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Effects of Alpha, Beta Momorcharin Extract of *Momordica charantia* Fruit Extract 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. This study was specifically designed to investigate the cellular mechanisms whereby α, β momorcharin an extract of *M. charantia* can induce cell death with the combination of temozolomide or with vinblastine. Different concentration (200µM - 1000µM) of the α, β momorcharin fruit extract were treated (24 hrs incubation) separately with five 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 µg) α, β momorcharin extract of *M. charantia*, result in significant decreases in cell viability for each cell line, these effects were additive compared to the individual effect of temozolomide or vinblastine.

INTRODUCTION

The water-soluble extract of the *M. charantia* can significantly reduce blood glucose concentrations in type-1 diabetic rats [1]. Several studies have reported that the water-soluble extract of *M. charantia* can exert anti-cancerous activity through inhibition of DNA, RNA and cellular protein synthesis. [2-6] The fruit juice of *M. charantia* has been found to increase glucose uptake by several tissues *in vitro* and moreover, it can increase the storage of glycogen by the liver.[6,7] Temozolomide (Temodal) is an alkylating agent derived from dacarbazine and first synthesised in 1984. Temozolomide (8-carbamoyl-3-methylimidazo [5,1-d]-1,2,3,5-tetrazin-4(3H)-one) is a bicyclic heterocycle and is chemically classed as an imidazotetrazinone. The defining characteristic of this class of compound is an imidazole ring that is fused with a tetrazinone ring system that contains three adjacently bonded nitrogen atoms. [8-9] 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. [10-12] FDA in the USA has approved TMZ for the treatment of glioma. It is easier to administer than other chemotherapeutic regimes for this indication and is given orally, once a day for 5 days in a 28-day cycle. [11-13]. It has high bioavailability and crosses the blood-brain barrier where it is spontaneously hydrolysed to its active form. It is toxic to cancer cells due to inhibition of tumour cell DNA replication. [13-16]. The Vinca alkaloids have become clinically useful since the discovery of their anti-tumour properties in 1959. [17] Vinblastine sulfate has the molecular formula of $C_{46}H_{58}O_9N_4 \bullet H_2SO_4$ and it is a dimeric alkaloid containing both indole and dihydroindole moieties. 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. [18-20] VIB is mainly used to treat bladder cancer and to a lesser extent to treat other cancers including lymphoma and Kaposls sarcoma.[21] The antitumor drug, VIB was analysed on the human tumour cell lines U-118 MG (glioma) and HTh 7 (Thyroid cancer).[20-22]. 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 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 spectrophotometry 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}C and 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 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 incubation.

Preparation and application of α, β momorcharin extracts of *M. charantia* on the cancer and L6 cell lines.

Amounts of 14.51 mg, 29.25 mg, 43.53 mg, 58.50 mg, and 72.57 mg of α, β momorcharin (9.7 kDa) were weighed out in 5 ml universal vials and initially dissolved in 500 µl of phosphate buffer by continuous stirring and with the brief use of a sonicator water bath. These were then made up to 5 ml by adding 4.5 ml of the cell medium to give concentrations 200 µM, 400 µM, 600 µM, 800 µM, respectively. The drug (extract) stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22 µm filters into another sterile 10 ml Universal bottles. These stock solutions were stored in a sealed tube in the fridge until required. Once removed from the fridge the prepared drug (extract) solutions were gently warmed in water bath at 37°C in order to ensure that the α, β momorcharins was in a complete solution, before aliquoting. Volumes of 40 µl, 80 µl, 120 µl, 160 µl, contained 200 µM, 400 µM, 600 µM, 800 µM, respectively. Different concentrations of α, β momorcharins were transferred in triplicate using a Gilson pipette to 96 wells plate and the volume made to 200 µl by adding the cell media to both treated and control cell wells. Both control (untreated) and treated with (α, β momorcharin) 96 well plates were incubated for 24 hrs.

Combined effects of either temozolomide or vinblastine with α, β momorcharin.

Since both the commercial anti-cancer drugs and α, β momorcharin could elicit significant decreases in cell viability; it was decided to investigate the combined effects of these anti-cancer agents (drug + extract) on cell viability. Different cancer cell lines (1231N1, Gos-3, U87-MG, Weri Rd-1, Corl-23, Sk and (800 µM) of the α, β momorcharin for 24 hours. Similarly, each cell was incubated with either vinblastine (30 µg) and (800 µM) of the α, β momorcharin for 24 hours. Control cell lines were also incubated for the same time but without any drug or extract. 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.

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

Figure1 shows the effect of different concentrations (200 - 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 effects of α, β momorcharin were also dose-dependent with maximal cell death occurring at 800 µM of α, β momorcharin. In contrast, α, β momorcharin had little or no effect on the death of healthy L6 skeletal muscle cell line. The results presented in figure 1. Again 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.

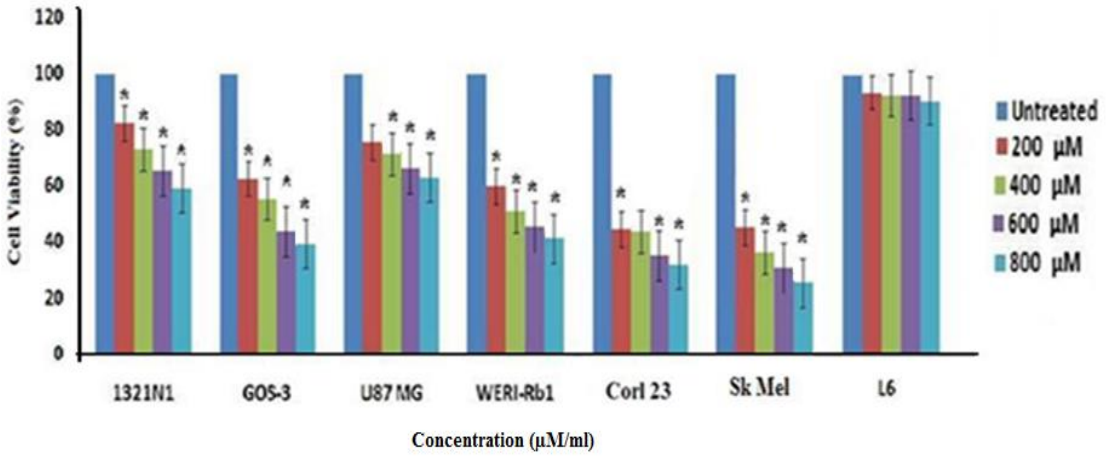

Figure No. 1: Dose-dependent effects of α, β momorcharin

Dose-dependent effects of VIB on cell viability

Figure 2 shows 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 healthy L6 skeletal muscle cell line compared to untreated L6 cell line but mainly at 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 crude water-soluble extract of *M. charantia, which* had no detectable effect on the viability of L6 cell line.

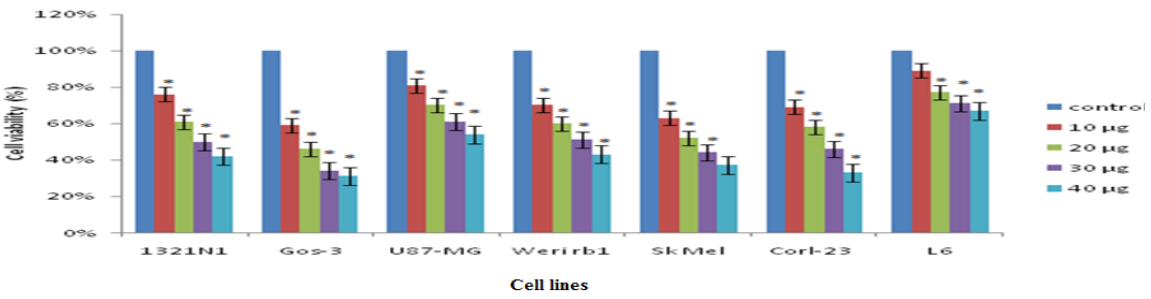

Figure No. 2: Dose-dependent effects of Vinblastine

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) TMZ evoked 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. Comparing the effects of TMZ with VIB (see figure 2). The results clearly show that VIB was more effective than TMZ in killing cancer cells.

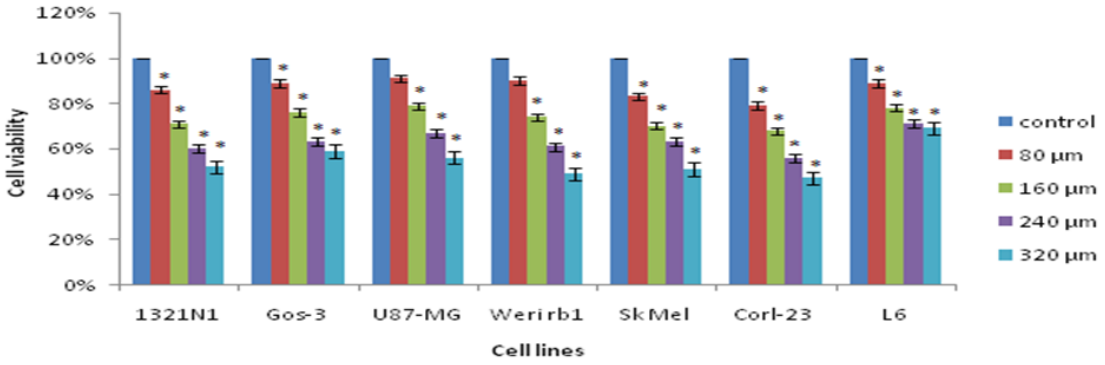

Figure No. 3: Dose-dependent effects of Temozolomide

Combined effects of α, β momorcharinwith either VIB or TMZ cell lines

Figure 4 shows the effect of either 40 µg of the vinblastine, 800 µM of α, β momorcharin 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, β momorcharin or 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 Weri Rb-1) either vinblastine, α, β momorcharin or combined 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 the entire cell lines vinblastine was more effective on cell viability compared to α, β momorcharin alone. Moreover, the results also show that when vinblastine was combined with α, β momorcharin 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, β momorcharin can elicit a significant decrease of L6 skeletal muscle cell line compared to untreated cells or treated with α, β momorcharin alone.

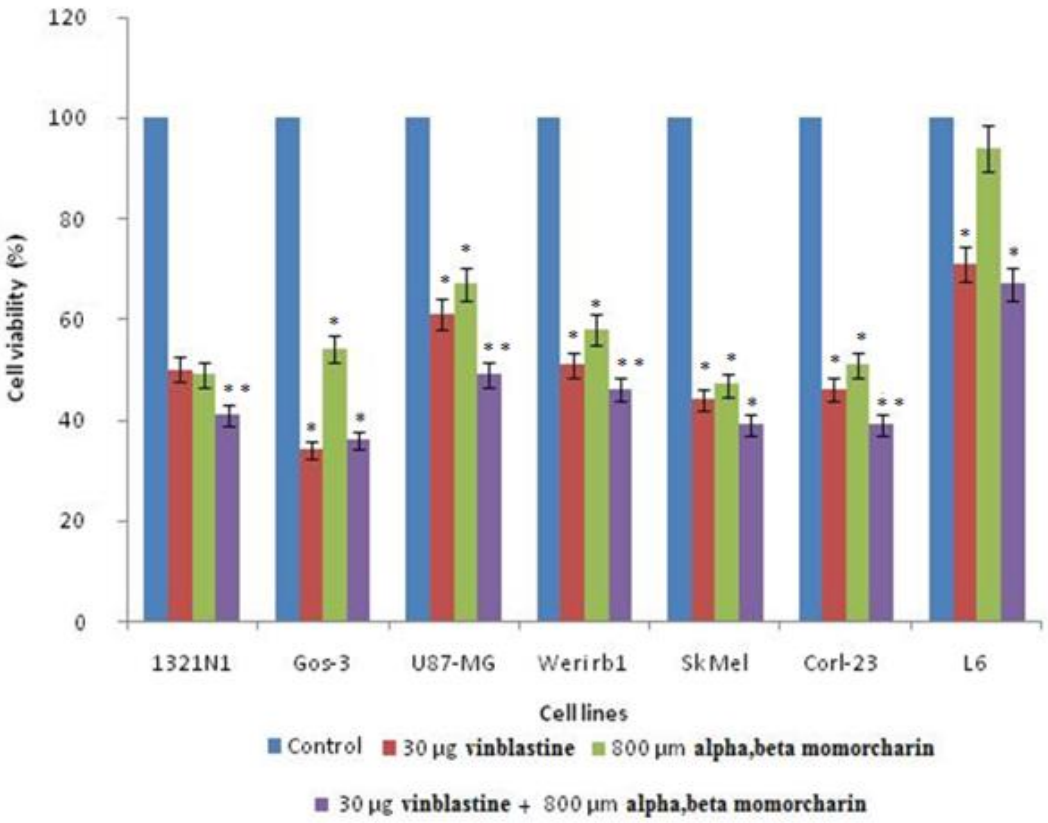

Figure No. 4: Combined effects of α, β momorcharin with VIB

Figure 5 shows the effect of either temozolomide 240 µM alone, or α, β momorcharin 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 figure 5 are the untreated (no temozolomide or α, β momorcharin) six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either temozolomide, α, β momorcharin 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 momorcharin or a combination of temozolomide 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, β momorcharin can evoke significant ($p < 0.05$) decreases in the viability of healthy L6 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, β momorcharin seem to be more effective on U87-MG cell line.

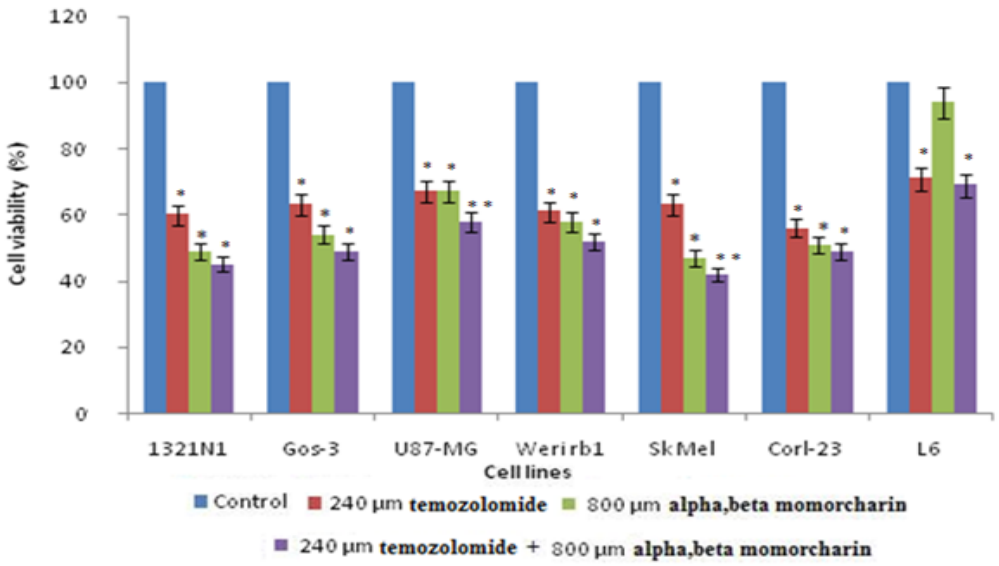

Fig. 5: Combined effects of α, β momorcharin with TMZ

Figure 5 bar charts showing effect of the temozolomide 240 µM alone or of α,β momorcharin 800 µM alone or a combination of α,β momorcharin (800 µM) and temozolomide (240 µM) on the viability of six different cancer cell lines (1321N1, Gos-3, U87- MG, Sk Mel, Corl - 23, Weri Rb-1) and healthy L6 muscle cell line for comparison. The untreated (no temozolomide, α, β momorcharin, or combined α, β momorcharin800 µM and temozolomide 240 µM) cell line for each (first bar chart) is also shown as 100% in the figure for comparison. Each treated or untreated cell line was incubated for 24 hrs. 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. Note that the combined effect of temozolamide with α, β momorcharin was more or less the same as α, β momorcharin alone, except for 1321N1, U87-MG, WeriRb-1 and Sk Mel cells lines, where there was only a small decrease in cell viability; ** $p < 0.05$ for either temozolomide or α, β momorcharin compared to the combination of both.

DISCUSSION

These commercial available drugs not only kill the cancer cells but also kill normal cells. This is also depends on the type of cancer and concentrations of the drugs employed by the

Physician. However, further search must go to find a safer plant based-medicine to treat cancer.

CONCLUSION

In conclusion, the results of this study have clearly demonstrated that the α, β momorcharin can 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 240 µM and 30 µg can also elicit dose-dependent decreases in cancer cell viability. Combining either TMZ or VIB with either α, β momorcharin had 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* α, β momorcharin possess anti-cancer properties since they can induce cell death.

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