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Uninfected agarwood branch extract possess cytotoxic and inhibitory effects on MCF-7 breast cancer cells

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ABSTRACT: Apart from the highly sought agarwood resin primarily for perfumery industry, agarwood or gaharu (*Aquilaria* spp.) in general has been one of basic components in traditional medicine including Ayurvedic, Traditional Chinese Medicine and other communities in the Asian region. While the resin is formed in infected trees, this present study reported the cytotoxicity and attachment inhibition effects of leaf and branch extracts from uninfected agarwood tree against breast cancer cells. Qualitative extraction screening process was first conducted to screen for suitable extraction solvents and parts of plant (leaf or branch). Then, the *in vitro* anti-cancer screening assays including cytotoxicity and attachment assays were conducted. Branch sample extracted using ethanol and distilled water resulted in higher yield and more potent cancer inhibiting effects as compared to other solvents. Crude extract obtained after drying process using ethanol as solvent yielded 9.47 % g/g (branch) and 13.2 % g/g (leaf); distilled water as solvent yielded 9.33 % g/g (branch) and 12.8 % g/g (leaf), respectively. However, branch extract exhibited more potent cancer-inhibiting effects with IC_{50} of 23 µg/mL (ethanol) and 38 µg/mL (distilled water) as compared to leaf (no intersection points in the plot). To this end, it can be concluded that extracts from uninfected agarwood tree (*Aquilaria subintegra*) possesses cytotoxic and anti-attachment effects on MCF-7 breast cancer cells with ethanolic branch extract being the most promising. The screening and selection of extraction solvent and plant type are crucial steps towards cost-effective extraction and further bioprocessing of bioactive compounds from agarwood tree.

KEYWORDS: Agarwood; cytotoxic; *Aquilaria subintegra*; anticancer.

1. INTRODUCTION

Agarwood is an important non-timber forest product originating from trees of the genus *Aquilaria* of the *Thymelaeceae* family [1-4]. Currently, small to large scale agarwood plantation employ certain techniques (such as wounding of the tree or infection via injection of microbial concoction) to imitate the process that triggers the tree defensive system that subsequently results in the formation of the resin (gaharu) [5]. In Malaysia, commonly, 'Gaharu' is used as generic term for both tree and resin, similar to the term agarwood [6]. It is noteworthy that the term 'gaharu' or 'agarwood' in this sense refers to the heavy fragrant wood that hardened from the resin. This fragrant product is expensive in the market since efforts imitating the natural mixture of sesquiterpenes and unique chromones are still not entirely successful [7-9]. Further, this unique profile of compounds has been thought to be contributing to the biological activities observed. Until recently, less has been focused on the compounds from the uninfected trees.

In China, Japan and India, agarwood has been used as sedative, analgesic and digestive remedy while in traditional Malay medicine it has been used as tonic, stimulant and carminative agent after childbirth [10, 11]. In Indonesia, agarwood has been reported to be used as treatment for joint pain by exposing the pain area to the wood smoke [12]. In Philippines, agarwood has been used to stop wound bleeding, treat skin disease and as substitute of quinine for malaria treatment [13, 14]. In Tibet, agarwood was reported to be used as a remedy to nervous and emotional disorders

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[15]. Meanwhile, in Japan and Korea, agarwood was described as ingredient for stomachic and sedative agent as well as remedy to treat cough, asthma, acroparalysis and croup [16, 6]. These ethnopharmacological evidences, combined with the current trends in bioprospecting, have incited the scientific community to make efforts and investigate claims by using modern tools. One of the earliest scientific study related to agarwood pharmacological activities reported that alcohol extract of *Aquilaria malaccensis* stem bark and stem exerted mild cardiotonic activity and anti-cancer effects against Eagle's carcinoma of the nasopharynx [17]. In another study, agarwood essential oil vapor was reported to possess sedative effects in mice [18]. Another work reported that *Aquilaria malaccensis* leaves extract exhibited potent antioxidant activity [19]. More recently, agarwood materials have also been tested against cancer and inflammation as the two conditions are interrelated; hence studies performed could help reveal target and potential treatment regimen [6]. However, in each of these studies, there was no information on the condition of the tree whether samples were collected from a healthy or infected tree. The act of infecting the agarwood tree is a practise to mimic the natural wounding of the tree to obtain the resin [6].

One study reported cytotoxicity (IC_{50} values of 44 µg/mL) of agarwood oil against MCF-7 breast cancer cells [20]. Another study reported the cytotoxic effects of ethanolic extract from *Aquilaria crassna* stembark with IC_{50} values of 38 µg/mL for HCT116 colorectal carcinoma cells, 72 µg/mL for PANC-1 pancreas/duct epithelioid carcinoma cells, 119 µg/mL for PC-3 prostate adenocarcinoma cells and 140 µg/mL for MCF-7 cells [21]. Other studies showed (i) methanolic extract from *Aquilaria agallocha* woody hull (from the fruit) possessed cancer inhibiting properties [22], and (ii) *Aquilaria malaccensis* stembark (clean and infected part) have cancer inhibiting activity (IC_{50} values of 44 µg/mL) against HCT116 cells [5].

In terms of anti-inflammatory properties, studies showed that (i) hexane extract of heartwood of *Aquilaria agallocha* (*in vitro* at 500 µg/mL) gave 78.5 % protection of human red blood cells in hypotonic solution while at 100mg/kg, it resulted in reduction of carrageenan-induced paw edema in rats [23], (ii) soxhlet-extracted (ethyl acetate) heartwood of *Aquilaria crassna* inhibited TNF-α gene expression when tested *in vitro* on human peripheral blood mononuclear cells (hPBMCs) [24], (iii) ethanolic extract of *Aquilaria sinensis* leaf caused 65 % writhing reduction and 32 % to 51 % inhibiton of paw edema when tested at 848 mg/kg [25] and (iv) *Aquilaria agallocha* heartwood possessed analgesic effects that reduced writhing while increasing the latency of tail flicking and paw licking in male albino mice [26].

Majority of previous biological studies did not report the status of materials (such as leaf and stem) whether they are from infected or uninfected trees. This present study specifically looked at the effects of uninfected leaf and branch extracts of agarwood tree on breast cancer based on their cytotoxicity and inhibition of cell attachment. The term uninfected refers to healthy tree prior to manual inoculation of fungus or bacterial concoction to imitate the agarwood formation process. Continuous supply of uninfected leaf and branch can be obtained from agarwood plantation up to five years after planting since the usual practice of inoculation starts at the tree age of 5 years old. During the first 5 years, leaf and branches will be trimmed in the pruning process to maintain the shape of tree and also to remove crossing branches [27]. Prior to the investigation of the anti-cancer effects, the plant samples must undergo extraction process to obtain the bioactive compounds. The extraction process needs to be flexible, simple and economical with the ability to extract and preserve most of the active compounds present in the plant matrix [28]. The current study is positioned as the platform to screen for a suitable extraction solvent system and plant part that will give extracts that have effective anticancer effects while also maintaining minimum cost of operation, ease of operation and repeatability of data. To the best of our knowledge, this is the first report on the anti-cancer effects of healthy and uninfected leaves and branch extract of *Aquilaria subintegra*.

2. RESULTS

2.1. Selection of solvent system and raw material

Yield of leaf and branch crude extracts obtained using different types of solvent is summarized in Table 1. Overall, data analysis pointed out that agarwood leaves produced higher yield of crude extract (range between 4.53 to 13.2 % (g/g)) compared to branch material (range between 2.10 to 9.47 % (g/g)) when extracted using six different solvents respectively. However, both material types, showed higher yield of extract when extracted using ethanol and distilled water, respectively. This suggests that the samples may have more polar compounds.

Table 1. Data analysis of agarwood crude extract yields by six different solvents.

Solvent	Yield			
	Branches		Leaves	
	Weight (gram)	Yield percentage (%)	Weight (gram)	Yield percentage (%)
Distilled Water	0.70 ± 0.02	9.33 ± 0.23	0.96 ± 0.08	12.8 ± 1.01
Ethanol	0.71 ± 0.09	9.47 ± 1.22	0.99 ± 0.05	13.2 ± 0.69
Acetone	0.59 ± 0.22	7.87± 2.88	0.60 ± 0.04	8.00 ± 0.58
Ethyl acetate	0.15 ± 0.04	2.00 ± 0.46	0.34 ± 0.16	4.53 ± 2.15
Benzene	0.26 ± 0.01	3.47 ± 0.13	0.89 ± 0.10	11.8 ± 1.29
Hexane	0.15 ± 0.04	2.00 ± 0.48	0.51 ± 0.12	6.80 ± 1.60

* Data presented is based on triplicate experiment. Values are expressed as means ± SD (n=3). Solvent is listed based on polarity with distilled water being the most polar.

2.2. Effects of agarwood leaf and branch crude extracts obtained from different solvent extraction on cell attachment

Crude extracts from both agarwood leaf and branch were subjected to cell attachment assay (CAA) to investigate their potential anti-attachment effects. The concentrations of crude extracts were adjusted to 100 µg/mL and introduced to the culture at the time of inoculation. CAA results showed that distilled water as solvent exhibited the highest attachment inhibition for both branch extract which is 89.04 % (10.96 % viability) and leaf extract which is 87.06 % (12.94 % viability), followed by absolute ethanol with 78.08 % inhibition (21.92 % viability) for branch extract and 67.66 % for leaf extract (32.34 % viability). Figure 1 shows the viability of MCF-7 cells after 24 hours of incubation for all tested solvents and tree parts.

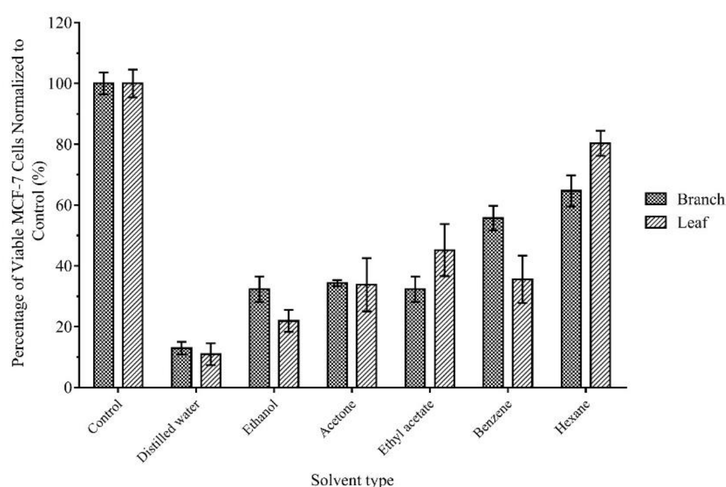

Figure 1. Percentage of viable cell percentages normalized to control group in Cell Attachment Assay (CAA). Viable cells indicated that the cells managed to attach after 24 hours treatment with 100 µg/mL agarwood crude extracts. Data is based on triplicate experimental sets (n = 3 ± s.d). Control is a set of MCF-7 cells treated with 10 % (v/v) DMSO.

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Figure 2 shows the morphology and population density representative images of MCF-7 cells in the CAA procedure for both branch and leaf type, respectively. In agreement with CAA data analysis in Figure 1, MCF-7 cells treated with crude extract from both branch and leaf material type extracted using either distilled water or absolute ethanol treated demonstrated lower population density with respect to control group as compared to crude extracts obtained from other solvents. In this case, extract may have interrupted cell attachment processes which is a prerequisite to cancer growing and spreading. Thus, the declining number of viable cells in this assay reflects the ability of the extracts to prevent cells from attaching to substrate and or cause cells to detach from substrate; cumulatively referred as anti-attachment activities. However, since this is an end-point based assay, the effects seen could also be due to cytotoxic effects (i.e. with the 24 hours treatment, cells may be affected by the cytotoxic effects masking the result as anti-attachment).

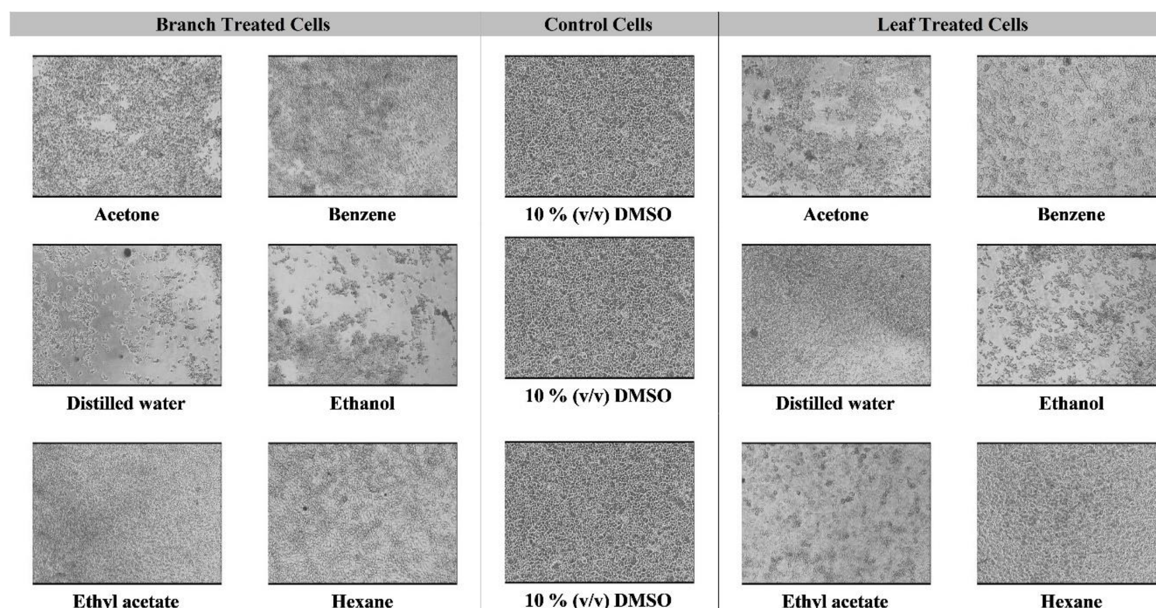

Figure 2. Representative images show viable MCF-7 cells at 24 hours incubation following the treatment of leaf and branch type crude extract from all six solvents respectively. Extracts were included in the growth medium during the cell seeding process to observe the potential anti attachment activity of agarwood. Control is a set of MCF-7 cells treated with 10 % (v/v) DMSO.

2.3. Effects of agarwood leaf and branch crude extracts obtained from different solvent extraction on cell viability

Cell viability assay (CVA) was designed in order to observe the effects of agarwood crude extracts as potential cytotoxic agent. Crude extracts obtained from all six solvents were subjected to this assay. After 24 hours of incubation, healthy and already adherent cells in culture flasks were treated with 100 µg/mL crude extract, incubated for another 24 hours and subjected to cell counting procedure. Figure 3 shows the percentage of viable cells normalized to control after 24 hours incubation with crude extracts. Overall, agarwood crude extracts from distilled water and absolute ethanol solvents for both leaf and branch type showed higher cell inhibition as compared to other solvents. Leaf type extracts from absolute ethanol and distilled water exhibited only 35.29 % and 4.52 % cell viability, respectively. Meanwhile, branch type extracts for absolute ethanol and distilled water showed 13.62 % and 16.73 % viability, respectively.

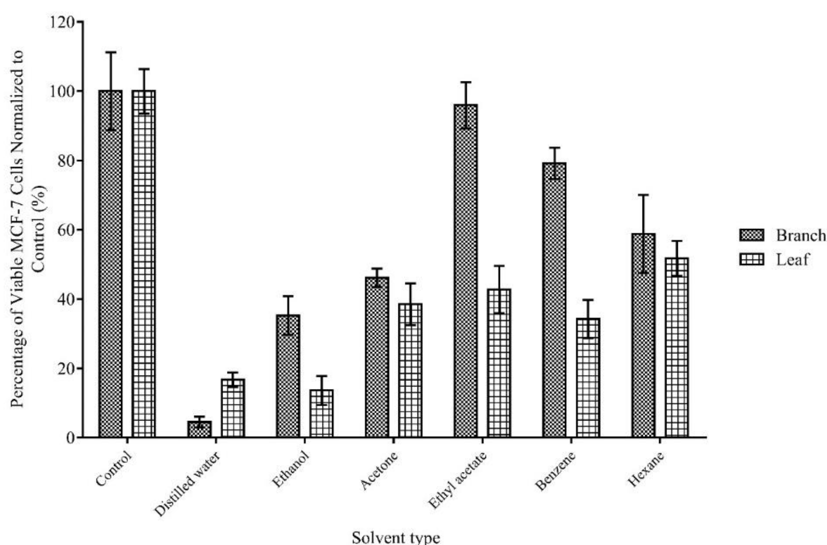

Figure 3. Percentage of viable cell percentages normalized to control group in Cell Viability Assay (CVA). Viable cells indicated that the cells managed to attach after 24 hours treatment with 100 µg/mL agarwood crude extracts. Data is based on triplicate experimental sets (n = 3 ± s.d). Control is a set of MCF-7 cells treated with 10 % (v/v) DMSO.

Figure 4 shows the population density of MCF-7 cells at the end of this assay. Population density showed that both leaf and branch type extracts from absolute ethanol and distilled water demonstrated higher cell inhibition confirming the CVA data plot. Taken together, at 100 µg/mL, agarwood branch and leaf (extracted using absolute ethanol and distilled water respectively) may possess anti-attachment and cytotoxic effects.

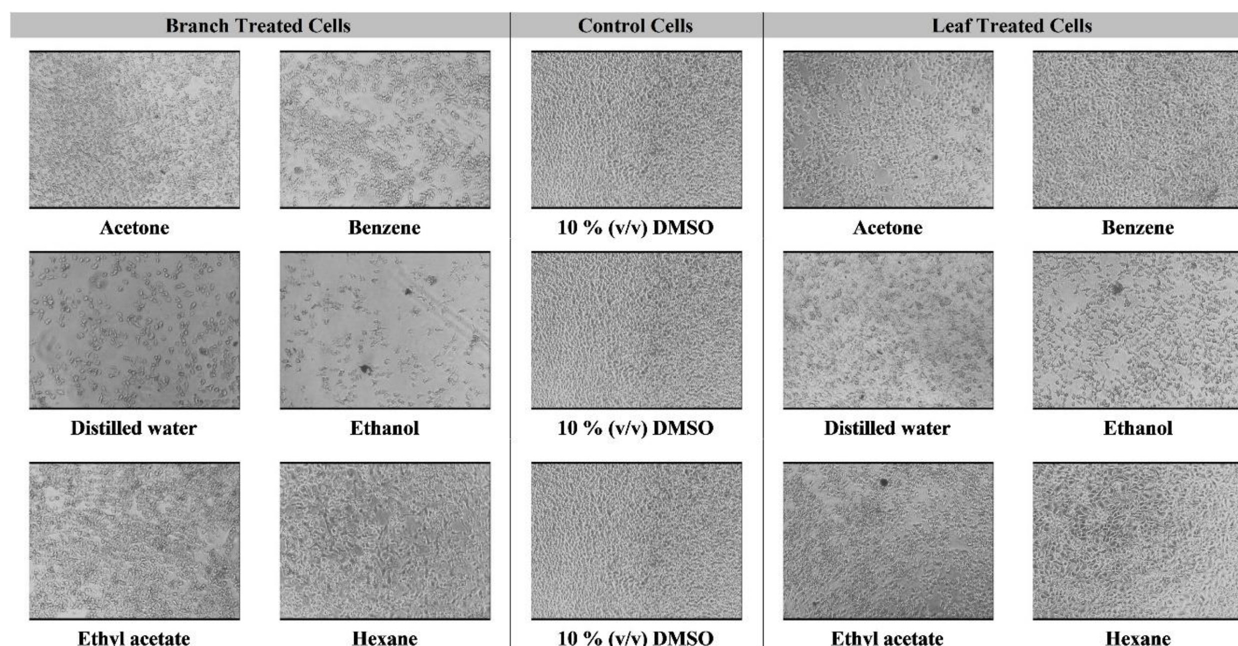

Figure 4. Representative images show viable MCF-7 cells at 24 hours incubation following the treatment of branch and leaf type crude extract from all six solvents, respectively. Cells were inoculated initially and allowed to adhere to culture flasks for 24 hours before treatment with extracts. Control is a set of MCF-7 cells treated with 10 % (v/v) DMSO.

2.4. Cytotoxic effects of agarwood leaf and branch crude extracts obtained from different solvent

Sulforhodamine B (SRB) assay was employed in the study to investigate the potential cytotoxic effects of agarwood leaf and branch extract. Cells were treated with agarwood crude extracts from all six different types of solvents. Extracts were subjected to a serial dilution with a starting working concentration of 50 µg/mL. Taxol was used as positive control group while 10 % (v/v) DMSO was used in negative control group. Classical method was opted to determine the IC_{50} values via the curve fitting of the percentage of controlled cell growth against the concentration tested as shown in Figure 5 [29]. Upon testing, branch extract from absolute ethanol extraction was able to cause 50 % cell kill at the concentration of 23 µg/mL, while IC_{50} for distilled water branch extract was 38 µg/mL. Taxol (positive control) showed IC_{50} of 2.3 µg/mL against MCF-7 cells when tested.

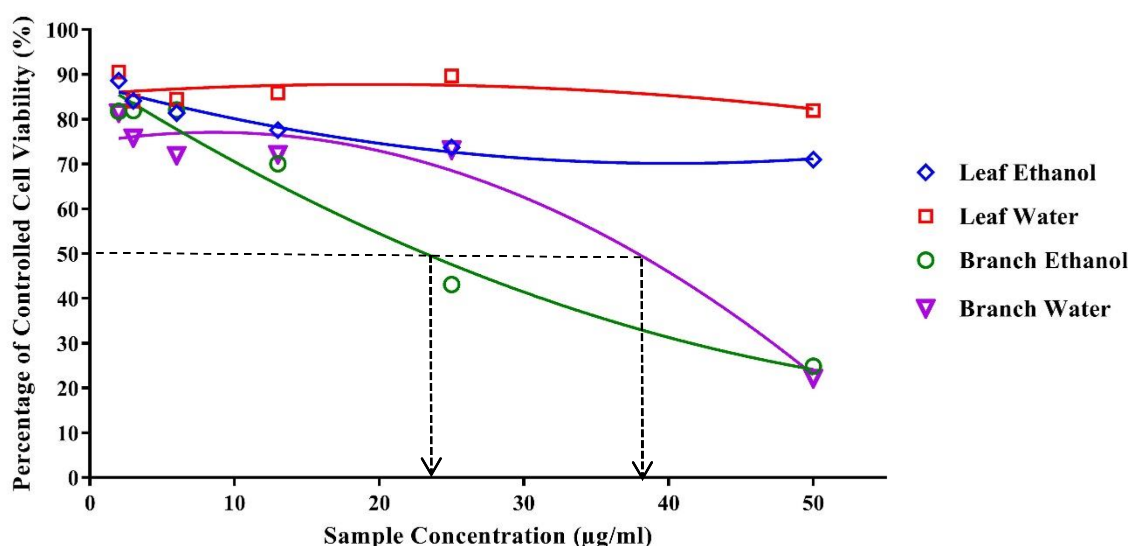

Figure 5. Fitted curve generated for agarwood crude extracts from branch and leaf. Branch ethanolic and distilled water extracts showed 50 % cell kill against MCF-7 cells at the concentration of 23 µg/mL and 38 µg/mL, respectively. IC50 for leaf extracts cannot be determined. Data is based on triplicate experimental sets (n = 3 ± s.d).

3. DISCUSSION

In this study, the cytotoxic and inhibitory effects of agarwood were observed when the extracts from both plant part (leaf and branch) from healthy and uninfected *Aquilaria subintegra* were tested against MCF-7 breast cancer cells. Most study conducted using agarwood did not state whether the sample originated from healthy or infected agarwood trees [14, 16, 19, 22, 24-26]. Extracts from leaf and branch demonstrate profound reduction of cell growth in both Cell Attachment Assay (CAA) and Cell Viability Assay (CVA) as well as showing significant IC_{50} value estimate via the Sulforhodamine B (SRB) Assay. The inhibiting effects for CAA and CVA were observed to be more severe for the ethanolic and water extracted leaf and branch at 100 µg/mL. This is similar to another study conducted to test agarwood oil effects against MCF-7 breast cancer cells that showed inhibition and anti-attachment effects at 200 µg/mL [20]. The results inferred that type of solvents and plant material contributed to different cytotoxic effects of agarwood crude extracts against MCF-7 cells. Yield data analysis showed that different solvent system resulted in different extraction yield result that may be due to different particle size between leaf and branch where the former is finer thus having more efficient mass transfer. In addition, different phytochemicals present in different parts of plants may also lead to the difference in yield [30]. However, both material types, showed higher yield of extract when extracted using ethanol and distilled water, respectively. This suggests that the samples may have more polar compounds. In more detail, branch was found to be the most suitable type of sample for the subsequent experimental design with better *in vitro* cytotoxic effects as compared to leaf although the yield obtained was lower. As for solvent

selection, both absolute ethanol and distilled water exhibited higher yield of crude extract with profound *in vitro* cytotoxicity effects (23 µg/mL for ethanolic branch extract; and 38 µg/mL for water branch extract) against MCF-7 cell when compared to extracts obtained from other types of solvent. The IC_{50} values obtained were comparable to another study conducted using the hulls of *Aquilaria agallocha* Roxb. on cell toxicity against T24 human bladder carcinoma cells (IC50 of 17.82 µg/mL), HT29 human colon adenocarcinoma cells (18.5 µg/mL), HeLa cervical carcinoma cells (35.19 µg/mL), AGS gastric epithelial cancer cells (43.13 µg/mL), and HepG2 human hepatocellular liver carcinoma cells (58.69 µg/mL) [22]. It is worth noting that IC_{50} values for the other types of extracts were not able to be determined in the selected range of concentration.

Generally, water is perceived as the best solvent since it is readily available, practically applicable and relatively cheap compared to other solvents [31]. However, the process of separating water from the extract was either considerably expensive or time consuming. Effective water removing process such as freeze drying and spray drying requires specialized equipment. Since freeze drying operates at low temperature; it is an excellent water removing technique for bioactive compounds. However, the drawbacks include (i) freeze stress that can damage samples, (ii) freezing rate that controls the overall heat transfer during drying, (iii) operational problem in relation to pressure applied during the drying process and (iv) eutectic formation that may disrupt the freeze-drying process [32]. Spray drying is another effective method to obtain 100 % dried product with a low residence time to preserve heat sensitive material. However, there are still concerns about the process since spray dryers operate at high temperatures (100 to 300 °C) which may affect the heat sensitive phytochemical compounds [33].

Ethanol has a moderately low environmental impact and is generally recognized as safe (GRAS). Safety is of paramount importance in any medical, pharmaceutical and nutraceutical research and development [35]. An earlier study was able to isolate cucurbitacins from fruits of *Aquilaria sinensis* using ethanol as the extraction solvent. In the same study, these compounds were reported to be the main constituents contributing to the cytotoxic effects of fruits of *A. sinensis* [34]. Another study also employed ethanol as the solvent system which successfully isolate twelve flavonoids from stems of *Aquilaria sinensis* [36]. To this end, agarwood branch and absolute ethanol were found to be more suitable candidates to be the manipulated and studied in cancer related studies.

4. CONCLUSION

Ethanolic agarwood branch crude extract showed higher *in vitro* cytotoxic effects towards MCF-7 breast cancer cells with IC_{50} value of 23 µg/mL. Results also showed that both distilled water and absolute ethanol gave high yield of extracts for either branch or leaf. However, due to the nature and drawbacks of water as well as versatility of ethanol as extraction solvent, to this end, ethanol is suggested as solvent for future investigation and development in elucidating agarwood role in cancer therapeutics.

5. MATERIALS AND METHODS

5.1. Plant material

Leaves and branches of healthy and uninfected trees of *Aquilaria subintegra* were obtained from a local Malaysian plantation located in Bangi, Selangor, Malaysia. As previously described, un-infected trees refer to healthy trees prior to manual inoculation of fungus or bacterial concoction to imitate the agarwood formation process. The raw materials were freshly collected, washed, rinsed and dried before being pulverized into fine powder for solid-liquid extraction process. The powder was stored in air-tight containers until further use.

5.2. Chemicals and reagents

Acetone, benzene, ethanol, ethyl acetate and hexane were purchased from HmbG Chemical, Germany. Dulbecco's modification of Eagle's medium, DMEM (with high glucose and L-glutamine) in powder form and fetal bovine serum (FBS) were supplied by Gibco® and were used as the culture medium throughout the experiment. Sulforhodamine B powder was purchased from Sigma-Aldrich Chemical, USA. Commercial drug, Taxol (Paclitaxel) was from Chemolab Supplies, Malaysia.

5.3. Cell lines

MCF-7 breast adenocarcinoma cell (ATCC® HTB-22™) was obtained from American Type Culture Collection (ATCC).

5.4. Selection of solvent system and raw material

Screening for solvent is crucial in order to determine the most suitable solvent system for an efficient extraction process that can actually extract the important bioactive compounds from samples of *Aquilaria subintegra*. In this first phase of study, types of solvent (acetone, benzene, ethanol, ethyl acetate, distilled water and hexane) and types of plant material (leaf or branch) were investigated while other quantitative parameters (solid-liquid ratio, agitation speed, temperature and time) were kept constant.

Amount of 7.5 g of leaf and branch powder was added to 100 mL of solvent, respectively. The conical flasks containing the mixture were capped with aluminium foil and placed in a temperature controlled incubator shaker at 40 °C and 100 rpm for 18 hours. Upon completion of the incubation, each mixture was filtered using the vacuum filtration apparatus. The filtrate was collected, concentrated by vacuum rotary evaporator and dried using nitrogen gas flushing. Weight of each crude extract was recorded before dissolving in 100 % (v/v) dimethylsulfoxide (DMSO) and stored in -20 °C freezer until further use. Response variables, namely yield and anti-cancer properties were determined for each set of experiment. Testing was carried out using three independent experiments for each type of solvent and plant material, respectively.

5.5. *In vitro* cytotoxic/anti-cancer screening assays

In order to study the potential anti-cancer effects of plant materials from *Aquilaria subintegra,* all extracts from both phases of study were subjected to *in vitro* cytotoxic/anti-cancer screening assay.

5.5.1. Cell Attachment Assay (CAA)

In this assay, crude extracts at adjusted concentrations were added to the culture media in the flask at the time of cell seeding with cell concentration of 1 x 10^5 cells/mL in a T-25 cm^2 culture flasks. 10 % (v/v) DMSO was added into the control flask. After 24 hours incubation at 37 °C/ 5 % CO_2, cells were washed with phosphate buffered saline (PBS), detached using accutase and then subjected to cell counting procedure using trypan blue dye exclusion method [29].

5.5.2. Cell Viability Assay (CVA)

Confluent cells were harvested and seeded into new T-25 cm^2 tissue culture flasks at the concentration of 1 x 10^5 cells/mL in 5 mL culture medium. The cultured flasks were then incubated at 37 °C/5 % $CO_2$for 24 hours. Following that, the spent media was discarded and fresh media containing crude extracts at adjusted concentrations were added into each flask. For control flask, 10 % (v/v) DMSO was added. Cells were subjected to final incubation at 37 °C/5 % CO_2 for 24 hours. Finally, cells were washed with phosphate buffered saline (PBS), detached using accutase and then subjected to cell counting procedure using trypan blue dye exclusion method [29].

5.5.3. Sulforhodamine B (SRB) Assay

This was a modified procedure [37], where crude extracts dissolved in 10 % (v/v) DMSO was prepared in two-fold serial dilutions with an initial concentration of 1000 μg/mL. 70-80 % confluent cells were detached from the culture flasks using accutase followed by trypan blue dye exclusion procedure in order to determine the seeding number for the 96-well tissue culture plate at 1 x 10^5 cells/mL in 190 μl culture media. Plates were then incubated at 37 °C/5 % CO_2 for 24 hours. Subsequently, 10 μl of each extract dilution was added into each well and incubated for 72 hours. Later, without removing the supernatant, 100 μl of cold trichloroacetic acid (TCA) was added into each well and plates were incubated at 4°C for an hour prior to washing under slow-running tap water. Plates were then allowed to dry at room temperature. For the staining step, 100 μl of 0.057 % (w/v) SRB solution was added into each well and left at room temperature for 30 minutes. Immediately afterwards, plates were rinsed four times with 1 % (v/v) acetic acid to remove unbound dye. Insufficient dye removal caused overestimation of cell mass whereas excessive washing caused reduced-estimation of cell mass due to bleaching of protein-bound dye. Then, 100 μl of 10 mM Tris base solution was added into each well containing the protein-bound dye for solubilisation. Plates were then placed on a gyratory shaker for 10

minutes. Optical density (OD) reading was measured for all plates at 510 nm wavelength. IC_{50} value (concentration of compound that yields 50 % fewer cells compared to control) was derived from curve-fitting methods following determination of percentage of controlled cell growth based on the Equation 1 below.

$$\%\ of\ controlled\ cell\ growth = \left(\frac{mean\ OD_{sample} - mean\ OD_{blank}}{mean\ OD_{negative\ control} - mean\ OD_{blank}}\right) \times 100 \qquad \text{(Eq. 1)}$$

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