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Cytotoxic activity of crude extracts and fractions from *Premna odorata* (Blanco), *Artocarpus camansi* (Blanco) and *Gliricidia sepium* (Jacq.) against selected human cancer cell lines

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ABSTRACT

Objective: To evaluate the cytotoxic activities of *Premna odorata* (*P. odorata*) leaves and bark, *Artocarpus camansi* (*A. camansi*) and *Gliricidia sepium* against selected human cancer cell lines by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Methods: The crude extracts of *P. odorata*, *A. camansi* and *Gliricidia sepium* were subjected to liquid–liquid partitioning by using hexane and ethyl acetate to separate compounds based on their polarity. The fractions were tested for their cytotoxic activity against human colon cancer cell line (HCT116), breast cancer cell line (MCF-7), lung adenocarcinoma cell line (A549) and Chinese hamster ovary cell line (AA8) by using MTT assay.

Results: Based on the standard values of toxicity set by the study of Suffness and Pezzuto, *P. odorata* leaves and *P. odorata* bark hexane fractions and *A. camansi* leaves were all considered highly cytotoxic against the selected human cancer cell lines. *P. odorata* bark hexane extract exhibited the highest selectivity index for HCT116, MCF-7 and A549 cancer cell lines.

Conclusions: The results obtained indicated that *P. odorata* leaves and bark and *A. camansi* leaves have excellent cytotoxic activity and warrant further studies to isolate novel compounds for chemotherapeutic use.

1. Introduction

Cancer greatly contributes to human mortality and is considered as a major threat to humankind. According to the Department of Health of the Philippines, cancer is the third leading cause of mortality and morbidity next to diseases of the heart and diseases of the vascular system [1]. One in every four deaths in the United States is due to cancer [2]. Overall, there are 27 prevalent types of cancers in the world [3]. Thus, there is a dire need to develop novel, effective and selective anticancer drugs to address the increasing threat of cancer to humankind.

Screening of medicinal plants and isolation of natural products provide pharmacologically active compounds against cancer. Some of the isolated compounds from plants are vincristine, vinblastine and paclitaxel [4]. Since then, research on plant natural products has actively contributed to drug development against cancer. Until 1990, almost 80% of approved drugs were natural products or their analogues [5].

There are several researches that evaluated the chemopreventive and chemotherapeutic properties of endemic and indigenous plants in the Philippines. Some of the plants that exhibited high cytotoxic activities against human cancer cell lines were *Aglaia loheri* (Blanco), *Ficus septica* (Burm.) and *Voacanga globosa* (Blanco) Merr [6]. Hexane extracts from *Cassia alata* exhibited remarkable cytotoxicity against MCF-7 (breast carcinoma), T24 (bladder carcinoma), and Col-2 (colorectal carcinoma) in a dose-dependent manner [7]. *Kalanchoe tubiflora* which is medicinally used in Indo-China and the Philippines exhibits anticancer properties. It disrupts centrosome integrity and induces multipolarity. It also inhibits chromosome alignment during metaphase [8].

Premna odorata (Verbenaceae) (*P. odorata*) is native to the Philippines and is locally known as alagaw. Leaves and roots of

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P. odorata are used in treating cough and colds [9]. Water decoction of its leaves is used to treat tuberculosis. Compounds isolated from bioactive fractions of *P. odorata* were observed to have antimycobacterial activity [10]. Other ethnomedicinal uses of *P. odorata* are remedies for head lice, cough, cuts and wounds [11]. It is also included in the seven components of a commercialized Philippine herbal preparation called "Pito-Pito" [12].

Artocarpus camansi (*A. camansi*) is locally known as kamansi in Philippines. It is utilized as vegetable. The decoction of *A. camansi* leaves is used for diabetes and baths of people with rheumatism. It was also found to be an effective natural product to treat allergic contact dermatitis [13]. β-Sitosterol propionate isolated from the hexane extract from the leaves of *A. camansi* showed antidiabetic properties [14]. There are compounds isolated from dichloromethane extract of the leaves of *A. camansi* but they were not cytotoxic against lung adenocarcinoma A549 cells, stomach adenocarcinoma AGS cells, colon adenocarcinoma HT29 cells and prostate cancer PC3 cells [15].

Gliricidia sepium (*G. sepium*) was introduced to the Philippines and is native to the American continent. It is used as shade for cocoa and coffee plantations in Mexico that is why it is called madre de cacao (mother of cacao). It is also used as a poison for rodents [16]. The traditional use of branches and leaves of *G. sepium* is against pruritic ailments, fever and it is one of the most frequently used plants for skin infections [17]. There are no literatures published regarding the anticancer properties of this plant species.

This study aimed to evaluate the cytotoxic activities of crude extracts and fractions from *P. odorata* leaves and bark, *A. camansi* leaves and *G. sepium* leaves against selected human cancer cell. In order to identify the plants with potential bioactive molecules against cancer cells, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by using their crude extracts and fractions.

2. Materials and methods

2.1. Extraction of crude extracts

The terrestrial plants *P. odorata*, *A. camansi* and *G. sepium* were collected from the author's backyard in Hermosa, Bataan and were identified by Dr. James V. La Frankie, curator of the Jose Vera Santos Memorial Herbarium in the Institute of Biology, UP Diliman. The plants were air dried at room temperature after collection. The dried samples were crushed by using a blender. Dried and crushed samples were soaked in 100% ethanol for three days and then filtered by using filter paper. The filtrate (crude ethanolic fraction) was concentrated by using rotary evaporation and reserved for solvent partitioning. Thereafter, the air dried samples were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 4 mg/mL for use in the subsequent assays [6].

2.2. Cell viability assay (MTT assay)

The assay to determine cell survival/toxicity was conducted after the method of Mosmann with some modifications [18]. In detail, AA8, A549, HCT116 and MCF-7 cells were seeded separately at 4×10^4 cells/mL/well in sterile 96-well microtiter plates. Cells were incubated overnight at 37 °C and 5% CO_2 at 98% humidity when they would have reached log phase of their growth curve.

The 4 mg/mL extracts were serially diluted to concentrations 1 000 μg/mL, 500 μg/mL, 250 μg/mL and 125 μg/mL in a master dilution plate (MDP). From the MDP, 10 μL were obtained and dispensed to the plated cells to obtain the final concentrations 50 μg/mL, 25 μg/mL, 12.5 μg/mL and 6.25 μg/mL. Doxorubicin, a cancer chemotherapeutic drug, served as positive control while DMSO, the solvent for the extracts, served as negative control. Three replicate wells were used per concentration. The treated cells were then incubated for 72 h at 37 °C and 5% CO_2.

After incubation, the media was withdrawn and 20 μL MTT dye at 5 mg/mL phosphate-buffered saline was added to every well. The cells were again incubated at 37 °C and 5% CO_2 for 4 h, after which 150 μL DMSO was added to each well. Absorbance was read by using the Ledetect reader at 570 nm. The concentration required to kill 50% of the cell population or IC_{50} was computed by using linear regression of the graph of absorbance against concentration. The selectivity index (SI) was calculated by dividing the IC_{50} value for the non-cancer cell line $_{AA8}$ by the value of the IC_{50} for cancer (A549, HCT116 and MCF-7) cell lines [19]. This value indicated the specificity of the extracts to cancer cells. A value of two or more indicated high specificity.

2.3. Solvent partitioning

Extracts with high cytotoxic activity were subjected to liquid–liquid partitioning by using hexane and ethyl acetate (EA). This procedure separated the compounds in the extracts based on their polarity thus the fractions with higher cytotoxicity could be determined.

Approximately 300 mL of the crude extract concentrated by using rotary evaporation was placed in a 1 000 mL separatory funnel. Equal amount of 95% *n*-hexane was added. After mixing, the solution was separated into layers; the upper organic layer contained the compounds which could be soluble in hexane. This procedure was done repeatedly until the organic layer turned colorless indicating that the entire hexane fraction was already acquired. It was then subsequently concentrated by rotary evaporation to obtain hexane fraction.

After the last round of hexane partitioning, the bottom layer was collected and subjected to EA partitioning. Collected extract at 300 mL was placed in a 1 000 mL separatory funnel and mixed with 300 mL of EA and 300 mL of distilled water. The solution was allowed to separate and the upper layer containing the EA fraction was collected and air dried to recover the EA extract.

2.4. Statistical analysis

The results were expressed as mean ± SD of three independent experiments. IC_{50} values from MTT assay were subjected to statistical analyses. Kolmogorov–Smirnov and Shapiro–Wilk test for normality was performed to determine if the IC_{50} values were in normal distribution. One way ANOVA was performed to determine whether there were any significant differences between the IC_{50} means of the extracts and the positive control, doxorubicin. Differences with $P < 0.05$ values were considered as significantly different. Tukey honest significant difference multiple comparison was also performed to determine which particular pair of extracts differed significantly from one another.

3. Results

Discovery of plant natural products with chemotherapeutic property had spurred numerous studies on plant extracts and eventually compounds with the potential for drug development. This research was a study of cytotoxic activities of crude extract, hexane and EA fractions of terrestrial plants *P. odorata* leaves and bark, *A. camansi*, *G. sepium* as compared to the standard drug doxorubicin against human cancer cell lines: colon carcinoma (HCT116), breast carcinoma (MCF-7), and non-small cell lung carcinoma (A549).

IC_{50} values of crude extracts and fractions against HCT116 were presented in Figure 1. *P. odorata* leaves crude extract [(12.37 ± 2.30) μg/mL], *P. odorata* bark crude extract [(6.06 ± 0.65) μg/mL] and *P. odorata* bark hexane fraction [(8.58 ± 3.56) μg/mL] were all considered as highly cytotoxic against HCT116 cell lines.

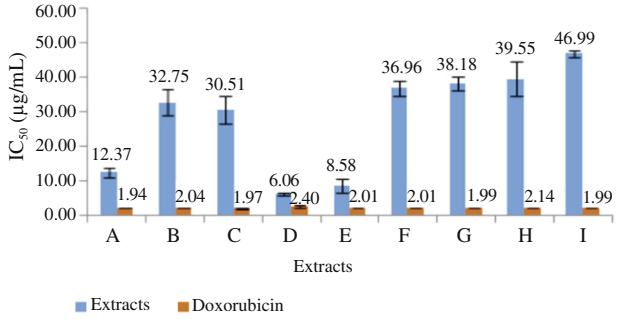

Figure 1. IC_{50} values of plant extracts against human colon carcinoma, HCT116.
Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration. Data were expressed as mean ± SD. A: *P. odorata* leaves crude; B: *P. odorata* leaves hexane; C: *P. odorata* leaves EA; D: *P. odorata* bark crude; E: *P. odorata* bark hexane; F: *P. odorata* bark EA; G: *A. camansi* leaves crude; H: *A. camansi* bark crude and I: *G. sepium* crude.

Figure 2 shows the IC_{50} values of the extracts against MCF-7 where *P. odorata* leaves crude extract [(4.30 ± 0.84) μg/mL], *P. odorata* leaves hexane fraction [(5.51 ± 0.65) μg/mL], *P. odorata* leaves EA fraction [(13.71 ± 1.39) μg/mL], *P. odorata* bark crude extract [(4.69 ± 0.63) μg/mL], *P. odorata* bark hexane fraction [(8.42 ± 2.11) μg/mL] and *A. camansi* leaves crude extract [(9.58 ± 3.29) μg/mL] exhibited high cytotoxic activities against MCF-7.

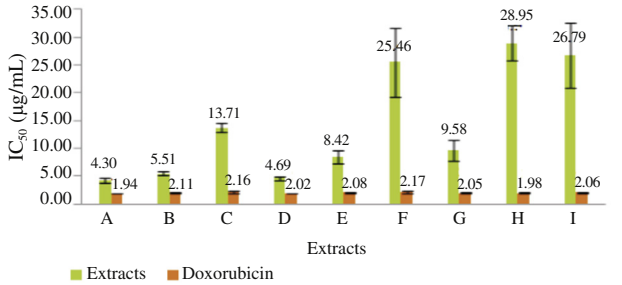

Figure 2. IC_{50} values of plant extracts against MCF-7.
Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration. Data were expressed as mean ± SD. A: *P. odorata* leaves crude; B: *P. odorata* leaves hexane; C: *P. odorata* leaves EA; D: *P. odorata* bark crude; E: *P. odorata* bark hexane; F: *P. odorata* bark EA; G: *A. camansi* leaves crude; H: *A. camansi* bark crude and I: *G. sepium* crude.

IC_{50} values against A549 were presented in Figure 3. *P. odorata* leaves hexane fraction [(16.78 ± 7.19 μg/mL)], *P. odorata* bark crude extract [(5.43 ± 1.72) μg/mL)] and *P. odorata* bark hexane fraction [(11.42 ± 0.92) μg/mL)] showed high cytotoxic activities.

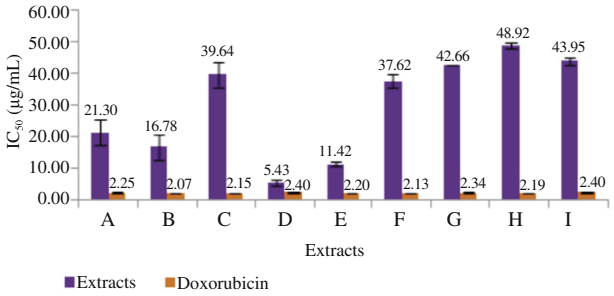

Figure 3. IC_{50} values of plant extracts against human lung non-small cell carcinoma A549.
Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration. Data were expressed as mean ± SD. A: *P. odorata* leaves crude; B: *P. odorata* leaves hexane; C: *P. odorata* leave EA; D: *P. odorata* bark crude; E: *P. odorata* bark hexane; F: *P. odorata* bark EA; G: *A. camansi* leaves crude; H: *A. camansi* bark crude and I: *G. sepium* crude.

Plant extracts that exhibited high cytotoxic activities should be selective against cancer cell lines and non-cytotoxic against normal cell lines. To determine the selectivity of the cytotoxic plant extracts, they were tested against the non-cancer cell line Chinese hamster ovary (AA8). Figure 4 shows the IC_{50} values of tested extracts against AA8. Comparatively, the cytotoxic activities of most of the plant extracts against AA8 (Figure 4) were lower than doxorubicin thus showing more selective cytotoxicity. *P. odorata* leaves crude extract [(11.28 ± 6.85) μg/mL], *P. odorata* leaves hexane fraction [(19.01 ± 5.12) μg/mL], *P. odorata* bark crude extract [(9.33 ± 1.55) μg/mL] and *P. odorata* bark hexane fraction [(18.09 ± 4.37) μg/mL] showed lowest cytotoxic activities against AA8.

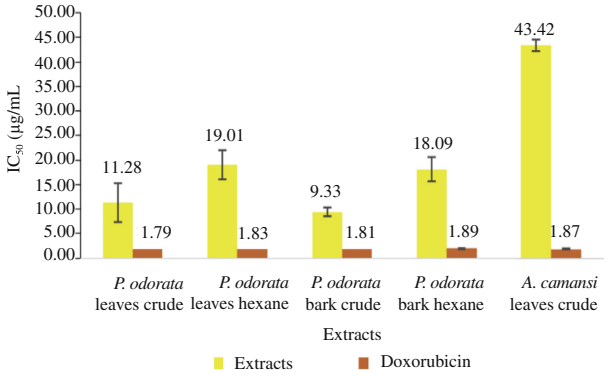

Figure 4. IC_{50} values of plant extracts against Chinese hamster ovary non-cancer cells, AA8.
Doxorubicin was used as positive control while DMSO was used as negative control. Three trials were done with three replicates for each concentration. Data were expressed as mean ± SD.

Figure 5 shows the SI of all the cytotoxic extracts used in this study. Crude *P. odorata* leaves, hexane fraction of *P. odorata* leaves, crude *P. odorata* bark and *A. camansi* leaves possessed high selectivity for MCF-7 cell line. However, only *P. odorata* bark hexane fraction showed high selectivity for HCT116.

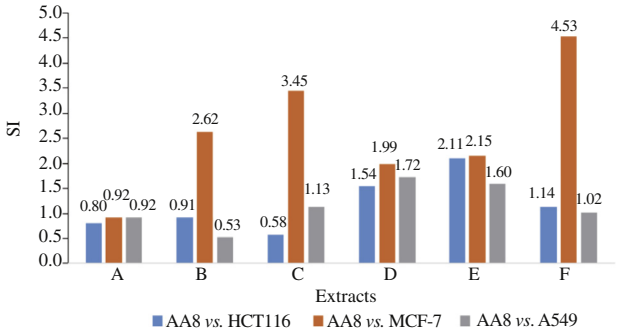

Figure 5. SIs of plant extracts against AA8, HCT116, MCF-7 and A549. A: Doxorubicin; B: *P. odorata* leaves crude; C: *P. odorata* leaves hexane; D: *P. odorata* bark crude; E: *P. odorata* bark hexane and F: *A. camansi* leaves crude.

P. odorata bark crude extract showed selective cytotoxicity. The SI of *P. odorata* bark crude extract for A549 was 1.72, 1.99 for MCF-7 and 1.54 for HCT116. On the other hand, the SI of *P. odorata* bark hexane fraction for A549 was 1.60, 2.11 for HCT116 and 2.15 for MCF-7.

4. Discussion

Statistical analysis revealed that there was no significant difference between the IC_{50} values of *P. odorata* leaves crude, *P. odorata* bark crude and *P. odorata* bark hexane extracts and doxorubicin for HCT116. No significant difference was found for the IC_{50} values between *P. odorata* leaves crude, *P. odorata* leaves hexane, *P. odorata* bark crude, *P. odorata* bark hexane, *A. camansi* leaves crude extracts and doxorubicin against MCF-7. Lastly, there was no significant difference between the IC_{50} values of *P. odorata* leaves hexane, *P. odorata* bark crude and *P. odorata* bark hexane and the positive control, doxorubicin against A549. This means that the cytotoxic activities of *P. odorata* leaves, bark and *A. camansi* leaves extracts are comparable to that of doxorubicin. These results support the impression of potential anticancer compounds present in these plant extracts.

P. odorata leaves hexane extract showed high cytotoxicity against MCF-7 and A549 cancer cell lines. This high cytotoxic activity could be due to certain phytochemicals in *P. odorata*. Phytochemical analysis of *P. odorata* crude extract revealed the presence of flavonoid, unsaturated sterol and triterpene, cyanogenic glycoside, anthraquinone, tannin and phenol. *P. odorata* was found to have mild antimutagenic activity [20]. Diosmetin and acacetin were proven to be present in the leaves of *P. odorata*. These two compounds are antimicrobial, anti-inflammatory and chemopreventive in nature. There are number of compounds present in *P. odorata* that are yet to be explored for biological activities [12].

Previous literature utilized *P. odorata* leaves ethanolic crude extract but did not show cytotoxic activity against HeLa cells using microtitration cytotoxicity assay [21]. This is in contrast to the results obtained in this study showing toxicity to other cell lines. The negative result obtained in the previous study might be due to heterogeneity in the sensitivity of different cancer cell lines to the same chemotherapeutic agent [22].

P. odorata bark hexane extract exhibited excellent cytotoxic activity against all cancer cell lines used in this study. To our knowledge, this is the first report for the biological activity of *P. odorata* bark.

A. camansi crude and hexane extract exhibited high cytotoxic activity against MCF-7 cell line. There were previous studies on other species of *Artocarpus* which showed high cytotoxic activities against cancer cell lines. *Artocarpus communis* methanol extract and its dichloromethane fraction induced apoptotic cell death in hepatocellular carcinoma cell lines [23]. A new furanodihydrobenzoxanthone, artomandin from *Artocarpus kemando* showed significant cytotoxic activity against HL-60 and MCF-7 cancer cell lines. It also exhibited antioxidant properties towards DPPH [24].

However, *A. camansi* did not show cytotoxic activity against HCT116 and A549. This is consistent with previous study which utilized compounds isolated from dichloromethane extract of the leaves of *A. camansi*. These compounds were not cytotoxic against lung adenocarcinoma A549 cells, stomach adenocarcinoma AGS cells, colon adenocarcinoma HT29 cells and prostate cancer PC3 cells [15].

G. sepium crude extract did not show cytotoxic activity against all cancer cell lines used in this study thus no fractionation or partitioning was conducted for its crude extract. It is commonly used as antimicrobial agent in different parts of the world [17]. Crude methanolic extract of *G. sepium* was studied by using Vitotox assay. It displayed no genotoxic and cytotoxic properties [25].

An index according to Al-Qubaisi *et al*. determined the selective cytotoxicity of an extract. SI beyond 2 means that an extract has selective cytotoxicity [19]. However, SI values lower than 2.0 indicates that the extract is a general toxin [26]. *P. odorata* leaves hexane extract exhibited high selective cytotoxicity against MCF-7 cell line whereas *P. odorata* bark hexane extract exhibited high selective cytotoxicity against all cancer cell lines used in this study.

To our knowledge, this work is likely the first study to report the cytotoxic activity of *P. odorata* and *A. camansi* against human cancer cell lines. Based on the standard values of toxicity set by Suffness and Pezzuto [27], they demonstrated high toxicity against selected human cancer cell lines. *P. odorata* bark hexane fraction exhibited the highest cytotoxicity against HCT116, MCF-7 and A549 cancer cell lines. It also exhibited high selectivity against these cancer cell lines.

Based on the results obtained, *P. odorata* and *A. camansi* warrant further studies to isolate novel compounds for chemotherapeutic use. Further studies are needed to evaluate the anticancer potentials of the *P. odorata* and *A. camansi* extracts when used alone or in combination with doxorubicin to lessen the toxic side-effects of the latter.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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