Apoptosis induction of crude water soluble *Momordica charantia* (chinese and indian bitter gourd) in human lung cancer cell and zebrafish embryo

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**ABSTRACT**

A therapy which can target apoptosis will be effective for cancer treatment. This preliminary study attempted in vitro and in vivo to target apoptosis capability of *Momordica charantia* extracts. Here, we describe that the crude hot and cold extraction of two varieties (Chinese and Indian) of *M. charantia*, induced apoptosis in human lung cancer cell line A549. This was obtained from the cell viability assay where crude extracts were incubated for 24 hours and tested with 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide (MTT) assay. The inhibitory concentration, IC₅₀ was obtained from the cell viability graph and further used in the upstream assays. The apoptotic morphology was presented by the crude extract-treated cells with a comparison to a positive control cisplatin. The percentage of apoptosis was obtained from a flow cytometric analysis using Hoechst 33342. Induction of apoptosis in vivo was tested using the extracts on dechorionated zebrafish embryo for 24 hours and monitored up to 72-hour post fertilization. Induction of apoptosis in A549 by crude water extracts revealed a decrease in cell viability giving a low IC₅₀. The IC₅₀ of cisplatin was 17.3 µg/ml, Chinese Hot Aqueous was 32.5 µg/ml, Chinese Cold Aqueous was 28.1 µg/ml, Indian Hot Aqueous was 36.9 µg/ml and Indian Cold Aqueous was 26.7 µg/ml significant to the control. The initiation of apoptosis by the crude water extracts was also evidence in vivo in zebrafish embryos with results demonstrating significant changes morphologically. There is a potential for the Indian Cold Aqueous to act as a chemo preventive agent as it has the overall best IC₅₀, percentage of apoptotic cells and changes in the morphology of cell and the zebrafish embryo.

**INTRODUCTION**

Nearly million of new cases of cancer diagnosed were contributed by lung cancer, making them the second most common malignancy after breast cancer (IARC, 2018). Even with new cancer therapy and operative techniques, lung cancer still remains to be the main cause of death worldwide (Anand et al., 2008). Amongst the advances in cancer researches throughout the last decade, researchers have been focusing on apoptosis and the cellular death pathways. This programmed cell death is of concern in
different action which involves normal cell turnover and also inducing chemical cell death. In fact, to halt apoptosis during carcinogenesis is the hallmark in the development and progression of cancers (Demir et al., 2016). Identification of apoptotic activity provides a conceptual order in detecting the defect and discovering profound treatments for various cancers.

The genus *Momordica* is found throughout the world mainly in the tropical and subtropical region with suitable temperature and humidity level (Mir et al., 2017). *Momordica charantia* or widely known as bitter gourd is notable for its medicinal values for the benefit of health. There are two variations of *M.charantia* which is the Chinese and the Indian *M.charantia*. Despite differ in appearance and chemical composition both *M.charantia* tastes bitter (Jia et al., 2017; Rashima et al., 2017). *M.charantia* is claimed to have high protein, carbohydrates, lipid, fibers, minerals, vitamin, saponins, phenolics, flavonoid and sterols which may help in promoting or controlling the cell cycle of apoptosis (Jia et al., 2017; Rashima et al., 2017).

*M.charantia* are locally consumed either prepared hot or cold, and either as a drink or for a meal (Deng et al., 2017). A great many clinical efficacies of *M.charantia* against diabetes, cancer, inflammations, viral infections, fungal infections and others are being observed (Ng et al., 1987; Li et al., 2012; Mir et al., 2017; Basch et al., 2003; Braca et al., 2008). To date, *M.charantia* has been speculated to depress tumor cells such as in the breast, prostate, skin, melanoma, and lymphoma to name a few. The main protein present in the bitter gourd is moromorcharins which include α-moromorcharins and β-moromorcharins, cucurbitacin B and momordin (Kumar et al., 2015). In other studies, α-moromorcharin was identified as a ribosome-in-activating protein which cleaves DNA making it capable to induce apoptosis in cancer cells (Wang et al., 2013).

There is also some speculation on anti-carcinogenic activity where the crude water-soluble*M.charantia* demonstrated disruption in the mitochondria which leads to apoptosis on different cell lines namely 1321N1, Sk Mel, U87-MG, Corl-23 and Weri Rb-1 (Manoharan et al., 2014). Furthermore, hot water extract depressed the uterine adenomyosis and mammary tumor growth in vivo (Singh et al., 1998; Nagasawa et al., 2002).

This study was intended to improve the understanding on two different variations of *M.charantia* which is the Chinese and the Indian *M.charantia*s potential in cancer therapy, specifically lung cancer cell, through the initiation of apoptosis in vitro and in vivo.

**MATERIALS AND METHODS**

**PLANT MATERIAL**

The Chinese (C) and Indian (I) varieties of bitter gourd were obtained from Selangor and were identified and authenticated by Institute of Bioscience, UPM (plant Voucher Number Chinese Bitter Melon: SK3160/17; Indian Bitter Melon: SK3157/17). With a slight modification in the protocol the whole fruit was washed thoroughly under running tap water and dried in a 40°C oven for 3 days. A fine powder was resulted from grinding the samples and sieved over a fine mesh sieve. The freshly prepared samples were stored in -20°C prior to extraction.

**Hot Aqueous Extraction (HA)**

Aqueous extraction was prepared by decoction where 100g of the fine powdered sample was soaked in 1L of distilled water at 70°C for 12 hrs. Thereafter, the extracted sample was filtered using a Whatman No. 1 filter paper and proceed to be freeze-dried. The freeze-dried samples were stored at -20°C prior to experiments (Tan et al., 2014).

**Cold Aqueous Extraction (CA)**

A 100 g of the fine powdered samples were soaked for 2 days in 1L of distilled water at room temperature. The aqueous extracts were filtered using Whatman No. 1 filter paper and freeze-dried. The extracts were stored at -20°C until needed in the experiment (Opara et al., 2014).

**Cell Culture**

A human lung cancer cell, A549 was used in this study. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (HyCloneTM,Sweden) supplemented with 10% of fetal bovine serum (FBS) (HyCloneTM,Sweden) and 1% penicillin-streptomycin (HyCloneTM,Sweden) at 37°C in a humidified incubator with 5% (v/v) CO2. A549 cell was used from passage 3 to passage 20 according to the ATCC product sheet.

**Cell Viability Assay**

The antiproliferative activity of both hot and cold extraction of Chinese and Indian bitter melon was evaluated by MTT assay. A549 cell was seeded at the density of 2.0 × 10^5 cells/well in 96-well plates and cultured for 24 hours at 37°C. The concentration of 0-200 μg/ml of Chinese Hot Aqueous (CHA), Chinese Cold Aqueous (CCA), Indian Hot Aqueous (HA), Indian Cold Aqueous (ICA), and cisplatin was added and tested at 24 hours. After incubation of 24 hours, twenty microliters of MTT reagent added and further incubated for 4 hours, and followed by
dissolving the formazan crystals with DMSO (100 μl). Then, the plate was measured at 570 nm using a microplate reader (Microplate reader E16, Hong Kong). Cell viability was calculated as the percentage of viable sample treated cells compared to controls (100%) of three independent experiments. The IC_{50} of each sample were identified and used for the following parameters (Manoharan et al., 2014).

Apoptotic Measurement and Morphology

A549 cells were seeded at 2.5 × 10^5 and incubated for 24 hours at individual IC_{50} of each sample. With manufacturer’s instruction, the cells were stained with Hoechst 33342 at the final concentration of 2 μg/ml and incubated for 30 minutes (Wang et al., 2013). The cells were then analyzed on the apoptotic activity using a Cell Reporter System (Molecular Devices®). Results were tabulated as the percentage of apoptotic cells and the morphology of the cells was also observed using cisplatin as a control.

In Vivo Apoptosis Assay

Zebrafish embryo

The wild-type zebrafish embryo (0 hpf) were purchased from Danio Assay Laboratories Sdn. Bhd. Two hundred 24 hours post fertilization (hpf) healthy embryos were selected and manually dechorionated. The embryos were then treated with samples and incubated for 24 hours. Cisplatin was used as a positive control. After being exposed for 24-hours to the samples, the embryos were then transferred into distilled water and observed up to 72 hpf by an image analyzer (DX51, Olympus, Tokyo, Japan) under ×4 magnification. The morphological changes on the tail and the whole fish was examined (Iman et al., 2016).

RESULTS AND DISCUSSION

The Chinese and Indian bitter melon of both hot and cold extractions inhibits A549 cell growth and proliferation. Previously it has been stated that *M. charantia* crude extract is found to halt cell proliferation and initiate apoptosis several cancer cell lines (Manoharan et al., 2014). The anti-cancer activity was portrayed in the decrease in cell viability at 24 hours of incubation. Figure 1, shows the cell viability at 50% which is justified as the inhibitory concentration, IC_{50}. Cisplatin showed the lowest IC_{50} which is 17.3 μg/ml, followed by ICA.
Figure 2: The morphological changes when a cell undergoes apoptosis when treated for 24 hours. A: control; B: cisplatin; C: CHA; D: CCA; E: IHA; F: ICA. The cells were viewed under the magnification of 50 μm

(26.7 μg/ml), CCA (28.1 μg/ml), CHA (32.5 μg/ml) and IHA (36.9 μg/ml). This shows that the samples were halting cell proliferation on the cancer cell line in contrast to the control.

The cells which were undergoing apoptosis were observed under a microscope for the change in their morphology, seen after the 24 hours treatment of crude extracts. Figure 2, shows the cell shrinking showing symptoms of cell death. The untreated cells showed a normal morphology which is fibro blast and the cell were intact (Figure 2 A). Apoptotic cells can be categorized when they are seen rounded up, shrink and detached from the bottom of the plate as seen in (Figure 2B, C, D, E, F). (Häcker, 2000).

A typical apoptotic feature was observed in cisplatin-treated cells at 24 hours Figure 2B. When the cells treated with crude extracts were in comparison to the positive control it is observed that CHA, CCA and ICA showed more apoptotic cells. However, IHA had lesser apoptotic cells with some normally attached cells. Furthermore, the total cell count was measured and the percentage of apoptotic cells were tabulated in Figure 3 using a flow cytometric analysis. The apoptotic activity was evaluated through the DAPI channel.

The population of cells affected was calculated
Figure 3: The effect of samples on A549 cell apoptotic cells. *p<0.05 and **p<0.01 when compared to control; #p<0.05 when compared to cisplatin. Data are mean ± SEM and n=3.

Figure 4: The zebrafish embryo at 24 hpf. A:embryo with chorion and B:embryo without chorion at the magnification of 500 µm.
Figure 5: The apoptotic features which can be seen in the tail of the 48 and 72 hpf zebrafish. The arrow indicates the apoptotic cells which are dominant at the tail part of the fish. A: cisplatin; B: CHA; C: CCA; D: IHA and E: ICA.

According to the number of apoptotic cells closely bonded to Hoechst 33342. The percentage of apoptotic cells were normalized to 100% of non-apoptotic cell or control. Consistent with the data of apoptotic features above, the percentage of apoptotic cells observed was highest and significant to the control was the cisplatin-treated cells at 41.3%. Next, ICA also had a significant percentage (37.9%) of the apoptotic cell when compared to the control and also cisplatin. Furthermore, it also has the highest amount of apoptotic cell when compared to all the other crude extracts. The lowest percentage of the apoptotic cell was in the CCA with 32.1%. The result suggested that ICA had triggered apoptosis at 24 hours in A549 cells.

Apoptosis induction by crude extract was examined in vivo using the zebrafish embryo model. The embryo of zebrafish has become a popular animal model in a fast and reliable assessment of drug tox-
icity and discovery (Pritchard, 2001; Sarvaiya et al., 2014). One of the advantages of using the embryos is they have rapid development in forming body organs by 48 hours which allows toxicological evaluation within days. Adding to that, the apoptosis rate is prominent during the development of zebrafish embryo to form their specific structures (Berry et al., 2007).

To observe the presence of the apoptotic cell by observing the tail of the zebrafish, healthy embryos were dechorionated. Figure 4A shows the embryo which is chorionated and Figure 4B shows dechorionated embryo.

Dechorionated embryos were introduced to crude extracts at each IC\textsubscript{50} to determine the apoptotic effects in vivo. The tails of crude extracts-exposed embryos showed apoptotic cellular death which is pointed by the arrow in Figure 5. The positive control used cisplatin showed deformities on the tail even at 48 hpf. The black dot on the tails (Iman et al., 2016) of 72 hpf zebrafish is more prominent in CHA treated zebrafish in Figure 5B. This is followed by the bending of the tail seen in Figure 5E which is treated with ICA. In general, all zebrafish treated with crude extract presented apoptotic feature when compared to cisplatin-treated embryos.

All crude water extracts demonstrated apoptotic activity in each parameter of apoptosis assay finding. When compared between the variations of \textit{M.charantia}, Indian bitter melon gave a better apoptotic activity with the lowest IC\textsubscript{50}, highest percentage of an apoptotic cell and the tail bending with the apoptotic body when compared to cisplatin. As studied previously, the protein content and total phenolic content of Indian bitter gourd are higher than the Chinese bitter gourd (Islam et al., 2011). When compared between extractions, hot extraction of Chinese bitter gourd has better apoptotic activity when compared to the cold extraction. However, this is in contrast with Indian bitter gourd where the cold extract posse higher apoptotic activity. This may due to the biochemical constituents of the different varieties of bitter melon and also the temperature of extraction. The hot aqueous extraction may yield higher compound such as cucurbitanes which induce apoptosis while the cold extraction reduces the yield of this active compound (Sultana et al., 2009).

CONCLUSIONS

This investigation gives the insight of the property of anticancer found in the two variations of \textit{M.charantia} with different extraction method on the initiation of apoptosis both in vitro and in vivo.

These findings were an only surface line of evidence on the pharmacological effect of the crude extracts. A further specific parameter in assessing apoptosis should be evaluated further for the future creation of the crude extracts as an anticancer agent.

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Conflict of Interest

None.

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