

Anticancer potential and chemical profile of agarwood hydrosol

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Article history

Received 24 March 2019
 Revised 8 April 2019
 Accepted 15 April 2019
 Published Online 1 October 2019

Abstract

This is the first report discussing the chemical profile of agarwood hydrosol and its potential anticancer effects. Agarwood hydrosols, from two batches, obtained from Negeri Sembilan, Malaysia, were used in this study. Calu-3 cancer cells were used as a model cell line for lung cancer. The cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). The study was carried out in two phases, namely the anticancer study via an attachment (AT) assay and cell viability (CV) assay, and then profiling the hydrosol from the two batches via GCMS and hSPME-GCMS. Design Expert software's two-factor face-centered central composite design (FCCCD) was used to study the effects of agarwood hydrosol amount and time of exposure on cell attachment (AT) and viability (CV). The findings suggest that agarwood hydrosols of *Aquilaria malaccensis* possess both anti-attachment and cytotoxic effects on Calu-3 lung cancer cells. A linear model was developed for anti-attachment effects and a quadratic model for the cytotoxic effects with exposure duration being significant in both cases. From the profiling data, 1-tricosene and 16-hentriacontanone were identified as potential contributors to the anti-attachment and/or cytotoxic activity observed. In conclusion, agarwood hydrosol holds a potential to be further investigated as a source of anti-cancer compounds.

Keywords: Agarwood hydrosol, *Aquilaria malaccensis*, GCMS, headspace solid phase micro-extraction (hSPME), lung cancer

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INTRODUCTION

The agarwood-producing *Aquilaria* trees that thrive in Malaysia, Indonesia, Thailand and the Indian subcontinents are known to have healing capacity in aromatherapy and traditional medicine for both topical application and oral consumption. As such, they stand as a potential halal and safe ingredients for development of food, nutraceutical and pharmaceutical as well as cosmeceutical products. Agarwood is normally described as the dense and fragrant resinous wood formed within the heartwood (Abbas *et al.*, 2017). The unique-smelling resinous wood obtained from these trees, possesses compounds such as sesquiterpenoids and their derivatives, and ethers and glycosides, many of which are known to be bioactive and to possess antimicrobial properties. Some compounds in agarwood have been shown to have medicinal benefits to treat Alzheimer's disease, hyperglycemia, fever, inflammation and constipation (Kakino *et al.*, 2010; Pranakhon *et al.*, 2011; Ibrahim *et al.*, 2011; Bahrani *et al.*, 2014; Manar Eissa *et al.*, 2018). Furthermore, other agarwood compounds were found to have neuroleptic and sedative properties. In addition to the above, agarwood is used in traditional medicine to treat lung and stomach tumors (Chetpattananondh, 2012). A review by Hashim *et al.* (2016) discussed agarwood materials as medicine in traditional practices as well as their pharmacologic evidences in modern science.

The common method of extraction of agarwood by hydrodistillation yields a costly essential oil fraction that is used in the perfume industry and a hydrosol fraction (distillate) that is used in

aromatherapy and traditional medicine. Hydrosols may contain some essential oils but are largely made up of water-soluble non-volatile organic compounds. In Malaysia, agarwood hydrosols are sold as health supplements for various unverified medicinal claims, including anticancer effects. Unfortunately, literature on agarwood hydrosols is limited and it is even more so on their anticancer effects. In a study, agarwood hydrosols, referred to as agarwood distillates, were found to possess anti-attachment effects as well as cytotoxic effects on MCF-7 breast cancer cells *in vitro* (Abbas *et al.*, 2014). These effects were limited to the cancer cells studied and were not observed on the control which was normal African green monkey (VERO) cells. An earlier study on the characterization of agarwood hydrosols for human consumption via brine shrimp lethality assay (BSLA) concluded that the hydrosol (pH 3.6) was safe for consumption with the highest lethal concentration value (LC₅₀) of 39.8% (v/v), equivalent to 398,000 ppm. This value was noted to be similar to that of caffeine, which is 306,000 ppm (Gameil, 2012).

Building on these previous findings, this paper presents some of the results on the anticancer potential of agarwood hydrosol against non-small cell lung cancer (NSCLC) cells, specifically Calu-3 cell line. NSCLC represents a large fraction of all pulmonary carcinomas. Despite advances in therapy, the high mortality of NSCLC patients still prevails, and this is due to the metastatic potential of these tumor cells. Inhibition of attachment and other processes of metastasis can incapacitate the tumor cells, limiting them to a specific location, and reducing mortality among NSCLC patients (Rasheed *et al.*, 2010). The anticancer potential of agarwood hydrosol on Calu-3 cells was

assessed by means of a cell attachment (AT) assay and a cytotoxicity (CT) assay. This paper reports on the effects of exposure time and concentration of agarwood hydrosol on Calu-3 cell inhibition of attachment and viability.

Literature on agarwood hydrosol composition is scarce and as such, determining their anticancer or antioxidant potential alone is not enough and must be supported up by the chemical profile of agarwood hydrosols in order to utilize agarwood hydrosols well. One of the problems in working with one hydrosol sample was that it was difficult to determine if it was a representative sample of agarwood distillates. This is because distillation parameters, season of collection, and storage conditions, vary and are often not properly documented and/or studied. However, by analyzing two hydrosols we have provided a more complete picture of the hydrosol composition.

EXPERIMENTAL

Materials

Agarwood hydrosol was obtained from two batches of hydrodistillation of *Aquilaria malaccensis* species from the state of Negeri Sembilan, Malaysia. The samples were filtered using a 0.45 μm syringe filter before use in cell culture experiments, and stored at room temperature.

Sample preparation

Calu-3 adenocarcinoma lung cancer cells (HTB-55, ATCC) were used as model cell line. ATCC's product sheet for the cell line was used as a guide. Calu-3 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), and 1% MEM non-essential amino acid solution (Sigma). Accutase (Innovative Cell Technologies) was used for cell detachment.

Experimental design

Design Expert (version 6.0.8) software's response surface methodology (RSM) was used to generate 13 experimental runs with two factors varied over three levels. The factors were amount of agarwood hydrosol (in ppm complete medium) and exposure duration (in hours). Face-centered central composite design was used for the experimental design, with five center points. There were two responses in the study, (i) number of cells attached (attachment assay), and (ii) percentage cell inhibition (cytotoxicity assay). Table 1 shows the experimental runs generated by the software. At each experimental plate, all the samples were repeated three times and all assays were performed in three replicates of plates.

Table 1 Experimental runs based on Central Composite Design (CCD) with two factors and three levels (-1, 0, +1). Actual values for each level are shown in parentheses. The CCD was generated by Design Expert Software v.6.0.8.

Run	Hydrosol amount (ppm complete medium)	Exposure duration (hours)
1	0 (150)	0 (24)
2	0 (150)	0 (24)
3	0 (150)	1 (36)
4	1 (250)	0 (24)
5	0 (150)	0 (24)
6	0 (150)	0 (24)
7	-1 (50)	-1 (12)
8	1 (250)	-1 (12)
9	0 (150)	0 (24)
10	-1 (50)	0 (24)
11	1 (250)	1 (36)
12	0 (150)	-1 (12)
13	-1 (50)	1 (36)

Cell attachment assay

Cells derived from multicellular organisms are able to adhere to an extracellular matrix or to other cells. This adhesion ability, in cancer cells, facilitates migration to a new site and eventually their metastasis. If these cancer cells can be prevented from adhering,

metastasis could be checked. Cell attachment assays, such as the trypan blue dye assay, are used to evaluate the metastatic ability of certain cancer cells as well as to assess the effect of certain stimuli or treatments with a compound of interest on the cell's ability to adhere. The cell attachment assay used in this study involved cell co-treatment with agarwood hydrosol. The method used was a modification of Freshney (2005) and Hashim *et al* (2014) where cells were plated at a seeding concentration of 5×10^4 cells/mL of medium in 6-well plates, to a volume of 2 mL per well. The medium already contained agarwood hydrosol at varying amounts to investigate the effects of the hydrosol on the adhering cells (as such called co-treatment). Cells were incubated for an exposure time of 12, 24 or 36 hours, respectively prior to counting using trypan blue dye exclusion method to measure the attachment of cells. DMSO (10% v/v) was used in positive control wells while negative control wells contained untreated cells only. The percentage of inhibition of cell attachment of Calu-3 cells by agarwood hydrosol was thereby calculated for attached (viable) cells, according to Equation (1) and compared with the controls.

$$\text{Percentage inhibition (\%)} = (NC - N/NC) \times 100 \quad (1)$$

where NC is negative control viable cell number, in cells/mL, and N is cell number after co-treatment, in cells/mL.

Cytotoxicity assay

Cytotoxicity assays such as the lactate dehydrogenase (LDH) assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), neutral red assay and sulforhodamine B (SRB) assay, are quick, affordable, reproducible *in vitro* methods to assess the effect of certain doses of a treatment on certain types of cells. Cytotoxicity can take on a variety of definitions depending on the nature of the study. It could be killing the cells, or altering their metabolism, cell signaling, or cell interaction. The MTT assay was chosen in this study since it was concluded to be the most sensitive in detecting cytotoxic events in a study by Fotakis and Timbrell (2006). The MTT assay is a colorimetric assay that assesses enzyme activity, where tetrazolium dye MTT, a yellow water-soluble tetrazole, is reduced to insoluble formazan, which is dark purple, by mitochondrial succinate dehydrogenase. This assay was conducted to determine the viability of cells after addition of agarwood hydrosol. This differs from the attachment assay in that it serves to determine if agarwood hydrosol is capable of killing already adherent Calu-3 cells.

The assay was carried out according to the method described by Freshney (2005) and Hashim *et al* (2014) with adjustments. The cells were initially plated at a seeding concentration of 2×10^6 cells/mL of media in flat-bottomed 96 well plates. Then, agarwood hydrosol was added to this sometime during the mid-log phase (determined from the growth profile), at varying amounts (according to the 13 runs), to a final volume of 200 μL per well. Different amounts of the hydrosol were prepared for cell treatment by diluting the stock with medium only (no serum added). This mixture was then vortexed for uniform mixing. Cells were incubated for an exposure time of 12, 24 or 36 hours, and then 20 μL MTT reagent were added, with mixing. The cells were then incubated for a further 4 to 8 hours, wrapped in aluminum foil, until the purple precipitate was visible. Then the medium containing MTT was removed and a solubilization buffer (150 μL DMSO) was added and they were left in the dark, at room temperature, for 2 hours. After mixing the 96-well plates to ensure uniform readings, absorbance at 570 nm was measured using a microplate reader and this gives a direct quantification of cells. DMSO (10% v/v) was used in positive control wells, while negative control wells contain untreated cells only. Fresh medium was used as a blank.

GCMS characterization

The samples were prepared for GCMS as described by the IUPAC method (IUPAC, 1987; Leon-Camacho *et al.*, 2013). First, 0.1 mL hydrosol was placed in 10 mL hexane. To this, 100 μL , 2 N potassium hydroxide in methanol, was added. This was mixed by vortexing for

30 seconds and was followed by centrifugation at 4500xg, at room temperature for 2 minutes. The resulting supernatant, containing fatty acid methyl esters (FAMES), was collected into 2 mL vials for GCMS.

The GCMS column used in this study was DB-WAX (Agilent Technologies; 30 m × 250 μm × 0.25 μm). The instrument used was a 7890A GC system (Agilent Technologies) coupled to a 5975C Inert XL MSD using the manufacturer’s software (MSD ChemStation). The carrier gas used was helium at a flow rate of 1.18 mL/min. The oven temperature program was 50°C (1 min), then 25°C/min to 200°C (0 min), and then 3°C/min to 230°C (18 min). The injector temperature was 250°C. The compounds obtained were matched using the NIST11 library.

Headspace SPME-GCMS characterization

Headspace SPME-GCMS analysis was carried out using the following technique. The SPME instrument (Supelco SPME Fiber, DVB/CAR/PDMS) was first pre-conditioned by placing it on the GCMS for 8 minutes. Then, 10 mL of the agarwood distillate sample was placed in a T-25 flask. The SPME instrument was used to pierce the septum of the T-25 cap. Then, the fiber was allowed to extract volatile samples from the headspace for 15 minutes. After that the SPME instrument was placed on the GCMS system to desorb, for 5 minutes (to prevent damage to the fiber), and then the system was left to run for a total of 24 minutes. The fiber was reconditioned before each analysis (Yu et al., 2009).

RESULTS AND DISCUSSION

Trypan blue dye attachment assay

Fig. 1 below displays the results of the trypan blue dye attachment assay. From the 13 runs conducted, the highest inhibition obtained was 100%, at runs 7 and 8, and the lowest inhibition, 22.22% was at run 11. The positive controls involved co-treatment with 10% (v/v) DMSO, gave 100% inhibition. Using the results, a response surface plot was obtained, as shown in Fig. 2. Meanwhile, based on the percentage inhibition of cells using the trypan blue dye attachment assay, the model equation was found to be that shown in Equation (2). It was expected that the model of this relationship would be quadratic, as suggested by the choice of experimental design (CCD). However, the model suggested by ANOVA is a linear model and is significant, with a model F value of 7.31 and its lack of fit is insignificant, with a value of 0.75. However, there is a 64.04% chance that this value could occur due to noise. The model suggests that the relationship between A, agarwood hydrosol amount, and B, exposure duration, to Calu-3 cell inhibition is linear, and is directly proportional in the former and inversely proportional in the latter and B is the significant term.

$$Cell\ Inhibition = 64.74 + 6.25A - 28.24B \tag{2}$$

Where A is agarwood distillate amount and B is exposure duration.

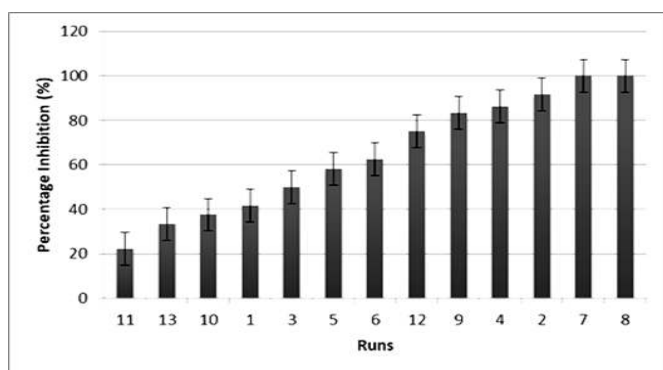


Fig. 1 Inhibition of Calu-3 lung cancer cell attachment upon treatment with agarwood hydrosol tested using Trypan blue dye attachment assay. Samples were prepared based on Central Composite Design (CCD) with two factors (hydrosol amount and exposure duration) at three levels (high, medium, low). Samples (agarwood hydrosol) were

added simultaneously with cells during cell seeding to observe the ability of samples to inhibit attachment of cells or cause cells to detach during the studied exposure time. Untreated cells were used as negative control while 10% DMSO (v/v) (positive control) showed 100 % inhibition.

DESIGN-EXPERT Plot

Cell Inhibition
X = A: Distillate concentration
Y = B: Exposure Time

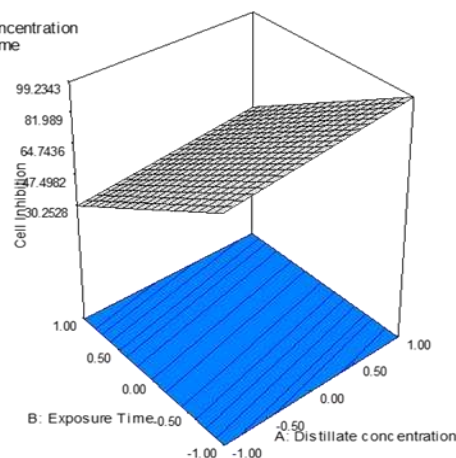


Fig. 2 Response surface plot showing the effects of hydrosol amount and exposure time on inhibition of cell attachment of Calu-3 cells as studied using Trypan blue dye attachment assay.

The results of the trypan blue dye attachment assay are very promising with 2 runs (runs 7 and 8) having 100% inhibition of cell attachment. The highest effect fluctuated between the middle and higher level of agarwood hydrosol, for the same exposure duration, and this suggests that while agarwood hydrosol has some anti-attachment effects on Calu-3 cells, this effect is less pronounced in higher amounts of agarwood hydrosol. This could be due to the presence of other compounds which, in higher concentrations cancel out the anti-attachment effect. It is therefore necessary to profile agarwood hydrosols to determine which components are synergistic to the anti-attachment effect and what compounds cancel it. Even though the results are insufficient to draw a conclusion on the IC₅₀, we note that addition of the lowest amount of agarwood hydrosol was able to inhibit cell growth. These results are consistent with previous findings of trypan blue attachment assays on MCF-7 cancer cells (Phirdaous et al., 2014). Results were significant (P-value of model is 0.0110, which is less than 0.05) with a linear model of cell inhibition with duration of exposure being a significant factor. This indicates that the cells’ ability to attach to the ECM of the tissue culture flask is impaired in the presence of agarwood hydrosol. Since attachment is a pre-requisite of metastasis, it could further be postulated that agarwood hydrosol potentially have anti-metastatic properties in general.

MTT cytotoxicity assay

Fig. 3 summarizes the results of the experiments for all runs, for the MTT cytotoxicity assay. The highest inhibition, 95.1%, was achieved in Run 7, whereas the lowest, 36.6%, was for Run 2. As with the trypan blue attachment assay, a response surface plot (Fig. 4) was obtained for the MTT assay.

Analysis of variance (ANOVA) suggested a quadratic model for the inhibition results obtained from the MTT assays conducted. This model’s F-value of 40.51 implies that the model is significant, whereas its "lack of fit F-value" of 0.43 implies that its lack of fit is not significant relative to the pure error. Here, B and B2 were found to be the significant terms. The model equation for cell inhibition, the calculated response of MTT assays, is:

$$Cell\ Inhibition = +43.77 + 1.15A - 17.57B + 0.043A^2 + 32.84B^2 + 0.28AB \tag{3}$$

where A represents agarwood hydrosol amount and B represents exposure duration.

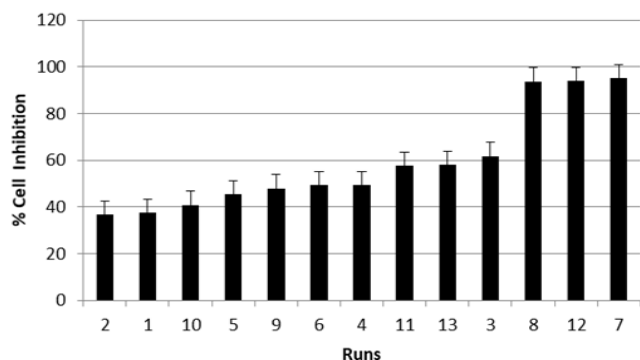


Fig. 3 . Cytotoxic effects of agarwood hydrosol against Calu-3 lung cancer tested using MTT assay. Samples were prepared based on Central Composite Design (CCD) with two factors (hydrosol amount and exposure duration) at three levels (high, medium, low). Samples (agarwood hydrosol) were added on already adherent cells to observe the ability of samples to kill the cells.. Untreated cells were used as negative control while 10% DMSO (v/v) was used as positive control.

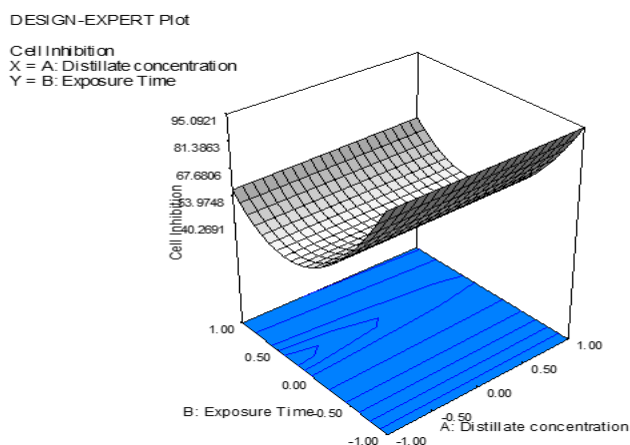


Fig. 4 Response surface for cell inhibition of distillates (MTT assay)

The cytotoxicity assay, on the other hand, also provides insight into the activity of agarwood hydrosol. The highest inhibition, 95.1%, was achieved in Run 7, as experienced with the attachment assay, whereas the lowest, 36.6%, was for Run 2. Run 7 represents the lowest concentration of agarwood (50 µL/mL) and the lowest exposure duration (12 hours). The agarwood hydrosol has the potential to alter the enzymatic activity of the mitochondria and initiate a preliminary injury that leads to cell death. This is observed in essential oils whereby they decrease the mitochondrial membrane potential and alter the permeability of membranes. Analysis of variance (ANOVA) suggested a quadratic model for the inhibition results obtained (P-value<0.0001, which is less than 0.05), with exposure duration being a significant factor.

The findings of both assays together suggest that the agarwood hydrosol works by several mechanisms and is able to both prevent adherence of Calu-3 cells as well as kill attached Calu-3 cells. This is useful since it would employ various mechanisms and may thus work on several cancer types. In retrospect, the factors and levels chosen provided us with useful information but they did not suffice for us to deduce an IC₅₀ value for agarwood hydrosol as statistical analysis did not find the dose significant as a factor. We note that an exposure duration longer than 12 hours would not enhance these two effects, which suggests that the hydrosol is degraded over time or consumed. Thus, further experimentation with dose must be explored to determine IC₅₀. One of the problems in working with one hydrosol was that it was difficult to determine if it was a representative sample of agarwood distillates. This is because distillation parameters, season

of collection, and storage conditions, vary and are often not properly documented and/or studied. However, by analyzing two hydrosols we have provided a better picture of the hydrosol composition.

GCMS and HSPME-GCMS

Fig. 5 (a) and (b) show the GCMS and HSPME profile obtained for agarwood hydrosol, respectively. Compounds with quality (confidence measure) greater than or equal to 70% are shown in Table 2.

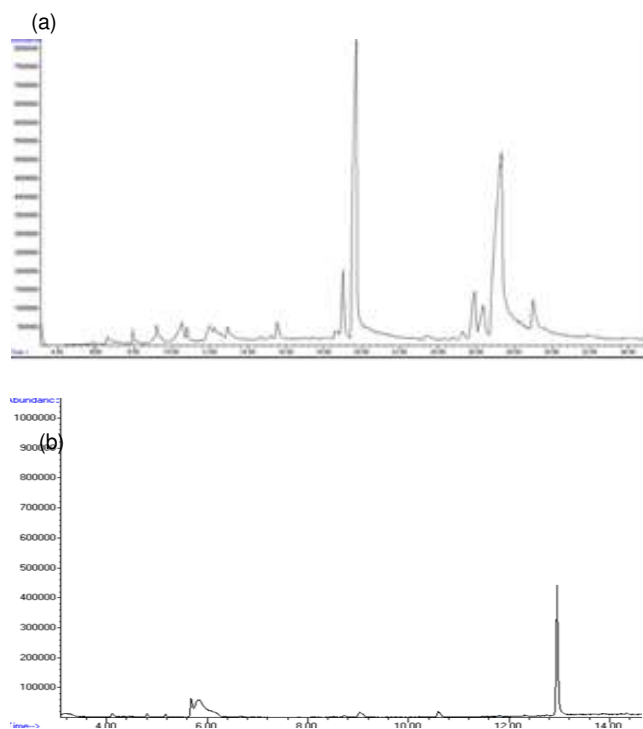


Fig. 5 Chromatograph of agarwood hydrosol using (a) GCMS and (b) HSPME-GCMS

Table 2 Compounds in agarwood hydrosol detected by GCMS and HSPME-GCMS respectively

GCMS		HSPME-GCMS		
No.	Compound Name	Area %	Compound Name	Area %
1	Octadecamethyl-cyclononasiloxane	1.36	4-methyl-2-heptanone	0.05
2	(Z)-9-Octadecenal	6.16	Styrene	1.47
3	(Z,E)-3,13-Octadecadien-1-ol	1.57	Benzaldehyde	0.16
4	(E)-9-Octadecenoic acid	2.59	2-Butanone, 4-phenyl-	1.49
5	Oleic acid	1.12		
6	6-Octadecenoic acid	32.18		
7	(Z,Z)-9,12-Octadecadienoic acid	8.63		
8	Glyceryl monooleate	0.21		
9	(E)11-Hexadecenal	0.54		
10	3-(hexahydro-1H-azepin-1-yl)-1,1-dioxide 1,2-Benzisothiazole	0.56		
11	1-Tricosene	0.50		
12	n-Hexadecanoic acid	24.92		
13	2,3-Dihydroxypropyl elaidate	0.75		
14	Octadecanoic acid	3.13		

Chemical characterization of the sample 1, via GCMS, identifies 49 peaks, many of which are fatty acids, such as octadecanoic (stearic) acid, 6-octadecenoic (petroselinic) acid, 9-octadecenoic (oleic acid), (Z,Z)-9,12-octadecadienoic (cis-linoleic) acid. These were in minute quantities, except for n-hexadecanoic (palmitic) acid, an antioxidant (Subavathy and Thilaga, 2016). Aside from these, fatty acid esters, alcohols, aldehydes, and alkenes are also present. Of interest, 9-octadecenal is an antimicrobial and anti-inflammatory agent (Subavathy and Thilaga, 2016). 1-Tricosene is a potential anticancer compound. Previous study revealed that octadecanoic acid and hexadecanoic acid were reported to possess antitumor activity (Kilonzo et al., 2017; Zainurin et al., 2018).

GCMS identifies 54 peaks for sample 2. Of these compounds, 16-hentriacontanone (also known as palmitone) and 6-(phenylmethyl)-5H-pyrrolo [3,4-b]pyrazine-5,7(6H)-dione make up a significant area. The former, is a potential anticancer compound (Lee, et al., 2008; Saibu, 2012), while very little information is available on the latter. Oleic acid, stearic acid and many of their derivatives possess anticancer effects (Falconer et al., 1994). In a study, stearic and oleic acids inhibited the colony-forming abilities of five human cancer cell lines in a dose-dependent manner (Fermor et al., 1992). Octadecanoic acids are conjugated linoleic acids (CLAs) and in a study, CLA was found to inhibit chemically-induced cancer where CLA-treated mice developed only about half as many papillomas and exhibited a lower tumor incidence compared with the control mice (Ha et al., 1987). In another study, CLA inhibited human hepatoma (HepG2) cell proliferation in a dose and time dependent manner (Igarashi and Miyazawa, 2001).

1-Tricosene, an alkene, and 16-hentriacontanone, an aliphatic ketone, are probable anti-tumor compounds (Lee et al., 2008; Olubunmi and Olatunji 2010; Saibu, 2012) as was concluded in other anticancer studies. In a study of *Solanum torvum* extract against Ehrlich ascites carcinoma (EAC) cell lines, 1-tricosene was found to be one of the major compounds in the extract and it could be responsible for the anti-tumor compound (Olubunmi and Olatunji, 2010; Panigrahi et al., 2014). 16-Hentriacontanone was found to possess anticancer activity against CaOV-3 ovarian adenocarcinoma cell line and to have no adverse effects on the growth of normal cells (Lee et al., 2008).

Headspace SPME profiling identifies 29 peaks from sample 1 such as benzaldehyde, 4-methyl-2-heptanone, and 4-phenyl-2-heptanone. Thirty five peaks were obtained from sample 2, belonging to various groups such as benzaldehyde, acetophenone, furfural, 3-furaldehyde, 2,5-dimethyl-2,5-hexanediol. The growth inhibition effect of benzaldehyde, one of the major compounds in both batches, on several cancer cells from inoperable cancer types has been reported in (Ariyoshi-Kishino et al., 2010). There is no literature on the cytotoxicity of furfural on cancer cells but its derivative flavanone exhibited significant anticancer activity with IC₅₀ values of 75.9, 51.0 and 59.3 µM for HT29, MCF7 and A498, respectively (Murthi and Mishra, 2014). In literature, such as Abbas et al. (2014), the potential anticancer compounds from agarwood essential oil were discussed but to the best of our knowledge; anticancer effects of agarwood hydrosols have never been reported. Headspace SPME-GCMS was found to be consistent but inadequate on its own for the purpose of characterizing the agarwood hydrosol. Factors such as headspace volume were also found to affect precision as well as sensitivity, which are crucial to obtaining an accurate profile of the distillates (Camarasu, 2000).

CONCLUSION

From the results of the experiments, we conclude that agarwood hydrosols possess anti-attachment and cytotoxic effects on Calu-3 lung cancer cells. Models for Calu-3 cell inhibition based on two factors, i.e. the exposure time and concentration of agarwood hydrosol were also obtained. From these models, the conditions can be optimized in order to obtain a higher inhibition of Calu-3 cells by agarwood hydrosol addition. Agarwood hydrosols from two batches of distillation were characterized via GCMS and hSPME-GCMS, and potential anti-tumor compounds were identified, namely 16-

hentriacontanone, benzaldehyde and 1-tricosene. It is recommended that further dose studies be undertaken to determine the IC₅₀ values of agarwood hydrosol, and that high performance liquid chromatography mass spectrometry (HPLC-MS) be used to profile agarwood hydrosols.

ACKNOWLEDGEMENT

The authors are grateful to the International Islamic University Malaysia and Asia Plantation Capital for the support which has made this research possible. This research is supported by International Islamic University Malaysia (IIUM) Special Grant for Agarwood Research and IIUM Research Initiative Grant Scheme (RIGS17-145-0720).

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