Original Article

EFFECTS OF α, β MOMORCHARIN EXTRACT OF *MOMORDICA CHARANTIA* IN INTRACELLULAR FREE CALCIUM ON CANCER CELL LINES

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ABSTRACT

The *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 its referred to as the African cucumber in many African countries. This study was specifically designed to investigate the cellular mechanisms whereby alpha, beta momorcharin an extract of *M. charantia* can induce cell death measuring the elevation in intracellular free calcium concentrations in three different cancer cell lines 1321N1, Gos-3 and U-87. The results show that incubation of the three cancer cell lines 1321N1, Gos-3 and U-87 with α, β momorcharin can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]_i$ in all three cancer cell lines compared to control (untreated) cells. Maximal increases in $[Ca^{2+}]_i$ was attained after 420 min of incubation. In control (untreated cell lines), $[Ca^{2+}]_i$ remained more or less stable in both cell lines after 420 min. The results also show that the increase in $[Ca^{2+}]_i$ in Gos-3 cell line was much more pronounced following incubation with α, β momorcharin compared to 1321N1 and U-87 cell line. The results show that incubation of the three cancer cell lines with α, β momorcharin can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]$ in all three cancer cell lines compared to control (untreated) cells. Maximal increases in $[Ca^{2+}]_i$ was attained after 420 min of incubation. In control (untreated cell lines), $[Ca^{2+}]_i$ remained more or less stable in all three cell lines after 420 min. These results clearly show that α, β momorcharin extract of *M. charantia* is exerting its anti- cancer effect via an insult to the mitochondria resulting in apoptosis, calcium overloading and subsequently, cell death.

Keywords: Cancer cells; Extract of *M. charantia* (α, β) alpha; Beta momorcharin; Intra cellular calcium; Cell viability

Introduction

Calcium is the most important physiological cation in cellular regulation [1, 2]. It is the trigger, the promoter and the regulator and moreover, a ubiquitous intracellular signaling molecule which controls a wide range of cellular processes including secretion, membrane transport, contraction, cell proliferation, gene transcription and even cell death [3-5]. In un-stimulated pancreatic acinar cells, as well as in other cells, the free intracellular calcium concentration $[Ca^{2+}]_i$ is between 50–100 nM. In order to maintain this low resting level, a variety of pumps and uptake systems are present in the plasma membrane and in intracellular organelles to buffer (Ca^{2+}) [6]. Calcium mobilisation is dependent upon intracellular calcium stores as well as extracellular calcium medium.

The intracellular level of $[Ca^{2+}]$i in resting cells is maintained within a normal range of 50 -100 nM. Ca^{2+} homeostasis is tightly controlled to prevent and overcome problems of cytotoxicity due to its very low diffusibility in the cytoplasm. Distributed throughout the cytoplasm is an extensive array of Ca^{2+} pumps [7, 8]. Many studies have demonstrated mitochondrial Ca^{2+} overload as the link between complement deposition and the observed changes in mitochondrial physiology and the triggering of programmed cell death (PCD) [9]. The mitochondrial Ca^{2+} overload is responsible for the increased O(2)(*-) production [10]. If cytosolic Ca^{2+} rise is not accompanied by the accumulation of the cation in the mitochondrion and consequent production of O(2)(*-), cells usually die by necrosis instead of PCD. For example, membrane attack complex assembly (MACA) on the parasit in cell surface allows Ca^{2+} entry in the cell and its accumulation in the mitochondrion can lead to O2 production. This in turn constitutes a PCD signal [11]. In the light of its different potential medicinal values and properties of *M. charantia*, this study was designed specifically to measure intracellular free calcium on four different cancer cell lines by the effects of α, β momorcharin extract of *Momordica charantia*.

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Material and Methodology

Extraction method for either of α or β momorcharin: 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. 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

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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. 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 DuoFlow system (BioRad, Hercules, CA) at 48°C. The purity of α and β momorcharin was examined by SDS-PAGE and gel filtration chromatography. The concentration of alpha momorcharin was determined by spectrophotometrically using optical absorbance at 280nm.

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 4° C 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 three different cancer cell lines 1321N1, Gos-3 and U-87 were incubated at 37°C incubator in an atmosphere of 5% CO2 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 cm2 flask and 2 ml if 25 cm2 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% CO2 in air for 3-5 min 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% CO2 incubation.

Measurement of intracellular free calcium $[Ca^{2+}]_i$.

Measurement of $[Ca^{2+}]_i$ was performed as previously described in the literature [12]. The measurement of $[Ca^{2+}]_i$ was performed by seeding a density of 10,000 cells/well in black 96 well plates (Griner, UK) in 100 µl of growth medium. At sub confluence, the cell culture medium was then replaced with FBS free medium for 24 hr. in order to synchronize cells into a non-proliferation stage. The cell were then loaded with 25 µM Fura-2 acetoxylmethyl ester (AM) in cell medium at 37°C for 30 min followed by 30 min at temperature to minimize dye leakage and sequestration into intracellular organelles. After loading, the cells were washed 2-3 times with 200 µl of Hank's Buffered Salt Solution (HBSS) to remove excess fluorescent dye. The cells were then treated with extract of *M. charantia* α, β momorcharin with concentration of 800 µM for different durations (0 min - 420 min) in a calcium free medium. The cells were washed twice with 200 µl HBSS and then a volume of 100 µl HBSS /wells was added to each well. The same procedure was carried out for the untreated cells. The intensity of the fluorescence's of Fura-2 AM was measured at emission wavelengths of 340 nm. The relative changes in calcium concentration using Fura-2 AM were determined by calculations of;

$$[Ca^{2+}]_i = Kd\ \beta\ (f - f\ min) / (f\ max - f).$$

Kd, the Ca-Fura-2 dissociation constant, β the fluorescence intensity ratio, excited at 380 nm without and with Ca; f min and f max [13]. All values were expressed as ratio units of the Fura-2 AM fluorescent intensity.

Statistical analysis: All control and test data collected from the different experiments were analysed using Statistical Package for Social Sciences (SPSS) version 17, Student's – t test and ANOVA test. Data obtained were expressed as mean ± standard deviation (S.D). Each experiment was repeated for 4-6 times in duplicate (6 for cell viability and 4 for cell signaling) to ensure the accuracy of results. A value of ($p < 0.05$) was taken as significant.

Results

Figure 1 and 2 shows the time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units, in (A) 1321N1 and (B) Gos-3 cancer cell lines (solid diamonds) following incubation with 800 µM of α, β momorcharin for 420 min. The changes in $[Ca^{2+}]_i$ in untreated (control) 1321N1 and Gos-3 cancer cell lines (solid squares) are also shown in the figure for comparison. The results show that incubation of the two cancer cell lines with α, β momorcharin can result in significant ($p < 0.05$) time-dependent increases in $[Ca^{2+}]_i$ in both cancer cell lines compared to control (untreated) cells. Maximal increases in $[Ca^{2+}]_i$ was attained after 420 min of incubation. In control (untreated cell lines), $[Ca^{2+}]_i$ remained more or less stable in both cell lines after 420 min. The results also show that the increase in $[Ca^{2+}]_i$ in Gos-3 cell line was much more pronounced following incubation with α, β momorcharin compared to 1321N1 cell line.

Figure 3 shows the time-course changes $[Ca^{2+}]_i$ in expressed as fluorescence ratio units (A) U87- MG cancer cell lines following incubation with 800 µM of α, β momorcharin (solid diamonds). The changes in $[Ca^{2+}]_i$ in untreated (control) U87- MG cancer cell lines are also shown in the figure for comparison (solid squares). In contrast, with untreated U87–MG cell line basal

$[Ca^{2+}]_i$ remained more or less the same over 420 min. However, in U87-MG cell line treated with α, β momorcharin $[Ca^{2+}]_i$ decreased slightly for 0 - 420 min compared to untreated control but then increase gradually for 360 min to 420 min when it was significant ($p < 0.05$) compared to control untreated cells.

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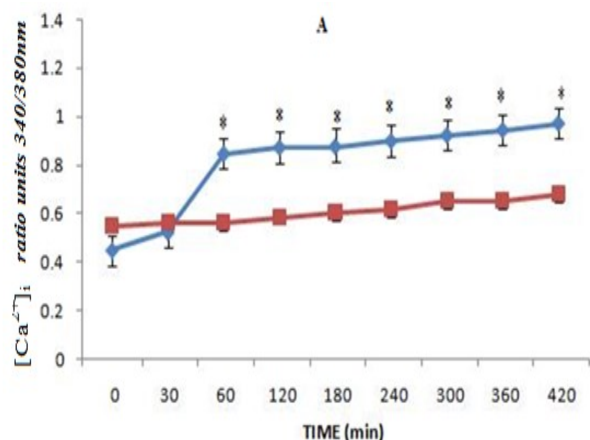

Figure 1: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded 1321N1 cell lines

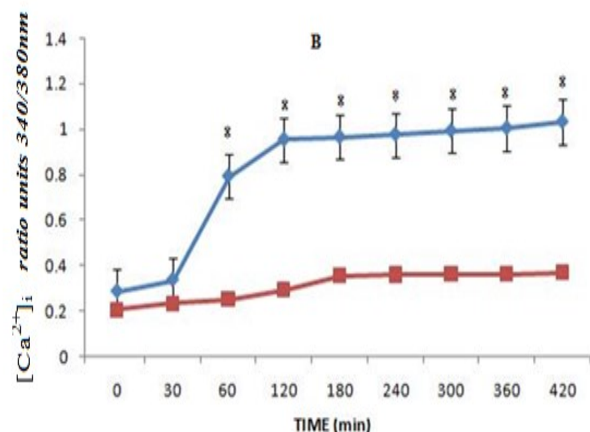

Figure 2: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura-2 AM loaded Gos -3 cell lines.

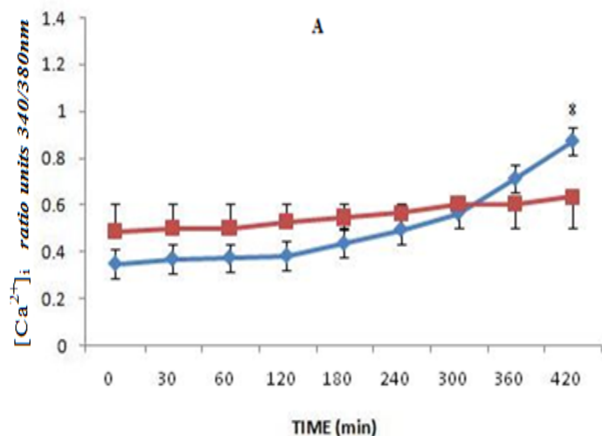

Figure 3: Time-course changes in $[Ca^{2+}]_i$ expressed as fluorescence ratio units (intensity) in Fura -2 AM loaded U87-MG cell lines.

Discussion

The results of this study have demonstrated significant anti-cancer effects of alpha, beta momorcharin of *M. charantia* on the three 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 [14-17]. This study employed extract of *M. charantia* alpha, beta momorcharin may exert their anti- cancer effects on cell death, namely, apoptosis and cellular calcium homeostasis.

The results presented in this study have shown that purified protein of *M. charantia*, namely alpha, beta momorcharin can elicit marked and significant changes in the activities of intracellular free calcium concentrations in all three 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 [18,19]. 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 [20,21]. There are two major apoptotic pathways in mammalian cells namely the receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [22]. The receptor-mediated pathway is triggered by activation of cell death receptors followed by the activation of caspase-8, which in turn cleaves and activates downstream caspase-3 [23]. 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 [24, 25].

The results of the present study have shown that alpha, beta momorcharin can evoke significant and time-dependent increases in $[Ca^{2+}]_i$ in all the three cancer cell lines. Their effect was less pronounced in U87-MG cell line compared to others. The question where now arises is: What is the significance of elevated $[Ca^{2+}]_i$ in these cancer cell lines following treatment with alpha, beta momorcharin. Previous studies have shown that sustained Ca^{2+} elevation can act as trigger for apoptosis or cell death [13]. In addition, Ca^{2+} overloading in mitochondria can induce a cell suicide programme by stimulating the release of apoptosis promoting factor like the release of cytochrome-c19,20. Cytosolic Ca^{2+} homeostasis in resting cells is achieved by balancing the leak of Ca^{2+} (entering from the outside of from the stores) by the constant removal of Ca^{2+} using pumps either on the plasma membrane or on the internal stores. These pumps ensure that cytoplasmic $[Ca^{2+}]_i$ remains low and that the stores are loaded with signal Ca^{2+}. In most cells, it is the internal stores (eg. ER, SR, mitochondria) which provide most of the signal calcium [7]. Furthermore, mitochondria have been found to play a pivotal role in Ca^{2+} signaling [13]. The cellular free Ca^{2+} is an important physiological mediator and regulator in the stimulus-secreting coupling process in different epitheli-

al cells [22], Many studies, have demonstrated mitochondrial Ca^{2+} overload as the link between complement deposition and the observed changes in mitochondrial physiology and the triggering of programmed cell death (PCD)6. The mitochondrial Ca^{2+} overload is responsible for the increased O(2)(*-) production8. The rates of mitochondrial membrane potential dissipation and mitochondrial Ca^{2+} uptake may determine cellular sensitivity to Ca^{2+} toxicity under pathological conditions, including ischemic injury [14]. It has also been suggested that only a subpopulation of mitochondria undergoes a permeability transition and releases apoptogens, whereas the remaining, undamaged mitochondria respire normally and produce ATP [21, 22].
The result presented in this study have demonstrated clearly that the anti-cancer effects of alpha, beta momorcharin are mediated via apoptosis and cellular Ca^{2+} overloading.

Conclusion

The results of this study have clearly demonstrated that the isolated and purified protein of *M. charantia*, namely alpha, beta momorcharin can evoke significant decreases in cancer cell viability (cell death) by exerting their anti-cancer effect on cells via damage of cell mitochondria body resulting in elevation in elevation of intracellular free calcium concentration $[Ca^{2+}]_i$. A combination of all these factors may lead to Ca^{2+} overloading in the mitochondria resulting in cancer cell death. However, further experiments are required to investigate the sub- cellular mechanisms associated with cell death including the involvement of kinase and gene expressions for apoptotic mediators.

Conflict of Interests: The author has not declared any conflict of interest.

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