RESEARCH ARTICLE

Inula viscosa (L.) Aiton Ethanolic Extract Inhibits the Growth of Human AGS and A549 Cancer Cell Lines

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The present study shows the chemical profile and cytotoxic properties of the ethanolic extracts of *Inula viscosa* from Northeast Algeria. The extract was obtained by maceration using ethanol. Its phenolic profile was determined using ultra-high-performance liquid chromatography coupled with a diode array detector and an electrospray mass spectrometer (UHPLC-DAD-ESI/MS), which allowed the identification and quantification of 17 compounds, 1,5-*O*-caffeoylquinic acid being the most abundant. The cytotoxic activity was assessed against human gastric cancer (AGS) and human non-small-cell lung cancer (A549) cell lines, whereas ethanolic extract elicited nearly 60% and 40% viability loss toward AGS and A549 cancer cells, respectively. Results also showed that cell death is caspase-independent and confirmed the involvement of RIPK1 and the necroptosis pathway in the toxicity induced by the *I. viscosa* extract. In addition, the ethanolic extract would not provoke morphological traits in the cancer cells. These findings suggest that *I. viscosa* can be a source of new antiproliferative drugs or used in preparation plant-derived pharmaceuticals.

Keywords: LC/MS analysis, cytotoxic activity, AGS and A549 cell lines, RIPK1, caspases, necroptosis.

Introduction

Treating cancer is considered one of the most challenging problems in medicine. In addition to conventional cancer treatments, patients increasingly resort to alternative and/or complementary treatments focusing in particular on the use of medicinal plants.[1–3] This practice is widespread in developing countries due to its low cost, easy access to these plants, and, above all, the concern about the harmful effects of synthesized drugs.

Previous studies have shown that the consumption of certain plants can promote chemopreventive and antineoplastic actions.[3–6] To improve cancer patients' survival and quality of life, research aiming at identifying new substances with anti-cancer properties has been steadily increasing in recent years.

Medicinal plants contain compounds that can be used either for therapeutic purposes or as precursors for pharmaceutical chemical synthesis.[7–9] *Inula viscosa* (L.) Aiton (currently accepted name *Dittrichia viscosa* subsp. *viscosa* (L.) Greuter)[10] belongs to the Asteraceae family found in the Mediterranean, Africa, Asia, and Europe.[11] It is known for its traditional use in treating several diseases, such as anti-inflammatory, astringent, vulnerary, antipyretic, antiseptic, and antiemetic properties. It is also used to treat gastrointestinal disorders and skin diseases.[12–17] Previous works reported some interesting activities displayed by *I. viscosa* extracts that can corroborate its traditional use. For example, antioxidant,[18–21] antifungal,[22] antibacterial,[21] anti-inflammatory,[23] hypoglycemic,[19,21] and cytotoxic[21,24–28] effects can be highlighted. The chemical composition for some *I. viscosa* extracts was reported before, namely the ethanol, methanol, water, and ethyl acetate fractions.[19–22,29,30]

Although *I. viscosa* has been the subject of some research works, there is still some missing information concerning the species growing in Algeria and its effect on some prevalent human cancer cells. So, in the present article, we aimed to investigate the chemical composition and cytotoxic properties of the *I. viscosa* extract against human gastric cancer (AGS) and human non-small-cell lung cancer (A549) cell lines due to the high prevalence of cancer from which they originate. The mechanisms of cell death associated were also assessed.

Results and Discussion

Total Bioactive Content

Total phenolic content (TPC) of *I. viscosa* extract were estimated using the Folin-Ciocalteu method,[31] while the total flavonoid content (TFC) was determined spectrophotometrically based on the AlCl$_3$ method.[32] The ethanolic extract showed contents of 145.3 ± 4.4 mg gallic acid equivalents/g of extract and 22.1 ± 0.6 mg quercetin equivalents/g of extract in terms of TPC and TFC contents, respectively.

The results herein discussed showed that ethanolic extract TPC is different from those previously cited for the same species and ranging from 75.3 ± 1.3 and 299.1 ± 34.5 mg GAE/g of extract.[22–24,29,30] These differences between our findings and those reported in the literature could be related to the solvents used but also to several other factors such as the geographical location, collection year, and extraction procedures.

LC/MS characterization of *I. viscosa* extracts

Inula viscosa ethanolic extract was further characterized by UHPLC-UV-MS/MS. The chromatogram was recorded at 280 nm (*Figure 1*), and the compounds identification was achieved by comparing the retention time and the MS data from samples and reference standards injected under the same chromatographic conditions, or by comparing their UV/VIS, MS and MS/MS spectra data with those reported in the literature. The peak characteristics and the assigned identification of compounds present in the ethanolic extract of *I. viscosa* are presented in *Table 1*, respecting their elution order.

The phenolic profile of *I. viscosa* ethanolic extract shows the presence of 17 compounds, 16 identified, and dominated by hydroxycinnamic acid and flavonol derivatives (*Table 1*). The hydroxycinnamic acid derivatives comprised, caffeoyl derivatives, mainly chlorogenic acid derivatives. One monocaffeoylquinic acid, compound **2**, was identified comparing the obtained data with previously reported one, mainly its pseudomolecular ion at *m/z* 353 and its typical base peak at *m/z* 191, which is correspondent to quinic acid fragment.[33] Naturally, the standard used to confirm the identification of caffeic acid also helped establish some fragment ions.

The other chlorogenic acids, compounds **5**, **6**, **7**, and **8**, are dicaffeoyl quinic acid derivatives, with the typical pseudomolecular ion at *m/z* 515 and exhibiting a base peak at *m/z* 353 due to the loss of one caffeoyl moiety (*Table 1*). Considering the retention time and their spectra data, these compounds were assigned to 1,4-*O*-diCQA, 1,5-*O*-diCQA, 4,5-*O*-diCQA.[33–36] Cytotoxic properties of caffeoylquinic acid and derivatives are well reported in the literature; for example, Ha and Park (2018) previously reported that six dicaffeoylquinic acids isomers, including 1,5-*O*-diCQA and 4,5-*O*-diCQA, showed an inhibitory effect on melanogenesis in B16F1 murine melanoma cells.[37] Additionally, 4,5-*O*-diCQA displayed therapeutic potential against prostate cancer by inactivating Bcl-2.[38] Using a modified TUNEL assay, Ooi *et al.* (2011) found that 3,4-*O*-diCQA can inhibit the growth of human lung adenocarcinoma cell lines (NCI–H23) by inducing apoptosis.[39] On the other hand, Teoh *et al.* (2016), reported that 3,5-*O*-diCQA displayed cytotoxic effects against colon cancer
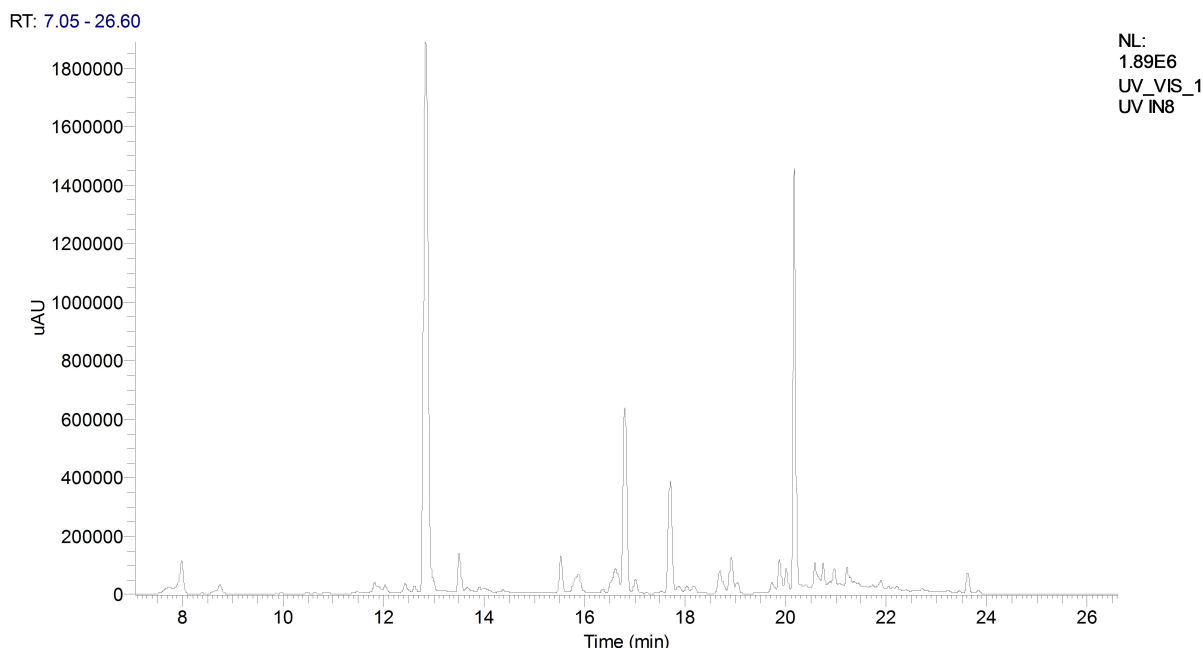
Figure 1. UHPLC chromatogram of *I. viscosa* ethanolic extract, recorded at 280 nm.

Table 1. Chemical composition of *I. viscosa* extracts by UHPLC-DAD-ESI/MS. Retention time (Rt), not quantified (NQ), caffeoylquinic acid (CQA).

Peak	Rt (min)	λ_{max}	$[M-H]^-$	MS^2 (*m/z*)	Identified compound	Quantification (µg/mg extract)
1	7.73	324, 239, 221	189	127, 115, 99	Unknown	NQ
2	7.99	325, 239, 218	353	191, 179, 173	1-*O*-CQA	7.397 ± 0.329
3	8.75	322, 239, 221	179	135	Caffeic acid	0.305 ± 0.069
4	11.82	352, 256, 203	477	301	Quercetin-*O*-glucuronide	1.192 ± 0.560
5	12.43	326, 241	515	353, 317, 299, 255, 235, 203	1,4-*O*-diCQA	4.674 ± 0.010
6	12.84	341, 311, 289, 245	515	353, 335, 191	1,5-*O*-diCQA	52.422 ± 4.035
7	13.51	326, 242, 220	515	353, 299, 203	3,4-*O*-diCQA	5.517 ± 0.067
8	13.91	326, 242	515	353, 335, 299, 255, 203	4,5-*O*-diCQA	4.213 ± 0.134
9	15.52	328, 244, 221	857	695, 533, 371, 353, 209	Tetracaffeoyl hexaric acid	5.486 ± 0.512
10	15.88	351, 267, 254	315	300, 287, 271	Methylquercetin isomer 1	2.315 ± 0.141
11	16.61	355, 255, 205	315	300, 287, 271	Methylquercetin isomer 2	3.962 ± 0.615
12	16.80	289, 222, 204	317	299, 289, 193	Myricetin	13.311 ± 0.876
13	17.70	334, 273, 217	299	284	Chrysoeriol	8.785 ± 0.497
14	18.67	340, 291, 269, 230	299	284	Diosmetin	2.847 ± 0.264
15	18.92	355, 254, 205	329	314, 301	Dimethylquercetin	3.174 ± 0.319
16	19.87	367, 256	315	300, 287, 193, 164	Isorhamnetin	2.945 ± 0.667
17	20.17	290, 229	359	341, 317, 299, 193	Trimethylmyricetin	26.682 ± 2.441

cells with minimal cytotoxic effects against normal colon cells.[40]

One of the central nuclei of these metabolites, caffeic acid, also occurs naturally in plants and is known to inhibited the growth of Ht-29 cell lines via induction of apoptosis[41,42] and has an antitumor effect against the human cutaneous melanoma cell lines (SK-Mel-28).[43]

The last caffeoyl derivative, compound **9**, showing a pseudomolecular ion at *m/z* 857, was assigned to tetracaffeoyl hexaric acid.[44] Not only it presents the four fragment ions typical of cleavage of the caffeoyl moieties, *m/z* 695 [M–$C_9H_7O_3$ (caffeoyl moiety)], *m/z* 533 [M–$C_{18}H_{13}O_6$ (2xcaffeoyl moiety)], *m/z* 371 [M–$C_{27}H_{19}O_9$ (3xcaffeoyl moiety)], and *m/z* 209 [M–$C_{36}H_{25}O_{12}$ (4xcaffeoyl moiety)], but also the UV/VIS

data identical to the one reported by Dudek *et al.* (2016).[45]

It is worth mentioning that Trendafilova *et al.* (2020) found that the extract of *Inula ensifolia* flowers with a higher concentration of chlorogenic and dicaffeoylquinic acids decreased the cell proliferation of A549 cancer cell lines.[46]

Additionally, to caffeic acid derivatives, the ethanolic extract profile shows several methylated flavone derivatives, from which the quercetin derivatives are the major ones (*Table 1*). Still, the flavones isomers, chrysoeriol, and diosmetin, with pseudomolecular ion [M−H]⁻ at *m/z* 299 and releasing a fragment ion at *m/z* 284, resulting from the loss of the methyl group, were also detected.[47] Compound **16** possessing a pseudomolecular at *m/z* 315 and a fragment ion at *m/z* 164 was tentatively identified as isorhamnetin since this fragment corresponds to the typical fragment $1,3B^+$.[48,49] On the other hand, compounds **10** and **11** (*Table 1*), also methylquercetin derivatives, could not be totally assigned, but due to their UV/VIS data, they seemed to be methylated in the A ring. The similarities in the UV/VIS data also suggest that compound **15** should be quercetin with *O*-methoxy groups in A ring and C-3. Finally, it should also be highlighted that a trimethylmyricetin (compound **17**) was also identified in this extract; it presents similar data to compound **12**, which is myricetin.[50–52]

Flavonoids, in general, are known to have important biological activities, including cytotoxic properties. Concerning the ones identified in *I. viscosa*, previous studies revealed that chrysoeriol significantly inhibits the growth of A549 lung cancer cells,[53,54] and exhibited significant anticancer effects against other tumor cell lines, including cervical cancer,[54,55] colon cancer cell lines,[55] HL-60 leukemia cells,[55] and human stomach cancer AGS cells.[56]

Myricetin is another flavonoid identified in the ethanolic extract of *I. viscosa* species, and this compound is endowed with remarkable cytotoxic properties. In many previous reports, it has been shown that myricetin-induced cytotoxicity against various types of cancer cell lines, including glioblastoma cells,[57] A2780, OVCAR3,[58] SKOV3 ovarian cancer cells,[59] and human cervical cancer (HeLa) cells.[60]

Quercetin methylated derivatives have also been found to inhibit the growth of cancer cell lines, namely human lung adenocarcinoma cell lines (A549 and HCC-44),[61] JB6 P + cells,[62] MDA-MB-231 cells,[63] HCT-116 cancer cell lines.[64] Finally, it can be highlighted diosmetin, which inhibits the growth of human squamous carcinoma cells obtained from the oral cavity.[65]

Overall, LC/MS analysis revealed that 1,5-*O*-diCQA was the major compound in the phenolic profile of the *I. viscosa* ethanolic extract. Our results agree with previous reports demonstrating that hydroxycinnamic acids were the major phenolic compounds isolated from the Asteraceae family.[66,67] Data showed that the phenolic compounds detected in this study differ from those reported in the literature by Brahmi-Chendouh *et al.* (2019).[30] Still, it should be emphasized that their phenolic profile was established for an ethyl acetate extract.

Cytotoxic Activity

Screening of Toxicity Towards Cancer Cells

Aiming the evaluation of the potential cytotoxic properties of the *I. viscosa* ethanolic extract, human gastric cancer (AGS) and human non-small-cell lung cancer (A549) cell lines were used due to the high prevalence of the cancer from which they originate. For the assessment of cell viability, we used a rezasurin-based assay, which has the advantage of not requiring cell lysis before reading when compared to assays like MTT, and additionally it can be measured by the means of fluorescence, while tetrazolium salts are read by the means of colorimetry. In the case of AGS cells, the *I. viscosa* ethanolic elicited more than 60 % viability loss (*Figure 2*).

A549 cells are known to be a drug-resistant cell lines, being frequently included in biological screenings for this reason.[68] In these cells, a different toxicity pattern was achieved, ethanolic extract cause nearly 40 % viability loss (*Figure 2*). According to the above reported results, the ethanolic extract was subjected to further mechanistic studies in AGS and A549 cells. Considering the extract phenolic profile, the significant cytotoxic effect found in this research could be attributed to the chemical compounds present in the *I. viscosa* ethanolic extract.

I. viscosa ethanolic extract-elicited cell death is caspase-independent

Aiming to assess the impact of the *I. viscosa* ethanolic extract upon cell morphology, the treated AGS and A549 cells were imaged to evaluate the chromatin status and overall cell morphology. Several techniques could be used to this end, including SEM and fluorescence microscopy. While SEM provides much
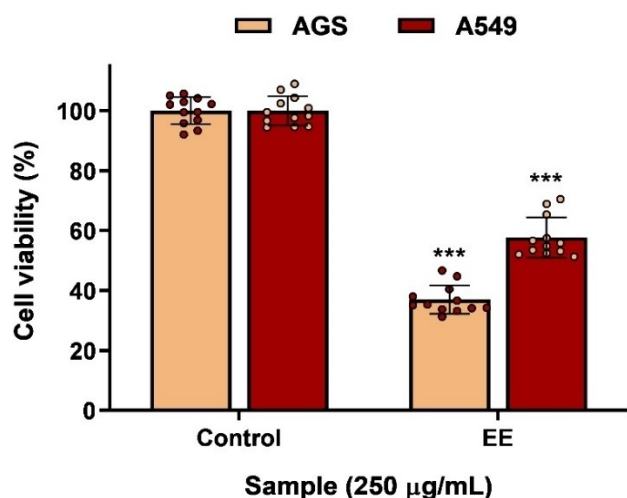
Figure 2. Viability of AGS and A549 cells exposed to the *I. viscosa* extract (250 µg/mL), or medium (control). EE: ethanolic extract. Cells were incubated for 24 h, after which viability was evaluated. Data represents the mean $\pm$ SD, of at least three independent experiments performed in triplicate. *** $p < 0.001$ compared to the respective control (Student's t-test).

higher magnification and resolution, the use of fluorescence microscopy can provide important insights when using actin- and chromatin-specific probes, as widely described in the literature.[69,70] In this work we the chromatin status of the cells incubated with ethanolic extract was evaluated using DAPI, while the cytoplasmic morphology was assessed using phalloidin, which binds to actin. The extract under study was tested at the concentration of 125 µg/mL and 250 µg/mL against AGS and A549 cells, respectively (*Figure 3*), due to the marked effect in the AGS cell lines at 250 µg/mL, that originate the loss of the majority of the cells after fixation. In both cases, it is noticeable that there is a marked decrease in cell density, however, were unable to find morphological traits that would be expected in case apoptosis was taking place, such as increased rate of cells exhibiting chromatin condensation or fragmentation.[71,72]

To further confirm that the loss of viability caused by the ethanolic extract was caspase-independent, cells were exposed to the extract under study in the presence Z-VAD-FMK, a pharmacological pan-inhibitor of caspase activity, which is capable of rescuing cell viability in cases where caspase-dependent pathways are involved. As shown in *Figure 4A*, co-incubation with Z-VAD-FMK did not result in a significant increase in cell viability, thus ruling out the involvement of these enzymes.

Toxicity of I. Viscosa Ethanolic Extract is RIPK1-Dependent

Resistance to apoptosis is often responsible for both tumorigenesis and drug resistance, resulting in chemotherapy failure.[73] There is an increasing number of pathways for programmed cell death. Necroptosis emerging as a novel approach to eliminate tumor cells, multiple therapeutic agents being reported as inducers or manipulators of this cell death pathway.[74,38] In contrast to apoptosis, necroptosis is a regulated necrotic cell death modality in a caspase-independent fashion and is mainly mediated by Receptor-Interacting Protein 1 (RIP1), RIP3, and Mixed Lineage Kinase Domain-Like (MLKL).[73] In order to evaluate the possible involvement of RIP1/RIP3 pathway to the mechanism of action of the *I. viscosa* ethanolic extract, cells were co-incubated with necrostatin-1 (nec-1), a serine/threonine kinase RIP1 inhibitor. As seen in *Figure 4B*, the inhibition of the RIP1/RIP3 (EE + nec-1) significantly rescued the cell death caused by the ethanolic extract treatment in A549 cells (47.5% vs. 57.4%, $p = 0.0103$), thus pointing to the involvement of RIPK1 and necroptosis pathway to the toxicity previously detected. In the case of AGS cells no significant differences have been noticed (48.0% vs. 55.7%, $p = 0.0965$).

The marked reduction in cell density shown in *Figure 3* could also suggest that an antiproliferative effect is taking place. Considering the quantification presented in *Table 1*, the major compounds are 1,5-O-diCQA, a myricetin derivative (trimethylmyricetin) and myricetin itself. Together, they account for nearly 60% of the total amount of phytochemicals identified and quantified in the extract. Several di-caffeoylquinic acids have been described for their antiproliferative effects in cancers cells, specifically in colon cancer and promyelocytic cells, with results showing only minor changes in activity according to the isomer involved.[75] Another study showed the antiproliferative of di-caffeoylquinic acids towards breast cancer cells *via* the IL6/JAK2/PI3 K pathway.[76]

In the case of myricetin, its antiproliferative effect has been described against several cancer cell lines ranging from esophageal carcinoma[77] to liver and bladder.[78] The mechanism of action involved in this effect seems to be cell type-dependent[79] and includes binding to p90 ribosomal S6 kinase (RSK2),[77] phosphorylation of the p38 MAPK[78] and binding to PI3 K.[80] Considering the significant amounts in which these molecules occur in the extract, it is highly
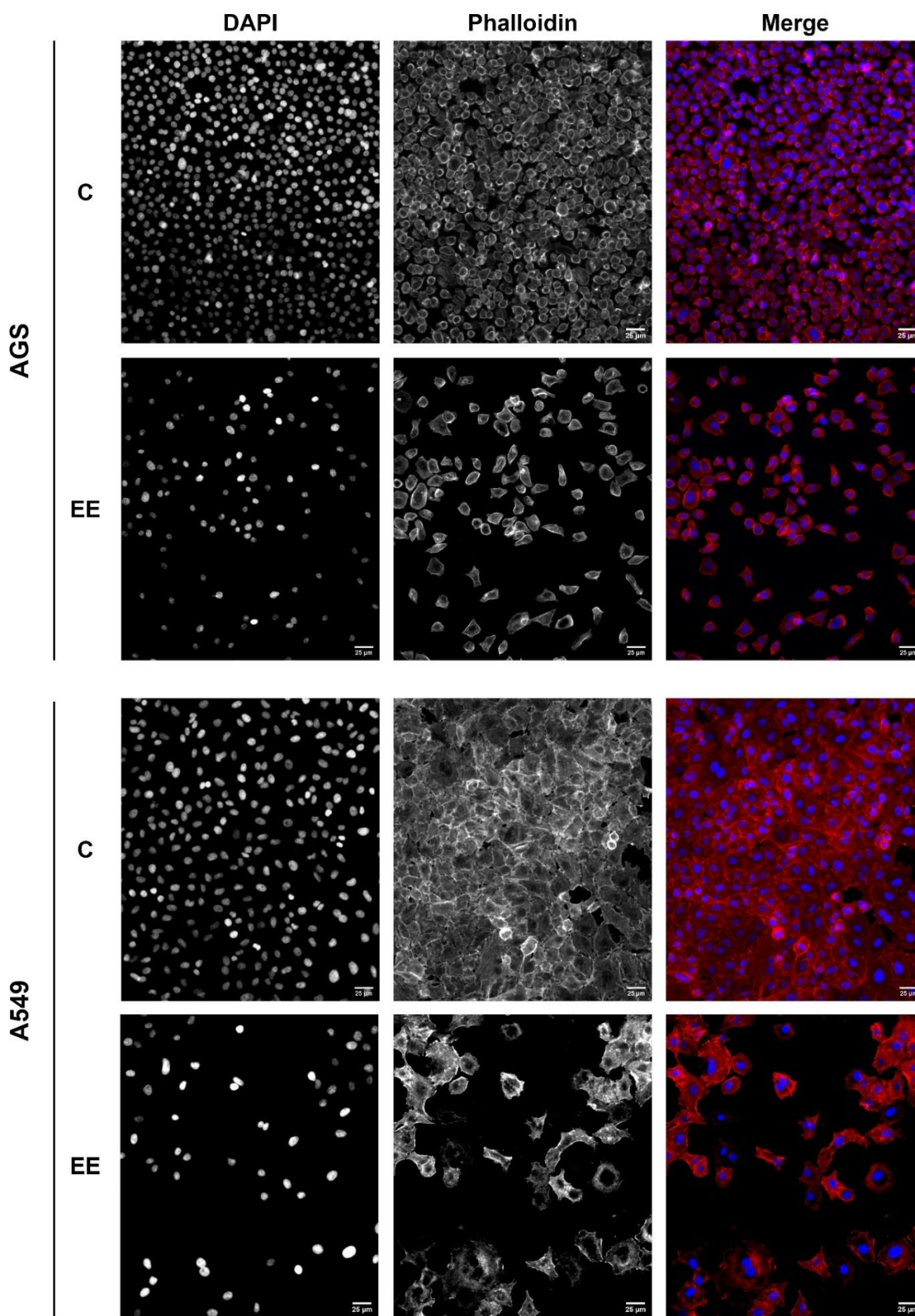
Figure 3. Morphology of AGS and A549 cells exposed to the *I. viscosa* ethanolic extract (EE) (AGS: 125 µg/mL; A549: 250 µg/mL) after 24 h of incubation (S Plan Fluor ELWD 20×DIC N1 objective). Overall cell morphology was evaluated using phalloidin (actin) and DAPI (chromatin status).
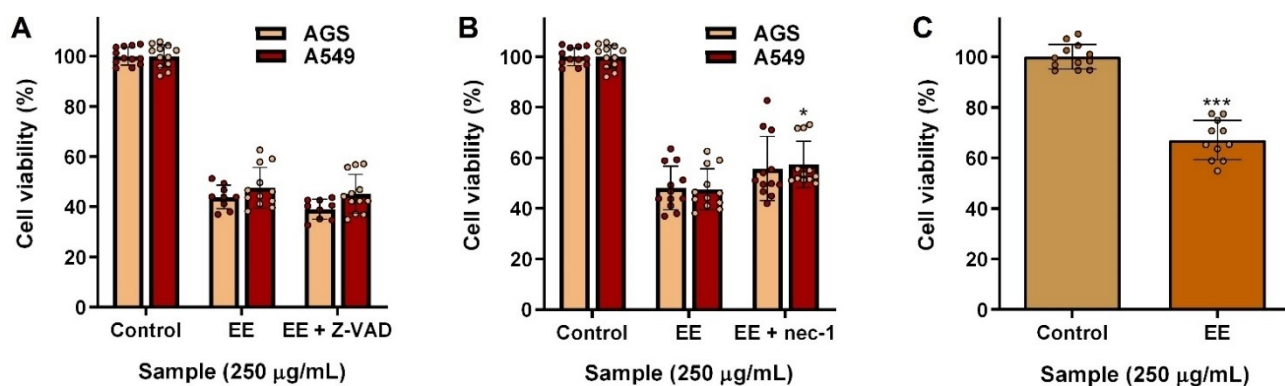
Figure 4. (A) Influence of Z-VAD-FMK (25 μM), a pan-caspase inhibitor, on the toxicity elicited by the *I. viscosa* ethanolic extract (EE) in AGS and A549 cells after 24 h of incubation. (B) Influence of nec-1 (9 μM), a serine/threonine kinase RIP1 inhibitor, on the toxicity elicited by *I. viscosa* ethanolic extract (EE) in AGS and A549 cells after 24 h of incubation. (C) Viability of HaCaT cells exposed to *I. viscosa* ethanolic extract (EE), or medium (control). Cells were incubated for 24 h, after which viability was evaluated. Data represents the mean ± SD, of at least three independent experiments performed in triplicate. * $p < 0.05$, *** $p < 0.001$ compared to the respective control (Student's t-test).

probably that can explain, at least in part, the biological effect we describe here.

Selectivity of I. Viscosa Ethanolic Extract Towards Cancer Cells

Therapeutic selectivity is one of the most important considerations in cancer chemotherapy. The design of therapeutic strategies to preferentially kill malignant cells minimizing the harmful effects to non-cancer cells is essential. For this reason and based on the cytotoxic results reported above towards cancer cells, the effect of the *I. viscosa* ethanolic extract in a human non-cancer cell lines, specifically HaCaT keratinocytes was evaluated. When compared with the HaCaT cell lines at the same concentration, *I. viscosa* ethanolic extract displayed 2-fold higher toxicity to AGS cancer cells which is a promising result (*Figure 2* and *4C*). Conversely, similar toxicity was displayed by this extract in A549 cells when compared with HaCaT cells (*Figure 2* and *4C*), suggesting a differential selectivity pattern according to the cancer cell lines tested. These results highlight the importance of monitoring the toxicological effect of new drug candidates against distinct cell lines, as they may encompass different selectivity profiles and even mechanisms of action.

Inula viscosa extracts' cytotoxic and anticancer effects have recently been shown in cervical cancer,[81,82] breast cancer,[83] Burkitt lymphoma cell lines,[84] and colorectal cancer cell lines.[85]

Although the cytotoxic effects of *I. viscosa* extracts have been the topic of previous research, some information is still missing. The chemical composition of the extracts and the mechanism of action are often not studied. Furthermore, the activity against typical human cancer cells, gastric and pulmonary cancers was not studied. Additionally, the species growing in Algeria and used in traditional medicine was not studied. As a result, our research provides new insights about the cytotoxic effect of *I. viscosa* species, confirms its traditional use as an anticancer remedy, and further highlights it as a potential source of anticancer compounds.

Conclusions

The present study assessed the phenolic profile of the *I. viscosa* ethanolic extract and its cytotoxicity against AGS and A549 human cell lines. LC/MS/MS allowed the identification of 17 compounds with 1,5-*O*-dicaffeoyl quinic acid as the major component. Results also showed that *I. viscosa* elicited nearly 60% and 40% viability loss toward AGS and A549 cancer cells, respectively. Further, a mechanistic study revealed the involvement of RIPK1 in the toxicity induced by the *I. viscosa* extract and discarded the fact that cell death was mediated by apoptosis. The results of this study suggest that the *I. viscosa* species could be considered a good candidate for developing new antiproliferative drugs. However, more studies are required to isolate and assess the main compounds responsible for this activity.

Experimental Section

Chemicals

Standards used for the elucidation of the phenolic compounds and for the elaboration of the calibration curves were obtained from EXTRASYNTHESE (Genay-Cedex, France). Acetonitrile HPLC-grade and formic acid were purchased from Panreac (Barcelona, Spain). All other chemicals were of analytical grade. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin solution (penicillin 10000 Units/mL and streptomycin 10000 µg/mL), Trypsin-EDTA (0.25%) and PrestoBlueTM were obtained from Invitrogen (Grand Island, NE, USA). Z-VAD-FMK and phalloidin (CF543) were provided by Santa Cruz (Heidelberg, Germany) and Biotium (CA, USA), respectively.

Extract Preparation

Aerial parts of *Inula viscosa* (L.) Aiton were collected in Jijel (Northeast of Algeria; 36°46'17'' N, 5°46'34'' E) and identified by Prof. Sarri Djamel (University of M'sila, Algeria). A specimen (AIV 11/18) was deposited in the Herbarium of the VARENBIOMOL research unit, Frères Mentouri University 1, Constantine, Algeria. The leaves were shade drying at ambient temperature (25 °C), and then were ground into a fine powder. Ethanol extract was obtained by maceration at room temperature for 48 h, followed by solvent replacement for 2 additional times. The solvent was then removed under reduced pressure.

Total Bioactive Content

Total Phenolic Content

The total phenolic content was determined using the Folin–Ciocalteu method described by Singleton *et al.* (1999).[31] 15 µL of plant extract (1 mg/mL) was mixed with 15 µL of Folin's reagent and 60 µL of water. After 5 min of incubation, 150 µL of Na_2CO_3 solution (20% w/v) was added. The mixture was once again incubated in the darkness for 60 min, and absorbance was recorded at 760 nm. The total phenolic content was measured as mg equiv. of gallic acid per g of extract (mg GAE/g of extract).

Total Flavonoid Content

The total flavonoid content was determined according to the method described by Türkoglu *et al.* (2007).[32] with a slight modification. An aliquot of 100 µL of each plant extract was mixed with 100 mL of $AlCl_3$ (2%) solution. After incubation at room temperature for 10 min, the absorbance was measured at 415 nm. The total phenolic content was measured as mg equiv. of quercetin per g of extract (mg QE/g of extract).

UHPLC/MS Characterization of I. Viscosa Extract

UHPLC-DAD-ESI-MS^n profiling of the *I. viscosa* ethanolic extract was carried out using an Ultimate 3000 (Dionex Co., San Jose, CA, USA) apparatus equipped with a binary pump, an automatic sampler and a diode array detector (Dionex Co., San Jose, CA, USA). The MS analysis was conducted using a Thermo LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA) outfitted with an electrospray ionization interface (ESI). The separation was performed at room temperature 25 °C with a Hypersil Gold (Thermo Scientific, USA) C18 column (100 mm length; 2.1 mm i.d.; 1.9 µm particle diameter, end-capped). Formic acid in water (A) and acetonitrile (B) were used as analyte solvents of the extract (1 mg/mL), with an injection volume of 10 µL and flow rate of 2 mL/min. UV/VIS spectral data were gathered in a range of 200 to 700 nm and chromatographic profiles documented at 280, 350, 470, 655 nm. MS and MS/MS data were processed using the Thermo XcaliburQual Browser data system (Thermo Scientific, USA). Spectra were recorded in negative-ion mode with electrospray ionization source of 5.00 kV and ESI capillarity temperature of 275 °C. The full scan covered a mass range of 50–2000 *m/z*. Collision-induced dissociation MS/MS experiments were simultaneously acquired for precursor ions. The identification of individual phenolic compounds by UHPLC/MS was achieved by comparing their retention times, UV/VIS spectra, and MS^n spectra data available on the literature. And also, with the data of reference standards or of the closest available standards, injected under the same UHPLC/MS conditions. The quantification of the individual phenolic compounds in the plant extract was performed by peak integration at 280 nm, through the external standard method, using the closest reference compounds available. The detection and quantification limits (LOD and LOQ, respectively) were determined from the parameters of the calibration curves (LOD=3 standard deviation/slope and LOQ=10 standard deviation/slope). The calibration curves were obtained by injection of five known concentrations with variable ranges and chosen in order to guarantee the quantification of each compound in the samples by intrapolation in the calibra-

tion curve. Values of correlation coefficients (> 0.99) confirmed linearity of the calibration plots (6 points; 3 assays). The results were expressed in µg of compound/mg of dried extract, as mean values ± standard deviation (MV ± SD) of four independent analyses.

Cytotoxic Activity

Cell Culture

Three human cell lines were used in this research: gastric carcinoma (AGS; Sigma–Aldrich), lung carcinoma (A549; ECACC, Salisbury, UK) and human keratinocytes (HaCaT; ATCC, Rockville, MD, USA). Cells were cultured as monolayer at 37 °C in a humidified incubator with 5 % CO_2. All cell lines were grown in glutamine-enriched DMEM (Gibco) supplemented with 1 % streptomycin/penicillin and 10 % FBS (Gibco). For subculture, cells were washed with HBSS, treated with 0.25 % trypsin-EDTA solution (Sigma–Aldrich) for 3 min at 37 °C, resuspended in 5 mL of culture medium and centrifuged at 1300 rpm for 3 min. The supernatant was removed, and the cell pellet was resuspended in culture medium. Cell passages were kept low for all cell lines, with a maximum of 12 passages. Unless otherwise stated, all assays were carried in 96-well plates.

Viability Assessment

I. viscosa ethanolic extract was solubilized in DMSO, in stocks of 50 mg/mL. For the assessment of viability, a resazurin-based method was used.[70] AGS and HaCaT cells were plated at a density of 1.5×10^4 cells/well, while A549 cell lines was seeded at a density of 1.0×10^4 cells/well. The cells were incubated for 24 h being then exposed to the extract under study (at 250 µg/mL; maximum DMSO concentration: 0.5 %) for another 24 h. After this period, a commercial solution of resazurin was added (1:10, final volume: 200 µL) and the plate incubated for 30 min, the fluorescence increase being monitored at 560/590 nm (excitation/emission wavelength) in a microplate reader (Cytation™ 3, BioTek, Winooski, VT, US). At least three independent experiments were performed in triplicate.

Caspase Pharmacological Inhibition Assay

AGS and A549 cells were seeded in 96-well plates at the same density used for viability experiments. After attachment, cells were pre-incubated with the pan-caspase inhibitor Z-VAD-FMK at 25 µM for 1 h, as

previously described.[72,86] Then, plant extract was added, and cells were co-incubated for 24 h. Cell viability was determined using a resazurin-based method as described above.

Involvement of the RIP1 Kinase

AGS and A549 cells were pre-incubated with 9 µM necrostatin-1 (nec-1) for 1 h, as described before.[68,86] Then, the plant extract was added, followed by co-incubation for 24 h and subsequent evaluation of viability by a resazurin-based method as described above.

Morphological Assessment

For morphological studies, AGS and A549 cells were cultured in 96-well plates at the same density used for viability experiments, in the presence of the plant extract under study. After incubation, cells were washed with HBSS and fixed in 10 % formalin solution for 30 min, at room temperature. CF543 phalloidin (5 U/mL) and DAPI (0.25 µg/mL) were added, and cells were stained for 25 min at room temperature and washed with HBSS.[70,87] Images were acquired in an inverted Eclipse Ts2R-FL (Nikon) equipped with a Retiga R1 camera and a S Plan Fluor ELWD 20x DIC N1 objective. Images were analyzed with Fiji.[88]

Statistical Analysis

Data were recorded as the mean ± standard deviation (m ± SD) from three independent assays. For the cytotoxicity assays, the Shapiro-wilks normality test was performed in the data to ensure that it followed a normal distribution. Comparison between the means of two groups (controls *vs.* each experimental condition) was performed using Student's t-test. Outliers were identified by the Grubbs' test. The analyses were performed using GraphPad Prism 7.0 or ggplot/R software and values were considered statistically significant with a $p < 0.05$.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

H.R.: Conceptualization, investigation, data curation, and writing-original draft preparation; A.H.: investigation, data curation and writing-original draft preparation; K.H.: conceptualization, supervision, validation, and writing-original draft preparation; D.M.P. and R.B.P.: investigation, data curation and writing; T.B.: investigation; D.C.G.A.P.: conceptualization, supervision, validation, software, and writing-reviewing the original draft; M.S.G.A.V.: software, methodology; A.M.S. S.: conceptualization, validation, resources, supervision, and writing-reviewing the original draft. All authors have read and agreed to the published version of the manuscript.

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