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Research Article

The Characterization of Phytochemical and GC-MS Analysis on Borneo Agarwood (*Aquilaria malaccensis* Lamk) Leaves and Its Utilization as an Anti- Browning in Apple Juice

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ABSTRACT
In consideration of the sustainability of agarwood production and its plants, besides resin of agarwood infected, others alternatives are use for agarwood derivative products i.e., agarwood leaves for pharmaceutical industries. The present study aimed to utilized agarwood leaves as a supplementation for anti-browning in apple juice. Here is also evaluated the presence of different phytochemical along with GC-MS investigations of water and ethanol soluble crude extracts, anti-oxidant, and organoleptic obtained from young and mature leaves of Borneo Agarwood (*A. malaccensis*). The results showed that *A. malaccensis* leaf extracts revealed presence of alkaloids and carbohydrate. GC-MS analysis of organic compound of Borneo Agarwood leaves identified that the most organic compound of young leaf (DM) is established ketones and benzoid compound, while nitrogen and benzoid compound also for mature leaf (DT). Antioxidant activity for all types of Borneo Agarwood leaves extract with hot water extraction showed a higher percentage than its ethanol, especially DM. DM solution was also best solution as an inhibition of browning in apple juices, as well as an essence for its. Thus, the present study has proved the usefulness of *A. malaccensis* for potential sources of active drugs.

Keywords: *Aquilaria malaccensis,* phytochemicals, GC-MS, anti oxidant, organoleptic

INTRODUCTION

The increasing number of publications on non-timber forest products (NTFPs) over the past decade indicates growing interest in the management of forest lands for a wider range of outputs than timber only. As many NTFPs, *Aquilaria* spp. (Thymelaeaceae) has been an important source of cash income and means of access to foreign goods. Agarwood products are the most valuable products in the world with higher prices and demands nowadays. The main markets for these products are in South, East Asia, and the Middle East[1]. *Aquilaria* spp. are locally known as agarwood, aloeswood, eaglewood, gaharu, kalamabak or oudh depending on the region[2]. Most of Thymelaeaceae family including *Aquilaria*, *Gonystilus*, and *Gyrinops* are agarwood production sources. Its having been listed in Appendix II of the Convention on Internal Trade in Endangered Species of Wild Fauna and Flora[3]. Therefore, to supply the demand increasing of agarwood, its cultivation efforts have been undertaken due to conservation program promoted for the sustainability of agarwood production. Thus, mainly agarwood resin produced from cultivation plants, not from nature[4]. In consideration of the sustainability of agarwood production and its plants, besides resin of agarwood infected, others alternatives are use for agarwood derivative products i.e., agarwood leaves for pharmaceutical industries. It has been widely identified as an important and excellent source of pharmaceutical products. Many parts of this plant, including leaves, skin, seeds, wood and roots are valuable in medicinal properties[5]. Utilization of agarwood leaves has very advantageous due to it can be available in a short time period (short cycle) compared with agarwood resin. In the field, it was indicated that the leaves harvested could be grew back normally within 3-4 months depending on habitat conditions. Aquilaria species (including A. *malaccensis*) have adapted to live in various habitats, including those that are rocky, sandy or calcareous, well-drained slopes and ridges and land mear swamps[6]. The most commonly utilization of leaves as a tea product or other beverages such as juices, i.e., apple juice. However, during pre-processing, fruits and vegetables have tendency to brown due to cutting, washing, bruising or other mechanical injury causing discruption of the cellular organisation[7]. Other study[8] showed that browning is responsible for degrading the color quality of apple juice since polyphenoloxidase in apple is highly active. Hence,

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the present study aimed to utilized agarwood leaves as a supplementation for anti-browning in apple juice. Here is also evaluated the presence of different phytochemical along with GC-MS investigations of water and ethanol soluble crude extracts and anti-oxidant analysis obtained from young and mature leaves of Borneo Agarwood (A. *malaccensis*).

MATERIALS AND METHODS

Sample Collection and Preparation

Two types of fresh leaves of Borneo Agarwood (*Aquilaria malaccensis*) were collected from Labanan Village (around Labanan Forest Research), Berau District, East Kalimantan, Indonesia (N $2^0$03.456' and E $117^0$18.644'). One was young leaves (DM) and the other was mature leaves (DT). Both of leaves were dried in Air Conditioner (AC) room for 3 days at 25 $\pm$ 2^0C and ground to a fine powder using crusher and sieved through 40 - 60 mesh. The powder samples were kept at AC room in a covered glass containers to protect them from humidity and light prior to extraction. Then these samples were prepared for further analysis.

Extraction of Borneo Agarwood Leaves Extracts

Maceration

5g dried powder leaves of each Boneo Agarwood (DM and DT) types were exhaustively extracted by maceration in 200ml ethanolic solvent for 24 hours at room temperature (28 $\pm$ 2^0C). Whereas, each extraction was concentrated from 200ml into 10ml concentrated crude ethanolic extracts, dried in oven at 50^0C to give dark green extracts. Further, these extracts were used for phytochemical and anti oxidant analysis (5ml), and GC-MS analysis (5ml).

Hot Water

5g dried powder leaves of each Boneo Agarwood (DM and DT) types were exhaustively extracted by 200ml H_2O for 2 hours at 100^0C. These extracts were divided into 2 parts, 100ml per each. 100ml was concentrated into 5ml concentrated crude ethanolic extracts for phytochemical and anti oxidant analysis. 100ml was used as additional solution for anti browning test.

Preliminary Phytochemical Analysis

Extracts were tested for the presence of active principles such as flavonoids, saponins, steroids, tannins, terpenoids, alkaloids and carbohydrate by using some following standard procedures[9,10]

Flavonoids Determination

About 1 ml of ethanolic extract was shaken with 1 ml of dilute ammonia solution. The layers were allowed to separate and the yellow color in the ammonical layer (bottom layer) indicates the presence of flavonoids.

Saponins Determination

5 ml of the filtrate was diluted with 20 ml of water and shaken vigorously (15 minutes). A stable froth (foam) up on standing indicates the presence of saponins.

Steroids Determination

1ml of ethanolic extract of each sample is boiled with 10ml chloroform, cooled, 1 to 2 drops of concentrated sulfuric acid were added slowly through the wall of the tube. Shake well and allow standing for some time, red color appears at the lower layer indicates the presence of Steroids.

Tannins Determination

Test solution (5ml ethanolic extract) with sodium hydroxide solution (1%) gives yellow to red precipitate within short time indicates of the presence of tannins.

Triterpenoid Determination

1ml of ethanolic extract of each sample is boiled with 10ml chloroform, cooled, 1 to 2 drops of concentrated sulfuric acid were added slowly through the wall of the tube. Shake well and allow standing for some time, reddish purple color appears at the lower layer indicates the presence of Triterpenoids.

Alkaloids Determination

5ml ethanolic extract was reacted with 2 drops Potassium bismuth iodide solution reagents in test-tubes. Development of creamy and an orange color respectively indicated positive result.

Carbohydrate Determination

Extract hydrolyzed with HCl in the water heater. Then, it was added with 1ml of pyridine and a few drops of a solution of sodium nitroprusside into the hydrolyzate, after it was etched with an alkaline solution of sodium hydroxide. The formation of a pink to red color indicates the presence of glycosides.

Antioxidant Assay

In this test used 100% of 5 concentrated sample with 50, 100, 200, and 400 times of dilution, respectively. Further, 1mg of vitamin C was weighed, then dissolved in 5000 µl of distilled water and regarded as a positive control. While, negative control was used its solvent (distilled water). 100 µl sample was mixed in cuvette with 400 µl of distilled water was added, and 500 µl of 2,2-diphenyl-1-picrylhydrazy (DPPH) radical scavenging activity. Mixing was stopped when the sample volume has reached 1000 µl (1ml). Samples were incubated for 20 minutes in indoor with minimum light. Antioxidant activity was determined by decolorization of DPPH with a wavelength of 517 nm using a spectrophotometer. The scavenging activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following equation: Free-radical scavenging activity (%) = A (blank) - A (extract) / A (blank) x 100

Gas Chromatography-Mass Spectrometry Analysis (GC-MS Analysis)

Gas Chromatography–Mass Spectrometry (GC–MS) analysis was carried out for the all ethanolic extracts. The analysis was performed according to the GC-MS equipments by Shimadzu QP 2010: RTX - column type is 5ms, Restek Corp (30 m length). The injector and detector temperatures were both maintained at 250^0C, while operation temperature at 50-300^0C. The column temperature was programmed at 50-120^0C, with 4^0C increase per min which was maintained for 1 min. Then it was programmed at 120-300^0C, with 6^0C increase per min and held on for 5 min, with retention time (Rt) totaled 60 min. Helium was used as a carrier gas is 50-500 atomic mass unit (amu). The compounds of eachs extracts were identified by using computer searchers in commercial libraries of Wiley.

Anti Browning Analysis

Watery apple juice was made using 50g apple demolished along with 200ml water. The solution was then poured into a glass container prior to use. While, 2, 4, 8ml of hot water extracted for each type of leaves separately and prepared as additional solution for anti browning analysis. Observations were made by recording time of color change of Borneo Agarwood Leaves solution mixed with apple juice, until the color coincides with pure solution of apple juice (control). The above mentioned processes were done, then organoleptic tests (taste and odor) were conducted to determine if substances used for pharmaceutical products.

RESULTS AND DISCUSSIONS

Preliminary Phytochemical Analysis of the Borneo Agarwood Leaves Ethanolic Crude Extracts

The presence of phytochemicals indicates its potential as a source of useful corrosion inhibitors. Factors affecting capture of active phytochemicals are the plant part being extracted, the types of solvents, the extraction period and extraction conditions[11,12]. Therefore, ethanol was chosen in this study as the organic solvent for its wide solubility properties for low molecular weight and moderately polar substances, including phenolic compounds. The qualitative screening of phytochemical compounds in Borneo Agarwood Leaves ethanolic extracts revealed the presence of alkaloids, flavanoids, saponins, tannins, triterpenoids, steroids and carbohydrate were shown in Table 1. Table 1 shown that all types of Borneo Agarwood Leaves extracted by maceration and hot water were indicated presence of alkaloids and carbohydrates. Recent study[13] have detected of alkoloids in Agarwood leaves extract for the traditional use for various health problems. Presence of alkaloids in Agarwood leaves extracts was of great justify for plant as some pharmaceutical uses, such as antimalaria, analgesic, antispasmodic, bactericidal, simultans and others**.** Further, present trends towards technologies and processes that increase the use of residues make carbohydrate (starchy) vegetal biomass an important alternative material in various applications. Starch is used as an excipient, a type of bonding agent to active drugs in the pharmaceutical industry[14].

Antioxidant Assay

Antioxidants play a major role in helping to protect our body from the formation of free radicals and prevent or delay the occurrence of lipid peroxidation[15]. Its have been able to destroy a single oxygen molecule and neutralize chemically active products of metabolism in order to protecting oxidative damage to cells, which cause several diseases such as cancer, ageing and diabetes[16,17]. Antioxidant activity of Borneo Agarwood Leaves was assessed by determining the percentage of inhibition of DPPH (Table 2). Generally, antioxidant activity for all types of Borneo Agarwood leaves extract with hot water extraction (400x dilution) showed a higher percentage than its ethanol. It is mainly due to the fact that boiling water could completely activate the degradative enzymes as against the ethanol solvent. The number of compounds with free hydroxyl groups was increased by exposure the plant materials at high temperature during extraction process[18,19]. Meanwhile, DM_HW extract (86.97%) was higher than IC_{25} (78.49%) indicated scavenging potential affected by phenolic compound on DPPH which able to donate hydrogen atoms to form stable[20].

Gas Chromatography-Mass Spectrometry Analysis (GC-MS Analysis)

Borneo Agarwood Young Leaf Extract (DM)

The chemical composistion of Borneo Agarwood young leaf extract (DM) identified by GC-MS is presented in Table 3. Most of the compound identified in DM is established aromatic or anti-microbial compound. It can be seen that ketones was indicated in 3 - Buten - 2 -one , 4- (2 - furanyl) compound amounted to 28.88 % and is the highest component of chemical compounds in DM. Ketones is one of aromatic compound in the nature and it could be a potential source of bioactive compounds. It can process a wide array of application in food, phamaceutical, and cosmetic industries either as anti-microbial agents, natural flavouring agent or as key ingredients in skin care and cosmetic products[21-24]. Meanwhile, other compound was identified in DM is a benzoid compound that it was indicated in 22.93% of 1-Phenanthrenol,1,2,3,4,4a,9,10,10a-octahydro-4a-methyl-, (1.alpha.,4a.alpha.,10a.alpha.). This compound would be beneficial for the anti-glucocorticoid theraphy, and it was indicated from activity of conscious rats and their ability, as an anti-diabetic and anti-obesity potential[25].

Borneo Agarwood Mature Leaf (DT)

Table 4 shown that the most chemical compound of Borneo Agarwood mature leaf extract (DT) is Methylpyridazine (31.47%). This nitrogen compound used widely in pharmaceutical and agricultural industries. [26]stated that such as compound was observed as an useful precursor to agrochemicals and antidotes for organophosphate poisoning. Furthermore, the obtained biological activity data indicated that some of the synthesized compounds were highly active antidotes to herbicides, as well as weak anti-microbial activity againts E.*coli* and St.*aureus*[26]. On the other hand, 24.22% of benzenoid compound in 1,3,5-Triazin-2(1H)-one, 4-(ethylamino)-6-[(1-methylethyl)amino]- was identified.

Table 1: Summary of the phytochemical screening of Borneo Agarwood leaves (DM and DT) by maceration and hot water extractions.

Extraction	Extract	Alkaloid	Flavanoid	Saponin	Tannin	noid	Steroid	Carbohydrate
Maseration	DM_MS	+	-	-	-	-	-	+
	DT_MS	+	-	-	-	-	-	+
Hot Water	DM_HW	+	-	-	-	-	-	+
	DT_HW	+	-	-	-	-	-	+

+ : Positive result (Presence of the phytochemical)

-: Negative result

Table 2: The Reducing power of ethanol and water extracts of Borneo Agarwood leaves extract (DM and DT).

Sample	Reducing antioxidant capacity of DPPH (%)				
	100% (concentrated)	50x Dilution	100x Dilution	200x Dilution	400x Dilution
DM_MS	-	51.56	71.74	80.16	84.43
DT_MS	-	-	-	34.49	59.63
DM_HW	65.28	77.16	83.04	85.93	86.97
DT_HW	59.05	72.32	80.16	83.16	85.70

Vitamin C: 100 ppm = 90.50%, 50 ppm = 89.92%, 25 ppm = 78.49%

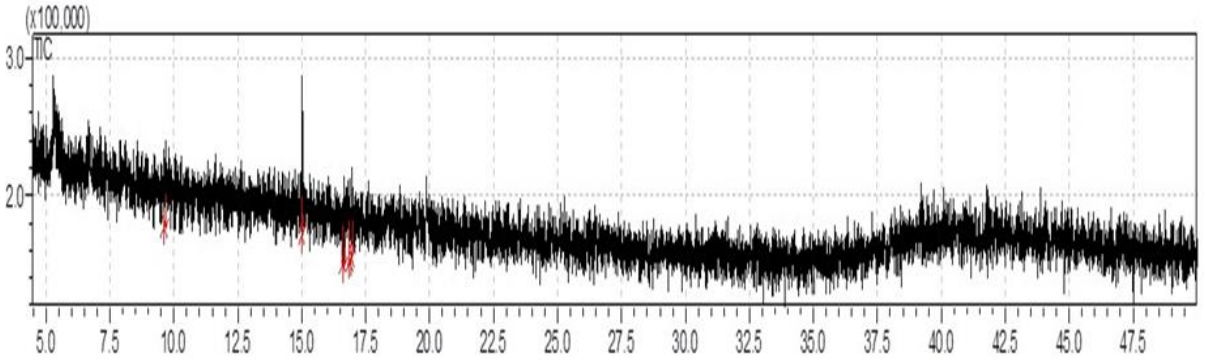


Figure 1: GC-MS Analysis of Borneo Agarwood young leaf extract (DM).

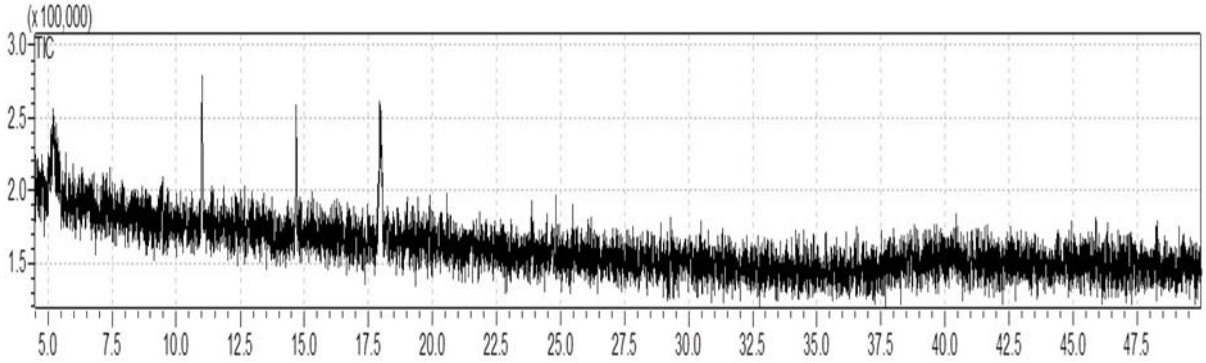


Figure 2: GC-MS Analysis of Borneo Agarwood mature leaf extract (DM).

Its have been developed for herbicides in agricultural industries as an inhibitor of photosynthesis in plants, as weel as anti-bacterial activity in pharmaceutical industries[27].

Anti Browning Test

Browning is a major problem for various fruit products due to lowering of quality, safety, and nutritional value. In this study, browning is responsible for degrading the color quality of apple juice since polyphenoloxidase in apple is highly active[8]. Generally, additional of Borneo Agarwood leaves solution can inhibit browning of apple juice along with the calculation of time addition shown in Table 5. The increasing of additional of Borneo Agarwood leaves solution can increase the brightness of apple juice. Other study by[8] determined that the increasing of onion addition in apple juice represented more brightness of apple juice.

Organoleptic Test

Organoleptic properties are the aspects of food, water or other substances that an individual experiences via the senses, including taste, sight, smell, and touch[28,29]. The organoleptic properties of Borneo Agarwood leaves solution including taste, and smell are shown in Table 6. In Table 6, it can be seen that DM especially with 2ml concentration was the most favourite due to it is the most tasty (sweet) and smelling fresh. According to the results of GC - MS analysis, DM extracts obtained D - Glucitol , 1 - deoxy -1- (octylamino), which was indicated as an anti-diabetic and anti-obesity potential[25].

CONCLUSSION

The antioxidant activity correlated with active compounds phytochemicals. Ethanol and water extracts of fresh leaves showed that it may account for its antioxidant in Borneo Agarwood (A. *malaccensis* Hull). Generally, all of types of Borneo Agarwood leaves (DM and DT) extracted with hot water were indicated higher in antioxidant activity than ethanol. Meanwhile, phytochemicals screening shown that all of leaf types extracted with both of ethanol and water extract were indicated presence of alkoloids and carbohydrate. The phytochemical constituents of the leaf extracts was established that the extracts used in this study also contain a mixture of organic compounds containing O, N or π-electrons in their molecules. GC-MS analysis of organic compound of Borneo Agarwood leaves identified that the most organic compound of DM is established

Table 3: Results of the GC-MS analysis of Borneo Agarwood leaf extract (DM).

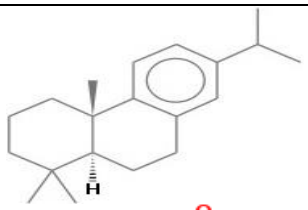
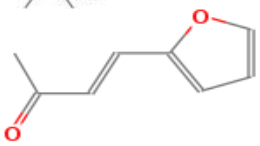
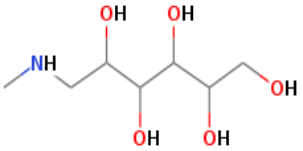
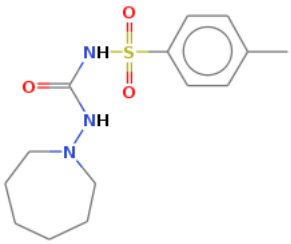
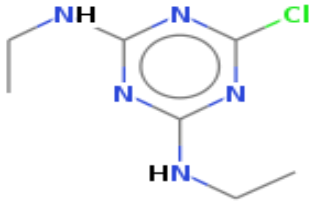
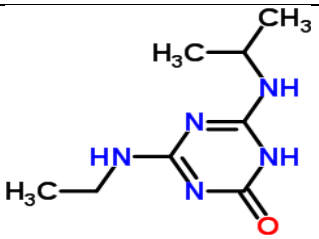
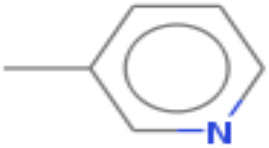
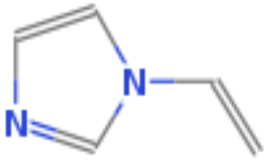
Retention Time (min)	Percent of total (%)	Compound	Structure
9,685	22,93	1-Phenanthrenol, 1,2,3,4,4a,9,10,10a-octahydro-4a-methyl-, (1.alpha.,4a.alpha.,10a.alpha.)-	
15,010	28,88	3-Buten-2-one, 4-(2-furanyl)-	
16,670	19,55	D-Glucitol, 1-deoxy-1-(octylamino)-	
16,865	16,25	Tolazamide	
16,965	12,39	1,3,5-Triazine-2,4-diamine, 6-chloro-N,N'-diethyl-	

Table 4: Results of the GC-MS analysis of Borneo Agarwood leaf extract (DT).

Retention Time (min)	Percent of total (%)	Compound	Structure
5,010	24,22	1,3,5-Triazin-2(1H)-one, 4-(ethylamino)-6-[(1-methylethyl)amino]-	
5,165	31,47	3-Methylpyridazine	
5,330	1,78	N-Vinylimidazole	

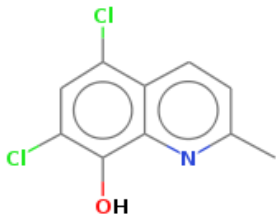
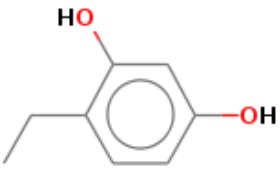
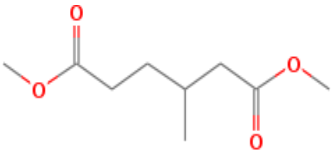
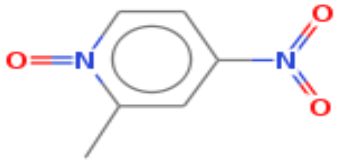
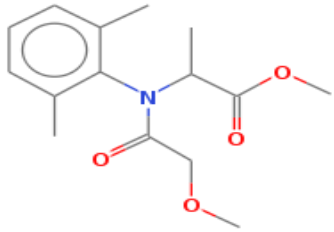
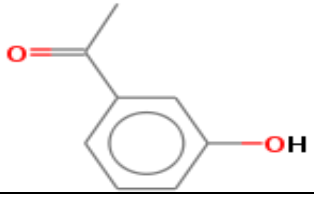
7,238	3,21	Chlorquinaldol	
11,009	19,78	1,3-Benzenediol, 4,4'-thiobis-	
11,050	3,19	Hexanedioic acid, 3-methyl-, dimethyl ester	
11,355	2,68	4-Nitro-2-picoline-N-oxide	
11,430	0,88	DL-Alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, methyl ester	
14,681	12,79	Ethanone, 1-(3-hydroxyphenyl)-	

Table 5: Anti browning test of Borneo Agarwood leaves solution (as a supplementation in apple juice).

Solution	Added Solution (ml)	Time of color change (min)
Control (Pure Aplle Juice)	-	04’00”
DM	2	09’19”
	4	12’75”
	8	18’39”
DT	2	06’01”
	4	06’49”
	8	11’05”

Table 6: Organoleptic test of Borneo Agarwood leaves solution.

Solution	Added Solution (ml)	Taste (%)	Smell (%)	Favourite /Savour (Rank 1-6)
DM	2	90	100	1
	4	90	100	2
	8	20	50	5
DT	2	90	90	3
	4	80	60	4
	8	0	0	6

ketones and benzoid compound, while nitrogen and benzoid compound also for DT. Consistenly, in our study, apple juices supplemented with Borneo Agarwood leaves solution showed inhibition of browning in apple juices, especially with more additional DM solution. The organoleptic properties were important visual characteristics which is a requirement for its utilization as a medicinal plants. As a medicinal plants, Borneo Agarwood leaf extract has an essence, tasty and smelling fresh due to it was obtained aromatic compound as an anti-diabetic and anti-obesity.

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