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Chromatographic determination of alpha beta momocharin and its effects with the combination of temozolamide and vinblastine in the treatment of glioma cancer *In-Vivo*

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ABSTRACT

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 M.charantia (Family: Cucurbitaceae), whose fruit is known as Karela or bitter gourd. M.charantia is believed to posse's anti-carcinogenic properties and it can modulate its effect via xenobiotic metabolism and oxidative stress. Different concentration (200µM - 800µM)of the alpha and beta momorcharin a protein extracted from bitter gourd fruit, were treated (24 hrs incubation) separately with six different cancer cell lines 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 and normal L6 muscle cell line. The results also show that combining either temozolomide (240 µM) or vinblastine (40 µg) with (800 µM) alpha and beta momorcharin, result in significant decreases in cell viability for each cell line, these effects were additive compared to the individual effect of temozolomide or vinblastine.

Keywords: Cancer cell lines, Momordicacharantia (MC)alpha, beta momorcharin (α, β),temozolomide (TMZ) andvinblastine (VIB).

INTRODUCTION

MC contains a number of biologically active plant-based chemicals including triterpenes, proteins and steroids,alkoloids, inorganic lipids and phenolic compounds.[1-2] Some proteins in bitter melon including MAP-30, MRK29, alpha-momocharin, beta-momocharin and momordicin and they have the ability to treat tumours and HIV.[3-4] A steroid called charantin is found mainly in the aerial parts of MC and it has been shown to possess anti-diabetic properties.[5-6]The phenolic compounds extracted from MC using a solvent extraction procedure were reported to exhibit anti-oxidant activity.[7-8]The ripe fruit of MC has been shown to exhibit some remarkable anti-cancer effects, especially leukemia.[9]A chemical analogue of a protein isolated from MC was developed and named MAP-30. It was reported that MAP-30 can inhibit prostate tumour growth. Several phytochemicals extracted from MC have been documented with cytotoxic activities and they include a group of ribosome-inactivating proteins named (α,βmomocharins, momordin and cucurbitacin B).[9-12]

Temozolomide (Temodal) is an alkylating agent derived from dacarbazine and first synthesised in 1984. Temozolomide (trade name: Temadol in Europe, Temador in the USA) is a new chemotherapy agent that has generated considerable interest as a treatment for glioma. It is recommended for the treatment of patients with malignant gliomas showing recurrence or progression after standard therapy.[13] FDA in the USA has approved TMZ for the treatment of glioma.

The Vinca alkaloids have become clinically useful since the discovery of their anti-tumour properties in 1959.[14]VIB is a chemotherapeutic drug that belongs to the class of microtubule depolymerising agents and binds specifically to tubulin, inhibiting its polymerization and the subsequent association of microtubules.[15-16]VIB is mainly used to treat bladder cancer and to a lesser extent to treat other cancers including lymphoma and Kaposls sarcoma.[15]

In the light of its different potential medicinal values and properties, this study was designed specifically to investigate its anticancer effects either combined with TMZ and VIB by employing six different cancer cell lines and a normal healthy cell line.

MATERIALS AND METHODS

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 mM sodium phosphate buffer at pH 7.5. The unbound proteins was 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 mMTris-HCl buffer, pH 7.8. The chromatography was performed on Bio Logic Duo Flow 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^{o}Cand subsequently placed in the water bath at 37^{o}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 cm^2 flask or 1 ml if 25 cm^2 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 minuntil 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 incubation.

Dose-dependent effects of*α, β* momorcharinon the cancer and L6 cell line viability

In this series of experiments, different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk Mel) and healthy L6 muscle cell line were incubated with the different concentrations (200 µM - 800 µM) of α, β momorcharinfor 24 hours. Control cell lines were also incubated for the same period of time but without any (200 µM - 800 µM) of α, β momorcharin. At the end of the incubation period, cell viability of each cell line was measured using the MTS assay.

Dose dependent effects of either TMZ or VIB on cancer cell line viability

In this series of experiments, different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk Mel) and healthy L6 muscle cell line were incubated with the different concentrations of either TMZ (80 - 320 µM) or VIB (10 - 40 µg) for 24 hours. Control cell lines were also incubated for the same period of time but without any TMZ or VIB. At the end of the incubation period, cell viability of each cell line was measured using the MTS assay.

Application of α, β momorcharineither with TMZ or with VIB on the cancer and L6 cell lines

Either VIB (40 µg) and TMZ (240 µM) alone or theα, β momorcharin(800 µM, a high dose) alone or combination of VIB(40 µg) or TMZ (240 µM) with theα, β momorcharin(800 µM) on the viability of the six different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. All the cells were treated with either VIM or TIM orα, β momorcharinor combined drugs (drug + α, β momorcharin) for 24 hours. Control cell lines were also incubated for the same time.

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

RESULTS

Dose-dependent effects of the α, β momorcharin

Figure 1 shows the effect of different concentrations (200 µM - 800 µM) of α, β momorcharinon the viability of six different cancer cell lines and healthy L6 muscle cell lines for comparison. All the cells were treated for 24 hours. Control cell lines were also incubated for 24 hrs but without any α, β momorcharin. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) α, β momorcharin evoked marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effect of α, β momorcharin was also dose-dependent with maximal cell death occurring 800 µM. In contrast, α,β momorcharinhad a little or no effect on the death of healthyL6 skeletal muscle cell line. α, β momorcharin was more effective in killing Sk Mel and Corl -23 cell lines compared to Gos-3 and U87-MG and 1321N1 cell lines.

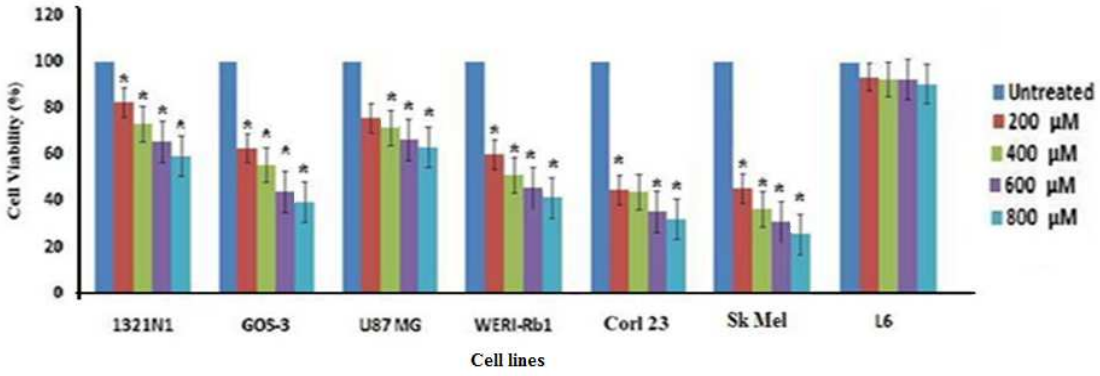

Fig.1 Dose-dependent effects of α, β momorcharin

Dose-dependent effects of VIB on cell viability

Figure 2shows the effects of different concentrations (10 - 40 µg) of VIB 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. All the cells were treated with VIB for 24 hours. Each control cell lines were also incubated for 24 hrs but with no VIB. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1), VIB can evoke marked and significant ($p < 0.05$)decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the VIB were dose-dependent with maximal cell death occurring with 40 µg. Similarly,VIB significantly ($p < 0.05$) decreased the viability of healthyL6 skeletal muscle cell line compared to untreated L6 cell line but mainly at a high doses. The results also show that VIB was more effective in killing 1321N1, Gos-3, Sk Mel and Corl -23 cell lines. It has less effective on U87-MG cell line, which seems to be more resistant to the drug. The surprised finding in this study was that VIB could also kill healthy L6 skeletal muscle cell compared to the α, β momorcharin which had no detectable effect on the viability of L6 cell line.

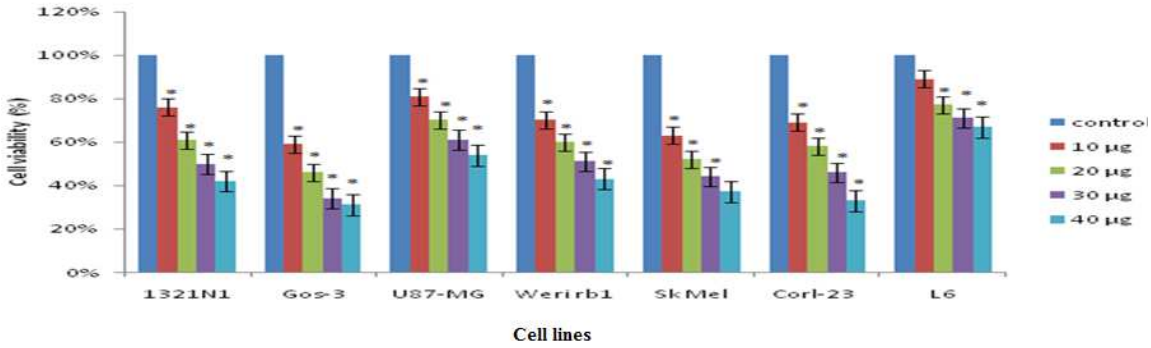

Fig.2: Dose-dependent effects of Vinblastine

Dose-dependent effects of TMZ on cell viability

Figure 3 shows the effects of different concentrations (80 µM - 320 µM) of TMZ 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 3 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were incubated for 24 hours either with or without TMZ. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23 and Weri Rb-1) TMZevoked marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). These effects of the TMZ were dose-dependent with maximal cell death occurring with 320 µM. Similarly, TMZ evoked a significant ($p < 0.05$) decrease in viability of healthyL6 skeletal muscle cell line but this was less compared to the cancer cell lines. The values reach significant levels ($p < 0.05$) compared to control (untreated) L6 cells. This effect of TMZ on L6 muscle cells was dose-dependent. The result also show that the TMZ was more effective in killing 1321N1, Gos-3, Sk Mel, Weri Rb-1 and Corl -23 cell lines. It was less effective on and U87-MG cell line.

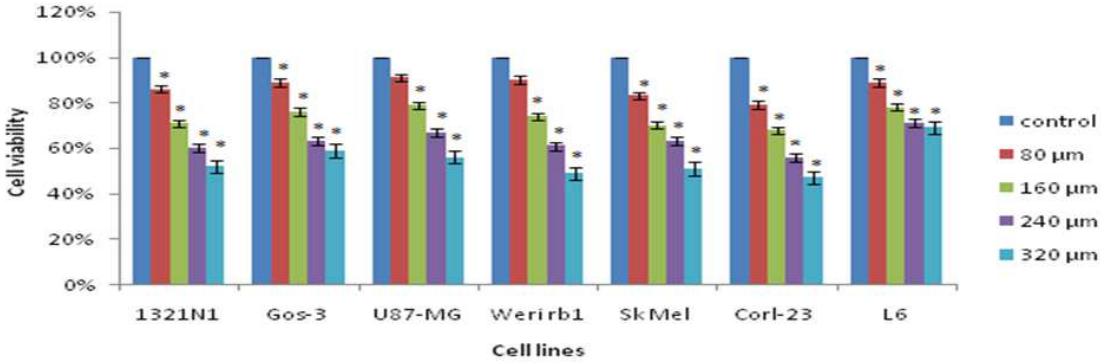

Fig.3: Dose-dependent effects of Temozolomide

Combined effects of α,β momorcharin with either VIB or TMZ

Figure 4 shows the effect of either 40 µg of the vinblastine, 800 µM of α,βmomorcharin alone or combining α,β momorcharin(800 µM) with vinblastine (40 µg)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 4 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison (100 % viability). All the cells were treated with either vinblastine,α,βmomorcharinor combined drugs (vinblastine + α,β momorcharin) for 24 hours. Control cell lines were also incubated for the same time of 24 hours. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23 and WeriRb-1) either vinblastine,α,βmomorcharinorcombined drugs (vinblastine + α,β momorcharin) can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). In all the cell lines vinblastine was more effective on cell viability compared to α,βmomorcharin alone. Moreover, the results also show that when vinblastine combined with α,βmomorcharin they were slightly more effective in killing 1321N1, U87-MG, Sk Mel and Corl -23 cell lines. In contrast, they were less effective on Gos-3 cell line. The result also show that either vinblastine or a combination of vinblastine with α,βmomorcharin can elicit a significant decrease of L6 skeletal muscle cell line compared to untreated cells or treated with α,β momorcharin alone.

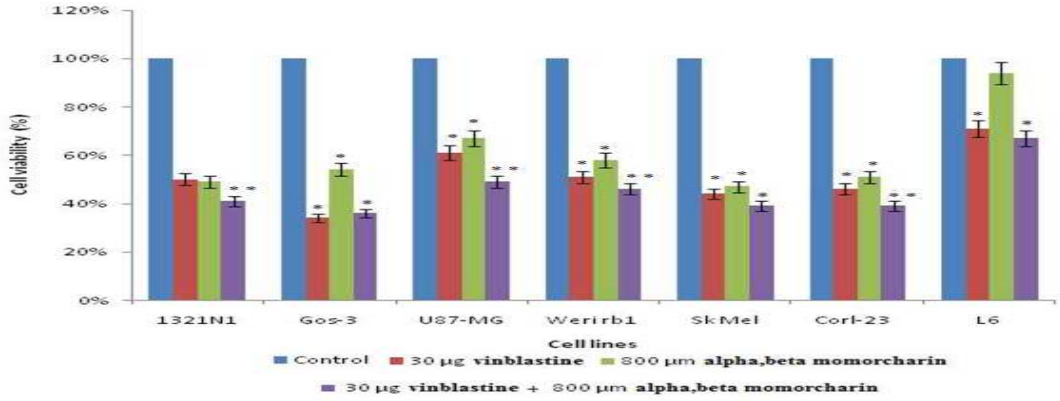

Fig.4: Effect of either of 40 µg VIBalone or 800 µM of α,β momorcharinalone or a combination of VIB (40 µg) and the α,β momorcharin800 µM on the viability of six different cancer cell lines

Figure 4bar charts showing effect of either 40 µg of the vinblastine alone or 800 µM α,β momorcharin alone or combining α,β momorcharin(800 µM) with vinblastine (40 µg) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl -23 and Weri Rb-1) and healthy L6 muscle cell line. The untreated (no vinblastine, α,βmomorcharin, or combined drugs) cell line for each (first bar chart) is also shown as 100% in the figure 4 for comparison. Each cell line was incubated with for 24 hrs either with or without drugs. Data are mean ± SD, n = 6 different experiments in duplicate; * $p < 0.05$ for untreated (100 % viability) compared to the treated cells for the different concentrations. Similarly, ** $p < 0.05$ for the combined effect of vinblastine + α,βmomorcharin compared with either vinblastine or αβ momorcharin alone. Note that vinblastine or combination of vinblastine with αβ momorcharin can also decrease the viability of healthy L6 skeletal muscle cell line.

Figure 5 bar charts showing the effect of either temozolomide 240 µM alone, or α,βmomorcharin 800 µM alone or a combination of α,β momorcharin(800 µM) with temozolomide (240 µM)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 5 are the untreated (no temozolomide or α,βmomorcharin) six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either temozolomide,α,βmomorcharin or a combination of temozolomide with α,β momorcharin for 24 hours. Control cell lines were also incubated for the same time. The result shows that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) either temozolomide,α,β momorcharinor a combination oftemozolomide with α,β momorcharin can evoke marked and significant ($p < 0.05$) decreases in the cell viability (cell death) compared to untreated cells (100% viability). Similarly, either temozolomide or a combination of temozolomide with α,βmomorcharin can evoke significant ($p < 0.05$) decreases in the viability of healthyL6 skeletal muscle cell lines. The results also show that a combination of temozolomide with α,β momorcharin was slightly more effective in killing 1321N1, Gos-3, Weri Rb-1, Sk Mel and Corl-23 cell lines compared to either temozolomide or α,β momorcharin alone. However, temozolomide with α,βmomorcharin seem to be slightly more effective on U87-MG cell line.

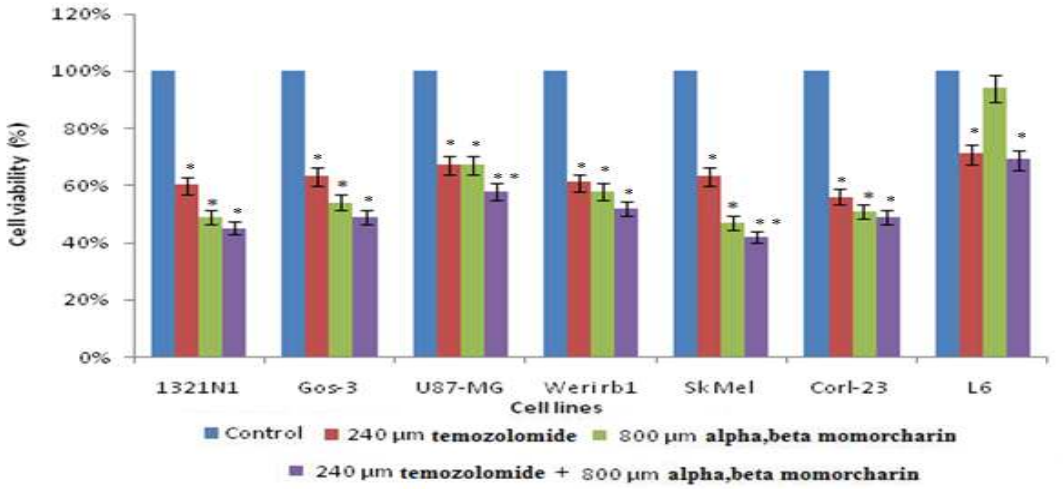

Fig.5:Effect of either TMZ240 µM alone or 800 µM of α,β momorcharin and a combination of the α,β momorcharin(800 µM) with TMZ(240 µM) on the viability of six different cancer cell lines

DISCUSSION

This study employed the α,β momorcharin(proteins isolated and purified from *M. charantia*) and two commercially available anti-cancer drugs namely, vinblastine and temozolomide to investigate their effects on the viability (cell death) of six different cancer cell lines compared to healthy L6 skeletal muscle cell line. Either α,βmomorcharin, vinblastine or temozolomide was tested alone measuring the viability of each cell line. In some experiments, either vinblastine or temozolomide was combined with α,βmomorcharinto investigate any potentiating or attenuating effect on cell viability. The dose-dependent experiments were carried out in this study. The rationale for this study was that *M. charantia*, a local plants-base (herbal) medicine could be used to treat different types of cancers. The results of the present study have demonstrated that the α,βmomorcharincan evoke significant time-dependent decreases (cell death) in the viability of six different cancer cell lines, namely 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 employed in this study. Maximal anti-cancer effect was obtained after 24 hours of incubation. In contrast, the α,βmomorcharinhad no significant anti-cancer effect of the viability of healthy L6 skeletal muscle cell line.

The results of this study have also shown that α,βmomorcharincan produce similar anti-cancer effects on the viability of each cell line.

Second, the present study also investigated the possibility in combining a high dose of α,βmomorcharinwith a low to moderate dose of commercially available anti-cancer drugs, vinblastine and temozolomide on the viability of each cell line. The rationale is that the commercially available anti-cancer drug in high concentration not only kill cancer cells, but also healthy cells in the body.[12] If a low to moderate dose of either temozolomide or vinblastine can be combined with a high dose of α,β momorcharinto produce maximal anti-cancer effect, without killing healthy cells, then they should be safer in treating cancer.

The results of the present study have shown that either vinblastine or temozolomide can significantly decrease the viability of 1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1 cancer cell lines. Both anti-cancer drugs also decreased the viability of healthy L6 skeletal muscle cell line. The effect of each drug was dose–dependent with maximal effect occurring at 40 µg for vinblastine and 360 µM for temozolomide. The results of this study also show that combining a moderate to a high dose of either vinblastine or temozolomide with a high dose of α,βmomorcharinonly produce a small, but significant decrease in the viability of each cancer cell line compared to the effect of either temozolomide, vinblastine, alpha, beta momorcharin alone. This small decrease in cell viability of each cell line was slightly significant, but it was neither additive nor synergetic compared to the separate effect of each. This was a rather surprising result in this study.

CONCLUSION

In conclusion, the results of this study have clearly demonstrated that the α,βmomorcharincan evoke significant decreases in cancer cell viability (an increase in cell death) without killing healthy cell line like L6 skeletal muscle cell line. Either temozolomide or vinblastine with maximal effect of 360 µM and 40 µg can also elicit dose-dependent decreases in cancer cell viability. Combining either TMZ or VIB with either the α,βmomorcharinhad no additive or synergetic effect on the viability of each cell line compared to the effect of either alone. It is concluded that extracts of *M. charantia* possess anti-cancer properties since they can induce cell death.

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