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Balinese *Usada* as a biocultural knowledge system revealed through the chemodiversity of two *Ocimum* species

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Abstract. *Arsana IN, Cahyaningrum PL, Widyantari AAASS. 2026. Balinese* Usada *as a biocultural knowledge system revealed through the chemodiversity of two* Ocimum *species. Biodiversitas 27 (3): d270337. https://doi.org/10.13057/biodiv/d270337. Ocimum tenuiflorum* and *Ocimum gratissimum* are widely used in the Balinese *Usada*, a biocultural knowledge system that integrates plant diversity and culturally transmitted medical practices. However, systematic documentation linking comparative phytochemical diversity with functional differentiation between *Ocimum* species within this context remains limited. This study aims to compare the phytochemical profiles, antioxidant activity, and antibacterial activity of *O. tenuiflorum* and *O. gratissimum* to elucidate species-level differentiation as a character of biocultural biodiversity. The research procedure began with the process of extracting separately the leaves of *O. tenuiflorum* and *O. gratissimum* using ethanol, methanol, and hexane solvents. The active compounds were analyzed with Gas Chromatography-Mass Spectrometry (GC-MS). Total phenols and tannins were analyzed using the Folin-Ciocalteu reagent. Total flavonoids using quercetin. Antioxidant activity was analyzed using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH). The antibacterial activity was tested using the diffusion method on Mueller-Hinton agar media, with four types of bacteria: *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The results revealed that clear interspecific phytochemical differentiation of *O. gratissimum* was characterized by a predominance of phenylpropanoids, particularly eugenol, whereas *O. tenuiflorum* was dominated by methyleugenol and sesquiterpenes such as caryophyllene. These chemical differences were accompanied by distinct functional responses, with *O. gratissimum* exhibiting better antioxidant capacity (IC_{50} 43.92±0.03 µg/mL) compared to *O. tenuiflorum* (IC_{50} 79.45±0.01 µg/mL). Both species exhibited differently antibacterial inhibition patterns against the tested bacteria, indicating species-specific functional traits rather than bioactivity. Overall, this study demonstrates that interspecific chemodiversity in *Ocimum* reflects species-level differentiation within the Balinese *Usada* system. Integrating comparative phytochemical and functional analyses within a biocultural framework, the findings contribute to biodiversity science by highlighting chemical variation as a functional character of biocultural biodiversity.

Keywords: Biocultural biodiversity, chemodiversity, differentiation, *Ocimum*, traditional medicine

INTRODUCTION

The traditional Balinese medical system, known as *Usada*, is derived from ancient palm-leaf manuscripts (*Lontar Usada*) that document the relationships between local communities, plant diversity, and culturally embedded healing practices (Arsana 2019; Muderawan et al. 2020). *Usada* represents a biocultural knowledge system in which biological diversity and cultural knowledge co-evolve through long-term interaction (Gavin et al. 2015; Caillon et al. 2017; Otamendi-urroz et al. 2025). This system has been shaped by historical continuity, ritual practices, and interaction with local flora, positioning medicinal plants not merely as therapeutic resources but as integral components of cultural identity and biodiversity management (Caneva et al. 2017).

Globalization and modernization have resulted in the commodification phenomenon of *Usada*, where this traditional medicine practice is not only used in spiritual and health contexts but is also adapted into products and services with economic value (Guritna et al. 2024), as part of wellness tourism (Suatama et al. 2019). Such transformations have been recognized as potential drivers of biocultural erosion, where shifts in the utilization and valuation of biological resources can weaken the linkage between biodiversity and traditional knowledge systems (Sujarwo et al. 2014). Nevertheless, the continued practice of *Usada* in traditional villages (*Baliaga*) indicates a persistent role of local institutions in safeguarding both plant diversity and ethnobotanical knowledge (Caneva et al. 2017).

One type of plant used in the treatment of *Usada* is *Ocimum tenuiflorum* L. and *O. gratissimum* L. These species are not only sources of therapeutic compounds, but also represent units of biological and cultural biodiversity, whose selection and use are shaped by species-specific traits that are empirically recognized and culturally transmitted (Gavin et al. 2015). Comparative studies have shown that *Ocimum* species exhibit substantial interspecific phytochemical variation, especially in secondary metabolite composition and essential oil profiles, which constitutes an important dimension of functional biodiversity (chemodiversity) (Dharsono et al. 2022; Sharma et al. 2024).

However, existing phytochemical research on *O. tenuiflorum* and *O. gratissimum* has largely focused on identifying dominant essential oil constituents, such as α-thujene, eugenol, and β-terpineol in *O. tenuiflorum* (Sharma et al. 2024), chavibetol, bicyclosesquiphellandrene, and α-pinene in *O. gratissimum* (Dharsono et al. 2022; Bhattarai et al. 2024). In addition, this study did not systematically compare *O. gratissimum and O. tenuiflorum*, and does not link phytochemical diversity to cultural species differentiation or biodiversity science more broadly. As a result, the role of comparative chemodiversity in explaining why specific *Ocimum* species are maintained, substituted, or combined within traditional medical systems remains insufficiently explored. This raises the urgent need for more systematic comparative chemodiversity studies. Pharmacological studies have reported antioxidant, antifungal, and antibacterial activities of individual *Ocimum* species (Chuks-Oguine et al. 2020; Akpo et al. 2023; Azuamah et al. 2024). However, such investigations only examine single species in isolation, making it difficult to conclude the relative potential of both as well as to understand species-level differentiation and comparative functional traits within culturally embedded systems of plant use. From a biodiversity perspective, this approach overlooks the role of biological variation among closely related species in contributing to cultural selection, knowledge transmission, and the maintenance of plant diversity within traditional medical systems such as Balinese *Usada* (Sterling et al. 2017).

The plant is valued not only for its religious and cultural value but also for its sustained use within traditional medical practices, reflecting long-term integration in local biocultural systems (Arsana and Suardana 2020). However, despite this persistence, systematic documentation of comparative phytochemical diversity and its integration within the *Usada* knowledge system remains limited. Addressing this gap is essential to clarify how chemical differentiation among *Ocimum* species contributes to biocultural biodiversity rather than merely demonstrating bioactivity. Therefore, integrating traditional knowledge with comparative phytochemical profiling and functional assays is necessary to interpret species-level differentiation. This study aims to compare the phytochemical profiles, antioxidant, and antibacterial activities of *O. tenuiflorum* and *O. gratissimum* to elucidate species-level differentiation as a character of biocultural biodiversity within the Balinese *Usada* system.

MATERIALS AND METHODS

Plant materials

Samples of *O. tenuiflorum* and *O. gratissimum* plants were obtained from the area of Abiansemal Sub-district, Badung District, Bali Province (8°34'46.2"S 115°14'45.2" E), on June 1, 2025. The plant has been identified at the Eka Karya Bali-BRIN Botanical Garden, and specimen vouchers are stored in the Biology Laboratory of the Universitas Hindu Indonesia, with collection numbers OT-301Unhi for *O. tenuiflorum* and OG-302Unhi for *O. gratissimum*. Fresh plant leaves are taken and then cleaned of impurities to be dried for two weeks in a refrigerated room, then ground until smooth and sifted with a sieve (60 mesh size) (Arsana et al. 2023).

Procedures

Extraction process

The extraction process uses ethanol 96%, methanol, and hexane (analytical grade, Merck, Germany) to obtain complementary fractions of phytochemical diversity and to examine species-level chemical differentiation. A total of 100 g of dried simplicia were separately macerated in 1000 mL of ethanol, methanol, and hexane for 48 hours at room temperature with daily stirring. The extracts were then filtered using filter paper. Maceration is repeated 4 times. The filtrates from four macerations are combined and then concentrated using a rotary evaporator at 45°C to obtain a viscous extract. All samples were extracted under identical conditions to ensure internal consistency. Extraction yield was calculated as:

$$\text{Extraction yield} = \frac{\text{Weight of dried extract}}{\text{Weight of initial plant material}} \times 100\%$$

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analysis was performed using a Thermo Scientific TRACE 1310 gas chromatograph coupled to an ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific, USA), operated with Chromeleon software version 7.2.10.24543. An HP-5MS UI capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness) was employed for compound separation. The sample was introduced using a split injection mode with a split flow of 10 mL/min, corresponding to an approximate split ratio of 10:1. The injector temperature was maintained at 230°C, with helium used as the carrier gas at a constant flow rate of 1.0 mL/min. The GC oven temperature program started at 60°C, held for 2 min, then ramped to 280°C and held for the remainder of the 32-min run. The MS transfer line and ion source temperatures were set at 250°C and 200°C, respectively. The analysis was conducted in Electron Ionization (EI) mode with an energy of 70 eV. Mass spectra were acquired in full scan mode over a mass-to-charge (m/z) range of 40-500, with a scan time of 0.2 s. Data acquisition started at 4 min post-injection to eliminate solvent interference. Sample injection was performed using a TriPlus RSH autosampler with a 1 µL injection volume. The syringe (10 µL, 57 mm needle) underwent automated pre- and post-injection rinsing with solvents A and B, followed by multiple cleaning cycles to prevent carryover and maintain analytical integrity. Compound identification was based on the comparison of acquired mass spectra against the NIST Mass Spectral Library. Identification was based on automatic spectral matching using both the Match Factor and Reverse Match Factor generated by the software, with ≥800 as the general acceptance threshold for tentative identification, consistent with standard GC-MS practice. Since a C7-C30 n-alkane ladder was not included in the original analytical run, Retention Indices (RI) were not experimentally determined. Compound assignments were instead supported by retention-time consistency and fragmentation pattern comparison with the literature. No

derivatization or internal standard was used. The percentage peak area (% area) was automatically calculated from the Total Ion Chromatogram (TIC) and reported as a semi-quantitative measure of relative abundance, not as absolute content. Compounds exhibiting the highest similarity indices and % areas were considered the dominant constituents of the extract (Adams 2017; Xie et al. 2023).

Total phenolic

Total phenolic content was determined using the Folin-Ciocalteu phenol reagent (Sigma-Aldrich, USA). Briefly, 50 mg of extract was dissolved in 10 mL of distilled water, from which 1 mL was mixed with 0.5 mL of Folin-Ciocalteu reagent and 5 mL of distilled water. After incubation for 10 min at room temperature, 1.5 mL of 20% sodium carbonate was added, and the volume was adjusted to 10 mL with distilled water. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Total phenolic content was expressed as mg Gallic Acid Equivalent (GAE)/g extract based on a gallic acid calibration curve prepared at concentrations of 0.5, 1, 2, 5, 10, 25, 75, and 100 ppm. The calibration equation was $y = 0.00888378x - 0.00191564$ ($r^2 = 0.99991$) (Chaovanalikit and Wrolstad 2004). All measurements were conducted in triplicate (n = 3), representing independent technical replicates of the same extract preparation, and results are expressed as mean±Standard Deviation (SD). Absorbance values were blank-corrected. Limits of Detection (LOD) and Quantification (LOQ) were calculated from calibration curve statistics following Majewska and Drużyńska (2025), yielding LOD and LOQ values of 0.49 mg/L and 1.48 mg/L, respectively. No defatting step was applied before extraction or analysis.

Total flavonoid

Total flavonoid content was analyzed using a standard solution of quercetin (Sigma-Aldrich, USA). A total of 50 mg of extract was hydrolyzed with 2 mL of 4 N HCl by autoclave at 110°C for 2 h. The hydrolysate was filtered, and the filtrate was extracted with diethyl ether three times and evaporated to dryness. The dried residue was reacted sequentially with 0.3 mL of 5% sodium nitrite (5 min), 0.6 mL of 10% aluminum chloride (5 min), and 2 mL of 1 M sodium hydroxide, then diluted with distilled water to a final volume of 5 mL. Absorbance was measured at 510 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). Total flavonoid content was expressed as mg Quercetin Equivalent (QE)/g extract using a quercetin standard curve (0.5-100 ppm) with the calibration equation $y = 0.00599857x - 0.00857346$ ($r^2 = 0.99965$) (Dalawai and Murthy 2021). All analyses were performed in triplicate (n = 3) as independent technical replicates of the same extract preparation, and results are expressed as mean±Standard Deviation (SD). Absorbance values were blank-corrected using reagent blanks. LOD and LOQ values were calculated following Majewska and Drużyńska (2025), resulting in LOD and LOQ values of 1.51 mg/L and 4.57 mg/L, respectively. No defatting procedure was conducted before extraction or analysis.

Tannin

Total tannin content was quantified using the Folin-Ciocalteu reagent (Sigma-Aldrich, USA). A total of 50 mg of extract was subjected to purification with 10 mL of diethyl ether for 20 h. This step functioned as post-extraction purification rather than defatting. The filtrate was evaporated to dryness and reconstituted with distilled water to a final volume of 10 mL. An aliquot of 1 mL was mixed with 0.1 mL of Folin-Ciocalteu reagent and incubated for 5 min, then 2 mL of 20% sodium carbonate was added and incubated for 5 min. Distilled water was added to reach a final volume of 10 mL, and the mixture was incubated at room temperature for 30 min. Absorbance was measured at 760 nm using a UV-Vis Spectrophotometer (Shimadzu UV-1800, Japan). Total tannin content was expressed as mg Tannic Acid Equivalent (TAE)/g extract based on a tannic acid calibration curve prepared at concentrations of 0.125-16 ppm. The calibration equation was $y = 0.0245196x + 0.00131617$ ($r^2 = 0.99996$) (Chanwitheesuk et al. 2005). Measurements were conducted in triplicate (n = 3) as independent technical replicates of the same extract preparation, and results are expressed as mean±Standard Deviation (SD). Absorbance values were background-corrected. LOD and LOQ values were calculated following Majewska and Drużyńska (2025), yielding values of 11.15 mg/L and 33.78 mg/L, respectively, indicating sufficient analytical sensitivity for tannin quantification.

Antioxidant activity

Antioxidant assays were used as standardized functional metrics to reflect chemical differentiation, rather than as indicators of therapeutic effectiveness. Antioxidant activity was analyzed using DPPH (analytical grade, Merck, Germany) as a free radical source, and absorbance was recorded at 517 nm with a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) equipped with a 1.00-cm quartz cuvette. The extracts were tested at 6-8 concentration points within the range of 26-921 µg/mL, adjusted to ensure coverage of the 20-80% inhibition linear response region. The sample solution is prepared by dissolving a specific amount of extract in the same solvent as the extract. The solution is then combined with 1 mL of 0.4 mM DPPH solution and the same solvent as the extract solvent, until the total volume reaches 5 mL. The solution is incubated for 30 minutes in a dark room. The sample solution was then measured for absorbance at 517 nm. The ability to scavenge DPPH is calculated by the equation:

$$\% \text{ Inhibition} = \frac{Ao - As}{Ao} \times 100$$

Where, Ao: The control absorbance, As: The sample absorbance (Al-Mutaani et al. 2025).

Antioxidant activity was expressed as IC_{50} and calculated by linear regression of % inhibition versus concentration. Ninety-five percent confidence intervals (95% CI) were derived from the regression parameters. All measurements were performed in triplicate (n = 3) as independent technical replicates of the same extract preparation. IC_{50} values were calculated for each replicate, and results are reported as mean±SD. Ascorbic acid was used as the reference antioxidant and assessed at a fixed

concentration range of 1.0-8.0 µg/mL under identical assay conditions. The IC_{50} of ascorbic acid served as the basis for calculating the comparative IC_{50} of all six extracts (Arsana et al. 2023).

Antibacterial

Antibacterial activity was evaluated using the paper disk diffusion method on Mueller-Hinton Agar (MHA) (Oxoid, UK). Sterile MHA (15 mL) at 45°C was poured into Petri dishes and allowed to solidify. Bacterial suspensions of *Streptococcus mutans* ATCC 25175, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were prepared and standardized to a 0.5 McFarland turbidity, then uniformly swabbed onto the agar surface. The extracts were prepared at a working concentration of 1 mg/mL in distilled water. A 25 µL aliquot of each extract was applied to sterile 6 mm paper disks (Oxoid). The disks were aseptically dried at room temperature for approximately 20-30 minutes, or until the solvent completely evaporated. Disks loaded with 25 µL distilled water served as the vehicle control, while chloramphenicol 0.5% served as the positive control. All disks were placed onto the inoculated agar and incubated at 35-37°C for 24 hours in an inverted position. Inhibition zone diameters were measured using a calliper. The inhibition zone was defined as the clear area surrounding the disk. The total diameter of the zone was first measured, and the disk diameter (6 mm) was then subtracted to obtain the net inhibition zone width. Inhibition zones were measured in millimeters. All assays were performed in triplicate (n = 3) as independent technical replicates of the same extract preparation, and results are expressed as mean±Standard Deviation (SD) (Cintrón et al. 2025).

Attempts to determine MIC and MBC values using the CLSI broth microdilution protocol were unsuccessful. All extracts showed very low solubility in aqueous Mueller-Hinton Broth (MHB) and did not form stable or homogeneous dispersions. The extracts remained as particulate aggregates or surface films, resulting in phase separation, uneven distribution, and artificial turbidity in the microdilution wells. These interferences prevented reliable endpoint determination. Therefore, valid MIC/MBC values could not be obtained, and the antimicrobial evaluation in this study is based solely on disk diffusion results (Mahendran and Vimolmangkang 2023).

Data analysis

Normality was assessed using the Shapiro-Wilk test, and homogeneity of variances was evaluated with Levene's test. Variables meeting both assumptions (total phenolic content, total flavonoid content, and tannin levels) were analyzed using One-Way ANOVA. When the omnibus ANOVA indicated significant group differences, Bonferroni-adjusted post hoc comparisons were performed. Variables that did not meet the normality or homogeneity assumptions (antioxidant activity and antibacterial activity) were analyzed using the Kruskal-Wallis test, followed by pairwise Mann-Whitney U tests with Bonferroni correction. The sample size (n) for each assay is reported, along with effect sizes for the Mann-Whitney U test. The effect size was reported as $r = Z/\sqrt{N}$, where Z is the standardized test statistic, and N is the total sample size of both groups. Statistical significance was set at $p\_adj<0.05$. Statistical conclusions were based on Bonferroni-adjusted p-values (p_adj), while unadjusted p-values are reported only descriptively. All statistical analyses were conducted using SPSS Statistics software version 22 (IBM, USA). Meanwhile, the chemical compound profile was analyzed descriptively.

RESULTS AND DISCUSSION

Phytochemical profile, antioxidant activity

The extraction yields varied according to plant species and solvent polarity. *O. tenuiflorum*, methanolic extract produced the highest yield at 8.43% (w/w), followed by the ethanolic extract at 6.77% (w/w), while the n-hexane extract resulted in 5.88% (w/w). In contrast, *O. gratissimum* showed higher extraction efficiency, with the highest yield obtained from the ethanolic extract at 13.11% (w/w), followed by the methanolic extract at 10.30% (w/w). The n-hexane extract of *O. gratissimum* yielded 6.01% (w/w).

GC-MS analysis for ethanol extracts of *O. tenuiflorum* (OT_EtOH), methanol *O. tenuiflorum* (OT_MeOH), n-hexane *O. tenuiflorum* (OT_Hexane), ethanol *O. gratissimum* (OG_EtOH), methanol *O. gratissimum* (OG_MeOH), and n-hexane *O. gratissimum* (OG_Hexane) successfully identified several metabolite compounds that represent the typical phytochemical profile of this plant. Based on the results of chromatogram peak deconvolution and matching with the NIST database, in OT_EtOH, there are 58 identifiable compound peaks (Figure 1), 49 compound peaks in OT_MeOH (Figure 2), 109 OT_Hexane compound peaks (Figure 3), 50 compound peaks in OG_EtOH (Figure 4), 33 compound peaks in OG_MeOH (Figure 5), and 91 compound peaks in OG_Hexane (Figure 6). The main compounds that can be determined in OT_EtOH are 10 compounds, 11 compounds in OT_MeOH, 17 compounds in OT_Hexane, 8 compounds in OG_EtOH, nine in OG_MeOH, and 17 main compounds in OG_Hexane (Table 1). The main compounds identified in each extract, along with their mass spectra, are detailed in the Supplement.

The results showed that the IC_{50} values of *O. gratissimum* were better compared to *O. tenuiflorum*, with the lowest value obtained in OG_MeOH. However, the value of IC_{50} is only 0.63% to 8.15% compared to the ascorbic acid used as a standard (Table 2). The replicate-specific IC_{50} values were highly consistent with overlapping 95% confidence intervals (Table 3). Kruskal-Wallis analyses revealed significant differences across groups for all measured parameters ($p<0.05$). Subsequent Mann-Whitney U pairwise testing initially suggested meaningful contrasts, with large effect sizes ($r>0.50$). Nevertheless, after rigorous control using Bonferroni adjustment, none of these contrasts retained statistical significance ($p\_adj>0.05$; Table 4).

The results showed that total phenols were highest in OT_MeOH and lowest in OT_Hexane. However, no statistically significant differences were observed between OG_MeOH and OT_EtOH, OG_Hexane and OT_EtOH, as well as OG_Hexane and OT_MeOH ($p>0.05$; Bonferroni

post hoc test). The flavonoid content tends to be higher in *O. gratissimum* compared to *O. tenuiflorum*, with the highest values observed in OG_Hexane and the weakest in OT_Hexane, but between OG_EtOH and OG_MeOH did not differ significantly ($p>0.05$). Tannin shows significant differences ($p<0.05$) between different species and solvents. Higher tannin concentrations were obtained using methanol as the solvent for both species (Table 2).

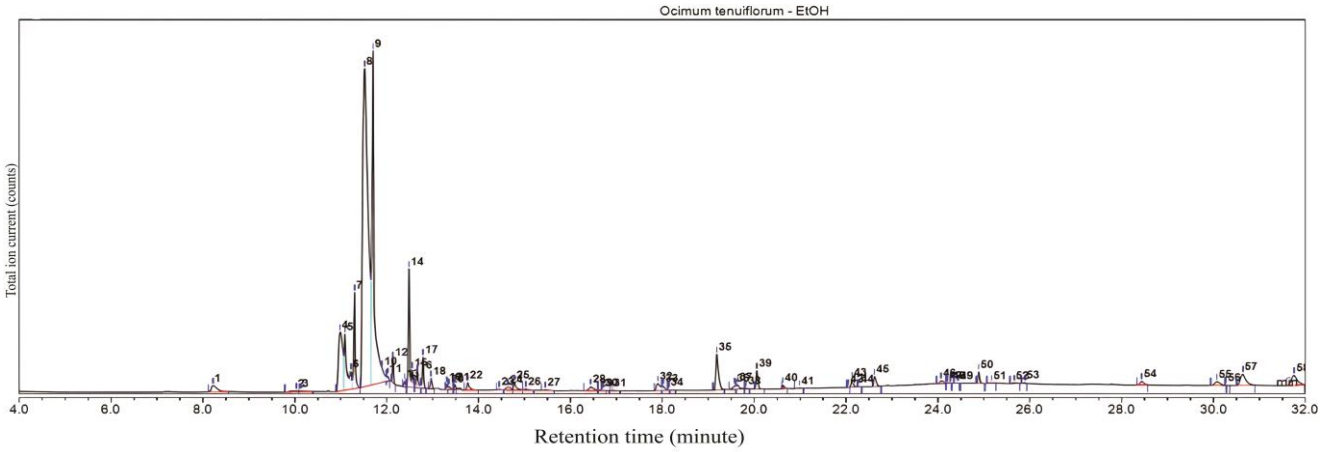

Figure 1. Chromatogram GC-MS of the ethanol extract of *Ocimum tenuiflorum* leaf

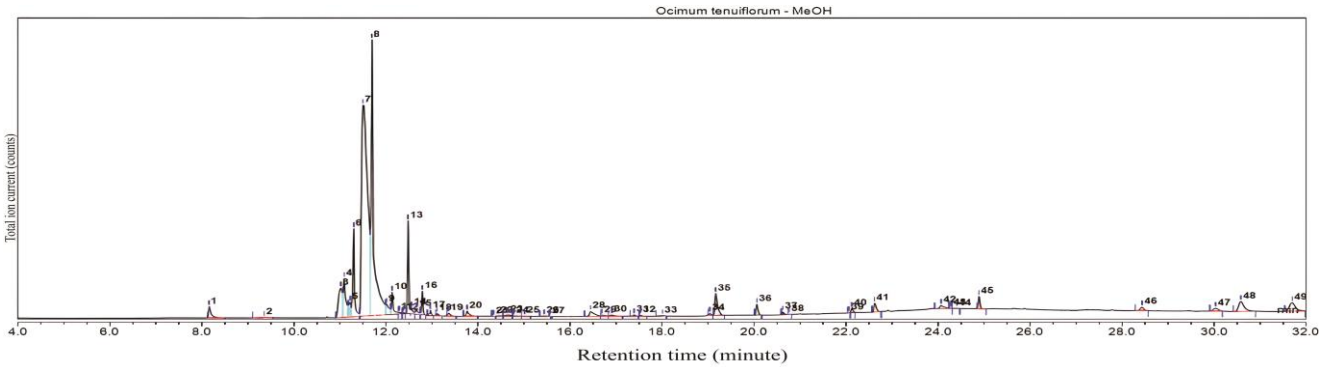

Figure 2. Chromatogram GC-MS of the methanol extract of *Ocimum tenuiflorum* leaf

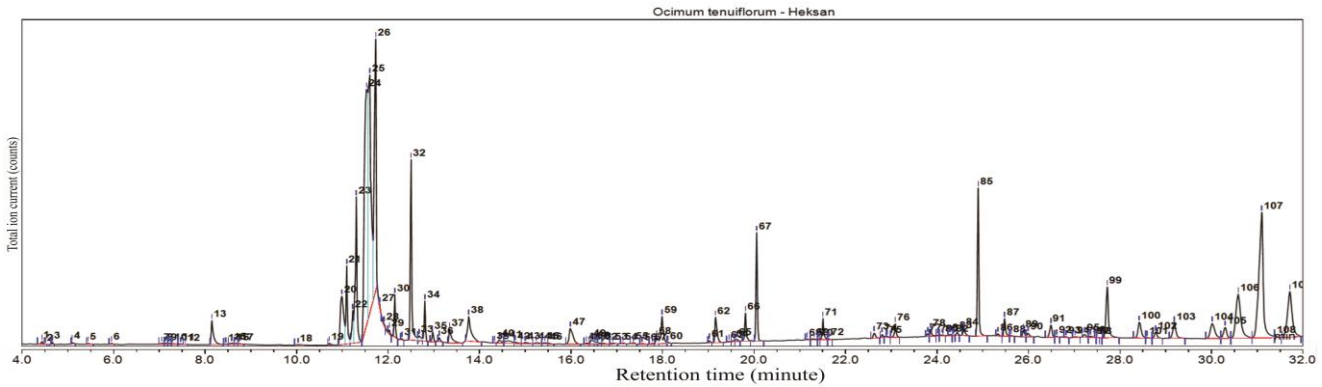

Figure 3. Chromatogram GC-MS of the hexane extract of *Ocimum tenuiflorum* leaf

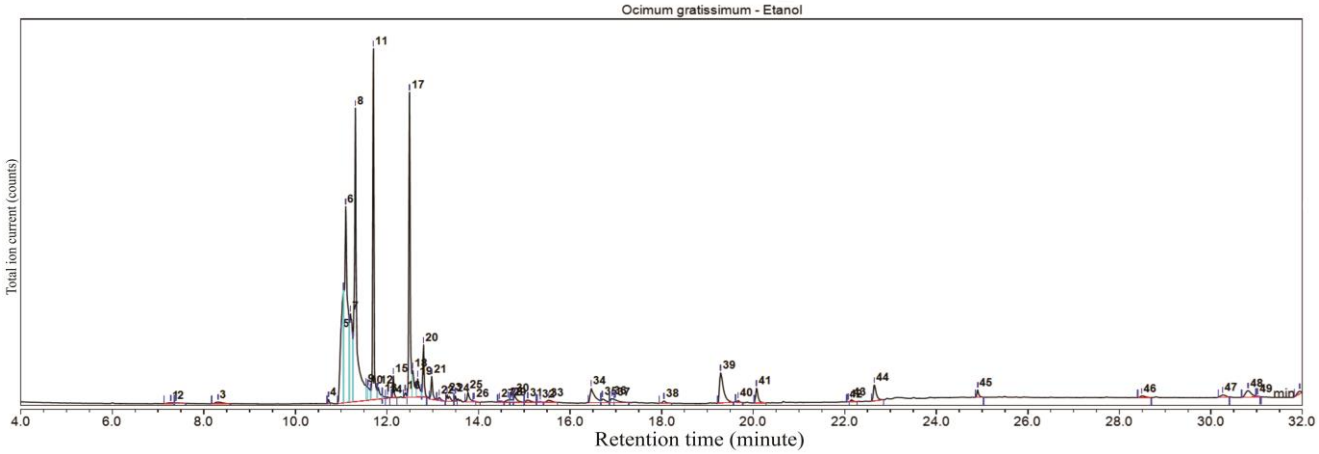

Figure 4. Chromatogram GC-MS of the ethanol extract of the leaves of *Ocimum gratissimum*

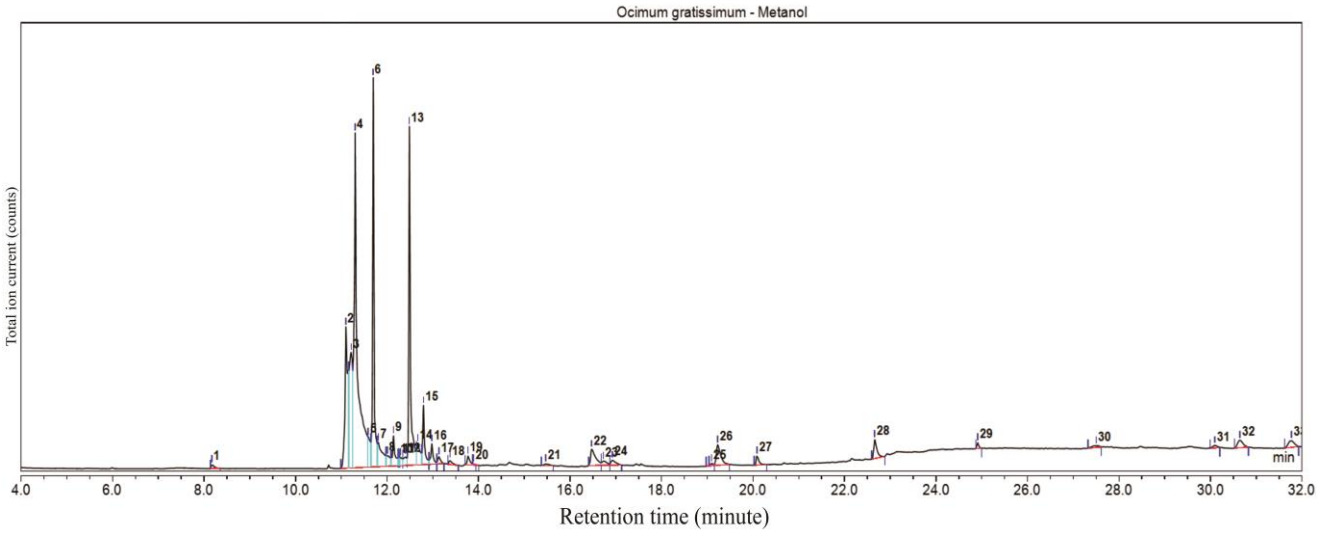

Figure 5. Chromatogram GC-MS of the methanol extract of the leaves of *Ocimum gratissimum*

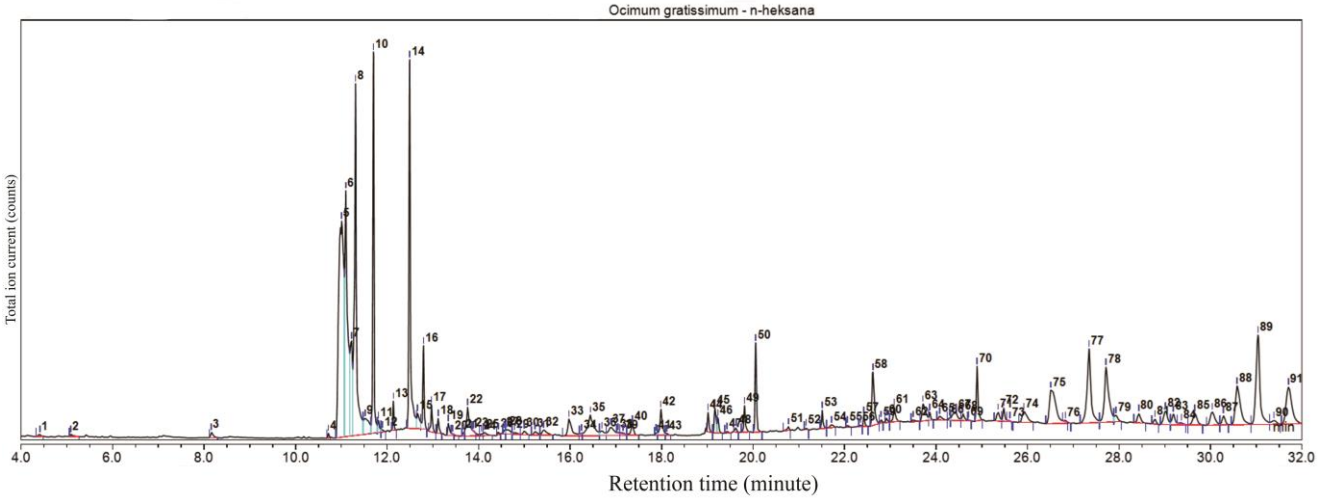

Figure 6. Chromatogram GC-MS of the hexane extract of the leaves of *Ocimum gratissimum*

Table 1. The main compounds identified in the ethanol extract of *Ocimum gratissimum* (OG_EtOH), the methanol extract of *Ocimum gratissimum* (OG_MeOH), the hexane extract of *Ocimum gratissimum* (OG_Hexane), the ethanol extract of *Ocimum tenuiflorum* (OT_EtOH), the methanol extract of *Ocimum tenuiflorum* (OT_MeOH), and the hexane extract of *Ocimum tenuiflorum* leaves (OT_Hexane)

Compound name	Formula	Relative area (%)						Group of compounds
		OG_EtOH	OG_MeOH	OG_Hexane	OT_EtOH	OT_MeOH	OT_Hexane	
Eugenol	$C_{10}H_{12}O_2$	8.7	8.8	14.6	6.1	3.7	3.1	Phenylpropanoid
Methyleugenol	$C_{11}H_{14}O_2$				42.8	41.5	14.9	Phenylpropanoid
Caryophyllene	$C_{15}H_{24}$	12.2	13.4	6.4	19.8	23.4	9.7	Sesquiterpene
Humulene	$C_{15}H_{24}$	0.7	1.8	0.4	0.7	1.3	0.9	Sesquiterpene
Germacrene D	$C_{15}H_{24}$	11.4	13.4	7.6	4.3	3.5	4.5	Sesquiterpene
ß-Elemene	$C_{15}H_{24}$	19.1	27.5	9.5	3.4	4.7	4.5	Sesquiterpene
Caryophyllene oxide	$C_{15}H_{24}O$		0.6	1.5	0.5	0.5	1.6	Sesquiterpene oxidation
Phytol	$C_{20}H_{40}O$	3.4	2.3	0.6	2.3	1.9	1.0	Alcoholic diterpenoids
Phytol, acetate	$C_{22}H_{42}O_2$	1.0		1.8			2.7	Diterpenoid ester
Squalene	$C_{30}H_{50}$			1.2			3.9	Triterpena
Stigmasterol	$C_{29}H_{48}O$			3.0	1.6	1.8	4.0	Phytostero
Tetratriacontane	$C_{34}H_{70}$			3.8			9.9	n-alkane
Alloaromadendrene	$C_{15}H_{24}$		1.7	0.5				Sesquiterpene hydrocarbon
Neophytadiene	$C_{20}H_{38}$		2.0	1.8				Diterpenoid hidrokarbon
Campesterol	$C_{28}H_{48}O$			0.8			1.3	Phytosterol
?-Sitosterol	$C_{29}H_{50}O$				1.5	1.8	3.9	Phytosterol
Borneol	$C_{10}H_{18}O$			0.2		0.9		Monoterpenoid alcohol
endo-Borneol	$C_{10}H_{18}O$						0.9	Monoterpenoid alcohol
alfa.-Copaene	$C_{15}H_{24}$						2.0	Sesquiterpene
Cubebol	$C_{15}H_{26}O$			0.3				Sesquiterpene alcohol
Elemol	$C_{15}H_{26}O$			0.4			0.7	Sesquiterpene alcohol

Table 2. Phytochemical and antioxidant activity of ethanol, methanol, and n-Hexane extracts from the leaves of *Ocimum tenuiflorum* and *Ocimum gratissimum*

Extracts	IC_{50} (µg/mL±SD; n=3)	Comparative IC_{50} (%)	Flavonoid (mg QE/g±SD; n=3)	Phenol (mg GAE/g±SD; n=3)	Tannin (mg TAE/g±SD; n=3)
OG_EtOH	56.95±0.06	6.29	16.17±0.3 [a]	90.45±2.60 [a]	38.53±0.22 [a]
OG_MeOH	43.92±0.03	8.15	16.27±0.16 [a]	104.86±1.96 [b]	49.53±0.27 [b]
OG_Hexane	67.25±0.03	5.32	33.61±0.82 [b]	112.94±2.05 [cd]	2.56 ±.0.01 [c]
OT_EtOH	565.88±0.71	0.63	11.03±0.09 [c]	110.58±0.56 [db]	31.36±0.21 [d]
OT_MeOH	79.46±0.01	4.51	13.10±0.41 [d]	116.69±2.04 [ec]	62.36±0.46 [e]
OT_Hexane	140.78±0.05	2.54	7.74±0.03 [e]	81.68±2.29 [f]	0.80±0.00 [f]
Ascorbic acid	3.58±0.01				

Note: Values within the same columns with different superscript letters (a, b, c, d, e) are significantly different between groups (p_adj<0.05, Bonferroni post hoc test)

Antibacterial activity

Antibacterial assays revealed variation in inhibition patterns between *O. gratissimum* and *O. tenuiflorum* extracts obtained using different solvents against four test bacteria (Table 5). Extracts of *O. gratissimum* showed larger inhibition zones against *E. coli* and *S. mutans* compared with those of *O. tenuiflorum*. In contrast, *O. tenuiflorum* extracts produced measurable inhibition against both Gram-positive and Gram-negative bacteria across multiple solvent types. Non-parametric analysis using the Kruskal-Wallis test indicated significant overall differences among groups ($p<0.05$). Although several pairwise comparisons using the Mann-Whitney test yielded nominally significant p-values and large effect sizes, none of these differences remained statistically significant after Bonferroni correction (Table 6).

Discussion

Phytochemical profile, antioxidant activity

The present study demonstrates clear interspecific phytochemical differentiation between *O. gratissimum* and *O. tenuiflorum*, characterized primarily by contrasting dominance of phenylpropanoids and sesquiterpenes. Both species exhibited phytochemical profiles typical of the genus *Ocimum*, with phenylpropanoids and sesquiterpenes constituting the major chemical groups. However, the relative abundance and composition of these compounds differed consistently between species, indicating stable species-level chemical differentiation. One of the main compounds consistently found in all types of extracts is eugenol, with a higher percentage in *O. gratissimum*, specifically in OG_Hexane at 14.6%. In contrast, the major component identified from *O. tenuiflorum* is methyleugenol with a very high relative area of 42.8% in OT_EtOH, 41.5% in OT_MeOH, and 14.9% in OT_Hexane (Table 1). This compound was not detected in *O. gratissimum*. These findings are consistent with previous reports classifying *O. tenuiflorum* into methyleugenol-rich chemotypes and *O. gratissimum* into eugenol-dominant profiles (Ríos-Rodríguez et al. 2021; Gurav et al. 2022; Mulugeta et al. 2024).

Eugenol is a phenolic compound with a free -OH group that can donate hydrogen atoms to free radicals, resulting in phenoxyl radicals that are stabilized through aromatic resonance. This mechanism makes eugenol a highly effective radical scavenger (Nenadis et al. 2021). The high concentration of eugenol in *O. gratissimum* (especially OG_MeOH) appears to be directly related to the low IC_{50} value of the methanol extract at 43.92±0.03 µg/mL (Table 2). Whereas methyleugenol is a methoxylated derivative of eugenol, and its biosynthesis in *O. tenuiflorum* is strongly influenced by the expression of the eugenol-O-methyltransferase gene, which is differentially expressed on a specific chemotype, resulting in a distinctive metabolite profile (Renu et al. 2014; Gurav et al. 2022).

The chemotypic differentiation, from a biocultural perspective, represents an important dimension of functional diversity (chemodiversity) that contributes to species distinctiveness within the *Ocimum* genus. The persistence of methyleugenol-rich *O. tenuiflorum* within Balinese *Usada* suggests that this chemical profile has been empirically recognized and culturally maintained over time. Rather than being interchangeable with *O. gratissimum*, *O. tenuiflorum* represents a chemically distinct entity, and continued use reflects species-level differentiation embedded within traditional knowledge systems. In addition, the identification of methyleugenol as a dominant constituent is also important for interpreting broader implications of traditional plant use, including considerations of functionality and safety. In this context, chemodiversity documentation contributes to biodiversity characterization rather than therapeutic endorsement. Such an approach aligns with numerous ethnobotanical studies emphasizing that the principal scientific value of phytochemical data lies in elucidating relationships between biological diversity, cultural practices, and local adaptation, rather than in validating medical claims (Bhandari et al. 2021; Kumar et al. 2021).

The results showed that the total phenols in OT_MeOH were higher compared to OG_Hexane and OG_MeOH (Table 2). However, this high phenol content does not necessarily imply a direct correlation with antioxidant activity. Although OT_MeOH has a high phenol content, the IC_{50} value of OT_MeOH is still higher than that of OG_MeOH (Table 2). This indicates that the phenol type is more decisive than the total. Phenols, such as eugenol, tend to be more active than methyleugenol in reducing the DPPH. This happens because eugenol has a phenolic group with free OH in the aromatic ring, so that it can donate hydrogen atoms to free radicals, resulting in a more stable form of phenoxyl radicals.

Table 3. IC_{50} estimates and associated 95% confidence intervals for three independent replicates

Extract	Replication	IC_{50} (µg/mL)	SE	95% CI	
				Lower	Upper
OG_EtOH	1	57.01	0.63	55.40	58.63
	2	56.92	0.61	55.34	58.49
	3	56.92	0.61	55.34	58.49
OG_MeOH	1	43.96	0.89	41.48	46.43
	2	43.91	0.91	41.40	46.42
	3	43.91	0.91	41.40	46.42
OG_Hexane	1	67.27	0.74	65.21	69.32
	2	67.27	0.74	65.21	69.32
	3	67.22	0.72	65.21	69.23
OT_EtOH	1	566.46	11.05	538.06	594.86
	2	566.09	10.89	538.11	594.08
	3	565.08	11.03	536.73	593.43
OT_MeOH	1	79.47	0.58	78.05	80.88
	2	79.45	0.59	78.02	80.89
	3	79.45	0.60	77.97	80.92
OT_Hexane	1	140.84	3.15	132.09	149.60
	2	140.76	3.13	132.08	149.43
	3	140.75	3.21	131.84	149.67
Ascorbic acid	1	3.59	0.08	3.41	3.77
	2	3.58	0.07	3.40	3.76
	3	3.58	0.07	3.40	3.75

Table 4. Effect sizes (r) of Mann-Whitney U and the Bonferroni adjustment (p_adj) for phytochemical and antioxidant activity of ethanol, methanol, and n-Hexane extracts from the leaves of *Ocimum tenuiflorum* and *Ocimum gratissimum*

Groups	IC_{50}			Flavonoid	Phenol	Tannin
	p_raw	r	p_adj	p_adj	p_adj	p_adj
OG_EtOH vs OG_MeOH	0.04	0.82	0.65	1.00	0.00	0.00
OG_EtOH vs OG_Hexane	0.04	0.82	0.65	0.00	0.00	0.00
OG_EtOH vs OT_EtOH	0.04	0.81	0.69	0.00	0.00	0.00
OG_EtOH vs OT_MeOH	0.04	0.82	0.65	0.00	0.00	0.00
OG_EtOH vs OT_Hexane	0.04	0.81	0.69	0.00	0.00	0.00
OG_MeOH vs OG_Hexane	0.04	0.82	0.65	0.00	0.00	0.00
OG_MeOH vs OT_EtOH	0.04	0.81	0.69	0.00	0.70	0.00
OG_MeOH vs OT_MeOH	0.04	0.82	0.65	0.00	0.00	0.00
OG_MeOH vs OT_Hexane	0.04	0.81	0.69	0.00	0.00	0.00
OG_Hexane vs OT_EtOH	0.04	0.81	0.69	0.00	1.00	0.00
OG_Hexane vs OT_MeOH	0.04	0.82	0.65	0.00	0.64	0.00
OG_Hexane vs OT_Hexane	0.04	0.81	0.69	0.00	0.00	0.00
OT_EtOH vs OT_MeOH	0.04	0.81	0.69	0.00	0.04	0.00
OT_EtOH vs OT_Hexane	0.05	0.80	0.75	0.00	0.00	0.00
OT_MeOH vs OT_Hexane	0.04	0.81	0.69	0.00	0.00	0.00

Table 5. Antibacterial activity of ethanol, methanol, and n-Hexane extracts from the leaves of *Ocimum tenuiflorum* and *Ocimum gratissimum*

Extract (1 mg/mL)	*Escherichia coli* (mm±SD, n = 3)		*Staphylococcus aureus* (mm±SD, n = 3)		*Streptococcus mutans* (mm±SD, n = 3)		*Pseudomonas aeruginosa* (mm±SD, n = 3)	
	Extract	Control+	Extract	Control+	Extract	Control+	Extract	Control+
OG_EtOH	2.83±0.29	18	2.50±0.00	19	3.00±0.00	29	0.00±0.00	14
OG_MeOH	3.33±0.58	17.5	1.67±1.15	18.5	5.00±0.00	18.5	3.50±0.00	17.5
OG_Hexane	2.16±0.29	17.5	1.00±0.00	19	1.00±0.00	18.5	0.00±0.00	17.5
OT_EtOH	0.50±0.00	18.5	0.50±0.00	19	1.67±0.29	19.5	5.00±0.00	9
OT_MeOH	0.83±0.29	18.5	3.50±0.00	19.5	4.33±0.76	28	8.00±0.00	5
OT_Hexane	0.50±0.00	26	1.00±0.00	20.5	1.00±0.00	18.5	0.50±0.00	5

Table 6. Effect sizes (r) of Mann-Whitney U and the Bonferroni adjustment (p_adj) for antibacterial activity of ethanol, methanol, and n-Hexane extracts from the leaves of *Ocimum tenuiflorum* and *Ocimum gratissimum*

Groups	*Escherichia coli*			*Staphylococcus aureus*			*Streptococcus mutans*			*Pseudomonas aeruginosa*		
	p_raw	r	p_adj	p_raw	r	p_adj	p_raw	r	p_adj	p_raw	r	p_adj
OG_EtOH vs OG_MeOH	0.19	0.53	1.00	0.48	0.29	1.00	0.03	0.91	0.38	0.03	0.91	0.38
OG_EtOH vs OG_Hexane	0.07	0.75	1.00	0.03	0.91	0.38	0.03	0.91	0.38	1.00	0.00	1.00
OG_EtOH vs OT_EtOH	0.03	0.87	0.51	0.03	0.91	0.38	0.03	0.87	0.38	0.03	0.91	0.38
OG_EtOH vs OT_MeOH	0.04	0.82	0.65	0.03	0.91	0.38	0.04	0.89	0.56	0.03	0.91	0.38
OG_EtOH vs OT_Hexane	0.03	0.87	0.51	0.03	0.91	0.38	0.03	0.91	0.38	0.03	0.91	0.38
OG_MeOH vs OG_Hexane	0.04	0.82	0.65	0.32	0.41	1.00	0.03	0.91	0.38	0.03	0.91	0.38
OG_MeOH vs OT_EtOH	0.03	0.87	0.51	0.03	0.87	0.51	0.03	0.87	0.51	0.03	0.91	0.38
OG_MeOH vs OT_MeOH	0.04	0.82	0.65	0.03	0.87	0.51	0.12	0.63	1.00	0.03	0.91	0.38
OG_MeOH vs OT_Hexane	0.03	0.87	0.51	0.32	0.41	1.00	0.03	0.91	0.38	0.03	0.91	0.38
OG_Hexane vs OT_EtOH	0.03	0.87	0.51	0.03	0.91	0.38	0.03	0.87	0.51	0.03	0.91	0.38
OG_Hexane vs OT_MeOH	0.04	0.82	0.65	0.03	0.91	0.38	0.04	0.85	0.56	0.03	0.91	0.38
OG_Hexane vs OT_Hexane	0.03	0.87	0.51	1.00	0.00	1.00	1.00	0.00	1.00	0.03	0.91	0.38
OT_EtOH vs OT_MeOH	0.11	0.65	1.00	0.03	0.91	0.38	0.05	0.81	0.69	0.03	0.91	0.38
OT_EtOH vs OT_Hexane	1.00	0.00	1.00	0.03	0.91	0.38	0.03	0.87	0.51	0.03	0.91	0.38
OT_MeOH vs OT_Hexane	0.11	0.65	1.00	0.03	0.91	0.38	0.04	0.85	0.56	0.03	0.91	0.38

Meanwhile, in methyleugenol, the OH group at the ortho position is methylated into a methoxy group, so that there are no more protons that can be donated to free radicals (Park 2006; Bhattarai et al. 2024). The presence of methyleugenol in *O. tenuiflorum* is consistent with a higher IC_{50} value compared to *O. gratissimum*, even at OT_EtOH reaching 566.30±0.74 µg/mL (Table 2). This suggests that the presence of bioactive compounds is not always directly proportional to antioxidant capacity but depends on the properties of the molecule. Several studies show that although eugenol and methyleugenol can act as hydrogen atom donors in radical suppression mechanisms, eugenol is more efficient and contributes more to total antioxidant activity than methyleugenol (Nenadis et al. 2021). Eugenol in clove extract also showed similar results, where eugenol had a better IC_{50} against DPPH compared to methyleugenol (Leem et al. 2011).

Antioxidant assays, from a biodiversity science perspective, function as standardized methodological tools to compare chemical and functional differences among species, rather than as indicators of therapeutic effectiveness (Subbiah et al. 2023; Papaefthimiou et al. 2024). Comparative antioxidant data primarily reflect differences in molecular composition, redox behavior, and structural properties of secondary metabolites, which are shaped by species-specific metabolic pathways and ecological adaptation, not by clinical effectiveness (Du et al. 2021). Antioxidant value, within a biocultural biodiversity framework, contributes to understanding how chemical variation among closely related species is maintained through long-term ecological processes and culturally based selection within traditional knowledge systems (Sterling et al. 2017; Saensouk et al. 2025). This functional differentiation explains why multiple plant species are maintained, distinguished, or combined in biocultural practices, without implying that higher antioxidant values necessarily indicate superior medicinal merit (Bhagawan et al. 2023). Accordingly, the observed differences in antioxidant activity between *O. gratissimum* and *O. tenuiflorum* can be interpreted as functional indicators associated with this chemodiversity. The distinct antioxidant responses observed between *O. gratissimum* and *O. tenuiflorum* reinforce the role of chemodiversity in functional differentiation among closely related species maintained within the Balinese *Usada* system.

The results showed that the flavonoid content in OG_Hexane was higher compared to OG_MeOH (Table 2), although theoretically non-polar solvents such as n-hexane are generally less effective in extracting polar flavonoids. This may be due to the presence of methylated flavonoids or lipophilic glycosides dissolved in non-polar solvents. Nevertheless, the IC_{50} values of OG_MeOH are

still better than those of OG_Hexane (Table 2). This indicates that the structure of flavonoids also strongly determines the bioactivity of *Ocimum* species (Moazzen et al. 2022; Shah et al. 2024). Meanwhile, the highest tannin content was found in OT_MeOH, which was 62.36±0.46 mg TAE/g, and OG_MeOH, 49.53±0.27 mg TAE/g (Table 2). This suggests that methanol solvents are very effective at extracting these compounds. Tannins are large molecular-weight polyphenols with many hydroxyl groups that are soluble in polar solvents. The presence of tannins in high concentrations in OT_MeOH and OG_MeOH indicates a synergy with simple phenolics such as eugenol. Eugenol can donate hydrogen quickly, while tannins with many hydroxyl groups act as more stable radical catchers and are capable of repeated redox regeneration (De Francesco et al. 2020). This combination resulted in a lower value of IC_{50} OG_MeOH than OT_MeOH, namely 43.92±0.03 µg/mL (Table 2). However, although the tannin content in OT_MeOH was higher than that of OG_MeOH, which was 62.36±0.46 mg TAE/g (Table 2), the dominance of methyleugenol, which has much weaker antioxidant activity than eugenol, caused IC_{50} in OT_MeOH to remain higher, namely 79.45±0.01 µg/mL (Table 2). This phenomenon shows that the dominant phenol type plays a more decisive role than the total number of polyphenols present (Santos et al. 2021).

Quantitative analysis of total phenolic content, total flavonoid, and total tannin revealed additional layers of chemodiversity between *O. gratissimum* and *O. tenuiflorum*, as well as among types of extract. These differences from a biodiversity perspective should be interpreted as species-specific functional traits reflecting divergent secondary metabolic strategies, rather than as indicators of pharmacological superiority. This finding aligns with research on *Gastrodia elata* Blume, which reveals that quantitative chemical traits often correspond to traditional classifications and their usage patterns, reflecting empirical knowledge accumulated through long-term interactions between communities and their local environments (Fan et al. 2025). Furthermore, conservation-oriented research highlights that documenting functionally distinct plants embedded in cultural landscapes supports biodiversity characterization, thereby reinforcing the argument for the integrated conservation of both biological and cultural diversity (Kulak et al. 2022). Biocultural theory also emphasizes that plant species are maintained, differentiated, and valued not only for single bioactivities but also for their broader functional characteristics, sensory attributes, and roles within culturally embedded knowledge systems (Argumedo et al. 2021; Franco 2022). Differences in total phenolics, flavonoids, and tannins between *O. gratissimum* and *O. tenuiflorum*, therefore, contribute to explaining why these species are recognized as distinct entities within the Balinese *Usada* system. The cultural value of this plant arises from its long-term ecological availability, consistent functional properties, distinctive chemical profile, and integration into culturally transmitted practices, rather than from any single demonstrable therapeutic effect. The quantitative assessment of total phenolics, flavonoids, and tannins contributes not to pharmacological validation, but to a deeper understanding of chemodiversity as a component of biocultural biodiversity. By situating these chemical differences within ecological and cultural contexts, such analyses strengthen biodiversity-oriented interpretations of medicinal plants and support the recognition of traditional knowledge systems as integral to biodiversity conservation.

Caryophyllene is also an important compound found in large quantities in both species, but the relative percentage is higher in *O. tenuiflorum* compared to *O. gratissimum* (Table 1). Caryophyllene is a group of sesquiterpenes that are lipophilic and are commonly found in *Ocimum* spp. (Fonseka et al. 2020; Kunihiro et al. 2022; Ahmad et al. 2024; Le et al. 2024). Theoretically, caryophyllene, which is non-polar in nature, is easier to extract by non-polar solvents such as n-hexane. However, the results of the study showed that the relative percentage was actually higher in methanol and ethanol extracts. This could possibly be due to the amphipathic properties of polar solvents that are capable of extracting semi-polar or non-polar compounds. In addition, it is possible that the interaction of compounds in the plant matrix and the effects of co-extraction with other compounds, thus help polar solvents attract non-polar compounds (Kustiati et al. 2022). Similar behavior has been reported in *Cannabis sativa* L., where ethanol and methanol recovered lipophilic terpenes and cannabinoids, despite their low polarity (Isidore et al. 2021; Haczkiewicz et al. 2025a, 2025b). Ethanol performed comparably to n-hexane in extracting plant oils (Magalhães et al. 2023), while aqueous ethanol enhanced triterpenoid recovery owing to improved solute-solvent interactions (Castellano et al. 2022). Moreover, its amphiphilic nature facilitates the simultaneous extraction of both hydrophilic and lipophilic constituents (Lee et al. 2024). Collectively, these observations indicate that solvent polarity alone cannot fully predict extraction efficiency, particularly in complex plant matrices, because the actual extraction outcome is influenced by solvent-solute affinity, matrix porosity, cell-wall permeability, and co-extraction effects that alter solubility and diffusion behavior.

The compound β-elemene is also found in fairly high percentages, especially in OG_MeOH and OG_EtOH, as well as in moderate amounts in OG_Hexane. The percentage of β-elemene in *O. tenuiflorum* is lower but still detectable. Another relevant component is germacrene D, found in high amounts in *O. gratissimum* extract, whereas in *O. tenuiflorum* it is lower (Table 1). These compounds are widely reported constituents of *Ocimum* species and contribute to their overall chemical identity (Gurav et al. 2022). Germacrene D, along with eugenol, contributes to the clove-like aroma profile (Fonseka et al. 2020; Du et al. 2023).

Solvent-dependent variation in obtaining compounds, from a biodiversity standpoint, does not undermine species-level differentiation; instead, it provides an additional analytical dimension for assessing the stability of chemodiversity. In this context, the consistent dominance of eugenol in *O. gratissimum* and methyleugenol in *O. tenuiflorum* from solvents of differing polarity demonstrates that interspecific phytochemical differentiation is robust

and not merely an effect of extraction method. Similar observations have been reported in comparative phytochemical studies of *Ocimum* species, where chemotypic identity remains stable despite variations in solvent systems, extraction protocols, and analytical platforms (Gurav et al. 2022).

Solvent polarity primarily influences the relative abundance and detectability of compounds, but does not fundamentally alter species-specific metabolic pathways that already exist (Subbiah et al. 2023; Stavropoulou et al. 2024). In this sense, solvent-dependent variation can be interpreted as revealing different chemical phenotypes, rather than obscuring interspecific differentiation. The persistence of eugenol- and methyleugenol-dominated profiles supports the interpretation of these compounds as species-associated chemical traits, which contribute to functional differentiation within the genus *Ocimum*. The existence of such a chemical, from a biocultural biodiversity perspective, aligns with the long-term recognition and maintenance of distinct *Ocimum* species within traditional knowledge systems. The stability of chemodiversity suggests that species differentiation is rooted in biological identity and ecological adaptation, rather than contingent on laboratory conditions. Accordingly, solvent effects should be regarded as a methodology that enriches phytochemical resolution, while the persistence of interspecific chemical patterns reinforces the role of chemodiversity as a meaningful biodiversity character (Braga 2021; Franco 2022).

Bacterial activity

Antibacterial assays revealed clear variation in inhibition patterns between *O. gratissimum* and *O. tenuiflorum* extracts across different solvents and bacterial strains. The OG_MeOH produced larger inhibition zones against *S. mutans* (5.00±0.00 mm) and *E. coli* (3.33±0.58 mm), whereas OT_MeOH showed measurable inhibition against *P. aeruginosa* (8.00±0.00 mm) and *S. mutans* (4.33±0.76 mm) as well as *S. aureus* (3.50±0.00 mm) (Table 5). These contrasting patterns indicate interspecific differences in antibacterial response rather than uniform activity across species. These differences, from a chemodiversity perspective, are interpreted in relation to species-specific phytochemical composition, particularly the dominance of eugenol in *O. gratissimum* (Tables 4 to 6) and methyleugenol in *O. tenuiflorum* (Tables 1 to 3), together with varying proportions of sesquiterpenes such as caryophyllene and germacrene D. Previous studies have documented antimicrobial properties associated with phenylpropanoids and sesquiterpenes commonly found in *Ocimum* species (Nisar et al. 2021; Obasi et al. 2023; Chen et al. 2024). However, in the present study, these compounds are discussed as contributors to interspecific chemical differentiation rather than as determinants of therapeutic efficacy. The observed variation in inhibition profiles thus reflects functional differentiation linked to chemical composition at the species level.

The observation shows that OT_MeOH also exhibited inhibition against both Gram-positive and Gram-negative bacteria, namely *S. mutans* (4.33±0.76 mm) and *S. aureus* (3.50±0.00 mm), whereas *O. gratissimum* extracts showed a more restricted pattern under the same assay conditions (Table 5). This aligns with reports describing broader antibacterial spectra in methyleugenol-rich *Ocimum* chemotypes, although such patterns are strongly influenced by assay conditions and extraction systems (Abdoul-Latif et al. 2022; Hao and Quoc 2024; Ren et al. 2024). Potential interactions between phenylpropanoids (eugenol or methyleugenol) and sesquiterpenes (caryophyllene, germacrene D) have also been proposed in much of the literature (Hernández-León et al. 2020; Shivakumar et al. 2022; Trivedi et al. 2024), but direct evidence of synergistic effects remains limited and cannot be inferred from disk diffusion assays alone. From a biodiversity perspective, such differences are functional indicators of chemical diversity rather than evidence of broader or superior antibacterial performance (Iskandar et al. 2022).

The results showed that extracts obtained with n-hexane (OG_Hexane and OT_Hexane) tended to exhibit lower inhibition against the four bacteria tested (Table 5). This is thought to be related to the properties of compounds extracted by n-hexane that are generally non-polar, such as sterols (stigmasterol, β-sitosterol), long-chain hydrocarbons (tetratriacontane), and squalene. These compounds have a large molecular weight, are highly lipophilic, have limited solubility in aqueous media, and are therefore less able to diffuse optimally in the agar matrix during the disc diffusion test. Although some components of hexane have biological potential, for example, squalene, which is known as an antioxidant and a precursor of sterols (Micera et al. 2020), or phytosterols, which are reported to have immunomodulatory effects (Jahanger et al. 2023), their antibacterial activity is difficult to detect by disc diffusion methods. This limitation underscores the importance of complementary quantitative approaches, particularly MIC and MBC assays using microdilution broth. In this study, attempts to determine MIC and MBC values were unsuccessful due to the very low solubility of all extracts in aqueous Mueller-Hinton Broth. Future studies should incorporate optimized solubilization strategies. Furthermore, further characterization of the active constituents is required. Future work should include phytochemical profiling and systematic fractionation to identify the compounds responsible for the antibacterial effects, along with cytotoxicity testing to assess their safety. In addition, synergistic interaction studies using checkerboard FIC assays, particularly against relevant oral pathogens, may clarify whether these extracts can potentiate the activity of conventional antibiotics.

It is essential to note that disk diffusion assays offer qualitative or semi-quantitative indications of antibacterial activity and are influenced by factors such as compound diffusion, solubility, and molecular size. Consequently, inhibition zone diameters should not be equated with potency or clinical relevance. Antibacterial activity within the biodiversity-oriented framework is interpreted as a functional indicator reflecting interspecific chemical variation rather than evidence of therapeutic application. In addition, explicit acknowledgment of this limitation in research reinforces the conservative interpretation adopted here, wherein antioxidant and antibacterial assays are treated as comparative functional indicators rather than

definitive measures of efficacy. Overall, the results demonstrate that *O. gratissimum* and *O. tenuiflorum* exhibit stable, species-specific chemical character encompassing both volatile compounds and polyphenolic constituents. This chemodiversity underpins species-level differentiation and supports the interpretation that both species represent distinct components of biocultural biodiversity. The continued use of both species within the Balinese *Usada* system suggests that such chemical differentiation has been empirically recognized and culturally transmitted. Integration of comparative phytochemical profiling with functional indicators and interpretation of their results within a biocultural biodiversity context, this study contributes to biodiversity science by demonstrating how chemical variation among closely related plant species can underpin species differentiation within traditional medical systems.

In conclusion, this study shows that *O. tenuiflorum* and *O. gratissimum* differ consistently in dominant volatile constituents and polyphenolic profiles, supporting interspecific chemodiversity as a meaningful biodiversity character within Balinese *Usada*. Antioxidant and antibacterial assays are interpreted here as comparative functional indicators linked to chemical composition and assay constraints, not as evidence of therapeutic efficacy. The continued use of both species in *Usada* can therefore be discussed in terms of culturally maintained plant differentiation and embedded chemical traits rather than pharmacological superiority.

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