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CYTOTOXIC EFFECT OF LOW MOLECULAR WEIGHT PROTEIN ISOLATED FROM *CROTALARIA PALLIDA*

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Abstract:
Low molecular weight proteins (LMWPs) were isolated from Crotalaria pallida using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The antimitotic assay has been investigated using onion root tips whereas apoptosis assay was conducted on MCF-7 cell lines. A 14 kDa protein has been identified as potent antitumor agent.

Key words: *Low Molecular weight proteins, SDS-PAGE, apoptosis, antitumor agent, Crotalaria pallida.*

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1. INTRODUCTION:

Breast cancer is a type of carcinoma. Incidence of breast cancer is increasing in Indian women. Cancer can be suppressed by the balance between proliferation and apoptosis. Investigation of novel agents against breast cancer is highly essential due to the adverse effect of conventional treatments. Several phytochemicals have been investigated as potent agents against cancer [1-6]. The investigation is aimed to study the effect of low molecular weight protein extracted from crotalaria pallida against breast cancer cell lines. MCF-7 cell lines are chosen for the study. The size of LMWP ranges up to 69 KDa. Elimination of cancer cells by early apoptosis is preferred over other forms of cell growth inhibition. Apoptosis directly leads to tumour regression and reduces the risk of selecting more aggressive and drug resistant phenotypes that are often responsible for tumour regrowth and treatment failure [7-10].

2. MATERIALS AND METHODS:

A. Separation of proteins by SDS PAGE:

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a reliable method for determining the molecular weight (MW) of an unknown protein.

SDS-PAGE gel electrophoresis was performed on resolving and stacking gel at 16% and 4% concentrations of acrylamide: bisacrylamide respectively. Sample buffer was added to 20 µl of partially purified protein, molecular weight marker (low range from Aristogene biosciences limited, Bangalore) and heated for 5 minutes at 95^0C in water bath. 20 µl of each mixture was loaded in the wells. The electrophoresis unit was filled with 1X running buffer and the electrophoresis was carried out at 50 V initially for 30 minutes and then at 100 V. Gels was placed in the staining solution for 2-3 hr at 37^0C with agitation, followed by its destaining in the destaining solution with agitation, overnight. The destained gels were immediately photographed. Next, a graph of log MW vs. relative migration distance (Rf) is plotted, based on the values obtained for the bands in the MW standard. The MW of the unknown protein band is then calculated by interpolation using this graph. The key to determining MW accurately is selecting separation conditions that will produce a linear relationship between log MW and migration within the likely MW range of the unknown protein [11-13].

B. Determination of antimitotic activity:

Healthy and equal sized bulbs of common onions were chosen and series of bulbs are grown in test chemicals. The loose outer scales of bulbs and old roots were removed with the help of sharp and pointed forceps so as to expose the root primodia. A series of bulbs were then placed distilled water at a temperature. When the roots are 2-3cm, the sample was added to the water and incubated for 24hr. After treatment, the bulbs were washed thoroughly under running tap water. The root tips from each bulb were plucked and fixed in Farmer's fluid (glacial acetic acid: ethanol:: 1:3) for 24 hours. The root tips were hydrolyzed in 1N HCl at 60^0C for 1minute and transferred to a watch glass containing aceto-orcein and 1 N HCl in 9:1 ratio. They were then heated intermittently for 5-10 minutes, covered and kept aside for 20 minutes. The tip of the root was then cut with sharp blade and placed on a glass slide in a drop of dye and covered with cover slip. The root tip was squashed gently by tapping with a dissection needle and observed under oil immersion objective [14-18].

C. Screening for Cytotoxicity activity of protein on breast cancer cell lines (MCF-7) [19-22]:

After trypsinization 120,000 cells were taken from the T- Flask for plating for MTT Assay in a 15ml centrifuge tube**.** The volume was made up to 6.5ml by adding 10% DMEM/F12 Media. Excluding the outermost rim, 100µl (Approx... 2000 cells) was add to each of the remaining wells in a 96- well microtitre plate. Media control, vehicle control, positive control and cell with different drug concentrations were incubated. 200µl of MTT dye was added and incubated. The plate was removed from the incubator. The purplish residue in most of the wells was due to formazan crystals. Carefully the spent media was removed from the wells, column wise, replacing it with 100 µl of crude DMSO. The crystals formed at the bottom of the wells were not disturbed. The plates were incubated for 10 minutes at 37°C and 5% CO_2. This allows time for the crystals to dissolve in the DMSO giving a purplish solution in most wells. Row by row, the contents of the wells was loaded into cuvettes to be used with the spectrophotometer. Using a Spectrophotometer, the absorbance of the cuvettes was measured at 545nm. The results were recorded.

D. Acridine orange assay [23-26]:

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.

3. Results and discussion:

A. Determination of molecular weight of LMW proteins:

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

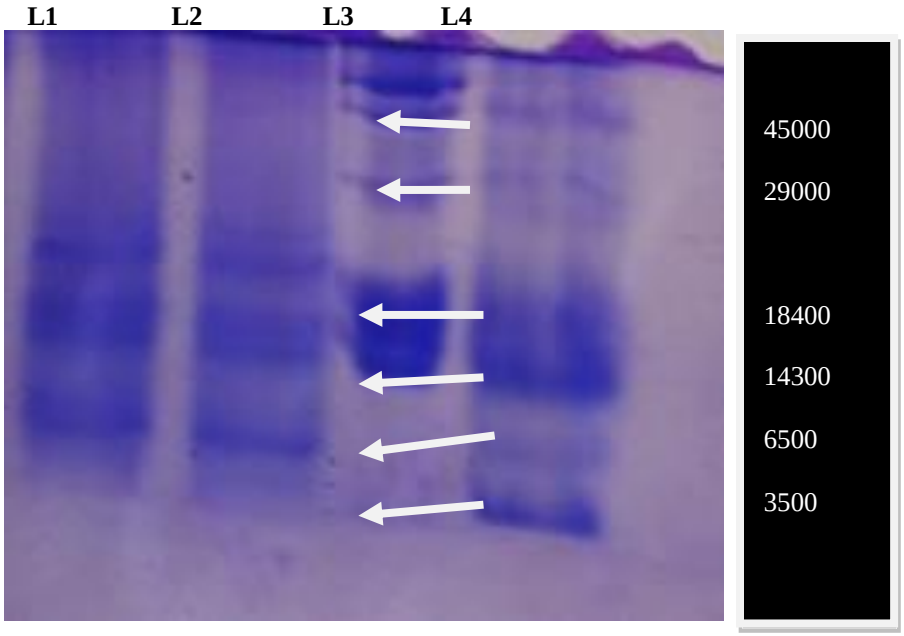

Fig 1: SDS PAGE: L1 and L2 are lane 1 and 2 with purified protein sample bands; Lane 3-Medium range molecular markers; Lane 4-low range molecular markers;
Distance of Dye front is 4.5cm

Table 1: 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
5	4.0	0.88	6610

B. Determination of antimitotic activity [17-30]:
The LMWP of 14 kDa (T1) and 18 kDa (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 antimitotic 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-2). 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.

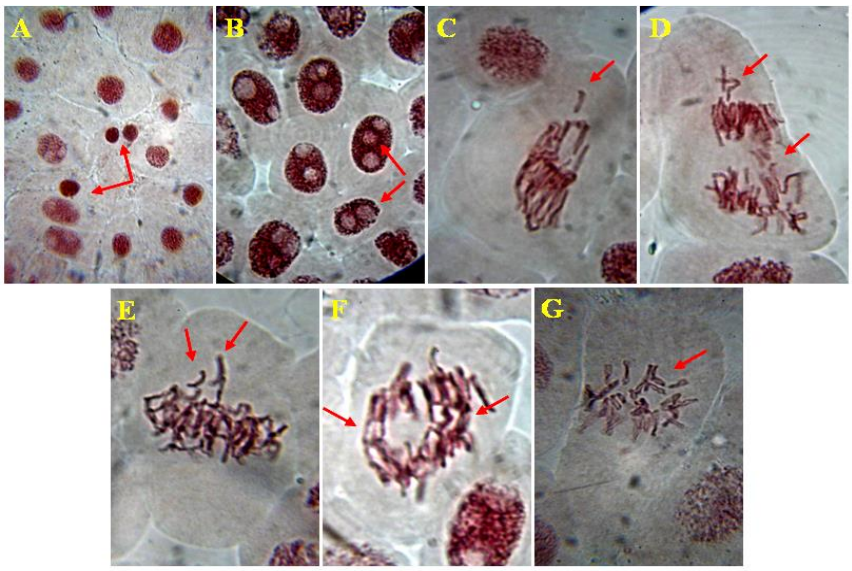

Fig 3: Cellular, Nucleolar and chromosomal abnormalities cells of treated onion root tips, A) Cell shrinkage, B) Binuclear and multi nucleolar cells, C) Chromosomal fragmentation at metaphase, D) Chromosomal bridge and diversions in early anaphase, E) Chromosomal fragmentation at metaphase, F) Chromosomal bridges at anaphase and G) Abnormal chromosomal distribution and fragments at metaphase.

C. Screening for Cytotoxicity activity of protein on breast cancer cell lines (MCF-7):

The LMWP has shown the cytotoxic effect on MCF cell lines. The cell viability decreased with increased concentration. The IC_{50} was determined as 150µg. The cell viability was reduced as the concentration increased

Table 2: OD values obtained at different concentration by MTT assay after 24hr incubation

24hour data			
Concentration	OD Value	Corrected	Viability
Blank (DMSO).	0.349		
Media Control	1.264	0.915	
Vehicle Control	1.243	0.894	97.70492
Positive Control	0.496	0.147	16.06557
50ug	1.158	0.809	88.4153
100ug	0.963	0.614	67.10383
150ug	0.894	0.545	59.56284
200ug	0.647	0.298	32.56831
250ug	0.514	0.165	18.03279

Table 3: OD values obtained at different concentration by MTT assay after 48hr incubation

48hour data			
Concentration	OD Value	Corrected	Viability
Blank	0.361		
Media Control	1.341	0.98	
Vehicle Control	1.296	0.935	95.40816
Positive Control	0.438	0.077	7.857143
50ug	1.203	0.842	85.91837
100ug	1.028	0.667	68.06122
150ug	0.849	0.488	49.79592
200ug	0.691	0.33	33.67347
250ug	0.527	0.166	16.93878

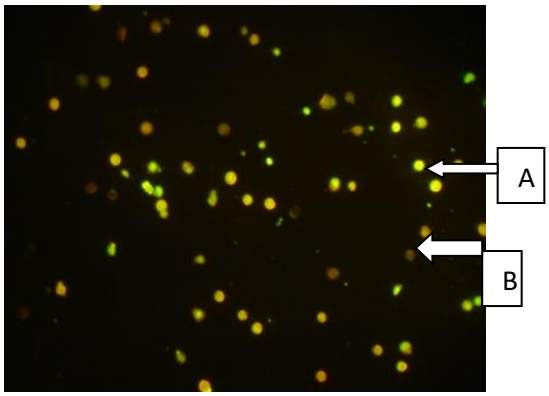

Fig. 4: Acridine orange assay: A) apoptotic cell; B) necrotic cell

D. Acridine orange assay [30-35]:

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.

4. CONCLUSION:

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

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