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CYTOTOXICITY AGAINST TUMOR CELL LINES OF A PURIFIED MIRABILIS ANTIVIRAL PROTEIN ISOLATED FROM ROOT OF *MIRABILIS JALAPA*

Dipti Kumar Chandraprabha Kale* and Usha Mukundan

Department of Biotechnology, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai 400086.

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***Correspondence for Author**
Dipti Kumar Chandraprabha Kale
Department of Biotechnology, Ramniranjan Jhunjhunwala College, Ghatkopar (W), Mumbai 400086.

ABSTRACT

Background: Root extract of *Mirabilis jalapa* (Nyctaginaceae) contain an antiviral protein which is type I Ribosomal inactivating protein (RIP), named as Mirabilis antiviral protein (MAP). **Objective:** The aim of the study was to isolate, purify, identify and study its potential application as an anticancer agent. **Methodology:** The protein was purified to homogeneity from roots of *Mirabilis jalapa* by ammonium sulfate precipitation and ion exchange chromatography with CM-Sepharose CL-6B column. The purified protein was identified with MALDI-TOF followed by MASCOT-search. Cytotoxic effect of purified protein against three cancer cell lines, namely MCF-7, A549, HCT 116 and Vero cells as normal cells by MTT assay was studied. **Results:** The list of selected ion peaks from the mass spectrum of the purified trypsin digested product was used to compare the result with database. The MASCOT software identified Mirabilis Antiviral Protein (MAP) as the most likely protein. The *in-vitro* anti-proliferative activity studies proved that HCT116, MCF7 and A549 cell lines were inhibited significantly with increasing of drug concentration. IC_{50} of MAP was observed to be 150 µg/ml, 175 µg/ml, 200 µg/ml to HCT116, MCF-7, A549 respectively. **Discussion:** There may be differential expression of MAP receptor type on the different cells used in the present study, which account for the difference in cytotoxic response against cancer cell lines. The cytotoxicity mechanism of isolated RIP from *M. jalapa* could be due to apoptosis. **Conclusion:** The present study showed that the plant *M. jalapa* contains MAP; Type I RIP. This could be utilized as therapeutic agents against cancer.

KEYWORDS: - Mirabilis Antiviral Protein, Ribosome Inactivating Protein.

INTRODUCTION

Traditionally, plants are used as source of treatment of diseases in different parts of the world and their use contributes significantly to primary health care delivery.[8,14,15] They are regarded as invaluable sources of pharmaceutical products like phytochemicals and protein source.[26]India is rich with natural biodiversity, and thousands of plants are used in folk medicine. One of them is *Mirabilis jalapa*, which is commonly known as Four o'clock plant. This plant has been utilized for traditional medicine in many countries for years. Leaves and roots of this plant were reported to contain a protein.[18] This protein belongs to Ribosome Inactivating Proteins (RIPs) that are widely distributed in higher plants, and hold promise for agricultural and pharmaceutical application.

Ribosome Inactivating Proteins (RIPs) are a group of plant enzymes that possess capability to inactivate ribosomes by modifying the 28S rRNA through their N-glycosidase activity, which is manifested by cleavage of the N-glycosidic bond at a specific adenine. The adenine at position 4324 of rat liver28S rRNA and corresponding adenine on other eukaryotic or prokaryotic models are the target sites.[34]Through this mechanism, the binding of elongation factor 2 is prevented, with the consequent arrest of protein synthesis leading to autonomous cell death.[11,12,29] They were thus classified as rRNA N-glycosidases (EC 3.2.2.22). Subsequently it has been observed that some RIP release more than one adenine from ribosomes, others act on RNA species apart from ribosomal, and on poly(A), and all RIP release adenine from DNA.Thus the enzymatic activity of RIP was defined as polynucleotide:adenosine glycosidase.[2,3]

RIPs from plants have been classified into three main types: Type I is composed of a single polypeptide chain of approximately 30 kDa, Type II is a heterodimer consisting of an A chain, functionally equivalent to the Type I polypeptide,[28] linked to a B subunit, endowed with lectin-binding properties,[23] while Type III are synthesized as inactive precursors (ProRIPs) that require proteolytic processing events to form an active RIP[30] and are not in use for therapeutic purposes.

Leaves and roots of the native Peruvian plant *M. jalapa*[24] were found to contain an antiviral protein which was active against the mechanical transmission of certain plant viruses, [16,18,35,36, 37] and the highest activity was found in roots.[18] This protein, is Type I RIP, named as Mirabilis Antiviral Protein (MAP).[13] MAP was purified to homogeneity and was revealed

to be lysine rich and basic, with a molecular weight close to 24.2 kDa as determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).[35]

RIPs are currently under study as therapeutic agents against cancer.[17] Potential applications of RIP isolated from *M. jalapa* was found to have cytotoxic activity on HeLa cell line,[33] and showed high cytotoxicity against T47D and SiHa cell lines with different extent and relatively less cytotoxic to mononuclear cells.[39] In other study they had demonstrated that the death of HeLa cells caused by the MAP protein fraction was due to induction of apoptosis, while on Raji cell-line was due to non-apoptosis way, presumably via necrosis.[40]

In this study, we investigated the cytotoxic effect of purified protein fraction isolated from *M. jalapa* root against three cancer cell lines, namely human breast cancer MCF-7, human lung cancer A549, human colon cancer HCT116 and Vero cells as normal cells.

MATERIALS AND METHODS

Plant material

*M. jalapa*plants were collected from Maharashtra, India. Fresh root materials from pink, yellow, orange and white color flower plant were used for extraction of protein.

Extraction of protein

A modified protein extraction protocol was used.[32] Root extracts were prepared by homogenizing 50 g of fresh tissue in 100 ml of ice-cold extraction buffer (25 mM Sodium phosphate buffer pH 7.0, with 250 mM NaCl, 10 mM EDTA, 10 mM Thiourea, 5 mM DTT, 1 mM PMSF and 1.5 % PVP). The recovered supernatant was filtered through muslin cloth and centrifuged at 10,000 rpm for 30 min at 4^{o}C, and subsequently precipitated in 80 % (w/v) ammonium sulfate at 4^{o}C for 1 hr with continuous stirring. The solution was centrifuged for 30 min at 10,000 rpm. The pellet was suspended in 25 mM Sodium phosphatebuffer pH 7.0, containing 50 mM NaCl and then dialyzed against 25 mM Sodium phosphatebuffer pH 7.0, until it was free from the sulphate ions. The extract was filter sterilized (0.22 μm; Millipore, Bedford, MA). The ammonium sulphate fraction was stored at –20°C until use.

Estimation of total protein content by Layne method

Total protein content was determined by measuring absorption at 280 nm and 260 nm as described by Layne.[20] And protein concentration was calculated with the help of following formula.

Protein concentration (in mg / mL) =(1.55 x A280) - (0.76 x A260)

Protein purification by Cation-exchange chromatography

The proteinaceous metabolite was purified in two steps by use of cation-exchange column chromatography. CM Sepharose CL-6B (Sigma, St. Louis, MO) packed in a polypropylene column (2.5 × 10 cm) was equilibrated with 25 mM Sodium phosphate buffer pH 6.0. Ammonium sulphate fraction was then loaded on to the column and incubated for 30 min. After this the column was washed with the same buffer.The bound proteins were eluted by a linear NaCl gradient (0 to 0.4 M) in 25 mM Sodium phosphate buffer pH 6.0 with the flow rate of 1 ml/min. The presence of protein in all fractions was assayed by Bradford's estimation[4] followed by SDS-PAGE.[19] The fractions which had protein with a molecular weight approximately 24 kDa were pooled, dialyzed against 25 mM Sodium phosphate buffer pH 7.0 and concentrated by lyophilisation.[38]

The lyophilised content was dissolved into 500 µl of 25 mM Sodium phosphate buffer pH 7.0 containing 50 mM NaCl, and again purified by same procedure as mention above. The bound protein was eluted by a linear NaCl gradient (0 to 0.3 M) in 25 mM Sodium phosphate buffer pH 6.0 with the flow rate of 1 ml/min. The presence of protein in all fractions was assayed by Bradford's estimation[4] followed by SDS-PAGE.[19] The fractions which showed protein with molecular weight approximately 24 kDa were pooled, dialyzed against 25 mM Sodium phosphate buffer pH 7.0 and concentrated by lyophilisation.[38]

In-gel tryptic digestion

Trypsin digestion was done according to the method of *Lazarev et al.*[21] with some modifications. Purified protein was resolved on a 10% SDS-PAGE and visualized by CBB R-250.[4] The protein bands were excised from the gel, sliced into 1 mm^3 cubes and incubated overnight at room temperature with 200 µl of 50 mM NH_4HCO_3 in 40% ethanol. Stain was removed and replaced with 50 µl of 50 mM NH_4HCO_3. Subsequently, NH_4HCO_3 buffer was removed and replaced with 150 µl of acetonitrile and incubated for 10 min. This step was repeated till it showed complete decolourizations, the gel pieces were dried for a few minutes. 20 µl of fresh trypsin solution (12.5 ng/µl) in 50 mM NH_4HCO_3 buffer was added to the tube on ice. Excess enzyme solution was discarded after 10 min of rehydration and the solution was replaced with an equivalent volume of fresh 25 mM NH_4HCO_3 buffer, followed by incubation for 12 hr at 36°C. The digestion was terminated by addition of 10 µl of 1% TFA for MALDI-TOF MS analysis.[21]

Sample preparation for MALDI-TOF MS

20 µl of trypsin digested purified protein solution was used for MALDI-TOF MS analysis, recorded on an AB SCIEX Voyager DE Pro MALDI-TOF (Applied Biosystems, Foster City, CA) time-of-flight spectrometer, with a pulsed nitrogen laser (337 nm; 3-ns pulse width). The spectra were recorded in the linear, positive high-mass mode. A saturated solution of $C_{10}H_7NO_3$ in a 1:1 mixture of $C_3H_6O$and H_2O along with 0.1 % TFA was used for obtaining the mass spectra.[10]

Peptide mass fingerprinting search

For identification of the protein fragments, selected peptide masses were submitted to MASCOT (http://www.matrixscience.com) for SwissProt databases search.[25]

Cytotoxicity Assay

Subcultures of MCF-7, A549, HCT116 and Vero cells in Dulbecco's Modified Eagle's Medium (DMEM) were trypsinized separately, after discarding the culture medium, to the disaggregated cells, 25 ml of DMEM with 10% FCS was added individually. 1 ml of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentration of lyophilized sample (0 to 250 µg/ml) and incubated at 37°C in a humidified CO_2 incubator with 5 % CO_2. After 48 hr incubation the cells were observed under an inverted tissue culture microscope. With 80% confluence of cells cytotoxic assay was carried out.[1] After 48 hr incubation, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added in all wells and incubated for 3 hr at room temperature. The content from all wells were removed and 100 µl SDS in DMSO was added to dissolve the formazan crystals, absorbances were read in Lark LIPR-9608 micro plate reader at 540 nm.[1]

RESULT

Total protein content of *M. jalapa*

Roots from different flower colour of *M. jalapa*viz. pink, white, yellow, orange were used for extraction of MAP. The total protein concentration was estimated by Layne method as follows.

Table 1:- Total protein concentration in all varieties as estimated by Layne method.

Sr. No.	*M.jalapa* varieties	Conc. of total protein (mg/ml)
1	White	11.6
2	Pink	12.3
3	Yellow	23.8
4	Orange	9.7

A yellow flower trait of *M. jalapa* showed highest concentration of total protein, therefore it was further utilized for protein purification. This difference may be due to the cultivar, since the growing environmental conditions of the all plants were same.

Protein purification by Cation-exchange chromatography

Mirabilis Antiviral Protein was purified to homogeneity in two steps by using CM-Sepharose-CL- 6B columns. The elution of bound protein on CM-Sepharose CL-6B-column one was eluted with alinear (0 to 0.4 M) gradient of NaCl in 25 mM Sodium phosphate buffer pH 6.0 (Fig.1-A). The first step of purification resulted in one major peak and few minor peaks were observed. Fractions 22-28 eluted at around 0.15 M NaCl showed a single band in SDS-PAGE, and pooled fractions sample was migrated at a position of about 24 kDa as revealed by SDS-PAGE analysis (Fig.1-B). In second step of purification the elution of bound proteins on CM-Sepharose CL-6B-column two was eluted with a linear (0 to 0.3 M) gradient of NaCl in 25 mM Sodium phosphate buffer pH 6.0 (Fig.2-A). This purification step removed the minor proteins, resulting in homogeneity of protein. Fractions 15-21 eluted at around 0.15 M NaCl showed single major peak and pooled fraction sample was migrated at a position of about 24 kDa as observed in SDS-PAGE (Fig.2-B).

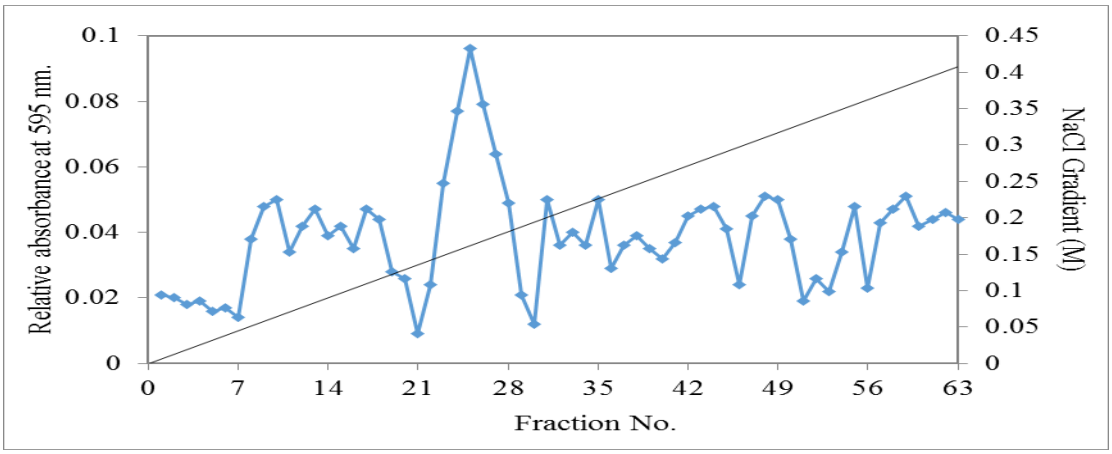


Fig.1-A: -CM-Sepharose CL-6B column chromatography (Column-1) of the crude extract from roots of *M. jalapa*. Protein was eluted at the fractions (♦): Relative absorbance at 595 nm; NaCl gradient (M).

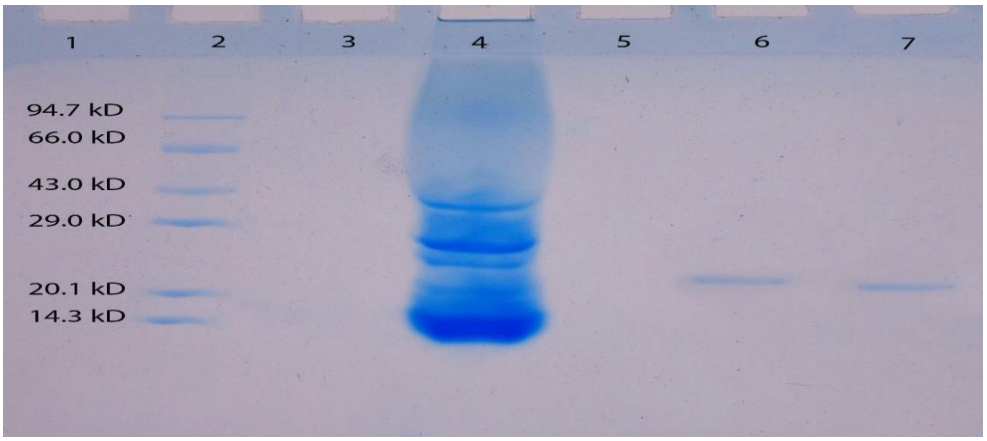


Fig.1-B: - SDS-PAGE profiles of fractions.
Lane 2- Molecular weight marker
Lane 4- Crude extract of *Mirabilis jalapa*
Lane 6 &7-Pooled purified protein from 22-28 fractions.

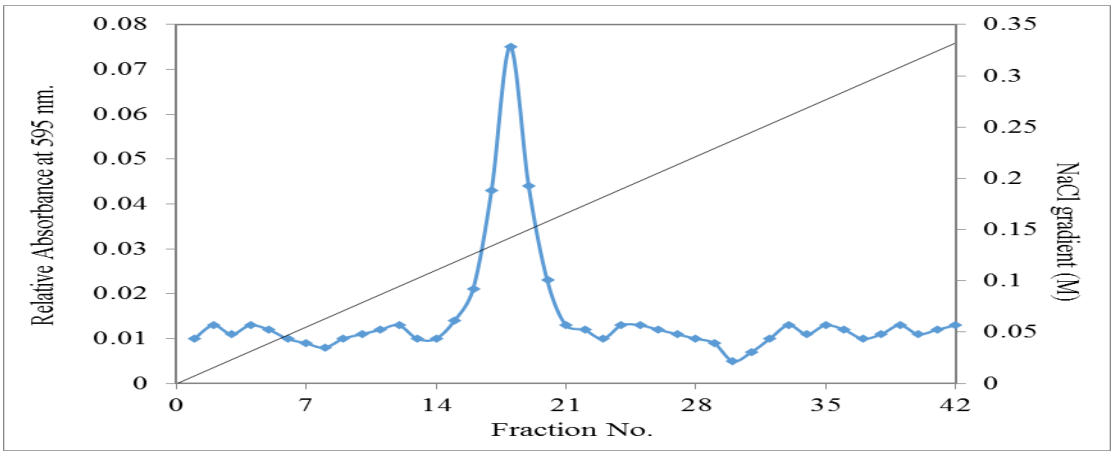


Fig.2-A: -CM-Sepharose CL-6B column chromatography (Column-2) of the purified protein from column 1. Protein was eluted at the fractions (♦): Relative absorbance at 595 nm; NaCl gradient (M).

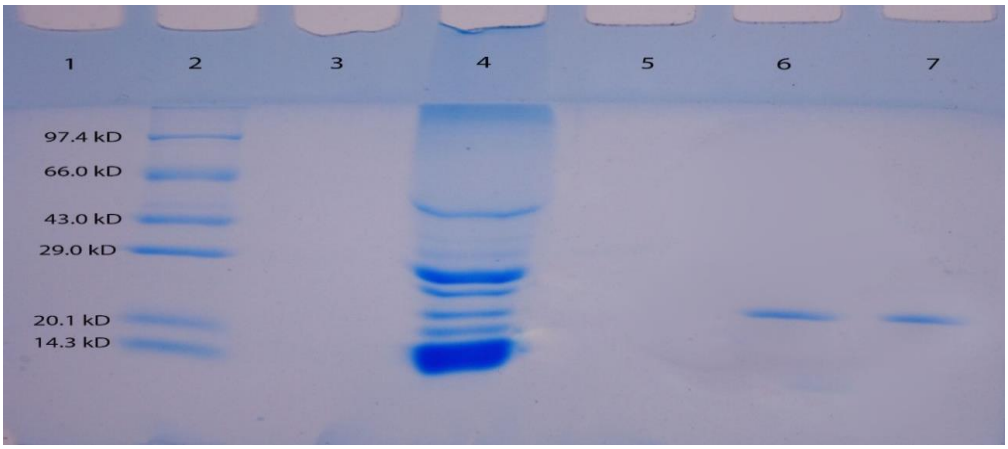


Fig.2-B: -SDS-PAGE profiles of fractions.
Lane 2-Molecular weight marker
Lane 4- Crude extract of *Mirabilis jalapa*
Lane 6 &7 - Pooled purified protein from 15-21 fractions.

MALDI-TOF

In-gel trypsin digestion was performed and extracted sample was utilized for MALDI-TOF analysis. The mass of peptides produced by the tryptic digestion of protein was measured by mass spectrometry with high accuracy (known as a peptide mass fingerprint). The list of selected ion peaks from the mass spectrum of purified digested product was used to compare the result with database. The MASCOT software identified Mirabilis Antiviral Protein as the most likely protein.

In vitro cytotoxicity

Purified RIP from *M.jalapa* showed different levels of cytotoxic activity against MCF-7, A549 and HCT116 cell lines, and very less toxic against Vero (Fig.8).It revealed that in 24 hr of treatment, increased MAP concentration significantly inhibit cancer cells lines as compared to normal cell line (Table 2). It was recorded that; IC_{50} of sample was 175 µg /ml, 200 µg/ml and 150 µg /ml to MCF-7, A549 and HCT116, respectively. About 87.47% of cell viability was seen in Vero cell at the maximum tested concentration, 250 µg/ml. This effect is due to the drug having more specific activity against cancer cell line (Fig.4-7).

Table 2:-In vitro cytotoxicity assay of MAP against HCT116, MCF-7, A549, and VERO Cell lines.

MAP Conc. (µg/ml)	% Cell Viability			
	HCT116	MCF-7	A549	VERO
0	100	100	100	100
25	97.24	98.35	99.24	99.61
50	88.64	93.74	96.38	98.71
75	79.24	87.68	93.67	97.24
100	67.95	79.59	88.92	95.96
125	58.94	70.23	79.54	94.58
150	**49.68**	64.95	70.65	92.37
175	40.95	**48.32**	59.65	91.01
200	34.92	42.71	**51.27**	89.68
250	26.39	36.92	42.36	87.74

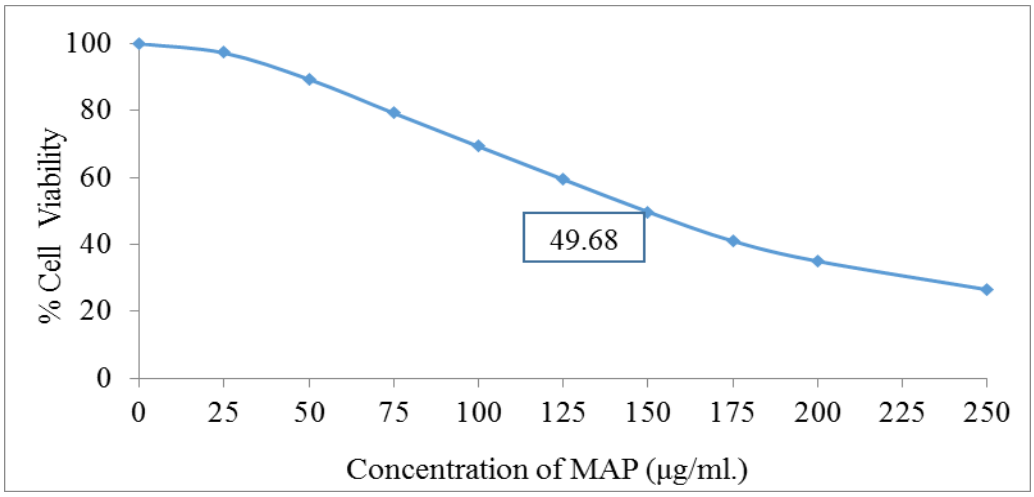


Fig.4:- Effect of different concentrations of Mirabilis Antiviral Protein on HCT116 cells line.

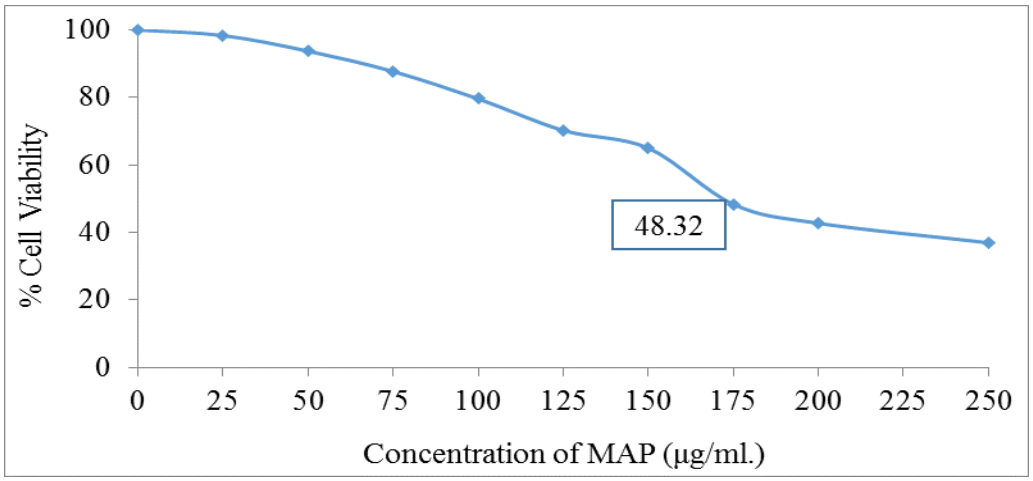


Fig.5:- Effect of different concentrations of Mirabilis Antiviral Protein on MCF-7 cells line.

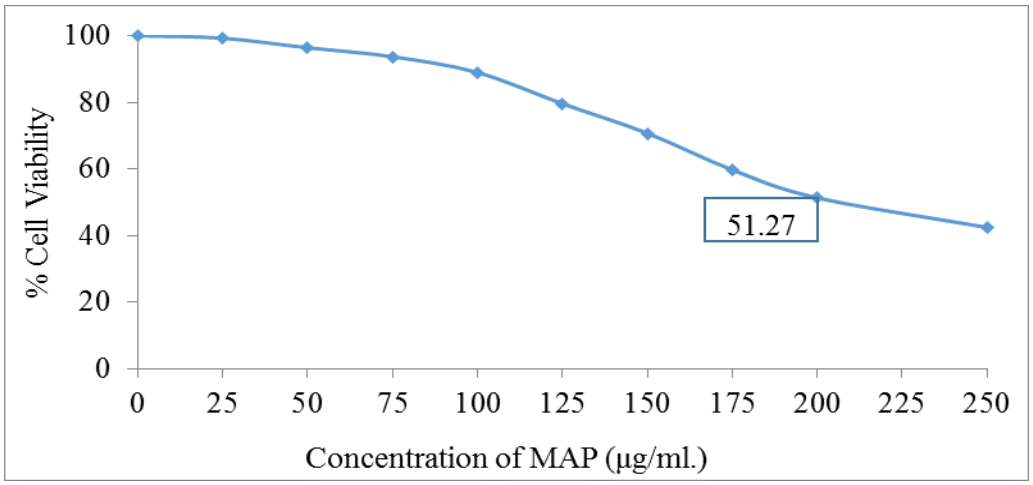


Fig.6:- Effect of different concentrations of Mirabilis Antiviral Protein on A549 cells line.

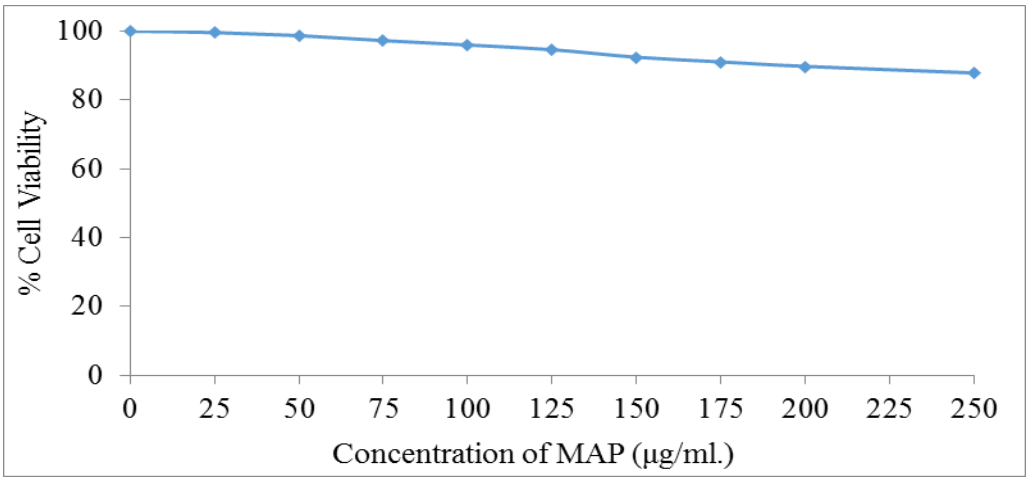
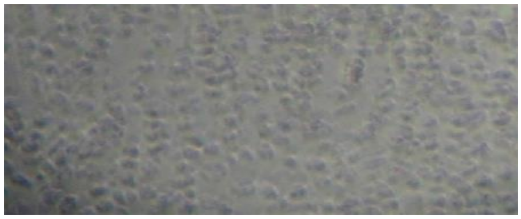
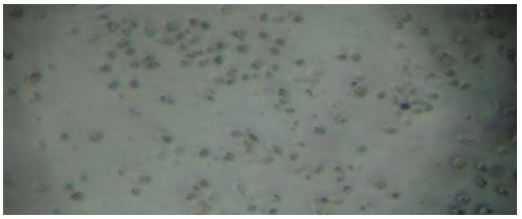
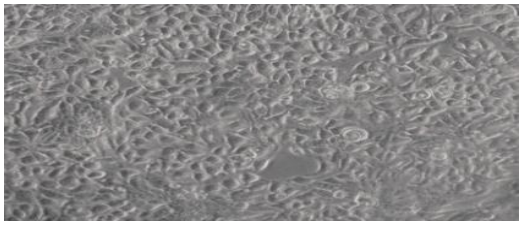
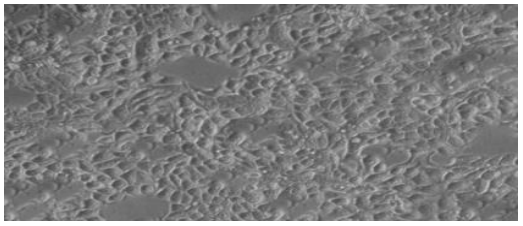


Fig.7:- Effect of different concentrations of Mirabilis Antiviral Protein on Vero cells line.


Control HCT116 cells line


Treated HCT116 cells line- IC_{50}


Control MCF7 cells line


Treated MCF7 cells line- IC_{50}


Control A549 cells line


Treated A549 cells line- IC_{50}


Control Vero cell line


Treated Vero cells line at 250 µg/mL

Fig.8:- Cytotoxic effect of Mirabilis Antiviral Protein against HCT116, MCF-7, A549, Vero cell lines.

DISCUSSION

The extraction, purification and In-gel digestion coupled with modern mass-spectrometric analysis is a powerful tool for analysis of protein. The results of this study showed that root of *M. jalapa* contain MAP which belongs to RIP family. The MAP from *M. jalapa* root showed different level of cytotoxic effect against MCF-7, A549 and HCT116 cell lines, but Vero cells were comparatively less sensitive.

The difference in cell type may account for the difference in cytotoxic response against anti-cancer agents. In case of RIP, variation of cytotoxicity against different cells was alsoobserved in another RIP, trichosantin.[7] It was observed that three different cell types IC21, JAR and Vero cell lines, which were shown to be high, medium and low sensitivity to trichosantin.[7] A good relationship was demonstrated between intracellular trichosantin concentrationand toxicity. It seemed that variation of cytotoxicity of RIPs in different cells may be depending upon the mechanisms affecting its internalization. This phenomenon may also be applied generally for other RIPs, including RIP isolated from *M. jalapa*. However, some observations, such as the difference in sensitivity of Type I RIPs among different cell types, and the organ-specific toxicity of Type I RIPs, indicate a specific mechanism for the entry of these proteins into target cells.[6] The receptor responsible for the binding and endocytosis of RIP is α2-macroglobulin receptor (α2MR).[6,7]All the human tumour cell lines so far examined express α2-macroglobulin receptor at different levels from ~300 to ~10000 sites per cell.[22,31]Despite the lack of data concerning with the expression of α2-macroglobulin receptors on various types of cells, it is assumed that there may be differential expression of RIP receptor types on the different cells used in the present study; therefore difference in cytotoxicity against different cells was observed.

Other possibility that may explain the different cytotoxicity of the RIPs toward different kind of cells is difference in the downstream death pathways. However, beside inhibition of protein synthesis, RIPs have been shown to induce apoptosis or programmed cell death.[27, 40]The p53 is a tumour suppressor gene, encode a protein which triggers repair of the damage or induces apoptosis.[5]In this study we had used MCF-7, A549 and HCT 116 which have wild type p53,[5,9] therefore the cytotoxicity mechanism of isolated RIP from *M .jalapa* could be due to apoptosis.

CONCLUSION

In this study we reported that an anticancer substance in roots of *M. jalapa* was purified to homogeneity by ammonium sulfate precipitation and cation exchange chromatography CM-sepharose CL-6B and was revealed to be a RIP with nominal mass (Mr) 31 kDa by MALDI-TOF analysis followed by MASCOT search. This purified RIP from *M. jalapa* showed different levels of cytotoxic activity against MCF-7, A549 and HCT116 cell lines, and very less toxic against Vero. The variation of sensitivity of these tumor cells employed in our study against RIP is not fully understood, however, these results give insight to development of anticancer agent derived from RIP for certain type of cancers.

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