DETERMINATION OF TANNIN CONTENT, PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF ROOIBOS TEA, GREEN TEA AND BLACK TEA FROM COMMERCIAL TEA BAG

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Abstract

Various teas like Rooibos, black, and green tea offer unique benefits through antioxidants that protect cells from free radicals. Tannin, an antioxidant in tea, has health advantages but may hinder iron absorption, reducing the nutritional value of tea. Thus, this study aims to determine the total tannin content (TTC), total phenolic content (TPC), and antioxidant activity of Rooibos tea, green tea, and black tea. This study also aims to determine the correlation between TPC and TTC with antioxidant activity. The total tannin content and total phenolic content were measured using the Folin-Ciocalteu method. The antioxidant activity was evaluated using the DPPH radical scavenging assay. Green tea exhibited the strongest antioxidant activity with an IC_{50} value of 15.01 μg/mL and possesses high phenolic content (56.57 mg GAE/g), and high tannin content (101.75 mg TAE/g). Black tea also showed strong antioxidant activity with an IC_{50} value of 18.93 μg/mL with a total phenolic content of 46.18 mg GAE/g and a total tannin content of 41.37 mg TAE/g. Similarly, Rooibos tea also showed strong antioxidant activity with an IC_{50} value of 25.11 μg/mL but Rooibos tea had a lower tannin content (38.75 mg TAE/g), and lower phenolic content (36.95 mg GAE/g) compared to green tea and black tea. Hence, the teas ranked by antioxidant strength are Rooibos < black < green tea. By using Pearson's correlation coefficient, total phenolic content was found closely associated with antioxidant activity with r = 0.984. Total tannin content, on the other hand, was weakly associated with antioxidant activity with r = 0.178. Consequently, the study's findings suggest that a higher total phenolic content corresponds to stronger antioxidant activity, whereas elevated total tannin content does not necessarily indicate higher antioxidant activity. Therefore, according to all values, Rooibos tea is a better option than green and black tea.

Keywords: Rooibos tea, green tea, black tea, tannin, antioxidant activity.

Introduction

Tea, mainly herbal tea is becoming increasingly popular as a nutritious beverage for people of all ages (Khan & Mukhtar, 2013). *Camellia sinensis*, which offers a variety of flavors and scents, is widely found in Southeast Asia's humid tropical climates such as China, India, Sri Lanka, Kenya, Japan, Vietnam, Indonesia, and Turkey. However, *Aspalathus linearis* (Rooibos tea) can only be found in South Africa. Green tea (unfermented *C. sinensis*) and black tea (fermented *C. sinensis*) are extracts from the *C. sinensis* plant. Rooibos tea or known also as red tea is distinct from others because it can only be derived from the leaves of the *A. linearis* (Onaolapo & Onaolapo, 2019). Tea exhibits several health benefits since it contains a variety of phytochemicals such as phenol and flavonoid, making it a nutritious drink.

Flavonoids can be classified into several types. The main flavonoid that can be identified in tea are rutin,

quercetin and orientin (Ligor et al., 2008). Tannin is a type of flavon-3-ol that is uncommon in herbal teas such as rooibos tea. Rooibos tea, green tea, and black tea all contain tannin. Tannin can be classified into hydrolyzable tannin and condensed tannin. Both types of tannin are found in a variety of human diets, including fruits, vegetables, and beverages such as tea and it exhibits various bioactive properties which are anti-oxidant, anti-carcinogen, anti-microbial, anti-mutagens (Chung et al., 1998), anti-inflammatory, anti-obesity (Sieniawska, 2015). However, tannin is also anti-nutritional (Chung et al., 1998). The toxic effects of phytochemicals linked to tannins include a reduction in feed efficiency, growth rate, and protein digestibility. In contrast, tannin extract from leaves has been reported to reduce the impact of waste disposal on the environment (Kavitha & Kandasubramanian, 2020). This shows that tannin is good for the environment, but not for human health. As an iron chelator, when someone consumes tea that contains a high dosage of tannin, a high amount of iron in the body will be absorbed by the tannin compound as tannin has a high affinity toward metal (Delimont et al., 2017).

As an antioxidant constituent, tannin helps to neutralize harmful free radicals in our body. With free radicals exposed in the human body, they may be exposed to diseases like cancer and heart disease (Lobo et al., 2010). Most commercialized tea is thought to have strong antioxidant activity due to the presence of polyphenol compounds (Kowalska et al., 2021). Still, the tea's total tannin content and antioxidant activity have not yet been correlated in any studies. Therefore, this study aims to determine the tannin content and phenolic content in Rooibos tea, green tea, and black tea using the Folin-Ciocalteu method. This study also will evaluate the antioxidant activity of Rooibos tea, green tea, and black tea using a DPPH radical scavenging assay. Eventually, the findings of this study can serve as solid scientific proof to promote the healthier tea option.

Methods

Materials
Commercial tea of Rooibos tea was purchased from Health Builders, green tea (Ahmad tea) and black tea (Sabah tea) were purchased from the local market in Kangar, Perlis.

Chemicals and Reagents
The chemicals used in this study were methanol, Folin reagent, sodium carbonate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, ascorbic acid, and tannic acid provided in Makmal Kimia Star Complex, UiTM Perlis Branch.

Preparation of tea extract
A 10 g tea sample (Rooibos tea, green tea, and black tea) was soaked in 100 ml of methanol and was left for 3 days. All the macerates were collected and filtered. Then, it was concentrated with a rotary evaporator until the solvent evaporated perfectly and a crude extract of tea was obtained (Indarti et al., 2019). Thus, the percentage yield of the crude extract of the tea sample was calculated by using Equation 1 (Ngamkhae et al., 2022).

$$\text{Percentage yield of extract} = \frac{\text{mass of crude extract}}{\text{mass of sample}} \times 100\% \qquad \text{Equation 1}$$

Total tannin content (TTC) assay
Total tannin content (TTC) was determined by using the Folin-Ciocalteu method. About 0.1 ml of tea extract was dissolved in 7.5 ml of distilled water. 0.5 ml of Folin reagent was added to the mixture. 1 ml of 35% Na_2CO_3 was added and diluted to 10 ml with distilled water. The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of tannic acid was prepared with a concentration of 10, 20, 30, 40, and 50 μg/ml. Absorbance for test and standard solutions was measured against the blank at 725 nm with a UV/Visible spectrophotometer. The total tannin content was expressed in terms of mg of TAE/g of extract (Lahare et al., 2021). Total tannin content was calculated using the Equation 2 (Siddiqui et al., 2017).

$$\text{Total tannin content (C)} = C1 \text{ x } \frac{V}{M} \qquad \qquad \text{Equation 2}$$

Where C = total tannin content in mg TAE/g, C1 = concentration of tannic acid obtained from the calibration curve in mg/ml, V= volume of extract in ml, and m = the weight of the extract in g.

Total phenolic content (TPC) assay
Folin–Ciocalteu method was chosen to measure the TPC of tea extracts. This method was conducted with slight modification. The crude sample was prepared by dissolving 10 mg of the extract in 10 mL of the solvent to yield a concentration of 1 mg/mL. About 1 ml of the extract was combined and mixed with 5 ml of a 1/10 dilution Folin–Ciocalteu reagent in distilled water. The liquid mixture was allowed to stand for 5 minutes at room temperature. The mixture was then added about 4.9 ml of 7.5% sodium carbonate solution and the test tube was shaken gently to mix them. After 60 minutes, the absorbance of the mixture was measured using the UV-Vis spectrophotometer at 765 nm. A calibration curve of standard reference was created using gallic acid with a range of concentration from 0.02 to 0.1 mg/mL as standard references plotted. Total phenolic content was expressed as gallic acid equivalents in milligrams per g of the extract (Johari & Khong, 2019). Total phenolic content was calculated using Equation 3 (Siddiqui et al., 2017).

$$\text{Total phenolic content (C)} = C1 \text{ x } \frac{V}{M} \qquad \qquad \text{Equation 3}$$

Where C = total phenolic content in mg GAE/g, C1 = concentration of gallic acid obtained from the calibration curve in mg/ml, V = volume of extract in ml, and m = the weight of the extract in g.

Determination of antioxidant activity by DPPH assay
To determine the antioxidant activity of tea samples, a DPPH assay was used. A 2 mL of tea extract solution of 20 to 100 μg/mL in methanol was added to 2 mL of DPPH (0.1 mM) solution. The mixtures were kept aside in a dark area for 30 minutes and absorbance was measured at λ_{max} 517 nm against an equal amount of DPPH and methanol as a blank. Ascorbic acid was prepared with concentrations from 20 to 100 μg/ml and used as standard reference. The percentage of DPPH radical scavenging (RSA%) will be estimated using Equation 4 (Aryal et al., 2019). A graph of percentage inhibition (%) against concentrations of the sample was plotted. From the graph, 50% inhibition (IC_{50} value) provided the value of concentrations for each sample.

$$\% \text{ Inhibition} = [(A_0 – A_1) /A_0] \text{ x } 100 \qquad \qquad \text{Equation 4}$$

Where A_0 = absorbance of blank and A_1 = absorbance of sample and standard

Statistical Analysis
All data were plotted and calculated using MS Excel (Microsoft Excel 2021). Total phenolic content and total tannin content were performed in triplicate, and results were expressed as mean ± standard deviation. Pearson's correlation coefficient was conducted to find the association between total phenolic content and total tannin content with antioxidant activity. The presence of a negative value for the correlation coefficient suggests a negative linear relationship, while a positive value suggests a positive linear relationship. A value of 0 signifies no linear correlation. A correlation coefficient between 0 and 0.3 implies a weak linear correlation, while a value between 0.3 and 0.7 indicates a moderate linear correlation. Finally, a correlation coefficient between 0.7 and 1 indicates a strong correlation (Ratner, 2009). Statistical significance was determined among teas with a one-way ANOVA test using MS Excel (Microsoft Excel 2021). A statistical significance of $p < 0.05$ was considered to be significant (Kumar & Ojha, 2014).

Result and Discussion

Extraction yield of Rooibos tea, green tea, and black tea

Sample of Rooibos tea, green tea, and black tea have been obtained from commercial tea bags of mentioned specific brand for a specific tea. Five tea bags (10 g) were used as each tea bag weighed 2 g. The powder of tea from the tea bag was removed and soaked into 100 ml of methanol. The maceration took 3 days and crude extracts of three types of tea were obtained using a rotary evaporator. Methanol was selected for this analysis because it enables the extraction of a wide range of components from tea samples. Additionally, it has been recognized as one of the favored solvents for the extraction of polyphenols. The yield of Rooibos tea is 9.76%, green tea is 10.959% and black tea is 11.032%. Black tea had the highest percentage yield compared to Rooibos tea and green tea. However, Nkubana & He (2008) argued that by using the methanolic method, green tea has the highest yield than black tea.

Total tannin content assay (TTC)

A total tannin content assay is used to determine and quantify the amount of tannin present in the sample. In this study, total tannin content is determined by the Folin–Ciocalteu method with tannic acid as a reference. The calibration curve of tannic acid was plotted, and the total tannin content was derived from the equation $y = 0.0008x + 0.0003$ and expressed in tannic acid equivalents (TAE) per gram.

Table 1 shows the total tannin content of Rooibos tea, green tea, and black tea. Tea samples were found significantly different in total tannin content with a p-value less than 0.05. According to Table 1, green tea has the highest amount of tannin content, which is 101.75 mg TAE/g. Black tea follows with a concentration of 41.37 mg TAE/g, whereas Rooibos tea has the least amount of tannin content, which is 38.75 mg TAE/g. Compared to this study, Sarkar et al. (2022) found that the total tannin content of green tea ranged from 3.754 mg GAE/g to 4.846 mg GAE/g, which is relatively low. However, Rahman et al. (2021) reported that the average total tannin content in black tea was found to be 6.47 mg TAE/g, while in green tea, it was 14.51 mg GAE/g. Therefore, the findings of this current study are consistent with Rahman et al. (2021) that green tea has higher tannin content compared to black tea. On the other hand, Oancea and Neamtiu (2018) found that the tannin content for Rooibos tea ranged from 487.80 to 2127.93 mg catechin 100 g^{-1} DM which is high. Some previous studies have reported significant variations in values and cannot be compared with the current study because of the differences in methods and standards used for total tannin content assay and solvent used to extract tannins from tea samples (Oancea and Neamtiu, 2018). This study used methanol as the solvent for tannin extraction, which is recognized as the most suitable solvent for this purpose. As Ng et al. (2020) reported, methanol extraction yields the highest tannin content in the sample.

Table 1. The total tannin content of Rooibos tea, green tea, and black tea.

Types of tea	Total tannin content (mg TAE/g)
Rooibos tea	38.75±12.32*
Green tea	101.75±12.12*
Black tea	41.37±9.71*

Notes: Values of total tannin content are means of triplicate measurements, mean ± SD, and expressed in mg TAE/g. P-value was determined using one-way ANOVA test.
* Indicates significant value when $p < 0.05$

This study aimed to determine the best tea in terms of tannin content, in which the lower the tannin content in the tea, the healthier the tea. This is because a higher concentration of tannin might have an adverse effect on humans as tannin may inhibit nutrient absorption, especially iron in the human body, and this has been proven in past studies (Delimont et al., 2017). Table 1 showed that Rooibos tea exhibits the lowest tannin concentration compared to green tea and black tea. Georgiev et al. (2014) as cited in Bhebhe et al. (2015), concluded that the tea with the least tannins value, approximately 3.84%, was considered the healthiest. Therefore, in this study, Rooibos tea was found to have a low tannin content

of 3.87% indicating it is a healthy tea. Green tea and black tea had higher tannin contents of 10.17% and 4.14%, respectively, suggesting they are comparatively less healthy.

Total phenolic content assay (TPC)
The term "total phenolic content" describes the total number of phenolic compounds that are present in a substance, such as a plant extract or a sample of food. A wide range of chemical substances known as phenolic compounds have antioxidant capabilities and are frequently linked to some health advantages. A sample's general antioxidant value or capacity can be determined by measuring the total phenolic content. The Folin–Ciocalteu reagent was used to measure the amount of phenolic content in each extract. The calibration curve of gallic acid was plotted and the concentration for total phenolic content of Rooibos tea, green tea, and black tea can be calculated using $y = 0.0079x + 0.0051$ from the calibration curve. Total phenolic content was expressed in gallic acid equivalents (GAE) per gram.

Table 2 shows the total phenolic content of Rooibos tea, green tea, and black tea. Tea samples were found significantly different in total phenolic content with a p-value less than 0.05. Based on Table 2, green tea showed the highest value of total phenolic content which is 56.57 mg GAE/g, followed by black tea with 46.18 mg GAE/g and Rooibos tea with 36.95 mg GAE/g. This indicates that green tea has the most phenolic compounds compared to black tea and Rooibos tea. Rahman et al. (2021) also observed that green tea exhibited a greater total phenolic content compared to black tea, with a relatively smaller difference between the two teas. The average total phenolic content of green tea found in the study was 763.42 mg GAE/g and as for black tea, the average value of TPC found was 242.46 mg GAE/g. Furthermore, Aktar (2020) conducted a study comparing the total phenolic content (TPC) of green tea and fermented Rooibos tea, and the results indicated that Rooibos tea had a lower TPC value. This is due to the solvent effect used to extract phenolic compounds in both teas. This study found that water as a solvent reduced the compound extraction. Green tea (cold brewing) exhibited a TPC value of 10.480 mg GAE/g, whereas fermented Rooibos tea (cold brewing) had a TPC value of 1.587 mg GAE/g. McAlpine et al. (2021) also found Rooibos tea had the lowest TPC value compared to green tea and black tea.

Table 2. Total phenolic content of Rooibos tea, green tea, and black tea

Types of tea	Total phenolic content (mg GAE/g)
Rooibos tea	36.95±0.71*
Green tea	56.57±2.90*
Black tea	46.18±1.07*

Notes: Values of total phenolic content are means of triplicate measurements, mean ± SD, and expressed in mg TAE/g. P-value was determined using one way ANOVA test
* Indicates significant value when $p < 0.05$

These findings indicate that green tea and black tea are richer in phenolic compounds compared to Rooibos tea. This is because of differences in processing methods and the species of the plant (Astill et al., 2001). The result showed green tea has higher phenolic content than black tea even though both green tea and black tea are derived from the same leaves of the *Camellia sinensis* plant. This is due to the different production of green tea and black tea in which for green tea, the leaves are quickly heated to prevent oxidation, preserving the natural phenolic compounds present in the leaves. Black tea, on the other hand, undergoes a process called oxidation, where the leaves are fully exposed to oxygen. This process leads to enzymatic reactions that result in the formation of different phenolic compounds, including theaflavins and thearubigins (Astill et al., 2001). This is also supported by Jyoti et al. (2021) Vastrad et al. (2022) mentioned that the phenolic compound of green tea is higher than black tea.

Antioxidant activity by DPPH radical scavenging assay
The scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) is rooted in a process known as one-

electron reduction (Johari & Khong, 2019). This process signifies the ability of antioxidants to reduce free radicals. Free radicals are highly reactive species that can cause oxidative damage to cells and tissues. In the DPPH assay, the DPPH radical, which is a stable free radical with a deep purple color, is used as a representative of free radicals. When antioxidants come into contact with DPPH, they transfer an electron, resulting in the reduction of DPPH to a non-radical form, which causes the color to fade. By measuring the extent of color change, the scavenging activity of antioxidants can be quantified, providing valuable insights into their potential as free radical scavengers and their overall antioxidant activity.

In this study, ascorbic acid was used as a standard reference with a concentration of 20 to 100 µg/mL. Figure 1 shows the percentage inhibition (%) vs. concentration which indicates the percentage of DPPH radical scavenging activity of Rooibos tea, green tea, and black tea extract with standard. The percentage of inhibition has been calculated using Equation (4). The results showed that green tea exhibits the highest percentage inhibition value which is 80.2%, followed by black tea (79.32%) and Rooibos tea (79.01%) at 100 µg/mL.
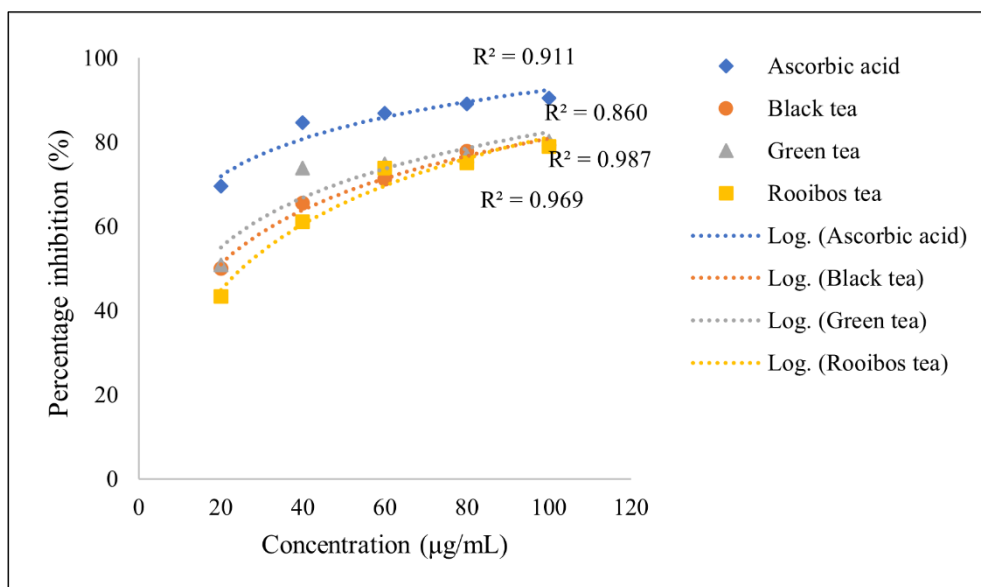

Figure 1. DPPH radical scavenging assay of Rooibos tea, green tea and black tea extract, and ascorbic acid at concentration 20-100 µg/mL

As shown in Table 3, the lowest IC_{50} is green tea, followed by black tea and Rooibos tea with values of 14.89, 18.93, and 25.11 µg/mL, respectively. Green tea possesses high antioxidant activity as it has the lowest IC_{50}. The lower the IC_{50} value, the stronger the antioxidant activity which also has the highest ability to scavenge free radicals compared to Rooibos tea and black tea. This is supported by Nuryana et al. (2021) which reported that green tea (5.65 µg/mL) was found to possess the lowest IC_{50} value compared to black tea (43.5 µg/mL). However, ascorbic acid (standard) remains the strongest antioxidant agent with the IC_{50} value obtained was 3.54 µg/mL. In the previous study, ascorbic acid which is the standard was found to be 2.74 µg/mL (Nuryana et al., 2021) and 6.49 µg/mL (Malongane et al., 2022). In this study, Rooibos tea demonstrates a notably higher IC_{50} value compared to other types of tea.

Table 3. IC_{50} value of three types of tea

Types of tea and standard	Logarithmic equation	IC_{50} value (μg/mL)
Rooibos tea	$y = 22.601\ln(x) - 22.852$	25.11
Green tea	$y = 17.027\ln(x) + 4.011$	14.89
Black tea	$y = 18.551\ln(x) - 4.550$	18.93
Ascorbic acid	$y = 12.706\ln(x) + 33.931$	3.54

The IC_{50} represents the concentration of the sample required to inhibit 50% of the activity being measured. By analyzing the graph, the IC_{50} value can be determined using the formula log obtained from the graph plotted in Figure 1. Using equation $y = a \ln x$ from Table 3 and substituting $y = 50$, the IC_{50} value can be calculated.

Correlation Analysis of TPC and TTC with DPPH
Linear correlation between TPC and DPPH and TTC and DPPH has been plotted. Pearson correlation coefficient or r value was used to express the linear relationship of correlation. Based on Figure 2, linear correlation showed a positive correlation in which three types of tea have high r value. The value obtained was 0.984. This positive correlation indicates that the higher the total phenolic content value, the higher the antioxidant activity. Additionally, in previous studies, the high antioxidant activity showed a positive association with total phenolic content value. This implies that a higher TPC value corresponds to greater antioxidant activity. The value of r obtained was 0.699 (Muflihah et al., 2021), 0.994 (Kumar & Ojha, 2014), and 0.9156 for 2 minutes of infusion (Dobrinas et al., 2021)
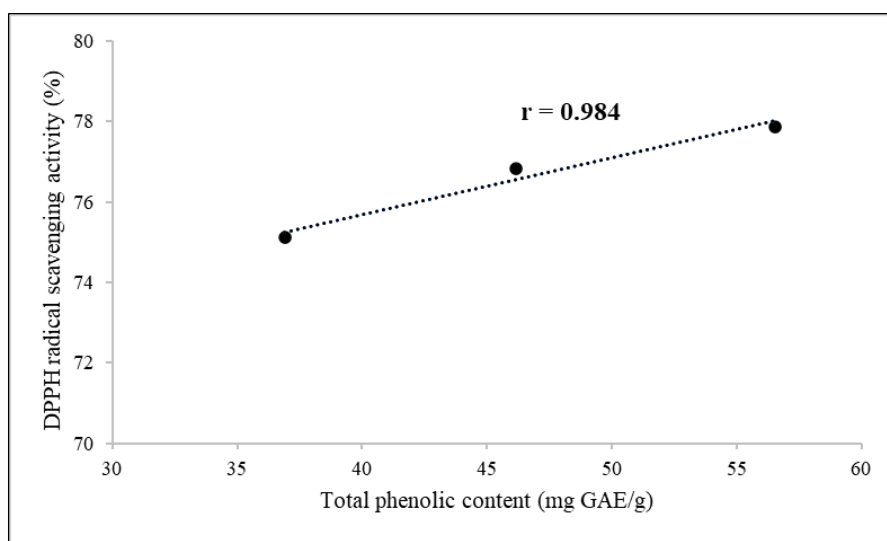

Figure 2. Linear correlation of total phenolic content (TPC) in mg GAE/g with DPPH radical scavenging activity (%) at a concentration of 80 μg/mL of tea extract.

On the other hand, the linear correlation between TTC and DPPH has been plotted. Based on Figure 3, results showed Rooibos tea, green tea, and black tea have a very weak correlation in which the r value obtained was 0.178. This indicates that there is less or no correlation between TTC and antioxidant activity. Therefore, a high level of tannins does not necessarily indicate a high level of antioxidant activity. However, Patel et al. (2018) reported there is a considerable correlation between TTC and DPPH assay in which the r value obtained was 0.792.
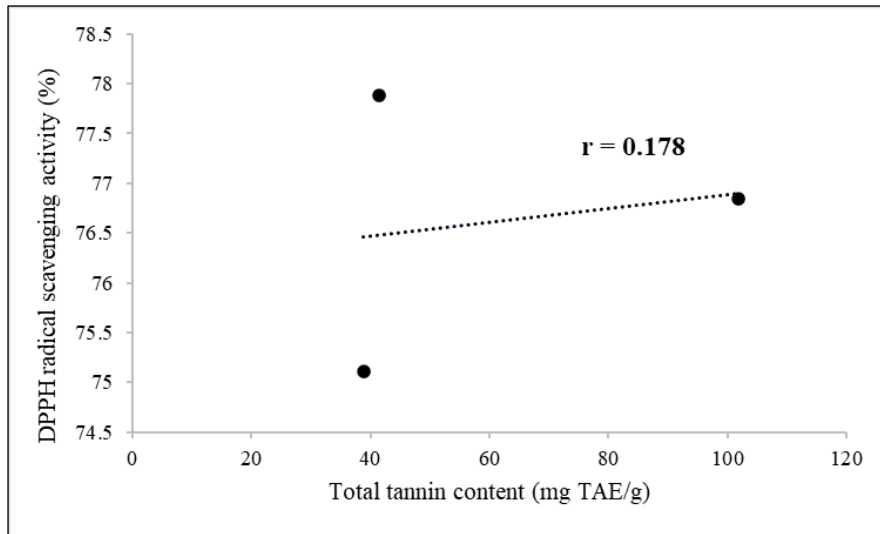
Figure 3. Linear correlation of total tannin content (TTC) in mg TAE/g with DPPH radical scavenging activity (%) at a concentration of 80 μg/mL of tea extract.

Conclusion

In conclusion, three types of tea which are Rooibos tea, green tea, and black tea had undergone maceration and the percentage yield obtained were 9.76, 10.96 and 11.03%, respectively. The total tannin content and total phenolic content of these teas have been determined using Folin-Ciocalteu reagent and the antioxidant activity of these teas has been evaluated using DPPH radical scavenging assay. Rooibos tea has the lowest total tannin content, followed by black tea and green tea in which the value was 38.75 mg TAE/g, 41.37 mg TAE/g, and 101.75 mg TAE/g. In the total phenolic content assay, green tea exhibited the highest value which is 56.52 mg GAE/g, followed by black tea of 46.18 mg GAE/g and Rooibos tea of 36.95 mg GAE/g. In addition, antioxidant activity has been analyzed and calculated using the equation given. The result showed that green tea exhibited the highest antioxidant activity as it has the lowest IC_{50} value which is 14.89 μg/mL, while black tea exhibited an IC_{50} value of 18.93 μg/mL, and Rooibos tea exhibited an IC_{50} value of 25.11 μg/mL. The percentage of radical scavenging activity in teas increased from Rooibos tea, black tea, and green tea with the values was 80.2, 79.32, and 79.01%, respectively. The correlation between total tannin content and total phenolic content with DPPH radical scavenging assay has been determined by using the Pearson correlation coefficient (PCC). Correlation between TPC and DPPH showed a positive correlation (r = 0.984) meanwhile correlation between TTC and DPPH showed weak or no correlation (r = 0.178). Overall, this study concluded that Rooibos tea has low tannin, low TPC, and low antioxidant activity compared to green tea and black tea. The lower tannin levels in Rooibos tea make it a favorable choice for individuals seeking a beverage with potentially fewer negative effects associated with high tannin consumption. Therefore, Rooibos tea has been chosen as a healthy alternative to traditional teas like green tea and black tea. This study can be improved by using various types of instruments to analyze the sample more efficiently such as high-performance liquid chromatography (HPLC) to measure the concentration of tannin and phenolic compounds. Ferric chloride test and gelatin test can be conducted to detect the presence of tannin in the teas. Antioxidant activity can be determined by using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay to evaluate the correlation of total phenolic content (TPC) and total tannin content (TTC) with antioxidant activity.

Author Contribution

AF Mohammad Taib – Conceptualization, data curation, formal analysis, methodology, writing – original draft; AD Hazmi – formal analysis; NS Rahim – Supervision, Writing – review&editing; NZ Alias – Supervision, Writing - review&editing

Conflict of Interest

Authors declare no conflict of interest.

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