Antioxidant Properties of *Curcuma longa* L. and *Curcuma xanthorriza* Rhizomes

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Abstract: Oxidative stress can lead to tissue damage and result in disease or aggravate existing disease. Antioxidants are required to protect cells from free radical damage. Temulawak (*Curcuma xanthorriza* L.) and turmeric (*Curcuma longa* L.) are natural ingredients with polyphenol compound. Polyphenols has antioxidants that can neutralize free radicals by donating an electron or hydrogen atom. This study was aimed to determine the antioxidant properties of temulawak extract (TLE) and turmeric extract (TE). The antioxidant activity were determined using total phenolic content (TPC), total flavonoid content (TFC), 2,2 diphenyl 1 picrylhydrazyl (DPPH), 2,2′-Azinobis(3-Ethylbenzthiazoline-6-Sulfonate) (ABTS), hydrogen peroxide (H_2O_2), NO (Nitrogen Oxide) scavenging and ferric reducing antioxidant power (FRAP). The result showed that the TPC of value was 10.93 µg GAE/mg extract, and the TFC value was 5.67 µg QE/mg extract. Meanwhile, TPC and TFC value of TLE were 4.83 and 2.68 µg GAE/mg, respectively. The IC_{50} value of DPPH, ABTS, H_2O_2, NO scavenging activity and FRAP activity of TE were 300.7; 39.19; 86.83; 88.03 µg/mL and 493.75 µm Fe (ii)/µg respectively compared to TLE 197.5; 82.55; 205.94; 164.25 µg/mL and 451.00 µm Fe (ii)/µg respectively. Turmeric has higher antioxidant properties than temulawak, both turmeric and temulawak are potential natural antioxidants.

1 INTRODUCTION

Free radicals are a highly unstable substance. Free radicals are generated in the body due to metabolic processes or environmental factors like industrial chemical exposure, X-ray exposure, smoking, ozone, and air pollution (Lobo et al., 2010). If free radicals are present in the human body, they can bind with other molecules to become stable, allowing these molecules to become free radicals (Phaniendra et al., 2015). As a result of this chain reaction, cells, tissues, and organs are damaged. Antioxidants can donate electrons to free radicals, causing oxidative stress through free radical chain reactions. Lipid peroxidation is caused by free radicals, which destroys liver cells. Antioxidants can minimize cell

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damage caused by the oxidative process, making them hepatoprotective (Lobo et al., 2010). However, synthetic antioxidants such as Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ) can give some side effects such as skin allergies, gastrointestinal tract, and even cancer (Caleja et al., 2017; Lourenço et al., 2019; Wang & Kannan, 2019).

In Asia, the Zingiberaceae family is the most commonly grown crop. This plant is beneficial to human health as a source of food, spices, dyes, food colouring, and herbal medicine. Some of the Zingiberaceae family are turmeric (*Curcuma longa* L.) and temulawak (*Curcuma xanthorriza* L.). Turmeric and temulawak are both available and can be consumed as a beverage or used as a cooking spice. Turmeric and temulawak have been shown in previous studies to have various health benefits, including anti-inflammatory, antibacterial, antioxidant, and hepatoprotective properties (Cavaleri, 2018; Lukitaningsih, 2020). Curcuminoid compounds in turmeric and temulawak (curcumin, demethoxycurcumin, bisdemethoxycurcumin) are the main components that function as antioxidants.

This research has done as preliminary data to prove turmeric extract (TE) dan temulawak extract (TLE) as antioxidants potential and this research will be continued to prove TE dan TLE with Good Manufacturing Practice (GMP) as hepatoprotective potential.

This study was aimed to determine the antioxidant properties of TLE and TE using method of 2,2 diphenyl 1 picrylhydrazyl (DPPH), 2,2′-Azinobis(3-Ethylbenzthiazoline-6-Sulfonate) (ABTS), hydrogen peroxide (H_2O_2), NO (Nitrogen Oxide) scavenging activities and ferric reducing antioxidant power (FRAP) potential.

2 METHODS (AND MATERIALS)

2.1 Samples

Temulawak and turmeric were extracted with 70% ethanol solvent. The standardized extract powder of turmeric and temulawak were produced based on current Herbal Good Manufacturing Practices by FAST Co. (Jakarta, Indonesia).

2.2 Total Phenolic Content

The total phenolic content (TPC) was determined using method described by Prahastuti and Utami with slight modification (Prahastuti et al., 2020; Utami et al., 2018). A 0,015 mL standard gallic acid (Sigma 398225) solution in 6 concentration level (50.00 - 1.56 µg/mL) and sample of TE and TLE in concentration of 2000; 1000; and 500 µg/mL were added into well in 96-well plate, respectively. Then, added 60 µl of Na_2CO_3 7.5% (Merck A897992745) and 75 µl Folin- Ciocalteu reagent 10% (Merck 1.090.010.500) into well. The mixed solution was incubated at 50ºC for 10 minutes, then the absorbance was measured in a wavelength of 760 nm using a microplate reader (Multiskan Go Reader, Thermo Fisher Scientific 1510). The phenolic content (TPC) calculation was compared to the gallic acid linear regression using equations 1.

$$y = 0.0429x + 0.152 \quad (1)$$

2.3 Total Flavonoid Content

The total flavonoid content (TFC) was performed using an $AlCl_3$ colorimetric assay method described by Prahastuti and Utami with slight modification (Prahastuti et al., 2020; Utami et al., 2018). An amount of 75 µL standard quercetin (Sigma Q4951) solution in 7 concentration level (500.00 - 7.80 µg/mL) and TE and TLE in concentration of 2000 and 1000 µg/mL, were added into well respectively and each well was mixed with 75 µl $AlCl_3$ 2% (Merck 449598). Using microplate reader (Multiskan Go Reader, Thermo Fisher Scientific 1510), the absorbance was measured in 415 nm of wavelength. The concentration of flavonoid content was calculated from calibration linear regression equation 2.

$$y = 0.0095x+0.037 \quad (2)$$

2.4 DPPH Free Radical Scavenging Assay

The antioxidant activity using DPPH free radical scavenging assay was perfomed using method described by Prahastuti and Widowati with slight modification (Prahastuti et al., 2020; Widowati et al., 2018). An aliquot of 0.05 mL of TE and TEE samples solution was poured into well respectively, then 200 µL of DPPH solution (D9132, Sigma Aldrich, Missouri, USA) was added to each well. The mixture was incubated at the dark room temperature for 30 mins. The absorbance was measured at 517 nm by the microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Massachusetts, USA). The IC_{50} of free radical

inhibition activity calculation was obtained from the Equation 3 scavenging activity.

$$\% \text{ scavenging} = \frac{Ac-As}{Ac} \times 100 \quad (3)$$

Ac : negative control absrobance
As : sample absorbance

2.5 FRAP Assay

The antioxidant activity using FRAP assay was perfomed using method described by Prahastuti and Widowati with slight modification (Prahastuti et al., 2020; Widowati et al., 2018). The FRAP reagent (mixture of 10:1:1 of 300mM sodium acetate buffer, pH 3.6; 10mM 2,4,6-tris(2-pyridyl)-1,3,5-triazine in 40mM HCl; 20mM $FeCl_3.6H_2O$), 142.5µL and 7.5µL of TE and TLE samples respectively were mixed into 96 well plate then incubated at 37°C for 30 min. The absorbance of the mixture was measured at 760 nm by microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Massachusetts, USA). FRAP analysis was measured by comparing a linear regression equation of $FeSO_4.7H_2O$ standard solution.

2.6 ABTS Reducing Activity Assay

The antioxidant activity using ABTS assay was perfomed using method described by Ginting, Prahastuti and Widowati with slight modification (Ginting et al., 2020; Prahastuti et al., 2020; Widowati et al., 2018). The solution of ABTS was made by reacting 14 mM 2,2'-Azino-bis (3-ethylbenzothiazoline-6- sulphonic acid)(ABTS•+) [Sigma Aldrich A1888-2G, USA] with 4.9 mM potassium persulfate [Merck EM105091, USA] in 1:1 volume ratio, for 16 h at the dark room temperature. Then, the mixture was diluted with 5.5 mM PBS (pH 7.4) until the solution's absorbance was was 0.70 ± 0.02 at 745 nm. The 2 µL of samples were added into microplate of 96 well, followed by 198 µL of ABTS solution. The mixture was then incubated at 30º C for 6 min and measured by microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Massachusetts, USA) at 745 nm. ABTS-reducing activity was then used to measure the median inhibitory concentration (IC50). The equation of ABTS reducing activity was calculated with equation 4.

$$\% \text{ reducing activity} = \frac{Ac-As}{Ac} \times 100 \quad (4)$$

Ac : control absorbance
As : sample absorbance

2.7 H_2O_2 Scavenging Activity Assay

H_2O_2 scavenging activity was performed using phenanthroline method by Mukhopadhyay et al. (2016) with slight modification (Mukhopadhyay et al., 2016). The samples (TE and TEE) 60 µL was added into plate of 96 well, respectively and followed 12 µL of 1mM Ferrous ammonium sulfate (215406, Sigma Aldrich. Then, mixed with 3 µL of 5mM H_2O_2 (1.08597.1000, Merck). The mixture were incubated at dark room temperature for 5 min. Then, 75µL of 1mM 1,10-phenanthroline (131377, Sigma Aldrich) was added to the mixture and incubated for 10 min at the dark room temperature. The mixture absorbance was measured by microplate reader at 510nm wavelenght. The IC_{50} scavenging activity of H_2O_2 was calcuated by Equation 3.

2.8 No Scavenging Activity Assay

The antioxidant activity using NO assay was perfomed using method described by Utami with slight modification (Utami et al., 2018). An amount of 10 µL of samples (TE and TLE, respectively) in various concentrations were mixed with 40 µL 10 mM sodium nitroprusside (106541, Merck, Germany) in phosphate buffered saline (PBS) (1740576, Gibco, Canada). Then the mixture was incubated at room temperature for 2 hours followed by the addition 100µL of Griess reagent (1:1 of 1% sulfanilamide [Merck 111799, Germany] in 2% H_3PO_4 [Merck 100573, Germany] and 0.1% N-(1-napththyl) ethylenediamine dihydrochloride) [Sigma 222488, USA]). The formation of chromophore absorbance due to diazotization of nitrite with sulfanilamide and coupling of Naphthylethylenediamine dihydrochloride was measured by a microplate reader (Thermo Scientific Multiscan GO) at a wavelength of 546 nm. The scavenging activity of NO was calculated by Equation 3.

3 RESULTS AND DISCUSSION

3.1 Total Phenolic Content and Total Flavonoid Content of TE and TLE

Curcuma longa and *C. xanthorrizha* are a rhizomes having a yellow or an orange color due to the present of curcuminoids (Rafi et al., 2015). This pigment belongs to the family of flavonoid and flavonoid is one group of polyphenols. The precence

of secondary metabolites phenolic and flavonoid in the *Curcuma longa* and *C. xanthorrhiza* were further used for the standardization of the extract by using determination of phenolic and flavonoid content (Ab Halim et al., 2012).

Total phenolic content and total flavonoid content of the sample TE and TLC are as shown in Table 1.

Table 1: The Average of TPC and TFC of TE and TLE.

Sample	TPC (µg/mg extract)	TFC (µg/mg extract)
TE	3.90 ± 0.04	1.86 ± 0.36
TLE	5.92 ± 0.33	2.68 ± 0.46

Note: The data was given in mean±SD, n=3

TLE had a higher polyphenol content, namely 5.92 ± 0.33 µg / mg, compared to TE (3.90 ± 0.04 µg / mg). Synchronous results were found on flavonoid levels. The level of flavonoids in TLE (2.68 ± 0.46 µg / mg) was higher than in TE (1.86 ± 0.36 µg / mg).

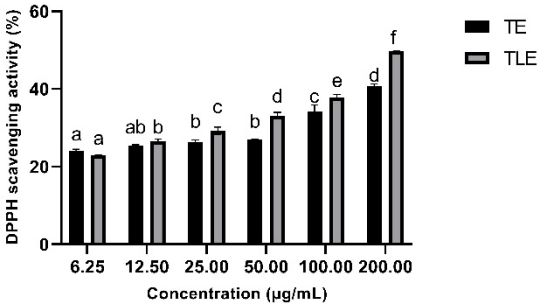
The aim of TPC was to bind phenolic compounds to a blue complex formed by Folin Ciocalteu's reagent. Using the gallic acid calibration curve, total polyphenols were measured (Prahastuti et al., 2020) . The analysis show that TPC of TLE is higher than TE.

The theory behind total flavonoid content is that $AlCl_3$ forms acid-stable complexes with flavonoid's keto groups and either hydroxyl groups, while it binds to curcuminoids through the β-diketon group (Indira Priyadarsini, 2013). The analysis show that theTFC of TE is less than TLE.

The quantity of polyphenol content of TE and TLE depend on sources, method of extraction, phytogeographic region, and time of the collection of rhizome (Adaramola & Onigbinde, 2017; Akinola et al., 2014).

3.2 The Antioxidant Capacity of TE and TLE using DPPH Assay

The results of antioxidant capacity of using DPPH Assay are obtained as shown in Figure 1 and Table 2. The color change purple to yellow that occurs when free radicals interact with antioxidant compounds in the extract, which is then measured using a spectrophotometer, is the basic concept of this process (Kedare & Singh, 2011). The reactive group in DPPH (1,1-diphenyl-2-picrylhydrazyl) is a nitrogen atom that forms a stable DPPH radical with the antioxidant's hydrogen atom (1,1-diphenyl-2-pikrihildrazil). The analysis that have been done show that TE has less DPPH scavenging activity than TLE, according to research findings. In the previous research (Widowati et al., 2011), the lowest DPPH scavenging activity was TE than TLE with the scavenging capacity 8.33 µg/mL and 39.58 µg/mL respectively. The turmeric extract was strongest antioxidant than temulawak (Widowati et al., 2011). The different of quantity of scavenging capacity of TE and TLE in our previous research in 2011 with this research due to the different method of extraction and the addition lactose in powder extract.



*Data are presented as means ± standard deviation, differences letter (a,ab,b,c,d) for TE and differences letter (a,b,c,d,e,f) for TLE show significant differences among concentrations at P <0.05 (Tukey HSD post hoc test)

Figure 1: DPPH scavenging activity of TE and TLE.

Table 2: The IC_{50} value of DPPH scavenging activity of TE and TLE.

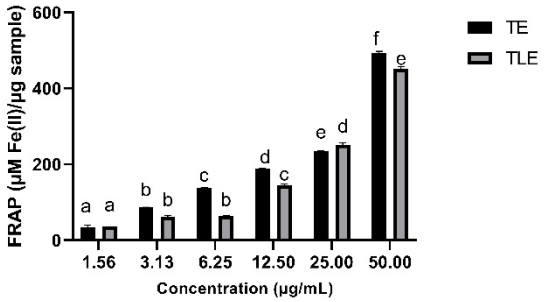
Sample	Equation	R^2	IC_{50} (µM)
TE	y = 0.0866x + 23.945	0.98	300.87
TLE	y = 0.1274x + 24.838	0.97	197.50

A lower IC_{50} correlate better with higher DPPH radical scavenging activity, which represents the concentration of the extract to decrease 50% of the DPPH solution initial absorbance. Antioxidant potency is usually associated with the content of phenolic compounds due to their extensive conjugated π-electron systems that facilitate the donation of electrons from the hydroxyl moieties to oxidizing radical species (Pratami et al., 2018).

3.3 The Antioxidant Capacity of TE and TLE using FRAP Assay

The FRAP assay determines the test sample's reducing potential by using antioxidants in the sample as reductants in a redox reaction. Antioxidants break the chain reaction of radicals by donating electrons or hydrogen atoms to the ferric complex, which then converts to the ferrous complex (Fe^{3+} to Fe^{2+} - TPTZ complex) (Bolanos De La Torre et al., 2015). The absorbance calculation can be linked to antioxidant activity and shows the amoun of Fe^{2+} that have been decreased (Al-Salahi et al., 2018). An increase in

absorbance indicates a high reducing power. Based on the research that have been done, the FRAP activity of TE, TLE respectively 493.75; 451.00 µm fe (II)/µg at the highest concentration (50 µg/mL) (Figure 2).



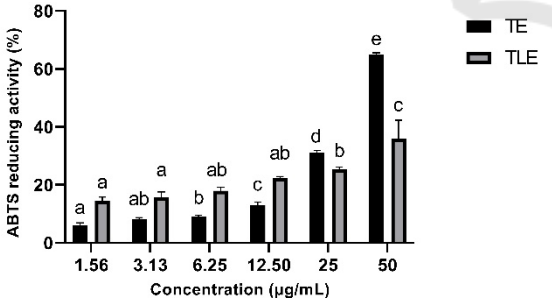
*Data are presented as means ± standard deviation, differences letter (a,b,c,d,e,f) for TE and differences letter (a,b,c,d,e,) for TLE show significant differences among concentrations at P <0.05 (Tukey HSD post hoc test)

Figure 2: FRAP activity of TE and TLE.

The reducing power obtained for the TE is greter than TLE. The reducing power capacity of the samples is probably due to the phytochemical compounds present in TE and TLE.

3.4 The Antioxidant Capacity of TE and TLE using ABTS Assay

The research show that TE has better antioxidant capacity with higher ABTS scavenging activity and lower IC_{50} compare to TLE (Figure 3 and Table 3).



*Data are presented as means ± standard deviation, differences letter (a,ab,b,c,d,e,) for TE and differences letter (a,ab,b,c) for TLE show significant differences among concentrations at P <0.05 (Tukey HSD post hoc test)

Figure 3: ABTS scavenging activity of TE and TLE.

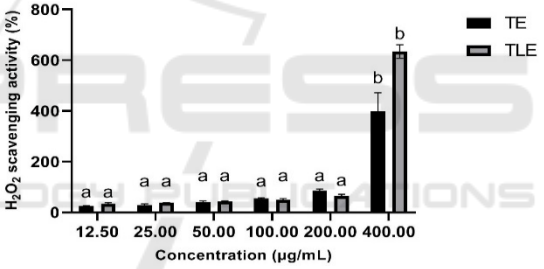
Table 3: The IC_{50} value of ABTS scavenging activity of TE and TLE.

Sample	Equation	R^2	IC_{50} (µM)
TE	y = 1.2269x + 1.9177	0.99	39.19
TLE	y = 0.4243x + 14.975	0.98	82.55

The ABTS assay determine the antioxidant capacity of samples by donating hydrogen to cation radical, then the solution become colorless (Pisoschi & Negulescu, 2011).

3.5 The Antioxidant Capacity of TE and TLE using H_2O_2 Assay

The result of antioxidant capacity of TE and TLE using H_2O_2 assay was shown in Figure 4 and Table 4. In H_2O_2 assay, the reaction between ferrous ammonium and phenantroline was inhibited by the presence of H_2O_2. Thus, it can determine the antioxidant capacity of the sample against H_2O_2. (Pisoschi & Negulescu, 2011). Turmeric has lower IC_{50} and higher ABTS scavenging activity. It means that TE has higher antioxidant capacity than TLE.



*Data are presented as means ± standard deviation, differences letter (a,b) for TE and differences letter (a,b) for TLE show significant differences among concentrations at P <0.05 (Tukey HSD post hoc test)

Figure 4: H_2O_2 scavenging activity of TE and TLE.

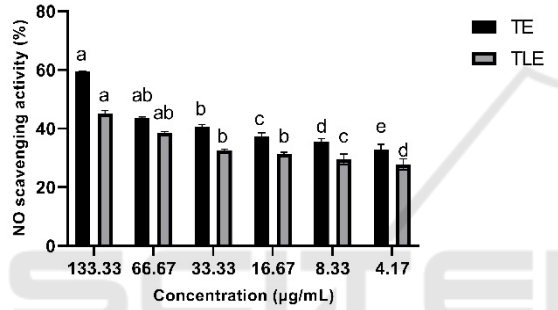
Table 4: The IC_{50} value of H_2O_2 scavenging activity of TE and TLE.

Sample	Equation	R^2	IC_{50} (µM)
TE	y = 0.3135x + 22.779	0.99	86.83
TLE	y = 0.0806x + 33.401	0.99	205.94

In our previous research the H_2O_2 scavenging activity of TE was greater than and TLE extract, with the value *C. longa* 55.82% than *C. xanthorrhiza* 49.04% (Widowati et al., 2011). The turmeric extract was strongest antioxidant than temulawak by using H_2O_2 scavenging assay.

3.6 The Antioxidant Capacity of TE and TLE using NO Scavenging Assay

The result of antioxidant capacity of TE and TLE using NO scavenging assay was shown in Figure 5 and Table 5. Determination of antioxidant by NO scavenging assay have been done. Specific nitric oxide synthases catalyze a biochemical reaction that creates NO in biological tissues, which is the basis of this assay. In buffered saline, sodium nitroprusside reacts with oxygen to form nitrite ions, which can be measured with Griess reagent (Alam et al., 2013). The higher NO scavenging activity and lower IC_{50} indicates that the sample has better antioxicant activity. The result show that turmeric has higher antioxidant activity than temulawak.



*Data are presented as means ± standard deviation, differences letter (a,ab,b,c,d,e) for TE and differences letter (a,ab,b,c,d) for TLE show significant differences among concentrations at $P < 0.05$ (Tukey HSD post hoc test)

Figure 5: NO scavenging activity of TE and TLE.

Table 5: The IC_{50} value of NO scavenging activity of TE and TLE.

Samples	Equation	R2	IC_{50} (µM)
TE	y = 0.1912x + 33.169	0.98	88.03
TLE	y = 0.1312x + 28.449	0.98	164.26

3.7 The Comparison of Antioxidant Capacity of TE and TLE

Curcumin, from family Zingiberaceae, has an unique conjugated structure that show a typical radical trapping ability as a chain-breaking antioxidant, including two methoxylated phenols and an enol form of β-diketon (Nurrochmad, 2004). The antioxidant activity of TE and TLE are due to curcuminoids (Widowati et al., 2011). Curcuminoid is a phenolic group, has benzene ring. Thus, it can function as free radical scavengers. A phenolic antioxidant has a distinct hydroxyl group (–OH) attached to its composition's benzene loop (Asouri et al., 2013). When reactive oxygen species (ROS) are present at a specific concentration, they affect the function of electron-releasing substituents contained as substituents on the phenyl ring in phenolic antioxidants. The O–H bond is broken as a result, and the hydrogen ion is released. This hydrogen ion was made accessible to nucleophilic free radicals, quenching their reactive tendencies in the process (Malik & Mukherjee, 2014). Previous studies have shown that the phenolic hydroxyl and the methoxyl groups on the phenyl ring and the 1,3-diketone system are essential structural features for antioxidant activity.

The reason that curcumin elicited the higher total phenolic content maybe since it contains two phenolic groups (Sepahpour et al., 2018). The number of phenolic groups present in an antioxidant molecule structure is not always the only factor to determine its antioxidant activity. Positions of the phenolic groups, presence of other functional groups in the molecules such as double bonds, and conjugation to phenolic and ketone groups also play essential roles in antioxidant activities (Borra et al., 2013).

Based on 7 antioxidant methods namely: total phenolic content (TPC), total flavonoid content (TFC), 2,2 diphenyl 1 picrylhydrazyl (DPPH), 2,2′-Azinobis (3-Ethylbenzthiazoline-6-Sulfonate) (ABTS), hydrogen peroxide (H_2O_2), NO (Nitrogen Oxide) scavenging and ferric reducing antioxidant power (FRAP) that have been done, TE have better antioxidant activity compared to TLE. This is due to curcuminoids in TLE is less than turmeric. Previous study have shown that curcumin in turmeric is 74.57%, while in TLE is 20.04 mg/g (Kusuma, 2012). Curcumin is strong anti-oxidant and anti-inflammatory effects and thus it possesses hepatoprotective properties. The damage cells in the liver is due to lipid peroxidation mechanism. Antioxidant inhibit lipid peroxidation and enhance antioxidant enzyme. Thus, it prevent the damage of cells in the liver.

4 CONCLUSIONS

In conclusion, turmeric has higher antioxidant properties than temulawak, but both turmeric and temulawak are potential natural antioxidants.

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