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SCREENING OF ANTICANCER ACTIVITIES FROM AGARWOOD'S METHANOL EXTRACT (GYRINOPS VERSTEGII [GILG.] DOMKE)

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ABSTRACT

Agarwood typifies as one of non-wood forest products with high values. It is a resin entity, contained/embedded in woods of e.g. Aquilaria spp. and Gyrinops spp, under Thymelaeaceae family. Gyrinops species serves as Indonesia's endemic plants and currently has been numerously planted/cultivated particularly in Eastern Indonesia's part. The research was conducted on Gyrinops verstegii species which aimed to look into possible uses of its agarwood, obtained through methanol extraction, for anticancer agent. Agarwood for such extraction presented the lowest quality based on the Indonesia's National Standards. The obtained agarwood's methanol extract was used for the screening-ability test to inhibit growth of MCF-7's breast cancer cells (ATCC HTB 22) that used MTT assay methods. IC_{50} values for agarwood extract ranged about 5.01-8.32 µg/mL, regarded effective for such inhibition. These prospective results can become a significant basis to further develop alternative efficacious drugs against cancer diseases.

KEYWORDS: *Agarwood, Gyrinops verstegii (Gilg.) Domke., methanol extract, MCF-7, alternative anticancer drugs*

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INTRODUCTION

Agarwood pertains merely to a resin substance characterized among others by its specific fragrant smell (pleasing odor), embedded in wood portion of particular tree species that belong to *Thymelaeaceae* family. Accordingly, those tree species could serve as agarwood sources or producers. In other names, agarwood is termed as consecutively kalambak, eaglewood, agarwood, aloeswood, agalloch, kanankoh, kyara, and jinkoh, or chen xiang. Agarwood is often made use as ritual incenses and traditional medicines (drugs)[1-3]. In addition, agarwood is useful as perfume ingredients, soothing (relieving) stomach pain, coughing drugs, rheumatic remedy, antitumor, anticancer, and antimicrobe agent [4-5] Agarwood-producing trees in record number more than six genera, which encompasses among others *Aquilaria, Wikstroemia, Enkleia, Aetoxylon, Gonystylus* and *Gyrinops* [6-7]. Of those six genera, *Aquilaria* dan *Gyrinops* refer to those able to produce agarwood with best or most satisfactory qualities in Indonesia, which by species comprise *A. malaccensis*, *A. filaria*, and *Gyrinops verstegii* [8]. *Gyrinops verstegii* trees from which the agarwood could be obtained presents as one of the endemic species in Indonesia, which grow numerously in consecutively Sumbawa, Alor, Flores, and Sumba islands [9]. Local community there have cultivated (planted) those trees numerously, but unfortunately study results and information about agarwood from such *Gyrinops* spp. are still very limited compared to those from *Aquilaria* spp. For example regarding the latter, methanol extract from *Aquilaria crassna*'s agarwood proves efficacious as anticancer agent for curing/overcoming cancers attacking digesting system (HCT1 16); as anticancer agent for pancreatic organs (PANC-1)[10]; agarwood oil from *Aquilaria malaccensis* as digestive anticancer agent (HCT 16) [11]; and ethanol extract from *Aquilaria sinensis* leaves as antidiabetic agent[12]. In relevant, accordingly research was already conducted focusing particularly on agarwood from *Gyrinops verstegii* species to look into its possible activity as efficacious anticancer agent to cure breast cancer disease (MCF-7). The detailed results and related narration are forthcoming.

MATERIALS AND METHODS

Materials

Agarwood with origin of *Gyrinops verstegii* tree species was obtained from particular traders in Lombok island, Province of Nusa Tenggara Barat (West South-East Insland), Indonesia. The obtained agarwood comprised three kinds of samples, each categorized as consecutively natural agarwood with "kemedangan" quality (**A**); inducted agarwood, or modified kemedangan quality II (**B1**); and inducted agarwood as well, or also modified kemedangan quality II (**B2**). The separation of agarwood qualities into those three types was based on Indonesia's National Standard (SNI), as depicted in Figure 1.

Figure 1
Visual illustration of agarwood with "kemedangan" quality; Natural agarwood (A); B1 and B2 = inducted natural agarwoods

Agarwood extraction

Initially, agarwood sample was shaped into powder form. The agarwood powder was then filtered through a screen having the holes of 80-mesh to obtain the filtered agarwood powder with uniform size. The resulting filtered powder was further ready for extraction using methanol solvent in a continuous hot extractor of the Soxhlet apparatus. As such, as much 10 grams as filtered agarwood powder was taken, and then packed into an extraction thimble (equipped with fritted-glass disk). Afterwards, the agarwood-filled thimble was inserted into the Soxhlet extractor apparatus, wherein the agarwood extraction was performed using as much 100 mL of methanol as extracting solvent. The temperature of the heater mantle equipped with the thermostat for heating of the Soxhlet flask (inside which the methanol solvent was placed) was maintained constant at 65-70^{o}C (which approximately corresponded to the boiling point of methanol). As such, the methanol extraction lasted for 24 hours. The obtained agarwood's methanol extract (liquid form) was then collected into a conical flask, while the agarwood powder that remained in the extraction thimble was discarded. Further, the collected agarwood's methanol extract was sieved using filter paper, and then repeatedly washed with distilled water (under suction). The washing was terminated when the residual washing water passing out through the sieving (filter-paper) looked apparently clear or colorless as indication that the agarwood extract became free of impurities; and in this way the obtained sieved extract was called as agarwood's crude extract. Afterwards, the impurity-free crude extract was stored

temporarily in the cool dark room for further investigation (particularly its possible activity as anticancer agent).

Cell line and culture medium

Breast cancer cells technically termed as MCF-7's breast adenocarcinoma cells (*ATCC HTB-22*) were used in this investigation. Those cancer cells were maintained in a culture medium, which comprised Dulbecco's modification of Eagle's medium, D-MEM (Gibco, USA); RPMI 1640, Fetal Bovine Serum, FBS 5% (Hyclone, USA); Penicillin-steptomisin 100 µg/mL (Invitrogen, USA), Phosphate buffer saline, PBS (Gibco, USA); Trypsin (Gibco, USA), DMSO (Sigma, USA); Ethanol (Merck, Germany); and MTT (Sigma, USA).

Monolayer cells

Breast cancer cells which had grown confluent should undergo the so-called subculturing procedure. The medium for cancer cells were discarded, and then added with PBS as much as 10 mL to cleanse the flask of medium residue; and afterwards the used PBS was discarded as well. Subsequently, as much as 5 mL of the trypsin (125%) was added into the flask, and then incubated at 37^{o}C for 5 minutes. The cancer cells that had become loose from their substrate were put into the 15 mL tube; and then centrifuged for 5 minutes, forming the so-called monolayer cancer cells which were further ready for the cell counting. The counting of cancer cells as such used the so-called haemocytometer device, and then the counted cells were prepared in accordance with the necessity for testing. Afterwards, the cancer cells were incubated again in the incubator with CO_2 concentration at 5%.

Suspension of cancer cells

As much as a half volume of the medium for cancer cells was discarded, and then the medium residue that still contained cancer cells was put into 15 mL tube. The tube with the cancer cells inside (weighing 500 g) was also centrifuged for 5 minutes, forming a suspension of cancer cells. Afterwards, the supernatant fluid that formed at the upper part of the centrifuged cancer-cell suspension was discarded. Meanwhile, the cancer cells which were separated from it suspension, after undergoing 5-minute centrifusion, and then precipitated at the bottom of the tube were ready for the cell counting. The counting of cancer cells used the so-called haemocytometer device as well, and then the already counted cancer-cells were also prepared in accordance with the necessity for testing. Afterwards, the cancer cells were also incubated again in the CO_2's incubator at 5% concentration.

Counting of the cancer cells

As much as 50 µl of the cancer-cell solution was added with 50 µL of trypan blue, and then flown into the haemocytometer device. Afterwards, observation was performed by counting the number of cancer cells which were still alive (indicated by not absorbing color), as viewed from the two big square shapes; and then average number of cells was taken from those two. The number of cells per unit volume (mL) could then be calculated using the following formula:

$$\text{Number of cancer cell per mL} = \text{average number of the counted cells} \times \text{dilution factor} \times 10^4$$

MTT assay [13]

The cancer cells which had been grown inside the flask T25 underwent the subculturing process; and then were grown (cultivated) at 96 well tissue culture plate, wherein their number corresponded to 5000 cells/well. Still in their cultivation/culture medium, the cancer cells were incubated for 24 hours at 37^{o}C and 5% CO_2 concentration. Meanwhile, bioactive compounds (agarwood's crude methanol extract with each quality origin, i.e. A, B1, and B2-type; refer to Figure 1) at varying concentrations (0; 0.625; 1.25; 2.5; 5; 10; 20; 40 and 80 µg/mL) was prepared and then added as much as 100 µL/well to the cancer cells (still in their cultivation/culture medium). After addition of such bioactive compounds, the resulting mixture was incubated again at 37 ºC for 48 hours. Following the 48-hour incubation, compound of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the mixture, and then incubated again for 4 hours at 37^{o}C and 5% CO_2 concentration. Afterwards, the supernatant (fluid) layer that formed and might contain cancer cells was discarded, while the formazan crystals left and formed underneath were dissolved in ethanol 70%. In this way, the reading of optical density (OD) value on the ethanol-dissolved crystals could be carried out using the so-called microplate reader at wavelength of 565 nm, which came up with the OD value of the treated MCF-7's breast cancer cells (i.e. cancer cells added with bioactive compounds). Meanwhile, the breast cancer cells without addition of bioactive compounds were also prepared in the same way as those with such addition, as control (untreated cancer cells). In this way also, the value of OD for untreated MCF-7's breast cancer cells (as control) could also be obtained through the same reading procedures. From the values of both OD of the treated cancer cells and OD of the untreated cancer cells (control) then could be calculated the so-called inhibition percentage (%), using the formula described below.

$$\%\ \text{inhibition} = \frac{\text{OD of the untreated cancer cell} - \text{OD of the treated cancer cell}}{\text{OD of the untreated cancer cell}} \times 100\%$$

After the values of % inhibition were obtained at each concentration of the bioactive compounds (i.e. agarwood's methanol extract at its particular quality origins [A, B1, dan B2 types]) using the equation as above, then the so-called probit analysis could be performed. Such analysis was merely shaping a response curve (trend plots) that related the value of inhibition percentage (in %, as probability unit) plotted against the logarithm of bioactive compound concentrations. The shape of response curve depended

on the tendency of trend plots. Further from the response curve (i.e. the linear or polynomial regression equations, depending on the resulting fitting-curve) then could be derived or determined the so-called IC_{50}, which in this case implied the effective inhibitory concentrations of agarwood's methanol extract capable of inhibiting 50% growth of MCF's breast cancer cells [14].

RESULTS AND DISCUSSION

Yield of agarwood extract

As described before, agarwood with origin of *Gyrinops vertegii* tree species used in this investigation belonged to the category of "kemedangan" qualities, based on Indonesia's National Standard (SNI 7631:2011)[15]. Agarwood with A type (kemedangan quality) (see Figure 1) typifies as the one which is obtained from nature (natural agarwood). Meanwhile, agarwood with B1 and B2 types refers to the one actually also with "kemedangan" quality but already modified through the induction process that used fusarium (a kind of microorganisms) which is previously infected into the stem portion of agarwood host trees (*Gyrinops vertegii*); and moreover the local community have already cultivated (planted) those trees intensively. Such induction aims to induce more agarwood formation inside the stem of its host trees (e.g. *Gyrinops vertegii* species) and concurrently to enhance its quality. Agarwood with "kemedangan" quality is regarded as the lowest quality. This is because such agarwood contains sesquiterpene compounds and does not contain chromone derivatives[16]. In general, agarwood with better quality contains sesquiterpene compounds as well as chromone derivatives[17-20]. Flavonoid, alkaloid, and terpenoid were reported having potency as anticancer and antioxidant[13]. Sesquiterpene compound is part of a group of compound terpenoids therefore agarwood extract has potential as an anticancer. The yield of agarwood's methanol extract at each type (quality) is disclosed in Table 1. It reveals that the highest extract yield was brought out from agarwood with B2-type quality (11.41%), followed in decreasing order by the ones with consecutively A-type and with B1-type qualities (2.45% and 2.36%, respectively). As related, Figure 2 depicts that agarwood extract with B2 type exhibited darker color compared to the one with other types (A and B1}. This indicatively makes possible for B2-type's agarwood to contain more resin.

Table 1
Yield of agarwood extract

No.	Agarwood qualities (types)	Average yield (%)
1.	A	2.45 ± 0.30
2.	B1	2.36 ± 0.08
3.	B2	11.41 ± 0.21

Remarks: Values are mean ± SD, n=3

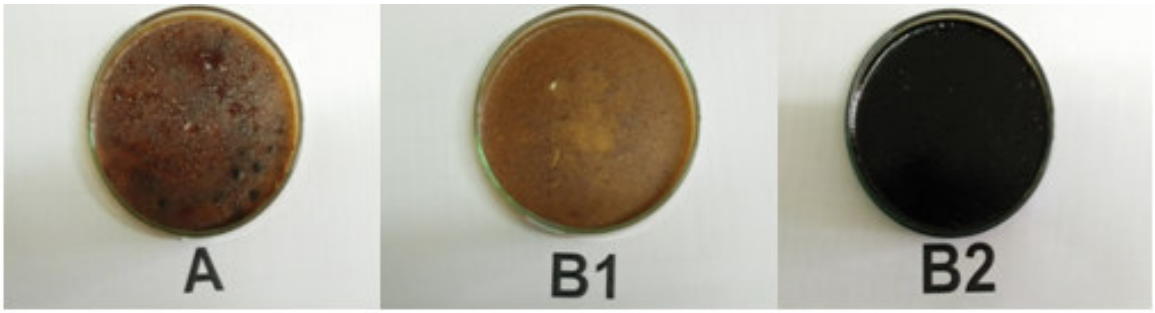
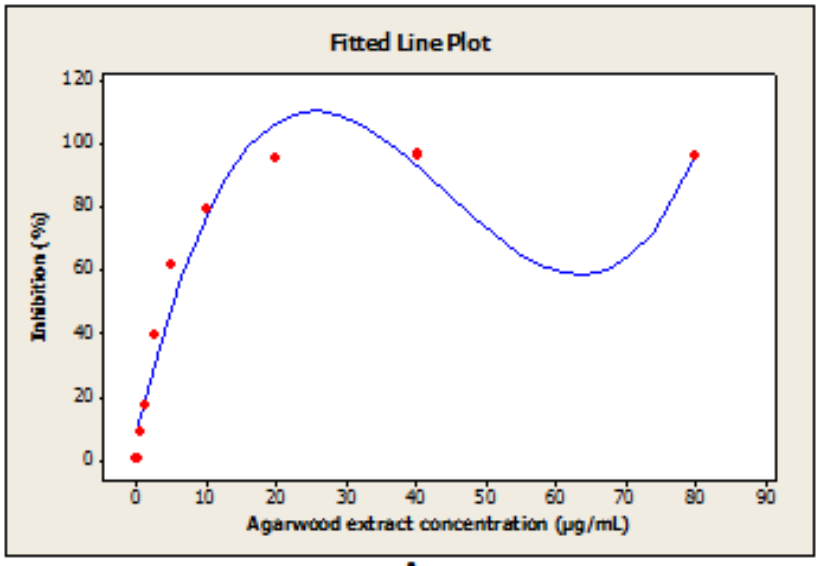


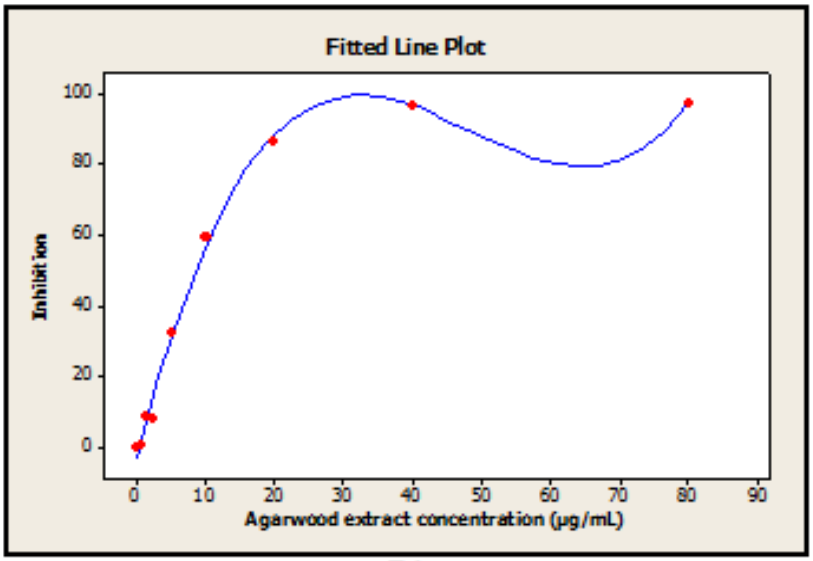
Figure 2.
Agarwood extract
Remarks: for the notations of A, B1, and B2, please refer to Figure 1

Anti-cancer effects inflicted by agarwood extract

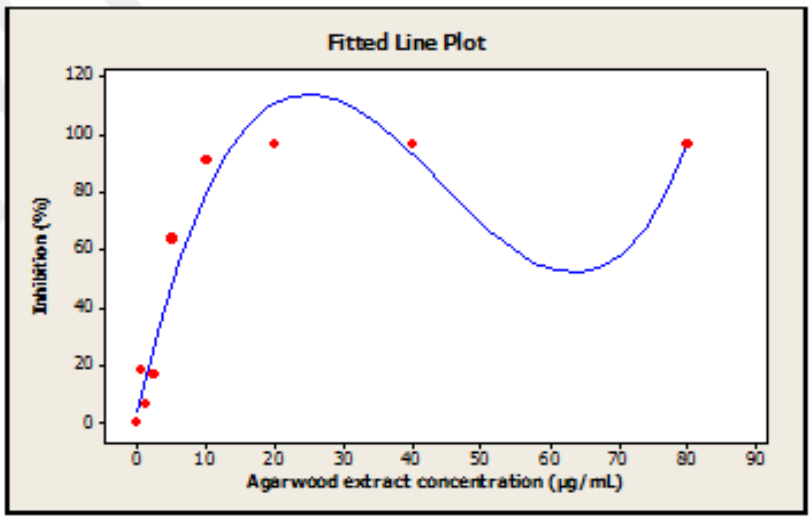
Figure 3 (A, B1, and B2) discloses the response curve which illustrates how the agarwood's methanol extract at its varying concentrations could inhibit the growth of MCF-7's breast cancer cells. It could be observed from the trend plots (response curve) and then analyzed that all types of agarwood's methanol extract (A, B1, and B2) was able to inhibit the growth of breast cancer cells. In general, the greater the dosages or concentrations of agarwood extract, then the higher would be its ability to inhibit (screen) growth of MCF-7's breast cancer-cells; and conversely for the lower concentrations (dosages). From the scrutiny results on the tendency of trend plots (Figures 3A, 3B1, and 3B2), it turned out that the equations in third order (power) polynomial regression (i.e. not linear regression equations) were the best fitting the relation between concentration of agarwoods and percentage (%) of inhibition, either for A1, B1 or B2 types; and this was based on the three determination coefficient (R^2) values which were significant. Accordingly, from those three polynomial regression equations (Figures 3A, 3B1, and 3B2), it could be determined that the IC_{50} value for agarwood extract with A-type corresponded to 5.01 µg/mL, while the IC_{50} values for agarwood extract with B1-type and B2-type were equal to consecutively 8.32 µg/mL and 5.13 µg/mL.



A



B1



B2

Figure 3
Relationship between agarwood extract concentration (µg/mL) nd growth inhibition (%)

As described before, those three different IC_{50} values imply the concentrations (dosages) of agarwood extract at particular type (A, B1, and B2) which were able to screen or inhibit as much as 50% growth activity of breast cancer cells. This suggests that agarwood's methanol extract of those tree types (A, B1, B2) could perform as anti-cancer agent, whereby in performance (intensity) the order was A > B2 > B1. Assessing the order, it also implies that the methanol extract from natural agarwood with "kemedangan" quality (A type; without induction) performed better than the fusarium-inducted agarwood (B1 and B2 types) despite originating also from "kemedangan" quality. Notwithstanding such different performances, those three IC_{50} values of agarwood's methanol extract (5.01-8.32 µg/mL) indicates that they were still more effective in inhibiting the growth of MCF-7's breast cancer cells than the agarwood's volatile oil which exhibited its IC_{50} value at 44 µg/mL[14]. Further, the volatile (essential) oil obtained from agarwood of *Aquilaria malaccensis* species afforded the IC_{50} value at 44 µg/ml, which was able to inhibit the growth of digestive cancer cells (HCT116)[11]. Meanwhile, agarwood's methanol extract of *Aquilaria crassna* species exhibited consecutively the IC_{50} value at 28.0 µg/ml for dealing also with digestive cancer cells (HCT116); IC_{50} value at 32.0 µg/ml for pancreatic cancer cells (PANC-1); IC_{50} value at 140 µg/ml for breast cancer cells (MCF-7); and IC_{50} value at 110.2 µg/mL for prostate cancer cells (PC3)[10]. Assessing the research results as above, agarwood from several particular host tree species (i.e. *Gyrinop verstegii*, *Aquilaria malaccensis*, and *Aquilaria crassa*) could indicatively be beneficial serving as anti-cancer agent. Such anti-cancer capability possibly owes to the presence of sesquiterpene compounds which becomes the specific characteristics of chemical components in agarwood, in addition to chromone derivatives.

CONCLUSION AND SUGGESTIONS

Methanol extract of agarwood with low "kamedangan" quality, comprising three types (A, B1, and B2), was originated from its host tree species (*Gyrinops verstegii*). The A type referred to the natural agarwood, while the B1 and B2 types resulted from the fusarium-inducted natural agarwood. The agarwood's methanol extract of all those three types (A, B1, and B2) was indicated able to perform as anti-cancer agent to screen or inhibit the growth of MCF-7's breast cancer cells, each with their different IC_{50} values. As such, the IC_{50} values for agarwood extract with A type corresponded to 5.01 µg/mL, while the values for agarwood extract with B1 and B2 types equal to consecutively 8.32 and 5.13 µg/mL; indicating that in inhibiting performance against breast cancer cells, the intensity order was: A > B2 > B1. Despite different performance, those three IC_{50} values (5.01-8.32 µg/mL) were much lower than the values for agarwood's volatile oil (44 µg/mL), indicating that anti-cancer performance of the overall agarwood's methanol extract (with A, B1, B2 type) was quite effective against breast cancer cells. These prospective results strongly suggest that further investigation deserves carrying out which focuses thoroughly on development of alternative drugs from *Gyrinops verstegii* 's agarwood to cope with cancer diseases.

CONFLICT OF INTEREST

Conflict of interest declared none.

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