

RESEARCH HIGHLIGHT

Effects of crude water-soluble extract of *Momordica charantia* on viability, caspase activity, cytochrome-c release and on cytosolic calcium levels in different cancer cell lines

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Prior to the availability of chemotherapeutic agents, dietary measures, including traditional medicines derived from plants, were the major forms of cancer treatment. One such plant is *Momordica charantia* (Family: Cucurbitaceae), whose fruit is known as corilla or bitter gourd/melon. *M. charantia* possesses anti-carcinogenic properties and it can modulate its effect via xenobiotic metabolism and oxidative stress. This study investigated the anti-cancer effect of an active water-soluble extract (s) of *M. charantia* on cell viability and its cellular mechanism(s) of action in inducing cell death. The fruit was washed and cut in to small pieces, liquidised in deionised water using a blender and subsequently, dried using a rota evaporator and oven. Both time course (800 µg/ml) and dose- dependent (200 µg/ml-800 µg/ml) experiments were performed treating six different cancer cell lines, namely 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and normal L6 muscle cell line with the crude fruit extract for 24-48 hours at 37 °C. Cell viability was measured using the MTT assay. The results show that the crude water soluble extract of *M. charantia* can evoke both time-course (800 µg/ml) and dose-dependent (200 µg/ml - 800 µg/ml) decreases in cell viability (cell death) with maximal increases in cell death employing 800 µg/ml over a period of 24 hours following incubation. The results of this study have also shown that the crude water-soluble extract of *M. charantia* (800 µg/ml) can elicit marked and significant ($p < 0.05$) increases in the activities of caspase - 3 and caspase - 9 in all the cell lines. The crude water soluble extract of *M. charantia* can stimulate the release of cytochrome-c and elevated intracellular free calcium concentrations $[Ca^{2+}]_i$ in the different cancer cell lines compared to untreated cell lines. These results clearly show that *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; crude water-soluble extract of *M. charantia*; cytochrome c; calcium; caspase; cell viability

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Introduction

A multitude of plants have been identified and used for the treatment of different diseases throughout the world, especially in poor countries. Much research has been focused on the scientific evaluation of traditional derived drugs from the tropical plant, *Momordica charantia* (*M. charantia*). The whole plant, its green fruits or extracts from both the plant and its fruits have been commonly or frequently used as an anti-cancer and anti-diabetic agents and each is often described as food of medicine [1]. *M. charantia* is commonly known as bitter melon, bitter gourd or corilla. Bitter gourd grows in all tropical parts of the world and it is cultivated throughout South America, Asia and Africa. The plant is a slender climbing annual vine with long-stalked leaves and yellow, solitary male and female flowers borne in the leaf axils [2]. It is related to squash and cucumber plants. The fruit looks like a warty gourd. The young fruit is emerald green, turning to orange-yellow when it is ripped.

The Latin name *Momordica* means “to-bite” referring to the jagged edges of the leaves, which appear as if they have been bitten. All parts of the plant, including the fruit, the stem and seed taste very bitter. In botanical terms, the plant is referred as Family: Cucurbitaceae, Genus: *Momordica*, Species: *charantia*, Synonyms: *Momordica chinensis*, *Momordica elegans*, *Momordica indica*, *Momordica operculata*, *Momordica sinensis* and *Silyos fauriei*. Common Names: Bitter Melon, *papailla*, *melaode saocaetano*, bittergourd, balsam apple, balsam pear, *karela* or *corilla*, *ku kua karela*, *kor-kuly*, *ku gua*, *para-aki*, *salsamino*, *Soru*, *Sorosis borossieb*, *pare*, *peria La at*, *peria*.

In the Amazon, local people grow bitter melon in their gardens for food and medicines [3]. They add the fruit for bitter or sour flavor by parboiling it first with a dash of salt to remove some of the bitter taste [4-6]. It is used as leaf tea for the treatment of diabetes, to expel intestinal gas, to promote menstruation and as an antiviral treatment for measles, hepatitis and feverish conditions [1, 7]. It is also used typically for sores, wounds, infections and also internally and externally to treat for worms and parasites. *M. charantia* is also used for the treatment of cancer tumours [8, 9]. In the last few decades, several studies that have been carried with *M. charantia* using modern tools and they have credited *M. charantia* with anti-diabetic, anti-viral, anti-tumor, anti-leukemic, anti-bacterial, anti-helminthics, anti-mutagenic, anti-mycobacterial, anti-oxidant, anti-ulcer, anti-inflammatory and hypochlolestromic, hypoglyceridemic, hypotensive, immune-stimulant and insecticidal properties [3, 10-12]. *M. charantia* seems to have universal medicinal properties for the

treatment of different diseases. In the light of its different potential medicinal values and properties, this study was designed specifically to investigate its anti-cancer properties employing six different cancer cell lines and a normal healthy cell line. In addition, its cellular mechanism(s) of action in inducing cancer cell death was also investigated for comparison.

Materials and methods

Extraction of crude water-soluble extract of *M. charantia*

The unripe green intact fruits of *M. charantia* were obtained from the local supermarket and subsequently cleaned and cut into small pieces. Approximately, one kilogram of chopped green fruit was liquidized in distilled water for 5-10 min using a blender. The juice was then kept in a hot water bath for 2 hours at the temperature of 67°C. The fruit juice was centrifuged at 5000 RPM (Beckman, UK) for 30 min. The suspension was removed and filtered through Whatmann filter paper (No: 4 Whatmann, UK). The filtered green sample (supernatant) was then transferred to the 1000 ml round bottom rotating flask. The flask was then connected to the Rota evaporator machine through a clamp. The rotating flask was then heated by partial emersion in a hot water bath at a temperature of 40°C. A typical 120-rpm speed was used for the flask rotation. The Rota evaporated sample was then scrapped using spatula and dried overnight in an oven at 43°C. This crude water-soluble extract (powder) was stored at 2°C for further use.

Preparation and application of crude water-soluble extracts of *M. charantia* on the cancer and L6 cell lines

An amount of 30 mg of the crude water-soluble extract of *M. charantia* was initially dissolved in 500 µl of phosphate buffer by continuous stirring and with the brief use of a sonicator water bath. This was made up to 5 ml by adding 4.5 ml of the cell medium. The water-soluble crude extract stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22 µm filters into other sterile 10 ml Universal bottles. These stock solutions were stored in sealed tubes in the fridge until required. Once removed from the fridge, the prepared crude water-soluble extract of *M. charantia* solutions were gently warmed in water bath at 37°C in order to ensure that the water-soluble crude extract was mixed complete in solution, before aliquoting. Volumes of 34 µl, 67 µl, 100 µl, 134 µl contained approximately, 200 µg/ml, 400 µg/ml, 600 µg/ml, and 800 µg/ml of the crude water-soluble extract of *M. charantia*, respectively.

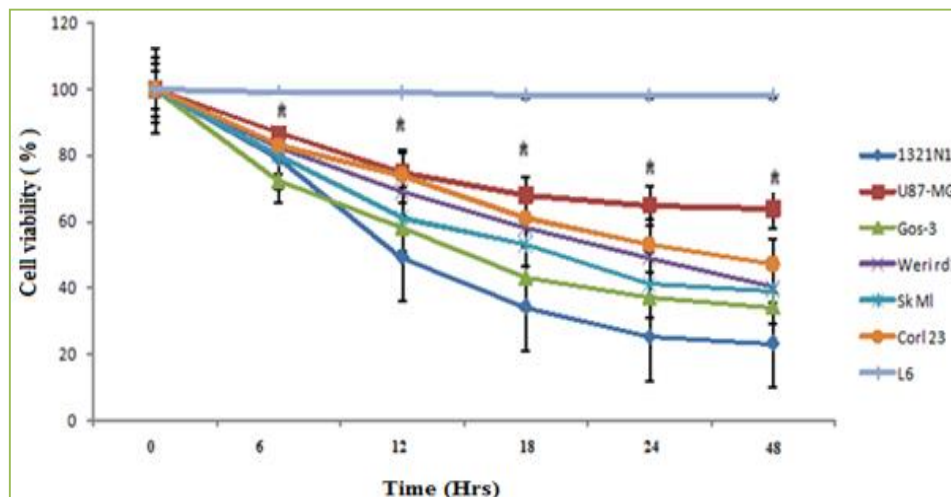


Figure 1. Time course effects of the crude water-soluble extract of *M. charantia*. Time-course effects of 800 µg/ml of crude water-soluble extract of *M. charantia* on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl -23, Weri Rb-1) and on healthy L6 muscle cell line for comparison. Cell lines also incubated at the same time points without any extract are also shown in the figure for comparison. Solid straight line shows the 100 % values for each time point (untreated cell lines). Cell viability for each time point was expressed as percentage of the respective control (no extract, but only cells in medium) at each time point of incubation. Each cell line was incubated with the crude water-soluble extract of *M. charantia* for up to 48 hrs. All Data are mean \pm SD, n = 6 different experiments in duplicate; (* p < 0.05) for all test samples compared to control (100 %).

Time-course effects of the crude water-soluble extract of *M. charantia* on cell viability

Each cell line (6 different cancer cell lines namely 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and healthy L6 skeletal muscle cell line) was incubated with 800 µg/ml of the extract for 6, 12, 18, 24 and 48 hours. In another series of experiments all six cell lines were grown alone in the medium for the same time points in the absence of any extract for comparison. At the end of each experiment, cell viability was measured by MTT assay [16-18]. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), the crude water-soluble extract of *M. charantia* was able to evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (see Figure 1). From these initial time- course experiments, it was established that cell viability (cell death) decreased to its maximal level after 24 hours of incubation with the crude water-soluble extract of *M. charantia*. No further significant effect on cell viability was observed at 48 hours of incubation compared to 24 hours. The incubation time of 24 hours was employed in all the dose- dependent experiments of this study as well as those involved in investigating the mechanism(s) of action of the extract on cell death.

Dose-dependent studies

Different concentrations (200 µg/ml, 400 µg/ml, 600

µg/ml, and 800 µg/ml) of water-soluble crude extract in the cell medium was transferred in triplicate using a Gilson pipette to 96 well plates to give a final volume of 200 µl to the treated cell wells. An equivalent volume of 200 µl of the medium was added to the control (untreated) well with cells. Both control (untreated) and treated (with extract) 96 well plates were incubated for 24 hours and the cell viability was measured by MTT assay as outlined earlier [16-18].

Measurement of caspase-3 activities in treated and untreated cells

The six different cancer cells lines and healthy L6 skeletal muscle cell line were incubated with 800 µg/ml crude water-soluble extract of *M. charantia*. In another series of experiments, the different cell lines were incubated with the medium alone. The extract-induced apoptosis cell suspension for each cell line contained roughly 10^7 cells. A sample of non-induced cells for a zero-time (control) also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hours at 37°C in 5 % CO₂ atmosphere. The induced cells and the control cells were transferred to 15 ml centrifuge tube and centrifugation at 600 x g for 5 min at 4 °C. The supernatant was removed by gentle aspiration for both induced and control cell suspensions. The cells were then pelleted and washed once with 1 ml of phosphate buffer solution (PBS) and centrifuged at 5000 rpm for 5 min and the supernatant was completely removed by gentle aspiration. The centrifuged

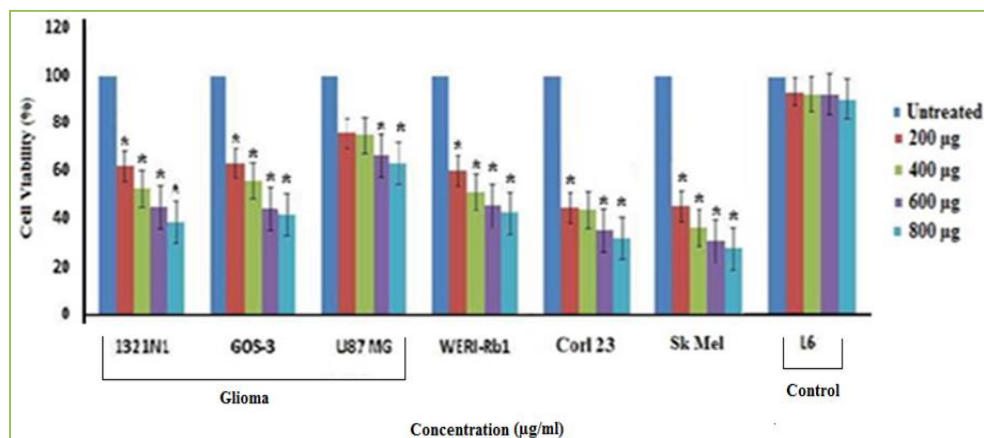


Figure 2. Effects of the crude water-soluble extract of *M. charantia*. Bar charts showing the effects of different concentrations (200 µg/ml - 800 µg/ml) of the crude water-soluble extract of *M. charantia* on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel-2, Corl-23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no crude extract) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each cell line was incubated with the crude water-soluble extract of *M. charantia* for 24 hrs. Data are mean \pm SD, n = 6 different experiments in duplicate; (* p<0.05) for untreated (100 % viability) compared to the treated cells for the different concentrations. Note the pronounced anti-cancer effects of the crude water-soluble extract of *M. charantia* on 1321N1, SkMel and Corl-23 compared to the other cancer cell lines.

cell pellets were then treated with 1 X lysis buffer at a concentration of 100 µl per 10^7 cells and the cells were incubated on an ice for 15-20 min. The lysed cells were centrifuged at 16,000 to 20,000 x g for 10 to 15 min at 4°C and the supernatants were transferred to new 1 ml tube and subsequently frozen in liquid nitrogen and stored in aliquots at -70°C for further use. All the values were expressed as µmol/min/ml.

Measurement of caspase-9 activities in treated and untreated cells

The six different cancer cells lines and healthy L6 skeletal muscle cell line were incubated either with (test) or without (control) with 800 µg/ml crude water-soluble extract of *M. charantia*. The extract-induced apoptosis cell suspension contained roughly 10^7 cells. A sample of non-induced cells for a zero-time control also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hours at 37°C in 5 % CO₂ atmosphere. Prior to starting the assay the prepared sample of Caspase-Glo® 9 reagents was equilibrated to room temperature. After 24 hours prior to the experiment, each plate (96 well plates GRE 96 fb) was removed from the incubator for 30 min to equilibrate to room temperature. A volume of 100 µl of Caspase-Glo® 9 assay reagent was added to each well of a white walled 96 well plate containing 100 µl of blank and treated cells in the culture medium. The plate was then covered with the aluminium foil. The covered plate was then mixed gently by using a plate shaker at 300-500 rpm for 2 min. The plate was then incubated at room temperature for 30 min. The Caspase-Glo® 9 assay was

carried in the absence of light. The Luminescence was measured on a Tecan Plate reader with the appropriate settings for the plate. The software used for the Luminescence assay was XFLUORHGENIOSPRO version V 4.53. The plate type used from the software was specified as GRE 96 fb Pdf. The temperature was stated to be 20 - 23°C. All the values were expressed as µmol/min/ml.

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

The six different cancer cells lines and healthy L6 skeletal muscle cell line were incubated either with (test) or without (control) 800 µg/ml crude water-soluble extract of *M. charantia*. The extract-induced apoptosis cell suspension contained roughly 10^7 cells. A sample of non-induced cells for a zero-time control also contained 10^7 cells. The induced and non-induced cells were incubated for 24 hours at 37°C in 5 % CO₂ atmosphere. The induced cells and the control cells were transferred to 15 ml centrifuge tube and centrifugation at 1000 x g for 5 min at 4 °C. The supernatant was removed by gentle aspiration of both induced and control. The cell pellets were then washed once with 1 ml of PBS. The microsomal pellet was subsequently obtained by centrifuging at 12, 000 x g supernatant for 1 hour at 100,000 x g and the supernatant was removed completely by gentle aspiration. The centrifuged cell pellets were then treated with 1X lysis buffer at a concentration of 100 µl per 10^7 cells, and the cells were incubated on ice for 15-20 min. The lysed cells were centrifuged at 16,000 to 20,000 x g for 10 to 15 min

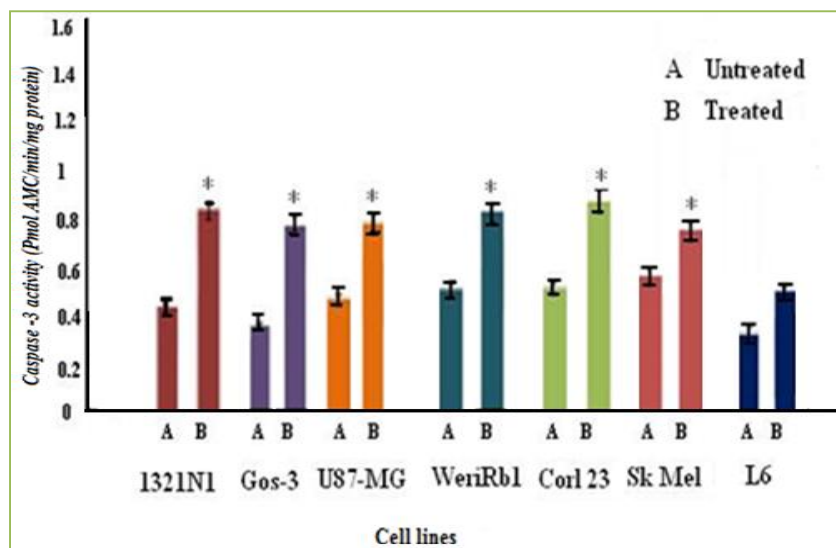


Figure 3. Measurements of caspase-3 activity in untreated and treated cells. Bar charts showing caspase-3 activity in (A) six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated alone in the medium without any crude extract for the duration of 24 hrs and (B) with six different cancer cell lines incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hours. Data are mean ± SD, n = 4 different experiments in duplicate. Note that caspase-3 activity increased significantly (* p < 0.05) in treated cells compared to untreated cells.

at 4°C and the supernatants were transferred to new 1 ml tube and subsequently frozen in liquid nitrogen and stored in aliquots at -70°C for further use. A volume of 950 µl of the working solution (9 mg of cytochrome-c to 20 ml of assay buffer to get a concentration of 0.45 mg/ml, 36 mM) was made up by adding 9 mg of cytochrome-c to 20 ml of the assay buffer in a 1 ml cuvette. A volume of 50 µl of the test sample was added to 1 ml cuvette containing the working solution. For the sample, which had interference from cytochrome-c oxidase activity, a volume of 20 µl of cytochrome-c oxidase inhibitor solution was used for the positive control reaction. The positive control was obtained by diluting an aliquot of the cytochrome-c reductase (NADPH) 10-fold with the enzyme dilution buffer. Each set of reactions required a total of 75 ml of the diluted positive control. A volume of 100 µl NADPH solutions was added to start the reaction. The blank reaction was measured by the value given by the reagents alone without enzyme present.

Time-course measurements of intracellular free concentrations $[Ca^{2+}]_i$ in untreated and treated cells

Measurement of $[Ca^{2+}]_i$ was performed as previously described in the literature [21]. 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 hours in order to synchronize cells into a non-proliferation stage. Cells

from each cell line (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1, L6 skeletal muscle) were then loaded with 25 µM Fura-2 acetoxyethyl 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, cells from each cell line 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 crude water-soluble extract of *M. charantia* (800 µg/ml) for different time points (0 min - 420 min) in a calcium free medium. Following incubation, 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-2AM were determined by calculations of

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

K_d , the Ca-Fura-2 dissociation constant, β the fluorescence intensity ratio, excited at 380 nm without and with Ca; f_{min} and f_{max} . 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 analyzed using Statistical Package for Social Sciences (SPSS) version 17, Student's t test and

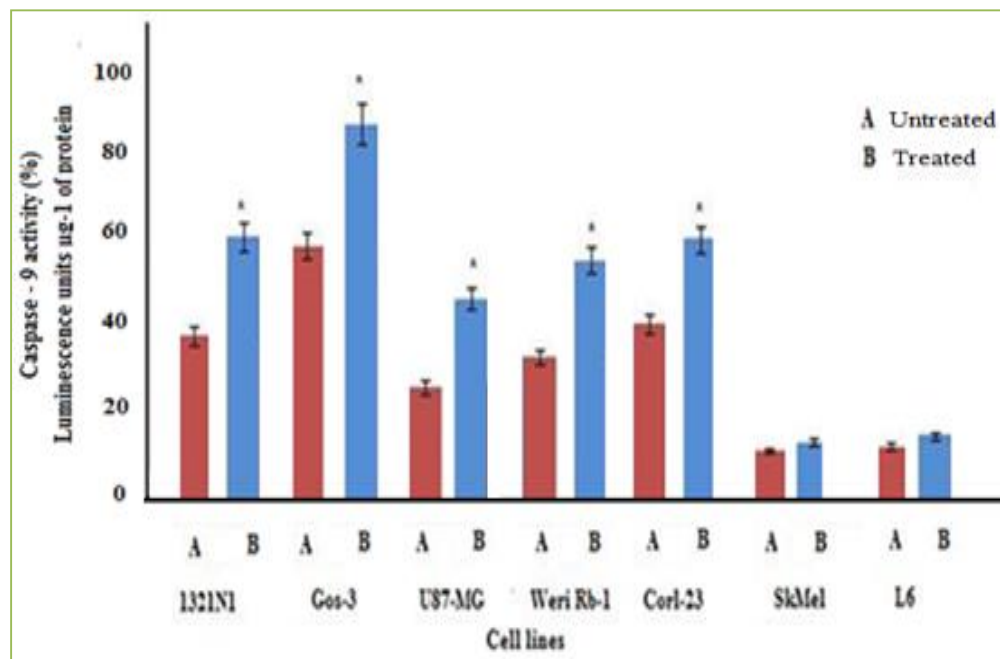


Figure 4. Measurements of caspase-9 activity in untreated and treated cells. Bar charts showing caspase-9 activity in (A) six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated alone in the medium without any crude extract for the duration of 24 hrs and (B) with six different cancer cell lines incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hours. Data are mean ± SD, n = 4 different experiments in duplicate. The results show that caspase-9 activity increased significantly (* p < 0.05) in treated (A) 1321N1, Gos-3, U87-MG, Corl- 23 and Weri Rb-1 cell lines compared to their respective untreated control (B). The crude water-soluble extract of *M. charantia* had little or no significant effect on caspase -9 activity in Sk Mel skin cancer and L6 skeletal cell lines.

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 signalling) to ensure the accuracy of results. A value of p < 0.05 was taken as significant.

Results

Figure 1 shows the time-course effects of 800 µg/ml of the crude water-soluble extract of *M. charantia* on the viability of different cancer cell lines (1321N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk Mel) and healthy L6 muscle cell line. Each cell line was incubated with the extract for 6, 12, 18, 24 and 48 hours. Also shown in the figure 1 are the different cell lines incubated with the medium alone for the same time for comparison. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), the crude water-soluble extract of *M. charantia* can evoke marked and significant (p < 0.05) decreases in the cell viability (cell death) compared to untreated cells. From these initial time- course experiments, it was established that cell viability (cell death) decreased to its maximal level after 24 hours of incubation with the crude water-soluble extract of *M. charantia*. This time point was used in

subsequent experiments.

Figure 2 shows the effects of different concentrations (200 µg/ml - 800 µg/ml) of the crude water-soluble extract of *M. charantia* on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 2 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), the crude water-soluble extract of *M. charantia* evoked marked and significant (p < 0.05) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the crude extract were dose-dependent with maximal cell death occurring with 600 µg/ml and which was not significantly (p > 0.05) different from 800 µg/ml. In contrast, the crude water-soluble extract of *M. charantia* had a little or no effect on the death of healthy L6 skeletal muscle cell line for comparison. The results also show that the crude extract was more effective in killing 1321N1, Sk Mel and Corl-23 cell lines compared to its effect on Gos-3 and U87-MG cell lines. Figure 3 shows caspase-3 activity in (A) the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6

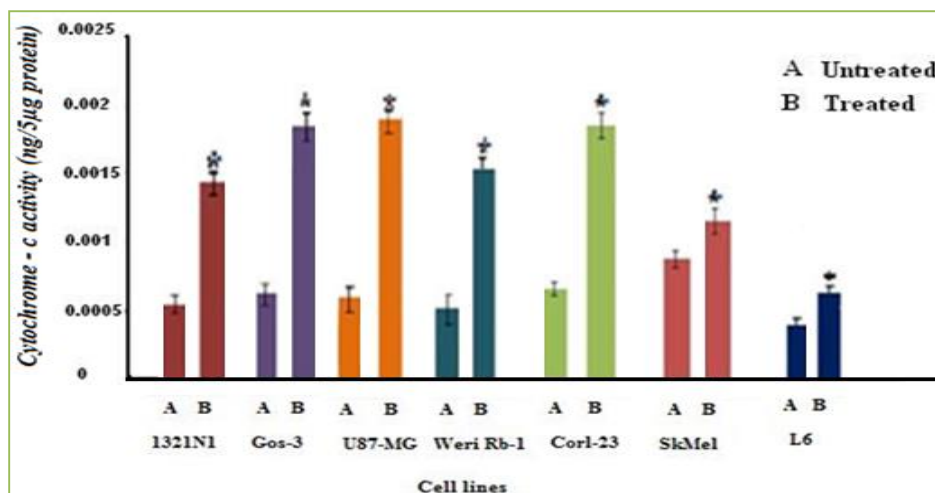


Figure 5. Measurement of cytochrome – c release (activity) in untreated and treated cell lines. Bar charts showing cytochrome-c release (activity) in (A) the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated alone in the medium without any crude extract for the duration of 24 hrs and (B) with six different cancer cell lines incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hrs.. Data are mean \pm SD, n = 4 different experiments in duplicate. Note the significant (* p < 0.05) increases in cytochrome-c release in treated cells (B) compared to untreated cells (A).

muscle cell line incubated individually in the medium without any crude extract for the duration of 24 hours and (B) with six different cancer cell lines and L6 muscle cells incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hours. The results show that the crude extract can elicit significant (p < 0.05) increases in caspase -3 activity in 1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 cancer cell lines compared to untreated cell line for each. There was little or no significantly increase in caspase-3 activity in either Sk-Mel or L6 healthy skeletal muscle cell line compared to the respective untreated cell lines.

Figure 4 shows caspase-9 activity in (A) the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl-23 and Weri Rb-1) and healthy L6 muscle cell line incubated alone in the medium without any crude extract for the duration of 24 hours and (B) with six different cancer cell lines and L6 skeletal cells incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hours. The results show that the crude extract can elicit significant (p < 0.05) increases in caspase -9 activity in 1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 cancer cell lines compared to untreated cell line for each. There was little or no significantly increase in caspase-9 activity in Sk Mel or L6 healthy skeletal muscle cell line compared to the respective untreated cell lines.

Figure 5 shows the activity (release) of cytochrome-c in (A) the six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl- 23 and Weri Rb-1) and healthy L6 muscle cell line incubated alone in the medium without any

crude extract for the duration of 24 hours and (B) with six different cancer cell lines incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* for 24 hours. The results show that the crude water-soluble extract of *M. charantia* can evoke significant (p < 0.05) increases in cytochrome-c activity in all the cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and L6) compared to untreated cell lines. The increase in cytochrome-c activity in 1321N1, Gos-3, U87-MG, Corl-23 and Weri Rb-1 cancer cell lines were much more pronounced compared to control cytochrome-c activity with Sk Mel and L6 health skeletal muscle cells.

Figure 6 shows the maximum elevation of $[Ca^{2+}]_i$ in 1321N1, Gos-3, U87-MG, Sk Mel, Corl-23, Weri Rb-1 and L6 cell lines incubated with 800 µg/ml of the crude water-soluble extract of *M. charantia* over a period of 420 min. The concentrations of $[Ca^{2+}]_i$ prior to extract treatment (control) of each cell line with the extract and in the absence of the extract after 420 min of incubation in the culture medium are also shown in figure 6 for comparison. The results show that the water-soluble extract of *M. charantia* can evoke large increases in $[Ca^{2+}]_i$ for the six different cancer cell lines with maximal increases in $[Ca^{2+}]_i$ after 420 min of incubation. In contrast, the extract had virtually little or no effect on $[Ca^{2+}]_i$ in healthy L6 skeletal cells. The results also show the basal $[Ca^{2+}]_i$ at 0 min and increases in $[Ca^{2+}]_i$ in untreated and treated cells for a duration of 420 min. The data clearly reveal that 800 µg/ml of the crude water-soluble extract of *M. charantia* can evoke significant (p < 0.05) increases in $[Ca^{2+}]_i$ compared to control at 0 min,

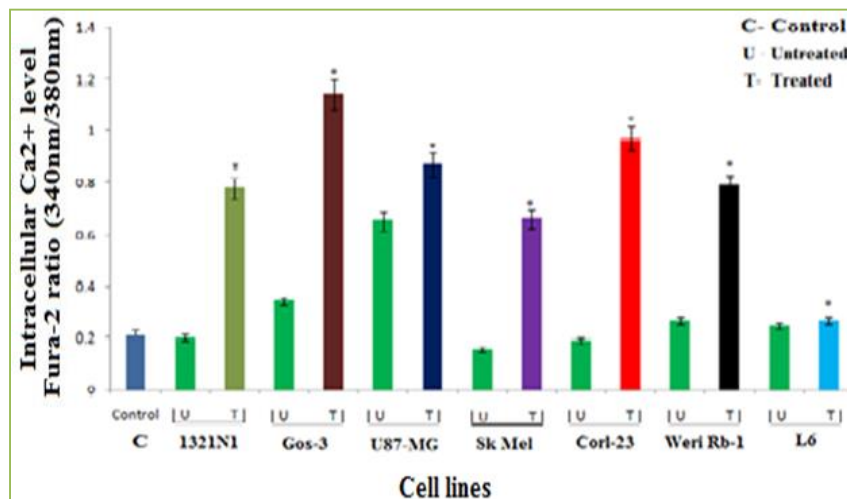


Figure 6. Elevation in intracellular free calcium concentration $[Ca^{2+}]_i$ in untreated and treated cells. Bar charts showing maximal increases in $[Ca^{2+}]_i$ in basal control (C), untreated (U) cells and treated (T) cancer cell lines as well as L6 cell line following incubation with 800 $\mu\text{g/ml}$ crude water-soluble extract of *M. charantia* for 420 min. The data are expressed as mean \pm SD (ratio units); n = 6; (* p < 0.01) for test (T) compared to either control (C) or (U) untreated cells. Note the large increases in $[Ca^{2+}]_i$ for 1321N1, Gos-3, Corl -23 and Weri Rb-1 cell lines and the significant (P<0.05) elevation for $[Ca^{2+}]_i$ in untreated GOS-3, and U87-MG compared to control (C). It is also noteworthy that the extract had no significant effect on $[Ca^{2+}]_i$ in healthy L6 skeletal muscle cell line.

as well as untreated cells over the same duration of incubation.

Discussion

The results of this study have demonstrated significant anti-cancer effects of the crude water-soluble extract of *M. charantia* on the six different cancer cell lines compared to untreated control cells and healthy L6 skeletal muscle cell line. The results show that the extract can elicit both time course and dose -dependent decreases in cell viability (cell death) with maximal effect on cell death after 24 hours of treatment. Anti-cancer drugs are believed to exert their 'killing' effects on cells via different cellular and sub-cellular mechanisms including damages to the cell membrane, mitochondria and microtubules, inhibition of kinases or by cellular calcium over-load [13-16]. This study employed two cellular pathways via which the crude water-soluble extract of *M. charantia* may exert its anti- cancer effects on cell death, namely, apoptosis and cellular calcium homeostasis.

The results also show that the crude water-soluble extract of *M. charantia* can elicit marked and significant changes in the activities of caspase-3 and caspase-9 and in the release of cytochrome-c in all the cell lines employed in this study compared to control untreated cell lines. In addition, the crude water-soluble extract of *M. charantia* can elevate intracellular free calcium concentrations in all six-cancer cell lines compared to untreated control cells.

Apoptosis is programmed cell death and it is associated with damage of cell mitochondria in the body to elevate such intra-cellular mediators as caspase-3 and caspase-9 and the release of cytochrome-c [17, 18]. 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 [19, 20]. There are two major apoptotic pathways in mammalian cells namely the receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [21]. 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 [22]. 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 [22- 26]. In this study, the activity of caspase-3 was determined using the caspase-3 activity kit. To evaluate the activity of caspase-3, the different cell lines were treated with the 3.3 mM RIP or RIP-PEG conjugate, the cell lysates were collected, respectively at 0, 12, 24 and 36 h after exposure. The caspase activities were measured as fold of enzyme activity in comparison with control [27].

The results of the present study have also shown that the crude water-soluble extract of *M. charantia* can evoke significant and gradual time-dependent increases in $[Ca^{2+}]_i$ in all the six cancer cell lines employed in this study compared to untreated cancer cells and. the L6 skeletal

muscle cell line. Maximum elevation in $[Ca^{2+}]_i$ occurred following 420 min of treatment with the extract. The present show that U87-MG cells released less cytosolic calcium compared to other cancer cell lines. The question which now arises is: What is the significance of elevated $[Ca^{2+}]_i$ in these cancer cell lines following treatment with the crude water-soluble extract of *M. charantia*? Previous studies have shown that sustained Ca^{2+} elevation can act as trigger for either apoptosis or cell death [21]. In addition, Ca^{2+} overloading in mitochondria can induce a cell suicide programme by stimulating the release of an apoptotic promoting-like factor which in turn triggers the release of cytochrome-c [28-30]. 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 and mitochondria), which provide most of the signal calcium [31].

Furthermore, mitochondria have been found to play a pivotal role in Ca^{2+} signaling [32]. The cellular free Ca^{2+} is an important physiological mediator and regulator in the stimulus-secreting coupling process in different epithelial cells [33, 34]. Many studies have previously 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) [35]. The mitochondrial Ca^{2+} overload is responsible for the increased O₂ (•) production [36]. 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 [37]. It has also been suggested that only a subpopulation of mitochondria undergoes a permeability transition and releases of apoptogens, whereas the remaining, undamaged mitochondria respire normally and produce ATP [34, 38].

Loss of Ca^{2+} homeostasis, often in the form of cytoplasmic increases, leads to cell injury. Depending upon the cell type and the intensity of Ca^{2+} toxicity, the ensuing pathology can be either reversible or irreversible [37-40]. Although Ca^{2+} activates multiple destructive processes, lethal outcomes are determined largely by Ca^{2+} -induced mitochondrial permeability transition [41, 42]. This form of damage is primarily dependent upon mitochondrial Ca^{2+} accumulation, which is regulated by the mitochondrial membrane potential [43-47].

In conclusion, the results of this study have clearly demonstrated that the crude water-soluble extract of *M. charantia* can evoke significant decreases in cancer cell viability (cell death) by exerting its anti-cancer effect on

cells via damage of cell mitochondria body resulting in elevation in such cellular mediators as caspase-3 and caspase-9 and the release of cytochrome-c and an 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. Similar findings were obtained when the different cell lines were treated with α , β momorcharin, an isolated protein from *M. charantia* [48]. However, the effect of α , β momorcharin was less pronounced on cell death compared to the water soluble extract as shown in this study. 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.

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