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The Effects of Metallochlorophyll Formation and Pretreatment on Color, Chlorophyll Content, Total Phenolic Content, and Antioxidant Activity of Sambiloto (*Andrographis paniculata*) Simplicia Powder

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Abstract This research aimed to produce sambiloto simplicia which is rich in chlorophyll and Zn through the process of forming metallochlorophyll by blanching either fresh or dry leaves. Sambiloto simplicia powder is produced through several stages including sorting, boiling in Zn acetate medium (0, 300, 400, 500 ppm), draining, drying, and grinding. The types of materials used are dry and fresh leaves material. The sambiloto simplicia powder was analyzed for chlorophyll content, bound Zn content, total phenolic content (TPC), and total flavonoid content (TFC). Analysis of the antioxidant activity of sambiloto simplicia powder included lipid peroxidation inhibition (LPI) activity and DPPH radical scavenging capacity. The results show that the greater Zn acetate concentration, the green color intensity, chlorophyll content, bound Zn content, and total phenolic and flavonoid content of sambiloto simplicia powder increased, but at concentrations more than 300 ppm in dry leaves and more than 400 ppm in fresh leaves it decreased again. Simplicia powder from dry leaves by blanching at a concentration of 300 ppm and fresh leaves material at a concentration of 400 ppm had the highest contents of chlorophyll, TPC, TFC, and the ability to inhibit lipid peroxidation. At a concentration of Zn acetate of 300 ppm, the simplicia powder from the dry leaves had a higher RSA and LPI than the fresh leaves, but at a concentration of 400 ppm, the LPI from the fresh leaves was higher. The sambiloto simplicia powder could be a natural source of antioxidants as it is high in zinc.

Keywords Metallochlorophyll, *Andrographis paniculata*, Phenolics, Flavonoids, Antioxidant Activity

1. Introduction

Changing to a diet rich in pro-oxidants and living in a polluted environment increase exposure to radicals, which might lead to degenerative diseases. Free radicals were responsible for the excessive oxidation reactions that occur in the body. Oxidation reactions may generate excessive free radicals that the body's antioxidants were unable to neutralize, necessitating a higher antioxidant intake. Likewise, in order to prevent diseases brought on by viruses that keep evolving, the COVID-19 pandemic has

made us more aware of how well our bodies were immune to disease. The body needs to consume a variety of nutrients that seem to be high in antioxidants and micronutrients that help boost immunity, like minerals like Zn, Cu, and Se, in order to stay healthy.

Due to its high phenolic and flavonoid content, the bitter plant or also known in Indonesia as sambiloto provides a natural source of antioxidants. Sambiloto (*Andrographis paniculata*) was a type of plant that includes phenolic and flavonoid compounds with antioxidant activity [1]. Antioxidants were compounds that could slow, delay, or prevent the oxidation of easily oxidized lipids and other compounds [2]. Chlorophyll would be another active component found in bitter plants. Chlorophyll has antioxidant and antimutagenic features [3]. Additionally, chlorophyll and its derivatives might boost the body's immunity, especially against virus infection [4]. It was considered that during the drying process, chlorophyll and other active components were degraded, hence reducing the antioxidant activity of the dried simplicia. Chlorophyll degradation may decrease the antioxidant activity of the substance [5].

The stability of chlorophyll could be improved by forming metallochlorophyll complexes with metals that could also form more stable complexes than Mg metals [6]. Chlorophyll did break down in the presence of heat, acid, light, a low pH atmosphere, and oxygen. Changes in chlorophyll molecules, such as the loss of phytol side chains or the loss of the central ion Mg^{2+}, could lead to chlorophyll degradation. The phytol side chain would be released due to the activity of the chlorophyllase enzyme or acidic conditions. The Mg^{2+} central ion could be liberated upon exposure to acids and high temperatures. The presence of a phytol group on the C-17 atom captures chlorophyll nonpolar and water-insoluble, so the removal of this group yields chlorophyll compounds that are water-soluble. Alkaline conditions and light exposure could also induce changes in chlorophyll molecules [7]. It does seem to be possible to increase the stability of chlorophyll and its anti-oxidation capacity by replacing Mg^{2+} with other metal ions [3]. Zn, Mn, and Fe were also alternative metals that may be used to form complex compounds that are more stable than magnesium. The Fe-chlorophyll complex would increase chlorophyll's ability to absorb light, whereas Mn would be less stable with ligands than Zn [8]. In this study, Zn metal was used because of its advantages as a micronutrient that can increase body immunity [9] and is considered safer than Cu metal [10]. Various methods of forming metallochlorophyll may be used to increase the stability of chlorophyll, including blanching leaves or fruit in $ZnCl_2$ solution and adding Zn^{2+} ions to the chlorophyll extraction media or to the chlorophyll extract while heating. The results of various investigations included the addition of 300 ppm Zn^{2+} ions in the processing of chickpea pulp [11], blanching beans at a concentration of 300 ppm $ZnCl_2$ [12], and blanching pears at a concentration of 2600 ppm $ZnCl_2$ [13], and the application of metallochlorophyll complex formation on pandan leaf chlorophyll [14]. In the previous research, $ZnCl_2$ was the only type of salt utilized, and pandan leaf pulp, not chlorophyll extract, was used for the formation of the metallochlorophyll complex. Yakimovskii et al. [15] showed that the intake of $ZnCl_2$ as much as 1 µg in rats for 15 days could interfere with the motor nerves while the intake of Zn acetate at the same dose had no effect. Consequently, it would be vital to explore the influence of various concentrations on the formation of metallchlorophyll complexes utilizing Zn acetate salts.

Additionally, the formation of the metallochlorophyll complex in chlorophyll should begin with the release of Mg^{2+} ions, which could be accomplished through acidification or heating. Hence, the pretreatment of leaves prior to the formation of the metallochlorophyll complex was crucial. If chlorophyll has released Mg^{2+} in the form of pheophytin, it might bind Zn^{2+}. The degradation of chlorophyll to pheophytin was believed to occur during the drying process involved in the development of leaf simplicia as well as during the boiling or blanching of the plant material. Still, these two processes also could result in the degradation of phenolics and flavonoids. This study aimed to determine the impact of the pretreatment and concentration of the complex-forming reagent Zn acetate metallochlorophyll on the chemical characteristics and antioxidant activity of sambiloto simplicia.

2. Materials and Methods

2.1. Materials

The materials used were fresh sambiloto leaves from the State-Owned Forestry Company (Perhutani), Ngawi, East Java and dried simplicia from the Tawangmangu Medicinal Plants Research Institute. Meanwhile, materials for the extraction process were distilled water, acetone, and Whatman papers No. 1 and 42. The chemical for the formation of the metallochlorophyll complex was Zn $(CH_3COO)_2.2H_2O$ (Sigma, Aldrich). Materials for analysis of chlorophyll content, analysis of phenolic and flavonoid content (standard gallic acid, quercetin, Folin-Ciocalteu reagent (Sigma Chemical Co.), 95% ethanol, 90% acetone, methanol, HCl, acetate buffer), chemicals for activity test antioxidants namely linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytolune (BHT), $FeSO_4.7H_2O$, $AlCl_3.6H_2O$, HCl, NaOH, ethanol, potassium phosphate buffer, ammonium thiocyanate, and ion-free water were obtained from Merck with standard specifications for analysis (pro analysis).

2.2. Pretreatment

Fresh leaf preparation

The process of making sambiloto simplicia began with sorting fresh and dry sambiloto leaves in order to obtain

good quality simplicia. Fresh sambiloto leaves were sorted according to the following criteria: the condition of leaves should be intact, unwilted, and in good condition. Thereafter, the hard stems and damaged leaves were removed, and the leaves were cleaned and drained. Afterwards, cut the material into pieces that were less than two centimeters (< 2 cm). The subsequent step would be the weighing of fresh and dry sambiloto leaves in accordance with the research requirements specifically at 100 g.

Dry leaf preparation

The process of making sambiloto simplicia at the Tawangmanggu Medicinal Plants Research Institute began with the fresh leaves being washed, drained, and then withered in the sun. If the leaves have withered, the next step would be to dry them in a cabinet drier at a temperature between 40-50 °C for 24 hours. Sambiloto simplicia was first sorted to eliminate hard leaf stalks before being ground for one minute in a dry blender. The material that did not pass through the sieve was then ground once more.

Formation of the metallochlorophyll complex

The next process was blanching or boiling fresh sambiloto leaf or dry leaf using Zn acetate medium at medium concentrations of 0, 300, 400 and 500 ppm for 15 minutes at 100 °C. The ratio between material and medium is 100:400 (w/v). Furthermore, boiling was carried out at 100 °C for 10 minutes, then drained [16]. The blanching was aimed to form metallochlorophyll that could increase the stability of chlorophyll during the processing.

The next process was dried one more time using a cabinet drier at a temperature of 50 °C for 8 hours to dry (it is said to be dry if the stalk is easily broken), then mashed by a dry blender, then sifted using a 60 mesh sieve. Those components that fail to pass through should be sieved again. This process aimed to produce a finer powder of sambiloto simplicia and to separate between coarse and fine powders. Furthermore, the simplicia powder was packaged in 0,5 mm PP plastic before being analyzed.

Color measurement

Measuring the color of the simplicia powder of sambiloto leaves using a colorimeter. The color measurement of the sample was according to the L a b color system. The L (lightness) value indicates the brightness content of the material, the a* (+a redness/ -a greenness) value indicates the intensity of red (+) or green (-) color, while b* (+b yellowness/ -a blueness) indicates yellow (+) or blue (-) intensity. Based on the data obtained, the hue (ho) of the sample was calculated with reference to the formula

$$hue(h^o) = arctan\frac{b}{a}$$

Zn content

Sambiloto simplicia powder was weighed as much as 2 g and ashed at 450 ^oC with a temperature rise rate of 50 ^oC/hour, after being dissolved in 10 ml of HCl 6 N and then the solution was evaporated by heating in an acid room until dry (at a temperature of 65 ^oC for 4 hours). The residue obtained was dissolved in 0,1 N HNO_3 5 ml and then analyzed by atomic absorption spectrophotometric (AAS).

Total chlorophyll content

Total chlorophyll content was measured by Vernon method [5]. Sambiloto simplicia powder weighed as much as 5 g, then extracted with 80% acetone, as much as 20 ml, homogenized then centrifuged at 8000 rpm for 15 minutes and filtered with Whatman paper no 1 and 42. The filtrate obtained was added with 80% acetone until the volume reached 25 ml in a volumetric flask. The absorbance of the extract obtained was measured at a wavelength of 663 and 645 nm using a UV-Vis spectrophotometer. The equation below was used to calculate the total chlorophyll contents.

$$Total\ chlorophyll\ \left(\frac{mg}{g} wet\ weight\right)$$
$$= (20.2A_{663} + 8.02A_{645})\ \text{x}\ Dillution\ factor/1000$$

Total phenolic content

Total phenolic content (TPC) was determined by the Folin Ciocalteu method [17] using gallic acid as standard. The acetone extract obtained from the analysis of chlorophyll content was also used for the analysis of phenolic and flavonoid contents. The extract was 50 µl, added with 250 µl of Folin-ciocalteu solution, then allowed to stand for 1 minute and added 750 µl of 20% NaCO3, then homogenized with a vortex, and added with distilled water to a volume of 5 ml. After 5 minutes of incubation at room temperature, the absorbance was measured at λ 760 nm. In this case, gallic acid was used as standard and a calibration curve was prepared with gallic acid. The TPC calculation results were mg Gallic Acid Equivalent (GAE) per gram of dry weight.

Total flavonoid content

Total flavonoid content (TFC) was measured using a technique developed by Wariyah & Riyanto [18]. Extract 50 µl plus 4 ml of distilled water and 0,3 ml of 10% $NaNO_2$. After settling for 6 minutes, 0,3 ml of 10% $AlCl_3.6H_2O$ was added, left for 5 minutes, then added 4 ml of 10% NaOH. Thereafter, added distilled water (to a total volume of 10 ml), homogenized with a vortex for 1 minute, and left for 15 minutes. Absorbance was measured at a wavelength of 510 nm. The blank used was distilled water. TFC was determined using standard quercetin at a concentration of 1,25-80 mg/L and calculated as mg quercetin equivalent (QE)/g dry weight.

Lipid peroxidation inhibitory activity

Determination of antioxidant activity was done by a linoleic acid emulsion system and using the FTC method [5]. Sambiloto extract was prepared by weighing 0,5 g

sample and adding 10 ml of 80% methanol, homogenized, and filtered using Whatman filter paper no 42, volume was adjusted until it reached 25 ml. Methanol extract of sambiloto simplicia powder or BHT standard (33,3µg/ml) mixed with linoleic acid emulsion. Linoleic acid emulsion was prepared by: 1 ml of 2,5% linoleic acid mixed with 0,1 ml of Tween 20 added with 4 ml of sambiloto simplicia extract sample and 0,02 M potassium phosphate buffer to reach a volume of 10 ml. The emulsion was incubated at 37 ± 1 °C in a dark room for 6 days and 0,1 ml of aliquot was taken every day for analysis. The degree of oxidation was measured by: 0,1 ml aliquot plus 5 mL ethanol (75% v/v), 0.1 mL ammonium thiocyanate (30% w/v), and 0,1 mL $FeCl_2$ (0,02 M in 3,5% HCl v/v), then homogenized. After being homogeneous, the absorbance was measured at 500 nm, and the measurement was repeated 3 times. A solution without extract was used as a blank and BHT was used as a comparison at the concentration of 100 ppm. The percentage of lipid peroxidation inhibition was determined by equation 3 with the absorbance A_o of the control and A1 of the sample absorbance.

$$LPI\ (\%) = 100 - [(A_1 / A_0) \text{ x } 100]$$

DPPH radical scavenging capacity

The scavenging capacity of DPPH free radicals was determined by the method developed by Xu & Chang and Kang et al. [19],[6] This method was chosen because the sample volume tested was smaller than the DPPH solution volume so that the chlorophyll color effect during measurement could be minimized. A sample of 0,2 ml of methanol extract of sambiloto simplicia powder, added with 3,8 ml of 0,1 mM DPPH solution in methanol solvent, mixed using a vortex for 1 minute, the resulting filtrate was incubated in the dark at room temperature for 30 minutes. Controls were made using methanol as a sample replacement and BHT as a comparison at a concentration of 100 ppm. After incubation, the absorbance of the filtrate was measured by UV-Vis spectrophotometry (Shimadzu) at a wavelength of 515 nm. The data obtained were A_0: absorbance of DPPH without sample, A_S: absorbance of the sample that has been added with DPPH and A_b: absorbance of sample extract without DPPH. Radical Scavenging Activity (RSA) expressed in percent (%). The RSA value indicates the ability of the sample to bleach the violet color of DPPH and could be calculated by equation 4 [6].

$$RSA(\%) = [A_0 - (A_S - A_b)/A_0) \text{ x } 100$$

3. Result and Discussion

Color

One of the parameters for the successful formation of the metallochlorophyll complex was the increase in the intensity of the green color [14]. The information in Table 1 indicates that the greater the concentration of Zn acetate, the greater the green color intensity (-a*) and hue value (H*) of sambiloto simplicia powder. Conversely, the intensity of the yellow color produces a lower value. The greater the Zn acetate concentration, the greater the total chlorophyll content (Table 2). The greater the chlorophyll content, the more metallochlorophyll complexes occur. Hue values in the range of 120^o in the HSL color system show green, while more than 120^o to close to 160^o shows green, bluish green to blue. The bluish green color was the color of chlorophyll *a*. Conversely, if it was less than 120^o to 60^o, it shows a yellowish green to yellow color [20].

Powder made from fresh leaves had a more hue value than powder made from dry leaves. This was due to the uncontrolled degradation of chlorophyll that occurred throughout the drying process, resulting in the formation of gray-colored. If pheophorbide binds to Zn, the resulting compound would be not green. This was in accordance with Canjura et al. [12] and Ngo & Zhao [13] that the regreening effect occurs if Zn-pheophytin, Zn-pyrophytin or Zn-pyrophytin was formed.

Bound Zn content

During the boiling process of fresh and dry leaves in Zn acetate solution, complexes will occur between chlorophyll derivatives and Zn^{2+}. The data in Table 2 showed that the greater the Zn acetate concentration, the greater the Zn content bound to the sambiloto simplicia powder. The Zn content of dried leaf simplicia powder was higher than that of fresh leaves. This was due to the degradation of chlorophyll in dry leaves being more than in fresh leaves so that more complexes were formed. Likewise, Schwartz et al. also showed that the complex between chlorophyll and Zn formed when heating green vegetables in Zn^{2+} solution was Zn-pheofitin or Zn-pyropheophytin [21].

Theoretical calculations show that the Mg^{2+} content bound by chlorophyll was 2,72% of the mass of chlorophyll *a* and 2,68% of the mass of chlorophyll *b* [22]. Analogous to these calculations, the Zn content that could be bound was 7% (70 mg/g) of the mass of chlorophyll *a* and 6,89% (68,9 mg/g) of the mass of chlorophyll b. If viewed from the data in Table 2, the contents of bound Zn range from 110-163 mg/g, thus it was higher than the theoretical ability of chlorophyll to bind Zn, which was 70 mg/g. This was due apart from chlorophyll, other components in the leaves could also bind to Zn. These components include phenolic components [23]–[26], flavonoids [27], cellulose [28], [29], and proteins [30]. The sambiloto simplicia powder formed a metallochlorophyll complex, which was less efficient than the extract due to competition in the binding of Zn^{2+}. This was indicated by a lower regreening effect with a hue value of 104.70-108.62 (see Table 1). The hue value was lower than the result of the formation of metallochlorophyll in pandan chlorophyll extract, namely 120.86-130.17 [14].

Table 1. Color characteristics of sambiloto simplicia powder at various concentrations of Zn acetate and leaf pretreatment

Pretreatment/concentration of Zn acetate (ppm)	Greenness (-a*)	Yellowness (b*)	Hue (H*)
Dried leaves			
0	-1.74±0.10a	10.51±0.01abc	99.40±0.54a
300	-2.85±0.14b	10.86±0.18abc	104.70±0.60b
400	-3.18±0.13c	9.45±0.29a	108.62±1.11d
500	-3.24±0.05c	9.93±0.21ab	108.08±0.46d
Fresh leaves			
0	-3.22±0.26c	11.34±1.68bc	105.96±1.17c
300	-4.24±0.14e	12.70±0.58d	106.60±0.39c
400	-3.57±0.37d	11.98±1.51cd	108.47±0.32d
500	-3.44±0.06cd	10.61±0.81abc	108.03±1.25d

Chlorophyll content

From the information in Table 1, it was clear that the greater the Zn acetate concentration, the greater the total chlorophyll content of sambiloto simplicia powder. The increase in chlorophyll content was due to the Zn-chlorophyll complex that was formed and was detected spectrophotometrically at a wavelength similar to that of chlorophyll. The greater the Zn acetate concentration, the more complexes formed. At a concentration of 400-500 ppm, the total chlorophyll content of sambiloto simplicia powder made from dried leaves was lower than fresh leaves. In other words, the drying treatment causes more chlorophyll degradation. At a concentration of 300 ppm, the chlorophyll content of sambiloto simplicia powder between the dried and fresh leaves was not significantly different.

The highest total chlorophyll content of sambiloto simplicia powder from fresh leaves was at a concentration of Zn acetate of 400 ppm, while the highest concentration of dry leaves was at a concentration of 300 ppm. This shows that at a concentration of Zn acetate of 400 ppm the formation of the Zn-chlorophyll complex has reached a maximum. This was consistent with the results of color analysis which shows that at concentrations above 400 ppm, the increase in green color was not significant. The color of simplicia powder by boiling treatment in 500 ppm Zn acetate medium was not significantly different from that of 400 ppm concentration. When compared with the chlorophyll content of fresh leaves, namely 492.62 ± 4.08 mg/100 g DW, the boiling or blanching process in Zn acetate medium could increase the total chlorophyll retention of sambiloto simplicia powder with a value of 84,90%. In this case, the chlorophyll content of simplicia produced was lower than the chlorophyll content of fresh leaves, possibly due to the dissolution of some chlorophyll in the boiling medium, which was shown from the observation that the boiling medium solution was green.

Total phenolic content

Based on the analysis of total phenolic content, it was known that the greater the Zn acetate concentration, the greater the total phenolic content. Hence, the greater the concentration of Zn acetate, the lower the degradation of the phenolic components. The phenolic content of sambiloto simplicia got closer to the total phenolic content of fresh leaves, namely 653.35 ± 6.89 mg EAG/100 g DW. Similar to the results of the analysis of chlorophyll content, the heating process in aqueous medium only (0 ppm) resulted in a very high degradation of phenolic components. The total phenolic content fell to 225.02±3.73 mg EAG/100 g DW. This lines up with the findings of the study performed by Turkmen et al. [31] which showed that the boiling process in boiling water for 5 minutes could reduce the total phenolic content of peas, squash, and leek ranging from 60-94% compared to fresh leaves. Boiling in Zn acetate medium could increase the stability of the phenolic component because the Zn^{2+} complex with the phenolic component was more stable. The boiling process in Zn acetate medium could increase the total phenolic retention of sambiloto simplicia powder by 73,69%.

Table 2. Characteristics of bound Zn content, total chlorophyll content, total phenolic content, and total flavonoid content of sambiloto simplicia powder at various Zn acetate concentrations and leaf pretreatment

Pretreatment/concentration of Zn acetate (ppm)	Zn content (mg/100 g DW)	Total chlorophyll content (mg/100 g DW)	TPC (mg EAG/100 g DW)	TFC (mg EQ/100 g DW)
Dried leaves				
0	9.11±1.02a	298.26±0.25a	389.18±31.07b	3.20±0.03b
300	110.63±6.16b	349.26±0.62bc	458.61±0.67cd	3.45±0.01bc
400	169.33±6.52c	325.50±0.43ab	458.74±1.602cd	3.51±0.13bc
500	263.00±3.92f	319.35±31.07ab	461.95±3.37cde	3.99±0.76c
Fresh leaves				
0	15.71±4.75a	291.47±14.92a	225.02±3.73a	2.32±0.15a
300	177.09±1.73c	381.10±3.79c	443.14±13.09c	2.93±0.51ab
400	207.66±7.79d	418.24±0.49d	465.41±13.55de	4.06±0.84c
500	226.32±8.85e	351.43±1.88bc	481.45±4.06e	3.44±0.59bc

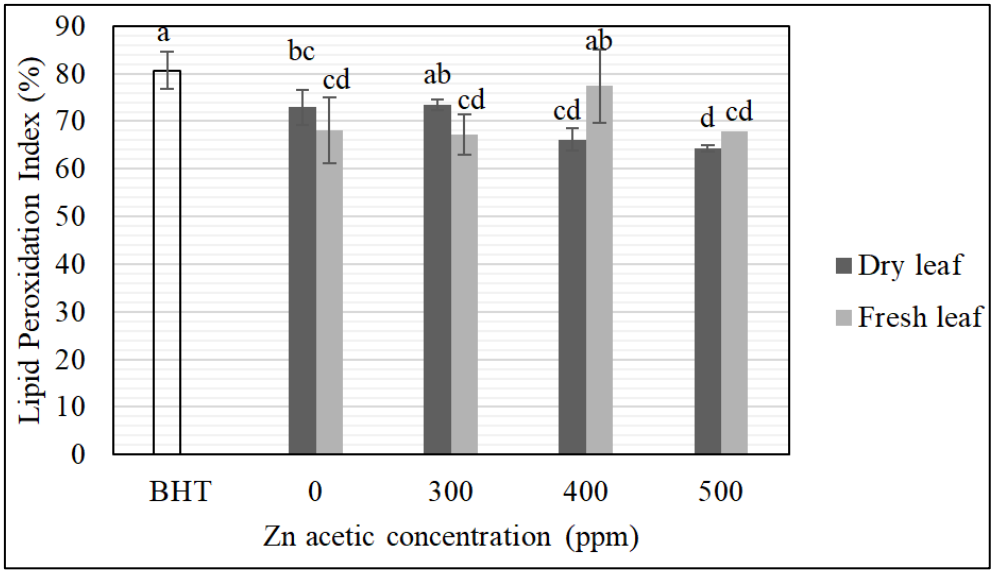

Figure 1. Lipid peroxidation index of sambiloto simplicia with metallochlorophyll complex formation and pretreatment of dry and fresh leaves (the letter a-d shows a significant difference $p < 0.05$)

Total flavonoid content

Similarly, the total flavonoid content of sambiloto simplicia powder increases with increasing Zn acetate concentration. The blanching effect on fresh leaves in aqueous medium (0 ppm) resulted in more flavonoid degradation. As such, boiling in Zn acetate medium could further increase the retention of flavonoids. This was due flavonoids were able to form complexes with Zn^{2+} [32] which was more stable. Flavonoid content in sambiloto simplicia powder at a concentration of 300 ppm was higher than that of the fresh material, although at a concentration of 400-500 ppm, the total flavonoid content was lower. This shows that the fresh material with a Zn ion concentration of 300 ppm has not been able to increase the stability of the flavonoid component.

Antioxidant activity

Figure 1 showed the effect of boiling or blanching in Zn acetate medium at concentrations of 0, 300, 400, and 500 ppm and the different types of material on the Lipid Peroxidation Index (LPI). It was known that the greater the concentration of Zn acetate, the higher the LPI, especially in the fresh leaves material, the highest increase in the percentage of LPI was at a concentration of 400 ppm, while in the dry leaves material the highest LPI was at a concentration of 300 ppm. The increase in lipid peroxidation inhibition was in line with the increase in TPC, TFC and total chlorophyll content. The decrease in the ability to inhibit lipid peroxidation again at a concentration of 400 ppm in the dry leaves material and 500 ppm in the fresh leaves material was due to the increasing number of bound Zn^{2+} ions which would increase lipid peroxidation because metals could function as pro-oxidants. This was in line with Kalinowska et al. [33] which shows that Zn(II) chlorogenic acid has better antioxidant activity than chlorogenic acid alone, but at high concentrations, the pro-oxidant properties increase again.

In contrast to the inhibition of lipid peroxidation, the data in Figure 2 shows that the scavenging power of the DPPH (RSA) sambiloto simplicia in the dry leaves material was higher than that in the fresh leaves. The results showed that in dry leaves material there was no significant difference ($p>0.05$) between concentrations of 0, 300 and 500 ppm. The fresh leaves showed a significant decrease in RSA as the Zn acetate concentration increased from 0 to 500 ppm. The diminishing ability of fresh leaf material to scavenge radicals may be attributed to the progressive formation of highly stable Zn-chlorophyll complexes, which become increasingly abundant over time, thereby reducing the availability of free chlorophyll molecules to participate in radical capture reactions. Endo et al. [34] highlight that the antioxidant activity of chlorophyll and its derivatives was mainly influenced by the presence of the phorpyrin ring, not by the presence of phytol, metal or isocyclic rings. The inhibition of lipid peroxidation was higher because the antioxidant activity of Zn-pheophytin was determined by two molecular structure factors, namely the presence of π-cation radicals in the structure of the porphyrin and the bound metal ion. The π-cation radical would induce electron donation from the porphyrin structure to break the radical chain reaction [34], whereas the presence of bound metal ions would increase the ability to donate electrons by concentrating the electron density towards centrally bound metal ions and away from the porphyrins structure [35].

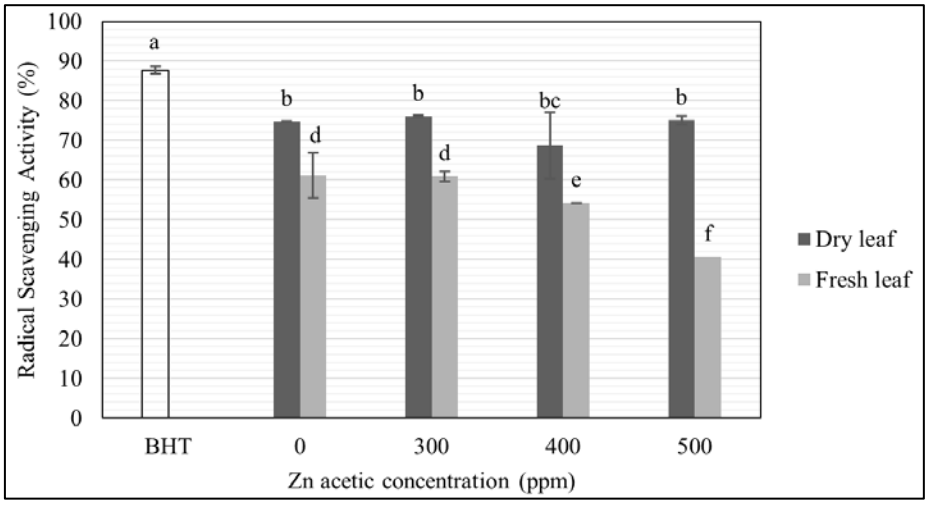

Figure 2. Radical scavenging activity of sambiloto simplicia with the formation of metallochlorophyll complex and pretreatment of dry and fresh leaves (the letter a-f shows a significant difference $p<0.05$)

Table 3. Correlation between Zn acetate concentration, bound Zn content, color, total chlorophyll content, phenolic content, and total flavonoids

	Chlorophyll Contents	**Zn Contents**	**TPC**	**TFC**	**LPI**	**RSA**
Zn Acetate Concentration	0.431*	0.937**	0.685**	0.497**	-0.223	-0.350*
	0.014	0.000	0.000	0.004	0.219	0.049
Chlorophyll Contents		0.321*	-0.008	-0.190	0.084	-0.849*
		0.037	0.966	0.297	0.646	0.000
Bound Zn Contents			0.693**	0.452**	-0.216	-0.317*
			0.000	0.009	0.235	0.047
TPC				0.610**	0.112	-0.211
				0.000	0.543	0.246
TFC					0.396*	0.058
					0.025	0.752
LPI						-0.051
						0.782

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed).

Correlation between parameters

Table 3 showed that the concentration of Zn acetate has a positive correlation with total chlorophyll content, bound Zn content, phenolic content, and total flavonoids, but has a negative correlation with RSA. This indicates that in general an increase in the concentration of Zn acetate would increase the number of complexes between Zn and chlorophyll, phenolics and flavonoids, but this increase results in a decrease in the ability to capture DPPH radicals. Likewise, bound Zn content also positively correlated with chlorophyll, TPC, and TFC contents, but negatively correlated with RSA. Lipid peroxidation inhibition activity was positively correlated with TFC. Hence, during the formation of metallochlorophyll, careful consideration should be given to the concentration of the reagent used, as excessive amounts may attenuate its antioxidant capacity, particularly with regard to its free radical scavenging ability.

4. Conclusions

The success of the metallochlorophyll formation process in sambiloto simplicia powder is proven by the increased contents of bound Zn and total chlorophyll contents. The greater the concentration of Zn acetate as a boiling or blanching medium, the higher the bound Zn, chlorophyll, phenolic and total flavonoid contents. Hence, the greater of Zn acetate concentration, the green color intensity, chlorophyll content, bound Zn content, and total phenolic and flavonoid content of sambiloto simplicia powder increased, but at concentrations more than 300 ppm in dry leaves and more than 400 ppm in fresh leaves it decreased again. The results of the antioxidant activity test show that the ability to inhibit lipid peroxidation of sambiloto simplicia powder in the dry leaves material was higher than in the fresh leaves material. The DPPH radical scavenging capacity of sambiloto simplicia powder in fresh leaves material is higher than in dry leaves material. Simplicia powder from dry leaves by blanching at a concentration of 300 ppm and fresh leaves material at a concentration of 400 ppm had the highest contents of chlorophyll, TPC, TFC, and the ability to inhibit lipid peroxidation. At a concentration of Zn acetate of 300 ppm the simplicia powder from the dry leaves had higher RSA and LPI than the fresh leaves, but at a concentration of 400 ppm the LPI from the fresh leaves was higher.

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Author Contribution

CLS: Conceptualized and designed the study, elaborated the intellectual content, performed literature search, data acquisition, data analysis, manuscript preparation, and manuscript revision.

IAF, EE, N, FXS: carried out experimental studies and manuscript review, elaborated the intellectual content, performed literature search, and manuscript review.

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Conflict of Interest

The authors have no conflict of interest

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