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Research Article

The effect of different sweeteners on the free radical scavenging activities, alcohol contents, sugar reductions, and hedonic properties of green tea kombucha

Muhammad Shidiq Rukman[1], Ayu Nala El Muna Haerussana[1,2*]

[1] Department of Pharmacy, Poltekkes Kemenkes Bandung, Bandung, Indonesia
[2] Center of Excellence on Utilization of Local Material for Health Improvement, Poltekkes Kemenkes Bandung, Indonesia

ABSTRACT

Green tea (*Camellia sinensis*) consumption has increased due to the compounds in green tea that have health benefits. Fermenting brewed green tea with a Symbiotic Culture of Bacteria and Yeast (SCOBY) to create green tea kombucha is a suitable strategy to raise that benefit. Free-radical scavenging activity, alcohol level, sugar reduction, and the hedonic test (color, aroma, and flavor hedonic test) were used to determine the quality of kombucha. This study determined how various sweeteners influence that parameter using DPPH (2,2-diphenyl-1-picrylhydrazyl) method to determine free-radical scavenging activity; distillation, and specific weight to determine alcohol level; refractometer to determine sugar reduction content; and scoring preference from 30 panelists to determined hedonic. This study revealed that the IC_{50} value of kombucha ranged between 33.44 and 38.85 ppm. The honey yielded the highest alcohol content, 2.78%, while stevia yielded the lowest, 2.36%. The range of reducing sugar content is 18.99 to 88.90 grams. The color, aroma, and flavor of green tea kombucha are enhanced by adding stevia, honey, palm sugar, and refined sugar, respectively. Our findings showed that green tea kombucha had very strong radical scavenging activity in various sweeteners. Stevia had the highest radical scavenging activity and was deemed the best by the panel. Stevia also showed the lowest levels of alcohol and sugar reduction. However, additional other sweeteners must be investigated to determine the health benefits of kombucha.

Keywords:
Kombucha, Stevia sweetener, Honey, Sugar, DPPH test, IC_{50}, Hedonic test

1. INTRODUCTION

Tea is the oldest beverage with a history spanning multiple cultures. Chinese legend asserts that tea has been consumed since 3,000 years ago[1], but archaeological research indicates that tea was first consumed around 5,000 years ago, during the early paleolithic period[2]. In addition to being the oldest beverage, tea is the second most consumed beverage in the world, behind mineral water[3]. By 2025, global consumption and production of tea are projected to increase by 3.08%, reaching 7,000 metric tons[4].

Previous studies indicated that the rise was influenced by public awareness of the health benefits of tea consumption[5]. Tea plays a role as an anti-inflammatory, antioxidant, anti-diabetic, cardiovascular disease risk reducer, and weight loss aid[6-7]. These advantages are due to the polyphenolic compounds found in tea, such as epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). EC, EGC, ECG, and EGCG substances have become anti-radical antioxidants[7].

The fermentation process by bacteria and yeast can potentially increase polyphenol levels[8-9]. According to Oyzurt green tea kombucha has a total phenol 472.09± 4.94 mM GAE, while green tea infuse contains 326.5± 52.68 mM GAE[10]. An increase in polyphenol compounds is associated with increased antioxidant activity[11-12].

***Corresponding author:**
*Ayu Nala El Muna Haerussana Email: ayunalael_farmasi@staff.poltekkesbandung.ac.id

During fermentation, bacteria and yeast can produce enzymes that break down complex polyphenols into less complex polyphenols[13]. In kombucha fermentation, polyphenols such as gallic acid increased with fermentation time, gallocatechin gallate increased up to the ninth day of fermentation, and epicatechin to the sixth day of fermentation whereas caffeine, an alkaloid, decreased with fermentation time[14]. Polyphenols can form stable phenoxy radicals by accepting an electron in oxidation reactions, such as the gallate moiety, which is esterified at the 3 positions of the C ring, disrupting chain oxidation reactions in cellular components[15].

Kombucha is produced by fermenting brewed tea with Symbiotic Culture of Bacteria and Yeast (SCOBY). Several species from the genera *Saccharomyces*, *Brettanomyces*, *Zygosaccharomyces*, and *Pichia* comprised the yeast group, which bacteria consisted of acetic acid bacteria[13,16]. The yeast invertase enzyme hydrolyzed sucrose and converted it to glucose-fructose. Fructose was converted into various organic acids, while glucose was converted back into gluconic acid and glucuronic acids by bacteria[6,16]. As a result, gluconic acid and other organic acids depend on the amounts of glucose and fructose found in each type of sugar. Yeast also produces alcohol during fermentation in the glycolysis cycle, which is the excess alcohol then oxidized by bacteria to produce various organic acids, including acetic acid and gluconic acid[15]. Depending on the carbon source (sugar) employed, the amount of alcohol that remains after fermentation may vary[17].

Yeast hydrolyzed carbon sources into simple sugars with the aid of invertase enzymes. After that, yeast and bacteria degrade the simple sugars[18]. SCOBY microorganisms' ability to degrade depends on the sweetener used as a carbon source, which has different saccharides. The remaining simple sugar in kombucha is calculated as sugar reduction, which not processes further during kombucha fermentation[19]. Kombucha's quality is also determined by its acceptance of color, aroma, and flavor[20]. Therefore, the effect of different sweeteners on free radical scavenging activity, alcohol content, sugar reduction, and the hedonic test of kombucha should be evaluated.

2. MATERIALS AND METHODS

2.1. Materials

Green tea leaves (*Camellia sinensis* L. Kuntze or *Camellia chinensis* (Sims) Kuntze) from the Gambung Tea and Quinine Research Center (PPTK), West Java Province, Indonesia, were used in this study. The determination was made at the Plant Taxonomy Laboratory of Pajajaran University, Indonesia, with the identification number 21/HB/06/2022. Kombucha starter consisted of sour broth and cellulosic layer floating on the liquid surface (SCOBY) from Wikikombucha Indonesia. The sweetener used were refined sugar (Gulaku), low-calorie sugar/stevia (Tropicana slim stevia; component: sorbitol, erythritol, and steviol glycoside), honey (Pure TJ Honey) and palm sugar (Palmsuiker). The materials used were DPPH (2,2-Diphenyl-1-Picrylhydrazyl), quercetin, ethanol (Merck®), methanol (Merck®), aquadest and water for brewing. The instruments used were spectrophotometer UV-Vis (Shimadzu®), analytical balance (Mettler Toledo®), distillation set, pycnometer, refractometer, and glassware.

2.2. Green tea fermentation

The green tea brewing process conforms to the Indonesian National Standard (SNI) 3945:2016 for green tea. Green tea kombucha was produced by infusing 0.8% green tea (8.0 g/L) with 10.0% (100.0 g/L) sweeteners (refined sugar, stevia, honey, and palm sugar) up to 1 liter in hot aquadest (90-100°C). The brewed green tea is then gradually cooled to 25-30°C before being filtered through a tea filter into a clean glass jar. Fermentation is accomplished by adding kombucha culture (200 mL sour broth and one cellulosic layer) aseptically into brewed green tea for five days at room temperature in the dark. The jar was carefully covered with a clean cloth and fastened properly. The kombucha obtained was filtered and analyzed in triplicate[12].

2.3. Free radical-scavenging activity

The maximum wavelength, sample, and standard solution were prepared using Kusmiyati and Sudaryat's (2015) methods with slight modifications[21]. The DPPH radical activity was determined using the spectrophotometric method and the synthetic radical DPPH (2,2-Diphenyl-1-picrylhydrazyl). The absorbance was measured in triples at its maximum wavelength (515 nm). The measurements were expressed as a percentage of DPPH radical inhibition by samples, and the free-radical scavenging activity of the test solution was expressed as a percentage of DPPH inhibition using the following formula[12,22]:

$$\% \text{ inhibition} = \frac{A0 - As}{A0} \times 100$$

Note:
A0 = absorbance of DPPH solution without test sample
As = absorbance of DPPH solution using a test sample

The DPPH-scavenging activity can be calculated by dividing the percentage inhibition of DPPH by the IC_{50} of each sample. Then, the % inhibition value against DPPH was calculated using Microsoft Excel, and then to determine the IC_{50}, a linear regression was performed between the percent inhibition and the concentration of kombucha, yielding the equation $y = bx+a$. The IC_{50} is calculated using the formula $IC_{50}(x) = (50-a)/b$(22).

2.4. Alcohol content

The alcohol content was determined using the distillation method. First, 25 mL of kombucha was pipetted into a distillation flask, and the temperature at the pipetting was described. Next, 25 mL of distilled water was added to the flask. The process was repeated until the distillate was approximately 2 mL smaller than the volume of the test liquid. The distillate was heated to the same temperature as the boiling point of the alcohol (78°C). Water was added until the volume equaled the test liquid (the liquid's specific gravity was 25°C). The percentage in alcohol volume was calculated using a specific gravity and alcohol content table[23].

The distillate was added until the pycnometer was completely filled, and the excess distillate at the top of the capillary tube was cleaned. Then, the weight of the pycnometer containing the distillate was recorded. As a comparison, the same treatment was performed on aquadest. Alcohol's specific gravity was calculated as follows:

$$\text{Destilate specific gravity} = \frac{\text{Destilate mass}}{\text{Aquadest mass}}$$

$$\text{Alcohol specific gravityin sample} = \frac{\text{Destilate mass}}{\text{Aquadest mass}} \times \text{Aquadest specific density (25°C)}$$

Which,

$$\text{Aquadest mass} = (\text{pycnometer} + \text{aquadest mass}) - \text{empty pycnometer (dry)}$$

$$\text{Destilate mass} = (\text{pycnometer} + \text{destilate mass}) - \text{empty pycnometer (dry)}$$

Then, the specific gravity obtained was compared to the predicted alcohol content in the alcoholometric tables listed in the Indonesian Pharmacopeia, and the alcohol content (v/v or w/w) was calculated using the formula below:[23]

$$\text{Alcohol content} = \text{lower limit percent} + \frac{\text{lower limit different}}{\text{upper limit different}}$$

2.5. Sugar reduction content

The Brix refractometer was used to measure sugar reduction levels in kombucha, which were determined by measuring the concentration of dissolved solids. The Brix refractometer was proportional to the solids concentration in the solution. The more solids a solution contains, the more concentrated it becomes. In addition, the movement of light in the solution was increasingly impeded (the speed of light in the solution decreases), indicating that the concentration of solids in the solution increases as the solid molecules become more densely packed together. Degassed kombucha was dripped on a refractometer prism glass, producing measurement results in units of %Brix. The %Brix value-the number of dissolved solids (grams) in every 100 grams of the solution-on the refractometer will show a different scale as the sugar content increases[23]. To calculate the sugar content of kombucha, first, determine the specific gravity of the tea, then use the following formula:

$$\text{Sample mass} = \text{sample specific gravity} \times \text{sample volume}$$

$$\text{Reducing sugar in sample} = \frac{\text{sample mass} \times \%\text{Brix}}{100}$$

2.6. Hedonic test

Thirty panelists carried out the kombucha hedonic test. Approval number 73/KEPK/EC/III/2022 declared it ethically appropriate by Health Research Ethics Committee Politeknik Kesehatan Kementerian Kesehatan Bandung. Each sample is served in a different glass coded. The panelists conducted sensory tests on the samples' color, aroma, and flavor and then recorded their responses as an assessment of the questionnaire provided. This organoleptic test was conducted based on the panelists' assessment criteria on a numerical scale, as shown in Table 1[24].

Table 1. Hedonic assessment scale.

Numeric scale	Preference scale
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely

2.7. Data processing and analysis

The obtained data were then analyzed using Microsoft Excel, and the hedonic test results were analyzed using IBM SPSS 20 univariate analysis of variance test Duncan's post hoc.

3. RESULTS AND DISCUSSION

3.1. Free radical scavenging activity

As presented in Table 2, kombucha had very strong activity. Sukandar et al. stated that very strong antioxidant activity if IC_{50} less than 50 ppm, and strong if IC_{50} 50-100 ppm[25]. Kombucha with the highest free radical scavenging activity used stevia with an IC_{50} value of 33.44 ppm. This is due to the bioactive compounds in stevia, such as phenolic compounds and flavonoids, which positively correlate with that activity. Adding stevia increased the phenolic component[26]. Polyphenols are chemical components with hydroxyl groups that can donate electrons to stabilize reactive free radicals, which explains the link between phenolic compounds and antioxidant activity[27].

Peasura and Sinchaipanit stated that antioxidant activity increased due to the high amount of ascorbic acid[28]. Meanwhile, with an IC_{50} value of 38.85 ppm, honey-sweetened kombucha has the lowest free radical scavenging activity. Some organic acids found in honey include gluconic, acetic, butyric, lactic, citric, and formic acids made lower the pH value[29]. Acidic conditions can reduce that activity because phenolic compounds become more stable, and difficult to release protons that can bind to DPPH[30]. Essawet et al. showed that the IC_{50} for black tea kombucha with coffeeberry extract ranged from 26.33 to 170.13 µl/ml[15]. The IC_{50} for soursop-leaf kombucha was higher, at 173.09 ppm[31]. The lower IC_{50} value indicates a greater capacity to scavenge free radicals.

3.2. Alcohol content

Table 3 shows that alcohol content ranges from 2.36% (stevia) to 2.78% (honey). Different substrates (sweeteners) result in alcohol levels that are not

Table 2. Free radical-scavenging activity of green tea kombucha.

Kombucha sample	Concentration (ppm)	% DPPH Inhibition	Linear Regretion	IC_{50} (ppm)	Category
Refined sugar	10	15.68±0.35	y = 1.4084x+0.6971	35.01	Very strong
	20	27.35±0.13	r = 0.9953		
	30	44.33±0.20			
	40	55.23±0.13			
	50	72.16±0.20			
Stevia	10	24.46±0.07	y = 1.1273x+12.3030	33.44	Very strong
	20	34.55±0.13	r = 0.9974		
	30	44.72±0.20			
	40	57.58±0.20			
	50	69.31±0.20			
Palm sugar	10	11.80±0.13	y = 1.4620x+(-2.6542)	36.02	Very strong
	20	25.29±0.15	r = 0.9956		
	30	42.63±0.13			
	40	57.55±0.28			
	50	68.77±0.13			
Honey	10	16.15±0.09	y = 1.1805x+4.1342	38.85	Very strong
	20	26.49±0.18	r = 0.9933		
	30	39.87±0.09			
	40	53.64±0.18			
	50	61.60±0.09			
Standard (quercetin)	3	18.66±0.15	y = 4.2352x+5.7792	10.44	Very strong
	6	30.39±0.13	r = 0.9973		
	9	43.94±0.33			
	12	58.23±0.33			
	15	68.27±0.20			

Table 3. Alcohol content of green tea kombucha.

Kombucha sample	Alcohol specific gravity (g/mL)	Alcohol content (%)
Refined sugar	0.9961	2.66±0.07
Stevia	0.9965	2.36±0.07
Honey	0.9959	2.78±0.04
Palm sugar	0.9964	2.45±0.11

substantially different. The kombucha fermentation process may contain a small amount of alcohol as a byproduct. The results agreed with those given by de Oliveira et al., alcohol in kombucha varies from 0.5% to 3% (v/v)[32].

3.3. Sugar reduction content

Different substates (sweeteners) have different effects on sugar reduction content (Table 4). The stevia-sweetened kombucha has the least amount of sugar at 18.99 grams. On the other hand, honey has the highest reducing sugar content, at 88.90 grams. Refined sugar was composed entirely of sucrose, while palm sugar comprised between 70 and 80% of sucrose. About 3-9% of palm sugar includes glucose and fructose[33]. Stevia derives its sweetness from the glycosides stevioside, rebaudioside, and dulcoside, which are produced by the reaction of steviol with monosaccharides[34]. On the other hand, a large percentage of honey's sugar content is fructose (41%), followed by glucose (34%) and sucrose (1%-2%)[35]. Therefore, honey kombucha may contain the least sucrose and the greatest amount of sugar reduction among all varieties. Fermentation begins with the most complex ingredients and gradually reduces to the simplest.

Table 4. Reducing sugar of green tea kombucha.

Kombucha sample	% Brix	Kombucha mass (g)	Sugar mass in kombucha (g)
Refined sugar	5.2	999.03	51.95±0.01
Stevia	1.9	999.45	18.99±0.01
Honey	8.9	998.86	88.90±0.00
Palm sugar	6.8	999.32	67.95±0.01

3.4. Hedonic test

Adding a different carbon source (sweetener) affects the sensory qualities of kombucha, including its color, aroma, and flavor[32], resulting in distinct color characteristics between samples before and after fermentation (Figure 1). Lactic acid bacteria (LAB) degraded catechins and polyphenols in green tea under acidic conditions, resulting in a brighter hue and distinct color characteristics[11]. The color and density of kombucha diminish as its constituents degrade. Acidic components reduce pH and reddish-brown pigment color. The degradation of tannins has increased kombucha's yellowish hue[11, 36].

The results of the panelists' preference for the color of kombucha (Figure 1) indicated that honey sweetener, with an average value of 6.27 and a golden yellow hue, was the most desired color. IBM SPSS 20 was used to assess the influence or significance of the difference between the four samples through data analysis. A univariate statistical test concluded that using different sweeteners had no significant effect (p>0.05) on the color of the kombucha produced, yielding a significance value of 0.688%; therefore, conducted no additional testing. The average total score of the panelists' evaluation results for the color is between 5 and 6, indicating that panelists did not reject the color of kombucha with various sweeteners.

In the aroma evaluation (Figure 2), palm sugar sweetener had the most favored aroma. Palm sugar kombucha had a distinct aroma that can balance the characteristic sour aroma of kombucha; however, honey and stevia produced a sour aroma with a sharpness that was still acceptable since the preference value varies from 5 to 6. Refined sugar permits olfactory rejection due to the potency of the sour aroma; a preference scale indicates a moderate to minor aversion. According to the univariate statistical test, the aroma of kombucha differs significantly (p<0.05) depending on the type of sugar used, with a significance level of 0.000. Duncan's post hoc test was used to identify samples with significant differences. According to the results of post hoc tests, refined sugar was significantly different from stevia, honey, and palm sugar. Honey and palm sugar did not significantly differ from stevia kombucha. Therefore, the panelists may reject the aroma of kombucha made with refined sugar based on their evaluation of the aroma.

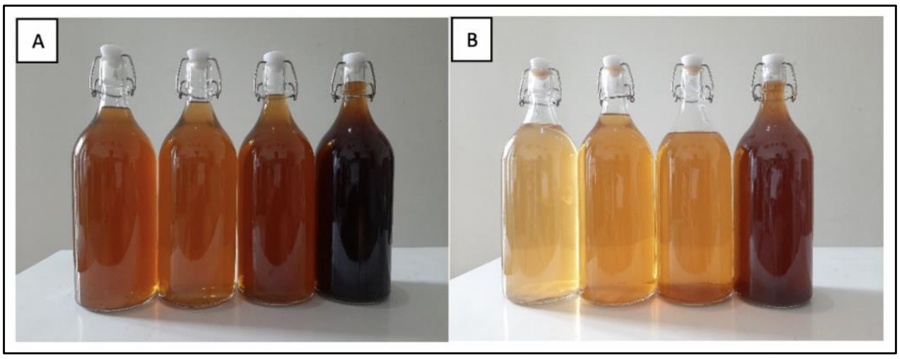

Figure 1. Appearance of kombucha prepared with refined sugar, stevia, honey, and palm sugar from left to right (A) before and (B) after fermentation.

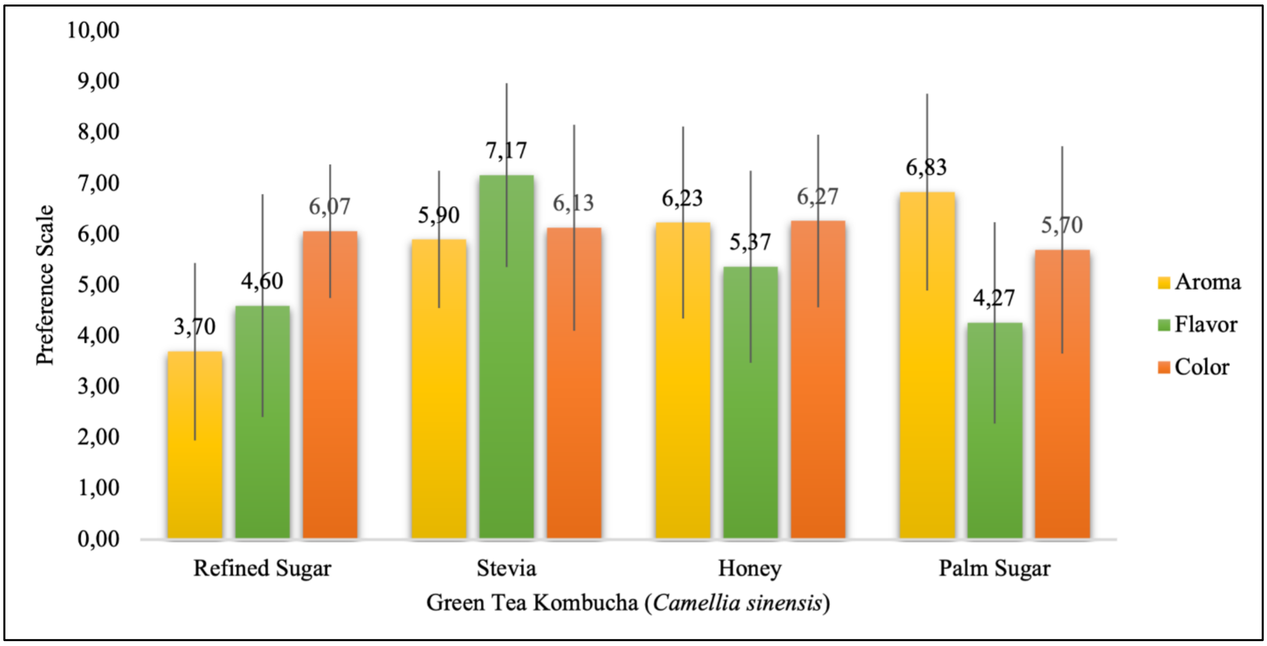

Figure 2. Hedonic test of green tea kombucha.

Aromatic chemicals found in green tea include linalool, geraniol, benzyl alcohol, indole, and coumarin from 200 volatiles components that are responsible for tea's aroma[37]. After fermentation, the aroma of brewed green tea turned into a distinct kombucha aroma sour and alcoholic aroma formed[38], produced from volatile molecules and organic acids formed during the fermentation process. In addition, adding various sweeteners throughout the fermentation process makes a kombucha aroma with varying degrees of sharpness and distinct features[32].

Although kombucha has a distinctive vinegar-like sour flavor[17], different sweeteners also provide distinct flavors and acidity levels. The more acidic kombucha is, the sourer it tastes[36]. Higher hydrogen ions are primarily responsible for the acidic perception at higher concentrations. The primary source of these ions is the fermentation-produced organic acids[39]. Different types of sweeteners in this study had a significant effect (p<0.05) on the flavor, with a significance value of 0.000.

There was no discernible difference between refined sugar and palm sugar and honey kombucha. Kombucha flavor preferences (Figure 2) shows that kombucha with stevia is the most popular and had a distinct flavor compared to refined sugar, palm sugar, and honey. Stevia kombucha had a sweet taste underneath its sour flavor, the sweetness mainly due steviol glycoside molecules. Peteliuk et al. stated that steviol glycoside is 250-300 times sweeter than sucrose[40]. Therefore, even in low quantities, stevia can provide a sweet flavor. The hedonic flavor test revealed that panelists would reject kombucha made with palm sugar and refined sugar. This was because the average value of the kombucha flavor evaluation with the addition of palm sugar and refined sugar is between 4 and 5, whereas the panelists do not reject the honey and stevia kombucha. Overall stevia was like slighty to like moderately, since the preference value varies from 6 to 7.

5. CONCLUSION

In this study, green tea kombucha demonstrated a very strong free radical scavenging activity in various sweeteners. In addition, alcohol content and sugar reduction vary according to the fermentation substrate. Regarding aroma and flavor, the panelists can accept the kombucha's color but reject the refined sugar. Panelists approved of the flavor of honey and stevia. Stevia had the highest radical scavenging activity and overall liking from hedonic, but another additional sweetener must be studied.

6. ACKNOWLEDGEMENT

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Author contribution
Muhammad Shidiq Rukman conducted all the assays, literature searches, and data for manuscript preparation; Ayu Nala El Muna Haerussana supervised the experiments, made relevant interpretations of the results, and wrote the manuscript.

Conflict of interest
None to declare.

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Ethics approval

The hedonic studies were declared ethically appropriate with approval number 73/KEPK/EC/III/2022, in accordance with the ethical guidelines of seven WHO standard, reviewed by "Health Research Ethics Committee Politeknik Kesehatan Kementerian Kesehatan Bandung", Indonesia.

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