AsPac J. Mol. Biol. Biotechnol. 2017
Vol. 25 (1) : 00-00

Cytokinetic Study of Uninfected Agarwood Branch Ethanolic Extract on Breast Cancer Cells

Phirdaous Abbas[1], Yumi Zuhanis Has-Yun Hashim[1], 2, Azura Amid[1]

[1]Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur.

[2]International Institute for Halal Research and Training (INHART), International Islamic University Malaysia, P.O. Box 50728, Kuala Lumpur Malaysia

Received XXXX / Accepted XXXX

Abstract. Breast cancer is the most common malignancy in women with over 500,000 global death toll annually. Ethnopharmaceutical practices provide evidences of *Aquilaria* spp. as traditional treatment for numerous illness. This study intended to look into the cytotoxic kinetics of uninfected agarwood branch ethanolic extract from *Aquilaria subintegra* against MCF-7 breast cancer cell line that could help grasp the effect and provide baseline towards development of agarwood-based therapeutics. Extract was subjected to a series of kinetic studies in order to investigate the growth rate, death rate, doubling time and death time of MCF-7 between the treated and control groups. Results obtained indicated that the extract lowered the growth rate from 0.0421 h^{-1} to 0.0158 h^{-1} and stretched the doubling time from 16.4 hours to 43.8 hours. Death rate was escalated from 0.0058 h^{-1} to 0.0563 h^{-1}. Closer inspection on the population density images revealed that the extract-treated MCF-7 cells showed abnormal appearance with blebs, volume reduction, cell shrinking and loss of cell-cell contact. These discoveries implied that agarwood uninfected branch ethanolic extract altered the kinetics of MCF-7 cells and additional study should be made to substantiate the hypothesis and elucidate the effects against cancer.

Keywords: agarwood, ethanolic extract, cytotoxic, MCF-7, breast cancer

INTRODUCTION

One of the forest resource known as agarwood (also known as aloeswood or eaglewood) normally refers to the dense and fragrant resinous wood produced inside tropical rainforest trees of the genera *Aquilaria, Gonystylus* and *Gyrinops.* This dark resinous wood has an intense aroma when burned and considered as an exclusive non-timber product that has been traded since ancient time. This luxurious substance is still being used to satisfy demands in religious, medical and aromatic preparation (Jayaraman *et al.*, 2014). Figure 1 displays the *Aquilaria subintegra* tree and agarwood merchandises traded in the market. The aromatic resin has many relations with cultures around the world such as Arabian, Chinese and Japanese, and also linked to religious history, customs and rituals in Buddhism, Christianity, Hinduism, and Islam (Barden *et al.*, 2000; Blanchete and Van Beek, 2005).

Agarwood economic point of view always been leaning to its pathological resin, that materialized in the woody tissues of the stem as a response to injuries or attacks. Still, other parts of the agarwood plant were also popular as ingredients in the traditional medicine procedures of the South East Asian communities; Chinese, Tibetan, Unanai and Ayurvedic (Hashim *et al.*, 2016). Agarwood was recorded to be utilized as sedative, analgesic and digestive remedy (Yagura *et al.*, 2005). One classic scientific work on agarwood was conducted by Gunasekara *et al.* (1981) reported that the alcoholic extract of *Aquilaria malaccensis* stem bark and stem exhibited mild cardiotonic activity and anti-cancer effects when used to treat Eagle's carcinoma of the nasopharynx. Recently, scientific studies on *Aquilaria* spp. have shown favourable outcomes in the pharmacological field. Feng *et al.*, (2009) studied the extract obtained from the leaves of *Aquilaria sinensis* and reported anti-inflammatory, antitumor, analgesic, therapeutic and prophylactic activity on constipation, intestinal obstruction, and obesity; therapeutic effect on

* Author for correspondence: Yumi Zuhanis Has-Yun Hashim
Department of Biotechnology Engineering, Kulliyyah of Engineering, International Islamic University Malaysia, Jalan Gombak, 53100 Kuala Lumpur. Emel : yumi@iium.edu.my

Figure 1: *Aquilaria subintegra* plant and agarwood product in the form of woodchips available for perfumery and incense industry (Photo: P. Abbas, 2010, Kajang, Selangor, Malaysia).

haemorrhage and cerebral ischemia; and blood glucose-reducing effect. A study found that wounding of agarwood tree initiates the defence system that produces secondary metabolites that are proven to be health beneficial which includes terpenes, phenolics and alkaloids (Mohamad *et al.*, 2012).

Ethnopharmacological facts combined with the advancement in biotechnology, have sparked interest in the scientific community to study the claims by modern tools. Agarwood related scientific publications in recent years have risen, especially associated with pharmacological actions including antidiabetic, anti-inflammatory, anticancer, antidepressant and antioxidant activities (Sattayasai *et al.*, 2012; Bahrani *et al.,* 2014; Hashim *et al.*, 2014b; Tay *et al.,* 2014; Phranakhon *et al.*, 2015).

Breast cancer is considered as the global common death causes among women with high mortality rate and projected death toll of 500,000 (Ferlay *et al.*, 2013). In Malaysia, the National Cancer Registry recorded as high as 18,219 diagnosed cancer cases in 2007 (Omar and Ibrahim, 2011). Treatment and therapy for the disease are usually accompanied by chemically synthesized drugs with serious side effects. These dangerous drugs was one of the driving force that shifted the attentions towards natural products as alternative remedies for cancer. In accordance, earlier study found that agarwood branch ethanolic extract (ABEE) was able to inhibit MCF-7 breast cancer cells (Hashim *et al.*, 2014a). The cytotoxicity effect was shown using sulforhodamine B (SRB) in vitro assay and the inhibition concentration (IC50) value of ABEE against MCF-7 cells was determined to be at 8 µg/ml. This present study was commenced to look deeper into the cytokinetic effects of ABEE on MCF-7 cells.

MATERIALS AND METHODS

In this study, branches selected from uninfected *Aquilaria subintega* was gathered from a local farm in Bangi, Malaysia. The samples were subjected to cleaning, drying and grounding for solvent extraction procedure using ethanol. Previously, unpublished screening assays conducted revealed that the extract may possess anti-attachment and cytotoxic effects against MCF-7 breast cancer cells with a profound inhibition concentration (IC50) of 8 µg/ml when tested using sulforhodamine B (SRB) assay. The extract that exhibited the best IC50 value was extracted using ethanolic solvent extraction under the following extraction conditions: time 24 hours, temperature 50 °C, agitation speed 200 rpm, solid-solvent ratio 1:20. These conditions were repeated to obtain fresh crude extract for the kinetics study. Follow-

ing solvent extraction, the mixture was filtered and dried using rotary evaporation and finally freeze dried to remove any residual solvent. Then, the agarwood branch ethanolic extract (ABEE) was diluted using dimethylsulfoxide (DMSO) to the working concentration of 8 µg/ml for testing.

The cytokinetic study was conducted on MCF-7 breast cancer cells HTB-22™ (ATTC). Cells were passaged a few times prior to the kinetic study to ensure that they are in stable and active conditions (Freshney, 2011). For the kinetic study, MCF-7 cells were divided into 3 sets of testing flasks (20 flasks in each group with triplicates) with seeding concentration of 1 x 105 cells/ml each. These 3 groups were designated as testing (treated using ABEE at 8 µg/ml), positive control (treated using commercial anticancer drug, Taxol at 2.8 µg/ml) and negative control group (treated using 10 % v/v DMSO). Cells were allowed to adjust to culture condition for 24 hours before introducing the treatment regimen. Cell counting procedure using trypan blue dye and population density observation were conducted every 8 hours until plateau phase was reached or zero viability was observed. Cell population data collected was used in calculation to obtain cell doubling time, growth rate, death rate and cell generation number. Growth and death rates were obtained from the log transformed plot for the MCF-7 cytokinetics growth curve. Doubling time was calculated using Equation (1) below as described in Freshney (2011):

$$T_D = \frac{1n2}{\mu} \quad (1)$$

Cell generation number was modified from the following Equation (2) below:

$$N = N_0 . 2^X \quad (2)$$

where N is the final cell concentration, N0 is the initial cell concentration and X is the cell generation number (Butler 2004). Rearranging Equation (2) to get the cell generation number, X, becomes Equation (3) as below:

$$X = \frac{\log_{10} N - \log_{10} N_0}{\log_{10} 2} \quad (3)$$

RESULTS AND DISCUSSION

Cell population data showed that in all groups, lag phase occurred around 8 to 20 hours. Control group showed rapid proliferation marking the log phase after 20 hours and started to enter the stationary phase after 70 hours of growth. On the contrary, ABEE treated and Taxol treated group showed reduction in cell population after 24 hours of growth. The population density data was used to generate MCF-7 growth profile as shown in Figure 2.

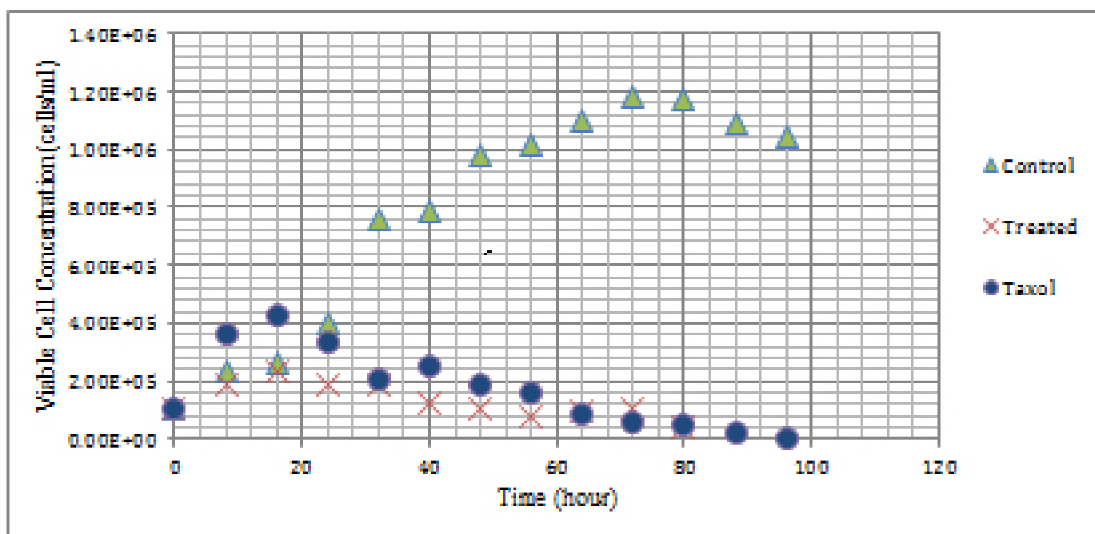

Figure 2: Cell growth curve generated from cell population density data. MCF-7 density was recorded every 8 hours interval from 3 different set of treatment. Control group (10 % v/v DMSO treated), ABEE group (8 µg/ml of ABEE) and Taxol group (2.3 µg/ml of Taxol).

The generated growth profile allowed the estimation of growth rate (μ), death rate (k_d), and cell generation number (X) as shown in Table 1. Control group achieved maximum growth within 70 to 80 hours of incubation time (1.19 × 106 cells/ml) while both treated groups achieved the maximum growth within the first 8 to 24 hours of incubation time. The number of generation (X) calculated for all groups demonstrated that ABEE inhibited MCF-7 cell growth as the number of generation was reduced from 3.56 generations to 1.22 generations (p-value = 0.032). Similarly, Taxol reduced the number of generations to 2.10 generations (p-value = 0.015). The slope of the plot represents the specific growth rate (μ) of cells at the exponential phase. Following the Equation 1, doubling time was then calculated. As compared to control, ABEE extended the doubling time from 16.4 hours to 43.8 hours indicating that proliferation rate was lower when cells were treated with agarwood extract. Similarly, Taxol also showed similar trend extending the doubling time to 42 hours. Meanwhile, looking at the death rate, negative slope ($\mu<0$) indicated an exponential decay and this part is usually calculated at the death phase of the plot. The death phase in control and treatment group showed negative slopes confirming the exponential decay and indicating cell death incidence. Control group, showed a death rate of 0.0058 h^{-1} with an estimated death time of 119.5 hours. Death rate for ABEE extract-treated group was 0.0563 h^{-1} (3 times larger than the control death rate, 0.0058 h^{-1}) and the estimated death time was 12.3 hours indicating that agarwood treatment on MCF-7 cells increases cell death incidence. Likewise, death rate of Taxol-treated group death rate was 0.0542 h^{-1} and the estimated death time was 12.8 hours indicating that Taxol induced a much severe killing effect by increasing the cell death incidence. Control group showed slow cell death which is not favourable since cancer may spread and metastasize. Similar inhibiting trend exhibited by ABEE extract strongly suggest that agarwood possesses potential anticancer characteristics.

Figure 3 showed the population density images for control group in the study in which normal growth trend was observed. Meanwhile, Figure 4 showed the population density images for ABEE group which demonstrated morphological changes after 8 hours of incubation. The changes became more apparent and cells were observed to experience volume reduction as incubation time continued. Finally, dead and floating cells were observed after 72 to 96 hours of incubation time in which cells were observed to be shrunk with appearance of blebs. These changes including irregular appearance with blebs, volume reduction, cell shrinking and loss of cell-cell contacts indicate cell death similar to apoptosis (Kumaraswamy *et al.*, 2013). This trend was also observed in the Taxol group in which cells

Table 1: Estimated specific growth rate (μ), death rate (kd), doubling time, death time and cell generation number obtained from plotted population density data.

Cell group	Specific growth rate, μ, (h^{-1})	Estimated doubling time, (hours)	Specific death rate, kd, (h^{-1})	Estimated death time, (hours)
Control	0.0421	16.4	0.0058	119.5
ABEE	0.0158	43.8	0.0563	12.3
Taxol	0.0165	42.0	0.0542	12.8
Cell group	**Initial cell seeding (cells/ml)**	**Final cell concentration (cells/ml)**	**Cell generation number (X)**	**p-value**
Control	1.0 x 105	1.19 x 106	3.56	-
ABEE	1.0 x 105	0.43 x 106	2.10	0.015
Taxol	1.0 x 105	0.23 x 106	1.22	0.032

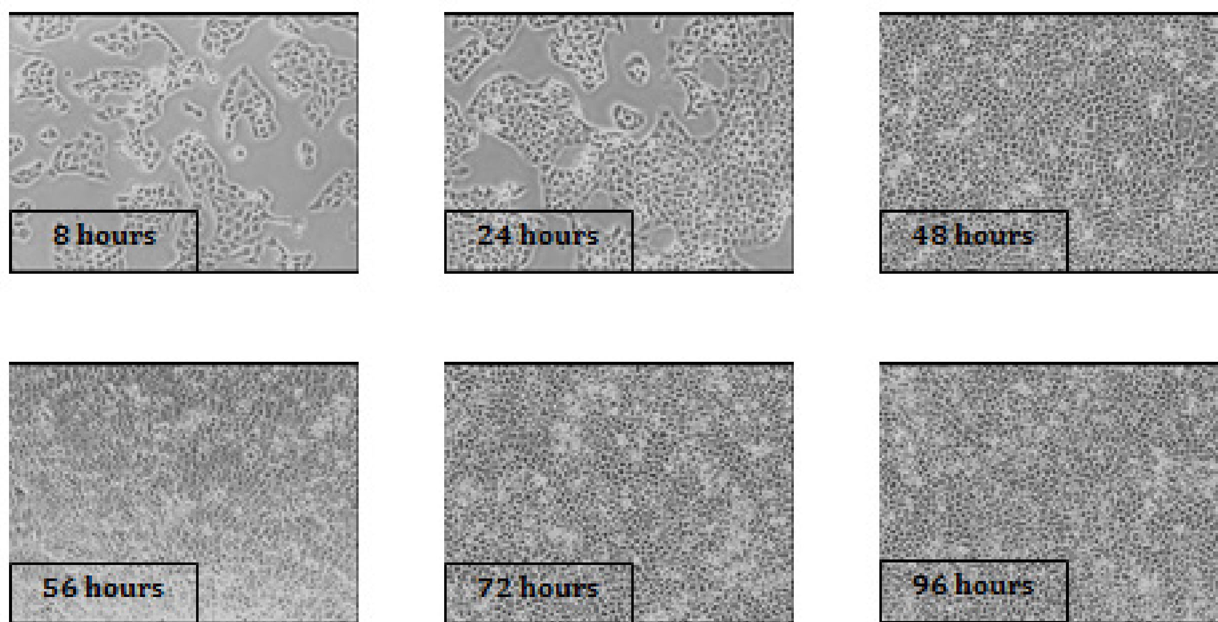

Figure 3: Representative population density images for control group. MCF-7 cells exhibited normal growth trend. Cell population increased as incubation time increased. Images were obtained using inverted phase contrast microscope at 40X magnification.

experienced more detrimental morphological changes as shown in Figure 5. In both Figure 4 and Figure 5, the presence of cell debris and dust-like particles were observed after 8 hours of incubation. Cytotoxicity of Taxol has been known due to its effect on microtubules of cells (Malik *et al.*, 2011). Given the similar morphology of ABEE-treated cells observed in this phase of study; future work in elucidating the mechanism is warranted.

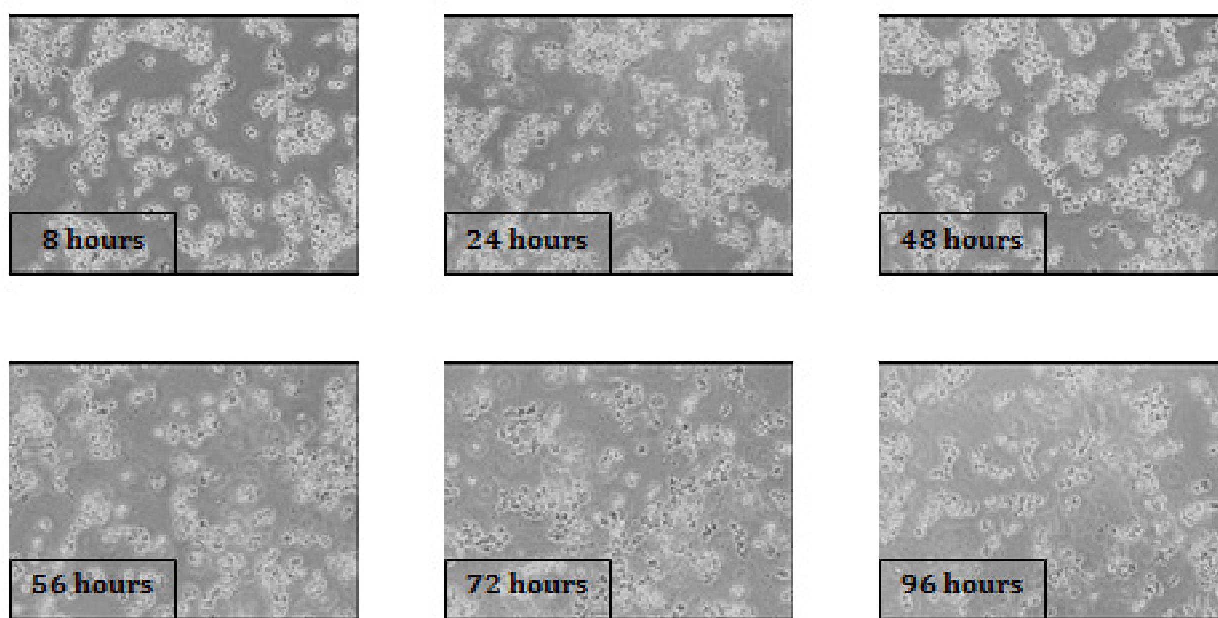

Figure 4 : Representative population density images for ABEE group. Cells showed morphological changes (blebs and reduced volume) and less adhering cell was observed. Maximum growth was achieved after 24 hours of incubation time followed by declining growth trend. After 72 hours of incubation, almost no viable cell was observed and flaskwas filled with dead cells and cell debris (dust-like). Images were obtained using inverted phase contrast microscope at 40X magnification.

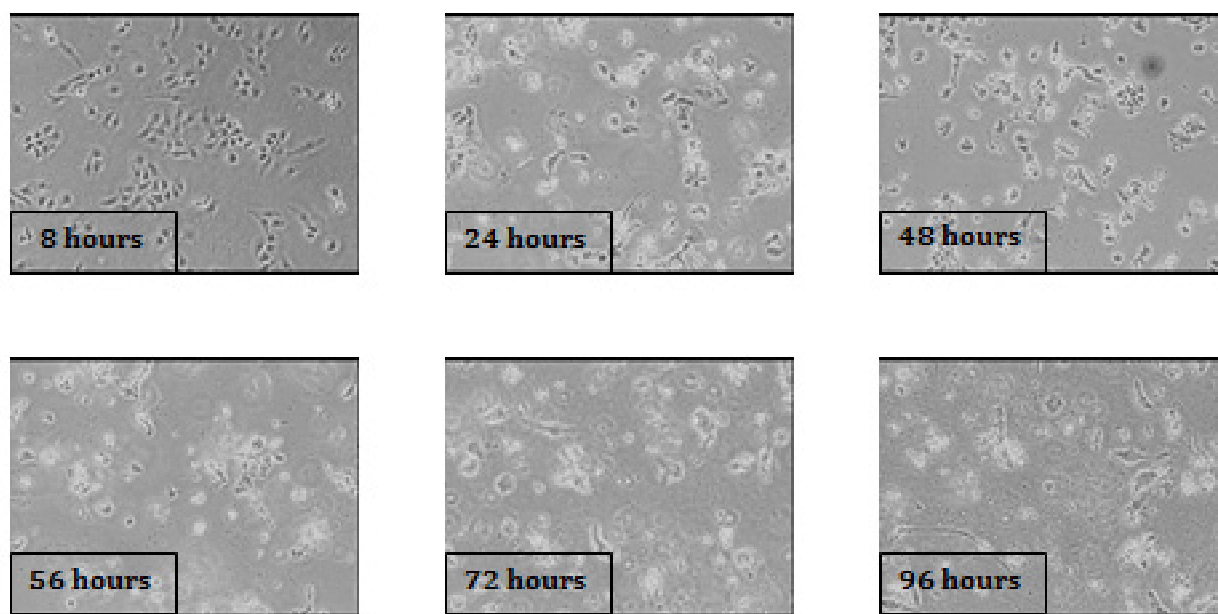

Figure 5: Representative population density images for Taxol group. Cells showed morphological changes (blebs and reduced volume) and less adhering cell was observed. Maximum growth was achieved after 24 hours of incubation time followed by declining growth trend. After 72 hours of incubation, almost no viable cell was observed and flask was filled with dead cells and cell debris (dust-like). Images were obtained using inverted phase contrast microscope at 40X magnification.

CONCLUSION

The cytokinetic study undertaken showed that ABEE exhibited inhibition of cell growth with reduction in cell generation number from 3.56 (control group) to 1.22. ABEE also reduced the specific growth rate of MCF-7 cells from 0.0421 h^{-1} to 0.0158 h^{-1} and extended the doubling time of cells from 16.4 hours to 43.8 hours. Data collected at later stage of growth phase showed that ABEE increases the death rate from 0.0058 h^{-1} to 0.0563 h^{-1} which is 3 times larger than the control death rate. These data suggested that ABEE potentially possessed both cytostatic and cytotoxic effects. Population density images showed morphological changes in cells including irregular appearance with membrane blebs, volume reduction, cell shrinking and loss of cell-cell contacts which is similar to the mechanisms of apoptosis. Therefore, it is only right to allocate more attention on agarwood plant materials as they possess a lot of potential to be developed into cancer therapeutics.

ACKNOWLEDGEMENT

This research was funded by a collaboration grant (CLB11-002-009) between Kayu Gaharu (M) Sdn Bhd and International Islamic University Malaysia (IIUM). We are also thankful to Mr. Mohd Hafizul Bin Shaibon (Assistant Engineer, IIUM), Mr. Azmir Bin Arifin, Mrs. Nur Husna Bt. Samsudin, Ms Irmanisha Bt. Ibrahim, Ms. Faqihah Bt Salleh and Mrs Rahilah Rahman for their useful ideas, kind assistance and support towards the completion of this study.

REFERENCE

Bahrani, H., Mohamad, J., Paydar, M. and Rothan, H.A. 2014. Isolation and characterisation of acetylcholinesterase inhibitors from *Aquilaria subintegra* for the treatment of Alzheimer's disease (AD). *Current Alzheimer Research* 2014. 11:1-9.

Barden, A., Anak, N.A., Mulliken, T., Song, M. 2000. Heart of the Matter: Agarwood Use and Trade and CITES Implementation for *Aquilaria malaccensis*, TRAFFIC SE Asia, Kuala Lumpur.

Blanchette, R.A., van Beek, H.H. 2005.Cultivated agarwood. US 6,848,211 B2, US.

Butler, M. 2004. Animal Cell Culture and Technology: BIOS Scientific Publishers.

Feng, J., Yang, X.-W., and Liu, T.H. 2009. RP-LC Quantification and Pharmacokinetic Study of Iriflophenone 2-O-α-Rhamnopyranoside in Rat Plasma. *Chromatographia.* 70(7-8):227-1231.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., … Bray, F. (2013). GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. Lyon, France: International Agency for Research on Cancer. Retrieved 21 August 2015 from http://globocan.iarc.fr.

Freshney, R. I. 2011. Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications: Wiley.

Gunasekara, S. P., Kinghorn, A. D., Cordell, G. A. and Farnsworth, N. R. 1981. Plant Anticancer Agents XIX Constituents of *Aquilaria malaccensis*. J*ournal of Natural Products*. 44(5):569-572.

Hashim, Y., Abbas, P. and Amid, A. 2014a. Anti-cancer activity of un-inoculated agarwood branch against MCF-7 breast cancer cells. In: 5th International Conference on Biotechnology for the Wellness Industry, 10th-11th June 2014, Kuala Lumpur (unpublished).

Hashim, Y., Abbas, P. and Amid, A. 2014b. Screening of anticancer activity from agarwood essential oil. *Pharmacognosy Research.* 6:191-194.

Hashim, Y., Kerr, P., Abbas, P. and Mohd Salleh, H. 2016. *Aquilaria* spp. (agarwood) as source of health beneficial compounds: A review of traditional use, phytochemistry and pharmacology. *Journal Of Ethnopharmacology.* 189:331-360. http://dx.doi.org/10.1016/j.jep.2016.06.055.

Jayaraman, S., Daud, N. H., Halis, R. and Mohamed, R. 2014. Effects of plant growth regulators, carbon sources and pH values on callus induction in *Aquilaria malaccensis* leaf explants and characteristics of the resultant calli. *Journal of Forestry Research*. 25(3), pp. 535-540.

Kumaraswamy, K., Archana, M., Bastian, and Yogesh, T. 2013. Various methods available for detection of apoptotic cells- A review. Indian *Journal of Cancer.* 50(3):274-283.http://dx.doi.org/10.4103/0019-509x.118720.

Malik, S., Cusidó, R. M., Mirjalili, M. H., Moyano, E., Palazón, J. and Bonfill, M. 2011. Production of the anticancer drug taxol in Taxus baccata suspension cultures: A review. *Process Biochemistry*. 46(1):23-34.

Mohamed, R., Hang, Y. T. and Chai, H. S. 2012. A real-time PCR method for the detection of trnL-trnF sequence in agarwood and products from A*quilaria (Thymelaeaceae)*. Conservation Genetics Resource. 4(3):803-806.

Omar, Z. A. and Ibrahim, N. S. T. (2011). National Cancer Registry Report: Malaysian Cancer Statistics-Data and Figure. Ministry of Health, Malaysia.

Pranakhon, R., Aromdee, C. andPannangpetch, P. 2015. Effects of iriflophenone 3-C-beta-glucoside on fasting blood glucose level and glucose uptake. Pharmacognosy Magazine. 11:82-89.

Sattayasai, J., Bantadkit, J., Aromdee, C., Lattmann, E., Airarat, W. 2012. Antipyretic, analgesic and anti-oxidative activities of *Aquilaria crassna* leaves extract in rodents. *Journal of Ayurveda and Integrative Medicine.* 3:175-179.

Tay, P.Y., Tan, C.P., Abas, F., Yim, H.S., Ho, C.W., 2014. Assessment of extraction parameters on antioxidant capacity, polyphenol content, epigallocatechin gallate (EGCG), epicatechin gallate (ECG) and iriflophenone 3-C-β-glucoside of agarwood *(Aquilaria crassna*) young leaves. Molecules. 2014. Volume 19, pp. 12304-12319.

Vichai, V. and Kirtikara, K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature Protocols*. 1(3):1112-1116.

Yagura, T., Shibayama, N., Ito, M., Kiuchi, F. and Honda, G. 2005. Three novel diepoxy tetrahydrochromones from agarwood artificially produced by intentional wounding. *Tetrahedron Letters.* 46(25):4395-4398.