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Antioxidant Activity of TEMON (*Clitoria ternatea* and *Citrus* sp.) as an Infused Herbal Tea

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ABSTRACT

Many people around the world believed that herbal tea had a lot of antioxidants contained in it, thus it was widely drunk all over the world. To increase the taste aromatic, color appearance, we combine taste and color, resulted tea with new color and taste and have high antioxidant. This research was conducted to evaluate antioxidant activities of butterfly flower or kembang telang (*Clitoria ternatea*), lemon (*Citrus sp*), combination of telang and lemon called TeMon. This study examines the antioxidant activity of herbal tea made from dried lemon (*Citrus sp.)* and telang (*C. ternatea*), telang tea, lemon tea. The antioxidant potential was assessed by performing on 2,2-diphenyl-1picrylhydrazyl (DPPH) and hydrogen peroxide (H_2O_2) scavenging activity assay, total phenolic and flavonoid content assay, 2,2-Azinobis 3-ethyl benzothiazoline 6-sulfonic acid ($ABTS^{\bullet+}$) reduction, and Ferric Reducing Antioxidant Power (FRAP) assay. Dried tea namely telang tea of 5 flower bud, lemon tea 5 g and mix of 5 flower bud telang and lemon 2 g (TeMon) were soaked in 200 ml hot water for 5 min resulted telang infusion, lemon infusion and TeMon infusion. All samples telang infusion, lemon infusion and TeMon infusion were assayed the antioxidant activity including scavenging activity of DPPH, H_2O_2, ABTS, FRAP assay and phenolic, flavonoid content. The median inhibitory concentration (IC_{50}) was used to measure the antioxidant activity. The IC_{50} value of DPPH scavenging activity of telang tea, lemon tea, TeMon tea were 17.07%, 25.34%, 28.66 % respectively. The IC_{50} value for H_2O_2 scavenging activity respectively were 26.62%, 38.60%, 15.98%. The IC_{50} value of ABTS scavenging activity respectively were 2.51%, 3.50%, 3.27%. The IC_{50} value of FRAP assay respectively were 5.56%, 10.84 %, 12.50%. The flavonoid content respectively were 16.20 µg QAE/100% sample; 0.82 µg QAE/100% sample; 2.97 µg QAE/100% sample. The phenol content respectively were 4.88 µg GAE/100% sample; 0.19 µg GAE/100% sample; 0.28 µg GAE/100% sample. Telang tea contain highest flavonoid, phenol, have highest antioxidant compared with TeMon and lemon tea. TeMon tea show lower antioxidant activity compared with telang tea but TeMon have tasty and attractive color.

Keywords: Antioxidants; Herbal Tea; *Citrus* sp.; *Clitoria ternatea;* Flavonoids

INTRODUCTION

Tea is the world's second most popular beverage after water, and a range of variables, including its refreshing flavor, enticing aroma, and potential health advantages, are thought to play a part. According to Quispe *et al*. (2012), there is a worldwide trend toward increased consumption of herbal teas because supplementing the human diet with herbal teas delivers a high concentration of antioxidant chemicals that may be advantageous. Furthermore, as technology has advanced and people's time has become more limited, they have begun to seek out convenient herbal products. Herbal tea has been used for thousands of years in many countries for health care and disease prevention (Zhao *et al*., 2013) because, as refer to Tschiggerl and Bucar (2012), herbal teas are easy to produce, mild in action, in most circumstances, have minor negative effects, as well as being inexpensive and abundant in resources.

Citrus fruits are well-known for their nutritional and health-promoting properties. This reputation stems from decades of research into the biological activities of phytochemicals found in citrus fruits and their derivatives. Citrus fruits are widely grown in both tropical and subtropical regions of the world, as well as in many other areas, with an annual production capacity of approximately 102 million tons (Mehl *et al*., 2014). Citrus fruits are popular among consumers all over the world due to their appealing colors, pleasant flavors, and aroma. Citrus fruits have now become a major nutritional source of nutrients for the

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Chinese people, thanks to increased production, advancements in storage and processing processes, and the attainment of a year-round supply. Citrus fruits' antioxidant activity and role in the prevention and treatment of numerous chronic and degenerative diseases in humans have received a lot of attention in recent years.

Clitoria ternatea Linn (Fabaceae) is a high nutrient legume that is widely used as a cattle fodder plant in many regions (Gomez and Kalamani, 2003). All components of the plant (roots, seedlings, and leaves) are used for medicinal purpose and have been found to improve cognitive performance and reduce dementia, cure respiratory illnesses including asthma and bronchitis, reduce inflammation, and work as a laxative and diuretic (Devi *et al*., 2003; Jain *et al*., 2003). Numerous investigations on the therapeutic characteristics of this plant have been done, with much of this study focusing on the study of *C. ternatea* neurological function. However, little of it is known about the extracts' additional actions or the flower's activities. The flowers contain anthocyanins and some other flavonoids that may be useful in medication, notably as antioxidants. (Kazuma *et al*., 2003).

A bioactive compound's ability to sustain cell structure and function by effectively removing free radicals, suppressing lipid peroxidation events, and preventing other oxidative damage is referred to as antioxidant activity (Widowati *et al*., 2018; Prahastuti *et al*., 2020). It also serves as the basis for many other biological functions, along with anti-cancer, anti-inflammation, and anti-aging (Cai *et al*., 2004; Ke *et al*., 2015). More importantly, antioxidant activity has been related to the avoidance of a wide range of chronic disorders, including cancer, diabetes, and cardiovascular disease (Rajendran *et al*., 2014; Yu *et al*., 2005). As a result, a thorough investigation of natural antioxidants found in fruits and vegetables, is important for human health.

Butterfly pea flowers are used as ornamental plants because of their attractive colors. Telang have various name, depending on the region such as blue flowers, teleng flowers for Java, talang flowers for Sulawesi and bisi for Maluku, kelenit flowers and telang flowers for Sumatra (Fitrilia *et al*, 2020). One of the unique characteristics of butterfly pea flower is bluish-purple at a low acidic condition, The combination of exotic color and health benefits will promote telang as functional drink (Marpaung *et al*., 2020).

Telang have various bioactivity, attractive blue color, but telang less tasty or tasteless this research was conducted to improve the taste and attarctive color, we combine dried telang and dried lemon called TeMon tea resulted purple color, little bit sour taste and fresh taste.

METHODOLOGY

Materials

The fresh specimen of *Citrus sp.* was collected from Pasar Sederhana Bandung, West Java, Indonesia, *C. ternatea* was collected from Kampung Herbal Desa Sukolilo, Prigen, Pasuruan, East Java, Indonesia. The specimens used in this study were lemons harvested at the age of 8 months after the flowers bloom or ripe lemons on the tree. As well as the telang flower specimens used are 49-73 days old flowers and harvested in the morning when the flowers are in full bloom.

Methods

Specimen Preparation

The fresh specimen of *Citrus sp.* was washed in the flowing water and then being manually thinly sliced. Both lemon (*Citrus sp.*) and telang (*C. ternatea*) were dried using food dehydrator at 50ºC for about 72 hours lemon and 36 hours telang until each slice and bud were fully dried and contained low water content (13-15%).

Formulation and TeMon Infusion

Dried lemon 2 g combined with 5 flower buds of telang were infused using 200 mL boiling hot water and then being left for 5 minutes until its ready to be used and consumed.

Total Phenolic Content Assay

The phenolic content was determined using a minor alterations to the Folin-Ciocalteu method. In the sample well, up to 15 µL of sample was inserted and mixed with up to 75 µL of 10 percent Folin-reagent Ciocalteu's and 60 µL of 7.5 percent sodium carbonate. The blank solution was made by combining 135 µL of 10% DMSO with 15 µL sample. It was then warmed for 10 minutes at 50ºC. In addition, the absorbance at 760 nm was measured using a microplate reader. Gallic acid equivalence (GAE) in g/mg sample was used to calculate total phenolic content. This test was repeated three times. (Nurhayati *et al*., 2018; Rusmana *et al*., 2017; Widowati *et al*., 2018; Prahastuti *et al*., 2019).

Total Flavonoid Content Assay

The altered previous method was used to determine flavonoid content (Kalita *et al*., 2013; Prahastuti *et al*., 2019). In this method,

6 concentration levels of a standard solution of sample at 200 µg/mL as well as 15 µL was used. It was blended with 75 µL $AlCl_3$ at a 2 percent concentration. The absorbance was measured at 415 nm wavelength. The linear regression equation was constructed using the standard (quercetin) absorbance value (y = 0.0174x + 0.0209). The flavonoid content of the sample was determined using a standard linear regression equation. The result of flavonoid content was expressed in terms of quercetin equivalence (QE) in µg/100% concentration of sample tea. The experiment was carried out in triplicate.

DPPH Scavenging Assay

a microplate reader spectrophotometer (Thermo Scientific MultiskanTM GO Microplate Spectrophotometer). The sample received a total of 200 µL of 0.0777 mmol 2,2-diphenyl-1-picrylhydrazil (DPPH) (Sigma Aldrich, D9132) in methanol in the 96-well microplate, with concentrations ranging from 2.5 % to 20 %L. It was then incubated at room temperature for 30 minutes. The absorbance value was then measured using a microplate reader at a wavelength of 517 nm using microplate reader spectrophotometer (Thermo Scientific MultiskanTM GO). The negative control solution contained 250 µL of DPPH, while the blank solution had 50 µL of the sample and 200 µL of DMSO (Merck, 1029522500) (Widowati *et al*., 2018). The DPPH scavenging activity was calculated using the formula below.

$$\text{DPPH scavenging activity (\%)} = \frac{A-B}{A}\ x\ 100$$

A: control solutions absorbance; B: sample absorbance

ABTS Reduction Assay

The free radical test $ABTS^+$ 2, 2-Azinobis - (3 ethylbenzothiazoline -6- sulfonic acid) diammonium salt was used to measure the ABTS reduction assay. ABTS (2,2'-Azinobis (3-ethylbenzo thiazoline-6-sulfonic acid) reagent was checked first, as much as 2 µL of the test sample was added to a 96-well plate followed by 198 µL of ABTS reagent, 200 µL of ddH2O was added to the control well and 200 µL of ABTS reagent was added to the blank well. The microplate was incubated for 10 min at 37°C. The absorbance was calculated with a wavelength of 745 nm. And for sample, 198 µL of ABTS and 2 µL of sample were used, whereas for the negative control, 200 µL of ABTS was being used, and for the blanks, 200 µL of an absolute ddH_2O was utilized. The following formula was used to calculate the ABTS decrease percentage:

$$\text{ABTS reduction activity (\%)} = \frac{A-B}{A}\ x\ 100$$

A: control absorbance; B: sample absorbance

H_2O_2 Scavenging Assay

The scavenging of H_2O_2 was measured using previous method (Prahastuti *et al*., 2020). Every sample well consist 60 µL sample, 12 µL ferrous ammonium sulphate (1mM, Sigma Aldrich 7783859), and 3 µL H_2O_2 (5mM, Merck 1.08597). Briefly 12 µL of ferrous ammonium sulphate and 63 µL of ddH2O were utilized for the negative control, the blanks were made with only 150 µL of ddH2O.

After adding H_2O_2, control, sample, and blank solutions were added to the 96-well plate and incubated for 5 minutes at room temperature in a dark room. After adding 75 µL of 1, 10-phenanthrolines to the sample and control solutions, they were incubated for 10 minutes in a dark room at room temperature. At 510 nm, the absorbance value was measured. The scavenging activity percentage was calculated as follows:

$$H_2O_2 \text{ scavenging activity (\%)} = \frac{A}{B}\ x\ 100$$

A: control absorbance; B: sample absorbance

FRAP Assay

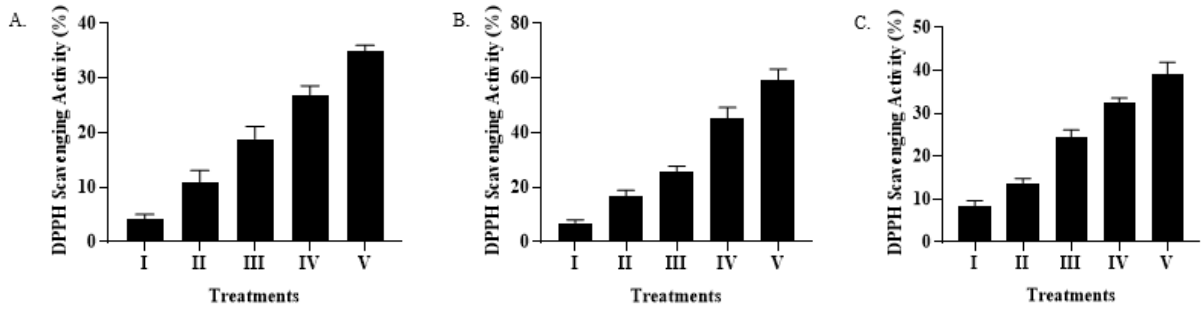
FRAP reagent consist 10 mL of 300 mM acetate buffer, 1 mL of ferric chloride hexahydrate, 20 mM diluted in distilled water, and 1 mL of 2,4,6-Tris-(2-pyridyl-5-Triazine) (TPTZ) (Sigma Aldrich, T1253). In a 96-well microplate reader, 7.5 µL of the sample was combined with 142.5 µL of FRAP reagent, then incubated at 37°C for 30 minutes. A microplate reader was used to detect the absorbance of samples at a wavelength of 593 nm (Prahastuti *et al*., 2020).

$$\text{FRAP activity (\%)} = \frac{A}{B}\ x\ 100$$

A: control absorbance; B: sample absorbance

Statistical Analysis

The results data were statistically analyzed in the SPSS program using analysis of variance (One Way ANOVA) and post hoc Tukey's Honest Significant Difference test (version 25). The goal of these investigations was to calculate notable differences in sample concentrations. The IC_{50}



* All data are presented in mean±standard deviation. Each sample was diluted in ddH_2O to create the final concentration: I: 2.5%, II: 5%, III: 10%, IV: 15%, and V: 20%. The assay was done triplicate for each treatment.

Figure 1. Effects of different concentrations on DPPH scavenging activities: TeMon tea (A), Telang tea (B), Lemon tea (C)

Table I. Phenolic and Flavonoid content TeMon tea, Telang tea, Lemon tea

Samples	Flavonoid Content (µg QE/100% concentration of tea infusion)	Phenolic Content (µg GAE/100% concentration of tea infusion)
Telang tea	4.88	16.20
Lemon tea	0.19	0.82
TeMon tea	0.28	2.97

from each assay (DPPH, H_2O_2, ABTS scavenging) was calculated based on the curve standard.

RESULT AND DISCUSSION

Result

Total Phenolic Content

The studies demonstrated that the extract contained a significant concentration of phenolics which was 2.97 µg GAE/100% concentration for TeMon, 16.20 µg GAE/100% concentration for telang, and 0.82 µg GAE/100% sample for lemon (Table I). The highest phenolic content was telang tea and the lowest was lemon tea.

Total Flavonoid Content

Its quantification revealed a significant concentration of flavonoids in each sample, which was 0.28 µg QE/100% concentration for TeMon, 4.88 µg QE/100% concentration for telang, and 0.19 µg QE/100% concentration for lemon (Table1). The highest flavonoid content was telang tea, moderate flavonoid was TeMon tea and the lowest flavonoid content was lemon tea.

DPPH Scavenging Activity

The IC-50 value is the concentration at which an antioxidant can scavenge 50% of the DPPH free radical; higher antioxidant activity is defined by a smaller IC_{50} value. In this study, the IC_{50} of TeMon was 28.66 %, while telang had an IC_{50} of 17.07 %, and lemon had an IC_{50} of 25.34 % concentration. Telang was found to be the most active of DPPH scavenging activity with the smallest IC_{50} value (Table II) and the DPPH scavenging activity (Figure 1), higher concentration of sample increased the DPPH scavenging activity.

ABTS Reducing Activity

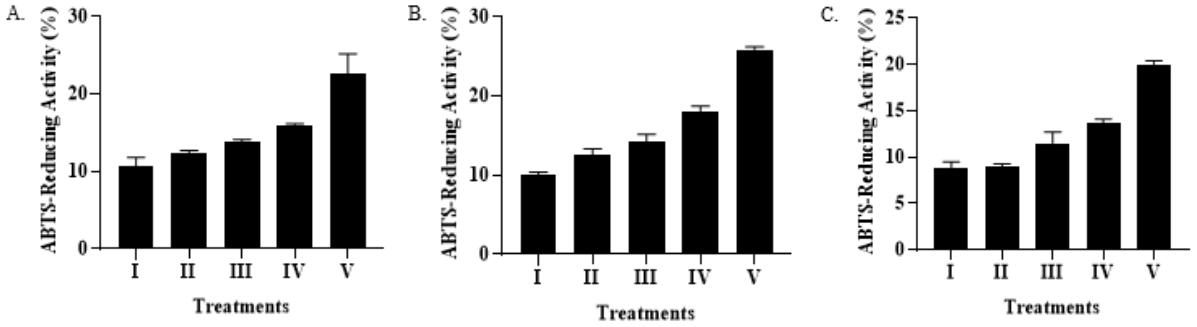
The ABTS-reducing activity percentages of TeMon, telang, and lemon were compared, which telang tea had the highest activity (Figure 2). The IC_{50} values were 3.27 %, 2.51 % and 3.50 % concentration, respectively (Table II). Higher concentration of tea sample increased ABTS reducing activity.

H_2O_2 Scavenging Activity

Table II shows the IC_{50} of TeMon, telang, and lemon in H_2O_2 radical scavenging activity. TeMon, telang, and lemon have IC_{50} values of 15.98 %, 26.62 % and 38.60 % concentration, respectively. Figure 4 further demonstrates that TeMon tea had highest activity compared telang tea and lemon tea.

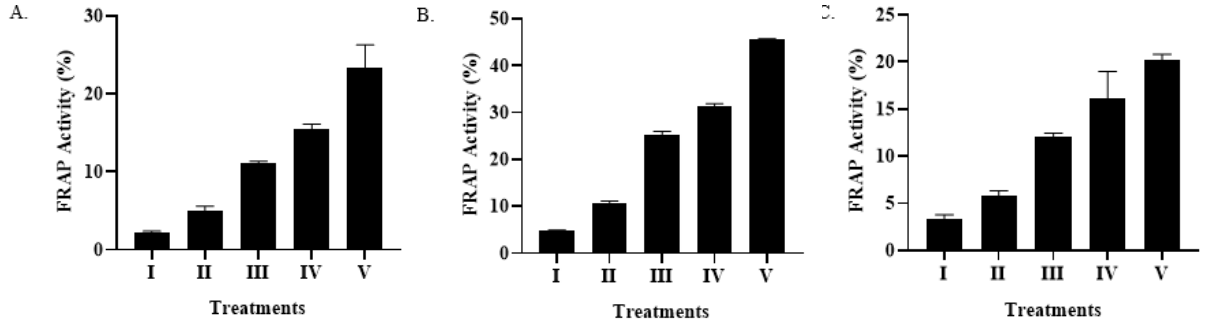
FRAP Activity

Table II shows the most active of FRAP activity was telang tea with IC_{50} 5.56 % mean while TeMon tea had IC_{50} 10.84 %, and lemon tea 12.50 % concentration. Figure 3 depicts the FRAP



* All data are presented in mean±standard deviation. Each sample was diluted in ddH_2O to create the final concentration: I: 6.25%, II: 12.5%, III: 25%, IV: 50%, and V: 100%. The assay was done triplicate for each treatment.

Figure 2. The IC_{50} values representing the antioxidant activites of TeMon tea, Telang tea, Lemon tea



*All data are presented in mean±standard deviation. Each sample was diluted in 10% ddH_2O to create the final concentration: I: 0.63%, II: 1.25%, III: 2.5%, IV: 3.75%, and V: 5%. The assay was done triplicate for each treatment.

Figure 3. Effects of different sample concentrations on FRAP activities: TEMON (A); *Clitoria ternatea* (B); *Citrus sp.* (C).

Table II. The IC_{50} values representing the antioxidant activites of TeMon tea, Telang tea, Lemon tea

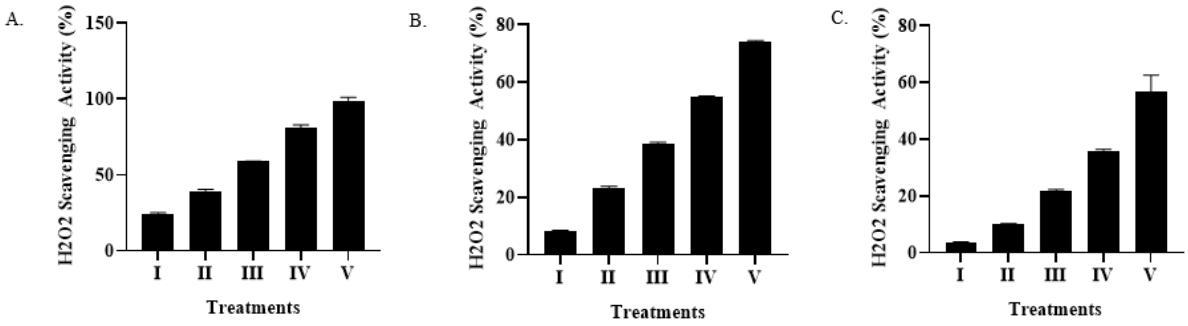
Assays	Samples	Linear Equation	R^2	IC_{50} (%)
DPPH Scavenging Activity	TeMon	y = 1.6987x + 1.3102	0.99	28.66
DPPH Scavenging Activity	Telang	y = 2.9396x - 0.1648	0.98	17.07
DPPH Scavenging Activity	Lemon	y = 1.7769x + 4.971	0.99	25.34
H_2O_2 Scavenging Activity	TeMon	y = 2.1029x + 16.399	0.99	15.98
H_2O_2 Scavenging Activity	Telang	y = 1.8076x + 1.8895	0.99	26.62
H_2O_2 Scavenging Activity	Lemon	y = 1.4228x - 4.9205	0.99	38.60
ABTS Reduction	TeMon	y = 12.108x + 10.392	0.99	3.27
ABTS Reduction	Telang	y =16.02x + 9.9844	0.99	2.51
ABTS Reduction	Lemon	y = 12.02x + 7.9517	0.99	3.50

*The data are presented in mean±standard deviation. The assays were run in triplicate. The coefficient of regression (R^2) and the IC_{50} of each sample were calculated by linear regression.

activity of TeMon, telang, and lemon tea. Higher concentration of sample increased FRAP activity.

Discussion

Total phenolics were determined using a colorimetric method adapted from a prior work. This was found based on entangling phenolic compounds with a blue complex created unanimously by reducing Folin-reagent. Ciocalteu's The total polyphenols were determined using the Gallic acid calibration curve (Widowati *et al*., 2018; Prahastuti *et al*., 2020). Phenolics of plants correlate with antioxidant activity (Turumtay *et al*., 2014), free radicals process was



*All data are presented in mean±standard deviation. Each sample was diluted in 10% ddH_2O to create the final concentration: I: 5%, II: 10%, III: 20%, IV: 30%, and V: 40%. The assay was done triplicate for each treatment.

Figure 4. Effects of different sample concentrations on H2O2 activities: TeMon tea (A), Telang tea (B), Lemon tea (C)

inhibited by the presence of phenolic and flavonoid compound (Aryal *et al*., 2019).

The total flavonoid content is determined according to the aluminium chloride ($AlCl_3$) colorimetric method (Kumar *et al*., 2013). Flavonoid compounds as antioxidants because of their ability to change and reduce the disease risk which can be caused by free radicals (Aryal *et al*., 2019). The flavonoids capacity as antioxidants affected by their molecular structure. The position of hydroxyl (OH) groups, other features of chemical structure are important for antioxidant, free radical scavenging activities (Kumar *et al*., 2013).

To measure antioxidant activity, various assays have been utilized, but the most generally used approaches are the formation of free radical species and their neutralization with antioxidant of chemical or pant extract. An unpaired electron resulted in the stable free radical DPPH. DPPH is a nitrogen-centered stable free radical that is commonly used to attribute radical scavenging activity to chemicals or plant extracts (Kedare and Singh, 2011; Sasikumar and Kalaisezhiyen, 2014). In the presence of a hydrogen donor, it pairs and decreases absorbance at 517 nm (Widowati *et al*., 2016). During the DPPH test, the antioxidant sample converted stable DPPH radicals to yellow-colored diphenyl picrylhydrazine (DPPH-H). The DPPH free radical is commonly utilized as a substrate in studies of antioxidant function (Widowati *et al*., 2016).

The activity of the ABTS-reducing assay measures the antioxidant's relative ability to scavenge the ABTS produced. In this work, ABTS was created through a reaction between a strong oxidant and an ABTS salt. The ABTS radical blue-green solution was reduced by a hydrogen-donating antioxidant and evaluated at a long-wave absorption spectrum (Widowati *et al*., 2016; Widowati *et al*., 2016; Prahastuti *et al*., 2018).

H_2O_2 is involved in a variety of *in vivo* processes such as energy production, phagocytosis, intercellular signal transfer, cell growth control, and the manufacture of vital biological components (Packer *et al*., 2008). H_2O_2 is produced and increased as a consequence of regular aerobic metabolism during illnesses, workouts, and stressful situations (Mukhopadhyay *et al*., 2016; Prahastuti *et al*., 2020).

The FRAP procedure was based on the inhibition of the ferroin analog TPTZ3+ in acidic media to the colored Fe2+ complex of Fe $(TPTZ)^{2+}$ (extremely blue) by antioxidants The absorbance of the Fe (II) complex leads in a reduction in the equivalent tripyridyltriazine Fe (III) complex at 593 nm. (Widowati *et al*., 2018; Prahastuti *et al*., 2020).

The result data showed that telang tea contain phenolic total content this result was in line with previous research that butterfly pea flower produced with a volume of 55.834 mL, contain 0.874 mg/mL of total phenolic content, and 10.42 mg/L of total anthocyanins (Izza & Tristantini, 2021). The total phenolic content was 1.9 mg/g extract as gallic acid equivalents (Kamkaen and Wilkinson, 2009). Blue color anthocyanins of *C. ternatea* flower is one anthocyanin source containing stable blue color polyacylated anthocyanins. The anthocyanins in telang increased the functional properties such as antioxidant and antimicrobial properties (Gamage *et al*., 2021).

Telang tea had antioxidant activities including DPPH, ABTS, H_2O_2 scavenging activity and FRAP capacity, this results were validated with previous research that *C. ternatea* extract could

protect canine erythrocytes from hemolysis and oxidative damage induced by 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) (Fitrilia *et al*., 2020). Methanol extract of the butterfly pea flower compound as an antioxidant in inhibiting ROS and the most potential to inhibit ROS, was caffeine (Fitrilia *et al*., 2020). DPPH scavenging activity methanol extract was more active than ethanol extracts (IC_{50} values were 1 mg/mL and 4 mg/mL, respectively) (Kamkaen, and Wilkinson, 2009). Total anthocyanin, the antioxidant activity of the butterfly pea flower drink remained stable during storage for 4 weeks (Marpaung *et al*., 2020).

The result data showed that lemon tea contained phenolic and flavonoid (Table I), had antioxidant activities (Table II), this results were in line with previous study that both two citrus cultivars including *Citrus sinensis* 'Siavaraz' and *Citrus limon* 'Lisbon' have total flavonoid content and total antioxidant capacity (TAC) (Mohammadian *et al*., 2011). *C. limon* 'Lisbon' possessed higher total flavonoid content and antioxidant activity based on the DPPH assay (Mohammadian *et al*., 2011). Total phenolic content of the *Citrus spp*. 66.5 to 396.8 mg GAE/g of extract and flavonoids content varied from 0.3 to 31.1 mg QE/g of extract (Mohammadian *et al*., 2011).

TeMon tea consist of telang tea and lemon tea had moderate antioxidant activities compared to telang tea which has highest antioxidant activities and lemon tea had lowest antioxidant activities toward DPPH, ABTS, H2O2 scavenging activities and FRAP capacity. Telang tea contained the highest phenolic and flavonoid total and followed by TeMon tea, lemon tea.

CONCLUSION

TeMon tea consist of telang tea and lemon tea had moderate antioxidant activities compared to telang tea which has highest antioxidant activities and lemon tea had lowest antioxidant activities toward DPPH, ABTS, H_2O_2 scavenging activities and FRAP capacity. Telang tea contained the highest phenolic and flavonoid total and followed by TeMon tea, lemon tea.

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