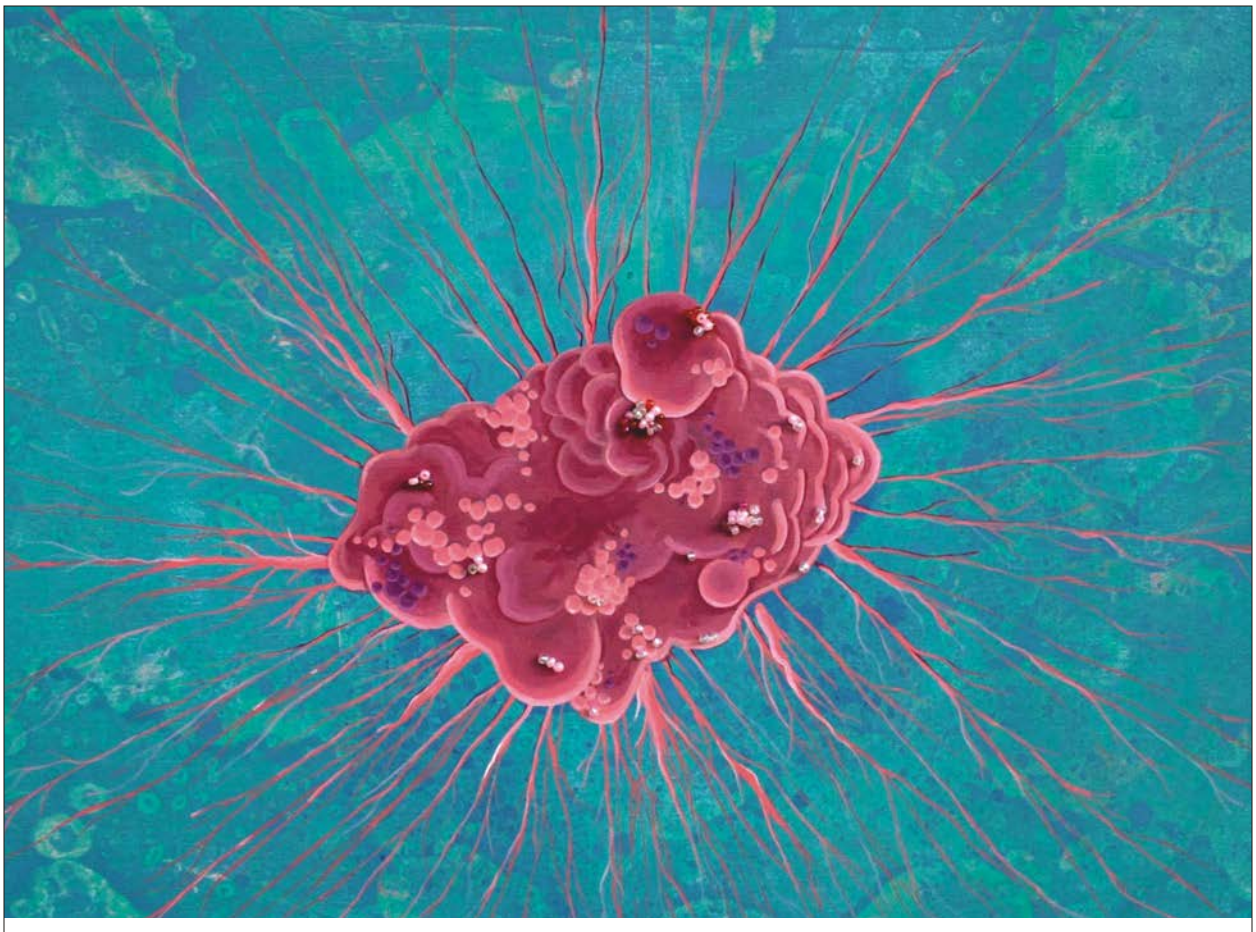

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Research Article
Cytotoxic Potential and Phytochemical Profile of Extracts from *Garcinia rubra* Merr. Leaves

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Abstract

Background and Objectives: Plant and marine extracts remain popular subjects for drug development. Exploring the potential of endemic Philippine plants as sources of cytotoxic compounds, this study looked into the cytotoxicity of *Garcinia rubra* Merr. against selected human cancer cell lines. **Materials and Methods:** Sub-fractions from the ethyl acetate fraction of *G. rubra* leaf were generated using vacuum liquid chromatography. Cytotoxicity of the fractions against colorectal, breast and lung adenocarcinoma cell lines was assessed and the most toxic sub-fraction was screened for selected hallmarks of apoptosis. Its selectivity for cancer was then determined. Moreover, the potential of the most active sub-fraction to act in synergism with doxorubicin was assessed using combinatorial assay. Lastly, the phytochemical constituents of the sample were screened. **Results:** Fractions 6, 7, 8 and 9 were active against the cancer cell lines tested. Fraction 7 induced apoptosis against colorectal cancer cells, but was not selective against cancer cells. It also proved to act synergistically with doxorubicin, allowing even sub-lethal concentrations to induce cell death. Lastly, phytochemical screening showed that the active fraction contained condensed tannins and phenolic compounds. **Conclusion:** *Garcinia rubra* was established to be a good source of cytotoxic and pro-apoptotic compounds.

Key words: Apoptosis, cancer cell line, phenolic compounds, doxorubicin, adenocarcinoma cell lines

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer remains one of the leading causes of death worldwide. The International Agency for Research on Cancer (IARC) estimated that 14.1 million new cases of cancer and 8.2 million deaths related to cancer have been recorded in 2012. Lung cancer is the leading cause of cancer deaths among males and has already exceeded the cancer deaths associated with breast cancer among females[1]. In more developed countries, colorectal cancer among males and females and prostate cancer among males are the leading causes of cancer deaths[2]. Natural products are still the leading sources of novel compounds with anticancer properties. The World Health Organization estimated that 65% of the population of the world in 1985 still turned to plant-derived traditional medicine to alleviate various diseases[3]. Various anticancer agents have already been developed from plant natural products including paclitaxel, cabazitaxel, camptothecin, vinblastine and vincristine[4,5]. However, treating cancer has proven to be complicated especially because of the various pathways which are being utilized to avoid cell death[6].

In an effort to explore Philippine flora as source of viable anticancer compounds, this study looked into the Philippine endemic species *Garcinia rubra* Merr., locally known as kamandiis. The *G. rubra* is a small tree belonging to the family Clusiaceae. It is usually found among rainforests at low to medium altitudes[7]. The genus *Garcinia* has members that have been found to contain anticancer compounds including *G. mangostana*, *G. atroviridis* and *G. braceata*[8-10]. The *G. rubra,* however, remains unexplored for its therapeutic properties based on available literature. This study was undertaken to assess the cytotoxicity of the fraction from its ethanolic leaf extract against human colorectal (HCT-116), breast (MCF-7) and lung adenocarcinoma (A549) cell lines. Its pro-apoptotic potential, selectivity against cancer cells and combinatorial interaction with doxorubicin were then determined. Lastly, the phytochemical constituents that may account for its bioactivity were also evaluated.

MATERIALS AND METHODS

This study was conducted from January-December, 2015 at the Institute of Biology, University of the Philippine, Diliman.

Preparation of plant samples: Leaves of *Garcinia rubra* Merr. were collected from Mt. Lamao, Bataan, Philippines and deposited at Jose Vera Santos Memorial Herbarium (PUH) Institute of Biology, University of the Philippines, Diliman. The leaves were washed with tap water, oven-dried (40°C) then ground into fine powder. These leaves were then soaked in absolute ethanol (10 mL ethanol per 1 g sample) for 48 h before being filtered using Whatman Filter No. 1. The filtrate was then concentrated *in vacuo*. The extract was partitioned exhaustively using hexane, water and finally ethyl acetate to obtain hexane fraction (3.43 g), ethyl acetate fraction (4.07 g) and aqueous fraction (1.51 g). The ethyl acetate fraction was further fractionated using vacuum liquid chromatography (VLC) with Merck silica gel 60 as the stationary phase and varying gradients of hexane and ethyl acetate as the mobile phase. The VLC afforded 8 sub-fractions.

Cell lines and cell culture: All cell lines were purchased from the American Type Culture Collection (ATCC). The human colorectal cancer cell line, (HCT-116 (ATCC CCL-247)) and the breast cancer cell line, (MCF7 (ATCC HTB-22)) were maintained in McCoy's 5a Medium (1X) Modified and MEM, respectively; the lung cancer cell line (A549 (ATCC CCL-185)) and the Chinese hamster ovarian fibroblast cell line (AA8 (ATCC CRL-1859)) were maintained in F-12 (1X) Nutrient Mixture (Ham) and RPMI, respectively. All base media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS); media components were purchased from Gibco Life Technologies. All cell lines were incubated at 37°C, 5% CO_2 and 95% relative humidity.

MTT assay: The MTT cytotoxicity assay was adapted from Mosmann[11] with some modifications. Briefly, HCT-116, MCF7 and A549 cells were seeded separately in 96-well plates at 4×10^4 cells/mL. Cells were incubated for 24 h before exposure to varying concentrations of the plant samples. Doxorubicin, an anticancer drug, and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively. Treated cells were then incubated for 72 h. After incubation, the spent media was discarded and 20 µL MTT solution was added to each well. The plates were again incubated for 4 h. The resulting formazan crystals produced by the reduction of the MTT by the live cells were then dissolved with DMSO before the plates were read with an ELISA microplate reader at 570 nm. The inhibition per concentration was calculated using the following Eq. 1:

$$\text{Inhibition (\%)} = \left(\frac{\text{Optical density of DMSO - Optical density of sample}}{\text{Optical density of DMSO}} \right) \times 100 \qquad (1)$$

Using the inhibition per concentration, the half maximal concentration (IC_{50}) values of the samples were computed with Graph Pad Prism 6, which employs non-linear regression curve fit.

Pro-apoptosis assays: Using HCT-116 cells, hallmarks of apoptosis namely: externalization of the phosphatidylserine (PS) residues from the cytosolic side of the cell membrane to the outer membrane and DNA laddering were assessed using annexin V staining and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL), respectively. HCT-116 cells were treated with the computed IC_{50} of the most active VLC sub-fraction. Invitrogen Alexa Fluor™ 488 Annexin V/Dead Cell Apoptosis Cat No. V13245 and Invitrogen Click-iT® TUNEL Alexa Fluor® Imaging Assay Cat No. C10245 kits were used in this study according to the manufacturer's protocols[12,13].

Selectivity against cancer cells: To assess if the most active VLC sub-fraction from *G. rubra* is selective against cancer cells, its cytotoxicity against the non-cancer Chinese hamster ovarian fibroblast cell line (AA8) was determined using MTT assay. The selectivity indices (SI) of the fraction for each cancer cell line were computed using the Eq. 2:

$$SI = \frac{IC_{50} \text{ for non-cancer cell line}}{IC_{50} \text{ for cancer cell line}} \quad (2)$$

Samples with SI values higher than 3 were considered to be highly selective[14].

Combinatorial assay: The protocol used to assess the potential of the active sub-fraction from *G. rubra* to have a synergistic effect with doxorubicin was adapted from Bojo *et al.*[15] with a few modifications. High and low doses of both the active fraction and doxorubicin were used in different combinations on HCT-116 cells. The high dose used for doxorubicin was six 5-fold dilutions starting from 0.41 μg mL^{-1} while the low doses were six 5-fold dilutions starting from 0.14 μg mL^{-1}. For the most active fraction of *G. rubra*, the high doses started from 25.5 μg mL^{-1} while the low doses started from 8.5 μg mL^{-1}. Table 1 summarized the combination of doses used as treatments. The IC_{50} values for each combination were determined using the MTT assay. The effect of the combinations on HCT-116 cells was determined using the Chou-Talalay Eq. 3[16]:

$$\text{Combination index } (CI) = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (3)$$

where, $(Dx)_1$ and $(Dx)_2$ are the IC_{50} values of either drug or plant fraction alone and $(D)_1$ and $(D)_2$ are the IC_{50} values of either drug and plant fraction used in combination. Compounds having a CI>1 are antagonistic to each other, compounds with CI = 1 have additive effects, while compounds having CI<1 are synergistic.

Phytochemical screening: The phytochemical screening employed in this study was based on the protocols of Edeoga *et al.*[17] and Onwukaeme *et al.*[18] with slight modifications (Table 2).

Statistical analyses: Three trials were performed for the cytotoxicity assays and IC_{50} values were reported as Mean±standard deviation.

RESULTS

VLC afforded 8 sub-fractions from the ethyl acetate fraction (GrEA): The ethyl acetate fraction (GrEA) had significant cytotoxicity against HCT-116, MCF7 and A549. The hexane fraction (GrH) was also cytotoxic against all three cancer cell lines, with IC_{50} values lower than that of GrEA (Fig. 1), but GrEA was pursued for further fractionation because it had higher

Table 1: Combination of concentrations of doxorubicin and *G. rubra* VLC fraction 7 (μg mL^{-1}) on colorectal adenocarcinoma cell line (HCT-116)

	Concentration					
Parameters	A	B	C	D	E	F
Separate treatments						
Doxorubicin	0.4181	0.0816	0.0163	0.0033	0.0007	0.0001
Active fraction	25.5	5.1	1.02	0.204	0.0408	0.0082
Low doxorubicin+low active fraction						
Doxorubicin	0.1360	0.0272	0.0054	0.0011	0.0002	0.00004
Active fraction	8.5	1.7	0.34	0.068	0.0136	0.0027
Low doxorubicin+high active fraction						
Doxorubicin	0.1360	0.0272	0.0054	0.0011	0.0002	0.00004
Active fraction	25.5	5.1	1.02	0.204	0.0408	0.0082
High doxorubicin+low active fraction						
Doxorubicin	0.4181	0.0816	0.0163	0.0033	0.0007	0.0001
Active fraction	8.5	1.7	0.34	0.068	0.0136	0.0027

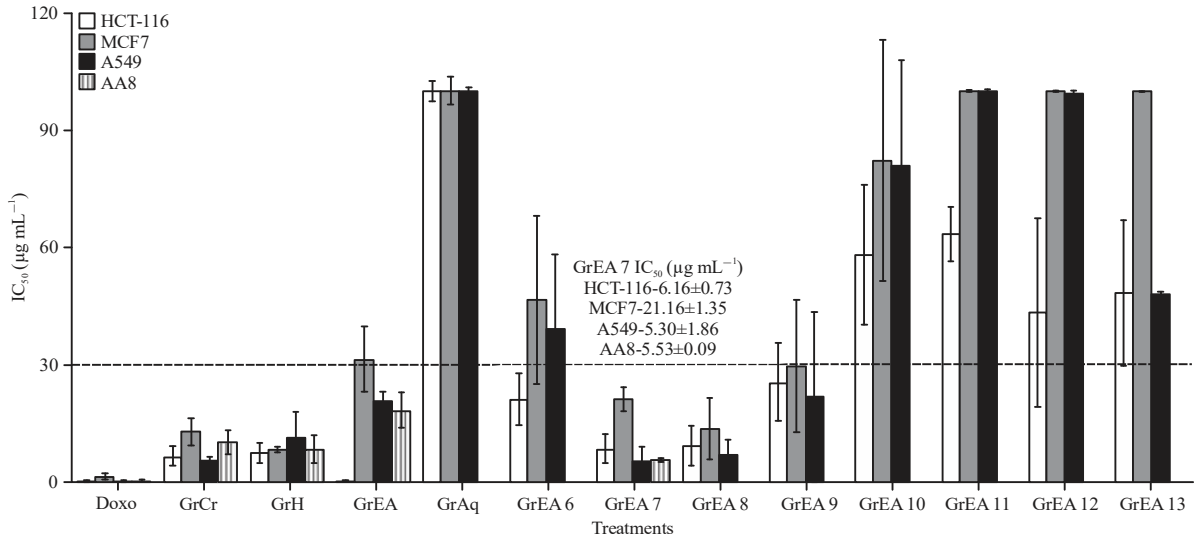

Fig. 1: IC_{50} values of the different solvent fractions and ethyl acetate sub-fractions from the leaves of *G. rubra.* All treatments with IC_{50} values below 30 µg mL^{-1} (red dashed line) are considered active. Fraction 7, which was active against all cell lines tested was used for apoptosis and combinatorial assays

Table 2: Step-wise procedures of the phytochemical tests done to *G. rubra* extracts and their subsequent positive responses

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

yield. When GrEA was subjected to VLC, 4 of the resulting 8 sub-fractions were active against at least one of the 3 cancer cell lines. GrEA 6 was active against HCT-116 while GrEA 7, GrEA 8 and GrEA 9 were active against all 3 cancer cell lines tested. GrEA 7, which was used for apoptosis and combinatorial assays had IC_{50} values of 6.16±0.73, 21.16±1.35 and 5.30±1.86 µg mL^{-1} against HCT-116, MCF7 and A549, respectively.

GrEA 7 has pro-apoptotic capabilities against HCT-116 cells: Externalization of PS and DNA laddering were assessed using annexin staining assay and TUNEL assay, respectively. DMSO-treated cells were still viable as indicated by the absence of both green and red fluorescence (Fig. 2b, c). All treatments did not exhibit red fluorescence after staining with propidium iodide (Fig. 2c, f). High intensity of green fluorescence was observed on the margins of the cells exposed separately to the anticancer drug paclitaxel and the test sample GrEA 7 for annexin V staining assay (Fig. 2e, h). Similarly, no significant fluorescence was observed in DMSO-treated cells (Fig. 3b, c) for TUNEL assay. Doxorubicin-treated and GrEA 7-treated cells, on the other hand, exhibited green fluorescence (Fig. 3e, h) and showed highly fluorescing blue-foci (Fig. 3f, i).

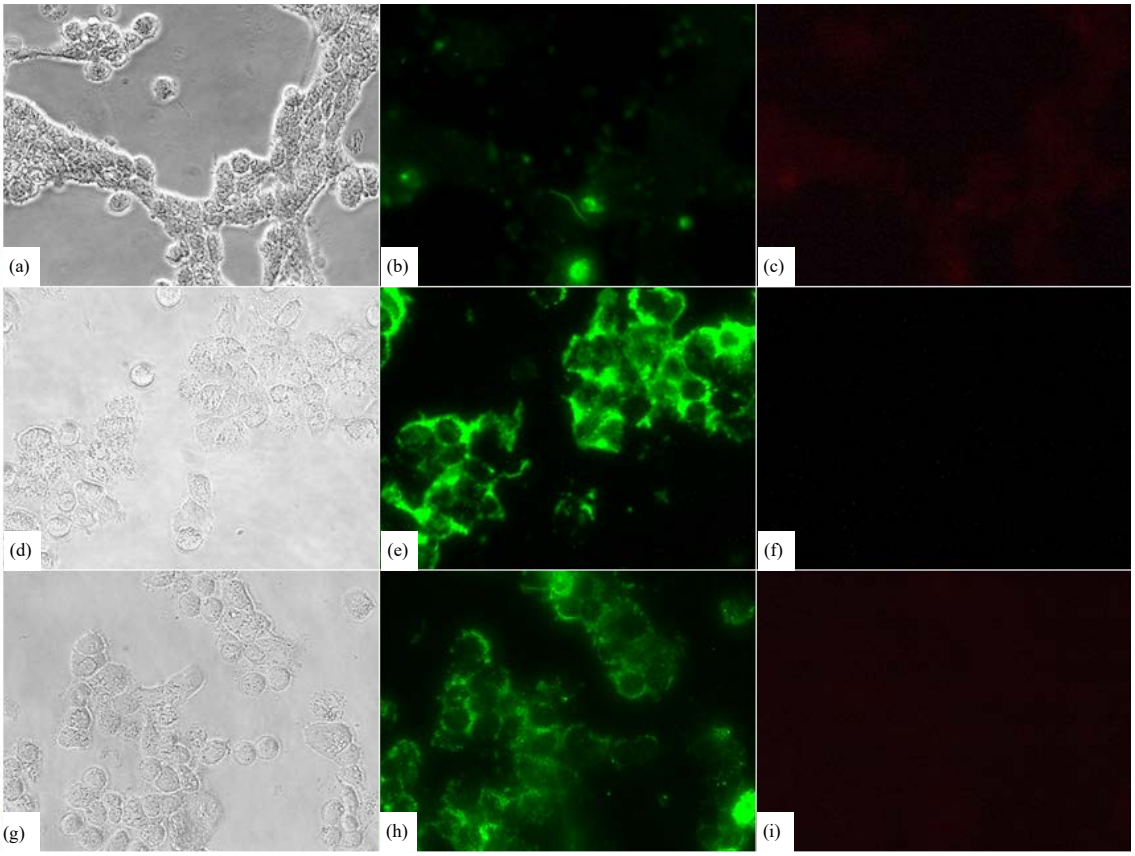

Fig. 2(a-i): HCT-116 cells treated with (a-c) Negative control DMSO, (d-f) Positive control paclitaxel and (g-i) *G. rubra* ethyl acetate extract GrEA 7 for annexin V staining. Green fluorescence localized mainly on the surface of paclitaxel-treated and GrEA 7-treated cells (e, h)indicate externalization of phosphatidylserine residues. Absence of red fluorescence (c, f, i) suggest that cell membranes were still intact 24 h after treatment

Table 3: Dose-dependent response of non-cancer AA8 cells treated with GrEA 7

Concentration (μg mL^{-1})	Inhibition (%)
100.00	77.48
50.00	48.18
25.00	53.11
12.50	39.15
6.25	44.47
3.13	36.18
1.56	12.00
0.78	4.68

All concentrations of GrEA 7 exhibited inhibitory effects in a dose dependent manner

Table 4: Selectivity indices (SI) of doxorubicin and GrEA 7 against all cancer cell lines tested

Parameters	HCT-116	MCF7	A549
Doxorubicin	2.02	0.20	1.41
GrEA 7	0.898	0.261	1.043

Treatments with SI greater than or equal to 3 are considered highly selective. Both doxorubicin and GrEA 7 were not selective against cancer cells

GrEA 7 is not selective against cancer cells: Although GrEA 7 was highly cytotoxic against all cancer cell lines tested, it also had high toxicity against the non-cancer (AA8). Even at 3.13 μg mL^{-1}, GrEA 7 still exhibited 36.18% inhibition (Table 3). It's selectivity indices against all cancer cell lines tested were low, reaching only up to 1.043 for A549 (Table 4).

GrEA 7 has a synergistic effect with the anticancer drug doxorubicin: The combined effect of the active fraction GrEA7 and doxorubicin against HCT-116 was assessed. Three combination schemes of the plant fraction GrEA 7 and doxorubicin were examined. The IC_{50} values of both GrEA 7 and doxorubicin were reduced in all combination ratios (Fig. 4a, b).

Condensed tannins and phenolic compounds may be the cytotoxic components of *G. rubra*: The results in Table 5 summarize the phytochemical composition of all solvent fractions and sub-fractions of *G. rubra*. Evidently, condensed tannins, terpenoids, flavonoids, cardiac glycosides and

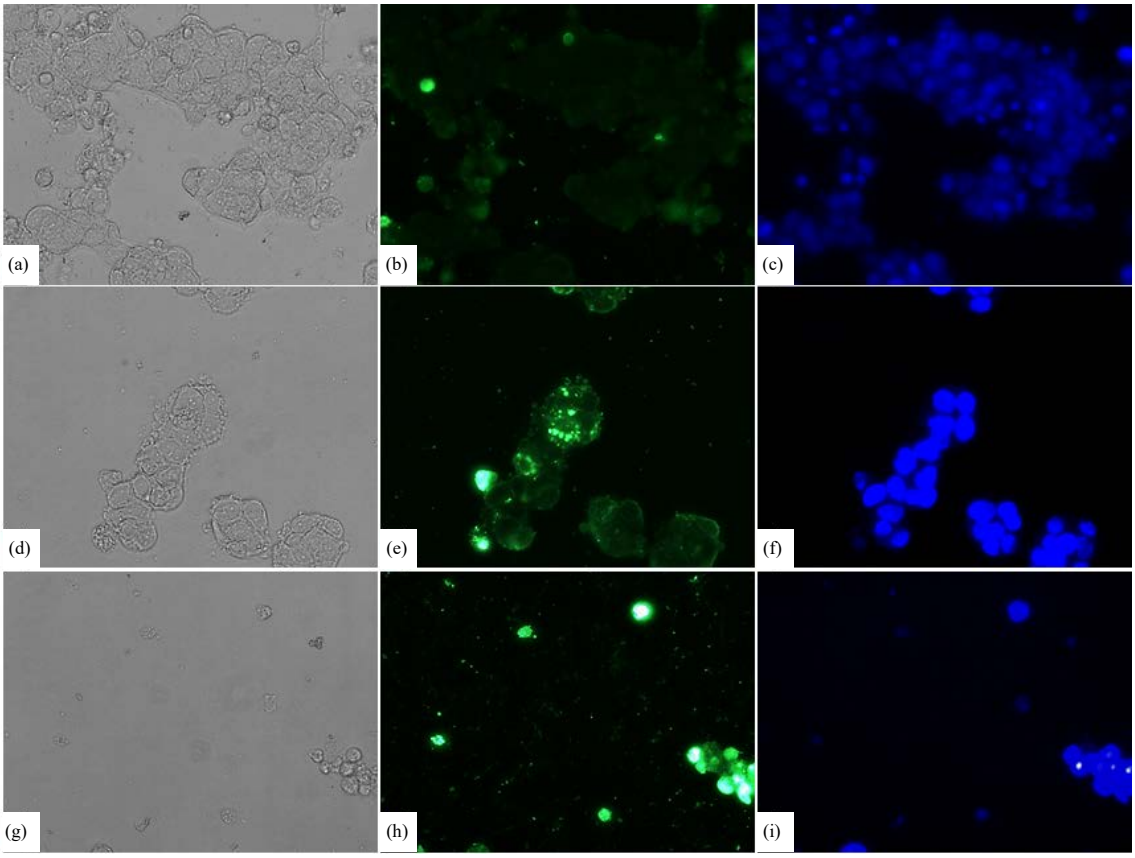

Fig. 3(a-i): HCT-116 cells treated with (a-c) Negative control DMSO, (d-f) Positive control doxorubicin and (g-i) *G. rubra* ethyl acetate extract GrEA 7 for TUNEL assay. Green fluorescence seen in doxorubicin-treated and GrEA 7-treated cells indicate DNA laddering, highly fluorescing blue foci indicate chromatin condensation

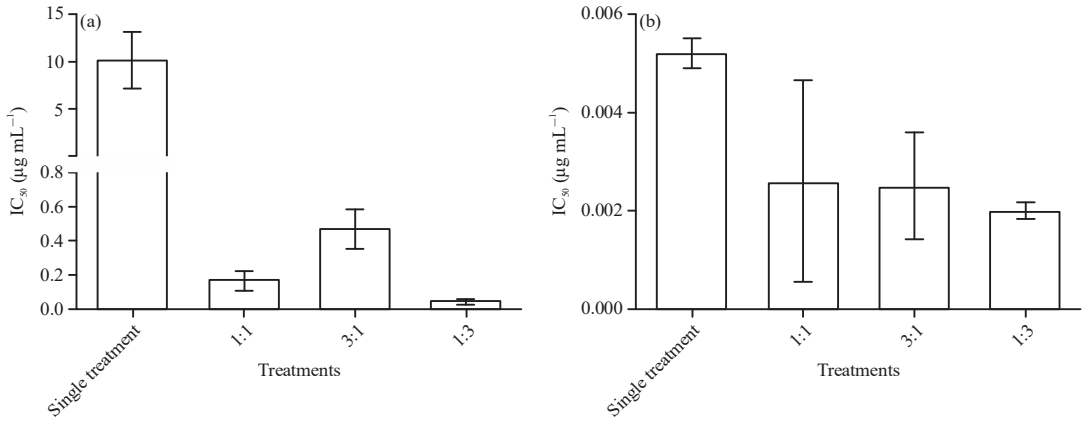

Fig. 4(a-b): Effect of combined treatments of the active fraction GrEA 7 and doxorubicin against HCT-116 cells. IC_{50} values of (a) GrEA 7 and (b) Doxorubicin were reduced when treated together. Combination indices (CI) were also computed and all combination ratios showed synergism between GrEA 7 and doxorubicin

phenolic compounds were present in GrEA7. However, terpenoids, flavonoids and cardiac glycosides were also present in the fractions that were not cytotoxic against the cancer cell lines tested. Thus, the possible active components of *G. rubra* can be narrowed down to two phytochemicals: condensed tannins and phenolic compounds.

Table 5: Phytochemical analyses of the different fractions of *G. rubra*

Variables	Tannins	Saponins	Terpenoids	Flavonoids	Cardiac glycosides	Phenolic compounds	Alkaloids
GrCr	Condensed tannins	+	+	+	+	+	-
GrH	Condensed tannins	-	+	+	+	-	-
GrEA	Condensed tannins	+	+	+	+	+	-
GrEA 6	Not enough sample						
GrEA 7	Condensed tannins	-	+	+	+	+	-
GrEA 8	Condensed tannins	-	+	+	+	+	-
GrEA 9	Condensed tannins	-	+	+	+	+	-
GrEA 10	-	+	+	+	+	-	-
GrEA 11	-	+	+	-	+	-	-
GrEA 12	-	+	-	-	+	-	-
GrEA 13	-	+	+	-	+	-	-

Positive results are indicated by "+". Active fractions 7, 8 and 9 all contained condensed tannins and phenolic compounds. Fraction 6 did not have enough yield for phytochemical screening

DISCUSSION

This study is the first to establish the potential of *Garcinia rubra* leaves as source of cytotoxic compounds. According to the American National Cancer Institute (NCI), cytotoxic extracts must have IC_{50} values less than 30 μg mL^{-1} to be considered active[19]. The ethyl acetate fraction (GrEA) showed significant activity against HCT-116 and A549, but not against MCF7. However, cytotoxicity was observed against MCF7 in 3 sub-fractions from GrEA after it was subjected to VLC. Potential antagonistic interaction of the phytochemicals in the crude extract may have caused the absence of toxicity in MCF7[20]. This is in contrast to the findings of Karna *et al*.[21], wherein phytochemicals from ginger root worked in an overlapping mechanism to enhance its bioactivity against prostate cancer.

The results of this study showed that *G. rubra* induced apoptosis in colorectal cancer cells. Apoptosis is the preferred mode of cell death in cancer treatment because it involved a regulated series of cellular responses that lead to cell suicide without triggering the immune system's inflammatory response. Thus, the neighbouring normal cells are not harmed[22]. The pro-apoptotic potential of the cytotoxic fraction of *G. rubra* (GrEA 7) was established based on the results of annexin V staining and TUNEL assays. The PS residues were translocated to the outside surface of the cells after exposure to the sample. This apoptosis hallmark happened during early apoptosis[23]. It is postulated that the externalization of the PS residues aids in the recognition of these apoptotic cells by nearby phagocytic cells *in vivo*[24]. The absence of the red fluorescence of both paclitaxel-treated cells and GrEA 7-treated cells further supported the claim that these cells were still in early apoptosis 24 h after exposure to treatment. Red fluorescence can be attributed to propidium iodide (PI) staining, but since the cell membrane were still intact during early apoptosis[25], the nuclear stain could not pass through. Chromatin condensation and DNA laddering happen during late apoptosis followed by the breakdown of the apoptotic cells to membrane-bound apoptotic bodies[26]. Other *Garcinia* species have already been established to induce apoptosis. Fruit methanolic extracts from *G. dulcis* were able to induced apoptosis of the liver cancer cell line HepG2[27]. The leaves of *G. cowa* contained compounds that arrested cell cycle, induced apoptosis and activated autophagy in the cervical cancer cell line HeLa, pancreatic cancer cell line PANC-1 and A549[28]. Fruit extracts from *G. mangostana* were also proven to inhibit the proliferation and induce cell cycle arrest of HeLa cells[29].

Although GrEA 7 exhibited pro-apoptotic potential, it is also important to highlight that it had no selectivity against cancer cells. This indiscriminate cytotoxicity also exhibited by current chemotherapeutic drugs which caused severe side-effects that reduce cancer patient's quality of life and survival[30]. Camptothecin, from *Camptotheca acuminata*, is indiscriminately toxic. One of its side-effects that prohibit its development into a marketable chemotherapeutic drug is severe urinary tract complications[31]. Doxorubicin is also known to cause cardiomyopathy by inducing increased oxidative stress and decreased levels of antioxidants[32].

The results of the combined treatments of doxorubicin and GrEA 7 suggested that sub-lethal doses of the anticancer drug and the plant fraction can be utilized to inhibit the adverse effects of synthetic anticancer medicine available in the market. This combination method can potentially alleviated side-effects of doxorubicin such as; severe nausea and cardiovascular complications.

Phytochemical screening confirmed that GrEA 7 contained secondary metabolites that were previously established to have anticancer properties[33,34]. Based on previous literature, it is more likely that the cytotoxicity of *G. rubra* is due to the presence of phenolic compounds. Xanthones, the major secondary metabolites present in other *Garcinia* species such as; *G. bracteata*, *G. cowa* and

G. mangostana are polyphenolic compounds that have antibacterial, anti-malarial and anti-tumor properties[35-37].

Isolation and structure elucidation of the chemical constituents of *G. rubra* are can be recommended for future studies. This can potentially decrease the toxicity of the bioactive phytochemicals and could lead to the development of analogs with higher cytotoxic selectivity against cancer cells. Chemical characterization can also give insights on the molecular targets of *G. rubra*.

CONCLUSION

This study proposed that tannins and/or phenolic compounds present in this Philippine endemic species may be responsible for its cytotoxicity against human colorectal, breast and lung cancer cells. Apoptosis was established to be the mode of cell death induced by the active fraction. It also acted synergistically with doxorubicin, a known anticancer drug.

SIGNIFICANCE STATEMENT

This study established the toxicity of a VLC fraction of the Philippine endemic, *Garcinia rubra,* which opens new avenues for research towards drug development. Future efforts may be directed towards purifying the active principle and determining the specific cell signalling pathways involved in cancer cell toxicity. Using *in vivo* models to replicate the synergism of doxorubicin and the active fraction observed in this study can also be explored.

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