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REVIEW ARTICLE

CYTOTOXIC EFFECTS AND RESPONSE SURFACE OPTIMIZATION OF SOLVENT EXTRACTION OF CRUDE EXTRACTS FROM Aquilaria subintegra UNINFECTED BRANCH

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ABSTRACT

Agarwood plant has been used in traditional medicine to treat wide range of disorders aside from the main function of its fragrant wood in perfumery. In this present study, we are interested to maximize the yield of extract and investigate the cytotoxic/anti-cancer effects of uninfected branch extracts. Uninfected branch is readily available from agarwood plantation particularly during the early years before inoculation process that formed the fragrant wood (resin); and as such is seen as an economical raw material. Uninfected branch from Aquilaria subintegra was subjected to response surface methodology (RSM) - guided ethanolic extraction to achieve maximum yield while maintaining the biological activity. Cytotoxicity/anti-cancer assays including cell attachment assay, cell viability assay and sulforhodamine B (SRB) assay were carried out on the extracts. Run 11 with 12 hours extraction time, 50 °C temperature, 100 rpm and 60 ml extraction volume gave the highest yield of 0.2130 ± 0.036 g/g agarwood branch extract (ABE). Experimental extract from run 16 showed the most promising cytotoxic effects against MCF-7 with IC_{50} of 8 µg/ml. The study showed that ABE possess potential cytotoxic/anti-cancer activities against MCF-7 cells and further research is warranted to identify the bioactive compounds and mechanism of action.

KEYWORDS

Agarwood, *Aquilaria subintegra,* branch, breast cancer, cytotoxic, optimization, response surface methodology.

1. INTRODUCTION

The fragrant-wood of the agarwood tree has been the subject of interest in the perfumery industry due to its unique odour profile [1]. This fragrant-wood is formed through resin development and deposition in wounded trees in response to the host defence system. The agarwood trees are from the genus *Aquilaria* and some other members of the *Thymelaeceae* family. Apart from its use in perfumery industry, the fragrant-wood (commonly referred to as agarwood) and its essential oil have also been reported to have various health beneficial effects. One of the first scientific study related to agarwood pharmacological activities reported that alcoholic extract of *Aquilaria malaccensis* stem bark and stem exhibited mild cardiotonic activity and anti-cancer effects against Eagle's carcinoma of the nasopharynx [2]. However, the full potential of agarwood as source of medicinal compounds is challenged by its unique and relatively slow formation process. Due to this, in more recent years, the scientific community has turned to other parts of agarwood tree in search for potential health beneficial compounds guided by ethnopharmacology evidences. A review on traditional and modern-based medicinal health benefits of various parts of agarwood tree [3].

In this present study, we are interested to specifically investigate the effects of uninfected branch extracts of agarwood tree on breast cancer based on their cytotoxicity. In a study, reported that ethanolic branch extract obtained through extraction based on selected processing conditions exhibited more potent cancer-inhibiting effects with IC_{50} of 23 µg/mL (ethanol) and 38 µg/mL (distilled water) as compared to leaf from uninfected trees [4]. The term uninfected refers to healthy tree prior to manual inoculation of fungus or bacterial concoction to imitate the agarwood formation process in plantation scale. In view of continuous supply of uninfected branch from agarwood plantation, particularly during the years before inoculation (commonly five years), the uninfected branch is seen as a more economical raw material as compared to resin which is the focus after the tree inoculation process. Prior to the investigation of the anti-cancer effects, the plant samples must undergo extraction process to obtain the bioactive compounds. In order to prevent loss of these bioactive compounds while also maintaining high yield, extraction method and parameters must be carefully selected and optimized [5].

To the best of our knowledge, to date there are no reports on the optimization of processing conditions to achieve high yield of uninfected branch extract of *Aquilaria subintegra* and investigation of its cytotoxic/anti-cancer effects against breast cancer and normal cell lines. In this study, significant extraction parameters were identified and the optimum conditions were predicted using the generated response surface methodology 2^4 full factorial design.

2. MATERIALS AND METHODS

2.1 Plant Material

Branches of uninfected trees of *Aquilaria subintegra* were obtained from a local Malaysian plantation located in Bangi, Selangor, Malaysia. The

branches 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.

2.2 Chemicals and reagents

Ethanol was purchased from HmbG Chemical, Germany. Cell culture media (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®. Sulforhodamine B powder was purchased from Sigma-Aldrich Chemical, USA. Commercial drug, Taxol (Paclitaxel) was from Chemolab Supplies, Malaysia.

2.3 Cell lines

MCF-7 breast adenocarcinoma cell (ATCC® HTB-22™) and African Green Monkey kidney VERO cell (ATCC® CCL-81™) were obtained from American Type Culture Collection (ATCC).

2.4 Ethanolic extraction

Amount of branch powder was added to ethanol in accordance to experimental design (see Section 2.5). The mixture (in capped conical flasks) was placed in a temperature-controlled incubator shaker at pre-determined temperature, agitation and time (Section 2.5); prior to filtration using vacuum filtration apparatus. Collected filtrate was then 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 [4].

2.5 Experimental design for response surface methodology

Optimization experiment was carried out using response methodology (RSM) to obtain maximum crude extract from agarwood branch. Extract from each run was also tested for cytotoxic effects against MCF-7 and VERO cells. Factors namely, solid-liquid ratio, agitation speed, temperature and time were varied based on a "2^4 full factorial design" generated by MODDE, SIMCA-P+ V12.0.1 (Umetrics, Umea, Sweden). The 2^4 factorial refers to factor (4 factors), levels (2 levels), and conditions (2^4 = 16), respectively [6]. This design was employed to study the possible cumulative effects of various parameters on the yield of extract in the most time and cost-effective way. For solid-liquid ratio, sample (branch) weight was maintained at 3 g per run while volume of solvent being varied. The experimental design generated using the software is shown in Table 1 (refer to result Section 3.1). Adopting the design, each individual experiment was subjected to similar process as in Section 2.4 (filtration, concentration via vacuum rotary evaporator, drying via nitrogen gas flushing, weight recording and finally dissolving in 100 % (v/v) DMSO for storage). Then, each crude extract was subjected to the cytotoxic/anti-cancer screening assays. Three independent experiments were carried out for each run.

2.6 *In vitro* cytotoxic/anti-cancer screening assays

In order to study the potential cytotoxic/anti-cancer effects of plant materials from *Aquilaria subintegra,* extracts from each experimental run were subjected to *in vitro* cytotoxic/anti-cancer screening assay. In this section, 10 % (v/v) DMSO was used as control as the extract was diluted using DMSO. As such this would confirm that the results from treated cells were not due to background effects of the DMSO

2.6.1 Cell Attachment Assay (CAA)

Cell attachment assay was conducted following methods in Abbas et al, 2018 [4]. 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), trypsinized using accutase and then subjected to cell counting procedure using trypan blue dye exclusion method.

2.6.2 Cell Viability Assay (CVA)

Cell attachment assay was conducted following methods in Abbas et al, 2018 [4]. 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. Spent media was then 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), trypsinized using accutase and then subjected to cell counting procedure using trypan blue dye exclusion method.

2.6.3 *Sulforhodamine B (SRB) Assay*

The cytotoxicity assay was modified from Vichai and Kirtikara, 2006 and as reported in Abbas et al, 2018 [4, 7]. Briefly, crude extracts were dissolved in 10 % (v/v) DMSO. Plates with 1 x 10^5 cells/ml in 190 µl culture media (in each well); were 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. Cold tricholoroacetic acid (TCA) was added prior to staining step using SRB solution and washing with acetic acid. Finally, 10mM 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 and optical density (OD) reading was measured at 510 nm. 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. In addition, commercial Taxol (Paclitaxel) was used as positive control for IC_{50} comparison purposes.

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

2.7 Statistical analysis

Results obtained were expressed as mean ± standard deviations of the replicates unless otherwise stated. Response Surface Methodology (RSM) based model fitting and statistical analysis; including analysis of variance (ANOVA) was performed using MODDE, SIMCA-P+ V12.0.1 (Umetrics, Umea, Sweden).

3. RESULTS AND DISCUSSION

3.1 Process conditions affecting the extraction of agarwood branch

Response surface methodology was employed to obtain the most influential parameters that affect the yield of ethanolic agarwood branch extracts (ABE). High yield of extract from optimal extraction process reflects the productivity level as well as facilitates subsequent product development. Four (4) factors which have shown high positive effects on yield in many plant extraction works reported by various groups; were selected in this model: (i) extraction time (h), (ii) agitation speed (rpm), (iii) temperature (°C) and (iv) solid to solvent ratio. The complete experimental run and yield obtained from each run are shown in Table 1. The mean yield of ABE obtained from the experiment varied from 0.0993 ± 0.056 g/g to 0.2130 ± 0.036 g/g. Run 11 with 12 hours extraction time, 50 °C temperature, 100 rpm and 60 ml extraction volume gave the highest yield of 0.2130 ± 0.036 g/g ABE.

Table 1: Experimental runs of "2^4 full factorial" design generated using MODDE (SIMCA-P12+ V12.0.1) with coded and actual values of four independent parameters of process conditions. Actual experimental yield of ABEs are also listed.

Exp No.	X_1[a]	X_2[b]	X_3[c]	X_4[d]	Actual Yield (g/g)
1	-1(12.00)	-1(30.00)	-1(100.00)	-1(30.00)	0.1535 ± 0.060
2	+1(24.00)	-1(30.00)	-1(100.00)	-1(30.00)	0.1499 ± 0.023
3	-1(12.00)	+1(50.00)	-1(100.00)	-1(30.00)	0.1774 ± 0.057
4	+1(24.00)	+1(50.00)	-1(100.00)	-1(30.00)	0.1971 ± 0.071
5	-1(12.00)	-1(30.00)	+1(200.00)	-1(30.00)	0.0993 ± 0.056
6	+1(24.00)	-1(30.00)	+1(200.00)	-1(30.00)	0.1200 ± 0.035
7	-1(12.00)	+1(50.00)	+1(200.00)	-1(30.00)	0.0895 ± 0.013
8	+1(24.00)	+1(50.00)	+1(200.00)	-1(30.00)	0.0888 ± 0.043
9	-1(12.00)	-1(30.00)	-1(100.00)	+1(60.00)	0.1305 ± 0.039
10	+1(24.00)	-1(30.00)	-1(100.00)	+1(60.00)	0.1370 ± 0.071
11	-1(12.00)	+1(50.00)	-1(100.00)	+1(60.00)	0.2130 ± 0.036
12	+1(24.00)	+1(50.00)	-1(100.00)	+1(60.00)	0.1841 ± 0.072
13	-1(12.00)	-1(30.00)	+1(200.00)	+1(60.00)	0.0999 ± 0.033
14	+1(24.00)	-1(30.00)	+1(200.00)	+1(60.00)	0.1155 ± 0.033
15	-1(12.00)	+1(50.00)	+1(200.00)	+1(60.00)	0.1438 ± 0.038
16	+1(24.00)	+1(50.00)	+1(200.00)	+1(60.00)	0.1094 ± 0.014

X_1-Extraction time (hour), X_2-extraction temperature (°C), X_3-agitation speed (rpm), X_4-volume based on solid to solvent ratio (ml). Sample weight was kept constant at 3 g. Actual yield (g/g) is based on triplicate data. Values are expressed as mean ± SD (n=3). Highlighted row represented the highest yield.

Summary of fit plot generated by the MODDE software fitted using the Partial Least Square (PLS) projection with a logarithmic transformation generated a model fit with the R^2 value of 0.9591 and Q^2 of 0.7355. Coefficient of determination (R^2), being the measure of the goodness fit and adequacy of the applied model, indicates that 95.91 % of the total variation was explained by the model. Meanwhile, Q^2 value denotes the goodness of prediction. In general, a $Q^2 > 0.5$ is accepted as good and the difference between R^2 and Q^2 should be in the range of 0.2 to 0.3 with substantially larger difference suggested an inappropriate model [8]. Here, R^2 and Q^2 difference was 0.2236 which still in the range ($0.2<R^2-Q^2<0.3$); suggesting that the model was appropriate.

Coefficient plot in Figure 1 was then generated to display the regression coefficient in a more convenient manner. In this plot, each bar represents regression coefficient while the error bar represents the confidence interval which indicates the uncertainty of each coefficient [8]. Agitation speed was found to have the largest negative effect followed by the temperature-agitation interaction coefficient. This suggests that that lower agitation speed may give more profound effects to the yield of ABE. Indeed, many studies in relation to bioactive compounds and cytotoxic effects of plant samples employ maceration method in which no shaking and agitation is involved [9-12].

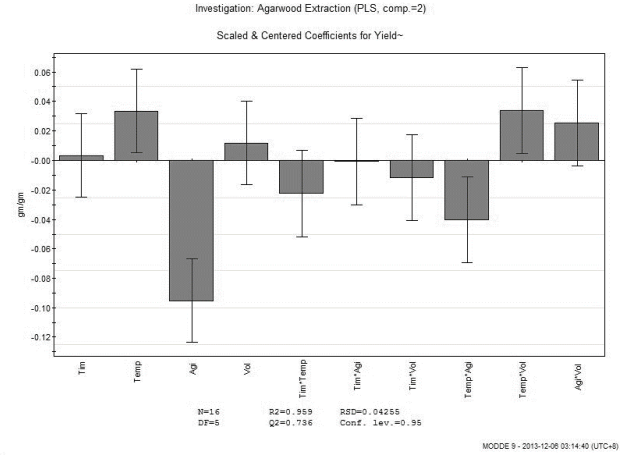

Figure 1: Regression coefficient plot generated for yield of ABEs model. Error bars represented confidence interval.

Temperature and temperature-volume coefficients were both positive significant effects suggesting that high temperature gave higher yield of ABE. Similar trend was also observed in another study in which higher temperature result in higher yield of chestnut tree wood extract [11]. Time, volume and other interaction coefficients were statistically insignificant since the confidence intervals crosses zero at y-axis [8].

The influential parameters of temperature and agitation on yield were further demonstrated in the response contour plot. Interaction between high temperature (>40 °C) and with low agitation (<100 rpm) is desired to achieve higher yield (Figure 2A). Meanwhile, analysis on the volume-temperature interaction in Figure 2B suggested that higher temperature combined with sufficient solid to solvent ratio resulted in higher yield of ABE. The contour plot demonstrated that at the range of 1:15 to 1:20 solid to solvent ratio combined with high temperature resulted in high yield of ABEs.

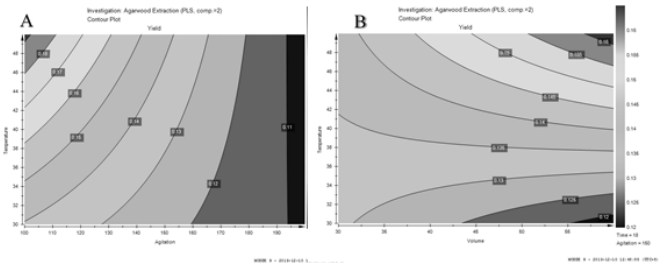

Figure 2: Response contour plot for experimental yield. (A) The interaction between temperature and agitation suggested that at high temperature and low agitation, more ABEs was produced. (B) The interaction between temperature and volume showing at high temperature and high solid to solvent ratio produced higher ABEs.

The model design was able to identify significant factors influencing the yield of ABE. Through partial least square projection (PLS), two-parameter interaction of temperature and agitation speed showed strong interaction suggesting that improvement to the model should be made by positioning new experiments with new ranges for these two terms. It is reported that higher working temperature favours extraction, enhancing both the solubility of solute and diffusion coefficient [11]. However, high temperature will have impact on the volatile compounds present in ABEs. A previous study revealed that hot water extraction produced lower biological activity of xanthine oxidase inhibitor extracted from leaf sample of banaba tree (a local Philippines tree) [13]. This issue was not considered by the software thus a maximum of 60 °C to 70 °C limit should be kept in future experiment. As for agitation speed, range lower than 100 rpm was suggested by the contour plot. Since most extraction study employs maceration method to obtain crude extracts, shifting the range of agitation speed between 0 rpm to 100 rpm is suggested for the next

modelling. Further, the model suggested that both solid to solvent ratio and extraction time were not significant to the model. Thus, for subsequent phase of research, both time and volume parameters may be kept constant in the experimental design.

3.2 *In Vitro* Cytotoxic/Anti-cancer Screening Activities of Agarwood Branch Extract (ABE)

ABEs from each experimental run were subjected to the three *in vitro* anti-cancer screening assays against MCF-7 breast cancer cells and VERO normal cells in order to observe the cytotoxic effects. The three *in vitro* assays included were Cell Attachment assay (CAA), Cell Viability assay (CVA) and Sulforhodamine B assay (SRB). Concentration of ABE from each experimental run was adjusted to 100 µg/ml for the CAA and CVA assays while in the SRB study, ABE from each run was serially diluted with a starting concentration of 50 µg/ml.

3.2.1 Effect of ABE on cell attachment

Cell counting procedure (trypan blue dye exclusion method) was not successful since the unattached but viable cells were washed away during the washing step using phosphate buffered saline. This suggests that ABE obtained from each experimental run at 100 µg/ml concentration may possess high anti-attachment and cytotoxic effects against MCF-7 and VERO cells. Figure 3 shows the morphology of MCF-7 cells after 24 hours of incubation in the presence of ABEs in the culture medium.

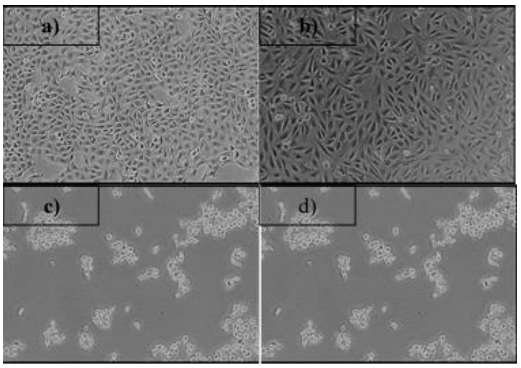

Figure 3: MCF-7 and VERO cells after 24 hours of co-incubation with ABE in the culture medium in CAA. (a,b) Control MCF-7 and VERO cells treated with 10 % (v/v) DMSO respectively. (c,d) ABE treated MCF-7 and VERO cells, respectively. Treated groups exhibited floating cells (viable and dead) due to failure to attach to the culture flask. Counting procedure was not successful since floating viable cells were rinsed during the washing step of the process.

3.2.2 Effect of ABE on cell viability

Figure 4 show the viable cell percentage plot for all ABE obtained from experimental runs against MCF-7 cells and VERO cells in CVA. Data obtained showed reduced cell number of both MCF-7 and VERO cells suggesting that at high concentration, ABEs were able to affect the viability of both cancer and normal cells. The inhibition percentage ranged from 66.18 % to 82.12 % for MCF-7 cells. ABEs also exhibited similar effect on VERO cells within the range of inhibition from 54.17 % to 83.85 %

Figure 4: Viable cell percentage plot normalized to control. Both MCF-7 and VERO cell number was reduced after 24 hours of treatment with ABEs at 100 µg/ml. Both cell lines were cultured initially without ABEs for 24 hours. Values are expressed as mean ± SD (n=3).

CVA was designed in order to observe the effects of ABE as potential cytotoxic agent which kills or inhibit viable cell growth. Additionally, the reduction of cell number seen in the CVA study could also be a cumulative effect of agarwood extract as anti-attachment agent (attribute of preventing cells to attach to substrate and or causing cells to detach from substrate). Taken together, the observed cumulative effect is particularly interesting for the development of anti-cancer treatment or prevention at pre-metastatic level. Changes in cell morphology and population density reduction of both MCF-7 and VERO cells were observed before and after treatment with ABEs at 100 µg/ml. After treatment with ABEs, no cells were able to adhere, whereby adhering is important for cell proliferation and spreading [14].

3.2.3 Cytotoxic effect of ABE

Table 2 listed the IC_{50} values obtained for both MCF-7 and VERO cells when curve fitting technique was applied to each plot from each respective run. It was found that ABE from run 16 with the extraction time of 24 hours, 50 °C extraction temperature, agitation speed of 200 rpm and 60 ml solvent volume give the lowest IC_{50} value of 8 µg/ml. It can also be observed that runs with high agitation speed (experimental run 7 and 8) and combination of high agitation speed and low temperature parameters (experimental run 5 and 6) resulted in non-determinable IC_{50} values which suggested that different concentration range was needed for extracts from these experimental settings to exhibit cytotoxicity against MCF-7 and VERO cells. Meanwhile, Taxol gave the IC_{50} values of 2.8 µg/ml and 2.4 µg/ml for MCF-7 cells and VERO cells, respectively, as expected from commercial drug. This also assured that the experimental work undertaken was reliable with credible results while allowing comparison of effectiveness of extract against commercial drug. Referring to Table 2 the lowest IC_{50} value fitted against MCF-7 cells was from experimental run 16 (IC_{50} = 8 µg/ml) and non-determinable for VERO cells as the percentage of controlled cell growth for VERO cells did not drop below 50 %. While the extract showed potent IC_{50} concentration towards cancer cells (MCF-7), interestingly it did not show toxicity towards normal cells (VERO) unlike Taxol at the concentration tested. This may point to development of safe anti-cancer drugs with minimal side effects towards normal cells; warrants further research.

Table 2: IC_{50} values determined using curve fitting technique for ABE from each experimental run.

	IC_{50} Values Obtained from Curve Fitting (µg/ml)	
Run	MCF-7 Cells	Vero Cells
1	ND	14
2	29	20
3	16	48
4	32	22
5	ND	ND
6	ND	ND
7	ND	ND
8	ND	ND
9	ND	29
10	40	49
11	43	ND
12	43	ND
13	48	ND
14	ND	ND
15	42	ND
16	8	ND
Taxol	2.8	2.4

*ND – Not determinable. Graph plotted showed trend higher than 50 % value at all ABE concentrations

3.2.4 Effect of ABE16 on cell attachment and viability

Based on the results, experimental run 16 (ABE16) was selected to undergo another set of Cell Attachment assay (CAA) and Cell Viability assay (CVA) at several concentrations (8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml and 100 µg/ml) to further observe the cytotoxicity effects due to the interestingly low IC_{50} value. As shown in Figure 5, at 8 µg/ml, MCF-7 viability was found to be at 35.71 % compared to control.

Meanwhile, no viable cells were observed when treated with high dose of extract (≥ 64 µg/ml) suggesting a profound anti-attachment and cytotoxic effects towards cells. Meanwhile, CAA on VERO cells demonstrated that at 64 µg/ml, ABE16 gave 38.89 % cell viability compared to control and 100 µg/ml, ABE16 gave 30.95 % cell viability. ABE16 at lower concentrations (< 64 µg/ml) result in higher viable cell percentages indicating that ABE16 has less profound effects on normal cells.

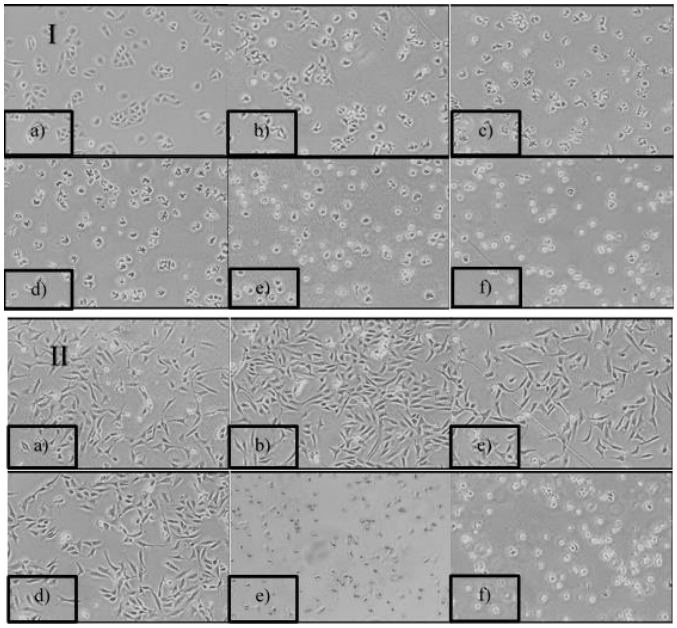

Figure 5: Viable cell percentage plot normalized to control for MCF-7 and VERO cells after 24 hours of treatment with ABE from experimental run 16 at different concentrations in CAA. Values are expressed as mean ± SD (n=3).

Figure 6 showed the population densities for MCF-7 cells VERO cells respectively, and at 8 µg/ml, MCF-7 cells already experienced morphological changes and volume reduction which indicates cell death initiation.

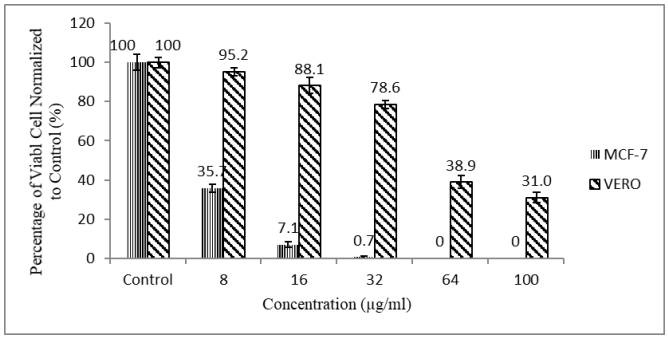

Figure 6: (I) MCF-7 cells and (II) VERO treated with extract from experimental run 16 at different concentrations in CAA. a) Control group cells, b) treated with 8 µg/ml extract, c) treated with 16 µg/ml extract, d) treated with 32 µg/ml extract, e) treated with 64 µg/ml extract, f) treated with 100 µg/ml extract.

CVA on MCF-7 cells using experimental run 16 also produced similar trend as shown in the CAA results in which inhibitory effect demonstrated by the extract from experimental run 16 was observed even at the lowest concentration (extract concentration 8 µg/ml resulted in only 28.62 % viable MCF-7 cells). Figure 7 presented the growth-inhibitory or cytotoxic effects of ABE from experimental run 16 on viable MCF-7 cells. CVA on VERO cells also showed similar result as in CAA study where, at 64 µg/ml, ABE showed 32.73 % cell viability and at 100 µg/ml, it showed 20.14 % cell viability as compared to control.

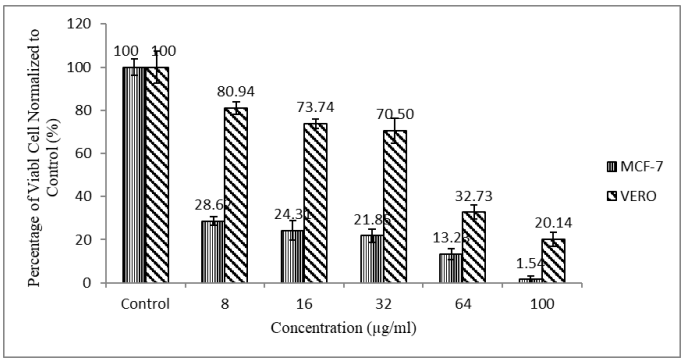

Figure 7: Viable cell percentage plot normalized to control for MCF-7 and VERO cells after 24 hours of treatment with ABE from experimental run 16 at different concentrations in CVA. Cells were cultured initially without treatment of ABE. Values are expressed as mean ± SD (n=3).

Figure 8 showed population densities for MCF-7 and VERO cells respectively and it was observed that at 8 µg/ml, MCF-7 cells had slight morphological changes and volume reduction which was less compared to the changes in the previous CAA section. However, this was expected since in CVA, cells were already adherent to the culture flask. As such, they are more stable and only affected by the cytotoxic effects. Similar findings were made in a study in which ethanolic extract of *Aquilaria crassna* stem bark showed potent cytotoxic effects with IC_{50} of 30, 72, 119 and 140 µg/ml against pancreatic, prostate, colorectal and breast cancer cell lines, respectively [15]. Another study found that methanolic extract of *Aquilaria agallocha* woody hull of fruit was effective in inhibiting colorectal cancer cell line [16]. *Aquilaria malaccensis* stem bark oleoresin extracted via supercritical fluid extraction also exhibited profound cytotoxic effects towards HCT116 human colorectal cancer cell line which further support the finding of this current study [17].

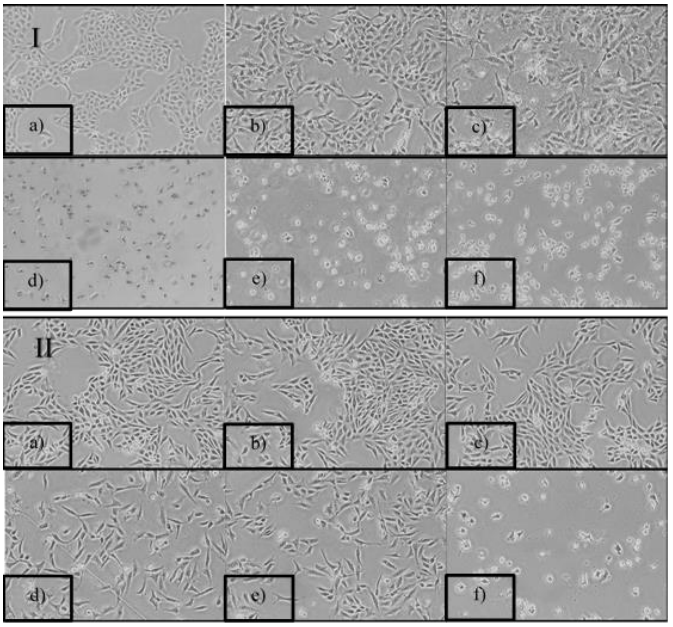

Figure 8: (I) MCF-7 cells and (II) VERO cells treated with extract from experimental run 16 at different concentrations in CVA. a) Control group cells, b) treated with 8 µg/ml extract, c) treated with 16 µg/ml extract, d) treated with 32 µg/ml extract, e) treated with 64 µg/ml extract, f) treated with 100 µg/ml extract.

4. CONCLUSION

Run 11 with 12 hours extraction time, 50 °C temperature, 100 rpm and 60 ml extraction volume gave the highest yield of 0.2130 ± 0.036 g/g ABE.

Agitation, temperature and temperature-volume coefficient were among the influential parameters affecting the yield of ABE extraction. However, extract from run 16 showed the most promising cytotoxic effects against MCF-7 with IC_{50} of 8 µg/ml; as compared to run 11 with 43 µg/ml. Run 16 (extraction time of 12 hours, temperature of 50 °C, agitation speed of 200 rpm and 1:20 solid to solvent ratio) yielded 0.1094 ± 0.014 g/g ABE. To this end, it can be concluded that ABE possess potential cytotoxic/anti-cancer activities against MCF-7 cells. It is important to note that optimal yield does not necessary correlate with maximum biological activity. Further purification and isolation of related bioactive compounds is warranted together with proper chemical characterization and biological investigations in order to understand the mechanism of action of the agarwood extracts on cancer cells.

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