SUPPLEMETARY MATERIAL

Biological activities of the aerial and undergound parts of *Gymnadenia nigra* Rchb.f. (syn. *Nigritella nigra* (L.) Rchb. f.) from the Italian Alps

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This study investigated the bioactivity of both aerial (GNAR) and underground (GNUG) parts of *Gymnadenia nigra* Rchb.f. (syn. *Nigritella nigra* (L.) Rchb. f.) (Orchidaceae). The obtained data proved interesting when the samples were tested in two adrenocortical cancer cell lines (SW13 and H295R). In particular, the GNAR 80% methanol extract distinctly inhibited their viability after 24 h at a concentration of 1 μg/μL by MTT assay and trypan blue dye exclusion method. Cell morphology evaluation by means Wright's staining also showed significant results, particularly in SW13 cells under the effect of both extracts. GNAR extract was able to scavenge the DPPH radical better than GNUG extract. It also was more active in albumin denaturation (a maximum % denaturation equal to 463.0±8.3 vs 77.3±13.3) and protease inhibition (a maximum % inhibition equal to 138.5±7.0 vs 2.1±2.0) tests. The results highlighted an important antitumor activity of *G. nigra in vitro* that deserves to be further studied.

Keywords: black vanilla orchid; antioxidant activity; anti-inflammatory activity; anti-proliferative activity.

Experimental

Materials and reagents

Fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), propidium iodide were purchased from Sigma Aldrich, Italy. DMEM-F12, 0.05% trypsin-EDTA, insulin, transferrin, selenium and antibiotics were from Life Technologies, Italy. Primary antibodies were: Akt (cod. 9272), phospho-Akt (Ser473) (cod. 9271), Erk1/2 (cod. 4695), phospho-Erk1/2 (Thr202/Tyr204) (cod. 4370) all from Cell Signaling Technology; Caspase 3 (cod. 110543) and 9 (cod. 112888) from GeneTex; anti-β-Actin antibody (cod. A5441) from Sigma-Aldrich. Pyridine and bis-(trimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich. Secondary antibodies were horseradish peroxidase-labeled goat anti-mouse or anti-rabbit (Jackson ImmunoResearch).

Plant material

G. nigra was collected on the south-facing slope of Passo del Tonale (Brescia, Italy), at an altitude above 2000 meters (46°28'22" N, 10°61'74" E) during the summer season 2021. The species was determined by the authors (Raffaele Pezzani and Sara Vitalini) according to Flora d'Italia (Pignatti et al. 2017) and a voucher sample (No. 011N) was deposited in the herbarium of the Department of Pharmaceutical Sciences of the University of Padova, Italy. The aerial (flowers) and underground (bulb and roots) parts were separated, then dried in the shade and stored until processing. Before use, they were powdered using an electric blender.

Extraction procedure

The powders of both plant parts were extracted with 80% methanol (1:4, w/v) by placing them on a shaker for 24 hours The mixture was then filtered using Whatman No. 1 filter paper to eliminate plant residues (Rubin et al. 2019; Patti et al. 2020). The solvent was removed under reduced pressure with a controlled temperature vacuum rotary evaporator (Rotavapor R-210, Flawil, Switzerland). DMSO (1:1, w/v) was added to the two obtained extracts (GNAR, *Gymnadenia nigra* aerial parts and GNUG, *Gymnadenia nigra* underground parts) to facilitate their solubility and to use them in all experiments, unless otherwise indicated.

DPPH radical scavenging assay

To measure antioxidant activity, the 1,1-diphenyl-2-picrylidrazyl (DPPH) radical scavenging test was performed according to a previously described procedure, with minor modifications (Zhang et al. 2018). Briefly, the scavenger potential of the GNAR and GNUG samples towards the stable radical DPPH was measured in a reaction mixture containing 0.9 ml of a 1 mM DPPH solution and 0.1 mL of different concentrations (20, 50, 100, 200, 400, 500, 800, 1000, 10000 µg/mL) of both extracts. After 30 min of incubation in the dark at room temperature, the absorbance was read at 517 nm. Methanol and DPPH solution were used as blank and control, respectively, while ascorbic acid was used a reference. Experiment was performed in quintuplicate and repeated 3 times.

In vitro anti-inflammatory activity

Both extracts were evaluated following the previous work of Khan and collaborators (2015) on albumin denaturation assay. Briefly, the albumin was added to the different concentrations of the extracts in a phosphate buffered saline at pH 6.4, then all incubated at 37±2 °C for 15 min and heated at 70 °C for 5 min. The mixture was cooled and the absorbance read at 660 nm. Acetylsalcylic acid was used as a standard. The % inhibition of protein denaturation was calculated with the formula: Protein denaturation (%) = (Control Absorbance - Test Absorbance)/(Control Absorbance).
Moreover, proteinase inhibitory assay was performed following previous published method (Aziz 2015). Briefly, trypsin in 0.025 M tris-HCl buffer was added to the different concentrations of the extracts and then incubated for 5 min at 37 °C. After that, 0.8% casein was added and incubated for 20 additional minutes. The 70% perchloric acid has been used to stop the reaction. Acetylsalcylic acid was used as a standard. The absorbance was measured at 280 nm and the percentage of proteinase inhibition was calculated with the formula: Proteinase inhibition (%) = [1 – Extract Absorbance/Control Absorbance] x 100. Each experiment was performed in triplicate and repeated 3 times.

Brine shrimp lethality bioassay (BSLB)

The assay was performed with GNAR and GNUG extracts at 10, 2, 1, 0.5, 0.25, 0.125, 0.062 μg/μL as previously described (Patti et al. 2020). Briefly, brine shrimps (*Artemia salina*) were hatched in a round shaped vessel with sterile artificial seawater and continuous oxygen supply, then added to wells containing or not GNAR and GNUG samples. Afterward, dead larvae were counted. The mean percentage mortality was plotted against the logrithm of concentrations and the LC_{50} (concentration that kills fifty percent of the naplii) was determined using the probit analysis described by Finney (1949) as well as linear regression equation using the software "Microsoft Excel 2010". Plant extracts were considered toxic when mortality rates were >50% (Mitsias et al. 2011). The experiments were performed in triplicates and repeated 3 times.

Cell cultures and maintenance

Adrenocortical cells (H295R and SW13 cells) were obtained from the American Type Culture Collection (ATCC, Rockwille, MD). The cells were cultured in the supplemented Dulbecco's Modified Eagle's Medium (DMEM)-F12 medium containing insulin (5 μg/mL), transferrin (10 μg/mL), selenium (20 mg/mL), antibiotics (100 μg/mL pen-strep), and 5% fetal calf serum (FBS) for H295R cells and 10% FBS for SW13 cells. Cells were grown at 37 °C in a humidified atmosphere with 5% CO2. All experiments involving cell manipulation were conducted incubating cell lines as previously de-scribed (Bertazza et al. 2019).

MTT assay and trypan blue dye exclusion method

SW13 and H295R cells were plated in 96-well plates at a density of 5x103 cells/well in supplemented medium with or without GNAR or GNUG samples. These extracts were used at 5, 2, 1, 0.5 μg/μL for 24 and 72h. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (2.5 mg/mL in PBS). The percentage of surviving cells was calculated from the absorbance values as follows: (A tested-A blank)/(A untreated control-A blank) × 100, where A blank refers to the absorbance of wells that contained only medium and MTT. Method has been described in detail previously (Pezzani et al. 2014). Each analysis was performed in quadruplicate and repeated 3 times.

The trypan blue dye (0.4% in PBS) exclusion method was also used to assess cell viability after treatment with both extracts (5, 2, 1, 0.5 μg/μL) as previously described

(Patti et al. 2020). Cells were incubated with trypan blue solution (1:1) for 5 min, then transferred to the Burker chamber and counted by light microscope. Dead cells were stained with the trypan blue dye. The percentage of living cells was calculated considering the number of viable cells and the total number of cells counted.

Assessment of cell morphology by Wright's staining

Cells were cultured on coverslips for 48h, incubated overnight in 0.1% FBS, then processed for 24h (for SW13) and 72h (for H295R) and treated with GNAR and GNUG extracts (1 and 5 μg/μL, respectively). Afterwards, SW13 and H295R cells were stained as previously described (Pezzani et al. 2014). Cell morphology was evaluated by light microscopy at x400 magnification. Cells were counted by 2 independent experts (MR and RP) and at least 600 cells were counted for every experiment in at least 10 different fields. Experiment was repeated twice.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel software. Experimental data were analyzed by two-tailed paired Student's t test and Kruskal–Wallis analysis followed by Dunn's post-test. Data are presented as mean ± standard error of the mean (SEM). A *p*-value <0.05 was considered statistically significant.

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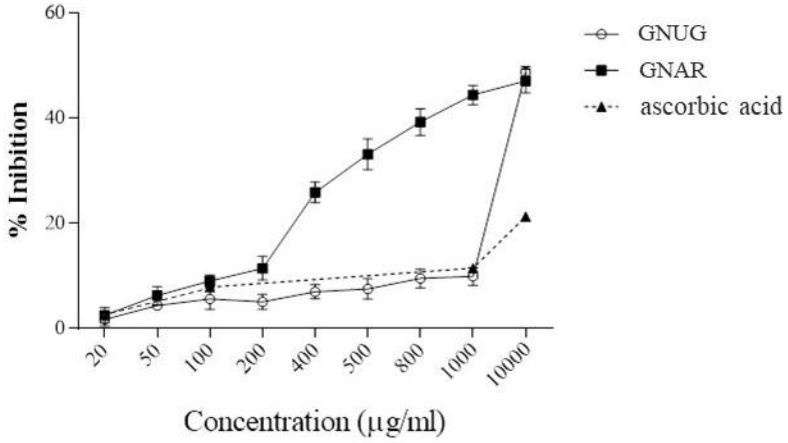

Figure S1. DPPH scavenging activity of GNUG and GNAR extracts.

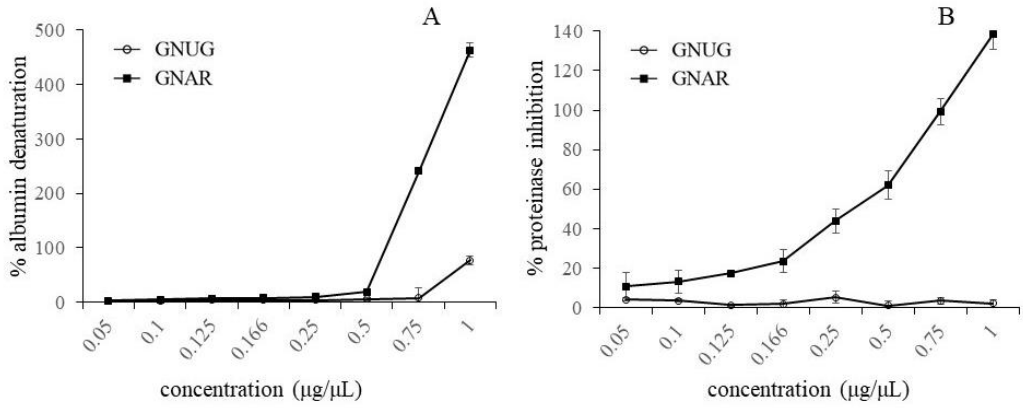

Figure S2. Anti-inflammatory activity of GNUG and GNAR extracts tested by albumin denaturation (A) and proteinase inhibition (B) assays at different concentrations.

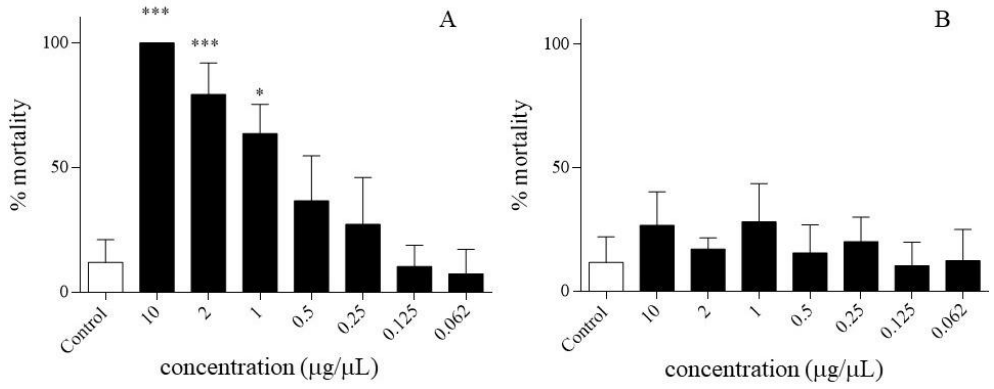

Figure S3. Cytotoxic effects of GNAR (A) and GNUG (B) extracts at different concentrations after 24 h as per protocol.

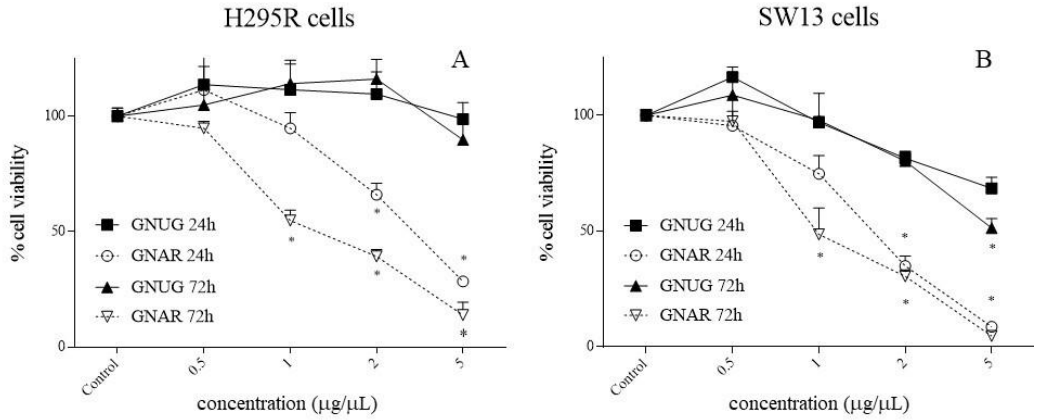

Figure S4. Cell viability evaluated by MTT assay for H295R (A) and SW13 (B) cells treated with GNUG and GNAR extracts. Results are expressed as percentage of control (100%). Treatment vs. control: * $p<0.05$.

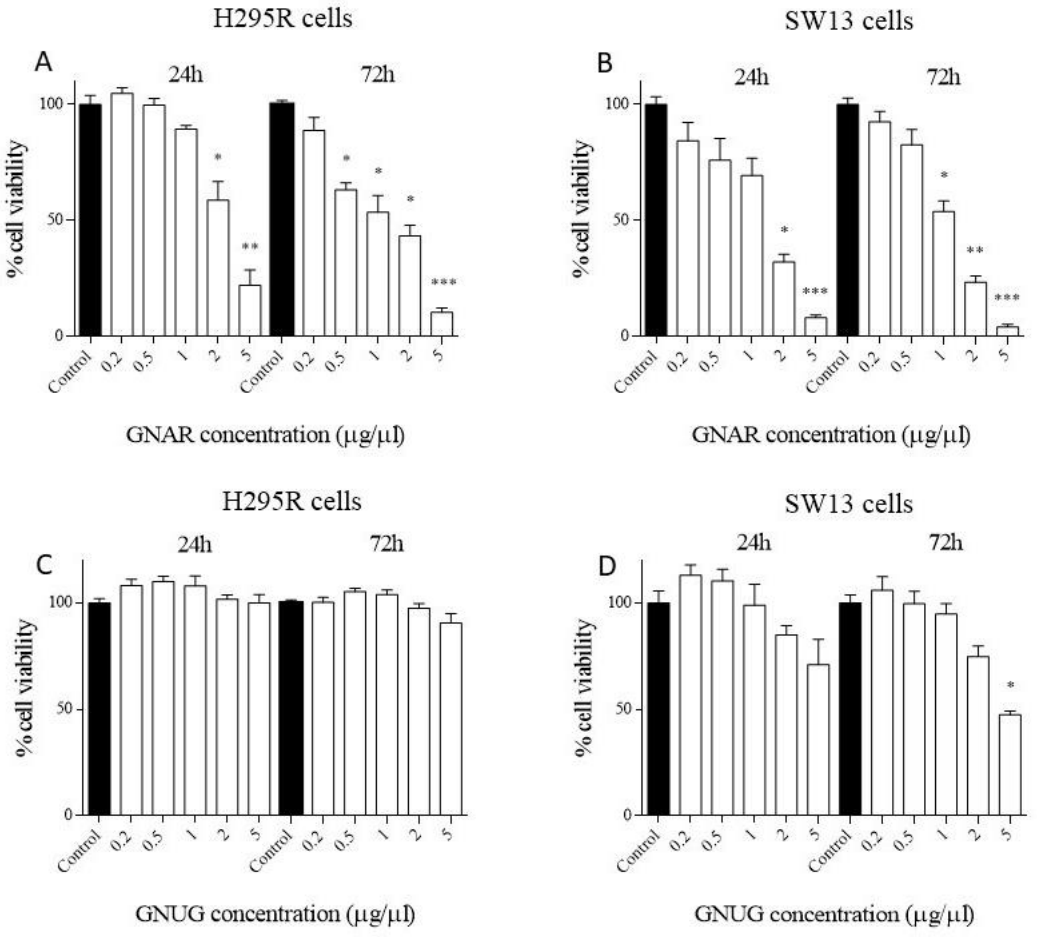

Figure S5. H295R (A-C) and SW13 (B-D) cell viability assessed by trypan blue assay at different con-centrations and times. The results are expressed as a percentage of control (100%). Treatment vs control: * p<0.05; ** p <0.01; *** p <0.001.

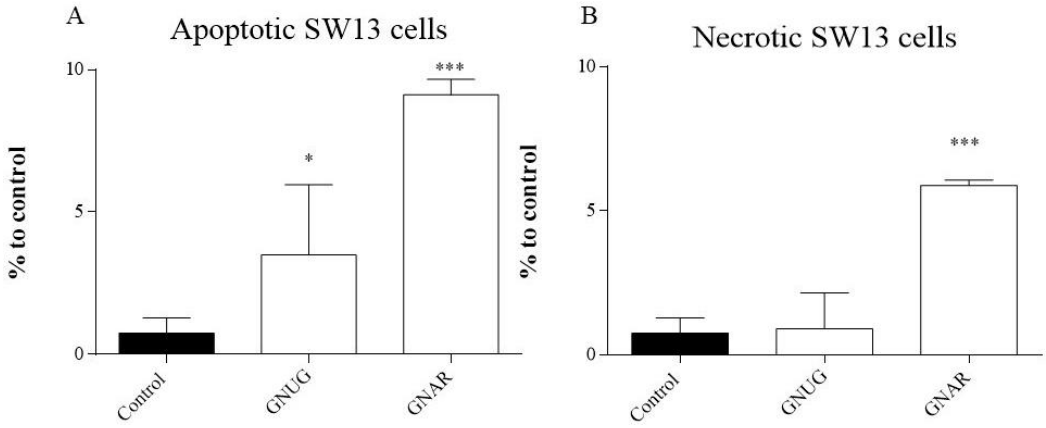

Figure S6. Morphology quantification (A: apoptosis and B: necrosis) in SW13 cells treated by GNAR extract at 1 µg/µL for 24 h and by GNUG extract at 5 µg/µl for 72 h.

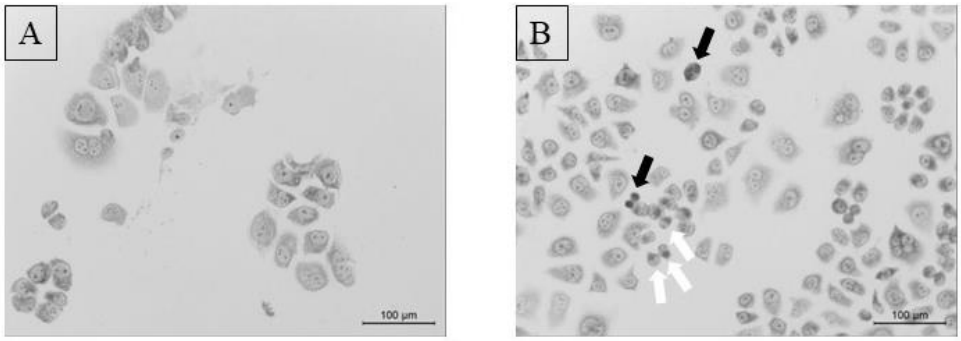

Figure S7. Representative pictures of SW13 cells untreated (A) and treated (B) by GNAR ex-tract. The arrows show apoptotic (white) or necrotic cells (black).

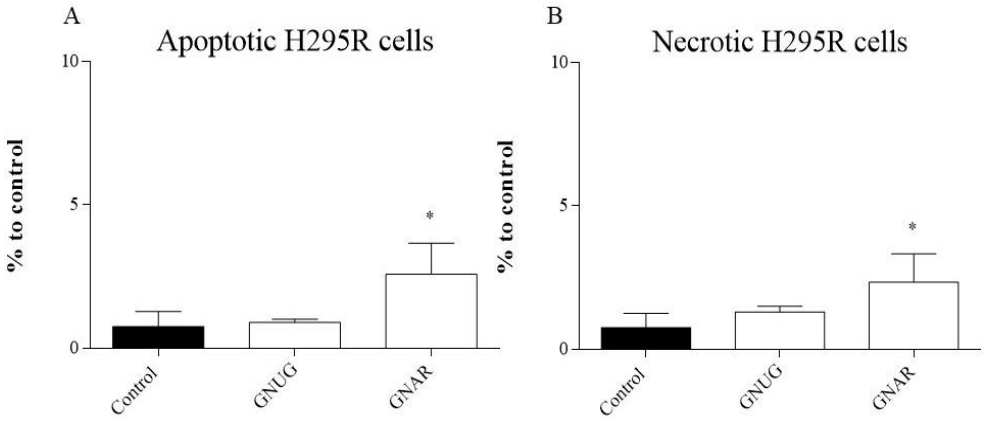

Figure S8. Morphology quantification (A: apoptosis and B: necrosis) in H295R cells treated by GNAR extract at 1 µg/µL for 24 h and by GNUG extract at 5 µg/µL for 72 h.

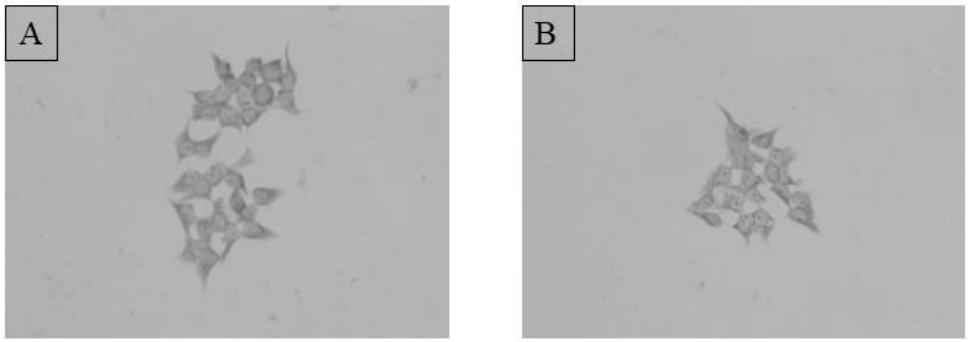

Figure S9. Representative pictures of H295R cells untreated (A) and treated (B) by GNAR extract.