RESEARCH ARTICLE

Apoptosis-Inducing Activity of HPLC Fraction from *Voacanga globosa* (Blanco) Merr. on the Human Colon Carcinoma Cell Line, HCT116

Alvin Resultay Acebedo[1]*, Evangeline Cancio Amor[2], Sonia Donaldo Jacinto[1]

Abstract

***Voacanga globosa* (Blanco), a plant endemic to the Philippines, is traditionally used especially by indigenous people of Bataan in the treatment of ulcers, wounds and tumorous growths. This study aimed to provide scientific evidence to therapeutic properties by determining cytotoxic and pro-apoptotic activity of HPLC fractions from leaves on HCT116 human colon carcinoma and A549 human lung carcinoma cell lines. Ethanolic extraction was performed on *V globosa* leaves followed by hexane and ethyl acetate partitioning. Silica gel column chromatography and high performance liquid chromatography (HPLC) produced MP1, MP2 and MP3 fractions. Cytotoxic activity of the fractions was determined through MTT assay against the cancer cell lines HCT116 and A549 and the non-cancer AA8 Chinese hamster ovarian cell line. Pro-apoptotic activities of the most active fractions were further assessed through DAPI staining, TUNEL assay and JC-1 mitochondrial membrane potential assay with HCT116 cells. While the MP1 fraction exerted no significant activity against all cell lines tested, MP2 and MP3 fractions demonstrated high toxicity against HCT116 and A549 cells. The MP3 fraction induced formation of apoptotic bodies, condensed DNA and other morphological changes consistent with apoptosis of HCT116 cells and TUNEL assay showed significant increase in DNA fragmentation over time. In these cells, the MP3 fraction also induced mitochondrial membrane destabilization, which is generally associated with the beginning of apoptosis. Phytochemical analysis demonstrated the presence only of saponins and terpenoids in the MP3 fraction. The results indicate that the MP3 fraction exerts cytotoxic activity on HCT116 cells via induction of apoptosis triggered by loss of mitochondrial membrane potential crucial for cell survival.**

Keywords: *Voacanga globosa* - cancer cell lines - cytotoxicity - apoptosis - saponins - terpenoids

Asian Pac J Cancer Prev, **15 (2)**, 617-622

Introduction

During carcinogenesis, certain mutations suppress apoptosis allowing the continued survival of cancer cells (Hanahan and Weinberg, 2000; Varfolomeev and Vucic, 2011). The Department of Health has reported malignant neoplasms to be the third leading cause of mortality in the Philippines from 2004-2008 and in 2009 (DOH 2013). Thus the push to find new and more efficacious chemotherapeutic drugs such as those which induce apoptosis. It is widely accepted that induction of apoptosis plays a role in killing cancer cells *in vitro* and is an attractive target for cancer therapy (Blagosklonny, 2004; Strasser et al., 2011).

In this study, leaves of *Voacanga globosa* were investigated for possible cytotoxicity against cancer cells. A previous study has demonstrated that extracts from *V globosa* leaves exhibit high toxicity against two cancer cell lines, HCT116 human colon carcinoma and A549 human lung carcinoma (Canoy, 2011). *V globosa* is thus likely to produce anti-cancer compounds. Using MTT assay, the study aimed to test HPLC fractions from the *V globosa* leaf extract for cytotoxicity against the HCT116 human colon carcinoma, A549 human lung carcinoma and the non-cancer AA8 Chinese hamster ovarian cell lines. Possible pro-apoptotic activity was also assessed using DAPI nuclear staining, TUNEL assay and the JC-1 mitochondrial membrane potential assay.

Materials and Methods

Production of the ethanolic crude extract

Mature leaves from *V globosa* (Blanco) Merr. were harvested from Morong, Bataan, Philippines. Identification was verified by the Jose Vera Santos Herbarium of the Institute of Biology, University of the Philippines Diliman (a voucher specimen sample submitted was assigned the accession number: 14609). Leaves were air dried until crispy and then macerated in 95% technical grade ethanol for at least 48 hours. The leaf suspension was filtered and

*[1]Mammalian Cell Culture Laboratory, Institute of Biology, [2]Institute of Chemistry, College of Science, University of the Philippines, Diliman, Quezon City, Philippines *For correspondence: eternal_alvin@yahoo.com*

concentrated by rotary evaporation at 37°C producing the ethanolic crude extract.

Solvent partitioning and column chromatography

For the next stages of extraction, all solvents used were of analytical grade. Ethanolic crude extract was partitioned with equal parts hexane. Hexane and ethanol partitions were concentrated by rotary evaporation. The ethanol fraction produced was then partitioned with equal parts ethyl acetate and distilled water. The resulting aqueous and ethyl acetate partitions were also concentrated separately by rotary evaporation. The hexane, ethyl acetate and aqueous partitions were reconstituted to final concentrations of 4 mg/mL DMSO then tested for cytotoxicity using the MTT assay.

The solvent partition with significant cytotoxic activity was further fractionated using normal silica gel column chromatography with 9:1 chloroform-methanol solvent system. Separation was monitored using thin layer chromatography (TLC) silica gel 60 F254 plates with 33% ethyl acetate-hexane solution as the mobile phase. Fractions with similar banding profiles were re-dissolved in 9:1 chloroform/methanol, pooled, air-dried and re-tested for cytotoxicity. The pooled fraction with the most significant cytotoxic activity was reloaded onto a silica gel column chromatography using a solvent gradient system of increasing polarity (elution starting at 35% ethyl acetate/hexane with increasing ethyl acetate concentrations at 10% increments). Fractions from the gradient silica gel column were subjected to TLC. Those with similar banding profiles were dissolved in 9:1 chloroform/methanol, re-pooled then tested for cytotoxicity. The pooled fraction with the most significant activity was then profiled and fractionated using high performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC)

The fraction was air-dried and dissolved in filtered HPLC grade methanol; 100 µL of the extract was then injected into the HPLC with a semi-preparative column using a filtered acetonitrile (HPLC grade) -deionized water gradient solvent system (Total flow: 3 mL/min; B. conc: 5; B. curve: 0; P max: 25 Mpa; P. min: 0 MPa). The resulting HPLC profile was monitored using the CLASS-VP 5.0 program. Fractions corresponding to each major peak were collected, dried using rotary evaporation and then lyophilized. Dried fractions were re-dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 4 mg/mL.

Phytochemical screening

Phytochemical screening was performed with modifications following protocols of Harborne (1984) and Edeoga et al. (2005). Screening was performed for the following organic compounds: tannins, saponins, terpenoids, flavonoids, cardiac glycosides, phenolic compounds, steroids and alkaloids. At least two trials were performed for each phytochemical test.

Methyl thiol tetrazolium (MTT) cytotoxicity assay

The MTT proliferation/cytotoxicity assay was performed with modifications according to the protocol of Mossman, 1983. Cells were washed, treated with 0.05% trypsin-EDTA and seeded at 4×10^4 cells/mL in sterile 96 well microtiter plates. The plates were incubated overnight at 37°C and 5% CO_2. Cells were then treated with DMSO (negative control), doxorubicin (positive control), or the *V globosa* plant extracts. After 72 hours of incubation, the media were replaced with 15 µL MTT solution (5 mg/mL PBS). Plates were then re-incubated for 4 hours then added with 150 µL DMSO to each well. Absorbances were then read using the LEDETECT reader at 570 nm. IC_{50} values were derived through linear regression. Three trials with at least two replicates per concentration were performed.

Diamidino-2-Phenylindole (DAPI) nucleic acid staining

HCT116 cells were washed, detached with 0.05% trypsin-EDTA and seeded into sterile 96 well plates at 4×10^4 cell/mL. Cells were incubated overnight at 37°C and 5% CO_2 then treated using the IC_{50} concentrations established by the MTT assay for MP3 (3.42 µg/mL), doxorubicin (positive control) and DMSO (negative control). Doxorubicin was the positive control at 1.92 µg/mL and DMSO was the negative control. After 12, 24, 48 and 72 hours incubation, 50 µL of 1X DAPI was applied per well then incubated at 37°C for at least 60 minutes. Cells were viewed under bright field and fluorescence microscopy. Cells with morphologies indicative of apoptosis (breaking up into apoptotic bodies) were scored as positive. Three trials with three replicates per concentration were done with at least 200 cells scored per replicate. Apoptotic index was computed as number of cells positive for apoptosis /total number of cells scored.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

DNA fragmentation was assayed following the manufacturer's instructions of the APO-BrdU TUNEL assay kit with some modifications. HCT116 cells were washed, trypsinized and seeded into sterile 96-well plates at 4×10^4 cell/mL then incubated overnight at 37°C and 5% CO_2. Test extracts were applied at IC_{50} concentration. Ttreated cells were incubated at 37°C for 48, 60 and 72 hours then trypsinized and harvested. Cells with similar treatment regimes were pooled into microfuge tubes. At least four wells per replicate for each treatment regime were pooled together. Cells were washed with PBS and fixed with cold 1% paraformaldehyde in PBS on ice for 30 minutes then with PBS and resuspended in 0.5 mL PBS with 5 mL cold 70% ethanol. The cell suspensions were then stored in ice for not less than twenty-four hours then centrifuged. The ethanol from each tube was discarded. The cell pellets were washed then incubated with 50 µL DNA labeling solution at 37°C for two hours and shaken every 30 minutes. Cell suspensions were then washed with the rinse buffer and incubated with 100 µL antibody-staining solution at room temperature for 30 minutes. Cells were viewed under fluorescence microscopy. Those displaying bright green fluorescence were scored as positive for DNA fragmentation and apoptosis. A minimum of five to ten fields of view per replicate was used for scoring of cells. Three trials with three replicates each concentration were performed and at least 200 cells per replicate were scored. The apoptotic index for each

replicate was computed as previously described for the DAPI nucleic acid staining.

JC-1 mitochondrial membrane potential assay

Mitochondrial membrane depolarization was assessed using the MitoProbe™ JC-1 Assay Kit following the manufacturer's instructions with some modifications. HCT116 cells were washed, trypsinized, seeded into sterile 96-well plates at 4×10^4 cell/mL then incubated overnight at 37°C and 5% CO_2. Test extracts were then applied to the cells at their respective IC_{50} concentrations, followed by re-incubation at 37°C for seventy-two hours. Cells were then trypsinized and collected. At least four wells per replicate for similar treatment regime were pooled together into microfuge tubes. Cells were centrifuged and the supernatant from each tube was removed. The resulting cell pellets were re-suspended in 1.0 mL warm PBS. JC-1 stock solution was added to each cell suspension at 10 µL then incubated at 37°C, 5% CO_2 for 45 minutes. Cell suspensions were then centrifuged, washed with PBS, transferred to a 96-well plate and then viewed with fluorescence microscopy using appropriate filters. Cells displaying green (positive for mitochondrial membrane depolarization) and red (negative for mitochondrial membrane depolarization) fluorescence were scored. Five to ten fields of view per replicate was used for scoring of cells. Three trials with three replicates each were performed and at least 200 cells per replicate were scored. Red-green fluorescence ratio was computed from number of red fluorescent cells / number of green fluorescent cells

Statistical analysis

Normality of distribution and homogeneity of the variances of means were assessed through the Shapiro-Wilk test and the Levene's test respectively. Means were then analyzed with One-way Analysis of Variance (ANOVA). The results of ANOVA from data sets with unequal variances of means were confirmed using the Brown-Forsythe test. Post hoc tests (Games-Howell test for data sets with unequal variance and Tukey's Honestly Significant test for data sets with equal variance) were then performed to determine significant differences between means. P-values less than 0.05 indicates significant differences between means. Three independent trials with at least two replicates each were performed for each experiment. All statistical analysis were performed using the SPSS Statistics 17.0 software.

Results

Extraction from voacanga globosa

A total of 2.5 kg of air-dried *V globosa* leaves were used for extraction. The crude ethanolic extract was subsequently partitioned producing the hexane, aqueous and ethyl acetate partitions. Isocratic column chromatography (9:1 chloroform/methanol) of the ethyl acetate partition produced nine column fractions (cf) as demonstrated by TLC analysis. The first fraction, Vg cf 1,2, was selected for further purification due to its observed bioactivity in the MTT assay (Table 1). Gradient elution of Vg cf 1,2 using an ethyl acetate/hexane solvent system yielded 11 gradient fractions. Gradient fraction, Vg G10 was eluted at the 95% ethyl acetate/5% hexane gradient mark and was determined to be the most bioactive gradient fraction (Table 1). HPLC analysis of Vg G10 produced three fractions: MP1, MP2 and MP3. Up to 9 mg of MP1, 2 mg of MP2 and 3 mg of MP3 were produced giving yields of 0.00035% for MP1, 0.00008% for MP2 and 0.00012% for MP3.

Methyl thiol tetrazolium (MTT) cytotoxicity assay

Table 1 shows *V globosa* crude ethanolic extract with an average IC_{50} of 12.17±0.69 µg/mL against the HCT116 cell line in the MTT assay. Solvent partitioning of the crude extract and subsequent MTT assays of the fractions, resulted to ethyl acetate and hexane fractions with average IC_{50} against HCT116 of 7.77 µg/mL±1.14 and 33.59±2.20 µg/mL respectively. Vg cf 1,2 had the most cytotoxic mean IC_{50} value (4.40 µg/mL±1.39) among the other column fractions. Gradient elution of Vg cf 1,2 produced Vg G10 fraction, which displayed the most cytotoxic mean IC_{50} of 3.38 µg/mL±0.03. Fractionation of Vg G10 through HPLC using a C18 semi-prep column produced three fractions (MP1, MP2 and MP3), MP1 (mean IC_{50} of 15.88 µg/mL±1.64): MP2 (mean IC_{50} of 7.63 µg/mL±1.86) and MP3 (mean IC_{50} of 3.42 µg/mL±0.03). MP3 was further tested against the A549 cell line (mean IC_{50} of 5.07 µg/mL±0.51) and against the AA8 cell line (mean IC_{50} of 3.38 µg/mL±0.02) (Table 2).

Table 1. IC_{50} (µg/mL) of the *V globosa* Leaf Extract against HCT116 and A549 Cancer Cell and the AA8 Non-cancer Cell Line as Assessed by MTT Assay

Extracts	HCT116	A549	AA8
Doxorubicin	1.92±0.09	2.19±0.08	1.78±0.04
Ethanol	12.17±0.69		
Ethyl Acetate	7.77±1.14		
Vg cf 1,2	4.40±1.39		
Vg G10	3.38±0.03		
MP3	3.42±0.03	5.07±0.51	3.38±0.02

*Data were derived from three independent experiments with at least three replicates each. Data are presented here as means±SD

Table 2. Qualitative Analysis of Phytochemicals in *V globosa* Extracts

Extracts	Tannins	Saponins	Terpenoids	Flavonoids	Glycosides	Phenolics	Steroids	Alkaloids
Ethanol	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(-)
Hexane	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
Aqueous	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
Ethyl Acetate	(+)	(+)	(+)	(-)	(-)	(-)	(+)	(-)
Vg cf 1,2	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
Vg G10	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)
Vg MP3	(-)	(+)	(+)	-	-	-	-	-

*Presence of the constituent is represented by (+); absence is represented by (-); nt: not tested

Phytochemical screening

Qualitative analysis for phytochemicals revealed the presence of saponins and terpenoids in the MP3 fraction and all of its parent fractions (ethanolic crude extract, ethyl acetate partition, Vg cf 1,2 and Vg G10). Condensed tannins, cardiac glycosides and steroids were found to be present in the initial ethanolic crude extract but were removed in successive stages of extraction, being absent in the Vg MP3 fraction. Tests for flavonoids, phenolic compounds and alkaloids did not yield a positive result for all extracts tested (Table 2).

Assessment of cellular morphology

HCT116 cells incubated with the MP3 fraction at 3.42 µg/mL for 72 hours demonstrated features consistent with apoptosis which include membrane blebbing, cell shrinkage, surface detachment, loss of contact with neighboring cells and loss of spindle shape. These were also observed in those treated with doxorubicin (positive control) at its IC_{50} of 1.92 µg/mL but not in HCT116 cells

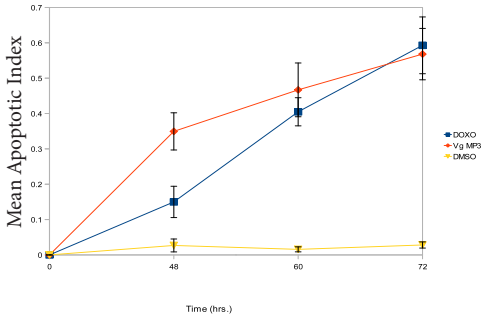

Figure 1. Mean Apoptotic Indices of HCT116 Human Carcinoma Cells as Assessed by TUNEL Assay. Cells were treated with IC_{50} concentrations of MP3 at 3.42 µg/mL, doxorubicin (positive control) at IC_{50} 1.92 µg/mL and the vehicle solution DMSO (negative control). Apoptotic index was computed as the number of TUNEL-positive cells per replicate divided by the total number of cells. Values are means of three trials with three replicates each, ±SD.

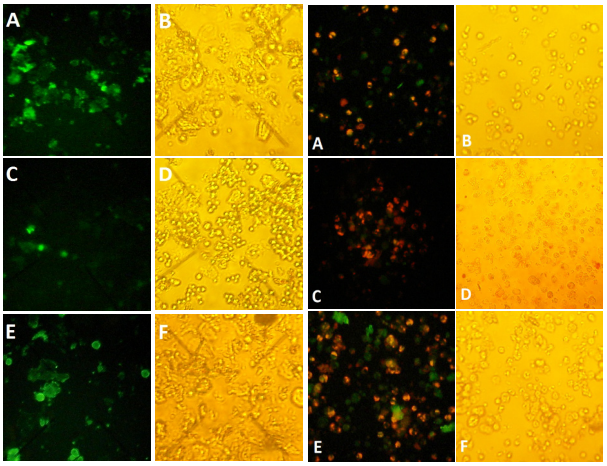

Figure 2. TUNEL Results (left with Anti-BrdU mouse monoclonal antibody PRB-1, Alexa flour in the TUNEL assay) and Mitochondrial Membrane Destabilization (right with JC-1). HCT116 Cells incubated for 72 hours with IC_{50} of MP3 (A, B) at 3.42 µg/mL, DMSO (C, D) and doxorubicin (E, F) at IC_{50} of 1.92 µg/mL. Cells were viewed under fluorescence (A, C, E) and bright field (B, D, F) at 200X magnification

treated only the DMSO (negative control). DMSO-treated cells demonstrated normal epithelia-like morphology, including spindle-shape, close attachment to neighboring cells and the substrate and were observed to grow in monolayers. Doxorubicin and MP3-treated cells also showed much reduced cell densities, implying cell death.

Diamidino-2-Phenylindole (DAPI) nucleic acid staining

In MP3-treated cells, significant increase (p=0.000) in apoptosis was observed at 24 hours of incubation with mean apoptotic indices of 0.238±0.083. The mean apoptotic indices for both treatment groups at 48 and 72 hours were found to be significantly different from the mean apoptotic indices of the DMSO-treated cells (negative control) at the same incubation periods (0.116±0.049 for 48 hours of incubation and 0.089±0.025 for 72 hours of incubation). At 12 hours of incubation, no significant differences were observed between the negative (0.094±0.032) and positive controls (0.139±0.059) and the MP3-treated cells (0.150±0.061). HCT116 cells treated with MP3 and doxorubicin (positive control) displayed apoptotic bodies and condensed DNA indicating the occurrence of apoptosis.

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

TUNEL assay screening of HCT116 cells treated with MP3 or doxorubicin (positive control) for 48, 60 and 72 hours demonstrated potent activity starting at the 48 hours and continuing to increase significantly up to 72 hours of incubation. Apoptosis was demonstrated by bright green fluorescence indicating DNA fragmentation. At all incubation periods, mean apoptotic indices of MP3 and doxorubicin were found to be significantly different (p=0.000) from the mean apoptotic indices of DMSO-treated cells. However, apoptotic indices of MP3- and doxorubicin-treated cells showed no statistical difference except at 48 hr when MP3 showed significantly higher apoptotic index (Figures 1 and 2 left).

JC-1 mitochondrial membrane potential assay

The MP3 fraction induced loss of mitochondrial membrane potential in HCT116 cells. Figure 2 left shows the collapse of mitochondrial membrane potential indicated by changes in the ratio of cells with red fluorescence (with viable mitochondria) to cells with green fluorescence (with collapsed mitochondrial membrane potentials). Cells incubated with the MP3 fraction for 72 hours at the IC_{50} of 3.42 µg/mL demonstrated significantly lower (p=0.000) red-green fluorescence ratios (mean fluorescence ratio of 1.208±0.155) compared to cells incubated with only the vehicle DMSO (mean fluorescence ratio of 4.950±0.256). The red-green fluorescence ratio in MP3-treated cells did not display significant differences from cells treated with the positive control, doxorubicin (mean fluorescence ratio of 1.180±0.134).

Discussion

Extracts of *Voacanga globosa* displayed toxicity against human cancer cell lines and are likely candidates

for development as chemotherapeutic drugs. Previous studies demonstrated that plant species related to *V globosa* produce compounds with significant anti-cancer activities. Examples of these include the chemotherapeutic drugs vincristine and vinblastine which are derived from *Catharanthus roseus* belonging to the same family (Apocynaceae) as *V globosa* (Cragg and Newman, 2005). Although many studies have demonstrated presence of bioactive compounds from the family Apocynaceae, very few studies have established bioactivity of compounds from *Voacanga globosa*. Here, the presence of anti-cancer substances in *V globosa* was confirmed and demonstrated to have pro-apoptotic activity against HCT116 cells.

Anti-cancer activity of the *V globosa* extract is supported by results from the MTT assay when it showed potent cytotoxicity against the HCT116 human colon carcinoma cell line. According to the American National Cancer Institute (NCI), the standard for IC_{50} values implying significant activity against cancer cell lines is <30μg/mL (Suffness and Pezzuto, 1990). This standard was used as an indicator of significant cytotoxic activity in several other studies focusing on the induction of cell death in cancer cell lines through the MTT cytotoxicity assay (Jokhadze et al., 2007; Chang et al., 2010). In the current study, the standard value of <30μg/mL falls within the concentration range (50.00 μg/mL, to 6.25 μg/mL) used in the cytotoxicity screening. The extract when pursued for further fractionation and bioassays demonstrated potent activity in the MTT assay according to this standard value with IC_{50} as low as <5μg/mL. Inhibitory activity of the extract against the HCT116 cells was observed at different stages of extraction, with generally increasing toxicity (decreasing IC_{50}) with further purification of the sample culminating in the MP3 fraction which displayed the most potent activity against HCT116 (average IC_{50} of 3.42 μg/mL). The MP3 fraction, because of its potent activity against HCT116 cells compared to the MP1 and MP2 fractions, was selected for further assays.

The MP3 fraction has cytotoxic activity against the very sturdy A549 human lung carcinoma cell line and against the non-cancer AA8 Chinese hamster ovarian cell line. The low IC_{50} values of MP3 against the HCT116, A549 and AA8 cell lines (<5μg/mL) again indicate an ability to inhibit cell growth even at very low concentrations.

MP3 demonstrated an ability to induce time-dependent changes in the nuclear morphology of HCT116 cells at 3.42 μg/mL as assessed by DAPI staining. Observed changes in nuclear and cell morphology culminate in the fragmentation of cells into membrane-bounded bodies (apoptotic bodies) which has been cited previously as a hallmark feature for apoptosis (Savill and Fadok, 2000; Kurosaka et al., 2003; Wu et al., 2013). Pro-apoptotic activity is also demonstrated in the changes in cellular morphology after 72 hours of incubation, with cells displaying loss of spindle shape, membrane blebbing, surface detachment and the formation of cellular granules.

Cytotoxicity of MP3 due to the induction of apoptosis in HCT116 is supported by results with the TUNEL assay. DNA double strand breaks due to extensive fragmentation of nuclear DNA in karyorrhexis is a distinguishing feature of apoptosis that can be identified with TUNEL assay (Kressel and Groscurth, 1994; Darzynkiewicz et al., 2008). TUNEL assay has been demonstrated to be specific for cells undergoing cell death and is a common assay in apoptosis research (Mazahery et al., 2009; Goh and Kadir, 2011; Hasan et al., 2011; Tan et al., 2013; Wu et al., 2013). In this study, the MP3 fraction induced significant DNA fragmentation from 48 to 72 hours of incubation.

MP3 demonstrates significant ability to induce mitochondrial membrane depolarization as assessed by the JC-1 mitochondrial membrane potential assay. JC-1 is a delocalized lipophilic cation that will either form aggregates within viable mitochondria leading to red fluorescence or, in the instance of mitochondrial membrane potential destabilization, localize throughout the cytosol yielding green fluorescence. Collapse of the mitochondrial potential is brought about by the formation of the mitochondrial permeability transition (MPT) pore in the mitochondrial membrane which facilitates discharge of pro-apoptotic factors from the intermembrane space into the cytosol (Saelens et al., 2004; Brenner and Moulin, 2012). Studies have demonstrated that collapse of the mitochondrial membrane potential due to opening of the MPT pore typically triggers apoptosis (Qanungo et al., 2005; Gao et al., 2013; Ramkumar et al., 2013). The loss of mitochondrial membrane potential in MP3-treated cells supports the observed cytotoxic activity of the fraction in the MTT assay. The MTT assay is based on the ability of viable cells to reduce MTT into formazan and this reaction occurs in the mitochondria. The use of JC-1 demonstrates that the mitochondria of MP3-treated HCT116 cells have been compromised hence, unable to reduce MTT leading to the observed toxicity in the MTT assay. The cytotoxic activity of the fraction may thus be facilitated by the induction of mitochondrial membrane depolarization.

The ability of MP3 to induce apoptosis is advantageous in the treatment of cancer. Unlike in necrosis, pro-inflammatory cytosolic contents are not released but are kept inside vesicles which are eventually phagocytosed when phagocytic markers are exposed during the terminal steps of apoptosis. The induction of apoptosis thereby avoids inflammation and tissue damage (Savill and Fadok, 2000; Kurosaka et al., 2003; Ravichandran 2011) thus reducing incidence of negative side effects on surrounding normal tissue.

Phytochemical analysis of the parent fractions of MP3 demonstrated the presence of several known bioactive compound groups. Further screening confirmed saponins and terpenoids to be present in the MP3 fraction. These may likely be the cause of the observed cytotoxicity as these substances have all been previously shown to possess anticancer activity (Polo and de Bravo, 2006; Tin et al., 2007; Lu et al., 2011; Thoppil and Bishayee, 2011).

In conclusion, this study demonstrated that the leaves of *V globosa* are possible sources of anti-cancer compounds validating its use by indigenous people in Bataan, Philippines as antitumor agents. Further fractionation of the extract produced successively more cytotoxic fractions culminating in the MP3 fraction. MP3 was demonstrated to have toxicity to A549 human lung carcinoma and the AA8 Chinese hamster ovarian cell lines,

indicating that the observed cytotoxicity is not unique to HCT116 but to other cell lines as well. Significant cytotoxic activity against A549 demonstrates an ability to resist drug resistance mechanisms present in the A549 cells. The MP3 fraction was also demonstrated through DAPI staining and TUNEL assay, to induce apoptosis due to a reduction of the mitochondrial membrane potential determined by treatment with JC-1. Cytotoxic and apoptosis-inducing activities of the MP3 fraction are likely to have been caused by saponins or terpenoids.

Acknowledgements

We gratefully acknowledge the research grants provided by the University of the Philippines Office of the Vice Chancellor for Research and Development (UP OVCRD), University of the Philippines, Natural Sciences Research Institute (UPNSRI) and the Department of Science and Technology-Philippine Council for Health Research and Development (DOST-PCHRD). We acknowledge further, the Institute of Biology and the Institute of Chemistry, University of the Philippines, Diliman for the facilities and work space where this research was done.

References

Blagosklonny MV (2004). Prospective strategies to enforce selectively cell death in cancer cells. *Oncogene*, **23**, 2967-75.

Brenner C, Moulin M (2012). Physiological roles of the permeability transition pore. *Circ Res*, **111**, 1237-47.

Canoy RJ, Lomanta JMJC, Ballesteros PM, et al (2011). Cancer chemotherapeutic potential of endemic and indigenous plants of Kanawan, Morong, Bataan Province, Philippines. *Asia Life Sciences*, **20**, 331-9.

Chang SC, Huang CT, Huang SM (2010). Experimental study on effect of compounds in inhibiting HCT-116 human colon cancer cells: the preliminary results. *J Soc Colon Rectal Surgeon*, **21**, 121-9.

Cragg GM, Newman DJ (2005). Plants as a source of anti-cancer agents. *J Ethnopharmacol*, **100**, 72-9.

Darzynkiewicz Z, Galkowski D, Zhao H (2008). Analysis of apoptosis by cytometry using TUNEL assay. *Methods*, **44**, 250-4.

DOH (2013). Leading Causes of Mortality. Philippine Health Statistics 1982-2009, DOH.

Edeoga HO, Okwu DE, Mbaebie BO (2005). Phytochemical constituents of some Nigerian medicinal plants. *Afr J Biotechnol*, **4**, 685-8.

Gao J, Sana R, Calder V, et al (2013). Mitochondrial permeability transition pore in inflammatory apoptosis of human conjunctival epithelial cells and T cells: effect of cyclosporin A. *Invest Ophthalmol Vis Sci*, **54**, 4717-33.

Goh BH, Kadir HA (2011). In vitro cytotoxic potential of Swietenia macrophylla King seeds against human carcinoma cell lines. *J Med Plants Res*, **5**, 1395-404.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell*, **100**, 57-70.

Harborne JB (1984). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. Chapman and Hall, London, UK.

Hasan TN, B LG, Shafi G, et al (2011). Antiproliferative effects of organic extracts from root bark of *Juglans regia* L. (RBJR) on MDA-MB-231 human breast cancer cells: role of Bcl-2/Bax, caspases and Tp53. *Asian Pac J Cancer Prev*, **12**, 525-30.

Jokhadze M, Eristavi L, Kutchukhidze J, et al (2007). Cytotoxicity of some medicinal plants from georgian *Amaryllidaceae*. *Phytother Res*, **21**, 622-4.

Kressel M, Groscurth P (1994). Distinction of apoptotic and necrotic cell death by in situ labeling of fragmented DNA. *Cell Tissue Res*, **278**, 549-56.

Kurosaka K, Takahashi M, Watanabe N, et al (2003). Silent cleanup of very early apoptotic cells by macrophages. *J Immunol*, **171**, 4672-9.

Lu C, Li CJ, Wu D, et al (2011). Induction of apoptosis by Rhizoma paridis saponins in MCF-7 human breast cancer cells. *Afr J Pharm Pharacol*, **5**, 1086-91.

Mazahery A, Dator R, Concepcion G, et al (2009). Murihexocin C from the leaves of *Annona squamosa* Linn. induces apoptosis in human colon carcinoma col2 cell line. *Philipp Agric Sci*, **92**, 122-32.

Mossman T (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxic assays. J Immunol Meth, 65, 55-63.

Polo MP, de Bravo MG (2006). Effect of geraniol on fatty-acid and mevalonate metabolism in the human hepatoma cell line Hep G2. *Biochem Cell Biol*, **84**, 102-11.

Qanungo S, Das M, Haldar S, et al (2005). Epigallocatechin-3-gallate induces mitochondrial membrane depolarization and caspase-dependent apoptosis in pancreatic cancer cells. *Carcinogenesis*, **26**, 958-67.

Ramkumar KM, Manjula C, Elango B, et al (2013). In vitro cytotoxicity of Gymnema montanum in human leukaemia HL-60 cells; induction of apoptosis by mitochondrial membrane potential collapse. *Cell Prolif*, **46**, 263-71

Ravichandran KS (2011). Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. *Immunity*, **35**, 445-55.

Saelens X, Festjens N, Vande Walle L, et al (2004). Toxic proteins released from mitochondria in cell death. *Oncogene*, **23**, 2861-74.

Savill J, Fadok V (2000). Corpse clearance defines the meaning of cell death. *Nature*, **407**, 784-8.

Strasser A, Cory S, Adams JM (2011). Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. EMBO J, 30, 3667-83

Suffness M, Pezzuto JM (1990). Assays related to cancer drug discovery. In Methods in Plant Biochemistry: Assays for Bioactivity, vol. 6, Hostettmann K (ed.). Academic Press: London.

Tan W, Zhou W, Yu HG, et al (2013) The DNA methyltransferase inhibitor zebularine induces mitochondria-mediated apoptosis in gastric cancer cells *in vitro* and *in vivo*. *Biochem Biophys Res Commun*, **430**, 250-5.

Thoppil RJ, Bishayee A (2011). Terpenoids as potential chemopreventive and therapeutic agents in liver cancer. *World J Hepatol*, **3**, 228-49.

Tin MMY, Cho CH, Chan K, et al (2007). Astragalus saponins induce growth inhibition and apoptosis in human colon cancer cells and tumor xenograft. *Carcinogenesis*, **28**, 1347-55.

Varfolomeev E, Vucic D (2011). Inhibitor of apoptosis proteins: fascinating biology leads to attractive tumor therapeutic targets. *Future Oncol*, **7**, 633-48.

Wu X, Cheng B, Cai Z, et al (2013). Determination of the apoptotic index in osteosarcoma tissue and its relationship with patients prognosis. *Cancer Cell Int*, **13**, 56.