

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Website: www.ijirset.com

Vol. 6, Issue 6, June 2017

In vitro Assay of Antimitotic and Apoptosis Effect of Low Molecular Weight Proteins

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ABSTRACT: Breast cancer is a leading cause of death among women in many nations. *Crotalaria pallida* was selected to isolate Low Molecular Weight Proteins (LMWPs) that have antimitotic and apoptosis effect. Proteins with molecular weights of 14 kDa and 18kDa were isolated by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and screened for their antimitotic activity on *Allium cepa* root tip cells. Apoptotic activity of proteins was screened by using *Saccharomyces cerevisiae* cell viability and MCF-7 malignant mammalian cell line/cells. Based on haemolytic assay, a 14kDa protein was identified as potent antitumor phytopeptide.

KEYWORDS: *Crotalaria pallida*, Low molecular weight proteins, SDS PAGE, Antimitotic activity, Apoptotic activity. *Saccharomyces cerevisiae*, Phytopeptide.

I. INTRODUCTION

Breast cancer is a very common disease in women. It is progressively increasing in India. The use of radiation therapy, surgery, and chemotherapeutic drugs can reduce invasive breast cancer. However, the patients remain traumatized by the unfavourable side effects [1-3]. Phytochemicals and plant peptides have been turned out to be successful small molecules against invasive cancer cells [4-7]. Various parts (roots, leaves, seeds, etc.) of *Crotalaria pallida* are used in the traditional medicine to treat urinary issues, fever, swelling of joints [8-13]. Low Molecular weight proteins size ranges from 8.2-66 kDa [14-15].

In this connection, the current investigation is aimed to extract and characterize the low molecular weight proteins from *Crotalaria pallida* against breast cancer cell lines. The anticancer activity of the Low Molecular Weight Proteins (14 and 18kDa) extracted was evaluated by their application against MCF-7 cells.

II. MATERIALS AND METHODS

1. Collection of plant species

Crotalaria pallida was collected from Shridevi Institute of Engineering and Technology campus, Tumakuru, Karnataka, India. Pods were collected and dried under the shade. Dried seeds were removed from pods and were used as source to identify the LMWP.

2. Extraction of proteins by precipitation

Dried seeds of *Crotalaria pallida* were powdered in grinder, and 0.5 gm of seed powder was used for the protein extraction by salting in and salting out. The seed powder was grinded with 0.2N NaOH and 0.3N sucrose, and centrifuged at 6000 rpm for 10 minutes at 4^oC. The pellet was discarded and supernatant was measured. Required amount of saturated ammonium sulphate solution was added to the supernatant to get 20% saturation, centrifuged at 6000 rpm for 10 minutes at 4^oC, and the pellet was collected. The supernatant was mixed with required amount of

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saturated ammonium sulphate solution to obtain 40% saturation, and the pellet was collected after centrifugation at 6000 rpm for 10 minutes at 4°C. The procedure was repeated till 60% saturation [2,16-17]. The desired ammonium sulphate solution was calculated by using the following formula:

$$\text{Volume of saturated ammonium sulphate required} = \frac{\text{Volume of the supernatant} \times \text{Desired final concentration (\%)}}{100 - \text{percentage of saturation required}}$$

3. Screening of Low Molecular weight proteins

The gel for SDS-PAGE was prepared by using 16% resolving gel and 4% stacking gel in a gel casting apparatus (glass plates with sandwiched gel). Twenty microlitres (20 µL) of protein sample was transferred into the vial labelled as “1” and 20µl of markers (low range markers from Aristogene biosciences limited, Bangalore) into vial labelled as “2”. Ten microlitres (10µL) of sample loading buffer (0.2% mercaptoethanol, Tris SDS-6.8, 20% glycerol) were added to both vials and the samples were heated at 95°C for 5 minutes. Gel casting apparatus was clipped to the electrophoresis unit. The comb was removed from the casting apparatus to make wells and the electrophoresis unit was filled with 1X running buffer (Tris Glycine SDS buffer). Samples (15 µL) from each vial were loaded into the bottom of the wells using microlitre pipette. The electrophoresis was carried out at 50V initially for 30 minutes and then at 100V. When the dye front was 0.5 cm above the bottom of the gel, the power pack was turned off. The gel was washed with deionised water and placed in gel stainer (0.1% Coomassie R250, 10% acetic acid, 40% methanol and distilled water) for 2-3 hrs at 37°C with gentle stirring, and subsequently destained by overnight placing in destaining solution (5% (v/v) methanol, 7% (v/v) acetic acid and distilled water). The so destained gel was immediately photographed [3, 16]. The distance from the well to each protein band in the marker’s lane was measured first, and consequently - the distance from the well and dye front. The Retardation factor (Rf) value for each protein was calculated using the following equation:

$$Rf = \frac{\text{Distance moved by the protein band from the well}}{\text{Distance moved by the dye front from the well}}$$

This step was repeated for the unknown protein bands in the sample lane. Using Grapher software, the molecular weight of unknown protein bands was determined by taking Rf values on X-axis and molecular weight of markers on the Y-axis.

4. Determination of antimutagenic activity

A series of fresh onion bulbs (totally 15) were placed in boiling test tubes, containing distilled water to produce roots. Onion bulbs with 2-3cm roots were transferred into fresh distilled water and incubated with purified protein samples (gel bands were excised and purified by cation exchange chromatography) for 24hrs. After incubation, the root tips were excised and treated with Farmer’s solution (glacial acetic acid and ethanol in 1:3 ratios) for 30 minutes, after which they were hydrolyzed with 1N HCl for 1 minute. The hydrolyzed roots were treated with acetoorcein and 1N HCl mixture (9:1) with intermittent heating for a period of 5-10 minutes and kept aside for 20 minutes in closed condition [18]. The root tip was squashed gently by tapping with a dissection needle on the cover slip and observed under oil immersion objective by immersion light microscope [15, 19].

5. Quantification of protein by Lowry’s method

Different dilutions of BSA solutions were prepared by mixing stock BSA solution (0.2 mg/ml) and water in the test tube as given in the table 4. The final volume in each of the test tubes was 2 ml. The BSA range was 0.04 to 0.2 mg/mL, to which 5 ml of alkaline copper sulphate solution was added and incubated at room temperature for 10 minutes. Then 0.5 ml of Folin Ciocalteu solution was added to each test tube, and the tubes were incubated in dark for 30 minutes. Optical density was measured at 660 nm against blank. Against protein concentration, this parameter was plotted to get standard calibration curve. The concentration of unknown samples (protein samples which has shown antimutagenic activity) was determined using the standard curve.

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6. Determination of Yeast (*Saccharomyces cerevisiae*) cell viability

Yeasts (0.5 gm) were cultured in 250 ml of Yeast extract Peptone Dextrose (YEPD) broth for 48 hours. Different protein dilutions were prepared by mixing stock T1 protein solution (200µg/ mL) and YEPD broth (500 µL). The protein range was 0.05 to 0.3 mg/mL. To this 100µL of yeast culture was added and incubated at room temperature for 24 hours. Subsequently, 50µL of culture from each dilution were transferred into the labelled vials and 20 µL of methyl blue dye (100 mL of citrate buffer by dissolving 2.4gm of disodium hydrogen citrate and 2.1gm of sodium dehydrogenate citrate was added to 30 ml of methylene blue solution prepared by dissolving 0.3gm in 95% v/v ethanol) was added and transferred on to the haemocytometer to check yeast cell viability under microscope. Yeast culture from each dilution was transferred on to the YEPD agar medium, containing plates for checking the growth. The same experiment was repeated for T2 protein sample.

7. Haemolytic assay

Membrane stabilization was assayed on human erythrocytes (blood group A). Blood sample was collected from Shridevi Medical College, Tumakuru. Stock solution was prepared by mixing 1 mL of erythrocytes with 9 mL of EDTA. 40 µL of this stock solution were incubated with various concentrations (5µg-40µg) of protein for 30 minutes at 37°C [9]. The volume of the reaction mixture was made up to 1 mL by adding 850 µL of 1XPB [20-22]. After 30 minutes incubation, the cells were resuspended, and the so prepared cell suspension was centrifuged at 3000 rpm for 3 minutes at 4°C. The supernatant was used to measure the absorbance of the liberated haemoglobin at 540 nm and 640 nm to determine the percentage of haemolysis (Table-5). The average was calculated from triplicate assay. The control was maintained without adding the protein sample. The percentage of haemolysis was calculated using the following formula:

$$\% \text{ of haemolysis} = \frac{\text{OD of Control} - \text{OD of Sample}}{\text{OD of Control}} \times 100$$

8. Acridine orange assay

Cells from malignant cell line MCF-7 were exposed to 14 kDa proteins (150µg/mL) for apoptosis induction, and then were stained with Acridine orange prior and examined to detect fluorescence [23-24].

III. RESULTS

1. Protein Extraction by precipitation

Maximum pellet was extracted by 60% saturation. The pellet obtained was dissolved in phosphate buffered saline (PBS) for further use, and the so prepared solution was stored in dried form (Table 1).

Table 1: Amount of saturated ammonium required for the extraction of protein by salting out for various percentages

% of saturation	Volume of supernatant (mL)	Volume of saturated ammonium sulphate required (mL)	Volume of saturated ammonium sulphate added (mL)
20%	10	2.5	2.5
40%	13	8.67	6.17
60%	19	28.5	19.83

2. Screening of LMWP by SDS-PAGE:

The protein bands, obtained in different lanes, are calculated by using Rf values (Fig-1). The molecular weight ranges from 6-27 KDa (Fig-2 & Table-2). The distance of dye front is 4.5cm.

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Table 2: Molecular weight and Rf values of protein sample after comparing with standard markers

Purified Protein bands from well to towards dye front	Distance travelled (mm)	Rf	Molecular weight (Da)
1	2.5	0.55	27610
2	2.8	0.62	24370
3	3.4	0.75	18230
4	3.5	0.77	14250

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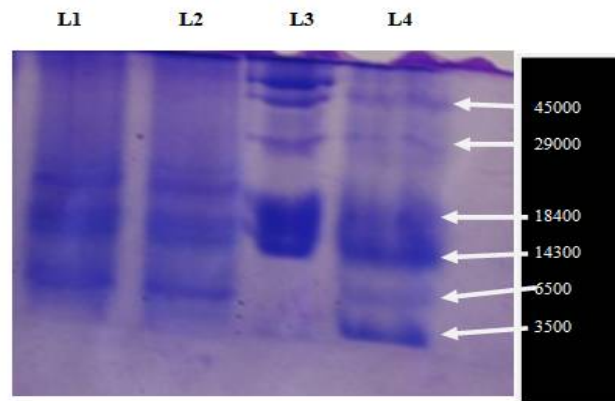


Fig-1: SDS-PAGE: L1 and L2 are lane 1 and 2 with purified protein sample bands; Lane 3 (L3) - Medium range molecular markers; Lane 4 (L4) - low range molecular markers

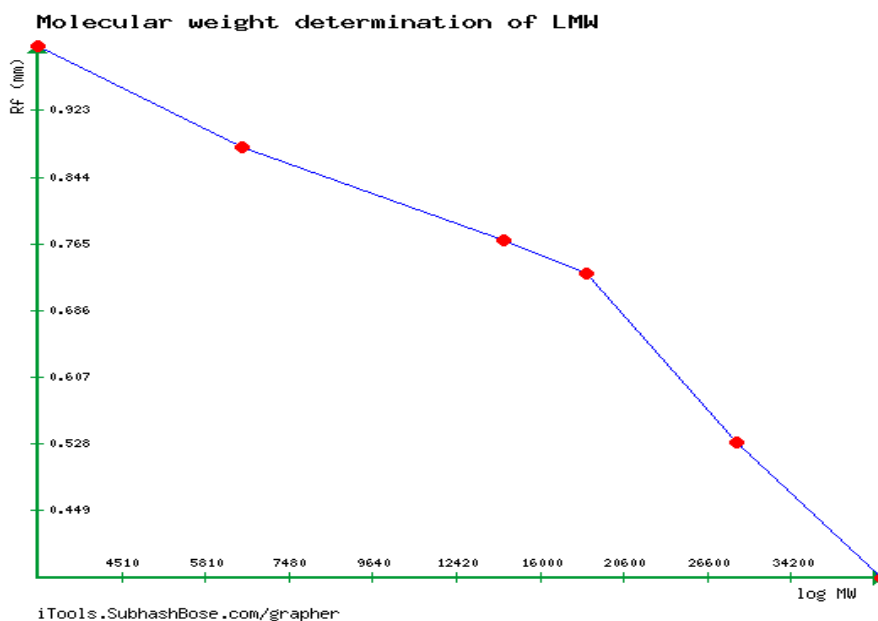


Fig-2: Determination of molecular weights of unknown proteins by using Rf values

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3. Determination of antimutagenic activity of LMWP using *Allium cepa* roots

The LMWP of 14 KDA (T1) and (T2) have restricted the cell division at various stages, mainly by induction of cell restriction in prophase. Chromosomal aberrations (CAs), arising at each level, proved that the sample has antimutagenic activity. The CAs were characterized by change in either total number of chromosomes or in chromosomal structure, which occur as a result of the exposure of chemical treatment. To evaluate the different chromosomal abnormalities, several types of CAs are considered in different stages of cell cycle (prophase, metaphase, anaphase and telophase) (Fig-3; Fig-4). Micro-nuclei can be spontaneously originated due to the development of the isolated chromosome that results from an unequal distribution of genetic material. Cells, bearing micronuclei, were observed at different stages of cell cycle, although most of them were involved in interphase and prophase stages. Chromatin bridges could happen during translocation of unequal chromatid exchange and cause structural chromosome mutations.

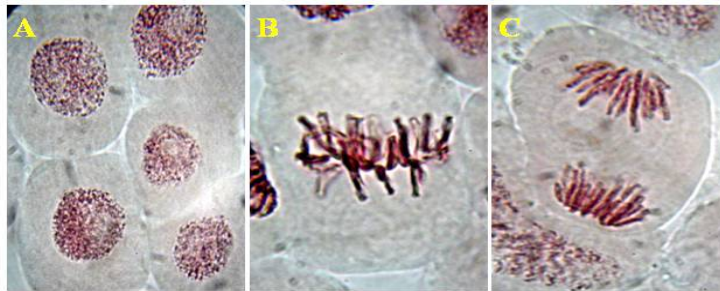


Fig-3: Normal mitotic cells, A) Prophase, B) Metaphase and C) Anaphase

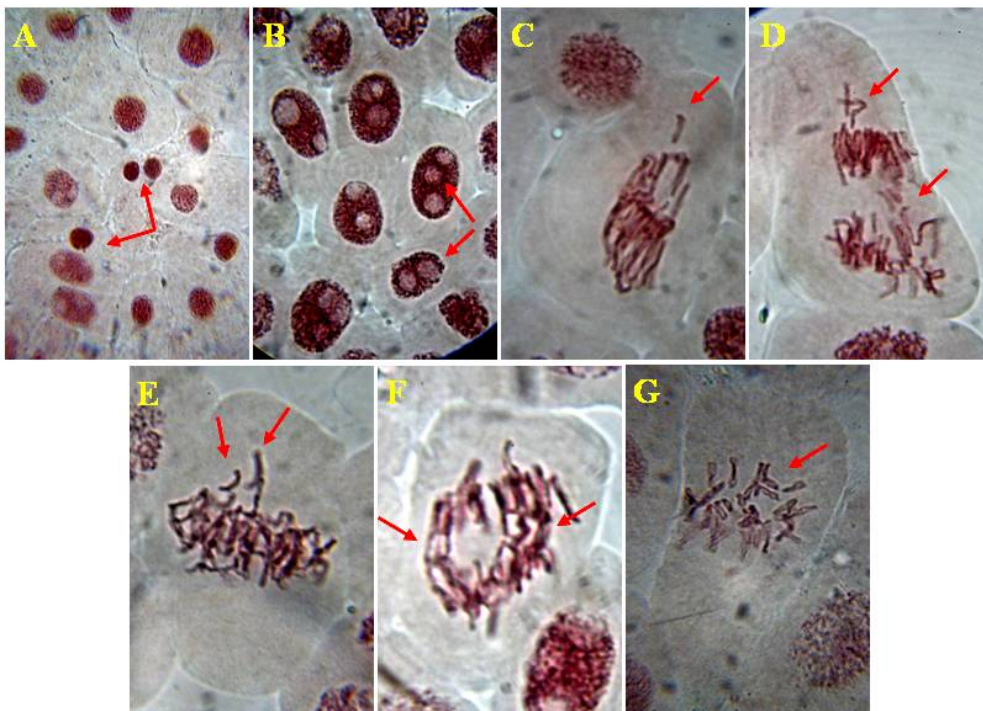


Fig-4: Cellular, Nucleolar and Chromosomal abnormalities in treated onion root tips, A) Cell shrinkage, B) Binuclear and multi-nucleolar cells, C) Chromosomal fragmentation at metaphase, D) Chromosomal bridge and diversions at early anaphase, E) Chromosomal fragmentation at metaphase, F) Chromosomal bridges at anaphase and G) Abnormal chromosomal distribution and fragments at metaphase.

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4. Determination of yeast cell viability

Lysis of yeast cells occurred from 120 µg/mL. Cell debris was observed at concentration of 250µg/mL (Fig-5A & 5B). Colonies were grown on the YEPD agar plates, inoculated with control alone.

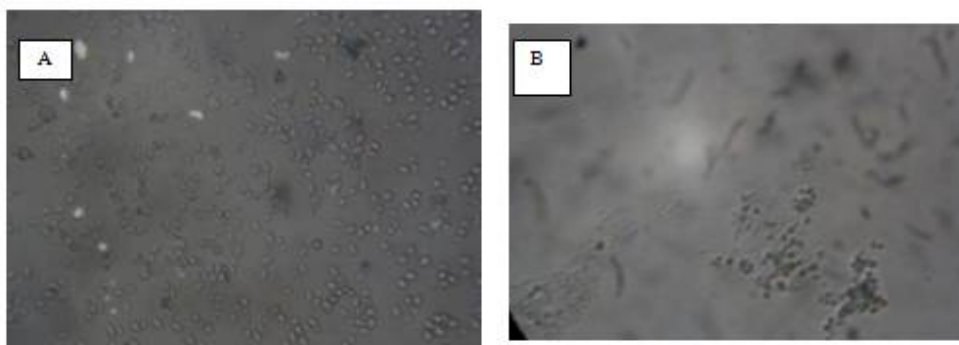


Fig-5: Yeast cell viability; A) living cells without sample; B) Cell debris after incubation with sample.

5. Quantification of protein:

The concentration of T1 and T2 samples (Table-3) were identified as 20 µg/ml and 154 µg/ml respectively using standard BSA curve

Table 3: Estimation of LMWP concentration by Lowry's method

SI No	Volume of BSA (mL)	Volume of Distilled Water (mL)	Volume of alkaline copper sulphate solution (mL)	Incubation at room temperature for 10 minutes	Volume of FC reagent (mL)	Incubation in dark for 30 minutes	OD at 660 nm	Concentration (µg/mL)
1(B)	0.0	1.0	5		0.5		0.00	00
2	0.2	0.8					0.08	40
3	0.4	0.6					0.10	80
4	0.6	0.4					0.14	120
5	0.8	0.2					0.21	160
6	1.0	0.0					0.24	200
7(T1)	0.5	0.5			0.24	200		
8 (T2)	0.5	0.5			0.19	154		

6. Haemolytic assay:

The in vitro haemolysis assay evaluates haemoglobin release in the plasma (as an indicator of red blood cell lysis), following the test agent exposure. T1 protein (14kDa) did not show any haemolysis, whereas T2 protein (18kDa) showed it (Table-5).

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Table-4: Percentage of Hemolysis at different concentrations of protein sample (T2)

Volume of sample (μL)	Absorbance at 540 nm	Absorbance at 640 nm	Difference between absorbance	Percentage of Haemolysis
100	0.671	-0.072	0.743	99
25	0.283	0.233	0.05	93.27
50	0.316	0.249	0.067	90.98
75	0.251	0.206	0.045	93.94
100	0.186	0.142	0.044	94.08
125	0.063	0.039	0.024	96.68
150	0.187	0.153	0.034	95.42
175	0.104	0.147	-0.043	105.787
200	0.082	0.112	-0.03	104.038

7. Acridine orange assay

Progressively, several nuclear apoptotic bodies, containing the fragmented DNA, were visible, appearing as green fluorescent patches (Fig-6). In an advanced apoptotic stage the disintegration of apoptotic bodies lead to total nuclear DNA fragmentations typical of late apoptosis (orange to red stained nuclei). These data confirm that apoptotic-like bodies are fundamental marks of apoptosis in plants and animals.

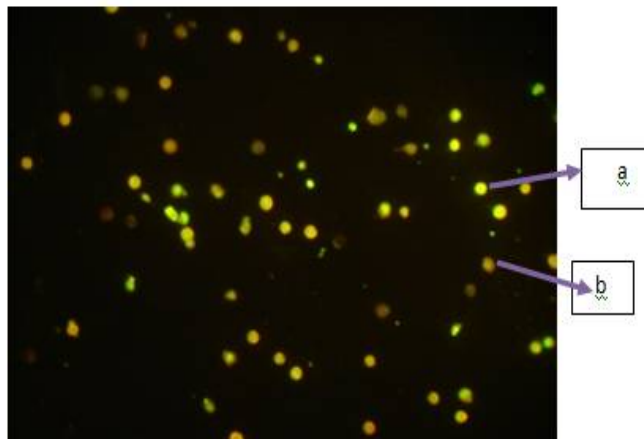


Fig-6: Acridine orange assay: a) apoptotic cell b) necrotic cell

IV. DISCUSSION

As per the US FDA recommendation, the toxicity of injectable formulations should be checked on the erythrocytes to determine the membrane toxicity. Erythrocyte mode has been chosen as its membrane has similarities with other cell membranes. The 18kDa protein lysed the erythrocytes at very low concentration (8.85μg/mL). However, the 14 kDa protein has not affected the erythrocytes' membrane stability, which characterizes it to be suitable for the preparation drug concentration to check on MCF cells. Yeast cell viability was checked as the cellular processes of yeasts are similar to those of mammalian cells. Yeast cells incubated with T₁ and T₂ proteins were completely lysed, which may be due to DNA-damage or loss of membrane integrity. The root tips cells, treated with the same proteins detected, were in prophase, which indicates the inhibition of transition from prophase to metaphase. Chromosomal aberrations (CAs) developed at different stages other than prophase indicates that the proteins have antimetabolic activity. MCF-7 cells, containing apoptotic bodies, are fundamental marks of apoptosis in plants and animals.

Viable cells were identified by bright green color. Apoptotic cells contain orange color due to chromatin degradation or fragmented chromatin whereas necrotic cells show orange nuclei with intact structures. Peptides that can assemble or form pores can disrupt cell membrane or organelle membranes and cause apoptotic or necrotic death [25]. The 14kDa

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protein which is responsible for the apoptosis may be a protein that can enter the cell by forming pores in the membrane.

V. CONCLUSION

The 14kDa protein, isolated from *Crotalaria pallida*, can be used to prevent the progression of cancer in susceptible individuals as it has shown antimetabolic properties and apoptotic activity.

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