Concentration and Time Dependent Cytotoxic effect of Methanolic Crude Extracts of *Pseuduvaria macrophylla* on the Human Cancer Cell Line

Atiqah Aziz[1#], Mohd Zukiflee Abu Bakar[1], Farida Zuraina Mohd Yusof[2]

1 Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, MALAYSIA.
2 Faculty of Applied Sciences, Universiti Technology MARA, Shah Alam, 40450, Selangor, MALAYSIA.
Corresponding author. E-Mail: eyqa@um.edu.my; Tel: +603-79672931; Fax: +603-79677887.

ABSTRACT The previous study demonstrated promising anti-cancer potential in methanolic crude extracts using single concentration, especially on breast cancer cells under 24 hours treatment. The present study investigates the effective concentration of bark and leaf methanolic extract on MCF-7 breast cancer cell line under 48 and 72 hours of treatment. The method employed was MTT assay to determine the half maximal inhibitory concentration (IC^{50}) of both extracts at different concentration under 48 & 72 hours of treatment. The IC50 was obtained by plotting the concentration (µg/mL) versus the percentage of inhibition of each extract. The MCF7 cell line had decreased response to both extracts within 72 hours but showing promising cytotoxicity within 48 hours especially for leaf methanolic extracts at concentration of 140 µg/mL ± 0.23 to inhibit 50% of tested cancer cell line, meanwhile the medium inhibitory concentration (IC^{50}) of bark methanolic extract on MCF7 cells was 242 µg/mL ± 0.13. The results showed that the the IC^{50} of leaf methanolic extracts was comparably lower than the IC^{50} of bark methanolic extracts. In fact, leaf methanolic extracts demonstrated better efficacy on the MCF7 after been treated within 48 hours compared to 72 hours. In other words, leaf methanolic extract more potent than bark methanolic extracts.

KEYWORDS: Annonaceae, MTT assay, IC^{50}, MCF-7 cell line, cell culture

Full Article - *Medical biotechnology*
Received 14 September 2017 **Revised** 6 November 2017 **Accepted** 14 November 2017 **Online** 28 December 2017

INTRODUCTION

Cancer remains one of the leading causes of morbidity and mortality globally. It also considered as an important health and socioeconomic problem where all of type of cancer are endemic. Cancer is the second leading cause of death, after cardiovascular disease (Mathers & Loncar, 2006; Lopez *et al*., 2001; Hoyert *et al*., 2006; Tunstall-Pedoe, 2005). Chemotherapy is the use of medicines or drugs to treat cancer and also works as routine treatment for cancer other than radiotherapy and surgery. Over the years, chemotherapy drugs have successfully treated many people with cancer. However, chemotherapy is not always satisfactory in terms of its lack of effectiveness and also due to the toxicity associated to long-term treatments with empirically discovered drugs for example 5-fluorouracil is known to cause cardiotoxicity (Macdonald, 1999) and myelotoxicity (Rexroth & Scotland, 1994). Furthermore, it also could act as a vasospastic agent in rare but documented cases (Rastogi *et al*., 1993).

One of the most significant features of cancer cells is it lose many of the regulatory function present in normal cells. The cancer cells will divide continuously when normal cells do not therefore makes cancer cells vulnerable to chemotherapeutic drugs. Therefore, new chemotherapies are urgently needed to prevent and control this chronic disease. Traditional medical systems such as the use of plant-derived products in cancer treatment are one of the alternative approaches in cancer treatment as it may reduce adverse side effects. Historically, more than 3000 plant species that have reportedly been used in the treatment of cancer. Over 60% of currently used anti-cancer agents are

derived from natural sources, including plants, marine organisms and microorganisms (Newman *et al*., 2003). The discovery of anticancer agents in a natural product has started since the 1950s with the finding and development of vinblastine and vincristine. Vincristine and vinblastine were two vinca alkaloids derived from family Apocynaceae which has been discovered from the Madagascar periwinkle. These vinca alkaloids were famous anticancer agents clinically used for the treatment of lymphoma and leukemia (Casey *et al*., 1973).

Previously, a preliminary study on the antioxidant and anti-cancer activities have been done on stem bark and leaf of Pseuduvaria macrophylla (Annonaceae) extracted by methanol and hexane solvent against breast cancer cell line (MCF-7). For antioxidant activity, the most active extract was leaf and bark methanolic with the IC50 value of 50.08±0.3 µg/mL ($p<0.05$). The methanolic crude extracts also show better proliferation on human breast cancer cell (MCF-7) in the range of 80.70 µg/ml±0.08 - 94.96 µg/ml±3.57 compared to hexane crude extracts (Aziz *et al*., 2016).

MTT assay is an established method that to observe the cell proliferation of a cell line after exposure to a compound of interest (Mosmann, 1983 and Twentyman & Luscombe, 1887). The cytotoxicity of the extracts measured using inhibitory concentration (IC 50) which indicates the concentration to inhibit proliferation of cancer cells by 50%. The lower the IC 50, the lower its value the greater its potency to inhibit cell proliferation. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase such as succinate, NADH and NADPH in viable cells. The MTT enters the cells and passes into the mitochondria where it is reduced the yellow color of MTT to an insoluble, colored (dark purple) formazan product with adsorption range between 510 and 570 nm (Berridge & Tan, 1993 and Dhanjaland & Fry,1997).The cells are then solubilized with an organic. Spectrophotometric will be used to measure the released solubilized formazan reagent. The reduction of MTT can only occur in metabolically active cells, therefore the level activity of this assay is a measured of the viability of the cells.

The reaction of NADH dehydrogenase is:

$$NADH + H+ + CoQ + 4H+ \text{ in} \qquad NAD+ CoQH2 + 4H+ \text{ out}$$

During this process, four protons will be translocated across the inner membrane per molecule of oxidized NADH in order to build the electrochemical potential used to produce ATP (Newman *et a*l., 2003). Those proton produced by mitochondrial dehydrogenase will be taken up by MTT dye containing tetrazolium salt to convert the tetrazolium to formazan and the cell culture will undergo decolourisation from yellow to purple color. This circumstance occurs when the culture contains live cells. If the culture contains cell death, there will be no more dehydrogenase activity in their mitochondria, thus zero protons will be produced in the cells which means tetrazolium cannot be converted to formazan and the cells will not change color.

IC50 represents the concentration of a particular drug or other substances (inhibitor) that is required for 50% inhibition of given biological process. This method was used to measure the effectiveness of each concentration of the sample to inhibit 50% of a compound in inhibiting biological or biochemical function. A serial dilution of samples and standards were prepared before being subjected to any assay. This quantitative measure indicates how much of a particular drug or other inhibitor is required to inhibit biological process by half. In this assay, the IC50 value will determine through plotting the graph.

The objectives of this study are to determine antiproliferative effect of leaf and bark methanolic extract on breast cancer cell line at different concentration under 48 and 72 hours of treatment, to compare the efficacy of leaf and bark concentration on the human breast cancer cell lines, to determine IC50 of these two extracts, and to compare the cytotoxicity of these two extracts on breast cancer cell line.

MATERIALS

Analytical grade Fisher Scientific 90% Methanol was used to extract the bark and leaf of Pseuduvaria macrophylla. This species was collected from the lowland forest area in Kenong Forest Park, Malaysia. Voucher specimen (HIR 0009) was deposited at the herbarium, Chemistry Department of the University of Malaya. The human breast cancer cells MCF-7 was obtained from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's Modified Eagle's medium (DMEM) from Sigma Aldrich (Germany), 25cm2 tissue culture flasks (Corning, USA), Fetal Bovine Serum (FBS) from GIBCO (Life technologies, USA), The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) substance and all the other reagents and substances were obtained from Sigma Aldrich (Germany).

METHODOLOGY

Extraction

P. macrophylla leaves and bark ground samples have been cold extracted using 90% methanol. About 500 g of the sample has been placed portion by portion into a flask containing about 2 L of solvent (methanol). The solution was left for three days. Once completed, supernatant will be removed. Using the same residue it will be mixed again with 2 L of particular solvent. The sample will be left submerged in solvent for three days, and then filtered to obtain the solvent extract. After the extraction, solvent has been removed by means of rotary evaporator, yielding the extracted compounds.

Cell Culture

MCF-7 cells was grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated Fetal bovine serum, 2 mM glutamine, 1% penicillin and streptomycin. An inverted microscope was used to check the percentage of confluence (80-90%) and to confirm the absent f fungal and bacterial contamination. Cells were cultured in 25cm2 tissue culture flasks and 1.5 mL of trypsin was added into the flask before incubated at 37 C in a humidified incubator under 5% CO2 for 5 mins to detach the cells.

MTT cell proliferation assay

An amount of 1×104 human cancer cells per well were seeded into 96-well plate overnight. Cells were treated with various concentrations (0.3,0.7,1.5,3.1,6.25,12.5,25,50,100,200 & 400 µg/ml) of extract (dissolved in dimethyl sulfoxide, DMSO) for 48 and 72 hours. As negative control, cells will be treated with vehicle (DMSO) only. Next, cells were incubated with 50 µl of MTT at 37°C and the plates were placed in a shaker incubator for 2 hours. After dissolving the formazan crystals in 10 µl DMSO, plates were read in microplate reader (Hidex, Turku, Finland) at 570 nm against 620 nm. After getting the absorbance value from the microplate reader, the percentage of inhibition was calculated using the following equation:

$$\text{Cell viability}(\%) = \frac{\text{OD}(\text{test}) - \text{OD}(\text{blank})}{\text{OD}(\text{negtive control}) - \text{OD}(\text{blank})} \times 100 \quad (1)$$

Statistical Analysis

The experiment has been repeated for 3 times. The data have been entered to Microsoft excel and were analyzed for significance by one-way ANOVA. The test value of $p<0.05$ was considered highly significant.

RESULT AND DISCUSSION

In Figure 1, MCF-7 breast cancer cells were incubated with bark methanolic extract of P.macrophylla for 48 hours (0.3-400 µg/mL). The cytotoxicity of leaf methanolic extract in MCF7 cells was evaluated based on its effect on cell proliferation by using the MTT assay. The cytotoxicity of leaf methanolic extract was dose-dependent, the maximum cell death shows at a concentration of 0.3 ± 0.66 µg/mL. The medium inhibitory concentration (IC50) of the extract on MCF7 cells was 242 µg/mL ± 0.13).

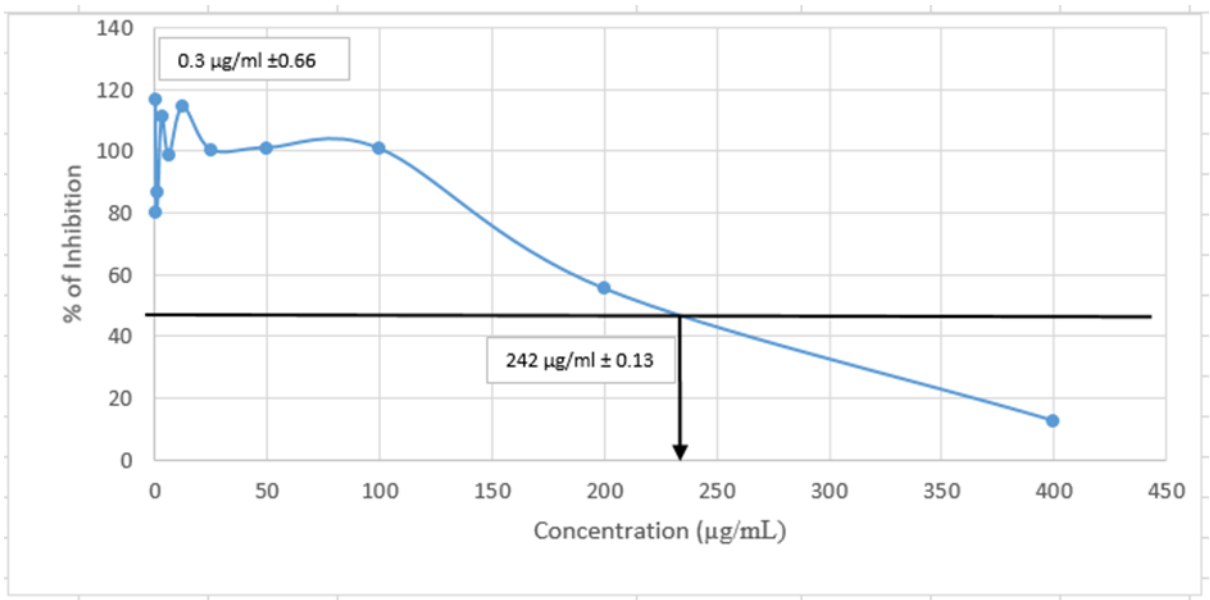

Figure 1: Effect of bark methanolic extract on MCF7 cell viability after 48 hours. Data are presented as mean S.D. (n=3). IC50= 242 ± 0.13 µg/mL.

MCF7 breast cancer cells were incubated with leaf methanolic extract of P.macrophylla (0.3-400 µg/mL) for 48 hours as shown in Figure 2. The cytotoxicity of bark methanolic extract in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay. The cytotoxicity of bark methanolic extract was dose-dependent, the maximum cell death seen at a concentration of 3.1 µg/mL ± 0.19. The medium inhibitory concentration (IC50) of the extract on MCF7 cells was 140 µg/mL ± 0.23.

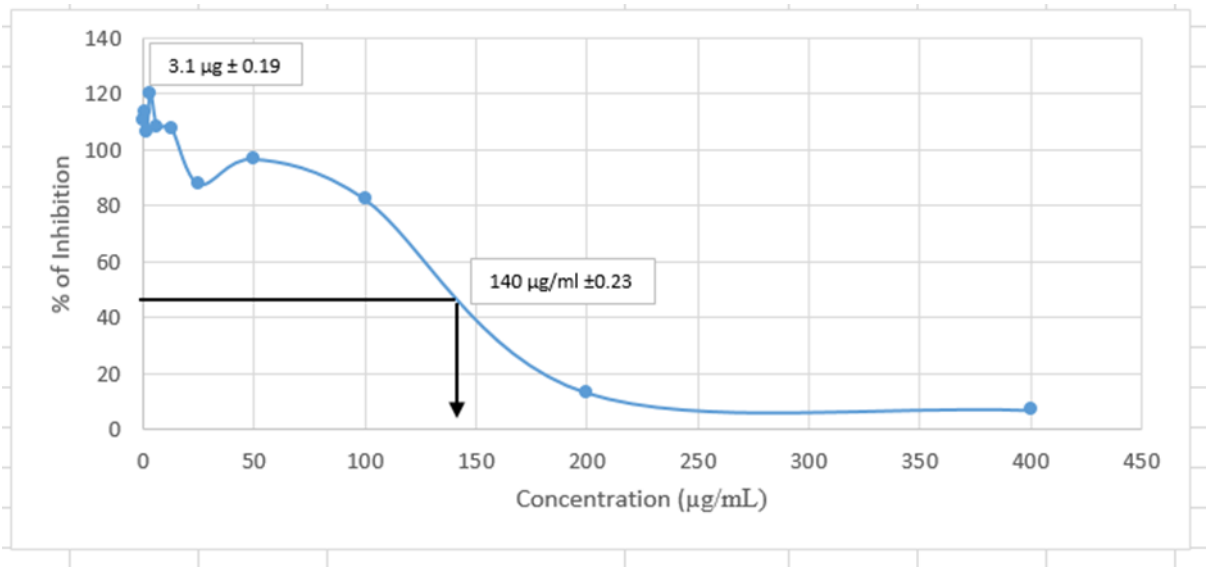

Figure 2: Effect of leaf methanolic extract on MCF7 cell viability after 48 hours. Data are presented as mean S.D. (n=3). IC50=140 µg/mL ± 0.23.

MCF-7 breast cancer cells were incubated with bark methanolic extract of P.macrophylla for 72 hours (0.3-400 µg/mL).Refer to Figure 3. The cytotoxicity of leaf methanolic in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay. The cytotoxicity of leaf methanolic extract was dose-dependant, the maximum cell death seen at a concentration of 1.5 µg/mL ± 0.57. The medium inhibitory concentration (IC50) of the extract on MCF7 cells was 260 µg/mL ± 0.11.

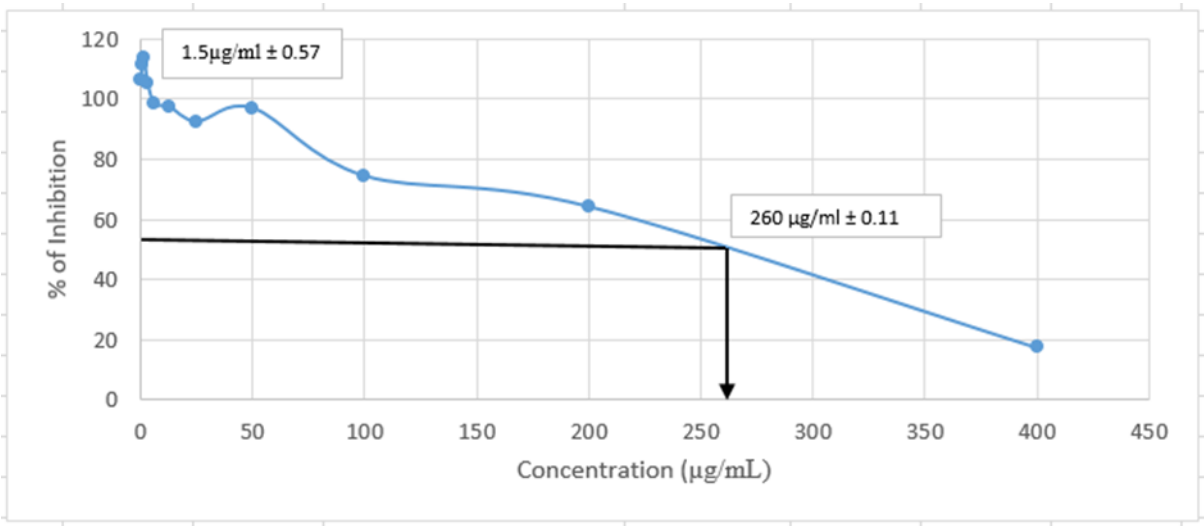

Figure 3. Effect of bark methanolic extract on MCF7 cell viability after 72 hours. Data are presented as mean S.D. (n=3). IC50= 260 µg/mL ± 0.11.

Based on Figure 4, MCF7 breast cancer cells were incubated with leaf methanolic extract of P.macrophylla (0.3-400 µg/mL) for 72 hours. The cytotoxicity of bark methanolic extract in MCF7 cells was evaluated based on its effect on cell growth by using the MTT assay. The cytotoxicity of bark methanolic extract was dose-dependent, the maximum cell death seen at a concentration 0.3 µg/mL ± 0.66. The medium inhibitory concentration (IC50) of the extract on MCF7 cells was 227 µg/mL ± 0.10.

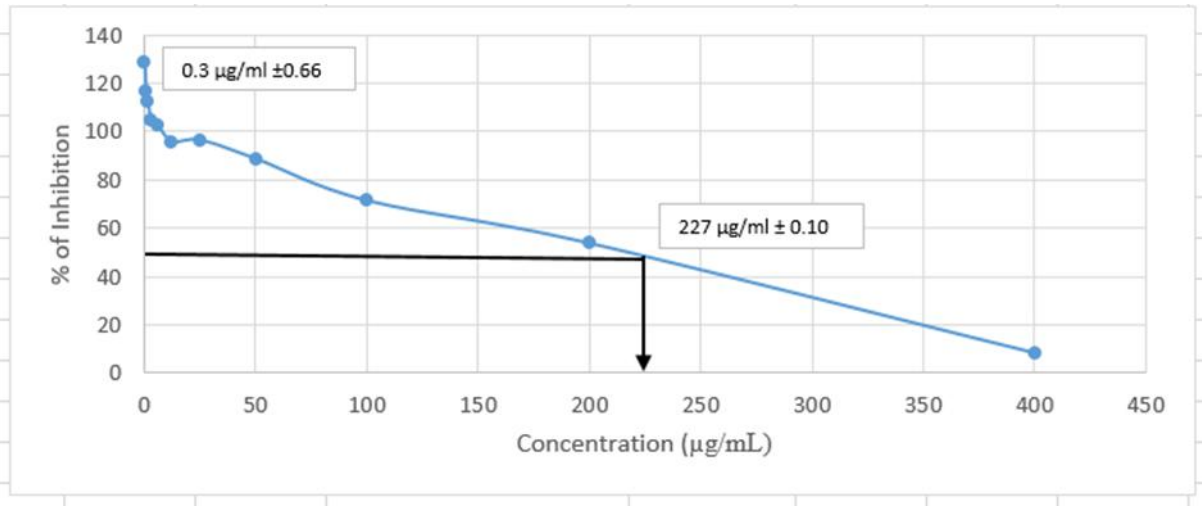

Figure 4. Effect of leaf methanolic extract on MCF7 cell viability after 72 hours. Data are presented as mean S.D. (n=3). IC50=227 µg/mL ± 0.10

In this study, two extracts with different concentrations have been applied on the MCF7 breast cancer cell line. The previous study shows that leaf methanolic extract is more toxic and demonstrated a lower IC50 value compare to bark methanolic extract of P.macrophylla (Aziz *et al*., 2016). Bark methanolic extract composed very little bioactive compound compared to leaf methanolic extract, thus lower toxicity, and higher IC50 is expected.

The MCF7 cell line has a promising response to leaf methanolic extract within 24 hours. The result was significant after 48 hours and decrease after 72 hours. According to the results, after 48 hours treatment of MCF7 cell line with leaf methanolic extract, in the concentration of 140 µg/mL, 50 % of cells have been inhibited. After 72 hours treatment of the cells with leaf methanolic extract, at the concentration of 230 µg/mL, 50% of the cell have been inhibited.

Bark methanolic extract also was effective on the MCF7 cell line after 48 hours and not after 72 hours. After 48 hours treatment of MCF7 with bark methanolic extract, in the concentration of 160 µg/mL, 50% of the cell have been inhibited. The leaf methanolic extract of the P.macrophylla was most effective in inhibition of the breast cancer cells proliferation as it requires lesser concentration in order to damage human cancer cells line.

Previous study of the other member of Annonaceae which are Polyalthia debris Finet & Gagnep, Polyalthia parviflora Ridl and Uvaria rufa also have shown anti-cancer criteria at lower concentration by revealing significant reduction of human oral epidermoid carcinoma cells and breast cancer cells at concentration of (EC50 0.42–20 lg ml) and (EC50 0.18– 20 lg ml) respectively (Wiyakrutta *et al*, 2004). Annonaceous acetogenins which also known as Annonacin is a group of potential antineoplastic agents isolated from the seeds of another species of Annonaceae called Annona reticulate has shown outstanding cytotoxicity potential on ovarian cancer (PA-1 and SKOV3), cervical cancer (HeLa and HeLa S3), breast cancer (MCF7), bladder cancer (T24), and skin cancer (BCC-1) at concentration between 0.452- 0.427 µg/mL (Yuan *et al*., 2003).

The inhibitory effect of Annonaceae species extract is not only due to its local cytotoxic effect but also due to its systemic action. For example, to confirm anti-tumor activity of Pseuduvaria longifolia, the effect of the methanolic extract on solid tumor was assessed using DLA cells. Significant reduction in tumor volume was observed with P. longifolia extract treatment, which implies inhibition of DLA tumor growth. This species also was assessed by MTT assay to determine

its effect on the growth of cancer cells (MCF-7 and HeLa). The cells were exposed to different concentrations of extract (5–200μg/mL) for 48h and absorbance was recorded at 540nm by ELISA reader. The IC50 value of the plant extracts against tested cancer cell lines were 25 and 50 ug/ml, respectively. Based on the study, in vitro cytotoxicity and antiproliferative data supported the in vivo antitumor activity of P. longifolia extract against EAC and DLA, showed extract caused significant cell death and inhibition of cancer cell growth. In addition, the leaf methanolic extract of this species has been reported to contain cytotoxic aporphine alkaloid liriodenine. Many studies have proven that alkaloid plays a major role in the anticancer activity.For example, four compounds were isolated from Annonaceae species known as Enicosanthum membranifolium. The structures of the compounds were confirmed by spectroscopic data which refer to N-trans-feruloyltyramine, R-(−)-mellein, clerodermic acid, and salicifoline chloride as a quaternary alkaloid compound ((Mosmann, 1983 and Twentyman & Luscombe, 1887).The presence of any of these components may be attributed to the antitumor property of the extract as the tumor selective action and characterization of the active component of P. longifolia extract responsible for the activity (Manjula *et al*., 2010).

It is also suggested that polar solvent methanol was most successful in extracting secondary metabolites which responsible for its anticancer property (Bobadilla et al., 2002). Moreover, high bioactivity usually belongs to the highly polar extract (Castillo *et al*, 2010) due to polar extract consisting of molecules having a polar water-soluble group attached to a water-insoluble hydrocarbon chain. In fact, the molecule of methanol consists of a single atom of a tetraedric carbon, linked to 3 hydrogens, and a -OH group. The -OH group is the polar group, and the three hydrogens, the water-insoluble hydrocarbon chain. Most methanol is used for extraction various polar compounds but the certain group of nonpolar compounds is fairly soluble in methanol if not readily soluble. Therefore methanol is commonly used for extraction of bioactive compounds (Eloff, 1998).

The high anticancer activity of P.macrophylla leaf may attribute to the presence of various bioactive constituent. This extract has revealed some fatty acids and terpenoids which include Linoliec acid, Palmitic acid, α- codinol and Oleic acid. Other compounds are neophytadiene and methyl ester which was previously identified in another member of Annonaceae known as Annona Squamosa (Vanitha *et al*., 2011).

Polar extracts of Annonaceae member really illustrated outstanding antitumor potential as shown in the previous experiment of two types of species belongs to Annonaceae family which are Polyalthia longifolia Benth.and Hook. These species were screened for it's in vitro antitumor activity towards murine cancer cells and in human cancer cells by Trypan blue exclusion assay. While in vivo antitumor activity was assessed by MTT assay under 48hours of treatment. In the MTT assay, the IC50 values of P. longifolia extract against HeLa and MCF-7 cells were 25.24 and 50.49 μg/mL, respectively which require lesser concentration of the plant extracts to inhibit proliferation in both tested human cell lines (Twentyman & Luscombe,1987). The results are in support of the previous study done on the analysis of cell cycle revealed high levels of early apoptotic on breast cancer cells under 48 hours exposure of P.macrophylla in polar extracts within the IC50 value of 82.35 μg/mL (Chu, 2014).

CONCLUSION

It may be concluded from study that bioactive compounds from methanolic extract of P.macrophyyla can be employed in the formulation of anticancer agents, thus lead to apoptosis to

decrease human breast cancer cells proliferation especially under 48 hours treatment. Moreover, isolation, identification and purification of theses phytoconstituents and determination of their respective anticancer apotencies and toxicological evaluation to determine novel chemotherapeutic agents should be the future direction for investigation.

ACKNOWLEDGEMENTS

This work was supported by the grant from UiTM.The author wish to express their thanks to Dr Cheng Yeng Looi and Miss Noorshin from University of Malaya for their assistance and for valuable suggestions during manuscript preparation.

REFERENCES

[1] Aziz, A., Taha, H., Mohebali, N., Chung, Y. L., Ismail, N. H., Bakar, M. Z. A., & Yusof, F. Z. M.(2016). Anti-Cancer Potential of Pseuduvaria Macrophylla in Human Cancer Cell Lines. *Journal of Advanced Research in Applied Sciences and Engineering Technology*, **4**(1), 1-11.

[2] Berridge, M. V. & Tan, A. S. (1993). Characterization of the cellular reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. *Archives of biochemistry and Biophysics*, **303**(2), 474-482.

[3] Bobadilla, M., Zavaleta, G., Gil, F., Pollack, L., Sisniegas, M. & Gonzales, M. (2002). Efecto bioinsecticida del extracto etanólico de las semillas de Annona cherimolia Miller" chirimoya" y A. muricata Linneaus" guanabana" sobre larvas del IV estadio de Anopheles sp. *Revista Peruana de Biología*, **9**(2), 64-73.

[4] Casey, E.B., Jellife, A. M., Le Quesne, P.M. & Millett, Y. L. (1973). Vincristine neuropathy clinical and electrophysiological observations. *Brain*, **96**(1), 69-86.

[5] Castillo, L. E., Jiménez, J. J. & Delgado, M. A. (2010). Secondary metabolites of the Annonaceae, Solanaceae and Meliaceae families used as biological control of insects. *Tropical and Subtropical Agroecosystems*, **12**(3), 445-462.

[6] Chu, J. H. Y. (2014). *Investigating in vitro anticancer properties of Malaysian rainforest plants: Acalypha wilkesiana Müll, Arg. Archidendron ellipticum (Blume) Hassk. Duabanga grandiflora Walp. Pseuduvaria macrophylla (Oliv.) Merr.* Doctoral dissertation, University of Nottingham.

[7] Hoyert, D. L., Heron, M. P., Murphy, S. L. & Kung, H. C. (2006). Deaths: Final Data for 2003. *National Vital Statistics Reports*, **54**(13), 1-120.

[8] Dhanjaland, P. & Fry, J. R. (1997). Determinants of MTT reduction in rat hepatocytes. *Biomarkers*, **2**(2), 111-116.

[9] Efdi, M., Itoh, T., Akao, Y., Nozawa, Y., Koketsu, M. & Ishihara, H. (2007). The isolation of secondary metabolites and in vitro potent anti-cancer activity of clerodermic acid from Enicosanthum membranifolium. *Bioorganic & Medicinal Chemistry*, **15**(11), 3667-3671.

[10] Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of ethnopharmacology*, **60**(1), 1-8.

[11] Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T. & Murray, C.J. (2001). Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *PubMed*. **367**(9524), 1747-57.

[12] Mathers, C.D. and Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Medicine*, **3**(11), e442.

[13] Manjula, S. N., Kenganora, M., Parihar, V. K., Kumar, S., Nayak, P. G., Kumar, N., Pai, K.S.R. & Rao, C. M. (2010). Antitumor and antioxidant activity of *Polyalthia longifolia* stem bark ethanol extract. *Pharmaceutical biology*, **48**(6), 690-696.

[14] Macdonald, J. S. (1999). Oral fluoropyrimidines: a closer look at their toxicities. *American Journal of Clinical Oncology*, **22**(5), 475-480.

[15] Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**(1-2), 55-63.

[16] Newman, D. J., Cragg, G. M. & Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products*, **66**(7), 1022-1037.

[17] Rexroth, G. & Scotland, V. (1994). Cardiac toxicity of 5-fluorouracil. *Medizinische Klinik*, **89**(12), 680-688.

[18] Rastogi, N., Chag, M. & Ayyagari, S. (1993). Myocardial ischemia after 5-fluorouracil chemotherapy. *International Journal of Cardiology*, **42**(3), 285-287.

[19] Tunstall-Pedoe, H. (2005). Preventing Chronic Diseases. A Vital Investment: WHO Global Report. Geneva: World Health Organization. *Chronic diseases and health promotion*. (http://www.who.int/chp/chronic_disease_report/en/). Accessed on 19th July 2016.

[20] Twentyman, P. R. & Luscombe, M. (1987). A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *British Journal of Cancer*, **56**(3), 279-285.

[21] Vanitha, V., Umadevi, K. J. & Vijayalakshmi, K. (2011). Determination of bioactive components of Annona squamosa L leaf by GC-MS analysis. *International Journal Of Pharmaceutical Sciences And Research*, **3**(4), 309-312.

[22] Wiyakrutta, S., Sriubolmas, N., Panphut, W., Thongon, N., Danwisetkanjana, K., Ruangrungsi, N. & Meevootisom, V. (2004). Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. *World journal of microbiology and biotechnology*, **20**(3), 265-272.

[23] Wu, Y. C., Duh, C. Y., Wang, S. K., Chen, K. S. & Yang, T. H. (1990). Two new natural azafluorene alkaloids and a cytotoxic aporphine alkaloid from Polyalthia longifolia. *Journal of Natural Products*, **53**(5), 1327-1331.

[24] Yuan, S. S., Chang, H. L., Chen, H. W., Yeh, Y. T., Kao, Y. H., Lin, K. H., Wu, Y. C. & Su, J. H. (2003). Annonacin, a mono-tetrahydrofuran acetogenin, arrests cancer cells at the G1 phase and causes cytotoxicity in a Bax-and caspase-3-related pathway. *Life Sciences*, **72**(25), 2853-2861.