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Measurements of Cytochrome – C Activity on Cancer Cell Lines by the Effects of Alpha or Beta Momorcharin an Extract of Momordica Charantia

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Abstract

The vegetable Momordica charantia L., (family: Cucurbitaceae) is a scientific name of the plant and its fruit. It is also known by other names, for instance in the USA it is known as Bitter gourd or balsam pear while it's referred to as the African cucumber in many African countries. M.charantia is believed to posse's anti-carcinogenic properties and it can modulate its effect via xenobiotic metabolism and oxidative stress. This study was specifically designed to investigate the cellular mechanisms whereby α, β momorcharin an extract of M. charantia can induce cell death. The activity of cytochrome-c in the cancer cell lines treated with 800 μM of the α, β momorcharin for 24 hours. The results show that α, β momorcharin can evoke significant ($p < 0.05$) increases in cytochrome-c activity in all the cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 and L6) compared to either untreated cell lines or control cytochrome-c activity. In L6 skeletal muscle cell line, cytochrome-c activity increased.

Keywords: Cancer cells; Extract of M. charantia (α, β) alpha; Beta momorcharin; cytochrome – c; Cell viability

Introduction

Cytochrome-c is an intermediate in apoptosis, which is a controlled form of cell death in the process of development or in response to infection or the induction of DNA damage of any apoptotic programme in cell free extracts [1-2]. NADPH-cytochrome c reductase (NADPH cytochrome P450 reductase, EC 1.6.2.4) is a flavoprotein localized in the endoplasmic reticulum (ER) of the cell. It transfers electrons from NADPH to several oxygenases. The most important of which is the cytochrome P450 family of enzymes, which are responsible for xenobiotic metabolism [3-4]. NADPH-cytochrome c reductase is widely used as an ER marker-3 and as a biomarker of ecological pollution and dietary lipid uptake [5-6]. Cytochrome-c is released by the mitochondria in response to pro-apoptic stimuli. Normally calcium levels are elevated and this in turn is preceded by the release of cytochrome-c from the mitochondria. The small amounts of releasable cytochrome-c lead to an interaction with the inositol triphosphate (IP3) receptor on the ER causing it to release calcium. The increase in cellular free calcium triggers a massive release of cytochrome-c which then maintains ER calcium release through the inositol 1, 4, 5-triphosphate receptors (IP3RS). ER calcium release can reach cytotoxic levels and cause calcium overloading. The release of cytochrome-c in turn enhances the activity of caspase-9, a cysteine protease. Cytochrome-c assay kit is designed to measure the NADPH cytochrome-c reductase activity in cell and in purified microsomes of the ER. The cytochrome-c measurement is based on a colorimetric assay that monitors the reduction of cytochrome-c by NADPH- cytochrome-c reductase in the presence of NADPH. The reduction of cytochrome-c results in the formation of distinct bands in the absorption spectrum and the increase in absorbance at 550 nm is measured with time [6].

Extraction method for either of alpha or beta momorcharin

In this study alpha, beta or alpha beta momorcharin was purchased as a purified compound from IMAM International

Group Pharmaceutical Company in China. According to the literature, the Company extracted, isolated and purified each compound using the following procedure. The whole fruit of bitter gourd was ground and homogenized in 2 mM sodium phosphate buffer, pH 7.5. The resulting slurry was then stirred for 3 hrs to extract the crude proteins [7]. The insoluble component from crude proteins was removed by the filtration and centrifugation at 30,000 x g for 1 hour at 48°C. By using 2 mM sodium phosphate buffer, pH 7.5, the crude protein solution was dialysed. The dialysed protein sample was applied to DEAE Sepharose column equilibrated with 2 mM sodium phosphate buffer at pH 7.5. The unbound proteins were then applied to Mono-S column which was equilibrated by 2 mM sodium phosphate buffer at pH 7.5 and eluted by 0.5 m of NaCl [7]. The fraction corresponding to either alpha and beta or alpha, beta momorcharin, which was confirmed the N-glycoside activity RNA, was concentrated and dialysed against 20 mM Tris-HCl buffer, pH 7.8. The chromatography was performed on Bio Logic Duo Flow system (Bio-Rad, Hercules, CA) at 48°C. The purity of alpha and beta or alpha, beta momorcharin was examined by SDS-PAGE and gel filtration chromatography. The concentration of alpha momorcharin was determined by spectrophotometrically using optical absorbance at A280 nm.

Cell Culture

Passaging of the cancer cell lines and Control cell line

The culture medium, phosphate buffer solution (PBS), and trypsin (sterile) were removed from the fridge at 4ºC and subsequently placed in the water bath at 37°C for 30 min in order to equilibrate. The Laminar flow hood was turned on for 15 min, prior to start of the experiment, in order to purge the air inside the cabinet and to reach the maximum cleanliness. The different cancer and normal cell lines were incubated at 37°C incubator in an atmosphere of 5% CO_2 in air. The cells were examined under the inverted contrast microscope to note the both confluence and general health of the cells. The flask was passaged when the cells had reached 70-80% confluence.

The medium was aspirated from the cultured flask and was washed with sterile PBS (5 ml if 75 cm^2 flask and 2 ml if 25 cm^2 flask) in order to remove any traces of serum from the cells. This prevented the serum from inactivating the trypsin which was used to detach adherent cells from the cell clump. Trypsin solution (2 ml if 75 cm2 flask or 1 ml if 25 cm2 flask) was pipetted in the flask and incubated at 37°C in an incubator in an atmosphere of 5% CO_2 in air for 3-5 mins until the cells began to detach. The detachment was confirmed by observing at intervals under an inverted microscope. The cells were left in the trypsin solution for the correct length of time. If the cells were left for a longer period of time then this would lead to damage of the cells. A volume of 3 ml complete growth medium was then added to the flask to inactivate the trypsin and the cells were pipetted up and down to break up any large cell aggregates. The cell suspension was transferred from flask into 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 min. Following centrifugation, the supernatant was aspirated and the cells were pellet at the bottom of the centrifuge tube. Based upon the cell pellet density volumes of 1 ml to 3 ml fresh medium were suspended in the centrifuge tube. The cell pellet was flicked properly in the medium containing 20 µl of trypsinised cell suspension and 80 µl of tryphan blue (used to detect dead cells in the cell suspension 1:5 ratio). The contents were mixed well together and a haemocytometer test was performed using 1 ml of cell suspensions. This process helped to assess the total number of the cell suspension present in the centrifuge tube and which was required to make 1 or 2 flasks and to do 96 well plates. Thereafter, the cells were frozen in liquid nitrogen depending on the number of cells present per ml. The cell suspension was divided in either one or several flasks (depending on the cell density) and fresh growth medium (10 ml to 12 ml if 75 cm^2 flask and 5 ml if 25 cm^2 flask) was added to the flasks. These were then placed in a 5% CO_2 incubator to continue cell growth.

Cell Counting Method

A volume of 20 µl of cell suspension and 80 µl of tryphan blue (1:5 ratio) were pipetted into a micro centrifuge tube and mixed together. A coverslip was gently pushed over the chambers of a haemocytometer and 20 µl of cell suspension was slowly pipetted against each short side of the coverslip so that the suspension could spread into each chamber. The haemocytometer was placed onto the stage of an inverted phase contrast microscope and focused on the central 25 squares of one chamber. The numbers of cells in these squares were counted. These steps were repeated for the other chambers. The average number of cells in the center grid (1 mm^2) of each chamber was calculated. This number was multiplied by 10^4 to obtain the number of cells per 1ml of suspension. The total number of cells was calculated by multiplying the number of cells per 1 ml by the total volume of the cell suspension.

Measurement of cytochrome – c activity in untreated and treated cell lines

Figure 1 shows the activity of cytochrome-c in (A) the cancer cell lines treated with 800 µM of the α, β momorcharin for 24 hours and (B) the same cell lines incubated with media alone but without any α, β momorcharin for the same duration of 24 hrs. The control response of cytochrome-c is also shown in the figure for comparison. The results show that α, β momorcharin can evoke significant ($p < 0.05$) increases in cytochrome-c activity in

all the cancer cell lines (1321N1, Gos- 3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 and L6) compared to either untreated cell lines or control cytochrome-c activity. In L6 skeletal muscle cell line, cytochrome-c activity increased significantly ($p < 0.05$) in treated (A) cells compared to untreated (B) cells but these values were less than the assay kit control cytochrome-c activity. Figure 1 shows the percentage difference (treated – untreated cell lines) or increase in cytochrome-c activity in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line treated with 800 µM of α, β momorcharin. The value for each untreated cell line was taken as 100 % and the value for the respected treated cell line was expressed as a percentage of the untreated cell line. The difference between treated and untreated for each cell line is plotted in figure 1. The results show that α, β momorcharin can evoke large and significant ($p < 0.05$) increases in cytochrome-c activity in 1321N1, Gos-3, Weri Rb-1 and Corl -23 cell line compared to the respective untreated cell lines. The results also show that α, β momorcharin had little effect on cytochrome – c activity in L6 skeletal muscle cell line (Figure 1).

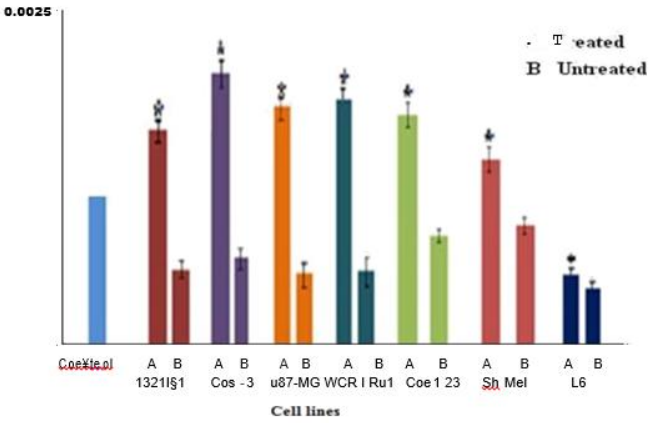

Figure 1: *Bar charts showing cytochrome-c activity in (A) six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel-2, Corl- 23, Weri Rb-1) and healthy L6 muscle cell line following incubation with 800 µM α, β momorcharin for 24 hrs. The cytochrome-c activity (B) for the untreated (no α, β momorcharin) cell lines is also shown in figure for comparison. Similarly, the background assay kit control cytochrome-c activity in the absence of any cells is shown in the figure. Data are mean ± SD, n = 4 different experiments in duplicate. Note that cytochrome-c activity increased significantly (* $p < 0.05$) in all the treated cells compared to untreated cells. The results also show that cytochrome-c activity was maximal in Gos-3 cell line where as 1321N1, U87- MG, Sk Mel-2 and Weri Rb-1 contain more or less the same activity. However, cytochrome-c activity in L6 muscle cell line was the least compared to all the cancer cell lines.*

Discussion

The results presented in this study have demonstrated significant anti-cancer effects of the isolated and purified proteins, alpha, beta momorcharin of M. charantia on the six different cancer cell lines compared to untreated control. Anti-cancer drugs are believed to exert their 'killing' effects on cells via different cellular and sub-cellular mechanisms including damages to the mitochondria and microtubules, inhibition of kinases or by cellular calcium over-load [8]. The results presented in this study have shown or the isolated and purified protein of M. charantia, namely alpha, beta momorcharin can elicit marked and significant changes in the release of cytochrome-c in all the cell lines employed in this study compared to control untreated cell lines. Apoptosis is programmed cell death and it is associated with damage of cell mitochondria in the body to elevate such intra-cellular mediators such as caspase-3 and caspase-9 and the release of cytochrome-c [9-10]. In previous studies, it was shown that anti-cancer drugs exert their lethality by inducing apoptosis in tumour cells in vitro and in vivo targeting both the mitochondrial and death receptor pathways [11]. There are two major apoptotic pathways in mammalian cells namely the receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [9]. The mitochondrial pathway is initiated by cytochrome-c release from the mitochondria which promotes the activation of caspase-9 through activated caspase-9 which is responsible for the activation of cell death proteases [12-13].

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