosome-inactivating protein Amaranthus *tricolor* (Fig. 3.4).

Fig 3.4: Phylogenetic analysis of amino acid sequences of SoRIP1

Characterization of the Protein Model

COFACTOR predict the functions of the gene such as functions at molecular level, the components of cell in which chances of presence of protein is more and all processes in which the protein is involved biologically. As per biological process SoRIP1 is involved in biological regulation, negative regulation of translation, defense response and changes in morphological form and functions of other organism. C-score for biological process is shown in Table 3.2. The functions forecasted by the server at molecular level showed that the gene SoRIP1 has rRNA N-glycosylase activity with C-score 0.98 and carbohydrate binding activity with C-score 0.55. SoRIP1 works in cellular part of cell. The fig. 3.5 shows molecular function and biological process.

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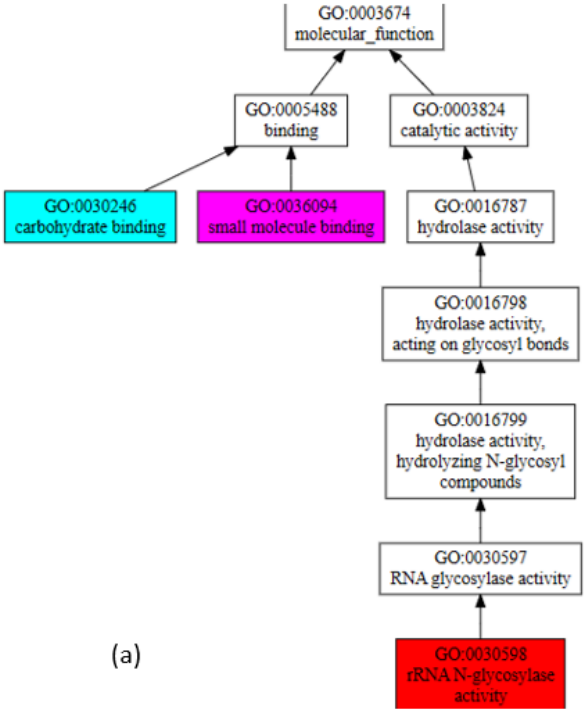
Table 3.2: Shows Functional Analysis of SoRIP1 protein by COFACTOR

Sr. No	Molecular Function		Biological process		Cellular component	
	GO term	C-score	GO term	C-score	GO term	C-score
1.	Carbohydrate binding	0.55	Biological regulation	0.99	Intracellular part	0.97
2.	Nucleotide binding	0.44	Negative regulation of translation	0.98	Cellular part	1.00
3.	Small molecule binding	0.50	Defense response	0.75	Endoplasmic reticulum	0.93
4.	rRNA N-glycosylase activity	0.98	Killing of cells of other organism	0.52	Cytoplasmic part	0.68
5.			Changes morphology of other organism	0.59		



(a)

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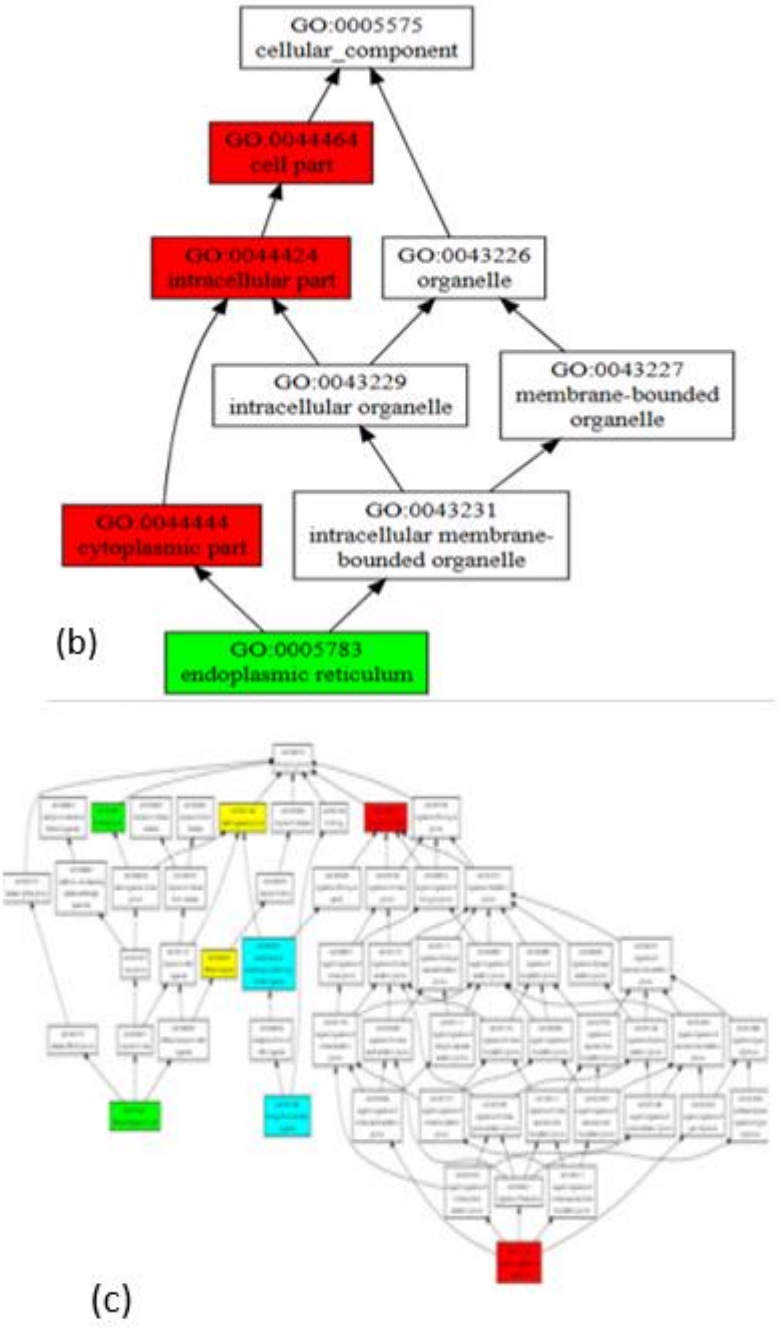

Fig 3.5: Functional analysis of SoRIP1 protein (a) Shows molecular function of SoRIP1 protein (b) Shows cellular component of SoRIP1 protein (c) Shows biological process of SoRIP1 protein

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3.2 Construction of Plasmid pET29a-SoRIP1

Full length nucleotide sequence of BP31/SoRIP1 protein (accession number AB435548.1) was regained from GenBank NCBI (Ishizaki et al., 2002). The gene sequence for complete open reading frame was checked by Expert Protein Analysis System (ExPASy) (Gasteiger et al., 2003). SoRIP1 gene (full length) translates into a protein of 319 amino acids. SoRIP1 gene with signal peptide sequence have size 960 bp and without signal peptide have size 867 bp. The pET29a-SoRIP1 was constructed (Fig. 3.6) and checked by colony PCR and restriction digestion (Fig. 3.6). The size of plasmid pET29a-SoRIP1 was 6108 bp.

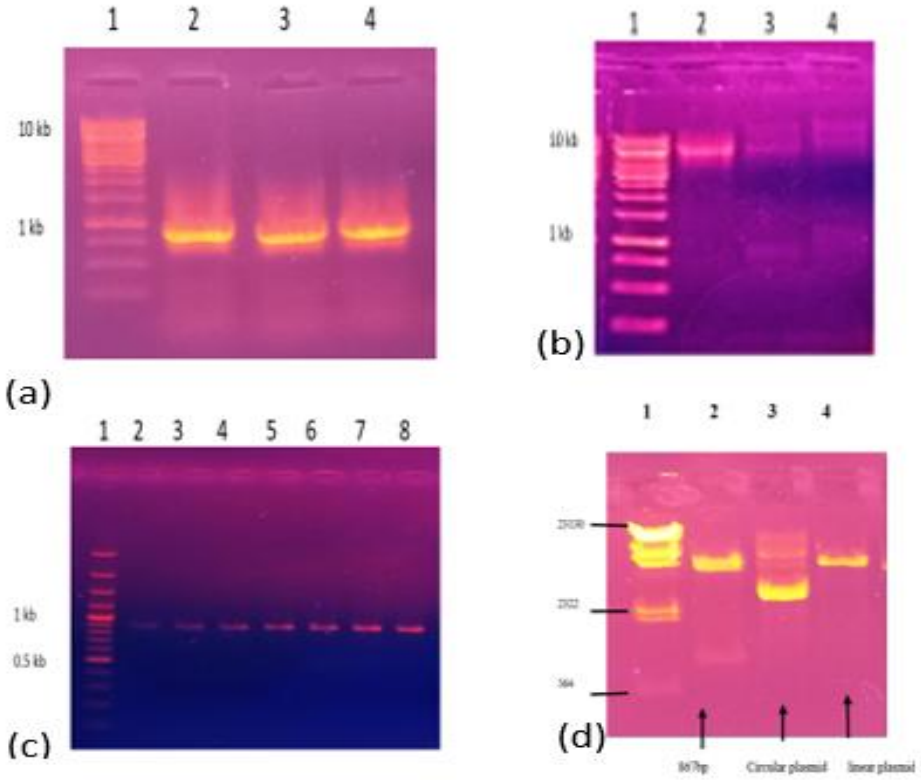

Fig 3.6: Construction of recombinant plasmid pET29a-SoRIP1. (a) Restriction digestion of PCR amplified SoRIP1 gene. Lane 1:1 kb DNA marker, Lane 2-4: Digested PCR product of SoRIP1. (b) Confirmation of Ligation of SoRIP1 gene in vector pET-29a (+). Lane 1: 1kb DNA marker. Lane 2: Digested pet-29a (+) vector, Lane 3-4: Ligated product. (c) Confirmation of recombinant plasmid (pET29a-SoRIP1) by PCR. Lane 1:100 bp plus DNA ladder, Lane 2- 8: PCR product. (d) Confirmation of recombinant plasmid pET29a-SoRIP1 by restriction digestion. Lane 1: lambda DNA\ hind2 marker, thermo: cat no: SM0103, Lane 2: Construct (BR2-SoRIP1) cut with both nde1 and xho1, Lane 3: Undigested plasmid DNA, Lane 4: Plasmid cut with nde1 only

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3.3 Expression and Purification of SoRIP1

To investigate the expression of the SoRIP1 was affected by IPTG, the conc., 0.1, 0.5, 1.0 mM of IPTG were tested. The results showed the different concentrations of IPTG effect the expression of SoRIP1 protein. Hence, the optimal expression of SoRIP1 was obtained with IPTG conc., 0.5 mM for 16 h incubation at temperature 37 ℃. So, the SoRIP1 protein expressed at temperature 37 ℃ and IPTG concentration 0.5 mM at its expected molecular weight (32.5 kDa) as shown in Fig. 3.7a.

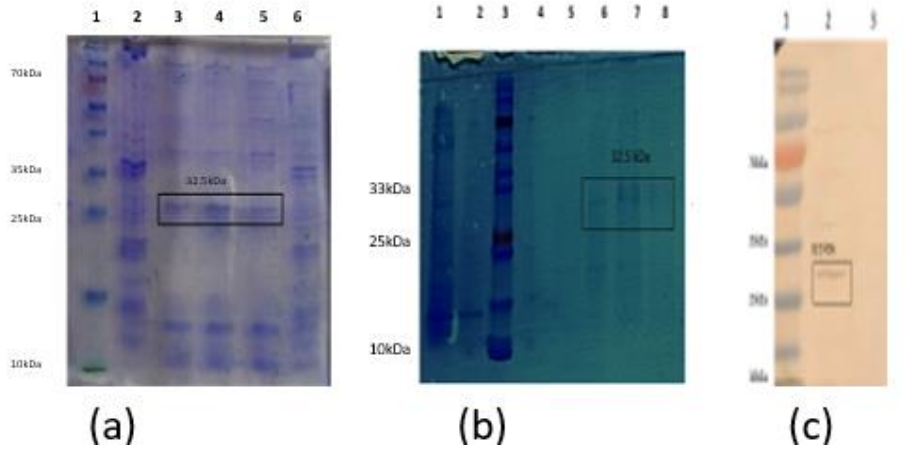

Fig 3.7: Analysis of expression of SoRIP1 by SDS PAGE. (a) Lane 1: Protein ladder thermo: 26616, Lane 2: induced sample of BL21, Lane 3-4: induced sample of SoRIP1, Lane 5: Un- induce samples of SoRIP1, Lane 6: Un- induce BL21 (b): Lane 1: crude extract, Lane 2: wash 1, Lane 3: protein ladder, Zokeyo: 025A1, Lane 4-5: wash 2, Lane 6-8: Protein fractions (c) Western Blot of SoRIP1 protein. Lane 1; Protein ladder, thermo: 26616, Lane 2: SoRIP1 protein, Lane 3; Negative control (untransformed BL21)

Cells were collected after induction and Lysis of cells was performed by exposing to sonication, and then the solubilized SoRIP1 was loaded to the affinity column. After the washing step, the fractions containing respective protein was eluted and the target SoRIP1 protein was confirmed by SDS PAGE analysis (Fig. 3.7 b). Western-blotting also confirmed the SoRIP1 protein (Fig. 3.7c). The purities of SoRIP1 was about 92% as estimated by analysis of SDS–PAGE gel. The yield of SoRIP1 was 1.8 mg/mL culture, respectively, based on Bradford assay.

Activity assessment assays of SoRIP1 protein

RNA glycosidase activity

The RNA glycosidase activity of the purified SoRIP1 protein, was determined with help of rabbit reticulocyte lysate (as28S rRNA source). In Fig. 3.8a acidic aniline treatment of the protein SoRIP1 treated rRNA resulted in the generation of a specific RNA fragment of

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about size 460bp approximately; this is because of its N-glycosidase activity of SoRIP1. Whereas in control, without SoRIP1 protein treatment, no RNA fragment released.

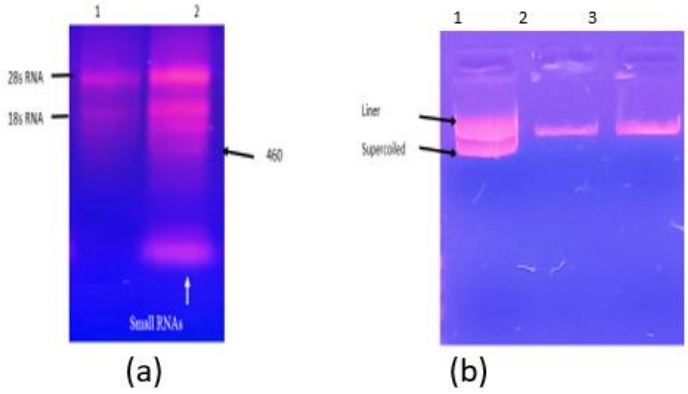

Fig 3.8: (a) N-glycosidase activity of SoRIP1. Lane 1, negative control (without SoRIP1); lane 2 treatment with SoRIP1 (1 ug) and aniline. The arrow showed the ~ 460-bp fragment generated as a result of treatment of modified rRNA with aniline (b) DNase activity of SoRIP1 protein (1% Agarose gel). Lane 1: Control (Plasmid DNA), Lane 2-3: Plasmid DNA incubated with SoRIP1 protein

Topological inactivation activity

The result showed that the DNA (double stranded) when incubated with SoRIP1 protein for 2 h, the DNA was cleaved and became linearized after incubation, which demonstrated that this protein exhibited DNase-like activity (Fig. 3.8b).

Hemagglutinating activity

Purified SoRIP1 protein indicated hemagglutination of erythrocytes in different blood groups of human (Fig. 3.9). At conc., of 90–150 µg/mL, it was seen that the erythrocytes were agglutinated. There was a mat shape formation at the well's bottom when incubated (with SoRIP1 protein) at room temperature for 1 h. Whereas in case of control and the wells having conc., < 90 µg/mL of protein, RBCs were fully sediment and these were seen as red spot at well's bottom. Hence the protein SoRIP1 was showing hemagglutinating activity.

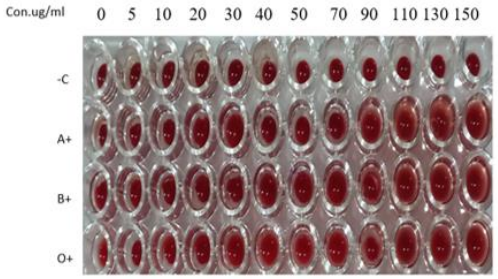

Fig 3.9: Hemagglutination assay of various blood groups (human) including A+, B+ and O+ in the presence of purified SoRIP1

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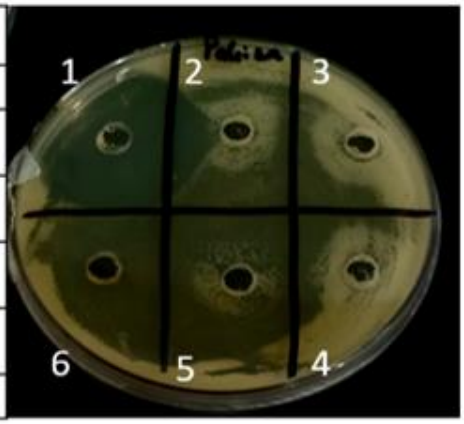
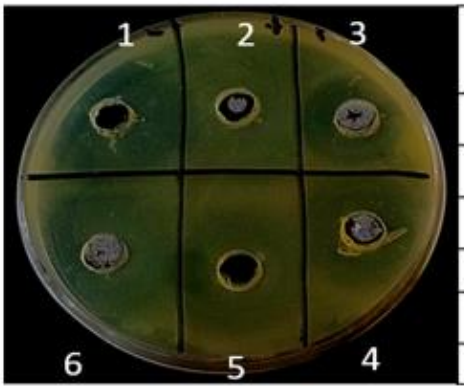
Antimicrobial activity

The antimicrobial activity of SoRIP1 was investigated against 2 pathogenic strains of bacteria that were *Ralstonia solanaceum* and *Acinetobacter lwoffii*. Significant bacterial growth retardation was observed when different concentrations 20, 40, 60 and 80 µg of SoRIP1 protein was applied as shown in Fig. 3.10. The inhibition zone increased with an increasing concentration of the SoRIP1 protein. However, no such zone was observed around the negative control, in which heat-deactivated SoRIP1 protein was used. An antibiotic drug chloramphenicol was used as positive control for antibacterial activity. SoRIP1 showed less inhibitory activity as compared to antibiotic drug (chloramphenicol) for both the tested bacteria.

NAME	Conc. µg	Zone of Inhibition
1 +ve C	20	35 mm
2 SoRIP1	20	11 mm
3 SoRIP1	40	12 mm
4 SoRIP1	60	13 mm
5 SoRIP1	80	13 mm
6 –ve C	-	-



(a)



NAME	Conc. µg	Zone of Inhibition
1. +ve Control	20	35 mm
2. –ve Control	-	-
3. SoRIP1	20	10 mm
4. SoRIP1	40	11 mm
5. SoRIP1	60	21 mm
6. SoRIP1	80	22 mm

(b)

Fig 3.10: Bacterial Inhabitation assay by SoRIP1. (a) Ralstonia solanaceum and different concentrations of purified SoRIP1 in µg. (b) Acinetobacter lwoffii and different concentrations of purified SoRIP1 in µg

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Antifungal activity

The antifungal activity of SoRIP1 was investigated against 2 pathogenic strains of fungi that were *Alternaria solani* and *Alternaria Alternata***.** Significant growth retardation of both fungi were observed when different protein concentrations that were 10, 20, 30µg were applied as shown in Fig. 3.11. The inhibition of growth at growing margins of fungus increased with an increasing concentration of the SoRIP1 protein. However, no such effect was observed around the negative control, in which heat-deactivated SoRIP1 protein were used.

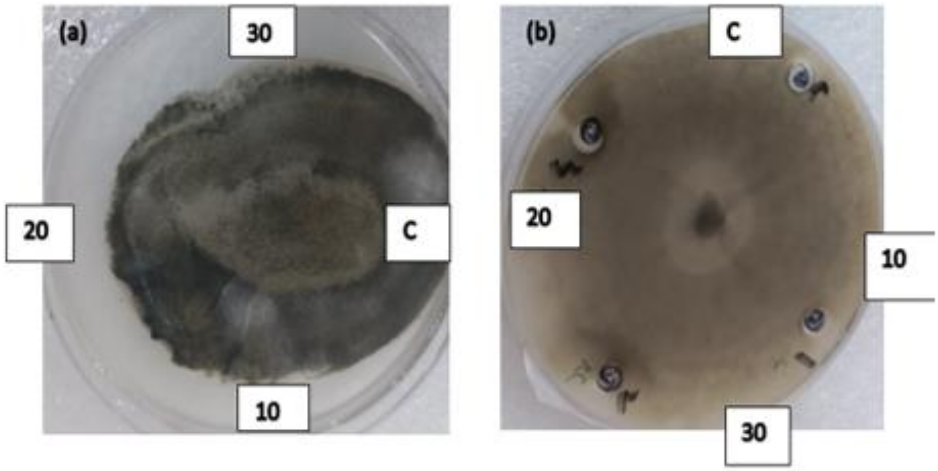

Fig 3.11: Fungal Inhabitation assay by SoRIP1, (a): *Alternaria solani* and different concentrations of purified SoRIP1 in µg (b): *Alternaria alternata* and different concentrations of purified SoRIP1 in µg

Cytotoxicity of SoRIP1 protein

For the cytotoxic effect of the SoRIP1 protein on tumor cells growth, viability assay was done (in vitro). The MCF-7 and HepG-2 cells were treated with SoRIP1 protein at concentrations ranging from 6.25 – 100 µg/mL and MTT test was used to calculate the resultant cell growth inhibition. The data obtained indicated that the SoRIP1 protein harbored cytotoxicity in vitro. It was seen that with increasing concentration of SoRIP1, cell viability get decreased.

With reference to control, the maximum cell proliferation inhibition was seen at conc., of 100 µg/mL of protein (after 72 h incubation). The IC_{50} values of SoRIP1 protein was calculated with Prism software (Graph Pad) by nonlinear regression (Fig. 3.12). It was revealed that SoRIP1 had IC_{50} value of 19.6µg/ml in case of MCF-7 cells and In case of HepG-2, IC_{50} of SoRIP1 was 22.98 µg/mL. Overall, the IC_{50} value of SoRIP1 protein was decreased against MCF-7cells as compared to HepG-2 cells.

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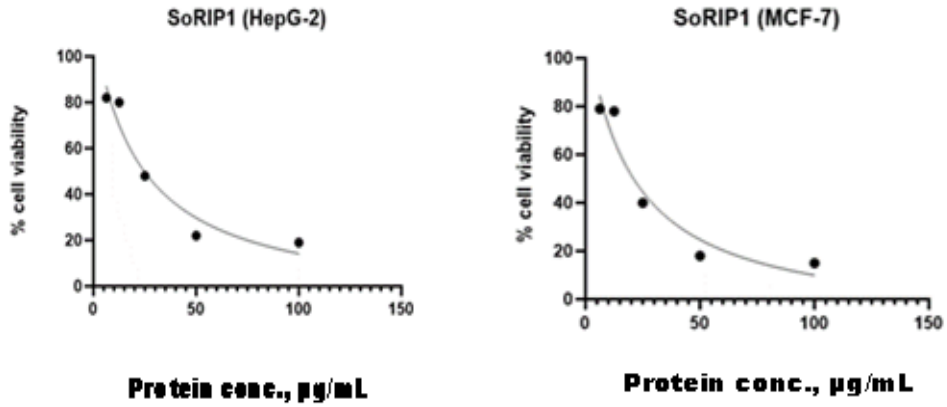

Fig 3.12: The anti- proliferative effect of different concentrations of SoRIP1 on MCF-7 and HepG-2 cell lines after 72 h incubation by MTT test

4. DISCUSSION

SoRIP1 that is a type-1 RIP was isolated from *Spinacia oleracea* (Spinach) and the full length nucleotide sequences of BP31/SoRIP1 protein (accession number AB435548.1) was retrieved from GenBank NCBI (Ishizaki et al., 2002). After that insilico analysis of SoIP1 protein was performed. The physico-chemical properties of SoRIP1 was predicted by protparam server (Gasteiger et al. 2005). One of the important property, the isoelectric point value of protein which shows the lowest solubility of the protein with zero flexibility value in an electro focusing system (Bjellqvist et al., 1993).

The isoelectric point of SoRIP1 protein was 5.44 which indicates its acidic nature. The predicted instability index value of SoRIP1 protein was 30.96 which confirmed their stability. The instability index value 40 or greater than 40.0 confirms the unstable nature of protein (Guruprasad et al., 1990). The GRAVY value shows how protein will interact with water (Kyte and Doolittle 1982). The GRAVY values of SoRIP1 protein was -0.340, which shows hydrophilic nature of the protein (Table 3.1).

For predication of secondary structure features PSIPRED server was used (McGuffin et al., 2000) which suggests that random coils are dominated in SoRIP1 protein (Fig. 3.3). Structure prediction shown in Figure 3.2 was done by I Tasser, Alpha fold and trRosetta. Based upon saves server and Ramachandran plot the best among these three models was the one obtained from I Tasser. (Pearce and Zhang, 2021). The phylogenetic tree made with mega11 revealed that the SoRIP1 is closed to ribosome-inactivating protein from Beta vulgaris and ribosome-inactivating protein Amaranthus tricolor (Fig. 3.4).

COFACTOR predict the major functions of the protein listed in table 3.2. Some of the major functions include its N-glycosidase activity, carbohydrate binding property, negative

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regulation of translation and defense response by changing morphology of other organism. All of these properties of SoRIP1 were verified in-vitro in current study. The plasmid pET29a-SoRIP1 was constructed (Fig. 3.6). The protein expressed by the construct named pET29a-SoRIP1 was recovered by Ni–NTA affinity chromatography (Fig.3.7b).

Western-blotting confirmed the SoRIP1 protein (Fig. 3.7c). The results showed in Fig. 3.8a that acidic aniline treatment of the protein SoRIP1 treated rRNA resulted in a RNA fragment of about size 460bp approximately; this is because of SoRIP1 glycosidase activity. Whereas in control, without SoRIP1 protein treatment, no such RNA fragment released. It means SoRIP1 protein impaired rabbit reticulocyte rRNA through strong N-glycosidase activity. SoRIP1, damages or inactivate ribosomal RNA because of its glycosidase activity as seen in case of many other plant based RIPs. Basically SoRIP1 cleaved the adenine (specific) and pentose glycosidic linkage in 28S rRNA and this produced rRNA fragment when treated with acidic aniline. When treated with Aniline, sensitivity of the phosphodiester bond breakage increased and results in release of adenine. The size of the released rRNA fragment was about 460 nucleotides, same as seen from many other type 1 RIPs (about 450–460 nucleotides) (Nuchsuk et al., 2013).

Along with N- glycosidase activity, Topological inactivation ability was one of the major biological activity of SoRIP1. Upon incubation of plasmid DNA with purified SoRIP1 protein, cleavage of plasmid DNA shows DNase activity of the protein SoRIP1 (Fig. 3.8b). All these results validated that the protein SoRIP1 reserved their inherent biological activities. The protein SoRIP1 was showing hemagglutinating activity as Jc-SCRIP, a type 1 RIP from the seed coat of Jatropha curcas Linn. (Villanueva et al., 2015). In case of SoRIP1, the least agglutinating conc., was 90 µg/mL. When compared to type 2 RIPs, SoRIP1 showed agglutination of human erythrocytes at higher concentrations, this indicates SoRIP1 less toxicity towards the cells (Villanueva et al., 2015).

Antimicrobial and Antifungal activity of RIPs have been proposed by several studies (Iglesias et al., 2016: Shih et al., 1997). In previous studies the antimicrobial activity of RIP1 was not clearly explained yet, whereas a possible mechanism for this purpose can be recommended. TRIP (type 1 RIP) from the leaf of *Nicotiana tabacum* inhibited *Pseudomonas solanacearum* and *Trichoderma reesei*, this is because of its N-glycosidase activity (Sharma et al., 2004). So, SoRIP1 antimicrobial activity may also because of its N-glycosidase action that cleaved microbial rRNA. Many of the antifungal proteins attacked the fungal cell wall and inhibits cell wall synthesis whereas there are some antifungal proteins that causes cell death by attacking cell membrane and cell wall of fungal cells that results in fungal cell death (Morais et al., 2010). A typical RIP, α-Momorcharin, showed antifungal activity towards *F. solani*. The RIP α- Momorcharin not only inhibit mycelial growth but also causes disruption of cell membrane and cell wall of fungus and degradation of genomic DNA of fungus as well (Wang et al., 2016). Additionally, another type 1 RIP BE27 isolated from sugar beet showed antifungal activity towards *Penicillium digitatum* (Citores et al., 2016).

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Although Type I RIPs lack B chain and therefore their capability of entering into the cells was not good but still they can be cytotoxic especially at higher concentrations. Earlier passive mechanisms e.g. Fluid phase uptake were considered as entry mechanism for type 1 RIPs (Stirpe et al., 1992).

Studies showed a close correlation between the intracellular routing of RIPs and the cytotoxicity that depends upon different binding molecules expressing on the cell surface, transport pathways available and ligands sorting to different compartments (Sandvig et al., 2002). SoRIP1 share the shiga/ricin toxic domain (Kawade et al., 2008). SoRIP1 demonstrated different cytotoxicity against both the cell lines MCF-7 and HepG-2. The cytotoxicity of SoRIP1 to MCF-7 cells was higher when compared to HepG-2 cells (Figure 3.12).

A number of reported RIPs show different levels of cytotoxicity towards various cell lines (Villanueva et al., 2015: Li et al., 2016). A RIP named α-Luffin that is a type 1 RIP shows variation of cytotoxicity when tested with three different cell lines that were HepG-2, JEG-3 and MCF-7 (Liu et al., 2010). The SoRIP1(IC_{50} = 19.6 µg/mL) is little less toxic as compared α-Luffin when tested on MCF-7 cells (IC_{50} < 10.42 µg/mL) (Liu et al., 2010). Cytotoxicity of SoRIP1 is less when compared to type 2 RIPs e.g. pebulin (IC50 < 0.1 µg/mL) (Rezaei-Moshaei et al., 2021).

5. CONCLUSION

In this study, we reported antimicrobial, antifungal and cytotoxic properties of SoRIP1 protein. The SoRIP1 was purified by Ni–NTA affinity chromatography and reserved its inherent biological activities such as topological inactivation and glycosidase activity. It also shows hemaglutination activity. Hence SoRIP1 is a potential candidate to be used in agriculture and medicine.

Conflicts of interest:

All contributing authors declare no conflicts of interest.

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University of Punjab.

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