METABOLITE PROFILING OF PERSIMMON VINEGAR RESIDUE EXTRACT (*Diospyros kaki*) USING UPLC-MS METHOD

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ABSTRACT

Persimmon (*Diospyros kaki* L.) is known as a plant with potential use as an herbal remedy to help address various health problems and as a natural source of antioxidants. In the process of producing vinegar, persimmon residue is generally discarded as waste, even though it still contains various valuable metabolite compounds. Therefore, metabolite profiling analysis is needed to identify the chemical components present in the vinegar residue of *D. kaki*. This study aims to determine the metabolite profile of the 96% ethanol extract of *Diospyros kaki* (L.) vinegar residue using UPLC-QToF-MS/MS. The thick extract was prepared using 96% ethanol at a ratio of 1:10 (w/v) through the Ultrasound-Assisted Extraction (UAE) method. The extract was then prepared using methanol and DCM before 5 µL was injected into the UPLC-QToF-MS/MS for analysis with the MassLynx 4.1 and ChemSpider software. The analysis showed that the vinegar residue of *Diospyros kaki* (L.) contains 25 metabolite compounds. The three major compounds detected were Harmane (12.83%), 9-Methylbistetrazolo[1,5-a:5′,1′-c]quinoxaline (8.60%), and Andrographolide (6.28%).

Keywords*: Diospyros kaki, vinegar residue, metabolite profiling*

Introduction

Indonesia is known as a country rich in diverse flora and fauna, which are widely utilized by its people in various sectors such as clothing, food, and shelter. In addition, Indonesians frequently use these natural resources as alternative medicines to treat various diseases based on empirical experience and traditional remedies passed down through generations. Medicinal materials derived from flora and fauna are categorized as traditional medicines (Hakim et al., 2018). One widely cultivated plant is persimmon (*Diospyros kaki* L.) from the Ebenaceae family, which grows in several regions including East Asia, Spain, Israel, and Indonesia (Apriani et al., 2022). Persimmon fruit is known to possess anticancer potential due to its abundance of bioactive compounds such as flavonoids, tannins, and carotenoids, which act as antioxidants capable of neutralizing free radicals and preventing DNA damage that may trigger the transformation of normal cells into cancerous ones (Bianchini et al., 2024).

Fermentation originates from the Latin word fervere, meaning “to boil,” and is commonly used in microbiology to describe the process of producing a substance through the activity of microorganisms. These microorganisms convert solid and liquid materials into a variety of products. The substrates used may vary, but must support the growth of the microorganisms (Tarigan, 2017). Fermentation has long been applied to enhance the value of natural materials, both in terms of nutrition and bioactive compound content. However, the residue produced during fermentation is often overlooked and considered waste, despite still containing bioactive compounds with potential pharmacological benefits. Therefore, it is important to identify the compounds present in the vinegar residue of *Diospyros kaki* (L.) so that it may serve as a foundational reference for future studies.

Metabolite profiling is an analytical technique based on a metabolomic approach aimed at describing the profile of secondary metabolite compounds in plants (Fiehn et al*.,* 2000). Various methods can be used in metabolite profiling, one of which is Ultra Performance Liquid Chromatography–Quadrupole Time of Flight–Mass Spectrometry (UPLC-QToF-MS/MS).

In this study, metabolite profiling of the ethanol extract of *D. kaki* vinegar residue was conducted using UPLC-QToF-MS/MS. This technique is an advanced form of liquid chromatography that enables a more detailed analysis of plant metabolites. It offers several advantages over other analytical methods, such as the ability to detect both volatile and non-volatile compounds, determine complete molecular formulas, and provide high sensitivity and selectivity toward target compounds. Additionally, the method requires only a small sample volume and allows analysis within a relatively short time (Khansa et al*.,* 2023). The double MS system also provides more accurate monoisotopic mass measurements, high-resolution spectra for confirming target compounds and unknown compounds, and rapid data acquisition without compromising mass resolution (Zhang et al*.,* 2015). Based on these advantages, UPLC-QToF-MS/MS was employed to obtain a more comprehensive and accurate metabolite profile of *D. kaki* vinegar residue compared to previous studies, enabling its use as a database for future research on *D. kaki*.

Method

Tools and Materials

The plant used in this study was *D. kaki*, obtained from Malang Regency, East Java. The part of the plant used was the fruit. The fruit was washed thoroughly with running water, then drained and cut. It was then fermented spontaneously for 6 months. After the fermentation process was completed, the vinegar and its residue were separated by filtration. The obtained residue was then dried in an oven at 40°C for 3–5 days and ground into powder. The prepared powder was then analyzed for its moisture content using a Moisture Content Analyzer.

The chemicals used included 96% technical ethanol, methanol (hypergrade for UPLC), formic acid (ultrapure for UPLC), acetonitrile (hypergrade for UPLC), and 0.05% water injection for UPLC.

The research equipment used included an Ultrasonic Cleaner (Sonica), a Rotary Evaporator (Heidolph), and a UPLC-MS instrument with specifications listed in Table 1. The UPLC-MS analysis was conducted at Pusat Laboratorium Forensik, Badan Research Kriminal Polri, Sentul, Bogor, Indonesia.

Extraction

The simplicia powder was extracted using 96% ethanol with a ratio of 1:10 (w/v), assisted by ultrasonic waves for 30 minutes (stirred every 10 minutes). The extract was then filtered, and

the obtained filtrate was evaporated using a rotary evaporator. Once the extract began to thicken, it was transferred into a porcelain dish and dried in an oven until it reached a paste-like consistency. The extract was then weighed at approximately 10 mg, placed into an Eppendorf tube, and prepared for the next analysis stage.

Metabolite Profiling using UPLC-MS

A total of 10.00 mg of *D. kaki* vinegar residue extract was accurately weighed and dissolved in methanol to a final volume of 10 ml using a volumetric flask. From this solution, 5 µl was taken with a microsyringe and injected into the sample vial to be introduced into the UPLC column. Each sample was analyzed in four replicates. As the sample passed through the needle with positive ESI ionization (+), the liquid phase converted into aerosol droplets. The ions formed were then separated by the Q-ToF analyzer. The separation process used a mixed eluent of (A) water:formic acid (99.9:0.1) and (B) acetonitrile:formic acid (99.9:0.1) with a gradient elution system according to Table 2 and a flow rate of 0.2 ml/min. More polar compounds were detected first, followed by compounds with lower polarity. The separation results were then read by the QToF-MS detector and displayed as chromatographic peaks, which were subsequently interpreted using MassLynx software.

Table 1. UPLC-MS instrument spesification

Instrument	Spesification	Detail
LC-System	ACQUITY UPLC H-Class system (Waters, USA)	UPLC (Ultra Performance Liquid Chromatog raphy)
LC-Coloumn	ACQUITY UPLC BEH C18 (1.8 µm 2.1 x 50 mm; Waters, USA)	UPLC Column BEH (Ethylene Bridge Hybride)
Mass Spectrometer	Xevo G2-S QToF (Waters, USA)	Quadropole time-of-flight mass spectrometry

Table 2. The solvent ratio used in the gradient elution system

Time (Minute)	Solvent A (%)	Solvent B (%)
0,00	95,0	5,0
2,00	75,0	25,0
3,00	75,0	25,0
14,00	0,0	100,0
15,00	0,0	100,0
19,00	95,0	5,0
23,00	95,0	5,0

Results and Discussion

Extraction

The moisture content of the *Diospyros kaki* vinegar residue powder, as shown in Table 3, averaged 8.32%. The acceptable moisture limit to maintain the quality of simplicia is ≤10% (Kemenkes, 2017). This requirement is set because moisture levels above 10% can promote the growth of molds and bacteria. The moisture content of the persimmon vinegar residue powder falls within the appropriate range and meets the established standard. The water present in the sample can trigger the growth of molds or other microorganisms, and if the moisture level remains high, various enzymes within the cells may still degrade active compounds even after the cells have

died. Therefore, the drying temperature plays a crucial role in determining the quality and integrity of simplicia (Handoyo and Pranoto, 2020).

Table 3. Water content test results of persimmon vinegar residue powder

Sample	Replication	Moisture Content (%)	Mean (%)
Persimmon vinegar residue powder (*Diospyros kaki* L.)	1	8.40	8.32
	2	9.00	
	3	7.58	

The extraction of *Diospyros kaki* (L.) vinegar residue was carried out using 96% ethanol with a sample-to-solvent ratio of 1:10 (w/v), meaning that 30 g of powder was dissolved in 300 ml of ethanol. This ratio ensures an adequate amount of solvent so that bioactive compounds can dissolve efficiently, prevents solvent saturation, and enhances both the effectiveness and stability of the extracted product (Faradilla et al., 2024). Ethanol 96% was selected because it functions as a universal solvent capable of extracting both polar and nonpolar compounds. It was preferred over 70% ethanol due to its higher volatility, which allows the concentration process to occur at lower temperatures and in a shorter time (Kindangen et al., 2018).

The Ultrasonic Assisted Extraction (UAE) method was employed to extract active compounds from the *Diospyros kaki* vinegar residue. UAE utilizes ultrasonic waves that generate cavitation, causing cell structures to rupture more rapidly and enhancing diffusion, which significantly shortens the extraction duration (Firdausia et al., 2025). This technique offers advantages such as producing more concentrated extracts, yielding higher levels of active constituents, and providing better time efficiency due to increased cell wall permeability and accelerated release of bioactive compounds (Susiloningrum and Sari, 2023).

The ultrasonic extraction yielded a yellowish-brown liquid extract, which was subsequently filtered and evaporated using a rotary evaporator to reduce the solvent. The evaporator works by lowering the pressure so that the solvent vaporizes at a reduced temperature and condenses separately from the extract (Wardaniati and Gusmawarni, 2021). Evaporation was performed at 175 mbar, 40°C, and 70 rpm to ensure that ethanol evaporated without degrading the active compounds, as the temperature remained below its boiling point (Muiz et al., 2021). Once solvent droplets ceased and the extract thickened, the process continued with oven drying at 40°C to remove residual moisture, resulting in a paste-like extract that was then weighed to determine its yield.

Figure 1. The concentrated extract of persimmon vinegar residue

Table 4. Yield of the *Diospyros kaki* L. vinegar residue extract.

Simplicia Weight(g)	Extract Weight (g)	Yield (%)
30	3,1222	10,407

Based on Table 4, the yield of the *Diospyros kaki* vinegar residue extract was 10.407%. This value is considered good because it meets the minimum required yield for concentrated extracts, which is not less than 10%. This result also indicates that the extraction procedure for the *Diospyros kaki* vinegar residue was carried out effectively (Kemenkes, 2017).

Metabolite Profiling using UPLC-QToF-MS/MS

The metabolite profile of the persimmon vinegar residue extract was analyzed using UPLC-QToF-MS/MS, with sample preparation performed through Solid Phase Extraction (SPE). The SPE technique relies on interactions between the solid and liquid phases to separate compounds from complex matrices, thereby improving analytical efficiency (Dalimunthe, 2021). A total of 10 mg of extract was dissolved, loaded into the cartridge, where organic compounds were adsorbed, and subsequently eluted with methanol to obtain the filtrate.

Chromatographic separation was carried out using a C18 column, which is capable of separating compounds ranging from nonpolar to highly polar (Hakim et al*.,* 2018). The separation utilized water:formic acid and acetonitrile:formic acid eluents, along with a gradient elution system that accelerates compound separation and enhances analytical efficiency (Farag et al., 2016). More polar compounds eluted earlier, while less polar ones appeared later in the chromatogram (Hakim et al., 2018).

A 5 µL volume of the filtrate was injected into the UPLC-QToF-MS/MS system. The UPLC platform, equipped with small particle-sized columns, provides high resolution and rapid separation (Lestari et al*.,* 2024). The separated compounds were ionized using positive-mode ESI, forming $[M+H]^+$ ions (Pratama et al*.,* 2024), which were then analyzed in the quadrupole and TOF analyzers to obtain accurate mass values. During MS/MS analysis, precursor ions were fragmented to produce characteristic fragmentation patterns for each compound (Lei et al., 2023). The separation results appeared as chromatographic peaks, which were processed using MassLynx 4.1 software to identify the spectra corresponding to each peak. Figure 2 presents the chromatogram generated from the metabolite profiling of *Diospyros kaki* vinegar residue.

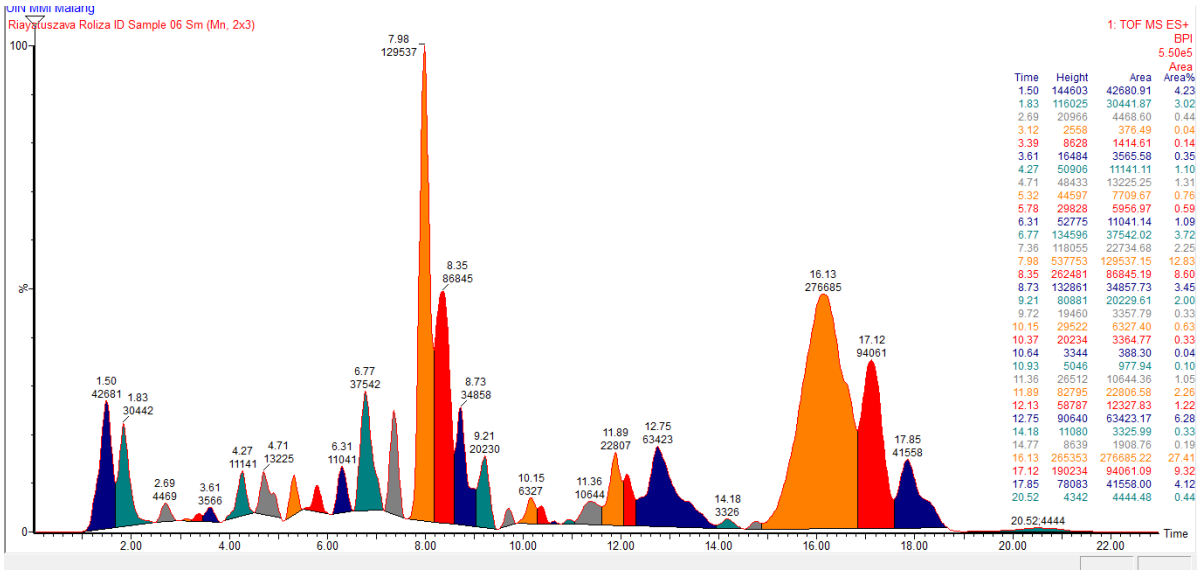

Figure 2. Chromatogram of the *Diospyros kaki* L. vinegar residue sample

The chromatogram was processed using MassLynx 4.1 to identify and predict the molecular formula of each peak by comparing the measured mass with the calculated mass, which had been adjusted by subtracting one hydrogen atom due to ESI (+) ionization. Molecular formulas were assigned based on the highest FitConf value, indicating the best match between theoretical and experimental isotope patterns. The selected formulas were then verified through ChemSpider after first removing one hydrogen atom, and the identified compounds were assigned IDs according to the number of supporting publications. The identification results are summarized in Table 5.

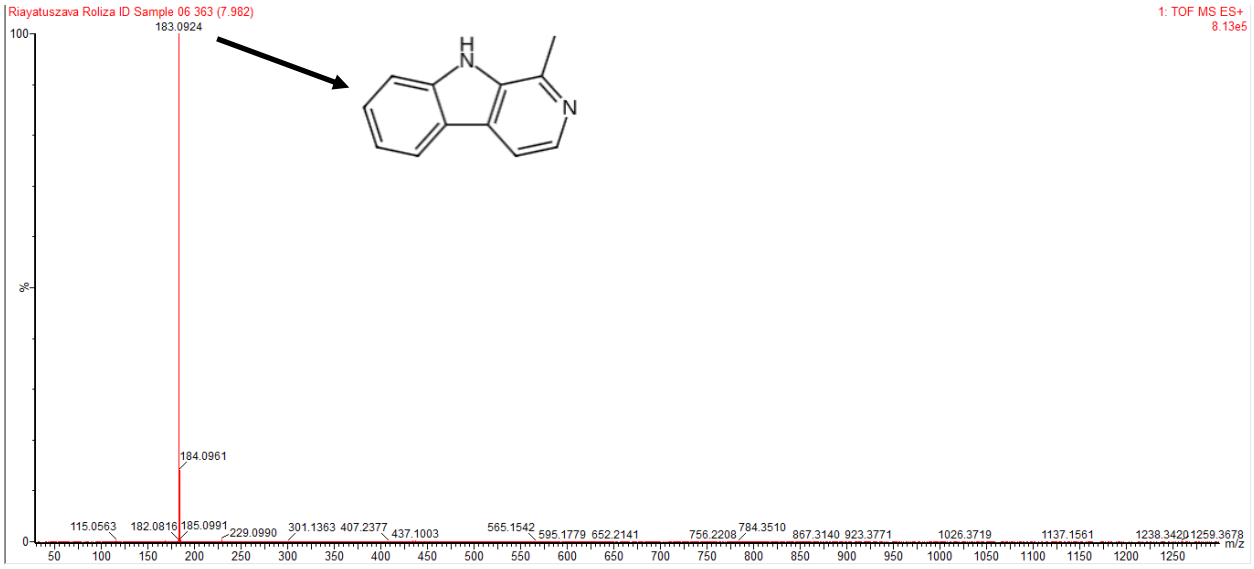

Figure 3. Mass spectra and chemical structures of the major compounds in *D. kaki* vinegar residue

For example, a peak detected at a retention time (RT) of 7.98 min exhibited an observed m/z value of 183.0926 in ESI (+) mode. The predicted molecular formula was C12H10N2, corresponding to harmane, with a calculated exact mass of 183.0922. The mass error was determined using the following equation:

$$\text{Mass error (ppm)} = \frac{183.0926 - 183.0922}{183.0922} \times 10^6$$

yielding a value of 2.18 ppm. The low mass error (<5 ppm) indicated excellent agreement between the measured and theoretical masses (Mokhtar et al., 2020). In addition, the isotope distribution pattern showed a high FitConf value, supporting the proposed molecular formula assignment. Verification through ChemSpider further confirmed the identity of the compound as harmane.

Table 5. Metabolite profile of *Diospyros kaki* L. vinegar residue

No	RT	%Area	*Measured Mass*	*Calculated Mass*	Molecular Formula	Compound Name
1	1,50	4,23	381,0800	381,0822	C17H16O10	CHEMBL479499
2	1,83	3,02	189,0769	189,0736	C4H8N6O3	bis(carbamoylamino)methylidene urea
3	2,69	0,44	180,0671	180,0634	C5H5N7O	8-nitroso-[1,2,4]triazolo[1,5-c]pyrimidine-5,7-diamine
4	3,12	0,04	230,1029	230,1028	C10H15NO5	1,2-Pyrrolidinedicarboxylic acid, 5-oxo-, 1-(1,1-dimethylethyl) ester, (2S)-
5	3,39	0,14	231,0877	231,0869	C10H14O6	Methacryloyloxyethyl succinate
6	3,61	0,35	294,1556	294,1553	C12H23NO7	N-Fructosyl isoleucine
7	4,27	1,10	358,1718	358,1713	C13H27NO10	CHEMBL1277063
8	4,71	1,31	158,0826	158,0790	C3H7N7O	2-(5-Amino-1H-tetrazol-1-yl)acetohydrazide
9	5,32	0,76	195,1140	195,1093	C5H14N4O4	2-Ethyl-2-{[ethyl(nitro)amino]methyl}-1-hydroxyhydrazine 1-oxide
10	5,78	0,59	158,0824	158,0790	C3H7N7O	2-(5-aminotetrazol-1-yl)acetohydrazide
11	6,31	1,09	210,1138	210,1103	C7H11N7O	2-[5-(1H-Tetrazol-1-ylmethyl)-1,3,4-oxadiazol-2-yl]-2-propanamine
12	6,77	3,72	231,1143	231,1107	C9H10N8	5-Amino-1-(6-hydrazino-3-pyridazinyl)-3-methyl-1H-pyrazole-4-carbonitrile
13	7,36	2,25	229,0992	229,0995	C12H17O2Cl	Fenpentadiol
14	7,98	12,83	183,0926	183,0922	C12H10N2	Harmane
15	8,35	8,60	227,0829	227,0794	C9H6N8	9-Methylbistetrazolo[1,5-a:5′,1′-c]quinoxaline
16	8,73	3,45	211,1456	211,1420	C7H14N8	N-[3-(4H-1,2,4-Triazol-3-yl)propyl]imidodicarbonimidic diamide
17	10,15	0,63	273,1137	273,1186	C9H20O9	1,2,3-Propanetriol - β-D-allopyranose (1:1)
18	10,37	0,33	273,1135	273,1127	C16H16O4	p-Anisoin
19	10,93	0,10	383,2048	383,2070	C20H30O7	T-2 triol
20	11,36	1,05	367,2102	367,2121	C20H30O6	Dibutoxyethylphthalate
21	11,89	2,26	181,1234	181,1202	C7H12N6	6-(1-Pyrrolidinyl)-1,3,5-triazine-2,4-diamine

22	12,75	6,28	351,2148	351,2171	C20H30O5	Andrographolide
23	14,18	0,33	379,2452	379,2484	C22H34O5	1,9-dideoxyforskolin
24	14,77	0,19	316,2847	316,2825	C14H33N7O	N-{4-[(4-Aminobutyl)amino]butyl}-N~5~-(diaminomethylene)-L-ornithinamide
25	20,52	0,44	203,0541	203,0556	C8H10O6	cis-Dihomoaconitic acid

Based on the interpreted UPLC-QToF MS data, a total of 25 compounds were identified in the persimmon vinegar residue extract. The interpretation also revealed the presence of several major constituents, defined as compounds with higher area percentages than the others (Hakim et al., 2018). The major metabolites detected in the *D. kaki* vinegar residue include:

1. Harmane, with an area percentage of 12.83%;
2. 9-Methylbistetrazolo[1,5-a:5′,1′-c]quinoxaline (ChemSpider ID: 45087548), with an area percentage of 8.60%; and
3. Andrographolide, with an area percentage of 6.28%.

The metabolite profiling analysis revealed that the detected compounds were predominantly represented by nitrogen-containing heterocyclic compounds and terpenoid derivatives. Harmane, which exhibited the highest relative abundance with an area percentage of 12.83%, was classified as a β-carboline alkaloid. This class of compounds has been widely reported to possess various biological activities, particularly antimicrobial and antioxidant properties (Nikam et al., 2025). Harmane showed a relatively high abundance in the fermentation sediment, suggesting that this compound may be generated during fermentation and subsequently accumulated in the residue rather than being released into the fermented liquid.

The second major compound, 9-methylbistetrazolo[1,5-a:5′,1′-c]quinoxaline (ChemSpider ID: 45087548), with an area percentage of 8.60%, belongs to the quinoxaline-derived heterocyclic compounds characterized by a high nitrogen content. In addition, andrographolide, accounting for 6.28% of the total peak area, was identified as a diterpenoid lactone compound known for its broad pharmacological activities, including antimicrobial, anti-inflammatory, and antioxidant effects (Patil and Jail, 2021).

Conclusion

The metabolite profile of *Diospyros kaki* (L.) vinegar residue showed moisture and yield values of 8.32% and 10.407%, respectively. UPLC-QToF-MS/MS analysis revealed the presence of 25 identified compounds. Among these, the major compounds were harmane, 9-methylbistetrazolo[1,5-a:5′,1′-c]quinoxaline (ChemSpider ID: 45087548), and andrographolide, with relative peak area percentages of 12.83%, 8.60%, and 6.28%, respectively.

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