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Triterpenoids isolated from *Jatropha macrantha* (Müll. Arg.) inhibit the NF-κB and HIF-1α pathways in tumour cells

Triterpenoids isolated from *Jatropha macrantha* (Müll. Arg.) inhibit the NF- κ B and HIF-1 α pathways in tumour cells

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ABSTRACT

Activity-guided fractionations of *Jatropha macrantha* Müll. Arg. led to the isolation of pomolic acid (**1**) and euscaphic acid (**2**). The potential for inhibition against NF- κ B and HIF-1 α production of these two compounds was tested in different tumour cell lines. Compounds **1** and **2** showed an inhibitory activity of HIF-1 α in the SK-MEL-28 (IC₅₀=3.01 $\pm$ 0.02 μ M and 3.78 $\pm$ 0.02 μ M), A549 (IC₅₀=9.97 $\pm$ 0.01 μ M and 10.25 $\pm$ 0.01 μ M) and U-373 MG (IC₅₀=6.34 $\pm$ 0.02 μ M and 8.85 $\pm$ 0.02 μ M) cell lines. In addition, compounds **1** and **2** showed an inhibitory activity on NF- κ B in SK-MEL-28 (IC₅₀=1.05 $\pm$ 0.02 μ M and 2.71 $\pm$ 0.01 μ M), A549 (IC₅₀=3.63 $\pm$ 0.01 μ M and 3.73 $\pm$ 0.02 μ M) and U-373 MG (IC₅₀=2.55 $\pm$ 0.02 μ M and 3.39 $\pm$ 0.01 μ M) cell lines. This is the first report that isolates these compounds from *J. macrantha* and tests their antitumor potential.

Keywords:

Euphorbiaceae, *Jatropha macrantha*, NF- κ B, HIF-1 α , Sterols, Triterpenoids

1. Introduction

Cancer development is a multifactorial process, resulting from mutations of cells that tend to increase in size, as they lack ions, glucose and nutrients, in addition to having inadequate oxygenation mediated by HIF (Wu *et al.*, 2018). HIF is a heterodimer complex, consisting of two subunits: HIF-1 α and HIF-1 β , the former being constitutive and extremely sensitive to changes in oxygen levels (Bandarra & Rocha, 2013). On the other hand, NF- κ B is a family of transcription factors that regulate immune, inflammatory and oncogenic responses (Hoesel & Schmid, 2013). Finally, the NF- κ B and HIF-1 α transcription factors are intimately connected by sharing many stimuli capable of activating and attacking genes, so they both cooperate to create the necessary conditions for the tumour to enlarge and become aggressive (Bandarra & Rocha, 2013).

Jatropha (Euphorbiaceae) is a genus of approximately 199 species. Its extracts are obtained from different parts such as leaves, stem, bark and roots, and have been used in the ethnomedicine of different cultures (Duke, 1985). Several compounds with antitumor activity have been isolated from these species, e.g., *J. isabellei* (Fröhlich *et al.*, 2013); *J. podagrica* (Ghali *et al.*, 2013); *J. multifida* (Das *et al.*, 2009); *J. neopauciflora* (García & Delgado, 2006) and *J. curcas* (Juan *et al.*, 2003). *Jatropha macrantha* Müll. Arg. also known as "Huanarpo Macho", is employed in the traditional medicine of the Peruvian region as a blood depurative and antidiabetic, in the treatment of respiratory diseases and as a stimulator of the nervous system (Condori *et al.*, 2018). It is also used in poultice form in traditional medicine, for the treatment of skin ulcers (Sabandar *et al.*, 2013).

Our study started by accounting for how other plants of this genus have shown promising potential for anti-tumour compounds, e.g., curcin isolated from *J. curcas* showed an IC₅₀ of 0.19 nmol/l in the SGC-790 tumour cell line; diterpenoids curcusone C, curcusone D, multidione, 15-epi-4Z-jatrogrossidentadion, 4E-jatrossidentadion, 4Z-jatrossidentadion, 2-hydroxyjatrogrossidion and 2-epi-hydroxyisojatrogrossidion isolated from *J. curcas* showed a strong action against the HeLa tumour cell line; calenduladiol and (3 β ,16 β)-16-hydroxylup-20-(29)-en-3-yl(E)-3-(4-hydroxyphenyl) prop-2-enoate isolated from *J. neopauciflora* exhibited activity potential against the K562 and U251 tumour cell lines (Cavalcante *et al.*, 2020). Thus, our study aims to identify and characterise the compound(s) responsible for inhibiting the production of HIF-1 α and NF- κ B in extracts of *J. macrantha* by chromatographic methods and spectrophotometric analysis.

2. Results and discussion

Pomolic acid (1) and euscaphic acid (2) (Figure 1) were isolated from the CH₂Cl₂ extract prepared from *J. macrantha* which was repeatedly subjected to several column chromatography. Both compounds are reported for the first time in the *J. macrantha* species.

Figure 1.

The extracts obtained from *J. macrantha* did not show cytotoxic effects in hypoxic conditions at 72 h of treatment in the SK-MEL-28, A549 and U-373 MG cells by means of the XTT assay. In addition, the pomolic acid (1) and euscaphic acid (2) compounds showed a cytotoxic effect against the SK-MEL-28, A549 and U-373 MG cancer cell lines (Table S1).

The inhibitory activity of the extracts and isolated compounds over NF- κ B under hypoxic conditions on different cancer cell lines at 72 h of treatment is reported in Table S2, and their effect was compared with that of the positive control, JSH-23 (IC₅₀=7.1 μ M). The CH₂Cl₂ extract revealed a greater inhibitory effect on NF- κ B compared to the *n*-hexane, MeOH and aqueous extracts.

Pomolic acid (1) inhibited the production of NF- κ B on the SK-MEL-28, A549 and U-373 MG cancer cell lines with IC₅₀ of 1.05 $\pm$ 0.02 μ M, 3.63 $\pm$ 0.01 μ M and 2.55 $\pm$ 0.02 μ M. Our data corroborates the antitumor activity of pomolic acid described by other authors on breast cancer cell lines (MCF7, MDA-MB-231) with an IC₅₀ of 25 μ M and 10 μ M, respectively (Kim *et al.*, 2016). Euscaphic acid (2) inhibited the production of NF- κ B with IC₅₀ of 2.71 $\pm$ 0.01 μ M, 3.73 $\pm$ 0.02 μ M and 3.39 $\pm$ 0.01 μ M on SK-MEL-28, A549 and U-373 MG cancer cells, respectively. The results show that euscaphic acid has NF- κ B dose-dependent inhibition activity in all the tested tumoral cell lines, inhibiting IKK α / β phosphorylation and I κ B α phosphorylation, which subsequently causes the blockage of NF- κ B p65 phosphorylation and nuclear translocation (Ouyang *et al.*, 2019; Kim *et al.*, 2012). Likewise, Dai *et al.* (2019), reported that euscaphic acid reduced cell proliferation and induced apoptosis and cell cycle arrest in Nasopharyngeal carcinoma cells by suppressing the PI3K/AKT/mTOR signalling pathway.

Analysing both mechanisms, we can indicate that there is a relationship between the NF- κ B and PI3K/Akt/mTOR signalling pathways. In this sense, mTOR is known to regulate Akt via a feedback mechanism. The regulation of NF- κ B by mTOR happens

through Akt regulation and by means of the mTOR-associated protein raptor. Likewise, this can occur due to a more direct regulation of NF- κ B by mTOR which involves IKK. In the activation of NF- κ B, IKK plays an important role by phosphorylating the inhibition of I κ B α leading to its dissociation from NF- κ B, and resulting in an activated NF- κ B that translocate to the nucleus. Thus, the mTOR mediated induction of IKK leads to the activation of NF- κ B (Ahmad *et al.*, 2013).

The inhibitory activity of the extracts and compounds over HIF-1 α under hypoxic conditions on different cancer cell lines at 72 h of treatment is reported in Table S3 and compared to the positive control, 2-MeOE2 (IC₅₀=0.5 μ M). The CH₂Cl₂ extract revealed a greater inhibitory effect on HIF-1 α compared to n-hexane, MeOH and aqueous extracts.

Pomolic acid (**1**) inhibited HIF-1 α with IC₅₀ of 3.01 $\pm$ 0.02 μ M, 9.97 $\pm$ 0.01 μ M and 6.34 $\pm$ 0.02 μ M on the SK-MEL-28, A549 and U-373 MG cells, respectively. We can compare these results to those obtained by Park *et al.* reporting an IC₅₀ of 10 μ M on the control cell lines (HUVEC) and the MCF-7 and MDA-MB-231 tumoral cell lines in normoxic conditions (Park *et al.*, 2016). Euscaphic acid (**2**) inhibited HIF-1 α with IC₅₀ of 3.78 $\pm$ 0.02 μ M, 10.25 $\pm$ 0.01 μ M and 8.85 $\pm$ 0.02 μ M on the SK-MEL-28, A549 and U-373 MG cells, respectively.

This is the first report on the inhibitory activity of euscaphic acid against the HIF-1 α transcription factor. Previous research showcased triterpenoids preventing HIF-1 α translocation in the nucleus, where they are heterodimised with HIF-1 β to form HIF. This together with the p300/CBP co-activators form a transcription complex that binds to target genes and activates the cascade of pro-angiogenic, prooncogenic and inflammatory events (Bahadori *et al.*, 2019). Since the isolated compounds are also terpenoids, we can reasonably assume that their mechanism of action is like that described by Bahadori *et al.*

Our results regarding the response of extracts and compounds (pomolic and euscaphic acids) isolated of *J. macrantha* on the HIF-1 α and NF- κ B targets confirm that there are positive correlations between these targets and that both are involved in cancer progression and tumour responses in hypoxic conditions. Since the HIF-1 α and NF- κ B regulatory pathways are 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.

Supporting Information

Supplementary data (NMR and MS of compounds) associated with this article can be found in the online version.

Disclosure statement

The authors declare no conflict of interest.

Acknowledgments

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Author contribution

LAT and ARS contributed to the analysis of the spectral data; LAT and GP contributed to the conception and experimental design of the pharmacological study and LAT and FA contributed to the writing and review of the manuscript.

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Legends of Figures

Figure 1. Absolute configuration of pomolic acid (1) and euscaphic acid (2)

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ABSTRACT

Activity-guided fractionations of *Jatropha macrantha* Müll. Arg. led to the isolation of pomolic acid (**1**) and euscaphic acid (**2**). The potential for inhibition against NF- κ B and HIF-1 α production of these two compounds was tested in different tumour cell lines. Compounds **1** and **2** showed an inhibitory activity of HIF-1 α in the SK-MEL-28 (IC₅₀=3.01 $\pm$ 0.02 μ M and 3.78 $\pm$ 0.02 μ M), A549 (IC₅₀=9.97 $\pm$ 0.01 μ M and 10.25 $\pm$ 0.01 μ M) and U-373 MG (IC₅₀=6.34 $\pm$ 0.02 μ M and 8.85 $\pm$ 0.02 μ M) cell lines. In addition, compounds **1** and **2** showed an inhibitory activity on NF- κ B in SK-MEL-28 (IC₅₀=1.05 $\pm$ 0.02 μ M and 2.71 $\pm$ 0.01 μ M), A549 (IC₅₀=3.63 $\pm$ 0.01 μ M and 3.73 $\pm$ 0.02 μ M) and U-373 MG (IC₅₀=2.55 $\pm$ 0.02 μ M and 3.39 $\pm$ 0.01 μ M) cell lines. This is the first report that isolates these compounds from *J. macrantha* and tests their antitumor potential.

Keywords:

Euphorbiaceae, *Jatropha macrantha*, NF- κ B, HIF-1 α , Sterols, Triterpenoids.

Abbreviations

2-MeOE2	2-Methoxyestradiol
ATCC	American Type Culture Collection
DTT	Dithiothreitol
DMEM	Dulbecco's Modified Eagle Medium
EPO	Erythropoietin
FBS	Fetal Bovine Serum
HRE	Hypoxia Response Element
HIF	Hypoxia-inducible factor
JSH-23	4-methyl-1-N-(3-phenylpropyl)-1,2-benzenediamine
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMCs	Peripheral blood mononuclear cell
PMS	Phenazine methosulfate
PBS	Phosphate-Buffered Saline
RLU	Relative light units
RPMI	Roswell Park Memorial Institute
TNF-α	Tumour necrosis factor-α

Experimental

Plant material

Jatropha macrantha was collected from in Paucarpata municipality, in the Arequipa province of Arequipa department, Peru (16°25'46" S 71°30'08" W), in September 2015, at an altitude of 2410 m. Botanical identification was confirmed by the National Herbal of Medicinal Plants, National Institute of Health, CENSI, and a voucher specimen was deposited (HINS 02334).

General experimental procedures

First grade organic solvents were used for isolating the compounds and they were purchased from Sigma-Aldrich. TLC was performed using Merck Silica gel 60-F₂₅₄ plates. Chromatograms thus obtained were visualised by UV absorbance (254 nm) and through heating a plate stained with phosphomolybdic acid. Column chromatography was performed with silica gel (20-45 μm and 40-63 μm, Merck). NMR experiments were performed on the Bruker Advance DRX 300, 500 and 600 spectrometers operating at 300 MHz, 500 MHz, 600 MHz (¹H) or 126 MHz, and 151 MHz (¹³C). The deuterated solvents were CDCl₃-d₁, MeOD-

d_4 and DMSO- d_6 . Spectrums were calibrated by assignment of the residual solvent peak to δ_H 7.26, δ_H 3.31 and δ_H 2.5, and δ_C 77.16, δ_C 49.00 and δ_C 39.52, for $CDCl_3$, MeOD and DMSO, respectively. The complete assignment of protons and carbons was done by analysing the correlated 1H - 1H COSY, HSQC and HMBC spectrums. Mass spectrums were recorded by the VG AutoSpec equipment from Waters, with magnetic sector analyser. The FAB ionisation technique used L-SIMS with cesium cation bombardment as an energy source, and the *m*-nitrobenzyl alcohol as a matrix.

Preparation of extracts and isolation of compounds

After air-drying and powdering *J. macrantha* (200 g), the result was repeatedly extracted at room temperature with *n*-hexane (13.60 g), CH_2Cl_2 (2.36 g) and MeOH (44.36 g), as well as from an aqueous extract (30.70 g). Each extract was evaluated for its cytotoxic effects and HIF-1 α /NF- κ B production on the SK-MEL-28, A549 and U-373 MG cells and the nontumorigenic PBMCs cell line. All extracts did show viability in the non-cancerous cell line (PBMCs). With respect to the activity assays, the dichloromethane and *n*-hexane extracts of *J. macrantha* showed a reduction in the production of HIF-1 α /NF- κ B. However, the methanol and aqueous extracts did not show activity in terms of reduction in the production of HIF-1 α /NF- κ B. Therefore, the dichloromethane extract was fractionated to identify the active compounds.

The bioactive CH_2Cl_2 extract (2 g) was analysed through bioassay-guided Silica gel (40-63 μ m) column chromatography (2x50 cm), using a step-wise gradient of *n*-hexane/ CH_2Cl_2 (1:1 to 3:0.5 v:v), obtaining four fractions (1-4), where **fractions 1** (43.7 mg) and **4** (21 mg) showed greater activity (inhibition of the activity of HIF-1 α and NF- κ B). **Fraction 1** was a pure compound—**compound 1**, while **fraction 4** needed a new separation through a Silica gel column chromatography (2x50 cm) (20-45 μ m) (*n*-hexane/ethyl acetate, 6:0.5), obtaining five fractions (4A-4E) that showed inhibition over HIF-1 α and NF- κ B, with the **fraction 4D—compound 2** (3.1 mg) being the most active.

Cell culture

Three human cancer cell lines were used in this study: Human skin melanoma (SK-MEL-28, ATCC® HTB-72), Human lung carcinoma (A549, ATCC® CRM-CCL-185™), Human brain astrocytoma (U-373 MG, ATCC® HTB-17) cancer cell lines were selected to carry out the assays on the extracts and compounds from *Jatropha macrantha*. We have selected these cancer cell lines according to their physiological link with particularly relevant diseases against which *Jatropha macrantha* is used in traditional medicine (Condori *et al.*, 2018;

Pardo, 2002; Desmarchelier *et al.*, 1996). In this sense, the SK-MEL-28 cell lines were selected due to *J. macrantha* traditional use in the treatment of skin ulcers, the A549 cell line was selected because of *J. macrantha* use as a cough suppressant and anti-asthmatic, and the U-373 MG cell line was selected in relation to *J. macrantha* use as a cerebral tonic. In addition, PMBCs was used to assess the safety of the compounds. PBMCs from healthy donors were isolated from the blood by Ficoll (Rafer) density gradient centrifugation in Leucosep tubes (Greiner Bio-One) following the manufacturer's recommendations. All experiments with human PBMC were carried out in accordance with the Declaration of Helsinki, and all investigations were approved by the ethics committee of the Hospital Universitario La Paz (No. 913/2018). All cell lines were obtained from the 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% O₂), thus mimicking the *in vivo* tumour microenvironment.

Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, USA) supplemented with L-glutamine (PanReac AppliChem, Barcelona, Spain), 10% FBS (Summit Biotechnology; Ft. Collins, CO), 100 U/mL penicillin and 100 µg/mL streptomycin (Fisher Scientific, Pittsburgh, USA). 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.

Viability assay

Inhibition of H₂O₂-induced viability by the extracts at various concentrations was tested by the method of XTT-formazan dye formation (Weislow *et al.*, 1989), using the above mentioned cell lines. These cells were sown (200 µL, 2x10⁴ cells/well) in a 96-well plate and allowed to grow at 37°C. After 72 h, medium was removed from all wells. 200 µL fresh medium (DMEM medium supplemented with L-glutamine, 5% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin) was added to the control wells. Cells in each test well were treated with 0.1 mM H₂O₂ (prepared in medium) along with extracts or compounds in different concentrations (100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.12 µM, 1.56 µM, 0.78 µM, 0.39 µM, 0.95 µM). Concerning viability, the results were compared with those of Docetaxel as positive control (IC₅₀=5.7 nM). For this assay, Docetaxel was chosen. It is a chemotherapy medication used to treat various types of cancer. The concentration used in the assay was based on previous work (Florento *et al.*, 2012). Cells in both control and test wells were re-incubated for 8 h maintaining the same conditions. After the treatment incubation

period, medium in each well was substituted by 200 μ L of fresh medium, followed by the addition of 50 μ L of XTT (0.6 mg/ml) containing 25 μ M PMS. The plate was further incubated for 4 h in the same conditions. Absorbance was measured at 450 nm (with a 630 nm reference filter) in a spectrophotometric ELISA plate reader (SpectraMax® i3, Molecular Devices, CA, USA).

NF- κ B inhibition assay

Cells were transfected using a lipofectamine plus transfection reagent (Thermo Fisher Scientific) with 0.3 μ g of the NF- κ B-promoted luciferase reporter gene plasmid (pGL2-NF- κ B-Luc) (Promega), and 0.03 μ g 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 extracts or the isolated compounds at different concentrations (100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.12 μ M, 1.56 μ M, 0.78 μ M, 0.39 μ M, 0.95 μ M) for 72 h of treatment in hypoxic conditions (1% O₂). With respect to the inhibition of NF- κ B, the results were compared to that of JSH-23, used as positive control (IC₅₀=7.1 μ M). It is an inhibitor of NF- κ B nuclear translocation. The concentration used in the assay was based on previous work (Kumar *et al.*, 2011). Subsequently, the cells were lysed in 25 mM Tris-phosphate, at pH 7.8, containing 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100 and 7% glycerol. The luciferase activity was measured by Dual-Luciferase Reporter Assay Kit (Promega), according to the manufacturer's instructions. Luminescence was measured immediately in triplicates using a GloMax 96 Microplate Luminometer (Promega). The experiments for each concentration of the samples were performed using 3 replicates of each well in three different experiments.

HIF-1 α inhibition assay

Cells have been stably transfected with EPO-Luc plasmid. EPO-HRE-luciferase plasmid reporter contained three copies of the HRE consensus sequence from the promoter of the erythropoietin gene in the pGL3 vector. Cells (1x10⁴) were seeded the day before the assay. The next day, the cells were stimulated with the extracts and the isolated compounds at different concentrations (100 μ M, 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M, 3.12 μ M, 1.56 μ M, 0.78 μ M, 0.39 μ M, 0.95 μ M). With respect to the inhibition of HIF-1 α , results were compared with those of the positive control, 2-MeOE2 (IC₅₀=0.5 μ M), an inhibitor of angiogenesis, microtubule disruption and upregulation of the extrinsic and intrinsic apoptotic pathway. The concentration used in the assay was based on previous work (Zhao *et al.*, 2017). After 72 h of stimulation in hypoxic conditions (1% O₂), the cells were lysed in 25

mM Tris-phosphate, at pH 7.8, 8 mM MgCl₂, 1 mM DTT, 1% Triton X-100 and 7% glycerol during 15 min at room temperature in a horizontal shaker. The luciferase activity was measured using a GloMax 96 microplate luminometer (Promega) following the instructions of the luciferase assay kit (Promega). The RLU (Relative light units) was then calculated and the results were expressed as percentage of inhibition induction/inhibition of EPO-luc activity. Luminescence was measured immediately in triplicates using a GloMax 96 Microplate Luminometer (Promega). The experiments for each concentration of the samples were performed using 3 replicates of each well in three different experiments.

Statistical analysis

The statistical significance of differences was calculated employing the GraphPad Prism software, version 8.2.1 (GraphPad Software Inc., San Diego, CA, USA), using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. Results were considered significant when $p < 0.01$. IC₅₀ values were determined by non-linear regression using GraphPad Prism, version 8.2.1. All the experiments were performed in triplicate.

Spectroscopic data

Pomolic acid (**1**): Colourless crystalline powder; m.p.=299°C; $[\alpha]^{20}_D +36.7$ (c 2.0, THF); ¹H-NMR (500 MHz, DMSO) δ_H =5.13 (1H, s, H-12), 2.99 (1H, m, H-3), 2.43 (1H, m, H-16 β), 2.37 (1H, m, H-18), 1.88 (1H, m, H-11 β), 1.83 (1H, m, H-11 α), 1.69 (1H, m, H-15 α), 1.68 (1H, m, H-2 β), 1.60 (1H, m, H-21 α), 1.58 (1H, m, H-22 β), 1.56 (1H, m, H-9), 1.52 (1H, m, H-1 β), 1.50 (1H, m, H-22 α), 1.49 (1H, m, H-1 α), 1.47 (1H, m, H-6 β), 1.45 (1H, m, H-7 β), 1.38 (1H, m, H-16 α), 1.31 (1H, m, H-6 α), 1.28 (3H, s, Me-27), 1.26 (1H, m, H-20), 1.22 (1H, m, H-7 α), 1.12 (1H, m, H-21 β), 1.06 (3H, s, Me-29), 0.93 (3H, s, Me-23), 0.90 (1H, m, H-15 β), 0.88 (3H, s, Me-25), 0.83 (3H, s, Me-30), 0.71 (3H, s, Me-26), 0.69 (3H, s, Me-24), 0.67 (1H, m, H-5); ¹³C-NMR (126 MHz, DMSO) δ_C =179.55 (C-28), 138.91 (C-13), 126.85 (C-12), 71.85 (C-19), 77.15 (C-3), 55.05 (C-5), 53.44 (C-18), 47.10 (C-17), 46.90 (C-9), 41.59 (C-20), 41.24 (C-14), 38.54 (C-4), 38.33 (C-10), 37.50 (C-1), 36.72 (C-22), 32.88 (C-7), 28.40 (C-23), 28.30 (C-2), 27.09 (C-15), 26.61 (C-29), 26.15 (C-21), 25.43 (C-16), 24.09 (C-27), 23.30 (C-11), 18.28 (C-6), 16.83 (C-26), 16.48 (C-30), 16.18 (C-24), 15.25 (C-25); HRESIMS m/z 472.3539 [M+H]⁺ (calcd for C₃₀H₄₈O₄). Data was compared to the reference from Zhao *et al.*, 2019.

Euscaphic acid (**2**): Colourless crystalline powder; m.p.=273°C; $[\alpha]^{23}_D +12.2$ (c 1.0, MeOH); ¹H-NMR (600 MHz, DMSO) δ_H =5.17 (1H, brs, H-12), 3.75 (1H, dt, H-2 β),

3.15 (1H, d, H-3 β), 2.47 (1H, ddd, H-16), 2.37 (1H, s, H-18), 1.29 (3H, s, Me-27), 1.08 (3H, s, Me-29), 0.88 (3H, s, Me-23), 0.88 (3H, s, Me-25), 0.84 (3H, d, Me-30), 0.78 (3H, s, Me-26), 0.69 (3H, s, Me-24); ^{13}C -NMR (151 MHz, DMSO) δ_{C} =179.01 (C-28), 138.70 (C-13), 126.79 (C-12), 77.94 (C-3), 71.67 (C-19), 64.72 (C-2), 53.21 (C-18), 47.68 (C-5), 46.91 (C-17), 46.57 (C-9), 41.61 (C-20), 41.43 (C-14), 41.20 (C-1), 38.01 (C-8), 37.83 (C-10), 37.31 (C-4), 32.66 (C-22), 28.91 (C-7), 28.03 (C-15), 26.45 (C-23), 25.97 (C-16), 25.20 (C-29), 24.10 (C-21), 23.19 (C-27), 21.87 (C-11), 17.75 (C-6), 16.68 (C-26), 16.32 (C-24), 16.32 (C-25), 16.14 (C-30); HRESIMS m/z 488.3428 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{30}\text{H}_{48}\text{O}_5$). Data was compared to the reference from Woo *et al.*, 2014.

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- Table S2. Inhibitory effect of *J. macrantha* extracts and compounds 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% O₂) conditions. The results are the means (±SD) of three separate experiments performed in triplicate. JSH-23 was used as positive control.
- Table S3. Inhibitory effect of *J. macrantha* extracts and compounds on HIF-1α in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic conditions. The results are the means (±SD) of three separate experiments performed in triplicate. 2-MeOE2 was used as positive control.

-Figure S1. ¹H-NMR spectrum of n-hexane extract of *J. macrantha* in CDCl₃ 300 MHz

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-Figure S4. ¹H-NMR spectrum of aqueous extract of *J. macrantha* in D₂O 300 MHz

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-Figure S8. ^1H - ^1H COSY spectrum of JMEDCM_F1 in DMSO 500 MHz.

-Figure S9. ^1H - ^1H COSY spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 1).

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-Figure S14. HMBC spectrum of JMEDCM_F1 in DMSO 500 MHz (Extension 1).

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Elemental Composition Report

Multiple Mass Analysis: 122 mass(es) processed – displaying only valid results

Tolerance = 10.0 PPM / DBE: min = - 1.5, max = 50.0

Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions
1270 formula(e) evaluated with 43 results within limits (all results (up to 1000) for each mass)

Element Used:

C: 5-30 H: 7-70 O: 1-6

JMEDCM_F1

18-Oct19

-Figure S15. HRMS spectrum of JMEDCM_F1.

-Figure S18. DEPT-135 spectrum of JMEDCM_F4D in DMSO 151 MHz.

-Figure S19. ^1H - ^1H COSY spectrum of JMEDCM_F4D in DMSO 600 MHz.

-Figure S20. ^1H - ^1H COSY spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).

-Figure S21. HSQC spectrum of JMEDCM_F4D in DMSO 600 MHz.

-Figure S22. HSQC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).

-Figure S23. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz.

-Figure S24. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 1).

-Figure S25. HMBC spectrum of JMEDCM_F4D in DMSO 600 MHz (Extension 2).

Elemental Composition Report**Multiple Mass Analysis: 354 mass(es) processed – displaying only valid results**

Tolerance = 10.0 PPM / DBE: min = - 1.5, max = 50.0

Selected filters: None

Monoisotopic Mass, Odd and Even Electron Ions

4057 formula(e) evaluated with 80 results within limits (all results (up to 1000) for each mass)

Element Used:

C: 5-30 H: 5-48 O: 0-5

JMEDCM_F4D

18-Oct19

-Figure S26. HRMS spectrum of JMEDCM_F4D.

- Table S1. XTT viability assay of *J. macrantha* extracts and compounds against a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic (1% O₂) conditions. Data presents the means ($\pm$ SD) of three separate experiments performed in triplicate. Docetaxel was used as positive control.

Samples	CC ₅₀			
	PBMCs	SK-MEL-28	A549	U-373 MG
<i>n</i> -Hexane extract ^(a)	97.09 $\pm$ 1.82	29.36 $\pm$ 1.27	51.97 $\pm$ 1.97	48.03 $\pm$ 1.77
CH ₂ Cl ₂ extract ^(a)	>100 $\pm$ 1.00	11.89 $\pm$ 1.04	36.88 $\pm$ 1.05	29.95 $\pm$ 1.09
MeOH extract ^(a)	>100 $\pm$ 1.61	9.05 $\pm$ 1.13	21.76 $\pm$ 1.03	12.05 $\pm$ 1.02
Aqueous extract ^(a)	>100 $\pm$ 1.45	88.94 $\pm$ 1.72	>100 $\pm$ 1.17	91.54 $\pm$ 1.11
Compound 1 ^(b)	61.23 $\pm$ 1.07	5.01 $\pm$ 0.02	20.80 $\pm$ 1.13	12.70 $\pm$ 1.10
Compound 2 ^(b)	73.00 $\pm$ 1.00	8.79 $\pm$ 0.03	26.73 $\pm$ 1.14	17.31 $\pm$ 1.15
Docetaxel ^(c)	52.94 $\pm$ 1.01	3.95 $\pm$ 1.02	5.67 $\pm$ 1.21	4.48 $\pm$ 1.25

(a)=CC₅₀ in μ g/mL; (b)=CC₅₀ in μ M; (c)=CC₅₀ in nM

- Table S2. Inhibitory effect of *J. macrantha* extracts and compounds 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% O₂) conditions. The results are the means (±SD) of three separate experiments performed in triplicate. JSH-23 was used as positive control.

Samples	IC ₅₀			
	PBMCs	SK-MEL-28	A549	U-373 MG
Control	11.99±0.01	12.14±0.07	12.13±0.01	12.01±0.05
DMSO	10.70±0.03	10.67±0.01	10.76±0.02	10.71±0.03
<i>n</i> -Hexane extract ^(a)	5.19±0.01	5.03±0.01	5.99±0.01	5.67±0.02
CH ₂ Cl ₂ extract ^(a)	4.46±0.02	2.78±0.04	4.39±0.02	4.55±0.01
MeOH extract ^(a)	8.85±0.01	8.65±0.03	8.87±0.01	8.80±0.02
Aqueous extract ^(a)	11.67±0.03	10.09±0.03	12.07±0.02	11.77±0.03
Compound 1 ^(b)	2.70±0.01	1.05±0.02	3.63±0.01	2.55±0.02
Compound 2 ^(b)	3.17±0.01	2.71±0.01	3.73±0.02	3.39±0.01
JSH-23 ^(b)	7.12±0.03	7.11±0.01	7.14±0.03	7.13±0.01

(a)=IC₅₀ in μg/mL; (b)=IC₅₀ in μM

- Table S3. Inhibitory effect of *J. macrantha* extracts and compounds on HIF-1α in a panel of human cancer cell lines and one noncancerous cell line, after 72 h of treatment under hypoxic conditions. The results are the means (±SD) of three separate experiments performed in triplicate. 2-MeOE2 was used as positive control.

Samples	IC ₅₀			
	PBMCs	SK-MEL-28	A549	U-373 MG
Control	96.10±0.04	94.47±0.02	97.45±0.01	96.506±0.01
DMSO	93.10±0.03	90.83±0.02	95.12±0.03	93.62±0.02
<i>n</i> -Hexane extract ^(a)	9.05±0.03	7.72±0.03	17.07±0.01	11.30±0.02
CH ₂ Cl ₂ extract ^(a)	6.19±0.01	5.83±0.01	13.14±0.02	9.17±0.01
MeOH extract ^(a)	88.01±0.01	79.00±0.01	87.02±0.02	85.01±0.01
Aqueous extract ^(a)	90.05±0.01	87.12±0.01	92.00±0.01	90.13±0.02
Compound 1 ^(b)	47.88±0.02	3.01±0.02	9.97±0.01	6.34±0.02
Compound 2 ^(b)	60.02±0.02	3.78±0.02	10.25±0.01	8.85±0.02
2-MeOE2 ^(b)	0.45±0.01	0.51±0.02	0.49±0.02	0.47±0.03

(a)=IC₅₀ in μg/mL; (b)=IC₅₀ in μM

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