

(RESEARCH ARTICLE)



## Effects of alpha, beta momorcharin fruit extract with the combination of Cyclophosphamide in the treatment of glioma cancer *In-vivo*

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### Abstract

The vegetable *Momordica charantia* L., (family: Cucurbitaceae) is a scientific name of the plant and its fruit. It is also known by other names, for instance in the USA it is known as Bitter gourd or balsam pear while it's referred to as the African cucumber in many African countries. *M. charantia* is believed to possess 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  $\alpha$ ,  $\beta$  momorcharin an extract of *M. charantia* can induce cell death with the combination of Cyclophosphamide. Different concentration (200 $\mu$ M - 1000 $\mu$ M) of the  $\alpha$ ,  $\beta$  momorcharin fruit extract were treated (24 hrs incubation) separately with three different cancer cell lines 1321N1, Gos-3, U87-MG and normal L6 muscle cell line. The results also show that Cyclophosphamide (250  $\mu$ g) with (1000  $\mu$ M) of the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia*, and result in significant decreases in cell viability for each cell line, these effects were additive compared to the individual effect of Cyclophosphamide.

**Keywords:** Cancer cell lines;  $\alpha$  momorcharin;  $\beta$  momorcharin extract of *M. charantia*; Cyclophosphamide (CYP)

### 1. Introduction

The water-soluble extract of the *M. charantia* can significantly reduce blood glucose concentrations in type-1 diabetic rats [1-2]. 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 [3-7]. 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 [8-10]. Cyclophosphamide (CP) is an alkylating agent and is usually administered orally or intravenously and much less frequently intramuscularly or intraperitoneally [11-14]. CP is hydrolyzed in the liver by cytochrome enzymes P450 to the active form 4-hydroxycyclophosphamide, which can diffuse out into circulation and affects the target cells [14-18]. In the cytosol of cell, through spontaneous  $\beta$ -elimination, acrolein and phosphoramidate mustard evolve [19-21]. These are the actual agents responsible for cytotoxic effects and DNA crosslinking [22-23].

### 2. Material and methods

#### 2.1. Extraction method for either of $\alpha$ or $\beta$ 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,

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pH 7.5, the crude protein solution was dialyzed. The dialyzed protein sample was applied to DEAE Sepharose column equilibrated with 2 mM sodium phosphate buffer at pH 7.5. The unbound proteins were then applied to Mono-S column which was equilibrated by 2 mM sodium phosphate buffer at pH 7.5 and eluted by 0.5 M of NaCl. The fraction corresponding to either  $\alpha$  and  $\beta$  or  $\alpha$ ,  $\beta$  momorcharin, which was confirmed the N-glycoside activity RNA, was concentrated and dialyzed 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  $\alpha$  and  $\beta$  momorcharin was examined by SDS-PAGE and gel filtration chromatography. The concentration of  $\alpha$ - momorcharin was determined by spectrophotometrically using optical absorbance at 280nm.

## 2.2. Passaging of the Cancer cell lines and Control cell line

The culture medium, phosphate buffer solution (PBS), and trypsin (sterile) were removed from the fridge at 4°C and subsequently placed in the water bath at 37°C for 30 min in order to equilibrate. The Laminar flow hood was turned on for 15 min, prior to start of the experiment, in order to purge the air inside the cabinet and to reach the maximum cleanliness.

The different cancer 1321N1, Gos-3, U87-MG and L6 muscle normal cell lines were incubated at 37°C incubator in an atmosphere of 5% CO<sub>2</sub> 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<sup>2</sup> flask and 2 ml if 25 cm<sup>2</sup> 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<sup>2</sup> flask or 1 ml if 25 cm<sup>2</sup> flask) was pipetted in the flask and incubated at 37°C in an incubator in an atmosphere of 5% CO<sub>2</sub> 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  $\mu$ l of trypsinised cell suspension and 80  $\mu$ l of trypan 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<sup>2</sup> flask and 5 ml if 25 cm<sup>2</sup> flask) was added to the flasks. These were then placed in a 5% CO<sub>2</sub> incubation.

## 2.3. Preparation and application of $\alpha$ , $\beta$ 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 either  $\alpha$  or  $\beta$  momorcharin and  $\alpha$ ,  $\beta$  momorcharin (9.7 kDa) were weighed out separately in 5 ml universal vials and initially dissolved in 500  $\mu$ l 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  $\mu$ M, 400  $\mu$ M, 600  $\mu$ M, 800  $\mu$ M, 1000  $\mu$ M, respectively. The drug (extract) stock solution was transferred to a 10 ml syringe and sterile filtered using 0.22  $\mu$ m 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  $\alpha$ ,  $\beta$  momorcharin was in a complete solution, before aliquoting. Volumes of 40  $\mu$ l, 80  $\mu$ l, 120  $\mu$ l, 160  $\mu$ l, 200  $\mu$ l contained 200  $\mu$ M, 400  $\mu$ M, 600  $\mu$ M, 800  $\mu$ M, 1000  $\mu$ M, respectively. Different concentrations of  $\alpha$ ,  $\beta$  momorcharin were transferred in triplicate using a Gilson pipette to 96 wells plate and the volume made to 200  $\mu$ l by adding the cell media to both treated and control cell wells. Both control (untreated) and treated with ( $\alpha$ ,  $\beta$  momorcharin) 96 well plates were incubated for 24 hrs.

## 2.4. Dose dependent effects of Cyclophosphamide on cancer cell line viability

In this series of experiments, different cancer cell lines (1231N1, Gos-3, U87-MG) and healthy L6 muscle cell line were incubated with the different concentrations of Cyclophosphamide (50 - 250  $\mu$ g) for 24 hours. Control cell lines were also incubated for the same period of time but without any Cyclophosphamide. At the end of the incubation period, cell viability of each cell line was measured using the MTS assay.

## 2.5. Combined effects of either Cyclophosphamide with the $\alpha$ , $\beta$ momorcharin extract of *M. charantia*

Different cancer cell lines (1231N1, Gos-3, U87-MG) and healthy L6 muscle cell line was incubated either with Cyclophosphamide (250  $\mu$ g) and the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* (1000  $\mu$ M) or Cyclophosphamide (250  $\mu$ g) and 1000  $\mu$ M) of the  $\alpha$ ,  $\beta$  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.

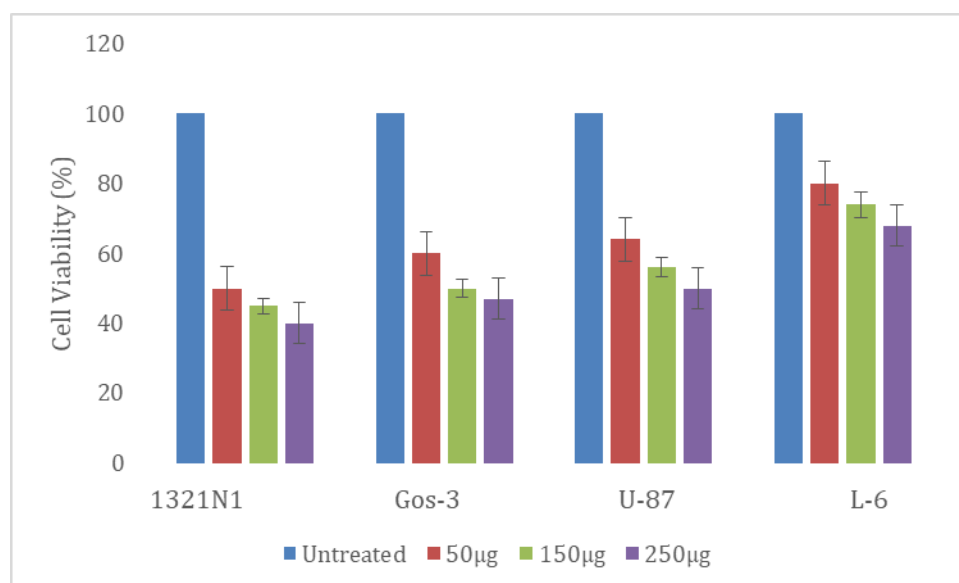
## 2.6. Statistical Analysis

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  $\pm$  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.

## 3. Results and discussion

### 3.1. Dose-dependent effects of Cyclophosphamide on cell viability

Figure 1 shows the effects of different concentrations (50 - 250  $\mu$ g) of Cyclophosphamide on the viability of the three different cancer cell lines and on healthy L6 skeletal muscle cell line employed in this study. Also shown in the figure 1 are the untreated three different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with Cyclophosphamide for 24 hours. Each control cell lines were also incubated for 24 hrs but with no Cyclophosphamide. The results show that in all three different cancer cell lines (1321N1, Gos-3, U87-MG), Cyclophosphamide 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 Cyclophosphamide were dose-dependent with maximal cell death occurring with 250  $\mu$ g. Similarly, Cyclophosphamide 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 dose. The results also show that Cyclophosphamide was more effective in killing 1321N1 and Gos-3, 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 Cyclophosphamide could also kill healthy L6 skeletal muscle cell compared to the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia*, which had less effect on the viability of L6 cell line.

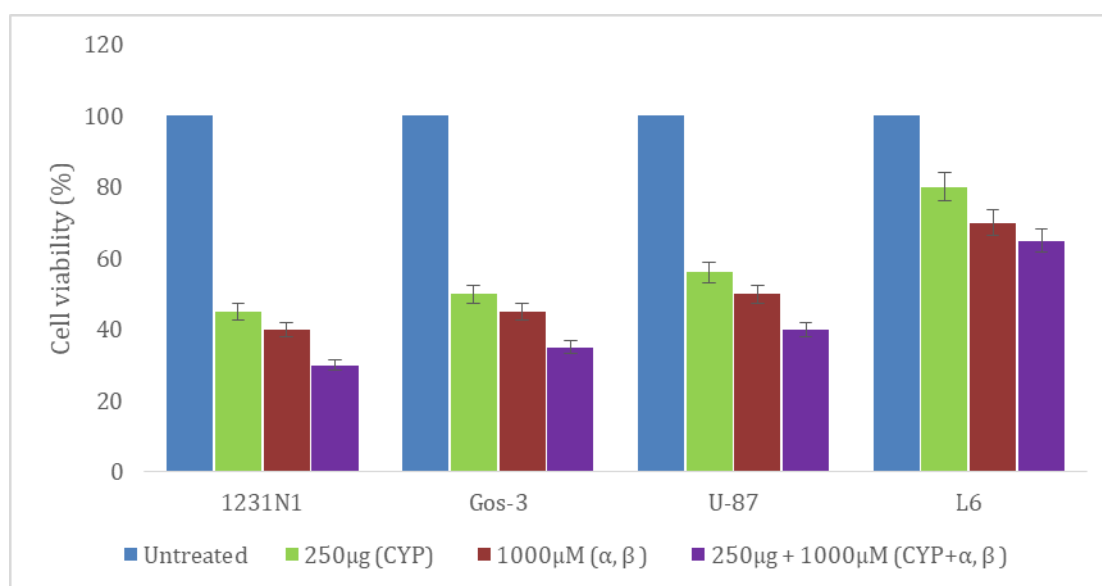


**Figure 1** Dose-dependent effects of Cyclophosphamide

Figure 2 shows the effect of Cyclophosphamide (250  $\mu$ g) alone or the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* (1000  $\mu$ M, a high dose) alone or a combination of Cyclophosphamide (250  $\mu$ g) with the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* (1000  $\mu$ M) on the viability of the three 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 three different cancer cell lines and healthy L6 skeletal muscle cell line for comparison. All the cells were treated with Cyclophosphamide and  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* (drug + extract) for 24 hours. Control cell lines were also incubated for the same time. The results show that in all three different cancer cell lines (1321N1, Gos-3, U87-MG,) either Cyclophosphamide or  $\alpha$ ,  $\beta$  momorcharin 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 Cyclophosphamide was combined with the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia*, there was a further decrease in cell viability. These values were significantly ( $p < 0.05$ ) different compared to either untreated cell (100%) or cell treated with either Cyclophosphamide or  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia*.

Similarly, Cyclophosphamide combined with the  $\alpha$ ,  $\beta$  momorcharin 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 + extract) were more effective in killing 1321N1, Gos-3, cell lines. It has less effective on U87-MG cell lines.



**Figure 2** Effect of either of 250 µg Cyclophosphamide alone or 1000 µM of  $\alpha$ ,  $\beta$  momorcharin of *M. charantia* alone and combination of Cyclophosphamide (250 µg) with  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* (1000 µM) on the viability of three different cancer cell lines

This study employed the  $\alpha$ ,  $\beta$  momorcharin extracts of *M. charantia*, and commercially available anti-cancer drugs Cyclophosphamide to investigate their effects on the viability (cell death) of three different cancer cell lines compared to healthy L6 skeletal muscle cell line. Either the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia*, Cyclophosphamide was tested alone measuring the viability of each cell line. In some experiments, Cyclophosphamide was combined with  $\alpha$ ,  $\beta$  momorcharin 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 Cyclophosphamide can significantly decrease the viability of 1321N1, Gos-3, U87-MG, 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 Cyclophosphamide. The results of this study also show that combining a moderate to a high dose of Cyclophosphamide with a high dose of either the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* only produce a small, but significant decrease in the viability of each cancer cell line compared to the effect of Cyclophosphamide and the  $\alpha$ ,  $\beta$  momorcharin extract of *M. charantia* alone. This small decrease in cell viability of each cell line was slightly significant, but it was neither additive nor synergistic compared to the separate effect of each. This was a rather surprising result in this study.

#### 4. Conclusion

In conclusion, the results of this study have clearly demonstrated that the  $\alpha$ ,  $\beta$  momorcharin 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 Cyclophosphamide with maximal effect of Cyclophosphamide can also elicit dose-dependent decreases in cancer cell viability. Combining Cyclophosphamide with either the  $\alpha$ ,  $\beta$  momorcharin 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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## Compliance with ethical standards

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### Disclosure of conflict of interest

Authors has declared that no competing interests exist.

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