



International Journal of
Cancer Research

ISSN 1811-9727



Academic
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Cytotoxic Potential on Breast Cancer Cells Using Selected Forest Species Found in Malaysia

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Abstract: *In vitro* studies were carried out to evaluate the cytotoxic potential of three selected forest herbaceous species: *Tectaria singaporeana* (TS), *Blechnum orientale* (BO) and *Tacca integrifolia* (TCI). Methanol/methylene chloride extracts of three plant parts viz. leaves, roots and stems were assessed for their cytotoxic potential against human breast cancer cells (MCF-7wt.). Screening of these extracts was done using the microculture, followed by tetrazolium assay after a period of 72 h. There were significant differences between different parts of plants and dilution levels in terms of cytotoxicity, with roots and concentration of 100 $\mu\text{g mL}^{-1}$ showing the highest cell mortality of 19.58 and 36.59%, respectively. However, the leaves and the stems of all three plant species did not induce any cytotoxic activity on the cells. Overall, the most promising material ($\text{IC}_{50} < 100 \mu\text{g mL}^{-1}$) were the methanolic extracts from the roots of all three plants. *Tectaria singaporeana* showed the highest cytotoxic potential with an IC_{50} value of $28.57 \pm 11.74 \mu\text{g mL}^{-1}$ followed by *Blechnum orientale*, $32.07 \pm 7.85 \mu\text{g mL}^{-1}$ and *Tacca integrifolia*, $95.03 \pm 17.49 \mu\text{g mL}^{-1}$. From this study, the extracts of these plants may prove to be useful in cancer treatment and prevention.

Key words: Medicinal plants, Apoptosis, breast cancer, *Tectaria singaporeana*, *Blechnum orientale*, *Tacca integrifolia*

INTRODUCTION

Malaysia is a country blessed with a broad spectrum of diversity in flora and fauna. Past and recent ethno-botanical studies suggest that at least 20% of the estimated 12 000 total higher plant species possess medicinal or other therapeutic properties (Parris and Latiff, 1997). The abundance of medicinal plants serves as an ideal resource that can unleash new discoveries in the medical breakthrough. They are proven beneficial because they contain various phytochemicals which are natural molecules produced by plants for protection. These plants serve as an alternative to modern medicine for most local and tribal communities. Traditional use of herbal medicine include herbs, herbal material, herbal preparations and finished herbal products that contain active ingredients in various parts of plants.

In the pursuit of determining the cure for cancer, especially breast cancer which results in 40,000 deaths yearly, making it the second leading cause of death from cancer in women (Lester and Cotran, 1999), various plants have been tested and have led to several therapeutically available anticancer drugs

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derived from plants such as vinca alkaloids from the *Catharanthus roseus* plant (Wendell *et al.*, 1993), damnacanthal from *Morinda citrifolia* (Hisawa *et al.*, 1999), diterpene lactone andrographolide, neoandrographolide and three minor diterpene constituents from *Andrographis paniculata* (Chan *et al.*, 1971; Balmain and Gonnolly, 1973). Phenolic compounds, like gallic acid, ethyl gallate and luteolin with antineoplastic activities against a panel of human cancer cell lines have been isolated from the stem bark and leaves of *T. arjuna* (Roxb.). It is estimated that of the 250,000 to 500,000 plant species available, only a small percentage has been properly studied in terms of their pharmacological properties (Rates, 2001). Cardellina *et al.* (1999) reported that cytotoxicity screening models are the preliminary methods for selection of active plant extracts against cancer.

Hence, in this study, three local herbs (*Tectaria singaporeana*, *Blechnum orientale* and *Tacca integrifolia*) which are commonly found in the lowland Dipterocarp forests of Malaysia as well as widely known for their medicinal properties through undocumented traditional preparations were screened for their cytotoxic potential on human breast cancer cells.

MATERIALS AND METHODS

Sample Collection

Three plant specimens *Tectaria singaporeana*, *Blechnum orientale* and *Tacca integrifolia* were collected from the Air Hitam Forest Reserve, a Lowland Dipterocarp Forest situated in Puchong, Selangor. The plants were randomly selected in areas located in Compartment 15. The plants, especially the roots were washed thoroughly. Plants were then separated into three portions i.e., roots, leaves and stems. The dried plants were cut or hammered into small pieces. The dried samples were then pulverized with grinders and the powder was kept in air-tight plastic bags.

Sample Extraction

Plant specimens that have been ground were soaked in a mixture of methylene chloride and methanol at a ratio of 1:1 at room temperature. The solvent was filtered using a Whatman filter paper No. 1 and the marc was then soaked in methanol for 30 min. The solution was then filtered and pooled together with methylene chloride-methanol extracts to increase the content of organic solvent fraction. The solvent of the extraction was removed using a rotor evaporator (IKA Rotary Evaporator, RV05-ST) with a water bath temperature not exceeding 55°C. The concentrate was then transferred into Bijour bottles and the residual solvent was removed. A final drying occurred by placing the concentrate extract in an incubator. The final dried extract was weighed and stored under -20°C.

Sample Preparations

Dried samples were measured and dissolved in DMSO (Dimethylsulphoxide) with 4X penicillin/streptomycin to make a stock concentration of 100 µg mL⁻¹. The stock solution were diluted 10-folds serial to prepare the extract concentration ranging from 0.1, 1, 10 and 100 µg mL⁻¹. These drug concentrations were then used for the cytotoxic analysis.

Cell Culture

Human Breast Cancer (HBC) cells, MCF-7wt., provided by the Cancer Research and Drug Discovery Laboratory, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, were grown in RPMI 1640 standard culture medium supplemented with 10% Heat Inactivated Fetal Calf Serum (HIFCS), 2 mM L-glutamine with 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin in 25 and 75 cm² tissue culture flask under conventional culture conditions, such as 37°C, 5%CO₂, 95% air and 100% relative humidity. Adherent cancer cells approaching 80% confluency were harvested with trypsin-EDTA to detach the cells from the culture flask. The cells were then collected in a fresh medium and were sub-cultured at densities of 1.0 -5.0×10⁵ cells/10 m into 25 cm² flask every 3-4 days as being adopted by Stanslas (1998).

Cytotoxic Assay

The log phase cells with viability of 95% were mildly trypsinized (trypsin 0.5 mg mL⁻¹; EDTA 0.2 µg mL⁻¹) to detach the cell from the culture flask. Cells were then collected in fresh medium. A syringe (21G) was used to prepare a suspension of single cells in order to determine the cell number. Appropriate number of cells in 180 µL of medium was transferred to a 96-well flat bottom tissue culture plate. The cells were allowed to attach for about 24 h before the drug was introduced. The extract concentrations were obtained by a serial dilution of the 100 mg mL⁻¹ of stock solutions in the culture medium, thus decreasing the concentrations to 1 mg mL⁻¹, 100, 10 and 1 µg mL⁻¹. A volume of 20 µL from these concentrations of extracts (1 µg mL⁻¹-1 mg mL⁻¹) was added into each well to yield a final concentration ranging from 0.1-100 µg mL⁻¹. The final mixture used for treating the cell contained not more than 1% of the solvent, the same for control wells as well. The plates were then incubated at 5% CO₂, 37°C, under high humidity for four days and the viability of the cells in the plates were determined using the Microculture Tetrazolium (MTT) assay. Fifty microliter of MTT (2 mg mL⁻¹) were added to each well, giving a final concentration of 0.4 mg mL⁻¹. The plates were incubated for 3-4 h for the living cells to convert the soluble MTT salt into insoluble purple formazon. The medium was then aspirated from the wells as completely as possible without disturbing the formazon crystals and cells on the plastic surface (Twentyman and Luscombe, 1987). Hundred microliter of DMSO were added to dissolve the purple formazon. The plate was then analyzed using the SOFTProMax spectrophotometer.

Data Analysis

The absorbance of the formazon solution at 550 nm was determined on the spectrophotometer. IC₅₀ values (dose of drug that produces a 50% reduction in the absorbance compared to the control) were determined from the dose-response cytotoxic curves. Data obtained were analyzed using the 2-Way Analysis of Variance (ANOVA) and Duncan's Mean Separation Tests using the percentage of cell viability and IC₅₀ values as parameters

RESULTS AND DISCUSSION

Cytotoxic Potential of Plant Extracts

Medicinal plants have proven to be an invaluable source of drugs for the advancement and efficacy of modern medicine. Indeed, despite all the advances in biotechnology and synthetic chemistry, plants are still an indispensable source of medicinal preparations, both preventive and curative. Accordingly, several therapeutic properties of medicinal plants are known in obstetrics and gynaecology (Abo *et al.*, 2000), in respiratory disorders (Neto *et al.*, 2002), in skin disorders (Graf, 2000) in cardiac cases (Ankli *et al.*, 2002) and many more. In addition, several studies have demonstrated that medicinal plants could bring to the identification of antitumor compounds as recently suggested by Fumoleau *et al.* (1995) and Mantle *et al.* (2000).

In the pursuit of breast cancer treatment and prevention, methanolic extracts of three different types of plants were tested on the MCF-7wt. breast cancer cell lines at various concentrations ranging from 0.1-100 µg mL⁻¹. The extracts were obtained from three different parts of the individual plants namely the leaves, stems and roots. MCF-7wt. cells containing oestrogen receptor (ER⁺) were used as a model for studying the events associated with response to chemotherapy of ER⁺ breast cancer cells (Perry *et al.*, 1995). These cells are able to exhibit fast responses such as inhibition effects and therefore serve as a suitable model.

There were significant differences in the mean values for the various plant parts and dilutions used (Table 1). However, there were no significant differences in the mean values for the plant types when tested against cell viability.

Table 1: ANOVA summary: Analysis of variance for 3 factors (species of plant, parts of plants, dilution levels) and their interactions

Source	df	Sum of square	F-value
Total	539	223102.89	
Species	2	88.08	0.45 ^{ns}
Parts	2	16982.79	87.33*
Dilution	4	91715.28	235.81*
Species×Parts	4	5767.76	14.83*
Species×Dilution	8	2849.94	3.66*
Parts×Dilution	8	44250.03	56.89*
Species×Parts×Dilution	16	13318.41	8.561*
Residual	495	48130.59	

*Significance level at $p < 0.05$, ns: non-significant

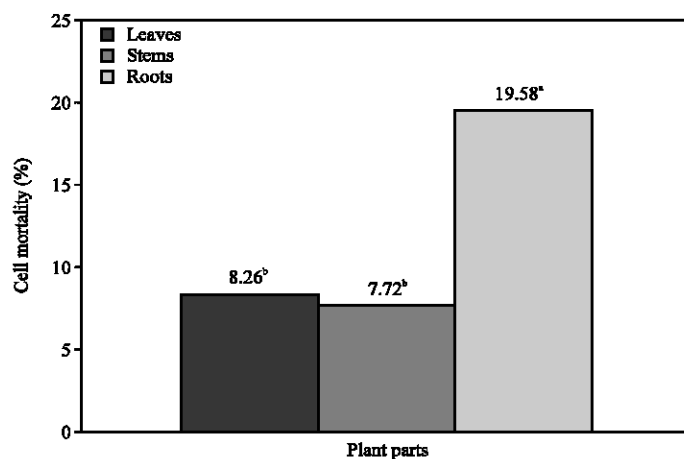


Fig. 1: Mean separation test for parts of plants. Different letter(s) indicate significant differences at $p < 0.05$

The roots displayed the highest cell mortality at 19.58% compared to the leaves and stems as shown in Fig. 1. As for the various drug dilutions that were tested against the cell line, the results show that the highest significance is at $100 \mu\text{g mL}^{-1}$ with a cell mortality of 36.59%. Generally, the mechanism of cell death induced after treatment is dependent on the drug, its concentration and the particular cell line used for the study. This was also suggested by Junaidah (1997), where observations were made between 10 and $30 \mu\text{g mL}^{-1}$ of drug concentrations on cell lines. The results indicated that a higher drug concentration caused much severe damages to the cell line than at a lower concentration.

The parameters measured were according to the National Cancer Institute (NCI) guidelines in which $\text{IC}_{50} < 100 \mu\text{g mL}^{-1}$ is considered active. The IC_{50} value shows the inhibition concentration at which only 50% of the cells are viable. The criteria of cytotoxic activity for the crude extracts, as established by the American National Cancer Institute (NCI) is an $\text{IC}_{50} < 30 \mu\text{g mL}^{-1}$ in the preliminary assay (Suffness and Pezzuto, 1990). According to Quah (1995), crude materials that require a drug concentration greater than $30 \mu\text{g mL}^{-1}$ to exhibit cytotoxicity were not considered as cytotoxic agents. Therefore, among all the three plants tested based on their roots, leaves and stems, the roots of *Tectaria singaporeana*, TS displayed the most cytotoxic potential with an IC_{50} value of $28.57 \mu\text{g mL}^{-1}$ as shown in Table 2.

As for *Tectaria singaporeana*, the results obtained suggest that the root of this fern is a cytotoxic agent since it is most effective against the breast cancer cell as compared to the other parts of the plants. The percentage of cell viability obtained from the root extracts were 16.62% which were far

Table 2: IC₅₀ values (µg mL⁻¹) of roots of TCI, BO and TS in MCF-7wt. breast cancer cell lines. Values are mean of 3 separate determinations and errors represent the SD values

Plant extracts	MCF-7wt.
<i>Tectaria singaporeana</i> (TS)	28.57±11.74
<i>Blechnum orientale</i> (BO)	32.07±7.85
<i>Tacca integrifolia</i> (TCI)	95.03±17.49

lower than those of the leaves (88.46%) and stems (90.68%). However, no studies were done on this particular fern species regarding their medicinal value or cytotoxicity against cancer cells. It is difficult to compare the findings from this study to other previous studies in terms of IC₅₀ and cell viability due to the lack of studies conducted.

In our screening of *Blechnum orientale*, the roots of the plant showed the lowest percentage of cell viability that is 31.47% as compared to the leaves and stems of the plant, 81.44 and 89.05%, respectively. The roots also displayed an IC₅₀ value of 32.065 µg mL⁻¹ where changes significantly occurred from 10 to 100 µg mL⁻¹ of drug concentrations. Earlier studies have shown that *Blechnum orientale* and *Blechnum spicant* contain presumed caffeic acid derived lignans, e.g., the 8-2-linked (-) blechnic and (-) brainic acids (Wada *et al.*, 1992; Davin *et al.*, 2003). Based on existing chemotaxonomic data, lignans are present in primitive plants, such as the fern *Blechnum orientale* (Wada *et al.*, 1992) in which these lignans take the role of phytoestrogens and thus are currently being studied for possible use in cancer prevention, particularly breast cancer.

As for *Tacca integrifolia*, significant changes occurred between 10 to 100 µg mL⁻¹ concentrations for the various parts. The methanol/methylene chloride extracts of TCI leaves and stems showed an average cell viability percentage of 52.36 and 80.78%, respectively. However, the root extracts displayed the lowest cell viability percentage of 46.48% with an IC₅₀ value of 95.03 µg mL⁻¹. No studies have been reported for the cytotoxic effects of this plant, therefore comparisons of this study were made based on research findings made by Tinley *et al.* (2003) in which a new class of microtubule-stabilizing agents, the taccalonolides were identified. Taccalonolide A was first isolated from *Tacca plantaginea* by Chen *et al.* (1987) and Taccalonolide E was isolated in 1991 (Shen *et al.*, 1991). In this study, the roots of *Tacca chantrieri* also contained the active constituents, taccalonolides E and A, which are complex, highly oxygenated steroids. These drugs caused the formation of abnormal mitotic spindles, thus inhibiting the mitotic progression and causing a G₂-M arrest in cancer cells. It is possible that taccalonolides could account for one of the active compounds responsible for the observed cytotoxic activity on the cancer cells.

CONCLUSIONS

From this study, we can conclude that different parts of the plants such as the roots, leaves and stems produce different effects on the MCF-7wt. cell cancer lines. Only the roots of *Tectaria singaporeana* showed potential cytotoxicity effects on MCF-7wt. cell cancer lines with the lowest IC₅₀ value of 28.57 µg mL⁻¹. Further investigation of the active extracts and isolated compounds in animal models for the therapeutic efficacy and toxicity would provide evidence to determine whether these medicinal plants could be beneficial for drug discovery.

REFERENCES

- Abo, K.A., A.A. Adeyemi and D.A. Adeite, 2000. Ethnobotanical survey of plants used in the treatment of infertility and sexually transmitted diseases in Southeast Nigeria. Afr. J. Med. Sci., 29: 325-327.

- Ankli, A., M. Heinrich, P. Bork, L. Wolfram and P. Bauerfeind *et al.*, 2002. Yucatec mayan medicinal plants: Evaluation based on indigenous uses. *J. Eth.*, 79: 43-52.
- Balmain, A. and J.D. Gonnolly, 1973. Minor diterpenoid constituents of *Andrographis paniculata* nees. *J. Chem. Soc. Perkin Trans.*, 1: 1247-1251.
- Cardellina, II, J.H., R.W. Fuller, W.R. Gamble, C. Westergaard and J. Boswell *et al.*, 1999. Evolving Strategies for the Selection, Dereplication and Prioritization of Antitumor and HIV-Inhibitory Natural Products Extracts. In: *Bioassay Methods in Natural Product Research and Development*, Bohlin, L. and J.G. Bruhn (Eds.). Kluwer Acad. Publ., Dordrecht, pp: 25-36.
- Chan, W.R., D.R. Taylor, C.R. Willis and R.L. Bodden, 1971. The structure and stereochemistry of neoandrographolide, a diterpene glucoside from *Andrographis paniculata*. *Tetrahedron*, 27: 5081-5091.
- Chen, Z.L., B.D. Wang and M.Q. Chen, 1987. Steroidal bitter principles from *Tacca plantaginea*. Structures of taccalonolide A and B. *Tetrahe. Lett.*, 28: 1673-1678.
- Davin, L.B., C.Z. Wang, G.L. Helms and N.G. Lewis, 2003. [¹³C]-Specific labeling of 8-2_ linked (-)-*Cis-blechnic*, (-)-*Transblechnic* and (-)-*Brainic* acids in the fern *Blechnum spicant*. *Phytochemistry*, 62: 501-511.
- Fumoleau, P., B. Chevallier, P. Kebrat, V. Dieras and N. Azli *et al.*, 1995. Current status of Taxotere as a new treatment in breast cancer. *Breast Cancer Res. Treat.*, 33: 39-46.
- Graf, J., 2000. Herbal anti-inflammatory agents for skin disease. *Skin Ther. Lett.*, 5: 3-5.
- Hisawa, T., Y. Arase, Z. Chen, K. Kita and K. Umezawa *et al.*, 1999. Stimulation of ultraviolet-induced apoptosis of human fibroblast UVr-1 cells by tyrosine kinase inhibitors. *Feder. Eur. Biochem. Soc. Lett.*, 444: 173-176.
- Junaidah, M., 1997. Cytotoxic activity of damnacanthal in human T-cell acute lymphoblastic leukaemia. B.Sc. Thesis, Faculty of Biomedical and Health Science, Universiti Putra Malaysia, pp: 57
- Lester, S. and R. Cotran, 1999. Breast Cancer. In: *Robbins Pathologic Basis of Disease*. Cotran, R., V. Kumar and T. Collins (Eds.). Saunders, Philadelphia, pp: 1093-1120.
- Mantle, D., T.W. Lennard and A.T. Pickering, 2000. Therapeutic applications of medicinal plants in the treatment of breast cancer: A review of their pharmacology, efficacy and tolerability. *Adverse Drug Reac. Toxi. Rev.*, 19: 223-240.
- Neto, C.C., C.W. Owens, R.D. Langfield, A.B. Comeau and J.S. Onge *et al.*, 2002. Antibacterial activity of some Peruvian medicinal plants from the Callejon de Huaylas. *J. Ethnopharma*, 79: 133-138.
- Parris, B.S. and A. Latiff, 1997. Towards a pteridophyte flora of Malaysia: A provisional checklist of taxa. *Malayan Nat. J.*, 50: 235-280.
- Perry, P.R., Y. Kang and B. Greaves, 1995. Effects of tamoxifen on growth and apoptosis of oestrogen-dependent and independent human breast cancer cells. *Ann. Surg. Oncol.*, 2: 145-238.
- Quah, B.A., 1995. Cytotoxic activity of *Goniothalamin* on different types of cancer cell lines. B.Sc. Thesis, Biotechnology, University Putra Malaysia.
- Rates, S.M.K., 2001. Plants as source of drugs. *Toxicon*, 39: 603-613.
- Shen, J., Z. Chen and Y. Gao, 1991. The pentacyclic steroidal constituents of *Tacca plantaginea*. Taccalonolide E and A. *Chin. J. Chem.*, 9: 92-94.
- Stanslas, J., 1998. Mechanisms of antitumour activities of novel polycyclic acridines. Ph.D Thesis, University of Nottingham.
- Suffness, M. and J.M. Pezzuto, 1990. Assays Related to Cancer Drug Discovery. In: *Methods in Plant Biochemistry: Assays for Bioactivity*, Hostettmann, K. (Ed.). Vol. 6. Academic Press, London, pp: 71-133.

- Tinley, T.L., D.A. Randall-Hlubek, R.M. Leal, E.M. Jackson and J.W. Cessac *et al.*, 2003. Plant-derived steroids with microtubule stabilizing activity. *Cancer Res.*, 63: 3211-3220.
- Twentyman, P.R. and M. Luscombe, 1987. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br. J. Cancer*, 56: 279-285.
- Wada, H., T. Kido, N. Tanaka, T. Murakami, Y. Saiki and C.M. Chen *et al.*, 1992. Chemical and chemotaxonomical studies of ferns. LXXXI. Characteristic lignans of *Blechnaceous ferns*. *Chem. Pharma. Bull.*, 40: 2099-2101.
- Wendell, K.L., L. Wilson and M.A. Jordan, 1993. Mitotic block in HeLa cells by vinblastine: Ultra structural changes in kinetochore-microtubule attachment and in centrosomes. *J. Cell Sci.*, 104: 261-274.