ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Targeted Isolation of Cytotoxic Sesquiterpene Lactones from *Eupatorium fortunei* by the NMR Annotation Tool, SMART 2.0

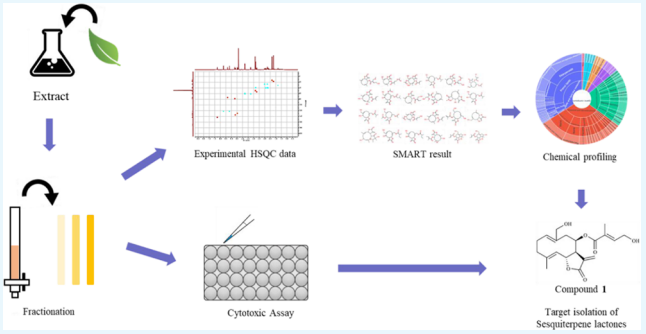
Jiho Lee, Jinyoung Park, Juyeol Kim, Birang Jeong, Seong Yeon Choi, Hyeon Seok Jang, and Heejung Yang*

Cite This: *ACS Omega* 2020, 5, 23989−23995

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Small Molecular Accurate Recognition Technology (SMART 2.0) has recently been introduced as a NMR-based machine learning tool for the discovery and characterization of natural products. We attempted targeted isolation of sesquiterpene lactones from *Eupatorium fortunei* with the aid of structural annotation by SMART 2.0 and chemical profiling. Eight germacrene-type (**1**−**7** and **10**) and two eudesmane-type sesquiterpene lactones (**8** and **9**) were isolated from the whole plant of *Eupatorium fortunei*. With the guidance of the results of the subfractions from *E. fortunei* obtained by SMART 2.0, their cytotoxic activities were evaluated against five cancer cells (SKOV3, A549, PC3, HEp-2, and MCF-7). Compounds **4** and **8** exhibited IC_{50} values of 3.9 ± 1.2 and 3.9 ± 0.6 μM against prostate cancer cells, PC3, respectively. Compound **7** showed good cytotoxicity with IC_{50} values of 5.8 ± 0.1 μM against breast cancer cells, MCF-7. In the present study, the rapid annotation of the mixture of compounds in a fraction by the NMR-based machine learning tool helped the targeted isolation of bioactive compounds from natural products.

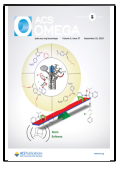


INTRODUCTION

Natural products have been spotlighted as potential sources providing new insights for the development of therapeutic agents. Multiple chemicals in natural products have hindered the discovery of novel druggable candidates. Recently, natural product chemists have shown interest in the development of new *in silico* approaches for the rapid annotation of novel compounds and dereplication of the known ones from natural products. *In silico* methods for spectrometric data generated using nuclear magnetic resonance (NMR) and mass spectrometry (MS), which are the two most popular techniques for characterizing natural products, are being developed.[1−4] Although high-resolution MS is the powerful technique for the measurement of tons of metabolites and production of their structural data in a short time, low reproducibility and interpretability have become the weak points for the accurate identification of compounds.[5] Meanwhile, NMR spectroscopy guarantees higher reproducibility compared to MS, but it also needs to acquire expertise for the interpretation of 1D and 2D NMR data, which has become a big barrier for novice chemists in the fields of natural products. Recently, Small Molecule Accurate Recognition Technology (SMART 2.0) has been introduced as the NMR-based machine learning study for the structural annotation of compounds in mixtures or unknown compounds.[6,7] SMART 2.0 trained on ^{1}H-^{13}C HSQC spectral data of tons of natural products provided the insights for elucidating unknown compounds. We expected this tool to help in simplifying the dereplication step and provide the bypass for the isolation of the targeted compounds in the natural product research.

Eupatorium fortunei Turcz. (Compositae) is widely distributed in Southeast Asian countries, such as Korea, Japan, and China. *Eupatorium fortunei* has been used as a traditional medicine for the treatment of cold, dropsy, chills, and fevers.[8,9] A wide range of secondary metabolites, such as alkaloids,[10] phenolic acids,[11] benzofuran,[12] triterpenoids,[13] and sesquiterpenes,[14] has been reported as the constituents found in *E. fortunei*. During the course of discovery of cytotoxic sesquiterpene lactones from *Eupatorium* species,[15] we focused on *E. fortunei*, which is native to Korea, and especially tried to isolate germacrane-type sesquiterpene lactones having 10-membered monocyclic ring with a cyclic ester structure.[16,17] In the present study, we utilized the state-of-art technique, the SMART 2.0 tool, to rapidly discover the targeted compounds in the extract of a natural product and tried NMR spectra-

Received: July 8, 2020
Accepted: August 26, 2020
Published: September 9, 2020



https://dx.doi.org/10.1021/acsomega.0c03270
ACS Omega 2020, 5, 23989−23995

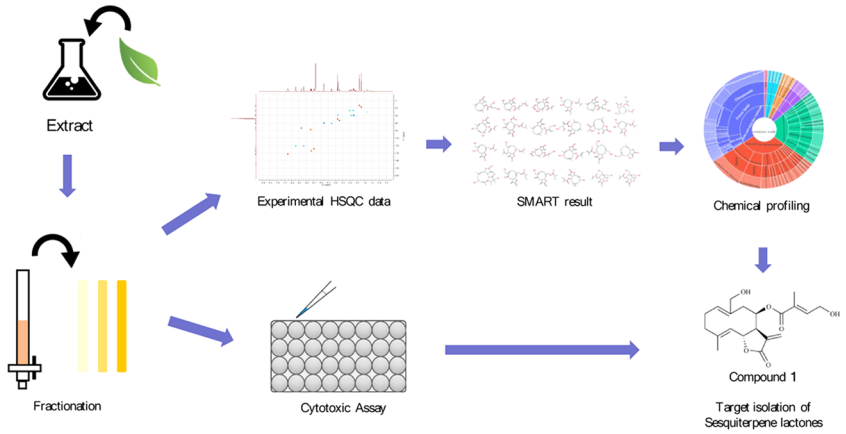


Figure 1. Overview of the steps of the target isolation study. *E. fortunei* crude extract was partitioned successively with *n*-hexane, EtOAc, *n*-BuOH, and H_2O. The subfractions were obtained from the EtOAc fraction. Subsequently, subfractions were analyzed by HMQC NMR spectroscopy and subjected to MTT assay. HSQC data of fractions were analyzed using SMART analysis. Then, the SMART result was processed with statistical data to target the sesquiterpene lactones.

guided isolation of the target compounds, which are expected to have biological activities. Consequently, we succeeded in the isolation of 10 sesquiterpene lactones having potent cytotoxicity from *E. fortunei*.

RESULTS AND DISCUSSION

The experimental steps for the isolation of cytotoxic sesquiterpene lactones from *E. fortunei* are summarized in Figure 1. For the targeted isolation of the cytotoxic sesquiterpene lactones from *E. fortunei*, the total extract was fractionated into *n*-hexane, EtOAc, *n*-BuOH, and H_2O fractions, sequentially. The EtOAc fraction of the subfractions showed the most potent cytotoxic activity against 5 cancer cell lines (A549, MCF-7, HEp-2, SKOV3, and PC3) at a concentration 100 μg/mL (Figure S1). The EtOAc fraction, which was expected to contain the cytotoxic constituents, was subjected to normal column chromatography and fractionated into five subfractions (E1 ∼ 5). Among the subfractions, E1 ∼ 5, E2, and E3, showed the most potent cytotoxic activities of 16.3−32.1 and 15.7−31.6%, respectively, at a concentration of 100 μg/mL against five cancer cell lines and were expected to contain the anticancer compounds of *E. fortunei* (Figure 2A). The HSQC NMR data of the subfractions E1, E2, and E3, were measured for the introduction into SMART 2.0 analysis (https://smart.ucsd.edu/classic), which resulted in the prediction of the structures of the mixed components in the samples (Figures 2B, S2, and S3). The SMART tool result of low cytotoxicity of subfraction E1 confirmed what was predicted for phenolic compounds and flavonoids (Figure S3). Although the subfraction E2 showed more diverse chemical profiles than E3, both fractions had terpenoid-type components including sesquiterpenes and diterpenes as major proportions of overall profiles. The subfraction E2 showing more complicated profiles was separated to yield seven subfractions (E2−1 ∼ 7), followed by evaluation of the cytotoxic activities against five cancer cells (Figure 2A). Three subfractions E2−4 ∼ 6 showing potent cytotoxic activities except E2−7, showing yellow major spots in the TLC experiment, which were regarded as nonterpenoid-type were studied in the HSQC NMR experiments (Figures 3 and S4). Interestingly, the results predicted by SMART 2.0 analysis showed quite different profiles of four subfractions. The subfractions E2−4, E2−5, and E2−7 showed intricate profiles

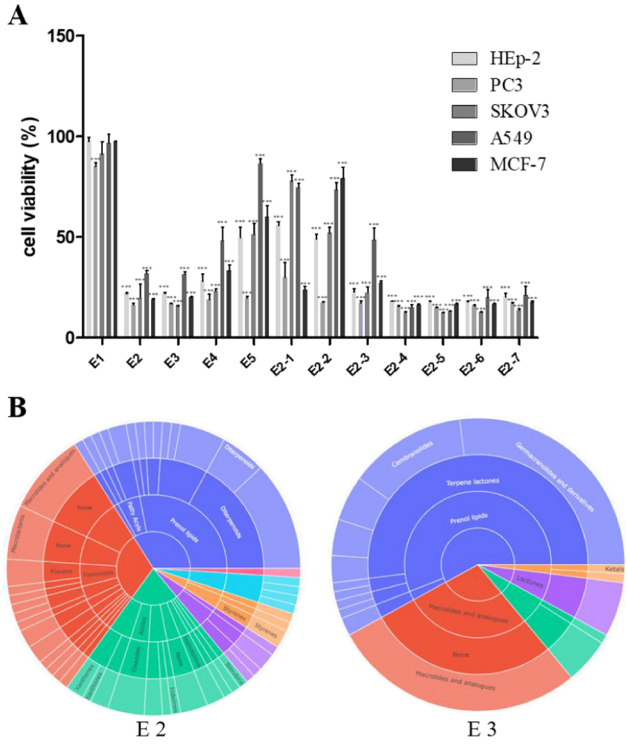


Figure 2. (A) Cytotoxic effect of subfractions (E1 ∼ E5 and E2−1 ∼ E2−7) on human lung cancer A549, breast cancer MCF-7, ovarian cancer SKOV3, laryngeal carcinoma HEp-2, and prostate cancer PC3 cells using MTT assay. Data indicates statistical significance at $p < 0.001$ compared to the negative control. (B) Various class level classifications of predictive compounds in the SMART tool are shown in the sunburst plot.

due to the diverse kinds of chemicals, while terpene lactones including of germacranolide-type occupied the largest portion for E2−6 (Figures 3, S5−S7 and Tables S1−S3). The SMART 2.0 predicted the structures of 24 prenylated sesquiterpene lactones including those of germacranolide-type sesquiterpene lactones with the highest cosine score of 0.88 (Figure 4 and Table S4). Next, we focused on the targeted isolation of the subfractions E2−6 and could isolate seven germacranolide-type sesquiterpene lactones (**1**−**7**), 14-hydroxy-8β-[4′-hydroxytigloyloxy]-costunolide (**1**),[18] 14-acetoxy-8β-[4′-hydroxyti-

https://dx.doi.org/10.1021/acsomega.0c03270
ACS Omega 2020, 5, 23989−23995

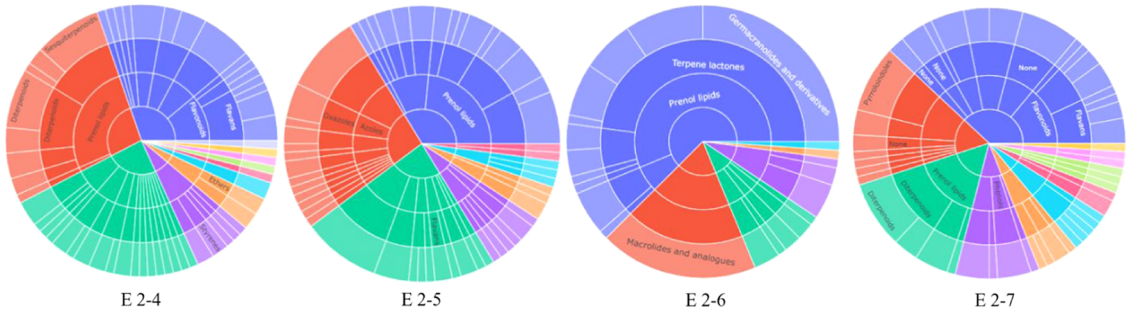


Figure 3. Various class level classifications of predictive compounds in the SMART tool are shown in the sunburst plot (E2−4 ∼ E2−7).

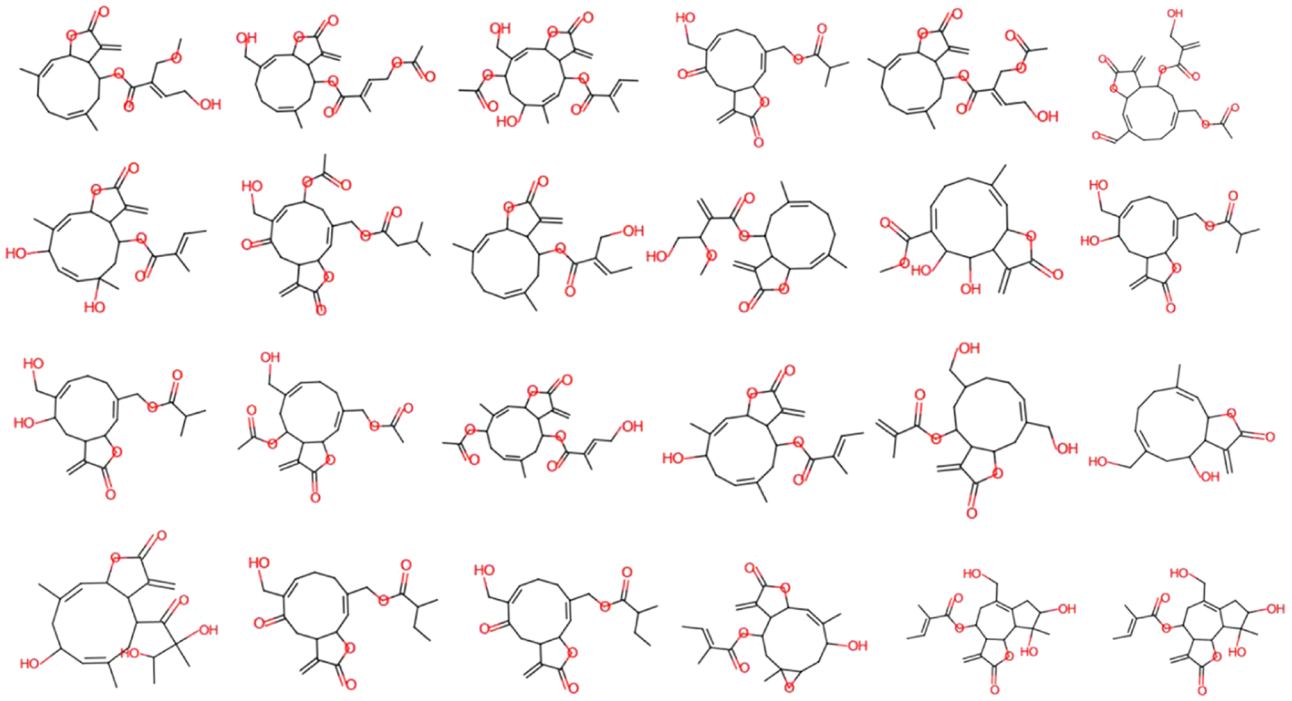


Figure 4. Structures of sesquiterpene lactones predicted by SMART 2.0.

gloyloxy]-costunolide (**2**),[19] 14-acetoxy-8β-hydroxy-costunolide (**3**),[20] 8β-[4′,5′-dihydroxytigloyloxy]-costunolide (**4**),[21] 8β-[4′-hydroxytigloyloxy]-14-oxo-costunolide (**5**),[18] 3β-acetoxy-8β-[4′,5′-dihydroxytigloyloxy]-costunolide (**6**),[22] and 2β-hydroxy-8β-[5′-hydroxytigloyloxy]-costunolide (**7**),[23] and two eudesmane-type sesquiterpene lactones **8** and **9**, 1β-hydroxy-8β-[4′-hydroxytigloyloxy]-β-cyclocostunolide (**8**)[24] and 1β-hydroxy-8β-[4′-hydroxytigloyloxy]-α-cyclocostunolide (**9**).[25] Interestingly, compounds **9** and **10** contained an exomethylene-γ-lactone and a prenylated ester, which are the same as the seven derivatives with germacranolide-type compounds **1**−**7**.

In the case of subfraction E3, the chemical profile showed that terpenoid-lactones including germacranolide-type compounds occupied the largest portion in the subclass level of the sunburst plot (Figure 3). We expected that the subfraction E3 has a wide range of the germacranolide-type compounds similar to E2 but only 8β-[4′-hydroxytigloyloxy]-costunolide (**10**)[26] was isolated as the major compound (Figure 5). These results indicated that the HSQC signals of compound **10** overwhelmed those of other minor components due to the majority of compound **10** in the subfraction E3. Consequently, we could successfully isolate 10 sesquiterpene lactones, expecting the potent cytotoxic activities using the SMART 2.0 tool. Since compounds **1**, **8**, and **9** were previously reported about 20−40 years ago, the full assignment of their structures was not determined in the literature. In the present study, the structures of these compounds were fully elucidated from the 1D and 2D NMR spectroscopic data. Among these, the determination of compound **1** is described below.

Compound **1** was obtained as a white powder and has a molecular formula of $C_{20}H_{26}O_6$, based on HR-ESI-MS analysis (*m*/*z* 363.1818 $[M + H]^+$, calcd for 363.1808). The ^1H spectral data and HSQC spectrum showed resonances of two methyl protons at δ_H 1.84 (3H, s, H-5′) and 1.71 (3H, s, H-15); two oxymethine protons at δ_H 5.84 (1H, d, J = 4.2 Hz, H-8) and 5.19 (1H, t, J = 9.3 Hz, H-6); three olefinic protons at δ_H 6.77 (1H, t, J = 11.6 Hz, H-3′), 5.15 (1H, dd, J = 12.3, 4.3 H-1), and 4.97 (1H, d, J = 9.9 Hz, H-5); two oxymethylene protons at δ_H 6.22 (1H, d, J = 3.2 Hz, H-13a) and 5.67 (1H, d, J = 3.2 Hz, H-13b); six methylene groups at δ_H 4.29 (2H, d, J = 6.0 Hz, H-14a, 4′a), 4.28 (1H, d, J = 3.8 Hz, H-4′b), 3.76 (1H, d, J = 11.9 Hz, H-14a), 3.32 (1H, m, H-3b), 2.51 (1H, m, H-2b), 2.40 (1H, m, H-9b), 2.27 (1H, m, H-2a), 2.20 (1H, m, H-3a), and 2.15 (1H, d, J = 5.7 Hz, H-9a); and one methine proton at δ_H 3.22 (1H, m, H-7). The ^{13}C NMR and HSQC

https://dx.doi.org/10.1021/acsomega.0c03270
ACS Omega 2020, 5, 23989−23995

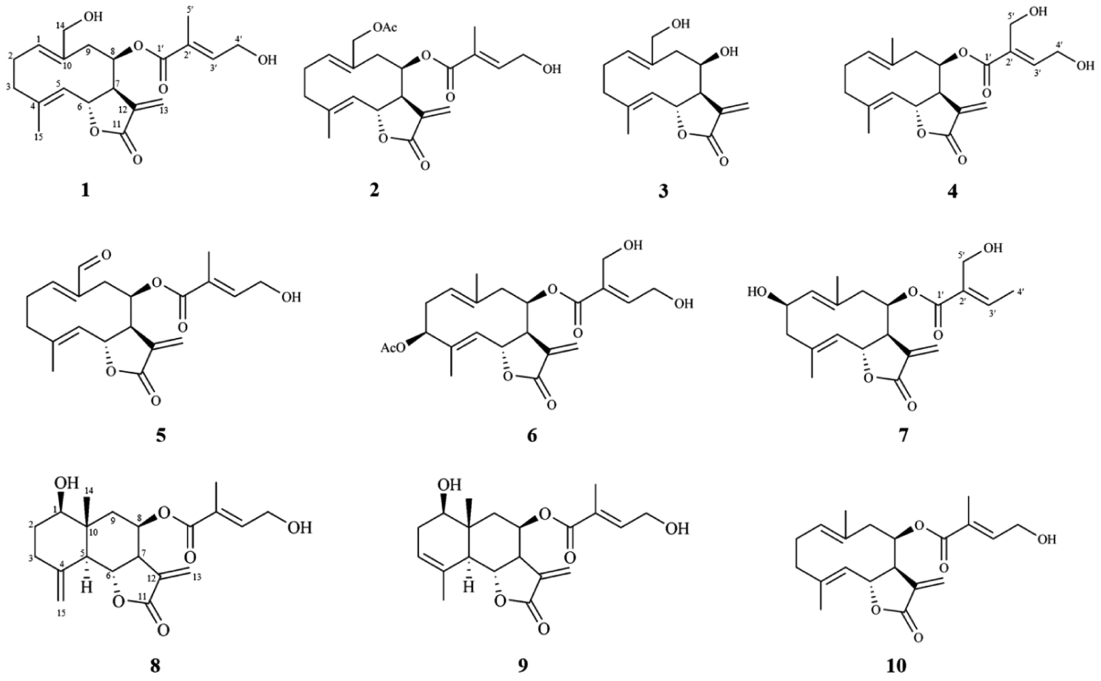


Figure 5. Structures of compounds **1**−**10** isolated from *Eupatorium fortunei*.

spectra showed resonance signals for 20 carbons, including two carboxylic carbons at δ_C 171.8 (C-11) and 167.7 (C-1′); five olefinic quaternary carbons at δ_C 128.9 (C-2′), 139.0 (C-12), 143.6 (C-4), 138.9 (C-10), and 121.5 (C-11); three olefinic methane carbons at δ_C 142.9 (C-3′), 134.5 (C-1), and 128.5 (C-5); two oxygenated methine carbons at δ_C 77.6 (C-6) and 73.5 (C-8); five methylene carbons including two oxygenated methylenes at δ_C 61.0 (C-4′), 59.8 (C-14), 40.1 (C-9), 39.0 (C-3), and 26.6 (C-2); a methine carbon at δ_C 53.5 (C-7); and two methyl groups at δ_C 17.2 (C-15) and 12.8 (C-5′). The COSY data showed correlations between H-1/H-2/H-3, H-5/H-6/H-7/H-8/H-9, and H-3′/H-4′. In addition, the key HMBC correlations between H-2 and C-10, H-3 and C-2/C-4 and C-15, H-3 and C-10, H-5 and C-3, and H-6 and C-8 are also shown. The exomethylene protons (H-13a and 13b) were correlated with a methine carbon (C-7) and a carboxylic carbon (C-11). These results suggested that the backbone of compound **1** is the germacranolide-type sesquiterpene lactone.[27] The prenyl group suggested by the additional correlations between H-3′ and C-1′, H-4′ and C-1′/C-2′ was attached to C-8 of the backbone indicated by the signal from H-8 to C-1′. The COSY, HMBC, and ROESY are detailed in Figures 6 and S8−S13. Consequently, compound **1** was identified as 14-hydroxy-8β-[4′-hydroxytigloyloxy]-costunolide, whose H NMR profile was reported in 1987.[18]

Additionally, compounds **1**−**10** were evaluated for their anticancer activities against five cell lines A549, MCF-7, HEp-2, SKOV3, and PC3 (Table 1). Compounds **2**, **4**, and **7**−**10** showed significant cytotoxic activities against human prostate cancer cell line PC3. Compounds **4** and **8** exhibited IC_{50} values of 3.9 ± 1.2 and 3.9 ± 0.6 μM, respectively. For human breast cancer cell line MCF-7, among compounds **2**, **7**, **8**, and **10** showing cytotoxic activities, compound **7** exhibited IC_{50} values of 5.8 ± 0.1 μM. In the case of compound **10**, it showed good cytotoxicity against five cancer cell lines (4.3 ± 0.8−20.2 ± 5.0 μM).

In the present study, we could isolate ten sesquiterpene lactones (**1**−**10**), which have been rarely reported in Nature with the guidance of an *in silico* NMR spectra annotation tool, SMART 2.0. Though SMART 2.0 did not identify the exact structures, the results were quiet reliable to guide the discovery of the targeted compounds with specific moieties among the mixed compounds in the levels of extracts or fractions of natural products.

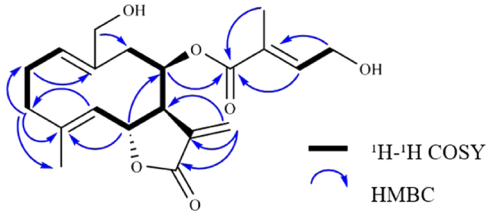


Figure 6. Key ^{1}H-^{1}H COSY and HMBC correlations of **1**.

EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra, including 2D NMR (^{1}H-^{1}H COSY, HSQC, and HMBC), were obtained using a Bruker Avance Neo 600 (Bruker, Germany) spectrometer at 600 MHz at the Central Laboratory of Kangwon National University (Chuncheon, Korea). Column chromatography procedures were performed on silica gel Kieselgel 60 (40−60 μm, 230−400 mesh, Merck, Germany) and Sephadex LH-20 gel (18−111 μm, GE Healthcare, Sweden). Thin-layer chromatography (TLC) was performed on precoated silica gel plates (Kieselgel 60 F_{254} Merck, Germany) and RP-C_{18} plates (Kieselgel 60 F_{254s}, Merck, Germany). Spots were detected by TLC using UV light or H_2SO_4-EtOH (v/v) spray followed by heating. Multiple preparative HPLC was performed on a LC-Forte/R system equipped with a YMC UV/Vis detector (YMC, Shimogyo-ku, Japan) using a YMC-Actus Triart C_{18} column (s-5 μm, 12 nm, 20 × 250 mm^2) at a flow rate of 50 mL/min and monitored at 210 nm. Extra high purity solvents were purchased from

https://dx.doi.org/10.1021/acsomega.0c03270
ACS Omega 2020, 5, 23989−23995

Table 1. Cytotoxicity Data of Compounds 1−10 against Five Cell Lines

	IC_{50} (μM)[a]				
compound	PC3	SKOV3	A549	MCF-7	HEp-2
1	15.4 ± 1.3	≥100	≥100	13.5 ± 3.5	56.3 ± 3.2
2	4.2 ± 1.3	32.5 ± 7.4	28.3 ± 0.9	6.1 ± 1.8	20.9 ± 6.2
3	15.2 ± 1.0	46.0 ± 6.8	52.3 ± 8.0	30.0 ± 7.4	54.9 ± 3.0
4	3.9 ± 1.2	33.8 ± 6.0	37.9 ± 7.5	12.8 ± 3.3	26.4 ± 6.5
5	12.6 ± 4.0	31.8 ± 4.6	25.9 ± 1.0	16.3 ± 3.4	25.0 ± 7.1
6	17.4 ± 2.3	68.6 ± 2.2	61.2 ± 3.3	28.9 ± 1.2	26.8 ± 1.1
7	8.1 ± 0.3	29.4 ± 2.9	29.3 ± 7.9	5.8 ± 0.1	27.6 ± 3.4
8	3.9 ± 0.6	36.9 ± 2.5	44.8 ± 6.1	8.3 ± 0.9	22.7 ± 0.3
9	4.7 ± 0.9	50.2 ± 6.5	45.6 ± 3.6	14.6 ± 5.0	25.6 ± 1.2
10	4.3 ± 0.8	20.2 ± 5.0	17.8 ± 3.8	6.1 ± 0.3	12.6 ± 2.7
docetaxel	0.6 ± 0.2	0.3 ± 0.3	1.0 ± 0.4	1.4 ± 0.2	0.2 ± 0.0

[a]The results are IC_{50} values of compounds against each cancer cell line: PC3 (prostate), SKOV3 (ovarian), A549 (lung), MCF-7 (breast), and HEp-2 (laryngeal); docetaxel was used as a positive control.

DaeJung (Sicheung, Korea) for extraction, fractionation, and separation.

Plant Material. The whole plants of *E. fortunei* were collected from the Medicinal Plant Garden, Seoul National University, Korea in May 2018. The plant was identified by Professor Yong Soo Kwon, the College of Pharmacy of Kangwon National University, and the specimen (KNUEF-01) was deposited in the Herbarium at the College of Pharmacy, Kangwon National University.

SMART 2.0 Analysis. For the HSQC analyses, sample aliquots of 10−20 mg dissolved in 650 μL of methanol-d_4 with 0.05 v/v TMS (tetramethylsilane) were ultrasonicated for 10 min and filtered. The dissolved sample was taken into a 5 mm NMR tube. For SMART 2.0 analysis, the measured HSQC spectrum was imported into MestReNova, and the ^{1}H-^{13}C correlation data were converted into a CSV file. The converted CSV file was uploaded to SMART 2.0 (https://smart.ucsd.edu/classic) to obtain the candidate structures. Sunburst was performed using these data. For sunburst, the annotation compounds in the SMART tool were classified based on ClassyFire.[28]

Extraction and Isolation. The aerial parts of *Eupatorium fortunei* (10 kg) were extracted three times with 100% MeOH and ultrasonic extraction for 3 h. The crude extract (552.25 g) was dissolved in H_2O and partitioned successively with *n*-hexane (116.53 g), EtOAc (163.68 g), and *n*-BuOH (56.12 g). A part of the EtOAc fraction (100 g) was chromatographed by RP C_{18} MPLC with MeOH-H_2O (1:3, to 1:0) to obtain subfractions (E1−E5). The subfraction E2 was resolved by silica gel MPLC with hexane−EtOAc (5:1 to 1:5) to give seven fractions. Subfraction E2−6 was separated into six subfractions using a Sephadex LH-20 CC with 70% MeOH. E2-6-3 was resolved by silica gel MPLC with hexane−EtOAc (3:1 to 1:5) to obtain six subfractions containing **1** (99.1 mg) and **2** (134.2 mg). Compounds **4** (4.2 mg) and **5** (2.1 mg) were purified from E2-6-3-3 by multiple preparative HPLC using a gradient solvent system of MeOH-H_2O (60%, 10.0 mL/min). Subfraction E2-6-3-4 was further chromatographed on multiple preparative HPLC with an isocratic solvent system of MeOH-H_2O (60%, 10.0 mL/min) to afford **3** (7.5 mg), **6** (7.3 mg), **7** (26.6 mg), **8** (10.1 mg), and **9** (3.1 mg). The subfraction E3 was separated by silica gel MPLC with hexane−EtOAc (5:1 to 1:5) and MeOH-CH_2Cl_2 (1:5 to 1:0) to obtain eleven fractions. Subfraction E3-5 was passed over the Sephadex LH-20 column with 100% MeOH to give five fractions. Compound **10** (29.0 mg) was obtained from E3-5-2 using silica gel MPLC with hexane−EtOAc (5:1 to 0:1).

*14-Hydroxy-8β-[4′-hydroxytigloyloxy]-costunolide (**1**).* ^{1}H NMR (CD_3OD, 600 MHz) δ_H 6.77 (1H, t, J = 11.6 Hz, H-3′), 6.22 (1H, d, J = 3.2Hz, H-13b), 5.84 (1H, d, J = 4.2 Hz, H-8), 5.67 (1H, d, J = 3.2 Hz, H-13a), 5.19 (1H, t, J = 9.3 Hz, H-6), 5.15 (1H, dd, J = 12.3, 4.3 Hz, H-1), 4.97 (1H, d, J = 9.9 Hz, H-5), 4.29 (2H, d, J = 6.0 Hz, H-14a, 4′b), 4.28 (1H, d, J = 3.8 Hz, H-4′a), 3.76 (1H, d, J = 11.9 Hz, H-14a), 3.32 (1H, m, H-3b), 3.22 (1H, m, H-7), 2.51 (1H, m, H-2b), 2.40 (1H, m, H-9b), 2.27 (1H, m, H-2a), 2.20 (1H, m, H-3a), 2.15 (1H, d, J = 5.7 Hz, H-9a), 1.84 (3H, s, H-5′), 1.71 (3H, s, H-15); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 171.8 (C-11), 167.7 (C-1′), 143.6 (C-4), 142.9 (C-3′), 139.0 (C-12), 138.9 (C-10), 134.5 (C-1), 128.9 (C-2′), 128.5 (C-5), 121.5 (C-11), 77.6 (C-6), 73.5 (C-8), 61.0 (C-4′), 59.8 (C-14), 53.5 (C-7), 40.1 (C-9), 39.0 (C-3), 26.2 (C-2), 17.2 (C-15), 12.8 (C-5′); HREIMS m/z 363.1818 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

*14-Acetoxy-8β-[4′-hydroxytigloyloxy]-costunolide (**2**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 6.70 (1H, t, J = 5.5 Hz, H-3′), 6.31 (1H, d, J = 2.9 Hz, H-13b), 5.84 (1H, d, J = 4.3 Hz, H-8), 5.62 (1H, d, J = 2.9 Hz, H-13a), 5.19 (1H, dd, J = 11.0, 5.6 Hz, H-1), 5.12 (1H, t, J = 9.3 Hz, H-6), 4.84 (1H, d J = 9.9 Hz, H-5), 4.62 (1H, d, J = 12.4 Hz, H-14), 4.35 (1H, dd, J = 14.9, 6.7 Hz, H-4′b), 4.29 (1H, d, J = 5.03, H-4′a), 4.25 (1H, m, H-14a), 3.24 (1H, dd, J = 14.8, 5.0 Hz, H-9b), 2.95 (1H, m, H-7), 2.42 (1H, m, H-2b), 2.37 (1H, m, H-3b), 2.34 (1H, m, H-2a), 2.22 (1H, m, H-9a), 2.17 (1H, m, H-3a), 1.94 (3H, s, -OAc), 1.82 (3H, s, H-5′), 1.75 (3H, s, H-15); HREIMS m/z 405. 1910 $[M + H]^+$ (calcd for $C_{22}H_{29}O_7$, 405.1913).

*14-Acetoxy-8β-hydroxy-costunolide (**3**).* ^{1}H NMR (CD_3OD, 600 MHz) δ_H 6.29 (1H, d, J = 3.4 Hz, H-13b), 5.71 (1H, d, J = 3.4 Hz, H-13a), 5.15 (1H, dd, J = 10.0, 8.4 Hz, H-1), 5.11 (1H, m, H-6), 4.96 (1H, d, J = 10.1 Hz, H-5), 4.51 (1H, d, J = 3.5 Hz, 1H, H-8), 4.14 (1H, d, J = 11.8 Hz, H-14b), 3.88 (1H, d, J = 11.8 Hz, H-14a), 2.95 (1H, m, H-7), 2.87 (1H, dd, J = 14.1 5.1 Hz, H-9b), 2.45 (1H, m, H-9a), 2.42 (1H, m, H-2b), 2.35 (1H, m, H-3b), 2.22 (1H, m, H-2a), 2.19 (1H, m, H-3a), 1.67 (3H, d, J = 1.2 Hz, H-15); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 172.7 (C-12), 143.3 (C-4), 140.4 (C-11), 137.9 (C-10), 134.8 (C-1), 128.3 (C-5), 121.3 (C-13), 77.1 (C-6), 71.7 (C-8), 60.9 (C-14), 54.6 (C-7), 45.2 (C-9), 40.4 (C-3), 26.7 (C-2), 17.3 (C-15); HREIMS m/z 265.1073 $[M + H]^+$ (calcd for $C_{15}H_{21}O_4$, 249.1440).

https://dx.doi.org/10.1021/acsomega.0c03270
ACS Omega 2020, 5, 23989−23995

*8β-[4′,5′-Dihydroxytigloyloxy]-costunolide (**4**).* ^{1}H NMR (CD_3OD, 600 MHz) δ_H 6.90 (1H, t, J = 5.9 Hz, H-4′), 6.20 (1H, d, J = 3.3 Hz, H-13b), 5.86 (1H, d, J = 4.0 Hz, H-8), 5.66 (1H, d, J = 3.3 Hz, H-13a), 5.28 (1H, t, J = 9.3 Hz, H-6), 4.97 (1H, m, H-1), 4.90 (1H, d, J = 9.9 Hz, H-5), 4.40 (2H, d, J = 5.9, H-5′), 4.30 (2H, s, H-3′), 2.82 (1H, dd, J = 14.3, 4.8 Hz, H-9b), 2.45 (1H, dd, J = 15.7, 1.4 Hz, H-9a), 2.41 (1H, m, H-2b), 2.38 (1H, m, H-3b), 2.21 (1H, m, H-2a), 2.15 (1H, m, H-3a), 1.80 (3H, s, H-14), 1.53 (3H, s, H-15); ^{13}C NMR (CD_3OD, 150 MHz) δ_C 171.9 (C-11), 167.0, (C-1′), 146.9 (C-3′), 143.6 (C-2′), 138.9 (C-12), 135.50 (C-4), 132.5 (C-10), 131.7 (C-1), 128.6 (C-5), 121.5 (C-13), 77.7 (C-6), 73.9 (C-8), 59.4 (C-5′), 56.9 (C-4′), 53.7 (C-7), 44.7 (C-9), 40.3, (C-3), 27.1 (C-2), 19.5 (C-15), 17.6 (C-14); HREIMS m/z 363.2518 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

*8β-[4′-Hydroxytigloyloxy]-14-oxo-costunolide (**5**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 9.45 (1H, s, H-14), 6.76 (1H, m, H-3′), 6.62 (1H, m, H-1), 6.46 (1H, t, J = 8.3 Hz, H-8), 6.22 (1H, d, J = 2.8 Hz, H-13b), 5.58 (1H, d, J = 2.8 Hz, H-13a), 5.09 (1H, m, H-6), 5.08 (1H, m, H-5), 4.36 (2H, d, J = 5.7 Hz, H-4′), 2.80 (1H, m, H-9b), 2.52 (1H, m, H-2b), 2.51(1H, m, H-7), 2.41 (1H, m, H-3b), 2.29 (1H, m, H-2a), 2.11 (1H, d, J = 14.2 Hz, H-3a), 1.99 (1H, m, H-9a), 1.95 (3H, s, H-5′), 1.82 (3H, s, H-15); HREIMS m/z 361.2351 $[M + H]^+$ (calcd for $C_{20}H_{25}O_6$, 361.1651).

*3β-Acetoxy-8β-[4′,5′-dihydroxytigloyloxy]-costunolide (**6**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 6.93 (1H, s, H-3′), 6.32 (1H, s, H-13b), 5.78 (1H, s, H-13a), 5.59 (1H, d, J = 7.4 Hz, H-3), 5.27 (1H, m, H-8), 5.25 (1H, m, H-6), 5.22 (1H, d, J = 10.1 Hz, H-5), 5.08 (1H, s, H-1), 4.45 (2H, t, J = 8.8 Hz, H-4′), 4.39 (2H, d, J = 13.7 Hz, H-5′), 2.98 (1H, s, H-7), 2.72 (2H, d, J = 13.9 Hz, H-2b, 9b), 2.41 (1H, d, J = 13.5 Hz, H-9a), 2.11 (1H, s, H-2a), 2.10 (3H, s, -OAc), 1.90 (3H, s, H-14), 1.79 (3H, s, H-15);HREIMS m/z 421.2561 $[M + H]^+$ (calcd for $C_{22}H_{29}O_8$, 421.1862).

*2β-Hydroxy-8β-[5′-hydroxytigloyloxy]-costunolide (**7**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 6.39 (1H, q, J = 7.04 Hz, H-3′), 6.33 (1H, d, J = 2.3 Hz, H-13b), 5.85 (1H, d, J = 2.7 Hz, H-8), 5.61 (1H, d, J = 2.3 Hz, H-13a), 5.14 (1H, t, J = 9.1 Hz, H-6), 5.00 (2H, t, J = 11.6 Hz, H-1, 5), 4.75 (1H, dd, J = 15.3, 9.5 Hz, H-2), 4.26 (1H d, J = 12.7 Hz, H-5′), 4.17 (1H, d, J = 12.7 Hz, H-5′), 2.97 (1H, m, H-7), 2.91 (1H, dd, J = 14.3, 4.7 Hz, H-9b), 2.73 (1H, dd, J = 10.9, 5.7 Hz, H-3b), 2.37 (1H, d, J = 14.1 Hz, H-9a), 2.12 (1H, t, J = 10.4 Hz, H-3a), 2.02 (3H, d, J = 7.1 Hz, H-4′), 1.78 (3H, s, H-14), 1.54 (3H, s, H-15); HREIMS m/z 363.2511 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

*1β-Hydroxy-8β-[4′-hydroxytigloyloxy]-β-cyclocostunolide (**8**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 6.77 (1H, t, J = 5.3 Hz, H-3′), 6.15 (1H, d, J = 2.7 Hz, H-13b), 5.81 (1H, s, H-8), 5.45 (1H, d, J = 2.7 Hz, H-13a), 5.03 (1H, s, H-15b), 4.96 (1H, s, H-15a), 4.54 (1H, t, J = 11.0 Hz, H-6), 4.37 (1H, d, J = 5.8 Hz, H-4′), 3.53 (1H, dd, J = 11.3, 4.3 Hz, H-1), 2.86 (1H, J = 8.7 Hz, H-7), 2.39 (1H, d, J = 10.4 Hz, H-9b), 2.37 (1H, J = 7.3 Hz, H-2b), 2.34 (1H, m, H-3b), 2.27 (1H, d, J = 10.8 Hz, H-5), 2.12 (1H, m, H-3a), 1.63 (1H, m, H-9a), 1.60 (1H, m, H-2a), 1.83 (3H, s, H-5′), 0.97 (3H, s, H-14); HREIMS m/z 363.2534 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

*1β-Hydroxy-8β-[4′-hydroxytigloyloxy]-α-cyclocostunolide (**9**).* ^{1}H NMR ($CDCl_3$, 600 MHz) δ_H 6.78 (1H, s, H-3′), 6.15 (1H, s, H-13b), 5.81 (1H, s, H-8), 5.44 (1H, s, H-13a), 5.36 (1H, s, H-3), 4.45 (1H, m, H-6), 4.37 (2H, s, H-4′), 3.68 (1H, d, J = 8.6 Hz, H-1), 2.82 (1H, m, H-7), 2.37-2.40 (4H, m, H-2a, 2b, 5, 9a), 1.89 (3H, s, H-15), 1.83 (3H, s, H-5′), 1.60 (3H, s, H-14), 1.58 (1H, m, H-9b); HREIMS m/z 363.1801 $[M + H]^+$ (calcd for $C_{20}H_{27}O_6$, 363.1808).

*8β-[4′-Hydroxytigloyloxy]-costunolide (**10**).* ^{1}H NMR (CD_3OD, 600 MHz) δ_H 6.80 (1H, td, J = 5.9, 1.3 Hz, H-3′), 6.20 (1H, d, J = 3.3 Hz, H-13b), 5.82 (1H, d, J = 4.1 Hz, H-8), 5.64 (1H, d, J = 3.3 Hz, H-13a), 5.22 (1H, dd, J = 9.7, 8.8 Hz, H-6), 4.97 (1H, dd, J = 11.0, 2.4 Hz, H-1), 4.90 (1H, d, J = 10.0 Hz, H-5), 4.27 (2H, d, J = 5.73 Hz, H-4′), 3.19 (1H, m, H-7), 2.81 (1H, dd J = 14.3, 4.9 Hz, H-9b), 2.45 (1H, m, H-9a), 2.40 (2H, ddt, J = 8.0, 4.7, 4.0 Hz, H-2b, 3b), 2.21 (1H, dd, J = 11.0, 5.5 Hz, H-2a), 2.14 (1H, m, H-3a), 1.83 (3H, d, J = 1.1 Hz, H-5′), 1.79 (3H, d, J = 1.2 Hz, H-15), 1.51 (3H, s, H-14); HREIMS m/z 347.1836 $[M + H]^+$ (calcd for $C_{20}H_{27}O_5$, 347.1858).

Cytotoxicity Assay. The A549 human lung cancer cell line, MCF-7 breast cancer cell line, SKOV3 ovarian cancer cell line, and HEp-2 laryngeal carcinoma and PC3 prostate cancer cell lines were purchased from ATCC (American Type Culture Collection). Cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; WelGENE, Deagu, Korea) and Dulbecco's modified Eagle's medium (DMEM; WelGENE) containing 10% fetal bovine serum with 1% penicillin/streptomycin (WelGENE) under 5% CO_2 conditions at 37 °C. For cell culture, the cells were placed in 96-well plates at 5 × 10^3 cells/100 μL. After incubation overnight, the cells were treated with samples that were dissolved in various concentrations (1−100 μM) of dimethyl sulfoxide with 1% DMSO as negative control and docetaxel as positive control. After 48 h of incubation, cell viability was evaluated using MTT (Sigma-Aldrich) assay. The MTT solution at a concentration of 0.5 mg/mL was added to each well for 4 h at 37 °C. The absorbance was measured at 490 nm on a SpectraMax i3 multimode microplate reader (Molecular Devices). The IC_{50} values were calculated using GraphPad Prism software (GraphPad Software Inc., CA) as the average of three cycles of anticancer tests.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03270.

Cytotoxic activities of the fractions and the isolated compounds from *E. fortunei*; ^{1}H and ^{13}C NMR spectral data of EtOAc subfractions and compound **1** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Heejung Yang − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea;* orcid.org/0000-0001-5986-9024; Phone: +82-33-250-6919; Email: heejyang@kangwon.ac.kr

Authors

Jiho Lee − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*

Jinyoung Park − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*

Juyeol Kim − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*
Birang Jeong − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*
Seong Yeon Choi − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*
Hyeon Seok Jang − *Laboratory of Natural Products Chemistry, College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.0c03270

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (NRF-2018R1C1B6002574 and NRF-2019R1A6A3A13096888).

■ REFERENCES

(1) Tsugawa, H.; Ikeda, K.; Tanaka, W.; Senoo, Y.; Arita, M.; Arita, M. Comprehensive identification of sphingolipid species by in silico retention time and tandem mass spectral library. *J. Cheminformatics* **2017**, *9*, 19.
(2) Li, Y.; Kuhn, M.; Gavin, A.; Bork, P. Identification of metabolites from tandem mass spectra with a machine learning approach utilizing structural features. *Bioinformatics* **2020**, *36*, 1213−1218.
(3) Bacalhau, P.; Fernandes, L.; Martins, M. R.; Candeias, F.; Carreiro, E. P.; López, Ó.; Caldeira, A. T.; Totobenazara, J.; Guedes, R. C.; Burke, A. J. In silico, NMR and pharmacological evaluation of an hydroxyoxindole cholinesterase inhibitor. *Bioorg. Med. Chem.* **2019**, *27*, 354−363.
(4) Singh, D. K.; Sahu, A.; Wani, A. A.; Bharatam, P. V.; Kotimoole, C. N.; Batkulwar, K. B.; Deshpande, A. Y.; Giri, S.; Singh, S. Stability behaviour of antiretroviral drugs and their combinations. 10: LC-HRMS, LC-MSn, LC-NMR and NMR characterization of fosamprenavir degradation products and in silico determination of their ADMET properties. *Eur. J. Pharm. Biopharm.* **2019**, *142*, 165−178.
(5) Kaufmann, A.; Teale, P. *Capabilities and Limitations of High-Resolution Mass Spectrometry (HRMS): Time-of-flight and Orbitrap Chemical Analysis of Non-antimicrobial Veterinary Drug Residues in Food*; Kay, J. F.; Kay, J. F.; MacNeil, J. D.; Wang, J., Eds.; John Wiley & Sons, 2016; p 1.
(6) Zhang, C.; Idelbayev, Y.; Roberts, N.; Tao, Y.; Nannapaneni, Y.; Duggan, B. M.; Min, J.; Lin, E. C.; Gerwick, E. C.; Cottrell, G. W. Small molecule accurate recognition technology (smart) to enhance natural products research. *Sci. Rep.* **2017**, *7*, No. 14243.
(7) Reher, R.; Kim, H. W.; Zhang, C.; Mao, H. H.; Wang, M.; Nothias, L.; Caraballo-Rodriguez, A. M.; Glukhov, E.; Teke, B.; Leao, T.; et al. A Convolutional Neural Network-Based Approach for the Rapid Annotation of Molecularly Diverse Natural Products. *J. Am. Chem. Soc.* **2020**, *142*, 4114−4120.
(8) Kim, A.; Im, M.; Yim, N.; Ma, J. Y. Reduction of metastatic and angiogenic potency of malignant cancer by *Eupatorium fortunei* via suppression of MMP-9 activity and VEGF production. *Sci. Rep.* **2014**, *4*, No. 6994.
(9) Choi, J.; Lee, H.; Hwang, Y.; Lee, J.; Cho, W.; Ma, J. Y. *Eupatorium fortunei* and its components increase antiviral immune responses against RNA viruses. *Front. Pharmacol.* **2017**, *8*, 511.
(10) Liu, K.; Roeder, E.; Chen, T.; Xiu, X. Pyrrolizidine alkaloids from *Eupatorium fortunei*. *Phytochemistry* **1992**, *31*, 2573−2574.
(11) Pham, T. N.; Pham, H. D.; Dang, D. K.; Duong, T. T.; Le, T. P. Q.; Nguyen, Q. D.; Nguyen Tien, D. Anticyanobacterial phenolic constituents from the aerial parts of *Eupatorium fortunei* Turcz. *Nat. Prod. Res.* **2019**, *33*, 1345−1348.
(12) Jiang, H. X.; Liu, Q.; Gao, K. Benzofuran derivatives from *Eupatorium fortunei*. *Nat. Prod. Res.* **2008**, *22*, 937−941.
(13) Jiang, H.; Li, Y.; Pan, J.; Gao, K. Terpenoids from *Eupatorium fortunei* TURCZ. *Helv. Chim. Acta* **2006**, *89*, 558−566.
(14) Chen, Y.; Jiang, H.; Gao, K. One novel nortriterpene and other constituents from *Eupatorium fortunei* Turcz. *Biochem. Syst. Ecol.* **2013**, *47*, 1−4.
(15) Picman, A. K. Biological activities of sesquiterpene lactones. *Biochem. Syst. Ecol.* **1986**, *14*, 255−281.
(16) Kupchan, S. M.; Maruyama, M.; Hemingway, R.; Hemingway, J.; Shibuya, S.; Fujita, T.; Cradwick, P.; Hardy, A.; Sim, G. Tumor inhibitors. LXVII. Eupacunin, a novel antileukemic sesquiterpene lactone from *Eupatorium cuneifolium*. *J. Am. Chem. Soc.* **1971**, *93*, 4914−4916.
(17) Kupchan, S. M.; Fujita, T.; Maruyama, M.; Britton, R. W. Tumor inhibitors. LXXXIV. Isolation and structural elucidation of eupaserrin and deacetyleupaserrin, new antileukemic sesquiterpene lactones from *Eupatorium semiserratum*. *J. Org. Chem.* **1973**, *38*, 1260−1264.
(18) Zdero, C.; Bohlmann, F.; Schmeda-Hirschmann, G. Beyerene derivatives and other terpenoids from *Stevia aristata*. *Phytochemistry* **1987**, *26*, 463−466.
(19) Spring, O.; Zipper, R.; Conrad, J.; Vogler, B.; Klaiber, I.; Da Costa, F. B. Sesquiterpene lactones from glandular trichomes of *Viguiera radula* (Heliantheae; Asteraceae). *Phytochemistry* **2003**, *62*, 1185−1189.
(20) Herz, W.; Kumar, N. Sesquiterpene lactones from *Helianthus grosseserratus*. *Phytochemistry* **1981**, *20*, 99−104.
(21) Jolad, S. D.; Hoffmann, J. J.; Bates, R. B.; Calders, S.; McLaughlin, S. Caudatol, a guaianolide from *Pericome caudata*. *Phytochemistry* **1990**, *29*, 3024−3028.
(22) Takahashi, T.; Eto, H.; Ichimura, T.; Murae, T. Hiyodorilactones A and B, new tumor inhibitory germacradienolides from *Eupatorium sachalinense* Makino. *Chem. Lett.* **1978**, *7*, 1345−1348.
(23) Herz, W.; De Groote, R. Desacetyleupaserrin and nevadensin from *Helianthus pumilus*. *Phytochemistry* **1977**, *16*, 1307−1308.
(24) Hernández, L. R.; De Riscala, E. C.; Catalán, C. A.; Díaz, J. G.; Herz, W. Sesquiterpene lactones and other constituents of Stevia maimarensis and *Synedrellopsis grisebachii*. *Phytochemistry* **1996**, *42*, 681−684.
(25) Hernández, L. R.; Catalán, C. A.; Cerda-García-Rojas, C. M.; Joseph-Nathan, P. Sesquiterpene lactones from *Stevia breviaristata*. *Phytochemistry* **1994**, *37*, 1331−1335.
(26) Bohlmann, F.; Schmeda-Hirschmann, G.; Jakupovic, J. Heliangolides and germacrolides from *Disynaphia multicrenulata*. *Phytochemistry* **1984**, *23*, 1435−1437.
(27) Buděšínský, M.; Šaman, D. Carbon-13 NMR spectra of sesquiterpene lactones. In *Annual Reports on NMR Spectroscopy*; Elsevier, 1995; pp 231−475.
(28) Feunang, Y. D.; Eisner, R.; Knox, C.; Chepelev, L.; Hastings, J.; Owen, G.; Fahy, E.; Steinbeck, C.; Subramanian, S.; Bolton, E. ClassyFire: automated chemical classification with a comprehensive, computable taxonomy. *J. Cheminformatics* **2016**, *8*, 61.