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Antioxidant activities of roots, leaves, and stems of carrot (*Daucus carota* L.) using DPPH and FRAP methods

Hartati R*, Amalina M N, Fidrianny I

Department of Pharmaceutical Biology, School of Pharmacy, Bandung Institute of Technology, Bandung-40132, Indonesia

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ABSTRACT

Free radicals are chemical species with unpaired electrons in their outer orbital that can attack other molecules, causing cell oxidative damage and degenerative diseases. Free radicals can be prevented by antioxidant. An antioxidant can be found in nature as secondary metabolites in plants, such as carrot (*Daucus carota* L.). This research was conducted to study the antioxidant activities of roots, leaves, and stems of carrot extracts using DPPH and FRAP methods, determine total phenolic content (TPC) and total flavonoid content (TFC), analyse the correlation between TPC and TFC with AAI DPPH and FRAP, and the correlation between two methods. The sample was extracted by reflux using n-hexane, ethyl acetate, and ethanol. Determination of TPC, TFC, AAI DPPH and FRAP was performed using UV-visible spectrophotometry. Correlation of TPC and TFC with AAI DPPH and FRAP and also the correlation between the two methods were conducted using Pearson's method. Ethyl acetate carrot leaves extract showed the highest TPC and TFC (8.88 ± 0.44 g GAE/100 g and 9.00 ± 0.31 g QE/100 g). AAI DPPH of carrot extract in the range of 0.16 – 1.42, meanwhile AAI FRAP 1.89 – 5.45. TPC and TFC of carrot roots extract showed a significantly positive correlation with AAI DPPH and FRAP. AAI DPPH and FRAP of carrot roots extract gave a significantly positive correlation. Ethyl acetate and ethanol carrot leave extracts were strong to very strong antioxidant by DPPH and FRAP methods. TPC and TFC in carrot roots extract contributed to antioxidant activities by DPPH and FRAP. DPPH and FRAP presented linear results in antioxidant activities of carrot roots extract.

*Corresponding Author

Name: Hartati R
Phone: +62-222504852
Email: rika@fa.itb.ac.id

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INTRODUCTION

Free radical are chemical species that have one or more unpaired electron on the outer orbital layer. The unpaired electron is very reactive and unstable, thus can attack other molecules like lipid, protein, and carbohydrate, causing oxidative stress. Oxidative stress occurs when the body fails to maintain homeostatic processes and production of free radical is beyond the capacity of the body defence system, leading to cellular injury and tissue damage. This process can start the initiation of aging process and the pathogenesis of cancer, cardiovascular diseases, and other degenerative diseases (El-Shahid *et al.*, 2018; Kim *et al.*, 2012). To prevent

diseases caused by free radicals, the body needs chemical species that can prohibit oxidation reaction. This chemical species is called antioxidant. An antioxidant is a compound that can inhibit or delay the oxidation process by blocking the initiation or propagation of oxidizing chain reaction, which may be destructive to cells. An antioxidant may function as free radical scavengers, quenchers of singlet oxygen formation, reducing agents, or complex pro-oxidant metals (Andlauer and Furst, 1998; Arafa *et al.*, 2016).

Naturally, an antioxidant can be found in plants as secondary metabolites, particularly as phenolic and flavonoid compounds. Secondary metabolites are synthesized by plants in response to environmental stresses, such as injuries, external attack by pathogens or insects, and UV radiation (Eugenio *et al.*, 2017). A widely used plant that possesses antioxidant activity is the carrot (*Daucus carota* L.), one of the top ten most economically important vegetable crops in the world. Carrot contains phenolic and flavonoid compounds like hydroxycinnamic acid, chlorogenic acid, and carotenoid (Eugenio *et al.*, 2017; Faisal *et al.*, 2017). Those compounds are stored in all parts of the plant, thus all parts of carrot have been used as food products (salad, soup, juice), dye, cosmetic, and traditional medicine to lower blood sugar level and reliever for muscle and back pain Ayeni *et al.* (2018). The most widely applied part of a carrot is the roots part because it contains most of its antioxidant compounds in its peel (Kähkönen *et al.*, 1999).

Antioxidant activity assay of a sample can be done enzymatically (*in vivo*) or nonenzymatically (*in vitro*). *In vitro,* antioxidant assays correspond to the amount of hydrogens/electrons exchanged by sample, which has antioxidant capacity in the reaction with the oxidant probe (Abramovič *et al.*, 2018). Some of *in vitro* antioxidant assays are DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power), and CUPRAC (cupric ion reducing antioxidant capacity). This research was conducted using DPPH and FRAP methods to measure the antioxidant activity of carrot's roots, leaves, and stems extracts in different polarities solvent and the correlation between total phenolic and flavonoid content with the antioxidant activity.

MATERIALS AND METHODS

Materials and instruments

Folin-Ciocalteu, sodium carbonate, methanol, aqua dest, aluminium (III) chloride, sodium acetate, and chloride acid, were purchased from Merck (Germany). Quercetin, gallic acid, ascorbic acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), and TPTZ (2,4,6-tripyrydyl-S-triazine) were purchased from Sigma-Aldrich (USA). Other reagents were analytical grades. The instruments used were rotary evaporator (Heidolph), micropipette (Thermo Scientific), and UV-visible spectrophotometer (Beckman Coulter DU 720), calibrated to standard before use.

Sample preparation

Roots, leaves, and stems of carrot were collected from Parongpong, West Bandung, West Java, Indonesia. The sample was washed with tap water, wet sorted, cut, dried 48 hours in 40-50^{o}C, and grinded into powder. All samples were stored at room temperature in polythene zip lock bags.

Extraction

Ten kilograms of powdered sample was extracted by reflux using increasing gradient polarity solvents that were n-hexane, followed by ethyl acetate, and ethanol. Each step was performed in 2-3 hours after the solvent was boiled, repeated triplicate per solvent. The residue of the previous step, then extracted by the next solvent. Extract then concentrated using a rotary evaporator.

Total phenolic content determination

Total phenolic content (TPC) was measured using the adopted method from (Pourmorad *et al.*, 2006). Reagents used were Folin-Ciocalteu 10% (v/v) and sodium carbonate 1 M. Standard solution of gallic acid was made within a concentration of 40-130 μg/ml. A 0.5 ml standard or sample solution was mixed with 5 ml Folin-Ciocalteu 10% and 4 ml sodium carbonate 1 M. Mixture was incubated in room temperature for 15 min. The absorbance was evaluated at wavelength 765 nm using UV-visible spectrophotometer, performed triplicate. The TPC was expressed as g gallic acid equivalent per 100 g extract.

Total flavonoid content determination

Total flavonoid content (TFC) was determined using the modification method (Chang *et al.*, 2002). Reagents used were aluminium (III) chloride 10% (w/v) and sodium acetate 1 M. Quercetin was utilised as standard and prepared in various concentrations of 60-130 μg/ml. A 0.5 ml standard or sample solution was mixed with 1.5 ml methanol, 2.8 ml distillate water, 0.1 ml aluminium (III) chloride 10%, and 0.1 ml sodium acetate 1 M. Mixture was incubated in room temperature for 30 min. The absorbance was seen at l 415 nm, performed triplicate. Gram quercetin equivalent per 100 g extract was applied for TFC.

Antioxidant activity determination by DPPH assay

Minor modification of Blois' method was utilised in antioxidant activity by DPPH assay (BLOIS, 1958). Reagents used were DPPH 39.4 μg/ml solution as a control. Ascorbic acid as standard, and methanol as blank. A 1 ml standard or sample solution was mixed with 1 ml DPPH 39.4 μg/ml solution, then incubated in room temperature for 30 min. It was done triplicate for each standard and sample concentration. The absorbance was read at a wavelength of 517 nm. The results were exposed to antioxidant activity index (AAI). AAI was calculated by final concentration DPPH divide IC_{50} DPPH.

Antioxidant activity determination by FRAP assay

Antioxidant activity by FRAP assay was adopted from. Reagents used were FRAP 467.5 μg/ml solution as a control. Preparation and test were done in a dark room. A 1 ml standard or sample solution was mixed with 1 ml FRAP 467.5 μg/ml solution. The mixture was incubated in room temperature for 30 min. Ascorbic acid was applied as standard and pH 3.6 acetic buffer as blank. Then absorbance was investigated at l 593 nm (Benzie and Strain, 1996). The final concentration of FRAP was divided by EC_{50} FRAP to determine AAI value.

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 25 software. Correlation between each sample was analysed using one-way ANOVA with post-hoc Tukey ($p < 0.05$) whereas the correlation between TPC, TFC, AAI DPPH, and AAI FRAP were analysed using Pearson's correlation method.

RESULTS AND DISCUSSION

Carrot produces secondary metabolites such as phenolics and flavonoids in abundance. A flavonoid compound can be classified as a phenolic compound, depending on the position of -OH group on its structure. Flavonoid that has -OH group on A or B ring is classified as phenolic compound (Fidrianny *et al.*, 2010). Phenolic compounds give antioxidant activity as free radicals terminator and metal chelator, preventing autoxidation from occurring (Shahidi *et al.*, 1992). Meanwhile, flavonoid compounds show antioxidant activity as a free radical scavenger and metal ion chelation to inhibit lipid peroxidation (John *et al.*, 2018; Pourmorad *et al.*, 2006).

Different parts of the carrot plant contain different compounds. A study by (Faisal *et al.*, 2017) reported that major phenolic compound found in carrot extract was hydroxyl cinnamic acid derivatives, among them are 3-caffeoylquinic acid, caffeic acid, 3-p-coumaroylquinic acid, and 3-feruloyquinic acid meanwhile a study by (Eugenio *et al.*, 2017) showed the phenolic compounds in carrot leaves were chlorogenic acid, rosmarinic acid, o-coumaric acid, quercetin, caffeic acid, and trans-cinnamic acid. Other than phenolic and flavonoid compounds, major compounds found in carrot are carotenoids, a tetraterpenoid, lipid soluble organic pigment that gives color to a carrot. Some of the derivates of carotenoids are xanthophylls which contain oxygen (lutein, zeaxanthin, astaxanthin) and carotenes which does not contain oxygen (α-carotene, β-carotene, lycopene) (Singh *et al.*, 2018). Carrot roots contain mostly α-carotene and β-carotene with a low level of lutein, whereas the leaves and stems contain a high level of lutein (Yoo *et al.*, 2020).

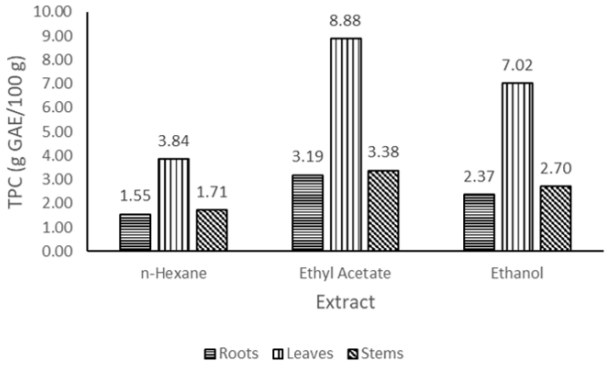

Figure 1: Total phenolic content of carrot roots, leaves, and stems extracts in different polarities

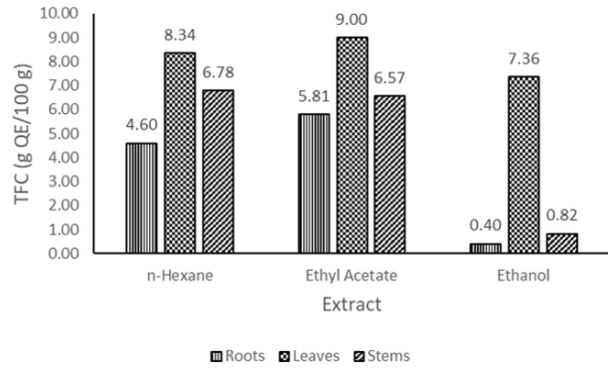

Figure 2: Total flavonoid content of carrot roots, leaves, and stems extracts in different polarities

Total phenolic and flavonoid content

Total phenolic content (TPC) determination was performed with Folin-Ciocalteu, a commonly used reagent to measure total phenolic content in natural products because it is simple, sensitive, and precise. This method is a colorimetric assay based on the oxidation of phenolic compounds of the sample by phosphotungstomolybdate in Folin-

Table 1: AAI by DPPH and FRAP of n-hexane carrot roots, leaves, and stems extracts

Sample	Antioxidant Activity	
	AAI DPPH	AAI FRAP
Roots	0.19 ± 0.01a	3.09 ± 0.03a
Leaves	0.31 ± 0.02b	4.53 ± 0.05b
Stems	0.28 ± 0.02b	1.95 ± 0.03c
Ascorbic Acid	29.49 ± 1.61	92.91 ± 4.53

Note: a-c =different letters in a column show significant difference ($p < 0.05$)

Table 2: AAI by DPPH and FRAP of ethyl acetate carrot roots, leaves, and stems extracts

Sample	Antioxidant Activity	
	AAI DPPH	AAI FRAP
Roots	0.62 ± 0.02a	5.27 ± 0.06a
Leaves	1.01 ± 0.05b	5.45 ± 0.19a
Stems	0.47 ± 0.01c	3.98 ± 0.06b
Ascorbic Acid	29.49 ± 1.61	92.91 ± 4.53

Note: a-c =different letters in a column show significant difference ($p < 0.05$)

Table 3: AAI by DPPH and FRAP of ethanolic carrot roots, leaves, and stems extracts

Sample	Antioxidant Activity	
	AAI DPPH	AAI FRAP
Roots	0.16 ± 0.01a	1.89 ± 0.01a
Leaves	1.42 ± 0.05b	3.12 ± 0.10b
Stems	0.82 ± 0.03c	2.79 ± 0.90c
Ascorbic Acid	29.49 ± 1.61	92.91 ± 4.53

Note:a-c = different letters in a column show significant difference ($p < 0.05$)

Table 4: Pearson's correlation between TPC and TFC with AAI DPPH and FRAP

Antioxidant Parameter	Pearson's Correlation Coefficient (r)				
	TPC	TFC	AAI FRAP Roots	AAI FRAP Leaves	AAI FRAP Stems
AAI DPPH roots	0,837**	0,714*	0,956**		
AAI DPPH leaves	0,083ns	-0,343ns		-0,462ns	
AAI DPPH stems	0,023ns	-0,936**			0,267ns
AAI FRAP roots	0,642*	0,879**			
AAI FRAP leaves	0,257ns	0,801**			
AAI FRAP stems	0,969**	0,072ns			

Note: ** = significant at $p < 0.01$, * =significant at $p < 0.05$, ns = not significant

Ciocalteu resulting phosphomolybdenum complex which yields blue color (Berker *et al.*, 2013). The standard curve equation y = 0.0056x + 0.00223, R^2 = 0.9983 was applied to investigate TPC of carrot roots, leaves, and stems extracts in different polarities solvent and stated as g GAE/100 g. The results are shown in Figure 1.

Total phenolic content (TFC) of carrot roots, leaves, and stems extracts in different polarities showed results within the range from 1.55 to 8.88 g GAE/100 g. The highest phenolic content was shown by ethyl acetate extract of carrot leaves with 8.88 ± 0.44 g GAE/100 g. The significant difference was shown between leaves with roots and stems on the three solvents ($p < 0.05$).

Total flavonoid content determination followed the method from (Chang *et al.*, 2002) based on the principle of complex formation between aluminium (III) chloride with flavonoid. The formed complex is acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Binding with ortho-dihydroxyl groups in B-ring of flavonoids can form an acid labile complexes (Chang *et al.*, 2002). Sodium acetate is used to prevent the breaking of the formed complexes. TFC of carrot roots, leaves, and stems extracts in different polarities solvent were calculated using the standard curve equation y = 0.0042x + 0.0739, R^2 = 0.9911 and represented as g QE/100 g (Figure 2).

Total flavonoid content of carrot roots, leaves, and stems extracts in different polarities solvent demonstrated results varied in the range of 0.40 - 9.00 g QE/100 g. The highest flavonoid content was given by ethyl acetate extract of carrot leaves with 9.00 ± 0.31 g QE/100 g. A significant difference was shown on ethyl acetate and ethanolic extracts between leaves with roots and stems ($p < 0.05$).

Antioxidant activity by DPPH and FRAP methods

DPPH is a stable organic nitrogen radical which can be stabilized by delocalizing free electron by a hydrogen-donating antioxidant, causing decoloration of the purple color into yellow color 2,2-diphenyl-1-picrylhydrazyne (nonradical DPPH-H) (Jovanova *et al.*, 2019; **?**). The DPPH antioxidant assay is based on measurement of the loss of DPPH color at 515-520 nm due to the reducing ability of antioxidant of the sample towards DPPH. This method is widely used in antioxidant screening because it is a simple and rapid method to perform (Prior *et al.*, 2005).

FRAP assay measures antioxidant activity by calculating the reduction of iron (III) into iron (II) and the formation of blue color iron (II)-TPTZ complex which can be measured at 593 nm. Acetic buffer pH 3.6 is needed to maintain the pH at 3.6 to keep the solubility of iron. FRAP assay is a simple, rapid, inexpensive, and robust method that does not need specialized equipment (Prior *et al.*, 2005).

DPPH and FRAP have a different mechanism in measuring the antioxidant capacity of a sample. DPPH uses hydrogen transfer of DPPH radical scavenging activity, whereas FRAP uses electron transfer which results in FRAP capacity value. From the measured capacity, the antioxidant activity of the sample can be exhibited in antioxidant activity index (AAI). Higher antioxidant activity of a sample gives higher AAI value. Antioxidant strength presented in AAI was classified by (Scherer and Godoy, 2009) into poor (AAI $\leq$ 0.5), moderate (0.5 $\leq$ AAI $\leq$ 1.0), strong (1.0 $\leq$ AAI $\leq$ 2.0), and very strong (AAI > 2.0) antioxidant activity.

Ascorbic acid was applied as standard to verify the DPPH and FRAP methods, which gave AAI value 29.49 ± 1.61 for DPPH, and 92.91 ± 4.53 for FRAP and showed very strong antioxidant activity on both methods. The results of AAI by DPPH and FRAP of carrot roots, leaves, and stems extracts in different polarities are shown in Tables 1, 2 and 3.

Roots, leaves, and stems of carrot extracts had AAI DPPH within in the range of 0.16 - 1.42 and AAI FRAP varied from 1.89 to 5.45. The top AAI DPPH value was displayed by ethanolic extract of carrot leaves with 1.42 ± 0.05, whereas ethyl acetate extract of carrot leaves had the top value AAI FRAP (5.45 ± 0.19). Ethyl acetate and ethanol extract of carrot leaves can be classified as strong to very strong antioxidant by two methods.

Correlation between TPC and TFC with AAI DPPH and FRAP

Quantitative correlation analysis between total phenolic and flavonoid content with AAI by DPPH and FRAP was conducted to know the contribution of phenolic and flavonoid compounds in antioxidant activity of carrot roots, leaves, and stems extracts. The positive and significant result suggested that the phenolic and flavonoid content contributed to antioxidant activity. The higher correlation value means the stronger relation between phenolic and flavonoid content in contributing to antioxidant capacity. The results are shown in Table 4.

Positive and significant correlation between TPC and TFC with AAI were shown by carrot roots, both on AAI by DPPH ($r = 0.837$; $p < 0.01$; $r = 0.714$; $p < 0.05$) and AAI by FRAP ($r = 0.642$; $p < 0.05$; $r = 0.879$; $p < 0.01$). Besides the roots, significantly positive correlation was shown between TPC with AAI

by FRAP of stems ($r = 0.969$; $p < 0.01$) and flavonoid content with AAI by FRAP of leaves ($r = 0.801$; $p < 0.01$). From the results, it can be concluded that phenolic and flavonoid content in carrot roots contributed in its antioxidant activity based on both DPPH and FRAP methods. The correlation between AAI DPPH and FRAP methods in measuring carrot roots, leaves, and stems extracts expressed positive and significant correlation on roots extract ($r = 0.956$; $p < 0.01$). Thus, it is concluded that antioxidant activity assay of carrot roots extract using DPPH and FRAP gave linear result.

DPPH and FRAP methods did not always show a correlation in measuring AAI of a sample because the two methods have a different mechanism and its own limitations. DPPH measures hydrogen transfer, whereas FRAP measures electron transfer. DPPH method has some disadvantages, such as complicated interpretation if the sample has spectra that overlap DPPH at 515-520 nm, such as carotenoids. The measured decolorization can happen by the radical reaction, reduction by reducing agent, or hydrogen transfer, determined by steric accessibility of the reaction. FRAP method is limited only to detect compounds with redox potential < 0.77 V (the redox potential of iron (III)/iron (II)). Besides, FRAP cannot detect compounds that act by radical quenching (H transfer), particularly thiols and proteins. Antioxidant activity measurement using the FRAP method should be followed by another method to know which mechanism is compatible with the sample (Prior *et al.*, 2005).

Different phenolic and flavonoid compounds present in different parts of carrot yield different antioxidant activity, both in value and mechanism of counteracting oxidation. The structure of the compound determines the antioxidant capacity. The -OH group in ortho position in C3' and C4' has the highest influence in contributing antioxidant power of flavonoid. Flavonoid will give greater antioxidant ability if it has a double bond at C2 and C3, oxo function in C4, -OH in C3, or di-OH in C 3',4'. The aglycone type of flavonoid has higher antioxidant capacity than glycosides type, giving conclusion that the presence of glycoside group in flavonoid can lessen the antioxidant capacity. Phenolic acid has a lower antioxidant activity than flavonoid (Fidrianny *et al.*, 2010).

A previous study by (Nguyen and Scarlett, 2016) measured the antioxidant activity of carrot peels in different polarities extract using DPPH and FRAP methods. The highest level of antioxidant capacity was shown by methanol extract, followed by ethanol, water, and hexane. The DPPH and FRAP assay showed correlation with phenolic content but not with saponin content, revealed that phenolic compounds highly contributed to the antioxidant activity of carrot peel with methanol as the most effective solvent. The result by DPPH stated that the antioxidant activity correlated with intermediate polarity of methanol that allowed the solvent to dissolve organic compounds with a low molecular weight that possess protonatable functional groups and the FRAP result revealed that phenolic compound of carrot peel increased ferric reduction ability of carrot peel.

According to a study by (Burri *et al.*, 2017), carrot leaves' antioxidant activity and radical scavenging activity correlated with its total phenolic contents, but each phenolic compound showed better correlation with the different antioxidant assay. Some of them are kaempferol-malonyl-glucoside and quercetin-3-O-malonyl glucoside A, which showed higher antioxidant capacity using the FRAP method. Meanwhile, other compound, namely rutin, cynarin, caffeic acid, neo-chlorogenic acid, were associated with another antioxidant activity assay, namely ABTS. A study by (Ayeni *et al.*, 2018) showed that ethyl acetate and methanolic extracts of carrot's aerial parts (leaves and stems) had the highest antioxidant activity using DPPH method.

In carrot, especially the roots part, antioxidant capacity is not only given by phenolic and flavonoid compounds, but also by carotenoid compounds by scavenging free radical. The increase of double bond amount in carotenoid structure gives higher free radical scavenging capacity. A study by (Müller *et al.*, 2011) reported that keto carotenoids showed high peroxyl radicals scavenging activity due to its large conjugated double bond systems. Based on FRAP assay, lycopene (11 conjugated double bonds) and hydroxy carotenoids could effectively reduce iron (III) while carotenoids with less double bond like neurosporene (9), phytofluene (5), and phytoene (3) did not reveal significant activity to reduce iron (III) due to steric hindrance and low chemical reactivity of cyclic carotenes and their carbonyl substituted derivates. None of the analysed carotenoids showed DPPH scavenging activity. The statement is supported by a study by (Sun *et al.*, 2009) which reported that carotenoid did not contribute to total antioxidant capacity but correlated with antioxidant capacity of hydrophobic extracts. Meanwhile, DPPH assay showed higher antioxidant capacity on hydrophilic extract than the hydrophobic extract. It can be concluded that carotenoid compounds in carrot extract showed higher antioxidant value when measured by FRAP method rather than the DPPH method.

CONCLUSION

Antioxidant activity of roots, leaves, and stems extracts of carrot using DPPH and FRAP methods showed AAI by DPPH within range of 0.16 - 1.42 and AAI by FRAP 1.89 to 5.45. Ethyl acetate leaves extract of carrot gave the top TPC and TFC. Ethyl acetate and ethanolic extracts of carrot leaves were considered as potent to very potent antioxidant using DPPH and FRAP methods. TPC and TFC of carrot roots showed positive and significant correlation with AAI DPPH and FRAP revealed that TPC and TFC of carrot roots contributed to antioxidant activity measured by DPPH and FRAP methods. AAI DPPH of carrot roots showed positive and significant correlation with AAI of FRAP, revealed that antioxidant activity assay using DPPH and FRAP methods showed linear result on carrot roots extract.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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