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Caspase dependent apoptotic inhibition of melanoma and lung cancer cells by tropical *Rubus* extracts

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ABSTRACT

Rubus fairholmianus Gard. inhibits human melanoma (A375) and lung cancer (A549) cell growth by the caspase dependent apoptotic pathway. Herbal products have a long history of clinical use and acceptance. They are freely available natural compounds that can be safely used to prevent various ailments. The plants and plant derived products became the basis of traditional medicine system throughout the world for thousands of years. The effects of *R. fairholmianus* root acetone extract (RFRA) on the proliferation of A375 and A549 cells was examined in this study. RFRA led to a decrease in cell viability, proliferation and an increase in cytotoxicity in a dose dependent manner when compared with control and normal skin fibroblast cells (WS1). The morphology of treated cells supported apoptotic cell death. Annexin V/propidium iodide staining indicated that RFRA induced apoptosis in A375 and A549 cells and the percentages of early and late apoptotic populations significantly increased. Moreover, the apoptotic inducing ability of RFRA when analysing effector caspase 3/7 activity, indicated a marked increase in treated cells. In summary, we have shown the anticancer effects of RFRA in A375 and A549 cancer cells via induction of caspase dependent apoptosis *in vitro*. The extract is more effective against melanoma; which may suggest the usefulness of RFRA-based anticancer therapies.

1. Introduction

Cancer is one of the major causes of death worldwide and characterized by uncontrolled growth of abnormal cells leading to a continuous rise in the number of cancer patients. Due to the inadequate competence of currently available cancer therapies the death rate among cancer patients remains high [1]. Various medicinal plant extracts are explored for bioactivities including anti-cancer activity [2]. The targeted or combination therapies are a promising approach for lung and skin cancer treatments [3]. The use of alternative medicine to eliminate the side effects and improve the efficacy of chemotherapy is popular in Western countries [4]. Apoptosis refers to programmed cell death which is a natural process. Generally, drug-induced apoptosis is one of the main principles of cancer treatment. Many signalling pathways including the intrinsic and extrinsic pathways are involved in the processes which converge in the mitochondria as it plays a vital role in apoptosis [5].

Melanoma is one of the cancers with an ever-increasing death rate. It accounts for less than two percent of skin cancer, but the majority of skin cancer deaths [6]. Skin cancer denotes around one to two percent of all cancers in Africans, Americans and Asian Indians [7]. Lung cancer is the second most diagnosed and one of the leading causes of cancer death worldwide due to its high incidence and rapid progression and accounts nearly 30% of all cancer deaths [8]. Currently, chemotherapy has been widely used for cancer treatment. However, many anticancer drugs have some serious side effects [9]. Thus, exploration of new compounds for treatment of skin and lung cancer is necessary.

A large number of natural products and dietary components have been evaluated as potential chemopreventive agents, and herbal remedies used in traditional folk medicine provide a largely unexplored source of novel drugs [10]. Evidence show that active principle compounds of plants serve as potent chemotherapeutic agents with less toxicity and few side effects. Plants have been used in cancer treatment since antiquity and over 60% of drugs used for the cancer therapies are derived from plant derived sources [11]. Around 80% of people in rural areas depend on plant products for their primary healthcare needs [12]. Anticancer plants are associated with induction of apoptosis, cell cycle arrest, inhibition of various signal transducers and signaling pathways [13,14]. Therefore, it is significant to screen the crude extracts or isolated compounds for their apoptosis inducing potentials.

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Berries from *Rubus* (cloudberry, raspberry and blackberry) are common in Mediterranean diets and are rich in bioactive phytochemicals [15]. It must be pointed out that a broad variety of food supplements consisting of 'berry extracts' is consumed worldwide due to their various health benefits [16–18]. On the basis of the previously reported, anti-inflammatory, wound healing [19] and anticancer properties [20,21], we have selected *Rubus* fairholmianus for this study. We have reported on the antiproliferative activity of this plant in human colorectal cancer cells (Caco-2) [21]. The evidence supports the idea that anticancer activities of berries are due to antioxidant, control over gene expression, alteration of cell signals and apoptosis induction properties [22,23]. This study examines the antitumour ability and cell death induction pathway of RFRA, and highlights the potential of developing a cost effective and competent drug for skin and lung cancer treatment.

2. Materials and methods

2.1. Extraction of plant material

R. fairholmianus was collected during September 2010 from Marayoor, India and authenticated (voucher specimen no: BSI/SRC/5/23/2010-11/Tech.1657) by Botanical Survey of India. The ground roots were extracted in Soxhlet using acetone. The extractability of phenolic compounds in the polar solvent like acetone was found to be high. The plants were collected from high altitude (2000 m above MSL) since the berries from high altitudes have higher antioxidant properties due to high polyphenolic content. The dried extract was then dissolved in 0.5% DMSO and used for further analysis. The preliminary screening of leaves, stem and root for total phenolics, tannin, flavonoids and antioxidant properties showed that root had the maximum activity [24].

2.2. Cell lines and culture conditions

Human skin fibroblast monolayer cultures (WS1-ATCC CRL1502) were grown in Eagle's minimal essential medium (Invitrogen 32360–026) that was modified to contain 2 mM L-glutamine (Gibco, 25030), 1 mM sodium pyruvate (Gibco, 11360), 0.1 mM nonessential amino acids (Gibco, 11140), 1% amphotericin-B (Gibco, 104813), 1% penicillin–streptomycin (Gibco, 15140) and 10% v/v foetal bovine serum (FBS; Gibco, 306.00301). The melanoma cells (A375-ATCC CRL1619) were cultured in Dulbecco's Modified Eagle's Media (DMEM, Sigma-Aldrich, D 6429) with 1.2 g/L sodium carbonate, 10% FBS, 10 mM non-essential amino acids, 0.5 mM sodium pyruvate, 2.5 mM L-glutamine, 1% penicillin-streptomycin and 1% amphotericin-B. Lung cancer cells (A549-ATCC CCL185) were cultured in Rosewell Park Memorial Institute 1640 medium (RPMI; Invitrogen, 21875–034, Life Technologies, South Africa) complemented with 10% FBS, 0.5% penicillin-streptomycin, and 0.5% amphotericin-B.

Once the cells reached 80% confluence, they were harvested and seeded in a 3.5 cm^2 diameter culture plates overnight at a concentration of 6.5×105 (WS1), 5×10^5 (A375) and 2×10^5 (A549) cells for experimental purposes. All the cultures were incubated at 37 °C with 5% CO_2 and 85% humidity.

2.3. Cellular morphology-Inverted microscopy

The effect of RFRA on cell morphology was determined using an inverted light microscope (Wirsam, Olympus CKX 41) after 24 h of incubation. Once digital images were recorded, cells were trypsinized using 1 ml/25 cm^2 of TrypLE Express (Invitrogen, 12605-028) and resuspended in Hank's Balanced Salt Solution (HBSS) before further tests.

2.4. Trypan blue dye exclusion assay

The trypan blue dye exclusion assay (Sigma-Aldrich T8154) is a quantitative method to determine the percentage of viable and non-viable cells. The viable cells with an intact cellular membrane do not take up the blue dye and the damaged cells take up dye as their membranes are damaged and stained blue. The cell suspension (10 μl) was carefully mixed with 0.4% trypan blue reagent (10 μl) in 1:9 ratio and the mixture was transferred to a Neubauer hemocytometer to determine the number of viable and non-viable cells. Cells were counted using automated cell counter (Countess™ Automated Cell Counter, Invitrogen).

2.5. Adenosine triphosphate (ATP) luminescent assay

The CellTiter-Glo[1] luminescent assay (Promega, G7571, Anatech Analytical Technology, South Africa) is used for the determination of cellular proliferation and quantification of ATP present in cells. Fifty microliters of reconstituted reagent was added to 50 μl of cell suspension and then mixed well to induce cell lysis. This was then incubated at room temperature for 10 min in dark to stabilize the luminescent signal. The luminescent signal was read using the 1420 Multilabel Counter Victor3 (Perkin-Elmer, Separation Scientific).

2.6. Lactate dehydrogenase (LDH) cytotoxicity

The Cyto-Tox96 X assay (Anatech, Promega G 400) was used to measure the level of LDH released. The membrane integrity was assessed by estimating the amount of LDH present in the culture media. The cytosolic enzyme LDH will be released when the cell membrane damages. Fifty microliters of reconstituted reagent and cell culture medium mixed and incubated in the ^{dark} at room temperature for 30 min. The colorimetric compound was measured spectrophotometrically at 490 nm (Perkin–Elmer, VICTOR3™).

2.7. Apoptosis-annexin V-FITC/PI staining

The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Becton Dickinson, 556570, Scientific Group, South Africa) was used to detect the percentage of apoptotic and non-apoptotic cells. One hundred microliters of cell suspension (1×10^6/ml) in 1x binding buffer was stained with 5 μl of Annexin V and 5 μl of propidium iodide (PI). The cells were gently vortexed and incubated for 10 min at room temperature and protected from light. After the addition of 400 ml of buffer to each tube the samples were run in Fluorescence Activated Cell Sorting (FACS) Aria flow cytometer (Becton Dickinson) by running 20000 events and analysed with Cell Quest Software (BD bioscience).

2.8. Caspase-3/7 apoptotic activity

Activity of caspases 3/7 in apoptosis was determined with the Caspase-Glo 3/7 luminescent assay (Whitehead Scientific, Bracken fell, South Africa; Promega G8091). Fifty microliters of treated cells were added to 96-well luminescent plate (Scientific Group Adcock Ingram, South Africa BD354651) containing equal volume of Caspase-Glo 3/7 substrate and the plate was then incubated at room temperature for 3 h. Luminescence was read using the Victor 3 (Perkin-Elmer, Separation Scientific, Johannesburg, South Africa).

2.9. Statistical analyses

Cancer cell lines between 15 and 20 passages were selected. Statistical analysis was done with SigmaPlot version 12.0. All