

CYTOTOXIC ACTIVITY OF
MOMORDICA CHARANTIA SEED EXTRACT

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Running Head: CYTOTOXIC ACTIVITY OF *MOMORDICA CHARANTINA*

Cytotoxic Activity of
Momordica charantina Seed Extract
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Abstract

Bitter melon extract has been used as a traditional medicine for several ailments. Modern techniques have authenticated its use for diabetes and also as an antibacterial, antiviral, anthelmintic and abortifacient agent. In this study, cytotoxicity of bitter melon seed powder extracts from water, ethanol, and a water- ethanol (1:1) mixture were tested on HEK293 cells and red blood cells (RBC). The water-ethanol extract showed the highest cytotoxic activity in inhibiting cell growth, but had less cytotoxic activity on RBC cell lysis. Whereas the ethanol extract had less cytotoxic activity in inhibiting cell growth but lysed RBC completely. To identify the active components in the water-ethanol extract, several separation methods were performed, and cytotoxicity was tested on HEK293 cells and RBC. The first method used ammonium sulfate precipitation and both the supernatant and precipitate were collected. The second method used size exclusion filtration and the filtrates were collected. Two fractions were isolated with highest cytotoxicity activity on cells. The fourth method used high performance liquid chromatography (HPLC), and several fractions were collected. The various fractions were tested on HEK293 cells and RBC. Two HPLC fractions exhibited their highest toxicity on HEK293 cells. Gas chromatography-mass spectroscopy (GC-MS) analysis of these two HPLC fractions identified potentially new type of low molecular weight cytotoxic compounds.

Introduction

All over the world, 80% of the population in developing countries still uses traditional medicine to treat primary medical problems (4). Pharmacognocny, one of the oldest scientific disciplines, has undergone major changes. Currently, plant based drugs are researched, formulated, modified and efficiently used in modern drugs. In the past decade, research has focused on the scientific evaluation of traditional drugs from plants (14). Experimental evidence suggests that free radicals (FR) and reactive oxygen species (ROS) can be involved in a large number of diseases (15). Plants produce antioxidants to control oxidative stress caused by light and ROS. These products represent a new source of compounds with antioxidant activity. Many studies (3, 15, and 16) have been performed to identify pharmacologically active and antioxidant compounds that have limited toxicity to normal cells (16).

Momordica charantia (*M. charantia*) is one such plant that has been frequently used for medicine (2, 9). *M. charantia*, a creeper belonging to the family Cucurbitaceae, is commonly known as bitter gourd or bitter melon. All parts of the plant, including the fruit, taste bitter. The fruit is oblong and resembles a small cucumber; young fruit is emerald green that turns orange-yellow when ripe (4).

M. charantia has various medicinal properties. It is an antidiabetic, abortifacient, anthelmintic, contraceptive, antimalarial and anticancer agent. In Potawale *et al.*, 2008 (14) reviewed the botanical, phytochemical, pharmacological and toxicological information of *M. charantia*. They observed *M. charantia* is rich in various biologically active chemicals including triterpenes, proteins, and steroids. Triterpenes of *M. charantia* has the ability to inhibit the enzyme guanylate cyclase that is thought to be linked to the

cause of psoriasis. In addition, guanylate cyclase is one of the important enzymes, necessary for the growth of leukemia and other cancer cells. In addition to these biologically active triterpenes, *M. charantia* proteins like momordin, alpha- and beta-momorcharin and cucurbitacin B were also tested for possible anticancerous effects. A chemical analog of these *M. charantia* proteins has been developed, patented, and named "MAP-30"; its developers reported that it was able to inhibit prostate tumor growth. Some of the proteins like alpha- and beta-momorcharins have been reported to inhibit HIV infections.

In vivo immunological responses of *M. charantia* plant extract on rats were studied by Jjiratchariyakul *et al.*, 2001 (5) by feeding them bitter melon for three weeks. They observed a decrease in the intestinal secretion of Interleukin (IL) -7 and a increase in the secretion of transforming growth factor (TGF) - β and IL-10. These effects reflect the changes induced by bitter melon in systemic immunity. The changes observed in the intestinal secretions results in a decrease in the lymphocyte population and an increase in immunoglobulin (Ig) production, T-helper cells (Th) and Natural Killer (NK) cell populations. Dietary bitter melon may also induce systemic anti-inflammatory responses in rats. Along with systemic induction, *M. charantia* consumption also inhibits the degenerative process called oxidative stress. Kubola *et al.*, 2009 (8) reported that *M. charantia* fractions were also rich in different types of phenolic compounds that have strong antioxidant activity. These phenol compounds also act as antimutagenic and antitumerogenic compounds.

Permeability-glycoproteins (P-gp) are the membrane proteins which belong to the ATP-binding cassette (ABC) transporter super family, which flushes out hydrophobic

drugs from a cell by using energy obtained by hydrolyzing ATP. Konishi *et al.*, 2004 and Limtrakul *et al.*, 2004 (7, 10) observed the high expression of P-gp in the cell membrane of tumor cells. Higher intracellular P-gp concentration will lower the intracellular drug concentration. *M. charantia* active compounds like 1-monopalmitin and its related compounds inhibit the P-gp activity in the tumor cells.

In other cancer studies, researchers (6, 22) found high levels of cyclic GMP in human leukemia cell line cultures. Several antileukemia factors which are competitive inhibitors of human lymphocytic guanylate cyclase, were extracted from *M. charantia*. Jilka *et al.*, 1983 (6) observed elevated levels of cyclic GMP alter cell growth and cell proliferation of normal cells and neoplastic cells. Higher levels of cyclic GMP were observed in human leukemic cells. *M. charantia* antileukemia factors potentially inhibited the human lymphocytic guanylate cyclase, which results in cyclic AMP: cyclic GMP to normal levels. Lai *et al.*, 2009 (1) isolated two novel peptides MCoCC1 and MCoCC2 from *M. charantia* seed extract. These peptides have a very high cytotoxic effect on human melanoma cell lines. Seed extracts were observed to be more toxic than the novel peptides. From these results, they confirmed the presence of more unidentified proteins which have high cytotoxic activity.

Several researchers (20, 21) have worked on various plant extracts that have cytotoxic activity on tumor cells. During these studies Jilka *et al.*, 1983 (6) found plant proteins from *Abrus precatorius* and *Ricinus communis* that have anticancer activity, but are toxic to normal cells. However, the seed extracts of plants like *Momordica* and *Modecin* have anticancer activity but are not toxic to normal cells. Lectins of *Momordica* play a vital role in the inhibition of tumor formation and are also involved in the

activation of peritoneal macrophages, which mediate tumor cell lysis. On the other hand, *M. charantia* proteins like Ribosome-Inactivating proteins (RIPs) inhibit tumor cell proliferation. RIPs belong to a family of enzymes that depurinate rRNA, which ultimately inhibits protein biosynthesis. Li *et al.*, 2009 (11) chemically modified RIPs from bitter melon by the addition of polyethylene glycol (RIP-PEG). RIP-PEG will reduce the antigenicity of the drug in host cells and ultimately increase the efficiency of the drug.

Purpose of the study

Several researchers have found active components from various parts of *M. charantia* that have antidiabetic, abortifacient, anthelmintic, contraceptive, antimalarial and anticancer activity. The anticancerous activity of *M. charantia* has attracted the attention of scientists. The combined extracts of leaves, green fruit and tendrils of *M. charantia* have high antioxidant activity (8) and also inhibit the P-gp activity in tumor cells (10). Whole fruit extract of *M. charantia* has anticancer activity and activates peritoneal macrophages to act against human leukemia (6). The whole fruit extract also induced systemic anti-inflammatory responses in rats (5). RIPS are a type of seed proteins isolated from *M. charantia* that inhibit the proliferation of tumor cells (11). Two other proteins were isolated with anticancer activity. It was found that the seed extract has the highest cytotoxic activity when compared with isolated proteins or peptides suggesting the presence of undetected proteins or molecules in the seed extract that may have similar or higher cytotoxic activity (1). In the present study, attempts were made to isolate and identify the cytotoxic active components present in seed extracts of *M. charantia* by extracting the seed powder in water, water-ethanol mixture (1:1) and ethanol. Cytotoxic activity of these seed extracts were tested on human embryonic kidney 293 cell lines (HEK293 tumor cells) and red blood cells (RBC).

Materials and Methods

Preparation of *M. charantia* seed powder extracts

Three types of *M. charantia* seed powder extracts were obtained using water, ethanol, and a water-ethanol mixture (1:1). Extracts were obtained by soaking 0.5g of *M. charantia* seed powder in 2 ml of each water, ethanol, and a (1:1) water-ethanol mixture (Mix) Solutions were mixed overnight at room temperature. Then, they were centrifuged at 5000 rpm for 5 mins and the clear supernatants were collected. These supernatants were tested for their cytotoxic effects.

Cytotoxic effect on HEK293 tumor cells and RBC

Cytotoxic activity of seed extracts were tested on HEK293 and RBC. The HEK293 cells were grown in Cellgro's Dulbecco's modification of Eagle's medium (DMEM containing 10% fetal bovine serum, 0.2% penicillin and streptomycin). These cells were grown in a monolayer in a 12 wellled plate that was incubated at 37°C. The RBC cell suspension was prepared by dissolving RBC in PBS (0.15 M NaCl, pH 8) in a 1:3 ratios.

The HEK293 cell cultures were treated with 1, 3, and 5 µl of each three different *M. charantia* seed extracts. These treated HEK293 cell cultures were incubated overnight at 34°C. The cytotoxic activity of these seed extracts was detected by observing tumour cell death under the microscope. The cell cultures which were incubated overnight were harvested using phosphate buffer saline (PBS), and a protein assay was performed using Pierce's bicinchoninic acid (BCA) reagent at a wavelength of 270 nm. The cytotoxic effect was also observed by performing a western blot. In the western blot technique, collected protein samples were separated by 10% sodium dodecyl poly acryl amide gel

(SDS-PAGE). These separated proteins were transferred on to a polyvinylidene fluoride membrane (PVDF) and treated with primary anti-bodies like rabbit-anti p-53. This primary antibody is tagged with a secondary antibody-horse radish peroxidase (HRP) enzyme linked to rabbit-anti IgG. The HRP cleaves a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. The proteins detected by the western blot were analyzed by exposing them to X-ray film.

RBC cell suspension was treated with *M. charantia* seed extracts which were incubated at room temperature for 1 hour. The cytotoxic activity of these seed extracts was quantified visually by observing under microscope.

Isolation of active components in the Mix extract

The cytotoxic active compounds from the Mix were separated using different procedures. To increase the efficiency, the extracts were concentrated by heating before separation.

The different separation procedures used were 1) salt precipitation, 2) filtration 3) ion exchange chromatography and 4) high performance liquid chromatography (HPLC).

1) Salt precipitation

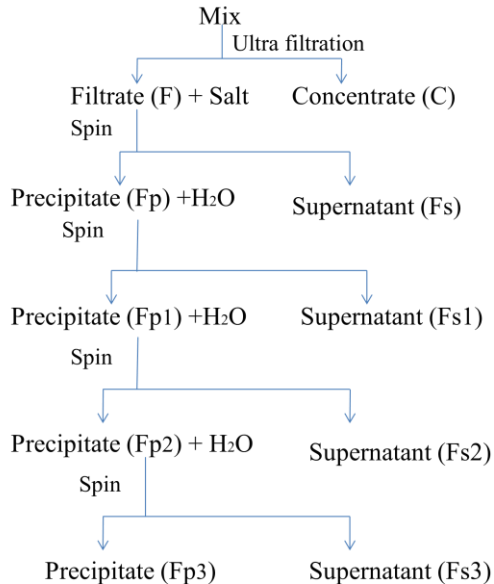
The Mix was ultra filtered, before salt precipitation, using a 5,000 nominal molecular weight limit membrane (NMWL) ultra filtration column. The resulting filtrates (F) and concentrates (C) were tested on HEK 293 cells and

the treated cells were incubated overnight at 37°C

The filtrate was subjected to salt precipitation. In this process, ammonium sulfate was added to the filtrate until saturation was obtained. The saturated

filtrate was centrifuged for 5-10 mins at 16,000 rcf and the resulting supernatant (Fs) and precipitate (Fp) were collected.

a) Flow Chart



As the collected samples have a high salt concentration which can kill tumor cells immediately, they were dialyzed using 6000-8000 dialysis bags in Tris (10 Mm

2) Filtration

In the filtration process, Mix was concentrated by heating and then filtered using 30,000 (30K) and 10,000 (10K) dalton centrifugal filters. In this process, Mix was first passed through a 30K filter producing a concentrate (30C) and precipitate (30F). The molecular weight of the resulting concentrate is greater than 30K daltons, while that of precipitate (30F) is less than 30K daltons. The 30F filtrate was then passed through a 10K filter which results in concentrate (10C) and filtrate (10F). The so, obtained filtrates and

of pH- 8.0) buffer for 24- 48 hours to remove the salt. Next, 150µl of water was added to the dialyzed precipitate (Fp) and it was mixed thoroughly. The resulting mixture was centrifuged at 16,000 rcf for 5 mins producing supernatant (Fs1) and precipitate (Fp1).

The above wash procedure was repeated twice producing two more supernatants (Fs2 and Fs3) and two more precipitates (Fp2 and Fp3). The supernatants and precipitates were then subjected to dialysis for 24 hours and then tested on HEK293 tumor cells.

concentrates were tested on HEK293 tumour cells which were incubated overnight at 37°C. Then, these cell cultures were PBS harvested and quantified using a protein assay.

A detailed study of 30F and 10C cytotoxic activity on HEK293 cells was made by incubating the cell cultures at 37°C for 72hours. Their effects on tumor cells were recorded by harvesting a set of samples for every 24 hours. Samples were stored at 4°C. Finally, a protein assay was performed on each of the harvested samples.

3) Ion Exchange Chromatography

Six different types of matrices made of sand, diethylaminoethyl (DEAE), Bio-Rad's agarose, sephadex G-75, cibacronblue(CB) and carboxymethyl (CM) were used for the separation of the active components of Mix. The beds of these matrices were prepared, and then 1 ml of Mix extract was added to each of the matrices. The resulting mixtures were shaken for two hours. These matrices were then centrifuged at 4000 rcf for 1 min and flow through was collected. One ml of PBS (PBS with 0.85M NaCl) was added equally to all the matrices and mixed for 30 mins. These matrices were then centrifuged at 16,000 rcf for 1 min and elutant was collected. The collected flow through and elutant was tested on HEK293 cells incubated overnight at 37°C. Then, these cell cultures were PBS harvested and quantified using a protein assay to determine flow through and elutant cytotoxicity.

4) High performance liquid chromatography (HPLC)

Chromatography is the process in which components present in sample extracts were separated based on their molecular weight. The concentrated Mix was subjected to HPLC for 20 minutes using the following settings: range-1.0, flow rate-1ml/min and a wavelength of 270 nm using HPLC buffer (50mM NaPo₄, 150 mM NaCl pH- 6.8). The

HPLC fractions were collected at each minute of the separation process. These fractions were then tested on HEK293 cells incubated at 37°C. These cells were harvested and quantified by performing a protein assay.

Gas chromatography-mass spectroscopy (GC-MS)

The identification of active compounds present in the collected HPLC fractions was performed using gas chromatography-mass spectroscopy (GC-MS). The inlet temperature was maintained at 350°C for 3 minutes and was steadily decreased to 180°C in 12 minutes.

Results

The cytotoxic activity of *M. Charantia* extracts:

The cytotoxic activity of *M. charantia* extracts of water, Mix and ethanol on HEK293 tumour cells were analyzed by performing protein assays. The protein percentages of the tumour cells are summarized in Fig 1. From the Fig 1, the lower protein percentages of HEK293 cells treated with Mix and water explain their cytotoxic activity on tumour cells. Among these two *M. charantia* extracts, the Mix has the highest effect. Their effects were also observed by performing a western blot, in which the protein samples were separated and were probed with anti-tumor P-53 and anti- actin. These probed proteins were analyzed using X-ray film and can be seen in Fig 2. By observing the X-ray film visually, the amount of protein present in tumor cells treated with 3 μ l of Mix was less when compared to the other extracts. The lower the amount of protein, the higher the cytotoxic activity of the extract. The cytotoxic activity of 3 μ l of Mix extract was higher when compared to 1 μ l and 2 μ l of extract; this explains the importance of the extract concentration.

The three *M. charantia* extracts were also tested on RBC and the effects of these extracts were visually observed and summarized in Table 1. The RBC cells were clumped and the cell surface of the RBC was serrated when the samples were observed under the microscope. From the visual observation, all the *M. charantia* extracts had a cytotoxic effect on the RBC.

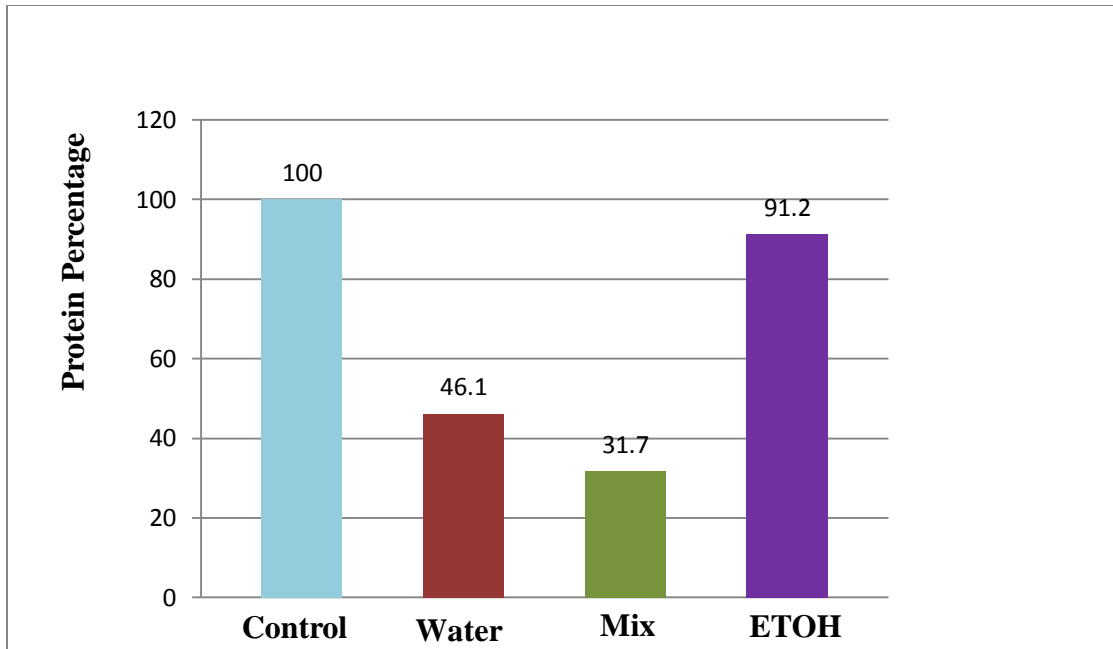


Fig 1. Effects of *M. charantia* seed extracts on HEK293 cells. The *M. charantia* extracts: Water, Mix and ETOH were tested on HEK293 tumor cells. The cytotoxic effects of these extracts were quantified using the BCA protein assay.

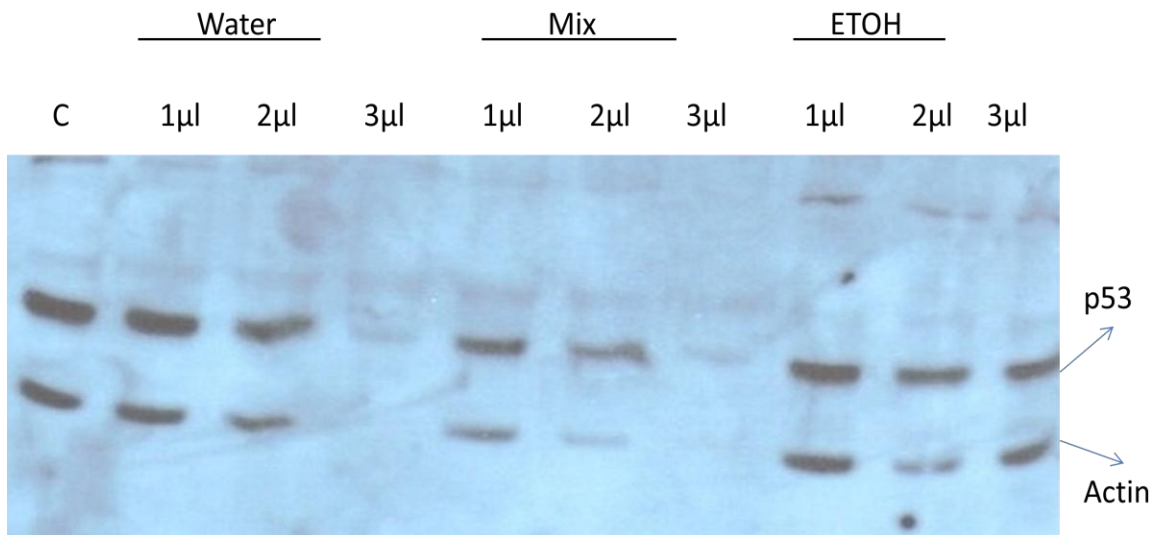


Fig 2. P-53 and actin proteins of HEK293 cells were treated with the three *M. charantia* extracts in various concentrations and analyzed by performing the western blot technique. An equal amount of protein was loaded in each well of the 10% SDS-PAGE. Primary antibody: rabbit anti-P-53, rabbit anti-actin and secondary antibody: rabbit anti-IgG were used for tagging the P-53 and actin proteins.

Table 1. Effect of *M. charantia* extracts: Water, Mix and ETOH were observed on RBC suspensions. Their cytotoxic activity on RBC was determined by cell lysing. Using +, ++, +++, +++++ (complete lysing) and -- (no lysing) to indicate results.

Samples	Water	Mix	Ethanol
Effect	++++	++++	++++

Cytotoxic activity of Mix extracts from *M. charantia*:

Attempts were made to isolate and identify the active compounds present in the Mix extract. Several separation processes like a) salt precipitation b) filtration c) ion-exchange chromatography and d) HPLC were used.

Isolation of active compounds by salt precipitation:

The Mix extract was subjected to ultrafiltration using a 5000 NMWL filter column. The filtrate (F) and concentrate (C) were obtained and tested on HEK293 tumor cells. Their effects were visually observed and summarized in Table 2. From the collected data, the Mix and filtrate each had a cytotoxic effect on tumour cells, while C did not show any activity.

Table 2. The filtrate (F) and concentrate (C) were tested on HEK293 tumor cells. The cytotoxic effects were visually observed and recorded. Using +, ++, +++, +++++ (maximum cell death) and -- (no cell death) to indicate results.

Sample	Mix	F	C
Effect	++++	+++	--

The collected filtrate (F) was then saturated with ammonium sulfate. Samples collected from this process were tested on HEK293 cells. The results were visually

observed and summarized in Table 3. In analyzing the results, Fs2 and Fs3 each had an effect on tumor cells whereas Fs1 has no effect.

Table 3. The filtrate (F) was subjected to salt precipitation; the obtained supernatants were tested on HEK293 cells. The cytotoxic effects were visually observed and recorded using +, ++, +++, +++++ (maximum cell death) and -- (no cell death) to indicate results.

Samples	Fs	Fs1	Fs2	Fs3
Effects	--	--	+++	+++

Isolation of active compounds by filtration:

After the filtration process, filtrates and concentrates collected at various levels were tested on HEK293 cells. The effects of these filtrates and concentrates were visually analyzed and summarized in Table 4. From this analysis, 30F and 10C each had a cytotoxic effect on tumor cells. This effect indicates the presence of cytotoxic active compounds in 30F and 10C samples whose molecular weights might be in between 10,000-30,000 Daltons.

Table 4. Filtrates and concentrates obtained by passing Mix through 30K and 10 K filters were tested on HEK293 cells. The cytotoxic effects based on the cell death were visually observed and recorded using +, ++, +++, +++++ (maximum cell death) and -- (no cell death) to indicate results.

Samples	30F	30C	10F	10C
Effect	++++	--	--	++++

Further information on the cytotoxic effect of 30F and 10C on tumour cells was obtained by incubating the cells with 30F and 10C for 78 hours and sampling the cells at

24 hour intervals and doing protein assays. The protein percentages of tumour cells are summarized in Fig 3. The 30F had the highest cytotoxic effect after 72 hours.

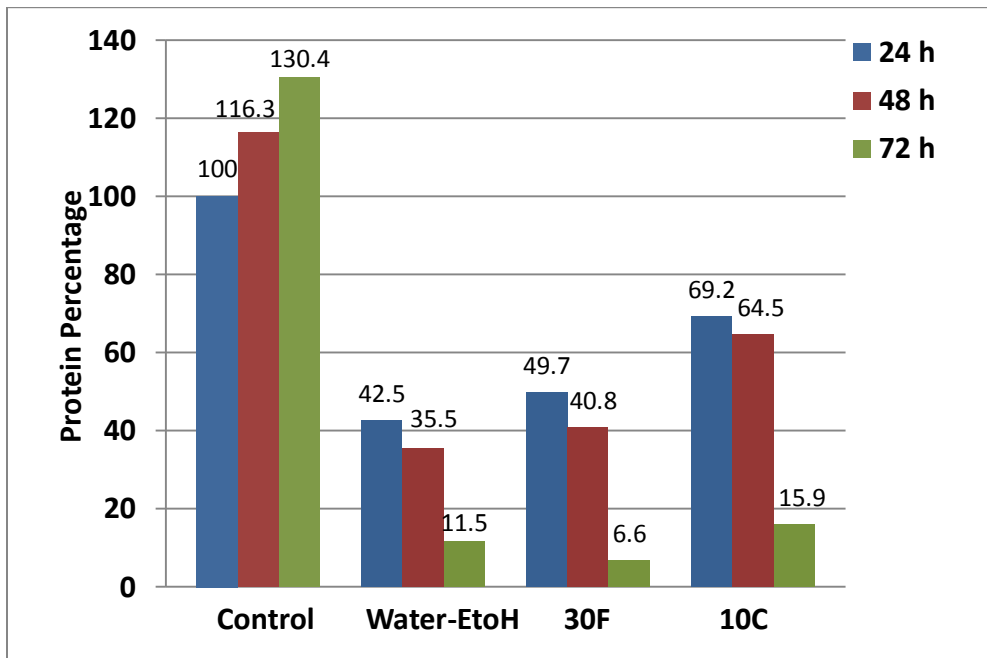


Fig 3. Effects of 30F and 10C on HEK293 cells. The 30F and 10C were collected by passing Mix extract through 30K and 10K filters. Collected samples were tested on HEK293 cells. The cytotoxic effects of these extracts were quantified using the BCA protein assay.

Isolation of active compounds by Ion exchange chromatography:

For a more precise separation of the active compounds, the Mix was subjected to Ion exchange chromatography. The Mix sample was mixed on six different types of matrices and the obtained flow through and elutant were tested on HEK293 cells and RBC. The cytotoxic effects of flow through and elutant on tumour cells were analyzed from the values of the protein assay and shown in Fig 4. The cytotoxic effects on RBC were summarized in Table 5. The cell surfaces of the RBC were serrated for the samples with flow through, while there was no effect on the RBC (smooth appearance) with elutant.

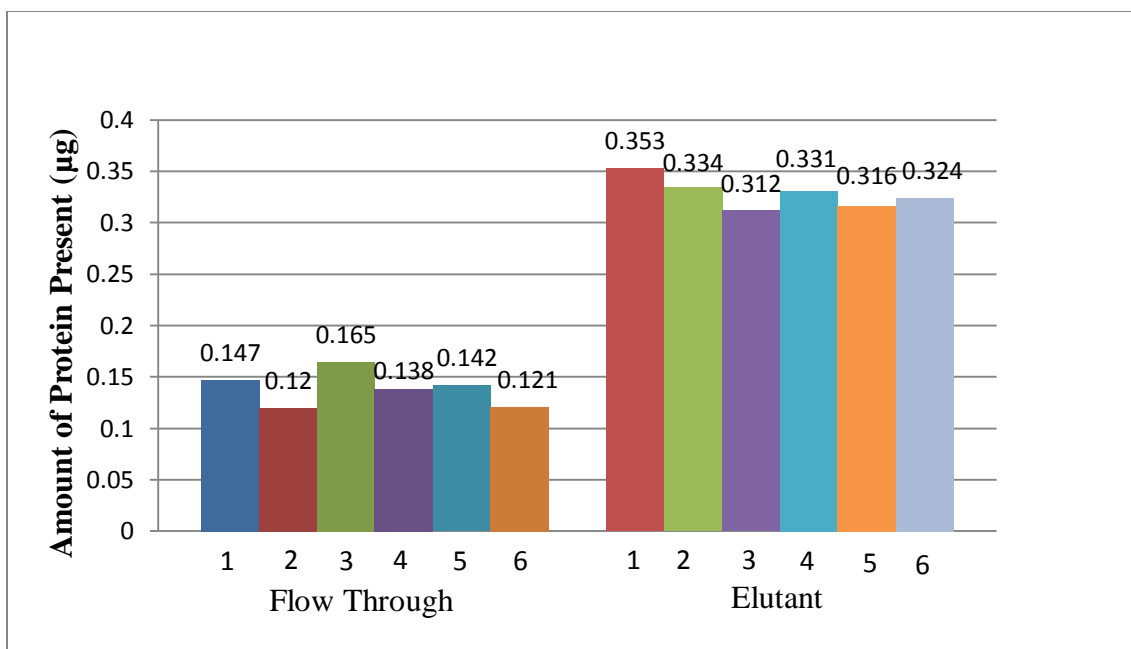


Fig 4. The effect of flow through and elutant on HEK293 cells: Cytotoxic effects of flow through and elutant from six different matrices were tested on HEK293 cells. The six different matrices which were used are 1. agar 2. Sand 3. G-75 4. DEAE 5. CB and 6. CM. The cytotoxic effects of these extracts were quantified using the BCA protein assay.

Table 5. Flow through and elutant from the six different matrices tested on RBC suspensions. The cytotoxic effects on RBC membranes were visually observed and recorded.

Matrices	Agarose	Sand	G-75	DEAE	CB	CM
Flow through	Serrated	Serrated	Serrated	Serrated	Serrated	Serrated
Elutant	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth

Isolation of active compounds from Mix by HPLC:

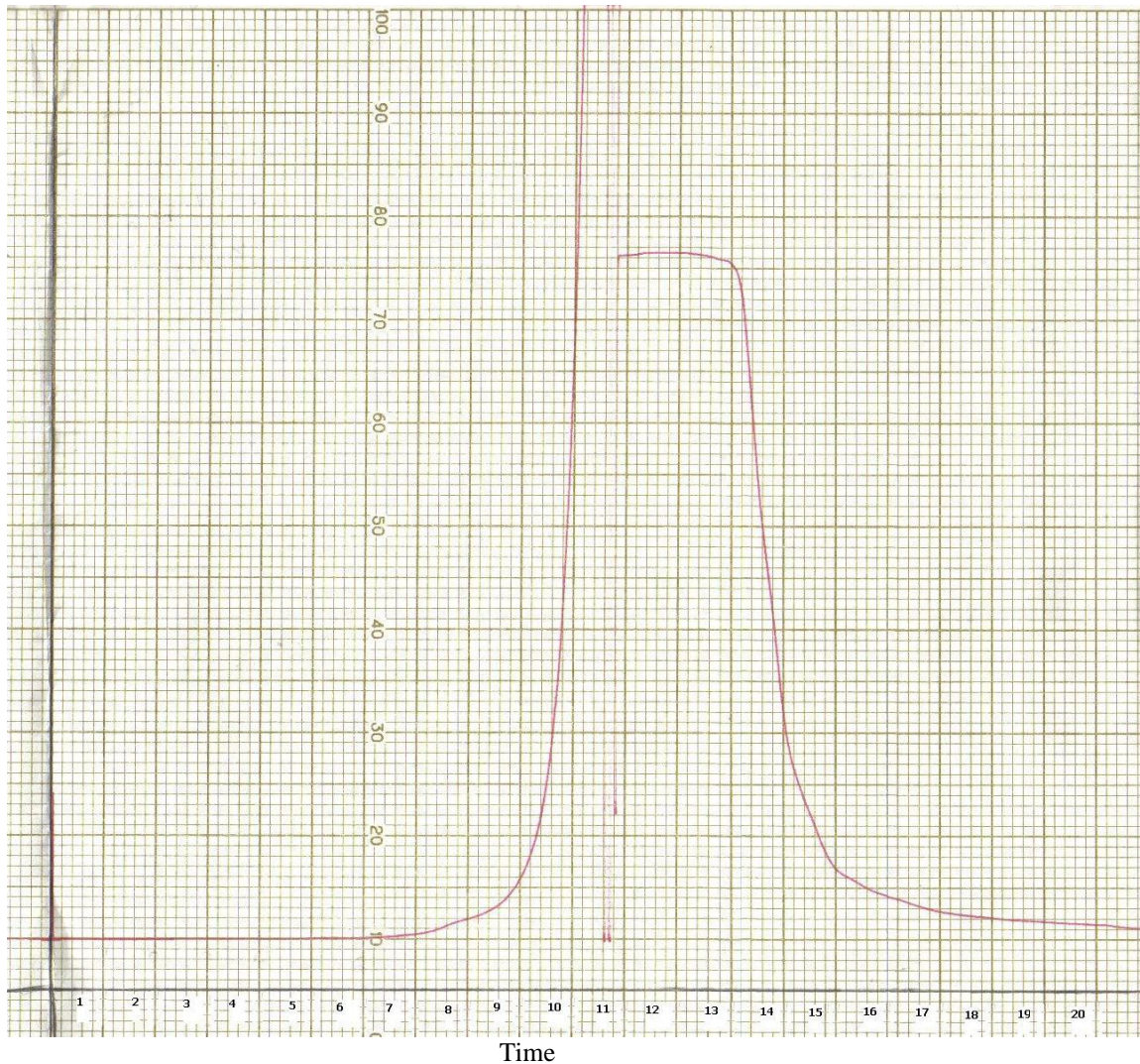
For a better separation of the active compounds, Mix was subjected to HPLC.

Mix has exhibited certain peaks in the HPLC chromatogram that can be seen in Fig 5.

H9-H16, are the HPLC fractions collected between 9 to 16 minutes of separation process were tested on HEK293 cells. The protein percentages of tumor cells from the protein assay are summarized in Fig 6. From the results, tumor cells treated with H11, H12 and

H13 HPLC fractions produced very low protein percentages when compared to those treated with other HPLC fractions. Among these three fractions, H12 and H13 had the highest cytotoxic effects on tumor cells. All three fractions were also tested on RBC. Their effects were observed visually and summarized in Table 6. The RBC cells were clumped and completely destroyed by the H12 and H13 HPLC fractions.

Ab



Ab: Absorbance.

Fig 5. The following peaks were observed when mix extract was subjected to size exclusion HPLC using a 300×7.8 column.

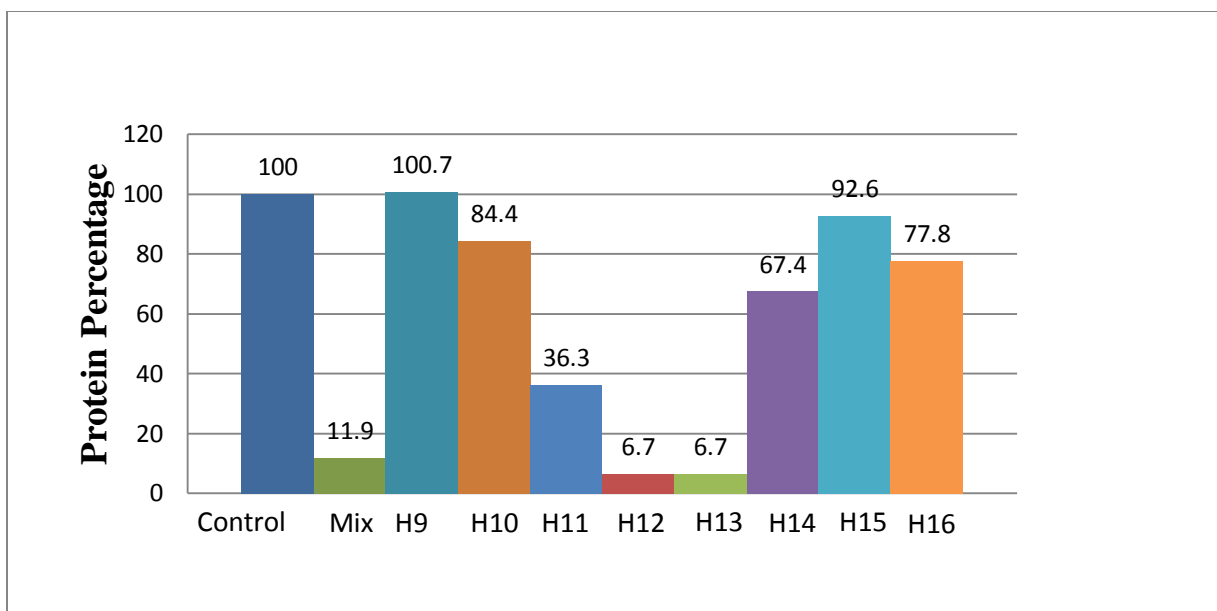


Fig 6. Effect of HPLC fractions on HEK293 cells. Mix extract was subjected to HPLC using HPLC buffer (50mM NaPo₄, 150 mM NaCl pH- 6.8). HPLC fractions were collected every minute for 20 minutes. Samples collected after 8 minutes, H9-H16, were tested for their cytotoxicity. Effects of the HPLC fractions were quantified using the BCA protein assay.

Table 6. The effects of the HPLC fractions of Mix were observed on RBC suspensions. The lysing of RBC was visually observed and recorded using +, ++, +++, ++++ (maximum cell lysis), -- (no cell lysis) to indicate results.

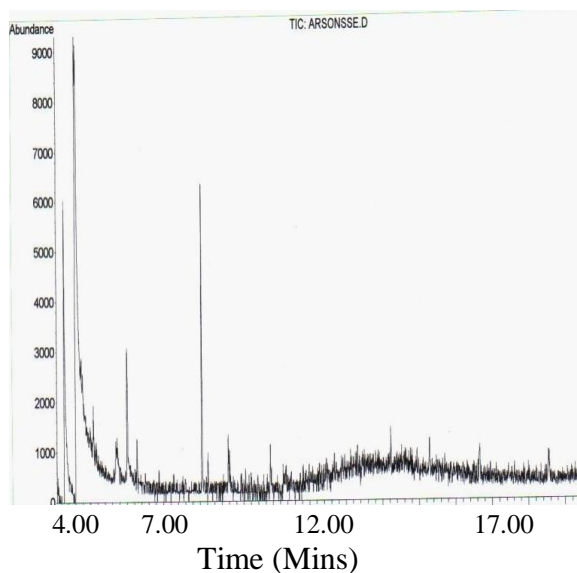
Samples	Mix	H11	H12	H13
RBC	++++	-- (Cells serrated)	++++	++++

RBC: Red Blood Cells, H11-H13: HPLC fractions.

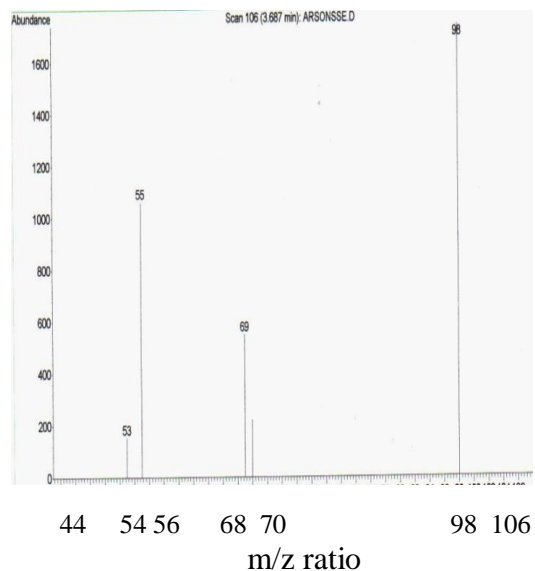
Identification of active compounds present in HPLC fractions:

The Mix HPLC fractions H12 and H13 were analyzed by GC-MS. During this analysis, low molecular weight active compounds were observed. The results for these two samples can be seen in Fig 7 and Fig. 8. In this analysis, GC separates compounds with high resolution, whereas MS provides detailed structural information on the compounds.

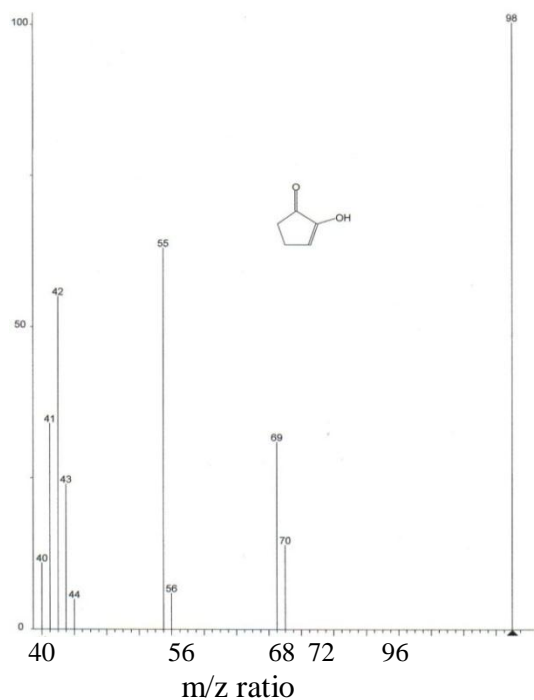
A



B



C



D (M) 2-hydroxy-2-Cyclopenten-1-one

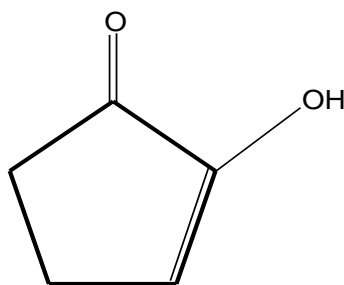


Fig 7. Analysis of HPLC fraction 12 by GC-MS. The total ion spectrum (A), the mass Spectrum (B) and fragmentation pattern (C) of the sample collected at 3.687 min in the total ion spectrum (A), and the predicted molecule (D) based on fragmentation pattern and total mass spectrum.

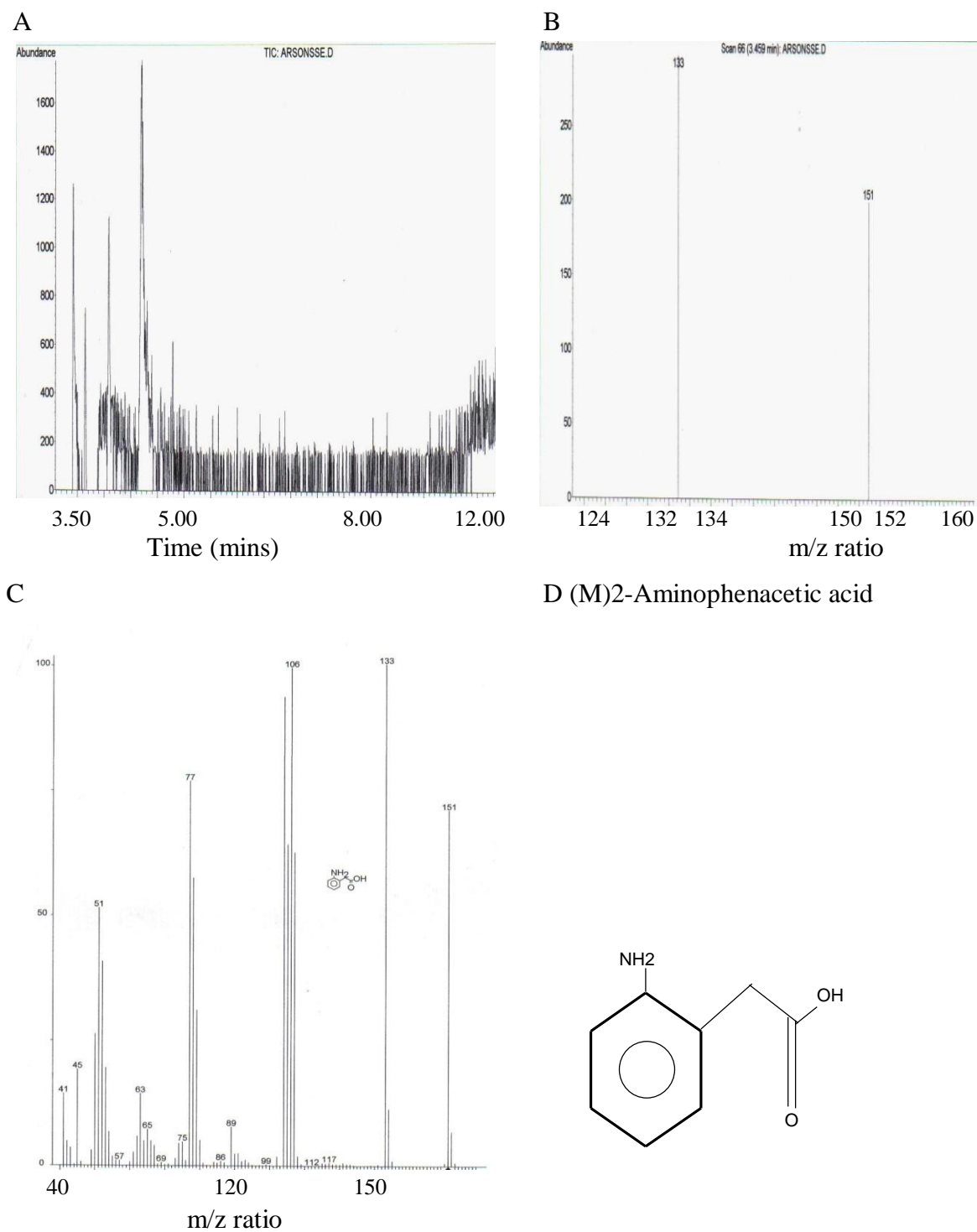


Fig 8. Analysis of HPLC fraction 13 by GC-MS. The total ion spectrum (A), the mass Spectrum (B) and fragmentation pattern (C) of the sample collected at 3.687 min in the total ion spectrum (A), and the predicted molecule (D) based on fragmentation pattern and total mass spectrum.

Discussion

Present study was focused on the identification and isolation of the cytotoxic active compounds from the seeds of *M. charantia*. Mix, water and ethanol extracts of *M. charantia* seed were prepared and tested on HEK293 cells and RBC. Mix has shown the highest cytotoxic effect on HEK293 and RBC. Cytotoxic effect indicates the presence of active chemical compounds in Mix. These results are in accordance with Tongia *et al.*, 2004 (23), who spotted different chemical compounds in phytochemical and chromatographic analysis of methanolic *M. charantia* fruit extract.

In various studies, different separation methods were used for isolation and identification of the active compounds present in *M. charantia* extracts. (19, 23, 24). In the present study, active compounds from Mix were precipitated by ammonium sulfate salt precipitation process. Supernatants Fs1, Fs2 and Fs3 were collected in the salt precipitation process and tested on HEK293 cells. From the results, Supernatants Fs2 and Fs3 has shown the cytotoxic effect whereas, Fs1 has no effect on HEK293 cells. Cytotoxic effect of supernatants Fs2 and Fs3 indicates the presence of active compounds in the dialyzed precipitate. If the active compounds were precipitated from the Mix, then Fs1 should have cytotoxic effect along with Fs2 and Fs3 supernatants. These results are not in accordance with the results of previous studies; in which researches has successfully precipitated active proteins from different parts of *M. charantia* plant. Among them Mahmood *et al.*, 2009 (12) worked on antimicrobial active proteins of *M. charantia* seed extract. These active proteins were precipitated in ammonium sulphate salt precipitation process and separated on SDS-PAGE. Electrophoretic profile of dialyzed seed extracts has shown seven protein bands ranging between 9 to 54Kda.

In the present study, active compounds from Mix were separated by filtration process. Mix was subjected to filtration using 30,000 (30K) and 10,000 (10K) dalton centrifugal filters. Obtained filtrates and concentrates were tested on HEK293 cells. 30F and 10C has cytotoxic effect on HEK293 cells. This effect indicates the presence of active compounds in 30F and 10C samples whose molecular weights might be in between 10 -30 KDa. These results are in accordance with Yuan *et al.*, 2008 (24), who identified hypoglycemic active proteins in *M. charantia* fruit extract. These active proteins were separated by passing through 10Kda ultra filtration column. The concentrate obtained in the filtration process was subjected to enzymatic hydrolysis using different enzymes. Peptides produced during alcalase enzyme hydrolysis had stronger hypoglycemic effect on diabetic mice.

Several researchers (5, 6, 7, and 11) identified biologically active compounds in different parts of *Momordica* plant species. After identification of these active compounds, different chromatographic techniques were used for the isolation and purification of the active compounds. These extracted active compounds have shown their therapeutic actions against different diseases. Among these researchers Lai *et al.*, 2009 (1) identified and isolated two novel peptides from whole fruit juice of *M. cochinchinensis*. These novel peptides were purified and extracted using reversed phase HPLC (RP-HPLC). Whereas Suresh *et al.*, 2012 (19) found antibacterial active compounds in *Terminalia chebula* extracts. Methanolic extracts of *T. chebula* were fractionated for the active compounds and the obtained fractions were pooled and analyzed by HPLC. Obtained fractions showed the maximum antibacterial activity against the multidrug resistant isolates of diabetic foot ulcer. Similar results were

observed in the present study, where Mix was subjected to the size exclusion HPLC. Fractions collected after 8 minutes of retention time were tested on HEK293 cells and RBC. Fractions H12 and H13 has shown the highest cytotoxic effect on HEK293 cells and RBC. These results indicate the presence of cytotoxic active compounds in the fractions H12 and H13. In order to determine the structure of active compounds, fractions H12 and H13 were subjected to GC-MS. From the GC-MS analysis, potentially new types of low molecular weight cytotoxic active compounds were identified. Similarly Zulbadli *et al.*, 2011 (25) also used GC-MS for analyzing the structure of valuable compounds present in the *M.charantia* fruit extract. Researchers identified few medicinal compounds like vitamin E, ascorbic acid, cucurbitacin B and gentisic acid in *M.charantia* fruit extract. Gentisic acid and vitamin E are known for their antioxidant activity. These medicinal compounds were known for their antioxidant activity, anti-proliferative activity and hypoglycemic effect.

Conclusion

It can be concluded from this study that *M. charantia* Mix can increase the cancer cell death. In contrast, either the water extract or methanol extract of *M. charantia* had less effect on tumour cells. Data obtained in the filtration and HPLC separation process indicates the presence of cytotoxic active compounds in the *M. charantia* seed extracts. Potentially new types of low molecular weight cytotoxic active compounds were identified in GC-MS analysis of the fractions obtained in the HPLC separation process.

Future studies

The isolation and testing of the active compounds recognized in GC-MS on HEK293 cells and RBC. The observed effect of these compounds on tumor cells and RBC would help in analyzing the cytotoxicity of *M. charantia* seed extracts.

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