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# Anti-cancer Potential of Agarwood Distillate

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**Agarwood or Gaharu by definition is the resin impregnated heartwood that produces unique aromatic scent when burnt. Apart from religious rituals and perfumes, agarwood has also been used as traditional medicines leading to the interest of this study which is to screen for anti-cancer properties. In this study, agarwood water distillate (hydrosol) obtained from distillation of agarwood (resin) were screened against MCF-7 cells, which are commonly used as *in vitro* model for breast cancer and VERO cells (normal cells). Distillate samples were collected, diluted and directly subjected to three anti-cancer screening assays (cell attachment assay, cell viability assay and sulforhodamine B assay). It was found that agarwood distillate possesses anti-cancer activity and exerts no significant effects on normal cells. This warrants further investigation with potential development of alternative remedy against cancer while adding more value to the agarwood industry.**

**Key words:** Agarwood, Gaharu, Distillate, Hydrosol, Anti-cancer, Cell culture.

The fragrant wood and resin of *Aquilaria* species is known as agarwood, eaglewood or gaharu depending on the country. It is used to fulfil demand in religious, medical and aromatic preparation[1, 2]. Traditionally, agarwood has been used as sedative, analgesic and digestive medicine[2]. Agarwood has also been used as a complex ointment for smallpox,various abdominal complaints and rheumatism[3].

More recently, scientific works on *Aquilaria spp*. have shown promising results in the pharmacological field. A study stated that extract obtained from the leaves of *Aquilaria sinensis* has anti-inflammatory, antitumor, analgesic, therapeutic, and prophylactic activity on constipation, intestinal obstruction, and obesity, therapeutic effect on hemorrhage and cerebral ischemia, and blood glucose-reducing effect[4]. Another study reported that alcoholic extract of *Aquilaria* malaccensis exhibited mild cardiotonic activity and significant activity against Eagle's carcinoma of the nasopharynx[5]. A recent work discovered that extract from *Aquilaria malaccensis* exhibit potent antioxidant activity[6]. Essential oil vapour from agarwood possessed sedative effects through evaluation of sedation and excitation activities by observing spontaneous motor activity of mice[7]. Agarwood was suggested to be involved in stimulation mechanism of intestinal motility via the acetylcholine receptors[8].

Agarwood distillate (AD) is obtained from the essential oil steam distillation process. Other plant hydrosols have been reported to exhibit therapeutic properties. For instance, rose distillate exhibited antibacterial property and cedarwood hydrosol possessed antiviral and antiseptic properties[9]. A previous study estimated that a

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maximum of 29 % of water from distillate comes from the plant material suggesting that plant distillate may demonstrate therapeutic properties possessed by the original plant9. Despite these findings, plant distillates are often regarded as waste by-products in the distillation process. This is also true in the agarwood industry where the sought resin is distilled to produce expensive aromatic oil, leaving the distillate as waste. While therapeutic properties of agarwood plant and its various parts have been reported, scarce information can be found on its distillate. This present study is therefore crucial in discovering the therapeutic potential of AD particularly its anti-cancer properties.

## MATERIALS AND METHODS

### Sample preparation

AD sample was collected and diluted with deionized distilled water ($ddH_2O$) according to the concentration desired. Both MCF-7 breast cancer cells (ATCC® HTB-22™) and Vero (green monkey kidney cells, ATCC® CCL-81™) representing normal cells were maintained at 37 °C/ 5 % $CO_2$ in Dulbecco's Modified Eagle medium (DMEM) with 10 % serum.

### Cell Viability Assay (CV)

This simple procedure is often carried out to determine the potential cytotoxicity of the compounds being studied, either because the compounds are being used as pharmaceuticals and must be shown to be non-toxic, or because they are designed as anti-cancer agents and cytotoxicity may be crucial to their action[10]. The principle of this assay is based on the breakdown of membrane integrity determined by the uptake of trypan blue into non-viable cells. In this study, the assay was used to study the effects of AD at different concentration on cell viability and the ability to cause loss of cell adhesion from plastic surface. Confluent cells were harvested and seeded into new T-25 $cm^2$ tissue culture flasks at adjusted concentration of $1 \times 10^5$ cells/ml. The cultured flasks were then incubated for 24 hours at 37 °C in a humidified incubator with 5 % $CO_2$. After 24 hours, the old media was discarded and new media containing samples was added while $ddH_2O$ was added into control flask. Cells were subjected to final incubation for 24 hours at 37 °C in a humidified incubator with 5 % $CO_2$. 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.

### Cell Attachment Assay (AT)

This simple procedure provides rough information about the adhesion system and overall effect of anti-attachment. Attachment is a process preceding cell proliferation, spreading and tumour invasion and metastasis[10]. Cell attachment or adhesion is mediated by specific cell surface receptors. Trypsin or accutase is used to dissociate cells from the cell-cell and cell- matrix interaction. After tissue disaggregation, adherent cells need to attach and spread out before proliferation can commence. In this assay, AD at adjusted concentrations using $ddH_2O$ was added to the culture media in the flask at the time of cell inoculation. Deionized distilled water was added into the control flask. After 24 hours incubation at 37 °C in a humidified incubator with 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. Anti-attachment effects from sample would result in reduced number of viable cells.

### Sulforhodamine B(SRB) Assay

SRB is a bright-pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues under mild acidic condition11. In this method, cell density was determined based on the measurement of cellular protein content. The assay relies on the ability of SRB to bind to protein components of cells that have been fixed to tissue culture plates by trichloroacetic acid (TCA). Initially, AD at adjusted concentrations in $ddH_2O$ was prepared in twofold serial dilutions with an initial working concentration of 100 % (v/v). Confluent cells were detached and counted in order to determine the seeding number for the 96-well tissue culture plate at $1 \times 10^5$ cells/ml in 190 µl culture media. Plates were then incubated at 37 °C in a humidified incubator with 5 % $CO_2$ for 24 hours. Subsequently, 10 µl of each dilution was added into each well and incubated for 72 hours. Later, without removing the cell culture supernatant, 100 µl of cold TCA was added into each well and plates were incubated at 4°C for an hour followed by the washing step using 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 can cause overestimation of cell mass whereas excessive washing can cause 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 and plates were then placed on a gyratory shaker for 10 minutes. OD reading was measured for all plates at 510 nm wavelength. IC50 value (concentration of compound that yields 50 % less cells compared to control) was derived from curve-fitting methods. OD data was used to plot the dose-response between the compound concentration and growth inhibition percentage[1].

## RESULTS AND DISCUSSION

Figure 1 shows CV and AT assay plot for VERO cells after exposure to different concentrations of AD samples. CV assay data analysis showed that culture exposed to 100 % (v/v) AD sample displayed viable VERO cells in the range of 88.5 % to 90.7 % as compared to the control. The AT assay data analysis exhibited consistent trend with the CV assay results where cell attachment was observed to be in the range of at 82 %, to 80.8 % compared to control.

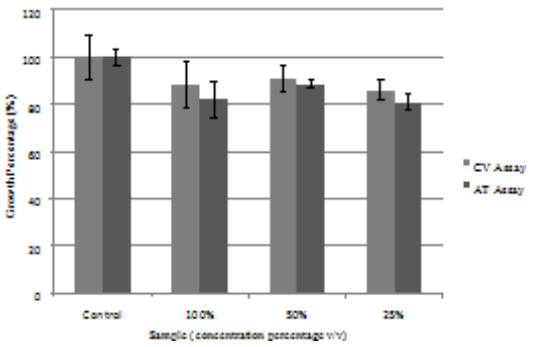


**Fig. 1.** VERO cells exposed to different concentrations of AD normalized to control group in CV and AT assay. Results are expressed as mean ± s.d; n=3

Meanwhile, Figure 2 shows the CV and AT assay data plot for MCF-7 cells normalized to control. Growth percentage in CV assay recorded was lower compared to VERO cells, in which only 63.3 %, 58.2 % and 60.6 % viable MCF-7 cells were observed after exposure to AD at 100 % (v/v), 50 % (v/v) and 25 % (v/v) respectively. Likewise, AT Assay data on MCF-7 cells also showed lower cell attachment percentage at 42 %, 46 % and 54 % when 100 % (v/v), 50 % (v/v) and 25 % (v/v) AD were added to the culture medium, respectively. In this instance, it can be suggested that AD possess anti-attachment effects in addition to cytotoxicity effects. This is particularly interesting for the development of anti-cancer treatment or prevention at pre-metastatic level.

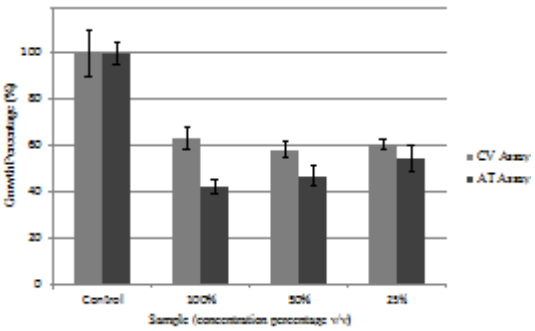


**Fig. 2.** MCF-7 cells exposed to different concentrations of AD normalized to control group in CV and AT assay. Results are expressed as mean ± s.d; n=3

Together, CV and AT study pointed out that an increase in the concentration of AD posed pronounced effects on MCF-7 cells as compared to VERO cells. When tested using SRB assay, the percentage of control cell growth showed decreasing trend as the AD concentration increased. Figure 3 shows the dose-response analysis of AD against MCF-7 cells. From the fitted curve, it was observed that at 35 % (v/v) concentration, AD caused 50 % cell inhibition

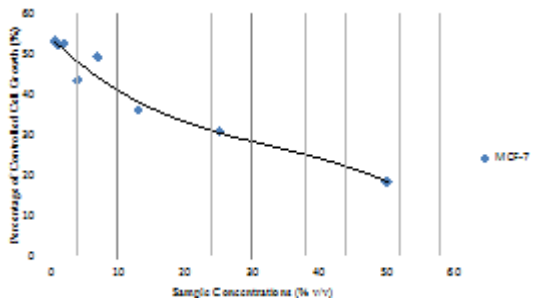


**Fig. 3.** Dose-response fitted plot for agarwood distillatel against MCF-7 breast cancer cell line following SRB assay. Agarwood distillate at 50 % (v/v) was able to inhibit 35 % of the controlled cell growth ($IC_{50}$: 35 % v/v)

suggesting that AD possesses the cytotoxic ability against MCF-7 cancer cells.

## CONCLUSIONS

Agarwood distillate (AD) has pronounced cytotoxic and anti-attachment activities on MCF-7 cells based on the CV and AT assay respectively. AD also showed cytotoxic effects against MCF-7 cells at $IC_{50}$ value of 35 % (v/v) when tested using SRB assay. Taken together, it can be suggested that AD possesses anti-cancer properties which warrants further investigation.

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