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Antioxidant potential of ethanol, ethyl acetate, n-hexane extracts from leaves, fruits, stems, and roots of red okra (*Abelmoschus esculentus*)

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ABSTRACT. *Abelmoschus esculentus* L. Moench has been known for its abundant and affordable antioxidant potential. This plant has different antioxidant levels regarding the parts of the plant. Red okra is rich in flavonoids and polyphenols, including strong antioxidant as quercetin ad anthocyanin. This research is intended to determine the IC_{50} value and compare the total content of phenolic and flavonoid from ethanol, ethyl acetate, and n-hexane extract of red okra (*A. esculentus*) leaves, fruits, stems, and roots. Extraction process was done by gradual maceration method using ethanol, ethyl acetate, and n-hexane. IC_{50} value interprets the antioxidant activity obtained from DPPH test. The results showed a very strong antioxidant activity from red okra stem extract with IC_{50} of 25.22 ppm and the lowest from fruit n-hexane extract with 821.55 ppm. From TPC test, the highest content was obtained from stem ethanol extract with 156.01 mg GAE/g and the lowest from stem n-hexane extract with 25.99 mg GAE/. From TFC test, the highest content was obtained from fruits ethanol extract with 108.15 mg QE/g and the lowest from stem ethanol extract with 2.26 mg QE/g. In conclusion, this research showed that red okra (*A. esculentus*) contains a high level of phenol and flavonoid compound and the highest antioxidant content was shown in ethanol extract.

Keywords: *Abelmoschus esculentus*; antioxidant; DPPH; flavonoid; phenolic

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INTRODUCTION

The normal level of free radicals would bring benefits to the body, however, if the level becomes excessive, it could lead to oxidative stress. Free radical has a high reactivity for its unpaired electron which tends to bond with another substance (protein, lipid, also DNA) and turn the electron to a new free radical. And if it finds other substances, the reaction will continue and form a free radical chain reaction. The immersion process in antioxidants could terminate the formation of free radicals (Phaniendra *et al*., 2015). An antioxidant is a crucial substance in the body that helps inhibit oxidation caused by excessive free radicals. Antioxidants are naturally obtained from extraction or by consuming natural ingredients, such as plants. This has been proven by numerous researches that antioxidants in plants can prevent the body from the risks of free radicals. This has been proven by numerous researches that antioxidants in plants can prevent the body from the risks of free radicals, such as vitamins, phenol, and flavonoids (Gulcin *et al*., 2012).

The phenolic compound is a secondary metabolite that is produced on the plant's shikimic acid and pentose phosphate pathway through phenylpropanoid metabolism. This has been categorized into seven groups that are flavonoid, phenolic acid, tannin (hydrolysable and condensed), stilbene, coumarin, lignan, and lignin. Flavonoids can prevent damage caused by free radicals, one of which is by directly scavenging free radicals. Flavonoid compounds can be found in every part of a plant such as leaves, root, stem, pollen, nectar, flower, fruit, and seed (Banjarnahor & Artanti, 2015).

Okra, *Abelmoschus esculentus* L. Moench, has been grown in tropical, subtropical, and warm temperate regions. It is a popular vegetable crop that has many bioactive compounds. Okra acts as an

immunostimulant, cytotoxic, bactericidal, and antioxidant in vitro from ethanol extracts obtained from various parts of okra leaves, fruits, and seeds (Guebebia *et al*., 2023) and anti-fatigue activity (Xia *et al*., 2015). Variants of okra grown in Indonesia are green, white, purple, and red. Red okra is one kind of plant that has high antioxidant content. Red okra is rich in flavonoid and polyphenols, including strong antioxidants such as quercetin, which also have medical benefit. Red okra contains high levels of anthocyanins, these anthocyanins contribute significantly to the overall antioxidant activity of red okra, providing additional health benefits compared to green okra (Son *et al*., 2023). Okra is a source of protein, carbohydrates, minerals, vitamins, and food fiber. Its leaves, flowers, and fruits contain several phenolic compounds that act as antioxidants (Bawa & Badrie, 2016; Wahyuningsih et al., 2021). These plants are easy to get in Indonesia at affordable prices and can be used as medicinal plants.

The preparation of medicinal plants for experimental purposes is the main key step to achieving quality research results. Preparation of medicinal plants for experimental purposes, including the correct and timely collection of plants, validation by experts, adequate drying, and milling. This is followed by the extraction, fractionation, and isolation of bioactive compounds, as well as the determination of the quantity and quality of bioactive compounds. Antioxidant compounds from plants can be obtained by extraction using solvents. The main steps to obtain plant bio-active molecules are the selection of appropriate solvents, extraction methods, phytochemical screening procedures, fractionation methods, and identification techniques. Appropriate solvents for the extraction of medicinal plants are polar (e.g. water, alcohol), intermediate polar (e.g. acetone, dichloromethane), and nonpolar (e.g. n-hexane, ether, chloroform) (Abubakar & Haque, 2020).

This study is using the DPPH method for the antioxidant test by measuring the IC_{50} value. This method is used because it is easy to use, quick, relatively affordable, and sensitive toward small-scale sample concentration, and if an error occurs, it will be quite easy to redo (Daulay *et al*., 2021). There have not been any studies comparing the antioxidant levels of *A. esculentus* leaves, fruits, stems, and roots using different solvent polarities (ethanol, ethyl acetate, and n-hexane) using the DPPH method. This study aims to determine IC_{50} values and compare the total phenolic and flavonoid contents of A. esculentus leaves, fruits, stems, and roots ethanol, ethyl acetate, and n-hexane extract.

MATERIALS AND METHODS

Plant material and preparation of extracts. Fresh red okra (*A. esculentus*) from leaves, fruits, stems, and roots in this study were collected from a local farm (Malang). Then, to remove foreign material, they were washed and dried and then cut into small pieces. All samples were air-dried in room temperature with no contact to direct sunlight. After that, the samples were mashed into powder. Extracts were made by the modification of the Wahyuningsih *et al*., (2020) method. Samples were extracted using a gradual maceration process with different solvent polarities. Samples were submersed in solvents with 1:1 ratio. Then stirred using vortex/spatula on a scale during maceration stage. The first extraction was using n-hexane (3x72 h). The second extraction was using ethyl acetate (3x72 h). The last extraction was using ethanol (3x72 h). In the drying process, simplicial were air-dried in room temperature with no contact to direct sunlight. Simplicial should have been dried from the previous solvent before changing the solvent in the immersion process for the solvent to absorb well. Each solvent was filtered using filter paper. Filtrate then was concentrated using a rotary evaporator at 60^{o}C.

Antioxidant activity test with DPPH (2,2-diphenyl-1-picrylhydrazyl) method. The antioxidant activity test was done using the DPPH method. The assays were carried out according to the previous study with slight modifications (Wahyuningsih *et al*., 2021). The making of DPPH 1000 ppm stock solution (1 mg DPPH dilution and 1 mL methanol p.a). The making of DPPH 50 ppm stock solution (100 mL DPPH 1000 ppm stock solution dilution and 1900 mL methanol p.a.). The main solution of 1000 ppm *A. esculentus* (leaves, fruits, stems, and roots) is ethanol, ethyl acetate, and n-hexane extract (1 mg sample extract in 1 mL methanol p.a.). The main solution of the sample

extract was diluted at the concentration of 5, 10, 20, 30, 40, and 50 ppm by mixing the main solution with methanol p.a. The amount of 200 µL sample solution was injected into the micro-plate. Added with 100 µL 50 ppm DPPH solution. Samples then were incubated in a dark room for 60 min. These steps were done in 3 repetitions. Sample absorbance using UV-vis microplate spectrophotometer at λ517 nm. Calculating percentages of antioxidant activity (% Inhibition) (Wahyuningsih *et al*., 2020):

$$\frac{Blank\ absorbance - Sample\ absorbance}{Blank\ absorbance} x\ 100\%$$

IC_{50} value determination. IC_{50} value was determined using the standard equation y = Ax + B. This equation was put into the linear regression in the Y variable and then substituted with 50 (IC_{50} coefficient) and X variable as the concentration level (ppm). This then will get the line equation with X as the concentration level (ppm) and Y as the percentage of antioxidant activity (%). The results then were categorized based on the value of their IC_{50} (Daulay *et al*., 2021). The IC_{50} values are classified into five categories, which are very strong (<50 ppm), strong (50-100 ppm), moderate (100-250 ppm), weak (250-500), and inactive (>500 ppm) (Indarti *et al*., 2019).

Total phenolic compound (TPC) assay. Sample stock solution was prepared with a concentration of 1000 ppm (1 mg in 1000 µL methanol p.a). An amount of 25 µL sample extract was injected into the micro-plate, added with Folin-Ciocalteu reagent (25 µL), and aquadest (75 µL). The sample was then incubated in a dark room for 5 min. Then, Na_2CO_3 solution (7%) was added to as much as 100 µL. The solution was homogenized and incubated for 90 min in a dark room. The absorbance was measured at λ753nm with a spectrophotometer micro-plate. The linear equation using the Gallic acid standard curve (0, 50, 100, 200, 300, 400, and 500 ppm). TPC was specified in milligrams of gallic acid equivalents per gram of extract dry weight (mg GAE/g) (Modification of Hidayati *et al*., 2019).

Total flavonoid content (TFC) assay. Sample stock solution was prepared with a concentration of 1000 ppm (1 mg in 1000 µL methanol p.a). An amount of 50 µL sample extract was injected into the micro-plate, added with 10 µL 10% AlCl3, 150 µL ethanol, and 10 µL 1 M CH_3COONa. The solution was homogenized and incubated for 40 minutes in a dark room. The absorbance was measured at λ430nm with a spectrophotometer micro-plate. The linear equation using quercetin standard curve (0, 50, 100, 200, 300, 400, and 500 ppm). TFC was specified in milligrams of quercetin equivalents/gram of extract dry weight (mg QE/g) (Modification of Nisa *et al*., 2017).

RESULTS AND DISCUSSION

Antioxidant activity of *A. esculentus* with DPPH method. The working principle for the DPPH method is that when the DPPH solution reacts with an antioxidant substance, the substance will donate its electron to DPPH to neutralize the free radical that exists in DPPH. The substance in the DPPH solution has the color of dark purple which will form a 2,2-diphenyl-1-picrylhydrazyl compound. The color of the solution will fade into lighter purple and even yellow. This resulting color change shows the level of antioxidant activity (Baliyan *et al*., 2022).

The Inhibition Concentration value (IC_{50}) interprets the antioxidant activity and indicates the samples ability to contain antioxidants to inhibit 50% of free radicals. Lower value indicates higher antioxidant activity (Olugbami *et al*., 2014; Wahyuningsih *et al*., 2021). The obtained IC_{50} values showed that ethanol had more antioxidants dissolved in the solvent rather than in ethyl acetate and n-hexane (Table 1). This happened because ethanol has a hydroxyl group in its structure that can form a hydrogen bond with hydroxyl groups from phenolic and flavonoid compounds. This reaction increased the solubility of phenolic and flavonoid compounds in ethanol (Prayitno *et al*., 2016).

Tabel 1. Result of antioxidant activity test and IC_{50} value of *A. esculentus* leaves, fruits, stems, and roots ethanol, ethyl acetate and n-hexane extract with DPPH method

Plant sample types	Solvent types	IC_{50} value (ppm)	Antioxidant activity
Leave	Ethanol	63.61	Very strong
	Ethyl acetate	119.08	Moderate
	N-hexane	165.10	Weak
Fruit	Ethanol	59.74	Strong
	Ethyl acetate	179.91	Weak
	N-hexane	821.55	Inactive
Stem	Ethanol	25.22	Very strong
	Ethyl acetate	105.77	Moderate
	N-hexane	358.48	Inactive
Root	Ethanol	89.53	Strong
	Ethyl acetate	85.63	Strong
	N-hexane	253.93	Inactive

The antioxidant activity of *A. esculentus* which is categorized as very strong was found in the stem ethanol extract with the value IC_{50} of 25.22 ppm. This result was supported by the result of total phenolic content (TPC) shown in Table 2. This showed that *A. esculentus* stems ethanol extract had the highest TPC among other samples. Yeats & Rose (2013) stated that the woody stem has layers of hydrophobic cuticles, saps, or resin to prevent bug bites and forms an indigestible cell wall, with the result that the woody stem has more secondary metabolites for its form of defense.

The previous study by Wahyuningsih *et al*. (2021) also showed that the fruit of *A. esculentus* had a high IC_{50} value in ethanol that was 38.8 ppm which was categorized as very strong. Whereas the ethyl acetate extract had an IC_{50} value of 89.5 ppm which was categorized as strong and the n-hexane extract had an IC_{50} value of 113.4 ppm which was categorized as moderate. The different results of antioxidant activity shown in this research could be resulted by environmental differences of where the samples were being collected. Environmental conditions can affect the contents of secondary metabolites in plants. The study by Martinez *et al*. (2022) stated that the factors that can affect the production of secondary metabolites are temperature, rainfall, and sunlight intensity obtained by plants. Thermal pressure such as high CO_2 levels can also be an influence. The higher the CO_2 level the higher the production of secondary metabolites will be. This showed that there is a form of defense for the plants to adapt to the conditions by producing secondary metabolite compounds. *A. esculentus* extract acts as a primary antioxidant based on its ability to scavenge DPPH free radicals. This activity of *A. esculentus* can explain its application in treating various diseases, especially those related to the formation of free radicals.

Total phenolic content (TPC). The working principle of TPC assay is the formation of blue-colored molybdenum-tungsten which is a complex substance for its aromatic core in phenolic compound that reduces phosphomolybdate–phosphotungstate (Perez *et al*., 2023). The addition of Na_2CO_3 7.5% aimed to form an alkaline solution that proton-dissociates in the phenolic compound into phenolic ion (Aswar *et al*., 2021). The linear regression of the gallic acid standard curve was y=0.0025x + 0.0533 with R^2 close to 1, which was 0.9962 (Fig. 1).

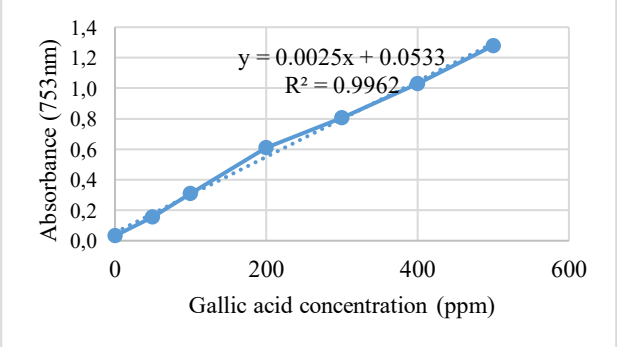

Fig. 1. Gallic acid standard curve and linear regression at the concentration of 0-500 ppm

The highest phenolic content was found in *A. esculentus* stem's ethanol extract with 156.01 mg GAE/g. The lowest phenolic content was found in the stems' n-hexane extract with 25.88 mg GAE/g. Phenolic compounds generally have a high extraction rate and are easier to dissolve in polar solvents than semi-polar or nonpolar (Mahardika & Roanisca, 2020). The TPC in plants positively correlates with its antioxidant activity. If the plant has a higher content of phenolic compounds, this means the higher activity of the antioxidant (Martinez *et al*., 2022). This statement supports the result that showed most of the plant parts that are studied had the most TPC in ethanol extract and the lowest in n-hexane. Because phenolic compounds are important in plant defense mechanism, including their antimicrobial and antioxidant properties, which help plant evade pathogenic infection and protect major tissues form effects of reactive oxygen (Salunke & Koche, 2023).

Table 2. Total Phenolic Content (TPC) of *A. esculentus* leaves, fruits, stems, and roots ethanol, ethyl acetate, and n-hexane extract

Plant sample types	Solvent types	Total phenolic (mg GAE/g)
Leaves	Ethanol	118.28 ± 9.70
	Ethyl acetate	79.21 ± 9.34
	N-hexane	52.95 ± 2.81
Fruits	Ethanol	69.88 ± 2.88
	Ethyl acetate	45.61 ± 1.51
	N-hexane	30.68 ± 2.00
Stems	Ethanol	156.01 ± 7.20
	Ethyl acetate	60.15 ± 13.03
	N-hexane	25.88 ± 1.83
Root	Ethanol	104.81 ± 17.45
	Ethyl acetate	114.81 ± 8.89
	N-hexane	31.48 ± 0.40

Total flavonoid content (TFC). Flavonoid is a typical compound found in almost every part of plants such as seed, leaf, stem, pollen, nectar, flower, and fruit (Panche *et al*., 2016). The principal of this assay is the formation of an aluminum chloride complex ($AlCl_3$) with a ketone group on the C4 atom and a hydroxyl group on the C3 or C5 atom (Ilmi *et al*., 2020). The linear regression of the quercetin standard curve was y = 0.0056x + 0.071 with R^2 close to 1, which was 0.9996 (Fig. 2).

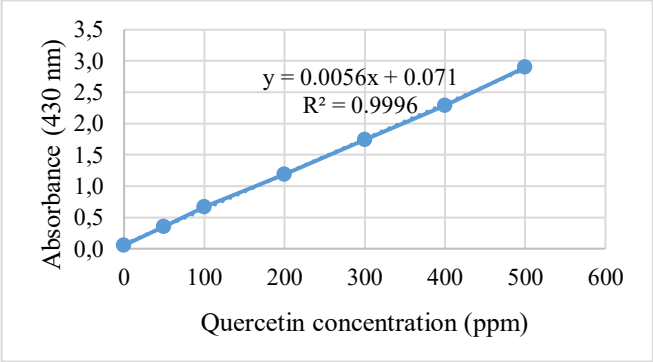

Fig. 2. Quercetin standard curve and linear regression at the concentration of 0-500 ppm

A. esculentus leaves ethanol extract had the highest TFC with 108.15 mg QE/g. Stems ethanol extract appeared to be the lowest TFC with 2.26 mg QE/g. Etika & Iryani (2019) stated that polar solvents, for instance, ethanol can be used for flavonoid extraction from plant tissues. However, flavonoid compounds are divided into several groups with different polarity depending on the number and position of the hydroxyl group (-OH) in each flavonoid group (Aisyah & Khoirul, 2022). This indicates that flavonoid compounds not only can be dissolved by polar solvents such as ethanol, but also by semi-polar solvents such as ethyl acetate, and nonpolar solvents such as n-hexane. This study showed that *A. esculentus* stem extract was more soluble in semi-polar solvent (ethyl acetate) than in

polar (ethanol) and nonpolar (n-hexane) solvents. The phenolic and flavonoid contents in this study were present in significant amounts in all okra extracts. However, there were observations of variations in the secondary metabolite content of the extract depending on the type of organ. Different parts of the okra plant, such as flowers, leaves, seeds, and pods, exhibit distinct profiles of secondary metabolites, which influence their antioxidant activities and other biological properties (Abdul-Razek *et al*., 2023).

The antioxidant potential of plant extracts is due to the presence of phytochemicals such as polyphenols and flavonoids. Both plant secondary metabolites are known to have the ability to absorb, neutralize, and extinguish singlet and triplet oxygen. Plants that produce phenolics have medicinal properties, including anti-carcinogenic, antioxidant, anti-mutagenic, free radical scavenging activities, etc. Flavonoids and phenolic compounds have a positive effect on human nutrition and health because of their strong antioxidant potential (Olugbami *et al*., 2014). A high TPC value indicates the potential therapeutic relevance of *A. esculentus* extract based on the fact that phenolics have been associated with diverse biological properties. Therefore, okra is a source of medicine because it is rich in bioactive compounds such as polyphenols and flavonoids.

Table 3. Total flavonoid content (TFC) of *A. esculentus* leaves, fruits, stems, and roots ethanol, ethyl acetate, and n-hexane extract

Plant sample types	Solvent types	Total flavonoid (mg QE/g)
Leaves	Ethanol	108.15 ± 5.10
	Ethyl acetate	28.75 ± 2.11
	N-hexane	15.71 ± 3.10
Fruits	Ethanol	55.00 ± 5.97
	Ethyl acetate	33.75 ± 2.77
	N-hexane	22.26 ± 1.72
Stems	Ethanol	2.26 ± 0.37
	Ethyl acetate	13.21 ± 0.18
	N-hexane	12.44 ± 1.54
Root	Ethanol	5.30 ± 1.27
	Ethyl acetate	9.70 ± 0.27
	N-hexane	3.99 ± 1.13

CONCLUSION

The results of this study indicated that the parts of the red okra plant (*A. esculentus*) contained large amounts of total phenolics and flavonoids and exhibited antioxidant activity. In addition, it has been indicated that the highest antioxidant content was found in *A. esculentus* stem ethanol extract with an IC_{50} value of 22.25 ppm. The highest TPC was found in *A. esculentus* stem ethanol extract with 156.01 mg GAE/g and *A. esculentus* leaves ethanol extract had the highest TFC with 108.15 mg QE/g.

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