

Research Article

Antioxidant Activity of 96% Ethanol Extract of Pepaya Jepang Leaves (*Cnidoscopus aconitifolius* (Mill.) I. M. Johnst) Using DPPH Method (1,1-diphenyl-2-picrylhydrazyl)

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*Pepaya jepang***Abstract**

Free radicals in the human body can be helped neutralized by natural antioxidants derived from plants. *Pepaya Jepang* (*Cnidoscopus aconitifolius* (Mill.) I. M. Johnst) contains antioxidant compounds such as flavonoids and polyphenols. This study examined the antioxidant activity of 96% ethanol extract of *C. aconitifolius* leaves. Maceration of *C. aconitifolius* leaves using 96% ethanol solvent, phytochemical screening, and antioxidant activity assay was carried out with 1,1-Diphenyl-2-Picryl Hydrazyl (DPPH) on 96% ethanol extract of *C. aconitifolius*. Extraction obtained a yield of 16.1834%, and phytochemical screening on the 96% ethanol extract of *C. aconitifolius* contained flavonoids, tannins, saponins, phenols, alkaloids, and steroids. The antioxidant activity assay on 96% ethanol extract of *C. aconitifolius* had an IC₅₀ value of 145.3855 ppm and an IC₅₀ value of vitamin C of 7.0806 ppm. The Antioxidant Activity Index (AAI) of 96% ethanol extract of *C. aconitifolius* was 0.4127, and the AAI of vitamin C was 8.4739. Based on the results obtained, although in a weak classification, a 96% ethanol extract of *C. aconitifolius* leaves has antioxidant activity.

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INTRODUCTION

Free radicals are compounds that have one or more unpaired electrons. Free radicals in the human body come from within the body (endogenous), such as the result of electron transport in mitochondria, and come from outside the body (exogenous), such as vehicle pollution, chemicals, and other pollutants¹. Free radicals that are not excessive can be neutralized with antioxidants in the body. However, antioxidants are required outside the body to help neutralize excess free radicals. These antioxidants can be obtained either naturally or artificially. Natural antioxidants can be obtained through the extraction of plants, while synthetic antioxidants are obtained from chemical synthesis processes².

The general public rarely uses synthetic antioxidants at this time due to their limited availability. Several studies have found that synthetic antioxidants may cause inflammation, liver damage, and carcinogenesis in laboratory animals when used long-term, such as BHA (Butylated Hydroxy Anisole)³. Concerns about the adverse effects of synthetic antioxidants have encouraged further research exploring alternative sources of antioxidants, such as antioxidants derived from plants⁴. Through His creation, God has provided plants that can be used as a source of nutrition and as medicinal ingredients by humans. Several studies have stated that consuming fresh vegetables and fruits rich in antioxidants and adopting a healthy lifestyle can reduce the risk of being attacked by degenerative diseases^{5,6}.

Plants containing antioxidant activities typically have significant quantities of polyphenolic chemicals. Polyphenol compounds are secondary metabolites derived from plants that have numerous health benefits. Polyphenolic chemicals

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provide antioxidants, protection against infections, and protection from UV radiation to plants⁷. Several prior investigations have indicated that several plants, including the leaves of *Cnidoscolus aconitifolius* (Mill.) I.M.Johnst (**Figure 1**), have significant polyphenol chemicals^{8,9}.

Cnidoscolus aconitifolius is a plant native to Yucatan, Mexico. This plant, sometimes known as "Tree Spinach," is an annual plant native to Mexico and Central America. It is also known as "Efo Inyana Ipaja" in Yoruba, Western Nigeria, and "Ogwonuoria" in Ibo, eastern Nigeria. Plants of *C. aconitifolius* are categorized as shrubs of the Euphorbiaceae family. This plant can reach a height of 6 M, with curled and pointed leaves, milky sap, and small flowers on dichotomous branched cymes¹⁰. *Cnidoscolus aconitifolius* is known as "Pepaya Jepang" in Java, Indonesia.

In several locations, *C. aconitifolius* is utilized as an alternative to ethnopharmaceutical therapy to treat various human diseases. This herb is widely used in Africa to treat diabetes, hypercholesterolemia, hyperlipidemia, obesity, atherosclerosis, and laxative kidney stones^{11,12}. Traditional treatment for diabetes mellitus and hypercholesterolemia in Nigeria uses polar solvent extracts from *C. aconitifolius* leaves^{9,13,14}.

Acute toxicity studies on various types of extracts from the *C. aconitifolius* plant have been conducted, with the ethanol extract of *C. aconitifolius* leaves having an LD₅₀ of 4000 mg/Kg BW, the methanol fraction having an LD₅₀ of 3500 mg/Kg BW, the *n*-hexane fraction having an LD₅₀ of 2000 mg/Kg BW, and the chloroform fraction having an LD₅₀ of 2500 mg/Kg BW. As a result of the high LD₅₀ derived from the results of this study, all *C. aconitifolius* leaf extracts show that this plant is non-toxic¹⁵.

The *C. aconitifolius* plant is commonly found in tropical and subtropical areas worldwide, including Africa, south of the Sahara, North and South America, India, and Indonesia. *Cnidoscolus aconitifolius* is commonly consumed as a vegetable in South Western Nigeria, called "Iyana Ipaja" in soups and salads, due to its good nutritional value¹⁶. Furthermore, in Indonesia, this plant also has utilized as a vegetable and salad. *Cnidoscolus aconitifolius* leaves have been shown to have a hematinic effect and stabilize erythrocyte membranes on protein-energy malnutrition in rats¹⁷. It also has anti-diabetic¹⁸ and antibacterial activity¹⁹. Further characterization showed that it contains phenols, saponins, cardiac glycosides, and phlobatannins²⁰.



Figure 1. *Cnidoscolus aconitifolius* (Mill.) I. M. Johnst.

Since the 1970s, people in Mexico have utilized the leaves of *C. aconitifolius* as a medicinal plant to effectively treat numerous diseases, such as diabetes, muscular disorders, arthritis, rheumatism, renal disorders, jaundice, and digestive issues²¹. According to research conducted by Obichi *et al.*⁹, *C. aconitifolius* leaves contain vitamins such as A, B3, B6, B12, C, and E. A large number of vitamins in these leaves will support an essential role in helping cure various diseases [5]. In addition to vitamins, *C. aconitifolius* leaves contain phytochemical compounds such as tannins, saponins, alkaloids, cyanogenic glycosides, oxalates, phenols, and flavonoids. It should be noted that the high content of phytochemicals in *C. aconitifolius* leaves is flavonoid compounds²².

Plants with antioxidant potential can be examined using various methods, one of which is the DPPH (1,1-diphenyl-2-picrylhydrazyl) method. A free radical, DPPH, can be used as a reagent in a free radical scavenging assay. IC₅₀ (inhibitory concentration) and AAI (antioxidant activity index) will be used as parameters in the DPPH assay. The IC₅₀ value is the concentration of the test extract appropriate to capture approximately 50% of the DPPH radicals. In contrast to the IC₅₀ parameter, the AAI parameter will correlate with the DPPH concentration. A sample with antioxidant activities will turn the DPPH solution color from purple to yellow²³. Based on the explanation above, although *C. aconitifolius* leaves have the potential to be a source of antioxidants, no scientific studies still show the antioxidant activity of *C. aconitifolius* leaves. This research aims to analyze the antioxidant activity of *C. aconitifolius* leaves extracted by 96% ethanol using the DPPH.

MATERIALS AND METHODS

Materials

The materials used in this research were amyl alcohol (Merck), ammonia (Merck), aquadest, *C. aconitifolius* leaves (obtained from Kelapa Dua District, Tangerang Regency, Banten, Indonesia, and determined at *Pusat Penelitian Konservasi Tumbuhan dan Kebun Raya, Lembaga Ilmu Pengetahuan Indonesia* (LIPI), Bogor with certificate No. B-1587/IPH.3/KS/XII/2020), DPPH (1,1-diphenyl-2-picrylhydrazyl), 96% ethanol (Merck), ether (Merck), iron trichloride (Merck), hydrogen chloride (Merck), chloroform (Merck), gelatin (Merck), sodium hydroxide (Merck), sodium acetate (Merck), Dragendorff's reagent, Liebermann-Burchard reagent, Mayer's reagent, Stiasny reagent, magnesium (Merck), and vitamin C. The tools used in this research were UV-Vis Spectrophotometer (Cary 60 Agilent), micropipette (Gilson), rotary evaporator (Heidolph), vortex mixer (Heidolph), oven (Mettler), and waterbath (Mettler).

Methods

Preparation of C. aconitifolius leaves herbs

Cnidocolus aconitifolius leaves weighing 15 kg were gathered, wet sorted, washed with running water, and drained. The drained leaves were cut into pieces and dried by air drying. After drying, the herbs were stored in a dry state, protected from light and insects.

Preparation of 96% ethanol extract of C. aconitifolius leaves

The dried powder of *C. aconitifolius* leaves was mashed, and 200 g were taken for maceration with 2 L of 96% ethanol. After maceration, the dregs and filtrate were separated using filter paper. The obtained dregs were macerated three times in a row. The filtrate obtained will be concentrated with a rotary evaporator and a water bath to get a dry extract⁶.

Phytochemical screening

Phytochemical screening was carried out to determine the components of bioactive compounds in the 96% ethanol extract of *C. aconitifolius* leaves. Phytochemical screening includes compounds of alkaloids, flavonoids, saponins, phenols, and tannins, according to the Harborne method with some modifications²⁴:

1. Alkaloid: 500 mg of sample was added with 5 mL of ammonia and 25 mL of chloroform, then homogenized. The mixture was filtered, and the filtrate was added 5 mL of 10% HCl and shaken in a test tube. After shaking, the upper part of the solution was taken and divided into two tubes. Dragendorff's reagent was added to the first test tube; a brick-

red precipitate indicated an alkaloid compound. Mayer's reagent was added to the second test tube; a white precipitate indicated an alkaloid compound.

2. Flavonoid: 500 mg of sample was added to 100 mL of hot water and boiled for 15 minutes. Then, 5 mL of the filtrate was filtered with magnesium powder, 1 mL of concentrated HCl, and 2 mL of amyl alcohol. The mixture was vortexed and let stand until two layers were formed. The formation of orange to pink in the amyl alcohol solution indicates the presence of flavonoid compounds.
3. Tannin: 500 mg of sample was added to 100 mL of hot water and boiled for 15 minutes. Then, 15 mL of the filtrate was filtered into three test tubes. In the first test tube, 1% FeCl₃ solution was added, and a dark blue/green/black color indicated a tannin compound. In the second tube, 1% gelatin solution was added, and a white precipitate indicated a tannin compound. In the third tube, Stiasny reagent was added, and a pink precipitate indicated catechol tannin compounds. The third tube mixture was filtered, and the filtrate was added with sodium acetate and FeCl₃; a blue precipitate indicated tannin gallic compounds.
4. Saponin: 500 mg of sample was added to 100 mL of hot water and boiled for 15 minutes. Then filtered, 10 mL of the filtrate was put into a test tube and vortexed, then allowed to stand, and a stable foam was formed. In the foam formed, 2-3 drops of 1% HCl were added, and the foam remained stable, indicating the saponin compound.
5. Steroid/Triterpenoid: 500 mg of sample was added to 100 mL of hot water and boiled for 15 minutes. Then filtered, 5 mL of the filtrate was added with 5 mL of 6 N NaOH. A red color indicated a quinone compound.
6. Phenol: 500 mg of sample was added with 5 mL of ammonia and 25 mL of chloroform and then homogenized. The mixture was filtered, and 5 mL of the filtrate was added with iron (III) chloride, forming a green-blue-black to black color, indicating a phenol compound.

Preparation of DPPH solution

1.5 mg of DPPH has dissolved with pro-analytical ethanol up to 25 mL in a volumetric flask to obtain a concentration of 60 ppm.

Determination of DPPH maximum wavelength

The maximum wavelength (λ) of DPPH was determined using 3 mL of 60 ppm DPPH solution. Then a blank solution was made by inserting 3 mL of pro-analysis ethanol into the cuvette, and the cuvette was inserted into the spectrophotometer. After inserting the blank, the maximum wavelength of DPPH was measured with a UV-Vis spectrophotometer at a wavelength of 400-600 nm to determine the optimal wavelength and absorbance value²⁵.

Measurement of vitamin C

A 100 g/mL stock solution was prepared by dissolving 2.5 mg of vitamin C in pro-analysis ethanol in a 25 mL volumetric flask and increasing the required volume. The comparison solution was prepared in a concentration of 1, 2, 4, 6, 8, and 10 ppm. 2 mL of 60 ppm DPPH solution was added to each concentration series. After adding 60 ppm DPPH solution, the concentration series was homogenized with a vortex mixer and incubated in a dark room for 30 minutes. After incubation, the absorbance at the maximum wavelength was measured. The absorption of the blank and control was measured by inserting 3 mL of pro-analysis ethanol into the spectrophotometer and then inserting 3 mL of 60 ppm DPPH solution⁶.

Measurement of 96% ethanol extract of C. aconitifolius leaves

A 1000 ppm stock solution was prepared by dissolving 25 mg of the extract in pro-analysis ethanol in a 25 mL volumetric flask. The solution was prepared in steps with concentrations of 5, 20, 40, 60, 80, and 160 ppm. 2 mL of 60 ppm DPPH solution was added to each concentration series. Following the addition of pro-analysis ethanol, 2 mL of 60 ppm DPPH solution was added to each of the six test tubes. After adding 60 ppm DPPH solution, the concentration series was homogenized with a vortex mixer and incubated in a dark room for 30 minutes. After incubation, the absorbance at the maximum wavelength was measured. Before measuring the extract's absorption, first measure the absorption of the blank

and control by inserting 3 mL of pro-analysis ethanol into the spectrophotometer, followed by 3 mL of 60 ppm DPPH solution⁶.

Determination of %inhibition

The %inhibition was calculated by the Equation 1⁹:

$$\%inhibition = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\% \quad [1]$$

Determination of IC₅₀

The concentration in the sample and the percent of inhibition were made on the *x* and *y* axes in the linear regression equation. The equation was used to determine the IC₅₀ value of each sample; the *y* value was 50, while the *x* value was obtained as IC₅₀²⁵.

Determination of AAI

The AAI value can be determined by the Equation 2⁶:

$$AAI = \frac{DPPH\ concentration\ (ppm)}{IC_{50}\ (ppm)} \quad [2]$$

RESULTS AND DISCUSSION

The dried herb of *C. aconitifolius* leaves yielded up to 2.77 Kg from 13.84 Kg of the fresh herb of *C. aconitifolius* leaves. The extraction results were obtained from 200 g of dried herbs powder of *C. aconitifolius* leaves macerated with 96% ethanol and obtained a dry extract of 32.3668 g with a yield of 16.1834%. The results of the extraction and yield can be seen in Table I.

Table I. The yield of 96% ethanol extraction of *C. aconitifolius* leaves

Herbs (g)	Dry extract (g)	Yield (%)
200	32.3668	16.1834

The screening of phytochemical components in herbs and a 96% ethanol extract of *C. aconitifolius* leaves revealed flavonoids, tannins, saponins, phenols, alkaloids, and steroids. These results are by those of Obichi *et al.*⁹, who reported that *C. aconitifolius* leaves contain phytochemical compounds such as alkaloids, flavonoids, saponins, and tannins. Table II shows the results of the phytochemical screening of herb and 96% ethanol extract of *C. aconitifolius* leaves. One of the elements influencing the phytochemical screening procedure is the selection of solvents that must match the target active chemicals for these molecules to be appropriately attracted^{27,28}.

Flavonoid compounds have antioxidant, anti-inflammatory, and antiseptic activity²⁹. Saponin compounds found in plants can significantly decrease cholesterol levels in the blood, serve as an inhibitor of tumor cell growth, and its non-sugar component can be used as an antioxidant³⁰. Plant phenolic compounds play a role in decreasing, donating hydrogen, and reducing free radicals. As a result, numerous plants with high phenolic compounds are employed as antioxidants³¹. Tannin compounds have been used to precipitate proteins and as metal chelators. Furthermore, because of their capacity to halt oxidation and serve as iron ion chelators, tannins can be used as secondary antioxidants in herbal medicine³². Alkaloid compounds have antibacterial, antidiabetic, and antimalarial activity³³. Steroid compounds found in natural ingredients can be used as a source of natural aphrodisiacs³⁴.

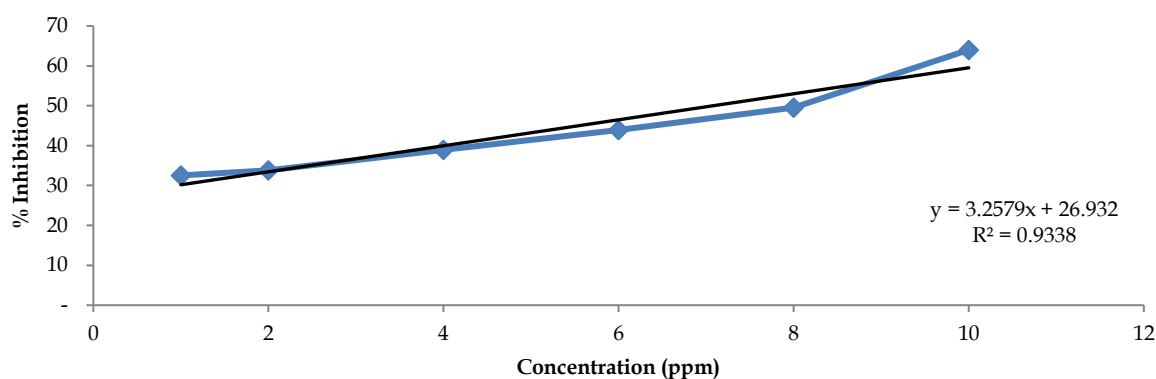
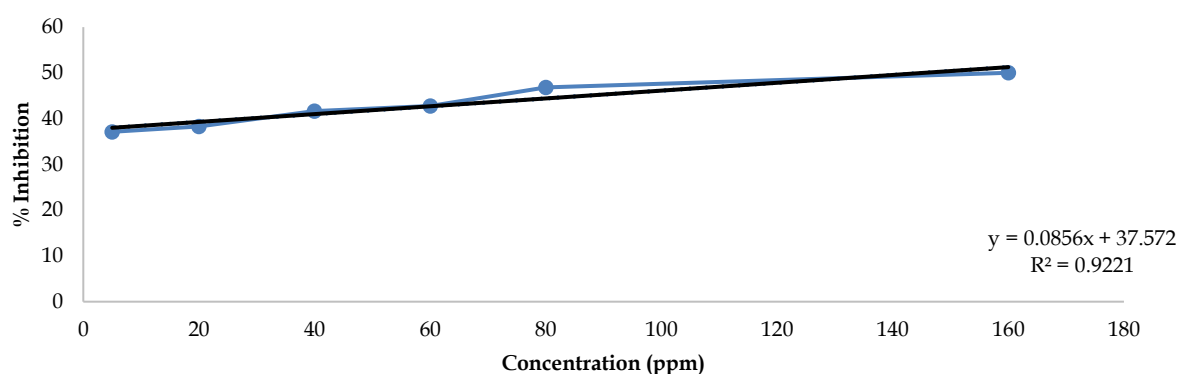
An antioxidant activity assay was carried out on 96% ethanol extract of *C. aconitifolius* leaves using the DPPH. This method was chosen because it is a simple, easy, and fast method of measuring antioxidant activity. In addition, using the DPPH method only requires a small sample of antioxidants from natural compounds³⁵.

Table II. The results of phytochemical screening of *C. aconitifolius*

Chemical compound	Herbs	Extract
Flavonoids	Orange (+)	Orange (+)
Saponins	Stable foam (+)	Stable foam (+)
Phenol		
• FeCl ₃	Green (+)	Green (+)
Tannins		
• FeCl ₃	Green (+)	Green (+)
• Gelatin	↓ White (+)	↓ White (+)
• Stiasny	↓ Pink (+)	↓ Pink (+)
• Sodium acetate	(-)	(-)
Alkaloids		
• Mayer's	↓ White (+)	↓ White (+)
• Dragendorff's	↓ Brick red (+)	↓ Brick red (+)
Steroids	Green (+)	Green (+)
Quinone	(-)	(-)

Vitamin C was a positive control in the antioxidant activity test of a 96% ethanol extract of *C. aconitifolius* leaves. Vitamin C was used as a comparison because it is a secondary antioxidant that traps free radicals and inhibits free radical oxidation processes. Furthermore, because it is a natural antioxidant component with the highest antioxidant activity when compared to other comparison compounds, vitamin C is a regularly used comparison in assessing antioxidant activity³⁶.

The correlation curve of concentration with %inhibition of vitamin C and 96% ethanol extract of *C. aconitifolius* leaves can be seen in **Figures 2** and **3**. The obtained R² value describes the linearity of concentration to %inhibition. A good correlation value between concentration and %inhibition will be close to +1. This indicates that with increasing concentration in the extract, the antioxidant activity also increases³⁵.

**Figure 2.** Graphic concentration vs. %inhibition of vitamin C.**Figure 3.** Graphic concentration vs. %inhibition of ethanol extract 96% *C. aconitifolius* leaves.

The DPPH method works on the principle that DPPH will be decreased after receiving electrons from antioxidant compounds. After all of the electrons in the free radicals have been coupled, the intensity of the purple color changes to a yellow color—this color shift results in a decrease in absorbance at the maximal wavelength of DPPH. The reduction in absorbance shows that the sample (vitamin C and extract) put to DPPH has antioxidant action. The decrease in absorbance can be evaluated using UV-Vis spectrophotometry to determine the antioxidant activity, which is reflected in the IC₅₀ and AAI values^{23,35}.

Compounds that have very strong antioxidant activity if the IC₅₀ value is <10 ppm, strong 10-50 ppm, moderate 50-100 ppm, weak 100-250 ppm, and inactive >250 ppm³⁷. The IC₅₀ value obtained for vitamin C is 7.0806 ppm, meaning that vitamin C has a very strong antioxidant activity. Meanwhile, the IC₅₀ obtained from the extract was 145.1869 ppm. A comparison of IC₅₀ values in both samples showed that the antioxidant activity of vitamin C was better than that of the 96% ethanol extract of *C. aconitifolius* leaves.

The IC₅₀ obtained in both samples can be used to calculate the AAI value of each sample. The AAI parameter will relate the concentration of DPPH used with the IC₅₀ results obtained. The results of obtaining the AAI value can be seen in **Tables III** and **IV**. An extract's antioxidant activity is weak if it has an AAI value of <0.5, moderate at 0.5 – 1.0, strong at 1.0 – 2.0, and very strong at >2.0²⁶. The AAI value obtained by vitamin C was 8.4739 and included in the very strong antioxidants category. Unlike the case with the AAI value obtained by the extract, which is 0.4132 and is categorized as having weak antioxidant activity.

Previous research on the antioxidant activity of *C. aconitifolius* revealed that the aqueous extract of the leaves of *C. aconitifolius* has antioxidant activity³⁸. The ethyl acetate and methanol extracts of the leaves of *C. aconitifolius* showed antioxidant and free radical scavenging activities DPPH IC₅₀ 12.14 and 93.85 g/mL, respectively. Phytols were abundant constituents in *n*-hexane, ethyl acetate, and methanol extracts, with corresponding proportions of 41.07%, 35.42%, and 35.07%, respectively³⁹. The ethyl acetate fraction of *C. aconitifolius* leaves contains coumaric acid, amentoflavone, hesperidin, protocatechuic acid, kaempferol, dihydromyricetin, quercetin, and rutin. The *C. aconitifolius* leaves have the extraordinary antioxidant potential⁴⁰. The difference in IC₅₀ and AAI values obtained by vitamin C and 96% ethanol extract of *C. aconitifolius* leaves was assumed to be attributable to the action of other extract components. Because the extract is not a pure antioxidant component, it exhibits limited antioxidant activity. This enables the presence of other molecules that operate in addition to antioxidants. Based on the criteria of Phongpaichit *et al.*³⁷ and Scherer *et al.*²⁶, 96% ethanol extract of *C. aconitifolius* leaves still has antioxidant activity, although in a weak category.

Table III. The results of antioxidant activity of vitamin C

Concentration (ppm)	Absorbance			Mean	% Inhibition	IC ₅₀ (ppm)	AAI
	1	2	3				
1	0.671	0.6388	0.7142	0.6747±0.03	32.53	7.0806	8.4739
2	0.6672	0.6248	0.6947	0.6622±0.03	33.78		
4	0.6552	0.5317	0.6458	0.6109±0.06	38.91		
6	0.6157	0.5161	0.5517	0.5612±0.04	43.88		
8	0.5819	0.4276	0.5057	0.5051±0.06	49.49		
10	0.3117	0.3188	0.4497	0.3601±0.06	63.99		
Control	0.9384	0.954	1.1076	1.0000±0.08			

Table IV. The results of antioxidant activity of 96% ethanol extract of *C. aconitifolius* leaves

Concentration (ppm)	Absorbance			Mean	% Inhibition	IC ₅₀ (ppm)	AAI
	1	2	3				
5	0.666	0.6708	0.5530	0.6299±0.05	37.13	145.1869	0.4132
20	0.6618	0.6529	0.5410	0.6186±0.05	38.26		
40	0.6210	0.6150	0.5179	0.5846±0.05	41.65		
60	0.5715	0.5791	0.5690	0.5732±0.04	42.78		
80	0.5616	0.5533	0.4837	0.5329±0.03	46.81		
160	0.5072	0.5244	0.4706	0.5007±0.02	50.02		

CONCLUSION

The 96% ethanol extract of *C. aconitifolius* leaves contains flavonoids, tannins, saponins, phenols, alkaloids, and steroids. The 96% ethanol extract of *C. aconitifolius* leaves has less antioxidant activity than vitamin C, with weak antioxidant activity.

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AUTHORS' CONTRIBUTION

R and **MW** determine the research topic. **MW** develops theory and performs calculations. **K** and **MA** verify methods and analyze. **R** directs, controls, and advises **MW** while conducting research. All authors discussed the results and contributed to the final manuscript.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

No potential conflict of interest is available to declare by the authors.

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