Pharmaciana
Vol.12, No.1, March 2022, Page. 136-146
ISSN: 2088 4559; e-ISSN: 2477 0256
DOI: 10.12928/pharmaciana.v12i1.20903

The antioxidant activity of *Zingiber officinale*, *Hibiscus sabdariffa*, and *Caesalpinia sappan* combination

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Submitted: 14-06-2021 ***Reviewed: 16-08-2021*** ***Accepted: 25-10-2021***

ABSTRACT

Medicinal plants have been used in traditional treatment since a long time ago by local people in Indonesia. Nowadays, the trend in the consumption of medicinal plants, especially herbal drinks, is increasing. *Zingiber officinale*, *Hibiscus sabdariffa*, and *Caesalpinia sappan* are their main materials of medicinal plants. They were chosen because of their high antioxidant contents. Nevertheless, there is no scientific research on the antioxidant activity of the combination of the three extracts. Therefore, the purpose of this study was to determine the total flavonoid contents and antioxidant activity, as well as to compare the antioxidant enhancement pattern of the combination. Samples were extracted by successive maceration with hexane and ethyl acetate as solvents. Total flavonoids contents were determined through colorimetric analysis and antioxidant activity was determined based on the DPPH method with the IC_{50} value as a parameter. Total flavonoids of ethyl acetate extract from *Z. officinale*, *H. sabdariffa*, and *C. sappan* were 30.28±0.04; 24.81±0.03; and 24.01±0.04 mg QE/ gram extract, and the IC_{50} value were 51.36±0.05; 83.37±0.06; and 35±0.04 ppm. Total flavonoid contents of their combination were 22.48±0.05 (0:1:1); 23.88±0.05 (1:1:1); 23.68±0.05 (1:4:1); 22.81±0.05; 28.81±0.04 (4:1:1); 27.55±0.03 (1:1:0); 24.41±0.04 mg QE/ gram extract (1:0:1). Antioxidant activities obtained from the combination were 57.50±0.05 (0:1:1); 52.25±0.06 (1:1:1); 71.50±0.06 (1:4:1); 45.74±0.05 (1:1:4); 54.36±0.05 (4:1:1); 68.97±0.06 (1:1:0); 40.52±0.05 ppm (1:0:1). The strongest antioxidant activity was *C. sappan*.

Keywords: *Antioxidant, C. sappan, flavonoid, H. sabdariffa, Z. officinale*

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INTRODUCTION

Antioxidants are compounds which can be used to overcome oxidative damage due to free radicals (Saefudin et al., 2013). Regular consumption of natural antioxidants provides good health benefits (Yoshihara et al., 2010). Antioxidants are needed by the body to overcome and prevent oxidative stress which might cause various degenerative diseases; one of them is cancer (Giacco & Brownlee, 2010). In cancer, free radicals that trigger oxidative stress can be stabilized and neutralized by antioxidants (Gupta et al., 2014). Antioxidants can be found in secondary metabolites of medicinal plants. Based on their structure and biogenetic pathways, secondary metabolites consist of terpenoids, steroids, phenylpropanoids, polyketides, alkaloids, and flavonoids (Lully, 2016).

Flavonoids are secondary metabolites with the largest number, which is about 5-10% of total secondary metabolites. Flavonoids are polar compounds. Thus, polar solvents such as ethanol, methanol, ethyl acetate, or a mixture of these solvents are needed to extract flavonoids from plant tissue (Anggraito et al., 2018). Flavonoids have biological activities such as anti-bacterial, antivirus, anti-inflammatory, and anti-cancer (Neldawati, 2013). Flavonoids can also act as antioxidants because they are good acceptors against free radicals (Sathishkumar et al., 2008).

Ginger (*Zingiber officinale*) is a medicinal plant that contains high bioactive compounds and strong in vitro and in vivo antioxidant properties. Ginger extract had significant scavenging effect of superoxide radicals (Morakinyo et al., 2011). Ginger is rich of antioxidants, compounds that prevent stress and damage to your body's DNA (Topic et al., 2002). Ginger also plays an important role as an anti-inflammatory process (Tjendraputra et al., 2001). It also acts as an antitumor through the modulation of genetic pathways. (Liu et al., 2012) showed that terpenoids, a constituent of ginger, induce apoptosis in cancer cells by p53 activation. (Ali et al., 2018) said that the highest flavonoid content is found in the rhizome. Ginger root contains a high level (3.85 mmol/100 g) of total antioxidants (Halvorsen et al., 2002). Therefore, this herb is often used to treat inflammation, cancer, and others.

Calyx of roselle (*Hibiscus sabdariffa*) is commonly used to treat inflammation, coughs, and hypertension. This plant contains phytochemical compounds such as anthocyanins and flavonoids (Me et al., 2019). Anthocyanins, commonly found in roselle flowers, are phenolic components which can be used as a source of antioxidants. Flavonoids are another source of antioxidants found in all parts of the roselle body (Mungole & Chaturvedi, 2011). Roselle extract contains high vitamin C, which has been known as an antioxidant compound. Research conducted by (Maksum & Purbowati, 2018) demonstrated that roselle extract contains vitamin C 10.74 ± 0.14 mg/g and IC_{50} 202.47 µL / mL. The content of these bioactive materials makes roselle have the ability as an antioxidant. Therefore, this plant has the potential to be used as an antioxidant and provides various health benefits.

Sappan wood (*Caesalpinia sappan*) extract is often used for treating external body wounds and heartburn (Rahmawati, 2011). Sappan wood extract contains alkaloid, flavonoid, and saponin groups compounds (Sufiana & Harlia, 2014). The active flavonoid compounds found in sappan wood act as primary antioxidants and secondary antioxidants. Brazilin and flavonoids are phytochemical compounds that act as antioxidants (Dwi & Suhartono, 2016). Brazilin showed the highest DPPH radical scavenging activity (IC_{50} = 57.2 µM) and ferric reduction activity compared to standard vitamin E (Sasaki et al., 2007).

The combination of these medicinal plants affects the up and down of antioxidant activity. The combination of ginger and roselle increases antioxidant activity (Yanis et al., 2016). According to (Junita et al., 2001), the combination of ginger and roselle has been found to provide a high antioxidant activity. The combination of ginger and sappan wood also increases antioxidant activity better than when they are separated (Nasrudin et al., 2019). (Sari et al., 2015) stated that increasing the concentration of ginger and sappan wood added to the formulation will increase the activity of antioxidants, polyphenols, and flavonoids. The combination of rosella and sappan wood can also synergistically increase antioxidant activity. The concentration of sappan wood extract in the combination affected the IC_{50} value (Yulianty et al., 2016).

Nowadays, the consumption of antioxidants, especially herbal drinks, is increasing. Ginger, roselle, and sappan wood are the main materials often used because of their high antioxidant content. However, there are no scientific studies that measure the antioxidant activity when they are combined. Therefore, this research aims to determine the total flavonoids and antioxidant activity of *Z. officinale*, *H. sabdariffa*, and *C. sappan* and to compare the antioxidant enhancement pattern of the combination of medicinal plants combination in different compositions.

MATERIALS AND METHOD

This research was conducted in the Laboratory of Biochemistry and Molecular Biology, Faculty of Biology, Universitas Kristen Satya Wacana for about four months. The experimental method with a completely randomized design was used in this research. Observation variables were total flavonoid content and antioxidant activity of *Z. officinale*, *H. sabdariffa*, and *C. sappan* that have been combined in certain compositions based on the Design of the Experiment (DOE).

Materials

The main materials in this research were rhizome of *Z. officinale var. Amarum* which has beige skin, small segment, and sharp odor (Wahyuni et al., 2015), calyx of *Hibiscus sabdariffa* which has brilliant red edible calyces (Keong et al., 2019), and stem of *Caesalpinia sappan* with red to red-brown color obtained from a local market in Salatiga, Jawa Tengah Province, Indonesia. That sample were identified in the Laboratory of Biology, Universitas Kristen Satya Wacana.

Methods

The research was conducted at the Laboratory of Biochemistry and Molecular Biology, Faculty of Biology, Universitas Kristen Satya Wacana, Salatiga. All samples (*Z. officinale*, *H.sabdariffa*, and *C. sappan*) were combined in certain compositions based on the DOE (*Design of Experiment*). The combinations are shown in Table 1.

Table 1. Combination of *Z. officinale, H. sabdariffa,* and *C. sappan* based on DOE

Group	*Zingiber officinale*	*Hibiscus sabdariffa*	*Caesalpinia sappan*
K1	0	1	1
K2	0	1	0
K3	1	1	1
K4	1	4	1
K5	1	1	4
K6	4	1	1
K7	1	0	0
K8	1	1	0
K9	1	0	1
K10	0	0	1

Note: The table above contains the ratio of the combination of the three medicinal plants based on DOE

Sample preparation

Z. officinale, H. sabdariffa, and *C. sappan* were air-dried. Thus, all samples had the same dryness level. They were then mashed in a blender (Philip HR 1538). Each sample (100 g) was macerated with hexane (200 mL) for 3 cycles, then macerated with ethyl acetate (200 mL) for 3

cycles. We used successive maceration for this method. Each cycle of maceration needed 1 hour (temperature 50 ^{0}C). We chose that time because while macerating, the sample was placed on a hot plate (50 ^{0}C) and stirred with a stirrer. The result of maceration was supernatant. Then, all supernatants were concentrated with a rotary evaporator (Rotavapor RE 100 Pro) until they become paste.

Qualitative analysis of flavonoids (Harborne, 1980)

A total of 15 mg of sample was dissolved in 10 ml of ethanol. Then, 2 mL was taken and acidified with 1% HCl as much as 3 drops. After that, it was dissolved in 20% NaOH (3 drops). The indicator for positive flavonoids is the formation of a canary yellow color.

Quantitative analysis of flavonoids (John et al., 2014)

Analysis of total flavonoid content was carried out using the aluminum chloride method. The calibration curves used quercetin solution. The series of quercetin concentrations were 40, 60, 80, and 100 mg/L. 15 mg of combination sample was dissolved in 10 mL of ethanol. Then 1 mL of it or quercetin (as standard) was mixed with 0.3 mL of 5% $NaNO_2$, then incubated (5 minutes). It was added with 0.3 ml of 10% $AlCl_3$ and incubated for 5 minutes. Then, it was added with 2 mL of 1 M NaOH and distilled water until reaching 10 mL. The absorbance of the solution was measured using a UV-Vis spectrophotometer (Hitachi UV mini 1240) at a wavelength of 510 nm. The concentration of standard quercetin was compared to its absorbance to obtain the linear regression equation, y=ax+b. The conversion of total flavonoid concentration (QE) is determined based on equation (1).

$$QE = c\ (V/m) \qquad (1)$$

where,
QE : total flavonoid concentration (mg/g)
c : total flavonoid concentration in the standard curve of quercetin (mg/L)
V : extract volume (L)
m : extract weight (g)

Analysis of antioxidant activity (Gomes de Melo et al., 2010)

Antioxidant activity was analyzed by DPPH and the results were expressed by the value of Inhibition Concentration 50 (IC_{50}). 10 mg sample extract was reconstituted with 95% ethanol (10mL) as stock solution. Combination sample was made based on Table 1. For example, in combination 1:1:1 (Z:H:C), we needed 3.33 mg of *Z. officinale*, 3.33 mg of *H. sabdariffa*, and 3.33 mg of *C. sappan* as sample extracts then mixed with 95% ethanol. The sample concentration series used were 20, 40, 60, 80, and 100 mg/L. A total of 0.5 mL of sample was added with 3.5 mL of DPPH 0.4 mM and incubated for 30 minutes in a dark room. The calculation of antioxidant activity was presented in equation (2):

$$\text{Antioxidant activity (\%)} = (\text{A blank} - \text{A sample})\ \text{A blank} \times 100\ \% \qquad (2)$$

where,
A blank is the absorbance of 0.4 mM DPPH.
A sample is the absorbance of 0.4 mM DPPH after treatment.

Data were analyzed by simple linear regression (y = a + bx). The lC_{50} value (x) was obtained by stating y value of 50. The smaller the IC_{50} value, the greater the antioxidant activity is (Rivero-Cruz et al., 2020).

Data Analysis

The research data were analyzed using SPSS 16.0 software. The IC_{50} value was obtained from the linear regression equation between the sample concentration and the antioxidant activity average percentage. Correlations among study variables were analyzed using Pearson correlation and

significant differences between samples were analyzed by ANOVA (P<0.05). Normality data were checked by Shapiro-Wilk's test. Then, the homogeneity was checked by Levene's test. Then, Anova test was carried out. To prove significance on data, we used Tukey's test.

RESULT AND DISCUSSION

Table 2. Result of qualitative flavonoid test (Ethyl Acetate Extract)

Sample	Flavonoid Test	
	Part	Result
Zingiber officinale	rhizome	 Canary yellow (+++)
Hibiscus sabdariffa	flower	 Canary yellow (++)
Caesalpinia sappan	stem	 Canary yellow (+)

Note: The plus sign (+) in the column indicates the level of color density of the test sample.

A thick yellow color was formed at the beginning when the sample was dripped by NaOH solution. It faded slowly and turned clearer like canary yellow when HCl solution was dripped into the samples (Saptarini et al., 2016). It was caused by the reaction between flavonoids, NaOH, and HCl. The addition of concentrated HCl is intended to hydrolyze flavonoids into their aglycones by hydrolyzing O-glycosides. This reduction with concentrated HCl produces canary yellow color complexes in flavonols, flavanones, and xanthones (Harborne, 1980). Table 2 shows that *Z. officinale* had the darkest canary yellow color, followed by *H. sabdariffa* and *C. sappan*. This indication was strengthened by the result of quantitative test presented in the Table 3.

From Table 3, flavonoids extraction using ethyl acetate was more effective. Previous research also stated the largest total flavonoids were from: ethyl acetate> 50% ethanol> butanol> hexane> water (Thavamoney et al., 2018). (Pratoko et al., 2018) also showed that the highest total flavonoid content obtained from ethyl acetate fraction of *Z. officinale* was 46.6 ± 1.8 mg QE/g extract while the one from hexane fraction was 26.1 ± 1.7 mg QE/g extract. Total flavonoid content, respectively from the largest: ethyl acetate> ethanol> n-hexane. It is because most flavonoids are less polar or semi-polar, such as quercetin, rutin, catechins, and epicatechin (Heim et al., 2002). The main difference in the content of flavonoids extracted from the solvent is attributed to the difference in polarity of the solvent. Therefore, the samples which were extracted using ethyl acetate solvent obtained more flavonoids than the ones extracted with hexane solvent (Prasad et al., 2010).

Table 3. Total flavonoid content of *Z. officinale, H. sabdariffa, C. sappan* combination

Combination Z:H:C	Total Flavonoid of Hexane Extract (mg QE/g)	Total Flavonoid of Ethyl Acetate Extract (mg QE/g)
0:1:1	6.82 ± 0.04[a]	22.48 ± 0.05[a]
0:1:0	8.34 ± 0.05[c]	24.81 ± 0.03[f]
1:1:1	7.49 ± 0.05[b]	23.88 ± 0.05[d]
1:4:1	7.44 ± 0.02[b]	23.68 ± 0.05[c]
1:1:4	7.25 ± 0.04[b]	22.81 ± 0.05[b]
4:1:1	9.53 ± 0.05[e]	28.81 ± 0.04[h]
1:0:0	10.10 ± 0.06[f]	30.28 ± 0.04[i]
1:1:0	9.02 ± 0.06[d]	27.55 ± 0.03[g]
1:0:1	8.54 ± 0.05[c]	24.41 ± 0.04[e]
0:0:1	8.24 ± 0.31[c]	24.01 ± 0.04[d]

Note: The combination above is based on DOE (Design of Experiment). The notation behind the flavonoid value shows a significant different ($p<0.05$) of each.

The ethyl acetate extract of *Z. officinale* (Z:H:C=1:0:0) had the highest total flavonoid content (30.28 ± 0.04 mg QE/gram). This result is in line with Ali et al. (2018) who stated that total flavonoid content of chloroform-methanol (CM) extract from *Z. officinale's* was 40.25 ± 0.21 mg QE /gram. Pratoko et al (2018) also found that total flavonoid content from ethyl acetate extract of *Z. officinale* rhizome was 46.6 ± 1.8 mg QE/g. These results corroborate the statement about the high total flavonoid content of *Z. officinale* rhizome. Rhizome of *Z. officinale* has the highest total flavonoid content than the other parts (Ali et al., 2018). Therefore, this part is frequently used in traditional treatment. The total flavonoid content of ethyl acetate extract from *H. sabdariffa* flowers was in the fourth position, which was 24.81 ± 0.03 mg QE/ g (Z:H:C=0:1:0). (Yusoff et al., 2017) support this data by showing that the highest total flavonoid content of *H. sabdariffa* extract was 14.4251 mg QE/gram. *C. sappan* was in the sixth position with the total flavonoid content of 24.01 ± 0.04 mg QE/g (Z:H:C=0:0:1). Research on the total flavonoids of *C. sappan* was also conducted by Siregar et al. (2019). In that study, it was found that the total flavonoids in *C. sappan* were 21.88 ± 0.33 mg QE/g. These results supported the statement that *C.sappan* contains high flavonoids.

The combination of those medicinal plants affected to the total flavonoids content (Sari et al., 2015). Not all medicinal plants showed synergism when combined. Total flavonoids decreased when added with *H. sabdariffa* or *C. sappan.* However, when *Z. officinale* was added, total flavonoid content increased. This showed that the presence of *Z. officinale* resulted in the increase of total flavonoids. The strong correlation between *Z. officinale* and total flavonoid content was also proved by Pearson-correlation test and it had a correlation of 86.9%. It means the higher concentration of *Z. officinale* added to the formulation, the higher the total flavonoids produced (Sari et al., 2015). These can be seen in Table 1 and Table 4 where the combination group with high *Z. officinale* extract would have a high total flavonoid content too.

Based on Table 4, ethyl acetate extract from *Caesalpinia sappan* had the strongest antioxidant activity with an IC_{50} value of 35 ± 0.04 ppm (Z:H:C=0:0:1). This is supported by several previous studies. (Wetwitayaklunga et al., 2005) measured the IC_{50} value of *Caesalpinia sappan* extract at 13.406 ppm. The same research conducted by (Utari, 2017) showed that *Caesalpinia sappan* extract contained very strong antioxidants (15.69 ppm). In other studies, the combination that only consisted of *Z. officinale* had a strong antioxidant activity (IC_{50}: 51.36 ± 0.05 ppm). It was supported by some researchers, such as (Yuliani et al., 2016) and (Kaban et al., 2016) who obtained data about the antioxidant activity of *Z. officinale* consecutively, which were 41.27 ± 0.3939 ppm and 25.69 ppm. These studies proved that *Z. officinale* had strong antioxidants. Other combination (Z:H:C=0:1:0) that only consisted of *Hibiscus sadariffa* has antioxidant activity of 83.37 ± 0.06 ppm (IC_{50} value). This

result is in line with (Inggrid et al., 2018) who stated that antioxidant activity of the *Hibiscus sabdariffa* extract was 97.4 ppm.

Table 4. Antioxidant activity of *Z. officinale, H. sabdariffa, C. sappan* combination

Combination Z : H : C	IC_{50} of Hexane Extract (ppm)	IC_{50} of Ethyl Acetate Extract (ppm)
0:1:1	68.51 ± 0.13^{g}	57.50 ± 0.05^{g}
0:1:0	93.5 ± 0.08^{j}	83.37 ± 0.06^{j}
1:1:1	62.71 ± 0.08^{e}	52.25 ± 0.06^{e}
1:4:1	81.44 ± 0.06^{i}	71.50 ± 0.06^{i}
1:1:4	56.44 ± 0.12^{c}	45.74 ± 0.05^{c}
4:1:1	65.57 ± 0.06^{f}	54.36 ± 0.05^{f}
1:0:0	62.25 ± 0.06^{d}	51.36 ± 0.05^{d}
1:1:0	80.40 ± 0.15^{h}	68.97 ± 0.06^{h}
1:0:1	51.76 ± 0.08^{b}	40.52 ± 0.05^{b}
0:0:1	44.34 ± 0.13^{a}	35.00 ± 0.04^{a}

Note: The notation behind the IC_{50} value shows a significant different ($p<0.05$) of each.

The correlation between total flavonoids and antioxidant activity was not strong. This is due to the presence of several chemical compounds (besides flavonoids) that take part in the resulting antioxidant activity. The relationship between these compounds is quite complex and they cannot be separated (Weckwerth, 2008). For example, in the combination 1:1:4 and 0:0:1 of *Z. officinale, H. sabdariffa, C. sappan*. These combinations had *C.sappan* is the largest composition so that the antioxidant activity was very strong, but the total flavonoid levels were not the highest. It is because *C. sappan* contains phenolic compounds (besides flavonoid) which also contribute to antioxidant activity in plants (Badami et al., 2003). (Widowati, 2011) also stated that sappan wood extract contains high terpenoids, such as monoterpenes and terpenes which cause high antioxidant activity. Another example was 1:1:0 (Z:H:C) combination which was only composed of *Z. officinale* extract. It was the highest total flavonoids but not the strongest antioxidant activity (Tables 3 and 4). The antioxidant activity in *Z. officinale* is not only supported by flavonoid compounds, but also by other compounds such as phenolic compounds (Mao et al., 2019). The active phenolic compounds in *Z. officinale* such as gingerol and shogaol have antioxidant effects. These compounds have antioxidant activity that exceeds tocopherols (Akhtar et al., 2011).

The combination of medicinal plants with a higher amount of *C. sappan* extract will increase antioxidant activity. Sample 1:1:4 (Table 4) had the strongest antioxidant activity compared to other samples (45.74 ppm). *C. sappan* is strongly correlated with antioxidant activity, which is 76.4% (Pearson correlation test). Nevertheless, when those three medicinal plants were combined, it did not show a clear synergism. The addition of *H. sabdariffa* into the combination did not increase the antioxidant activity as expected. It even worked antagonistically to antioxidant activity. The addiction of *Z. officinale* and *H. sabdariffa* had not even made the antioxidant activity stronger. We could see on table 4 that sample 0:0:1 had the strongest antioxidant although it only consisted of *C. sappan*. Other extracts that had been added into the combination did not increase the antioxidant activity.

All of the combinations of these herbal plants generally produce antioxidants from strong to very strong ranges (IC_{50} value<100 ppm). This powerful antioxidant has the potential to cure several degenerative diseases, one of which is cancer. Antioxidants can overcome and prevent oxidative stress triggered by free radicals (Giacco & Brownlee, 2010). In cancer, antioxidants will help stabilize and neutralize free radicals so that they can reduce the risk of damage to body cells (Gupta et al., 2014). This research is expected to help producers that need scientific data about total flavonoid and antioxidant activity of the combinations of medicinal plants to be used as their supporting data in production products, for example, herbal drinks or traditional herbal medicine. Furthermore, this study

can increase readers' knowledge about the total flavonoid content and antioxidant activity of a combination of medicinal plants, like *Z. officinale*, *H. sabdariffa*, and *C. sappan.*

CONCLUSION

The strongest antioxidant activity was owned by *C. sappan* with combination of Z:H:C was 0:0:1. The antioxidant activity will be stronger if the proportion of *C. sappan* is greater in its combination. Combination of *Z. officinale*, *H. sabdariffa*, and *C. sappan* didn't show a synergist effect in increasing antioxidant activity.

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