CHARACTERISATION OF GAHARU HYDROSOL: PHYSICAL, CHEMICAL AND MICROBIOLOGICAL PROPERTIES

PENCIRIAN HIDROSOL GAHARU: SIFAT FIZIKAL, KIMIA DAN MIKROBIOLOGIKAL

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Abstract

Gaharu hydrosol is produced during the hydrodistillation of resinous wood part of Aquilaria sp. This aromatic water is being considered as a by-product in the industry. There is interest to turn this aromatic by - product into aromatherapy products. The present study is carried out in order to understand the properties of gaharu hydrosol, physically, chemically and microbiologically. Gaharu hydrosol from two different extraction facilities i.e. at Kedaik Agarwood Sdn. Bhd. and Malaysian Nuclear Agency were characterised in this study. All the gaharu hydrosol samples displayed acidic nature, with pH in the range of 3.62 – 4.53. Four antioxidant assays were carried out to ascertain the antioxidant capabilities of two gaharu hydrosol samples through the total phenolic content assay, $ABTS^{\cdot+}$ radical scavenging activity, DPPH· radical scavenging activity and ferric reducing activity (FRAP). The results revealed that the samples exhibited lower antioxidant capabilities as compared to the positive control. For microbial population study, fungi was not present in the samples as there was no growth observed on the Plate Sabouraud Dextrose Agar (SDA) using membrane filtration technique. The antibacterial activity of the gaharu hydrosol against Staphylococcus aureus and Pseudomonas aeruginosa was determined using agar dilution method and disk diffusion method. The results showed that the gaharu hydrosol did not inhibit the growth of both the bacteria. The results obtained from this study will be further evaluated for the development of new products using this aromatic gaharu by-product.

Abstrak

Air sulingan atau hidrosol gaharu dihasilkan semasa penyulingan air bahagian kayu Aquilaria sp yang mengandungi oleoresin gaharu. Air beraroma ini dianggap sebagai satu produk sampingan dalam industri gaharu. Terdapat pihak yang berminat untuk menjadikan produk sampingan beraroma ini sebagai produk-produk aromaterapi. Kajian ini dijalankan untuk memahami ciri-ciri fizikal, kimia dan mikrobiologi hidrosol gaharu. Hidrosol gaharu daripada dua kemudahan ekstraksi iaitu di Kedaik Agarwood Sdn. Bhd. dan di Agensi Nuklear Malaysia telah dicirikan dalam kajian ini. Adalah didapati bahawa semua sampel air sulingan gaharu menunjukkan sifat berasid dengan pH dalam julat 3.62 – 4.53. Empat asai antioksidan telah dijalankan untuk menentukan keupayaan antioksidan pada dua sampel air sulingan gaharu melalui kaedah 'total phenolic content assay', 'ABTS·+ radical scavenging activity', 'DPPH· radical scavenging activity' and 'ferric reducing activity (FRAP)'. Keputusan daripada asai-asai ini menunjukkan sampel-sampel yang diuji mempamerkan keupayaan antioksidan yang lebih rendah berbanding kawalan positif. Bagi kajian populasi mikroorganisma, fungi didapati tidak hadir dalam sampel-sampel yang diuji memandangkan tiada pertumbuhan yang dicerap di atas 'Sabouraud Dextrose Agar (SDA)' dengan menggunakan teknik penurasan membran. Aktiviti antibakteria hidrosol gaharu ke atas Staphylococcus aureus dan Pseudomonas aeruginosa telah ditentukan menggunakan kaedah difusi agar dan difusi cakera. Keputusan-keputusan yang diperolehi menunjukkan bahawa air sulingan gaharu tidak berupaya merencat pertumbuhan kedua-dua bakteria tersebut. Kesemua keputusan yang diperolehi daripada kajian ini akan dinilai untuk pembangunan produk-produk baru mengunakan hasil sampingan gaharu yang beraroma ini.

Keywords/Kata kunci: gaharu hydrosol, acidic, antioxidant, antibacterial, product development

INTRODUCTION

Gaharu hydrosol is a by-product produced during the hydrodistillation of the resinous wood wood part of *Aquilaria* sp. This by-product is unique as it has the aroma of the essential oil of *Aquilaria* sp. even after storage for 1 year. Generally, hydrosol contains a small fraction of the aromatic compounds recovered from the plant in the distillation process which ends up in the distillation water. Every aromatic substances has a maximum solubility in water and only after this point is reached will these aromatic compounds, the essential oils starts to separate into a distinct layer on the top of the distillation water (Catty, 2007).

Aromatherapy is a branch of phytotherapy or plant therapy just as herbalism, homeopathy, flower remedies, traditional Chinese medicine and many other treatments. Aromatherapy in the truest sense of the word is the use of 100 % natural, whole, unadultered, aromatic essences obtained from specific botanical sources by steam distillation or expression for the benefits of mind, body and spiritual health. These essences may be pure essential oils, the non-water soluble, volatile, aromatic compounds found in flowers, leaves, branches, seeds, roots, barks, resins and fruits and obtained by gentle steam distillation. They may also be the expressed oils found in the rinds of citrus fruits like lemon, orange, bergamot and grapefruit which are gathered by squeezing the oil from the peel. Aromatherapy also involves the use of the nonvolatile fatty oils (carrier oils) found in avocados, sesame seeds and exotics such as rose hips and hazelnuts. Last but not least, aromatherapy uses hydrosols, the aromatic waters coproduced during the steam distillation of essential oils (Catty, 2001).

A recurring concern in all of aromatherapy is the potential of essential oils to irritate the various tissues with which they come in contact and the discussion about the proper dilution of essential oils to avoid such irritation is still raging. The appropriate application of aromatic substances in their dissolved state can avoid these problems. As hydrosols are dissolved, there is no oily or lipophilic phase to irritate tissues (Catty, 2001). Hydrosols are milder and safer to use than essential oils, as long as they are derived from nontoxic plants (Today's herbs, 1999).

The attempt to exploit this by-product into aromatherapeutic product is an innovative move. This attempt does not only turning waste into wealth, but also overcoming the problems caused by the use of essential oil in aromatherapy. Some of the properties of gaharu hydrosol were determined in this study in order to have a better understanding of this unique aromatic water. This initial investigation of gaharu hydrosol properties include the determination of pH, determination of the presence of bacteria and fungi using membrane filtration method and the determination of its antimicrobial activity. The antioxidant activity of gaharu hydrosol was also screened.

PROCEDURE

Sample of gaharu hydrosol

Gaharu hydrosol was taken mainly from Kedaik Agarwood Sdn Bhd extraction factory in Rompin. There were also samples from gaharu oil extraction facility in Agensi Nuklear Malaysia, Bangi. The samples were taken using aseptic technique and kept in sterile bottle unless being mentioned. The samples were stored in fridge or cold room.

pH determination

The pH of gaharu hydrosol was determined using pH meter (Mettler Toledo 320).

Microbial load analysis

The presence of bacteria and fungi in the gaharu hydrosol samples were determined using membrane filtration method. Three mL of the sample was pipetted onto sterile membrane filter (0.45 μm pore size, diameter 47 mm, cellulose nitrate, chmlab group) which was placed onto filtration unit. This filtration unit was autoclaved prior to use. After the hydrosol solution passed through the membrane filter with the aid of vacuum pump, the membrane filter was carefully removed using a flamed forcep and placed onto agar. For analysis of bacteria, Plate Count Agar

(Merck) was used while for fungi, Sabouraud Dextrose Agar (Difco) was used. The plate count agar was incubated at 37°C for 48 hours while the Sabouraud Dextrose Agar was incubated at 22°C for 7 days. After incubation, the number of colony forming unit was calculated and recorded. Each sample was replicated trice and all the work was carried out in laminar air flow cabinet.

Determination of antimicrobial activity

In this study, antimicrobial activity was determined using two methods i.e. the well diffusion assay and the disk diffusion assay. The antimicrobial activity of gaharu hydrosol against *Staphylococcus aureus* and *Pseudomonas aeruginosa* was determined in this study. Overnight broth cultures were freshly prepared for each assay. The cultures were grown in nutrient broth (Fluka) and incubated at 37°C.

Well diffusion assay

Fifty mL of molten Mueller-Hinton Agar (Oxoid) was inoculated with 0.33 mL of overnight grown bacterial culture. The inoculated agar was sswirled to mix thoroughly and poured into a glass petri dish (diameter 15 cm). When the agar solidified, the petri dish was kept upside-down in fridge overnight prior to use. Using a template, 13 wells were cut into the agar with sterile cork borer (6 mm). Gaharu hydrosol sample was pipetted into five wells until full while three wells were filled with gentamycin solution as positive control (Fig. 1). The petri dishes were incubated for 24 hours at 37°C and the zones of inhibition were measured and recorded.

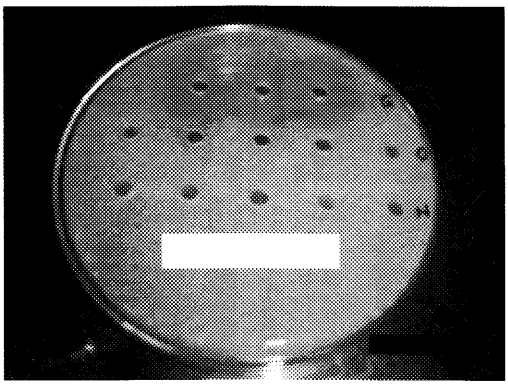

Fig. 1. Well diffusion assay using glass petri dish.

Disk diffusion assay

Twenty mL of Mueller-Hinton Agar (Oxoid) plates were inverted and dried at 37°C for 30 minutes. An overnight culture of bacteria (0.5 mL) was spread over the surface of the agar plate using a sterile glass spreader. Inoculated plates were inverted and incubated at 37°C for a further 30 minutes. A maximum of five discs (Oxoid) per plate was placed onto the inoculated surface of the agar plate. Two were the gentamycin containing disc while three were the 6mm blank antimicrobial susceptibility discs (Oxoid). Ten µL of gaharu hydrosol samples was pipette onto the blank discs. The agar plates were incubated overnight at 37°C and the zones of bacterial inhibition were measured and recorded. Each sample was replicated using three agar plates. This procedure is a modification from Hood *et al.*(2003).

Determination of antioxidant activity

The antioxidant activity of gaharu hydrosol was screened using four assays in this study. Two different gaharu hydrosol samples were analysed i.e. sample A is ANM Batch A from Agensi Nuklear Malaysia and sample B is Kedaik B from Kedaik Agarwood Sdn Bhd.

Total phenolic content assay

Analysis for total phenolic content was performed based on the Folin-Ciocalteau method. Gallic acid was used for the calibration curve and a concentration range of 50-200 mg/L was prepared. Results were expressed as mg gallic acid equivalents per mL sample.

Improved ABTS radical cation decolorization assay

Determination of the 2,2'-azinobis-(3-ethyl-benzothiozoline-6-sulfonic acid) radical cation scavenging effect of the gaharu hydrosol samples was done based on the method by Re et al. (1999). $ABTS^{\cdot+}$ was generated by reacting 7 mM $ABTS^{\cdot+}$ solution in water with 2.45 mM potassium persulfate in the dark for 12 hours. The $ABTS^{\cdot+}$ scavenging activities of the samples were measured using a Trolox standard curve and results were expressed as mmol Trolox equivalent antioxidant capacity per mL sample. Quercetin was used as a positive control.

DPPH· radical scavenging activity

Radical scavenging activities by antioxidants in plant extracts were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radicals. The DPPH· radical scavenging activity of the gaharu hydrosol samples was measured using a Trolox standard curve results were expressed as mmol Trolox equivalent antioxidant capacity per mL sample. Ascorbic acid was used as a positive control.

Ferric reducing ability of plasma (FRAP) assay

The ferric reducing activity of the gaharu hydrosol samples was estimated based on the ferric reducing ability of plasma (FRAP) assay detailed by Benzie and Strain (1996), owing to the formation of blue colored Fe(II) tripyridyltriazine compound from colorless oxidised Fe(III) form by the action of electron donating antioxidants. Standard curve was prepared using different concentrations of $FeSO_4.7H_2O$ (100-1000 µmol/L). The ferric reducing activity was estimated by monitoring the change in absorbance in the 0 to 4 minute time reaction and was plotted against the absorbance changes of Fe (II) standard solution. Results were expressed as mmol ferric reducing activity of the samples per mL. Quercetin was used as a positive control.

RESULTS AND DISCUSSION

pH of gaharu hydrosol

The pH of the difference batches of gaharu hydrosol samples were shown in Table 1. All the samples displayed acidic nature with the pH ranging from 3.15 to 4.53. The pH for the different types of hydrosol has been documented by Catty (2001) and it can be concluded that the pH of hydrosol varies according to plant types. The pH of sandalwood hydrosol is in the range of 5.9-6.0. Hydrosol from another softwood tree, the cedarwood exhibits pH ranging between 4.1-4.2 (Catty, 2001).

The acidic nature of hydrosols can be used to make great toners as they can restore the acid mantle to skin after cleansing with soap. This can continue to protect the skin from bacteria that do not live well in acidic condition. Besides this, this alcohol free toner can be an alternative for those who don't want alcohol in their toner, as alcohol strips away too much of the acid mantle and cause pores to overporduce oil in attempt to rebalance the skin. Another advantage of using hydrosol as toner is that it can avoid the exposure to synthetic fragrances in other toner which are potential irritants as hydrosol itself contain natural fragrance (Mare, 2008).

Table 1. The description of the different batches of gaharu hydrosol samples and their initial pH.

Gaharu hydrosol sample	Date of sampling	Description	pH
Kedaik A	29 April 2009	Distillation of wood chip from wild tree. After distillation for 1-3 days, sample was taken non-aseptically and kept in sterile glass bottle.	3.83
Kedaik B	29 April 2009	Distillation of wood chip from wild tree. This sample had been collected 1 month earlier non-aseptically by the factory worker and was kept in plastic bottle. The sample was then transferred aseptically into sterile glass bottle.	3.62
ANM Batch A	23 Jun 2009	Distillation of wood chip from ANM tree, sample was taken aseptically.	3.83
ANM Batch B	8 Julai 2009	Distillation of wood chip from ANM tree, sample was taken aseptically.	3.71
ANM Batch C	6 August 2009	Distillation of wood chip from tree belongs to other, sample was taken aseptically.	4.53
Kedaik A	3 March 2010	Distillation of wood chip from wild tree. This sample was collected non-aseptically and filtered by factory worker and kept in plastic mineral bottle.	3.24
Kedaik G	3 March 2010	Distillation of wood chip from wild tree. This sample was collected non-aseptically and not filtered by factory worker and kept in plastic container.	3.15

Microbial load of gaharu hydrosol

Table 2 shows the bacteria and fungi count for the different batches of gaharu hydrosol. Gaharu hydrosol samples from ANM showed the absence of bacteria and fungi. The aseptic technique used in sampling procedure explained this result. On the other hand, most of the samples from Kedaik Agarwood Sdn Bhd showed the presence of very low bacterial count using plate count agar. As describe in Table 1, all the samples from Kedaik were not sampled aseptically. The source of bacteria could be from the non-sterile container being used as well as from the environment. Thus, it can be concluded that, method of sampling will influence the sterility of the gaharu hydrosol. As for fungi, there was no growth observed on SDA plate. This indicates that all the gaharu hydrosol samples in this study were free from fungi either aseptic technique is used or not in the sampling procedure.

Table 2. The initial microbial load in the different batches of gaharu hydrosol samples. (ND : Not determined)

Gaharu hydrosol Sample	Initial microbial load (cfu/mL)	
	Plate count agar	Sabouraud dextrose agar
Kedaik A	0.7	-
Kedaik B	1.8	-
ANM Batch A	-	-
ANM Batch B	-	-
ANM Batch C	ND	ND
Kedaik A	-	-
Kedaik G	1	-

Antimicrobial activity of gaharu hydrosol

Table 3 shows the antimicrobial activity of the different batches of gaharu hydrosol samples. Both well diffusion and disk diffusion assays did not detect the presence of antimicrobial property in all the gaharu hydrosol samples as no inhibition zone was observed around the well or disk.

Table 3. The antimicrobial activity of the different batches of gaharu hydrosol samples determined by well diffusion assay and disk diffusion assay. (- : no inhibition zone; ND : Not determined)

Gaharu hydrosol sample	Diameter of inhibition zone (mm)			
	Well diffusion assay		Disk diffusion assay	
	S. aureus	*P. aeruginosa*	*S. aureus*	*P. aeruginosa*
Gentamycin	24	34	15	13
Kedaik A	-	-	-	-
Kedaik B	-	-	-	-
ANM Batch A	-	-	-	-
ANM Batch B	-	-	-	-
ANM Batch C	-	-	-	-
Kedaik A	ND	ND	ND	ND
Kedaik G	ND	ND	ND	ND

However, there are several hydrosols that have been documented to have antimicrobial properties but most of them are hydrosols from herbs and spices (Chorianopoulos et al. 2008; Boyraz and Özcan, 2006; Boyraz and Özcan, 2005; Sağdiç, 2003; Sağdiç and Özcan, 2003). It is known that the composition of hydrosols and their antimicrobial effects depend on plant species and regional conditions (Sağdiç and Özcan, 2003). Thus this might explain the results of this study whereby all the different batches of gaharu hydrosol sample did not showed antimicrobial effect on *S. aureus* and *P. aeruginosa.*

Antioxidant activity of gaharu hydrosol

A number of assays have been introduced for the measurement of the total antioxidant activity and each method relates to the generation of a different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time point or over a range (Re et al. 1999). In this study, four assays were used to screen the antioxidant activity of two sources of gaharu hydrosol sample.

Total phenolic content assay can determine the presence of phenolic compounds which in plants are synthesized as secondary metabolites. Phenolic compounds posses several biological properties and one of them is as an antioxidant (Han et al. 2007). Figure 2 shows that total phenolic content in sample B was higher than sample A but both sample showed lower total phenolic content as compared to the positive control. Thus this indicates that both gaharu hydrosol samples contained antioxidant properties due to the presence of phenolic compounds.

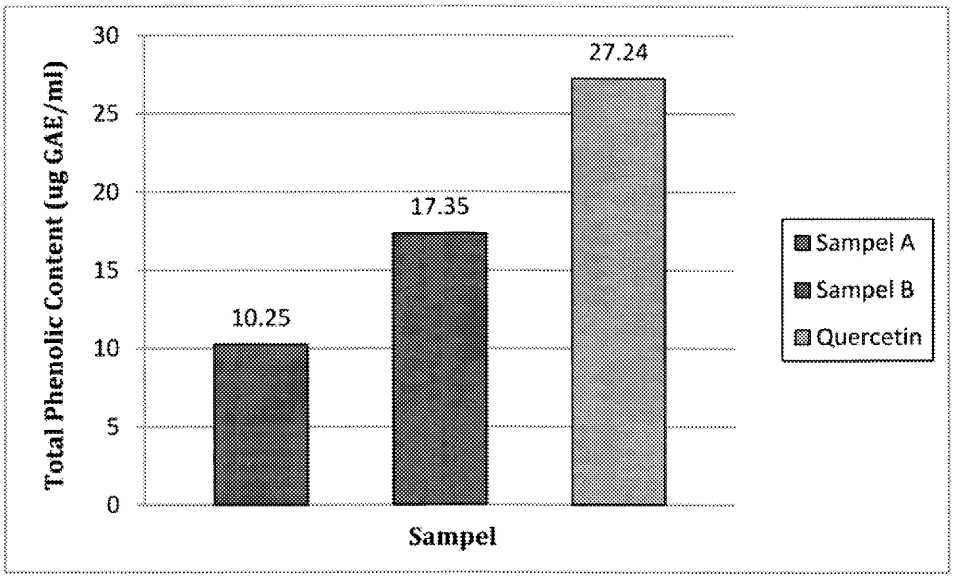

Fig. 2. Total phenolic content of gaharu hydrosol sample A and sample B as compared to positive control.

Improved ABTS radical cation decolorization assay is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds and food extracts. This assay gives a measure of the antioxidant activity, determined by the decolorization of the $ABTS^{\cdot+}$, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm (Re et al. 1999). In this study, sample A exhibited a slightly higher antioxidant activity than sample B but both show very much lower antioxidant capability than positive control (Fig. 3).

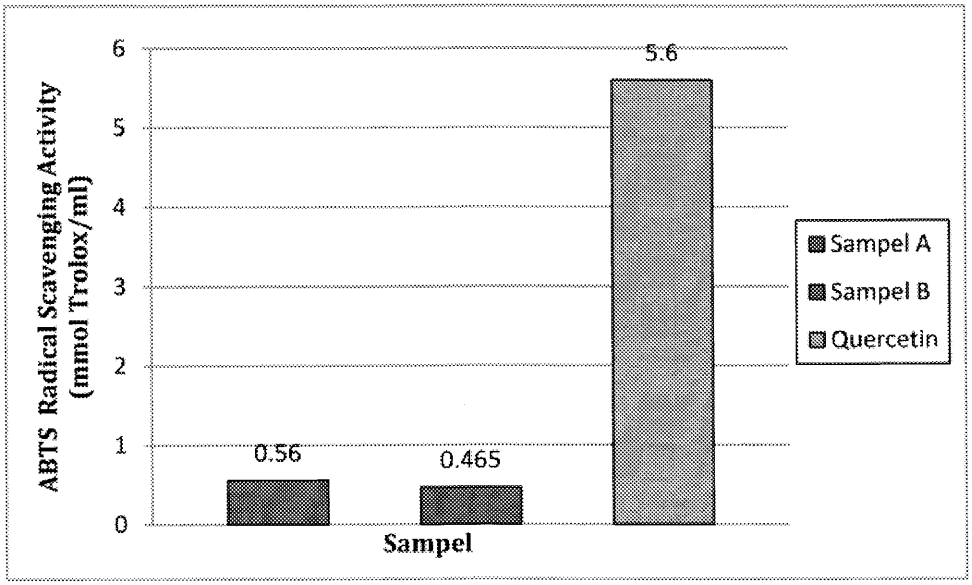

Fig. 3. ABTS radical scavenging activity of gaharu hydrosol sample A and sample B as compared to positive control.

DPPH radical scavenging activity by antioxidants in gaharu hydrosol sample A and sample B was shown in Fig. 4. Both the hydrosol samples showed very low DPPH inhibition than positive control.

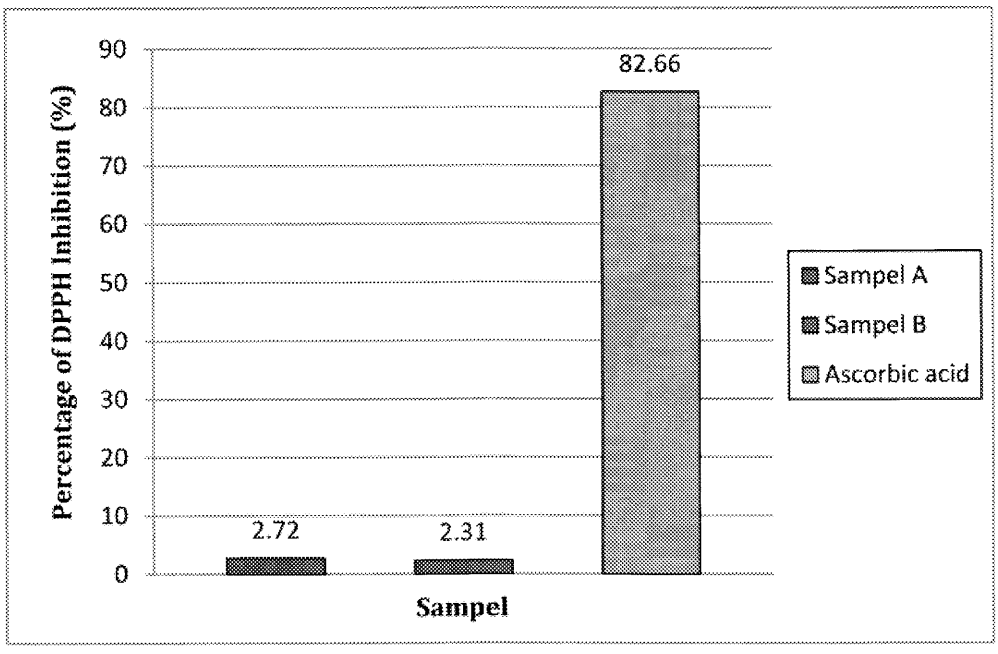

Fig. 4. Percentage of DPPH inhibition of gaharu hydrosol sample A and sample B as compared to positive control.

From FRAP assay, further indication of the antioxidant capability of gaharu hydrosol sample A and sample B can be obtained. As shown in Fig. 5, it clearly shows that both sample A and sample B posed ferric reducing activity lower than positive control. Sample A showed slightly higher ferric reducing activity than sample B.

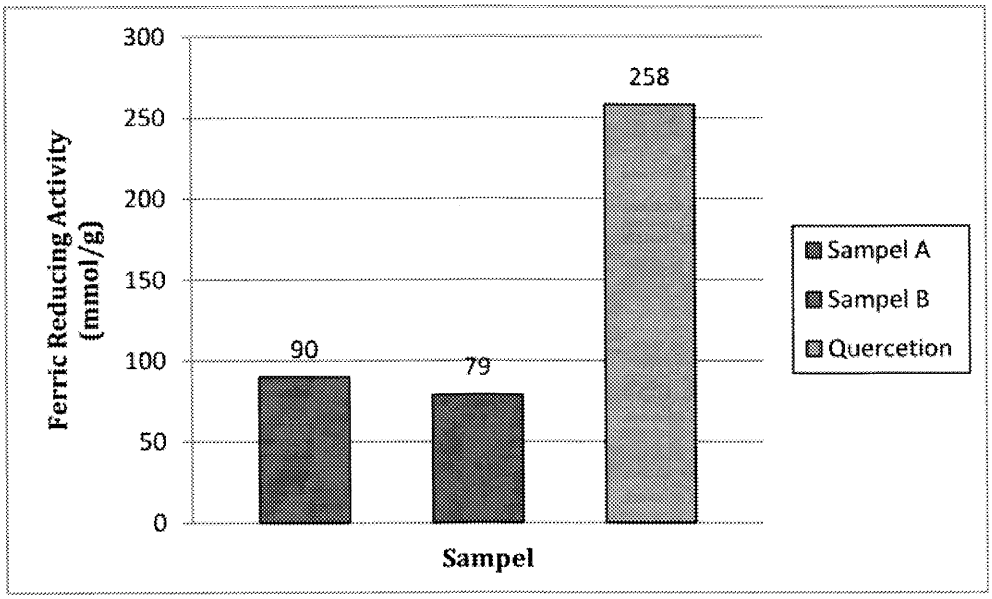

Fig. 5. Ferric reducing activity of gaharu hydrosol sample A and sample B as compared to positive control.

Based on the results from all the antioxidant analysis, it can be concluded that both gaharu hydrosol from Kedaik Agarwood Sdn Bhd extraction factory (sample B) and Agensi Nuklear Malaysia extraction facility (sample A) showed much lower antioxidant activity than positive control. Sample A which is ANM Batch A from Agensi Nuklear Malaysia showed slightly higher antioxidant activity in all the assays except the assay on total phenolic content. The higher antioxidant activity in sample A could be due to the tree oleoresin inducement treatment prior to harvest for sample A while sample B came from wild tree which is not treated. The treatment might have changed the properties or proportion of the antioxidant compounds in the plant, particularly the wood part.

CONCLUSION

Based on the results from this initial investigation study, some of the physical, chemical and microbiological properties of gaharu hydrosol are obtained. The results showed that gaharu hydrosol are acidic in nature, having the pH ranging from 3.15 to 4.53, particularly for the samples used in this study. In terms of microbial load, fungi was found absence while bacteria were found presence in very low number. The presence of bacteria was influenced by the technique used during sampling, either aseptic technique being applied or not. The gaharu hydrosol used in this study did not showed antimicrobial effect on *S. aureus* and *P. aeruginosa* in both the well diffusion and disk diffusion assays. Lastly, it is found that all the gaharu hydrosol samples in this study exhibited very low antioxidant activity compared to the positive control. The results obtained from this study will be further evaluated for the development of new products using this aromatic gaharu by-product.

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