

Research

Exploring *Hornstedtia scyphifera*: an extensive multimethod phytochemical investigation reveals the chemical composition and bioactive potential

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Abstract

Hornstedtia scyphifera (J.Koenig) Steud. represents a lesser-known member of the ginger family (Zingiberaceae) that is used in Malaysia as spice and traditional medicine. The phytochemical investigation of leaves from this species utilizing diverse analytical methods has provided comprehensive insights into its chemical profile for the first time. Headspace gas chromatography-mass spectrometry (HS-GCMS) and GCMS analyses of essential oil and nonpolar extracts verified α -pinene, camphene, *p*-cymene, and camphor as main volatile compounds. Metabolite profiling of the crude extract by ultra-high-performance-liquid chromatography-high resolution mass spectrometry (UHPLC-HRMS) unveiled terpenoids, flavonoids and other phenolics as major compound classes. Isolation and follow-up structure elucidation, involving 1D and 2D NMR, HRMS, UV and CD analysis, yielded two new sesquiterpenoids, (1*R*,5*S*,6*S*,7*R*,10*R*)-mustak-14-oic acid (**1**) and (1*R*,6*S*,7*S*,10*R*)-6-hydroxy-anhuienosol (**2**), along with 24 known compounds (seven terpenoids, seven flavonoids, ten phenolics), 21 of these never reported for *H. scyphifera*. Additionally, the crude extract and fractions from the purification process were screened for antibacterial and antifungal activity. This is supplemented by an extensive literature research for described bioactivities of all isolated compounds. Our results support and explain previously detected antimicrobial, antifungal and neuroprotective effects of *H. scyphifera* extracts and provide evidence for its potential pharmacological importance.

Keywords *Hornstedtia scyphifera* · Phytochemical investigation · Sesquiterpenes · Antimicrobial

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1 Introduction

The Zingiberaceae family, commonly known as the ginger family, represents the largest family within the Zingiberales order and comprises 53 genera and over 1200 species [1]. The family is subdivided into four major tribes: Alpinieae, Globbeae, Hedychieae, and Zingibereae, distinguished by morphological features only [1]. They grow in tropical regions such as India, Asia, Africa, and Australia [1, 2], characterized as perennial herbaceous species with fleshy-thickened rhizomes [3]. While many species have traditional applications as spices or ornamentals, they are also valued as herbal medicine [2]. The therapeutic properties of numerous members of this family are largely attributed to their abundant bioactive compounds [4, 5], such as terpenes, terpenoids, and flavonoids [6–8]. In the plant, some of these compounds serve to deter herbivores, combat microbial infections, or protect against solar radiation, while others attract pollinators through color or scent [6, 7, 9]. For humans, these secondary metabolites offer various pharmaceutical advantages. So far, extracts and isolates from Zingiberaceae members demonstrated antioxidative [10–12], antimicrobial [11], anti-inflammatory [10, 12] and anticancer [11, 12] properties or even neuroprotective activity [13], indicating considerable potential for application in contemporary medicine.

One of the lesser-known representatives of this highly potent family is *Hornstedtia scyphifera* (J.Koenig) Steud., also known as *Amomum scyphiferum* J.Koenig, *Cardamomum scyphiferum* (J.Koenig) Kuntze, *Greenwaya scyphifer* (J.Koenig) Giseke or *Greenwaya scyphiferus* (J.Koenig) Giseke [14]. The genus *Hornstedtia* comprises about 43 species found in tropical Southeast Asia from the Malay peninsula to the Himalayas [15, 16]. *H. scyphifera* is morphologically characterized by a robust rhizome, positioned just below or at the ground surface and large leaf shoots reaching 2–5 m in height, adorned with green leaves covered at the base by sheaths. The plant bears short inflorescences with a series of scales surrounding the floral bracts. The red flowers open individually for one day and develop smooth, elongated fruits. *H. scyphifera* appears to be the most common species of the genus in Malaysia [15], where it is traditionally used as a spice and for insect repellency [15, 17]. The essential oils from various organs are well researched and predominantly consist of mono- and sesquiterpenes as well as terpenoids, such as camphor and germacrene D, but also borneol and β -selin [17]. However, studies on non-volatile compounds are lacking except for two brief reports describing the isolation of four flavonoids (quercetin, 5-hydroxy-3,7,4'-trimethoxyflavon, kumatakenin, 3,5-dimethylkaempferol), two phytosterols (stigmast-4-en-3-one, 6-hydroxy-stigmast-4-en-3-one) and a fatty acid (dodecanoic acid) from *H. scyphifera* leaves [18, 19]. Extracts of *H. scyphifera* leaves show promising antibacterial and antioxidative activities [18], as well as anti-inflammatory and neuroprotective properties [20]. These effects may be ascribed to a high concentration of sesquiterpenes, which have been linked to antidepressant and neuroprotective properties [13], although other substances may also be involved.

In this study, we conducted a comprehensive phytochemical investigation focusing on both the volatile compounds and the isolation of the non-volatile compounds to fully evaluate the chemical composition and biological activity of *H. scyphifera*.

2 Materials and methods

2.1 General experimental procedures

Thin layer chromatography (TLC) analyses were performed on silica gel 60 normal phase (SG60), silica gel 60 reversed phase 18 F₂₅₄ (Merck, Darmstadt, Germany) or silica gel 60 reversed phase 2 UV₂₅₄ (Macherey–Nagel, Düren, Germany) using different solvent systems. To visualize the compound spots, long-wavelength UV light (366 nm), short-wavelength UV light (254 nm) and spraying with vanillin–H₂SO₄ reagent, followed by heating or spraying with natural product spray reagent (1 g 2-aminoethyl diphenylborinate/200 mL methanol) were applied.

Low-resolution ESI–MS spectra were performed on a Sciex API-3200 instrument (Applied Biosystems, Concord, Ontario, Canada) combined with a HTC-XT autosampler (CTC Analytics, Zwingen, Switzerland). The UV spectra were recorded on a Jasco V-770 UV–Vis/NIR spectrophotometer (Jasco, Pfungstadt, Germany), using a 10 mm quartz glass cuvette. The specific rotation was recorded on a Jasco P-2000 digital polarimeter (Jasco, Pfungstadt, Germany), using the software Spectra Manager 2 (version 2.14.02). The circular dichroism (CD) spectra were recorded on a Jasco J1500 CD spectrometer (Jasco, Pfungstadt, Germany), using the software Spectra Manager 2 (version 2.15.04). The

ultrasound assisted extraction of plant material was performed on a Bandelin Sonorex RK 510 H (9.7 L) (Bandelin electronic GmbH & Co. KG, Berlin, Germany) at 35 kHz for 60 min per cycle.

The semi-preparative HPLC was performed on a Shimadzu prominence system which consists of a SPD-M20A diode array detector, a FRC-10A fraction collector, a CBM-20A communications bus module, a DGU-20A5R degassing unit, a LC-20AT liquid chromatograph, and a SIL-20A HT auto sampler. The following columns were used for separation: column 1 (YMC Pack Pro C18, 5 μ m, 120 Å, 150 $\times$ 4.6 mm I.D., YMC, USA), column 2 (YMC Pack Pro C18, 5 μ m, 120 Å, 150 $\times$ 10.0 mm I.D., YMC, USA) and column 3 (Agilent Eclipse XDB-C18, 5 μ m, 80 Å, 250 $\times$ 9.4 mm I.D., Agilent, USA). Water (A)/methanol (B) (solvent system 1) or water (A)/acetonitrile (B) (solvent system 2) each with 0.1% formic acid were used as mobile phases at a temperature of 25 °C applying different gradients.

^1H and ^{13}C NMR spectra were recorded on an Agilent DD2 400 NMR spectrometer at 399.917 and 100.570 MHz, respectively. Chemical shifts are reported relative to TMS (^1H NMR) or peaks of solvent. For samples with low concentration, 1D ^1H and ^{13}C NMR spectra and 2D spectra (HSQC, HMBC, COSY, TOCSY, NOESY) were recorded on a Bruker Avance Neo 500 NMR spectrometer at 500.234 and 125.797 MHz, respectively, using a 5 mm prodigy probe with the TopSpin 4.0.7 spectrometer software or on an Agilent VNMR5 600 MHz NMR spectrometer equipped with 5 mm inverse detection cryoprobe, using standard CHEMPACK 8.1 pulse sequences implemented in Varian VNMRJ 4.2 spectrometer software.

2.2 Plant material

The plant material of *Hornstedtia scyphifera* (J.Koenig) Steud. was taken from the greenhouse collection of the IPB in Halle (Saale). Dried voucher material is stored at IPB (Identification code DFK214 and DFK221). For the investigations, fresh leaves as well as dried leaves were used. Fresh leaves were washed with water after being harvested and used for extraction immediately. For storage, the leaves were frozen in liquid nitrogen, then kept in a freezer at – 22 °C and finally freeze-dried. The dry material was stored at room temperature until being used for further treatment.

2.3 Extraction and isolation

2.3.1 Isolation from fresh leaves

Fresh leaves (66.40 g) from *H. scyphifera* were ground, followed by an exhaustive ultrasound assisted extraction with methanol (5 $\times$ 500 mL) to give 7.18 g of dried crude extract after evaporation of the solvent. The extract was five times partitioned by liquid–liquid-extraction between water (450 mL) and ethyl acetate (5 $\times$ 500 mL) yielding the ethyl acetate fraction (2.02 g) and the water fraction (2.90 g). The ethyl acetate fraction was submitted to a polyamide SC-6-AC column (l: 21 cm, d: 7 cm) and eluted according to the eluotropic series (*n*-hexane, ethyl acetate, acetone, methanol). The acetone fraction (320.5 mg) was further purified on a RP18 column (l: 10 cm, d: 7 cm) with an increasing methanol gradient (10–100% in water), followed by a preparative RP18 TLC (d: 1 mm, 40% methanol) and final purification by semi-preparative reversed phase HPLC (column 1, solvent system 1, isocratic, 27.5 min, 15% B, flow rate of 1.6 mL/min) to yield compound **22** (*p*-hydroxybenzoic acid, 0.7 mg, R_t = 12.36 min, R_f = 0.67 in MeOH/H₂O (1:1, v/v) on RP18). The methanol fraction of the polyamide column was submitted to a SG60 column (l: 9 cm, d: 5 cm) and eluted with an increasing methanol/chloroform gradient (5–100% MeOH, in 5% steps). Compound **12** (isokaempferid, 6.3 mg, R_f = 0.45 in MeOH/CHCl₃ (1:19, v/v) on SG60) eluted with 5% methanol. The fraction obtained with 10% methanol was further submitted to repetitive column chromatography on RP18 with an isocratic water–methanol-mixture (7:4, v/v) and finally purified by HPLC (column 1, solvent system 1, gradient 0–2.5 min, 30% B; 2.5–12.5 min, 30–50% B, isocratic for 2 min, flow rate of 0.7 mL/min) to yield compound **8** (epicatechin, 0.6 mg, R_t = 11.82 min, R_f = 0.44 in MeOH/H₂O (4:7, v/v) on RP18).

2.3.2 Isolation from dried leaves

Dried pulverized leaves (92.00 g) from *H. scyphifera* were exhaustively extracted assisted by ultrasonication with 80% aq. methanol (6 $\times$ 1 L) to give 18.02 g of dried crude extract after evaporation. The extract was successively partitioned by liquid–liquid-extraction between water and *n*-heptane, followed by ethyl acetate. A water-insoluble residue was later combined with the *n*-heptane fraction due to TLC profiles. This resulted into three fractions: 1) *n*-heptane (3.03 g), 2) ethyl acetate (1.50 g) and 3) water (8.59 g).

The *n*-heptane fraction was submitted to a silica gel 60 column (l: 90 cm, d: 4 cm), eluted with an increasing ethyl acetate/*n*-hexane gradient (0–100% ethyl acetate in 10% steps, each 1.5 L). The fraction eluted with 20% ethyl acetate was

submitted to a preparative RP18 TLC (d: 1 mm, 10% methanol) to yield compounds **14** (5-hydroxy-3,7,4'-trimethoxyflavon, 9.3 mg, R_f =0.78 in *n*-hexane/EtOAc (3:2, v/v) on NP) and **9** (0.9 mg, R_t =9.07 min, R_f =0.51 in MeOH/H₂O (9:1, v/v) on RP18). The fraction eluted with 70% ethyl acetate from the silica 60 column was further purified by preparative HPLC (column 3, solvent system 2, gradient 0–3.0 min, 70% B; 3.0–23.0 min, 70–100% B (isocratic for 6.5 min), flow rate of 1.8 mL/min) to yield compound **3** ((*E*)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid, 0.1 mg, R_t =23.51 min).

The ethyl acetate fraction was submitted to a Sephadex LH20 column (l: 99 cm, d: 2 cm) with an isocratic dichloromethane-methanol-mixture (3:1, v/v) yielding seven fractions (A1–A7) based on the TLC profile (NP, toluol/EtOAc/FA, 10:5:3, v/v), of which A2 (R_f 0.57), A4 (R_f 0.42), A6 (R_f 0.37) and A7 (R_f 0.28) were further purified.

A2 was submitted to a RP18 column (l: 51 cm, d: 3 cm) and eluted with a mixture of methanol and water (7:3, v/v) which yielded six fractions (B1–B6) based on the TLC profile (RP18, MeOH/H₂O, 7:3, v/v), of which B2 (R_f 0.70) and B6 (R_f 0.06) were further purified. B2 was repeatedly submitted to reversed phase HPLC (column 3). The separation with solvent system 1 (gradient 0–3.0 min, 33% B; 3.0–23.0 min, 33–38% B) and a flow rate of 1.0 mL/min yielded compounds **18** (evofolin B, 0.6 mg, R_t =15.39 min), **5** (*cis*-pinononic acid, 0.9 mg, R_t =17.11 min), **21** (syringaldehyde, 1.6 mg, R_t =18.82 min) and **20** (vanillin, 1.2 mg, R_t =19.53 min). In addition, a second gradient system was applied (solvent system 1; gradient 0–3.0 min, 30% B; 3.0–23.0 min, 30–52% B, flow rate of 1.4 mL/min) to yield compound **2** (6-hydroxy-anhuienosol, 2.7 mg, R_t =9.42 min), compound **7** (2-oxo-3–4,5,5-trimethylcyclopentynyl acidic acid, 2.3 mg, R_t =15.42 min) and compound **6** (*cis*-pinonic acid, 2.2 mg, R_t =16.85 min).

B6 was purified by preparative HPLC (column 2, solvent system 1; gradient 0–2.5 min, 80% B; 2.5–17.5 min, 80–100% B; flow rate of 2.64 mL/min) to yield compound **13** (kumatakenin, 2.3 mg, R_t =9.16 min).

A4 was purified by preparative HPLC (column 3, solvent system 1; gradient 0–3.0 min, 35% B; 3.0–23.0 min, 35–100% B, flow rate of 1.8 mL/min) to yield compounds **19** (protocatechualdehyde, 3.6 mg, R_t =11.38 min), **24** (vanillic acid, 3.2 mg, R_t =13.87 min), **25** (protocatechuic acid methylester, 0.1 mg, R_t =16.10 min) and **16/17** (*trans*- and *cis*-ferulic acid, 1.1 mg, R_t =16.70 min).

A6 was submitted to a RP18 column (l: 36 cm, d: 2 cm) and eluted with a methanol–water-mixture (3:7, v/v) which yielded three fractions (C1–C3) based on the TLC profile (RP18, MeOH/H₂O, 3:7, v/v), of which C1 (R_f 0.83) and C2 (R_f 0.80) were further purified by preparative HPLC. C1 was separated on column 3 using solvent system 1 (gradient 0–3.0 min, 8% B; 3.0–20.0 min, 8–32% B, 20.5–28.0 min, 50–70% B) and a flow rate of 1.8 mL/min to yield compound **23** (protocatechuic acid, 2.8 mg, R_t =16.60 min) and compound **26** (5-methoxy salicylic acid, 0.1 mg, R_t =21.95 min). C2 was purified on column 2 using solvent system 1 (gradient 0–2.5 min, 40% B; 2.5–22.5 min, 40–100% B) and a flow rate of 4.4 mL/min to yield compound **1** (mustak-14-oic acid, 0.6 mg, R_t =18.03 min).

A7 was purified by preparative HPLC (column 3, solvent system 1; gradient 0–3.0 min, 35% B; 3.0–23.0 min, 35–100% B, 23.0–30.0 min, 100% B; flow rate of 1.8 mL/min) to yield the compounds **15** (*E*-*p*-coumaric acid, 3.5 mg, R_t =16.60 min), **11** (rutin, 2.3 mg, R_t =17.86 min), **4** (verbenon-10-oic acid, 5.6 mg, R_t =19.23 min) and **10** (quercetin, 2.5 mg, R_t =21.71 min).

2.3.3 Spectral data

(1*R*,5*S*,6*S*,7*R*,10*R*)-Mustak-14-oic acid (**1**): colorless oil; $[\alpha]_D^{20} = -69$ (c 0.0001, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 202 (4.00), 225 (3.71), 277 (3.14); ¹H NMR (500 MHz, Methanol-*d*₄) and ¹³C NMR (obtained from HSQC and HMBC, Methanol-*d*₄) see Table 2; HR-ESI-MS (Orbitrap) *m/z* [M – H][–] 247.1338 (calc for C₁₅H₁₉O₃[–], 247.1329); MS²-Fragmentation (CE = –30 V) *m/z* 221 (8), 203 (100).

(1*R*,6*S*,7*S*,10*R*)-6-Hydroxy-anhuienosol (**2**): yellow oil; $[\alpha]_D^{20} = -17$ (c 0.0001, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 202 (3.87), 238 (3.63); ¹H NMR (500 MHz, Methanol-*d*₄) and ¹³C NMR (obtained from HSQC and HMBC, Methanol-*d*₄) see Table 3; HR-ESI-MS (TOF) *m/z* [M + FA][–] 313.1669 (calc for C₁₆H₂₅O₆[–], 313.1651); MS²-Fragmentation (CE = –20 V) *m/z* 313 (12), 267 (50), 209 (100), 191 (18), 121 (17).

2.4 Computational CD spectral analysis

The absolute configuration of compounds **1–6**, **8**, and **9** was determined by aligning experimental electronic circular dichroism (ECD) spectra with calculated CD spectra obtained by quantum chemical calculation. The initial 3D structures for the molecules, including all possible stereoisomers, were built using Molecular Operating Environment (MOE) 2020 software and the accompanying ChemDraw add-in. All structures and conformational analyses were executed utilizing the Amber10:EHT force field [21]. The energy minimum structures obtained from the force field were subsequently optimized employing the DFT method (B3LYP/def2-TZVP) [22–25] with the ORCA 5.0 program

package [26], incorporating the conductor-like polarizable continuum model (CPCM) solvent field [25] for methanol. For the optimized structures CD spectra were calculated via the ORCA software. The first 25 excited states for each compound and stereoisomers were computed using the TD B3LYP [22–25] with the def2-TZVP basis set [27] and CPCM model for methanol. The SpecDis software (version 1.71) [28] was employed for visualizing and contrasting the calculated spectra with the experimental data. The best similarity factor for the different stereoisomers was calculated and the one with the highest similarity factor was used for comparison at the same shift and sigma factor for a Gaussian band shape.

2.5 MS measurements

2.5.1 High resolution

The UHPLC-ESI-HRMS spectra of compound **2**, **3**, **5–9**, **12**, **14**, **18**, **20–23** and **26** were acquired using a TripleTOF (time of flight) 6600–1 mass spectrometer (Sciex, Darmstadt) in positive and negative ion modes. Samples (2 µL) were loaded on an Waters Acquity UPLC® BEH C18 column (1.7 µm, 130 Å, 50 × 2.1 mm I.D., Waters GmbH, Eschborn, Germany) under isocratic conditions (3% eluent B, 1 min), and separated using a linear gradient from 3 to 95% eluent B in 5 min. Separation was performed on an ACQUITY UPLC I-Class UHPLC System (Waters GmbH, Eschborn, Germany) with a flow rate of 0.4 mL/min and 55 °C column temperature. Eluents A and B were water and acetonitrile, with 0.1% formic acid. The mass spectrometer was equipped with an ESI-DuoSpray ion source (spray voltage: 5.5 kV (positive mode), 4.5 kV (negative mode), nebulizer gas: 60 psi, source temperature: 450 °C, drying gas: 70 psi, curtain gas: 55 psi) and was controlled by Analyst 1.7.1 TF software (Sciex, Darmstadt). Data acquisition was performed in MS1-TOF mode in a mass range of m/z 65 to 1250 with an accumulation time of 75 ms. The mass spectrometer was externally calibrated with calibration solutions provided by the manufacturer for positive and negative modes. For MS²-TOF mode the declustering potential was 35 V and the collision potential was 10 V, respectively. The product ion spectra (tandem mass spectra, MS/MS) were acquired in the high sensitivity mode (accumulation time 20 ms) in the m/z range of 50–1000 using unit Q1 resolution with mass resolution above 30,000. Collision potential (CE) was set from –80 to –20 V in negative ion mode and set from 20 to 80 V in positive ion mode, whereas collision energy spread (CES) was 15 V. The data were evaluated by Peak View 1.2.0.3 software (AB Sciex GmbH, Darmstadt, Germany).

The UHPLC-ESI-HRMS spectra of compounds **1**, **4**, **10**, **11**, **13**, **15–17**, **19**, **24** and **25** were acquired using an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), coupled with a UHPLC system (Dionex UltiMate 3000, Thermo Scientific) and a photodiode array detector (PDA, Thermo Scientific). The spectra as well as the collision-induced dissociation mass spectra (CID, collision induced dissociation) were recorded in positive and negative ion mode. Samples (3 µL) were loaded on an Waters Acquity UPLC® BEH C18 column (1.7 µm, 130 Å, 50 × 2.1 mm I.D., Waters GmbH, Eschborn, Germany) under isocratic conditions (5% eluent B, 1 min), and separated using a linear gradient from 5 to 95% eluent B in 10 min. Separation was performed on an ACQUITY UPLC I-Class UHPLC System (Waters GmbH, Eschborn, Germany) with a flow rate of 0.4 mL/min and 40 °C column temperature. Eluents A and B were water and acetonitrile, with 0.1% formic acid. Ionization was carried out by electrospray ionization (HESI ion source; spray voltage 4.0 kV in positive ion mode, 3.5 kV in negative ion mode; nebulizing and auxiliary gas: nitrogen; evaporation temperature: 300 °C; capillary temperature: 325 °C, FTMS resolution Full MS: 30,000; MSn: 15,000). The CID mass spectra were recorded automatically using a data-dependent fragmentation mode and a normalized collision energy (NCE) of 35%. The mass spectrometer was externally calibrated with calibration solutions provided by the manufacturer for the negative and positive modes. The spectra were analyzed using Xcalibur 2.2 SP1 software (Thermo Fisher Scientific).

The MSⁿ high-resolution electrospray mass spectra in negative ion mode of kumatakenin (**13**) were acquired using an Orbitrap Elite mass spectrometer (ESI ion source: spray voltage 4.0 kV, capillary temperature 275 °C, nebulizing gas: nitrogen, FTMS resolution 30,000). The sample solution was continuously injected using a syringe pump (flow rate: 5 µL/min). The mass spectrometer was externally calibrated (Pierce® ESI negative ion calibration solution, Product No. 88324, Thermo Fisher Scientific, Rockford, IL, 61,105 USA). The spectra were analyzed using Xcalibur 2.7 SP1 software. The CID-MSⁿ measurements were acquired with relative collision energies: 25% for MS² of m/z 313 in the m/z range of 85–350, 25% for MS³ of m/z 298 in the m/z range of 80–325, 30% for MS⁴ of m/z 283 in the m/z range of 75–300, 35% for MS⁵ of m/z 255 in the m/z range of 75–300.

2.5.2 Headspace GCMS

The headspace GCMS method was adopted from Farhadi et al. [29] with modifications. Ground leaf powder (0.5 g) was submitted into 20 mL headspace vials and then incubated at 100 °C for 30 min with constant shaking in a headspace assembly of CTC GC PAL Injector (Shimadzu Deutschland GmbH, Duisburg, Germany). 500 µL head space of each sample were injected into a ZB-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Aschaffenburg, Germany) and analyzed by gas chromatography-electron ionization-quadrupole-mass spectrometry (GC-EI-Q-MS) using a GC2010 gas chromatograph coupled online to a quadrupole mass selective detector Shimadzu GCMS QP2010. The temperature program started at 50 °C kept constant for 5 min, then a ramp of 3 °C/min to 140 °C followed by the second ramp of 10 °C/min to 240 °C and finally kept constant for 10 min. MS detection was performed in positive ion mode in m/z range 40–500 Da. Analysis was performed under the control of GCMS Real Time Analysis software (Shimadzu Deutschland GmbH, Duisburg, Germany). The compounds were annotated using the NIST17 library.

2.5.3 GC–MS of essential oil and *n*-hexane extract

To obtain essential oil, dried leaf powder (10 g) of *H. scyphifera* was subjected to hydrodistillation in a Dean Stark Apparatus for 12 h adding 400 mL deionized water into a 1000 mL flask. The pale-yellow essential oil (1.2 mg) was picked using a micro pipette (10 µL, BLAUBRAND, Wertheim, Germany), transferred to a vial and stored in the fridge.

To obtain the *n*-hexane extract, dried leaf powder (8 g) of *H. scyphifera* was subjected to a cellulose thimble and transferred to a Soxhlet extractor. The extractor was filled with 150 mL of *n*-hexane and heated under reflux for 3 h. Afterwards, the solvent was carefully removed using rotary evaporator.

Samples (1 µL) were injected on ZB-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness, Phenomenex, Aschaffenburg, Germany) and analyzed by gas chromatography-electron ionization-quadrupole-mass spectrometry (GC-EI-Q-MS) using a GC2010 gas chromatograph coupled online to a quadrupole mass selective detector Shimadzu GCMS QP2010. The temperature program was starting at 50 °C kept constant for 5 min, then a ramp 10 °C/min to 120 °C followed by the second ramp 5 °C/min to 300 °C and kept constant for 15 min. MS detection was performed in positive ion mode in m/z range 40–600 Da. The compounds were annotated using the NIST17 library.

2.6 X-Ray analysis

Single crystals of compound **13** (Kumatakenin, C₁₇H₁₄O₆) were obtained by slow evaporation of the solvent from a concentrated methanolic solution in the cold. A suitable crystal was selected and fixed on a STOE IPDS II diffractometer. The crystal was kept at 170 K during data collection. Using Olex2 [30], the structure was solved with the SHELXT [31] structure solution program and refined with the SHELXL [32] refinement package using Least Squares minimization.

2.7 Biological assays

2.7.1 Antibacterial Assays

The compounds were evaluated against the Gram-negative *Aliivibrio fischeri* (DSM507) and the Gram-positive *Bacillus subtilis* 168 (DSM 10), as described by Kappen et al. [33] and Manurung, Kappen et al. [34]. The tests were performed in 96-well plates based on the bioluminescence (*A. fischeri*) or absorption (*B. subtilis*) read-out. Chloramphenicol (100 µM) was used as a positive control to induce complete inhibition of bacterial growth. The results (mean ± standard deviation value, $n = 6$) are given in relation to the negative control (bacterial growth, 1% DMSO without test compound) as relative values (percent inhibition). Negative values indicate an increase in bacterial growth.

2.7.2 Antifungal Assay

The antifungal activity was tested on the phytopathogenic ascomycetes *Botrytis cinerea* Pers. and *Septoria tritici* Desm. and the oomycete *Phytophthora infestans* (Mont.) de Bary in 96-well microtiter plate assays according to protocols from the Fungicide Resistance Action Committee (FRAC) with minor modifications as described by Ware et al. [35, 36]. Briefly, the isolated compounds were tested at the highest concentration of 500 µg/mL, while the solvent DMSO in buffer was used as a negative control (max. concentration 2.5%). The commercially used fungicides, epoxiconazole and terbinafine

(Sigma-Aldrich, Darmstadt, Germany), served as reference compounds. The pathogen growth was evaluated seven days after inoculation by measurement of the optical density (OD) at L 405 nm with a TecanGENios Pro microplate reader (five measurements per well using multiple reads in 3 × 3 square). Each experiment was carried out in triplicate.

3 Results and discussion

3.1 Characterization of crude extracts and volatiles

A first phytochemical investigation of the leaves from *H. scyphifera* involved multiple methodologies. An exhaustive extraction with methanol was performed to yield a crude extract, which was characterized by ultra high-performance liquid chromatography high-resolution mass spectrometry analysis (UHPLC-ESI-HRMS). Additionally, the volatile compounds were screened on one hand by submitting ground plant material to a headspace gas chromatography-mass spectrometry-system (HS-GCMS) (Table S1) and on the other hand by extracting essential oil and a *n*-hexane extract from leaves to compare and verify results with published data (Table S2). Further, the crude extract was submitted to liquid-liquid partitioning and resulting fractions were screened by TLC and for bioactivity in selected assays.

The findings of the HS-GCMS analysis are largely consistent with existing reports on the characterization of the essential oils of *H. scyphifera*. Specifically, the main peaks were identified as α -pinene, camphene, *p*-cymene, and camphor, as reported by Hashim et al. in 2018 [17]. Notably, the presence of butylbenzene (Table S1, H6), constituting 18% of the composition, appears to be previously unreported based on available literature.

Analysis via GC-MS facilitated the tentative annotation of 71 compounds in total, while 49 are abundant in the essential oil (EO) and 24 compounds in the *n*-hexane extract from the leaves. Quantitatively relevant compounds in the EO included *trans*-dihydroagarofuran (14.91%, Table S2, G17), α -caryophyllene (6.77%, Table S2, G36) as well as *cis*-verbenol acetate (5.54%, Table S2, G53) and (*E*)-15,16-dinorlabda-8(17),11-dien-13-one (3.49%, Table S2, G48). The latter was previously isolated from the rhizome of *Globba schomburgii* by Suekaew et al. [37] but here is annotated for the first time in the EO of *H. scyphifera*. In the *n*-hexane fraction, *cis*-verbenone (13.49%, Table S2, G8), camphor (3.95%, Table S2, G4), camphene (4.23%, Table S2, G2), and α -pinene (1.49%, Table S2, G1) were the most abundant compounds. Most compounds identified through headspace analysis were also present in the *n*-hexane extract but were absent in the essential oil (EO). This might be attributed to the high volatility of these compounds, potentially resulting in incomplete condensation during EO distillation.

TLC screening of the crude extract and fractions from liquid-liquid-partitioning showed the highest variety of spots for the *n*-heptane and ethyl acetate fraction, corresponding to individual compounds (Fig. 1, lane 3). This indicated a complex matrix for these extracts suggesting challenging isolation and characterization. The TLC profile of the *n*-heptane fraction as well as the water insoluble residue of the crude extract (Fig. 1, 366 nm, lane 4 and 5) showed both bright yellow spots attributed to compounds **9** and **14** and were therefore combined for further isolation. Both the *n*-heptane and the ethyl

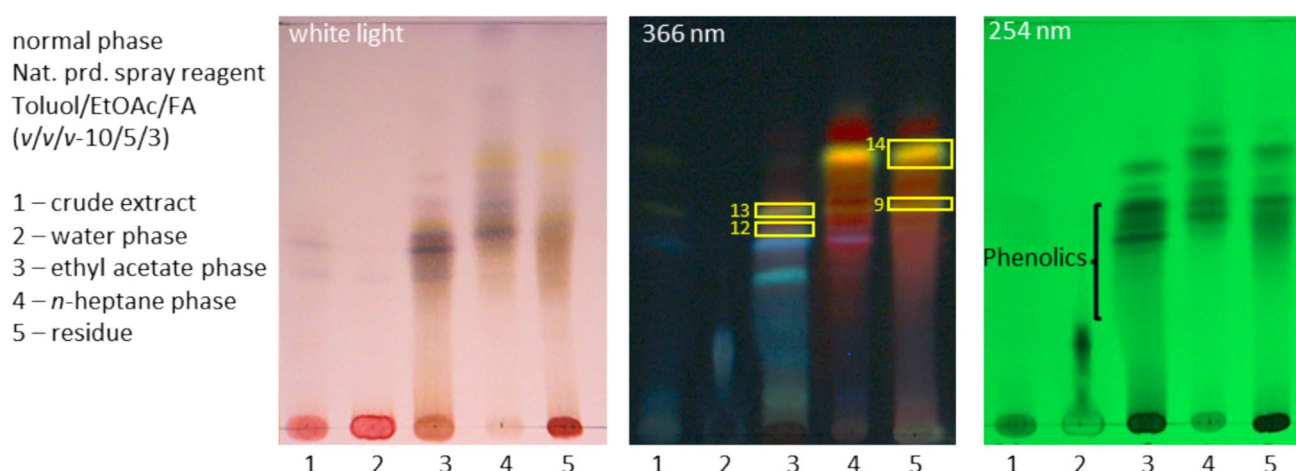


Fig. 1 TLC of crude extract and fractions from liquid-liquid-partitioning of dried leaves after derivatization with natural product reagent. Numbered boxes correspond to isolated compounds

acetate fractions exhibited strong activity against the Gram-negative bacterium *Allivibrio fischeri* (complete inhibition at a concentration of 500 µg/ml) (Fig. S1.1.B) and were therefore considered for further isolation. All fractions inhibited the growth of the phytopathogenic fungus *Botrytis cinerea* (Fig. S1.2.B).

The UHPLC-HRMS analysis allowed for the annotation of 27 individual metabolites from the total ion chromatogram (TIC) of the crude extract (Table 1 and Fig. 2). The most intense signal at R_t 8.2 min (overlapping peaks P22 and P23) was assigned to the previously unknown mustak-14-oic acid (**1**) and the flavonoid kumatakenin (**13**). Further dominating signals could be attributed to ferulic acid derivatives (P9 and P11), the flavonol glycoside rutin (**11**) (P13) and the flavonoid isokaempferide (**12**) (P19). Most signals were assigned to compounds that could also be isolated and identified within the scope of this work. However, not all isolated compounds were detected in the crude extract. This could be due to matrix effects leading to ion suppression of individual substances in the complex crude extract, low solubility in the applied solvent methanol or low substance concentration. Since two thirds of the crude extract mass is allocated to the aqueous fraction, a relatively low concentration of nonpolar and middle polar compounds in the crude extract can be expected. In contrast, the compounds behind other detected peaks were not obtained by isolation, as e.g. P9 and P11 (Table 1 and Fig. 2), which exhibited identical mass and MS^2 spectra and were thus attributed to *cis* and *trans* ferulic acid derivatives. This is supported by the isolation of both *trans*- and *cis*-ferulic acid (**16** + **17**). In conclusion, *H. scyphifera* possesses a multitude of metabolites, the majority of which can be classified into the three groups of (1) terpenes and terpenoids, (2) flavonoids and (3) other phenolics.

3.2 Purification and structure elucidation

The ethyl acetate and the *n*-heptane fraction obtained from successive liquid–liquid partition were further purified by different chromatographic techniques. This resulted in the isolation of 26 compounds (Fig. 3). Beside the two previously undescribed compounds mustak-14-oic acid (**1**) and 6-hydroxy-anhuienosol (**2**), the remaining 24 compounds were identified as (*E*)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid (**3**) [38, 39], (1*S*,5*R*)-verbenon-10-oic acid (**4**) [40, 41], *cis*-pinonic acid (**5**) [42, 43], *cis*-pinonic acid (**6**) [44], 2-oxo-3-4,5,5-trimethylcyclopentynyl acetic acid (**7**) [45, 46], epicatechin (**8**) [47, 48], (2*S*)-sakuranetin (**9**) [49, 50], quercetin (**10**) [51], rutin (**11**) [52, 53], isokaempferid (**12**) [54], kumatakenin (**13**) [19, 55], 5-hydroxy-3,7,4'-trimethoxyflavone (**14**) [19, 56], (*E*)-*p*-coumaric acid (**15**) [57], *trans*-ferulic acid (**16**) [58], *cis*-ferulic acid (**17**) [58], evofolin B (**18**) [59], 3,4-dihydroxybenzaldehyde (**19**) [60], vanillin (**20**) [61, 62], syringaldehyde (**21**) [63], *p*-hydroxybenzoic acid (**22**) [64], protocatechuic acid (**23**) [65], vanillic acid (**24**) [66], protocatechuic acid methyl ester (**25**) [67, 68] and 5-methoxy-salicylic acid (**26**) [69, 70], after comparison with NMR data from literature. Further, spectroscopic data, including NMR and MS for compounds **1** to **26** can be found in the supporting information. The absolute configuration of compounds **1**, **2**, **3**, **4**, **5**, **6**, **8**, and **9** were verified by comparison of experimental and calculated CD spectra (Fig. 4 and 7; Fig. S5–S10; Tables S5, S8–S14). To the best of our knowledge, this is the first report on isolation and identification of compounds **3–9** and **11**, **12**, **15–26** from *H. scyphifera*.

Interestingly, the monoterpene derivatives **5**, **6** and **7** so far were never isolated from a plant source. Compounds **5** and **6** were previously obtained as synthetic oxidation products from α -pinene [42, 44] while **7** was described as degradation product of camphor, induced by plant pathogens [46]. Both potential precursors were abundant in the GC analysis (H4 and H19 in Table S1; G1 and G4 in Table S2). At this point, the biological origin of **5**, **6** and **7** in *H. scyphifera* is unknown. The compounds might be derived from plant biosynthesis or by involvement of associated microorganisms. Similarly, a biosynthetic connection of the isolated (*E*)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid (**3**) and (*E*)-15,16-dinorlabda-8(17),11-dien-13-one (G48, Table S2) detected in GC–MS experiments might be expected due to their structural similarity.

Compound **1** was isolated as a colorless oil in a poor yield of 0.6 mg from 92.0 g of dried material, which may be attributed to its volatile nature and the drying methods employed, e.g. rotatory evaporation and lyophilization. The molecular formula was determined as $C_{15}H_{20}O_3$ from the molecular ion peak at m/z 247.1338 [$M - H$][−] (calcd. for 247.1329), obtained from the HR-ESI–MS spectra. The ^1H NMR spectra (Table 2) displayed a single signal for a proton attached to a conjugated double bond (δ_H 6.26, dd, brt, $J = 1.6$ Hz, H-3), three signals for protons in central positions (δ_H 2.72, brs, H-6; δ_H 2.69 dd, $J = 6.6, 1.3$ Hz, H-1; δ_H 2.66 dd, $J = 6.6, 0.9$ Hz, H-5), followed by two multiplets encompassing six overlapping signals, later resolved by HSQC (δ_H 1.69–1.95, m, H-7, H-8b, H-9a, H-9b; δ_H 1.50–1.66, m, H-8a, H-11). Additionally, three signals annotated to CH_3 groups emerged, with two displaying the same coupling constants, indicating an isopropyl group (δ_H 0.98, s, H-15; δ_H 0.89, d, $J = 6.8$ Hz, H-13; δ_H 0.87, d, $J = 6.8$ Hz, H-12). Due to quantitative limitations, no ^{13}C NMR spectra could be acquired. Therefore, ^{13}C shifts were obtained from 2D NMR experiments, HSQC and HMBC, which confirmed the presence of all fifteen carbon atoms postulated by HRMS (Table 2). These included a carbonyl group signal (δ_C 209.0, C-2), signals indicative of a double bond (δ_C 124.3 for a CH group, C-3 and δ_C 168.3 for a quaternary carbon, C-4), and a

Table 1 Metabolites annotated in methanolic leaf extract from *H. scyphifera* by reversed phase ultra-high-performance chromatography-tandem mass spectrometry (RP-UHPLC-MS/MS) on Orbitrap

Peak	t _R (min)	m/z measured [M–H] [–]	Error (ppm)	Molecular formula*	MS ² product ions m/z (rel. intensity [%])	Annotation
P1	0.26	341.1086	– 1.0	C ₁₂ H ₂₂ O ₁₁	341.1086 (100), 215.0328 (14)	Sucrose
P2	0.63	167.0350	0.2	C ₈ H ₈ O ₄	123.0454 (100)	Vanillic acid (24)
P3	0.72	167.0351	0.8	C ₈ H ₈ O ₄	123.0454 (100)	Protocatechuic acid methyl ester (25)
P4	0.82	153.0194	0.6	C ₇ H ₆ O ₄	109.0297 (100)	3,4-Dihydroxybenzoic acid (23)
P5	1.38	137.0246	1.0	C ₇ H ₆ O ₃	119.0252 (100), 93.0349 (52), 66.0352 (62)	p-Hydroxybenzoic acid (22)
P6	1.51	137.0245	0.7	C ₇ H ₆ O ₃	119.0252 (10), 110.0251 (9), 93.0348 (100), 66.0354 (8)	3,4-Dihydroxybenzaldehyde (19)
P7	2.19	151.041	0.5	C ₈ H ₈ O ₃	–	Vanillin (20)
P8	3.79	577.1339	– 1.3	C ₃₀ H ₂₆ O ₁₂	559.1238 (7), 451.103 (34), 425.0874 (100), 407.0767 (42), 289.0716 (19)	Procyanidin (Epicatechin dimer)
P9/	4.03/	399.0928	– 1.2	C ₁₇ H ₂₀ O ₁₁	381.0824 (7), 223.0459 (51), 205.0353 (100), 187.0248 (3), 129.0195 (15), 111.0090 (3)	Ferulic acid derivatives, e.g (E) and (Z)-Feruloyl-4-O-methoxyglucaric acid
P10	4.24	289.0716	– 0.6	C ₁₅ H ₁₄ O ₆	245.0819 (100), 205.0506 (32), 179.0351 (15)	Epicatechine (8)
P12	4.99	163.0402	0.57	C ₉ H ₈ O ₃	–	(E)-p-Coumaric acid (15)
P13	5.15	609.1453	– 1.4	C ₂₇ H ₃₀ O ₁₆	301.035 (100)	Rutin (11)
P14	5.38	179.0714	0.1	C ₁₀ H ₁₂ O ₃	135.0817 (100)	15,5 <i>R</i> -Verbenon-10-oic acid (4)
P15	5.69	187.0976	0.1	C ₉ H ₁₆ O ₄	125.0973 (100)	unknown
P16	6.24	301.0352	– 0.6	C ₁₅ H ₁₀ O ₇	273.0768 (100)	Quercetin (10)
P17	6.60	181.0870	0.1	C ₁₀ H ₁₄ O ₃	–	2-(2,2,3-Trimethyl-5-oxocyclopent-3-en-1-yl) acetic acid (7)
P18	6.67	183.1026	– 0.2	C ₁₀ H ₁₅ O ₃	–	2-(3'-Acetyl-2,2'-dimethyl-cyclobutyl) acetic acid (6)
P19	6.98	299.0559	– 0.9	C ₁₆ H ₁₂ O ₆	284.0326 (100)	Isokaempferid (12)
P20	7.18	431.1707**	– 1.0	C ₂₃ H ₂₈ O ₈	371.1498 (100)	Diarylheptanoid derivative, e.g. Tetrahydrocurcumin
P21	8.00	267.1601	– 0.3	C ₁₅ H ₂₄ O ₄	–	6-Hydroxy-anhuienosol (2)
P22	8.22	247.1339	– 0.4	C ₁₅ H ₂₀ O ₃	203.1441 (100)	Mustak-14-oic acid (1)
P23	8.25	313.0717	– 0.3	C ₁₇ H ₁₄ O ₆	298.0481 (100)	Kumatakenin (13)
P24	9.17	287.2015	– 0.4	C ₁₉ H ₂₈ O ₂	259.2067 (100)	Sterol derivative
P25	9.81	333.2069	– 0.8	C ₂₀ H ₃₀ O ₄	289.2172 (100), 271.2067 (43)	Diterpenoid, e.g. Agathic acid [71]
P26	10.63	555.2840	5.3	C ₂₈ H ₄₄ O ₁₁	–	Diterpenoid glycoside, e.g. Viteoside A [72]
P27	10.96	823.4689	–	–	–	unidentified

* Refers to non-ionized compound

**[M+CH₃COO][–]

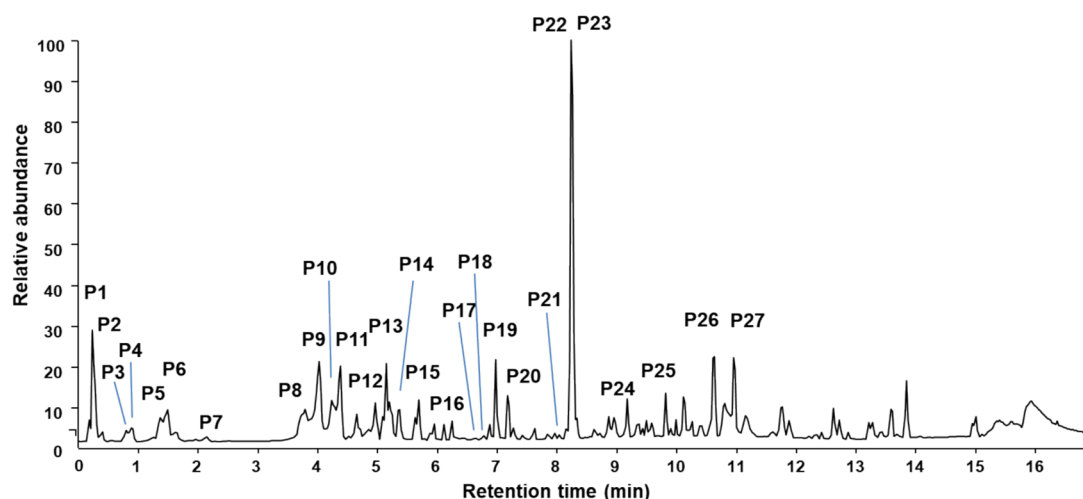


Fig. 2 Total ion chromatogram (TIC), acquired from the methanolic extract obtained from leaves of *H. scyphifera*. Please note that peak numbers are not identical to compound numbers. See Table 1 for assignment

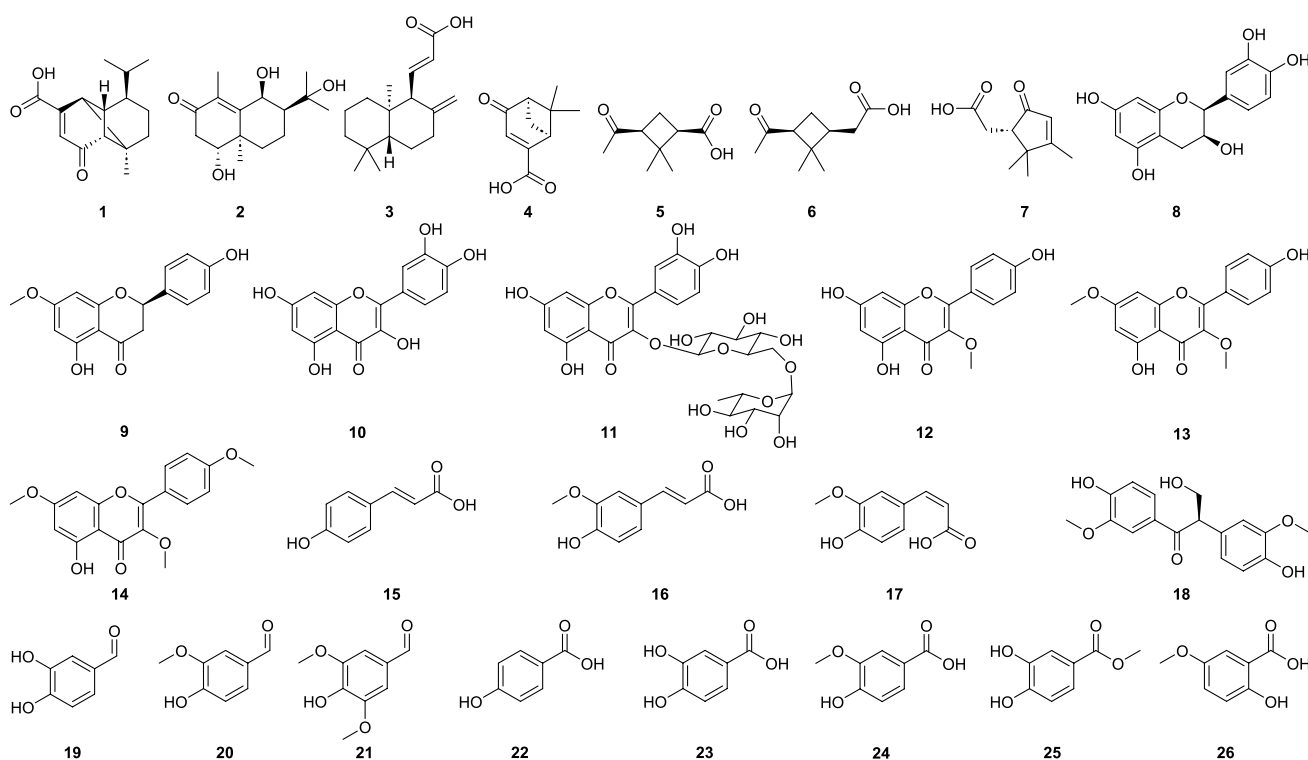


Fig. 3 Structures of all compounds isolated from *H. scyphifera* in this work

carboxylic group signal (δ_C 173.8, C-14). Additional signals observed comprised three CH_3 groups (δ_C 20.8, C-15; δ_C 20.2, C-12 and δ_C 19.9, C-13), two CH_2 groups (δ_C 37.9, C-9 and δ_C 23.1, C-8), five more CH groups (δ_C 58.8, C-6; δ_C 57.0, C-1; δ_C 53.5, C-5; δ_C 46.7, C-7 and δ_C 33.2, C-11), and one more quaternary carbon (δ_C 59.8, C-10). All 1D and 2D NMR spectra are shown in the supporting information (Fig. S2.1-S2.7).

Altogether, the NMR dataset of **1** exhibits a significant resemblance to a cadinane-type sesquiterpene [73] also found in the volatile fraction of *H. scyphifera*. However, the numerous strong HMBC correlations of H-5 and H-6 indicate an intramolecular third ring in accordance with a copaene skeleton [74]. One known copaene, trivially named mustakone, comprises NMR data with high similarity to **1**. This compound has been first isolated from the essential oils of *Cyperus*

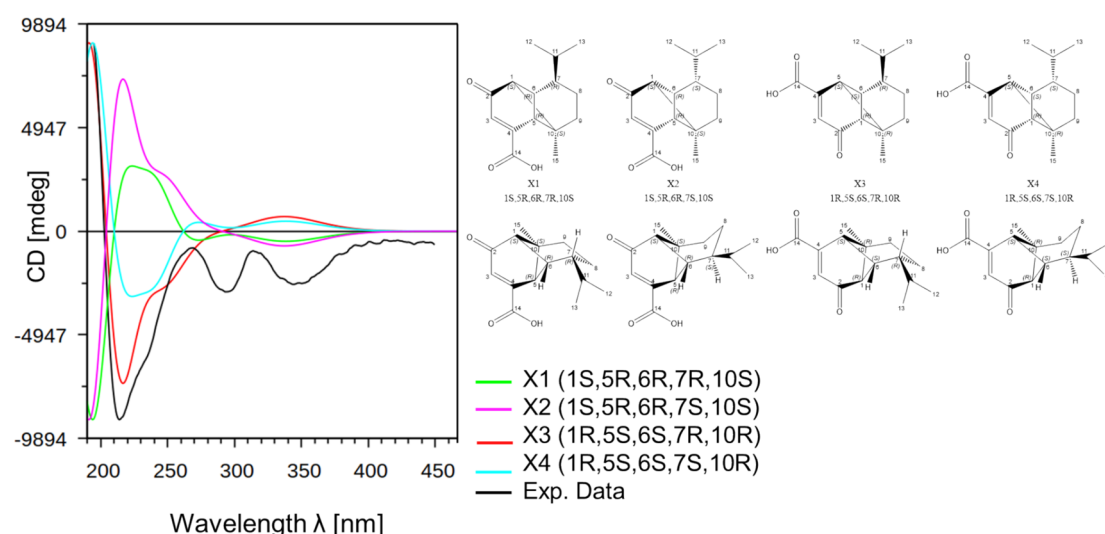


Fig. 4 CD spectrum and calculated spectra of the four stereoisomers X1-X4 of compound **1**

Table 2 NMR data of compound **1** (MeOD, 500 MHz, 25 °C)

No	δ_c^a	δ_H (multiplicity, J)	COSY	NOESY	HMBC
1	57.0, CH	2.69 (dd, 6.6, 1.3 Hz, 1H)	3, 5	8a, 12, 15	2, 3, 5
2	209.0, C				
3	124.3, CH	6.26 (brt, 1.6 Hz, 1H)	1, 5		1, 5, 14
4	168.3, C				
5	53.5, CH	2.66 (dd, 6.6, 0.9 Hz, 1H)	1, 3	7, 9a, 15	1, 3, 4, 6, 7, 9
6	58.8, CH	2.72 (brs, 1H)		7, 11, 12	2, 4, 5, 8, 10, 11, 15
7	46.7, CH	1.77 (m, 1H)	8a, 8b, 11	5, 8a, 13	1, 5, 12
8	23.1, CH ₂	a) 1.61 (m, 1H) b) 1.83 (m, 1H)	7, 8b, 9a, 9b 7, 8a, 9a	1, 8b, 9b, 12 5, 8a, 13	10
9	37.9, CH ₂	a) 1.86 (m, 1H) b) 1.90 (m, 1H)	8a, 8b, 9b 8a, 8b, 9a	5, 8a, 15 1, 8a, 15	5, 10 7
10	59.8, C				
11	33.2, CH	1.55 (m, 1H)	7, 12, 13	6, 8b, 12, 13	12, 13, 8, 7, 6
12	20.2, CH ₃	0.87 (d, 6.8 Hz, 3H)	11	1, 6, 11	13, 11, 7
13	19.9, CH ₃	0.89 (d, 6.8 Hz, 3H)	11	7, 11	7, 11, 12
14	173.8, C				
15	20.8, CH ₃	0.98 (s, 3H)		1, 5, 9a, 9b	1, 5, 9, 10

^a obtained from HSQC and HMBC

rotundus L., an Indian nutgrass variety known as Mustaka in Sanskrit language, by Kapadia et al. in 1963 [75]. To date, it has only been found in five additional species, none of which belong to the Zingiberaceae family [76]. In this study, we could detect mustakone (G27, Table S2) in the *n*-hexane extract by GCMS. The structure of mustakone was primarily determined through chemical modification in combination with IR and ¹H NMR [75], and a strong similarity with verbenone was noted. Full NMR data were later provided by Nyasse et al. [77]. Direct comparison of NMR data from compound **1** and mustakone revealed a missing CH₃ group in **1** (at δ_H 2.02 and δ_C 20.2), as well as an additional significantly shifted carbon signal at 173.8 ppm. The molecular formula of **1** includes two additional oxygen atoms compared to mustakone, implying carboxylation of the methyl group at position 14. This is notable, as a carboxylated derivative of verbenone, (1*S*,5*R*)-verbenone-10-oic acid (**4**), was also isolated, bearing the carboxyl function at the same position instead of methylation.

The cyclobutane ring in **1** exhibits increased ring strain due to the carbon-carbon bonds being required to adopt angles close to 90°, rather than the typical 109.5° angle of *sp*³ hybridized carbon in a tetrahedral configuration. This results in a highly rigid structure showing unique H-H couplings. A strong COSY coupling as observed between H-1 and

H-5 ($J=6.6$ Hz) can occur in case of a sterically fixed M or W-shaped arrangement of the coupling nuclei and is typical for $^4J_{HH}$ coupling of *cis* protons situated as bridgehead protons on the opposite side of cyclobutane rings [78, 79]. In contrast, COSY long-range couplings in saturated systems over more than 3 bonds typically have a J value of <1 Hz and are therefore often undetected.

In **1** the signals of both bridgehead protons H-1 (δ_H 2.69, dd, 6.6, 1.3 Hz) and H-5 (δ_H 2.66 dd, $J=6.6, 0.9$ Hz) appear consistent with the data from Nyasse et al. [77] on mustakone as doublet of doublets (dd) due to the long-range coupling to H-3. H-3 appears as a pseudo broad triplet with a J value of 1.6 Hz, resulting from the overlaying effect of the theoretically possible doublet of doublet splitting. In contrast, H-6 appears like in mustakone as a broad singlet which implies in accordance with the Karplus curve an approximate angle of 90° between H-6 and all three neighboring protons H-1, H-5 and H-7. While this may pose a challenge for structure elucidation, it provides key information for the relative configuration of these compounds. Mustakone, like **1**, features 5 stereocenters, which theoretically leads to 32 possible stereo isomers. However, due to the intramolecular cyclobutane ring, the associated steric constraints, and the multiplicity of H-6, only 4 possible stereo isomers remain after geometrical optimization (Fig. 4, X1–X4). A Minimize Energy MM2 calculation in Chem3D to optimize the spatial structure of the stereo isomers, followed by angle determination and calculation of theoretical coupling constants, did not lead to the exclusion of any of the possible stereoisomers (Table S3).

For further determination of the relative stereochemistry, NOESY data were employed (Table 2, Fig. 5). Generally, NOESY coupling is possible for protons at distances less than 4 Å apart. Distances between selected protons and crucial NOESY couplings of compound **1** are shown in Fig. 5. Respective values of all four stereoisomers of **1** are summarized in Table S4. The observed strong NOESY correlation between H-1 and the isopropyl methyl group H₃–12 (distance of 2.6 Å, Table S4) can be only explained by the relative configuration of the enantiomers X2 (possessing 1*S*,5*R*,6*R*,7*S*,10*S*-configuration) or X3 (possessing the 1*R*,5*S*,6*S*,7*R*,10*R*-configuration). To determine the absolute configuration, the CD spectrum of **1** was recorded and compared with calculated CD spectra of the possible enantiomers (Fig. 4), which verified X3 unequivocally as the correct structure. Thus, the new compound **1** has the absolute 1*R*,5*S*,6*S*,7*R*,10*R*-configuration and was given the trivial name mustak-14-oic acid. The absolute stereochemistry of **1** is in agreement with that of (–)-mustakone, as described by Kapadia et al. [80].

Compound **2** was isolated as yellow oil in a yield of 2.7 mg. The molecular formula was determined as C₁₅H₂₄O₄ from the formic acid adduct of the molecular ion peak at m/z 313.1669 [$M + FA$][–] (calcd. for C₁₆H₂₆O₆, 313.1651), obtained from the HR-ESI–MS spectra. The analysis of 1D and 2D NMR spectra (Fig. S2.1–S2.7.) implied that the basic structure of compound **2** is sesquiterpene-like and especially close to the structure of anhuienosol (C₁₅H₂₄O₃) by comparison with literature data [81–83] (Table 3, Table S8). However, the CH₂ group present in anhuienosol at position 6 (δ_C 28.6, δ_H 2.91 (brd, $J=13.7$ Hz) + 1.94 (1H, br, $J=13.7$ Hz)) (Table S8) [83] in **2** is replaced by an oxygenated CH group (δ_C 68.05, δ_H 5.26 (d, $J=2.7$ Hz, 1H) implying a hydroxylation at this position. With four stereocenters present in this molecule, theoretically, there could exist 16 possible stereoisomers. None of these can be eliminated based on geometric limitations. Nevertheless, targeted exclusion of isomers through NOESY correlations is attainable. The protons of the methyl group at position 14 (H₃–14) exhibit NOESY correlations to only one hydrogen of each CH₂ group at positions 2, 8, and 9, indicating the same spatial alignment of the respective protons 2a, 8a and 9a. Thus, the absence of NOESY correlation between H₃–14 and H-1 necessitates an orientation in the opposite direction. This is further supported by NOESY correlations between H-1 and H-9b. Therefore, only either 1*R*,10*R* or 1*S*,10*S* configuration is possible. With two additional unspecified stereocenters at positions 6 and 7, eight potential structures remain (Y1–Y8, Fig. S4, Table S6). Following geometric optimizations and MM2 minimize energy calculations, 3D structures of all stereoisomers were created to measure the distances of protons and to compare with observed NOESY correlations. The H₃–15 methyl group appears relatively

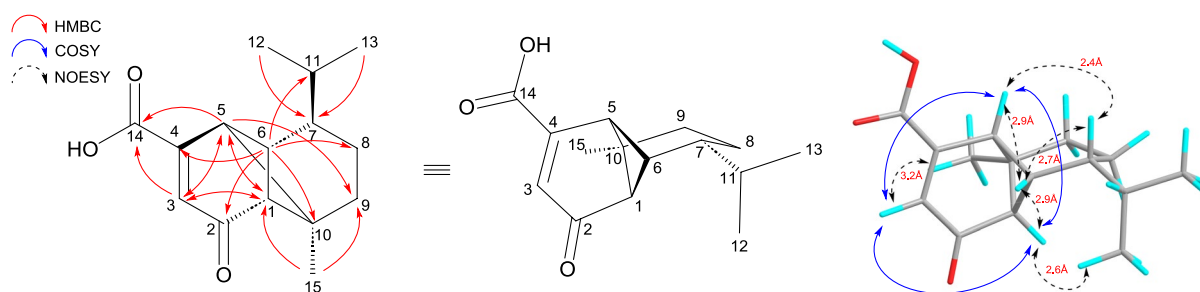


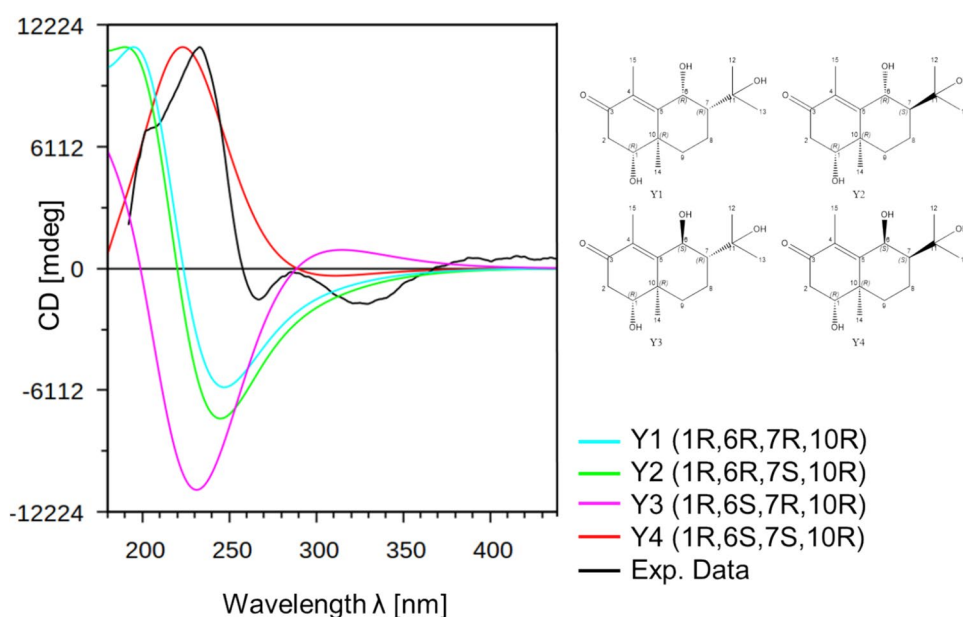
Fig. 5 2D NMR correlations for X3/compound **1**

Table 3 NMR data of compound **2** (MeOD, 500 MHz, 25 °C)

No	δ_C^a	δ_H (multiplicity, J)	COSY	NOESY	HMBC ^b
1	75.2, CH	3.66 (dd, 5.1, 13.1 Hz, 1H)	2a, 2b	2a, 2b, 9b	2, 9, 10, 14
2	43.0, CH ₂	a) 2.60 (dd, 13.1, 16.7 Hz, 1H) b) 2.52 (dd, 5.1, 16.7 Hz, 1H)	1, 2b 1, 2a	1, 2b, 14 1, 2a	1, 3, 10 1, 3, 4, 10
3	201.1, C				
4	132.1, C				
5	161.8, C				
6	68.0, CH	5.26 (d, 2.7 Hz, 1H)	7	7, 13, 15	4, 5, 7, 8, 10, 11
7	50.7, CH	1.30 (m, 1H)	6, 8a, 8b	6	8, 9, 13
8	16.6, CH ₂	a) 2.02 (m, 1H) b) 1.70 (m, 1H)	7, 8b, 9b 8a	8b, 14 8a, 9a, 12	6, 7, 9 6, 7, 9, 10, 11
9	38.3, CH ₂	a) 2.15 (m, 1H) b) 1.25 (m, 1H)	9b 8a, 9a	9b 1, 9a	5, 7, 8, 10, 14 8, 110, 14
10	41.5, C				
11	73.8, C				
12	27.9, CH ₃	1.27 (s, 3H)		8b, 13	7, 11, 13
13	28.2, CH ₃	1.38 (s, 3H)		6, 7, 12	7, 11, 12
14	17.7, CH ₃	1.31 (s, 3H)		2a, 8a, 9a	1, 5, 9, 10
15	9.9, CH ₃	1.85 (s, 3H)		6	3, 4, 5, 10

^a obtained from HSQC and HMBC, ^b600 MHz, 160 scans

isolated and only displays NOESY correlation with H-6. Notably, the orientation of H-6 is irrelevant in this context, as the distance to H₃–15 is less than 3 Å in all stereoisomers (2.0–2.4 Å, Table S6). In addition, H-6 shows NOESY correlations with H-7 (2.3–3.0 Å in all stereoisomers, Table S6) and H₃–13 (2.1–2.3 Å in all stereoisomers, Table S6), but none with any of the ring-bound protons H-1, H-8a/b or H-9a/b. Therefore, an equatorial rather than an axial position for H-6 is suspected. A comparison of distances between H-6 and all ring-bound protons for all stereoisomers revealed that only the enantiomers Y4/Y8 have consistently distances of > 4 Å and the expected equatorial position for H-6 and thus possess the correct relative configuration (Table S6). The elucidation of the absolute stereochemistry was done by comparison of experimental CD spectra with the calculated CD spectra of the possible stereoisomers (Fig. 6), which verified Y4 with the absolute 1*R*,6*S*,7*S*,10*R* configuration unequivocally as correct structure for the here isolated compound **2**, trivially named 6-hydroxy-anhuienosol (Fig. 7).

Fig. 6 CD spectra + calculated spectra of stereoisomers Y1–Y4 of compound **2**

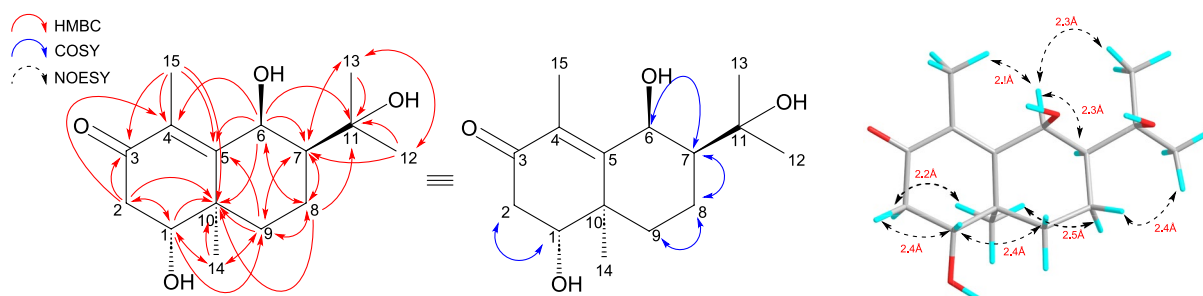


Fig. 7 2D NMR correlations for Y4/compound **2**

Beside terpenoids and simple phenolics, flavonoids constitute a major compound class of *H. scyphifera* with kumatakenin (**13**) as main representative. In addition to structure identification by NMR data, (SI, Spectroscopic data of isolated compounds, No. 13), we describe here for the first time the structure of kumatakenin by X-ray crystallography. The X-ray analysis was performed on a crystal with the size of $0.295 \times 0.029 \times 0.015$ mm (Fig. S11). It is a monoclinic crystal that shows a $P2_1/c$ space group. From the data it was possible to obtain all atom bonding which results in verification of the molecular structure (Fig. 8). Nevertheless, due to the small dimension of the crystal, only a weak dataset could be achieved, which is visible among others in the Goof of 0.863 (Table S15).

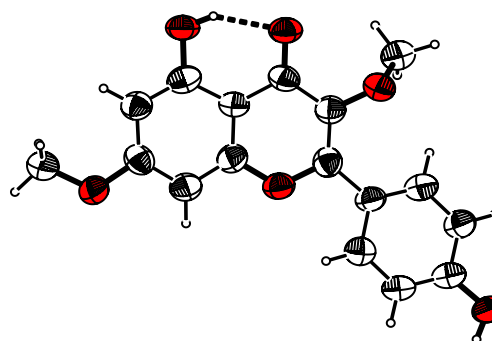
Exemplarily, we performed an extensive MS^n analysis of kumatakenin and present a fragmentation pathway (Fig. 9) in accordance with well researched fragmentation mechanisms of flavonoids [84–88]. From HRMS the molecular formula of $C_{17}H_{14}O_6$ was known. The follow-up MS^n experiments, performed in four steps from MS^2 to MS^5 , showed the subsequent losses of methyl radicals followed by characteristic CO and CO_2 elimination (Fig. 9).

3.3 Considerations on biological activity

Most of the compounds isolated from *H. scyphifera* leaves are widely distributed in plants and known for decades. Therefore, their biological effects were already exhaustively evaluated in previous studies. In addition, limitations in the yield of new compounds did not allow the submission to bioassays. Thus, we performed a literature review and focused on known sources and reported bioactivities for all 24 known compounds and on structurally related compounds for the two new isolates **1** and **2** (Table 4).

The Zingiberaceae family is known for various biological activities like e.g. anti-inflammatory and neuroprotective properties. Inflammatory and neurological diseases commonly arise in the presence of proinflammatory and proliferation-inducing cytokines, as well as through an escalation in the proliferation rate of immune system cells, or as a result of high levels of free radicals being released [20]. Cytokines are small proteins that act as messengers between cells, that are involved in the inflammatory response. In Zingiberaceae the pungent constituents like gingerols and shogaols, form one characteristic compound classes of this family, and are considered to be responsible for many pharmaceutical effects [89, 90]. These flavor components are phenolic ketones and structurally related to diarylheptanoids, like curcumin. Their anti-inflammatory effect arises from the inhibition of the synthesis of prostaglandins [91] as well as the inhibition of enzymes that produce NO radicals [92]. They also inhibit the synthesis and secretion of proinflammatory cytokines [90, 93, 94] such as interleukin-2 (IL-2). The extracts obtained from leaves of *H. scyphifera* were reported to

Fig. 8 Structure of kumatakenin (**13**), obtained from X-Ray



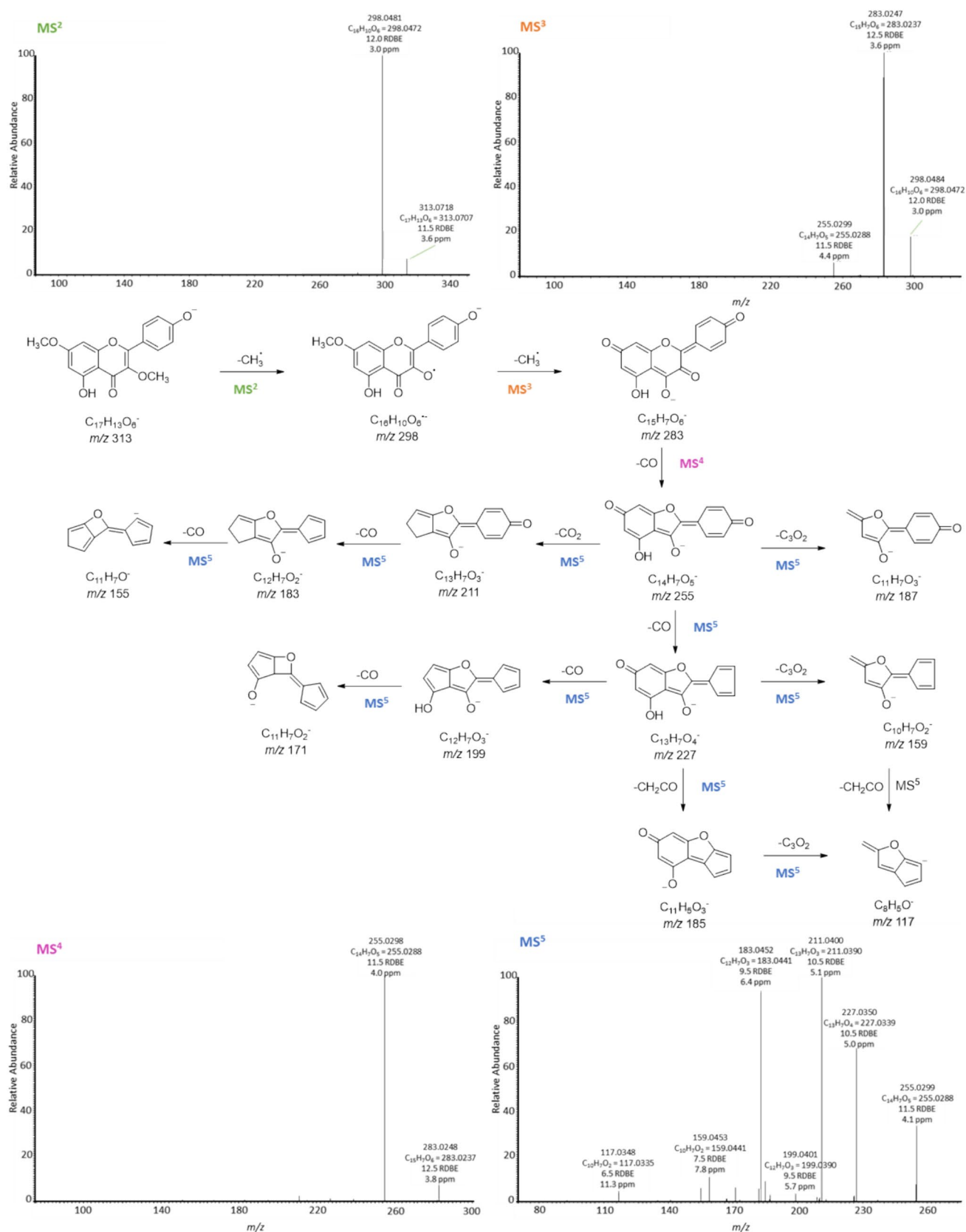


Fig. 9 Spectra and fragmentation pathway of the MSⁿ analysis of kumatakenin (**13**) in negative ion mode

Table 4 Reported sources and biological properties of compounds isolated from the crude extracts of *H. scyphifera* leaves

No	Name (Chemical Formula)	Compound class	Also known from: Family (Species)	Bioactivity described
3	(E)-14,15,16-Trinorlabda-8(17),11-dien-13-oic acid (C ₁₇ H ₂₆ O ₂)	Labdane-type diterpene	Zinigeraceae (<i>Curcuma amada</i> [123], <i>Alpinia pahangensis</i> [124],)	Antibacterial [124]
4	Verbenon-10-oic acid (C ₁₀ H ₁₂ O ₃)	Monoterpene	Lamiaceae (<i>Hyssopus cuspidatus</i> [40])	Antimicrobial [40], antifungal [40]
5	cis-Pinonic acid (C ₉ H ₁₄ O ₃)	Ketoacid	Synthesis product of Verbenon [42]	–
6	cis-Pinonic acid (C ₁₀ H ₁₆ O ₃)	Ketoacid	Synthesis product Verbenon [44]	–
7	2-(2,2,3-Trimethyl-5-oxocyclopent-3-en-1-yl) acetic acid (C ₁₀ H ₁₄ O ₃)	Ketoacid	Degradation product of Camphor [46]	–
8	Epicatechin (C ₁₅ H ₁₄ O ₆)	Flavonoid/Flavanol	Rosaceae (apple pomace [125])	Anticancer [126], antiinflammatory [126], antioxidant [105, 125, 127], neuroprotective [128, 129]
9	Sakuranetin (C ₁₆ H ₁₄ O ₃)	Flavonoid/Flavanon	Rubiaceae (<i>Gardenia ternifolia</i> [130]) Heliotropiaceae (<i>Heliotropium glutinosum</i> [131]) Asteraceae (<i>Ageratina havanensis</i> [132], <i>Baccharis retusa</i> [109])	Antioxidant [130, 131], antiviral [132], antimicrobial [109]
10	Quercetin (C ₁₅ H ₁₀ O ₇)	Flavonoid/Flavanol	Amaryllidaceae (onions (<i>Allium</i>) [104, 133]) Rutaceae (<i>Citrus aurantifolia</i> [104, 134]) Apiaceae (<i>Petroselinum crispum</i> [104, 135]) Oleaceae (olives (<i>Ole</i>) [104, 136]) Hypericaceae (<i>Hypericum perforatum</i> [104, 137]) Ginkgoaceae (<i>Ginkgo biloba</i> [104, 138]) Zingiberaceae (<i>Hornstedtia scyphifera</i> [18], <i>Zingiber officinale</i> [95])	Antibacterial [104, 139], antiinflammatory [104, 140] neuroprotective [141, 142], anticancer [95, 104, 141], antioxidant [95, 104, 134, 135]
11	Rutin (C ₂₇ H ₃₀ O ₁₆)	Flavonoid/Flavanol glycoside	Rutaceae (<i>Citrus aurantifolia</i> [134]) Oleaceae (olives (<i>Ole</i>) [136]) Fabaceae (<i>Sophora japonica</i> [143]) Polygonaceae (<i>Fagopyrum esculentum</i> [144]) Rutaceae (<i>Ruta graveolens</i> [145]) Strelitziaceae (<i>Strelitzia reginae</i> [146, 147]) Marantaceae (<i>Maranta leuconeura</i> [146, 147]) Solanaceae (tobacco leaves (<i>Nicotiana</i> [146, 147])	Antidiabetic [143], neuroprotective [142], hepatoprotective [148], anticancer [146], antiinflammatory [147, 149], antioxidant [134, 147, 150]
12	Isokaempferide (C ₁₆ H ₁₂ O ₆)	Flavonoid/Flavanol	Zinigeraceae (<i>Hornstedtia reticulata</i> [19], Fabaceae (<i>Amburana cearensis</i> [151]) Lamiaceae (<i>Dracocephalum subcapitatum</i> [152], <i>D. kotschy</i> [153]) Zingiberaceae (<i>Alpinia galanga</i> [96]) Asteraceae (<i>Centaurea jacea</i> [154]) Fabaceae (<i>Amburana cearensis</i> [155])	Antiproliferative [151], antitumor [154], anti-inflammatory [155], antioxidant [153]
13	Kumatakenin (C ₁₇ H ₁₄ O ₆)	Flavonoid/Flavanol	Zinigeraceae (<i>Hornstedtia scyphifera</i> [19]) Lamiaceae (<i>Ballota glandulosissima</i> [110]) Boraginaceae (<i>Alkanna orientalis</i> [156]) Myrtaceae (<i>Syzygium aromaticum</i> [157]) Scrophulariaceae (<i>Buddleja albiflora</i> [158])	Antimicrobial [110], antifungal [110], antiviral [156], anticancer [157], insecticidal [158]

Table 4 (continued)

No	Name (Chemical Formula)	Compound class	Also known from: Family (Species)	Bioactivity described
14	5-Hydroxy-3,7,4'-trimethoxyflavon ($C_{18}H_{16}O_6$)	Flavonoid/Flavonol	Zinigeraceae (<i>H. scyphifera</i> [19], <i>Kaempferia parviflora</i> [98]) Tamaricaceae (<i>Tamarix gallica</i> [159]) Lamiaceae (<i>Ballota inaequidens</i> [110])	Antimicrobial [110], antifungal [110], antioxidant [159]
15	(E) p-Coumaric acid ($C_9H_8O_3$)	Phenolic acid	Poaceae (barley [160]) Polygonaceae (buckwheat [161]) Rubiaceae (<i>Oldenlandia diffusa</i> [162])	Antioxidant [161], antimicrobial [111], anti-inflammatory [162], anticancer [163], antibacterial [164]
16	trans-Ferulic acid ($C_{10}H_{10}O_4$)	Phenolic acid	Poaceae (<i>Zea mays</i> hybrid maize [165])	Anti-inflammatory [166], antioxidant [112], antimicrobial [112]
17	cis-Ferulic acid ($C_{10}H_{10}O_4$)	Phenolic acid	Pinaceae (white spruce [167]) Poaceae (<i>Triticum aestivum</i> [168])	
18	Evofofin B ($C_{17}H_{18}O_6$)	Phenolic	Malvaceae (<i>Sida acuta</i> [113], <i>Sida rhombifolia</i> [169]) Fabaceae (<i>Caesalpinia bondu</i> [170])	Antibacterial [113], antioxidant [169], cytotoxic [170]
19	Protocatechualdehyde ($C_7H_6O_3$)	Phenolic aldehyde	Musaceae (green cavendish banana [121]) Rosaceae (Japanese apricot, <i>Prunus mume</i> [171]) Hymenophyllaceae (<i>Trichomanes chinense</i> fern [114]) Rhodamelaceae (<i>Polysiphonia morrowii</i> [172])	Fungistatic [121], antioxidant [114], antiallergic [172]
20	Vanillin ($C_8H_8O_3$)	Phenolic aldehyde	Orchidaceae (<i>Vanilla planifolia</i> [173], <i>V. tahitensis</i> [174])	Anticancer [175], antioxidant [176], anti-inflammatory [177], antibacterial [178], neuroprotective [179]
21	Syringaldehyde ($C_9H_{10}O_4$)	Phenolic aldehyde	Asteraceae (<i>Mikania laevigata</i> [180], <i>M. glomerata</i> [180])	Antioxidant [181], antimicrobial [182], antifungal [182], anticancer [183]
22	p-Hydroxybenzoic acid ($C_7H_6O_3$)	Phenolic acid	Apiaceae (<i>Daucus carota</i> [184]) Cucurbitaceae (cucumber, <i>Cucumis sativus</i> [185]) Arecaceae (<i>Phoenix dactylifera</i> [186])	Antimicrobial [187, 188], anti-inflammatory [189], antioxidant [188]
23	Protocatechuic acid ($C_7H_6O_4$)	Phenolic acid	Zingiberaceae (<i>Roscoe purpurea</i> [99], <i>Alpinia zerumbet</i> [190]) Aquifoliaceae (<i>Ilex chinensis</i> [191])	Anti-inflammatory [190, 192, 193], antinociceptive [190], antipyretic [190], antioxidant [191], analgesic [193]
24	Vanillic acid ($C_8H_8O_4$)	Phenolic acid	Lamiaceae (<i>Origanum vulgare</i> [194]) Amaranthaceae (<i>Chenopodium murale</i> [195])	Antioxidant [194, 195], antinociceptive [196], nephroprotective [197], antidiabetic [198]
25	Protocatechuic acid methylester ($C_8H_8O_4$)	Phenolic ester	Zingiberaceae (<i>Amomum xanthioides</i> [100])	Pulmonary protective [199]
26	5-Methoxy salicylic acid ($C_8H_8O_4$)	Phenolic acid	Phyllanthaceae (<i>Antidesma bunius</i> [200])	

inhibit the release of cytokine IL-2 from activated T-cells, reduce the proliferation rate of these T-cells and also minimize the release of nitric oxide radicals (NO) from activated microglial cells [20]. However, in our study of *H. scyphifera* we did not find any of the characteristic pungent compounds. Thus, the observed effects should result from other compound classes. We detected in *H. scyphifera* seven components known to occur in various species from the ginger family such as (*E*)-14,15,16-trinorlabda-8(17),11-dien-13-oic acid (**3**) from *Zingiber ottensii* [39], quercetin (**10**) from *Zingiber officinale* [95], isokaempferide (**12**) from *Alpinia galanga* [96] or *H. reticulata* [19], kumatakenin (**13**) from *Curcuma aromatica* [97] or *H. scyphifera* [19], 5-hydroxy-3,7,4'-trimethoxyflavone (**14**) from *Kaempferia parviflora* [98] or *H. scyphifera* [19], protocatechuic acid (**23**) from *Roscoea purpurea* [99], and protocatechuic acid methyl ester (**25**) from *Amomum xanthioides* [100]. These compounds reflect all three major compound groups identified in this study for *H. scyphifera*, namely terpenoids (**3**), flavonoids (**10**, **12**, **13** and **14**) and other phenolics (**23** and **25**). All three groups are known for anti-inflammatory effects [101]. Flavonoids such as epicatechin, sakuranetin, and quercetin are known to actively inhibit the release of cytokines like IL1 β , and consequently passively suppress the IL1 β -induced expression of NO radicals [102–104]. They are also able to trap radicals actively by donating phenolic hydrogen [105]. The same ability of free radical scavenging is reported for many phenolic compounds including isolates from *H. scyphifera*, namely ferulic acid (**16**, **17**), vanillin (**20**), vanillic acid (**24**), *p*-coumaric acid (**15**) and protocatechuic acid (**23**) [106]. Recently, El Omari et al. [107] showed, that sesquiterpene lactones are able to reduce specifically the IL-2 secretion, like Zipp, Ullrich et al. [20] reported earlier for *H. scyphifera* extracts. The same effects might be attributed to the newly found sesquiterpene lactone mustak-14-oic acid (**1**). In conclusion, the presence of flavonoids, phenols and terpenoids in combination could explain the reported anti-inflammatory and neuroprotective activities of *H. scyphifera* extract [20].

There is also a number of reports concerning the antifungal and antimicrobial activity of *H. scyphifera* isolates which aligns with the results of the growth-inhibition assays against the Gram-negative bacterium *A. fischeri* (Fig. S1.1.B) and the fungus *Botrytis cinerea* (Fig. S1.2.B) in the present study and with reports from literature [18, 20]. These bioactivities probably arise mainly from flavonoids and phenolics. Antimicrobial compounds are **4** [40], **8** [101], **9** [108, 109], **10** [18, 104], **13** [110], **14** [94, 110], **15** [111], **16** [112], **18** [113], **19** [114], **21** [115, 116], **22** [117], and **23** [118], and antifungal compounds are **9** [119], **13** [110], **14** [120], **19** [121], **20** [121], **21** [115, 116, 122]). However, terpenoids are often relevant for antifungal activity, like verbenon-10-oic acid (**4**) [40] and mustakone [77], a compound structurally closely related to sesquiterpenoid **1**. Applications of 20 μ g in standardized disc assays resulted in the inhibition of *Candida albicans* [40] and *Penicillium crustosum* [77].

Further potential effects *H. scyphifera* might exhibit based on the discovered constituents can be taken from the literature review (Table 4). Based on this, the species appears to be a promising study subject for more intensive investigations to unravel its full pharmaceutical potential. In summary, among the 26 isolated compounds, the predominant biological activities found so far cover antimicrobial and antifungal as well as anti-inflammatory and neuroprotective potentials, the latter effect often linked to the anti-inflammatory action. However, the high number of different active compounds suggests, that effects of the extract also might result from the combined powers of several active constituents, rather than being exerted by a single component with high specificity.

4 Conclusion

In conclusion, the phytochemical investigation of *H. scyphifera* leaves using multiple analytical methods has provided valuable insights into its chemical composition. Through GCMS analysis of headspace and of the essential oil, the presence of common aroma components such as α -pinene, camphene, *p*-cymene, and camphor were confirmed, along with the identification of a previously unreported compound, butylbenzene. Further analysis via UHPLC-HRMS allowed for the annotation of additional metabolites, identifying flavonoids, terpenoids and phenolics as the major compound classes of *H. scyphifera*. Purification of the crude extract led to the isolation of the two new sesquiterpenoids mustak-14-oic acid (**1**) and 6-hydroxy-anhuienosol (**2**) along with 24 known compounds. Structural elucidation of these compounds was achieved through a combination of extensive spectroscopic techniques, including 1D and 2D NMR and HRMS as well as comparison with literature data. Furthermore, the determination of stereochemistry was facilitated by NOESY correlations and CD spectroscopy. As an example of the flavonoid class, a four step MSⁿ analysis of kumatakenin (**13**) was performed and based on known fragmentation mechanisms, a full annotated fragmentation pathway could be established. This is also the first time that the keto acids **5**, **6** and **7** were isolated from plant origin. While the possibility that they originate from plant-associated bacteria or fungi cannot be ruled out, the quantities observed strongly suggest a plant origin.

Bioassays revealed antimicrobial and antifungal activities in fractions enriched with nonpolar isolated compounds. Intensive literature research of all isolated compounds showed that many of them are known for mild activities in such assays. For *H. scyphifera* activity, this implies an additive, maybe synergistic, combination of several constituents rather than one individual highly active compound with high selectivity being responsible. Along with the antimicrobial activity mentioned, there are further beneficial effects known for the isolated compounds, namely anti-inflammatory and neuroprotective activities which support observed effects of *H. scyphifera* extracts described in the literature. Overall, the comprehensive phytochemical investigation of *H. scyphifera* leaves has contributed to a deeper understanding of their chemical composition and increased the molecular basis for understanding its observed biological activities, laying the foundation for further studies on pharmacological and therapeutic applications.

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Author contributions J.K. performed the main phytochemical investigations, data analysis and visualization and wrote the main manuscript. A.D. performed studies on volatiles and literature searches on biological activities of known compounds leading to Tables S2 and 4, respectively. K.P., A.W. and I.W. contributed to the isolation and structure elucidation of constituents. D.D. and M.D. performed computational CD spectral analysis and prepared Figs. 6, S5, S6, S7 and Tables S3–S6; S9–S14. C.W. performed X-Ray crystallography of kumatakenin connected to Figs. 8, S11 and Table S15. K.F. was involved in data curation and validation and supervised the work. L.A.W. designed the study, acquired funding, provided resources and supervision. All authors reviewed and edited the manuscript.

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Data availability Data are included in the article and the supplementary material. The raw data supporting the conclusions of this article will be made available by the corresponding authors on request. The plant material is deposited at IPB, Germany. All primary data and reference compounds are stored at the IPB primary data storage for 10 + years, and in the compound depository to the extent available or stable.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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