JFSP Vol.10, No.3, September-December 2024, Page: 259-266
pISSN: 2549-9068, eISSN: 2579-4558
Jurnal Farmasi Sains dan Praktis
(JFSP)
http://journal.unimma.ac.id/index.php/pharmacy

(2,2-Diphenyl-1-picrylhydrazyl) ASSAY of KENIKIR LEAVES (*C. caudatus* K.), BELUNTAS LEAVES (*Pluchea indica* L.), AND PURPLE CORN (*Zea mays*) AS A SOURCE OF ANTIOXIDANTS

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https://doi.org/10.31603/pharmacy.v10i3.9275

Article info:
Submitted : 07-06-2023
Revised : 19-08-2024
Accepted : 17-10-2024

Publisher:
Universitas Muhammadiyah Magelang

ABSTRACT
The purpose of this study is to assess and compare the antioxidant activity of the ethanol extract and ethyl acetate fraction of the leaves of Kenikir (*C. caudatus* K.), Beluntas (*Pluchea indica* L.), and purple corn seed (*Zea mays* L.) in an effort to find new sources of antioxidants. The samples were extracted with a 96% ethanol as the solvent. Afterward, the extract was separated using n-hexane and ethyl acetate to get ethyl acetate fraction. The antioxidant concentration was determined using the DPPH free radical technique using spectrophotometer UV-Vis. The results indicate that the purple corn's ethyl acetate fraction has the highest antioxidant activity as a free-radical scavenger, with an IC_{50} value of 10.47 µg/mL. The ethyl acetate fraction of *P. indica* leaves, the *P. indica* 96% ethanol extract, the *C. caudatus* leaves 96% ethanol extract, the purple corn's 96% ethanol extract, and the *P. indica* 96% ethanol extract are all listed after that. The purple corn's 96% ethanol extract has an IC_{50} value of 21.80 µg/mL. According to these findings, purple corn has the highest antioxidant activity and may be a new source of antioxidants.

Keywords: *C. caudatus* K.; *Pluchea indica* L.; *Zea mays* L.; Antioxidant; DPPH

1. INTRODUCTION

Degenerative disease is one of the world's most significant causes of death. The World Health Organization (WHO) identifies cardiovascular disease, cancer, and diabetes as the three most prevalent degenerative diseases. According to statistics, it kills 41 million people annually, or 71% of the global population. Oxidative stress and the excessive number of oxidation reactions occurring within the body produce degenerative disease (Werdhawati, 2014). Oxidative stress is typically used to describe an imbalance between the manifestation of free radicals in the systemic and the ability of cells to detoxify and negate the effects of damage to proteins, fats and DNA (Ahmadinejad et al., 2017). An antioxidant is needed to reduce the amount of excess free radicals and prevent the damaging effects of *oxidative stress* that can cause various diseases. The process of forming free radicals can occur through oxidation reactions. Antioxidants can inhibit oxidation reactions, even if only in small concentrations (Yadav et al., 2017). In order to stop the free radical reaction, antioxidants are chemical substances that can provide free radicals one or more electrons (electron donors). Due to its low molecular weight, this substance can inhibit the growth of oxidation reactions by inhibiting the production of radicals (De Araújo et al., 2014).

One such compound is a phenolic compound, a secondary metabolite that protects plant organs from oxidation. Therefore, phenolic compounds are referred to as natural antioxidants. In

addition to their activity as antioxidants, phenolic compounds in plants are known to have anticarcinogenic, antibacterial, antiallergic, antimutagenic, and anti-inflammatory properties (Sukweenadhi et al., 2020). Other phytochemicals that possess antioxidant activity are flavonoids. Flavonoids are a group of polyphenols and are classified by their chemical structure and biosynthesis. The basic structure of flavonoids consists of two aromatic groups combined by a carbon bridge (C6-C3-C6). The mechanism of flavonoids as antioxidants is divided into three, namely slowing down the formation of Reactive Oxygen Species (ROS), breaking down ROS and regulating / protecting with antioxidants. Flavonoids also stimulate internal antioxidant enzymes, suppress enzymes related to the formation of free radicals, and bind metals. Secondary metabolites of flavonoids derived from plants and some of them from *C. caudatus* leaves, *P. indica* leaves, and purple corn kernels since they have high level of flavonoids. Based on the study by (Mediani et al., 2013), the IC_{50} value of an ethanol extract of *C. caudatus* leaves at 80% concentration is 32 µg/mL (Werdhawati, 2014) revealed that the IC_{50} values for *P. indica* leaves ethanol extract and ethyl acetate, respectively, were 37.25 µg/mL and 3.33 µg/mL. The ethanol extract of purple corn kernels has an antioxidant activity with an IC_{50} value of 48.5 mg/mL. Because of this, the study's goal is to use the DPPH method to find out if the ethyl acetate and 96% ethanol extracts from these three samples have any antioxidant properties. The experiment selected three plants from highland areas in September, potentially offering a novel approach to this research.

2. METHODS

2.1. Materials

Plant materials: *C. caudatus* leaves were collected in Gunung Kidul, Yogyakarta, *P. indica* leaves were collected in UPT Materia Medica Batu, and purple corns were collected in Sukabumi, East Java. All of them were identified in Materia Medica, Batu, East Java, Indonesia. The specimen numbers are 074/555/102.7-A/2021.

Chemical materials: Ethanol p.a Merck (Germany), ethyl acetate p.a Merck (Germany), n-hexane p.a Merck (Germany), ascorbic acid Merck (Germany), *2-2-diphenyl-1-picrylhydrazyl*, Sigma-Aldrich (United States).

Instrument: UV-Vis Shimadzu 2700 Spectrophotometer (Tokyo, Japan).

2.2. Extract Preparation

Using 96 percent ethanol as the solvent, the dry powder of *C. caudatus*, *P. indica* leaves, and purple corn kernels was extracted with ratio 1:20 using ultrasound technology (Sonica® Ultrasonic Cleaners).

Three times within 10 minutes, the extraction was repeated. The extract was filtered through a membrane filtrate, and the resulting filtrate was evaporated at 40 °C in a rotary evaporator to get a 96 percent ethanol extract of *C. caudatus*, *P. indica* leaves, and purple corn kernels.

2.3. Fraction Preparation

The 96% ethanol extract of *C. caudatus*, *P. indica* leaves and purple corn kernels was first fractionated with n-hexane and aqueous with ratio 1:1. The aqueous phase was taken and fractionated with ethyl acetate until it turned colorless. The ethyl acetate phase was collected and evaporated at 40 °C in a rotary evaporator to obtain ethyl acetate fraction of *C. caudatus*, *P. indica* leaves, and purple corn kernels.

2.4. Flavonoid Test

The ethyl acetate fraction and 96% ethanol extract of *C. caudatus*, *P. indica* leaves, and purple corn seed were dissolved in 96% ethanol. A few drops of concentrated hydrochloride acid

and a pinch of magnesium powder were also added. The color of solution turning to reddish-orange shows that the test was successful (Suharyanto & Hayati, 2021).

2.5. Antioxidant Test (Damanik et al., 2014)

2.5.1. DPPH Stock Solution

A 100-ppm DPPH solution is prepared with 96% ethanol solvent and then incubated for 30 minutes at room temperature.

2.5.2. C.caudatus Extract and Fraction

Extract - Sample was prepared by weighing 100 mg of the extract and dissolved with 96% ethanol to 100 mL volumetric flask, then pipette 0.2 mL, 0.3 mL, 0.4 mL, and 0.6 mL into a 10.0 mL volumetric flask.

Fraction - A total of 25.0 mg of the ethyl acetate fraction sample was dissolved with 96% ethanol up to 25,0 mL. Then pipette 0.5 mL, 1.0 mL, 2.0 mL, and 3.0 mL into a 50.0 mL volumetric flask

Each extract and fraction sample were added a 0.5-mL solution of DPPH and then incubated for 30 minutes at room temperature.

2.5.3. Pluchea indica Extract and Fraction

Extract - Sample was prepared by weighing 25 mg of the extract and dissolved with 96% ethanol to 25 mL volumetric flask, then pipette 0.1 mL, 0.2 mL, 0.3 mL, and 0.4 mL into a 10.0 mL volumetric flask.

Fraction - A total of 25.0 mg of the ethyl acetate fraction sample was dissolved with 96% ethanol then transferred in a 25,0 mL volumetric flask. Then pipette 0.05 mL, 0.5 mL, 0.6 mL, and 0.7 mL into a 10.0 mL volumetric flask

Each extract and fraction sample were added a 0.5-mL solution of DPPH and then incubated for 30 minutes at room temperature.

2.5.4. Purple corn Extract and Fraction

Extract - Sample was prepared by weighing 25 mg of the extract and dissolved with 96% ethanol to 25 mL volumetric flask, then pipette 0.2 mL, 0.5 mL, 1.0 mL, and 2.0 mL into a 25.0 mL volumetric flask.

Fraction - A total of 0.07 g of the ethyl acetate fraction sample was dissolved with 96% ethanol up to 50.0 mL. Then pipette 0.1 mL, 0.3 mL, 0.5 mL, and 1.0 mL in to a 25.0 mL volumetric flask

Each extract and fraction sample were added a 0.5-mL solution of DPPH and then incubated for 30 minutes at room temperature.

2.6. Ascorbic Acid

Stock solution was prepared with concentration 1000 ppm and standard solution with range 10-50 ppm. Each solution was added DPPH solution 0.5 mL and incubated for 30 minutes.

2.7. Determination of Antioxidant Activity

The absorbance of the secondary solutions were collected by using spectrophotometer UV-Vis on the maximum wavelength. This process was repeated three times on each concentration. The percentage of inhibition was calculated from the following equation:

$$\%\ Inhibition = \frac{(A.Control - A.Sample)}{A.Sample} x100\% \quad (1)$$

The data of % inhibition was plotted with absorbance to obtain a linear equation. The equation was made to calculate Inhibition Concentration 50% (IC_{50}) to determine antioxidant activity.

2.8. Data Analysis

The data of the study were analyzed by a one-way ANOVA. The data were considered significant if P-value was less than 0.05.

3. RESULTS AND DISCUSSION

The imbalance between free radicals and antioxidants can lead in oxidative stress, which can develop to degenerative illnesses. Consequently, antioxidants are required to avoid degenerative illnesses. According to the classification based on source, antioxidants are classified into two types: endogenous, which are derived from the body's enzymes, and exogenous, which are derived from outside the body, such as plants. If the body's endogenous antioxidants are insufficient, exogenous antioxidants are required. According to this study, *C. caudatus*, *P. indica* and purple corn plants have sufficient antioxidant activity to meet demand.

3.1. Yield Value of Extract and Fraction

The result of extraction process using ultrasonic-assisted extraction method with 96% ethanol as the solvent was shown in **Table 1**. Pulling the desired compound was conducted by an extraction process using Ultrasonic Assisted Extraction (UAE) with 96% ethanol as a solvent. Extraction was carried out using UAE because it's a green extraction method, meaning it doesn't need a lot of solvents. It took an advantage of using ultrasonic wave to decrease the time of extraction without increasing the temperature. Using heat may resulting on degradation of some flavonoid. The 96% ethanol solvent was chosen because it is able to optimally attract nonpolar to polar flavonoid compounds. From the extraction process, the yield was 6.8% in *C. caudatus*, 14.7650% in *P. indica* and 5.37% in purple corn (**Table 1**). Based on the yield results, it was found that the yield obtained met the requirements with the respective yield value. *C. caudatus* yield value is the highest among the samples.

The fractionation process was carried out because the extraction results still consist of secondary metabolites with various levels of polarity. The extraction results were separated by the fractionation method using a solvent that can attract and optimally dissolve flavonoid compounds. The fractionation process was carried out using the stratified fractionation method. In the first stage, the process was conducted using n-hexane as solvent. The use of n-hexane solvent aims to separate non-polar compounds such as fatty acids with a carboxyl group because the carboxyl group inhibits the hydrogen atom donation reaction to neutralize free radicals. The next stage is fractionation using ethyl acetate solvent, intending to attract the flavonoid group compounds in the sample because the solvent is semi-polar. The process shows the principle of solubility, which means that compounds will be attracted to solvents with the same polarity level (Mamuaja, 2017).

Table 1. The 96% ethanol extract yield value of *C. caudatus, P. indica* leaves and purple corn

Name of the plant	Dry Powder Weight (g)	The obtained extraction (g)	Yield value
C. caudatus leaves	1099.96	82.45	7.50%
P. indica leaves	699.02	103.21	14.77%
purple corn	1649.67	58.14	5.37%

The result of fractionation process using liquid-liquid method with ethyl acetate as the solvent was shown in **Table 2**.

Table 2. The ethyl acetate fraction yield value of *C. caudatus, P. indica* leaves and purple corn.

Name of the plant	The obtained ethyl acetate fraction (g)	Yield value
C. caudatus leaves	12.16	0.88%
P. indica leaves	0.22	0.03 %
purple corn	0.08	0.005%

The *C. caudatus* secondary metabolite composition has been widely documented. Flavonoids and terpenoids are this plant's major secondary metabolites. Quercetin and catechin are some of the flavonoids that have been discovered (Widiyantoro & Harlia, 2020). *P.indica* leaves, which are rich in antioxidants, belong to the class of chemicals called flavonoids. It may be inferred from the Ultraviolet-Visible spectrum that the flavonoid chemicals found in beluntas leaves are of the flavonol type (Fitrya et al., 2023). Purple corn contains anthocyanins. Anthocyanins are naturally occurring pigments that are part of the flavonoid group. Their primary structure consists of two benzene aromatic rings (C6H6) joined by three carbon atoms joined by an oxygen atom (Rahmah et al., 2022).

Flavonoids include -OH groups that can establish hydrogen bonds; they are polar compounds. Nonetheless, a number of flavonoid types are less polar, including isoflavones, flavones, flavanones, aurones, chalcones, anthocyanins, and flavonols. Flavonoid extraction often uses a polar solvent, such as ethanol, due to the polar nature of most flavonoid molecules, including flavonoid glycosides and aglycones. However, to extract some less polar flavonoids, use ethyl acetate. Consequently, we use ethyl acetate solution to extract less polar flavonols (Widyawati et al., 2010). In this research, a yield percentage of 0,88% *C.caudatus* leaves, 0,03% *P.indica* leaves and 0,005% purple corn were obtained from the fractionation of 82,45 g of *C.caudatus* leaves ethanol extract, 103,21 g of *P.Indica* leaves's extract and 58,14 g of purple corn's extract.

3.2. Flavonoid Assay

The result of flavonoid assay on 96% ethanol extract and ethyl acetate fraction was shown in **Table 3**. Positive result was being indicated by the changing color of solution to reddish orange solution.Flavonoid assay was conducted to determine the presence or absence of antioxidant-functioning flavonoid compounds in three samples. The Wilstater method was used to conduct the test, and it was determined that the reddish orange-colored test results indicated the presence of flavonoid compounds in the three positive samples **(Table 3)**. This is due to the reduction of bonds caused by breaking glycoside bonds by flavonoids (Muthmainnah, 2019).

Table 3. The result of flavonoid test of *C. caudatus, P. indica* leaves and purple corn

Name of the plant	96% Ethanol Extract	Ethyl Acetate Fraction
C. caudatus leaves	(+)	(+)
P. indica leaves	(+)	(+)
purple corn	(+)	(+)

3.3. IC_{50} Value

IC_{50} value of *C. caudatus, P. indica leaves,* and purple corn by calculated the obtained linear equation of each sample was shown in **Table 4**.

Table 4. The obtained IC50 value of *C. caudatus, P. indica* leaves and purple corn.

Name of the plant	96% Ethanol Extract (µg/mL)	Ethyl Acetate Fraction (µg/mL)	P-value
C. caudatus leaves	93.98	33.90	
P. indica leaves	34.19	22.98	
purple corn	21.80	10.47	0.000
Ascorbic acid	4.792		

Antioxidant test was conducted using radical DPPH, colorimetric method based on the measurement of the scavenging capacity of antioxidants towards DPPH. DPPH is a free radical in the form of a stable organic nitrogen. it has pi electrons of the aromatic systems present in the molecule can compensate for the lack of an electron (Santos-Sánchez et al., 2019). A quick and accurate way to test for antioxidants is the DPPH method. The fundamental premise behind this approach is that antioxidants act as hydrogen donors. If the substance under test is an antioxidant, it will provide hydrogen to the radical DPPH and neutralize it (Tejaputri et al., 2019). In this

method, the 96% ethanol extract and ethyl acetate fraction of *C. caudatus, P. indica* leaves and purple corn were tested by adding DPPH solution. This study utilised a wavelength of 516.50 nm. The test results indicate the absorbance value of the test solution for each sample, after which the DPPH inhibition percentage is calculated. After obtaining the percent inhibition, linearity regression is calculated to determine the IC_{50} value. Purple corn ethyl acetate fraction had the highest IC_{50} value at 10.47 µg/mL.

This is followed by the purple corn's 96% ethanol extract, which has an IC_{50} value of 21.80 µg/mL., the *P. indica* leaves ethyl acetate fraction with an IC_{50} value of 22.98 µg/mL, the *P. indica* 96% ethanol extract with an IC50 value of 34.19 µg/mL., the *C. caudatus* leaves ethyl acetate fraction, which has an IC50 value of 33.90 µg/mL., and *C. caudatus* leaves 96% ethanol extract with an IC50 value of 93.98 µg/mL **(Table 4)**. The highest IC_{50} value is found in the ethyl acetate fraction of purple corn due to the presence of flavonoids from the anthocyanin group. Meanwhile, the compound which play as antioxidant agent of *C. caudatus* and *P. indica* are flavonoid as well, to be exact, catechin and quercetin. These flavonoids have conjugated double bonds in their structure, allowing them to be highly reactive and function as free radicals. Anthocyanins are capable of reacting with various free radicals derived from reactive oxygen, hydroxyl, and singlet oxygen (Priska et al., 2018). The mechanism for free radical inhibition is the termination of the chain of free radical propagation and the donation of electrons by all hydroxyl groups in ring B (Nurtiana, 2019). Catechins represent antioxidant activity by scavenging free radicals, chelating redox active transition-metal ions, inhibiting redox active transcription factors, inhibiting pro-oxidant enzymes, and inducing antioxidant enzymes (Zanwar et al., 2014). Quercetin works as antioxidant agent by maintaining oxidative balance. It regulates levels of glutathione (GSH) to neutralize free radical by donating a hydrogen (Xu et al., 2019).

Results indicate that temperature, pH, solvent system, solvent-to-solid ratio, and number of extractions are important factors that should be optimized for each sample to maximize extraction efficiency. The obtained IC_{50} in this study was analyzed using one-way ANOVA. The result show that there is a significant difference between the obtained IC_{50} because the p was less than 0.05. The limitation of this study was only analyzing antioxidant activity of ethanol extract and hexane fraction from *C. caudatus, P. indica leaves,* and purple corn. This study's limitation is that it didn't evaluate additional solvents, such n-hexane.

4. CONCLUSION

Purple corn's ethyl acetate fraction has the highest antioxidant activity as a free-radical scavenger with an IC_{50} value of 10.47 µg/mL., followed by its ethanol extract which has an IC_{50} value of 21.80 µg/mL. These three plants can be turned into preparations that act as antioxidants, according to the findings.

5. ACKNOWLEDGMENTS

All authors would like to thank to Hang Tuah University for supporting funds to conduct this study.

6. AUTHOR DECLARATION

Authors' Contributions and Responsibilities

The authors made substantial contributions to the conception and design of the study. The authors took responsibility for data analysis, interpretation, and discussion of results. The authors read and approved the final manuscript.

Funding

Hang Tuah University.

Availability of Data and Materials
All data are available from the authors.

Competing Interests
The authors declare no competing interest.

Additional Information
No additional information from the authors.

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