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Isolation of Phenolic Acids from Land Kale (*Ipomoea reptans* Poir) and Antioxidant Activity

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Abstract

Land kale or *Ipomoea reptans* Poir is widely consumed by Indonesian people. Land kale plants can be used as natural antioxidants because they contain polyphenolic compounds, one of which is phenolic acid. This research was carried out to determine the antioxidant activity and isolate phenolic acid compounds contained in land kale plants (*Ipomoea reptans* Poir). The sample used was an ethanol extract of land kale. The total phenolics were determined using the Folin-Ciocalteu method. Phenolic acids were isolated using alkaline hydrolysis and acid hydrolysis and without hydrolysis methods. Separation of isolates was carried out using the TLC method. The structure was identified using UV-Vis spectrophotometry and LC-MS/MS. Antioxidant activity was measured using the DPPH method. The total phenolics of land kale ethanol extract were 71.2420 ± 0.0791 mg/g GAE. TLC showed that the HB, HA, and TH fractions contained caffeic acid and ferulic acid compounds. Separation of the (HB) fraction produced three isolates: B2 with a yellow color, B3 with a slightly yellowish color, and B4 which is colorless. B2 isolate was identified as potentially containing caffeic acid, while B3 isolate was indicated to potentially contain ferulic acid through analysis using a UV-Vis spectrophotometer. B4 isolate was thought to have a hydroxybenzoic acid framework after being determined using LC-MS/MS. The ethanol extract of land kale has strong antioxidant activity because it produces an IC_{50} value of 94.83 mg/L.

1. Introduction

Land kale or *Ipomoea reptans* Poir is widely consumed by Indonesian people as a vegetable, either stir-fried or boiled. Land kale contains vitamins and minerals that are good for human health and have secondary metabolite compounds in the form of phenolic acids, flavonoids, saponins, alkaloids, steroids, and triterpenoids [1]. Based on the content of secondary metabolites, kale plants are reported to have several activities, including antioxidant [2], anti-diabetic [3], anti-cancer [4], anti-inflammatory, anti-microbial [5], anti-ulcers [6], and hypolipidemic agents [7].

Plants with a high content of phenolic acid compounds, such as land kale (Convolvulaceae), can be used as natural antioxidants. *Ipomea aquatica* plants contain chlorogenic acid [8], salicylic acid, dihydroxy benzoic acid pentoside, dihydroxy benzoic acid di-pentoside [9], ellagic acid, caffeic acid, and gallic acid [10]. The phenolic acid compounds present in kale plants play a crucial role in chelating ions and scavenging free radicals, particularly superoxide (O•), peroxyl (ROO•), and hydroxyl radicals (•OH). This mechanism helps inhibit DNA damage and lipid peroxidation, thereby preventing potential membrane damage [11]. The greater the total phenolic content in a plant, the greater its antioxidant activity [12].

The phenolic acid content in plants exists in various forms, including free phenolic acids, phenolic acids bound as esters, and phenolic acids bound to sugars [13]. This research was conducted to determine the antioxidant activity and isolation of phenolic acid

compounds contained in land kale plants (*Ipomoea reptans* Poir).

2. Experimental

The procedures employed in this study encompassed the determination of land kale plants, the conversion of the plants into a dried form, the phytochemical screening of ethanol-water extract and the dried material, maceration utilizing ethanol followed by dechlorophyllation and defatization, the quantification of total phenolics within the ethanol extract, the isolation of phenolic acids employing various hydrolysis methods (alkaline hydrolysis, acid hydrolysis, and non-hydrolysis), the separation of phenolic acid isolates, analysis for purity, structural determination (UV-Vis Spectrophotometer and LC-MS/MS), and assessment of antioxidant properties utilizing the DPPH method.

2.1. Materials and Tools

The materials used in this research were land kale plants obtained from Bandungan, Semarang Regency. Sodium hydroxide, sulfuric acid, sodium bicarbonate, hydrochloric acid, chloroform, benzene, methanol, sodium carbonate, n-hexane, ethanol, ethyl acetate, chlorogenic acid, ferulic acid, gallic acid, caffeic acid, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were of analytical grade reagents. GF254 silica gel plate, ethanol, distilled water, iron (III) chloride 1%, acetic acid anhydride, ether, n-hexane, hydrochloric acid, chloroform, Mg metal scrap, Meyer's reagent, Dragendorff's reagent, Folin-Ciocalteu reagent, and universal indicator.

The tools used were analytical balance, beakers, Erlenmeyer flasks, test tubes, measuring cups, glass funnels, measuring pipettes, dropper pipettes, spatulas, stirrers, drop plates, vials, capillary tubes, porcelain flasks, separating funnels, filter paper, rotary evaporator Buchi R-114, chamber, water bath, UV detector lamp (254 nm and 366 nm), Genesys 10S (Thermofisher Scientific) UV-VIS spectrophotometer, LC-MS type 6530 Agilent Qtof specification sun shell column C18 2.6 µm (3.0 mmID × 150 mm).

2.2. Preparation and Production of Extracts

The cleaned land kale plants were cut into pieces, air-dried, and powdered. The powder was extracted through maceration using a 96% ethanol solvent until the solvent became clear, with replacement occurring every 24 hours. The chlorophyll was removed from this crude ethanol extract by adding distilled water in a volume ratio of ethanol extract to distilled water (1:1). Subsequently, the contained lipid was eliminated using n-hexane.

2.3. Phytochemical Screening

Phytochemical screening of land kale powder was conducted following a modification of the method outlined by Joshi *et al.* [14]. Additionally, ethanol extract screening was performed using thin-layer chromatography (TLC).

2.4. Determination of Total Phenolics

A total of 0.5 mL of 1000 ppm ethanol extract was put into a 10 mL volumetric flask, then added with 2.5 mL distilled water and 2.5 mL of 10% Folin-Ciocalteu reagent. The solution was incubated for 15 minutes, and then 2 mL of 7.5% sodium carbonate was added to the volumetric flask. The flask was shaken and incubated in the dark for 30 minutes. The absorbance of the solution that had been incubated was measured using a UV-Vis spectrophotometer at a wavelength of 758 nm. A standard curve was prepared using gallic acid standards of various concentrations [15, 16, 17] with slight modifications.

2.5. Isolation of Phenolic Acids

Isolation of phenolic acids was carried out using three methods: alkaline hydrolysis, acid hydrolysis, and without hydrolysis [13].

2.5.1. Alkaline Hydrolysis

Four grams of ethanol extract was dissolved in 40 mL of 96% ethanol and then filtered. The filtrate was hydrolyzed using 100 mL of 1 N NaOH and left for 24 hours at room temperature and in the absence of light. The hydrolyzed results were filtered and acidified using 10% H_2SO_4 until reaching a pH of 3, as verified with a universal indicator. The acidified extract was then underwent liquid-liquid extraction with 40 mL of ether four times to separate the phenolic compounds from the extract. The upper layer (nonpolar fraction) was subsequently evaporated until 40 mL remained, and it was then extracted with 8 mL of $NaHCO_3$ to isolate phenolic acid compounds from other phenolic compounds. The lower layer (polar fraction) was taken and re-acidified to pH 3 using 10% H_2SO_4, followed by extraction with 40 mL of ether four times. The upper layer (nonpolar fraction) was further evaporated to dryness. The resulting residue was dissolved in 1 mL of methanol, referred to as the HB fraction henceforth.

2.5.2. Acid Hydrolysis

A total of 4 grams of ethanol extract was dissolved in 40 mL of 96% ethanol and filtered. The filtrate was hydrolyzed using 40 mL of 2 N H_2SO_4 for 2 hours in a water bath at 60°C. The hydrolysis results were subjected to liquid-liquid extraction with 40 mL of ether four times to separate phenolic compounds from the extract. The upper layer (nonpolar fraction) underwent evaporation until 40 mL remained, followed by extraction with 8 mL of 20% $NaHCO_3$ to separate phenolic acid compounds from other compounds. The lower layer (polar fraction) was collected, re-acidified to pH 3 using 10% H_2SO_4 and then extracted with 40 mL of ether four times. The upper layer (nonpolar fraction) was evaporated to dryness. The resulting residue was dissolved in 1 mL of methanol, and referred to as the HA fraction.

2.5.3. Without Hydrolysis

A total of 4 grams of ethanol extract was dissolved in 40 mL of 96% ethanol and filtered. The filtrate was acidified with 10% H_2SO_4 until it reached pH 3. The acidified extract was underwent liquid-liquid extraction with 40 mL of ether four times to separate the phenolic compounds from the extract. The upper layer (nonpolar fraction) was evaporated until 40 mL remained and extracted with 8 mL of 20% $NaHCO_3$ to separate the

phenolic acid compounds from other compounds. The lower layer (polar fraction) was taken and re-acidified to pH 3 using 10% H_2SO_4 and then extracted using 40 mL of ether four times. The upper layer (nonpolar fraction) was evaporated until dryness. The resulting residue was dissolved in 1 mL of methanol, referred to as the TH fraction.

2.6. Separation of Isolates

The HB, HA, and TH fractions were then analyzed using TLC, and the R_f value was determined and compared with several phenolic acid standards. The eluent used was chloroform: ethyl acetate: acetic acid (30:50:3), and the stationary phase used was silica gel 60 GF254. The standard phenolic acids used in TLC were gallic acid, pyrogallol, ferulic acid, salicylic acid, and caffeic acid. TLC results were observed under 254 nm and 365 nm UV lamps.

The HB fraction stain that was parallel to the phenolic standard was separated using preparative TLC. The eluent used in preparative TLC was chloroform: ethyl acetate: acetic acid (30:50:3), and the stationary phase was silica gel 60 GF254 (glass plates). The results obtained from preparative TLC were isolate bands. The resulting isolate band was then scraped and dissolved in methanol and filtered to obtain the filtrate. The filtrate was then evaporated, and an isolate of phenolic acid B was obtained [18].

2.7. Purity Test

Purity tests were conducted employing TLC (Thin-Layer Chromatograph) with various eluents, including both single and mixed eluents. Additionally, a two-dimensional TLC analysis was performed. The eluents used were ethyl acetate, ethanol, ethyl acetate: ethanol (1:1), chloroform: ethyl acetate: acetic acid (30:50:3), and chloroform: ethyl acetate: acetic acid (40:50:3). The criteria for purity determination involved observing a single stain on the two-dimensional TLC plate; the presence of only one stain indicated the purity of the obtained isolate [19].

2.8. Identification of Phenolic Acid Structure

The structural analysis of the isolate was performed using a UV-Vis spectrophotometer and LC-MS/MS.

2.9. Antioxidant Analysis of Land Kale Extract

The sample solution was varied in concentration, then 0.5 mL of the solution of various concentrations was reacted with 3.5 mL of 0.1 mM DPPH solution and then shaken. The solution was left in the dark for 30 minutes. The absorbance at the maximum wavelength was measured using a UV-Vis spectrophotometer [20, 21] with slight modifications.

3. Results and Discussion

3.1. Determination, Extraction, Phytochemical Screening, and Determination of Total Phenolics of Land Kale

Land kale was determined at the Ecology and Biosystematics Laboratory, Department of Biology, FSM UNDIP. The results of the land kale plant determination showed that the sample used was *Ipomoea reptans* Poir. The ethanol extract obtained was 191.152 grams or 9.558%. The ethanol extract from which chlorophyll and fat have been removed produces a dark brown solid of 59.26 grams or 2.96%.

The secondary metabolites of dried land kale are flavonoids, tannins, saponins, steroids, and triterpenoids. The secondary metabolites of ethanol-water extract of land kale are tannins and flavonoids. The secondary metabolites are shown in Table 1, similar to the results of phytochemical screening in a previous study [1].

Determination of total phenolics using the Folin-ciocalteu method is based on the bluish color produced by the reaction between the phenolic compounds of the sample and the Folin-ciocalteu reagent (consisting of phosphotungstic acid and phosphomolybdic acid) [20]. Total phenolics were measured using gallic acid absorbance data of various concentrations, which produced a standard curve. The standard curve of gallic acid is shown in Figure 1.

Table 1. Phytochemical screening results of land kale

Secondary metabolite	Dried powder	Ethanol-water extract
Alkaloids		
Dragendorff	-	-
Mayer	-	-
Tannin/phenolic	+	+
Flavonoids	+	+
Saponin	+	+
Steroids	+	-
Triterpenoids	+	-

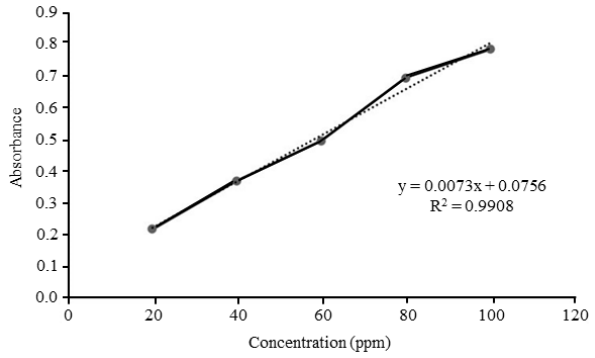

Figure 1. The gallic acid standard curve

The total phenolic acid of the ethanol extract of land kale obtained was 71.2420 ± 0.0791 mg/g GAE. The total phenolic results obtained in this study exceeded those reported in prior research by Mariani *et al.* [22], where the value was 31 ± 0.849 mg/g GAE, and in the research conducted by Kurniawan *et al.* [2], which reported a value of 0.86 mg/g GAE.

3.2. Analysis of Phenolic Acid Isolation Results

The results of phenolic acid isolation are shown in Table 2. Hydrolysis with a base (HB fraction) yielded the highest number of isolates, prompting subsequent separation procedures.

The results of separating the HB fraction by TLC obtained four stains, the HA fraction six stains, and the TH fraction three stains. The results of TLC analysis of phenolic acid isolates are shown in Figure 2. Table 3 shows the R_f values of phenolic acid isolates from the HB, HA, and TH fractions compared to standard phenolic acids.

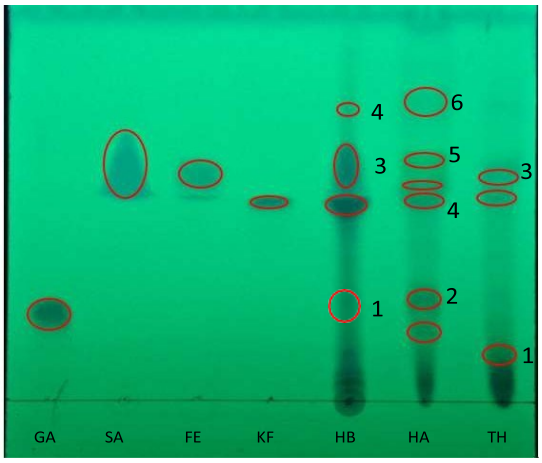

Figure 2. TLC results of HB, HA, TH, and standard phenolic acid fractions at λ = 254 nm using chloroform: ethyl acetate: acetic acid (30:50:3) (GA: gallic acid; SA: salicylic acid; FE: ferulic acid; KF: caffeic acid)

Table 2. Phenolic acid isolation results

Fraction	Result
HB	338 mg (8.45%
HA	321 mg (5.77%)
TH	335 mg (8.37%)

Table 3. R_f values of HB, HA, TH fractions, and standard phenolic acid

Compound name	Stain 1	Stain 2	Stain 3	Stain 4	Stain 5	Stain 6
gallic acid	0.21	-	-	-	-	-
salicylic acid	0.58	-	-	-	-	-
ferulic acid	0.55	-	-	-	-	-
caffeic acid	0.51	-	-	-	-	-
HB Fraction	0.25	0.51	0.55	0.74	-	-
HA Fraction	0.17	0.27	0.51	0.55	0.67	0.76
TH Fraction	0.12	0.51	0.55	-	-	-

In Table 3, it can be seen that the R_f values produced by the three fractions are in line with the standard R_f value for caffeic acid of 0.51 and standard ferulic acid of 0.55. It can be concluded that the ethanol extract of land kale contains caffeic acid and ferulic acid compounds. The results obtained are in accordance with research by Roy *et al.* [10], which reported that land kale contains caffeic acid. Im *et al.* [23] reported that sweet potato plants (*Ipomoea batatas*), which are in the same family as land kale, contain ferulic acid compounds. The HB fraction was then separated using preparative TLC. The results of preparative TLC for the HB fraction are shown in Figure 3.

Separating the HB fraction using preparative TLC produced four bands, which were then scraped off and dissolved in methanol. Band 1 has an orange color, band 2 and band 3 have a yellowish color, and band 4 is colorless. The four bands are referred to as B1, B2, B3, and B4 isolates. The structures of B2 and B3 isolates were identified using a UV-Vis spectrophotometer, and B4 isolate was identified using LC-MS/MS.

B2, B3, and B4 isolates were tested for purity using single eluent or mixed eluent TLC and separated using 2- dimensional TLC. The purity test outcomes indicated a single stain, confirming the purity of each isolate. B2, B3, and B4 isolates were analyzed using UV Vis. The results are shown in Figures 4, 5, and 6.

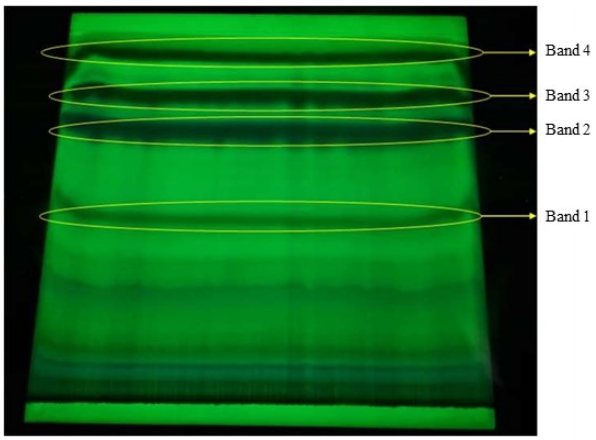

Figure 3. HB fraction preparative TLC result

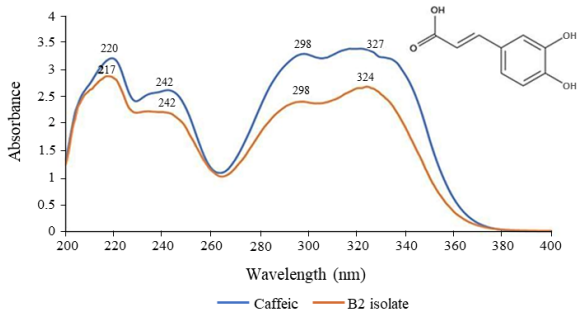

Figure 4. Standard UV-Vis spectra of caffeic acid and B2 isolate

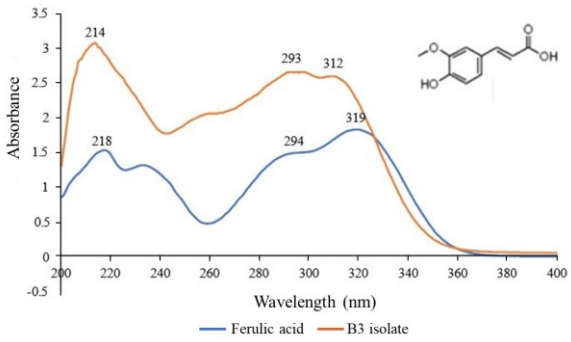

Figure 5. Standard UV-Vis spectra of ferulic acid and B3 isolate

The maximum wavelength of B2 isolate is similar to the wavelength of standard caffeic acid (Figure 4). Based on research by Catauro et al. [24], the wavelengths of caffeic acid produced are 216, 242, 296, and 324 nm. B3 isolate has a maximum wavelength similar to the wavelength of standard ferulic acid (Figure 5).

Based on Holser [25], the wavelengths of ferulic acid are 215, 285, and 312 nm. B4 isolate produces a peak at a wavelength of 252 nm (Figure 6). The peak wavelength of B4 isolate is similar to the peak wavelength of p- hydroxybenzoic acid, which is reported to be 256 nm by Rasmussen *et al.* [26]. The results of the LC-MS/MS spectrogram of B4 isolate at a retention time of 2.123-2.275 minutes are shown in Figure 7.

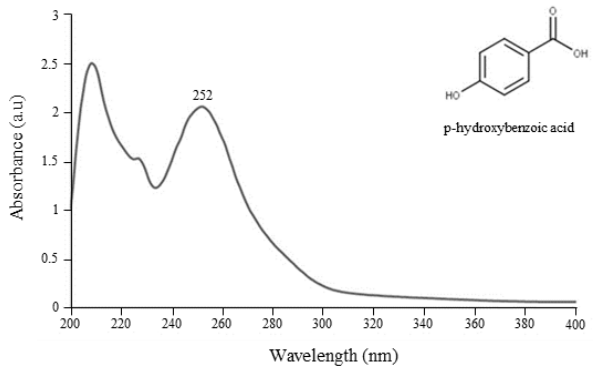

Figure 6. UV-Vis spectrum of B4 isolate

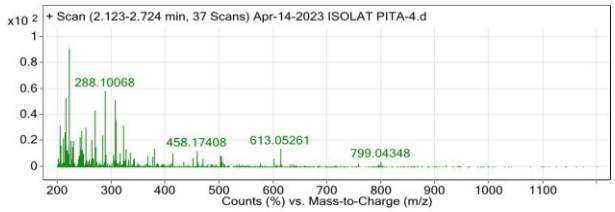

Figure 7. LC-MS/MS spectrogram of B4 isolate

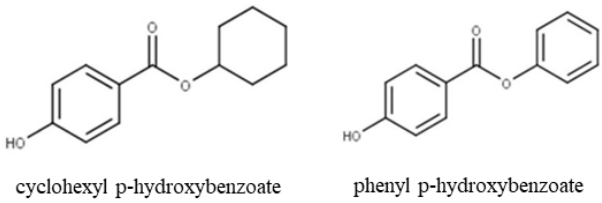

Figure 8. Structure of p-hydroxybenzoic acid derivatives

The molecular formula of B4 isolate can be calculated using rule 13 [27] on the most stable ions, namely $[M+H]^+$ 220 and 214, which are p-hydroxybenzoic acid derivatives bound as esters, namely cyclohexyl p-hydroxybenzoate ($C_{13}H_{16}O_3$) and phenyl p-hydroxybenzoate ($C_{13}H_{10}O_3$). Based on the resulting LC-MS/MS spectrogram, it can be concluded that the B4 isolate with a retention time of 2.123-2.724 minutes contains a p-hydroxybenzoic acid framework. The structure of p-hydroxybenzoic acid is shown in Figure 8.

3.3. Antioxidant Activity Test Results

The antioxidant activity of ethanol extract from land kale was tested using the DPPH method, with the positive control being the gallic acid standard. DPPH solution is a purple-colored solution that functions as a source of free radicals, which are reduced by the electron or hydrogen donor process, resulting in a pale purple to pale yellow color [28]. The dampening reaction of the DPPH solution by antioxidant compounds is shown in Figure 9.

The antioxidant activity of a compound is expressed in the IC_{50} value. If a compound has IC_{50} >200 µg/mL, it means no activity, IC_{50} >150-200 µg/mL is weak activity, IC_{50} >100-150 µg/mL means moderately strong, IC_{50} >50-100 µg/mL is strong, and IC_{50} <50 µg/mL is very strong [29]. The IC_{50} values for ethanol extract of land kale and gallic acid are presented in Table 4.

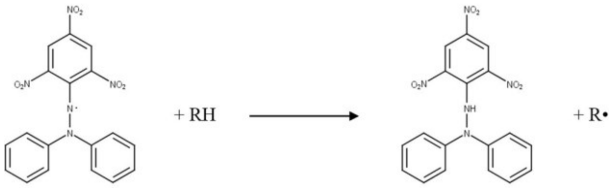

Figure 9. DPPH dampening reaction by the antioxidant compound RH [30]

Table 4. IC_{50} value of gallic acid and ethanol extract of land kale

Sample	IC_{50} (mg/L)	Classification
Gallic acid	6.68 ± 0.011547	Very strong
Ethanol extract	94.83 ± 0.00577	Strong

In Table 4, it can be seen that the gallic acid standard produces an IC_{50} value of 6.68 ± 0.011547 mg/L and the ethanol extract of 94.83 ± 0.00577 mg/L. The IC_{50} value of ethanol extract is greater than previous research by Dedi *et al.* [31], which was 157.72 mg/L. The ethanol extract of land kale has strong antioxidant activity because the IC_{50} value is below 100 mg/L and has the potential as a natural antioxidant.

4. Conclusion

The ethanol extract of land kale is brownish in color. Land kale plant powder contains tannin, phenolic, flavonoid, steroid, and triterpenoid compounds. The ethanol-water extract of land kale contains tannin, phenolic, flavonoid, and saponin compounds. The HB fraction predominantly produced most phenolic acid isolates. Identification using a UV-Vis spectrophotometer indicated that B2 isolate is suspected to be caffeic acid, and B3 isolate is suspected to be ferulic acid. LC-MS/MS identification suggested that B4 isolate, with a retention time of 2.123-2.724 minutes, is a phenolic acid compound with a p-hydroxybenzoic acid framework. Land kale plants exhibited total phenolics of 71.2420 ± 0.0791 mg/g GAE and an IC_{50} value of 94.83 ± 0.00577 mg/L.

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