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In-vitro Cytotoxicity of *Wrightia pubescens* (Blanco) Merr., *Aphanamixis polystachya* (Wall.) Parker, and *Platymitra arborea* (Blanco) against selected human cancer cell lines

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Abstract

Cancer is the second leading cause of mortality worldwide. Anticancer studies are centered on natural products as these have been found to exhibit properties that activate cell signaling pathways and cell aging and senescence. As such, there is a need for continuing research to explore these natural products especially those that do not only exhibit cytotoxicity but also specificity against cancer cells. This study evaluated the cytotoxic activity of the crude extracts of three Philippine indigenous plants, *Wrightia pubescens* (Blanco) Merr. *Aphanamixis polystachya* (Wall.) Parker, and *Platymitra arborea* (Blanco) against selected human cancer cell lines using *3-(4,5- dimethylthiazol-2-yl)-2-5-diphenyl-2H-tetrazolium bromide* (MTT) assay. Extracts were partitioned using hexane and ethyl acetate to determine their active fractions based on their polarity. These active fractions were then tested for their cytotoxicity against human colorectal cancer cell line (HCT116), human adenocarcinoma cell line (A549) and non-cancer Chinese hamster ovary cell line (AA8). Cytotoxic activities of the extracts were found in the ethyl acetate fractions of *W. pubescens* and *P. arborea* and hexane fraction of *A. polystachya*. All active fractions were highly cytotoxic to HCT116 and A549, with *A. polystachya* exhibiting the highest selectivity against cancer over the non-cancer cells. Results of this study imply that these extracts, especially that of *A. polystachya*, have a potential use in anti-cancer research due to their selectivity against cancer cell lines.

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Introduction

Cancer is the second leading cause of death globally, after heart disease (World Health Organization, 2017). In 2014, it has overtaken heart disease as the leading cause of death in persons younger than 85 years worldwide (World Health Organization, 2017). Mortality associated with cancer was recorded at 8.8 million in 2015, 70 percent of which occurring in low to middle income countries (World Health Organization, 2017). Mortality in the United States alone was expected to be 1,650 per day for the year 2017 (Siegel *et al.,* 2017).

Cancer develops once normal cells fail to communicate with one another due to an alteration in their genetic make-up (Kasper *et al.,* 2015). These alterations are due to mutations in the DNA upon exposure to certain carcinogens or due to random replication errors and faulty DNA repair processes. At present, treatment of different cancers is done in a case-to-case basis utilizing combinations of surgery, radiotherapy, chemotherapy, and targeted therapy (Kasper *et al.,* 2015).

Natural products, such as plant extracts, have been found to activate cell signaling pathways and cell aging and senescence and thus are used in anticancer studies for decades. In a review in 2006, 47% of the 155 approved anticancer drugs used in chemotherapy originated from plant-based compounds or their analogues (Pan *et al*., 2012). Examples of which are taxol, docetaxel, podophyllotoxin, vincristine, vinblastine, among others (Cragg and Newman, 2005). Despite its limited selectivity against cancer cells, chemotherapy is still widely used due to its cost-effectiveness (Brown *et al.,* 2013); hence, there is a need for continuing research on natural products for drug development.

Wrightia pubescens (Blanco) Merr. is an endemic plant in the Philippines and known locally as *lanete*. The genus *Wrightia* has three species endemic to the country namely *W. hanleyi, W. palawensis* and *W. candollei,* a synonym of *W. pubescens* subsp. *Candollei* (Middleton, 2005).

Several chemical constituents have been isolated from the leaves and twigs of *W. pubescens* namely oleanolic acid, squalene, B-sitosterol, a-amyrin acetate (Ragasa *et al.,* 2014a*)* and isoflavone (Ragasa *et al.,* 2015*). In vitro* and *in vivo* studies on its anticancer properties however are yet to be published. Other species belonging to the same genus were found to exhibit cytotoxic activities against cancer cells and also possess antimicrobial activity. The most studied species is *W. tinctoria*, which was found to exhibit cytoprotective properties against HIV-1 in lymphocytes (Selvam *et al.* 2009) Ethanolic and methanolic leaf extracts of the same species were also found to be potent antimicrobials against *B. subtilis, S. epidermidis* (Kannan *et al.,* 2006*), P. aeruginosa, S. aureus, K. pneumonia, B. pumilus* and *C. albicans* (Jain and Bari, 2009). *W. tomentosa* on the other hand, was found to display cytotoxic activity against the murine P388 lymphocytic leukemia cell line (Lin *et al.,* 1992) and breast cancer cell lines (Chakravarti *et al.,* 2012).

Aphanamixis polystachya (Wall.) Parker (*Amoora rohituka* (Roxb) Wight & Arn) is known in the Philippines as *kangko*. It has been traditionally used as an astringent, and an oral medication for liver and spleen diseases, rheumatism, tumors (Graham *et al.,* 2000*)* and cardiac and hepatic disorders (Rahmatullah *et al.,* 2010). Studies show that the stem and bark of *A. polystachya* is cytoprotective against radiation-induced chromosome damage in bone marrow cells of Swiss albino mice (Jagetia and Venkatesha, 2006). Its bark extracts were also found to have cytotoxic activity against Ehrlich ascites carcinoma in mice (Jagetia and Venkatesha, 2005), and the breast cancer cell line MCF-7 (Chan *et al.,* 2011). In addition, its methanol partition was found to demonstrate dose-dependent reduction in hepatic malondialdehyde, a marker of lipid oxidation, with simultaneous improvement in hepatic glutathione and catalase levels in *in vivo* animal studies (Krishnaraju *et al.,* 2009). Jagetia and Venkatesha in 2016 demonstrated the antineoplastic activity of the stem bark extract of *A.* polystachya against human cervical carcinoma (HeLa S3) at an IC50 of 25 ug/ml.

The antioxidant and anticancer activity of *A. polystachya* is attributed to certain alkaloids (Harmon *et al.,* 1979), limonoids (Mulholland and Naidoo, 1999), and terpenoids and sterol (Ragasa *et al.,* 2014b) found in its the bark and leaves.

Platymitra arborea (Blanco) Kessler of family Annonaceae on the other hand, is known in the Philippines as *bolon*. No cytotoxicity studies have been found for this specific genus. Other genera under this family however, exhibit anticancer potential such as that of *Goniothalamus, Uvaria* and *Annona*. Several species of *Goniothalamus* were found to exhibit antiproliferative and apoptotic properties against A549, GLC4, K562 (Pradupsri *et al.,* 2009), MCF-7 and HCT-8 cancer cell lines (Abdul *et al.,* 2009), while *Uvaria* species had documented activity against KB, HCT-8, human hepatoma and human ovarian cancer cell lines (Abdul *et al.,* 2009). Finally, *Annona* species were found to be cytotoxic against human hepatoma and breast cancer cell lines (Yu, 1999).

This study aimed to explore the cytotoxic activity of extracts from the Philippine indigenous plants *Wrightia pubescens* (Blanco) Merr., *Aphanamixis polystachya* (Wall.) Parker and *Platymitra arborea* (Blanco) Kessler against selected human cancer cell lines namely HCT116 or human colorectal carcinoma and A549 or human lung adenocarcinoma using *3-(4,5- dimethylthiazol-2-yl)-2-5-diphenyl-2H-tetrazolium bromide* (MTT) assay.

Materials and methods

Collection, proper identification and extraction of crude extracts

The indigenous plants *W. pubescens, A. polystachya* and *P. arborea* were collected from Bacnotan, La Union and submitted to the Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines-Diliman for verification of taxonomic details before depositing specimens. Accession numbers are as follows: *W. pubescens* 21190, *A. polystachya* 15382, *P. arborea* 5055. Leaves of the plants were dried, weighed, homogenized and soaked for 48 hours in 95% ethanol.

The suspension was filtered and the filtrate was then concentrated using a rotary evaporator. The concentrated paste was then collected, air-dried, weighed and dissolved in dimethyl sulfoxide (DMSO) to a concentration of 4 mg/mL for use in the assays.

Cell maintenance

All cell lines were purchased from the American Type Culture Collection Manassas, Virginia (American Type Culture Collection, 1999). HCT116 was grown in McCoy's 5a medium with 10% fetal bovine serum (FBS) and 1% Penicillin and Streptomycin (PS). A549 was grown in Ham's F12K medium, adjusted to contain 1% sodium bicarbonate and 10% FBS. Lastly, AA8 cell line or the Chinese hamster ovary cell line was grown in Rosswell Park Memorial Institute (RPMI) medium with 2% $NaHCO_3$, 10% FBS, 1% PS. All cell lines were incubated in 5% CO_2, 37°C at 90% humidity and passaged during the late-log phase or when the cells reach about 90% confluency.

In vitro cytotoxicity assay

Pretreatment

Cancer cells were seeded on 96-well titer plates at 4 x10^4 cells per well and were incubated for 24 hours. Two fold serial dilution of test samples were made up to concentrations of 1000, 500, 250 and 125 ug/ml in a separate 96 well plate. Ten (10) ul from the serially diluted samples were added to preincubated cells to make final screening concentrations of 50 ug/ml, 25 ug/ml, 12.5 ug/ml and 6.25 ug/ml with 3 replicate wells per concentration. Cells with DMSO were used as the negative control while those with Doxorubicin, an anthracycline antibiotic, were used as the positive control. The plates were then incubated for 72 hours at 37°C at 90% humidity in 5% CO_2.

MTT [3-(4,5- dimethylthiazol-2-yl)-2-5-diphenyl-2H-tetrazolium bromide] assay

The protocol used in this assay was adopted from that of Mosmann (Mosmann, 1983). Following incubation with the extracts, the medium in each well was discarded followed by the addition of 20 uL of the MTT reagent (5 mg/ml PBS) to each well. Plates were wrapped in aluminum foil and incubated in the same standard conditions as stated, for 4 hours.

Termination of the assay involved the addition of DMSO at 150 uL per well to dissolve the purple formazan crystals, which are the products of the mitochondrial metabolism of the live cells from the yellow MTT. Finally, spectrophotometric reading was done at 570 nm. Treatment of cells per cell line was done in at least three trials with three replicate wells per concentration in each trial to ensure validity of data before data analysis. The IC50 or the minimum concentration of extracts at which 50% of the cell population are already killed was then generated through linear interpolation using the software "Linear Interpolation Method for Sublethal Toxicity: The Inhibition Approach (Version 2.0).

Test for selectivity of the plant extracts

To determine if the plant extracts are selective against cancer cells over non-cancer cells, the MTT assay was also employed on the non-cancer cell line, AA8 derived from Chinese hamster ovary. Only the active fraction of each plant, which came from either the ethyl acetate partition or the hexane partition, was tested. The same protocol and data analysis were employed. Selectivity Index (SI) of the test sample per cell line was computed by dividing the IC_{50} of the non-cancer cell line by the IC_{50} of the cancer cell line used (Suffness and Pezzuto, 1990; Oliveira *et al.,* 2015). The selectivity index is then compared to the selectivity indices of the standard anticancer drug (Lopez-Lazaro, 2015), which is in this case doxorubicin. In previous studies, a selectivity index of >3 of plant extracts were considered as high selectivity (Machana *et al.,* 2004; Prayong *et al.,* 2008).

Fractionation

All plant extracts with cytotoxicity against any of the cancer cell lines were subjected to fractionation to determine polarity of their active fractions. Crude extracts were partitioned exhaustively with hexane and ethyl acetate into two separate set- ups using a 1000 ml separatory funnel. Both hexane and ethyl acetate fractions were concentrated, dried and assayed for cytotoxicity using MTT.

Data analysis

A. MTT assay

Results were expressed as mean ± SD of three independent experiments. IC_{50} values, the minimum concentration of the plant extracts at which 50% of the cell population are killed, were calculated from recorded absorbances. The software used was the "Linear Interpolation Method for Sublethal Toxicity: The Inhibition Concentration (ICp) approach (Version 2.0)".

Test for normality of data sets was done using the Kolmogorov-Smirnov and Shapiro-Wilk test. One-way ANOVA was also used to compare data sets among groups followed by Tukey's Honestly Significant Difference (HSD) Test to determine which particular pair of extracts differs significantly from one another. Confidence level for both statistical tests was set at 95%. Cytotoxicity is set at ≤ 30ug/ml for extracts (Jokhadze *et al.*, 2007).

Results

MTT Assay

HCT116

Crude extracts from the leaves of the plants *W. pubescens*, *A. polystachya* and *P. arborea* showed cytotoxic effects on the HCT116 cells with average IC_{50} values of 27.95 ± 2.93 ug/ml, 24.93 ug/ml ± 3.04 and 23.82 ug/ml ± 2.25 respectively (Fig. 1).

Doxorubicin, the positive control, had an average IC_{50} of 2.05 ug/ml ± 0.06. Higher activity was observed in the hexane fraction of *A. polystachya* having an average IC_{50} of 19.32 ± 3.76 ug/ml. *W. pubescens* and *P. arborea*, on the other hand, exhibited cytotoxicity in their ethyl acetate fractions with average IC_{50s} at 23.30 ± 5.85 ug/ml and 19.23 ug/ml ± 3.20 respectively.

A549

As seen in Fig. 2, all the crude extracts exhibited cytotoxicity against A549 cells. *W. pubescens* had an average IC_{50} of 22.34 ± 4.01 ug/ml while *A. polystachya* and *P. arborea* had 27.18 ± 0.33 ug/ml and 26.78 ± 2.87 ug/ml respectively.

Doxorubicin, on the other hand, had an average IC_{50} of 2.26 ± 0.62 ug/ml. Due to limited availability of cells for culture, only the fractions, which exhibited cytotoxicity against HCT 116 were tested.

The ethyl acetate fractions of *W. pubescens* and *P. arborea* were found to be more active against this cell line based on their average IC_{50}s recorded at 14.16 ± 3.69 ug/ml and 17.25 ± 0.76 ug/ml respectively.

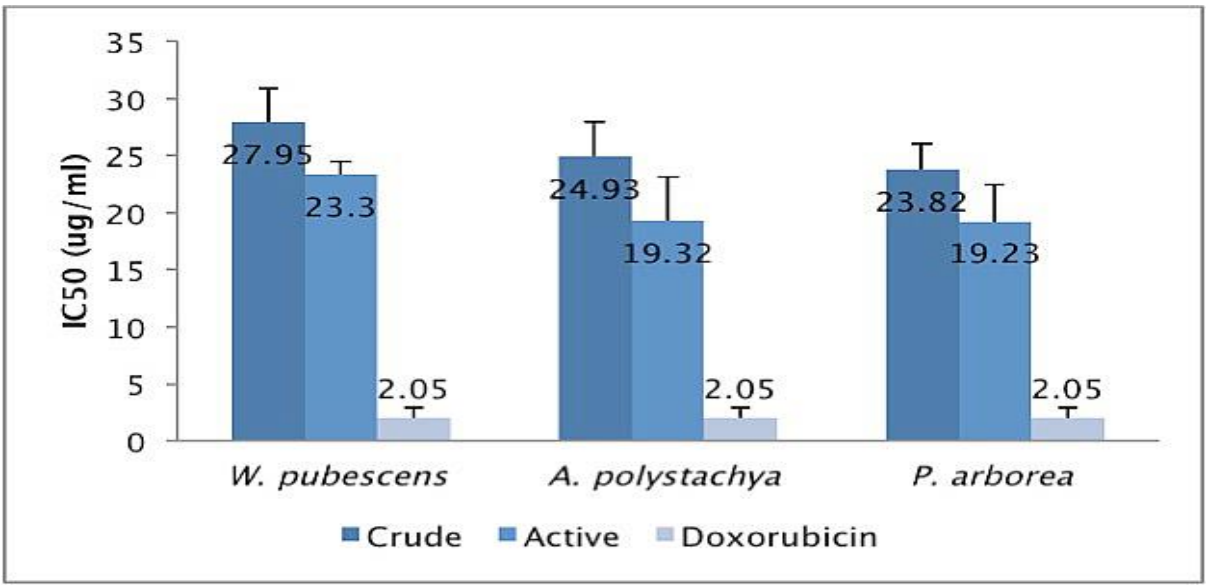

Fig. 1. Cytotoxic activity of the crude extracts and fractions from each plant extract based on average IC_{50} against human colorectal carcinoma, HCT116. Reported values for each treatment are means (±SD) of three independent trials. Bars represent standard deviations. Active fractions were found in the ethyl acetate partitions of *W. pubescens* and *P. arborea* and the hexane fraction of *A. polystachya*. Doxorubicin was used in the positive control set ups.

The hexane fraction of *A. polystachya,* on the other hand, was found to have an average IC_{50} of 15.04 ± 1.43 ug/ml (Fig.2).

Test for selectivity of active fractions against AA8

The selectivity of the active fractions against cancer cells over non-cancer cells was tested using non-cancer cell line, Chinese hamster ovarian cells, AA8. A high selectivity against cancer cells (Selectivity index of >3) may indicate that toxicity is limited to cancer cells with minimal or no effect on normal cell lines. The ethyl acetate fraction of *P. arborea* was found to be cytotoxic to normal cells with an average IC_{50} of 5.39 ± 0.03 ug/ml, a significantly low IC_{50} almost comparable to the positive control doxorubicin with an IC_{50} of 2.49 ± 0.52 ug /ml. Ethyl acetate partition of *W. pubescens* on the other hand is cytotoxic only at an IC_{50} of 32.30± 1.08 ug/ml (Fig.3). Selectivity indices (SI) for *P.* arborea and *W.* pubescens against HCT116 were found to be 0.28 and 1.39 respectively, while that against A549 was 0.31 and 2.28 respectively. Notably, the hexane fraction of *A. polystachya* did not display cytotoxicity against this cell line. The absorbances obtained using this extract were higher than the negative control, DMSO, resulting to no linear interpolation of its IC_{50} . At an arbitrary value of 50 ug/ml however, the selectivity index is computed at 2.59 for HCT116 and 3.32 for A549. Fig. 4 shows the selectivity indices of the active fractions of the plant extracts.

Discussion

Cytotoxicity of crude extracts against A549 and HCT116

IC_{50} values of the crude extracts of *W. pubescens, A. polystachya* and *P. arborea* showed a normal distribution. Statistical analysis showed a significant difference from that of doxorubicin when tested against HCT116 ($p=0.000$) and A549 ($p=0.000$). This means that although these plant extracts exhibited

cytoxocity against these cancer cell lines, their level of cytotoxicity is not as good as doxorubicin since these are still in the crude extract stage. It is expected that the IC_{50} values will considerably improve with every step of the purification process. Purification of the extracts could be done through a repeated, sequential chromatographic fractionation in order to extract and isolate the compounds responsible for cytotoxicity.

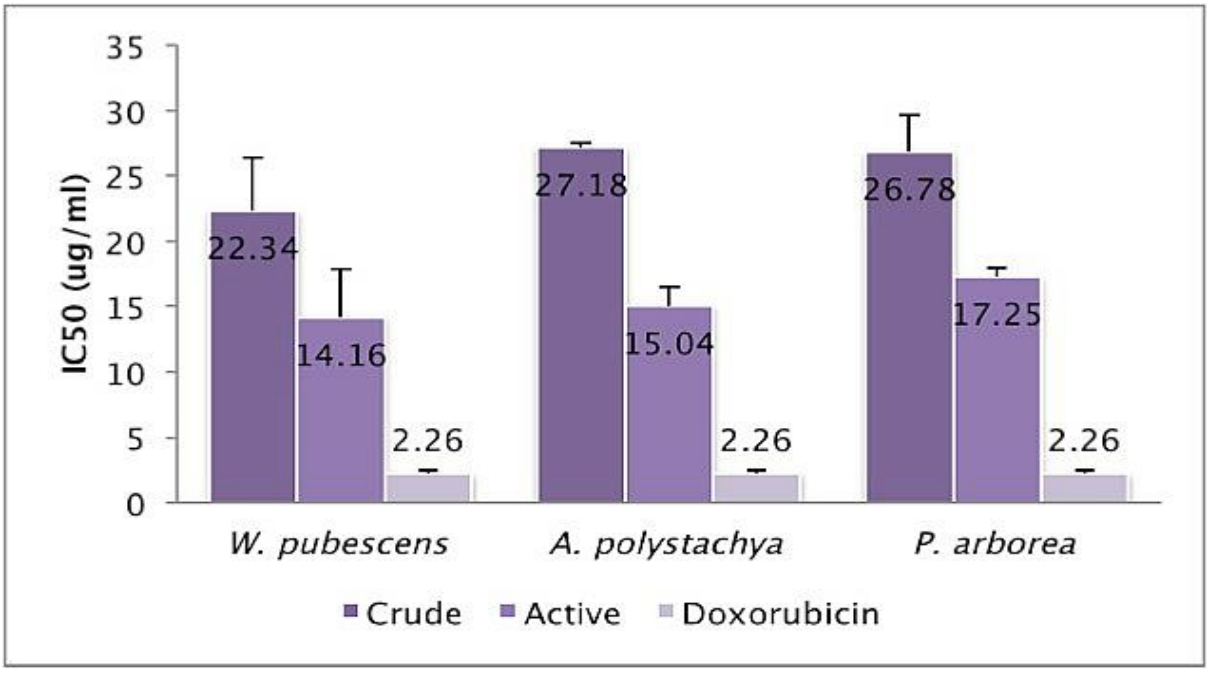

Fig. 2. Cytotoxic activity of the crude extracts and the fractions from each plant extract based on average IC_{50}s against human lung adenocarcinoma, A549. Reported values for each treatment are means (±SD) of three independent trials. Bars represent standard deviations. Active fractions were found in the ethyl acetate partitions of *W. pubescens* and *P. arborea* and the hexane partition of *A. polystachya*. Doxorubicin was used in the positive control set ups.

Cytotoxicity of Wrightia pubescens

Based on the literature cited, certain species of the genus *Wrightia* were found to have cytotoxic effects on cancer cells due to the presence of isoflavones such as wrightiadone (Lin *et al.,* 1992).

In 2012, oleanolic acid and urosolic acid were isolated from the extracts of *W. tomentosa* and were found to exhibit apoptotic effects when tested against MCF-7 and MDA-MB-231 breast cancer cell lines (Chakravarti *et al.,* 2012). *W. pubescens* in particular, was also found to contain oleanolic acid.

In addition, it was found to contain squalene, B-sitosterol, a-amyrin acetate (Ragasa *et al.,* 2014a*)* and isoflavone (Ragasa *et al.,* 2015*);* however, *in vitro* and *in vivo* investigations of its anticancer potential are yet to be published.

Cytotoxicity of Aphanamixis polystachya

Based on the results of this study, *A. polystachya* not only exhibited cytotoxicity against both HCT116 and A549 but also demonstrated its selectivity against cancer cells, an important factor in cancer drug development.

Cytotoxicity of Platymitra arborea

No prior literature has demonstrated activity of *P. arborea.* The ethyl acetate fraction of this extract exhibited cytotoxic activity against both HCT116 and A549. Although, based on its very low specificity index (SI), it did not show specificity for cancer cell lines in its toxicity, further evaluation must still be done to identify the active compounds and the specific mechanism by which this extract exerts its cytotoxic potential against the selected cancer cell lines.

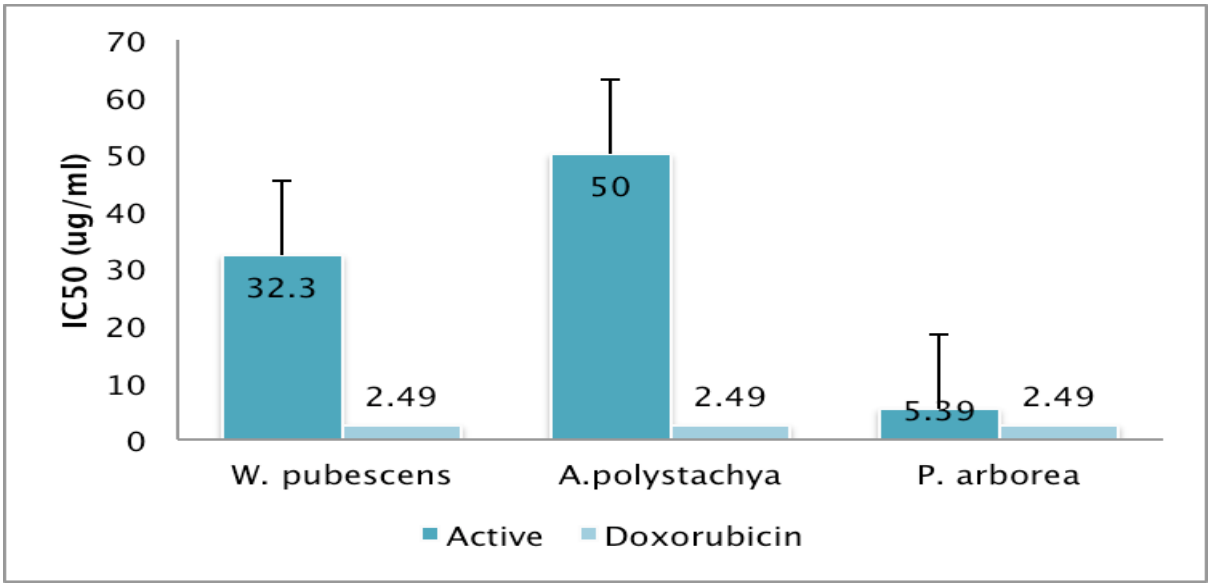

Fig. 3. Comparison of the cytotoxic activity of the ethyl acetate fractions of both *W. pubescens* and *P. arborea* based on average IC50s against AA8. Reported values for each treatment are means (±SD) of two independent trials. Bars represent standard deviations. Hexane fraction of *A. polystachya* did not show cytotoxicity but rather promoted growth of cells (absorbance readings greater than the negative control (DMSO)) hence an arbitrary value of 50 ug/ml. Doxorubicin was used as the positive control.

Activity against A549 compared to HCT116

Notably, the active ethyl acetate partitions of *W. pubescens* and *P. arborea* and the hexane partition of *A. polystachya* showed higher toxicity against A549 than HCT 116. A549 is considered the more sturdy cell line compared to HCT116 because of the overexpression of heat shock proteins (hsp70) in the cell membrane and cytosol that inhibits lysosomal membrane permeabilization (Mijatovic *et al.*, 2006).

These cytoprotective heat shock protein (hsp70), when upregulated, cause resistance to both radiotherapy and chemotherapy (Schilling *et al.*, 2013).

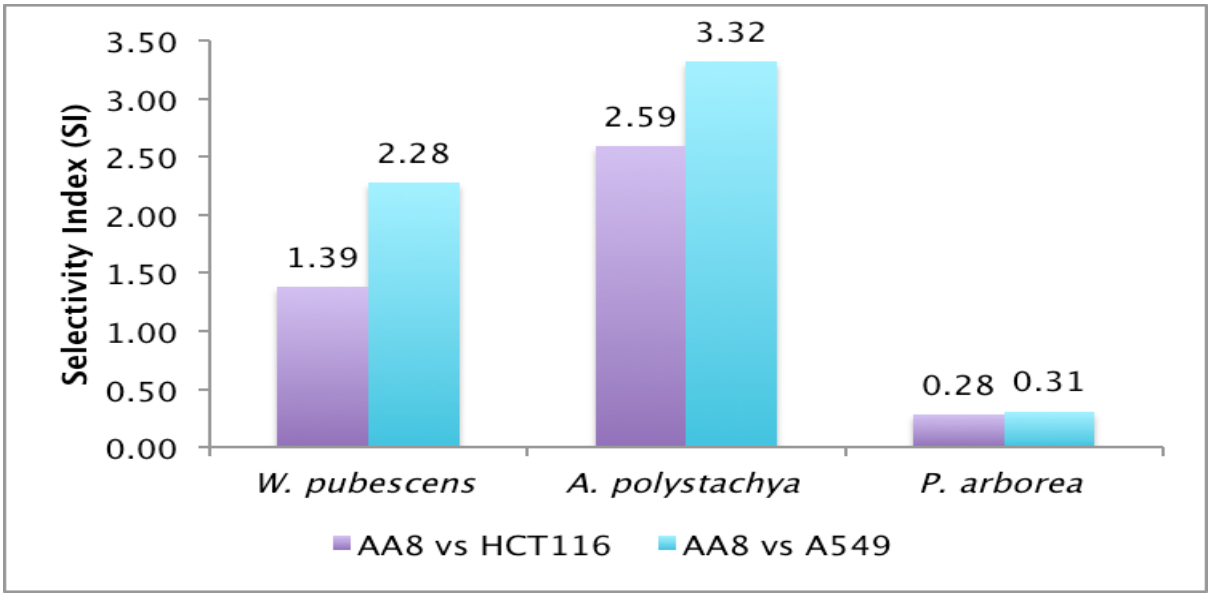

Fig. 4. Selectivity indices of the active fractions of the plant extracts. The ethyl acetate fraction of *W. pubescens* has a higher selectivity compared to *P. arborea*. The hexane fraction of *A. polystachya* is highly selective based against the lung adenocarcinoma (with low IC_{50} value on A549) and on the absence of toxicity to AA8.

To our knowledge, this work is likely the first study to report the cytotoxic activity of *P. arborea* against human cancer cell lines. *W.* pubescens is known to contain several active chemical constituents although *in vitro* and *in vivo* anticancer studies are yet to be published. *A. polystacha,* on the other hand, is already established as an active extract against certain human cancer cell lines although the exact mechanism of its anticancer activity is still unknown. Results of this study establish that the ethyl acetate fractions of both *Wrightia pubescens* and *Platymitra arborea* as well as the hexane fraction of *Aphanamixis polystachya* have a potential for further studies and may warrant the isolation of the active compounds for chemotherapeutic use. *A. polystachya* in particular is a promising candidate since it does not only kill cancer cells but also promote growth of normal cells. As well, it is recommended that in future confirmatory studies, normal human cell lines should be used for computing the selectivity indices.

References

Abdul A, Abdel-Wahab S, Fong HK, Mohan SM, Al-Zubair AS, Elhassan MM. 2009. *In vitro* Response of Cancer Cells to the Growth-Inhibitory Effects of Dichloromethane Extract of *Goniothalamus umbrosus*. Research Journal of Pharmacology **3**, 1-6. www.medwelljournals.com/abstract/?doi=rjpharm.2009.1.6

American Type Culture Collection. 1999. www.atcc.org/

Brown T, Pilkington G, Bagust A, Boland A, Oyee J, Tudur-Smith C, Blundell M, Lai M, Martin Saborido C, Greenhalgh J Dundar Y, Dickson R. 2013. Clinical effectiveness and cost-effectiveness of first-line chemotherapy for adult patients with locally advanced or metastatic non-small cell lung cancer: a systematic review and economic evaluation. Health Technology Assessment **17,** 1-278. http://dx.doi.org/10.3310/hta17310

Chan L, George S, Ahmad I, Gosangari S, Abbasi A, Cunningham B, Watkin K. 2011. Cytotoxicity Effects of *Amoora rohituka* and *chittangonga* on Breast and Pancreatic Cancer Cells. Evidence based Complementary and Alternative Medicine. Epub 2011 Apr 26. http://dx.doi.org/10.1155/2011/860605

Chakravarti B, Maurya R, Siddiqui JA, Bid HK, Rajendran SM, Yadav PP, Konwar R. 2012. *In vitro* anti-breast cancer activity of ethanolic extract of *Wrightia tomentosa*: role of proapoptotic effects of oleanolic acid and urosolic acid. Journal of Ethnopharmacology **142,** 72-79. http://dx.doi.org/10.1016/j.jep.2012.04.015

Cragg GM, Newman DJ. 2005. Plants as a Source of Anti-cancer agents. Journal of Ethnopharmacology **100,** 72-29. http://doi.org/10.1016/j.jep.2005.05.011

Graham JG, Quinn ML, Fabricant DS, Farnsworth NR. 2000. Plants used against cancer-an extension of the work of Jonathan Hartwell. Journal of Ethnopharmacology **73**, 47-377. http://dx.doi.org/10.1016/S0378-8741(00)00341-X

Harmon AD, Weiss U, Silverton JV. 1979. The structure of rohitukine, the main alkaloid of *Amoora rohituka* (syn. *Aphanamixis polystachya*) (Meliaceae. Tetrahedron Letters **8,** 721-724.

Jagetia G, Venkatesha V. 2006. Treatment of mice with stem bark extract of *Aphanamixis polystachya* reduces radiation-induced chromosome damage. International Journal of Radiation Biology **82,** 197-209. http://dx.doi.org/10.1080/09553000600621413

Jagetia GC, Venkatesha VA. 2005. Enhancement of radiation effect by *Aphanamixis ploystachya* in mice transplanted with Ehrlich ascites carcinoma. Biological & Pharmaceutical bulletin **28,** 69-77. http://dx.doi.org/10.1248/bpb.28.69

Jagetia GC, Venkatesha VA. 2016. Determination of Antineoplastic activity of Rohituka, *Aphanamixis polystachya* (Wall) RN Parker in Hela Cells: Correlation with Clonogenicity and DNA Damage. International Journal of Complementary & Alternative Medicine **3,** 1-11. http://dx.doi.org/10.15406/ijcam.2016.03.00083

Jain P, Bari S. 2009. Antibacterial and Antifungal activity of extracts of woody stem of *Wrightia tinctoria* R. Br. International Journal of Pharmaceutical Sciences and Research **1,** 18-21.

Jokhadze M, Eristavi L, Kutchukhidze J, Chariot A, Angenot L, Tits M, Jansen O, Frederich M. 2007. In vitro cytotoxicity of some medicinal plants from Georgian Amaryllidaceae. Phytotheraphy Research **21**, 622-624. www.dx.doi.org/10.1248/bpb.28.6910.1248/bpb.28.69

Kannan P, Shanmugavadivu B, Petchiammal C, Hopper W. 2006. In vitro antimicrobial activity of *Wrightia tinctoria* leaf extracts against skin microorganisms. Acta Botanica Hungarica **48,** 323-329. http://dx.doi.org/10.1556/ABot.48.2006.3-4.7

Kasper D, Fauci A, Hauser S, Longo D, Jameson J. 2015. Cancer genetics. In Kasper,DL Ed. Harrison's principles of internal medicine. New York. McGraw-Hill, Medical Pub. Division. 102e-1- 102e9 P.

Krishnaraju A, Rao C, Rao T, Reddy KN, Trimurtulu G. 2009. In vitro and In vivo Antioxidant Activity of *Aphanamixis polystachya* Bark. American Journal of Infectious Diseases **5,** 60-67.

Lin L, Topcu G, Lotter H, Ruangrungsi N, Wagner N, Pezzuto J, Cordell G. 1992. Wrightiadone from *Wrightia tomentosa*. Phytochemistry **31,** 4333-4335. http://dx.doi.org/10.1248/bpb.28.69

Lopez-Lazaro, M. 2015. Two precliicial tests to evaluate anticancer activity and to help validate drug candidates for clinical trials. Oncoscience **2,** 91-98. http://dx.doi.org/10.18632/oncoscience.132

Machana S, Weerapreeyakul N, Barusrux S, Nonpunya A, Sripanidkulchai B, Thitimetharoch T. 2011. Cytotoxic and apoptotic effects of six herbal plants against the human hepatocarcinoma (HepG2) cell line. International Society for Chinese Medicine **6,** 39. http://dx.doi.org/10.1186/1749-8546-6-39

Middleton DJ. 2005. A Revision of *Wrightia* (Apocynaceae: Aposcynoideae) in Malesia. Harvard Papers in Botany **10,** 161-182.

Mijatovic T, Mathieu V, Gaussin J-F, De Neve N, Ribaucour F, Van Quaquebeke E, Dumont P, Darro F, Kiss R. 2006. Cardenolide-induced lysosomal membrane Permeabilization demonstrates therapeutic benefits in Experimental Human Non-small cell lung cancers. Neoplasia **8**, 402-412. http://dx.doi.org/10.1593/neo.05850

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immulogical methods **65,** 55-63.

Mulholland DA, Naidoo N. 1999. Limonoids from *Aphanamixis polystachya*. Phytochemistry **51,** 927-930. http://dx.doi.org/10.1016/S0031-9422(99)001570

Oliveira PFd, Alves JM, Damasceno JL, Oliveira RAM, Júnior HD, Crotti AEM, Tavares DC. 2015. Cytotoxicity screening of essential oils in cancer cell lines. Revista Brasileira de Farmacognosia **25,** 183-188. http://dx.doi.org/10.1016/j.bjp.2015.02.009

Pan L, Chai HB, Kinghorn AD. 2012. Discovery of new anticancer agents from higher plants. Frontiers in Bioscience **4,** 142-156. http://dx.doi.org/10.2741/257

Pradupsri P, Loetchutinat C, Nuntasaen N, Meepowpan P, Tuntiwechapikul W, Pompimon W. 2009. Anticancer Activities of Styrylpyrone from the Leaves and twigs of *Goniothalamus maewongensis* via cell cycle arrest. American Journal of Applied Sciences **6,** 2018. http://dx.doi.org/10.3844/ajassp.2009.2018.2023

Prayong P, Barusrux S, Weerapreeyakul N. 2008. Cytotoxic activity screening of some indigenous Thai plants. Fitoterapia **79**, 598-601.

Jagetia GC, Venkatesha VA. 2016. Determination http://dx.doi.org/10.1016/j.fitote.2008.06.007

Ragasa C, Ng VA, De Los Reyes M, Mandia E, Shen CC. 2014a. Chemical constituents of *Wrightia pubescens* (R.Br.). Der Pharmacia Lettre **6**, 14-19.

R**agasa C, Ng VA, De Los Reyes M, Mandia E, Shen CC.** 2015. An Isoflavone from *Wrightia pubescens*. International Journal of Pharmacognosy and Phytochemical Research **7,** 353-355.

Ragasa C, Aguilar MLT, Ng VA, Bugayong ML, Jacinto SD, Wen-Tai L, Shen CC. 2014b. Terpenoids and sterol from *Aphanamixis polystachya*. Journal of Chemical and Pharmaceutical Research **6,** 65-68.

Rahmatullah M, Rahman L, Rehana F, Azad AK. 2010. A scientific evaluation of Medicinal plants used in the Folk Medicinal System of Five villages in Narsinghdi District, Bangladesh. American-Eurasian Journal of Sustainable Agriculture **4,** 55-64.

Schilling D, Duwel M, Molls M, Multhoff G. 2013. Radio sensitization of wild type p53 cancer cells by the MDM2-inhibitor PXN727 is associated with altered heat shock protein 70 (Hsp70) levels. Cell Stress Chaperones **18**, 183-191.
http://dx.doi.org/10.1007/s12192-012-0369-8

Seigel R, Miller K, Jemal A. 2017. Cancer Statistics, 2017. CA: A Cancer Journal for Clinicians **67,** 7-30.
http://dx.doi.org/10.3322/caac.21387

Selvam P, Murugesh N, Witvrouw M, Keyaerts E, Neyts J. 2009. Studies of antiviral activity and cytotoxicity of *Wrightia tinctoria* and *Morinda citrifolia*. Indian Journal of Pharmaceutical Sciences **71,** 670-672.
http://dx.doi.org/10.4103/0250-474X.59550

Suffness M, Pezzuto JM. 1990. Assays related to cancer drug discovery. In: Hostettmann, K, Ed. Methods in Plant Biochemistry, vol. **6**. London: Academic Press. p 71-133.

World Health Organization. February 2017. Cancer Fact Sheet.
www.who.int/mediacentre/factsheets/fs297/en/

Yu D. 1999. Recent works on anti-tumor constituent from Annonaceae plants in China. Pure and Applied Chemistry **71,** 1119-1122.
http://dx.doi.org/10.1351/pac199971061119