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Original Research Article

Anticancer Activity of Hispidulin from *Saussurea simpsoniana* and Lupeol from *Vincetoxicum arnottianum*

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ABSTRACT

Saussurea simpsoniana (Field & Gard.) Lipsch (Asteraceae) is a herb, locally known as *Boshi Phonar* in Gilgit, Pakistan. This herb is used for the treatment of asthma, cough, pneumonia, fever, snakebite, and dysmenorrhoea. The plant *Vincetoxicum arnottianum* (Wight) Wight (Syn: *Cyanchum arnottianum* Wight), (Apocynaceae) is used for the treatment of asthma, gastritis, malaria, cholera, skin diseases, wounds, ulcer and constipation. It has recently been reported for its antitumor activity. The present study was aimed to evaluate the methanol extracts of the plants species; *S. simpsoniana* and *V. arnottianum* for isolation of the phytoconstituents. As a result of our study, *β*-sitosterol, hispidulin and *β*-sitosterol-*β*-D-glucoside were isolated and characterized from *S. simpsoniana*. While *β*-sitosterol, *β*-sitosterol-*β*-D-glucoside, and lupeol were isolated and characterized from *V. arnottianum*. Hispidulin and lupeol were tested against breast cancer MCF-7, osteosarcoma MG-63, pancreatic PACA-44, and alveolar rhabdomyosarcoma Rh30 cell lines and hispidulin showed a moderate reduction of cell viability in the range of 20 to 50%.

***Keywords*:** Antiasthmatic herbs, Isolation, Antitumor activity, Metabolic viability assay.

Introduction

Medicinal plants show bioactivities against various ailments due to the presence of biologically active phytochemical constituents.[1] Our present study is on *Saussurea simpsoniana* (Field & Gard.) Lipsch and *Vincetoxicum arnottianum* (Wight) Wight. *Saussurea simpsoniana* (Field & Gard.) Lipsch (Asteraceae) is a herb, and its local name is *Boshi Phonar* in Gilgit, Pakistan. The decoction of *S. simpsoniana* has been reported for the treatment of respiratory diseases like asthma, cough, and pneumonia.[2] Furthermore, this plant species has been reported to exhibit antibacterial,[2] and insecticidal[3] properties. The preliminary phytochemical screening[2] has revealed the presence of saponins, flavonoids, alkaloids, etc. in *S. simpsoniana*. *Vincetoxicum arnottianum* (Wight) Wight (Syn: *Cyanchum arnottianum* Wight), (Apocynaceae), has recently been reported for its antitumor activity.[4] Apocynaceae consists of 375 genera and 3700-5100 species, which are mainly found in tropical and subtropical areas all over the world.[5] There are 19 genera and 26 species of family Apocynaceae in Pakistan, mostly found in North Punjab, Azad Kashmir, Hazara, Rawalpindi, Attock, Swat, Bajaur, Waziristan and Salt range area.[6] The plant *V. arnottianum* is also used for the treatment of various diseases like asthma, gastritis, malaria, cholera, skin diseases, ulcer and constipation. It is also used to treat external wounds and injuries in humans and animals.[7]

The present study was aimed to evaluate the methanol extracts of. *S. simpsoniana* and *V. arnottianum* for isolation of the phytoconstituents, and anticancer activity of the isolated compounds from the plants.

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Materials and Methods

General Experimental Procedure

NMR spectra were recorded on JEOL JNM-A500 and/or Varian INOVA-500 spectrometers in $CDCl_3$ with tetramethylsilane (TMS) as an internal standard. Silica gel 60 (Merck, 60-230 mesh size) was used for column chromatography. TLC was done using pre-coated silica gel plates (Merck, silica gel 60 F254, 0.20 mm). Spots were visualized by spraying with ceric sulphate reagent followed by heating.

Plant materials

The plant material (*S. simpsoniana*) was collected from Astor and was authenticated by a plant taxonomist at Department of Biological Sciences, Karakoram International University, Gilgit, Pakistan. The voucher specimen (SKN-12) was deposited at the Department of Biological Sciences, Karakoram International University. The plant material (*V. arnottianum*) was collected from Baluchistan, and was identified by plant taxonomist at Department of Botany, University of Baluchistan. Each plant material was dried in shade and crushed to powder using a grinder.

Extraction and fractionation

The crushed form of whole plant material of *S. simpsoniana* (3.4 kg) was macerated in methanol (13 L) for 7 days under ambient conditions. The extract was filtered and evaporated to dryness using rotary evaporator at 40°C under reduced pressure. Similarly the plant

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material of *V. arnottianum* was dried under shade, ground into powder and extracted (2 kg material) three times with methanol (10 L) for 5 days each. The solvent was evaporated to get methanol extract.

Isolation

The methanol extract (130 g) of *S. simpsoniana* was chromatographed over silica gel 60 (Merck, 60-230 mesh size) with different solvents starting with *n*-hexane, dichloromethane and methanol. A total of 80 fractions (f1-f80) were obtained from the main column. The fractions f6-f22 (20% DCM:*n*-Hexane) were combined and re-chromatographed over normal silica gel. As a result, sf1-sf71 fractions were obtained from the sub column elution. The sub fraction sf6-sf22 (20% EtOAc:*n*-hexane) yielded compound **1** (63.01 mg), sf32-sf40 (70% EtOAc:*n*-hexane) yielded compound **2** (17.5 mg), while the sub fraction sf50-sf56 (2% MeOH:EtOAc) yielded compound **3** (21.65 mg).

The methanol extract (60 g) of *V. arnottianum* was chromatographed over silica gel 60 (Merck, 60-230 mesh size) with different solvents starting with *n*-hexane, ethyl acetate and methanol. A total of 55 fractions (Fr1-Fr55) were obtained from the main column. The fraction Fr27 (30% ethyl acetate:*n*-hexane) yielded lupeol as compound **4** (9.32 mg). While *β*-Sitosterol (compound **1**, 13 mg) was isolated from Fr31 (50% ethyl acetate:*n*-hexane) and *β*-sitosterol-*β*-D-glycoside (compound **3**, 5.99 mg) was isolated from fraction F46 (absolute ethyl acetate). The structures of compounds **1-4** were confirmed through spectral data and by comparison with data reported in literature.

Cell viability assays

The cell lines MCF-7 (ACC 115) and Rh30 (ACC-489) were purchased from the German biological resource bank ´DSMZ´(https://www.dsmz.de). MG-63 (ATCC® CRL-1427™) cell line was purchased from American Type Culture Collection (ATCC). Paca-44 was a gift from Dr. Petra Müller, Department of cell biology, University Medical Hospital Rostock, Germany. All cell lines were cultured in Dulbecco's modified Eagle's medium plus Ultraglutamine 1 (Lonza, Verviers, Belgium), with 10% fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) and 1% Antibiotic-Antimycotic-Solution (Gibco, Paisley, UK). The cell viability measurements were quantified with the CellTiter 96®AQueous One Solution Cell Proliferation Assay (MTS-assay) according to the manufacturer's instructions (Promega Corp., Madison, USA) as described previously.[8]

Statistical Analysis

Statistical significance was determined by the unpaired student's t-test (*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$) using the software GraphPad Prism Version 5 (http://www.graphpad.com/scientificsoftware/prism).

Results and Discussion

The phytochemical investigation of *S. simpsoniana* resulted in the isolation of three compounds namely *β*-sitosterol (**1**), hispidulin (**2**) and *β*-sitosterol-*D*-glucoside (**3**) and their structures (Figure 1) were determined by means of spectral analysis and compared with available literature.

*NMR data for β-Sitosterol (**1**)*

^{1}H-NMR ($CDCl_3$, 250 MHz) (δ ppm): 0.68 (3H, s, H-18), 0.84 (3H, d, *J* = 2Hz, H-26), 0.85 (3H, d, *J* = 7.5 Hz, H-27), 0.89 (3H, t, *J* = 10, H-29), 0.92 (3H, d, *J* = 6.5 Hz, H-21), 1.01 (3H, s, H-19), 1.07 (2H, m, H-22), 1.17 (2H, m, H-28), 1.24 (2H, m, H-23), 1.29 (1H, m, H-24), 1.32 (1H, m, H-17), 1.33 (2H, m, H-1), 1.35 (1H, m, H-14), 1.35 (1H, m, H-2), 1.46 (2H, m, H-11), 1.49 (1H, m, H-9), 1.56 (2H, m, H-25), 1.59 (2H, m, H-15), 1.65 (1H, m, H-20), 1.68 (1H, m, H-8), 1.84 (2H, m, H-16), 1.96 (1H, s, 3-OH), 2.01 (2H, m, H-7), 1.42 (2H, m, H-12), 2.27 (2H, m, H-4), 3.52 (1H, m, H-3), 5.35 (1H, t, *J* = 5 Hz, H-6). ^{13}C-NMR ($CDCl_3$, 125 MHz) (δ ppm): 140.7 (C-5), 121.7 (C-6), 71.8 (C-3), 56.7 (C-14), 56.0 (C-17), 50.1 (C-9), 29.1 (C-25), 42.3 (C-13), 42.3 (C-4), 39.7 (C-12), 37.2 (C-1), 36.5 (C-10), 36.1 (C-20), 33.9 (C-22), 31.9 (C-7), 31.9 (C-8), 31.6 (C-2), 45.8 (C-24), 28.2 (C-16), 26.1 (C-23), 24.3 (C-15), 33.0 (C-28), 21.0 (C-11), 19.0 (C-27), 19.8 (C-26), 19.3 (C-19), 18.7 (C-21), 11.8(C-29), 11.9 (C-18). MS *m/z*: 414, 381, 303, 145, 43.

*Characterization of Hispidulin (**2**)*

^{1}H-NMR ($CDCl_3$, 400 MHz) (δ ppm): 13.07 (1H, s, OH), 13.06 (1H, s, OH), 13.06 (1H, s, OH), 7.78 (2H, d, *J* = 9.0 Hz, H-6′, H-2′), 6.94 (2H, d, *J* = 8.8 Hz, H-3′, H-5′), 6.55 (1H, s, H-8), 6.47 (1H, s, H-3), 4.02 (3H, s, 6-OCH_3). ^{13}C-NMR ($CDCl_3$, 400 MHz) (δ ppm): 163.8 (C-2), 102.3 (C-3), 182.1 (C-4), 152.7 (C-5), 131.3 (C-6), 157.2 (C-7), 94.2 (C8), 152.3 (C-9), 104.0 (C-10), 121.2 (C-1′), 128.4 (C-2′), 115.9 (C-3′), 161.1 (C-4′), 115.9 (C-5′), 128.4 (C-6′), 59.9 (OCH_3). MS *m/z*: 300.2, 285.2, 270.2, 257.2, 242.2, 167.1, 153.1, 139.1, 119.1, 69.0, 44.0.

*Characterization of β-sitosterol-β-D-glycoside (**3**)*

^{1}H-NM R ($CDCl_3$, 400 MHz) (δ ppm): 5.32 (s, 1H, H-6), 4.20 (d, 1H, J= 7.6 Hz, H-1′), 3.63 (m, 1H, H-2′), 3.46 (m, 1H, H-4′), 3.39 (m, 1H, H-3′), 3.12 (m, 1H, H-5′), 3.02 (m, 2H, H-6′), 2.88 (m, 2H, H-3), 2.36 (d, 1H, H-25), 2.11 (m, 1H, H-4), 1.93 (m, 2H, m, H-7), 1.79 (m, 2H, H-15), 1.62 (m, 1H, H-17), 1.54 (m, 2H, H-16), 1.47 (m, 2H, H-22), 1.42 (m, 2H, H-23), 1.37 (m, 2H, H-2), 1.31 (m, 2H, H-12), 1.24 (m, 2H, H-11), 1.17 (m, 2H, H-28), 1.14 (m, 1H, H-20), 1.08 (m, 2H, H-1), 1.03 (m, 1H, H-8), 1.01 (m, 1H, H-9), 0.98 (m, 1H, H-14), 0.95 (s, 3H, H-19), 0.89 (t, 3H, J = 6.4 Hz, H-29), 0.82 (d, 3H, *J* = 6.8 Hz, H-21), 0.78 (d, 3H, *J* = 6.8Hz, H-26), 0.74 (d, 3H, H-27), 0.65 (s, 3H, H-18). ^{13}C-NMR ($CDCl_3$, 100 MHz) (δ ppm): 140.4 (C-5), 121.1 (C-6), 76.7 (C-3), 56.1 (C-14), 55.4 (C-17), 49.5 (C-9), 45.1 (C-24), 41.8 (C-13), 39.2 (C-4), 38.2 (C-12), 36.8 (C-1), 36.2 (C-10), 35.4 (C-20), 33.3 (C-22), 31.4 (C-7), 31.3 (C-8), 29.2 (C-2), 28.6 (C-25), 27.7 (C-16), 25.4 (C-23), 23.8 (C-15), 22.5 (C-28), 20.5 (C-11), 19.6 (C-26), 19.0 (C-19), 18.9 (C-2), 18.5 (C-21), 11.7 (C-29), 11.6 (C-18), glycoside carbons at 100.76, 76.88, 76.73, 73.44, 70.09, 61.1. MS *m/z*: 396.0, 382.0, 367.0, 296.0, 255.0, 213.0, 147.0, 105.0, 81.0, 55.0

*Characterization of lupeol (**4**)*

^{1}H NMR ($CDCl_3$, 300MHz): δ 4.66, 4.54 (2H, s, H-29a, 29b), 3.16 (1H, m, H-3), 2.35 (1H, *dt*, H-19); 1.62 (2H, *m*, H-2); 1.49 (1H, *m*, H-11); 1.36 (2H, *m*, H-6); 1.33 (2H, *m*, H-21); 1.27 (4H, *m*); 1.23 (2H, *m*); 1.01 (2H, *m*); 0.97 (1H, *m*); 0.74, 0.76, 0.80, 0.92, 0.94, 1.01, 1.65 (each 3H, s, H-24, 28, 25, 27, 23, 26, 30 respectively). ^{13}C NMR($CDCl_3$, 75 MHz): δ 150.9 (C-20), 109.3 (C-29), 78.9 (C-3), 55.2 (C-5), 50.4 (C-9), 48.3 (C-18), 47.9 (C-19), 42.9 (C-17), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (C-4), 38.7 (C-1), 38.0 (C-13), 37.1 (C-10), 35.5 (C-16), 34.2 (C-7), 29.8 (C-21), 28.2 (C-23), 27.4 (C-2), 27.4 (C-15), 25.1 (C-12), 20.9 (C-11), 19.3 (C-30), 18.3 (C-6), 18.0 (C-28), 16.1 (C-26), 16.0 (C-25), 15.3 (C-24), 14.5 (C-27).

Compound (**1**) was isolated as white crystalline solid. The ^{1}H-NMR spectrum showed a signal at δ 5.35 (1H, t, *J* = 5.0 Hz) which was assigned for the olefinic proton H-6, and 3.50 ppm (1H, m) was observed for H-3. The signals at 2.27 ppm (2H, m) and 2.01 ppm (2H, m) were assigned to the two allylic protons at carbon 4 and 7, respectively. Chemical shift at 0.68, 1.01, 0.92, 0.84, 0.85 and 0.89 ppm were assigned to the six methyl groups at positions C-18, C-19, C-21, C-26, C-27 and C-29, respectively. The proton at position C-18 and C-19 showed singlet peaks due to the absence of neighboring proton. The protons at C-21, C-26 and C-27 showed doublet peaks because of coupling with one adjacent proton for each. The proton at position C-29 displayed as triplet because of coupling with 2 protons of CH_2 at position C-28. The ^{13}C-NMR broadband spectrum showed 29 carbon signals. This includes 6 methyl, 11 methylene, 9 methine, and three quaternary carbons. Quaternary carbons include carbon-5, 10 and 13. The most downfield chemical shift was at 140.7 ppm and 121.7 ppm that was observed for olefinic carbons at position 5 and 6, respectively. The chemical shift 71.8 ppm was assigned to the oxygenated carbon (C-3). The signals at 56.7 ppm, 56.0 ppm, 50.1 ppm were assigned to the three methine carbons at C-14, C-17 and C-9, respectively. The chemical shift (δ) for six methyl carbons at positions C-18, C-19, C-21, C-26, C-27 and C-29 resonated at the

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range 11.8 ppm to 19.8 ppm. The most down field chemical shift was observed for the methyl group at position C-29. ^{1}H and ^{13}C-NMR data of the isolated compound **1** was compared with the data from literature,[9, 10] which supported compound-1 as *β*-sitosterol.

Compound (**2**) was isolated as a yellow crystalline solid. The ^{1}H-NMR spectrum showed a doublet signal at δ 7.78 ppm for 2H (H-2′, H-6′) with *J* = 9.0 Hz and another doublet at 6.94 ppm for 2H (H-3′, H-5′) with *J* = 8.8 Hz. A singlet signal at 6.55 ppm was observed for 1H (H-8). The singlet signal at 6.47 ppm was assigned to H-3. The chemical shift for methyl protons of -OCH_3 was observed at 4.02 ppm as a singlet. The ^{13}C-NMR broadband spectrum showed 16 signals including 8 quaternary carbons (C), 7 tertiary carbons (CH), and 1 primary carbon as methyl (CH_3). The quaternary carbons include carbon-2, 6, 7, 9, 10, 1′, 4′ and C=O and their chemical shift (in ppm) were observed at 163.7, 131.3, 157.2, 152.3, 104.0, 121.1, 161.1 and 182.0, respectively. Tertiary carbons include carbon-3, 5, 8, 2′, 3′, 5′ and 6′ and their chemical shift (in ppm) were observed at 102.3, 152.7, 94.1, 128.4, 115.9 and 128.4, respectively. The most downfield chemical shift was 182.08 ppm and was assigned to the carbonyl carbon. Chemical shift for methoxy carbon was observed at 59.90 ppm. ^{1}H and ^{13}C-NMR data of compound 2 was compared with the data from literature[11-13] which supported compound **2** as hispidulin.

Compound (**3**) was isolated as a yellow semisolid, which was not crystallizable through any solvent. ^{1}H-NMR (400Hz, DMSO) spectrum showed one olefinic, 13 methine, 12 methylene, and six methyl protons.. The more deshielded proton was observed at 5.32 ppm for olefinic proton at carbon number 6. A doublet at 4.20 ppm (*J* = 7.6 Hz) was observed for the H-1′ of sugar coupling with H-2′. The glucosidic protons were observed in the chemical shift range δ 3.02 to 3.63 ppm. The signal at 2.36 (d, 1H) was assigned to H-25. The upfield singlet signals at 0.65 and 0.95 were assigned to H-18 and H-19, respectively. Chemical shift values 0.82 (d, 3H, J = 6.8 Hz), 0.78 (d, 3H, J = 6.8 Hz) and 0.74 were assigned to protons at positions 21, 26 and 27, respectively. The ^{13}C-NMR signals indicate that there were 35 carbons, including 3 quaternary carbons (C), 14 tertiary carbons (CH), 12 secondary carbons (CH_2) and 6 primary carbons as methyl (CH_3). Quaternary carbons include carbon-5, 10 and 13. The most downfield chemical shift were 140.4 and 121.1 ppm which were ascribed to the vinyl carbons at position 5 and 6, respectively. The chemical shift at 100.76 ppm was observed for carbon 1' which is attached to two oxygen atoms. The chemical shift values ranging from 11.6 to 19.6 ppm were due to the methyl protons. ^{1}H and ^{13}C-NMR data of compound 3 was compared with the data from literature[14] which supported compound 3 as *β*-sitosterol-glucoside.

Compound (**4**) was isolated as a white solid. ^{1}H and ^{13}C-NMR spectra revealed typical signals of a pentacyclic lupane-type triterpene with olefinic protons (H-29a, 29b) resonating at δ 4.66 and 4.54 ppm. The hydroxymethine proton (H-3) was observed at δ 3.16 ppm, and seven singlet signals were assigned to the methyl groups at δ 0.74, 0.76, 0.80, 0.92, 0.94, 1.01, and 1.65 ppm (each 3H, each s, H-24, 28, 25, 27, 23, 26, and 30, respectively). The ^{13}C-NMR spectrum indicated that there were 30 carbons with distinct signals in the compound. The olefinic carbons (C-29, C-20) were observed at δ 109.3 and 151.0 ppm, respectively. The hydroxymethine carbon (C-3) was observed at δ 78.9 ppm. The comparison of ^{1}H and ^{13}C-NMR chemical shifts with that of the reported data[15] led to the conclusion that the compound was lupeol.

Initial anticancer screening was performed by using a general, metabolic viability assay, which monitors the overall reductase activity within the cells. Compared to the control treatments the pure compound hispidulin reduced the viability of different cancer cell lines, significantly. For the breast cancer MCF-7, osteosarcoma MG-63, pancreatic PACA-44, and alveolar rhabdomyosarcoma Rh30 cell lines, the cell viability was reduced by lupeol to 98.13%, 80.74%, 98.83% and 98.50%, respectively, and by hispidulin to 79.95%, 67.56%, 50.15% and 75.11%, respectively. This means that the

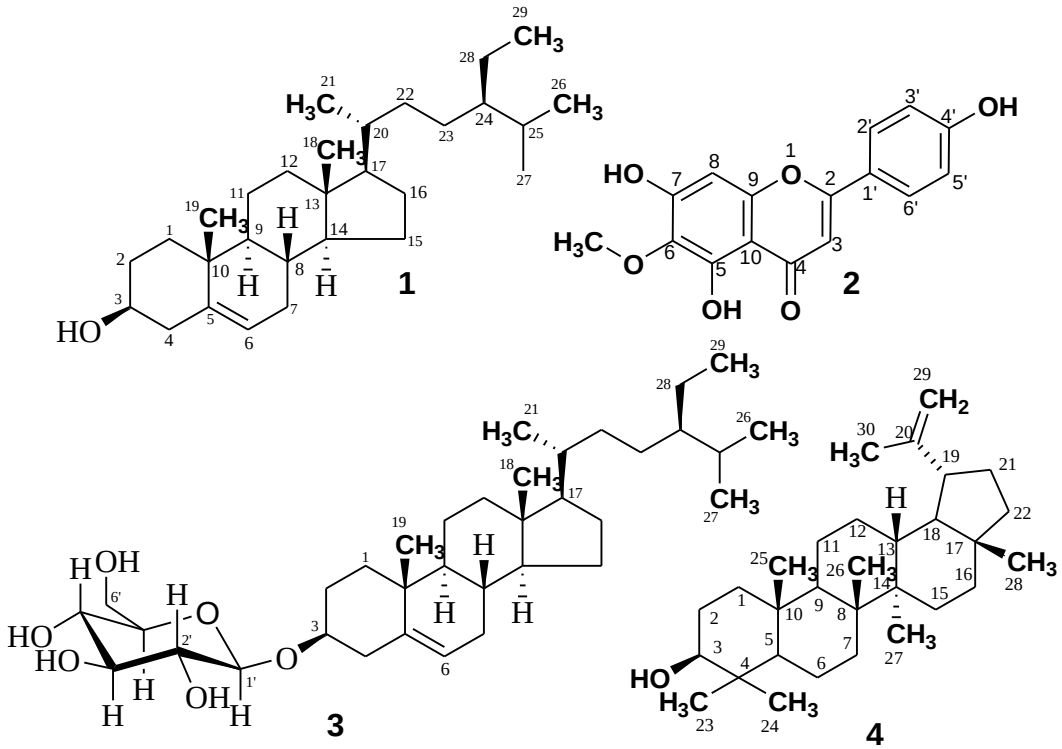

Figure 1: Structures of compounds **1-3** isolated from *S. simpsoniana* and **1**, **3-4** isolated from *V. arnottianum*

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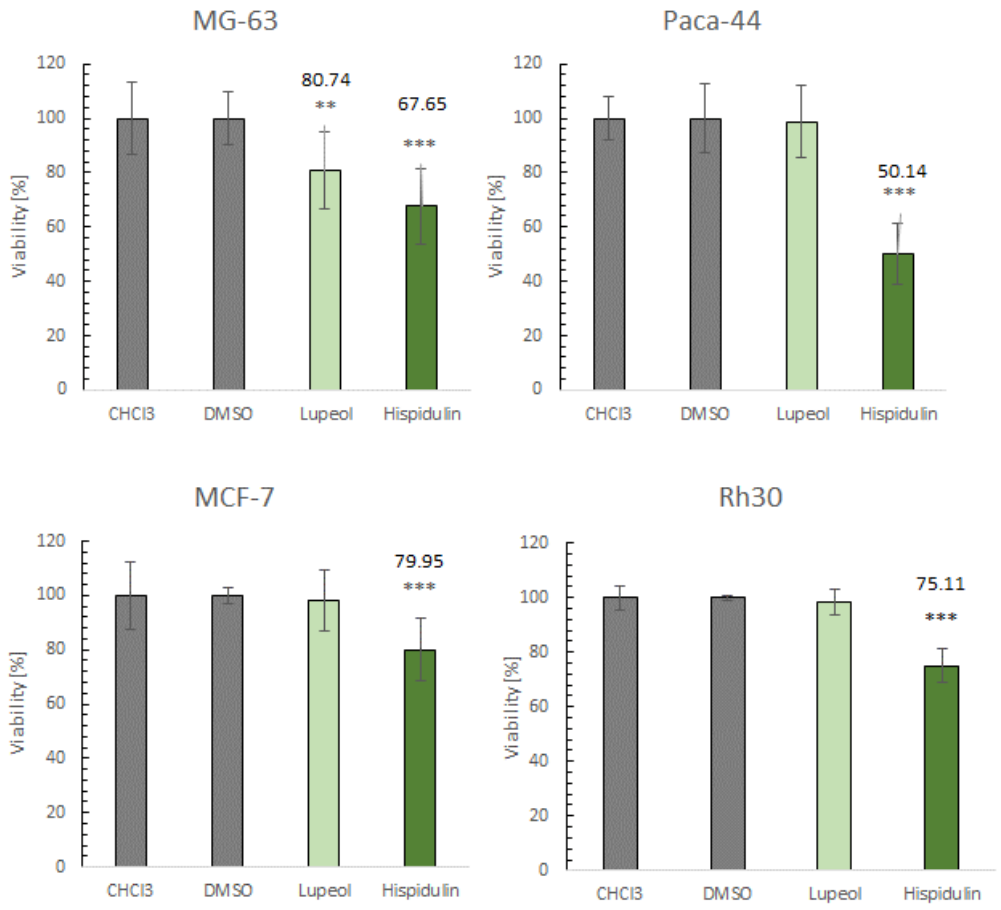

Figure 2: Determination of cell viability. Cell viability measurement via MTS-assay of the osteosarcoma cell line MG63 (A), pancreatic carcinoma cell line Paca-44 (B), mamma carcinoma cell line MCF-7 (C), and alveolar rhabdomyosarcoma cell lines Rh30 after treatment with solvents 0.1% $CHCl_3$ and DMSO as well as 50 µg/mL Hispidulin and Lupeol for 24 h. The solvent DMSO was used as negative control and was set to 100%. Mean±SD, n=6–8, *** $P < 0.001$, significantly different compared to vehicle control, unpaired t-test.

metabolic activity of cancer cells has been reduced in a range from 20 to 50% by hispidulin. Notably, lupeol only had a significant effect on the metabolic vitality of the bone cancer cell line MG-63.

Hispidulin also known as 4,5,7-trihydroxy-6-methoxyflavone is a phenolic natural compound, a flavonoid, and was first isolated in 1963 from *Digitalis lanata* L.[16] Hispidulin has been reported to exhibit anti-neoplastic activity against different cancer cell types e.g. hepatocellular carcinoma.[17] Hispidulin has been found to significantly inhibit tumor growth and metastasis in colorectal cancer[18] and cancer cell-induced circular chemorepellent-induced defects.[19] The cytotoxic and apoptotic properties of a plant *Centaurea fenzlii* have been determined on the breast cancer MCF-7 cell line and it has exhibited significant cytotoxic effects and it was found that the major component of the plants sample was hispidulin.[20] In our study, Hispidulin reduced the viability of four different cancer cell lines, significantly. The breast cancer MCF-7, osteosarcoma MG-63, pancreatic PACA-44, and alveolar rhabdomyosarcoma Rh30 cell lines showed a moderate reduction of cell viability in the range of 20 to 50%. These first results indicate that hispidulin has potential in cancer therapy. Therefore, we recommend further experiments *in vitro* as well as *in vivo*.

Conclusion

In the present study, the phytochemical investigation of *S. simpsoniana* and *V. arnottianum* led to the isolation of *β*-sitosterol (1), hispidulin (2) and *β*-sitosterol-*β*-D-glucoside (3) from *S. simpsoniana*, and *β*-sitosterol (1), *β*-sitosterol-*β*-D-glucoside (3), and lupeol (4) from *V. arnottianum*. The compounds were isolated for the first time from the respective plants. In addition, the pure compounds hispidulin (2), and lupeol (4) were tested for their cytotoxic effect against breast cancer (MCF-7), osteosarcoma (MG-63), pancreatic (PACA-44), and alveolar rhabdomyosarcoma (Rh30) cell lines and the compounds showed moderate reduction of cell viability in the range of 20 to 50%. The initial results on anticancer activity of hispidulin proved that this compound has potential in cancer therapy. Therefore, we recommend further experiments *in vitro* as well as *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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