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Effects 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. Different concentration (200µg - 800µg) of the crude water soluble fruit extract 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 µg) of the crude water- soluble 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.

Keywords: Cancer cell lines, crude water-soluble extract of *M. Charantia*, temozolomide (TMZ) and vinblastine (VIB)

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.[2-5] The fruit juice of *M. charantia* has been found to increase glucose up take 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 (figure 1). 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] The USA FDA 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. 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.[11-13]

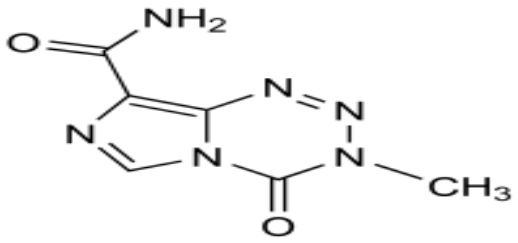

Fig.1: Chemical structure of Temozolomide (TMZ)

The Vinca alkaloids have become clinically useful since the discovery of their anti-tumour properties in 1959.[14] Vinblastine sulfate has the molecular formula of $C_{46}H_{58}O_9N4{\bullet}H_2SO_4$ and it is a dimeric alkaloid containing both indole and dihydroindole moieties (figure 2). 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.[17] The anti tumour drug, VIB was analysed on the human tumour cell lines U-118 MG (glioma) and HTh 7 (Thyroid cancer).[18-19]

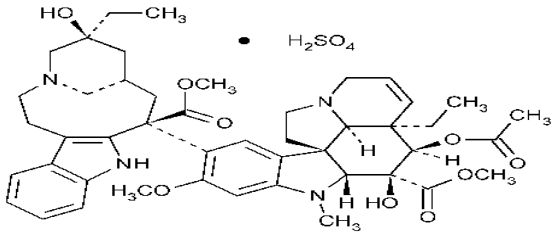

Fig. 2: Chemical structure of Vinblastine (VIB)

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 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.

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 2^{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 crude water-soluble extracts of *M. Charantia* either with TMZ or with VIB 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 a sealed tube 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 and methanol soluble crude extract was mixed complete in solution, before aliquoting. Volumes of 34 µl, 68 µl, 102 µl, 136 µl contained 200 µg, 400 µg, 600 µg, and 800 µg of the crude water-soluble extract of *M. charantia* respectively. Different concentration of either water or methanol soluble crude extract in 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. In this study, both time course and dose-dependent experiments were performed. The time-course experiments were done over a period of 48 hours, where the dose dependent experiments were done during incubation period of 24 hrs.

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

Dose-dependent effects of the crude water extracts of *M. Charantia*

Figure 3 shows the effects of different concentrations (200 µg - 800 µg) 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 3 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with the crude water-soluble extract of *M. charantia* for 24 hours. Control (untreated) cell lines were also incubated for 24 hrs but without any extract. 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 and which was not significantly $p > 0.05$ different from 800 µg. 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. The result 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.

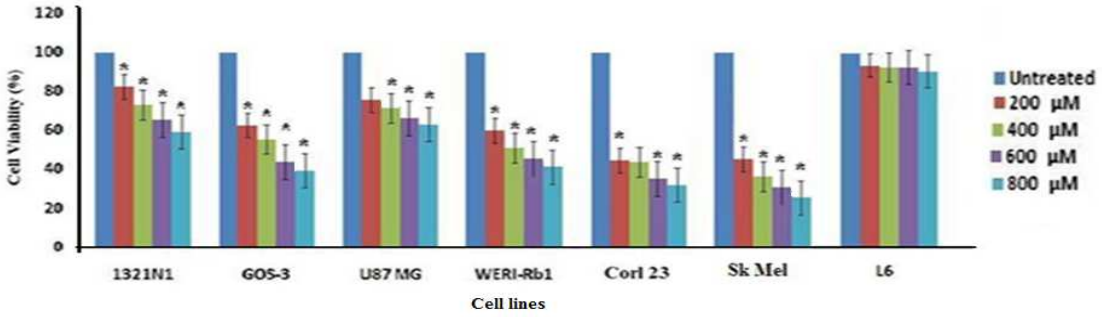

Figure 3: Dose-dependent effects of the crude water soluble extracts of *M. Charantia*

Dose-dependent effects of VIB on cell viability

Figure 4 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 4 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 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 crude water-soluble extract of *M. charantia* which had no detectable effect on the viability of L6 cell line.

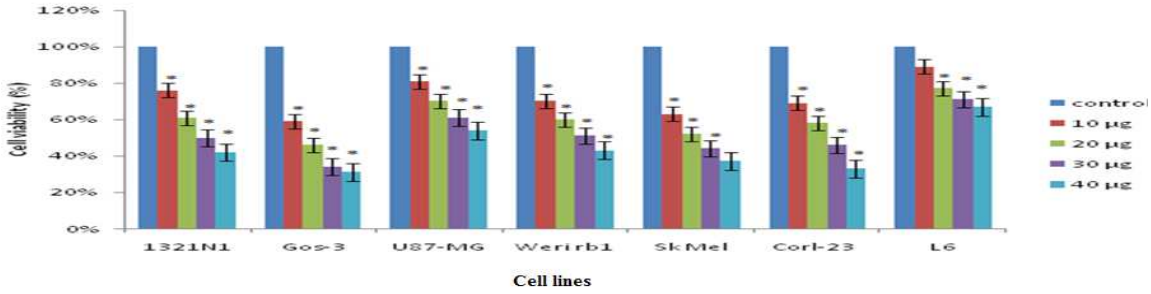

Fig. 4: Dose-dependent effects of Vinblastine

Dose-dependent effects of TMZ on cell viability

Figure 5 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 5 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 healthy L6 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 4). The results clearly show that VIB was more effective than TMZ in killing cancer cells.

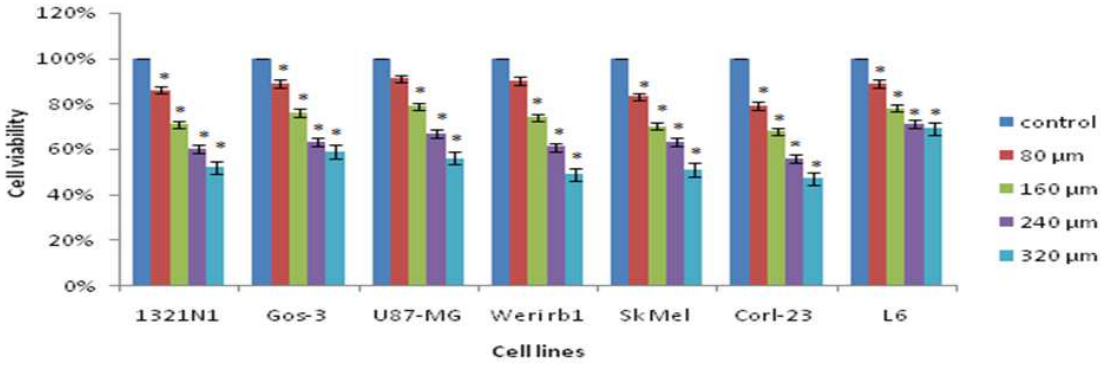

Fig. 5: Dose-dependent effects of Temozolomide

Combined effects of crude water-soluble extract of *M. charantia* with either VIB or TMZ

Figure 6 shows the effect of either VIB (40 µg) alone or the crude water-soluble extract of *M. charantia* (800 µg, a high dose) alone or a combination of VIB (40 µg) with the crude extract soluble extract of *M. charantia* (800 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 6 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either VIB or the crude water-soluble extract of *M. charantia* or combined drugs (drug + crude extract) for 24 hours. Control cell lines were also incubated for the same time. The results show that in all six different cancer cell lines (1321N1, Gos-3, U87-MG, Sk Mel, Corl -23, Weri Rb-1) either VIB, or crude water-soluble extract of *M. charantia* can evoked marked and significant $p < 0.05$ decreases in the cell viability (cell death) compared to untreated cells (100% viability). However, when VIB was combined with the crude water-soluble extract of *M. charantia*, there was a further decrease in cell viability. These values were significantly ($p < 0.05$) different compared to either untreated cells (100%) or cell treated with either VIB or crude water-soluble extract of *M. charantia.*

Similarly, either VIB combined with the crude water-soluble extract of *M. charantia* can evoke significant ($p < 0.05$) decrease in the death of healthy L6 skeletal muscle cell line. The results also show that combined drugs (drug + crude extract) were more effective in killing 1321N1, Gos-3, Sk Mel, Weri Rb-1 and Corl -23 cell lines. It has less effective on U87-MG cell lines.

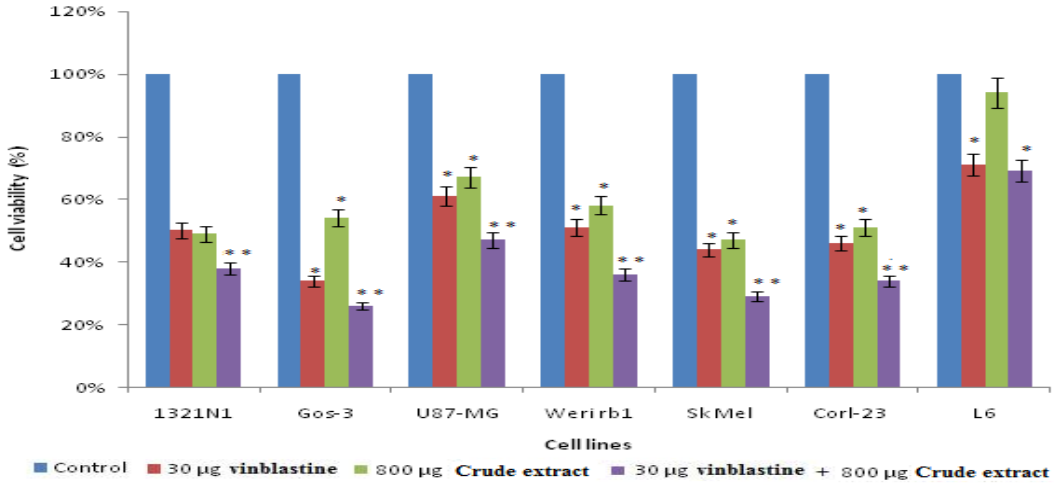

Fig. 6: Effect of either of 40 µg VIB alone or 800 µg of crude water-soluble extract of *M. charantia* alone or a combination of VIB (40 µg) and the crude extract soluble extract of *M. charantia* (800 µg) on the viability of six different cancer cell lines

Figure 7 shows the effects of 240 µM of the TMZ alone, 800 µg of crude water-soluble extract of *M. charantia* alone or a combination crude extract soluble extract of *M. charantia* (800 µg) with TMZ (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 7 are the untreated six different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with either TMZ alone, crude water-soluble extract of *M. charantia* alone or a combination of both (TMZ + crude extract) 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, Weri Rb-1)

either TMZ, the crude water-soluble extract of *M. charantia* or combined drugs (TMZ + crude extract) can evoke marked and significant $p < 0.05$ decreases in the cell viability (cell death) compared to untreated cells (100% viability). Similarly, TMZ alone but neither the crude extract soluble extract of *M. charantia* nor a combination of both, can evoke a significant ($p < 0.05$) decrease in the viability of healthy L6 skeletal muscle cell line compared to untreated cell. The results also show that a combination of TMZ with the crude extract was more effective in killing 1321N1, Gos-3, Sk Mel and Corl -23 cell lines. They are less effective on Weri Rb-1 U87-MG cell lines.

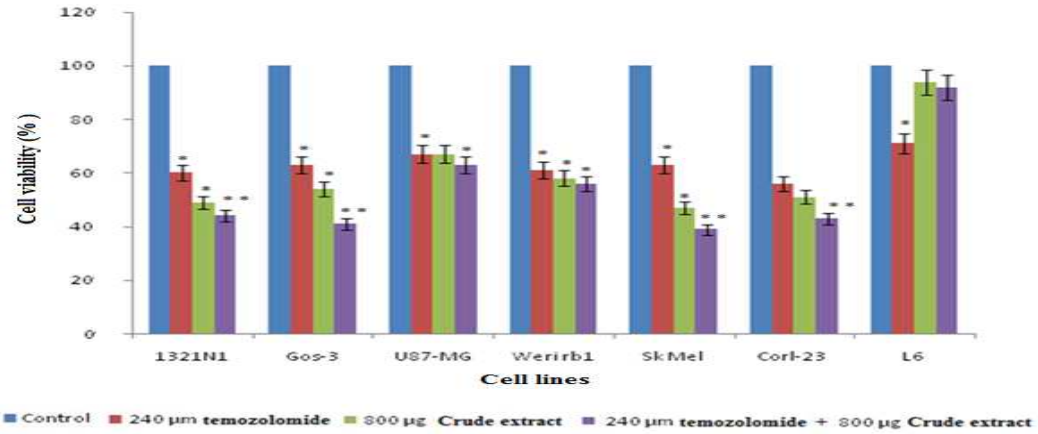

Fig.7: Effect of either TMZ 240 µM, or the crude water-soluble extract of *M. charantia* 800 µg and a combination of the crude extract soluble extract of *M. charantia* (800 µg) with TMZ (240 µM) on the viability of six different cancer cell lines

DISCUSSION

This study employed the crude water and methanol soluble extracts of *M. charantia*, and two commercially available anti-cancer drugs namely, VIB and TMZ to investigate their effects on the viability (cell death) of six different cancer cell lines compared to healthy L6 skeletal muscle cell line. Either the crude water soluble extract of *M. charantia*, VIB or TMZ was tested alone measuring the viability of each cell line. In some experiments, either VIB or TMZ was combined with crude water-soluble extracts of *M. charantia*, to investigate any potentiating or attenuating effect on cell viability. 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 shown that either VIB or TMZ 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 VIB and 360 µM for TMZ [15]. The results of this study also show that combining a moderate to a high dose of either VIB or TMZ with a high dose of either the crude water-soluble extract of *M. charantia* only produce a small, but significant decrease in the viability of each cancer cell line compared to the effect of either TMZ, VIB, the crude water-soluble extract of *M. charantia* 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. Nevertheless plant-based medicines have been shown to exert little or no effects on the cancer cells and tissues when they combine with drugs like, cisplatin, temozolomide, vinblastine or 5-fluorouracil [19]. 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 crude water-soluble extract of *M. charantia* 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 360 µM and 40 µg can also elicit dose-dependent decreases in cancer cell viability. Combining either TMZ or VIB with either the crude water-soluble extract of *M. charantia* 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* possess anti-cancer properties since they can induce cell death.

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