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Original Article

Purity and chemical marker profiling of *Ocimum basilicum* L. across Malaysian cultivation systems for quality specification

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ABSTRACT

Ocimum basilicum L. is prioritised under the Malaysian Traditional and Complementary Medicine (T&CM) Blueprint 2018-2027 for development into high-value healthcare products. Although it has been included in the Malaysian Herbal Monograph (MHM), the absence of quality specifications that account for local agronomic variations creates a significant regulatory gap in the development of the Malaysian Herbal Pharmacopoeia (MHP). This study established comprehensive quality standards by evaluating pharmacognostic purity and validating an HPLC-UV method for 3,4-dihydroxybenzoic acid, vicenin-2, and rosmarinic acid. By assessing the impact of hydroponics, indoor vertical farming, and soil fertigation, the research found that while all samples maintained high hygiene, the total ash content in ML-GH (17.14%) and PC-CH (15.41%) exceeded the MHM limit of 15.0%. As evidenced by the low acid-insoluble ash, this difference was not caused by contamination but by the physiological luxury consumption of minerals in nutrient-rich systems. Chemometric profiling showed that the indoor vertical farming UG-KL sample had higher levels of vicenin-2 (0.95 ± 0.02 mg/g DW) and rosmarinic acid (3.65 ± 0.06 mg/g DW), mainly driven by osmotic stress and LED spectral optimisation. In contrast, the highest concentrations of 3,4-dihydroxybenzoic acid (0.47 ± 0.01 mg/g DW) were found in the high-altitude planted ML-GH sample, indicating a potential role in UV-B photoprotection. The results support validation of the HPLC approach as a reliable tool for routine quality monitoring and the updating of pharmacopoeia ash limits to account for hydroponic growing conditions.

Keywords: *Ocimum basilicum* L, Malaysia herbal pharmacopoeia, Purity test, High-performance liquid chromatography (HPLC), Cultivation systems, Phytochemical profiling

Introduction

The global integration of herbal medicine into national healthcare systems requires rigorous standardization to overcome variations caused by genetic, environmental, and agricultural factors, ensuring consistent batch-to-batch efficacy and safety [1]. The World Health Organization (WHO) has identified the lack of standardization as a primary barrier to the integration of traditional medicine into national healthcare systems [2]. While the World Health Organization (WHO) outlines baseline global standards for botanical purity, robust localized regulatory frameworks are essential [3]. Crucially, the WHO guidelines emphasize the stability of active ingredients and the absence of harmful impurities like foreign matter or heavy metals [4]. Accordingly, Malaysia's Traditional and Complementary Medicine (T&CM) Blueprint 2018-2027 initiated the development of the legally binding Malaysian Herbal Pharmacopoeia (MHP) to enforce stricter product registration standards and the Malaysian Herbal Monograph (MHM) that provides comprehensive raw material specifications [5-7].

Ocimum basilicum L. (sweet basil), a medicinally valuable herb of the Lamiaceae family, is traditionally used across many cultures to treat respiratory, digestive, and skin conditions [8, 9]. Its

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therapeutic efficacy is primarily driven by volatile essential oils and non-volatile phenolic compounds, specifically rosmarinic acid, 3,4-dihydroxybenzoic acid, and vicenin-2, that serve as stable chemical markers for quality control [10-12].

Although *O. basilicum* is formally recognized in the MHM, the existing framework lacks the granularity to address site-specific agronomic variables. Current thresholds rely on international datasets that fail to account for Malaysia's intense ultraviolet exposure, high humidity, or the unique mineral accumulation observed in modern hydroponic cultivation [13-15].

Consequently, the primary objective of this study is to establish localized physicochemical and chromatographic quality specifications for *O. basilicum*. By evaluating key pharmacopoeial purity constants and validating a high-performance liquid chromatography (HPLC) chemical profile across three distinct cultivation systems, this study assesses the impact of modern agronomic factors on phytochemical consistency. Ultimately, these findings will serve as an empirical basis to update existing monograph limits and support MHP development, bridging the gap between modern agricultural practices and pharmaceutical quality control [16-19].

Materials and Methods

Plant materials and powder preparation

O. basilicum leaves were sourced from three commercial plantations in Malaysia. The ML-GH sample was collected from Monoluxury Sdn. Bhd. (Genting Highlands, Pahang), which utilizes industrial hydroponics [20]. The UG-KL sample was obtained from UrbanGrow (Bandar Sri Damansara, Kuala Lumpur), utilizing indoor vertical farming [21]. The PC-CH sample was sourced from Pun Chou Sdn. Bhd. (Cameron Highlands, Pahang), employing protected soil-based cultivation [22]. A voucher specimen (ID193/2026) was deposited at the Herbarium Universiti Kebangsaan Malaysia (UKMB). Leaves were oven-dried at 50 °C for 24 hours, pulverized, and sieved (355 µm mesh) to obtain a moderately fine powder (355/180) [3].

Chemicals and equipment

Analytical-grade 2N HCl, 95% ethanol, 0.1% formic acid, and HPLC-grade reagents methanol (ChemFaces) and acetonitrile (Fisher) were utilized. Reference standards included 3,4-dihydroxybenzoic acid (CAS 99-50-3, ≥ 98%), vicenin-2 (CAS 22666-13-9, 98%), and rosmarinic acid (CAS 20283-92-5, ≥ 98%). HPLC analysis was performed on a Waters Arc™ system (Quaternary Solvent Manager-R, Sample Manager FTN-R, 2489 UV/Vis detector) equipped with a Phenomenex Luna C18 column (5 µm, 250 × 4.6 mm). Evaporation was conducted using a Büchi Rotavapor R-II with V-100 vacuum pump, I-100 interface, B-491 heating bath and Julabo Vivo RT4 chiller. Other equipment included a Waring blender, Carbolite AAF 1100 muffle furnace, Favorit HS0707V2 hotplate stirrer, Memmert oven and water bath, Shimadzu ATX224R balance, IKA KS 501 orbital shaker, Whatman HMFT heating mantle, Hettich Rotofix 32 centrifuge, Branson 3510 ultrasonic cleaner, ELGA PURELAB Quest water system, VELP ZX3 vortex mixer, and Sun-Sri Titan nylon filters (0.45 µm, 47 mm).

Purity tests

All purity tests were conducted in triplicate according to the standard operating procedure for preparing Malaysian herbal monographs. Foreign matter was determined via visual macro-inspection of 250 g fresh and 5 g powdered samples [23]. Total ash was determined by incinerating 4.0 g of powder at 600 °C until a constant weight (difference < 0.5 mg) was achieved. The acid-insoluble ash was quantified by boiling the total ash with 25 mL of 2 M HCl for 5 minutes, then filtering using ashless filter paper and washing the insoluble residue until neutral, lastly re-igniting the residue at 600 °C [23]. Loss on drying was assessed by heating 2.0 g of powder at 100 °C to a constant weight (difference < 5 mg) [23].

For extractive values, water-soluble and ethanol-soluble (95% v/v) contents were determined using both hot and cold methods. The hot method involved macerating 2.0 g of powder in 50 mL of solvent for 1 hour, followed by refluxing for 1 hour, after which 12.5 mL of the filtrate was evaporated at 60 °C and heated at 100 °C for 6 hours. Conversely, the cold extraction method involved agitating 2.0 g of powder in 50 mL of solvent on an orbital shaker for 6 hours, followed by an 18-hour standing period before subjecting the filtrate to identical drying steps [23]. All purity tests were expressed as the mean percentage and relative standard deviation (RSD) to evaluate the consistency and quality of the raw materials across different cultivation regions.

Sample and standard solutions preparation

Sample extracts were prepared via ultrasound-assisted extraction (UAE) by suspending 1.0 g of powder in 40 mL of 60% (v/v) ethanol at 25 °C for 40 minutes. After centrifugation (3000 rpm, 20 minutes), the supernatant was filtered, evaporated under reduced pressure, and weighed. The dried extract was reconstituted in 80% (v/v) methanol to a standardized concentration of 10 mg/mL. Final HPLC results were expressed as milligrams per gram of the original dried plant weight (mg/g DW). Standard stock solutions (1 mg/mL) of 3,4-dihydroxybenzoic acid, vicenin-2, and rosmarinic acid were prepared in HPLC-grade methanol. All solutions were filtered through 0.45 µm nylon membranes and stored at 4 °C until analysis.

Chromatographic conditions

Chromatographic separation was performed on a Phenomenex Luna C18 column. The mobile phase consisted of 0.1% (v/v) aqueous formic acid (A) and acetonitrile (B) at a flow rate of 1.0 mL/min. The gradient elution program was as follows: 90% A at 0 min, decreasing to 85% A at 15 min, 80% A at 25 min, 70%

A at 30 min, and 50% A at 42 min, before returning to the initial conditions of 90% A at 45 min. The column and sample temperature were 25 °C, and the injection volume was 10 µL. Detection wavelengths were 272 nm (3,4-dihydroxybenzoic acid, vicenin-2) and 320 nm (rosmarinic acid), with peak areas expressed in mAU·s.

Method validation and system suitability

The HPLC method was validated according to ICH Q2(R2) guidelines [24]. Specificity was confirmed by comparing retention times between standard solutions and sample solutions to ensure the absence of peak interference. Linearity (R^2) was established using five-point calibration curves: 1.25, 2.5, 5.0, 10.0, and 20.0 µg/mL for 3,4-dihydroxybenzoic acid; 2.5, 5.0, 10.0, 20.0, and 40.0 µg/mL for vicenin-2; and 12.5, 25.0, 50.0, 100.0, and 200.0 µg/mL for rosmarinic acid. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated from the standard deviation of the response (σ) and slope (S) of the calibration curves. LOQ was further verified as the lowest concentration with acceptable repeatability (RSD ≤ 2.0%), and LOD as approximately one-third of the LOQ. Accuracy was evaluated via standard addition recovery studies by spiking samples with 100 µg/mL standards, followed by a 10-fold dilution. Precision was assessed through repeatability (six injections of a 100 µg/mL mixed standard on the same day) and intermediate precision (five concentrations in triplicate over three consecutive days).

System suitability testing (SST) was evaluated from six injections by manually calculating the theoretical plate number (N), capacity factor (k'), and tailing factor (Tf) from printed chromatograms, necessitated by software limitations [24].

Results and Discussion

Geographical and agricultural context

To properly evaluate the pharmacognostic purity and chemical profile of the local *Ocimum basilicum* L. samples, their distinct cultivation backgrounds must be considered. The ML-GH sample was cultivated in the Genting Highlands using advanced, soil-free hydroponic systems in controlled-environment greenhouses, designed for rapid vegetative growth [20]. The UG-KL sample was sourced from an indoor vertical farm in Kuala Lumpur, utilizing optimized LED lighting and inert media within a fully enclosed environment [21]. Conversely, the PC-CH sample was grown in the Cameron Highlands using traditional protected soil-based cultivation (polybag fertigation) under rain shelters, exposing the plants to the region's natural temperate microclimate and soil microbiome [22].

Foreign matter

The determination of foreign matter is the primary test to assess hygiene, purity, and adherence to Good Agricultural Practices (GAP) and Good Collection Practices (GCP). All three *O. basilicum* samples were entirely free of macroscopic contaminants, strictly complying with the MHM and Codex Alimentarius standards as shown in **Table 1** [10, 25]. For the ML-GH and UG-KL samples, the absence of foreign matter is directly attributed to the Controlled Environment Agriculture (CEA) techniques used. By eliminating soil, these enclosed hydroponic and vertical farming systems remove the primary vectors for mineral and biological contamination. Achieving 0.00% foreign matter in the soil-grown PC-CH sample indicates highly effective post-harvest handling, reflecting the strict manual sorting and hygiene standards expected of facilities registered under MyFood Tag certifications [26]. The consistent achievement of zero foreign matter across varying Malaysian agricultural systems indicates that local producers surpass international minimums, which is crucial for preventing the introduction of microbial contaminants and mycotoxins.

Ash content

Total ash represents the physiological and non-physiological inorganic residue remaining after the complete thermal oxidation. As shown in **Table 1**, the ML-GH and PC-CH samples exceeded the MHM total ash limit, while the UG-KL sample marginally complied with the MHM threshold [10, 25]. This excessively high ash content is likely due to physiological mineral accumulation rather than environmental contamination. Hydroponic systems (ML-GH) utilize nutrient solutions rich in nitrogen, phosphorus, potassium, calcium, and magnesium. Plants grown in these systems often exhibit luxury consumption, a physiological response wherein nutrient uptake exceeds metabolic requirements when minerals are highly accessible in the rhizosphere [27]. Similarly, the continuous fertigation utilized in the soil-based PC-CH system facilitates elevated mineral accumulation.

This physiological hypothesis is proven by the acid-insoluble ash, which selectively measures siliceous contaminants like soil and sand. As shown in **Table 1**, all samples yielded exceptionally low acid-insoluble ash values, which fell well below regulatory limits [10, 25]. Counterintuitively, the soil-grown PC-CH sample exhibited the lowest acid-insoluble ash, demonstrating the effectiveness of agricultural rain shelters and rigorous washing. Conversely, the highest value observed in the indoor UG-KL sample (0.68 ± 0.01%) reflects the use of silicate-based substrates (e.g., perlite) and potassium silicate hydroponic supplements intended to strengthen plant cell walls [28]. Because the acid-insoluble ash is negligible, the high total ash represents a cultivation signature of nutrient-dense crops. This suggests that pharmacopoeial limits, traditionally based on field-grown plants in nutrient-limiting environments, may need to be broadened to accommodate the mineral profiles of modern high-tech agriculture. The precision of these assessments was generally robust (RSD < 2.0%), though ML-GH recorded an RSD of 3.03% for acid-insoluble ash due to the heterogeneous nature of extrinsic silica particles within the plant matrix.

Loss on drying

Loss on drying measures the moisture and volatile matter content, which predicts post-harvest stability and essential oil retention. As shown in **Table 1**, all samples complied with the MHM and Codex limits [10, 25]. The results demonstrate a clear correlation between the cultivation environment and final moisture content. The PC-CH sample, grown in the cool and highly humid Cameron Highlands, yielded moisture content near the upper limit, which poses a slight stability risk by potentially facilitating enzymatic chlorophyll degradation (browning) or xerophilic mold growth [29]. In contrast, the ML-GH sample achieved a very low drying loss due to controlled greenhouse drying tunnels. While excellent for microbial stability, the aggressive drying necessary to drastically reduce moisture content risks volatilizing therapeutic essential oils, like linalool and eugenol, thereby compromising the herb's aromatic quality. The UG-KL sample represents an optimal middle ground, ensuring stability while suggesting a gentler drying process that likely preserves delicate volatile constituents.

Extractive values

Extractive values quantify extractable constituents, serving as an index of drug potency. As shown in **Table 1**, all samples met the MHM extractive value limits, except for the PC-CH sample in water-soluble hot extraction. In general, water-soluble yields significantly exceeded ethanol yields, indicating *O. basilicum* biomass is dominated by polar metabolites like carbohydrates, mucilage, and rosmarinic acid [30]. In the hot extraction method, the hydroponically cultivated UG-KL and ML-GH samples consistently produced higher water-soluble values than the soil-grown PC-CH sample. This aligns with agricultural literature suggesting that hydroponic systems provide optimal nitrogen and water availability, promoting the accumulation of soluble solids in large vacuoles, whereas soil plants exposed to environmental stress tend to develop more insoluble structural fibers [31].

For ethanol-soluble values, soil-grown PC-CH exhibited a significant thermal inversion, where the hot extraction value was lower than the cold extraction value. This suggests thermolabile, volatile essential oils were lost during boiling reflux. Soil cultivation induces environmental stresses that stimulate higher secondary volatile metabolite production compared to protected hydroponic environments [31]. Therefore, while water-soluble extraction is the best indicator of physiological yield, cold maceration extraction is the gold standard for accurately assessing the therapeutic potency of volatile-rich herbs to prevent the underestimation of product quality.

Table 1. Pharmacognostic purity and extractive values of *O. basilicum* samples across different cultivation systems compared to regulatory standards.

Test	Sample	Value (%)	RSD (%)	MHM limit	Codex limit	Compliance
Foreign matter	ML-GH	0.00	0.00	Not more than 2.0%	Not more than 0.1 % for powder	Compliant
	UG-KL	0.00	0.00			Compliant
	PC-CH	0.00	0.00			Compliant
Total ash	ML-GH	17.14 ± 0.13	0.75	Not more than 15.0%	Not more than 16.0%	Non-compliant
	UG-KL	14.60 ± 0.22	1.53			Compliant
	PC-CH	15.41 ± 0.07	0.44			Non-compliant (MHM)
Acid-insoluble ash	ML-GH	0.51 ± 0.02	3.03	Not more than 1.0%	Not more than 2.0%	Compliant
	UG-KL	0.68 ± 0.01	1.52			Compliant
	PC-CH	0.21 ± 0.00	1.84			Compliant
Loss on drying	ML-GH	5.80 ± 0.11	1.85	Not more than 10.0%	Not more than 10.0%	Compliant
	UG-KL	7.38 ± 0.11	1.50			Compliant
	PC-CH	9.95 ± 0.18	1.76			Compliant
Water-soluble extractive value (cold extraction method)	ML-GH	22.14 ± 0.44	1.99	Not less than 20.0%	-	Compliant
	UG-KL	25.55 ± 0.19	0.74			Compliant
	PC-CH	21.07 ± 0.38	1.78			Compliant
Water-soluble extractive value (hot extraction method)	ML-GH	29.91 ± 0.56	1.87	Not less than 27.0%	-	Compliant
	UG-KL	29.92 ± 0.45	1.52			Compliant
	PC-CH	26.96 ± 0.11	0.42			Non-compliant
Ethanol-soluble extractive value (cold extraction method)	ML-GH	10.20 ± 0.19	1.86	Not less than 5.0%	-	Compliant
	UG-KL	8.11 ± 0.15	1.79			Compliant
	PC-CH	12.01 ± 0.20	1.70			Compliant
Ethanol-soluble extractive value (hot extraction method)	ML-GH	14.20 ± 0.18	1.29	Not less than 11.0%	-	Compliant
	UG-KL	16.88 ± 0.19	1.11			Compliant
	PC-CH	11.94 ± 0.11	0.88			Compliant

Chemical markers

To evaluate the therapeutic potential and quality of *O. basilicum*, rosmarinic acid, vicenin-2, and 3,4-dihydroxybenzoic acid were selected as chemical markers. Rosmarinic acid is the most abundant phenolic compound that serves as the primary reference for antioxidant capacity and a chemotaxonomic marker for the Lamiaceae family [8, 10]. Its high concentration is critical

for therapeutic efficacy, providing potent antiviral and anti-inflammatory effects via inhibition of the cyclooxygenase (COX) and lipoxygenase pathways [9]. Vicenin-2 is a hydrolysis-resistant flavonoid C-glycoside that persists through extraction to offer multi-target therapeutic utility [32]. Pharmacologically, it shows potential in managing diabetes by inhibiting PTP1B and RLAR, and demonstrates synergistic anti-cancer properties by suppressing prostate cancer proliferation via the EGFR/Akt/mTOR pathway [33, 34]. Finally, 3,4-dihydroxybenzoic acid (protocatechuic acid) is a highly bioavailable phenolic acid that exerts neuroprotective and anti-atherogenic effects by scavenging ROS and inhibiting monocyte adhesion [35]. Ecologically, its presence indicates an adaptive, photoprotective metabolic response to environmental stressors, such as UV-B radiation [36].

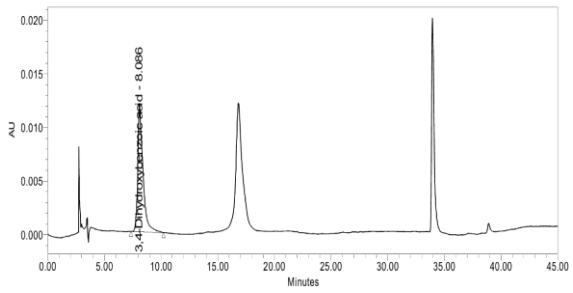
Identification of O. basilicum

To ensure consistent therapeutic efficacy, chromatographic results in **Figures 1-3** demonstrated precise retention time (t_R) alignment between sample matrices and reference standards. Specifically, mean tR values for 3,4-dihydroxybenzoic acid (8.12–8.19 min), vicenin-2 (16.51–16.56 min), and rosmarinic acid (34.03–34.04 min) showed excellent agreement with an RSD < 2.0%, as shown in **Table 2**. A spiking method further validated marker identity, eliminating co-eluting matrix interference by yielding proportional peak area enhancement without splitting or shoulder formation. To maintain the analytical range, spiked samples were diluted 10-fold, resulting in lower AUC values than the unspiked samples [37-40].

This specificity satisfies ICH Q2(R2) criteria [24]. Crucially, despite quantitative fluctuations driven by environmental conditions, the qualitative presence of these three markers remained consistent across all samples. This multi-component validation proves the HPLC method is a reliable tool for *O. basilicum* authentication regardless of agricultural origin.

Table 2. Specificity results showing mean retention times and peak areas for mixed standard solutions, and unspiked and spiked *O. basilicum* samples.

Samples		3,4-dihydroxybenzoic acid		Vicenin-2		Rosmarinic acid	
		AUC (mAU·s)	t_R (min)	AUC (mAU·s)	t_R (min)	AUC (mAU·s)	t_R (min)
Standard	**Mean ± SD**	380,238.00 ± 5,528.33	8.08 ± 0.01	514,322.83 ± 8,788.47	16.79 ± 0.04	801,220.33 ± 5,913.58	33.91 ± 0.03
	RSD (%)	1.45	0.15	1.71	0.25	0.74	0.09
Unspiked ML-GH	**Mean ± SD**	76,054.67 ± 1,252.34	8.19 ± 0.08	68,614.33 ± 823.11	16.52 ± 0.02	113,664.00 ± 821.98	34.03 ± 0.01
	RSD (%)	1.65	1.00	1.20	0.13	0.72	0.02
Unspiked UG-KL	**Mean ± SD**	27,536.33 ± 425.06	8.12 ± 0.06	142,447.00 ± 2,523.00	16.51 ± 0.11	1,381,289.33 ± 23,291.36	34.04 ± 0.01
	RSD (%)	1.54	0.75	1.77	0.65	1.69	0.03
Unspiked PC-CH	**Mean ± SD**	33,661.33 ± 359.49	8.12 ± 0.00	54,493.67 ± 697.38	16.56 ± 0.01	893,544.00 ± 11,310.58	34.03 ± 0.01
	RSD (%)	1.07	0.04	1.28	0.04	1.27	0.02
Spiked ML-GH	**Mean ± SD**	44,115.00 ± 469.54	8.11 ± 0.02	40,026.33 ± 603.91	16.51 ± 0.02	88,776.00 ± 1,026.38	34.01 ± 0.01
	RSD (%)	1.06	0.26	1.51	0.12	1.16	0.02
Spiked UG-KL	**Mean ± SD**	38,172.67 ± 192.68	8.11 ± 0.02	45,665.00 ± 569.89	16.53 ± 0.02	219,309.67 ± 1,795.04	34.03 ± 0.01
	RSD (%)	0.51	0.20	1.25	0.09	0.82	0.02
Spiked PC-CH	**Mean ± SD**	38,533.33 ± 367.69	8.1 ± 0.01	36,060.67 ± 580.90	16.55 ± 0.01	170,303.33 ± 2,581.51	34.02 ± 0.00
	RSD (%)	0.95	0.11	1.61	0.07	1.52	0.01



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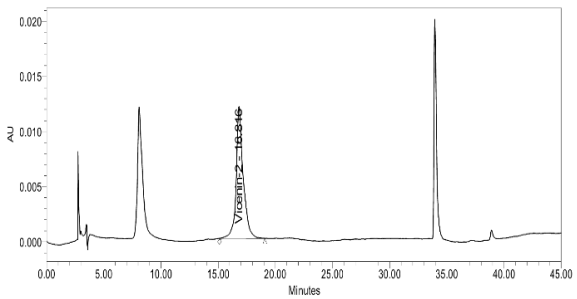

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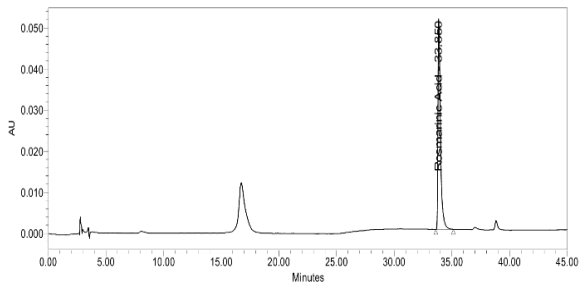
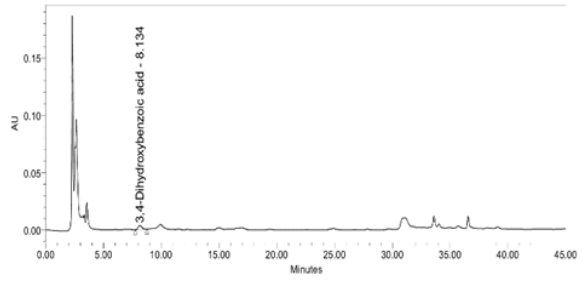


c)

Figure 1. Representative HPLC chromatograms of the mixed standard solution (100 µg/mL) showing (a) 3,4-dihydroxybenzoic acid (t_R = 8.086 min) and (b) vicenin-2 (t_R = 16.816 min) detected at 272 nm, and (c) rosmarinic acid (t_R = 33.850 min) detected at 320 nm.



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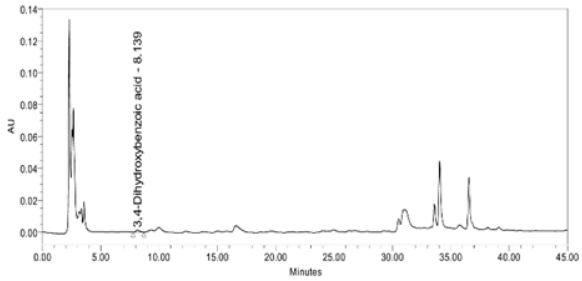

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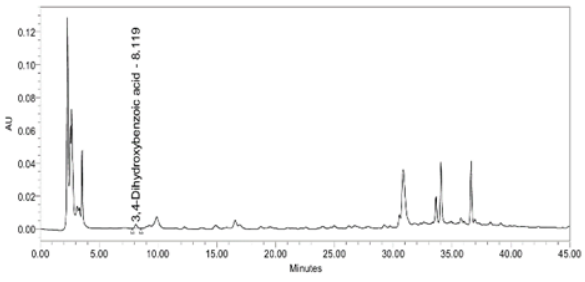

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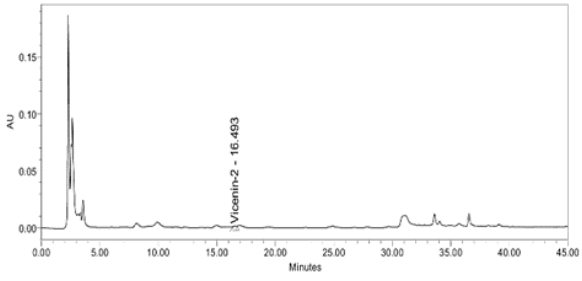

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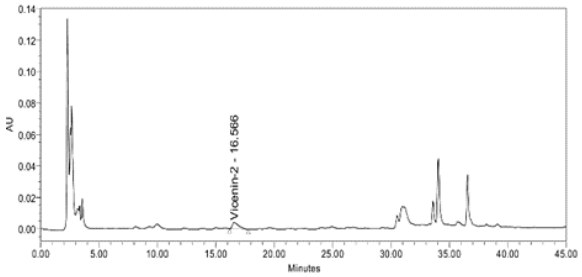

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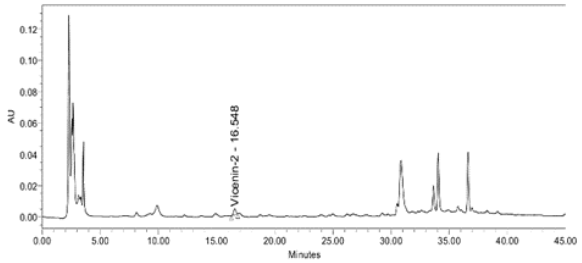

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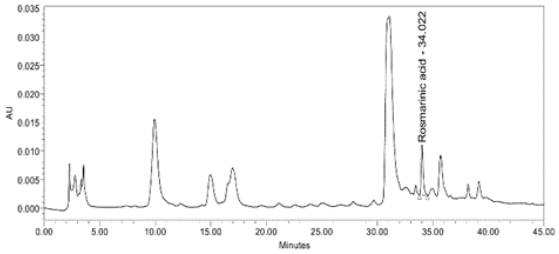

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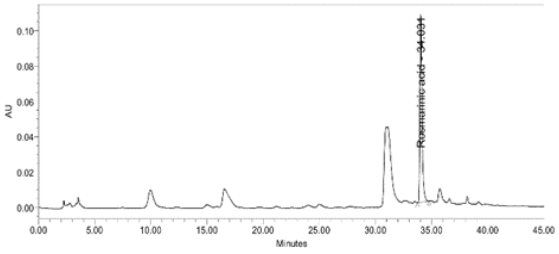

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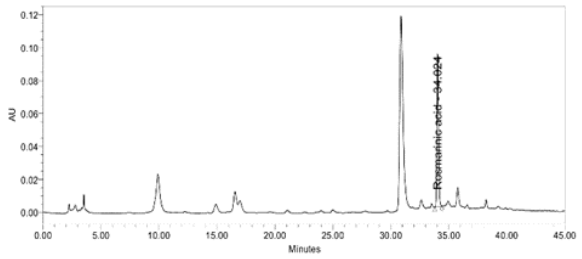

i)

Figure 2. HPLC chromatograms of targeted compounds in ML-GH, UG-KL, and PC-CH samples. Panels a–c show 3,4-dihydroxybenzoic acid detected at 272 nm in (a) ML-GH, (b) UG-KL, and (c) PC-CH. Panels d–f show vicenin-2 detected at 272 nm in (d) ML-GH, (e) UG-KL, and (f) PC-CH. Panels g–i show rosmarinic acid detected at 320 nm in (g) ML-GH, (h) UG-KL, and (i) PC-CH.

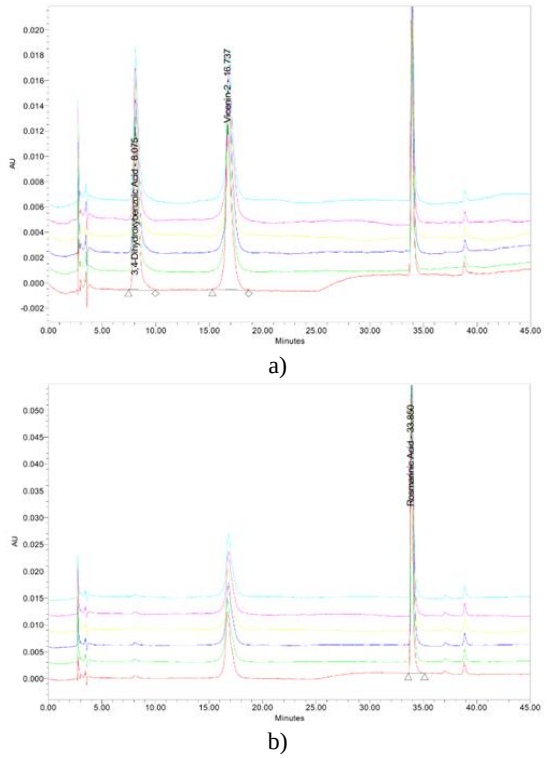

Figure 3. Overlay HPLC chromatograms of six replicate injections of the mixed standard solution (100 µg/mL) demonstrating method precision for (a) 3,4-dihydroxybenzoic acid and vicenin-2 at 272 nm, and (b) rosmarinic acid at 320 nm.

Quantitative analysis of rosmarinic acid

As shown in **Table 3**, rosmarinic acid is the predominant phenolic that peaked in the UG-KL sample. Its biosynthesis via the phenylpropanoid pathway, which involves PAL and RAS, is regulated environmentally [41]. The high concentration in the indoor vertical farm is attributed to high-energy blue LED lighting that acts as eustress, perceived by plant cryptochromes to upregulate PAL and scavenge ROS [21, 42]. Furthermore, targeted modulation of electrical conductivity (EC) in the hydroponic solution induces mild osmotic stress, driving the plant to synthesize rosmarinic acid as an osmoregulator [43]. Warm lowland temperatures also accelerate PAL/RAS kinetics, directing carbon flux toward complex polyphenols [44].

Conversely, the ML-GH sample showed severe pathway downregulation. According to the Growth-Differentiation Balance Hypothesis (GDBH), the automated, nitrogen-rich hydroponic system prioritizes rapid vegetative biomass turnover over carbon-based secondary metabolism, creating a dilution effect [45, 46]. Additionally, the highland's cold stress (14–22°C) reduces enzymatic turnover, while persistent cloud cover limits the Daily Light Integral (DLI) reaching the leaf surface, bottlenecking rosmarinic acid biosynthesis [47, 48]. PC-CH sample's intermediate concentration likely reflects rhizosphere interactions; beneficial soil microbes such as plant growth-promoting rhizobacteria (PGPR) act as biotic elicitors that trigger systemic acquired resistance (SAR) and defense priming, driving broad-spectrum secondary metabolites accumulation, though partially limited by cool highland temperatures [49, 50].

Quantitative analysis of 3,4-dihydroxybenzoic acid

An inverse trend was observed for 3,4-dihydroxybenzoic acid, which peaked in the ML-GH sample, as presented in **Table 3**. At 1800 m, the plant is exposed to significantly higher fluxes of high-energy ultraviolet-B radiation due to reduced atmospheric attenuation, and magnified by broken cloud scattering [51, 52]. Upon UVR8 photoreceptor activation, the plant prioritizes simpler, lower-energy benzoic acid derivatives as epidermal sunscreens to absorb UV-B and release it as heat, protecting delicate mesophyll cells and genomic DNA from photodamage [53]. Furthermore, cold stress bottlenecks the complex enzymatic steps required for rosmarinic acid synthesis, shunting carbon toward this immediate, simpler UV-protectant [41, 48, 54]. It also provides osmoregulation to maintain turgor pressure during cold-induced physiological drought and offers strong antifungal properties against highland pathogens like *Botrytis cinerea* [55].

UG-KL sample exhibited the lowest levels because indoor LEDs emit negligible UV-B. Without this trigger, warm temperatures drive the C6-C3 pathway toward complex end products like rosmarinic acid, minimizing C6-C1 simple C6-C1 side products [44, 47]. The PC-CH sample showed intermediate levels because the agricultural plastic rain shelters act as optical filters, blocking a large portion of UV-B wavelengths necessary for maximal phenolic acid upregulation.

Quantitative analysis of vicenin-2

Vicenin-2 peaked in the UG-KL sample, followed by the ML-GH and PC-CH samples, as presented in **Table 3**. Its C-C glycosidic bond is resistant to hydrolysis by acids, heat, or enzymes, allowing it to persist in harsh environments [32]. UG-KL sample's high accumulation results from the blue LED spectrum. When plant cryptochromes perceive high-intensity blue light, they trigger the upregulation of chalcone synthase (CHS) and chalcone isomerase (CHI). This mimics highlight stress, prompting the plant to produce photoprotective flavonoids without suffering thermal damage, an effect heavily supported by the facility's stable enzymatic temperatures [42, 56-58].

ML-GH sample presented intermediate concentration, reflecting a balance between environmental induction and physiological trade-offs. The high-altitude UV-B triggers flavone synthase (FNS) expression to accumulate vicenin-2 for photo-oxidative protection [51, 59]. However, abundant hydroponic nitrogen causes rapid leaf expansion that dilutes flavonoid concentration [20, 45, 46, 60]. The PC-CH sample exhibited the lowest vicenin-2 content due to compounded suppression, as rain shelters filter out the necessary high-energy UV photons required for biosynthesis, and continuous soil fertigation drives vegetative dilution, preventing vicenin-2 accumulation from matching growth [42, 45,46].

Table 3. Chemical marker concentrations in *O. basilicum* samples.

Marker	Sample	Concentration (mg/g DW)
Rosmarinic acid	ML-GH	0.34 ± 0.00
	UG-KL	3.65 ± 0.06
	PC-CH	2.04 ± 0.03
3,4-Dihydroxybenzoic acid	ML-GH	0.47 ± 0.01
	UG-KL	0.18 ± 0.00
	PC-CH	0.19 ± 0.00
Vicenin-2	ML-GH	0.44 ± 0.01
	UG-KL	0.95 ± 0.02
	PC-CH	0.32 ± 0.00

Method validation and system suitability

The HPLC method was validated according to ICH Q2(R2) guidelines and shown in **Table 4** [24]. The method demonstrated excellent linearity ($R^2 > 0.99$) across broad concentration ranges: 1.25 to 20.00 µg/mL for 3,4-dihydroxybenzoic acid, 2.50 to 40.00 µg/mL for vicenin-2, and 12.50 to 200.00 µg/mL for rosmarinic acid. This dynamic range accommodates the chemical plasticity of *Ocimum*, avoiding multiple dilutions. Because the calibration curve slopes method can theoretically overestimate LOD and LOQ due to heteroscedasticity, the limits were experimentally validated at lower concentrations. The established LOQs were 1.25, 2.50, and 12.50 µg/mL for the respective markers with RSD ≤ 2.0%. Accuracy, assessed via the standard spiking method, yielded mean recoveries of 95.39%, 86.57%, and 87.06%. These fell within the 80-120% acceptable range, confirming high extraction efficiency without matrix interference. Repeatability and intermediate precision studies proved highly robust, with retention time and peak area RSD values strictly below 2.0%.

System suitability testing (SST) verified optimal chromatographic performance. Theoretical plate counts (N) for 3,4-dihydroxybenzoic acid (2,487.03) and vicenin-2 (4,673.84) significantly exceeded the 2,000 acceptance criterion. Due to software limitations, SST parameters were calculated manually using a physical ruler on printed chromatograms. The highly narrow peak width at an extended retention time falls below reliable physical measurement resolution, resulting in an artificially inflated theoretical plate for rosmarinic acid (602,353.86). Despite this inflated value, the tailing factors (1.36) and capacity factor (11.40) for rosmarinic acid confirmed its high column efficiency. Overall peak symmetry was excellent, indicating that 0.1% formic acid effectively suppressed free silanol ionization to prevent secondary interactions [61]. The excellent capacity factors confirmed adequate analyte retention and separation from the void volume. Finally, all RSD for AUC and t_R remained within the 2.0% acceptance limit [24].

Table 4. HPLC method validation and system suitability parameters for the quantification of 3,4-dihydroxybenzoic acid, vicenin-2, and rosmarinic acid.

Parameter		3,4-Dihydroxybenzoic acid					Vicenin-2					Rosmarinic acid				
Precision																
RSD of t_R (%)		0.149					0.250					0.094				
RSD of AUC (%)		1.454					1.709					0.738				
Intermediate precision																
Concentration of marker (µg/mL)		1.25	2.50	5.00	10.00	20.00	2.50	5.00	10.00	20.00	40.00	12.50	25.00	50.00	100.00	200.00
Day 1	RSD of t_R (%)	0.402	0.098	0.922	0.948	0.949	0.375	0.095	0.740	0.693	0.766	0.023	0.021	0.018	0.035	0.021
	RSD of AUC (%)	0.853	1.310	1.998	1.414	0.950	1.920	1.203	1.613	1.441	1.261	0.424	1.199	0.209	0.402	1.235
Day 2	RSD of t_R (%)	0.955	0.985	0.994	0.901	0.147	0.722	0.719	0.699	0.612	0.167	0.009	0.032	0.032	0.012	0.006
	RSD of AUC (%)	0.820	0.435	1.381	0.816	0.262	1.371	0.856	0.375	1.386	0.324	1.639	0.483	0.231	1.184	1.256
Day 3	RSD of t_R (%)	1.119	0.815	1.004	0.940	1.007	0.558	0.936	0.886	1.023	0.880	0.006	0.006	0.024	0.006	0.012
	RSD of AUC (%)	1.870	1.568	1.402	1.551	0.313	0.741	0.576	1.289	1.122	1.467	1.459	1.284	1.947	0.341	0.171
Accuracy																
Percentage of recovery (Mean % ± RSD %)		95.39 ± 1.995					86.57 ± 4.115					87.06 ± 2.646				
Linearity and range																
Regression equation		y = 3,742.57x - 894.06					y = 3,665.47x – 3,119.85					y = 9,171.70x - 24,063.41				
Correlation coefficient (R2)		0.9982					0.9952					0.9999				
Analytical range (µg/mL)		1.25 to 20.00					2.50 to 40.00					12.50 to 200.00				
Sensitivity																
Limit of detection (µg/mL)		0.42					0.83					4.17				
Limit of quantitation (µg/mL)		1.25					2.50					12.50				
System suitability																
Theoretical plate, N		2,487.03					4,673.84					602,353.86				
Tailing factor, T_f		1.45					1.40					1.36				
Capacity factor, k'		2.03					5.22					11.40				
RSD of AUC (%)		1.45					1.71					0.74				
RSD of tR (%)		0.15					0.25					0.09				

Conclusion

This study of *Ocimum basilicum* across three Malaysian ecosystems demonstrates that while all samples met safety standards, their physicochemical and phytochemical profiles varied significantly by altitude and cultivation method. Notably, hydroponic samples exceeded the MHM total ash limit. However, negligible acid-insoluble ash confirmed this stems from luxury mineral consumption rather than soil contamination, highlighting a need to update MHM limits for nutrient-dense, high-tech crops. Furthermore, thermal inversion in extractive values establishes cold maceration as the optimal extraction method to preserve thermolabile volatile oils during quality control.

HPLC chemical profiling revealed that environmental factors heavily dictate secondary metabolism. The indoor vertical farming system, utilizing optimized LED spectra, maximized the accumulation of rosmarinic acid and vicenin-2. Conversely, high-altitude hydroponics with elevated UV-B maximized 3,4-dihydroxybenzoic acid. These distinct fingerprints emphasize the necessity of multi-marker profiling for accurate botanical authentication.

Ultimately, controlled environment agriculture (CEA) consistently produces high-value metabolites but alters classical purity constants. While future macroscopic and microscopic profiling remains necessary, this study provides a crucial empirical foundation for updating O. basilicum specifications in the Malaysian Herbal Pharmacopoeia, ensuring that high-tech botanical products meet stringent global standards.

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