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# Antiproliferative and Cytotoxic Effects of Methanol-Water Leaf Extracts of *M. balsamina* and *M. foetida* from Eswatini on Human Liver Hepatocellular Carcinoma (Hepg2) Cell Line

**Thabile P. Nkambule[a], Christine E. R. Dodd[b], and Gregory Tucker[c]**

[a] *University of Eswatini, Department of Food and Nutrition Sciences, Faculty of Consumer Sciences, M205, Manzini, Eswatini.*
[b] *University of Nottingham, School of Biosciences, Nottingham, LE12 5RD, UK.*
[c] *University of Nottingham, School of Biosciences, Nottingham, LE12 5RD, UK.*

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***Corresponding Author:***
Thabile P. Nkambule

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**ABSTRACT**

*Cancer is the second leading cause of death after heart diseases in many countries. This area signifies the need to exploit medicinal plants as a source of potential treatment. Drug affordability is another concern in many developing countries including Eswatini, thus a large proportion of the population is mainly dependant on medicinal plants to meet primary health care needs. This validates the interest in search of potential anti-cancer agents from the indigenous vegetables found in Eswatini. Momordica balsamina (inkakha) and Momordica foetida (inshubaba) are used both for food and as traditional medicine. The aim of this study was to investigate antiproliferative and cytotoxic activity of methanol-water extracts of M. balsamina and M. foetida leaves, well-known indigenous vegetables in Eswatini, on the proliferation of human liver hepatocellular carcinoma (HepG2) cell line. HepG2 cells were treated with different doses of individual extracts (0.06 to 1 mg/mL for M. balsamina and 0.25 to 4 mg/mL for M. foetida) for 24 and 48 hr. Cell proliferation was measured using Hoechst fluorometric assay. The tested Momordica plants showed antiproliferative activity at different doses. The most active plant was M. balsamina which displayed an initial cytotoxic response at 0.5 mg/mL and antiproliferative activity at 0.25 mg/mL after 24 hours of exposure. M. foetida leaf extracts only displayed antiproliferative activity at concentrations above 1 mg/mL both at 24 and 48 hours. These results indicate the potential use of Momordica extracts as anti-cancer agents and also provide the basis for isolation and identification of biologically active substances.*

## Introduction

Since plants have long been considered to have a wide variety of biologically active compounds, it is important to explore their possible medicinal applications (Talib *et al.* 2010). Cancer is a disease that is a result of many factors including environment, physical, metabolic and genetic factors. There is evidence suggesting that consumption of fruits and vegetables rich in antioxidants help reduce the risk of many cancers. In the past years a large part of cancer research has focused on identification and development of these antioxidants. Phenolics are a diverse group of plant metabolites many of which exhibit antioxidant and other biological functions that may contribute to the modulation of carcinogenesis (Dai & Mumper 2010). These facts were supported by Kim *et al.* (2010) who found high correlation between anti-proliferative activity and phenolic as well as flavonoid content indicating that the anti-proliferative effects of mango extracts may be due to a combination of polyphenols and flavonoids present in the extracts. Sun *et al.* (2002), however, concluded that even though total phenolic content and antioxidant activity were highly correlated ($R^2 = 0.979$), there was a weak correlation of total phenolic content and anti-proliferative activity of HepG2 cells ($R^2 = 0.415$) as well as antioxidant activity and anti-proliferative activity of the fruits tested ($R^2 = 0.369$).

Plants from tropical areas are considered to be one possible source for the evaluation of anti-cancer agents (Manosroi *et al.* 2006). *M. balsamina* (*inkakha*) and *M. foetida* (*inshubaba*) are used in several countries, including Eswatini, both for food and as a traditional medicine to treat several diseases. These plants have been used in Eswatini traditional medicine for patients suffering from diabetes and hypertension (Masarirambi *et al.* 2012), are often recommended to patients with

HIV/AIDS to strengthen their immune system and for the treatment of boils and other skin diseases. Furthermore, research have showed that *Momordica* plants have demonstrated a high antimicrobial activity against Gram positive bacteria. Exploring these indigenous vegetables for other potential bioactivities is important for their potential applications. Currently, there is limited knowledge of the potential anti-proliferative activity of *M. balsamina* and *M. foetida* extracts. Most available literature reports anti-proliferative activity of *M. charantia*. Jilka *et al.* (1983) reported that crude extracts of *M. charantia* fruits inhibited tumour formation in DBA/2 mice. Hsiao *et al.* (2013) reported that isolated cucurbitanes from *M. charantia* fruits displayed cytotoxic activity on MCF-7 (human breast adenocarcinoma), Doay (human medulloblastoma), Hep-2 (human laryngeal carcinoma) and WiDr (human colon adenocarcinoma) tumour cell lines. An iridoid lactone (plumericin) isolated from *M. charantia* vine which displayed antibacterial activity against *Enterococcus faecalis* and *Bacillus subtilis* was also found to inhibit proliferation of two leukemic cancer cell lines namely NB4 (acute promyelocytic leukemia (APL)) and K562 (human chronic myeloid leukemia) (Saengsai *et al.* 2015). Anti-proliferative activity has been assayed from other plants by many researchers using methods such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), MTS-based (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), ATP-based (adenosine triphosphate) and DNA (deoxynucleic acid) based assays. The cell viability and proliferation assay used may significantly influence the results (Wang *et al.* 2010). When using the MTT- assay, it is not possible to differentiate between inhibition of cell growth and increase in cell death (García-Alonso *et al.* 2006). In other research comparing anti-proliferative activity assays, MTT and MTS- based methods resulted in underestimation of the anti-proliferative effects of EGCG ((-)-epigallocatechin-3-gallate), yet ATP- and DNA- based methods were highly correlated (r = 0.95) and both consistently demonstrated anti-proliferative activity of EGCG (Wang *et al.* 2010).

For the current study, the ability of *M. balsamina* and *M. foetida* leaf extracts to inhibit the proliferation of human liver hepatocellular carcinoma (HepG2) cells was determined by estimating cell number by DNA content using the Hoechst Fluorometric method. The HepG2 cell line has been widely used in biochemical and nutritional studies as it is considered an experimental model that closely resembles the human hepatocyte in culture thus also permits the study of anti-proliferative activity for liver cancer research (Ramos *et al.* 2005; García-Alonso *et al.* 2006). The DNA-based method is based on the measurement of cellular DNA to show the relative cell number as cellular DNA content is highly regulated (Jones *et al.* 2001). In the DNA-based assay, the fluorescent dye binds to cellular nucleic acid and displays strong fluorescence with an excitation wavelength at 355 nm.

## Materials and Methods

*Plant material*

*Momordica balsamina* and *M. foetida* tissues were obtained either air dried under shade or oven dried (50$^{o}$C for 72 h) from the Malkerns research centre, Eswatini or from plants grown in the tropical glass house at the University of Nottingham. For planting in the glass house, *M. balsamina* and *M. foetida* seeds were obtained from ripe yellow wild fruits collected at Malkerns research station, Eswatini. These were initially air dried and then the red coating was removed before further drying. Dried seeds were pre-treated by soaking on filter paper soaked with distilled water for 24 h. Plants were grown in a glass house with temperatures between 27 – 31$^{o}$C. Seeds were sown into high nutrient compost (Levington R M3) in 9 cm round pots which were then maintained by irrigating every other day to keep the compost moist until plants germinated and were ready for transplanting. Transplanting took place between 4-6 weeks when plants were about 10-15 cm tall or when 4 true leaves were showing. Coarse potting compost (C2) with a pH 5.5 to 6 was used in 10-inch pots (young plants of *M. balsamina* and *M. foetida* are shown in Figure 1).


**Fig 1.** *M. balsamina* (A) and *M. foetida* (B) young plants at 6 weeks of planting in 10-inch round pots.

Seedlings were removed from the 9 cm pots together with compost to avoid disturbing the roots. Plants were watered every two days. At two to three weeks after plants were transplanted, they were supplied with a patterned release fertiliser (Osmocote exact tablet, Everris Ltd, Ipswich, UK). Five tablets were supplied to each pot. Plants were also provided with support by cane sticks (trellis). Once plants had started producing both male and female flowers, pollination was performed physically. For plant material preparation, plant samples (leaves, stem and fruits) were harvested when plants were about three months old. Tissue samples were washed with running water, drained to remove excess water and spread on oven trays covered with foil paper. Plant samples were then dried in an oven-drier set at 50$^{o}$C for 72 h. After drying, leaves were carefully separated from the stem and each were crushed and weighed separately. Fruits were also weighed and all samples were stored in airtight containers at -20$^{o}$C.

*Preparation of methanol-water (7:3 v/v) extracts*

Twenty grams *Momordica* tissue was weighed and added to a 500 mL Duran bottle containing 200 mL of methanol-water 7:3 (v/v). Samples were left on a stirrer overnight then filtered through Mira cloth and poured into a 500 ml round flask. Samples were then rotary evaporated at a rotation speed 70%, water bath temperature 50$^{o}$C for about 35 minutes or until the volume of concentrate was about 30 ml. The remaining methanol was evaporated under nitrogen until only the aqueous part remained. An appropriate volume of millipore water was added to make a final volume of 100 mL. Samples were centrifuged at 1167 x g for 7 minutes. The supernatant containing the *Momordica* extract was then transferred to foil plates. Extracts were stored at -40$^{o}$C before they were freeze dried. Freeze dried samples were then weighed, transferred to a sterile container and stored at -20$^{o}$C until required.

*Anti-proliferative activity determination in cell cultures*

Human liver hepatocellular carcinoma (HepG2) cell lines were obtained from the Nutritional Science Department, University of Nottingham, United Kingdom. These were originally sourced from the European Collection of Cell Cultures. Stock of HepG2 cells were kept in liquid nitrogen. For analysis 1 mL of the cells was defrosted and grown in 10 mL Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma D6546), and supplemented with 10% foetal bovine serum (FBS) (Gibco), 1% L-Glutamate (Gibco), 1% Penicillin-Streptomycin (Sigma) and maintained in a humidified atmosphere of 5% $CO_2$ at 37 °C.

The medium was changed every 2 days and cells cultured to approximately 80% confluence. Once cells had reached the desired confluence, they were sub-cultured by removing medium and washing with phosphate buffer saline (PBS) (10mL). Cells were then treated with 2 mL of 0.25% trypsin (Sigma) and 1 mM EDTA for 3 minutes. Trypsinized cells were then re-suspended in 8 mL of fresh DMEM and centrifuged at 200 x g for 5 minutes. Cells were then transferred to a new flask at 1:5 split of the original cell concentration. These were maintained in a humidified atmosphere of 5% $CO_2$ [generated by supply of $CO_2$ from a gas cylinder through a valve that is triggered to draw $CO_2$ whenever the level falls below 5% (Wilson & Walker 2010)] at 37 °C and the medium was changed every 2 days. Cells were then plated in 96-well round-bottomed tissue culture plates (Costar, Corning) for the cytotoxicity test. Cells were seeded at a density of approximately 80 000 cells/well (100µL).

The cell density was counted using a haemocytometer by adding 10 µL of harvested cells and placing on an inverted microscope and using the contrast to distinguish the cells. HepG2 cell lines were maintained for one day at 37$^{o}$C, to allow cell stabilization. Cells were then treated with different doses of individual extracts (100 µL) dissolved in PBS at concentration ranges of 0.06 to 1 mg/mL for *M. balsamina* and 0.25 to 4 mg/mL for *M. foetida*. Phosphate buffered saline (PBS) was used as a control and each test sample consisted of five replicates. Treated cells were incubated at 37$^{o}$C and the effects of extracts on the cells were determined at 0, 24 and 48 h. At each time point, media were carefully removed and the cells washed with 200 µL PBS then 100 µL of Sigmaclean water (Sigma) was added to the wells before incubation at 37$^{O}$C for 30 minutes. Cells were subjected to three freeze-thaw cycles of 30 minutes at -20$^{o}$C and room temperature until thawed, respectively to assist in DNA release by rupturing the cell membrane.

Cell number in each case was estimated by DNA content using the Hoechst Fluorometric method (Rage *et al.* 1990). 100 µL of Hoechst dye mixture (consisting of 2µg/mL Hoechst 33258 [Sigma] and 2X TNE buffer [20 mM Tris, pH 7.4, 2 mM EDTA and 0.2 M NaCl]) was added to each well and fluorescence was measured at an excitation wavelength of 355 nm and emission of 460 nm (BMG Labtech Fluorstar Optima). Calf thymus DNA (Sigma) was used for the standard curve and was achieved by preparing serial dilutions ranging from 0.6 µg/mL to 40 µg/mL.

**Results**

Anti-proliferative activity determination was performed on crude extracts of *M. balsamina* and *M. foetida* leaf. This study was performed to determine if the leaf extracts have the ability to reduce or stop multiplication of human liver hepatocellular carcinoma (HepG2) cells. The extracts were tested for anti-proliferative activity at a concentration range of 0.6 to 1 mg/mL for *M. balsamina* and 0.25 to 4 mg/mL for *M. foetida*. These concentration ranges were selected following an initial screening using a wider range and determining the range that impacted on proliferation. A concentration of *M. balsamina* extract above 1 mg/mL was found to result in cell death (data not shown) whilst for *M. foetida* extracts cell death was noticed at concentrations above 2 mg/mL.

*Morphology of untreated and treated HepG2 cells*

The morphological images of untreated HepG2 cells and those that had been treated with crude methanol-water extracts of 0.5 mg/mL for *M. balsamina* leaf and 1 mg/mL for *M. foetida* leaf, either sourced from Eswatini or UK glasshouse grown, after 24 h of incubation are presented in Figures 2 to 5.

In all instances, untreated HepG2 cells exhibited normal structure. The HepG2 cell line treated with *M. balsamina* for 24 h displayed a remarkable decrease in the number of cells (Figure 2 and Figure 3). Also, the treated cells looked different from untreated cells, particularly in those cells treated with extracts of *M. balsamina* leaf sourced from Eswatini, where cells seemed to have lost some of their neuronal-like features and were slightly smaller in size (Figure 2). The treated cells also seemed to have separated from each other with less clumping.

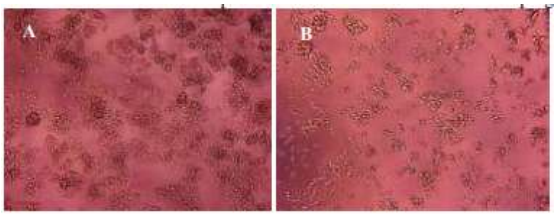


**Fig 2.** Effect of Eswatini sourced *M. balsamina* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 0.5 mg/mL of crude methanol-water extracts of *M. balsamina* leaf sourced from Eswatini for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

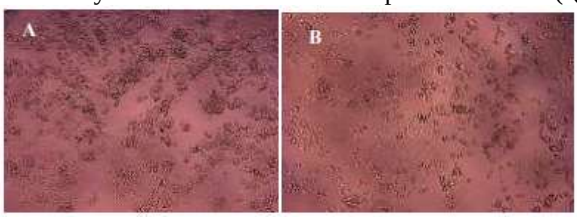


**Fig 3.** Effect of UK glasshouse grown *M. balsamina* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 0.5 mg/mL of crude methanol-water extracts of *M. balsamina* leaf grown in the UK glasshouse for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

The HepG2 cell line treated with *M. foetida* extract for 24 h displayed a slight decrease in the number of cells (Figure 4 and Figure 5). Unlike the morphological images of cells treated with *M. balsamina* extracts, the *M. foetida* treated cells did not look very different from the normal cells. They did however show signs of separation from each other with less clumping. The differences were particularly evident with the cells treated with extracts from UK glasshouse grown *M. foetida* leaf (Figure 5).

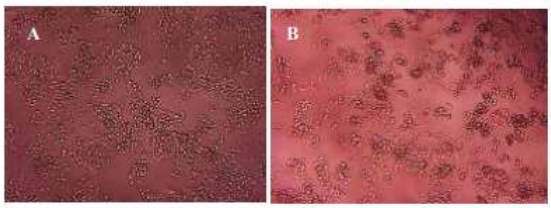


**Fig 4.** Effect of Eswatini sourced *M. foetida* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 1 mg/mL of crude methanol-water extracts of *M. foetida* leaf sourced from Eswatini for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

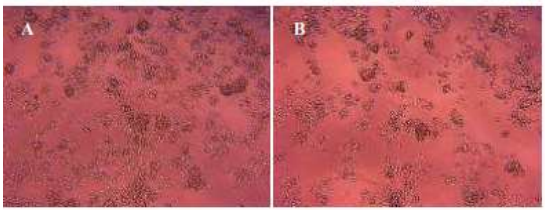


**Fig 5.** Effect of UK glasshouse grown *M. foetida* leaf extract on HepG2 cell morphology.

HepG2 cell lines were treated with 1 mg/mL of crude methanol-water extracts of *M. foetida* leaf grown in the UK glasshouse for 24 h. The figure shows confocal images at 100x total magnification, showing morphology of untreated (A) and treated (B) cells. Pictures were taken by a Leitz inverted microscope with camera (Qcapture).

*Anti-proliferative activity and cytotoxic effects of M. balsamina and M. foetida leaf extracts*

The results for the anti-proliferation assays are displayed in Figures 6 to 8. All the controls showed a clear increase in DNA concentration over the 48 h of incubation. This demonstrated that cells were properly proliferating. Anti-proliferative activity was observed in both *M. balsamina* and *M. foetida* extracts but at different concentrations in each case. At 0 h DNA concentrations of untreated and treated cells were not significantly different ($P>0.05$) however, significant differences ($P<0.05$) were observed in DNA concentrations of HepG2 cells at 24 and 48 h. The treatment results displayed a time-dependant dose response, where even after 24 h of incubation cytotoxic activity was observed. The highest anti-proliferative activity was observed in cells treated with extracts of *M. balsamina* leaf (Figure 6). Anti-

proliferative activity was noticed at 0.25 mg/mL after both 24 and 48 h of incubation of cells treated with *M. balsamina* extracts sourced from Eswatini after which cytotoxic activity was observed. For the cells treated with UK glasshouse grown extracts of *M. balsamina* leaf, anti-proliferative activity was noticed at 0.5 mg/mL and cytotoxic effects were observed at 0.75 mg/mL after 24 h of incubation and at 0.5 mg/mL after 48 h of incubation.

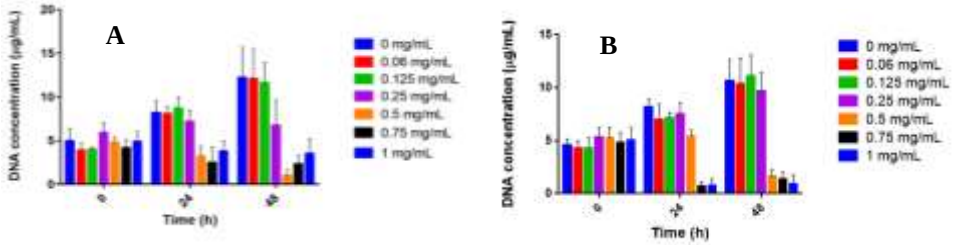


**Fig 6.** Effect of extracts of *M. balsamina* leaf on the proliferation of HepG2 cells. Extracts from *M. balsamina* leaf either (A) sourced from Eswatini or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 44) at concentrations of 0.06 to 1 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

Cells treated with extracts of *M. foetida* leaf displayed lower anti-proliferative activity (Figure 7 and Figure 8). Inhibition of proliferation was observed at higher concentrations compared to *M. balsamina* leaf extracts. Initially anti-proliferative activity was observed at 1 mg/mL after 48 h of incubation for both Eswatini sourced and UK glasshouse grown *M. foetida* extracts while cytotoxic effects were displayed at 2 mg/mL for these extracts. Passage number seemed to have an effect on the anti-proliferative activity of *M. foetida* extracts. Results showed that with the higher passage cell line (passage 47) of HepG2, anti-proliferative activity of extracts may be lowered compared to cells treated at lower passage (passage 33). In this regard, anti-proliferative of *M. foetida* extracts were observed at even higher concentrations of 1.5 to 2 mg/mL and there were no cytotoxic effects displayed in this instance.

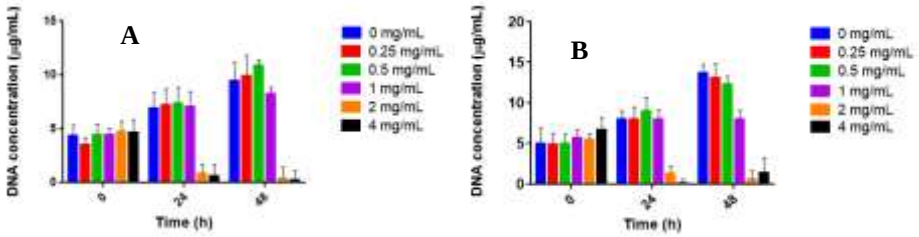


**Fig 7.** Effect of extracts of *M. foetida* leaf on the proliferation of HepG2 cells. Extracts from *M. foetida* leaf either (A) sourced from Eswatini or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 33) at concentrations of 0.25 to 4 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

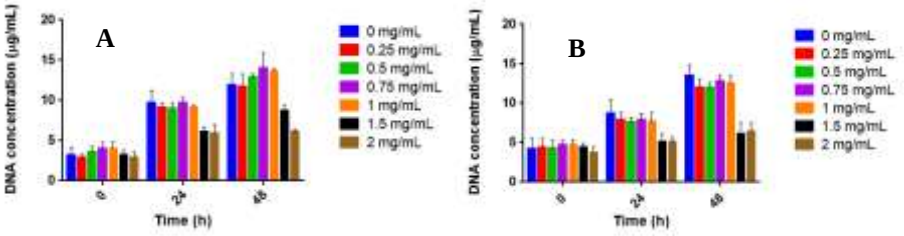


**Fig 8.** Effect of extracts of *M. foetida* leaf on the proliferation of HepG2 cells. Extracts from *M. foetida* leaf either (A) sourced from Eswatini or (B) UK glasshouse grown were applied to HepG2 cells (Cell passage 47) at concentrations of 0.25 to 2 mg/mL and DNA concentration monitored over 48 h of incubation. Results are the mean ± SD (n=6)

**Discussion**

It is well known that leaves of plants have been used for food flavouring, food preservation and medicinal purposes since ancient times. Today, strategies to improve health are one of the driving forces for market growth in the food and beverage industry and this is one of the reasons for the increased interest in the use of natural products (Kaefer and Milner 2008). In Eswatini about 85% of the population is dependant mostly on medicinal plants to meet primary health care needs (Amusan 2009; Nguta *et al*. 2011). This study investigated the potential anti-proliferative activity, against a HepG2 cell line, of methanol-water extracts of *M. balsamina* and *M. foetida* leaves either sourced directly from Eswatini or UK glasshouse grown. Neither of these *Momordic*a plants had previously been used traditionally for the treatment of cancer in Eswatini. This study revealed two main findings. First, anti-proliferative activity of *Momordica* plants varied depending on species and growth conditions. Effect of the extracts was also shown to be time and dose dependant with maximum effect observed mostly after 48 h of treatment. Extract of *M. balsamina* leaf sourced from Eswatini was the most effective and showed anti-proliferative activity at the lowest dose of 0.25 mg/mL and cytotoxicity at 0.5 mg/mL.

Extract of *M. foetida* leaf was the least potent and showed anti-proliferative activity at a dose of 1 mg/mL and cytotoxicity at 2 mg/mL. The morphological data of this study clearly displayed some changes in structure of the HepG2 cell line when comparing treated with untreated cells after 24 h. This set of results has shown that methanol-water extracts of *Momordica* leaves initiate visible changes to the structure of the cells indicating either cell death or a decrease in cell viability. The results are in agreement with findings of a study which showed that methanol extracts of *M. charantia* had a higher anti-proliferative effect against HepG2 cells compared to HCT116 (human colorectal cancer cells) and MCF-7 cell lines, showing an IC50 of *M. charantia* in HepG2 of 0.77 µg/mL while IC50 for HCT116 and MCF-7 were 0.81µg/mL and 135 µg/mL, respectively (Alshehri 2016). It is worth noting that the study displayed anti-proliferative activity of extracts at much lower concentrations compared to this current study and this may be due to the different *Momordica* species studied or cell passage number. Contrary to this, another study reported that methanol extracts of *M. charantia* did not display any effect on all cell lines studied except for a human astrocytoma (1321N) cell line where, at high concentrations, a small decrease in cell viability was observed (Manoharan 2011). This may mean that methanol extraction in the current study does not denature the active compound responsible for the activity.

The second and last observation was that cell passage may have an effect on anti-proliferative activity of extracts. This was particularly evident in cells treated with extracts of *M. foetida* leaves where at much higher passage number (P47) of the HepG2 cells, growth inhibition took place without any cytotoxicity, even at higher concentrations of the extract, compared to results with lower passage (P33) cells which displayed cytotoxicity. This may imply that when working with HepG2 cells it may be necessary to work within a less variability range for continuous cell lines. The differences may indicate altered genetic expression of the cells, affecting their susceptibility. O'Driscoll *et al.* (2006) compared low passage (P18) and high passage (P40) MIN-6 cells (insulinoma cell line) and found significant differences in the expression of several mRNAs involved in regulated secretion, adhesion and proliferation. Another study compared LNCap (human prostate cancer cells) at P25 and P60 and demonstrated that the regulation of androgen receptor activity by P13K/Akt (phosphoinositide 3-kinase / serine/threonine kinase) pathway was dependant on the passage number and this may affect the various stages of prostate cancer (Lin *et al.* 2003).

These results clearly suggest a strong potential medicinal value of the *Momordica* extracts particularly *M. balsamina* leaf. A report suggested a strong connection between plants known from indigenous cultures to have medicinal properties compared to empirically determined cytotoxicity (Booth *et al.* 2012) however, this would rather depend on the particular medicinal properties claimed. Mackeen *et al.* (2000) also reported that *G. atroviridis* extracts displayed antimicrobial, anti-tumour-promoting and antioxidant activities which rendered them appropriate as potential therapeutics.

**Conclusion**

This study observed a dose and time dependant anti-proliferative activity on HepG2 cells after incubation with several concentrations of the *Momordica* extracts. In general *M. balsamina* leaf extracts were cytotoxic at lower doses than *M. foetida* extracts. It was also demonstrated that passage number may have an effect on anti-proliferative activity especially for *M. foetida* extracts. Lastly, *M. balsamina* leaf extracts resulted in a greater reduction in HepG2 cell proliferation than *M. foetida* These results confirm the potential beneficial uses of *M. balsamina* and *M. foetida* in cancer prevention treatments. The preliminary anti-proliferative activity of the *Momordica* leaf extracts observed for the first time in this study against HepG2 cells requires further investigation to determine compounds that may be responsible for this activity and their potential mechanism(s). Studying the effects of these extracts on normal cell line is necessary to ascertain selectivity of anti-proliferative and cytotoxic activity to cancer cells.

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