Inhibition of HIF-1α through Suppression of NF-κB Activation by Compounds Isolated from Senecio graveolens

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ABSTRACT

One of the characteristics of cancer is that the lack of oxygen in the cancer cells triggers changes in their gene expression. This hypoxia activates hypoxia-inducible factor 1-alpha and this in turn sets in motion the whole family of important angiogenic genes for the tumour. Hypoxia-inducible factor 1-alpha therefore increases the density and vascular permeability within the tumours, facilitating their rapid growth and, later, the metastasis. *Senecio graveolens* is a South American medicinal plant commonly used for mountain sickness (lack of adaptation of the organism to hypoxia). Additionally, pharmacological studies showed that its alcoholic extracts have cytotoxic properties. This research aimed to perform a guided phytochemical study of *S. graveolens* to identify compounds capable of inhibiting hypoxia-inducible factor 1-alpha through suppression of nuclear factor kappa-light-chain-enhancer of activated B cell activation. The isolation led to the characterisation of phanurane (1), damsine (2), and scoparone (3), first reported in the *S. graveolens* species. Phanurane (**1**) showed inhibitory activity of hypoxia-inducible factor 1-alpha on the cancer cell lines U-373 MG (IC_{50} = 20.66 ± 0.04 μM), A549 (IC_{50} = 25.80 ± 0.04 μM), Hep G2 (IC_{50} = 29.21 ± 0.03 μM), and Caco-2 (IC_{50} = 38.58 ± 0.02 μM). Damsine (**2**) hypoxia-inducible factor 1-alpha displayed inhibitory activity of hypoxia-inducible factor 1-alpha on the cancer cell lines U-373 MG (IC_{50} = 2.29 ± 0.07 μM), A549 (IC_{50} = 4.13 ± 0.04 μM), Hep G2 (IC_{50} = 6.40 ± 0.03 μM), and Caco-2 (IC_{50} = 9.80 ± 0.04 μM). Finally, scoparone (**3**) displayed inhibitory activity of hypoxia-inducible factor 1-alpha on the cancer cell lines U-373 MG (IC_{50} = 15.22 ± 0.01 μM), A549 (IC_{50} = 17.47 ± 0.02 μM), Hep G2 (IC_{50} = 18.26 ± 0.06 μM), and Caco-2 (IC_{50} = 19.75 ± 0.04 μM). In addition, phanurane (**1**) displayed inhibitory activity over nuclear factor kappa-light-chain-enhancer of activated B cells on cancer cell lines U-373 MG (IC_{50} = 7.13 ± 0.03 μM), A549 (IC_{50} = 8.64 ± 0.03 μM), Hep G2 (IC_{50} = 8.87 ± 0.04 μM), and Caco-2 (IC_{50} = 15.11 ± 0.01 μM). Likewise, damsine (**2**) showed inhibitory activity over nuclear factor kappa-light-chain-enhancer of activated B cells on cancer cell lines U-373 MG (IC_{50} = 2.28 ± 0.01 μM), A549 (IC_{50} = 3.79 ± 0.02 μM), Hep G2 (IC_{50} = 3.98 ± 0.05 μM), and Caco-2 (IC_{50} = 6.41 ± 0.02 μM). Lastly, scoparone (**3**) displayed inhibitory activity of nuclear factor kappa-light-chain-enhancer of activated B cells on cancer cell lines U-373 MG (IC_{50} = 3.62 ± 0.06 μM), A549 (IC_{50} = 4.48 ± 0.03 μM), Hep G2 (IC_{50} = 5.25 ± 0.01 μM), and Caco-2 (IC_{50} = 11.90 ± 0.02 μM). This study corroborates the cytotoxic activity of the isolated compounds through the inhibition of hypoxia-inducible factor 1-alpha as well as its modulator nuclear factor kappa-light-chain-enhancer of activated B cells.

ABBREVIATIONS

ANGPT1	angiopoietin 1
BIRC5	baculoviral inhibitor of apoptosis repeat-containing 5
CA9	carbonic anhydrase IX
EPO	erythropoietin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLUT-1	glucose transporter 1
HIF-1α	hypoxia-inducible factor 1-alpha
ILs	interleukins
LDHA	lactate dehydrogenase A
LOX	lysyl oxidase
MMP2	matrix metalloproteinase-2
MMP9	matrix metalloproteinase-9
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PDK1	pyruvate dehydrogenase lipoamide kinase isozyme 1
PGK1	phosphoglycerate kinase 1
PKM2	pyruvate kinase isozymes M2
pVHL	von Hippel-Lindau protein
RelA	transcription factor p65
SDF1	stromal cell-derived factor 1
VEGF	vascular endothelial growth factor

Introduction

Senecio graveolens Wedd. (synonym: *Senecio nutans* Sch. Bip) belongs to the family Asteraceae. *S. graveolens*, also known as "Chachakuma", is a perennial shrub about 20–60 cm high that grows in habitats with heights fluctuating between 3500–5000 metres above sea level in the Andes Chile, Argentina, Peru, and Bolivia. It is a medicinal plant widely used by the communities living in these areas in the form of an infusion and decoction. The infusion of leaves and stems is used to ameliorate altitude sickness (principally as vasodilator preparations), headaches, stomach pain, intestinal inflammations, colitis, nausea, fever, pulmonary diseases, and bronchitis [1–4]. The decoction of leaves, stems, and roots is used against all types of pulmonary affections such as a brain tonic and against nervousness and excessive sweating [1, 3–5]. Pharmacological studies have shown that the ethanolic extract of *S. graveolens* possesses cytotoxic activity in MCF-7 cancer cells at a concentration of 200 μg/mL [6].

Human cancers frequently contain areas of necrosis in which cancer cells have died due to inadequate oxygenation [7, 8]. Cells closest to a perfused blood vessel are exposed to relatively high O_2 concentrations, which decline as distance from the vessel increases. Although such gradients exist in normal tissues, in cancers, the gradients are much steeper and O_2 concentrations drop to near zero in areas of necrosis. In addition to physical gradients, temporal fluctuations in oxygenation also commonly occur within tumours [9]. This activates HIF-1α, which in turn sets in motion the whole family of important genes that collaborate with different aspects essential for tumourigenesis and tumour progression, i. e., genes associated with angiogenesis (VEGF, SDF1, ANGPT1), survival (BIRC5, EPO), reprogramming from an oxidative metabolism to a glycolytic metabolism (GLUT-1, LDHA, PDK1, GAPDH, PGK1, PKM2), and epithelial-mesenchymal transition and metastasis (LOX, MMP2, MMP9) [10, 11].

HIF-1α therefore increases the density and vascular permeability within the tumours facilitating their growth and later the feared metastasis, since the vessels are the outlet for cancer cells capable of colonising other tissues [12, 13]. Regardless of hypoxia, in some tumours, such as hemangioblastomas and renal carcinomas, the activation of HIF-1α is related to the functional inactivation of the suppressor gene for pVHL, which decreases degradation and increases the total HIF-1α of the cell, even in normoxia [14, 15].

Under hypoxic conditions, phosphorylation of IκB and the subsequent activation of the NF-κB subunits p50 and p65 (RelA) has been reported to contribute to basal levels of HIF-1α mRNA and protein, and to mediate HIF-1α expression and promoter activity in response to thrombin, H_2O_2, and even short-term hypoxia. Indeed, the NF-κB subunits p50 and p65 have been shown to directly interact with HIF-1α at an NF-κB consensus site in the HIF-1α promoter at -197/-188 bp under these conditions. In addition to the stabilisation of HIF-1α under hypoxia conditions, van Uden et al. [16] described how the NF-κB complex is a direct modulator of HIF-1α expression in a manner dependent on stimulation with TNF-α in normoxic conditions [17–19].

The aim of the present research was to perform a bioguided phytochemical study to identify compounds of *S. graveolens* with the capacity to inhibit HIF-1α through suppression of NF-κB activation as future antitumour compounds.

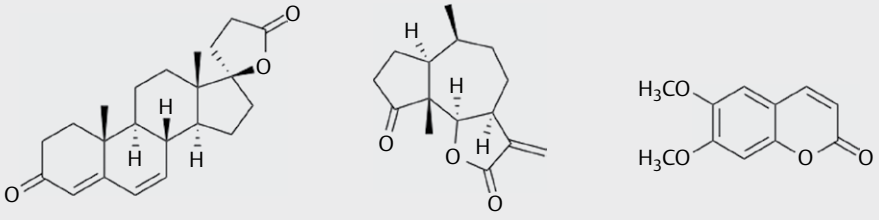
Results

Previously isolated compounds of the *S. graveolens* species belong to a range of structural groups such as monoterpenoids, germacrenes, eremophilanes, sesquiterpenes, *p*-hydroxyacetophenone derivatives and shikimic acid derivatives, benzofurans, and benzopyrans, which show a structural relationship with the previously unreported compounds isolated in this work [20–22]. Their structures were elucidated through ^{1}H and ^{13}C NMR, 2D spectroscopic, and HR-ESIMS analyses. Based on the reported data, they were identified as spironolactone (phanurane), sesquiterpene lactone (damsine), and coumarin (scoparone) (▶ **Fig. 1**), isolated for the first time from this plant species.

U-373 MG (human glioblastoma astrocytoma), A549 (human Caucasian lung carcinoma), Hep G2 (human Caucasian hepatocyte carcinoma), and Caco-2 (human Caucasian colon adenocarcinoma) cancer cell lines were selected to carry out the *in vitro* cytotoxicity and activity assays of the *S. graveolens* extracts and compounds. We selected these cancer cell lines according to their physiological relationship with certain diseases that are treated with *S. graveolens* in traditional medicine [1–4]. Therefore, the relationship is for mountain sickness, characterised by symptoms such as headache (U-373 MG cell line), respiratory diseases (A549 cell line), stomach pain, and colitis (Hep G2 and Caco-2 cell lines). In addition, a non-cancerous cell line (PMBC) was used to assess the safety of the compounds.

After examining the cytotoxicity of the *S. graveolens* extracts in hypoxic conditions (1 % O_2) after 72 h of treatment (▶ **Table 1**), we observed that all of them had a greater cytotoxic effect on the cancer cell line U-373 MG, which is a human glioblastoma astrocytoma cell line. We also observed that the dichloromethane/methanol extract was more active than the aqueous extract and the *n*-heptanic extract. These results corroborate those shown by Echiburú-Chau et al. [6], who by means of the neutral red uptake test observed that at a concentration of 200 µg/mL ethanolic extract of *S. graveolens* exhibited cytotoxicity in breast cancer cell lines ZR-75–1, MCF-7, and MDA-MB-231 at 24 h of treatment under hypoxic conditions, without damaging the MCF-10F cell line. However, at this concentration, the ethanolic extract of *S. graveolens* showed a greater cytotoxic effect on the cell line MDA-MB-231 (50 %) compared to the other cancer lines ZR-75–1 (9 %) and MCF-7 (6 %) [6]. Finally, both MTT and LDH assays showed that dichloromethane/methanol and aqueous extracts have cytotoxic effects higher than 100 µg/mL in the PBMCs. On the contrary, the *n*-heptanic extract was discarded due to its cytotoxic effect in PBMCs with a CC_{50} of 99.62 ± 2.22 µg/mL and 91.02 ± 3.60 µg/mL measured by the MTT and LDH assays, respectively.

Afterwards, we evaluated the dichloromethane/methanol and the aqueous extracts inhibitory activity over NF-κB (▶ **Table 2**) and HIF-1α (▶ **Table 3**) on the same cell lines under equal conditions. The dichloromethane/methanol extract presented a higher inhibitory effect on both transcription factors in all cell lines. Again, the lower IC_{50} values were those shown on the brain cancer cell line



(1) (2) (3)

▶ **Fig. 1** Chemical structures Phanurane (1), Damsine (2) and Scoparone (3).

▶ **Table 1** MTT and LDH cytotoxicity assays of *S. graveolens* extracts against a panel of human cancer cell lines and one noncancerous cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. The results are the means (± SD) of three separate experiments performed in triplicate. Control = untreated cells.

Samples	CC_{50} (µg/mL)				
	PBMCs	U-373 MG	A549	Hep G2	Caco-2
Control (untreated cells)	100.01 ± 3.45	100.00 ± 1.23	99.95 ± 1.87	99.98 ± 2.67	100.10 ± 2.21
Heptanic extract[a]	99.62 ± 2.22	41.74 ± 1.62	57.15 ± 2.42	78.66 ± 2.67	98.79 ± 4.62
Dichloromethane/methanol extract[a]	> 100 ± 1.64	31.40 ± 2.32	46.52 ± 2.26	68.97 ± 3.66	98.05 ± 3.67
Aqueous extract[a]	> 100 ± 1.75	33.48 ± 4.81	47.84 ± 2.67	74.73 ± 2.69	98.46 ± 1.65
Control (untreated cells)	99.96 ± 3.45	99.90 ± 2.65	100.11 ± 3.11	99.92 ± 2.87	100.09 ± 2.74
Heptanic extract[b]	91.02 ± 3.60	40.08 ± 1.46	54.24 ± 4.36	76.21 ± 3.50	95.71 ± 2.67
Dichloromethane/methanol extract[b]	> 100 ± 1.09	31.38 ± 1.95	42.02 ± 1.71	66.18 ± 2.05	94.93 ± 1.85
Aqueous extract[b]	> 100 ± 1.56	31.94 ± 2.09	47.12 ± 2.40	74.50 ± 1.37	95.63 ± 3.48

[a] Cytotoxicity values of the MTT assay; [b] Cytotoxicity values of the LDH assay

▶ **Table 2** Inhibitory effect of *S. graveolens* extracts on NF-κB activation in a panel of human cancer cell lines and one noncancerous cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. The results are the means (± SD) of three separate experiments performed in triplicate. Control = untreated cells.

Samples	CC_{50} (µg/mL)				
	PBMCs	U-373 MG	A549	Hep G2	Caco-2
Control	11.98 ± 0.01	12.43 ± 0.01	12.23 ± 0.08	12.45 ± 0.09	12.65 ± 0.08
DMSO	5.96 ± 0.05	6.35 ± 0.02	6.45 ± 0.03	6.23 ± 0.07	6.62 ± 0.08
Dichloromethane/methanol extract	3.89 ± 0.01	4.40 ± 0.02	5.35 ± 0.01	9.58 ± 0.04	10.44 ± 0.01
Aqueous extract	14.56 ± 0.02	20.29 ± 0.05	27.24 ± 0.02	30.23 ± 0.04	39.89 ± 0.03
JSH-23	7.09 ± 0.01 µM	6.99 ± 0.02 µM	7.10 ± 0.02 µM	7.10 ± 0.03 µM	7.11 ± 0.01 µM

U-373 MG in comparison with the rest of the cancerous cell lines. Nevertheless, the inhibitory effect was even higher on the PBMCs.

For the fractionation and isolation of the compounds of *S. graveolens*, the dichloromethane/methanol extract was selected because it showed no cytotoxic effect on the PBMCs, besides showing greater inhibitory activity over NF-κB and HIF-1α with respect to the aqueous extract.

Regarding the compounds (phanurane, damsine, and scoparone) isolated from the dichloromethane/methanol extract of *S. graveolens*, they were individually more cytotoxic than the crude extract in the same hypoxic conditions (1 % O_2) at 72 h of treatment. U-373 MG cell line viability remained as the most affected one by the three compounds with respect to the cancer cell lines A549, Hep G2, and Caco-2 (▶ **Table 4**). Besides, the isolated compounds did not show a cytotoxicity higher than 100 μM on the noncancerous cell line (PBMCs) by the MTT assay. However, in the LDH assay, phanurane, damsine, and scoparone compounds showed CC_{50}s of 90.11 ± 0.99, 79.62 ± 2.23 , and 89.79 ± 1.12 μM on the PBMCs.

Phanurane (**1**) showed a cytotoxic effect by means of the MTT assay under hypoxic conditions (1 % O_2) on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 with CC_{50}s of 23.63 ± 1.47, 28.54 ± 2.25, 45.33 ± 1.09, and 68.49 ± 3.50 μM at 72 h of treatment. However, using the LDH assay under hypoxic conditions (1 % O_2) at 72 h of treatment, phanurane showed CC_{50}s of 21.30 ± 1.09, 27.44 ± 3.18, 44.47 ± 1.77, and 67.89 ± 1.38 μM on the cancer lines mentioned above. There are no reports of phanurane cytotoxicity assays on cell lines.

Damsine (**2**) showed a cytotoxic effect by means of the MTT and LDH assays under hypoxic conditions (1 % O_2) at 72 h of treatment on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 with CC_{50}s of 12.40 ± 2.28, 23.80 ± 1.06, 39.07 ± 2.43, 63.79 ± 1.48 μM and 12.25 ± 1.10, 21.91 ± 2.66, 35.09 ± 3.05, 60.76 ± 1.12 μM, respectively. Our results corroborate the cytotoxic activity of damsine described by Saeed et al. [23], who observed cytotoxic activity (as measured by the resazurin reduction assay) at 24 h of damsine treatment on brain and colon cancer cell lines U87 MG/U87 ΔEGFR and HCT116. $P53^{+/+}$/HCT116 $P53^{-/-}$ with CC_{50}s of 154.4 ± 8.9/24.1 ± 1.5 μM and 32.5 ± 6/133.6 ± 18.4 μM, respectively. In addition, there are reports that damsine has cytotoxic activity at 72 h of treatment on breast cancer cell lines MCF-7, JIMT-1, and HCC1937 with CC_{50}s of 3.7 ± 0.4, 3.3 ± 0.6, and 6.8 ± 0.4 μM, respectively [24].

Scoparone (**3**) showed a cytotoxic effect under hypoxic conditions (1 % O_2) with CC_{50}s of 18.47 ± 1.49, 24.67 ± 1.34, 41.92 ± 2.39, and 66.39 ± 2.75 μM by means of the MTT assay and 16.84 ± 2.80, 23.82 ± 2.40, 41.59 ± 2.66, and 64.42 ± 1.76 μM by means of the LDH assay on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 after 72 h of treatment. There are also reports that scoparone significantly inhibited the proliferation of DU145 cells, with a CC_{50} value of 41.3 μM after 72 h of treatment [25].

Concerning the inhibition of NF-κB production in hypoxic conditions (1 % O_2), the isolated compounds of the dichloromethane/methanol extract of *S. graveolens* were compared with respect to the positive control JSH-23 (IC_{50} = 7.1 μM) [26]. Phanurane, damsine, and scoparone compounds showed a greater effect on the brain cancer cell line with respect to the lung, liver, and colon cancer cell lines after 72 h of treatment (▶ **Fig. 2**).

Phanurane (**1**) inhibited the production of NF-κB under hypoxic (1 % O_2) conditions on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 with IC_{50}s of 7.13 ± 0.03, 8.64 ± 0.03, 8.87 ± 0.04,

▶ **Table 3** Inhibitory effect of *S. graveolens* extracts on HIF-1α in a panel of human cancer cell lines and one noncancerous cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. Control = untreated cells.

Samples	CC_{50} (μg/mL)				
	PBMCs	U-373 MG	A549	Hep G2	Caco-2
Control	90.97 ± 0.02	96.71 ± 0.05	98.84 ± 0.05	97.82 ± 0.06	99.97 ± 0.03
Dichloromethane/methanol extract	6.26 ± 0.09	9.39 ± 0.03	15.72 ± 0.06	19.63 ± 0.05	32.51 ± 0.07
Aqueous extract	20.25 ± 0.09	25.87 ± 0.08	30.22 ± 0.07	41.89 ± 0.02	84.89 ± 0.05
2-MeOE2	0.49 ± 0.02 μM	0.48 ± 0.01 μM	0.50 ± 0.01 μM	0.49 ± 0.02 μM	0.51 ± 0.02 μM

▶ **Table 4** MTT and LDH cytotoxicity assays of *S. graveolens* compounds against a panel of human cancer cell lines and one noncancerous cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. Control = untreated cells.

Samples	CC_{50} (μg/mL)				
	PBMCs	U-373 MG	A549	Hep G2	Caco-2
Control (untreated cells)	99.98 ± 2.12	99.97 ± 3.43	100.01 ± 2.16	100.18 ± 3.07	99.90 ± 3.11
Compound 1[(a)]	> 100 ± 2.51	23.63 ± 1.47	28.54 ± 2.25	45.33 ± 1.09	68.49 ± 3.50
Compound 2[(a)]	> 100 ± 3.28	12.40 ± 2.28	23.80 ± 1.06	39.07 ± 2.43	63.79 ± 1.48
Compound 3[(a)]	> 100 ± 1.25	18.47 ± 1.49	24.67 ± 1.34	41.92 ± 2.39	66.39 ± 2.75
Control (untreated cells)	100.03 ± 2.11	100.97 ± 3.11	100.91 ± 2.91	100.95 ± 3.43	99.91 ± 3.56
Compound 1[(b)]	90.11 ± 0.99	21.30 ± 1.09	27.44 ± 3.18	44.47 ± 1.77	67.89 ± 1.38
Compound 2[(b)]	79.62 ± 2.23	12.25 ± 1.10	21.91 ± 2.66	35.09 ± 3.05	60.76 ± 1.12
Compound 3[(b)]	89.79 ± 1.12	16.84 ± 2.80	23.82 ± 2.40	41.59 ± 2.66	64.42 ± 1.76

[(a)]Cytotoxicity values of the MTT assay; [(b)]Cytotoxicity values of the LDH assay.

and 15.11 ± 0.01 μM at 72 h of treatment. There are no reports that phanurane has inhibited the production of NF-κB in cancer cell lines.

Damsine (**2**) inhibited the production of NF-κB under hypoxic (1 % O_2) conditions at 72 h of treatment with IC_{50}s of 2.28 ± 0.01, 3.79 ± 0.02, 3.98 ± 0.05, and 6.41 ± 0.02 μM on cancer cells U-373 MG, A549, Hep G2, and Caco-2, respectively. Also, there are reports that damsine inhibited the expression of NF-κB on cancer cell line Caco-2 with an IC_{50} of 7.2 μM after 6 h of treatment [27].

Scoparone (**3**) inhibited the production of NF-κB under hypoxic (1 % O_2) conditions at 72 h of treatment on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 with IC_{50}s of 3.62 ± 0.06, 4.48 ± 0.03, 5.25 ± 0.01, and 11.90 ± 0.02 μM, respectively. Scoparone has also been reported to inhibit the expression of NF-κB on cancer cell line U-937 with an IC_{50} of 10 nM after 24 h of treatment [28].

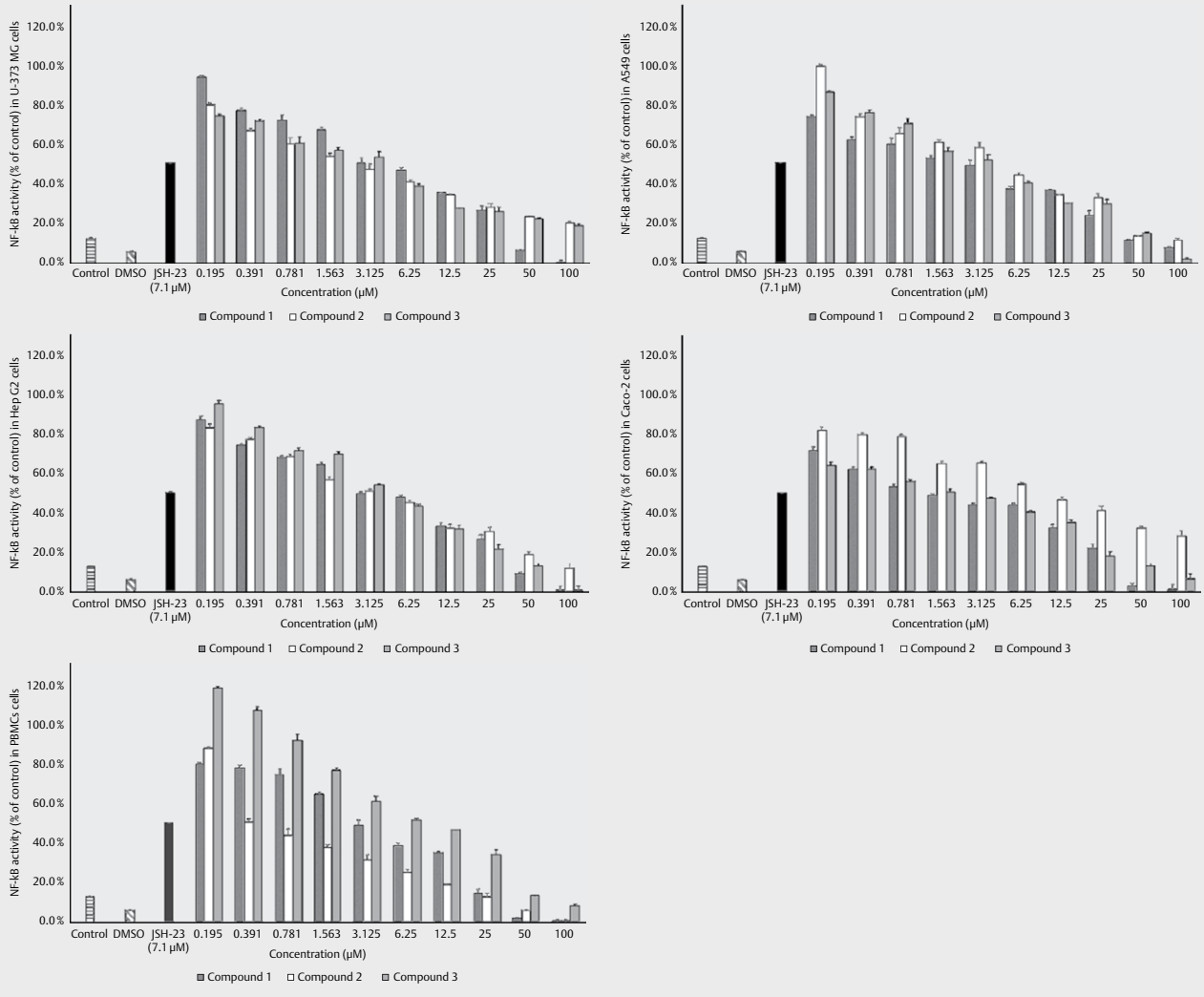
Finally, under hypoxic conditions, the isolated compounds of the dichloromethane/methanol extract of *S. graveolens* were compared with respect to the positive control 2-MeOE2 (IC_{50} = 0.5 μM) [29]. Phanurane, damsine, and scoparone compounds showed an inhibitory effect on HIF-1α on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 at 72 h of treatment (▶ **Fig. 3**).

Phanurane (**1**) inhibited HIF-1α under hypoxic (1 % O_2) conditions at 72 h of treatment with IC_{50}s of 20.66 ± 0.04, 25.80 ± 0.04, 29.21 ± 0.03, and 38.58 ± 0.02 μM on cancer cells U-373 MG, A549, Hep G2, and Caco-2, respectively.

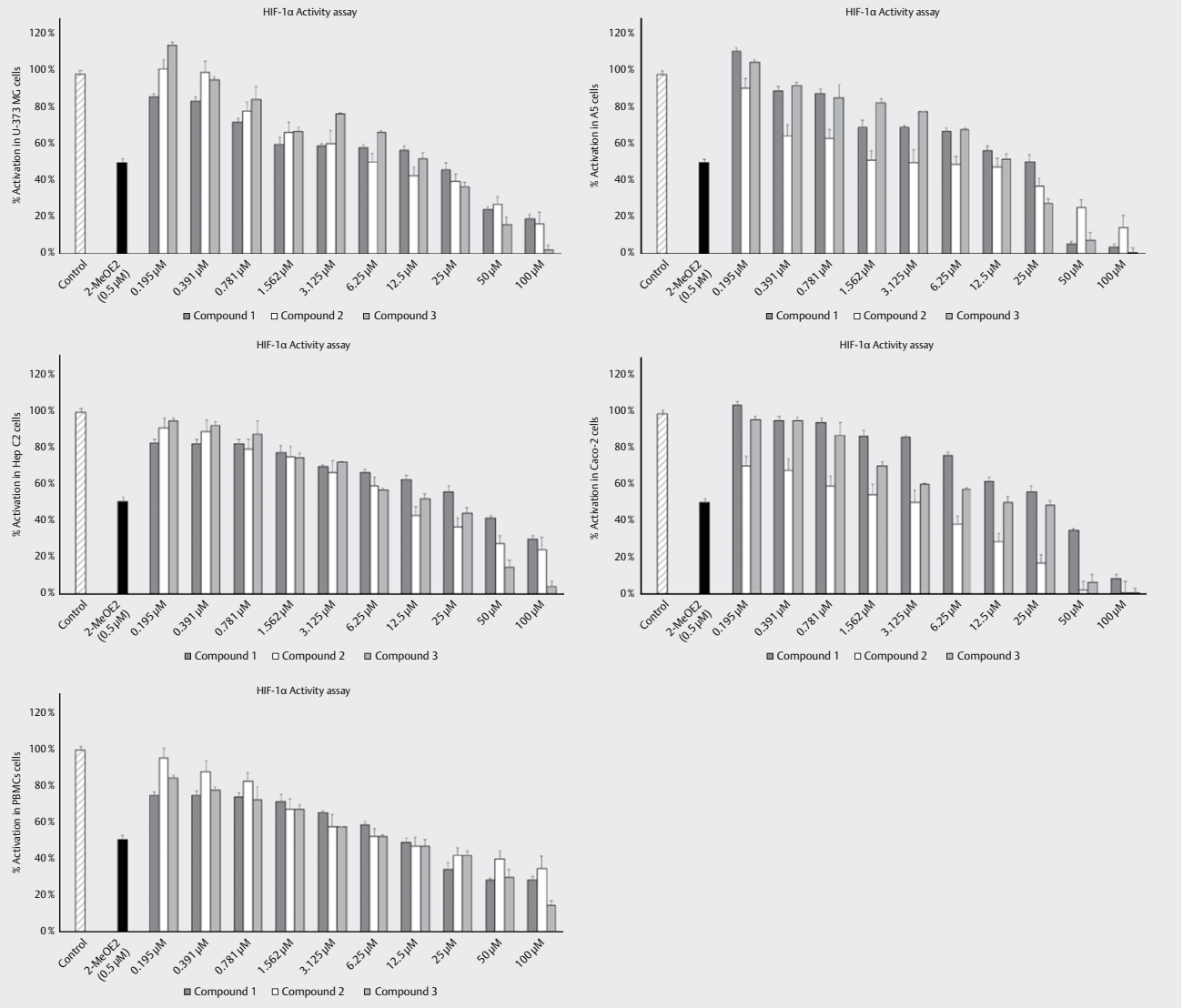
Damsine (**2**), under hypoxic (1 % O_2) conditions at 72 h of treatment on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2, inhibited HIF-1α with IC_{50}s of 2.29 ± 0.07, 4.13 ± 0.04, 6.40 ± 0.03, and 9.80 ± 0.04 μM, respectively.

Scoparone (**3**) inhibited HIF-1α under hypoxic (1 % O_2) conditions on the cancer cell lines U-373 MG, A549, Hep G2, and Caco-2 with IC_{50}s of 15.22 ± 0.01, 17.47 ± 0.02, 18.26 ± 0.06, and 19.75 ± 0.04 μM, respectively, at 72 h of treatment.

There are no reports on the inhibition of HIF-1α by means of the phanurane, damsine, and scoparone compounds.



▶ **Fig. 2** Inhibitory effect of *S. graveolens* compounds on NF-κB activation in a panel of human cancer cell lines and one noncancer cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. Control = untreated cells.



▶ **Fig. 3** Inhibitory effect of *S. graveolens* compounds on HIF-1α in a panel of human cancer cell lines and one noncancer cell line after 72 h of treatment under hypoxic (1 % O_2) conditions. Control = untreated cells.

Discussion

There is an extensive phytochemical analysis on the essential oil (46 compounds) of *S. graveolens,* where monoterpene hydrocarbons predominate, with sabinene and α-terpinene as the main components [30, 31]. The main activity of the essential oil of *S. graveolens* is antimicrobial [32, 33]. On the other hand, only 14 compounds present in the hydroalcoholic extract have been identified from the aerial parts of *S. graveolens*. The compounds dihydroeuparin, 4-hydroxy-3-(isopenten-2-yl) acetophenone, 3-hydroxy-2,2-dimethyl-6-acetylchromane, 5-acetylsalicylaldehyde, 4-hydroxy-3-(3'-hydroxyisopentyl) acetophenone, scopoletin, 3-phenyl-4-hydroxyacetophenone, 4-hydroxyacetophenone, 4-hydroxy-3-methoxyacetophenone, 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone, 5-acetyl-6-hydroxy-2-isopropenyl-2,3-dihydrobenzofurane, 4-acetylphenyl-α-D-glucopyranoside, 1-(3-hydroxy-2,2-dimethyl-3,4-dihydro-2H-chromen-6-yl) ethanone, and 1-(2,2-dimethyl-3,4-dihydro-2H-chromen-6-yl) ethanone are polyphenolic in nature [20–22, 34]. There are few works on the pharmacological activities of *S. graveolens*. With respect to the hydroalcoholic extract of *S. graveolens*, only antimicrobial, antioxidant, vasodilator, hypotensive, and antihypertensive activities have been tested. [34–37].

Regarding the cytotoxicity of *S. graveolens*, Echiburú-Chau et al. [6] reported that the alcoholic extract could induce cytotoxicity under hypoxic conditions in different breast cancer cell lines (ZR-75–1, MCF-7, and MDA-MB-231), but not in non-tumourigenic cells (MCF-10F). However, compound 4-hydroxy-3-(3-methyl-2-butenyl) acetophenone, mostly present in the alcoholic extract, did not show effective anticancer activity compared to the complete extract. The mechanisms responsible for the cytotoxicity exerted by the alcoholic extract of *S. graveolens* are not clear. It is very likely that the relative contribution of apoptosis and autophagy to the

cytotoxic effects of *S. graveolens* depends mainly on the genetic profile of the analysed cells, since alterations in the known oncogenic pathways (for example, P53) modify the way in which a cell responds to external stimuli.

Our study revealed that the dichloromethane/methanol extract of *S. graveolens* showed a greater inhibitory activity on NF-κB and HIF-1α than the aqueous extract of *S. graveolens* in all tumour cell lines (U-373 MG, A549, Hep G2, Caco-2) tested. Besides, both extracts did not show cytotoxicity over PBMCs non-tumourous cell line. We also observed that the *n*-heptanic extract of *S. graveolens* presented cytotoxicity over PBMCs non-tumourous cell line, and its activity over NF-κB and HIF-1α was lower with respect to the dichloromethane/methanol and aqueous extracts. For that reason, we selected the dichloromethane/methanol extract for subsequent fractionation. The dichloromethane/methanol extract was separated by bioguided analysis, determining the activity of each fraction over NF-κB and HIF-1α (data not shown).

The interest in the role of HIF-1 in cancer biology has grown exponentially because the usual conditions within most solid tumour masses occur are anoxia (absence of oxygen) or hypoxia (low oxygen concentrations). These situations activate HIF-1 protein, which consists of two subunits, α1 (inducible), and β1 (constitutively expressed). Under normoxia conditions (normal oxygen concentrations), the α1 subunit is very labile, and degrades at the same rate at which it is synthesised [38]. However, under hypoxia conditions, this subunit is stabilised and dimerizes with the β1 subunit. This heterodimer migrates to the nucleus, where it binds to specific DNA sequences, activating genes involved in adaptation to hypoxia, cell survival, angiogenesis, and metastasis, such as, for example, VEGF, TGF-a, GLUT-1, or CA9, among many others, which we know are involved in tumour development and aggressiveness [39–41]. Also, HIF-1α has been shown to be regulated by cytokines such as ILs, TNF-α, and NF-κB [42].

NF-κB is a central molecule that regulates the expression of various target genes that promote cell proliferation, modulate immune responses, and play a role in the pathogenesis of various diseases, including cancer [43, 44]. When analysing the possible mechanism by NF-κB inhibition of the compounds isolated from the dichloromethane/methanol extract of *S. graveolens*, we could indicate that damsine acts on sulfhydryl groups of cysteine residues in the DNA-binding domain of the NF-κB subunit through its exomethylene group [45]. Likewise, the unsaturated carbonyl group and the methylene-lactone ring present in damsine can exert a direct alkylation effect on the p65 subunit of NF-κB without inhibiting the degradation of IκB [46]. In the case of scoparone, the presence of its lactonic ring can inhibit the levels of the NF-κB-DNA complex. It can also inhibit dose-dependent phosphorylation of IκBα and nuclear translocation of NF-κB1 [47]. Finally, the low activity of phanurane can be attributed to its lactonic ring, which partially inhibits one of the stages of the cytoplasmic signalling pathway of NF-κB and the nucleus, namely, the phosphorylation of IκBα in the cytoplasm [48]. The damsine and scoparone compounds had a better inhibitory activity on NF-κB than the JSH-23 control (IC_{50} = 7.1 μM) in all tumour cell lines, except in the Caco-2 cell line (▶ **Fig. 2**).

Regarding the mechanism of inhibitory action over HIF-1α by the compounds isolated from the dichloromethane/methanol extract of *S. graveolens*, we could indicate, based on the bioinformatic experiment conducted by Dawood et al. [50], that sesquiterpene lactones inhibiting NF-κB also possess the ability to inhibit HDAC (histone deacetylase). The latter leads to the inhibition of HIF-α in different tumour cell lines. This phenomenon is suggested as a mechanism of collateral sensitivity [49]. In the case of scoparone, like other coumarins, it would cause degradation of HIF-1α, weakening glycolysis and all activities related to apoptosis in tumour cells [50]. Finally, regarding phanurane, there is no clear mechanism of its activity on HIF-1α. There is a report by Yim et al. [51] which mentions that spironolactone (compound structurally similar to phanurane) improved the hypoxic response and increased the expressions of HIF-1α and Ets-1 in the kidneys of newborn rats. However, they indicate that to clarify the hypoxic changes, it is necessary to investigate what types of signal cascades exist between RAAS (renin-angiotensin-aldosterone system) and renal hypoxia. These results corroborate our results, since the phanurane compound did not inhibit the activity of HIF-1α in the tumour cell lines analysed. On the contrary, we can indicate that phanurane increased its activity. None of the compounds showed a better inhibitory activity over HIF-1α than the 2-MeOE2 control (IC_{50} = 0.5 μM) in all tumour cell lines (▶ **Fig. 3**).

It should be noted that in the work of Echiburú-Chau et al. [6] they mentioned that the majority compound [4-hydroxy-3- (3-methyl-2-butenyl) acetophenone] of the alcoholic extract presented less cytotoxic activity on different tumour cellular lines than the complete extract. However, in our case, the major compounds (phanurane, damsine, and scoparone) of the dichloromethane/methanol extract showed greater cytotoxic activity over different tumour cellular lines than the complete extract (▶ **Table 3**).

Both HIF-1α and NF-κB are involved in cancer progression and have been implicated in the tumour responses to hypoxia [52]. Moreover, positive correlations between HIF-1α and NF-κB have been shown in various cancer cells, including lung cancer cells, colorectal cancer cells, osteosarcoma, and gastric cancer cells [53, 54]. Our results corroborate that both HIF-1α and NF-κB are involved in the progression of cancer and have been implicated in tumour responses to hypoxia. In addition, we have shown positive correlations between HIF-1α and NF-κB in cancer cells lines U-373 MG, A549, Hep G2, and Caco-2. Likewise, it is the first time that the inhibitory activity over HIF-1α is reported by means of the phanurane, damsine, and scoparone compounds, in addition to reporting for the first time the inhibitory activity of phanurane in NF-κB. Finally, we indicate, based on our results, that the inhibition of NF-κB could regulate both HIF-1α signalling and the progression of the epithelial-mesenchymal transition induced by hypoxia.

In conclusion, considering the multiple roles of HIF-1α in tumour progression and metastasis, phanurane, damsine, and scoparone compounds must be considered prototype molecules for the development of novel drugs aimed at inhibiting this pathway, due to their high cytotoxic capacity over different cancer cell lines. Since the HIF-1α regulatory pathway is a highly complex network involving several signalling cascades and overlapping mechanisms, each one of them could serve as a promising target or step to intervene tumours in the future.

Materials and Methods

Cell lines, chemicals. and biochemicals

Four human cancer cell lines were used in this study: U-373 MG, A549, Hep G2, and Caco-2, and the non-tumourigenic PBMCs cell line. All cell lines were obtained from ATCC. Cells were cultured in specific media according to ATCC recommendations. PMBCs were obtained from whole blood by density gradient centrifugation using Lymphoprep (StemCell Technologies) according to the manufacturer's instructions. In both cases, the incubation condition was established at 37 °C in hypoxic conditions (1 % O_2), thus mimicking the *in vivo* tumour microenvironment.

We used first grade organic solvents for isolating the compounds. TLC was performed using Merck Silica gel 60-F_{254} plates. Chromatograms thus obtained were visualised by UV absorbance (254 nm) and through heating a plate stained with phosphomolybdic acid. Manual flash chromatography was performed with flash grade Silica gel 60 (20–45 and 40–63 μM; Merck).

DMEM, RPMI, FBS, and PBS were obtained from Sigma-Aldrich. L-Glutamine was obtained from Applichem. Penicillin and streptomycin were purchased from PAA. Test compounds were dissolved in DMSO (Merck) at a 10 mM concentration, while the extracts and fractions were dissolved at 20 mg/mL in DMSO.

Plant material

A sample of *S. graveolens* was collected from the Pongo community (Murillo province, La Paz, Bolivia) in August 2018 at an altitude of 3800 m. Botanical identification was confirmed by the National Herbarium of Bolivia (No. 13898).

Extraction and isolation

S. graveolens leaves (2 kg) were extracted by repeated maceration with 2.5 L of different solvents, increasing the polarity: *n*-heptane, dichloromethane/methanol (1:1), and water. As a result, three extracts of 120, 95, and 375 g, respectively, were obtained.

Each extract was evaluated for its cytotoxic effects and its influence on the inhibition of HIF-1α as well as on its modulator NF-κB. Only the *n*-heptanic extract showed significant cytotoxicity on the noncancerous cell line (▶ **Table 1**), whereas the dichloromethane/methanol extract showed a greater inhibition of NF-κB and HIF-1α, being more active than the aqueous extract (▶ **Tables 2** and ▶ **3**). Therefore, the chemical constituents of the dichloromethane/methanol extract were investigated to identify specific compounds with cytotoxic potential.

The dichloromethane/methanol extract was analysed through bioassay-guided Silica gel (40–63 μM) column chromatography (2 × 50 cm) using a step gradient of *n*-heptane, dichloromethane and ethyl acetate (50/50/0 to 50/0/50) to produce 15 fractions (I-XV). Among them, III and IV were the fractions that showed the highest activity (inhibition of HIF-1α as well as of its modulator NF-κB).

As a result, fractions III and IV were combined and a second bioassay-guided Silica gel (40–63 μM) column chromatography (2 × 50 cm) was performed using step gradient *n*-heptane and dichloromethane (50/0 to 0/50). Nine fractions were obtained (1–9). Nine fractions showed inhibition of HIF-1α as well as of its modulator NF-κB, but fractions 1, 5, and 7 were the most active.

Fraction 1 was separated in a column (2 × 30 cm) of Silica gel (40–63 μM) with *n*-heptane/ethyl acetate, 80:20, from which six fractions were obtained (1a-1f). Fraction 1b was the most active and it was further chromatographed in a column (2 × 30 cm) of Silica gel (20–45 μM) (*n*-heptane/ethyl acetate, 60:40), obtaining compound **1** (14 mg).

Next, fraction 5 was chromatographed on a column (2 × 30 cm) of Silica gel (40–63 μM) with *n*-heptane/ethyl acetate, 60:40, to produce nine fractions (5a–5i), with the 5f fraction being the most active one. Fraction 5f was chromatographed in a column (2 × 30 cm) of Silica gel (20–45 μM) (*n*-heptane/ethyl acetate, 50:50) to isolate compound **2** (11 mg).

Finally, fraction 7 was fractionated by a column (2 × 30 cm) of Silica gel (40–63 μM) with *n*-heptane/ethyl acetate, 20:80, providing ten fractions (7a–7j), from which fraction 7i was chromatographed in a column (2 × 30 cm) of Silica gel (20–45 μM) (*n*-heptane/ethyl acetate, 40:60) to obtain compound **3** (5 mg).

NMR/MS analysis

NMR experiments were performed on a Bruker BioSpin GmbH spectrometer operating at 700 MHz (^{1}H) or 175 MHz (^{13}C). Deuterated solvents were methanol-d_4 and chloroform-d_1. Spectra were calibrated by assignment of the residual solvent peak to δ_H 3.31 ppm and δ_C 49.0 ppm for methanol-d_4, and δ_H 7.2 ppm and δ_C 77.0 ppm for chloroform-d_1. Complete assignment of protons and carbons was done by analysing the correlated ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, and ^{1}H-^{13}C HMBC spectra. HREIMS analyses were performed using a mass spectrometer with QTOF hybrid analyser model Q-Star Pulsar I from the commercial house Applied Biosystems. MS samples were prepared in MeOH + formic acid 0.1 % and analysed by the electrospray ionisation technique in the positive ion detection mode.

Cytotoxic assay

Cell viability was determined in a panel of four human cancer cell lines (U-373 MG, A549, Hep G2, Caco-2) and one noncancerous cell line (PBMCs) by MTT (≥ 97.5 %, Sigma-Aldrich) and LDH (Cytotoxicity Detection Kit; Sigma-Aldrich) assays. Cells were seeded in 96-well plates at a density of 2×10^4 cells/well, and then they were left for 24 h at 37 °C in a humidified atmosphere with 5 % CO_2. After that, cells were treated with various concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.95 μM) of the isolated compounds in hypoxic conditions (1 % O_2).

MTT cytotoxicity assay: Following a 72-h incubation, a volume of 20 μL PBS containing 4 mg/mL MTT was added to each well. After this, plates were incubated for 4 h, before they were centrifuged at 1500 rpm at 4 °C for 10 min, followed by the removal of the supernatant. Then, DMSO (150 μL) was added to each well for colouration, and the plates were subsequently shaken vigorously to ensure complete solubilisation for 10 min at room temperature. The light absorption (OD, optical densities) was recorded on a spectrophotometric ELISA plate reader (SpectraMax i3, Molecular Devices) at a wavelength of 590 nm.

LDH cytotoxicity assay: After 72 h of treatment with the compounds, 100 μL of culture supernatants were collected and incubated in the reaction mixture from the LDH kit (Innoprot Company). After 30 min, the reaction was stopped by adding HCl 1 N, and

the absorbance at a wavelength of 490 nm was measured using a spectrophotometric ELISA plate reader (SpectraMax i3; Molecular Devices).

Nuclear factor kappa-light-chain-enhancer of activated B cells inhibition assay

Cells were transfected using a lipofectamine plus transfection reagent (Thermo Fisher Scientific) with 0.3 μg of the NF-κB-promoted luciferase reporter gene plasmid (pGL2-NF-κB-Luc) (Promega), and 0.03 μg of the Renilla luciferase reporter plasmid (pTK-Renilla) (transfection normalisation vector; Promega). After 1 day, the cells were incubated with TNF-α (5 ng/mL) in the absence or presence of the isolated compounds at different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, and 0.95 μM) for 72 h of treatment in hypoxic conditions (1 % O_2). JSH-23 (≥ 98 %; Sigma-Aldrich) was used as a positive control at a concentration of 7.1 μM. Next, the cells were lysed in 25 mM Tris-phosphate, pH 7.8, containing 8 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 % Triton X-100, and 7 % glycerol. Luciferase activity was measured by a Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer's instructions.

Hypoxia-inducible factor 1-alpha inhibition assay

Cells were stably transfected with plasmid Epo-Luc plasmid. EPO-hypoxia response element (HRE)-luciferase reporter plasmid contains three copies of the HRE consensus sequence from the promoter of the erythropoietin gene in the pGL3 vector. Cells (1×10^4) were seeded the day before the assay. The next day, the cells were stimulated with the test compounds at different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1563, 0.781, 0.391, and 0.95 μM). 2-MeOE2 (≥ 99 %; Sigma-Aldrich) was used as a positive control at a concentration of 0.5 μM. After 72 h of stimulation in hypoxic conditions (1 % O_2), the cells were lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, and 7 % glycerol during 15 min at RT in a horizontal shaker. Luciferase activity was measured using a GloMax 96 microplate luminometer (Promega) following the instructions of the luciferase assay kit (Promega). The RLU was calculated and the results are expressed as percentage of inhibition induction/inhibition of EPO-luc activity. Experiments for each concentration of the test items were done in triplicate wells.

Statistical analysis

Statistical significance of differences was calculated employing GraphPad Prism software, version 8.2.0 (GraphPad Software Inc.) using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Results were considered different when $p < 0.0001$. IC_{50} values were determined by nonlinear regression using GraphPad Prism, version 8.2.0. All the experiments were performed in triplicate.

Phanurane (**1**): white amorphous powder; m.p. = 149°C; ^{1}H-NMR (700 MHz, $CDCl_3$) δ_H = 6.14 (1H, dd, *J* = 9.8, 2.7 Hz, H-6), 6.09 (1H, dd, *J* = 9.7, 2.0 Hz, H-7), 5.69 (1H, s, H-4), 2.58 (1H, m, H-21), 2.55 (1H, m, H-2), 2.50 (1H, m, H-21'), 2.42 (1H, m, H-2'), 2.37 (1H, ddd, *J* = 13.0, 9.1, 6.1 Hz, H-20), 2.31 (1H, m, H-16), 2.30 (1H, m, H-8), 2.02 (1H, ddd, *J* = 13.1, 5.4, 2.1 Hz, H-1), 1.92 (1H, ddd, *J* = 12.8, 9.2, 7.3 Hz, H-20'), 1.89 (1H, m, H-15), 1.87 (1H, m, H-16'), 1.68 (1H, m, H-1'), 1.66 (1H, m, H-11), 1.63 (1H, m, H-12), 1.57 (1H, m, H-15'), 1.47 (1H, qd, *J* = 12.7, 3.6 Hz, H-11), 1.37 (1H, m, C-12'), 1.36 (1H, m, H-14), 1.20 (1H, m, *J* = 13.2, 7.5, 2.5 Hz, H-9), 1.13 (3H, s, H-19), 1.04 (3H, s, H-18); ^{13}C-NMR (176 MHz, $CDCl_3$) δ_C = 199.57 (C-3), 176.74 (C-22), 163.20 (C-5), 139.56 (C-7), 128.50 (C-6), 124.14 (C-4), 95.52 (C-17), 50.58 (C-9), 47.17 (C-14), 46.59 (C-13), 37.95 (C-8), 36.19 (C-6), 35.63 (C-16), 34.09 (C-1), 34.05 (C-2), 31.78 (C-12), 31.33 (C-20), 29.41 (C-21), 22.62 (C-15), 20.25 (C-11), 16.49 (C-19), 14.58 (C-18); HRESIMS *m/z* 341.2117 [M + H^+] + 363.1943 [M + Na^+] + 703.3992 [2M + Na^+] (calcd. for $C_{22}H_{28}O_3$). Data compared to the reference Preisig et al. [55].

Damsine (**2**): white crystalline solid; m.p. = 107°C; ^{1}H-NMR (700 MHz, MeOD) δ_H = 6.18 (1H, d, *J* = 3.2 Hz, H-14), 5.64 (1H, d, *J* = 2.8 Hz, H-14'), 4.59 (1H, d, *J* = 8.7 Hz, H-6), 3.40 (1H, dddd, *J* = 11.7, 6.1, 4.6, 3.1 Hz, H-7), 2.40 (2H, H-3, H-3'), 2.19 (1H, m, H-10), 2.18 (1H, m, H-1), 2.03 (2H, m, H-8, H-8'), 1.90 (1H, dddd, *J* = 14.8, 7.8, 4.6, 1.8 Hz, H-2), 1.84 (1H, ddd, *J* = 11.8, 5.5, 1.4 Hz, H-2'), 1.79 (2H, m, H-9, H-9'), 1.08 (3H, d, *J* = 7.2 Hz, H-15), 1.03 (3H, s, H-11); ^{13}C-NMR (176 MHz, MeOD) δ_C = 222.13 (C-4), 172.65 (C-13), 141.65 (C-12), 121.58 (C-14), 83.68 (C-6), 56.22 (C-5), 47.09 (C-1), 45.57 (C-7), 36.83 (C-3), 35.76 (C-10), 34.41 (C-9), 26–61 (C-2), 24.99 (C-8), 16.22 (C-15), 14.23 (C-11); *m/z* 249.1496 [M + H^+] + 271.1316 [2M + Na^+] (calcd. for $C_{15}H_{20}O_3$). Data compared to the reference Li et al. [56].

Scoparone (**3**): amber crystalline solid; m.p. = 144°C; ^{1}H-NMR (700 MHz, $CDCl_3$): δ_H = 7.62 (1H, d, *J* = 9.4 Hz, H-4), 6.85 (d, *J* = 5.4 Hz, H-5 and H-8), 6.29 (1H, d, *J* = 9.4 Hz, H-3), 3.95 (3H, s, 6-OMe), 3.92 (3H, s, 7-OMe); ^{13}C-NMR (176 MHz, $CDCl_3$) δ_C = 161.57 (C-2), 152.99 (C-7), 150.19 (C-9), 146.49 (C-6), 143.44 (C-4), 113.72 (C-3), 111.58 (C-10), 108.09 (C-5), 100.17 (C-8), 56.53 (OMe-6), 56.50 (OMe-7); *m/z* 207.0598 [M + H^+] + 229.0480 [M + Na^+] (calcd. for $C_{11}H_{10}O_4$). Data compared to that of Ma et al. [57].

Supporting Information

NMR and MS data of the compounds are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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