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RESEARCH ARTICLE

# A new sesquiterpenoid from the shoots of Iranian *Daphne mucronata* Royle with selective inhibition of STAT3 and Smad3/4 cancer-related signaling pathways

Mustafa Ghanadian[1,2] · Zulfiqar Ali[2] · Ikhlas A. Khan[2] · Premalatha Balachandran[2] · Maryam Nikahd[1] · Mahmoud Aghaei[3] · Mahmoud Mirzaei[4] · Seyed Ebrahim Sajjadi[1]

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## Abstract

**Purpose** *Daphne mucronata* Royle grown in Iran has shown anticancer activities against different cancer cell lines. Therefore, within this study, we investigate the phytochemical pattern of this plant.

**Method** Phytochemical investigation was done using standard column chromatography system: The structures were recognized by the interpretation of one and two-dimensional nuclear magnetic resonance (NMR) spectra and the help of High-Resolution Electrospray Ionization Mass spectroscopy (HR-ESIMS) and Infrared spectroscopy (IR) data. Stereochemistry was determined using 2D and 3D NOESY, and comparison of coupling constant values with literature. The absolute configuration was determined and confirmed using specific rotation and electronic circular dichroism experiments. Cytotoxicity was done against HeLa cells by standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Luciferase assay was used to check if the compounds can inhibit the activation of cancer-related signaling pathways. Molecular docking simulation was done for biological activity evaluation and to examine the interaction of the ligand with each of the proteins.

**Results** A new sesquiterpenoid, 4,11(12)-guiadiene-1-ol-3-one (**4**), together with eight specialized metabolites, betulinic acid (**1**), coniferyl aldehyde (**2**), oleanolic acid (**3**), daphnetoxin (**5**), apigenin (**7**), syringin (**8**), and genkwanol A (**9**) were isolated and reported for the first time from the shoots of the plant. Compound **4** as an undescribed compound was submitted for cytotoxicity assay and showed moderate activity with the $IC_{50}$ value of 51.3 ± 4.2 μM against HeLa cancer cells. It showed selective inhibition of Interleukin-6 mediated signal transducer and activator of transcription 3 pathway (STAT-3/ IL-6), and Smad protein / transforming growth factor beta (TGF-β) transcription factors when screened through an array of cancer signaling pathways. Molecular docking confirmed biological tests and showed the interaction with STAT3 and Smad proteins.

**Conclusion** An undescribed sesquiterpenoid: 4,11(12)-guiadiene-1-ol-3-one in addition to eight known compounds were isolated. The new sesquiterpene was evaluated for the luciferase assay on 14 main cancer-related signaling pathways and showed selective inhibition of STAT3/IL6, and Smad/ TGF-β transcription factors. Molecular docking simulation showed more interactions with STAT3 than Smad, which confirms better interaction of compound **4** with STAT3 than Smad proteins.

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s40199-020-00336-x) contains supplementary material, which is available to authorized users.

✉ Mustafa Ghanadian
ghannadian@gmail.com

✉ Maryam Nikahd

[1] Department of Pharmacognosy, Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

[2] National Center for Natural Products Research, University of Mississippi, University, Oxford, MS 38677, USA

[3] Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

[4] Biosensor Research Center, School of Advanced Technologies in Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

**Keywords** *Daphne* ***mucronata*** · Luciferase assay · Molecular docking · Cancer signaling · Smad · STAT

## Introduction

Natural products have been used as medicine for several centuries, and herbal medicine has been increasingly used as a pain reliever and for curing various diseases. Thymelaeaceae is one of the large plant families with 44 genera and 500 species, including *Daphne* genus. The *Daphne* species are characterized as shrub either straight or recumbent with clustered or scattered alternate and leathery leaves. Flowers are hermaphrodite in a cluster or umbrella form, pink and fragrant, and fruits are fleshy, ovoid, or almost spherical [1]. Daphne fruits are not edible and reported to be toxic [2]. *Daphne mucronata* Royle (common name Kheweshk) is a wild shrub that grows in different parts of Iran [3, 4]. It is used in ethnomedicine for musculoskeletal disorders, rheumatoid, and wound healing [3]. Recently it has shown antituberculosis and anticancer activities against different cancer cell lines [3, 5]. During the last decade, various compounds were reported from *D. mucronata* grows in Pakistan, which include daphnecin, aquillochin, mucronin A, and mucronin B, cinnamic acid, 7,8-dimethoxycoumarin, 7,8-dihdroxycoumarin, umbelliferone, cumarin, daphnin, lupeol, β-amyrin, betulin, 3-O-β-glucostigmasterol, 5-hydroxy-3,6,7,4′-tetramethoxyflavone, 5,7,3′,4′-tetrahydroxyflavone, and 5,3′,4′-trihydroxyflavone-7-O-β-D-glucopyranoside [5–7]. The last two flavonoids showed good antioxidant, whereas mucronin A and mucronin B showed antituberculosis activities [5, 7]. Previous phytochemical research on the leaves of this plant, as well as anticancer biological studies of this plant, made us interested in a selection of other parts of the plant (shoots) for further phytochemical investigation with the aim of the discovery of new anticancer structures Figs 1, 2 and 3.

## Methods

### General experimental procedures

The specific rotation was recorded by AutoPol IV polarimeter (Rudolph, Hackettstown, NJ, USA). IR spectrum was obtained by Rayleigh WQF-510 FTIR spectrometer (China) on NaCl pellets. NMR spectra were taken at 25 °C on AVANCE DRX-400 and 500 NMR spectrometers (Bruker, Ettlingen, Germany). High-resolution mass spectra were acquired on an Agilent 1100 SL series mass spectrometer (Agilent Technologies Inc., Wilmington, DE, USA). Electronic circular dichroism data was taken by Olis DSM 20 CD digital spectrophotometer (Bogart, GA, USA). Flash silica gel (40–63 μm; Fisher Scientific, Fair Lawn, NJ, USA), and Sephadex LH-20 (Sigma-Aldrich) were used for gravity column chromatographies (CC) and size exclusion chromatographies (SEC). Spots were detected using UV light cabinet and visualized by 1% vanillin in 5% sulfuric acid solution-processed by hair dryer heating. Methanol (MeOH), chloroform ($CHCl_3$), hexanes, ethyl acetate (EtOAc), acetone ($Me_2CO$), and all other solvents or reagent were purchased from Fisher Scientific International, Inc. (China) or Sigma-Aldrich (USA) otherwise it is mentioned in the text.

### Plant material

The shoots of the plant were collected in September from Padena region located in Isfahan province, Iran. Mohammad Taghi Feizi recognized the plant and a specimen (herbarium No. 3411) has been deposited in the Samsam Shariat Herbarium, Department of Pharmacognosy, School of Pharmacy, Isfahan University of Medical Sciences.

### Extraction and isolation

The shade dried shoots were crushed to a fine powder (2180 g) and extracted in a percolator using chloroform: methanol (2:1) with a flow rate of 3 mL/min for 10 days. The extract was filtered, concentrated in a rotary evaporator at 40 °C, and yielded a gummy material (205 g). It was then applied on VLC in a sintered glass filter funnel (10*7 cm) containing reversed-phase $RP_{18}$ using MeOH: $H_2O$ (30:70, 2 L; 60:40, 6 L; 100:0, 2 L) as a solvent. Fraction eluted by MeOH: $H_2O$ (60:40, 163 g) free from fats and sugars was selected, coated on Celite and applied on flash silica gel (40–63 μm; 10 × 13 cm) using $CHCl_3$ (3 L, Fr. 3–4, 6.6 g); $CHCl_3$:MeOH 9:1 (6 L, Fr. 3–6, 66.5 g); $CHCl_3$:MeOH 8:2 (5 L, Fr. 7–8, 81.3 g); $Me_2CO$ (3 L, Fr. 9, 21.0 g); and MeOH (3 L, Fr. 10, 12.0 g). Based on TLC analysis fraction 4 (45.5 g) was applied on a flash silica gel (40–63 μm; 7 × 45 cm) using hexanes: EtOAc (5 → 100%); EtOAc: MeOH (85:15); and MeOH to yield Fr-4-1 to Fr-4-25 subfractions. Fr-4-6 (250 mg) was applied on SEC (hexanes: acetone: methanol, 3:1:6, 3× 100 cm) and yielded compound **1** (156 mg). Fr-4-9 (300 mg) was applied on SEC (hexanes: acetone: methanol, 3:1:6, 3 × 100 cm) and yielded compound **2** (178 mg). Fr-4-11 (333 mg) was applied on SEC (hexanes: acetone: methanol, 3:1:6, 3× 100 cm) and yielded compound **3** (72 mg) and **4** (47 mg). Fr-4-19 (1.8 g) was applied to the SEC and got three subfractions. Fr-4-19-3 (100 mg) was submitted on CC (chloroform: methanol, 96:4, 2× 100 cm) and yielded compound **5** (27 mg). Fr-4-20 (1.5 g) was applied on SEC (hexanes: acetone: methanol, 3:1:6, 7 × 50 cm) and yielded four subfractions, from which Fr-4-20-4 (100 mg) was submitted on CC (chloroform: methanol, 97:3,

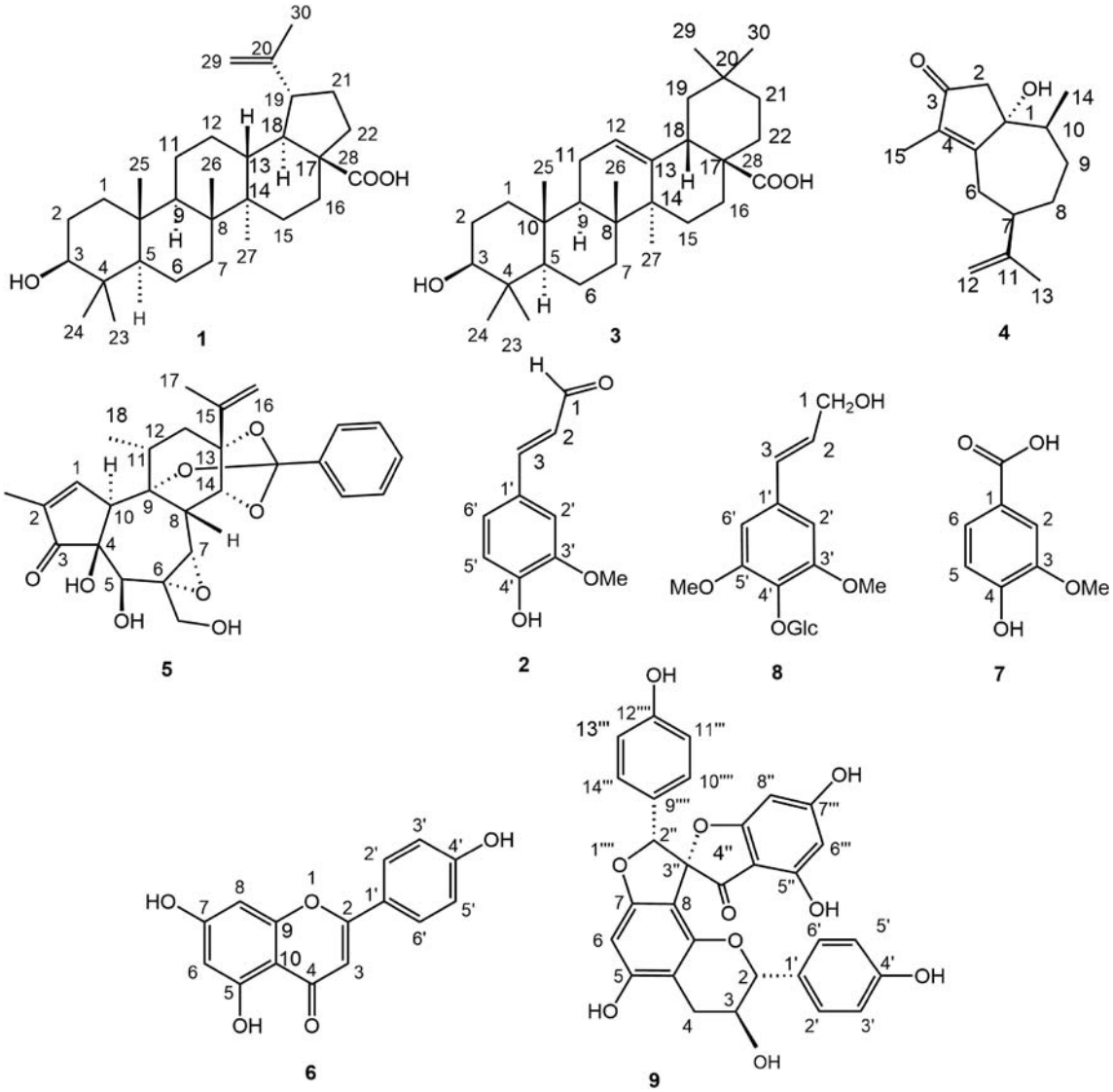


**Fig. 1** Terpenoid and phenolic compounds from the shoots of Iranian *Daphne mucronata*

1.5× 120 cm) and yielded compound **6** (5 mg), and **7** (4 mg). Fr-4-6 (20 g) was submitted on CC (ethyl acetate: chloroform: methanol: water, 15: 8:2; 0.5 followed by 15:8:4:1, 5× 70 cm) and yielded seven subfractions including compound **8** (570 mg). Fr-4-6-2 (350 mg) was injected into preparative recycling HPLC on Shimadzu's prep-Shim-pack column

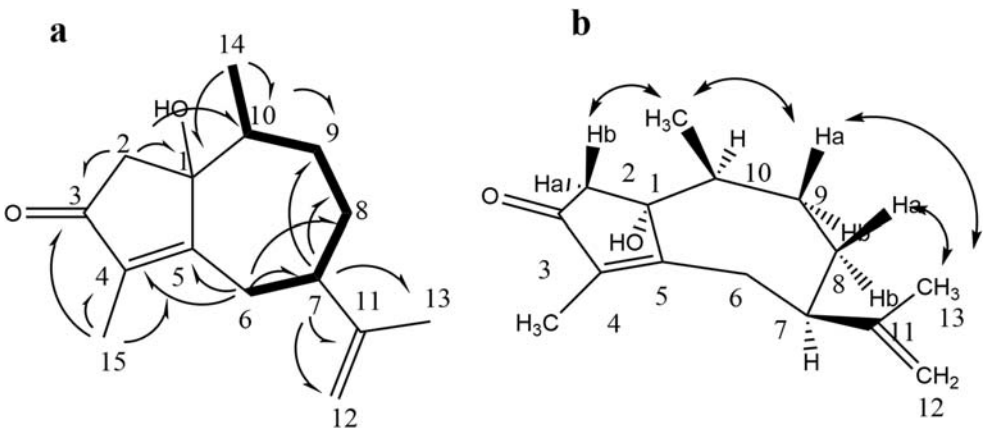


**Fig. 2** a) $^{1}H$-$^{1}H$ COSY (black bold line), HMBC correlations (C → H), and b) calculated molecular structure and spatial proximities through NOESY (H↔H) correlations of compound **4**

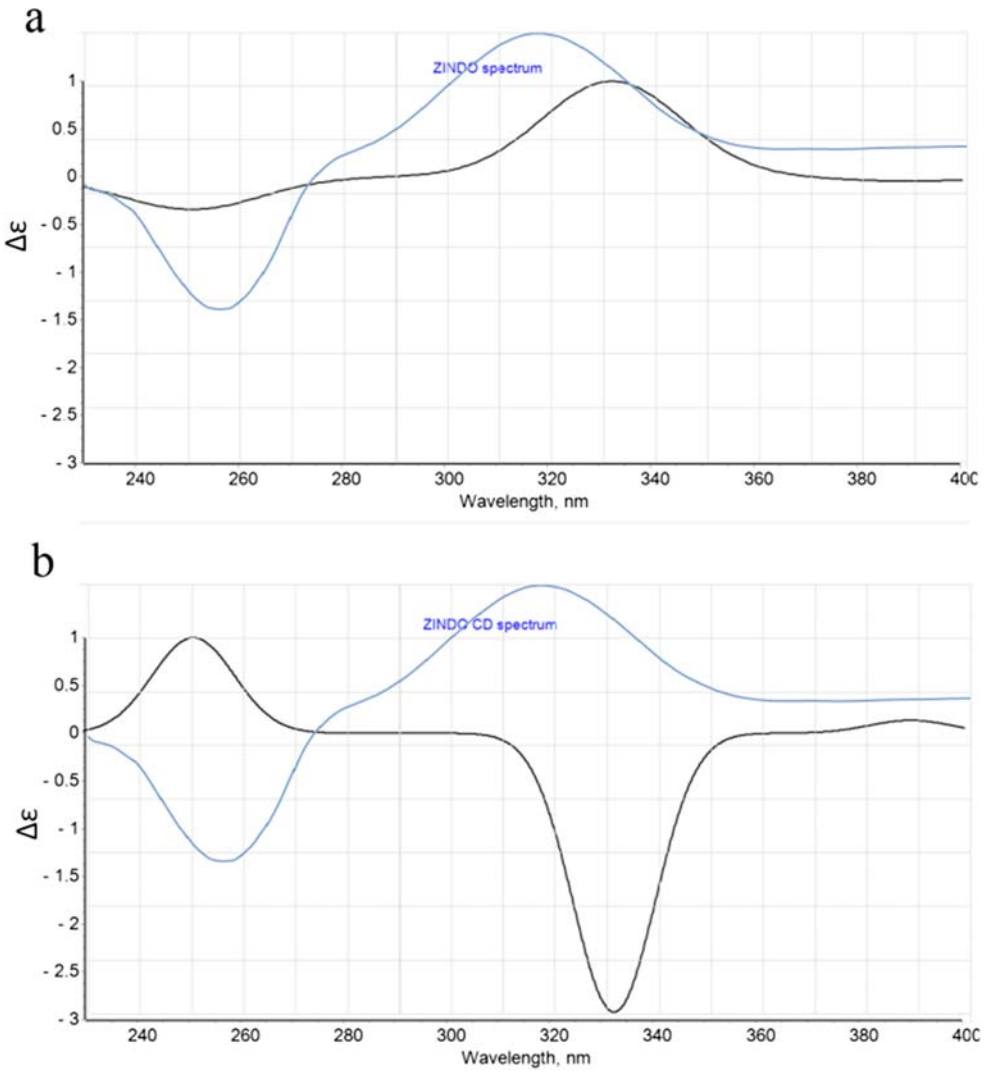


**Fig. 3** Determination of the absolute configuration of compound **4** by comparison of the experimental CD spectrum (Blue line) with the spectra calculated (Black line) for a) C-1(R) and b) C-1(S) enantiomers using the ZINDO keyword in Gaussian 09

(5 μm, 22 × 250 mm) using acetonitrile: water (2:1) and processed for three recycle stages to get compound **9** (23 mg) in a pure state. The structures were recognized by the interpretation of 1 and 2D NMR spectra and the help of HR-ESIMS and IR data. Stereochemistry was determined using 2D, and 3D NOESY, and comparison of coupling constant values with literature. The absolute configuration was determined and confirmed using specific rotation and electronic circular dichroism experiments.

## HSQC-NOESY experiment

The stereochemistry was determined through the three-dimensional HSQC-NOESY experiment, comparison with similar compounds, and taking ECD spectra. NOESY spectrum correlates spatially close protons not those that are attached through bonds and identify relative stereochemistry. However, we encountered a problem in the region of some overlapped protons. In this case, HSQC-NOESY lets us clearly find $^{1}$H-$^{1}$H NOE correlations using the deconvoluted $F_2$($^{13}$C) axis. In this way, $F_1$ ($^{1}$H)–$F_2$ ($^{13}$C) dimension refers to $^{1}$H-$^{13}$C HSQC, and $F_1$ ($^{1}$H)–$F_3$ ($^{1}$H) projection refers to $^{1}$H–$^{1}$H NOESY.

## Conformational and ECD predictions of compound 4 by computations

The conformational analyses were performed using the Gaussian program [Gaussian, Inc., Wallingford CT, 2009] by optimizing the structure of compound **4** by computing at the # HF/6-31G* geometry of the theoretical level. For each single geometries, a CD spectrum was calculated using the ZINDO procedure as implemented in the Gaussian program, and the CD spectra were visualized using Chemcraft program [Chemcraft ver 1.8, https://www.chemcraftprog.com].

### Cell cytotoxicity assay

HeLa cancer cells (Institute Pasteur, Tehran, Iran) were cultured in RPMI-1640 (fetal bovine serum: 10%, penicillin: 100 U/mL, and streptomycin: 100 μg/mL) in criteria of 5% $CO_2$ at 37 °C temperature. Cells were seeded separately in 96-well plates ($5 \times 10^3$ cells/well) using the same media and incubated in 5% $CO_2$ at 37 °C. Next day, fresh media was replaced and doxorubicin hydrochloride (Ebewe Pharma, Austria) in the concentration of 1 μM as positive control, vehicle-treated cells as negative control and compound **4** (new compound) as sample in serially diluted concentrations (0.1, 1, 10, 50, 100, and 200 μM) were added and incubated for 48 h. MTT (5 mg/mL in PBS) was added to each well and incubated again for four hours. Finally, well supernatants were taken out, dimethyl sulfoxide (150 μL) was added, and well plates were shaken for 10 min. Optical density values (OD absorption) were read at 570 nm using Bio-Rad microplate reader (Hercules, CA, USA), and cell viabilities were evaluated by the following equation [8].

$$\text{Viability } (\%) = 100 \times (OD_{sample} - OD_{blank})/(OD_{negative\ control} - OD_{blank}).$$

### Transfection and luciferase assay

HeLa cells from ATCC (CCL-2) were seeded in 384 well plates (density of 4300 cells/well) in 30 μL of growth medium containing Dulbecco's Modified Eagle Medium (DMEM) with fetal bovine serum (10%), penicillin, and streptomycin (each 100 μg/mL). After 24 h, the media were aspirated and replaced with fresh DMEM (containing 10% FBS). Then cells were transfected by different and appropriate plasmids using X-treme GENE HP transfection reagent (Roche, Germany). Next day, samples were added to the transfected cells, followed by addition of appropriate inducing agents (IL-6 for Stat 3, TGF-β for Smad, m-wnt3a for Wnt, and phorbol 12-myristate 13-acetate (PMA) for Activator protein 1 (AP-1), Nuclear factor kappa B (NF-κB), transcription factors E2F, ETS, Myc, Notch, and Hedgehog) 30 min later. No inducers were added for the Forkhead family of transcription factors (FOXO), K-Ras protein, microRNA 21 (miR-21) and Aryl hydrocarbon receptor (AhR). Empty vector without gene of interest was used as the negative control. Depend on each signaling pathway, after 4 h or 6 h of induction, the cells were lysed by the addition of the One-Glo luciferase assay system from Promega Corporation (Madison, WI, USA). The light output was detected in a Glomax Multi+ detection system with Instinct Software (Promega). Luciferase assay determines if the test agent is able to inhibit the induction of cancer-related signaling pathways by tumor environment-relevant inducing agents [8, 9].

### Molecular docking

For computational evaluation of the biological activity of compound **4**, we have performed molecular docking simulation. For this purpose, the 3D model of compound **4** as a ligand was first optimized at the hf/6-31 g* level of theory to obtain a minimized energy structure. The 3D file of the enzyme (receptor) was taken from Protein Data Bank (PDB) archive (STAT3 PDB id: 6QHD and Smad PDB id: LU7F). Molecular docking simulations were performed with a grid box of 126 × 126 × 126 with the spacing of 0.7 A to contain protein structure. The ligand was processed by configuration changes for 300 steps based on the genetic algorithm as implemented in the AutoDock4 program [10].

## Results

### Spectral data of isolated compounds

Compound **1**. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta_H$ 3.32–3.20 (m, H-3), 2.96 (td, $J$ = 9.1, 5.5 Hz, H-19), 0.87–0.86 (bs, H-23, 27), 0.65 (s, H-24), 0.76 (s, H-25), 0.93 (s, H-26), 4.68 (brs, H-29b), 4.56 (brs, H-29a), 1.64 (s, H-30). $^{13}$C-NMR data (DMSO-$d_6$, 125 MHz): $\delta_C$ 38.7 (C-1), 25.5 (C-2), 77.2 (C-3), 39.0 (C-4), 55.3 (C-5), 18.4 (C-6), 34.1 (C-7), 40.7 (C-8), 50.4 (C-9), 37.2 (C-10), 20.8 (C-11), 25.0 (C-12), 38.0 (C-13), 42.4 (C-14), 30.5 (C-15), 31.8 (C-16), 55.9 (C-17), 49.0 (C-18), 47.1 (C-19), 150.8 (C-20), 36.8 (C-21), 29.7 (C-22), 27.6 (C-23), 16.2 (C-24), 15.5 (C-25), 16.4 (C-26), 14.8 (C-27), 177.7 (C-28), 110.1 (C-29), 19.4 (C-30). Neg. HR-ESIMS at $m/z$ 455.3543 (calculated: 455.3531, $C_{30}H_{47}O_3$ $[M\text{-}H]^-$, Δ 2.64 ppm).

Compound **2**.$^1$H NMR (500 MHz, Chloroform-$d_1$): $\delta_H$ 9.61 (d, $J$ = 7.8 Hz, H-1), 6.60 (dd, $J$ = 15.7, 7.8 Hz, H-2), 7.41 (d, $J$ = 15.8 Hz, H-3), 7.06 (d, $J$ = 2.0 Hz, H-2′), 6.95 (d, $J$ = 8.2 Hz, H-5′), 7.10 (dd, $J$ = 2.0, 8.4 Hz, H-6′), 3.91 (s, 3′-OMe). $^{13}$C NMR (126 MHz, Chloroform-$d_1$) $\delta_C$ 194.2 (C-1), 126.1 (C-2), 153.9 (C-3), 126.5 (C-1′), 109.8 (C-2′), 147.2 (C-3′), 149.2 (C-4′), 115.1 (C-5′), 124.2 (C-6′), 56.0 (3′-OMe). Neg. HR-ESIMS at $m/z$ 177.061 (calculated: 177.0577, $C_{10}H_9O_3$ $[M\text{-}H]^-$, Δ −9.03 ppm).

Compound **3.** $^1$H NMR (500 MHz, Chloroform-$d_1$): $\delta_H$ 3.24 (dd, $J$ = 11.2, 4.3 Hz, H-3), 5.29 (t, $J$ = 3.4 Hz, H-12), 2.84 (dd, $J$ = 13.6, 4.0 Hz, H-18), 1.00 (s, H-23), 0.93 (s, H-25), 0.77 (s, H-26), 0.79 (s, H-27), 1.15 (s, H-28), 0.92 (s, H-29), 0.94 (s,H-30). $^{13}$C-NMR (126 MHz, Chloroform-$d_1$): $\delta_C$ 38.4 (C-1), 27.1 (C-2), 79.1 (C-3), 38.8 (C-4), 55.2 (C-5), 18.3 (C-6), 32.6 (C-7), 39.3 (C-8), 47.6 (C-9), 37.1 (C-10), 22.9 (C-11), 122.6 (C-12), 143.6 (C-13), 41.6 (C-14), 27.3 (C-15), 23.4 (C-16), 45.9 (C-17), 41.0 (C-18), 46.5 (C-19), 30.7 (C-20), 33.8 (C-21), 32.4 (C-22), 28.1 (C-23), 15.6 (C-24), 15.3 (C-25), 17.0 (C-26), 26.0 (C-27), 183.9 (C-28),

33.1 (C-29), 23.6 (C-30). Neg. HR-ESIMS at *m/z* 455.3533 (calculated: 455.3531, $C_{30}H_{47}O_3$ $[M-H]^-$, Δ 0.44 ppm).

Compound **4.** Amorphous white powder, $[\alpha]^D$ 225.9 (c 0.1%, MeOH); UV (MeOH) λmax: 256, 317 nm; IR (NaCl) $\nu_{max}$: 3452, 3080, 2966, 2939, 2877, 1704, 1651, 1456, 1334, 1107, 891, 758 $cm^{-1}$. $^1$H-NMR (Chloroform-$d_1$, 500 MHz, *J* in Hz) see Table 1.$^{13}$C-NMR data (Chloroform-$d_1$, 125 MHz): see Table 1. Pos. HR-ESIMS at *m/z* 235.1703 (calculated: 235.1693, $C_{15}H_{23}O_2$ $[M+H]^+$, Δ 4.25 ppm).

Compound **5.** $^1$H NMR (500 MHz, Methanol-$d_4$): $\delta_H$ 7.62 (s, H-1), 4.15 (brs, H- 5), 3.49 (brs, H-7), 3.08 (brd, *J* = 2.5 Hz, H-8), 3.93 (t, *J* = 2.67 Hz, H-10), 2.67–2.58 (m, H-11), 2.36 (dd, *J* = 14.3, 8.7 Hz, H-12a), 1.66 (d, *J* = 14.2 Hz, H-12b), 4.63(d, *J* = 2.6 Hz, H-14), 5.08 (s, H-16a), 4.91 (s, H- 16b), 1.83 (br s, H-17), 1.23 (d, *J* = 7.1 Hz, H-18), 1.78 (d, *J* = 2.5 Hz, H-19), 4.05 (d, *J* = 12.3 Hz, H-20a), 3.63 (d, *J* = 12.3 Hz, H-20b), 7.75 (dd, *J* = 7.7, 2.1 Hz, H-3 ‘, H-7’), 7.38 (overlapped, H-5′, H-4′, H-6′). $^{13}$C NMR (126 MHz, Methanol-$d_4$): $\delta_C$ 160.8 (C-1), 138.0 (C-2), 210.1 (C-3), 74.5 (C-4), 71.4 (C-5), 63.0 (C-6), 64.8 (C-7), 37.8 (C-8), 81.7 (C-9), 49.8 (C-10), 36.2 (C-11), 37.6 (C-12), 86.3 (C-13), 83.6 (C-14), 148.0 (C-15), 111.5 (C-16), 19.4 (C-17), 20.9 (C-18), 10.0 (C-19), 65.2 (C-20), 118.5 (C-1′), 130.3 (C-2′), 127.2 (C-3′, 7′), 128.8 (C-4′, 6′), 137.7 (C-5′). HR-ESIMS at *m/z* 505.1858 (calculated: 505.1833, $C_{27}H_{30}O_8$ + Na $[M+Na]^+$, Δ 4.94 ppm).

Compound **6**. $^1$H NMR (400 MHz, Acetone-$d_6$): $\delta_H$ 6.64 (s, H-3), 6.26 (d, *J* = 2.1 Hz, H-6), 6.53 (d, *J* = 2.1 Hz, H-8), 7.93 (d, *J* = 8.8 Hz, H-2′,6′), 7.02 (d, *J* = 8.8 Hz, H-3′,5′), 12.98 (s, OH). $^{13}$C NMR (126 MHz, Acetone) $\delta_C$ 165.0 (C-2), 104.1 (C-3), 183.1 (C-4), 105.3 (C-4a), 163.4 (C-5), 99.7 (C-6), 165.1 (C-7), 94.7 (C-8), 160.8 (C-8a), 123.2 (C-1′), 129.3 (C-2′, 6′), 116.9 (C-3′, 5′), 158.8 (C-4′). Neg. HR-ESIMS at *m/z* 269.0469 (calculated: 269.0455, $C_{15}H_9O_5$ $[M-H]^-$, Δ 5.20 ppm).

Compound **7**. $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta_H$ 7.43 (d, 1.9, H-2), 6.84 (d, *J* = 7.9 Hz, H-5), 7.45 (dd, *J* = 1.9, 7.9 Hz, H-6), 3.81 (s, 3-OMe). $^{13}$C NMR (126 MHz, DMSO-$d_6$) $\delta_C$ 167.7 (COOH), 122.0 (C-1), 115.5 (C-2), 147.7 (C-3), 151.5 (C-4), 113.1 (C-5), 123.9 (C-6), 55.9 (OMe). Neg. HR-ESIMS at *m/z* 167.0349 (calculated: 167.0350, $C_8H_7O_4$ $[M-H]^-$, Δ −0.6 ppm).

Compound **8**. $^1$H NMR (500 MHz, Methanol-$d_4$): $\delta_H$ 4.22 (d, *J* = 5.5 Hz, H-1), 6.30 (dt, *J* = 15.9, 5.5 Hz, H-2), 6.53 (d, *J* = 15.9 Hz, H-3), 6.74 (s, H-2′, 6′), 4.87 (d, *J* = 7.6 Hz, Glc-1′′), 3.82–3.2 (6H, Glc-2′′,3′′,4′′,5′′, 6a′′, and 6b′′), 3.85 (s, 3′-OMe, 5′-OMe). $^{13}$C NMR (126 MHz, Methanol-$d_4$): $\delta_C$ 63.5 (C-1), 130.0 (C-2), 131.2 (C-3), 135.2 (C-1′), 105.3 (C-2′), 154.2 (C-3′, 5′), 135.7 (C-4′), 105.2 (Glc-1′′), 78.2 (Glc-5′′), 77.7 (Glc-3′′), 75.6 (Glc-2′′), 71.2 (Glc-4′′), 62.4 (Glc-6′′), 57.0 (3′-OMe, 5′-OMe). Pos. HR-ESIMS at *m/z* 395.1340 (calculated: 395.1312, $C_{17}H_{24}O_9$ + Na $[M+Na]^+$, Δ 7.08 ppm).

Compound **9.** $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$ 4.61 (d, *J* = 6.7 Hz, H-2), 3.79–3.67 (m, H-3), 2.41 (dd, *J* = 16.0, 7.2 Hz, H-4a), 2.54 (dd, *J* = 16.2, 4.6 Hz, H-4b), 6.10 (s, H-6), 6.97 (d, *J* = 8.0 Hz, H-2′, H-6′), 6.62 (d, *J* = 7.9 Hz, H-3′, H-5′), 5.45 (s, H-2′′), 5.73 (d, *J* = 1.8 Hz, H-6′′), 5.45 (d, *J* = 1.9 Hz, H-8′′), 7.03(d, *J* = 7.8 Hz, H-10′′, H-14′′), 6.66 (d, *J* = 7.8 Hz, H-11′′, H-13′′). $^{13}$C-NMR data (DMSO-$d_6$, 125 MHz): $\delta_C$ 79.8 (C-2), 66.1 (C-3), 27.2 (C-4), 101.1 (C-4a), 159.1 (C-5), 89.6 (C-6), 160.0 (C-7), 102.0 (C-8), 151.3 (C-8a), 125.1 (C-1′), 127.6 (C-2′, C-6′), 115.1 (C-3′, C-5′), 156.3 (C-4′), 91.6 (C-2′′), 93.7 (C-3′′), 193.2 (C-4′′), 102.0 (C-4a′′), 157.3 (C-5′′), 96.4 (6′′), 167.0 (C-7′′), 89.3 (C-8′′), 172.2 (C-8a′′), 123.0 (C-9′′), 129.3 (C-10′′, C-14′′), 115.1 (C-11′′, C-13′′), 157.6 (C-12′′). Neg. HR-ESIMS at *m/z* 541.1149 (calculated: 541.1129, $C_{30}H_{21}O_{10}$ $[M-H]^-$, Δ 3.40 ppm).

## Cytotoxicity test

In the biological part, compound **4** showed moderate cytotoxic activities with the $IC_{50}$ value of 51.3 ± 4.2 μM against HeLa cancer cells (Fig. 4).

## Inhibition of cancer signaling pathways

Compound **4** was tested through an array of 14 cancer-related signaling pathways, as specified in the experimental part. It showed apparently selective inhibition of STAT3 and Smad3/4 with no significant response in other pathways up to the concentration of 100 μM.

STAT3 and Smad3/4 transcription factors are regulators of cell proliferation, survival, and self-renewal, which are significantly activated in several cancers. The luciferase assays showed inhibition of STAT3 with an $IC_{50}$ value of 84 μM, and Smad signaling with the $IC_{50}$ value of 75 μM (Fig. 5).

## Molecular docking

We performed molecular docking simulation to examine the interactions of compound **4** with each of STAT3 and Smad proteins. Values of energy binding were evaluated as interaction strengths and their schematics are represented in Fig. 5. The results showed the interaction of compound **4** with STAT3 with a binding energy of −7.04 kcal/mol, close to Smad with a binding energy of −6.55 kcal/mol. The visualized complex of ligand and interacting amino acids in receptor sites, along with qualities of interactions were presented in Fig. 6, in which both systems have either hydrogen or non-hydrogen bond interactions.

**Table 1** $^{1}H$ and $^{13}C$ NMR Data of Compound **4**, at 500 and 125 MHz in $CDCl_3$

| Position | $\delta_H$ (mult., J in Hz) | $\delta_C$ | *Position* | $\delta_H$ (mult., *J* in Hz) | $\delta_C$ |
|---|---|---|---|---|---|
| 1 | – | 82.9 (s) | 8a | 1.46–1.54 (overlapped) | 30.7 (t) |
| 2a | 2.50 (d, 18.2) | 51.2 (t) | 8b | 2.17 (dddd, 4.2, 3.5, 10.6, 12.4) | |
| 2b | 2.59 (d, 18.2) | | 9a | 1.45–1.53 (overlapped) | 30.0 (t) |
| 3 | – | 205.4 (s) | 9b | 1.61–1.70 (overlapped) | |
| 4 | – | 138.5 (s) | 10 | 2.28 (brdq, 3.7, 7.1) | 39.9 (d) |
| 5 | – | 171.6 (s) | 11 | – | 150.9 (s) |
| 6a | 2.51 (dd, 11.7, 19.1) | 35.7 (t) | 12 | 1.73 (s) | 20.3 (q) |
| 6b | 2.73 (brd, 19.1) | | 13a | 4.69 (brs) | 109.1 (t) |
| 7 | 2.85 (brddd, 12.4, 11.7, 4.5) | 42.8 (d) | 13b | 4.74 (brs) | |
| | | | 14 | 0.75 (d, 7.1) | 14.4 (q) |
| | | | 15 | 1.66 (s) | 7.9 (q) |

## Discussion

Briefly, we investigated for the first time, the phytochemical pattern of the shoot parts of the plant and report four terpenoids one being an undescribed sesquiterpene, and five known phenolic compounds.

Compound **1** exhibited $[M - H]^-$ ion peak at *m/z* 455.3543 in the HR-ESIMS which corresponded to the molecular formula $C_{30}H_{47}O_3$. The $^{1}H$-NMR (500 MHz, DMSO-$d_6$) spectrum revealed six methyl singlets at $\delta_H$ of 1.64 s, 0.93 s, 0.87 s, 0.86 s, 0.76 s and 0.65 s, a pair of olefin protons at $\delta_H$ of 4.68 and 4.56 (each one H, brs) characteristic of exocyclic methylene group, and carbinolic proton at $\delta_H$ of 3.25. The $^{13}C$-NMR data (experimental section) in addition to $^{1}H$-NMR data which were characteristic for betulinic acid [11].

Compound **2**, a white solid, showed positive reaction to FeSO4/$NH_4OH$ reagent (pH = 7) and exhibited $[M-H]^-$ ion peak in the HR-ESIMS at *m/z* 177.061 indicative of $C_{10}H_{10}O_3$ formula. The $^{1}H$ NMR (500 MHz, Chloroform-*d*) showed an ABX system δ 7.10 (1H, dd, *J* = 1.9, 8.2,, H-6′), 7.06 (1H, d, *J* = 2.0, H-2′), 6.95 (1H, d, *J* = 8.2,, H-5′), a trans substituted double bond δ 7.41 (1H, d, *J* = 15.8,, H-3), and 6.60 (1H, dd, *J* = 15.8, 7.8, H-2), attached to an aldehyde group δ 9.61 (1H, d, *J* = 7.8, H-1) and an aryl methoxy group δ 3.91 (3H, s) similar to those reported for 3-(4′-hydroxy-3′-methoxyphenyl)-2-propenyl aldehyde or coniferyl aldehyde [12].

Compound **3** was obtained with the molecular formula of $C_{30}H_{48}O_3$, derived from $[M-H]^-$ ion peak at *m/z* 455.3533 in the HR-ESIMS. The $^{13}C$-NMR and DEPT spectral data revealed thirty carbons consisted of seven methyls, ten secondary, five tertiary, and eight quaternary carbons. The $^{1}H$-NMR of an olefin proton at $\delta_H$ of 5.26 (t, *J* = 3 Hz, H-12), a carbinolic proton at $\delta_H$ of 3.24 (dd, $J_{ax,\ a\ x}$ = 11.2, $J_{ax,\ eq}$ = 4.3 Hz, H-3), one proton at $\delta_{H:}$ 2.84 (dd, *J* = 13.6, 4.0 Hz, H-18) along with seven singlet methyls at $\delta_H$ of 1.15, 1.00, 0.94, 0.93, 0.92, 0.79 and 0.77 were all in agreement with oleanolic acid [11].

Compound **4** was obtained as a white powder with $[M + H]^+$ at *m/z* 235.1703 (calculated: 235.1693, Δ 4.25 ppm) in the HR-ESIMS, corresponding to the molecular formula of $C_{15}H_{22}O_2$. The $^{13}C$-NMR (broadband, DEPT 90 and 135) spectral data and five degrees of unsaturation with regard to two olefin groups, and presence of one carbonyl carbon (Inspired from NMR data) suggested the presence of a sesquiterpene skeleton with two rings in the structure. The IR, $^{1}H$-, and $^{13}C$ NMR spectra showed signals attributed to a terminal methylene group [$\nu_{max}$ 1651 and 891 $cm^{-1}$; $\delta_H$ 4.75, 4.69 (each 1H, brs); $\delta_C$ 109.1 (t), and 150.9 (s)], a tetra-substituted double bond [$\delta_C$ 138.5 and 171.6 (each s)], conjugated with an α,β unsaturated keton [$\nu_{max}$ 1704 $cm^{-1}$; $\delta_C$ 205.4], two methines, one attached to a methyl [$\delta_H$ 2.28 (1H, br dq, *J* = 3.7, 7.1 Hz); $\delta_C$ 42.8], and one allylic [$\nu_{max}$ 1456 $cm^{-1}$; $\delta_H$ 2.85 (1H, br ddd, *J* = 12.4, 11.7, 4.5 Hz); $\delta_C$ 42.8], four methylene groups, one connected to two unprotonated carbons [$\delta_H$ 2.59, 2.50 (each 1H, d, *J* = 18.2 Hz); $\delta_C$ 51.2], one allylic [$\delta_H$

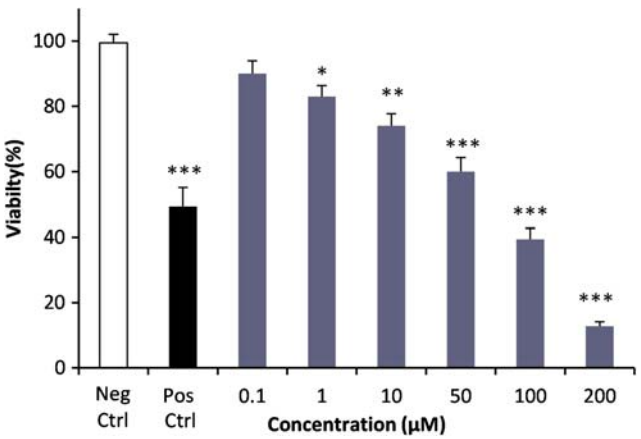


**Fig. 4** Cytotoxicity effect of compound **4** against HeLa cancer cells. Cells were incubated with six different concentrations of compound **4** (0.1, 1, 10, 50, 100, and 200 μM) in three replicates. Doxorubicin (1 μM), and vehicle-treated wells were considered as positive and negative controls. (* $p < 0.5$; ** $p < 0.01$; *** $p < 0.001$ versus control)

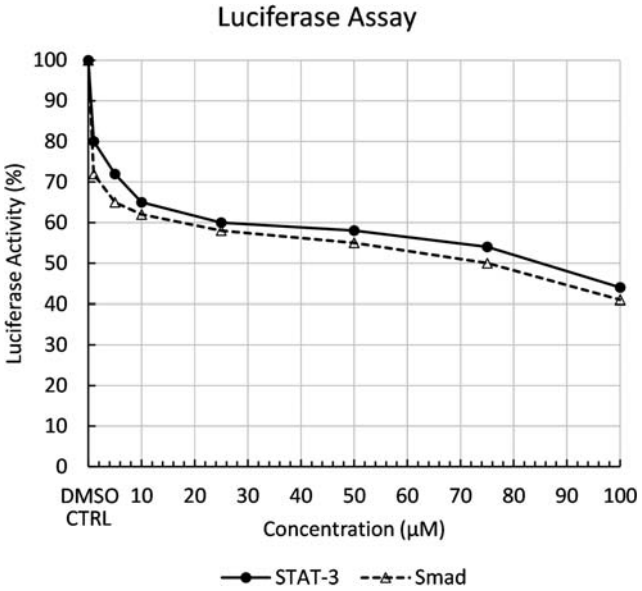


**Fig. 5** Transfection and Luciferase assay of compound **4** against STAT3/IL6, and Smad/TGF-beta cancer-related signaling pathways in HeLa cells

2.73 (1H, brd, $J = 19.1$ Hz), 2.51 (1H, dd, $J = 11.7$, 19.1 Hz); $\delta_C$ 35.7], and two secondary carbons [$\delta_H$ 2.17 (1H, dddd, $J =$ 4.2, 3.5, 10.6, 12.4 Hz), 1.46–1.54 (1H, overlapped); $\delta_C$ 30.7 (t); and $\delta_H$ 1.45–1.53 (1H, overlapped), 1.61–1.70 (1H, overlapped); $\delta_C$ 30.0], and three methyl groups, two connected to unprotonated carbons [$\delta_H$ 1.73, 1.66 (each 3H, s); $\delta_C$ 20.3, 7.9], and one connected to a tertiary carbon [$\delta_H$ 0.75 (3H, d, $J = 7.1$); $\delta_C$ 14.4]. Application of $^1$H-$^1$H correlation spectroscopy (COSY) allowed detecting the spin system of H-6 to H-10, and H-10 attached to H14 [-$CH_2$-CH-$CH_2$-$CH_2$-CH($CH_3$)-] which is shown as a bold black line in Fig. 2. The HMBC correlations of $H_2$–6/C-5, C4; $H_1$–7/C-11, C-12, C-13; $H_3$–14/C-1, C-14; $H_2$-2a/C-1,C-10, C-3; $H_3$–15/C-3, C-4, C-5 clarified that C-6 ($\delta_H$ 2.51, 2.71) is connected to a tetra-substituted double bond in ring A, α,β unsaturated ketone group ($\delta_C$ 205.4) is located at C-3 in ring A, C-7 ($\delta_H$ 2.85) is connected to isopropenyl group $CH_2 = C$-$CH_3$, and C-10 ($\delta_H$ 2.28) is bonded to quaternary oxycarbon C1 ($\delta_C$ 82.9). This information confirmed the structure of **4** as 4,11(12)-guiadiene-1-ol-3-one.

The stereochemistry was determined through the three-dimensional HSQC-NOESY experiment, comparison with similar compounds, and taking ECD spectra [13–15].

Using HSQC-NOESY, the NOE interactions (Fig. 2b) of $H_3$–13/H-8a, H-9a; H-9a/$H_3$–14; $H_3$–14/H-2b suggested that they are co facial and beta oriented. Consequently, H-7 and H-10 were assigned as alpha. The stereochemistry of hydroxyl group at C-1 was suggested as alpha from the ECD spectra and small chemical shift of H-14 in comparison with similar compounds which would be larger if a *cis* oriented hydroxyl was present. The absolute configuration was confirmed by a comparison of calculated and experimental CD spectra. The compound was optically active, and in the CD spectrum, a negative Cotton effect at 256 nm and a positive one at 319 nm were observed. Arbitrarily starting with C-1 (R), and then C-1 (S) enantiomers, the molecule was submitted to conformational optimizations with Gaussian 09 program by corresponding OPT keyword in the route section of the Gaussian input file. For every single geometry, a CD spectrum was calculated, and in both cases, the individual spectra were then taken through Chemcraft 1.8. The two calculated CD spectra were compared with the experimental CD curve (Fig. 3). A good agreement

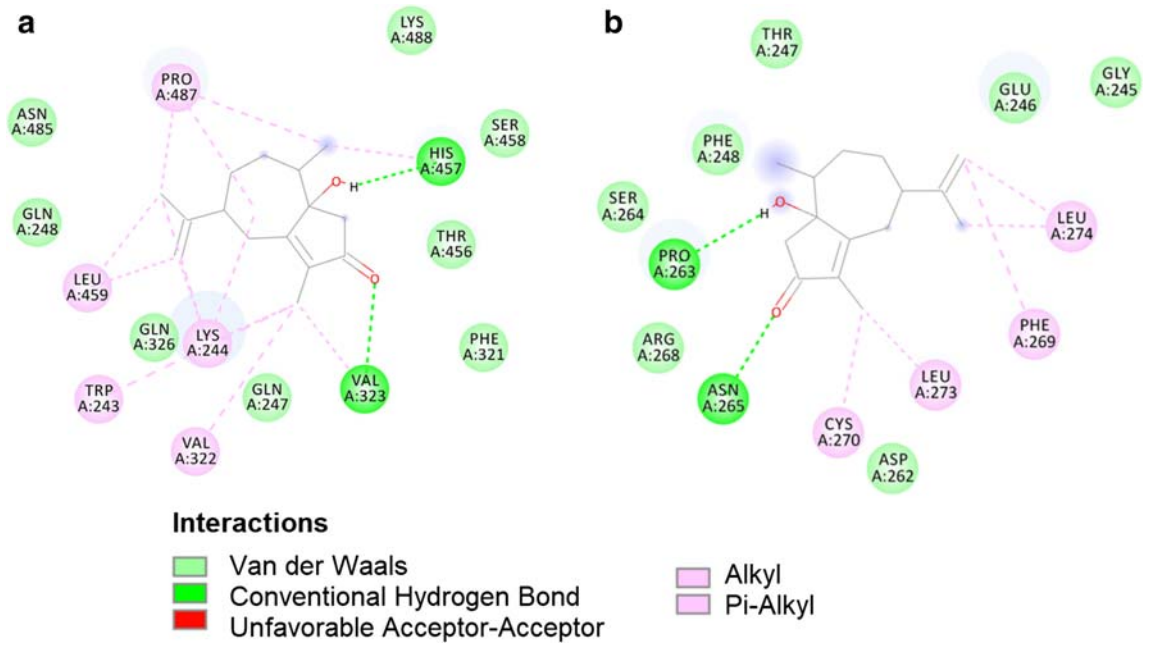


**Fig. 6** Molecular docking simulation of compound **4** with (A) STAT3 with the PDB ID of 6qhd and (B) Smad with the PDB ID of 1u7f

between the experimental and those predicted with Gaussian software was obtained for C-1(R): Fig. 3, left, whereas the CD spectra computed for C-1 (S): Fig. 3, right was virtually opposite as compared to the experimental one.

Compound **5** was obtained as oil with the molecular formula determined to be $C_{27}H_{30}O_8$ from its $[M+Na]^+$ pseudo-ion peak at *m/z* 505.1858 (calcd. 505.1833) in the HR-ESIMS. The $^{13}C$- and DEPT 90 and 135 NMR spectra displayed one orthoester benzoate group and a diterpene core comprising three methyls, three methylenes (one olefin, and one oxygenated), eight methines (three oxygenated and one olefin ones), and seven quaternary carbons (one α,β unsaturated ketone, two olefin, and four oxygenated). The $^1H$-NMR showed signals for one monosubstituted benzene ring $\delta_H$ 7.75 (2H, dd, *J* = 7.7, 2.1 Hz, H-3 ‘, H-7’), 7.38 (3H, overlapped, H-5′, H-4′, H-6′), in addition to two singlet methyls $\delta_H$ 1.83 (3H, br s, H-17), 1.78 (3H, d, J = 2.5 Hz, H-19), one secondary methyl 1.23 (3H, d, J = 7.1 Hz, H-18), one methylene at $\delta_H$ 2.36 (1H, dd, J = 14.3, 8.7 Hz, H-12a), 1.66 (1H, d, J = 14.2 Hz, H-12b), one exo methylene 5.08 (1H, s, H-16a), 4.91 (1H, s, H- 16b), one methylene attached to oxygen function 4.05 (1H, d, *J* = 12.3 Hz, H-20a), 3.63 (1H, d, *J* = 12.3 Hz, H-20b), three oxymethine 4.63 (1H, d, *J* = 2.6 Hz, H-14), 4.15 (1H, brs, H-5), 3.49 (1H, brs, H-7), and three methines including 3.08 (1H, brd, *J* = 2.5 Hz, H-8), 2.67–2.58 (1H, m, H-11), and 3.93 (1H, t, *J* = 2.67 Hz, H-10) and one olefin methine 7.62 (1H, s, H-1), which were in agreement with Daphnetoxin [16].

Compound **6** was isolated with a pale yellowish color UV absorption maxima at 266 and 339 nm characteristic of flavones. The molecular formula was determined as $C_{15}H_9O_5$ using negative HR-ESIMS at *m/z* 269.0455 $[M-H]^-$. The $^1H$-NMR (400 MHz, Acetone-$d_6$) spectrum displayed two meta doublets at δ 6.26 (1H, d, *J* = 2.1, H-6), 6.53 (1H, d, *J* = 2.1, H-8), a singlet proton at 6.64 (1H, s, H-3), and two ortho--coupled proton signals at δ 7.93 (2H, d, *J* = 8.8, H-6′,2′) and 7.02 (2H, d, *J* = 8.8 Hz, H-5′,3′) characterized for 4′,5,7-trihydroxyflavon or apigenin [17].

Compound **7** was obtained as a white solid with Neg. HR-ESIMS at *m/z* 167.0349 indicative of $C_8H_7O_4$ $(M-H)^-$. The $^1H$-NMR spectrum showed spin pattern of ABX for H-5, H-2, and H-5 at $\delta_H$ 7.43 (2H, overlapped, A and B of ABX), 6.84 (d, *J* = 7.9 Hz, X of ABX), in addition to one downfield singlet signal due to the aryl methoxy group at $\delta_H$ 3.81 (s) like vanillin type compounds. The $^{13}C$ NMR (BB and DEPT) spectrum supports six aromatic peaks: three sp2 methine $\delta_C$ 123.9, 115.5, 113.1, one sp2 quaternary $\delta_C$ 122.1, and two sp2 oxygenated quaternary carbons $\delta_C$ 151.5, 147.7, one methoxy $\delta_C$ 55.9, and one α,β unsaturated carboxylic group $\delta_C$ 167.7 similar to those reported for 4-hydroxy-3-methoxybenzoic acid or vanillic acid [18, 19].

Compound **8** was yielded as a white powder, with the molecular formula of C17H24O9 based on its positive HR-ESIMS at *m/z* 395.1340 ($C_{17}H_{24}O_9 + Na^+$). Its $^1H$ NMR (500 MHz, Methanol-$d_4$) spectrum showed a typical singlet integrating two protons appearing at $\delta_H$ 6.74 (2H, s, H-2′, H-6′), referring to a symmetric tetra-substituted phenolic ring, a *trans* substituted double bond δ 6.30 (1H, dt, *J* = 15.9, 5.5 Hz, H-2), 6.53 (1H, d, *J* = 15.9 Hz, H-3), $^1H$-$^1H$ coupled (*J* = 5.5 Hz) to an oxymethylene δ 4.22 (2H, d, *J* = 5.5 Hz, H-1), and two symmetrical aryl methoxy groups $\delta_H$ 3.85 (6H, s, 3′-OMe, 5′-OMe). Also observing glycopyranoside moiety peaks with characteristic anomeric doublet $\delta_H$ 4.87 (1H, d, *J* = 7.6 Hz, 1″), and corresponding sugar protons at 3.82–3.2 ppm (6H, Glc-2″, 3″, 4″, 5″, 6a″, and 6 b″) similar to data reported for synapyl alcohol-*4′*-O-glucoside or syringin [20].

Compound **9** was obtained in yellowish color with negative HR-ESIMS at *m/z* 541.1149 corresponded to the molecular formula $C_{30}H_{21}O_{11}$ $[M-H]^-$. Twenty degrees of unsaturation, the $^1H$ and $^{13}C$ NMR spectra signals of fourteen methines (eleven olefin and three oxygenated), one methylene, fifteen quaternary carbons including one oxygenated, five olefin, eight oxygenated double bond, and one carbonyl carbon, indicated seven rings in structure. The $^1H$ NMR showed signals assignable to two pairs of A2B2 aromatic protons at $\delta_H$ 6.97 and 6.62 (each 2H, d, *J* = 7.9 Hz, H-2′,6′ and H-3,5′) and $\delta_H$ 7.03 and 6.66 (each 2H, d, *J* = 7.8 Hz, H-10″,14″ and H-11′ ′,13″), indicating the presence of two *para* aromatic rings (4-oxyphenyl group), $\delta_H$ 5.73 (1H, d, *J* = 1.8 Hz) and 5.45 (1H, d, *J* = 1.9 Hz) suggested two *meta*-coupled aromatic protons at H-6″and H-8″, which confirmed the existence of a 2,4,6-trisubstituted phenyl group, and a 3-hydroxy-2,8 (or 2,6)-disubstituted 5,7-dioxy-3,4-dihydrobenzopyran system [$\delta_H$ 4.61 (d, *J* = 6.7 Hz, H-2), 3.79–3.67 (m, H-3), 2.41 (dd, *J* = 16.0, 7.2 Hz, H-4a), 2.54 (dd, *J* = 16.2, 4.6 Hz, H-4b), 6.10 (s, H-6)]. COSY and HMBC correlations assigned the structure as a biflavonoid structure similar to those of Genkwanol A reported before in other *Daphne* species [21].

In the biological part, compound **4** as an undescribed sesquiterpene was submitted for cytotoxicity assay and showed moderate activity against HeLa cancer cells. Results indicated differential sensitivity towards cancer signaling pathways for the new sesquiterpene when screened through luciferase gene reporter assays. It showed selective inhibition of STAT3/IL6, and Smad/TGF-β transcription factors when screened through an array of cancer signaling pathways. Molecular docking confirmed biological tests and showed the interaction with STAT3 and Smad proteins.

Compound **4** belongs to the guaianolide sesquiterpene lactones. They are a large group of sesquiterpene lactones which are currently investigated as a source for the discovery of selective anticancer agents [22, 23]. Previously, the authors described 13-O-acetylsolstitialin A, another guianolide structure, with apoptotic and cell cycle arrest against breast cancer cells with decreasing Bax to Bcl-2 protein ratio as well as expression of cyclin D1 and Cdk-4 [23]. But, it is the first report of guaianolide sesquiterpenes as inhibitors of Smad/

TGF-β and STAT3 transcription factors. Paul A. Johnston1,3 and Jennifer *R. grandis* has reported earlier other natural products like Guggulsterone (a phytosterol), honokiol (5, 5″-diallyl-biphenyl-2, 2″-diol), curcumin, resveratrol, flavopiridol (Alvocidib: a flavonoid alkaloid), and cucurbitacin to inhibit STAT3 activation, and finally suppress tumor cells [24]. However, STAT3 and Smad inhibitors are largely considered in the development of anti-cancer drugs in several contexts. They are important signal transducers which regulate cell growth, and any defect or inhibition in their signaling cause dysregulation of cell growth [24].

## Conclusion

A new sesquiterpenoid: 4,11(12)-guiadiene-1-ol-3-one was isolated along with eight known compounds. This undescribed sesquiterpene was evaluated for cytotoxicity against HeLa cells, and the luciferase assay on 14 different cancer-related signaling pathways. It showed cytotoxicity against HeLa cells with selective inhibition of STAT3/IL6, and Smad/TGF-beta transcription factors. Molecular docking simulation showed interactions with both STAT3 and Smad, which confirms biological results. STAT3 and Smad inhibitors are recently targeted in cancer treatment. Therefore, the discovery of their inhibitors is valuable in cancer treatment.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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