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Total phenolic and flavonoid contents and antioxidant activity of red ginger (*Zingiber officinale* var. *rubrum*) rhizomes from seven different regions in the island of Java, Indonesia

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Abstract

Red ginger from different growth locations with diverse elevation and geographic conditions may possess different levels of phenolic, flavonoid and antioxidant activity. Different altitudes and soil characteristics in the island of Java, Indonesia, are also one of the factors which can affect that level. This research aimed to study the differences in phenolic, flavonoid, and antioxidant activity of red ginger's methanolic extracts from seven different regions in Central Java, Special Region of Yogyakarta, and East Java, Indonesia. The seven samples were collected from a different altitude range, from 10 to 1300 m above sea level. The rhizomes were macerated with methanol, and the evaporated extracts were tested for total phenolic content, total flavonoid content, DPPH radical-scavenging and FRAP assay. All tests were performed in triplicate, and the data were statistically analysed with one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at a probability level of 0.05. The result showed that red ginger from Kajoran-Magelang-Central Java has the highest total phenolic content with 195.25 µg/mL GAE, and the lowest was from Lendah-Kulonprogo-Yogyakarta with 130.77 µg/mL GAE. The highest total flavonoid content was possessed by the sample from Bumiaji-Batu-East Java with 11.62 µg/mL QE, and the lowest was Pakem-Sleman-Yogyakarta with 4.74 µg/mL QE. The DPPH and FRAP assays showed a similar antioxidant activity exhibited by the sample from Lendah-Kulonprogo-Yogyakarta. This sample showed the lowest IC_{50} to inhibit DPPH radical activity at 10.02 µg/mL and the highest ferric-reducing activity at 54.52 µg/mL. The results showed that the altitude is relatively not to affect the level of phenolic, flavonoid, and antioxidant activities. The weak correlations among those parameters indicate that there may be other factors that independently influence the bioactive compound accumulation in red ginger rhizomes.

1. Introduction

Ginger belongs to the Zingiberaceae family with the scientific name *Zingiber officinale* Roscoe. It is a Monocotyledoneae plant with various rhizome morphologies. Common ginger was categorized into three varieties based on the color and size of the rhizome. There are big or giant white ginger (*Z. officinale* var. *officinale*), small white ginger or emprit ginger (*Z. officinale* var. *amarum*), and red ginger (*Z. officinale* var. *rubrum*) (Zhang *et al.*, 2022). Red ginger is well-known by several names in some countries, like stem ginger in England; kanchiang, chiang, and ganjiang in China; zingibil and zenjabil in Arabic; also, jahe merah in Indonesia (Suciyati and Adnyana, 2017). It is an annual plant, growing up to 50-100 cm tall, with 5-25 cm long, lancet-shaped leaves. It stems grow perpendicular and are flat without branches. The flowers are ovoid and crowned with a purple crown. Its fleshy rhizomes are thick with reddish-brown skin. The single

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root gets bigger along with its age to form the rhizome and shoot to grow into a new individual plant. The roots grow from the bottom while the buds grow from the top of the rhizomes (Supu *et al.*, 2018).

Generally, red ginger is known as one of the herbal medicines in Asia to relieve some health problems, such as coughs, sore throats, fever, flu, chills, sinusitis, motion sickness, arthritis, muscular pains and aches, colic, diarrhea, loss of appetite, indigestion, and also for several infectious diseases (Ma and Gang, 2006). Zingiberaceae, including red ginger, has medicinal properties as antioxidant, antimicrobial, anti-inflammatory, antitumorigenic, antihyperglycemic, hepatoprotective activities, and many others. Red ginger contains some pungent and biologically active compounds, 1.5-3% essential oil, 2-11% fixed oil, 8-10% ash, 9-12% water, and 40-70% starch (Ghasemzadeh *et al.*, 2015; Jan *et al.*, 2022). One of the bioactive compounds in red ginger's rhizomes, known as the chief pharmacologically active phenolic compound with antioxidant activities, is 6-gingerol (Alsahli *et al.*, 2021). Several gingerols, like 6-, 8-, 10, and 12-gingerol, are possessed by red ginger, but the most abundant and biologically active is 6-gingerol. Gingerol forms through intermediates of the phenylpropanoid pathway (Rani *et al.*, 2022). Red ginger is a rich source of antioxidant and antimicrobial polyphenols and flavonoids (Jan *et al.*, 2022).

The level of phenolic and flavonoid content in red ginger is affected by various factors, including environmental factors like the technique of cultivating, harvesting, post-harvesting process (Styawan *et al.*, 2022), planting location, weather, soil characteristics, climate, air temperature, and area humidity. Due to those factors, red ginger from different locations with diverse elevation and geographic conditions may possess different levels of antioxidant activity, phenolic, and flavonoid content. Those secondary metabolites naturally occur in red ginger's rhizomes, which provide a defense mechanism against abiotic stress, pathogen attack, and other environmental conditions without affecting the plant's normal growth and development (Tiwari *et al.*, 2023).

Red ginger is propagated vegetatively through its rhizomes and can be grown at various altitudes up to 1500 m above sea level with well-distributed rainfall. In general, irrigated and rain-fed conditions along with well -drained, clay, sandy, red, or lateritic soil are the most suitable for its cultivation (Jabborova *et al.*, 2022). Finding the best combination of environmental conditions and plant care systems is necessary to improve the production of secondary metabolites in red ginger. Luckily, Indonesia has diverse topography, including on the island of Java. Different altitudes and soil characteristics may affect the level of phenolic, flavonoid, and antioxidant activity of red ginger among different regions in Java. This research provided a study about the differences in phenolic, flavonoid, and antioxidant activity of red ginger's methanolic extracts from seven different regions in Central Java, Special Region of Yogyakarta, and East Java. Hopefully, we will find the most active red ginger rhizomes to further study their potency as a traditional herbal medicine.

2. Materials and methods

2.1 Plant materials

Healthy fresh rhizomes were collected from seven different regions in Java. Those seven regions are Bumiaji-Batu-East Java (BB), Sumowono-Semarang-Central Java (SS), Kaloran-Temanggung-Central Java (KT), Pakem-Sleman-Yogyakarta (PS), Kajoran-Magelang-Central Java (KM), Pengasih-Kulonprogo-Yogyakarta (PK) and Lendah-Kulonprogo-Yogyakarta (LK). All of those seven locations were at different altitudes above sea level. The rhizomes were cleaned, cut into thin slices, and then dried in a dryer cabinet for 48 h based on procedures from (Nurdyansyah *et al.*, 2023) with modifications.

2.2 Extraction process

The dried rhizomes were then ground and sifted through a 60 mesh sieve to obtain powdered dried rhizomes. Methanol (Merck) was added to the powdered sample (1:10) for each sample, for 24 h maceration with two re-macerations. The macerated samples were then filtered for residue separations. The filtrates were evaporated with a vacuum rotary evaporator (DLab) at 40°C and 40 rpm to concentrate the extract. The yield percentage of the extract was calculated using the equation below, based on (Putra *et al.*, 2021):

$$Yield\ percentage\ (\%) = \frac{Weight\ of\ extract\ obtained}{Weight\ of\ sample} \times 100$$

The drying loss of the extracts was measured according to the procedure stated in the Indonesian Herb Pharmacopoeia from the Ministry of Health, Republic of Indonesia (Hikmawanti *et al.*, 2021).

2.3 Measurement of total phenolic content

The total phenolic content (TPC) of red ginger extracts was determined with Folin-Ciocalteu method according to Bong *et al.* (2021) with some modifications. A 250 µL of either gallic acid as standard or extract was mixed with 200 µL Folin-Ciocalteu reagent (Merck). This solution was then incubated at room temperature for 5 min. Then, 2 mL of 7% sodium carbonate (Na_2CO_3)

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(Merck) was added into the solution and the aquadest was added to final volume of 5 mL. The mixed solutions were incubated for 30 min at room temperature. The absorbances were measured at 730 nm with UV-Vis spectrophotometer (Hitachi UH5300). The calibration curve (0-300 µg/mL; y = 0.0029x + 0.0071; R^2 = 0.979) was plotted using gallic acid (Sigma-Aldrich) as the standard. The TPC level was expressed as µg of gallic acid equivalent per mL. The measurement was performed in triplicate.

2.4 Measurement of total flavonoid content

The total flavonoid content (TFC) of red ginger extracts was determined with aluminium chloride method as described by Bong *et al.* (2021) with some modifications, using quercetine as standard. A 500 µL of either quercetine or extracts was added to 1.5 mL methanol (Merck). Then, 100 µL of 10% aluminium chloride hexahydrate ($AlCl_3$) (Merck) and 100 µL of 1M sodium acetate trihydrate (Merck) were added to the solution. The aquadest was added to final volume of 5 mL. The mixed solutions were incubated for 30 min at room temperature. The absorbances were measured with UV-Vis spectrophotometer (Hitachi UH5300) at 428 nm. The calibration curve (0-30 µg/mL; y = 0.0268x + 0.0136; R^2 = 0.9967) was plotted using quercetine (Sigma) as the standard. The TFC level was expressed as µg of quercetine equivalent per mL. The measurement was performed in triplicate.

2.5 Measurement of antioxidant activity

2.5.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay

The free radical scavenging activity of seven extracts were determined according to Ghasemzadeh *et al.* (2015) with some modifications. The 3.9 mg of DPPH was dissolved in 100 mL of methanol to give final concentration of 0.1 mM. Following that, 4 mL of DPPH solution was added to different concentration of red ginger extracts (5, 10, 15, 20, and 25 µg/mL) and the methanol was added to final volume of 5 mL. The mixture was homogenized gently and incubated at room temperature in the dark for 30 min. Methanol was used as a blank. The absorbance of the samples was read with UV-Vis spectrophotometer (Hitachi UH5300) at 516 nm. The measurement was performed in triplicate. The scavenging activities was calculated using the following formula:

$$\%\ inhibition = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} \times 100$$

The % inhibitions were then used to calculate the IC_{50} based on equation from calibration curve.

2.5.2 Ferric reducing antioxidant power assay

FRAP assay was performed according to Maryam *et al.* (2018) with modifications. The stock solutions included 0.2 M phosphate buffer (2 g sodium hydroxide in 250 mL CO_2-free aquadest and 6.8 g potassium dihydrogen phosphate in 250 mL CO_2-free aquadest), pH 6.6, 1% $K_3[Fe(CN)_6]$, 0.1% $FeCl_3$, and 10% trichloroacetic acid (TCA). The 5 mg extracts were added to 5 mL of ethanol (Merck). The 1 mL of extract solution was added with 1 mL phosphate buffer and 1 mL of 1% $K_3[Fe(CN)_6]$, incubated for 20 mins at 50°C. After the incubation, 1 mL of TCA was added to the solution, and then centrifuged for 10 min at 3000 rpm. The upper layer of the solution was pipetted (1 mL), then added with 1 mL of aquadest and 0.5 mL of 0.1% $FeCl_3$, incubated for 10 min at room temperature. The absorbances were measured with a UV-Vis Spectrophotometer (Hitachi UH5300) at 720 nm. The measurement was performed in triplicate.

2.6 Statistical analysis

All data were analyzed with one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test at a probability level of 0.05. The analysis was performed with SPSS version 24.

3. Results and discussion

All data are represented in Table 1, which shows the yield of all seven extracts, the drying loss, the total phenolic and flavonoid content, and also the IC_{50} of the DPPH assay and the FRAP activity.

3.1 The extract yields

The extraction process for the seven samples of red ginger was performed by maceration with methanol (Merck). Extraction is one important step to separate the bioactive compounds of natural raw materials. The extraction process was carried out in several steps, which include penetration of solvent into the solid matrix of the samples, dissolution of solute in the solvents, diffusion of solute out of the solid matrix, and collection of extracted solutes (Zhang *et al.*, 2018). Once the extraction process is done, its characterization is important in order to determine the composition and quality (Barbero, 2021).

The rhizome extract of red ginger, as shown in this research, was a brown, thick paste with a ginger scent. The pungent scent was still lingering, indicating that the compounds for pungent odor still exist. The highest yield was obtained from sample BB, and the lowest was from LK. The extract yields from those seven samples of red ginger range from 12 to 22% (Figure 1). Those yields

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Table 1. The yield, drying loss, total phenolic content, total flavonoid content, IC_{50} of DPPH assay, and FRAP activity of seven red ginger extracts from seven different regions.

Samples	Extract yield (%)	Drying loss (%)	TPC (µg/mL)	TFC (µg/mL)	DPPH IC_{50} (µg/mL)	FRAP activity (µg/mL)
BB	22.12	33.13	178.47^{bc}	11.62^{d}	20.48^{e}	22.20^{a}
SS	20.97	29.67	181.34^{bc}	11.37^{d}	16.42^{d}	28.74^{b}
KT	21.73	26.81	186.06^{bc}	6.11^{b}	15.07^{c}	45.61^{c}
PS	21.45	22.07	171.23^{b}	4.74^{a}	16.83^{d}	32.20^{b}
KM	20.42	37.15	195.25^{c}	6.87^{b}	12.50^{b}	45.81^{c}
PK	16.34	32.25	168.59^{b}	9.02^{c}	16.53^{d}	23.77^{a}
LK	12.77	25.48	130.77^{a}	6.74^{b}	10.02^{a}	54.52^{d}

Values with different superscripts in the same column are statistically significantly different ($p<0.05$).

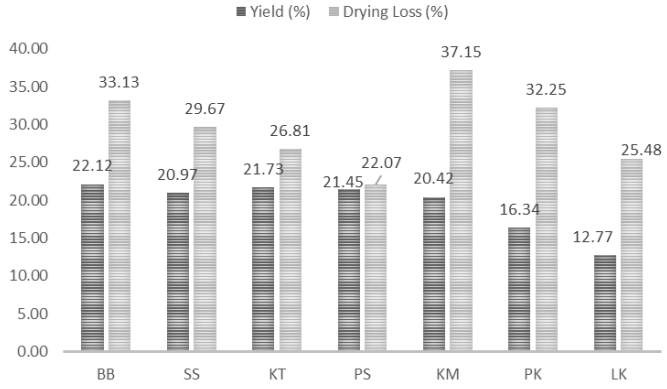

Figure 1. The yield and drying loss of seven samples, with the highest yield possessed by sample BB from Bumiaji-Batu-East Java and the lowest yield is LK from Lendah-Kulonprogo-Yogyakarta. The highest drying loss is in samples KM from Kajoran-Magelang-Central Java, and the lowest is in PS from Pakem-Sleman-Yogyakarta.

were obtained from 100 g of powdered dried rhizomes dissolved in 1 L of methanol. The methanol was used because it is a universal solvent for phytochemical investigation. Solvents with a polarity value near to the polarity of the solute perform better, and vice versa. The extraction efficiency is also influenced by particle size. Small particle size is known to be an enhancer for extraction efficiency (Zhang *et al.*, 2018).

Red ginger with many volatile compounds was suitable enough to be extracted by maceration. This technique is convenient for thermolabile plant material (Abubakar and Haque, 2020). The extraction efficiency increases due to the increase in extraction duration. However, the increasing time will not affect the extraction efficiency after the solute reaches equilibrium. The greater the solvent-to-solid ratio, the higher the extraction yield is (Zhang *et al.*, 2018).

For the drying loss, sample PS has the lowest and KM has the highest drying loss among the seven samples, that is 22.07% and 37.15%, respectively (Figure 1). The drying loss value describes the amount of water loss during heating at 105°C for several hours until a constant weight (Hikmawanti *et al.*, 2021). Although the LK has the lowest yield, it has a small drying loss value after PS, that is 25.48%. The higher drying loss value indicates that the sample has high water content, evaporated during the 105°C heating. The three highest drying loss values were KM, BB, and PK, respectively. Those indicate higher water content compared to others.

3.2 Total phenolic content

TPC of seven samples were measured spectrophotometrically. The highest TPC was possessed by KM with 195.25 µg/mL GAE, and the lowest was LK with 130.77 µg/mL GAE. The TPC of the seven samples are presented in Figure 2. This research showed that altitude did not affect the TPC level. The highest TPC level is the extract of red ginger's rhizome (KM) cultivated at a medium altitude that is 578 m above the sea level, and it is not significantly different from the rhizome which was cultivated at 1300 (BB), 955 (SS), and 715 m (KT) above the sea level. The significant differences are shown among KM, PK, and LK with TPC levels at 195.25, 168.59, and 130.77 µg/mL GAE, respectively. The extract from PS is not significantly different from PK, although the two growth locations have very different altitudes, 600 and 33 m above sea level.

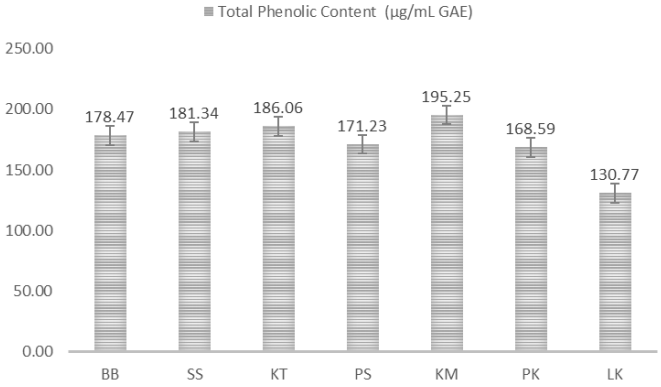

Figure 2. The total phenolic content of seven red ginger extracts was compared with gallic acid standard. The highest level is sample KM from Kajoran-Magelang-Central Java, and the lowest is LK from Lendah-Kulonprogo-Yogyakarta.

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The findings from this research are different from previous studies in *Acacia* honey samples, which reported that the honey collected from high altitude exhibited a higher mean of TPC and TFC. But, still in the same study, for *Zizipus* honey, the TPC and TFC mean values are higher in the honey from a lower altitude (Suleiman *et al.*, 2020). The lack of altitude influence on TPC was also reported by Gülsoy *et al.* (2023), who studied the Turkish hazelnut cultivars. They said that the altitude had a relatively limited impact on the TPC, even though the TPC was gradually decreased with the increase of elevations, which was also supported by the study from Nataraj *et al.* (2022).

The polyphenols in plants are mostly derived from the shikimic acid pathway. It is known to have antioxidant properties due to the presence of -OH groups in its chemical structure (Bong *et al.*, 2021). The phenolic compounds are generally produced as defense mechanism against pathogen attack or in order to respond to the stressful environment. They are not directly related to the growth and development of plant tissues (Ali *et al.*, 2018). The TPC has also been used for early screening of antioxidant activity in plants (Rachkeeree *et al.*, 2020). However, the antioxidant activity of natural products is not only influenced by TPC but also by some other bioactive compounds which activated during the activity to combat free radicals. The TPC obtained in this research may have been influenced by the characteristics of each location where the red gingers were cultivated.

3.3 Total flavonoid content

The largest part of polyphenol compounds is represented as flavonoids (Mošovská *et al.*, 2015), which have a basic flavone structure. They occur in plant stems and fruits in quite high amounts, and have been reported to have several functions, such as antioxidant and anti-inflammatory activities (Nam *et al.*, 2017). In this research, the result showed that the highest TFC was possessed by BB, and the lowest was PS. The TFC of BB (11.62 µg/mL QE) was not significantly different to SS (11.37 µg/mL QE), but it was different to PK (9.02 µg/mL QE), KM (6.87 µg/mL QE), LK (6.74 µg/mL QE), KT (6.11 µg/mL QE), and PS (4.74 µg/mL QE) as shown in Figure 3. The three samples, KM, LK, and KT, showed nearly similar TFC with no significant differences. Those may be influenced by a similar type of soil for cultivation.

BB has the highest TFC because the red gingers were grown under the pine trees surrounding the waterfall. The pine trees may act as a natural threat, so the red gingers were stimulated to produce more flavonoid compounds as defense mechanism. The TFC of BB was not significantly different from SS, but it was significantly different from the other five samples, significantly. Some samples with no significantly different TFC from one another are KM, LK, and KT. It showed that the KM, LK, and KT may face a similar environmental condition, so the production of flavonoid was nearly similar.

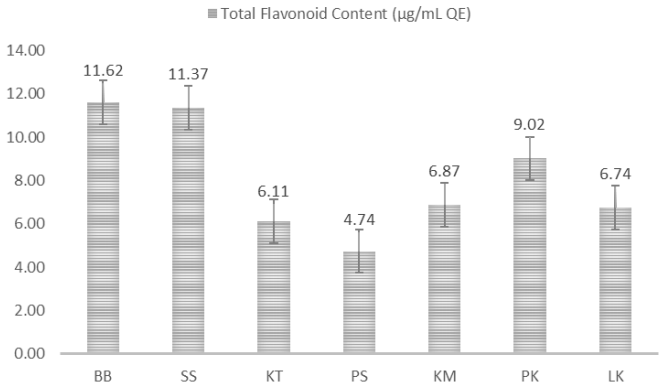

Figure 3. The total flavonoid content of seven red ginger extracts with quercetin standard. The highest level is sample BB from Bumiaji-Batu-East Java, and the lowest is PS from Pakem-Sleman-Yogyakarta.

The BB samples were cultivated at 1300 m above sea level, which is the highest altitude among the collected samples. The highest TFC levels of BB were supported by Suleiman *et al.* (2020), who reported that *Acacia* honey samples collected from higher altitudes possessed higher mean TFC values.

3.4 Antioxidant activity

3.4.1 DPPH radical scavenging activity

The DPPH assay is one of the spectrophotometric methods that is mostly applied to measure the antioxidant activity in natural products, including red ginger extract. This assay promotes the measurement of the free radical (DPPH) ability to react with hydrogen donor (Tena *et al.*, 2020). Antioxidants delay and prevent the oxidation of oxidizable substances, so oxidative stress can be reduced (Kadum *et al.*, 2019). In this research, the LK possessed the smallest IC_{50} of radical scavenging activity (10.02 µg/mL), which is significantly different from the other six samples.

The IC_{50} of all samples from the lowest to the highest, in order, are LK, KM, KT, SS, PK, PS, and BB, with the levels being 10.02, 12.50, 15.07, 16.42, 16.53, 16.83 and 20.48 µg/mL, respectively (Figure 4). It relatively fits the previous study by Gülsoy *et al.* (2023), who reported that the Turkish hazelnut cultivated at a lower altitude exhibited higher DPPH activity than those cultivated at higher altitudes. However, Pandey *et al.* (2018) reported that the extract of *Thalictrum foliolosum* from higher altitudes possessed higher scavenging activity than samples from lower altitudes.

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Although the LK has the lowest TPC and middle TFC, it has the highest radical scavenging activity among others. The other bioactive compounds may provide this activity, so the radical scavenging activity was not only supported by phenolic and flavonoid compounds but also by other compounds. The LK's radical scavenging activity was correlated to the FRAP activity (Figure 4), which was also shown as the highest among others. It strengthens the fact that LK is proven to possess high antioxidant activity, although the TPC and TFC are a low and middle levels.

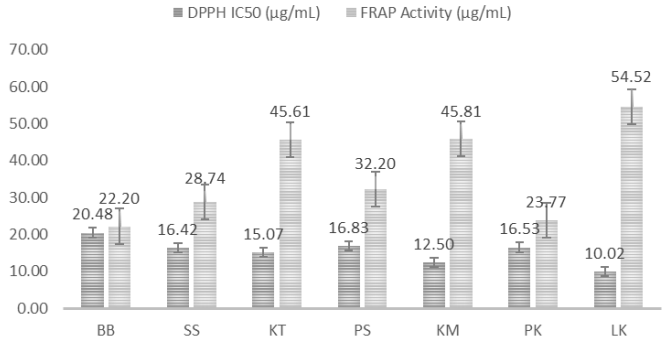

Figure 4. The antioxidant activity of seven red ginger extracts, measured with the DPPH and FRAP assays. Samples LK from Lendah-Kulonprogo-Yogyakarta have the lowest concentration to inhibit 50% of free radicals DPPH and have the highest ferric reducing antioxidant power activity.

3.4.2 Ferric reducing antioxidant power activity

The FRAP assay is a colorimetric method that can be used for the determination of antioxidant activity in red ginger extract. This assay is based on the reduction of Fe^{3+} to Fe^{2+} (Tena *et al.*, 2020). The FRAP antioxidant activity was reported to have a close correlation to DPPH radical scavenging activity (Kadum *et al.*, 2019). It was proven by this research, which showed that the highest DPPH radical scavenging activity and FRAP activity are possessed by LK, and the lowest is BB.

The FRAP activity of LK was 54.52 µg/mL, followed by KM (45.81 µg/mL), KT (45.61 µg/mL), PS (32.20 µg/mL), SS (28.74 µg/mL), PK (23.77 µg/mL), and BB (22.20 µg/mL) as shown in Figure 4. The high antioxidant activity of LK was influenced by the good cultivation system provided by the farmers in Lendah, Kulonprogo, Indonesia, where the samples were collected. The farmers cultivate it in the fertilizing soil with enough light so the rhizomes grow optimally and possess high antioxidant activity. The FRAP activity of LK was significantly different from other six samples. However, there are some samples with no significant differences between them, like the FRAP activity of KM was not significantly different from KT, PS and SS, and also PK and BB.

4. Conclusion

The analysis of seven samples of red ginger's extracts indicates that the TPC, TFC, and antioxidant activity, either DPPH radical scavenging activity or FRAP activity, are influenced by many factors. The altitude was relatively not too much to affect the TPC, TFC and antioxidant activities. The lack of strong correlations among TPC, TFC, and antioxidant activities among samples indicates that there may be other factors that independently influence their accumulation in red ginger's rhizomes.

Conflict of interest

The authors declare no conflict of interest.

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