AGARWOOD BRANCH ETHANOLIC EXTRACT: OPTIMAL EXTRACTION PROCESS CONDITIONS AND CYTOTOXIC EFFECTS

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ABSTRACT: Uninfected agarwood branch is readily available as raw material in agarwood plantation as new practices of agarwood plantation scheme were opted as substitute to the endangered wild type agarwood. The uninfected branch can be easily obtained during pruning process (one of plantation's common maintenance procedure), throughout the years before inoculation stage. This current study aimed to investigate the optimal extraction process conditions of agarwood branch using ethanol as solvent system for maximal yield, and assess its cytotoxic effects towards MCF-7 breast cancer cells. Uninfected branch of *Aquilaria subintegra* was subjected to One Factor at a Time (OFAT) and Response Surface Methodology (RSM)-guided ethanolic extraction to achieve maximal yield. The extract was then subjected to cytotoxicity, cell attachment and cell viability assays, respectively. Optimization Run 2 (temperature 45 °C, solid-liquid ratio of 1:30, 16 hours maceration) gave the highest agarwood branch ethanolic extract (ABEE) yield of 44.70 ± 18.9 mg/g dried material (DM). Meanwhile Run 7 (temperature 45 °C, solid-liquid ratio of 1:10, 16 hours of maceration) gave the lowest yield (19.34 ± 14.1 mg/g DM). However, while maintaining the 16 hour-maceration, the model predicted a slightly lower yield of 30.232 ± 0.266 mg/g DM of ABEE with process conditions of 45 °C and solid-liquid ratio of 1:19 when the desirable parameters were factored in namely using (i) the most suitable temperature (that does not risk the bioactivities of the extract), and (ii) an economical volume of solvent. Crude ABEE obtained from the optimal process conditions resulted in cytotoxicity effects on MCF-7 breast cancer cells with IC_{50} estimate of 3.645 ± 0.099 µg/mL. The extract also affected MCF-7 cell attachment and viability with altered morphology. More work to elucidate the mechanism of actions of the extract are warranted; which could further lead to development of breast cancer natural product-based therapeutics.

KEY WORDS: *Agarwood, Cytotoxic, Ethanolic extract, MCF-7 Breast Cancer Cells, Response Surface Methodology*

1. INTRODUCTION

Cancer is a disease that can be characterized by the repetitively uncontrollable multiplying cells in the human body leading towards malignant tumor formations forming tumors with high metastatic potential [1, 2]. Breast cancer is reported as the second most common case among women and in Malaysia, this disease accounted for 31 % of total

female cancer cases [3]. This type of cancer was reported to develop in breast tissues consist of glands for milk production, lobules, and the connecting duct or lining that bridges these lobules to the nipple [4]. The risk factors that may trigger breast cancer predisposition include carcinogens (substances that can trigger cell mutation leading to cancer), lifestyle choices (e.g., unhealthy diet, smoking, and alcohol consumption), hormones, and hereditary [5, 6].

Recently, more than 60 % of clinically approved anti-cancer drugs derived from medicinal plants due to their efficacy and safety as well as being eco-friendly and low-cost [7]. Complemented with new technology and development, more work is being directed towards natural product investigation. One of the interesting native plants in Malaysia is agarwood, which has been used in many culture and communities [8, 9]. Agarwood, or its other names in various languages include gaharu, agar, chim-hyuang, Oudh, Jin-koh, and chen-xiang, is highly sought for its aromatic resin which is formed within the woody tissues in the heartwood of *Aquilaria* species such as *A. malaccensis*, *A. crassna*, *A. subintegra*, and *A. sinensis* [10]. It is one of the most valuable non-timber products due to its application in three main areas: perfume, incense, and medicine [11]. Due to the diminishing wild trees as a result of the high demand, agarwood has been listed in Appendix II of CITES under conservation action [12]. In response to this, there has been an emergence of sustainable agarwood plantations and resin imitation efforts via fungal/bacterial injection into the woody tissues [11].

Ethnopharmacological activities of agarwood have been reported throughout the years and this led to many modern medicinal-based investigations including anti-cancer. Our earlier studies have demonstrated that healthy uninoculated agarwood branch ethanolic extract was able to inhibit MCF-7 breast cancer cells shown via *in vitro* sulforhodamine B cytotoxicity assay (SRB) with IC_{50} concentration of 8 µg/mL [13, 14]. The solvent screening was conducted eliminating distilled water, acetone, ethyl acetate, benzene, and hexane as the data showed ethanol to be a more versatile, safe, and suitable extraction solvent [14]. A follow-up cytokinetics study conducted using the same IC_{50} concentration then showed a reduced growth rate and increased death rate of MCF-7 cells [15]. Uninoculated branch used in the study refers to whitewood healthy tissues from trees without any resin imitation attempt via fungal/bacterial means. Thus, this current study aimed to investigate the optimal extraction process conditions of agarwood branch using ethanol as the solvent system; and assess its cytotoxic effects towards confirming the cytotoxicity towards MCF-7 cell growth. Findings from this may lead to the development of breast cancer natural product-based therapeutics.

2. METHODOLOGY

2.1. Plant Materials

Healthy uninoculated agarwood branch from the *Aquilaria subintegra* species was collected from a local farm located in Sungai Kembong Hilir, Semenyih, Selangor identified by voucher specimens #HBL707[VS-2] by the Herbarium of Kulliyyah of Architecture and Environmental Design (KAED), International Islamic University Malaysia (IIUM). Materials were collected from the trees with an average age of 5 to 6 years old and cleaned on-site and delivered to the laboratory. Upon arrival at the lab, these branches were further inspected for any irregularities and cleaned to remove dirt/dust. Tap-water was used to wash them followed by oven-drying at 40 °C for 48 hours before grinding. Prepared agarwood branch samples were kept at 4°C until use.

2.2. One Factor at a Time (OFAT) for Process Conditions Screening

OFAT was employed in this study as an initial strategy to investigate the effects of selected process conditions guiding the center point selections for subsequent optimization phase aiming for maximal yield while reducing the overall cost of operation. Powdered agarwood branch (5 g) was extracted with designated agitation speed (0, 50, 100, 200, 300 rpm) [16, 17, 18], extraction time (8, 16, 24, 32, and 48 hours) [19, 20, 21], solid-liquid ratio (1:10, 1:20, 1:30, 1:40, and 1:50 w/v) [22. 23, 24], and temperature (ambient, 35, 45, 55, and 65 °C) [22, 24, 25] selected from the review of agarwood and other plant extraction studies.

Accordingly, one studied parameter was varied at 5 different levels selected while other parameters were kept constant at their respective mid-point. Solvent extraction was conducted using Schott bottles placed in an incubator shaker (Ecotron, Infors, Switzerland) with absolute ethanol (99 %, HmbG Chemicals) as the extracting solvent. Once completed according to the required process conditions, the extraction mixture was filtered through a vacuum filter set equipped with Whatman No. 1 filter paper (pore size 11 µm) and the collected filtrate was dried using low temperature drying rotary evaporation (BÜCHI Labortechnik AG, Switzerland) and nitrogen gas flushing [26, 31, 32].

2.2.1. Data collection and analysis

Once the extraction process was completed, dried agarwood branch ethanolic extract (ABEE) was subjected to analysis of yield where each crude extract weight was recorded. For safe storage, dried ABEE was placed in 15 mL centrifuge tubes (sterile) with air displacement using nitrogen gas flushing method. This method will help reduce the risk of sample contamination as well as material oxidation by the oxygen present in the air [26]. Weight data were analyzed using Microsoft® Excel and statistical analysis of yield data (mg/g dried material, DM) was analyzed using One Way Analysis of Variance (ANOVA): Tukey test (T-test) via the Minitab® 17 with significance level, $\alpha = 0.05$. Significant difference implied that the tested parameter level may have affected the extraction of ABEE while no significant difference analysis suggested that the parameter tested exerted no effect on the extraction process.

2.3.Optimization of Process Conditions (Response Surface Methodology)

Optimization procedure for highest agarwood extract yield was carried out based on experimental design generated using Response Surface Methodology (RSM) by Design-Expert® version 7.0.0 (Stat-Ease, Inc., USA) selecting temperature (*A*) and solid-liquid ratio (*B*) as the assessed parameters with the ABEE crude extract yield (mg/g DM) as the response. Face Centered Central Composite Design (FCCCD) function was employed with 1 axial value and 5 center points consisting of a total of 13 experiments. A random experimental run was employed to reduce the unexplained variability effect. This design was used to investigate the log-transformed response based on the second-order polynomial model.

Dried ABEE was weighed for analysis of yield analysis (mg/g DM) and stored at 4°C until further use. Data analysis was conducted in Design-Expert® version 7.0.0 (Stat-Ease, Inc., USA) via ANOVA and 3D response surface. Also, model validation experiments were

conducted to assess the optimal conditions estimated and recommended by the extraction model, factoring in the desirable parameters namely using (i) the most suitable temperature (that does not risk the bioactivities of the extract), and (ii) an economical volume of solvent.

2.4. *In vitro* cytotoxic and anti-cancer screening assays

ABEE obtained from the extraction using the recommended (validated) process conditions was subjected to cytotoxic and anti-cancer screening assays (cell attachment and cell viability).

2.4.1. Cell lines

MCF-7 breast cancer cell line (ATCC® HTB-22™) and VERO cell line (ATCC® CCL-81™) were obtained from American Type Culture Collection.

2.4.2. Chemicals and reagents

Powdered and liquid Dulbecco's modification of eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics (100 U/ml penicillin, 0.1 g/l streptomycin), accutase (cell detachment solution) and Cell Count Reagent SF were obtained either from Gibco (United States) or Necalai Tesque (Japan). Other chemicals such as sodium bicarbonate, hydrochloric acid, sodium hydroxide and trypan blue dye were from Sigma-Aldrich (USA). Industrial grade ethanol that was used for sanitization was also obtained from local suppliers. Dimethylsulfoxide (DMSO) was obtained from Merck Millipore (Germany).

2.4.3. Cell attachment assay (CAA)

The modified assay manipulated the trypan blue dye exclusion method to assess the ability of ABEE in cell attachment disruption and inhibition whereby cell attachment is crucial for adherent-type cells for proliferation [27]. Any resistance and interruption of the attachment process may result in cell death and population density reduction. In this assay, ABEE was adjusted to 100 µg/mL using dimethylsulfoxide (final DMSO concentration less than 1 % v/v) and deionized distilled water (ddH_2O) followed by introduction to the culture flask at the time of cell inoculation (seeding). After 24 hours of incubation (37 °C, 5 % CO_2), trypan blue dye exclusion procedure was conducted for viable cell number estimation and data was analyzed between treated and control group (cell flask with 1 % v/v DMSO as sample). DMSO percentage of more than 1 % (v/v) may affect the reliability of data since higher concentration of DMSO is toxic to cells [28]. Viability reduction in treated cells may point to the ability of the ABEE in preventing cells from attaching and/or causing cells to detach from the substrate; cumulatively referred to as anti-attachment activities.

2.4.4 Cell viability assay (CVA)

This modified protocol also manipulated the trypan blue dye exclusion method to investigate the cytotoxic or ability of ABEE to kill or inhibit an already adherent/active cells [27]. Cells were seeded into growth flasks at 1 x 10^5 cells/mL and allowed to proliferate for 24 hours before ABEE introduction at 100 µg/mL. Then, the culture flask was incubated for another 24 hours (37 °C, 5 % CO_2) followed by the trypan blue dye exclusion protocol. Viable cell numbers compared to the control (1 % v/v DMSO as sample) in this assay can be inferred as the ability of the sample to inhibit active cells.

2.4.5 Cell cytotoxicity assay using Cell Count Reagent SF (CCRSF)

This protocol followed the provided guidelines by Necalai Tesque for their CCRSF product. ABEE cytotoxicity potential in this work was investigated using Cell Count Reagent SF. This *in vitro* screening assay was established by Dojindo Molecular Technologies Inc. that utilized the highly water-soluble tetrazolium salt (WST-8 [2-(2-methoxy-4- nitrophenyl)-3- (4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt]) [29]. The WST-8 salt produces water-soluble formazan dye reduced in presence of electron mediators such as dehydrogenases in viable cells resulting in orange colored dye formazan. The amount of formazan measured is directly proportional to the viable cell number. The assay started with viable cells seeding into a 96-well culture plate at 5 x 10^4 cells/mL in 90 µL of DMEM-FBS. Cell-loaded plates were incubated for 24 hours (37 °C/5 % CO_2) followed by the addition of serially diluted concentrations of ABEE (in triplicates) at 10 µL/well and further incubation for 48 hours. After that, CCRSF dye was introduced to the system at 10 µL/well and plates were further incubated for 4 hours. Data acquisition was done by optical density (OD) measurement at 450 nm using Multiskan™ Go (Thermo Scientific™, USA) and further analyzed through the Graphpad Prism 7 software to obtain the non-linear regression IC_{50} values and the dose-response curve for the sample.

3. RESULTS AND DISCUSSION

In the natural product investigation, extraction is considered the gatekeeper or the critical step that allows the optimal collection of desired compounds related to the bioactivities [30, 31]. The basic requirement of plant-type extraction includes pre-treatment of the sample that includes cleaning, drying, and grinding of selected plant samples followed by the extraction process based on the design selected. In accordance, the first step towards optimized natural product extraction was to conduct a process conditions selection through the One Factor at a Time (OFAT) method. This method varies one factor at a time while the other parameters were kept constant to observe the singular parameter effect of the process.

In this study, conventional solid-liquid extraction was selected due to its versatility, simplicity, and economical aspects. This versatile technique may be further improved by process conditions manipulation aiming towards an economical-safe optimized operation that includes the time of extraction, the temperature of extraction, sample-solvent ratio, and agitation requirement for yield as target function [32].

3.1 Process Conditions Screening via One Factor at a Time (OFAT) Method

A previous study in our lab has concluded that ethanol was the most suitable and versatile solvent system for rapid solid-liquid extraction of agarwood branch with minimal loss and risk of contamination [13, 14, 15]. Following this, the current study attempted to screen and select the optimal process conditions (agitation, time, temperature, and solid-liquid ratio)

required to obtain high yield of ABEE before proceeding to the optimization phase. Table 1 summarizes the effects of process conditions on the yield of agarwood branch ethanolic extract (ABEE). The yield obtained throughout the experiments was between 2.008 to 5.120 g/g (%).

The yield data were then analyzed statistically using One Way Analysis of Variance (ANOVA): Tukey test (T-test) via the Minitab® 17 significance level, $\alpha = 0.05$ to determine any significant difference of the process conditions studied against the yield of ABEE.

Table 1. Summary of the effects of process conditions on yield of agarwood branch ethanolic extract (ABEE). Experiments were conducted in triplicate sets (n = 3 ± s.d.).

Parameters	Agitation (rpm)	Time (hours)	Temperature (°C)	Solid-liquid ratio (w/v)	Yield percentage (mg/g DM)	Standard deviation
Agitation (rpm)	0	24	45	1:30	22.42	3.00
	50	24	45	1:30	27.00	5.58
	100	24	45	1:30	22.14	3.92
	200	24	45	1:30	28.72	1.15
	300	24	45	1:30	29.52	5.66
Time (hours)	100	8	45	1:30	27.95	4.83
	100	16	45	1:30	37.25	5.96
	100	24	45	1:30	32.48	8.25
	100	32	45	1:30	32.41	7.15
	100	40	45	1:30	26.50	0.71
Temperature (°C)	100	24	Ambient	1:30	20.08	2.40
	100	24	35	1:30	30.39	5.38
	100	24	45	1:30	28.08	0.64
	100	24	55	1:30	34.42	2.23
	100	24	65	1:30	27.76	8.09
Solid-liquid ratio (w/v)	100	24	45	1:10	39.52	3.34
	100	24	45	1:20	35.12	3.35
	100	24	45	1:30	28.07	0.87
	100	24	45	1:40	51.20	9.59
	100	24	45	1:50	34.46	6.13

3.1.1 Agitation Effect on ABEE Yield

Fig. 1a shows the generated bar chart of the T-test of mean yield against agitation speed (0, 50, 100, 200, and 300 rpm). It can be observed that all five-agitation speed tested shared the same designation 'A' indicating no significant difference ($p > 0.05$). The data can be interpreted as similar yield may be achieved when using any of these 5 agitation speeds (including 0 rpm; no agitation) for the extraction of agarwood branch using ethanol solvent system. Hence, following the economical point of view, agitation speed was suggested to be excluded from the optimization phase thus omitting the use of the shaker function.

For a simple and economical extraction process, many researchers opted for the maceration technique that required almost zero operating conditions. Samples were mixed with the solvent of choice and left to diffuse into the surrounding solvent at room temperature. The extraction process halted when equilibrium was reached between the solute inside the plant material and the concentration in the solvent [26]. In the case of agarwood extraction, several studies have been observed to employ simple maceration and percolation techniques [16, 17 18, 33]. However, experimental comparison data was not provided by these studies.

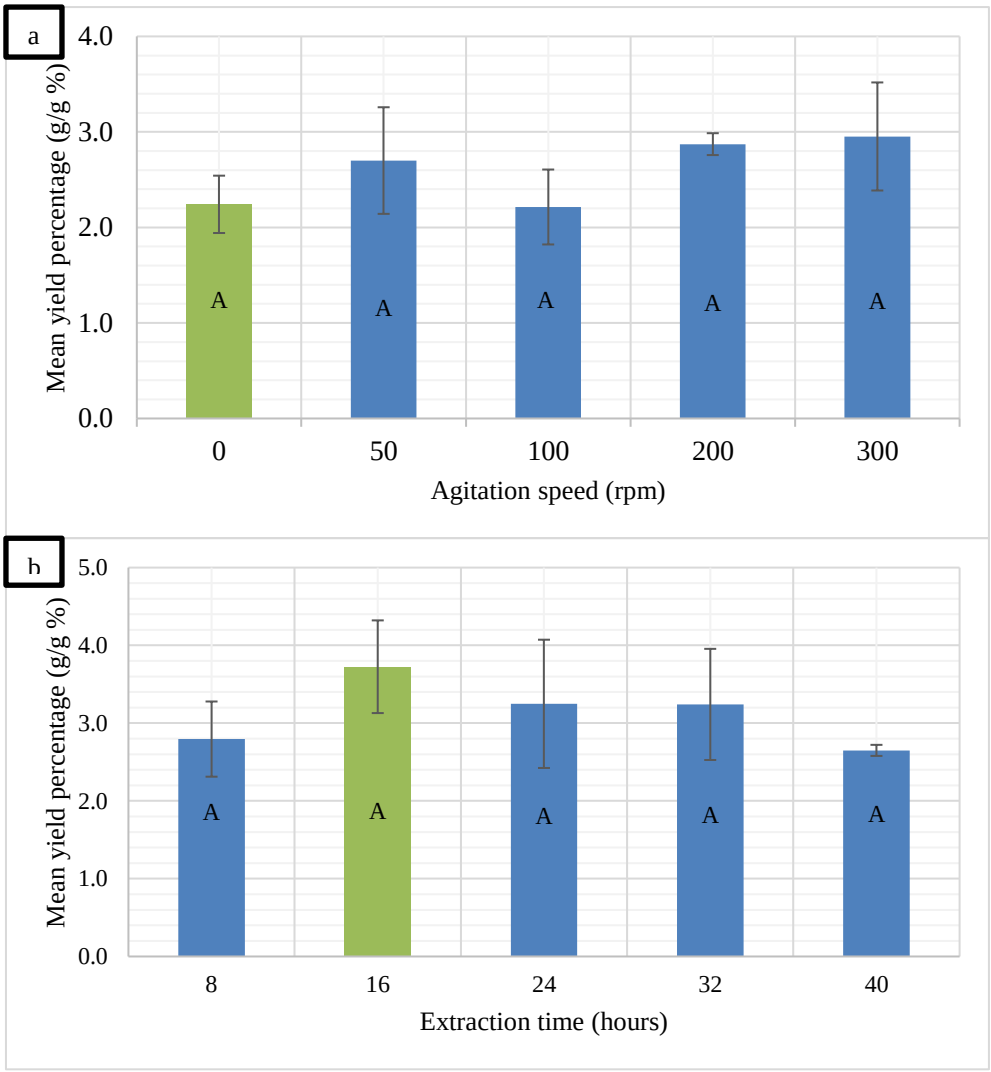


Fig.1. The mean yield percentage (g/g %) vs a) agitation speed (rpm) and b) extraction time plots generated based on the One Way Analysis of Variance: T-test analysis. All tested agitations were in the same 'A' group indicating no significant difference between them. Thus, both parameters were excluded from the optimization phase. Zero agitation and 16 hours (green bars) were selected constants in the optimization phase.

3.1.2 Extraction Time Effect on ABEE Yield

Fig. 1b shows the generated bar chart of the T-test of mean yield against extraction time tested (8, 16, 24, 32, and 48 hours). When observed, all extraction time tested resulted in the same category 'A' indicating no significant difference when analyzed statistically. It can also be suggested that after 8 hours of extraction, the solute and solvent have reached equilibrium. In this case, 16 hours point was selected to be used as a constant in the optimization stage to ensure exhaustive extraction and solute-solvent equilibrium.

The efficiency of extraction time is highly dependent on the temperature, agitation speed employed, and solid-liquid ratio used [26]. In this study, five different time points (Table 1) were selected based on previous studies [19, 20, 34]. Longer extraction time indicated a less efficient extraction process since longer exposure to high temperature increases the risk of degradation of heat-sensitive compounds resulting in extracts void of or with suboptimal bioactive properties [21, 35].

3.1.3 Extraction Temperature Effect on ABEE Yield

Fig. 2 shows the generated bar chart of the T-test of mean yield against temperature tested. The analysis showed that at the temperature of 55 °C, the mean yield obtained was the highest noted as 'A' while other temperature levels with lower mean yield were categorized as 'B'. The presence of two distinct categories denotes that there is a significant difference between the levels of temperature tested and values may affect the overall efficacy of ethanolic extraction of agarwood branch. Thus, 55 °C was selected as the mid-point for further optimization process conforming to the idea of lower temperature extraction reduce the risk of heat-sensitive compounds thermal degradation.

Temperature plays a significant role in effective extraction process and proper selection of temperature levels would dictate the end-product obtained [36]. Higher temperature will increase the kinetic energy of the solvent allowing more effective molecule breaking and increase the vibration of solute molecules expediting the diffusion process into the solution. Plant samples are weakened and softened when exposed to the high temperature allowing the compounds in the cell membrane to dissolve more readily [37]. Higher temperature usually enhances the solubility and increases the diffusion of solute in the plant material into the surrounding solvent reducing the overall extraction process duration [22, 37]. However, high-temperature extraction could result in heat-sensitive compounds and nutritional degradation, non-enzymatic browning reactions, and accumulation of unstable extraction products [23, 37]. In this study, five temperature levels were selected based on previous studies [23, 24, 37].

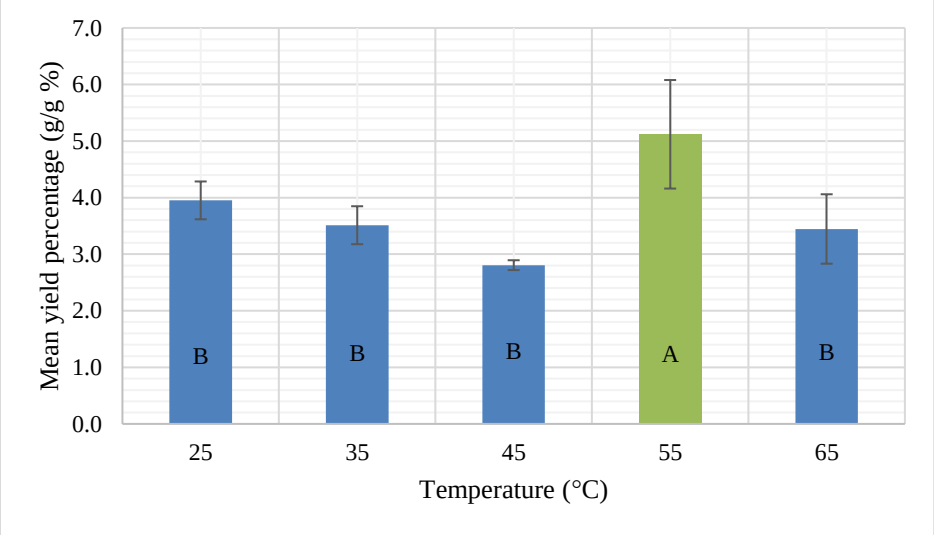


Fig. 2. The mean yield percentage (g/g %) vs temperature (°C) plot generated based on the One Way ANOVA: T-test analysis. Tested parameters were categorized in the two different groups indicating significant difference. Thus, 55 °C (green bar) in 'A' category was selected as mid-point targeting maximum yield in the subsequent optimization phase.

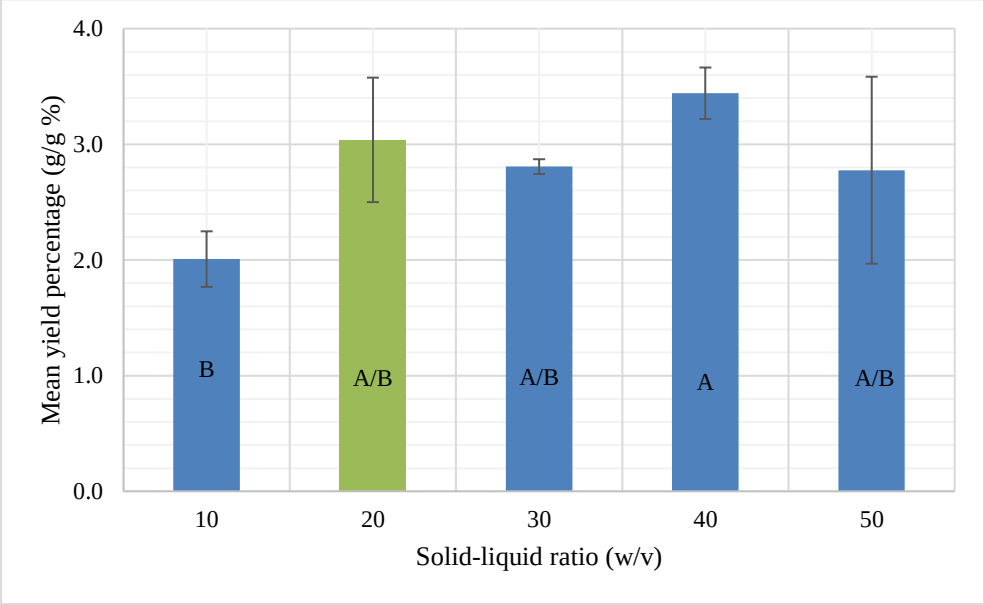
3.1.4 Solid-liquid (SL) Ratio Effect on ABEE Yield

Fig. 3 shows the generated bar chart of the T-test of mean yield percentage vs solid-liquid ratio tested. Interestingly, the ratios analyzed were divided into 3 categories i.e., i) category A with the highest mean yield, ii) category AB with the intermediate mean yield and iii) category B as the lowest mean yield obtained. Different categories result suggested that solid-liquid ratio play a significant role in the efficacy of ethanolic extraction of agarwood branch. While three conditions of solid to liquid ratio (1:20, 1:40, and 1:50) were found to

be in the same category as AB, indicating no significant differences, ratio 1:20 was selected as the mid-point for the subsequent optimization phase. This is to reduce solvent consumption, abiding by the economical extraction process with the target to achieve a maximal yield of extract.

A suitable solid-liquid ratio would help increase the maximum yield of extraction since higher substance diffusion can be achieved with a reduced mass transfer barrier [23]. Despite the favorable effect, a suitable limit must be identified to reduce solvent waste by observing the optimal ratio since solvent abundance can lead to waste while too much solid (plant material) can lead to incomplete extraction. Thus, analysis of the solid-liquid ratio was crucial to observe and select the suitable value for an efficient extraction process. In this study, five different ratios were selected based on previous work [22, 23, 24, 37]. In this current work, the solid-liquid ratio of 1:20 was selected as the mid-point of the optimization phase in the effort to reduce the solvent consumption while targeting to achieve a maximal yield of extract.

Fig. 3. The mean yield percentage (g/g %) vs SL ratio (w/v) plot generated based on the One Way ANOVA:



T-test analysis. Tested parameters were categorized in the three different groups indicating the significant difference. Thus, SL 1:20 ratio (green bar) in category 'AB' was selected to reduce the consumption of solvent while targeting maximum yield in the subsequent optimization phase.

3.1.5 Summary on the Effects of selected Process Conditions on Extraction of ABEE using OFAT

Natural product investigation usually involves a good extraction process that serves as a fundamental starting point for the identification of compounds and their bioactive properties. Since no universal extraction method can be directly applied due to the diversity of the phytochemicals in the plant material, it is practical to conduct a preliminary screening of effective process conditions [37]. The idea of having a good extraction is that the process must be rapid, maximal, and economical while maintaining the efficiency of the target yield or biological response. It is noteworthy that this study focuses only on the yield as the response for the OFAT extraction phase. In compliance, the designed OFAT phase has successfully screened and selected SL ratio and temperature as the potential process

conditions for optimization while excluding the extraction time and agitation where they were kept constant during the optimization process at 16 hours and 0 rpm, respectively. The temperature of 55 °C was selected as mid-point for the optimization process conforming to the idea of lower temperature extraction reduce the risk of heat-sensitive compounds thermal degradation. Meanwhile, an SL ratio of 1:20 was selected as the mid-point of the optimization phase in the effort to reduce the solvent consumption while targeting to achieve a maximal yield of extract.

3.2 Optimization of Selected Process Conditions for ABEE Extraction

Based on the process conditions obtained through OFAT experiments; an optimization study was designed to obtain the maximum yield under the most suitable process conditions. Response Surface Methodology (RSM) in Design-Expert® version 7.0.0 (Stat-Ease, Inc., USA) with FCCCD function was used with temperature (*A*) and solid-liquid ratio (*B*) as the parameters to be assessed. Coded levels of independent variables and responses (yield of ABEE) are displayed in Table 4.

Table 2 shows that Run 2 (temperature of 45 °C, solid-liquid ratio of 1:30, 16 hours of maceration period) gave the highest ABEE yield of 44.70 ± 18.9 mg/g DM while Run 7 (temperature of 45 °C, solid-liquid ratio of 1:10, 16 hours of maceration period) gave the lowest yield (19.34 ± 14.1 mg/g DM).

Table 2. Coded Face Centered Central Composite Design (FCCCD) for optimization of process conditions to obtain maximum ABEE yield (mg/g dried material, DM). A represented temperature with midpoint 55 °C and B represented SL ratio with midpoint 1:20 both from previous selection and screening section. Extraction was performed using maceration technique for 16 hours. Responses recorded were based on triplicate experimental sets (n = 3).

Exp. run	Extraction temperature (°C)		Solid-liquid ratio (w/v)		ABEE yield (mg/g DM)
	A	Code	*B*	Code	
1	65	+1	30	+1	35.31 ± 1.74
2	45	-1	30	+1	44.70 ± 18.9
3	55	0	20	0	29.54 ± 0.283
4	65	+1	20	0	31.55 ± 2.91
5	55	0	10	-1	20.21 ± 2.73
6	45	-1	20	0	29.34 ± 0.53
7	45	-1	10	-1	19.34 ± 14.1
8	55	0	30	+1	35.89 ± 1.47
9	55	0	20	0	28.41 ± 1.04
10	55	0	20	0	30.69 ± 3.08
11	55	0	20	0	30.73 ± 4.63
12	65	+1	10	-1	21.67 ± 4.38
13	55	0	20	0	31.16 ± 1.29

Note: To simplify analysis and reporting, solid-liquid ratio is denoted as the liquid part of the ratio only, with the solid part maintains at 1 in all cases.

3.2.1 Model Prediction and Statistical Analysis

Applying natural logarithmic transformation on the model, the correlation between the response variable *Y* (ABEE yield) and the parameters assessed was expressed by the following second-order polynomial quadratic equation:

$$\ln Y = +2.504 - 0.021A + 0.1152B - 8.7431 \times 10^{-4}AB + 3.3430 \times 10^{-4}A^2 - 8.868 \times 10^{-4}B^2 \quad (1)$$

where Y is the ABEE extraction yield (mg/g DM), A is the coded variable for extraction temperature (°C), and B is the coded variable for solid-liquid ratio (w/v). Table 5 shows the summary of ANOVA that was used to examine the adequacy of the model and identify the significant factors.

Based on Table 3, the model F-value was 58.22 (p-value < 0.0001) implying a significant model with a 0.01 % chance that a model F-value this large could occur due to noise. The F-value and p-value confirmed that the model could represent the actual relationship between parameters investigated and response with significance. The lack of fit F-value analyzed was 2.35 indicating non-significant relative to pure error and p-value 0.2138 indicating that there is only a 21.38 % chance that a lack of fit this large could occur due to noise. The lack of fit analysis evaluates the failure of the model and insignificant analysis dictates that the model fits the actual data [25, 38]. The model R^2 value was 0.9765 and the adjusted R_{adj}^2 value was 0.9597 indicating that the model fits well to the actual data. The difference between R^2 and R_{adj}^2 was very small, suggesting close agreement between the theoretical and experimental data for the polynomial model. The predicted R^2_{pred} value was 0.8424 indicating a good model prediction ability that was able to predict the response value. The difference between R_{adj}^2 and R_{pred}^2 was less than 0.20 indicating reasonable agreement between the model and response collected. The coefficient of variation percentage (CV %) was 1.41 % in which a model with CV % < 5% is considered acceptable suggesting precision, reliability, and reproducibility of data [25, 39].

Meanwhile, the solid-liquid ratio (B and B^2) and temperature/solid-liquid ratio interaction (AB) were found to be significant factors affecting the extraction. However, temperature (A and A^2) were not significant factors. These findings agree with a previous study that found the interaction between temperature and solid-liquid ratio to be positively affecting the ethanolic extraction of agarwood branch [14].

Table 3. ANOVA data for the regression model for ABEE extraction.

Source	Sum of squares	DF	Mean square	*F*-value	*p*-value
Model	0.6516	5	0.13	58.22	<0.0001[a]
A	0.0004	1	4.071 x 10^{-4}	0.180	0.6833[b]
B	0.6020	1	0.60	267.66	<0.0001[a]
AB	0.0306	1	0.031	15.590	0.0078[a]
A^2	0.0031	1	3.087 x 10^{-3}	1.370	0.2798[b]
B^2	0.0217	1	0.022	9.660	0.0171[a]
Residual	0.0157	7	2.249 x 10^{-3}		
Lack of fit	0.0100	3	3.348 x 10^{-3}	2.35	0.2138[b]
Pure error	0.0057	4	1.425 x 10^{-3}		
Cor. total	0.6700	12	0.13		
CV % =1.41 %					
R^2 = 0.9765					
R^2_{adj} = 0.9597					
R^2_{pred} = 0.8424					

A – temperature (°C), B – SL ratio (w/v), [a] significance p-value < 0.05, [b] significance p-value > 0.05

3.2.2 Response Surface Analysis

To visualize the interaction of parameters on ABEE yield, a three-dimensional (3D) response surface diagram was plotted using Design-Expert® version 7.0.0 software (Fig. 4).

As shown in Fig. 5, solid-liquid and temperature have an inverse proportional relationship towards the yield of ABEE in which a combination of higher solid-liquid ratio (1:30) and lower temperature (45 °C) resulted in a higher yield of ABEE. It is generally accepted that a higher solvent volume will increase the extraction yield and higher temperature also promotes extraction of natural product. Additionally, solvent polarity may also affect the overall process in which ethanol solvents have been reported to promote the extraction of mid to high polar compounds such as phenolics, from plant sample matrix [23, 37, 41, 42, 43]. However, high-temperature extraction may also result in degradation of heat-sensitive compounds which may be the case in this instance [22, 23, 41, 44]. To this end, though the previous section (OFAT experiments) have demonstrated that temperature level at 55 °C has a significant effect towards the yield of ABEE, the optimization data managed to highlight the interaction effects with solid-liquid ratio. With the inverse relationship of these two parameters, data collected showed a higher yield of ethanolic ABEE may be achieved at lower temperature and higher solid-liquid ratio. This is an important and interesting outcome as lower temperature extraction would allow better recovery of heat-sensitive products.

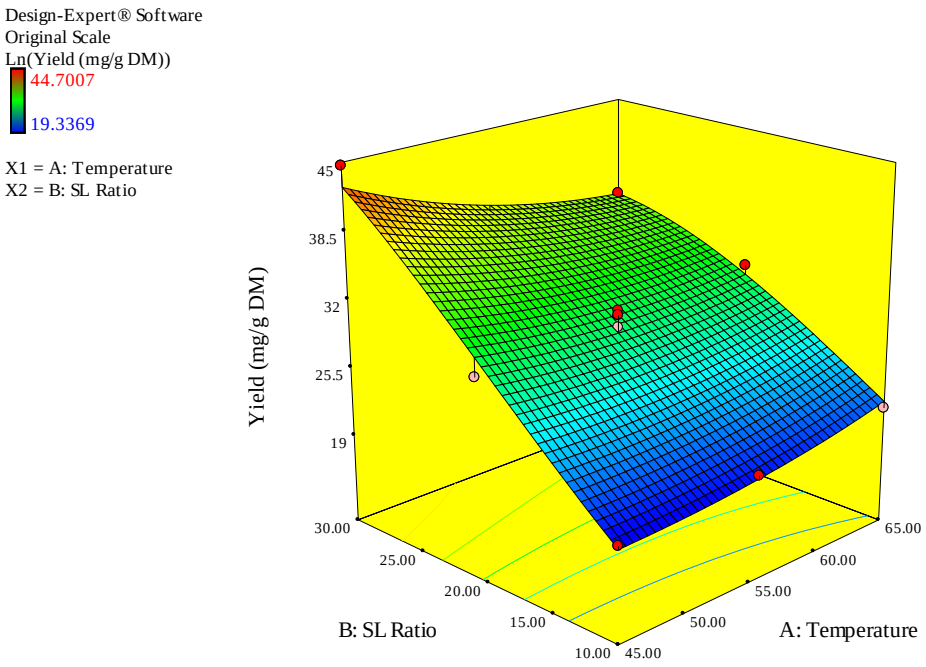


Fig. 4. The 3D contour response surface plot was generated to illustrate the interaction between temperature and solid-liquid ratio towards ABEE yield. As shown, a higher solid-liquid ratio and lower temperature led to higher ABEE yield indicating solid-liquid ratio may be inversely proportional to temperature in terms of ABEE ethanolic extraction.

3.2.3 Model Validation

With the aim to obtain a maximal yield of ABEE using the most suitable temperature (that does not risk the bioactivities of the extract), and an economical volume of solvent, the

model predicted yield of 30.232 ± 0.266 mg/g DM of ABEE with optimal process conditions of 45 °C and the solid-liquid ratio of 1:19. Three validation experiments were then conducted to evaluate the adequacy of the model equation as shown in Table 4. By applying these optimal conditions, the experimental value obtained was 25.35 ± 1.19 mg/g DM, which was in good agreement with the predicted value (*p*-value = 0.007, less than 0.05 indicated statistical significance). The results obtained through the validation experiments suggested the reliability of the developed polynomial quadratic model and these optimal values are valid within the specified range of process parameters.

Table 4. Three validation experiments were conducted based on the optimal process conditions provided by the software resulted in the following actual yield (mg/g DM).

	Predicted Yield (mg/g DM)	Actual Yield (mg/g DM)
Run 1	30.251	24.236
Run 2	30.488	26.617
Run 3	29.956	25.198
Average	30.232	25.350
Error	0.266	1.19
p-value	0.007	

3.3 Effects of ABEE on Cell Viability and Cell Attachment

The agarwood branch ethanolic extract (ABEE) obtained from the optimal process conditions (model validation phase) was tested for its biological activities. Three simple *in vitro* screening methods were conducted namely cytotoxicity assay, cell attachment assay (CAA), and cell viability assay (CVA).

3.3.1 Cytotoxicity Effects of ABEE

U.S National Cancer Institute and Geran protocol guidelines stated that plant sample with $IC_{50} \leq 20$ µg/mL is considered highly cytotoxic, between 21 and 200 µg/mL as moderate cytotoxic, between 201 and 500 µg/mL as weakly cytotoxic, and ≥ 501 µg/mL as having no cytotoxic activity [45]. Generally, crude extract can be considered as cytotoxic when the IC_{50} values ≤ 100 µg/mL when treated on cells between 48 and 72 hours while fractions and purified compounds should have lower $IC_{50} \leq 30$ µg/mL [46, 47, 48, 49, 50, 51, 52]. Therefore, based on Fig. 5, ABEE was found to be highly potent against both MCF-7 cells with IC_{50} estimate of 3.645 ± 0.099 µg/mL ($R^2 = 0.7453$) and VERO cells with IC_{50} estimate of 5.939 ± 0.019 µg/mL ($R^2 = 0.9786$).

A similar test was conducted using Taxol against MCF-7 cells as the comparison between commercial drugs and ABEE. In this study, as the positive control, Taxol was found to have IC_{50} of 0.195 ± 0.0265 µg/mL ($R^2 = 0.834$) showing potent cytotoxic effects against MCF-7 cells which was expected from a commercial chemotherapy drug that targets rapidly growing cancer.

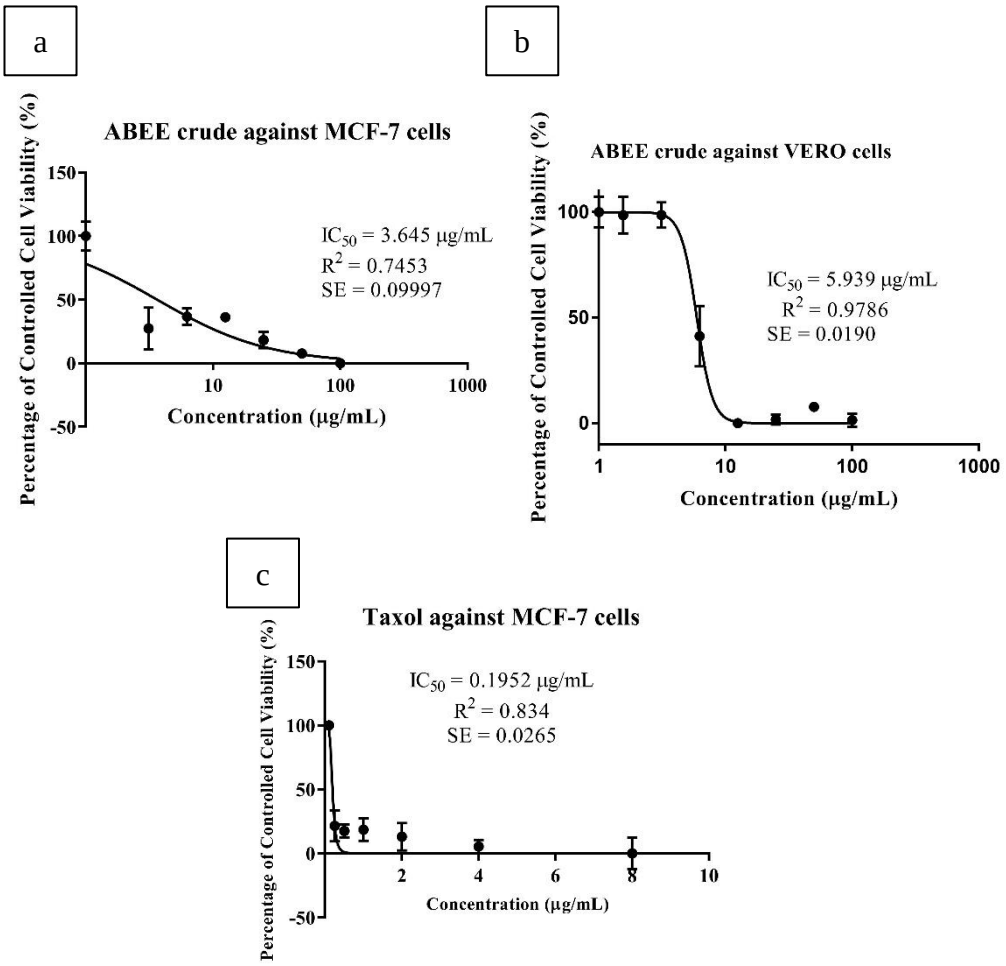


Fig. 5. Dose-response curve developed using GraphPad Prism 7 for (a) MCF-7 and (b) VERO cells treated with ABEE. The non-linear regression IC_{50} estimate for MCF-7 suggested ABEE as a potent cytotoxic agent. The positive control used against MCF-7 cells was (c) Taxol and the effect of this commercial drug was severe as expected from an anti-microtubule agent. Experiment was conducted in three independent experiments (n = 3 ± s.d) with 1 % (v/v) DMSO as negative control for normalization.

3.3.2 Effects ABEE on Cell Attachment and Cell Viability

Cell Attachment Assay (CAA) was designed to investigate the cytotoxic effects of ABEE on the cell attachment process [13, 14, 53]. Fig. 6 shows the CAA-CVA viability plot of MCF-7 after 24 hours of treatment with 100 µg/mL normalized to control. ABEE-treated cells were observed to have only 50 % cell viability compared to control. This points to the ABEE potential in hindering 50 % of the overall attachment process of MCF-7 cells which is a prerequisite to cancer growing and spreading [53, 54]. This finding reflects the ability of the extracts to prevent cells from attaching and/or cause cells to detach from the substrate; cumulatively referred to as anti-attachment activities. However, since this is an end-point based assay, the effects observed may also be due to cytotoxic effects (that could be present in the cell population during the treatment period). Meanwhile, it can also be seen in Fig. 7 that ABEE was able to inhibit 40 % of viable MCF-7 (already adhered and growing for 24 hours) after 24 hours of treatment indicating its potential as a cytotoxic agent.

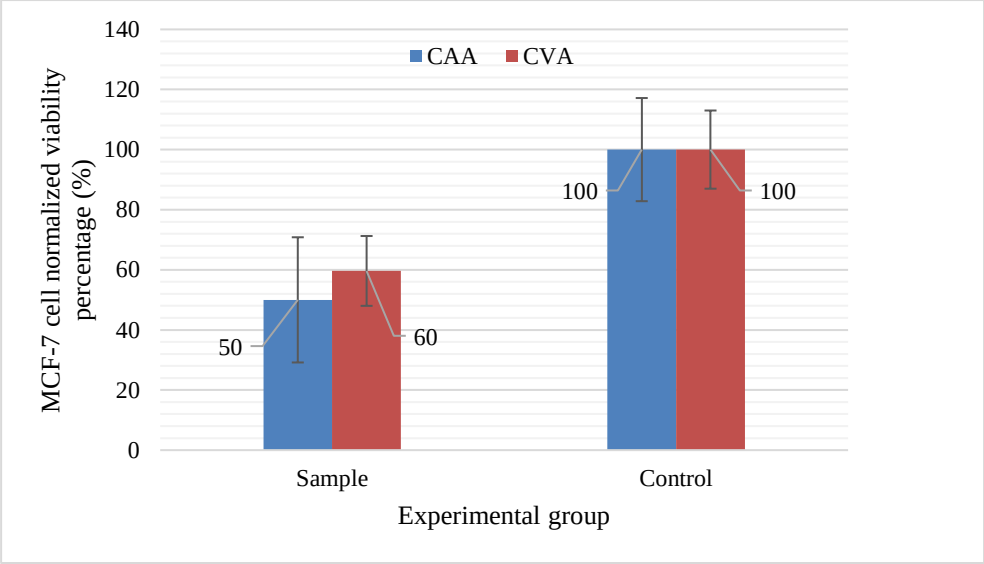


Fig. 6. Bar chart representing cell attachment assay-cell viability assay (CAA-CVA) data analysis. CAA analysis showed that after 24 hours of treatment with 100 µg/mL ABEE, the MCF-7 viable cell population was reduced by 50 %. CVA analysis showed that ABEE was able to reduce the viable cell population by 40 % at the end of the experiment. Data were analyzed from triplicate experimental runs (n = 3 ± s.d.).

Figs. 7 and 8 show representative population density images for CAA and CVA experiments. Viable cells observed in the treated set appeared to be characteristically different compared to the control set in which ABEE-treated cells appear as a single-cell colonies. Representative population density images of treated samples showed low viable cell presence compared to the control set. Treated cells had altered morphology and were unable to grow normally as cluster-like formation similar to the situation where cells undergo apoptosis (programmed cell death) [55, 56, 57]. These images demonstrated the severity of ABEE treatment on MCF-7 and can be inferred as a potent cytotoxic agent confirming the data analysis in Fig 7.

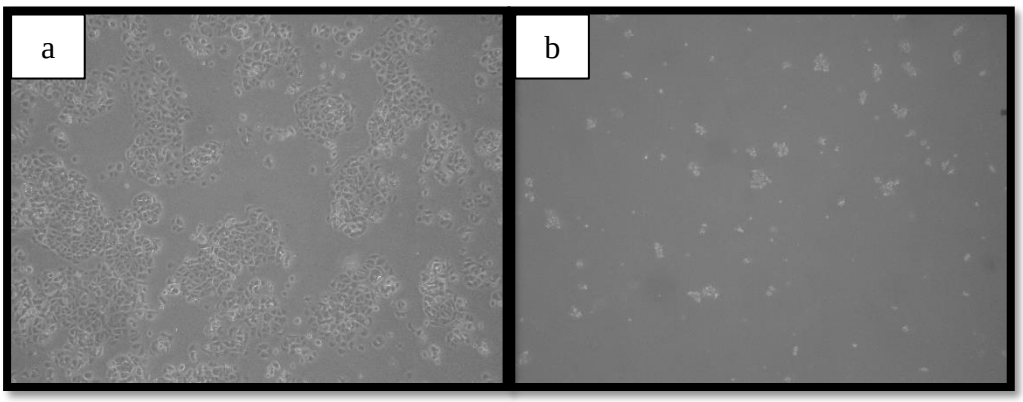


Fig.7: Representative population density images of MCF-7 in cell attachment assay (CAA). a) 24 hours control MCF-7 treated with 1 % (v/v) DMSO showing normal cell characteristics, b) 24 hours MCF-7 treated with 100 µg/mL ABEE showing less viable cells with altered cell characteristics indicating the potential anti-attachment effect. ABEE was introduced to the system during the cell seeding process to observe any effect on the cell attachment process.

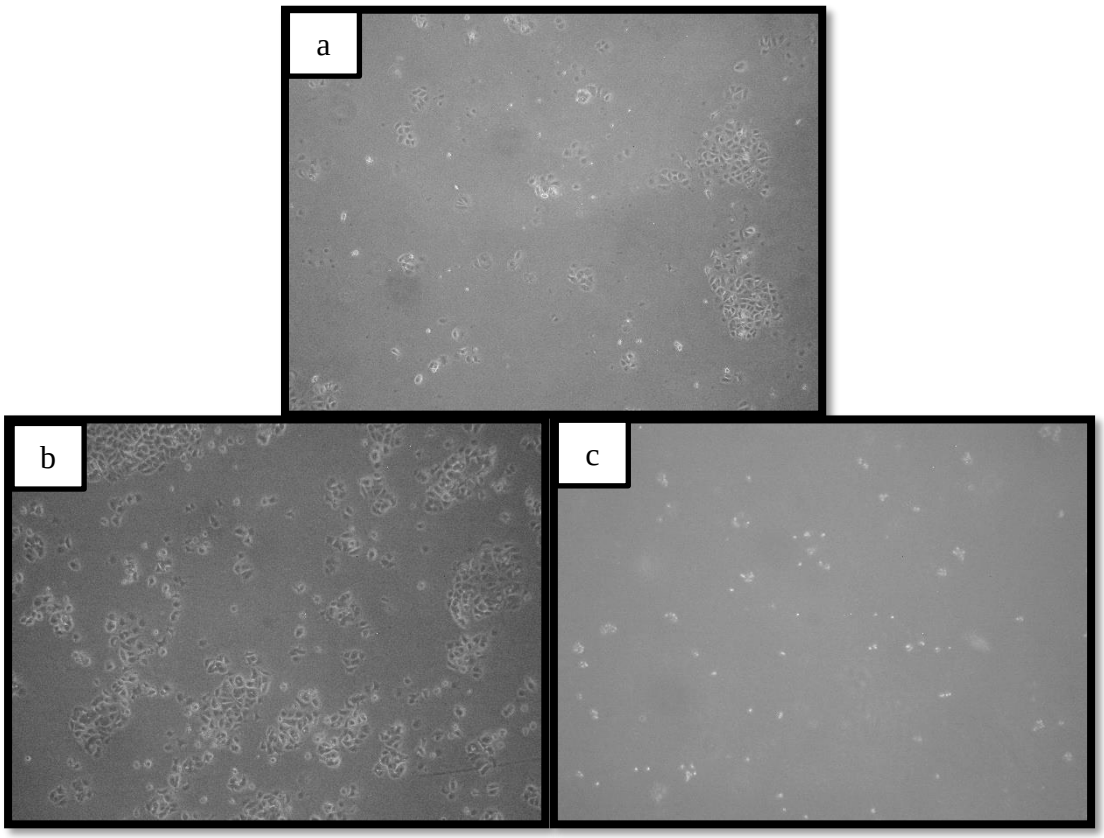


Fig. 8. Representative population density images of MCF-7 cells in CVA. a) Active and adherent MCF-7 cells before any treatment, b) control set MCF-7 after 24 hours treated with 1 % (v/v) DMSO showing increased population density and normal cell characteristics, c) ABEE treated MCF-7 after 24 hours showing significantly reduced viable cells with altered characteristics indicating the severe effect of ABEE treatment.

4. CONCLUSION

Optimization Run 2 gave the highest ABEE yield of 44.70 ± 18.9 mg/g DM. Meanwhile Run 7 (temperature of 45 °C, solid-liquid ratio of 1:10, 16 hours maceration period) gave the lowest yield (19.34 ± 14.1 mg/g DM). However, while maintaining the 16 hour-maceration, the model predicted a slightly lower yield of 30.232 ± 0.266 mg/g DM of ABEE with process conditions of 45 °C and the solid-liquid ratio of 1:19 when the desirable parameters were factored in namely using (i) the most suitable temperature (that does not risk the bioactivities of the extract), and (ii) an economical volume of solvent. The ABEE obtained from the extraction using the recommended process conditions showed cytotoxicity effects on MCF-7 breast cancer cells with IC_{50} estimate of 3.645 ± 0.099 µg/mL. The extract also affected MCF-7 cell attachment and viability with altered morphology. More work to elucidate the mechanism of actions of the extract are warranted; which could further lead to the development of breast cancer natural product-based therapeutics.

ACKNOWLEDGEMENT

The authors would like to thank the Department of Biotechnology Engineering, Kulliyyah of Engineering; and the International Institute for Halal Research and Training (INHART), IIUM for providing the necessary facilities and support to carry out this work.

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