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CYTOTOXIC EFFECTS AND EVIDENCE OF APOPTOSIS FROM *Avecennia alba EXTRACTS* ON HUMAN BREAST CANCER CELL LINE (MCF-7)

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Abstract: Breast cancer is the second leading cause of cancer death and the most common form of cancer affecting women worldwide. The search for natural products for cancer therapy is an area of great interest. The main aim of this study was to determine the cytotoxic effects and the mechanisms of cell death of the crude extracts from mangrove plant, *Avecennia alba* on human breast carcinoma cell line, MCF-7. Diethyl ether, butanol and methanol extracts were prepared from the leaves of *Avecennia alba*. Cytotoxicity study using MTS assay demonstrated that all the three extracts produced dose-dependent inhibition on MCF-7 cell growth, albeit at different levels. Diethyl ether extracts produced the most potent cytotoxicity, followed by butanol and methanol extracts with IC_{50} values of 25.1, 27.1 and 28.9 µg/mL, respectively, after 72 h incubation. The DeadEnd™ Colorimetric Apoptosis Detection System suggested that all the three extracts exerted cytotoxicity on MCF-7 cells via apoptosis. TLC profiling demonstrated the presence of phenolic and alkaloid compounds in methanol, diethyl ether and butanol extracts which may be responsible for mediating the cytototoxicity. The methanol, diethyl ether and butanol extracts of *Avecennia alba* may contain potential compounds to be developed as anti-cancer agents against breast cancer.

Keywords: *Avecennia alba,* extract, MCF-7 cells, cytotoxicity, apoptosis.

Introduction

Cancer is fast becoming one of the leading causes of death in the developed countries. However, in the past few decades, the incidence of breast cancer has increased considerably in the developing countries, where about 82% of world population resides, due to the growth and aging population (Parkin, 1998; Ferlay *et al*., 2015). It is estimated that 59% of the total breast cancer cases in 1990, occur in developed countries which account for less than 25% of the total female population (Jemal *et al*., 2011; Youlden *et al*., 2012). In less developed countries, the rate of breast cancer incidence in female population increases to 50% in 2008 and 53% in 2012 (Ferlay *et al*., 2015). Breast cancer has been the most common type of cancer in Asia-Pacific region, and in Malaysia, breast cancer has been identified as having the highest incidence rates in female as well as in the overall population. The statistics suggests that out of 40 persons that have been diagnosed with female breast cancer in 100,000 population, 19 involve mortality cases (Youlden *et al*., 2014).

Natural products and related drugs are used to treat 87% of human diseases including bacterial infection, cancer and immunological disorders. About 25% of prescribed drugs in the world originate from plants and over 3000 species of plants have been reported to possess anti-cancer properties (Sheikh *et al.,* 2009). Mangrove trees are used traditionally as sources of non-wood forest products, food and pharmacological agents (Gerd & Monica, 2006). More than 200 bioactive metabolites have been isolated from the tropical and subtropical mangroves (Wu *et al.,* 2008). Based on studies on the chemical structures, most of the isolated compounds belong to steroids, triterpenes, saponins, flavonoids, alkaloids, tannins and phenolics, all of which have a wide range of therapeutic possibilities (Bandaranayake, 1998).

Conventional chemotherapy, surgery or radiation show some successes but with side-effects. It becomes pertinent to explore and discover the alternatives to conventional treatment with anti-tumor potential. It is reported that some mangrove leaves, fruits and bark have been used in traditional Asian folk remedies for the treatment of blood pressures, cancer, ulcer, wounds and AIDS (Premanathan *et al.,* 1999). Research is currently under way to understand whether the compounds produced by the mangrove plants may actually possess anti-cancer properties through the gene activation encoding the proteins which trigger cancer cells into self-destruction by apoptosis (Fulda & Debatin, 2006). The bioactive compounds present in mangroves may play important role in inhibiting the growth of breast cancer cells via apoptosis, thus, may be potentially developed as the candidate for chemotherapeutics.

In this study, the cytotoxic effects and cell death mechanisms of the mangrove plant *Avecennia alba* extracts, on human breast carcinoma cell line (MCF-7) were investigated.

Materials and Methods

Sample Collection and Extracts Preparation

The leaves of *A.alba* from the family of Avicenniaceae, were collected from Kuala Terengganu, Terengganu, Malaysia (N 05º 24' 54.2"; E 103º 05' 12.9"). Subsequently, 10 g of the powdered dried plant was extracted with methanol. The extract was then filtered, concentrated and fractionated with diethyl ether and butanol to produce methanol, diethyl ether and butanol extracts.

Determination of Cytotoxic Activity

Human breast carcinoma MCF-7 cell line (ATCC, USA) was seeded overnight at a density of 6×10^4 cells/well in 96-well plate in Roswell Park Memorial Institute (RPMI) 1640 culture medium. Cells were treated with different concentrations of *A. alba* extracts (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 µg/mL). Negative and positive control cells were treated with the DMSO and vincristin sulphate (Sigma, USA), respectively. The cells were then incubated in CO2 incubator at 37ºC for 24, 48 and 72 h. The number of live cells present in each well was determined using MTS assay (CellTiter 96® AQueous One Solution Proliferation Assay, Promega, USA) as described in manufacturer's instructions. The absorbance was read at 490 nm using the plate reader and the percentage of inhibition was measured using the following formula:

$$Inhibition\ (\%) = \frac{[(OD\ control - background) - (OD\ test - OD\ background)]}{(\text{OD } control - \text{OD } blank)} \times 100\%$$

Equation 1

Detection of DNA Fragmentation

MCF-7 cells (6×10^4 cells /mL) were cultured in 8 well-slide chamber and incubated in CO_2 incubator at 37 ºC. After overnight incubation, the cells were treated with the extracts at IC_{50} concentrations for 72 h. Untreated control cells were treated with DMSO. Positive control cells were treated with DNase I (1U/mL). Cells were subsequently incubated for 12, 24 and 36 hr. After treatment, cells were washed with Phosphate Buffered Saline (PBS) twice and subsequently processed according to the Deadend™ Colorimetric Apoptosis Detection System (Promega, USA) protocol as described by the manufacturer's instruction.

Chemicals Profiling Using Thin Layer Chromatography (TLC)

The *A. alba* crude extracts were spotted on the TLC plate and developed in a TLC chamber using suitable mobile phases which were hexane: ethyl acetate (70:30) for diethyl ether extract, dichloromethane: methanol (80:20) for methanol extract and ethyl acetate: methanol: water (30:6:5) for butanol extract. The developed plates were air dried, heated and observed

under UV light at both 254 nm and 365 nm. The plate was also sprayed with iodine vapour, aniseldehyde and Dragendorff's reagents.

Statistical Analysis

The mean and standard error mean (SEM) were calculated using Graph Pad Prism software (Graphpad, USA). The significance of differences were determined using one way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test using SPSS 20.0 software (IBM SPSS Software, US). The IC_{50} values were derived from a non-linear regression model (curvefit) based on sigmoidal dose response curve (variable) and computed using GraphPadPrism.

Results

Cytotoxic Effects

Cytotoxicity study was carried out using methanol, diethyl ether and butanol extracts of *A. alba* on human breast carcinoma cell line, MCF-7. In all cases, there was a dose-dependent inhibition of MCF-7 cell growth especially when the cells were treated with higher concentrations (Figure 1). The methanol extracts (Figure 1a) at 3.12 µg/mL inhibited MCF-7 cells by 18%, 19% and 27% as compared to the untreated control for 24, 48 and 72 h treatment, respectively. The levels of inhibition increased from 20% to 67%, 27% to 67%, and, 39% to 69%, for 24, 48 and 72 h treatment, respectively, at 12.5 µg/mL and 100 µg/mL extract concentrations. The IC_{50} values of 56.2 µg/mL, 50.1 µg/mL and 28.9 µg/mL were determined for 24, 48 and 72 h treatment, respectively, indicated that only after 72 h treatment, the extract exhibit potent cytotoxicity against MCF-7 cells. Based on the criteria set by the National Cancer Institute, USA, an extract with IC_{50} value of only less than 30 mg/mL can be categorised as cytotoxic against the treated cancer cells (Geran *et al*., 1972).

Similar pattern of cell growth was observed when the cells were treated with diethyl ether extract where the levels of inhibition increased at higher concentrations. The MCF-7 cell growth was inhibited in the range of 15-23% and increased to 40-52% and 64-81% at 0.39, 25 and 100 µg/mL, respectively, for all incubation periods. The extracts produced potent cytotoxic activity at 72 h treatment with IC_{50} value of 25.1 µg/mL, and not at 24 and 48 h treatments which exhibited the values of 47.7and 31.6 µg/mL, respectively (Figure 1b).

The cell growth inhibition also increased when MCF-7 cells were treated with butanol extracts at 1.56 µg/mL (17%, 17% and 10%), 25 µg/mL (39%, 41% and 43%), and 50 µg/mL (47%, 56% and 71%) after 24, 48 and 72 h treatments, respectively. At 100 µg/mL, the levels of inhibition were less than 50% when the cells were treated for 24 h as compared to 58% and 76% at 48 and 72 h, respectively. Therefore, no IC_{50} value was determined at 24 h incubation. However, the IC_{50} values of the extract were 47.7 and 27.1 µg/mL at 48 h and 72 h, respectively, suggesting that the butanol extracts also produced potent cytotoxic activity on MCF-7 cells after 72 h treatment (Figure 1c).

Apoptosis Assay

All the three crude extracts of *A. alba* produced potent cytotoxicity on MCF-7 cell line after 72 h treatment. All extracts were therefore subjected to apoptosis assay and the concentrations of IC_{50} at 72 h were used to treat the cells for 12, 24 and 36 h. Figure 2 shows that the methanol extract produced dark brown stained nuclei of MCF-7 cells at all the three time points clearly indicating the presence of DNA fragmentation (Figure 2a-c). Similarly, diethyl ether and butanol extracts triggered DNA fragmentation in MCF-7 cells judging from the presence of dark-stained nuclei. The positive control cells treated with DNase I also indicated the presence of DNA fragmentation but no distinct nuclei-stained cells were observed in the DMSO-treated negative control cells. It is widely demonstrated that DNA fragmentation is the hallmark of apoptosis (Bowen *et al*., 1999). These results strongly suggest that all the three extracts of *A. alba* killed MCF-7 cells via apoptosis.

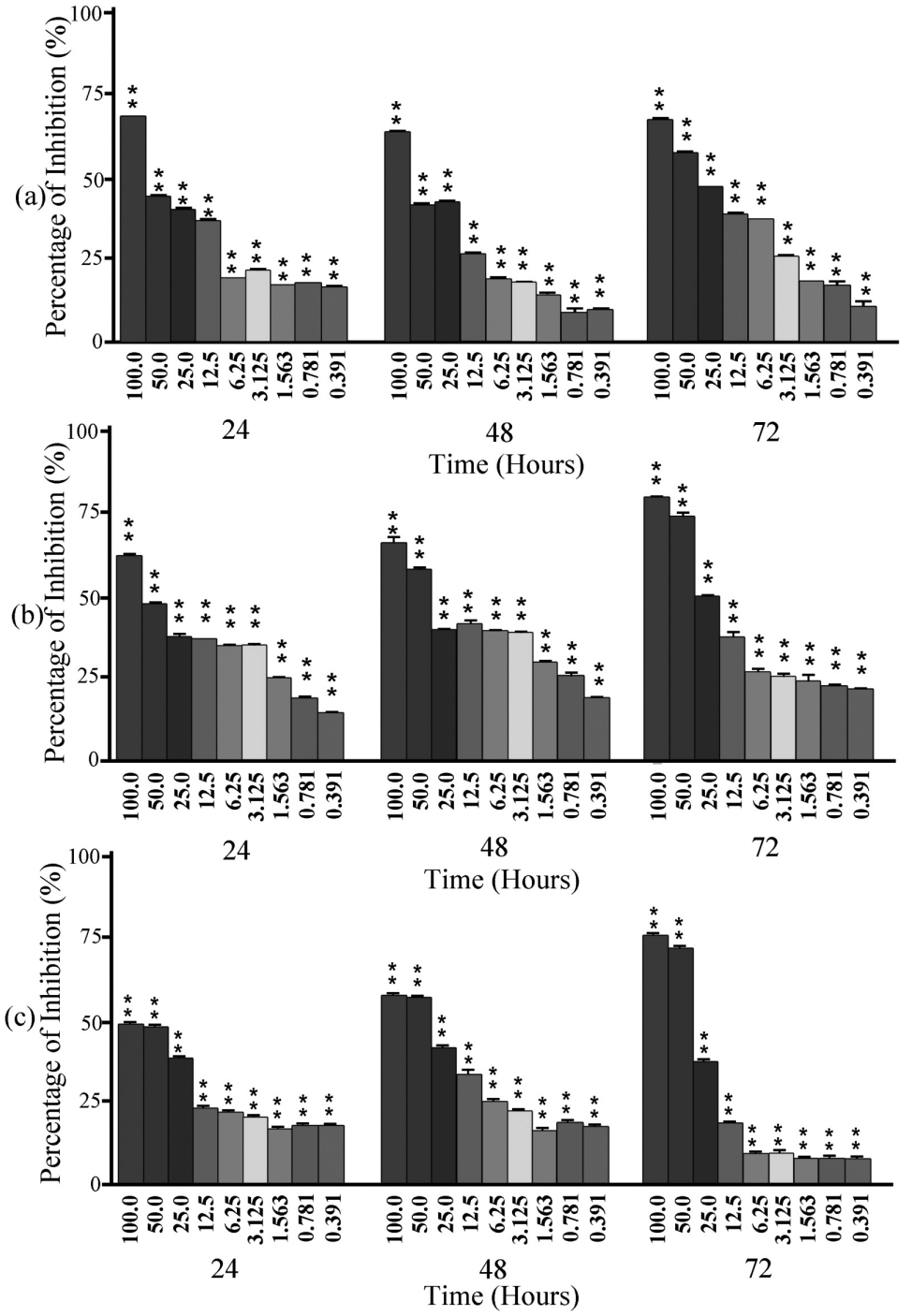

Figure 1: Cell growth inhibition for *A. alba* (a) methanol, (b) diethyl-ether and (c) butanol extracts against MCF-7 cell at 24, 48 and 72 h. Each value represents mean ± SEM of nine replicates (n=3) (three independent experiments). ANOVA ***P<0.01* (Dunnett post-test). The value below each bar represents the concentration of extracts used to treat the cells (mg/mL)

Thin Layer Chromatography Analysis

As shown in Figure 3(a-c), the separation of compounds was observed at different solvent system ratios. When observed under the wavelengths of 254 and 365nm, the presence of spots indicated that all extracts contained conjugated carbon double bonds (C=C) and/or benzene ring. Based on iodine vapour profiling, the extracts were found to contain organic compounds, and the reaction with Dragendorff's and Anisaldehyde reagents suggested the presence of alkaloids and phenol-based compounds. In addition, the grey spots representing terpenes were also found in the crude extracts.

Discussion

Natural products play an important role in the discovery of new drugs. More than half of the newly approved drugs are of natural product origin or designed based on the structure of natural product, whereas the synthetic and synthetic with natural product mimic compounds contribute 40% of the new drugs (Newman & Cragg, 2007). Various clinically useful anti-tumor agents are also derived from plants which include paclitaxel, vincristine and camptothecin through various screening platforms (Pezzuto, 1997). Identification of medicinal plants with significant cytotoxic potential useful for the development of cancer therapeutics has gained increasing importance in the last decade, and

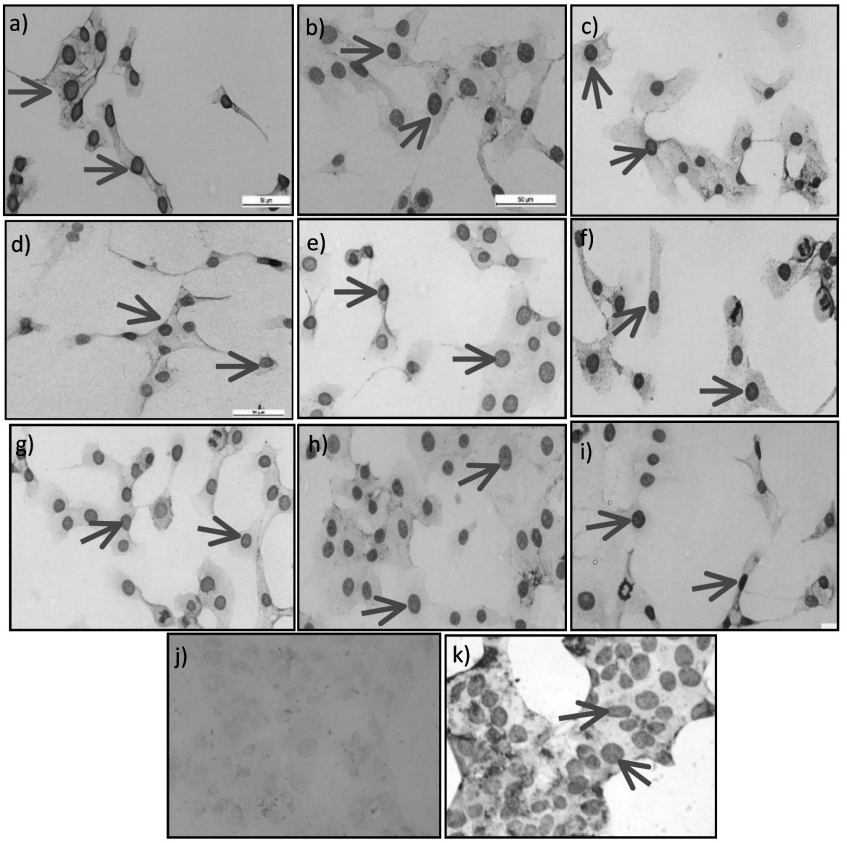

Figure 2: The presence of apoptotic cells in the MCF-7 cell line after the treatment of *A. alba*: methanol extract for (a) 12 h, (b) 24 h, (c) 36 h; diethyl ether extract for (d) 12 h, (e) 24 h, (f) 36 h; butanol extract for (g) 12 h, (h) 24 h, (i) 36 h; (j) untreated control [1% (v/v) DMSO] and (k) positive control [1 U/mL DNase] for 24 hr and stained with the Deadend™ Colorimetric Apoptosis Detection System. The image was observed under light microscope using 40X magnification. Apoptotic cells with stained nuclei are marked by arrows

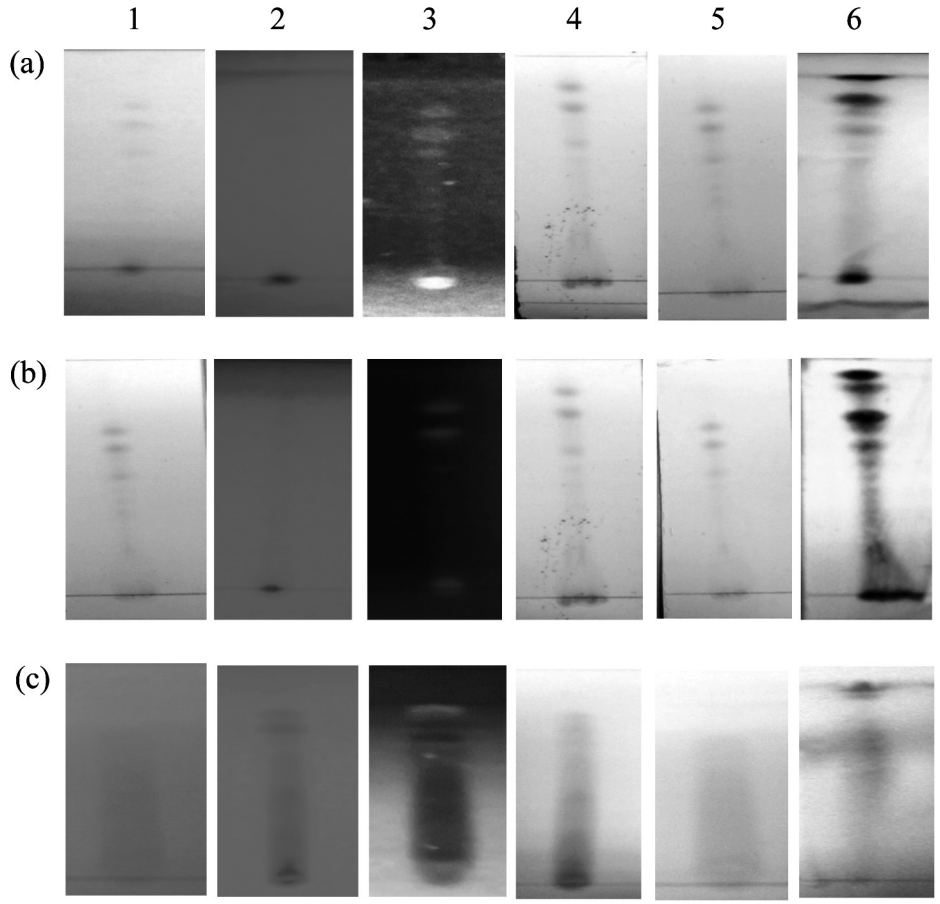

Figure 3: TLC profiling of *A. alba* from (a) methanol extract, (b) diethyl ether extract, (c) butanol extract under; (1) before spray, (2) UV_{254}, (3) UV_{365}, (4) Iodine vapour, (5) Dragendorff's reagent, (6) Anisaldehyde reagent

research in this field is expanding (Al-Kalaldeh *et al*., 2010).

The cytotoxic activity of various mangrove species against cancer cell lines has been widely studied. A study carried out by Samarakoon *et al.* (2016) demonstrates that out of 116 crude extracts of hexane, chloroform, ethyl acetate and methanol prepared from the leaves and stem barks of fifteen mangrove plants, 84 crude extracts produce the IC_{50} values of more than 100 mg/mL while others exhibit between 30 and 100 mg/mL, indicating that none of these mangrove species exert potent cytotoxicity on MCF-7 and human hepatocarcinoma cell line, HepG2. However, *A. alba* is not included in the study (Samarakoon *et al*., 2016). The methanol extract prepared from *A. alba* leaves reportedly produces selective cytotoxic activity on human breast cancer cell lines (MCF-7 and MDA-MB-231) even though the IC_{50} values are still above 30 mg/mL (Akter *et al*., 2013). The high IC_{50} values may be due to shorter treatment period of 48 h as compared to 72 h in our study. In fact, all extracts produced IC_{50} values of more than 30 mg/mL when MCF-7 cells were treated for 24 and 48 h in our study. Other mangrove species that have exhibited potent cytotoxic activity include the methanol extract from pneumatophore of *Xylocarpus mollucensis* with IC_{50} of 0.62 and 1.08 mg/mL against gastric and breast cancer cells, respectively (Sheikh *et al*., 2009).

It has been widely reported that the induction of apoptosis is one of the active strategies to arrest the proliferation of cancer cells (Steele, 2003). Apoptosis is the major focus and target for cancer research since the cells killed via this mode of cell death do not induce an inflammatory reaction which may lead to various adverse side effects (Steele, 2003). One of the hallmarks of apoptosis is the fragmentation of genomic DNA (Bowen *et al*., 1999; Elmore, 2007). The presence of dark brown stained nuclei of MCF-7 cells treated with the extracts strongly suggests that the cells are killed via apoptotic pathway. *Avicennia marina*, the most abundant and common mangrove species used as a traditional medicine has been found to have its leave extracts exerting cytotoxic effects on human cancer cell lines via apotosis due the presence of high phenolic and flavonoid contents (Huang *et al*., 2016).

Some of mangroves plants such as *Adiantum caudatum* and *Aegiceras corniculata* produce alkaloid compounds with anti-tumor activity. (The presence of terpenes and phenolic compounds explain the cytotoxicity effects of the extracts on MCF-7 cell line (Rouf *et al.,* 2007). Our study has shown the presence of the phenolics and terpenoids in the methanol, diethyl ether and butanol extracts of *A. alba* as also similarly reported in other mangrove plants (Achmadi *et al*., 1994; Raiham, 1994; Misra *et al*., 1984; Kato & Numata, 1972; Han *et al.,* 2004). The cytotoxicity and apoptotic effects exhibited by the extracts of *A. alba* on MCF-7 cell line may be due to the presence of similar compounds.

Conclusion

In this study, the extracts of mangrove plant *A. alba* were investigated for their cytotoxic effects on the human breast cancer cell line, MCF-7. The methanol, diethyl ether and butanol extracts of *A. alba* produced potent cytotoxicity with IC_{50} values of less than 30 µg/mL at 72 h. All the crude extracts exhibited potent cytotoxicity on MCF-7 cells via apoptosis. TLC profiling showed that the crude extracts contained alkaloid, phenolic and terpenoid compounds which may play important roles in killing the cells via apoptosis.

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References

Achmadi, S., Syahbirin, G., Choong, E. T., & Hemingway, R.W. (1994). Catechin-3-O-rhamnoside Chain Extender Units in Polymeric Procyanidins from Mangrove Bark. *Phytochemistry,* 35(1): 217-219.

Akter, R., Uddin, S. J., Grice, I. D., & Tiralongo, E. (2013). Cytotoxic Activity Screening of Bangladeshi Medicinal Plant Extracts. *Journal of Natural Medicines,* 68(1): 246-252.

Al-Kalaldeh, J. Z., Abu-Dahab, R., & Afifi, F. U. (2010). Volatile Oil Composition and Antiproliferative Activity of *Laurus nobilis, Origanum syriacum, Origanum vulgare,* and *Salvia triloba* Against Human Breast Adenocarcinoma Cells. *Nutrition Research,* 30: 271-278.

Bandaranayake, W. M. (1998). Traditional and Medicinal Uses of Mangroves. *Mangroves and Salt Marshes,* 2(3): 133-148.

Bowen, J. M., Towns, R., Warren, J. S., & Landis Keyes, P. (1999). Luteal Regression in the Normally Cycling Rat: Apoptosis, Monocyte Chemoattractant Protein-1, and Inflammatory Cell Involvement. *Biology Reproduction,* 60(3):740-746.

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology,* 35: 495-516.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., & Bray, F. (2015). Cancer

Incidence and Mortality Worldwide: Sources, Methods and Major Patterns in GLOBOCAN 2012 *International Journal of Cancer,* 136: E359-E386.

Fulda, S., & Debatin, K.M. (2006). Extrinsic Versus Intrinsic Apoptosis Pathways in Anticancer Chemotherapy. *Oncogene,* 25: 4798-4811.

Geran, R. I., Greenberg, N. H., Macdonald, M. M., Schumacher, A. M., & Abott, B. J. (1972). Protocols for Screening Chemical Agents and Naturals Products Against Animal Tumours and Other Biological Systems. *Cancer Chemotheraphy Reports,* 3(2): 1-61.

Gerd, L., & Monica, T. R. (2006). New Guinean Mangroves Traditional Usage and Chemistry of Natural Products. *Senckenbergiana Maritime,* 36(1): 1-10.

Han, L., Huang, X., Sattler, I., Dahse, H. M., Fu, H., Lin, W., & Grabley, S. (2004). New Diterpenoids from the Marine Mangrove *Bruguiera gymnorrhiza*. *Journal of Natural Products,* 67: 1620-1623.

Huang, C., Lu, C. K., Tu, M. C., Chang, J. H., Chen, Y. J., Tu, Y. H., & Huang, H. C. (2016) Polyphenol-rich *Avicennia* Marina Leaf Extracts Induce Apoptosis in Human Breast and Liver Cancer Cells and in a Nude Mouse Xenograft Model. *Oncotarget,* 7(24): 35874-35893.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). "Global Cancer Statistics". *Cancer Journal for Clinical,* 61(2): 69-90.

Kato, A., & Numata, M. (1972). Brugierol and Isobrugierol, Trans- and cis-1, 2-dithiolane-1-oxide, from Brugiera Conjugate. *Tetrahedron Letter,* 13: 203-206.

Misra, S., Choudhury, A., Datta, A. K., & Ghosh, A. (1984). Sterols and Fatty Acids from Three Species of Mangrove. *Phytochemistry,* 23(12): 2823-2827.

Newman, D. J., & Cragg, G. M. (2007). Natural Products as Sources of New Drugs Over the Last 25 Years. *Journal of Natural Product,* 70: 461-77.

Parkin, D. M. (1998). The Global Burden of Cancer. *Seminar in Cancer Biol*ogy, 8: 219-235.

Pezzuto, J. M. (1997). Plant-derived Anticancer Agents. *Biochemical Pharmacology,* 53(2): 121-133.

Premanathan, M., Kathiresan, K., Yamamoto, N., & Nakashima, H. (1999). *In vitro* Anti-human Immunodeficiency Virus Activity of Polysaccharide from *Rhizophora mucronata* Poir. *Biological Science, Biotechnological, Biochemistry,* 63: 1187-1191.

Raiham, S. M. A. (1994). *ACGC Chemical Research Communication,* 2: 21-24.

Rouf, R., Uddin, S. J., Shilpi, J. A., & Alamgir, M. (2007). Assessment of Antidiarrhoeal Activity of the Methanol Extract of *Xylocarpus granatum* Bark in Mice Model. *Journal of Ethnopharmacology,* 109:539.

Samarakoon, S. R., Shanmuganathan, C., Ediriweera, M. K., Piyathilaka, P., Tennekoon, K. H., & Thabrew, I. (2016). Screening of Fifteen Mangrove Plants Found in Sri Lanka for *in-vitro* Cytotoxic Properties on Breast (MCF-7) and *Hepatocellular carcinoma* (HepG2) Cells. *European Journal of Medical Plants,* 14(4): 1-11.

Uddin, S. J., Grice, I. D., & Tiralongo, E. (2011). Cytotoxic Effects of Bangladesh Medicinal Plant Extracts. *Evidence-Based Complementary and Alternative Medicine,* 2011: 1-7.

Steele, V. E. (2003). Current Mechanistic Approaches to the Chemoprevention of Cancer. *Journal of Biochemistry and Molecular Biology,* 36(1): 78-81.

Wu, J., Xiao, Q., Xu, J., Li, M. Y., Pana, J. Y., & Yang, M. (2008). Natural Products from True Mangrove Flora: Source, Chemistry and Bioactives. *Natural Product Report,* 25: 955-981.

Youlden, D. R., Cramb, S. M., Dunn, N. A., Muller, J. M., Pyke, C. M., & Baade, P. D. (2012). The Descriptive Epidemiology of Female Breast Cancer: An International Comparison of Screening, Incidence, Survival and Mortality. *Cancer Epidemiol,* 36: 237-248.

Youlden, D. R., Cramb, S. M., Yip, C. H., & Baade, P. D. (2014). Incidence and Mortality of Female Breast Cancer in the Asia-Pacific Region *Cancer Biology and Medicine,* 11:101-115.