INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 15, No. 5, p. 518-526, 2019

RESEARCH PAPER OPEN ACCESS

Cytotoxicity of fractions from *Quisqualis indica* Linn. against selected human cancer cell lines

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Key words: Apoptosis, Bioassay-guided fractionation, Colorectal cancer, Column chromatography, Natural products.

http://dx.doi.org/10.12692/ijb/15.5.518-526 Article published on November 28, 2019

Abstract

Recent studies on treatment and prevention of cancer focus on the cytotoxic activity of natural products and their derivatives. *Quisqualis indica* Linn. Or locally known as *niyog-niyogan*, is a woody vine with ethnobotanical uses containing bioactive chemical compounds that confer it a myriad of properties. In this study, the cytotoxicity of *Q. indica* leaf extracts was tested on selected human cancer cell lines. Crude ethanolic leaf extract of *Q. indica* was subjected to bioassay-guided fractionation. Hexane partition was determined to be the most active and most selective partition, being cytotoxic only against human colorectal cancer (HCT-116) cells. Isocratic and gradient column chromatography techniques were performed to further separate the components of the hexane partition. Sub-fractions from gradient column chromatography proved to by cytotoxic to all three cancer cell lines tested and was highly selective against cancer cells as indicated by its inactivity against the non-cancer cell line Chinese hamster ovarian fibroblast (AA8). Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) Assay further highlighted that the sub-fraction of interest induced its cytotoxicity *via* apoptosis as indicated by the presence of DNA laddering. Lastly, Phytochemical screening narrowed down the possible bioactive compounds of *Q. indica* leaves to alkaloids, cardiac glycosides, and flavonoids. The findings of the study proved that *Q. indica* may be a source of compound/s with potential for development into cytotoxic agents, which were highly selective against colorectal cancer.

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Introduction

Cancer is complex disease affecting millions of men and women worldwide. Its ability to sustain unregulated proliferation of aberrant cells, to upregulate production of pro-survival transcription factors, to deregulate cellular functions, and to evade cell death signals are just a number of ways that contribute to difficulty in eradicating the disease (*Sethi et al.,* 2018). Exposure to chemicals, radiation, and viruses may induce mutations, causing the cells to divide uncontrollably. Internal factors such as inherited mutations, hormones and metabolic disorders may also promote cell survival by suppressing apoptosis (Huang and Freter, 2015). Cancer remains as one of the leading causes of mortality worldwide. Breast, lung, and colorectal cancer accounts for the most number of cases in the Philippines in 2018. In the same year, approximately 30% of prevalent cases of cancers in the Philippines resulted in deaths (The Global Cancer Observatory, 2018). Primary cancer treatment procedures include a combination of adjuvant surgery, radiotherapy and use of chemotherapeutic drugs that alter the cancer cells' resistance to treatments (Jurj *et al.,* 2017). While existing therapies for cancer are shown to be effective in destroying tumor cells, they also harm normal cells in the process (Liu *et al.*, 2015). Current chemotherapeutic agents have negative side effects that impose harm to the patient. High cost of treatment and the unavoidable side-effects of chemotherapy drive the search for alternative forms of medicine.

Recent studies on treatment and prevention of cancer focus on cytotoxic activity of natural compounds and their derivatives against cancer cells (Moustafa *et al.*, 2014; Solowey *et al.*, 2014; Catalani *et al.,* 2016). Phytochemicals from terrestrial and marine plants are able to inhibit the growth and migration of abnormal cells, therefore preventing the formation of tumors. Bioactive natural products, when their chemical structure is optimized for human use, has the advantage of being less toxic against non-target cells (David *et al.,* 2015). The rich biodiversity in the Philippines is an attractive source of such natural products. This study determined the cytotoxic activity of the leaves of *Quisqualis indica* Linn, a woody vine belonging to family Combretaceae. Other plants from the same family tested positive for antioxidant and cytotoxic activity against Jurkat (human T lymphocyte) cells (Bhatnagar, 2010). *Terminalia angustifolia* and *Terminalia arjuna* exhibited cytotoxicity against breast carcinoma, colon carcinoma, hepatocellular carcinoma, and lung carcinoma cells. Presence of chemical compounds such as alkaloids, flavonoids, glycosides, saponins, steroids, tannins, and terpenoids are validated through phytochemical screening (Lim, 2014; Stuart, 2013).

This study aimed to establish the cytotoxicity of samples from *Q. indica* leaves against human colorectal cancer cell line (HCT-116), human breast cancer cell line (MCF-7), and human lung cancer cell line (A-549). Its selectivity against cancer cell lines and potential to induce apoptosis were also assessed.

Materials and methods

Preparation of plant samples

Leaves of *Quisqualis indica* Linn. Were collected from Mt. Lamao, Limay, Bataan and plant identity was authenticated by staff of the Jose Vera Santos Memorial Herbarium (PUH). A voucher specimen of the plant was deposited in the same herbarium. The leaves were then airdried before midribs were removed and leaf blades were homogenized using a blender. For crude extraction, ground leaves were soaked in absolute ethanol for at least 48 hours before filtration. Ethanolic extracts were filtered and concentrated using a rotary evaporator. The crude extracts were left to air dry in petri plates and stored in 4 °C until further use. The crude extract was then partitioned exhaustively using 95% analytical n-hexane, distilled water, and lastly ethyl acetate. The aqueous layer was further dried using an Alpha Christ 1-4 LD plus lyophilizer. The resulting solvent fractions were stored in 4 °C until further use. Cytotoxicity of the solvent fractions were then determined using 3-(4,5-dimethylethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and

the most active partition was further purified using isocratic column chromatography. Chloroform (100%) was used as the mobile phase and Merck Silica Gel 60 (0.063-0.22 mm pore size) as the stationary phase. Isocratic column chromatography fractions (labeled as IF) were again screened for their cytotoxicity. Subsequently, the most active isocratic fraction was subjected to gradient column chromatography using varying concentrations of hexane and ethyl acetate as the mobile phase and Merck Silica Gel 60 (0.063-0.22 mm pore size) as the stationary phase. Eluents, which were labeled as GF, were then tested using MTT assay.

Cell lines and cell culture

All cell lines were procured from the American Type Culture Collection (ATCC). The human colorectal cancer cell line (HCT-116) and the human breast cancer cell line (MCF-7) were maintained in McCoy's 5a Medium (1X) Modified and MEM, respectively; the lung cancer cell line (A-549) and the Chinese hamster ovarian fibroblast cell line (AA8) were maintained in F-12 (1X) Nutrient Mix (Ham) and RPMI, respectively. All base media were supplemented with 1% sodium bicarbonate and 10% (v/v) heat-inactivated fetal bovine serum (FBS). All media components were procured from Gibco Life Technologies. Cell cultures were kept at 37 °C, 5% CO_2, and 95% relative humidity.

MTT assay

HCT-116, MCF-7, and A-549 cells were seeded separately in culture treated 96-well plates (Corning) at 4×10^4 cells/mL (for HCT-116 and AA8) or 6×10^4 cells/mL (for MCF-7 and A-549). Plates were then incubated for 24 h before treatments of different concentrations of the extracts/fractions were administered. The cells were incubated with the treatments for another 72 h before the termination of the assay. Briefly, the spent media was discarded and 5 mg/mL MTT solution was dispensed to the cells. The plates were again incubated for 4 h before the resulting formazan crystals were dissolved by adding 150 µL DMSO. Absorbance of each well was read at 570 nm with a LEDETECT96 microplate reader. Absorbance values were plotted against different concentrations of the treatment to calculate for the half maximal inhibitory concentration (IC_{50}).

Three independent trials with triplicate wells per concentration of each treatment were performed.

Selectivity against cancer cells

The selectivity index (SI) was calculated following cytotoxicity assays. SI is the ratio of the IC_{50} of the samples against AA8 to the inhibitory concentration observed against each cancer cell line. The index reflects the differential activity of an extract; values equal or greater than two are considered twice more cytotoxic to the cancer cell line in contrast with the normal cell line (Suffness and Pezzuto, 1990).

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) Assay

The process of apoptosis is an essential event to maintain physiological balance between cell death and cell growth. Dysregulation of the said mode of cell death leads to cancer progression and resistance to cancer treatment. Testing the potential of the fraction of interest to induce apoptosis, HCT-116 was utilized to determine possible induction of DNA laddering. TUNEL assay was performed using Invitrogen Click-iT® TUNEL Alexa Fluor® Imaging Assay kit (Cat No. C10245). The manufacturer's protocol was followed for this assay.

Phytochemical screening

Crude, hexane partition, and active isocratic and gradient fractions of *Q. indica* were subjected to phytochemical screening. The methods performed were adapted from Harborne (1984), Edeoga *et al.* (2005), and Onwukaeme *et al.* (2007) with minimal modifications (Table 1).

Statistical analysis

Results were presented as mean values ± standard deviation (SD). The significance between the treatments and controls was analyzed using one-way analysis of variance (ANOVA) with post-hoc by Tukey's Honest Significant Difference (HSD) Test.

The Levene's statistic was also determined to assess homogenous groupings of values obtained from the different fractions. $P < 0.05$ was considered as significant. Non-homogenous data were subjected to Kruskal-Wallis Test and Mann-Whitney U Test.

Results and discussion

The crude extract of *Q. indica* exhibited significant cytotoxicity against HCT-116 but not against MCF-7 and A-549 (Fig. 1). Similarly, the hexane partition exhibited cytotoxicity against HCT-116 only.

Table 1. Step-wise procedures for phytochemical screening done to *Q. indica* samples and their subsequent positive responses.

Phytochemical being screened	Procedure	Expected chemical reaction
Tannins	• Dissolved 2 mg of sample in 5mL water • Added 15% $FeCl_3$ drop-wise	• Formation of blue-black precipitate indicates presence of hydrolysable tannins. • Formation of brownish-green precipitate indicates presence of condensed tannins
Saponins	• Dissolved 5 mg of sample in 5 mL distilled water. • Allowed to boil. • Cooled solution vigorously shaken	• Occurrence of frothing indicates presence of saponins
Terpenoids	• Dissolved 2 mg of sample in 2 mL $CHCl_3$ • Layered with H_2SO_4	• Formation of reddish brown interface indicates presence of terpenoids
Flavonoids	• Dissolved 2 mg of sample in 1.0 M NaOH • Added 1.0 M HCl	• Formation of yellow to orange solution with NaOH that turns colourless with addition of HClindicatespresence of flavonoids
Cardiac glycosides	• Dissolved 2 mg of sample in 2 mL distilled water • Added 1% $FeCl_3$ drop-wise • Added 1 mL concentrated H_2SO_4 without disturbing the solution	• Formation of brown ring indicates presence of cardiac glycosides
Alkaloids	• Dissolved 5mg of sample in 2 mL distilled water • Added 3 drops of Wagner's reagent (2 g of I_2 and 6_g of KI dissolved in 100mL distilled water)	• Formation of blue-black precipitate indicates presence of alkaloids

The ethyl acetate and aqueous partitions did not exhibit cytotoxicity against any of the cancer cell lines tested. Isocratic column chromatography of the hexane partition yielded 8 fractions. Among these, 5 fractions proved to be cytotoxic. IF 1, IF 2, IF 4, IF 5, and IF 6 were all cytotoxic against HCT-116 (Fig. 2). Even if their parent partition did not exhibit cytotoxicity against MCF-7 and A-549, IF 4 and IF 5 were cytotoxic against breast and lung cancer cells. Although fractions 4 and 5 seemingly exhibited better cytotoxicity among the 3 cancer cell lines tested, fraction 6 (IF 6) was still chosen to be further purified based on its better yield and lower activity against the non-cancer cell line AA8. Gradient column chromatography afforded 6 sub-fractions, 4 of which were proven to be cytotoxic.

Table 2. Selectivity indices observed in all cell lines treated with gradient fractions of *Q. indica*.

Treatment	Cell line		
	A-549	HCT-116	MCF-7
Doxorubicin	0.92	1.02	0.95
GF 6a	1.00	0.55	1.18
GF 6b	0.45	0.46	0.46
GF 6c	0.87	0.89	0.88
GF 6d	0.93	1.07	0.51
GF 6e	5.73	4.77	5.15
GF 6f	2.24	3.25	5.96

The last 3 sub-fractions eluted (GF 6d, GF 6e, and GF 6f) were cytotoxic against HCT-116, MCF-7, and A-549. Sub-fraction 6a was only active against MCF-7 (Fig. 3). Among the active gradient sub-fractions of *Q. indica*, sub-fractions 6e and 6f exhibited the highest selectivity against all three cancer cell lines (Table 2).

Table 3. Phytochemical analyses of the different samples from *Q. indica*.

Sample	Alkaloids	Cardiac glycosides	Flavonoids	Saponins	Tannins	Terpenoids
Crude	(+)	(+)	(+)	(-)	(+)	(+)
Hexane	(+)	(+)	(+)	(-)	(-)	(+)
IF 6	(+)	(+)	(+)	(-)	(-)	(+)
GF 6d	(+)	(+)	(+)	(-)	(-)	(-)

Positive results indicated by "+". Gradient fraction 6d contained alkaloids, cardiac glycosides, and flavonoids, with no terpenoids.

The selectivity of the active sub-fractions against cancer cells provide possibility of developing anticancer drugs that can circumvent harmful effects on normal cells, which bring about the various side-effects observed with the current chemotherapeutic drugs in the market (Addington and Freimer, 2016; Damrauer *et al.*, 2018). However, low yield prevented the use of these sub-fractions for the apoptosis assay in this study. Fraction 6d, which exhibited significant cytotoxicity and selectivity against HCT-116, was used to screen for pro-apoptotic potential instead.

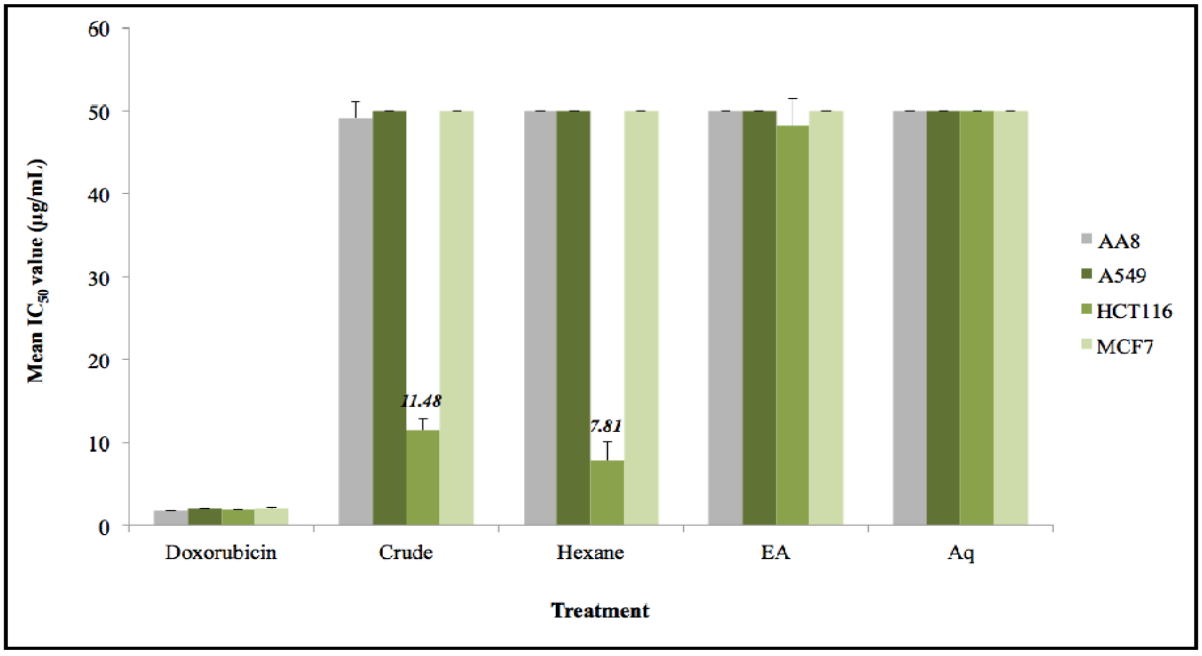

Fig. 1. IC_{50} values of the different solvent partitions from the leaves of *Q. indica*. All treatments with IC_{50} values below 30 µg/mL were considered active. Hexane partition, which was the only partition that exhibited significant cytotoxicity against HCT-116, was further purified.

TUNEL assay confirmed that GF 6d-treated cells underwent DNA fragmentation, one of the hallmarks of apoptosis. Highly fluorescing green foci marked the site of DNA strand breaks, which were present in doxorubicin-treated cells and GF 6d-treated cells. DMSO did not exhibit the said green fluorescence (Fig. 4).The DNA-binding Hoeschst 33342 dye marked areas within apoptotic cells that underwent nuclear condensation, another hallmark of apoptosis (Crowley *et al.*, 2016).

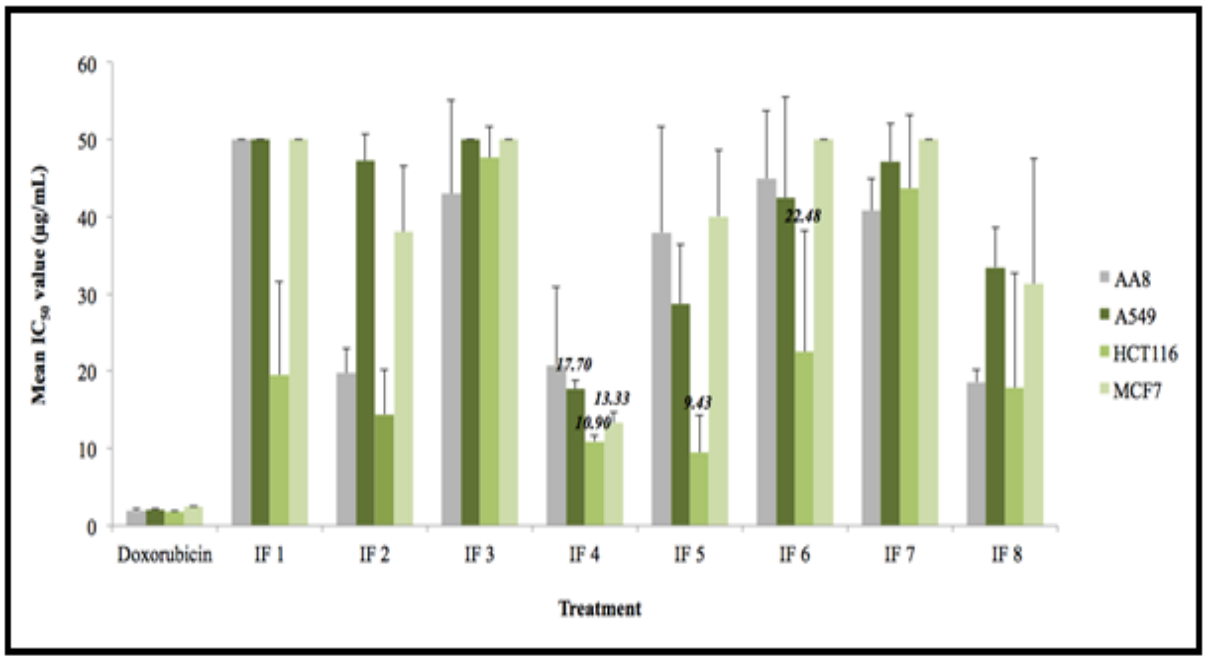

Fig. 2. IC_{50} values of the 8 different eluents from isocratic silica gel column chromatography. Cytotoxicity of some fractions against MCF-7 and A-549 was evident even though their parent fraction did not exhibit the same activity. All treatments with IC_{50} values below 30 µg/mL were considered active. IF 6 was further purified using gradient silica gel column chromatography.

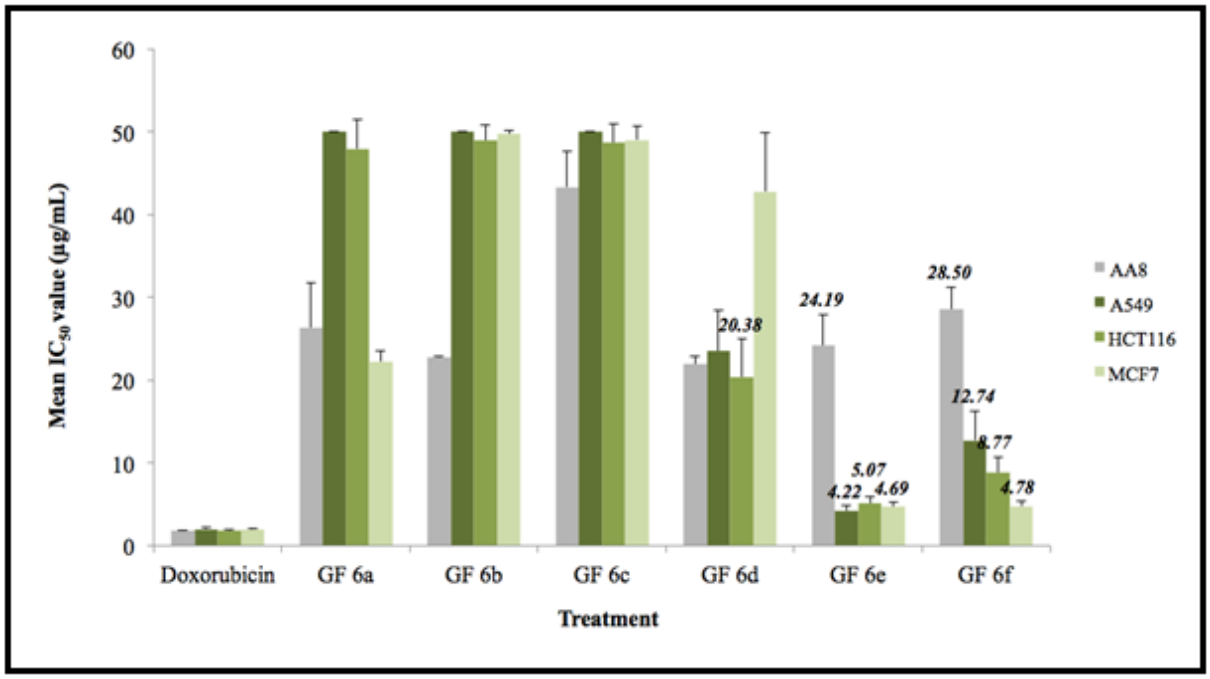

Fig. 3. IC_{50} values of the 6 different sub-fractions from gradient silica gel column chromatography. Cytotoxicity of some sub-fractions against MCF-7 and A-549 was evident even though their parent fraction did not exhibit the same activity. All treatments with IC_{50} values below 30 µg/mL were considered active. GF 6d was used for the apoptosis assay.

The crude extract, its hexane partition, and the most active isocratic and gradient fractions were subjected to phytochemical screening to determine the present bioactive chemical compounds that may induce the observed cytotoxic activities. Table 3 summarizes the secondary metabolites found in the samples. All

phytochemical compounds present in the crude sample were also found in the hexane partition and isocratic fraction, IF 6, except tannins.

The gradient fraction, GF 6d, contained all compounds present in the isocratic fraction except terpenoids. This narrows down the possible cytotoxic phytochemicals present in *Q. indica* leaves to alkaloids, cardiac glycosides, and flavonoids. Existing literature on *Q. indica* mainly focuses on the cytotoxicity of its leaves. Ethyl acetate extracts of both leaves and flowers of *Q. indica* exhibited high toxicity against L269 fibroblast cell line (Samu *et al.*, 2013).

Copper nanoparticles formed from the aqueous extract of *Q. indica* leaves proved to induce apoptosis in B16F10 melanoma cells (Mukhopadhyay *et al.*, 2018). This study mainly explored the cytotoxicity of the hexane partition and therefore mainly non-polar components of the leaves of *Q. indica*.

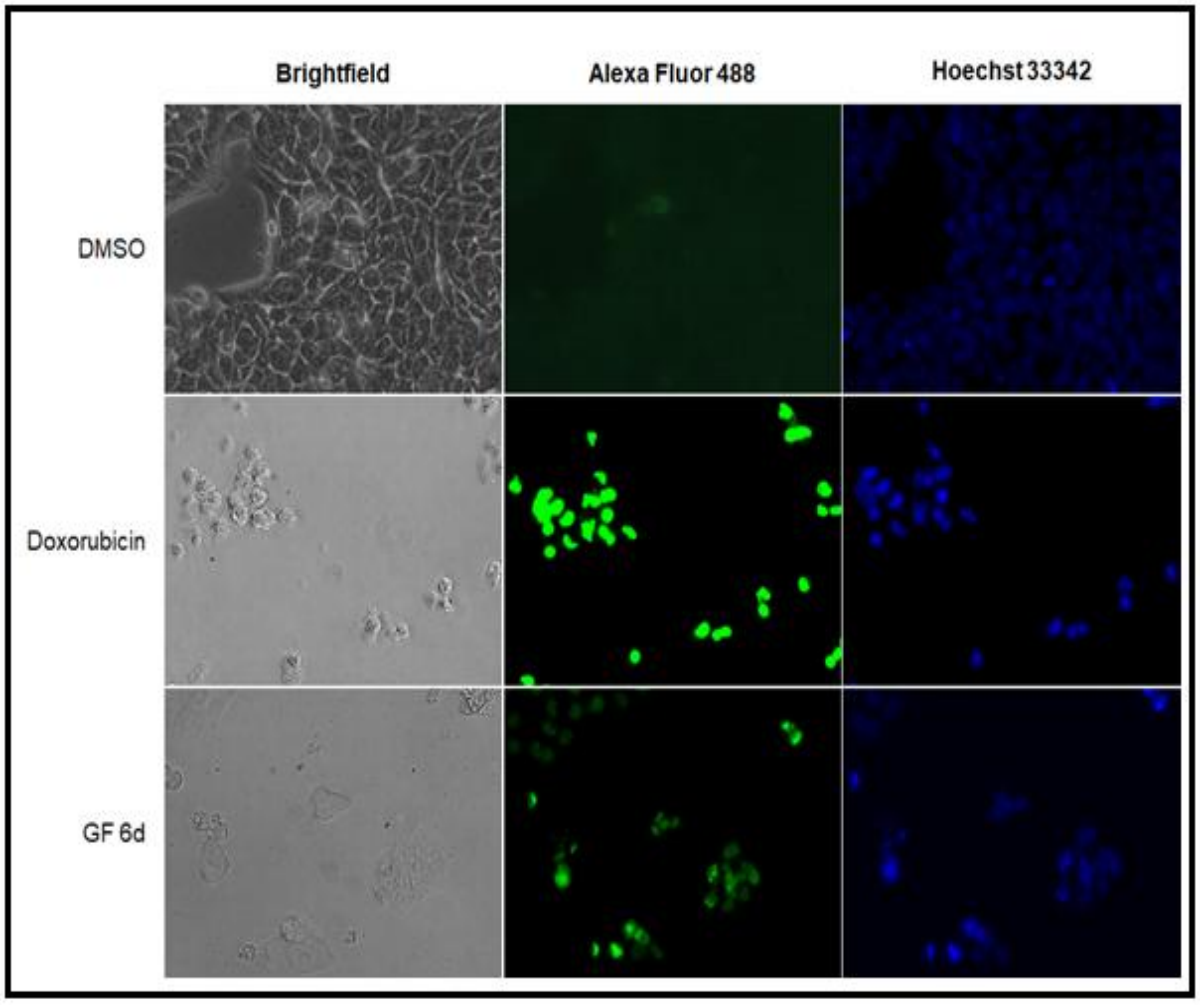

Fig. 4. Detection of DNA laddering in HCT-116 cells using TUNEL assay. Green fluorescence (middle panel) indicates site where DNA breaks were detected. Cells treated with doxorubicin and cells treated with gradient fraction 6d exhibited highly fluorescing green foci, which were absent in the DMSO-treated cells. Cells were viewed at 100x magnification using inverted fluorescence microscope.

The sudden emergence of cytotoxicity of fractions and sub-fractions even though their parent fraction did not exhibit significant cytotoxicity on that particular cell line may be an evidence of antagonistic interactions of these fractions (Sang *et al.*, 2006). The apparent cytotoxicity was only observed when these fractions were treated separately. More studies, whoever, are needed to support this claim. The observed selectivity of some of the fraction from *Q. indica* poses an attractive target for development of a

chemotherapeutic drug that not only is non-toxic against normal cells but also inhibits cancer cells to develop treatment resistance (Liu *et al.*, 2015).

Conclusion

The results of this study highlighted the potential of *Q. indica* leaves as a source of highly specific and highly selective cytotoxic compounds. Phytochemicals from *Q. indica* may be developed into more effective and selective chemotherapeutic agents.

Isolation and elucidation of these compounds is recommended and can prove useful in the development of less toxic anticancer drugs. Understanding cell death mechanisms triggered by these cytotoxic compounds can help push cancer research forward.

Acknowledgments

This research was funded by the Natural Sciences Research Institute, University of the Philippines, Diliman (Project No. BIO-15-1-02). The authors would also like to thank the Mammalian Cell Culture Laboratory of the Institute of Biology, UP Diliman for use of the instruments in implementing this project.

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