



# Novel Ribosome-inactivating Protein (RIP) Isolated from *Trichosanthes dioica* Induces Apoptosis in HeLa Cell Line

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## Abstract

Ribosome-inactivating proteins (RIPs) are toxic N-glycosidases that depurinate eukaryotic and prokaryotic rRNAs and thus interrupt protein synthesis during translation. In the present study, a protein of around 32 kDa, supposedly a RIP isolated from *Trichosanthes dioica*, was assessed for its potential to induce apoptosis in HeLa cells. Cell viability assay was done to measure cell proliferation and survivability. It was observed that cells viability decreased with the increase of decrease in dilution, i.e. when the sample was an undiluted one, the viability decreased drastically and almost came to less than 10%. To further check whether the isolated RIP could induce apoptosis, HeLa cells were treated with the test RIP. Immunoblotting was carried out using PARP poly (ADP-ribose) polymerase (PARP-1), a 113 kDa nuclear enzyme, which is considered a hallmark of cells undergoing apoptosis. HeLa cells were further analyzed for loss of mitochondrial membrane potential with JC-1 dye, which is an early event during apoptosis. Increased PARP breakdown in the RIP treated cells indicates that cells undergoing apoptosis and progressive loss of red J-aggregate fluorescence indicate that the isolated RIP from *Trichosanthes dioica* induces apoptosis in HeLa cells. The ability of apoptosis induction is comparable to another known RIP from *Momordica charantia*, which was used as a positive control. Promising results from the present study warrants the isolated RIP to be further explored for anticancer activities.

**Keywords:** *Trichosanthes dioica*, ribosome-inactivating protein, HeLa cells, apoptosis

## 1. Introduction

Plants from ancient times are used by all cultures as well as the source of medicines for health benefits. It has been estimated that approximately 80-85% of the world's population relies on traditional medicines mainly for their healthcare needs, and it is believed that a large part of traditional therapy requires the use of plant extracts or their active principles (Ignacimuthu et al., 2006; Tomlinson and Akerele, 1998). Numerous researches have been done and still going on to investigate the possible properties and uses of terrestrial plant extracts to prepare new plant-based medicines for diseases like cancer. Though a number of recent

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studies have been performed for improvements in cancer progression diagnosis and control, but still significant work and room for improvement remain. The biggest drawbacks of synthetic drugs are the side effects associated with them. The search for anticancer agents from plant sources started in the 1950s when the discovery and development of the vinca alkaloids (vinblastine and vincristine) and the isolation of the cytotoxic podophyllotoxins was carried out (Gordon and David, 2005). This led to the discovery of many novel chemo-types showing a range of cytotoxic activities (Cassady and Douros, 1980), including the taxanes and camptothecins, but the development of these into clinically active agents took around 30 years, i.e., from the early 1960s to the 1990s. Some plant families, such as Cucurbitaceae, are sources of proteins such as ribosome-inactivating proteins (RIPs) that have shown promising effects against microbes and are also being investigated for anticancer activity. This could also play a significant role in blocking the spread of certain pathogens. RIPs fall into the category of proteins that are studied for their selective toxicity. Some researchers indicated that the toxicity was attributable to the protein's ability to inhibit protein synthesis by inactivating ribosomes by cleaving the N-glycosidic bond at the 28S RNA position A324 (Li et al., 1997).

There are three types of RIPs reported until now, namely type 1, type 2, and type 3. Type 1 RIPs are monomeric enzymes with a molecular weight of around 30 kDa. Some examples include antiviral protein PAP, saporin (from soapwort, *Saponaria officinalis* L.), and bRIP (from barley, *Hordeum vulgare*) translation inhibitor (Asano et al., 1984; Barbieri et al., 1993; Irvin et al., 1994). Most of the RIPs which have been reported to date are type 1 RIPs (Asano et al., 1984). These RIPs are basic proteins and have been found to share several highly conserved active cleft residues and secondary structure within the active site region (Barbieri et al., 1993; Husain and Tickle., 1994; Mlsna et al., 1993; Monzingo and Robertus, 1992). Type 2 RIPs are heterodimeric proteins, i.e., it has two subunits. One is having enzymatic and the other having lectin properties. Each subunit has a molecular weight of approximately 30 kDa. Ricin and abrin are examples of type 2 RIP (Olsnes and Pihl, 1973; Stirpe et al., 1978). Type 3 RIPs are mostly inactive precursors (proRIPs) that require the occurrence of proteolytic processing events between amino acids involved in active site formation and are far less prevalent than type 1 or type 2 RIPs (Mundy et al., 1994). Most type 3 RIPs have been characterized from maize and barley (Bass et al., 1992; Chaudhry et al., 1994; Reinbothe et al., 1994; Walsh et al., 1991).

Plant proteins like RIPs are explored for potential drugs against many diseases. A plant-based approach for treatment is a necessity for developing countries like India and may pave the way for cheaper and better treatment for many diseases. Also, the side effects of plant-derived drugs have been found to be way less or minimal as compared to synthetic ones.

Additionally, some of the properties of RIPs, such as antiviral and anticancer can be used to develop drugs for certain specific viruses or tumour cells against which drugs are yet to be discovered or to improve existing ones by using RIPs as immunotoxins in immunotherapy (Griffiths et al., 1987). Some species of the Cucurbitaceae family are yet to be explored for new RIPs that could help better understand the mode of action of these proteins and will also prove to be more effective. RIPs like Balsamine from *M. balsamina* seeds are being tested for their DNase activity and their antioxidant properties (Ajji et al., 2010).

In the present study, a RIP isolated from *T. dioica* of the Cucurbitaceae family was assessed for its potential to induce apoptosis in HeLa cells. This may pave the way for the exploration of these proteins for potential use as anticancer agents in the future.

## 2. Materials and Methods

The experiments were conducted during the period of 2018 to 2019.

### 2.1. MTT assay

The effect of the RIP on the viability of HeLa cells was tested by using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. HeLa cells were seeded in 96-well flat-bottom tissue culture plates at a density of  $10^5$  cells/well (per 100  $\mu$ l) containing 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C in 5% CO<sub>2</sub>. After 16 h, the media was discarded, and each well was washed with phosphate-buffered saline (PBS) followed by the addition of RIP to each well at different concentrations with media. After 24 h treatment, 10  $\mu$ l of MTT solution (5 mg ml<sup>-1</sup>) was added to each well, and the plates were incubated for another 4 h. MTT containing media was removed from each well, and 100  $\mu$ l of DMSO was added, dissolving the formazan crystals. The absorbance was recorded in a microplate reader (Multiskan™ GO) at the wave length of 570 nm. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated cells (Freshney, 2010).

### 2.2. Study of apoptosis

Cultured HeLa cells were grown on 6-well plate and treated with 30  $\mu$ g of isolated RIP RIP from *M.charantia* was used as a positive control. After 24 h, cells were washed with PBS. 100  $\mu$ l of sodium dodecyl sulfate (SDS) loading dye was added to each well for immediate cell lysis and increased viscosity of the sample. The extract was transferred to a microcentrifuge tube and heated at 95°C for 10 min. Total cell protein was separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked in phosphate-buffered saline 5% dry milk for 2 h and incubated with primary antibody overnight at 4°C with shaking. The membranes were washed several times



with phosphate-buffered saline/Tween-20 and incubated with horseradish peroxidase (HRP)-labelled secondary antibody for 1 h at room temperature with shaking. Band intensity was detected by ECL detection reagent (Biorad) with  $\beta$ -actin as an internal control.

Also, further to confirm apoptosis, analysis of mitochondrial membrane potential was done using 5,5,6,6'-tetrachloro-1,1',3,3' tetraethyl benzimidazolyl carbocyanine iodide (JC-1) dye. HeLa cells were seeded in 6 well plates at a density not exceeding  $10^6$  cells/cm containing 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37°C in 5% CO<sub>2</sub>. After 16 h, the media was discarded, and each well was washed with phosphate-buffered saline (PBS) followed by the addition of the RIP test sample to each well at 30  $\mu$ g concentration. RIP from *M. charantia* was used as a positive control. After 24 h, 2  $\mu$ M (final concentration) of JC-1 dye was added to each well and incubated at 37°C, 5% CO<sub>2</sub> for 15-30 min. Cells were then washed with PBS and observed under a fluorescence microscope (Floyd Cell Imaging Station, Thermo Fischer Scientific) for the progressive loss of red J-aggregate fluorescence and cytoplasmic diffusion of green monomer fluorescence.

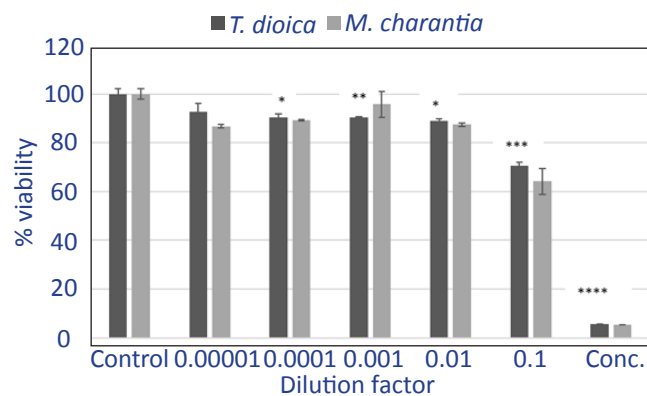
### 3. Results and discussion

#### 3.1. MTT test

The MTT test, which was performed against HeLa cell lines, showed a decrease in viability with an increase in the concentration of the test protein. The viability decreases as low as around 5% when test protein of 30  $\mu$ g concentration was used. MTT test is a routine assay to study the viability and thus determine the anticancer property of compounds. Results obtained from our study suggests that the test RIP can be a potential anticancer agent. Thus, MTT assay demonstrated that the RIP from *T. dioica* significantly affects HeLa cell lines and thus proves that it has anti-cancer activity against HeLa cell lines too. In addition, a known RIP from *M. charantia* was used in this analysis as a positive control, which helped to explain or compared the efficacy of the test RIP on different parameters with another RIP. (Figure 1).

#### 3.2. Apoptosis

During apoptosis, the poly (ADP-ribose) polymerase (PARP-1), a 113 kDa nuclear enzyme, is cleaved into 89 and 24 kDa fragments. This cleavage has become a hallmark of apoptosis, and DEVD-ase caspases, a family of proteases activated during apoptosis, have proven to be doing so. This property of PARP has been used in the present study to prove that the protein being studied induces apoptosis (Oliver et al., 1998). Apoptosis study by PARP cleavage analysis demonstrated apoptosis of the HeLa cell line when treated with the isolated RIP. The result showed that both the positive control and the isolated RIP could lead to apoptosis, and the isolated RIP could lead to apoptosis as good as the positive control (Figure 2).



\*\* $p < 0.0005$ ; \*\* $p < 0.001$ ; \* $p < 0.005$

Figure 1: Viability of isolated RIP in HeLa cells treated with varying concentrations for 24 h. RIP from *M. charantia* was used as a positive control. Values are expressed as mean  $\pm$  SEM

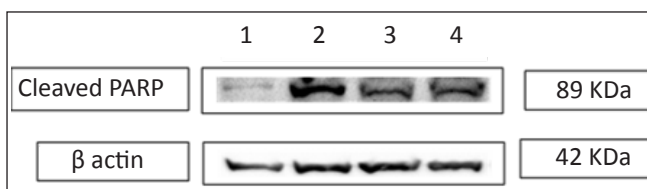


Figure 2: Immunoblotting of cleaved PARP gene proving apoptosis. Lane 1 is mock control. Lane 2 is a positive control. Lane 3-4 are two different concentration of isolated test RIP.  $\beta$ -actin was used as an internal control

Also, the study of the mitochondrial membrane potential with the help of JC-1 (Sivandzade et al., 2019) dye further gave a clear result of cells undergoing apoptosis on treating with the isolated RIP to that of the negative control. Here, a decrease in the red (~590 nm)/green (~529 nm) fluorescence intensity ratio was observed for both test sample and positive control. This decrease in intensity is indicative of depolarization/disruption of the mitochondrial membrane i.e. apoptosis (Figure 3).

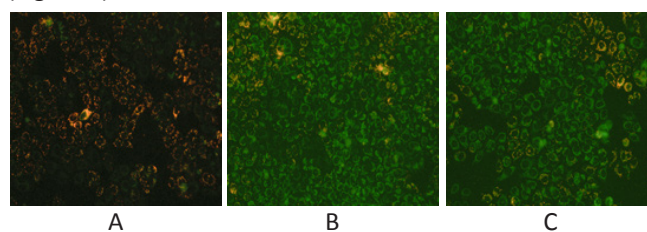


Figure 3: Assessment of mitochondrial depolarization/disruption using JC-1 dye. A. Mock control; B. Positive control (RIP from *M. charantia*); C. RIP sample (isolated from *T. dioica*). A decrease in the red (~590 nm)/green (~529 nm) fluorescence intensity ratio is indicative of depolarization/disruption of the mitochondrial membrane, i.e. apoptosis

Both the studies for apoptosis in HeLa proved that the isolated protein is capable of inducing apoptosis in HeLa cells. It is worth mentioning that, unlike most previous investigations, a known RIP from *M. charantia*, which was used in the present study as a positive control, helped to explain or compare the

efficacy of the test protein on different parameters with that of a known RIP.

As stated earlier, RIPs derived from natural sources can be used to treat many diseases for which treatment is still not available or for which there are only synthetic medicines. Hence, this RIP isolated from *T. dioica*, which demonstrated the ability to induce apoptosis in HeLa cells, could be useful for further studies in many areas like antimicrobial, anti-viral etc. Also, various parts of *T. dioica* could be investigated for more such RIPs, as before this present study *T. dioica* has not been well investigated for RIPs except the one published study of a 55 kDa lectin TDSL (Ali et al., 2004).

The results of the present study will pave the way for further analysis of the protein thus isolated. Also, other uses such as the use of RIP as immunotoxin may also have the potential to be explored in the future following protein structural analysis. Many new plant species should also be explored for new RIPs, such as a recently isolated type-I RIP from an ornamental plant *G. elegans* (Kokorina et al., 2019).

#### 4. Conclusion

The RIP from *T. dioica* affects the viability of HeLa cell lines which is comparable to that of another RIP, i.e. *M. charantia*. Also, the present study proves that the test RIP has the ability to induce apoptosis, and further research and exploration of this protein or related RIPs may one day aid in the development of a potential cancer drug.

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