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Original Article

Evaluation of cytotoxic activity of protein extracts from the leaves of *Morinda pubescens* on human cancer cell lines

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ABSTRACT

Biologically active proteins isolated from plant species can be used in traditional medicine as prolific resources for new drugs *Morinda pubescens* Sm., Rubiaceae, is a promising medicinal plant which is widely used in folk medicine to treat fever due to primary complex, ulcer and glandular swellings. In this study, proteins were extracted from the leaves of *M. pubescens*, and precipitated with ammonium sulphate at various saturation concentrations ranging from 20 to 80%. The precipitated protein sample obtained with 80% saturation was further purified using ultrafiltration membrane (<10 kDa). SDS-PAGE analysis identified the presence of crude and ultrafiltered protein bands. FTIR spectrum of the ultrafiltered protein fractions depicted the presence of hydroxyl and carbonyl groups of proteins. The ultrafiltered proteins exhibited increased cytotoxic activity on A549 cells at the concentrations ranging from 15 to 100 μg/ml. About 98% cell viability was also observed in Vero cells treated with the maximum concentration of 100 μg/ml of ultrafiltered protein extract. DNA fragmentation was observed in A549 cells treated with 10 μg/ml of ultrafiltered proteins, indicating the onset of apoptosis.

Introduction

Bioactive proteins and peptides from plant sources exhibit different activities, such as antimicrobial, antioxidant, antithrombotic, antihypertensive, hypocholesterolemic, hypoglycemic, immunomodulatory, opioid, and antiproliferative activities. These activities can affect the condition of major body systems, like cardiovascular, digestive, endocrine, immune and nervous system (Ledesma et al., 2009). Plants possessing anticancer activity were found in Violaceae, Rubiaceae, Fabaceae and Cucurbitaceae families (Gerlach and Mondal, 2007). Macrocyclic proteins, such as circulins A (32.8 kDa) and B (31.5 kDa), were isolated from *Chassalia parvifolia* and cyclopsychotride from *Psycho trialongipes* which belong to Rubiaceae family. These cyclic proteins show cytotoxic activity, antiHIV and hemolytic activity (Gustafson et al., 1994; Witherup et al., 1994). Anticancerous byproducts are derived from *Morinda citrifolia* (noni) fruit (A549 human lung carcinoma cells) (Jang, 2012) and b romelain, a protein found in several members of Rubiaceae family are reported to have anti-tumor activity (Marshall and Golden, 2012).

Morinda pubescens Sm., commonly known as Aalis a species of flowering plant of the family Rubiaceae, native to Southern Asia. The bark of *M. pubescens*, is useful in treating eczema, fever due to primary complex, ulcer and glandular swellings, while leaves are useful for digestive disorders and venereal diseases (Nivas et al., 2011). The preliminary phytochemical study of the methanol extracts of leaf and stem bark of *M. pubescens*, exhibited antimicrobial and antioxidant properties (Murugan et al., 2012). The aim of this study is to isolate and purify cytotoxic proteins from the leaves of *M. pubescens*.

Materials and methods

Plant material and reagents

The leaves of *Morinda pubescens* Sm., Rubiaceae, were collected and authentified (PARC/2012/1384) by Dr. P. Jayaraman, Director of Plant Anatomy Research Centre, Chennai. All buffers and chemicals used were of analytical grade. Human cancer cell line such as A549 (adenocarcinomic human alveolar basal epithelial cells) and Vero cell lines (African green monkey kidney cells) were purchased from NCCS, Pune.

Protein extraction

Leaves of *M. pubescens* were washed with distilled water and shade dried. The dried leaves were ground to fine powder. About 5 g of powdered leaf sample was extracted with 50 ml of extraction

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buffer (Ribeiro et al., 2007) consisting of 10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 10 mM KCl, 2 mM EDTA (pH 7.0) and kept in constant stirring for 3 h at 4 °C. Then the contents were filtered and centrifuged at 5000 × *g* for 20 min. The crude supernatant was lyophilized and stored for further use. The crude supernatant (lyophilized) was further treated with ammonium sulphate for precipitation of proteins with various saturation limit from 20 to 80%. The concentrations of proteins present in the precipitated samples were estimated by Bradford assay (Bradford, 1976).

Ultrafiltration of protein extracts

The precipitated proteins obtained using 80% saturation of ammonium sulphate from the seeds of *M. pubescens* were fractionated using ultrafiltration membrane (10 kDa cut-off membrane, Amicon). The concentrated filtered solution containing proteins smaller than the pore size which is less than 10 kDa, was lyophilized and stored at 4 °C for further use (Ketnawa, 2011).

SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a widely used technique to separate proteins according to their electrophoretic mobility (Mahmood et al., 2012). About 40 μl of the crude supernatant was loaded into Lanes 1–4, 7,8 and 40 μl of the ultrafiltered protein sample was loaded into Lanes 9, 10. Lanes 5, 6 represents the protein marker of size ranging from 3.5 kDa to 43 kDa. The gel was kept under electrophoretic run for 2 h at 100 V and the protein bands were identified.

FTIR analysis

Identification of functional groups of the ultrafiltered proteins isolated from the leaves of *M. pubescens* was performed using Shimadzu Fourier transform infrared spectrophotometer (Surewicz and Mantsch, 1988) Ultrafiltered proteins were homogenized with potassium bromide to obtain a pellet. The pellet was scanned in the infrared absorption region between 400 and 4000 cm^{-1} with a resolution of 4 cm^{-1} (Widjanarko et al., 2011).

Cytotoxic activity of protein extracts

The ultrafiltered protein fractions were tested for cytotoxic activity against the selected cancer cell lines using MTT assay (Pascariu et al., 2011). Different concentrations of ultrafiltered proteins ranging from 2 to 100 μg/ml were added to each well of 96 well plates. The cells were cultured in 96-well plates (2×10^5 cells per well) in DMEM supplemented with 10% FBS for 24 h. After 24 h the cells were observed under phase contrast microscope and morphology of cells were observed. The medium containing positive control and test samples were removed. MTT (50 μl) dye was added to the wells containing 200 μl of fresh medium. The cell lines were incubated in CO_2 incubator for 4 h. After 4 h of incubation, medium containing dye was removed and 200 μl of DMSO was added to dissolve the formazan crystal. The absorbance was recorded at 570 nm and the percentage of cell viability was calculated using the formula:

$$\%\text{ of cell viability} = \frac{\text{At}}{\text{Ac}} \times 100$$

where At, absorbance of treated cell; and Ac, absorbance of control (untreated cells).

DNA fragmentation assay

DNA fragmentation analysis (Kalinina et al., 2002) was carried out to evaluate the mechanism of cell death in A549 cancer cell line treated with 10 μg/ml, of ultrafiltered protein extract from the leaves of *M. pubescens* and incubated at −20 °C overnight. Cells were freezed and thawed three times for detachment of cells from the flasks. Cells (300 μl) from the flasks was taken in an eppendorf and added with 800 μl of proteinase K buffer was added. About 4 μl of proteinase K was added and kept for 1 h incubation at 56 °C in a water bath. After incubation, 700 μl of phenol:chloroform:isoamylalchohol (25:24:1) and 100 μl of 5 M sodium acetate were added to the mixture. The mixture was centrifuged for 15 min at 5000 × *g*, 4 °C. The supernatant was added with 200 μl of isopropyl alcohol and incubated at −20 °C for 1 h. After incubation, the contents were centrifuged again at 5000 × *g*, 4 °C for 15 min. The supernatant was discarded and 1 ml of 70% ethanol was added to the pellet which was centrifuged for 15 min at 6000 × *g*, 4 °C. The supernatant was again discarded and air dried. Nuclease free water (20 μl) was added and stored at −20 °C. Agarose gel electrophoresis (0.8%) was performed at 100 V.

Statistical analysis

The experiments were carried out in triplicates. The results were calculated as mean along with standard error values. Statistical significance was calculated using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered as statistically significant.

Results and discussion

Plant-based products including proteins and small molecular compounds have been suggested as the favorable drugs for cancer treatment in regard to many adverse effects exerted by current cancer treatments, namely chemotherapy and radiation therapy (Ledesma et al., 2005). The concentration of proteins present in the crude supernatant (1 mg/ml), 80% precipitated sample (0.8 mg/ml) and ultrafiltrate (0.5 mg/ml) were estimated by Bradford assay. Similar studies have been performed by extracting bioactive proteins from various plant sources (Maurya et al., 2011; Kumar and Santhi, 2012). SDS-PAGE gel (12%) showed the presence of protein

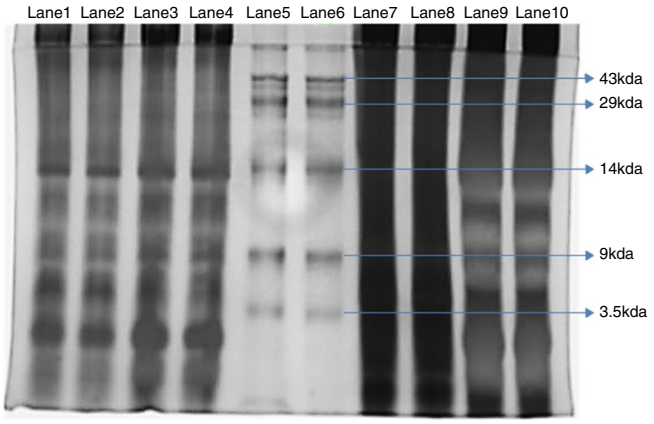

Fig. 1. SDS-PAGE analysis of protein samples from the leaves of *Morinda pubescens* Sm.

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