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EFFECT OF ETHANOL-WATER CONCENTRATION AS EXTRACTION SOLVENT ON ANTIOXIDANT ACTIVITY OF *Acalypha indica*

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Abstract

Acalypha indica is an abundant and obtainable wild weed plant in Indonesia. This is a possible opportunity to enhance its utility. The effect of free radicals is one of the factors that contribute to oxidative stress in the body, which can lead to cell damage and trigger various diseases. Plants rich in antioxidants can inhibit free radical reactions and protect the body from oxidative stress. In this study, we conducted the identification of major metabolite compounds using UHPLC-Q-Orbitrap HRMS from 70% ethanol extracts of *Acalypha indica*. We also performed phytochemical and antioxidant activity tests with various concentrations of ethanol-water solvents including water, 30%, 50%, 70% ethanol, and absolute ethanol to determine the optimal extraction conditions and the influence of solvent concentration on the antioxidant activity of *Acalypha indica* extract. The results of UHPLC-Q-Orbitrap HRMS identification showed that the 70% ethanol extract of *Acalypha indica* contains 10 major compounds suspected to correlate with its antioxidant activity. These include five flavonoids compounds (palasitrin, vitexin 2''-o-p-coumarate, isorhamnetin 3- (3'',6''-di-p-coumarylglucoside), peucenin, and sulfuretin), two amino acid compounds (2-amino-3-carboxymuconic acid semialdehyde and N-Acetyl-L-phenylalanine), two carboxylic acid compound (citric acid and traumatic acid), and one phenolic compound (quinic acid). Phytochemical tests show positive results for flavonoids, tannins, alkaloids, and triterpenoids in each of water, 30%, 50%, 70% ethanol, and absolute ethanol extracts. The best antioxidant activity was obtained from the 70% ethanol extract with an IC_{50} value of 47.064 ppm, categorizing it as an extremely strong antioxidant.

INTRODUCTION

Free radicals are compounds typically produced by the human body as part of a defense response to dangerous substances. When production of free radicals exceeds the ability of the body's antioxidants to neutralize, it can cause oxidative stress. Oxidative stress has hazardous effects on body systems because it plays a role in the pathogenesis of various diseases, including atherosclerosis, hypertension, diabetes mellitus, and cancer [1]. Antioxidants are substances that can prevent the process of oxidative stress and can slow the development of the body's degenerative diseases [2]. Antioxidants can be synthetic or natural. Natural antioxidants derived from plants are preferred because it's considered safer. According to epidemiological studies, consuming natural antioxidants regularly can reduce the risk of cardiovascular disease and cancer [3]. Based on previous research, *Acalypha indica* contains various secondary metabolites, including steroids, triterpenoids, saponins, flavonoids, phenolics, tannins, and alkaloids [4] [5] [6] which can act as an antioxidant compound.

Antioxidant activity increases with the rise in secondary metabolites especially phenol and flavonoid content [7]. Phenolic compounds exhibit strong antioxidant properties and can inhibit the formation of Reactive Oxygen Species (ROS) [8]. The antioxidant potential of flavonoid compounds is linked to their molecular structure, conjugation effects, including the position and total number of –OH groups, and resonance effects that modify specific antioxidant mechanisms of a compound [9]. In specific phenolic, the oxidation of the

flavonoids group is important to see the ability of antioxidant activity not only from one flavonoids molecule but also through the synergism of several compounds (one or more) from the extract's secondary metabolites that function as active antioxidant species [10].

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was selected to measure the antioxidant activity of natural plant herbal extracts due to its relatively high stability, low cost, and ease of experimental procedures compared to other methods [11]. The DPPH method works by reducing the unpaired valence electron of the nitrogen atoms in the DPPH radical with hydrogen atoms from the antioxidant molecule to form DPPH-H hydrazine [12]. Antioxidant activity is quantified as the amount of antioxidants in a solution that is capable of reducing the concentration of 50% DPPH free radicals, expressed as IC_{50}.

The polarity of solvent extraction has an impact on the solubility of specific metabolite compounds that are associated with their biological activity [13]. Differences in the metabolites extracted can influence the level of biological activity, including antioxidant activity, due to potential synergistic or antagonistic effects among these metabolites [14]. Various solvents, such as water, methanol, acetone, and ethanol have been used to extract bioactive compounds from plant materials. Out of these several solvents, ethanol provides a benefit as it is comparatively more suitable for human consumption in terms of safety. The ethanol-water mixture for phenolic extraction has different polarities to produce a solvent property that is more polar than absolute ethanol. It is expected to have a positive influence on the extraction results, especially regarding the phenolic content, which is closely related to antioxidant activity [15][16]. Consequently, it is essential to research the effect of variations in ethanol-water solvent concentration (water, 30%, 50%, 70% ethanol, and absolute ethanol) on the antioxidant activity of *Acalypha indica*. This evaluation purpose is to identify the most suitable optimal conditions for *Acalypha indica* extraction and achieve the highest level of antioxidant activity.

EXPERIMENT

Material

The materials used for this research include ethanol (Merck Darmstadt, Germany), purified water *for analytical laboratory use* (OneMed), DPPH reagent (Sigma Aldrich), Whatman membrane filters (pore size 0.22 μm; PTFE; P/N E252, Little from Sigma Aldrich (St Louis, MO, USA), and *Acalypa indica* samples collected from Tuban, East Java.

Instrumentation

The instruments used in this research include a rotary evaporator IKA RV10 (Labfirts Scientific, Shanghai) used for evaporation of solvent extraction, a visible spectrophotometer (Genesys 150 Thermo Scientific) for the analysis of antioxidant activity, and UHPLC-Q-Orbitrap HRMS (Thermo Fisher, Waltham, MA, USA) to identification the metabolites of *Acalypa indica.*

Procedure

Sample Preparation

Samples of *Acalypha indica* are washed, dried in an oven at 30-50 °C, and mashed into 60 mesh in the Laboratory of Materia Medica Batu, East Java. The samples were kept at room temperature with silica gel and protected from direct sunlight until the extraction process.

Extraction of Acalypa indica by maceration method

Extraction of *Acalypa indica* was carried out by maceration method with variations in the concentration of ethanol-water solvent (water, ethanol 30%, 50%, 70%, and absolute ethanol). Ten grams of sample powder was extracted in 150 mL solvent for three days with a powder: solvent ratio of 1:15 (w/v). Then, the ethanol extract was filtered using Whatman membrane filter paper. The resulting filtrate then concentrated utilizing a rotary vacuum evaporator with a speed of 100 rpm and set at a temperature of 50°C to obtain a concentrated extract. After that, the concentrated extract was weighed, and the % yield value using this equation:

$$\% \text{ yield } = \frac{Extract\ weight}{Sample\ weight} \times 100\ \% \quad (1)$$

Separation of Acalypha indica Metabolite using UHPLC-Q-Orbitrap HRMS

Determination of metabolite types from the ethanol 70% extract of *Acalypa indica* was conducted using the UHPLC-Q-Orbitrap HRMS. For this purpose, 5 mg of samples were dissolved in 1 mL MeOH. After filtering using a 0.2 μm PTFE membrane, and identify the existing

metabolites in *Acalypha indica* extract by employing Vanquish Flex UHPLC-Q Exactive Plus Orbitrap-High Resolution Mass Spectrometer. The metabolites are separated using the stationary phase column of Accucore C18, 100 xs 2.1 mm, 1.5 μm (Thermo Scientific). The shallow gradient elution consisting of the mobile phase mixture of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was applied as the following step: 0.0–1.0 minutes (15%B), 1.0–22.0 minutes (15–70%B), 22.0–24.0 minutes (70–95% B), 24.0–27.0 minutes (95% B), 27.0–32.0 minutes (15%B). The injection volume was 2.5 μL with the mobile phase flow rate was set at 0.25 mL/min. The MS system utilized an ESI ionization source, supporting both positive and negative ionization modes within the 100-1500 m/z range. Operating conditions included a gas sheath and sweep gas velocities of 15 and 3 mL/min, a spray voltage of 3.8 kV, a capillary temperature of 320°C, and automatic gain control (AGC) set at 3×10^6 with an injection time (IT) of 100 ms. Impact energies of 18, 35, and 53 eV were applied, and the scanning mode employed was MS/dd MS2 with a resolution of 70,000 FWHM.

Phytochemical Test

The presence of flavonoids was tested according to the reference [17] with a few modifications. Two milliliters of extract were combined with a small amount of hot methanol and then boiled for 5 minutes. Subsequently, the filtrate was mixed with 1 mL of concentrated HCl (36%) and a small amount of Mg powder, followed by shaking the mixture. The presence of flavonoids is indicated by the appearance of yellow, red, or orange colors.

A tannin test was performed using 2 mL of the sample by adding a few drops of 1% $FeCl_3$. The positive results are indicated by the color changes into green, purple, blue, or black. For the alkaloid test, 2 mL of the samples are placed into two separate test tubes. After transferring 1 mL HCl 2M to each tube, 3 drops of Dragendorff's reagent are added into the first tube and 3 drops of Mayer's reagent are added into the second tube. The formation of an orange precipitate in the first tube and a yellowish-white precipitate in the second tube show the existence of alkaloids [18].

A steroid test is performed using 2 mL of the extract and subsequently added with 10 drops of glacial CH_3COOH and 2 drops of concentrated H_2SO_4. After slowly stirring, the solution is allowed to stand for a few minutes. The presence of steroids is indicated by the formation of blue or green rings, while triterpenoids show through the formation of red, purple, or brownish rings [17].

Antioxidant Assay using the DPPH Method

A stock solution of 100 ppm was prepared by weighing 2 mg of concentrated extract and dissolving it in 20 mL of ethanol. Subsequently, the stock solution was diluted to concentrations ranging from 10, 20, 30, 40, to 50 ppm. Three milliliters of each concentration extract were pipetted and added with 1 mL of 0.2 mM DPPH. After that, vortex the mixture at room temperature until homogenized. Next, incubate the samples for 30 minutes at a temperature of 37 °C. Then, determine the absorbance using a visible spectrophotometer at the λ_{max} of DPPH (515-517 nm) and calculate the radical scavenging activity using the following formula:

$$\%\text{Antioxidant Activity} = \frac{A\ control - A\ sample}{A\ control} \times 100\% \quad (2)$$

The IC_{50} values for each sample are calculated by establishing a linear regression equation using Microsoft Excel, representing the correlation between extract concentration and percentage (%) of antioxidant inhibition, where a smaller IC_{50} value indicates higher antioxidant capability.

Data Analysis

The data obtained from measurements were analyzed using SPSS 25.0 software using One-Way ANOVA and Tukey's test for statistical processing. A significance level used is $p<0.05$. % Yield and antioxidant activities of each *Acalypa indica* extract were statistically analyzed using SPSS software to determine significant differences among the various groups at $p<0.05$ and then compared with the control group.

RESULT AND DISCUSSION

Extraction Yield Results

Ten grams of the sample were extracted using 150 mL solvent in a 1:15 ratio for 3x24 hours using the maceration method. Maceration was carried out at room temperature to avoid degradation or damage to compounds due to high temperatures [19]. Increasing the duration of contact between the sample and the solvent in a certain range of time was supposed to increase the extraction efficiency. The optimal

time for the extraction maceration process is 3x24 hours [20]. The abundance of metabolites contained in *Acalypha indica* could also create potential synergistic or antagonistic effects among metabolites, which might influence the resulting antioxidant activity.

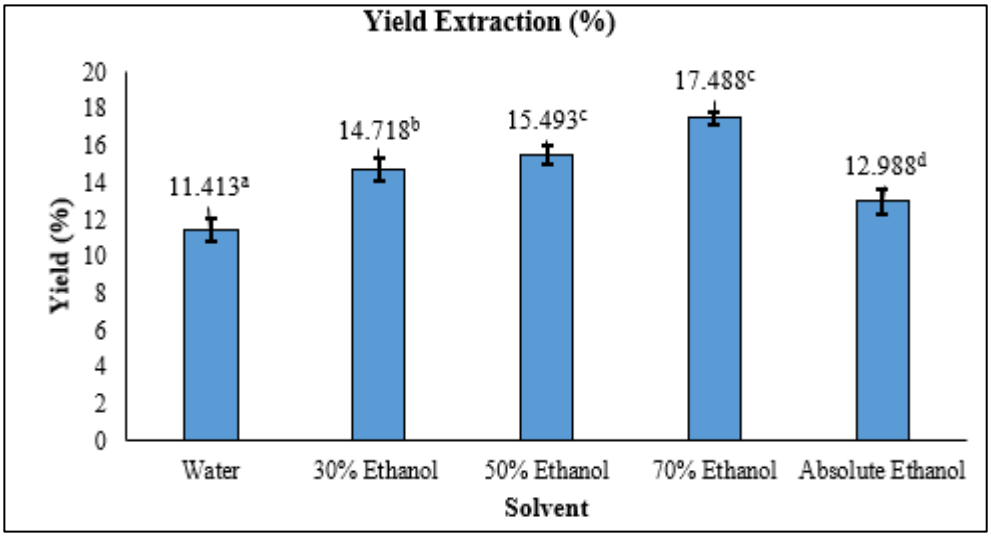

Figure 1. Yield extraction (%) of *Acalypha indica* extraction results from each variation of solvent concentration. (The values are mean ± SD of the 3 replicates test for each sample. The $^{a-d}$ mean ± SD for each extract in the same column followed by different superscript letters showed a significant difference).

Based on the obtained yield values, it is evident that different ethanol concentrations resulted in variations in the quantity of extracted metabolites. The extraction yields of *Acalypha indica* extracts ranged from 11.413 ± 0.597 to 17.488 ± 0.311, as shown in **Figure 1**. Analysis of variance results showed that the type of solvent used significantly influenced the extraction yield at a 5% level of significance ($p < 0.05$). Furthermore, Tukey's post hoc test indicated significant differences among the extraction solvents (water, ethanol 70%, and absolute ethanol), with the ethanol 70% extract exhibiting the highest extraction yield. Meanwhile, ethanol 50% and ethanol 30% extracts had extraction yields that were not significantly different. These findings suggest that the use of ethanol-water at a certain concentration of solvents is more effective in extracting metabolites, indicating the existence of secondary metabolites in the plant. Additionally, the use of hydro-alcoholic solvents (ethanol-water) as the extraction solvent can facilitate the extraction of chemical constituents, as these components may possess solubility in both water and organic solvents [21][22].

Identification of Major Metabolite Compounds from Ethanol 70% Extract of Acalypha indica

Ethanol 70% extract has the highest % yield value which is possibly containing the most extracted metabolite compounds compared to other solvent variations. The chromatogram profile of the ethanol 70% extract *Acalypha indica* can seen in **Figure 2**. The analysis result using UHPLC-Q-Orbitrap HRMS gives information about the metabolite content in *Acalypha indica*. A total of ten major metabolites were obtained in ESI^- (negative ion) mode, including flavonoids, phenolic, amino acids, and carboxylic acids compounds. These compounds are five flavonoids compounds: palasitrin, vitexin 2''-o-p-coumarate, isorhamnetin 3- (3'',6''-di-p-coumarylglucoside), peucenin, and sulfuretin; two amino acid compounds: 2-amino-3-carboxymuconic acid semialdehyde and N-Acetyl-L-phenylalanine; two carboxylic acid compound: citric acid and traumatic acid; and one phenolic compound, quinic acid.

The area under the curve (**Table 1**) represents the abundance of each type of compound by their peaks and retention times (**Figure 2**). The abundance of compounds in the ethanol 70% extract of *Acalypha indica* from highest to lowest is as follows: quinic acid > traumatic acid > 2-amino-3-carboxymuconic acid semialdehyde > citric acid > palasitrin> N-acetyl-L-phenylalanine> isorhamnetin 3- (3'',6''-di-p-coumarylglucoside) > peucenin > sulfuretin > vitexin 2''-o-p-coumarate. Quinic acid has an area under the curve of 4.81×10^8, indicating the highest abundance of compounds in the sample compared to other

compound types. Quinic acid is a phenolic compound likely that significantly contributes to the antioxidant activity in the *Acalypha indica* extract. phenolics can provide H atoms to free radical substrates and produce non-radical substrate species (RH, ROH, or ROOH) and antioxidant free radicals [23]. The UHPLC-Q-Orbitrap HRMS used in this study is a reversed-phase system with a polar mobile phase and a non-polar stationary phase. Therefore, compounds that appear in the chromatogram at the early retention times are polar, and as the retention time progresses, the compounds that appear become increasingly non-polar.

Based on the UHPLC-Q-Orbitrap HRMS results listed in **Table 1**, the polarity sequence of compound types in the absolute ethanol extract of *Acalypha indica* (from the most polar compound) is as follows: 2-amino-3-carboxymuconic acid semialdehyde > quinic acid > citric acid > N-acetyl-L-phenylalanine > palasitrin > vitexin 2"-o-p-coumarate > traumatic acid > isorhamnetin 3-(3",6"-di-p-coumarylglucoside) > peucenin > sulfuretin.

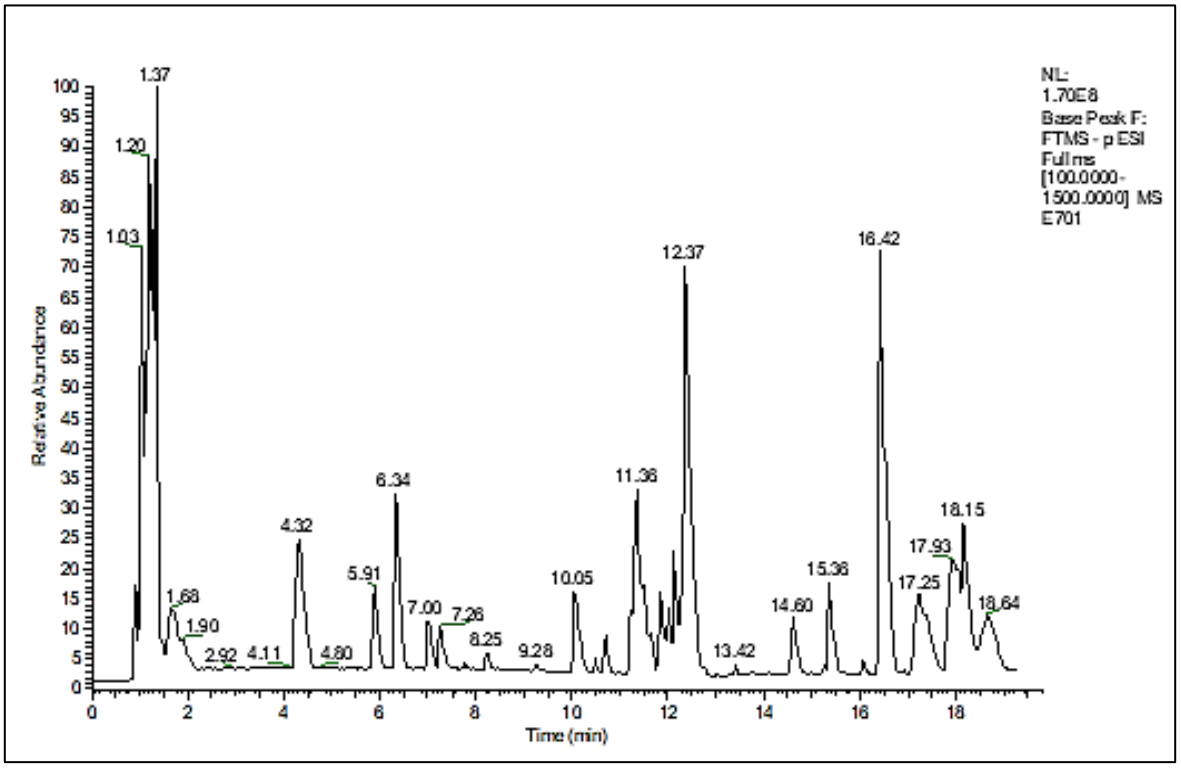

Figure 2. Chromatogram profile of absolute ethanol extract *Acalypha indica.*

Table 1. UHPLC-Q-Orbitrap HRMS analysis results of *Acalypha indica* ethanol extract.

RT (min)	Formulas	Calc. MW	Compound Name	Area (AUC)	Compound class
1.03	$C_7 H_7 N O_5$	185.0318	2-amino-3-carboxymuconic acid semialdehyde	4.21×10^8	Amino acid
1.20	$C_7 H_{12} O_6$	192.0629	Quinic acid	4.81×10^8	Phenolic
1.37	$C_6 H_8 O_7$	192.0265	Citric acid	3.96×10^8	Carboxylic acid
4.32	$C_{11} H_{13} N O_3$	207.0891	N-Acetyl-L-phenylalanine	2.96×10^8	Amino acid
6.34	$C_{27} H_{30} O_{15}$	594.1587	Palasitrin	3.66×10^8	Flavonoids
11.36	$C_{30} H_{26} O_{12}$	578.1424	Vitexin 2"-O-p-coumarate	39907067	Flavonoids
12.37	$C_{12} H_{20} O_4$	228.1359	Traumatic acid	4.50×10^8	Carboxylic acid
15.36	$C_{40} H_{34} O_{16}$	770.1849	Isorhamnetin 3- (3",6"-di-p-coumarylglucoside)	1.74×10^8	Flavonoids
16.42	$C_{15} H_{16} O_4$	260.1048	Peucenin	60527750	Flavonoids
18.8	$C_{15} H_{10} O_5$	270.0528	Sulfuretin	42142792	Flavonoids

Phytpchemical Screening

Qualitative tests on the *Acalypha indica* extract are to determine its secondary metabolite content. In this study, qualitative testing by taking a small amount of *Acalypha indica* extract, which was then mixed with reagents specific to the compounds to be identified. The results of the qualitative tests on the *Acalypha indica* extract from each solvent are presented in **Table 2.**

Table 2. Qualitative test results of *Acalypha indica* extract from each variation of solvent concentration.

Solvent	Flavonoids	Tanin	Alkaloid		Terpenoid
			Mayer	Dragendoft	Steroid/ Triterpenoid
Water	 (+)	 (+)	 (+)	 (+)	 (+) Terpenoid
30% Ethanol	 (+)	 (+)	 (-)	 (+)	 (+) Terpenoid
50% Ethanol	 (+)	 (+)	 (-)	 (+)	 (+) Terpenoid
70% Ethanol	 (+)	 (+)	 (+)	 (+)	 (+) Steroid
Absolute ethanol	 (+)	 (+)	 (-)	 (+)	 (+) Steroid

The flavonoids test in the *Acalypha indica* extract has positive results in all extracts, including water extract, 30% ethanol, 50% ethanol, 70% ethanol, and absolute ethanol. This was indicated by a color change to reddish-brown. The complex formed results in a reddish-brown color due to the covalent coordination bond between magnesium ions and the phenolic OH groups of flavonoid compounds. The reaction equation is as follows (**Figure 3**) [24]:

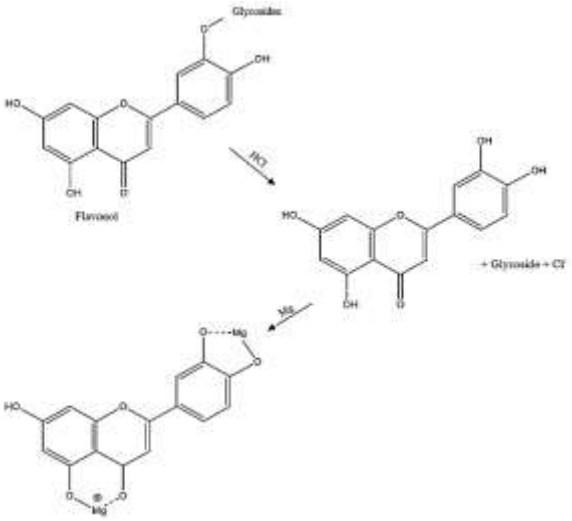

Figure 3. Reaction in the flavonoids test [24].

Qualitative tannin testing in this study was performed by adding 3 drops of $FeCl_3$ to the *Acalypha indica* extract. The results showed that all water extracts, 30% ethanol, 50% ethanol, 70% ethanol, and absolute ethanol extracts contained tannins, which was evident from the observed color change to a deep green shade. The color change to dark green occurs due to the formation of a covalent coordination bond between iron (III) ions and phenolic OH groups (**Figure 4**). The reaction equation is as follows:

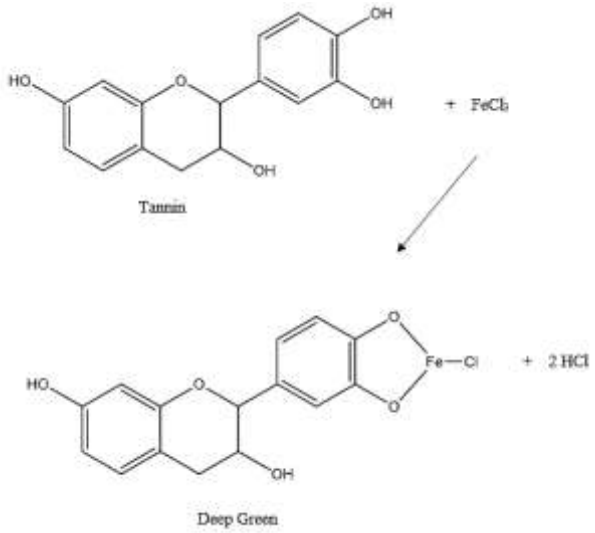

Figure 4. Reaction in the tannin test [18].

The alkaloid testing in this study was conducted by adding 3 drops of Meyer's reagent for the Meyer alkaloid test and 3 drops of Dragendorff's reagent for the Dragendorff alkaloid test. The formation of precipitates in the Meyer and Dragendorff tests indicates the presence of alkaloid compounds in the *Acalypha indica* extract. However, not all *Acalypha indica* extracts in the concentrations of water, 30%, 50%, 70% ethanol, and absolute ethanol showed positive results in all alkaloid tests. *Acalypha Indica* extracts in 70% ethanol and water showed positive results in the Meyer alkaloid test, as indicated by the formation of an orange-yellow precipitate in the test tube. The Dragendorff alkaloid test showed positive results in all concentrations of *Acalypha indica* extract, including water, 30%, 50%, 70% ethanol, and absolute ethanol, as indicated by the formation of an orange precipitate.

The results of the qualitative test for terpenoid compounds in this study were conducted by adding 3 drops of Bouchardat's reagent. Testing for steroids or triterpenoids is based on the compounds' ability to form colors from a mixture of concentrated H_2SO_4 in acetic anhydride solvent. A positive test indicates the presence of terpenoid compounds, with triterpenoids forming an orange or reddish-brown color and steroids forming a green-blue color [25]. The samples of absolute ethanol and 70% ethanol showed positive results for steroids, while the samples of water, 30% ethanol, and 50% ethanol showed positive results for triterpenoids.

Antioxidant Activity Test of Acalypha indica Extract

The antioxidant activity test using the DPPH reagent involves a color change from purple to yellow, indicating the process of hydrogen atom donation to the DPPH radical. This change was measured using a visible spectrophotometer. A higher percentage of antioxidant activity inhibition indicates the compound's ability to inhibit free radicals. Better antioxidant activity is expressed by a lower IC_{50} value. The results of the activity at various concentrations of *Acalypha indica* ethanol extract can be seen in **Figure 5.**

Extraction using different solvents shows variations in antioxidant activity results. The polarity of the solvent used in this research is influenced by the increased water content in ethanol [26]. *Acalypha indica* extract has IC_{50} values ranging from 47.064 ± 0.787 to 90.884 ± 0.911 µg/mL and a significant difference at a 5% significance level ($p < 0.05$) based on Tukey's post hoc test (**Figure 5**). The 70% ethanol extract has the lowest IC_{50} value compared to other extracts (<50 ppm), indicating its very strong antioxidant activity [27]. The results showed a correlation that

ethanol 70% had the highest yield about 17.488%. The higher yield percentage indicates that more metabolites are extracted, and they synergistically enhance antioxidant activity.

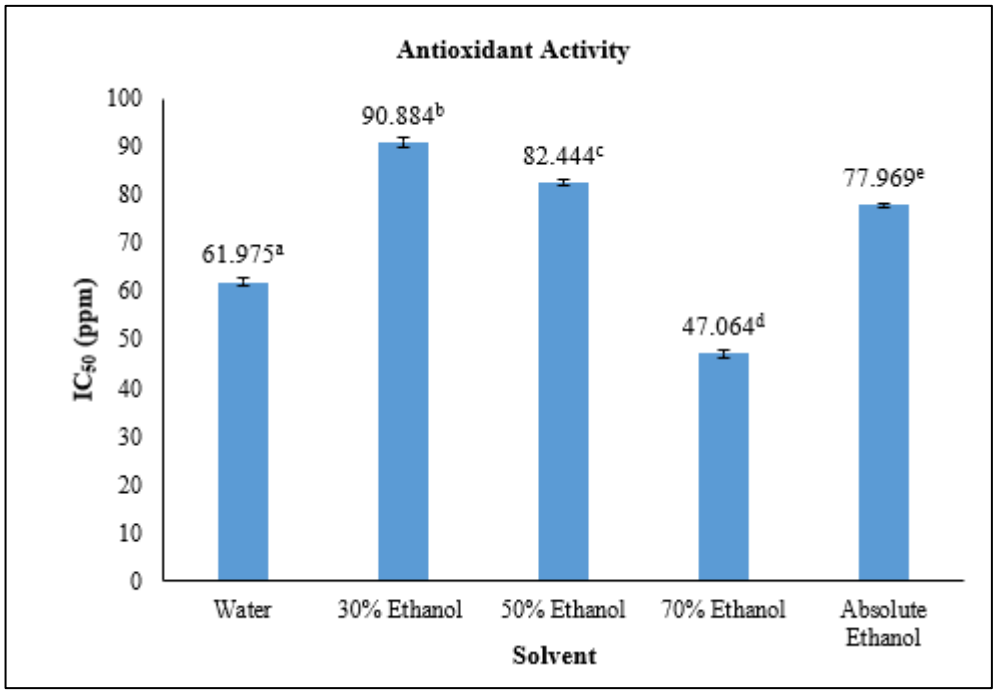

Figure 5. Antioxidant activity of *Acalypha indica* based on the difference in solvent concentration for extraction (The values are mean ± SD of the 3 replicates test for each sample. The $^{a-e}$ mean ± SD each extract in the same column followed by different superscript letters showed a significant difference).

CONCLUSION

The UHPLC-Q-Orbitrap HRMS chromatogram profile of ethanol 70% extract from *Acalypha indica* identified ten major compounds suspected to play a role in the antioxidant activity of this plant. These compounds include five flavonoids compounds: palasitrin, vitexin 2"-o-p-coumarate, isorhamnetin 3- (3",6"-di-p-coumarylglucoside), peucenin, and sulfuretin; two amino acid compounds: 2-amino-3-carboxymuconic acid semialdehyde and N-Acetyl-L-phenylalanine; two carboxylic acid compound: citric acid and traumatic acid; and one phenolic compound, quinic acid. Statistical analysis using One-Way ANOVA and Tukey's test ($p < 0.05$) showed that variations in ethanol-water solvent concentrations significantly affected the extraction yield (except for 50% and 30% ethanol, which did not show significant differences). Furthermore, the statistical test results also indicated that the antioxidant activities of all *Acalypha indica* extracts differed significantly. The antioxidant activity values of water, 30% ethanol, 50%, 70%, and absolute ethanol extracts were 61.975 ± 0.912, 90.884 ± 0.911, 82.444 ± 0.561, 47.064 ± 0.787, and 77.969 ± 0.406 ppm respectively. Ethanol 70% extract showed the highest extraction yield (17.488%) and the best antioxidant activity with an IC_{50} value is 47.064 ppm categorized as extremely strong antioxidant activity.

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