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Antioxidant Activity, Total Phenolic and Flavonoid Content, and Metabolite Profiling of *Kalanchoe Ceratophylla* Haw. Leaf Ethanol Extract

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ABSTRACT. *Kalanchoe ceratophylla* Haw. is a species from the Crassulaceae genus known for its various pharmacological benefits, including antioxidant and antidiabetic effects, due to the high contents of flavonoids, tannins, and other phenolic compounds. This study aims to determine the antioxidant activity, total phenolic and flavonoid content, and metabolite profiling of ethanol extracts from *K. ceratophylla* Haw. leaves. The study utilized *K. ceratophylla* Haw. leaves, which were pre-identified and authenticated. Extraction was performed using maceration with 70% and 95% ethanol as solvents. The leaves were dried at 40 °C and freeze-dried at -20 °C to -30 °C. The metabolite profiles of the extracts were analyzed using high-resolution mass spectrometry (LC-HRMS) and gas chromatography-mass spectrometry (GC-MS). The HR-MS and GC-MS analyses confirmed the presence of antioxidants, flavonoids, and phenolics in the extract. The findings indicate that the drying method and solvent type significantly influence the total phenolic and flavonoid content and antioxidant activity. The total phenolic content varied from 1.37±0.59 to 5.23±0.87 mg GAE/mL extract, the total flavonoid content varied from 4.26±0.05 to 12.33±0.19 mg QE/g extract, and the antioxidant activity varied from 1.07±0.04 to 3.03±0.18 mg/mL. Based on these results, the ethanol extract of *K. ceratophylla* Haw. leaves demonstrate significant potential as a source for medicinal applications. The optimal extraction condition in this study was maceration with 95% ethanol, followed by freeze-drying, which produced the highest levels of antioxidant and flavonoid content. Future research should investigate the clinical implications and potential pharmaceutical formulations derived from *K. ceratophylla* Haw.

Keywords: Antioxidants, *K. ceratophylla* Haw. leaf extracts, metabolite profiling, total flavonoids, total phenolics

INTRODUCTION

Natural substances are a significant source of active pharmaceutical compounds. This is supported by research revealing that over one-third of all small-molecule active compounds in globally approved medications from 1981 to 2010 originated from natural substances or semi-synthetic derivatives of natural substances (Newman & Cragg, 2012). The antioxidant activity of medicinal plants is a crucial parameter for evaluating their therapeutic potential.

According to Lobo et al. (2010), antioxidants help the body combat free radicals, which can lead to oxidative stress and contribute to various degenerative diseases, including cancer, heart disease, and neurological disorders. Meanwhile, Agil et al. (2018) suggested that flavonoid molecules act as antioxidants that help prevent cellular damage and the beginning of degenerative disorders like diabetes by opposing or inhibiting free radicals.

Kalanchoe ceratophylla Haw., a member of the *Crassulaceae* family, is a promising plant that has not yet been investigated scientifically. In many tropical nations, traditional medicine has long utilized the genus *Kalanchoe* to treat digestive issues, inflammation, and infections (Nascimento et al., 2023). According to the previous study by Assis de Andrade et al. (2023), plants belonging to the *Kalanchoe* family contain flavonoids, alkaloids, and phenolic compounds, which may have antidiabetic effects since these substances can aid in enhancing glucose metabolism and lowering insulin resistance. In addition to serving as beneficial nutrition supplements for diabetic patients, medicinal plants can be formulated as foods that help prevent the consequences of diabetes (Deb et al., 2023).

According to ethnomedicine in Northeast India, the leaves of *Kalanchoe pinnata* Pers. and *Bryophyllum pinnatum* (Lam.) Oken in the Assam region, belonging to the Crassulaceae plant family, are utilized as an antidiabetic. In Assam, Manipur, Nagaland, and Tripura, Kalanchoe pinnata Pers is used to cure diabetes. Local names for these plants include *mana hidak, hohlongkak (phom), kophpata* or *patharkuchi*, and *dupoor-tenga* (Deb et al., 2023). In addition to being effective supplements for diabetic patients, medicinal plants can be formulated as foods to help prevent the symptoms of diabetes. Certain Kalanchoe species have also been used in traditional Asian medicine. Fresh leaves of *K. pinnata* and *K. prolifera* are typically applied topically as poultices for fever, burns, and wounds in Indonesia (Khairiah et al., 2022). Manalu et al. (2025) studied drying methods and their effects on bioactive, phenolic, and flavonoid contents, as well as antioxidant activity. They used GC-MS analysis to identify major compounds, such as γ-sitosterol, glutinol, friedelan-3-one, squalene, ergost-5-en-3-ol, erythritol, and neophytadiene.

However, these studies had not yet comprehensively combined HR-MS (untargeted) techniques with GC-MS to obtain a complete profile of volatile and non-volatile metabolites, let alone correlate % area data (GC-MS) with total flavonoids, total phenols, and antioxidant activity through multivariate analysis (e.g., PCA). This study aims to fill that gap by conducting a comprehensive metabolite profiling using HR-MS and GC-MS and examining the quantitative relationships between metabolite profiles (% area), flavonoids, phenolics, and antioxidant activity of ethanol extracts from *K. ceratophylla* Haw. leaves. Thus, this study not only expands the range of known bioactive compounds but also provides a deeper understanding of the structural and compositional contributions of compounds to the antioxidant potential of this species.

This study analyzed the chemical components on the ethanol extract of *K. ceratophylla* Haw. leaves using HR-MS and GC–MS. These methods were applied to this specific plant for the first time to reveal both volatile and non-volatile compounds. Rather than using just one method, both methods also provide a better picture of what is contained in this plant. This study also checked how these chemicals relate to antioxidant activity as well as phenolic and flavonoid levels. It is found that flavonoids are the main part of what makes the extract fight free radicals. Thus, this research contributes to new findings about the chemical content of *K. ceratophylla* Haw. and its potential as a good source of natural antioxidants with unique chemical traits.

EXPERIMENTAL SECTION

Materials and Experimental Design

The *K. ceratophylla* Haw. The plant was identified at ITB Bandung with the number 1284/IT1.C11.2/TA.00/2024. The part of the plant used in this research is the leaves. The *K. ceratophylla Haw* plant originates from Riau but has been propagated and cultivated in Depok, West Java. The chemicals used included ethanol 96% (Merck), methanol p.a. (Merck), Mayer's reagent, Dragendorff's reagent, Bouchardat's reagent, reagent for Shinoda test, AlCl3, Folin-Ciocalteu reagent, quercetin (Sigma Aldrich), sodium acetate (Merck), sodium carbonate (Merck), gallic acid (Sigma Aldrich), dimethyl sulfoxide (Sigma Aldrich), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich). Meanwhile, the instruments were a maceration chamber, blower oven (MERCK), rotary evaporator (IKA), Microplate reader (Versamax). HRMS analysis was performed using the Vanquish™ UHPLC Binary Pump liquid chromatography system (Thermo Scientific™, Germany), Q Exactive™ Hybrid Quadrupole-Orbitrap™ High Resolution Mass Spectrometer (Thermo Scientific™, Germany). Liquid chromatography was carried out using a Thermo Scientific™ Accucore™ Phenyl-Hexyl 100 mm × 2.1 mm ID × 2.6 μm analytical column (Windarsih et al., 2022). GC-MS (Shimadzu QP2010, Autosampler: AOC 20i, Column: RTX-5MS).

Sample Preparation and Extraction

The preparation of simplicia from the leaves of *K. ceratophylla* Haw. began with an initial sorting process, washing, draining, weighing, wilting in a refrigerated room, cutting, drying, and size reduction of the simplicia. The drying process used a blower oven at 40°C and a freeze dryer at -20°C to -30°C. The extraction method used the maceration method with 70% and 95% ethanol as solvents. The finely ground simplicia of *K. ceratophylla* Haw. were weighed at 50 grams and then dissolved in each solvent with a ratio of 1:20 (simplicia: solvent). The collected filtrate was then evaporated using a rotary evaporator to obtain an extract of *K. ceratophylla* Haw. leaves in ethanol. In this study, there were 4 treatment combinations based on the solvent used for the maceration and the type of drying equipment for *K.ceratophylla* Haw. leaves. The treatments were A (70% ethanol; oven blower), B (95% ethanol; oven blower), C (70% ethanol; freeze dryer), and D (95% ethanol; freeze dryer). The leaf extracts were then screened for phytochemicals, tested for antioxidant activity as well as total phenol and flavonoid contents, and profiled using GC-MS and HR-MS.

Phytochemical Screening

Identification of secondary metabolite content was carried out for alkaloid, flavonoid, tannin, and saponin compounds. Phytochemical screening was conducted according to Materia Medica Indonesia and Harborne. Alkaloid screening was performed using Mayer, Dragendorff, and Bouchardat reagents; flavonoid screening was conducted using the Shinoda test; tannin screening was carried out using FeCl3; and saponin screening was performed using the foam test (Sembiring et al., 2018).

Total Phenolic Content

The total phenols were examined using the Folin-Ciocalteu method on a microplate. The absorbance was measured at 765 nm using a microplate reader. The total phenol content is expressed as gallic acid equivalent (GAE) in mg per g of extract (Sembiring et al., 2018).

Total Flavonoid Content

The total flavonoids were determined using a colorimetric test with $AlCl_3$. Absorbance was measured at 415 nm with a microplate reader. The total flavonoid content isis expressed as quercetin equivalent (QE) in mg per gram of extract (Sembiring et al., 2018).

Antioxidant Activity by DPPH Assay

The method used to test antioxidant activity is the DPPH free radical scavenging method (Bobo-García et al., 2015). The absorbance of the mixture was determined using a UV-2450 Spectrophotometer (Shimadzu Corp, Japan) at a wavelength of 517 nm. Positive control is a mixture of solution without the sample, considered 100% radical. The ability of the ethanol extract of *K. ceratophylla* Haw leaves to donate hydrogen (IA%) was measured using the following formula: IA% % = 100 − (Abs sample Abs control) × 100. IC_{50} represents the sample concentration required to scavenge 50% of free radicals. The IC_{50} value is calculated using the formula: y = bx + a and is expressed as mg/mL.

Identification of Volatile and Non-volatile Compounds in *K. ceratophylla* Haw.

The volatile components in extracts were separated and identified using a Shimadzu GC-2010 Ultra equipped with column Rtx-5MS (Crossbond 5% diphenyl/95% dimethyl polysiloxane, 30 m x 0.25 mm I.D. with 0.25 μm film thickness) and a mass spectrophotometer detector. The carrier gas, helium, was employed at a constant flow rate of 1 mL/min. Using splitless mode, 1 μL of material was automatically fed into the GC-MS system while maintaining the inlet temperature at 250°C. After three minutes at 80°C, the GC oven temperature was increased to 270°C for the following five minutes, at a rate of 10°C per minute.

Principal Component Analysis (PCA)

Principal Componen Analysis (PCA) analysis is a statistical method that involves a linear combination of observed variables to emphasize variation in existing variables or parameters, thereby producing the largest variance with specific weights that carry the most significant information in the data set (Rahimah et al., 2021). Thus, PCA facilitates the visualization of diverse data distributions. The PCA method is also often used to select appropriate combinations of classification parameters. In the process, PCA uses orthogonal transformation to convert correlated observation variables into a set of values of uncorrelated variables, known as principal components (Mattjik & Sumertajaya, 2011). PCA also simplifies data interpretation by reducing the factors and variables from the data structure, visualizing the patterns of similarity between observations and variables as points on a map. The purpose of using the PCA method in this research is to see whether there is a relationship between the group type variables (A, B, C, and D) and the parameters that have been taken (antioxidants, flavonoids, and phenols).

Statistical Analysis

The study employed a completely randomized design (CRD) for its experimental design. The data were presented as the mean standard deviation of three replicate analyses. Statistical analysis was performed using ANOVA and Duncan's Multiple Range Test (DMRT). PCA data were processed using XLSTAT Version 2014.5.03.

RESULTS AND DISCUSSION

Phytochemical Screening

The results of the phytochemical screening can be seen in **Table 1**. The screening results showed that the ethanol extract samples of *K. ceratophylla* Haw. leaves, namely samples A, B, C, and D, contained alkaloid, flavonoid, and tannin compounds, while saponin compounds were not detected. This suggests that *K. ceratophylla* Haw. is rich in secondary metabolites, particularly compounds with biological activity, including antioxidants, anti-inflammatory agents, and antimicrobials.

Total Phenolic and Flavonoid Content and Antioxidant Activity

The results of the analysis of total phenol and flavonoid and antioxidant activity tests can be seen in **Table 2**. The results of the total phenol measurement generally showed that the ethanol extract of *K. ceratophylla* Haw. leaves had a total phenol value of 1.37±0.59 - 5.23±0.87 mg GAE/g extract. The freeze-dry treatment with 70% ethanol extract showed higher total phenol content than other treatments.

The measurement results of total flavonoids showed that the total flavonoid content of *K. ceratophylla* Haw. leaf extract ranged from 4.26±0.05-12.33±0.19 mg QE/g extract. The freeze-dry treatment with 95% ethanol extract showed higher total flavonoid content than other treatments. The antioxidant activity test analysis results showed that the highest antioxidant activity was found in the freeze-dryer drying treatment with 95% ethanol extract at 3.03±0.18 mg/ml. The lowest antioxidant activity value was found in the oven blower drying treatment with 70% ethanol extract, measuring 1.07±0.04 mg/ml.

The variations in total phenolic and flavonoid contents using different maceration methods can be linked to differences in solvent properties, conditions of extraction, and the physicochemical properties of the phenolic and flavonoid compounds in question. Different maceration methods varying in the choice of

solvents, concentration, temperature, and extraction time can impact the solubility and diffusion of bioactive compounds within the plant matrices and solvent extracting media. There are peculiarities in the extraction of phenolic and flavonoid compounds due to differences in their polarity and molecular structure; hence, the polarity of extraction solvent can be a determining factor. Phenolic compounds are more hydrophilic, and polar solvents like ethanol or methanol are more effective in their extraction, while the less polar solvents are more effective in extracting the hydrophobic flavonoids. Further, the extraction time and temperature can aid in reaching the solvent and diffuse compounds within the plant matrices, and, inversely, concentrate heat-labile phenolic and flavonoid compounds by oxidation and thermal degradation, leading to lower extraction yields. Other factors are the maceration time, size of the plant particles, and the solvent-to-sample ratio, or the degree of agitation, which affect the rate of diffusion, or mass transfer. Small particles and vigorous agitation will promote extraction by increasing contact of the solvent and disrupting plant cell walls. Thus, the differences in extraction parameters for each maceration technique translate to different phenolic and flavonoid profiles and concentrations in the extract and, in turn, their antioxidant activity. This is because the free radical scavenging ability is usually higher for extracts containing greater phenolic and flavonoid concentrations (Savitri et al., 2019; Muflihah et al., 2021; Wakeel et al., 2019).

The results of the ANOVA test showed that the type of drying treatment and ethanol concentration significantly affect the total phenol and flavonoid content and antioxidant activity (sig. < 0.05). The post hoc test results using the DMRT (Duncan's Multiple Range Test) showed that in the total phenol test, treatment C was significantly different from the other treatments. The total flavonoid test showed that all treatments exhibited significant differences. In contrast, in the antioxidant activity test, treatments a and b did not show significant differences, as did treatments b and d.

As all extracts were obtained using ethanol as the solvent, the differences observed in total phenolic and flavonoid content and antioxidant activity for samples A–D justify the need for careful study design in extraction using different concentrations of ethanol, altering the temperature and duration of the extraction, and changing the solvent-to-sample ratio. Variation in isolation conditions may affect the polarity, solubility, and overall content of the phenolic and flavonoid compounds. Furthermore, differences in the plant material—such as the maturity of sampled leaves and the environmental and physiological conditions in which the plant was grown-could influence the biosynthesis of secondary metabolites. Differences in quantity, structure, and biological activity of the individual compounds in a mixture may also explain in part the differences in antioxidant activity exhibited in a sample. In addition, loss of compounds through oxidation or degradation and the presence of bioactive compounds that exert synergistic or antagonistic effects may explain the differences in antioxidant activity (Lohvina et al., 2022; Mandache et al., 2024;Muflihah et al., 2021).

Identification of Volatile and Non-volatile Compounds in *K. ceratophylla* Haw.

The classification of the dominant compounds from the identification of volatile compounds in *K. ceratophylla* Haw leaf extract can be seen in **Table 3**. Sample A is dominated by phenolic, alkaloid, terpenoid, and organosilicon compounds. Sample B contains phenolics, heterocyclic alkaloids, and organosilicon compounds. Sample C has phenolics, alkaloids (tyrosine/cinnoline type), organic acids, and organosilicon compounds, while sample D contains terpenoids, phenolic derivatives, organometallic, and organosilicon compounds. Table 4 shows the volatile compounds identified in the *K. ceratophylla* Haw leaf extracts with a similarity index above 50.

The presence of identified non-volatile compounds with antioxidant, flavonoid, and phenolic properties in the ethanol extract of *K. ceratophylla* Haw. leaves (**Table 5**) indicate that compounds with antioxidant properties originate from the group of oxygenated fatty acids (hydroxy/oxo fatty acids) and terpenoid derivatives, such as lupeol and pheophorbide A. Phenolic compounds such as 4-coumaric acid and 3,4-dihydroxybenzaldehyde were mainly found in sample D, suggesting a potential increase in reductive (phenolic) activity under these conditions. Flavonoid compounds identified include 3-methoxy-5,7,3',4'-tetrahydroxyflavone, which is an analog of kaempferol/quercetin and contributes strongly to the total antioxidant activity.

Table 1. Phytochemistry screening results of the ethanol extract of *K. ceratophylla* Haw. leaves

Samples	Alkaloids	Flavonoids	Tannins	Saponins
A	+	+	+	-
B	+	+	+	-
C	+	+	+	-
D	+	+	+	-

Table 2. The analysis results of the total phenolic and flavonoid content and antioxidant activity of *Kalanchoe ceratophylla* Haw. leaf extract

Sample	Total phenol content mg GAE/mL	Total flavonoid Content (mg QE/g extract)	Antioxidant activity (mg/ml)
A	3.01 ± 0.07^{ab}	4.26 ± 0.05^{a}	1.07 ± 0.04^{a}
B	3.47 ± 1.49^{b}	6.97 ± 0.06^{b}	2.79 ± 0.11^{b}
C	5.23 ± 0.87^{c}	8.14 ± 0.12^{c}	1.12 ± 0.24^{a}
D	1.37 ± 0.59^{a}	12.33 ± 0.19^{d}	3.03 ± 0.18^{b}

Note: Data are presented as means. Values in the same columns followed by different letters are significantly different from each other according to Duncan's test ($p<0.05$)

Table 3. Classification of dominant compounds in each treatment of *K. ceratophylla* Haw. leaf ethanol extract

Sample	Compounds	Chemical group	Bioactivity
A	• 4-[(Dimethylamino)methyl]-2,6-dimethylphenol; • Tricyclo[4.2.1.0(2,5)]non-7-ene; 3,4-di(tris(trimethylsilyloxy)silyl)-; • 2-(Pyridin-2-ylformamido) acetic acid; • Methyltris(trimethylsiloxy)silane; • Ethoxy(phenyl)silanediol; • Tris(tert-butyldimethylsilyloxy)arsane	Phenolic derivative, alkaloid (pyridine type), terpenoid, organosilicon compound	Antioxidant, antimicrobial, structural analog of alkaloids
B	• 4-[(Dimethylamino)methyl]-2,6-dimethylphenol; • Silicic acid, diethyl bis(trimethylsilyl) ester; • p-Cyanophenyl p-(2-propoxyethoxy) benzoate; • Ethoxy(phenyl)silanediol; • Tricyclo[4.2.1.0(2,5)]non-7-ene; 3,4-di(tris(trimethylsilyloxy)silyl)-; • Methyltris(trimethylsiloxy)silane; tert-Nonylphenol; • 1H,2H,3H,4H-Pyrido[3,4-b] pyrazine; • 3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, • ethyl ester	Phenolic, alkaloid (pyrazine & quinoline type), organosilicon compound	Antioxidant, potential enzyme inhibitor, heterocyclic bioactive compound
C	• 4-tert-Amylphenol; Ethoxy(phenyl)silanediol; • 4,5-Dimethyl-1,3-benzenediol; • 2,6-Dimethyl-L-tyrosine; • Silicic acid, diethyl bis(trimethylsilyl) ester; • 2-oxovaleric acid; • 1,2-Cinnolinedicarboxylic acid; • 2-(2-Chloroanilino)-2-oxoethyl 2-hydroxy-4-methylbenzoate; • 2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	Phenolic, alkaloid (tyrosine & cinnoline derivatives), organic acid, organosilicon compound	Antioxidant, nitrogenous metabolite, aromatic acid derivative
D	• Silicic acid, diethyl bis(trimethylsilyl) ester; • Ginsenol;	Terpenoid (triterpenoid, ginsenol), phenolic derivative,	Antioxidant, terpenoid-derived bioactive, potential detoxifying compound

Sample	Compounds	Chemical group	Bioactivity
	• Tris(tert-butyldimethylsilyloxy) arsane; (IR)-1-(2,6-Dichloro-3-fluorophenyl)ethanol, methyl ether; • 1,4-Bis(trimethylsilyl)benzene; • Stannane, tetrakis(1-methylethyl)-	organometallic, and organosilicon compounds	

Table 4. Volatile compounds identified in the ethanol extract of *K. ceratophylla* Haw. leaves

No.	Compound	Molecular formula	Sample [area (%)]				Similarity index			
			A	B	C	D	A	B	C	D
1	Decane	$C_{10}H_{22}$	1.3	4.66	3.69	3.12	93	92	92	91
2	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	0.94	2.74	2.57	1.96	72	75	76	73
3	Tris(tert-butyldimethylsilyloxy) arsane	$C_{18}H_{45}AsO_3Si_3$	5.03	-	4.87	6.78	66	-	45	66
4	Tricyclo [4.2.1.0(2,5)] non-7-ene, 3,4-di(tris(trimethylsilyloxy)silyl)-	$C_{27}H_{64}O_6Si_8$	6.72	6.96	2.24	-	57	58	47	-
5	Methyltris(trimethylsiloxy)silane	$C_{10}H_{30}O_3Si_4$	5.74	6.55	-	-	59	57	-	-
6	Silicic acid, diethyl bis(trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	4.34	8.68	5.79	10.55	61	60	57	62
7	Methyl 6,6,8,8,10,10-hexamethyl-3-oxo-2,5,7,9,11-pentaoxa-6,8,10-trisilatridecan-13-oate	$C_{12}H_{28}O_8Si_3$	0.62	1.36	-	-	54	66	-	-
8	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-	$C_{15}H_{22}$	1.88	4.07	3.67	2.7	83	81	86	80
9	6,6,8,8,10,10-Hexamethyl-2,5,7,9,11,14-hexaoxa-6,8,10-trisilapentadecane	$C_{12}H_{32}O_6Si_3$	4.81	3.1	1.06	2.09	54	68	66	50
10	2,2',2"-Nitrilotriethanol, triethyl ether	$C_{12}H_{27}NO_3$	0.33	2.96	-	-	73	70	-	-
11	3-Quinolinecarboxylic acid, 6,8-difluoro-4-hydroxy-, ethyl ester	$C_{12}H_9F_2NO_3$	4.14	5.07	-	-	50	54	-	-
12	Arsenous acid, tris(trimethylsilyl) ester	$C_9H_{27}AsO_3Si_3$	4.88	3.6	2.07	-	53	57	54	-
13	1,4-Bis(trimethylsilyl)benzene	$C_{12}H_{22}Si_2$	3.15	4.24	-	5.15	55	55	-	55
14	Loliolide	$C_{11}H_{16}O_3$	-	-	2.42	4.45	-	-	82	91
15	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	$C_{13}H_{22}OSi_2$	-	-	5.06	-	-	-	59	-
16	1,3-Cyclohexadiene, 5-(1 ,5-dimethyl-4-hexenyl)-2-methyl-, [S	$C_{15}H_{24}$	-	1.8	2.98	2.05	-	73	88	73
17	Ginsenol	$C_{15}H_{26}O$	-	-	-	10.43	-	-	-	62
18	2-Amino-N-(2,3-dihydro-1H-inden-2-yl) acetamide	$C_{11}H_{14}N_2O$	-	-	1.39	-	-	-	67	-
19	Phenol, 2,2'-methylenebis [6-(1,1-dimethylethyl)-4-methyl-	$C_{23}H_{32}O_2$	-	-	1.68	1.89	-	-	71	66
20	2,4,6-Cyc10heptatrien-1-one, 3,5-bis-trimethylsilyl-	$C_{13}H_{22}OSi_2$	4.76	-	4.02	3.14	60	-	59	65
21	(1 S,5S)-2-Methyl-5-((R)-6-methylhept-5-en-2-yl) bicyclo[3.1	$C_{15}H_{24}$	-	3.64	-	-	-	80	-	-
22	(+)-2-(Diethylamino)butyl acetate	$C_{10}H_{21}NO_2$	-	1.57	-	-	-	65	-	-
23	p-Cyanophenyl p-(2-propoxyethoxy) benzoate	$C_{19}H_{19}NO_4$	-	8.58	-	-	-	56	-	-
24	Cyclohexene, 3-(1 ,5-dimethyl -4-hexenyl)-6-methylene-, [S-(R	$C_{15}H_{24}$	-	2.51	-	-	-	70	-	-
25	1 -Butyl(dimethyl)silyloxypropane	$C_9H_{22}OSi$	-	-	3.87	-	-	-	76	-

No.	Compound	Molecular formula	Sample [area (%)]				Similarity index			
			A	B	C	D	A	B	C	D
26	2-(1,4,5,7-Tetramethyl-6H-pyrrolo [3 ,4-d]pyridazin-6-yl) anilin	$C_{16}H_{18}N_4$	-	-	3.61	-	-	-	64	-
27	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	-	-	2.4	-	-	-	81	-
28	5-Ethyl- 1 ,3-dioxane-5-methanol, tert-butyldimethylsilyl ether	$C_{13}H_{28}O_3Si$	-	-	1.68	-	-	-	58	-
29	Triacontanoic acid, methyl ester	$C_{31}H_{62}O_2$	-	-	1.43	-	-	-	70	-
30	1-Ethylsulfanylmethyl-2,8,9-trioxa-5-aza-1-sila-bicyclo[3.3.3] undecane	$C_9H_{19}NO_3SSi$	-	-	2.82	3.57	-	-	73	72
31	4,4'-bi-4H-pyran, 2,2',6,6'-tetrakis(1,1-dimethylethyl)-4,4'-dimethyl-	$C_{28}H_{46}O_2$	-	-	3.6	-	-	-	62	-
32	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	-	-	1.65	-	-	-	79	-
33	5,5'-Di(ethoxycarbonyl)-3,3'-dimethyl-4,4'-dipropyl-2,2'-dipyrrylmethane	$C_{23}H_{34}N_2O_4$	-	-	1.85	-	-	-	53	-
34	7-Hydroxy-7,8,9,10-tetramethyl-7,8-dihydrocyclohepta[d,e] naphthalene	$C_{18}H_{20}O$	-	-	3.01	-	-	-	54	-
35	Methanol, [4-(1,1 -dimethylethyl) phenoxy]-, acetate	$C_{13}H_{18}O_3$	-	-	3.18	-	-	-	56	-
36	2-Diisopropylsilyloxy methyltetrahydrofurane	$C_{11}H_{24}O_2Si$	-	-	-	1.91	-	-	-	50
37	2-Ethylbutyric acid, eicosyl ester	$C_{26}H_{52}O_2$	-	-	-	2.07	-	-	-	52
38	Propan-l -one, 2-methylthio-l-phenyl-, oxime	$C_{10}H_{13}NOS$	-	-	-	2.15	-	-	-	51

Table 5. Identified non-volatile compounds with antioxidant, flavonoid, and phenolic properties in the ethanol extract of *K. ceratophylla* Haw. leaves

No	Compound	Molecular formula	Chemical group	Bioactivity	Sample RT [Min]
1	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	$C_{18}H_{34}O_5$	Oxylipin (fatty acid derivative)	Antioxidant, anti-inflammatory	A(8.871), B(8.87), C(8.866)
2	12-oxo Phytodienoic Acid	$C_{18}H_{28}O_3$	Jasmonate derivative	Antioxidant, a signaling molecule in plants	A(8.455), B(12.365), C(8.493)
3	13(S)-HOTrE	$C_{18}H_{30}O_3$	Hydroxy octadecatrienoic acid	Antioxidant, anti-inflammatory	A-D (11.95-11.97)
4	2,4-Undecadien-1-al	$C_{11}H_{18}O$	Unsaturated aldehyde	Antioxidant (radical scavenger)	A(8.871), B(8.863), D(8.859)
5	2-monolinolenin	$C_{21}H_{36}O_4$	Glycerol ester of linolenic acid	Antioxidant, lipid mediator	A-D (11.011-11.83)
6	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	Oxidized linoleic acid	Antioxidant, anti-inflammatory	A(13.202), B(13.217)
7	Arachidonic acid	$C_{20}H_{32}O_2$	Polyunsaturated fatty acid	Antioxidant precursor,	A(14.22), B(14.206), D(14.216)

No	Compound	Molecular formula	Chemical group	Bioactivity	Sample RT [Min]
				signaling molecule	
8	Corchorifatty acid F	$C_{18}H_{32}O_5$	Polyhydroxy fatty acid	Antioxidant, anti-inflammatory	A(8.461), B(8.781), D(8.543)
9	Lupeol	$C_{30}H_{50}O$	Triterpenoid	Strong antioxidant, anti-inflammatory	A(18.915), B(18.901), D(18.886)
10	Pheophorbide A	$C_{35}H_{36}N_4O_5$	Chlorophyll derivative	Potent antioxidant, photosensitizer	C(14.753)
11	2-(3-Hydroxy-3,7,11,15-tetramethylhexadecyl)-3,5,6-trimethyl-1,4-benzoquinone	$C_{29}H_{50}O_3$	Quinone derivative	Antioxidant (vitamin E analog)	D(16.827)
12	3,4-Dihydroxybenzaldehyde	$C_7H_6O_3$	Phenolic compound	Phenolic antioxidant, radical scavenger	D(2.791)
13	3-Methoxy-5,7,3',4'-tetrahydroxyflavone	$C_{16}H_{12}O_7$	Flavonoid (flavone type)	Antioxidant, flavonoid marker	D(8.478)
14	4-Coumaric acid	$C_9H_8O_3$	Phenolic acid	Antioxidant, anti-inflammatory, UV absorber	D(7.329)
15	5-hydroxy-2,2,6,6-tetramethyl-4-{2-methyl-1-[2,4,6-trihydroxy-3-(2methylpropanoyl)phenyl] propyl}cyclohex-4-ene-1,3-dione	$C_{24}H_{32}O_7$	Polyphenolic derivative	Strong antioxidant, phenolic ketone	D(5.834)
16	2,6-Dimethoxy-4-allylphenol	$C_{11}H_{14}O_3$	Phenolic (methoxy-substituted)	Volatile phenolic compounds with antioxidant properties	D(7.789)
17	(2E)-3-(3,4-Dimethoxyphenyl) acrylic acid	$C_{11}H_{12}O_4$	Phenolic acid (ferulic acid analog)	Antioxidant, anti-inflammatory	D(7.648)

Principal Component Analysis (PCA) of Volatile Compounds

The connection between the sample groups (A, B, C, and D) was analyzed through Principal Component Analysis (PCA) regarding % area (GC–MS results), antioxidant activity, and total flavonoids and phenols (**Figure 1**). The first (F1) and second (F2) principal components together accounted for 89.50% of the total variance, with 61.08% and 28.42% attributed to the first and second components, respectively. This means that for the parameters analyzed, these two components fairly captured the total variability. The direction and proximity of the variable vectors in the biplot showed the degree of correlation between them. A strong positive correlation of antioxidant activity and flavonoid content was noted, suggesting that the samples with greater antioxidant activity had higher levels of flavonoids. This correlation is expected since flavonoids are strong free radical scavengers, donating free hydroxyl groups and stabilizing free radicals through resonance delocalization in their aromatic structures.

Phenolic content exhibited a negative correlation with both antioxidant activity and the value of flavonoids. This indicates that not every phenolic compound has the same value concerning an antioxidant. Some simple phenolics may have a lower redox potential and may not exert efficient radical scavenging action compared to flavonoid-type phenolics. The phenol and flavonoid negative association may also suggest the occurrence of a metabolic shift with respect to the biosynthesis of the flavonoids and other phenolic derivatives like hydroxycinnamic acids. With respect to the sample grouping, Group D was positioned closest to both the antioxidant and the flavonoid axes, meaning that it

held the greatest values for both axes and, consequently, the most bioactive profile. Group C was in close alignment with the phenol variable, meaning that it mainly contained phenolic compounds of relatively low antioxidant value. Group B was associated with a larger % area (GC–MS), denoting a higher abundance of the volatile or semi-volatile metabolites. This was contrasted by Group A, which had a smaller % area and lower activity overall. The PCA results demonstrated an antioxidant activity in *K. ceratophylla* Haw. In ethanolic leaf extracts, flavonoid content is the primary influence. This shows that the primary value of antioxidant extracts is determined by the qualitative composition and structure of the antioxidant compounds, not by their overall abundance.

Figure 2 shows the distribution of individuals within each group based on the parameters of antioxidants, flavonoids, phenols, and area. The variation explained by the first component (F1) is 52.98% and by the second component (F2) is 25.63%, so the total variation explained by both components is 78.61%. Samples A and C tend to have similar characteristics when compared to samples B and D, as seen from the proximity of the distribution of individuals within each sample.

The connection between total flavonoid content and the level of antioxidant activity shows that these compounds are the chief constituents of the radical scavenging ability of *K. ceratophylla* Haw. leaf extracts. Different flavonoids have several hydroxyl moieties that can donate hydrogen atoms or electrons to free radicals and disarm them. The conjugated ring structures of the radicals will then stabilize the radical to some extent by resonance. This is the reason sample D and other extracts with the most concentrated flavonoids are the most active in scavenging. Other works have also documented similar findings across many other plants(Tang et al., 2024; Dai & Mumper, 2010; Liu et al., 2021).

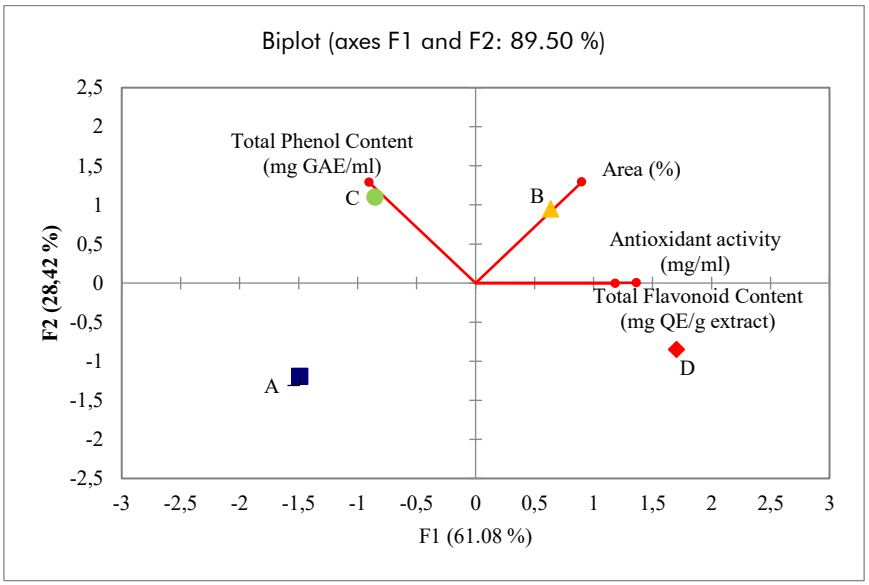

Figure 1. Biplot of PCA results for samples A, B, C, and D based on antioxidant, flavonoid, phenol, and % area parameters

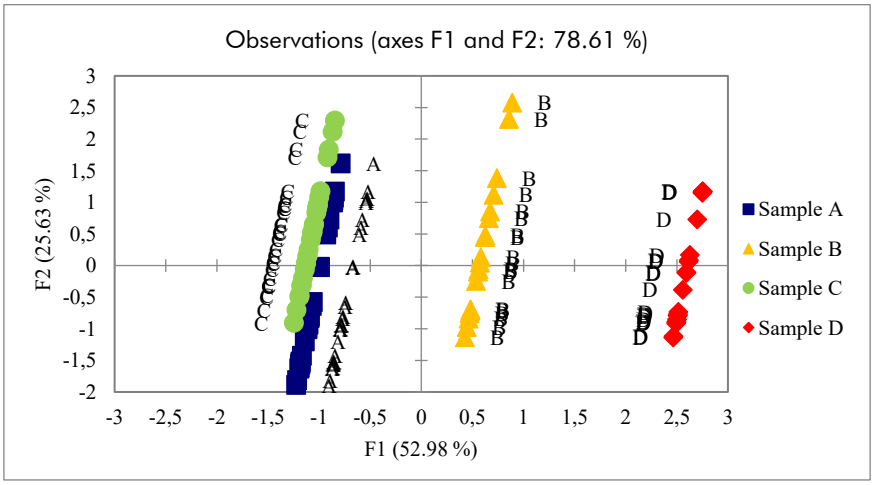

Figure 2. Distribution of individuals in each sample A, B, C, and D

A negative correlation was observed between total phenolic content and antioxidant activity, indicating that not all phenolic compounds have the same potential for radical scavenging. Since the Folin–Ciocalteu method determines the total phenolic content by measuring the total reducing capacity of the sample and not by determining the phenolic structures, a high total phenolic content may not always indicate the presence of compounds with high antioxidant activity. Moreover, certain phenolic acids at high concentrations may have antagonistic effects and even pro-oxidant effects, reducing the overall antioxidant activity (Wojdyło et al., 2007). This may explain what is observed in sample C. Although sample C had high phenolic content, its antioxidant activity was low.

In addition, the negative relationship between total phenolic content and total flavonoid content suggests that metabolites may compete for resources along the phenylpropanoid biosynthetic pathway, where precursors are allocated toward either phenolic acid or flavonoids, depending on the environment or extraction conditions (Cheynier, 2012). This selective enrichment may explain differences in solvent polarity and extraction efficiency. All in all, PCA results suggest that the antioxidant potential of *K. ceratophylla* Haw. extracts are predominantly determined by the composition of the flavonoids rather than the overall concentration of phenols, illustrating both biochemical and methodological components of the extraction and assay processes.

Principal Component Analysis (PCA) for non-Volatile Compounds

The relationship between variables A, B, C, and D is mapped based on the measured parameters, namely RT [min] (HR-MS), antioxidant activity, total flavonoids, and total phenols. Based on **Figure 3**, the variance explained by the first component (F1) is 55.34% and the second component (F2) is 31.49%, so the total variance explained by these two components is 86.82%. The relationship between the variables can be seen from the angle formed between them; the smaller the angle, the stronger the relationship between the variables. Conversely, if the angle formed between the variables approaches a right angle, the relationship becomes weaker or nonexistent.

The observed relationship, where antioxidant activities improve with increased levels of flavonoids, can be explained because flavonoids contain multiple hydroxyl groups, which means they can donate electrons and/or hydrogen atoms to free radicals and neutralize them. On the other hand, the faster rate of diminution of antioxidant activity with total phenols and the lower antioxidant activity with simple phenols suggests that some phenolic compounds lack sufficiently effective antioxidant properties, possibly due to weak structural elements and lower redox potentials. The lower presence of flavonoids and simple phenols could indicate a change in regulation in the phenylpropanoid pathway, such that the biosynthesis of flavonoids is inhibiting the accumulation of simple phenols. Group D had the highest level of flavonoids and antioxidants, Groups A and C were highest in phenolic components, and Group B showed the highest antioxidant activity, although they had a lower RT [min]. The results collectively suggest that in the case of *K. ceratophylla* Haw., the structural and compositional properties of the flavonoids primarily determine the antioxidant potential of the extracts, not the total phenolic content.

Figure 4 shows the distribution of individuals within each group based on antioxidant, flavonoid, phenol, and RT [min] parameters. The variation explained by the first component (F1) is 60.33%, and the second component (F2) is 25.02%, so the total variation explained by these two components is 85.35%. Groups A and C tend to have similar characteristics when compared to samples B and D, as seen from the proximity of the distribution of individuals within each sample.

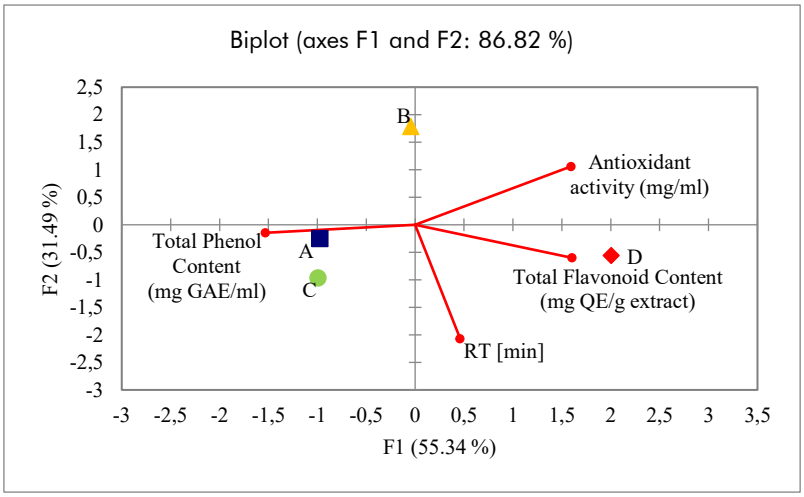

Figure 3. Biplot of PCA analysis results for samples A, B, C, and D based on antioxidant, flavonoid, phenol, and RT [min] parameters

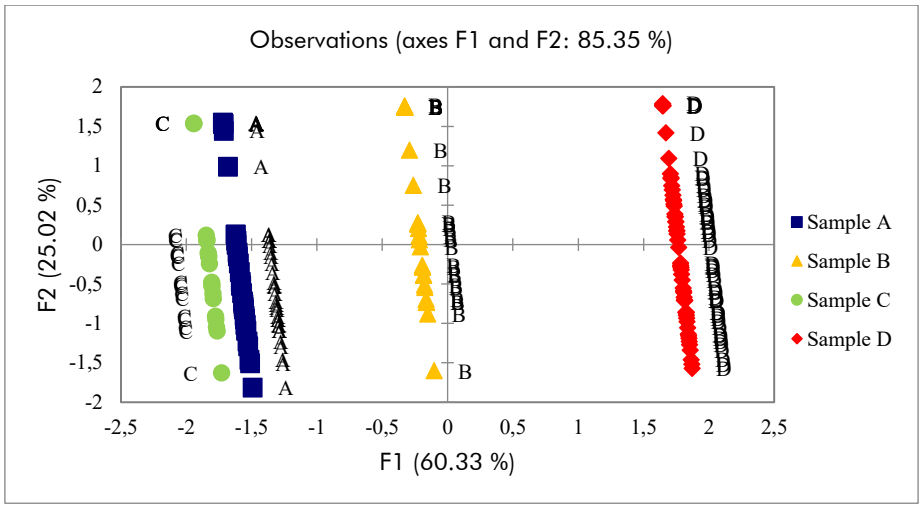

Figure 4. Distribution of individuals in each sample A, B, C, and D

The PCA results revealed a strong positive correlation between total flavonoid content and antioxidant activity, indicating that flavonoids play a dominant role in determining the radical-scavenging potential of *K. ceratophylla* Haw. leaf extract. This relationship is attributed to the structural characteristics of flavonoids, which contain multiple hydroxyl groups capable of donating hydrogen atoms or electrons and stabilizing free radicals through resonance. The presence of an ortho-dihydroxyl (catechol) group on the B-ring and the C2–C3 double bond conjugated with a 4-oxo function enhances the delocalization of electrons, making flavonoids particularly effective antioxidants (Dai & Mumper, 2010; Liu et al., 2021). Consequently, an extract with higher flavonoid concentrations, such as sample D, exhibited greater antioxidant capacity compared to other samples.

Conversely, a negative correlation was observed between total phenolic content and antioxidant activity, suggesting that not all phenolic compounds contribute equally to radical-scavenging activity. Certain phenolic compounds can exert pro-oxidant effects at high concentrations or in the presence of metal ions, thereby diminishing overall antioxidant activity (Wojdyło et al., 2007). This phenomenon explains why samples A and C, despite their high total phenolic contents, demonstrated lower antioxidant activities than sample D.

The negative association between total phenolic and flavonoid contents suggests a competitive relationship in the phenylpropanoid biosynthetic pathway, where metabolic flux may shift toward either phenolic acid or flavonoid synthesis depending on environmental and physiological factors (Cheynier, 2012). Furthermore, variations in solvent polarity and extraction efficiency could selectively enrich one compound group over another, resulting in differential compound distribution among samples. Collectively, these findings indicate that the antioxidant potential of *K. ceratophylla* Haw. The extract is primarily determined by the qualitative and quantitative composition of flavonoids rather than the overall phenolic content.

CONCLUSIONS

The results of this study demonstrate that the ethanol extract of *K. ceratophylla* Haw. leaves contain diverse bioactive compounds, including flavonoids, phenolics, and other antioxidant constituents identified through GC–MS and HR–MS analyses. Variations in solvent concentration and drying method significantly affect the total flavonoid and phenolic content as well as antioxidant activity. Among all treatments, an extract obtained through maceration with 95% ethanol followed by freeze-drying (sample D) produces the highest flavonoid content and antioxidant activity, whereas samples with higher phenolic content exhibit comparatively lower antioxidant potential. These findings indicate that *K. ceratophylla* Haw. leaves represent a promising natural source of antioxidant compounds with potential applications in the development of herbal formulations, nutraceuticals, or antioxidant-based pharmaceutical products. The metabolite profile identified in this study can serve as a reference for future research focusing on bioactivity testing, compound isolation, and formulation optimization to support the utilization of *Kalanchoe ceratophylla* Haw. in preventive and therapeutic medicine.

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