e-ISSN: 2807-6257

Open Access Indonesian Journal of Medical Reviews

[OAIJMR]

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Antioxidant Activity of Cubeb Fruit Extract (*Piper cubeba* L.) Using the ABTS Method and Determining Total Phenolic and Flavonoid Levels

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ARTICLE INFO

Keywords:
ABTS
Antioxidant
Cubeb fruit
Piper cubeba L.
Total flavonoids

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All authors have reviewed and approved the final version of the manuscript.

https://doi.org/10.37275/oaijmr.v4i3.566

A B S T R A C T

Cubeb fruit (*Piper cubeba* L.) is a plant that is rich in flavonoid compounds and has the potential to be a source of natural antioxidants. Free radicals can cause various dangerous diseases. This study aims to determine the antioxidant activity and total flavonoid content of ethanol extract of fruit cubes using the ABTS method. Cubeb fruit is extracted with ethanol using the maceration method. The antioxidant activity of the extract was measured using the ABTS and Trolox methods as a comparison. Total flavonoid levels were determined using the UV-Vis spectrophotometric method using gallic acid and quercetin as a comparison. The total flavonoid content of the ethanol extract of cubed fruit was 3.5302 ± 0.0918 mgQE and the total phenolic content was 183.0396 ± 0.991 mgGAE. The IC_{50} value of the ethanol extract of cubed fruit was 82.1023 ± 0.072062 µg/mL and trolox 20.1368 ± 0.2367 µg/mL. In conclusion, the ethanol extract of cubed fruit has strong antioxidant activity (IC_{50} in the range 50-100 µg/mL) and is rich in total flavonoids.

1. Introduction

In the modern era, health is a top priority for every individual. Unhealthy lifestyles, environmental pollution, and chronic stress contribute to an increase in free radicals in the body. These free radicals, if not controlled, can cause cell damage and various chronic diseases such as cancer, diabetes, and heart disease. In the midst of this global health crisis, the search for effective and safe sources of natural antioxidants is becoming increasingly important. Cubeb fruit (*Piper cubeba* L.), a spice plant popular in cooking and traditional medicine, emerged as a potential candidate. Cubeb fruit is rich in active compounds, especially flavonoids, which have the ability to neutralize free radicals and protect the body from oxidative damage. Cubeb fruit (*Piper cubeba* L.), also known as Javanese pepper, is a spice plant that has long been used in cooking and traditional medicine in various parts of the world. In Indonesia, cubeb fruit is easy to find in traditional markets and is often used as a cooking spice to add its distinctive taste and aroma. More than just a flavor enhancer, cubeb has extraordinary potential as a source of natural antioxidants that can protect the body from various chronic diseases. Antioxidants play an important role in warding off free radicals, reactive molecules produced by the body naturally, and external exposure such as air pollution, cigarette smoke, and UV rays.

These free radicals, if not controlled, can trigger oxidative stress and cell damage, leading to various chronic diseases such as cancer, diabetes, heart disease, and neurodegenerative diseases. Cubeb fruit is rich in various active compounds, including flavonoids, phenolics, and alkaloids, which have been shown to have strong antioxidant activity. Flavonoids, such as quercetin, luteolin, and apigenin, are the most studied group of plant compounds due to their ability to neutralize free radicals, increase antioxidant enzyme activity, and protect cells from oxidative damage. Scientific research on the antioxidant potential of cubeb fruit is still relatively limited. Existing studies show promising results, but further research is still needed to strengthen scientific evidence and reveal the benefits of cubed fruit comprehensively.[1-3]

In the modern era with a fast-paced lifestyle full of pollution, exposure to free radicals is increasing. This triggers an increase in the number of chronic diseases which become a global health burden. Finding natural sources of antioxidants that are easily accessible and affordable is an urgent need. Cubeb fruit, as a spice plant that is easy to find and relatively cheap, offers great potential as a source of natural antioxidants. Revealing the antioxidant activity and health benefits of cubeb fruit in a comprehensive manner can open up new opportunities in the development of preventive and alternative therapies for various chronic diseases.[4-6] This research aims to fill the knowledge gap regarding the antioxidant potential of cubeb fruit. Using the ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) method, this research will measure the antioxidant activity of the ethanol extract of fruit cubes accurately and reliably. In addition, this research will also determine the total flavonoid content of the ethanol extract of cubed fruit, the main compound that contributes to its antioxidant activity. It is hoped that the results of this research will provide important information about the potential of cubed fruit as a source of natural antioxidants that are beneficial for health.

2. Methods

The place of research was carried out at the Pharmaceutical Chemistry Laboratory of Wahid Hasyim University. The materials used in this research were ethanol extract from cubeb fruit, ethanol solvent p.a. The ingredients for determining total phenolics are gallic acid, Na_2CO_3 7%, Folin-Ciocelteu, and ethanol p.a. As well as the material used to test antioxidant activity, namely ABTS (2,2- Azinobis (3-Ethylbenzothaizoline)-6- Sulfonic Acid) p.a (Merck), trolox (Sigma), and potassium persulfate ($K_2S_2O_8$). The materials used for determining flavonoids are ethanol p.a., distilled water, aluminum chloride ($AlCl_3$) 10% (Merck), potassium acetate 1M (CH_3COOK), and quercetin (sigma). The tools used in phytochemical screening tests are analytical scales, a set of glass tools, filter paper, and a magnetic stirrer. The tools used to determine the levels of total flavonoids, total phenolics, and antioxidant activity tests are micropipette (blue and yellow), yellow tipe, blue type, cuvettes, and Shimadzu brand UV-Vis Spectrophotometer.

Extraction was carried out using the maceration method with 96% ethanol solvent. Put 1 kg of cubed fruit powder into a jar, then add 3 L of 96% ethanol solvent to get a ratio of 1:3, then close the jar tightly and cover the jar with brown paper to avoid direct sunlight and cover with aluminum foil. Soak for 3 days and stir for 15 minutes every 8 hours. After 3 days, filtration was carried out and macerate 1 was obtained. Then the dregs from the filtration were added to 712 mL of 96% ethanol solvent and re-soaking (remaceration) was carried out for 2 days. After 2 days, filtration was carried out again to obtain maserate 2. Maserate 1 and 2 were deposited overnight then separated from the residue and concentrated using a vacuum device. Rotary evaporator at a temperature of 50°C to obtain 96% ethanol extract.

Preparation of ABTS Solution: ABTS powder and potassium persulphate powder were weighed at 100 mg and 165.6 mg respectively, then each powder was dissolved in 5 mL of ethanol. The two solutions were

taken and mixed in a 1:1 ratio, then the solution was wrapped in aluminum foil so that it was not exposed to light and incubated in a dark room for 12-16 hours until the solution was completely mixed. Preparation of Trolox Mother Solution: 25 mg of Trolox is weighed then put into a 25 mL measuring flask and filled with ethanol p.a. to the limit mark. Preparation of Trolox Standard: The standard solution is made in 5 mL with concentrations of 5 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm. Each concentration was taken as 25 µL, 50 µL, 75 µL, 100 µL, and 125 µL, then filled with ethanol p.a until the limit mark. The wavelength is determined by adding the ABTS solution, and then the absorbance of the solution is read using a UV-Vis spectrophotometer with a wavelength of 600-800 nm. Determination of operating time by mixing ABTS and Trolox solutions with a concentration of 15 ppm with 1 mL each. Then the absorbance was read using a UV-Vis spectrophotometer at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes with the maximum wavelength obtained. Operating time can be seen from the results of the most stable absorbance and the highest absorption. The highest absorption indicates that the sample has reacted completely.

Preparation of 10,000 ppm sample stock solution: 1,000 mg of cubeb fruit ethanol extract was taken and put into a 50 mL beaker glass, then dissolved with 25 mL of ethanol p.a. using a magnetic stirrer with a speed of 300 rpm until completely dissolved. The solution was filtered using filter paper and put into a 100 mL volumetric flask and made up to the limit using ethanol p.a. Preparation of sample stock solution 1,000 ppm: The mother solution of ethanolic extract of cubeb fruit with a concentration of 10,000 ppm is taken as much as 1 mL, then put into a 10 mL volumetric flask and made up to the mark with ethanol p.a. A concentration series was made with concentrations of 20 ppm, 40 ppm, 60 ppm, 80 ppm, 100 ppm, and 120 ppm, put into a volumetric flask and filled with ethanol p.a up to the limit mark.
Measurement of antioxidant activity: 1. Trolox Activity Test: Trolox solution with levels of 5 ppm, 10 ppm, 15 ppm, 20 ppm, and 25 ppm, 1 mL is taken and 1 mL of ABTS solution is added. The solution was incubated in a dark place for operating time, then the absorbance was read using a UV-Vis spectrophotometer with a maximum wavelength of 730 nm. Do replication 3 times. 2. Antioxidant activity test of cubeb fruit extract: Sample solutions were prepared with concentrations of 20, 40, 60, 80, 100, and 120 ppm, 1 mL of each concentration was pipetted, then added with 1 mL of ABTS solution. The solution was homogenized and incubated for operating time, then the absorption was read using a UV-Vis spectrophotometer with a maximum wavelength of 730 and replicated 3 times.

Determination of total phenolic content using a UV-Vis Spectrophotometer: Preparation of gallic acid stock solution: 10 mg of gallic acid is weighed, put into a 10 mL measuring flask, then added ethanol p.a. until the limit mark. Preparation of 7% Na_2CO_3 solution: Sodium carbonate weighed as much as 7 grams and was put into a 50 mL beaker glass and then dissolved in 25 mL Aquadest. The solution was put into a 100 mL measuring flask and then added with distilled water to the mark. Preparation of cubeb fruit ethanol extract mother solution: 1000 mg of cubeb fruit ethanol extract was weighed using a porcelain cup, put into a 50 mL beaker, and dissolved in 25 mL ethanol using the help of a magnetic stirrer at a speed of 300 rpm until completely dissolved, filtered using filter paper into a 100 mL volumetric flask, then added ethanol p.a. up to the limit mark. Preparation of Gallic Acid Concentration Series: Concentration series were prepared at concentrations of 50, 100, 150, 200, 250, and 300 ppm in a 5 mL volumetric flask. The gallic acid stock solution taken from each concentration series was 250 µL, 500 µL, 750 µL, 1000 µL, 1250 µL, and 1500 µL put into a 5 mL measuring flask and then dissolved with ethanol p.a. up to the limit mark. Determination of the Maximum Phenolic λ Wavelength: Take 200 µL of a gallic acid solution with a concentration of 150 ppm into a test tube, then add 400 µL Folin-Ciocalteu, add 4 mL of Na_2CO_3 7% shake until homogeneous. Absorbance was read using UV-Vis spectrophotometry in the wavelength range (λ) 600

nm – 800 nm. Measurement operating time (OT): Take 200 µL of a gallic acid solution with a concentration of 150 ppm and put it in a test tube then add 400 µL Folin-Ciocalteu, and add 4 mL of Na_2CO_3 7% shake until homogeneous. Absorbance was read using UV-Vis Spectrophotometry in the time range 0 - 180 minutes at a maximum λ of 743 nm until a stable absorption time was obtained. Preparation of gallic acid curve: A series of gallic acid concentrations of 50, 100, 150, 200, 250, and 300 ppm, 200 µL each taken, put into a test tube then 400 µL added Folin–ciocalte, then added 4 mL of Na_2CO_3 7%. Absorbance is read using spectrophotometry at the maximum wavelength (λ), namely 743 nm, and operating time 105th minute – 135 minutes. Determination of Total Phenolic Content of ethanol extract of cubed Fruit: 200 µL of sample stock solution was taken into a test tube. Added 400 µL Folin-ciocalteu. Added 4 mL Na_2CO_3 7% shake until homogeneous. Dilution is carried out, 1 mL of the solution is taken, then put into a 5 mL measuring flask, and the volume is adjusted to the mark. Absorbance was read using a UV-Vis spectrophotometer at a maximum wavelength of 743nm and operating time minutes 105, 120, and 130 minutes. Replication was carried out 3 times.

Determination of total flavonoid content using a UV-Vis Spectrophotometer: Preparation of 10% $AlCl_3$ solution: 500 mg of $AlCl_3$ was taken and dissolved in ethanol p.a., then put into a 5 mL measuring flask and filled with ethanol p.a. to the limit mark. Preparation of 1 M Potassium Acetate: 500 mg of potassium acetate is taken dissolved in ethanol p.a, then put into a 5 mL measuring flask and filled with ethanol p.a to the limit mark. Preparation of 400 ppm quercetin solution: 20 mg of quercetin is taken and dissolved in 5 mL of ethanol. The solution was put into a 50 mL measuring flask and filled with ethanol p.a. to the mark. Preparation of quercetin concentration series: Quercetin concentration series were prepared with concentrations of 2 ppm, 4 ppm, 6 ppm, 8 ppm, 10 ppm, and 12 ppm in 5 mL ethanol p.a. Determination of maximum wavelength using a UV-Vis spectrophotometer using a quercetin comparator. 1000 µL of a concentration series solution at a level of 6 ppm was taken, then 200 µL of 10% $AlCl_3$ and 200 µL of 1M CH_3COOK were added until a yellow color was formed. Read on a UV-Vis spectrometer with a wavelength of 400-500 nm. Determination of operating time using a UV-Vis spectrophotometer and quercetin as a comparison. 1000 µL of the series solution at a concentration of 6 ppm was taken then 200 µL of 10% $AlCl_3$ and 200 µL of 1M CH_3COOK were added until a yellow color was formed. Read using a UV-Vis spectrometer with a wavelength of 436.2 nm at 0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 minutes. Determination of the Quercetin Standard Curve: Solution The concentration series of 2, 4, 6, 8, 10, and 12 ppm were taken 1000 µL each and added with 200 µL of 10% $AlCl_3$ and 200 µL of 1M CH_3COOK until a yellow color was formed. Then wait for the solution operating time of 30 minutes and the absorbance was read using a UV-Vis spectrophotometer at a wavelength of 436.2 nm. Preparation of Sample Solution: 1000 mg of ethanol extract from cubeb fruit was taken, then put into a 50 mL backer glass and dissolved with ethanol p.a. using a magnetic stirrer at a speed of 300 rpm until completely dissolved. Filter using filter paper then put into a 100 mL measuring flask and fill to the limit. Do replication 3 times. Measurement of Total Flavonoids: 1000 µL of the stock solution of ethanol extract of cubed fruit was taken, and added with $AlCl_3$ 10% and CH_3COOK 1M 200 µL each. The absorbance was read using a UV-Vis spectrophotometer at a wavelength of 436.2 nm and waited for an operating time of 30 minutes. Do replication 3 times.

3. Results and Discussion

Table 1 presents the results of research on the antioxidant activity and total flavonoid content of ethanol extract of cubed fruit. These findings indicate that cubeb fruit has potential as a source of natural antioxidants that are beneficial for health. The total flavonoid content of the ethanol extract of cubed fruit reached 3.5302 ± 0.0918 mgQE, indicating a fairly high flavonoid content. This is in line with other

research that identified cubeb fruit as a rich source of flavonoids. The total phenolic content in the ethanol extract of cubed fruit was also relatively high, reaching 183.0396 ± 0.991 mgGAE. Phenolic compounds, apart from flavonoids, also have antioxidant activity that is beneficial for health. The ethanol extract of cubeb fruit showed strong antioxidant activity with an IC_{50} value of 82.1023 ± 0.072062 µg/mL. The low IC_{50} value indicates the extract's ability to effectively neutralize ABTS free radicals. As a comparison, the IC_{50} value of Trolox, a standard antioxidant compound, is 20.1368 ± 0.2367 µg/mL. Although Trolox has stronger antioxidant activity, these results indicate that the ethanol extract of cubed fruit has the potential as a promising source of natural antioxidants.

Table 1. Antioxidant activity and total flavonoid content of ethanol extract of cubed fruit (*Piper cubeba* L.).

Parameter	**Value**	**Unit**
Total flavonoid levels	3,5302 ± 0,0918	mQE
Total phenolic levels	183,0396 ± 0,991	mgGAE
IC_{50} ethanol extract of cubed fruit	82,1023 ± 0,072062	µg/mL
IC_{50} trolox	20,1368 ± 0,2367	µg/mL

This research shows that the ethanol extract of cubed fruit has strong antioxidant activity with an IC_{50} value in the range of 50-100 µg/mL. This finding is in line with other research which shows that flavonoids, compounds that are abundant in cubeb fruit, have high antioxidant activity. The low IC_{50} value indicates that the ethanol extract of cubed fruit requires a small concentration to neutralize 50% of ABTS free radicals. This shows that the ethanol extract of cubeb fruit is effective in combating oxidative stress caused by free radicals. Oxidative stress can trigger various chronic diseases such as cancer, diabetes, and heart disease. The high level of total flavonoids in the ethanol extract of cubeb fruit (3.5302 ± 0.0918 mgQE) is thought to play an important role in its antioxidant activity. Flavonoids have a chemical structure that can donate electrons or hydrogen to neutralize free radicals. Flavonoid antioxidant mechanisms may also involve metal chelation, modulation of antioxidant enzymes, and inhibition of free radical signaling pathways. Even though the IC_{50} value of the ethanol extract of cubed fruit is higher than Trolox, a standard antioxidant compound, its antioxidant activity is still relatively strong. This shows that the ethanol extract of cubed fruit has the potential as a source of natural antioxidants that are beneficial for health. Overall, the results of this study indicate that the ethanol extract of cubed fruit has strong antioxidant activity and is rich in total flavonoids. These findings support the potential of cubeb fruit as a natural source of antioxidants that can help prevent various chronic diseases related to oxidative stress. Further research is needed to examine the antioxidant mechanism of action of cubed fruit ethanol extract in more detail and evaluate its potential as a preventive or alternative therapy for chronic diseases.[7-11]

Flavonoids, compounds found in many ethanol extracts of cubed fruit, have several antioxidant mechanisms that contribute to their activity in neutralizing free radicals and protecting the body from oxidative stress. Flavonoids have a chemical structure that is rich in hydroxyl groups (-OH) which can donate electrons or hydrogen to neutralize free radicals. Free radicals are unstable molecules with one extra electron, which can damage cells and body tissues. By donating electrons or hydrogen, flavonoids can stabilize free radicals and prevent cell damage. Free radicals can be formed through redox reactions involving transition metals such as iron (Fe) and copper (Cu). Flavonoids can bind these transition metals, making them unavailable for redox reactions and preventing the formation of free radicals.

Flavonoids can increase the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. These enzymes play an important role in detoxifying free radicals and protecting cells from damage. Flavonoids can inhibit the free radical signaling pathway, which is an intercellular communication mechanism involved in oxidative stress. By inhibiting this signaling pathway, flavonoids can reduce the production of free radicals and protect cells from damage. The high total flavonoid content in the ethanol extract of cubed fruit (3.5302 ± 0.0918 mgQE) is thought to play an important role in its antioxidant activity. The more flavonoids contained in the extract, the greater its potential to donate electrons or hydrogen, stabilize free radicals, and protect cells from damage. The flavonoids in the ethanol extract of cubeb fruit have various complex antioxidant mechanisms and are effective in neutralizing free radicals and protecting the body from oxidative stress. The high total flavonoid content in this extract makes cubeb fruit a potential source of natural antioxidants for various health applications.[12-16]

The strong antioxidant activity of the ethanol extract of cubeb fruit, as demonstrated in this study, offers a variety of potential benefits for human health, especially in preventing chronic diseases related to oxidative stress. Oxidative stress contributes to DNA damage and cell mutation, which are major risk factors for cancer. Antioxidants such as flavonoids in the ethanol extract of cubed fruit can help neutralize free radicals and protect DNA from damage, thereby reducing the risk of cancer. Chronic diabetes is linked to increased oxidative stress that can damage pancreatic cells and interfere with insulin production. The antioxidants in the ethanol extract of cubed fruit can help protect pancreatic cells and increase insulin sensitivity, making it useful in controlling diabetes. Oxidative stress plays a role in the formation of arterial plaque and blood vessel damage, which can trigger coronary heart disease and stroke. The antioxidants in the ethanol extract of cubed fruit can help reduce oxidative stress, protect blood vessels, and improve heart health. Oxidative stress can also damage brain cells and contribute to cognitive decline and neurodegenerative diseases such as Alzheimer's and Parkinson's. The antioxidants in the ethanol extract of cubed fruit can help protect brain cells from oxidative damage and improve cognitive function. Free radicals play a role in the aging process by damaging body cells and tissues. The antioxidants in the ethanol extract of cubed fruit can help neutralize free radicals and slow down the aging process, thereby improving health and vitality.[17-20]

4. Conclusion

The ethanol extract of cubeb fruit has strong antioxidant activity and is rich in total flavonoids. The high flavonoid and phenolic content, as well as good antioxidant activity, make the ethanol extract of cubed fruit a potential source of natural antioxidants that are beneficial for health.

5. References

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