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RESEARCH ARTICLE

Inhibitory effects of cynaropicrin on human melanoma progression by targeting MAPK, NF-κB, and Nrf-2 signaling pathways in vitro

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Malignant melanoma is the deadliest skin cancer, due to its propensity to metastasize. MAPKs and NF-κB pathways are constitutively activated in melanoma and promote cell proliferation, cell invasion, metastasis formation, and resistance to therapeutic regimens. Thus, they represent potential targets for melanoma prevention and treatment. Phytochemicals are gaining considerable attention for the management of melanoma because of their several cellular and molecular targets. A screening of a small library of sesquiterpenes lactones selected cynaropicrin, isolated from the aerial parts of *Centaurea drabifolia* subsp. *detonsa*, for its potential anticancer effect against melanoma cells. Treatment of human melanoma cells A375 with cynaropicrin resulted in inhibition of cell proliferation and induction of caspase-3-dependent apoptosis. Furthermore, cynaropicrin reduced several cellular malignant features such migration, invasion, and colonies formation through the inhibition of ERK1/2 and NF-κB activity. Cynaropicrin was able to reduce intracellular reactive oxygen species generation, which are involved in all the stages of carcinogenesis. Indeed, cynaropicrin increased the expression of several antioxidant genes, such as glutamate–cysteine ligase and heme oxygenase-1, by promoting the activation of the transcription factor Nrf-2. In conclusion, our results individuate cynaropicrin as a potential adjuvant chemotherapeutic agent for melanoma by targeting several protumorigenic signaling pathways.

KEYWORDS
chemoprevention, cynaropicrin, MAPK, melanoma, oxidative stress, sesquiterpene lactones

1 | INTRODUCTION

Consumption of plant-based foods, that is, fruits, vegetables, and whole grains, rich in diverse phytochemicals, is inversely associated with cancer incidence (Allen, 2018). During the last decades, the use of phytochemicals as adjuvant chemotherapeutic agents in heterogeneous human carcinomas has shown an upward trend (Sak, 2012). Malignant melanoma is the most aggressive form of skin cancer, originating from melanocytes aberrant proliferation. In melanoma, multiple signaling pathways are constitutively activated and lead to uncontrolled melanocytes proliferation, survival, differentiation, and, lastly, metastasis formation. Hyperactivation of the mitogen-activated protein kinase (MAPK) signaling pathway, following mutations in BRAF and NRAS oncogenes, has been reported in 90% of the melanomas (Wellbrock, 2016).

$BRAF^{V600E}$ is the most common BRAF mutation in melanoma, and it has been implicated in melanoma progression by activation of the downstream extracellular signal–regulated kinase (ERK)/MAPK signaling pathway. In fact, constitutive BRAF activation promotes the replicative potential of melanoma cells, reduces apoptosis, and

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stimulates cell invasion and metastasis (Wan et al., 2004). In addition, oncogenic BRAF has been shown to induce aberrant activation of the transcription factor nuclear factor kappa-light-chain enhancer of activated B-cells (NF-κB) (Liu et al., 2007) that in turn contributes to melanoma cell proliferation, survival, and resistance to apoptosis (Madonna, Ullman, Gentilcore, Palmieri, & Ascierto, 2012). Direct activation of the MAPK pathway is also induced by ultraviolet (UV) radiation throughout reactive oxygen species (ROS) formation (Syed, Afaq, & Mukhtar, 2012), which might be associated with melanoma development and progression.

Numerous diet-derived phytochemicals such as epigallocatechin-3-gallate, resveratrol, curcumin, proanthocyanidins, silymarin, apigenin, capsaicin, genistein, indole-3-carbinol, and luteolin have shown promising chemopreventive effects in melanoma, due to their antioxidant property (Ombra et al., 2019). In addition, most of these molecules are safe, widely available, high tolerable, and cost-effective, making them ideal agents to improve cancer chemoprevention and treatment.

Among plant-derived phytochemicals, sesquiterpene lactones (SQLs) represent one of the largest and most intensely studied class of compounds (Chadwick, Trewin, Gawthrop, & Wagstaff, 2013). They have received wide attention because of their potent bioactivities, including antibiotic, antitumor, antiinflammatory, insect-feeding deterrent, phytotoxic, and schistosomicidal potential (Adekenov, 2017). In the present study, we investigated a small library of sesquiterpene lactones obtained from four different plants (*Vernonia amygdalina*, *Daucus virgatus*, *Centaurea drabifolia* subsp. *detonsa*, *Onopordon illyricum*) for their anticancer activity against melanoma cells. This screening identified cynaropicrin, a sesquiterpene lactone of the guaianolide type, as the most promising agent, worthy of further investigation. Cynaropicrin is highly concentrated in the edible plant artichoke (*Cynara scolymus* L.), where it is coresponsible for the characteristic bitter taste (Eljounaidi et al., 2015); however, it has also been isolated from several species of the genus *Saussurea* and *Centaurea* (Formisano et al., 2017a; Milošević Ifantis et al., 2013; Pandey, Rastogi, & Rawat, 2007; Ren, Yu, Chen, Wu, & Fu, 2007; Saito et al., 2012). Cynaropicrin has been shown to possess various bioactivities such as antihepatitis C virus, antihyperlipidemic, antiprotozoal, antifeedant, antispasmodic, antiphotoaging, as well as antitumor and antiinflammatory activities (Elsebai, Mocan, & Atanasov, 2016). In the present study, the protective and therapeutic potential of cynaropicrin in human melanoma cells was explored for the first time, selecting the highly invasive A375 human melanoma cell line as clinical model expressing the $BRAF^{V600E}$ oncogenic mutation.

2 | MATERIALS AND METHODS

2.1 | Reagents and cell culture

The human melanoma cell lines A375 cells were purchased from Sigma-Aldrich (Milan, Italy), Sk-Mel-28 were purchased from IRCCS AOU San Martino—IST (Genoa, Italy), WM3060 and WM983B cells were purchased from Rockland (Limerick, Ireland). A375 and Sk-Mel-28 were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 μmol/L nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 1 mmol/L sodium pyruvate (all from Sigma-Aldrich, Milan, Italy). WM3060 and WM983B were cultured in Tumor Specialized Media (1:5 Leibovitz's—MCDB153), containing 2% inactivated FBS and 1.68 mM $CaCl_2$. Normal human epidermal melanocytes (NHEMs) were purchased from Lonza (Walkersville, Maryland) and were grown in melanocyte growth medium 2 (Lonza). Cells were grown at 37°C in a humidified incubator under 5% CO_2. All cell lines used in this study were characterized by the cell bank where they were purchased.

Cynaropicrin was diluted in DMSO to produce a stock solution of 10 mM for in vitro experiments.

2.2 | Sesquiterpene lactone isolation

Centaurea lactone and cynaropicrin were obtained from *Centaurea drabifolia* subsp. *detonsa* as reported (Formisano et al, 2017a). Vernodalol and vernolide were obtained from *Vernonia amygdalina* as reported (Sinisi et al., 2015). Daucovirgolides B, G, and F were obtained from *Daucus virgatus* as reported (Sirignano et al., 2017). Hydroxytigloyldeacylaguerin (HTDA), acydehydromelitensin (ADM), onopordopicrin, and elemacarmanin were obtained from *Onopordon illyricum* as reported (Formisano et al., 2017b).

2.3 | MTT assay

Cell proliferation was measured by the 3-[4,5-dimethyltiazol2yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. In preliminary experiments, A375 cells were seeded on 96-well plates at a density of 2×10^3 cells/well and treated with different concentrations (3–10-30-μM) of several sesquiterpene lactones (centaurea lactone; cynaropicrin; vernodalol; vernolide; daucovirgolides B, G, and F; HTDA; ADM; onopordopicrin; and elemacarmanin) for 72 hr. Afterwards, several melanoma cell lines (A375, Sk-Mel-28, WM3060 and WM983B at $2–5 \times 10^3$ cells/well) and one nonmelanoma cell line (NHEM at 1×10^4 cells/well) were seeded on 96-well plates and treated with cynaropicrin (0,1–3–10-30-μM) for 24 and 48 hr. After the treatment, MTT (Sigma, Milan, Italy) (25 μl of 5 mg/ml in saline) was added to each well. Cells were incubated for additional 3 hr at 37°C. Thereafter, cells were lysed and dark-blue crystals were solubilized with a solution containing 50% (vol/vol) N,N-dimethyl formamide, 20% (wt/vol) sodium dodecylsulfate with an adjusted pH of 4.5. The OD of each well was obtained by measuring the absorbance at 620 nm using Multiskan GO microplate reader (Thermo Fisher Scientific, MA, USA).

2.4 | Apoptosis assay

A375 were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with cynaropicrin (30 μM) for 24–48 hr, collected, and washed twice with PBS. Apoptosis was

detected using the Annexin V-FITC apoptosis detection kit (eBioscience. Thermo Fisher Scientific MA, USA) according to the manufacturer's instructions. Following the Annexin V and PI double staining, the cells were subjected to flow cytometric analysis. A minimum of 20,000 events for each sample were collected, and data were analyzed using BriCyte E6 (Mindray, P.R. China).

2.5 | Caspase-3/7 activity assay

ApoTox-Glo™ Triplex Assay (Promega Corporation, Madison, WI, USA) was used to assess the caspase-3/7 activity upon cynaropicrin treatment in A375 cells. Therefore, A375 were seeded in 96-well opaque-walled plates (3×10^3 cells/well) and treated with cynaropicrin (3–10-30 μM) for 24–48 hr. ApoTox-Glo assay was executed according to the manufacturer's instructions.

2.6 | Preparation of cellular extracts and western blot analysis

A375 cells were treated with cynaropicrin 30 μM for 24 and 48 hr. Whole-cell or nuclear extracts were prepared as previously described (De Cicco et al., 2017). The protein concentration was measured by the Bradford method (Bio-Rad, Milan, Italy). Equal amounts of protein (40 μg/sample) from whole or nuclear cell extracts were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes (Trans-Blot Turbo Transfer Starter System, Biorad). The membranes were blocked for 2 hr in 5% low-fat milk in PBS with 0.1% Tween 20 (PBST) at room temperature. Then the filters were incubated with the following primary antibodies: Bcl-2 (#2876; Cell Signaling, USA; diluted 1:1000), caspase-3 (#9662; Cell Signaling, USA; diluted 1:1000), PARP (#9542; Cell Signaling, USA; diluted 1:1000), p44/42 MAPK (Erk1/2) (#9102; Cell Signaling, USA; diluted 1:1000), phospho-p44/42 Erk MAPK (Erk1/2, Thr202/Tyr204) XP (#4370; Cell Signaling, USA; diluted 1:2000), NF-κB p65 XP (#8242; Cell Signaling, USA; diluted 1:1000), Nrf2 (sc-722; Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:100), β-actin (sc-47,778; Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:1000), GAPDH (#2118; Cell Signaling, USA; diluted 1:1000), and α-tubulin (#3873; Cell Signaling, USA; diluted 1:1000), overnight at 4°C. The membranes were washed three times with PBST and then incubated with antimouse or anti-rabbit horseradish peroxidase–conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA; diluted 1:2000) for 2 hr at room temperature. The immune complexes were visualized by the ECL chemiluminescence method and acquired by the Image Quant 400 system (GE Healthcare).

2.7 | Invasion assay

The assay was performed using chambers with polycarbonate filters with 8-μm nominal pore size (Millipore, USA) coated on the upper side with Matrigel (Becton Dickinson Labware, USA). Briefly, the chambers were placed into a 24-well plate, and A375 cells (2.5×10^5/ml) were plated in the upper chamber, with or without cynaropicrin (3–10 μM), in serum-free DMEM. After the incubation period (16 hr), the filter was removed, and nonmigrant cells on the upper side of the filter were detached with the use of a cotton swab. Filters were fixed with 4% formaldehyde for 15 min, and cells located in the lower filter were stained with 0.1% crystal violet for 20 min and then washed with PBS. The filters were examined microscopically and cellular invasion was determined by counting the number of stained cells on each filter in at least four–five randomly selected fields.

2.8 | Wound healing assay

A375 cells were seeded in 12-well plates ($2x10^5$ cells/well) and grown to full confluence. A 200 μl pipette tip was used to create a wound in the monolayer. Cynaropicrin (3–10 μM) was added after imaging of the wells with the microscope at 0 hours (wound induction). Imaging was repeated after 24 and 48 hr. An ImageJ macro, the MRI Wound Healing Tool (MRI Redmine), was used for calculating the area of the cell-free gap at 0–24-48 hr after wound induction.

2.9 | Clonogenic assay

A375 cells ($1x10^3$ cells/well) were seeded in six-well plates and treated with cynaropicrin 3 μM for 48 hr. After, fresh medium (drug-free) was changed every 2 days. Cells were cultured for 14 days to allow the colonies to form. Formed colonies were washed twice with 1xPBS, fixed by 4% paraformaldehyde, and stained with 0.5% crystal violet and colonies containing more than 50 cells (established by microscopy) were counted manually. Images of the colonies were obtained using a digital camera.

2.10 | RNA purification and quantitative real-time PCR (RT-qPCR)

A375 cells were treated with cynaropicrin 30 μM for 24 and 48 hr. Total RNA was isolated by using TRI-Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's instructions, followed by spectrophotometric quantization. Final preparation of RNA was considered DNA- and protein-free if the ratio between readings at 260/280 nm was ≥1.7. Isolated mRNA was reverse-transcribed by use of iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Milan, Italy). Then, quantitative real-time-PCR was performed using CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy). Primer sequences were:

HMOX-1:5′-GCCGTGTAGATATGGTACAAGGA-3′; 5′-AAGCCGAGAATGCTGAGTTCA-3′

GCLM: 5′-AGGAGCTTCGGGACTGTATCC-3′; 5′-GGGACATGGTGCATTCCAAAA-3′

GCLC: 5′GTTGGGGTTTGTCCTCTCCC-3′; 5′-GGGGTGACGAGG TGGAGTA-3′

Samples were amplified in triplicate using SYBR Green Master Mix kit (Bio-Rad, Milan, Italy). A control blank for each primer pair was used to control for contamination or primer nontemplate dimers formation, and the Ct value for each experimental group was determined. The S16 housekeeping gene was used as an internal control to normalize the Ct values, using the $2^{-\Delta Ct}$ formula.

2.11 | Intracellular ROS measurement

The generation of intracellular reactive oxygen species (ROS) was estimated using the fluorescence probe 2′,7′-dichlorfluorescein-diacetate (H2DCF-DA). For the experiments, A375 cells were plated in 96-multiwell black plates (Corning, USA) at the density of 3×10^3 cells per well and incubated with cynaropicrin (3–10-30 μM) for 24 hr. After washing, cells were incubated for 1 hr with 200 μl of 100 μM H2DCF-DA in HBSS containing 1% FBS. Finally, cells were rinsed and incubated with the Fenton's reagent (H_2O_2/Fe^{2+} 2 mM) for 3 hr at 37°C. The DCF fluorescence intensity was detected using a fluorescent microplate reader (excitation 485 nm and emission 538 nm; GloMax®-Multi Detection System, Promega). The intracellular ROS levels were expressed as fluorescence intensity after normalizing to viable cell numbers.

2.12 | Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed with a number of $n \geq 3$. Data were analyzed with GrapdPad Prism 6.0 software program (GraphPad Software, Inc., San Diego, CA, United States). Significance was determined using Student's two-tailed *t*-test. Results were considered significant at *p*-values ≤.05 and are labeled with a single asterisk. In addition, *p*-values ≤.01 and .001 are designated with double and triple asterisks, respectively.

3 | RESULTS

3.1 | Screening of a library of sesquiterpene lactones on melanoma cells

A small library of sesquiterpene lactones has been built up for evaluation in a screening test for anticancer activity against melanoma cells (Figure 1). The compounds included in the library belong to different structural classes and possess different acyl decorations on the sesquiterpene core. In particular, we have selected vernolide and vernodalol, obtained from *Vernonia amygdalina* (Sinisi et al., 2015), centaurea lactone, and cynaropicrin, obtained from aerial parts of *Centaurea drabifolia* subsp. *detonsa* (Bornm) Wagenitz (Formisano et al., 2017a), a plant endemic of Turkey, very popular to prepare infusions to be used both as refreshing drink and as traditional remedy with antiinflammatory applications (Honda et al., 1996). Daucovirgolides B, G, and F have been obtained from *Daucus virgatus* (Sirignano et al., 2017); hydroxytigloyldeacylaguerin (HTDA), acydehydromelitensin (ADM), onopordopicrin, and elemacarmanin from *Onopordon illyricum* (Formisano et al., 2017b). These compounds belong to the guaianolide, germacranolide, and elemanolide structural classes.

Antiproliferative activity of these compounds was evaluated against A375 human melanoma cells (Table 1) at 72 hr using the MTT assay. The most potent compound was cynaropicrin (IC_{50} = 25 μM) followed by onopordopicrin, vernolide, and vernodalol (IC_{50} = 35–42 μM).

3.2 | Cynaropicrin inhibits proliferation and induces apoptosis in human melanoma cells

To corroborate the antiproliferative effect of cynaropicrin an MTT assay was performed on several human metastatic melanoma cells (A375, Sk-Mel-28, WM983B, and WM3060). Treatment with cynaropicrin significantly reduced proliferation of melanoma cells in a time- and concentration-dependent manner (Table 2) which was also confirmed by the BrdU incorporation assay (Figure S1). The IC_{50} values for each cell line were, respectively, 25, 57, 27, and 162 μM following 48 hr of incubation with cynaropicrin. By contrast, cynaropicrin did not affect the proliferation of normal melanocytes NHEM (Table 2) demonstrating its sensitivity for malignant cells. On the basis of the IC_{50} values, we selected the A375 cell line for further experiments. Although similar values of the IC_{50} parameters were obtained for both A375 and WM983B cells, we decide to use A375 cells to conduct additional analysis because they show enhanced invasion capacity suggesting a more aggressive phenotype (Widmer et al., 2012). To further confirm the antiproliferative effect of cynaropicrin, we performed Annexin V/PI double staining analysis. A375 cells were treated with cynaropicrin (30 μM), and the percentage of cells undergoing apoptosis or necrosis was evaluated by flow cytometric analysis at 24 and 48 hr. Cynaropricrin treatment–induced apoptosis in a time-dependent manner (Figure 2a,b). In addition, our results showed that apoptosis was mediated by caspase-3/7 activation (Figure 2C), as also confirmed by the cleavage of caspase-3 in cynaropicrin-treated A375 cells. Figure 2D showed that the level of the cleaved active form of caspase-3 and of its substrate poly (adenosine diphosphate-ribose) polymerase (PARP) increased in response to cynaropicrin. We further assessed the expression of two antiapoptotic proteins, X-chromosome-linked inhibitor of apoptosis protein (XIAP), and B-cell lymphoma gene-2 (Bcl-2), whose expression is modulated by the transcriptional activity of NF-κB. Western blot experiments showed that cynaropicrin markedly decreased the expression of both antiapoptotic genes (Figure 2e) in A375 human melanoma cells.

3.3 | Cynaropicrin inhibits cell motility, invasion, and colony formation of human melanoma cells

Antimetastatic activity of cynaropicrin was further investigated by evaluating its ability to inhibit some of the malignant features of

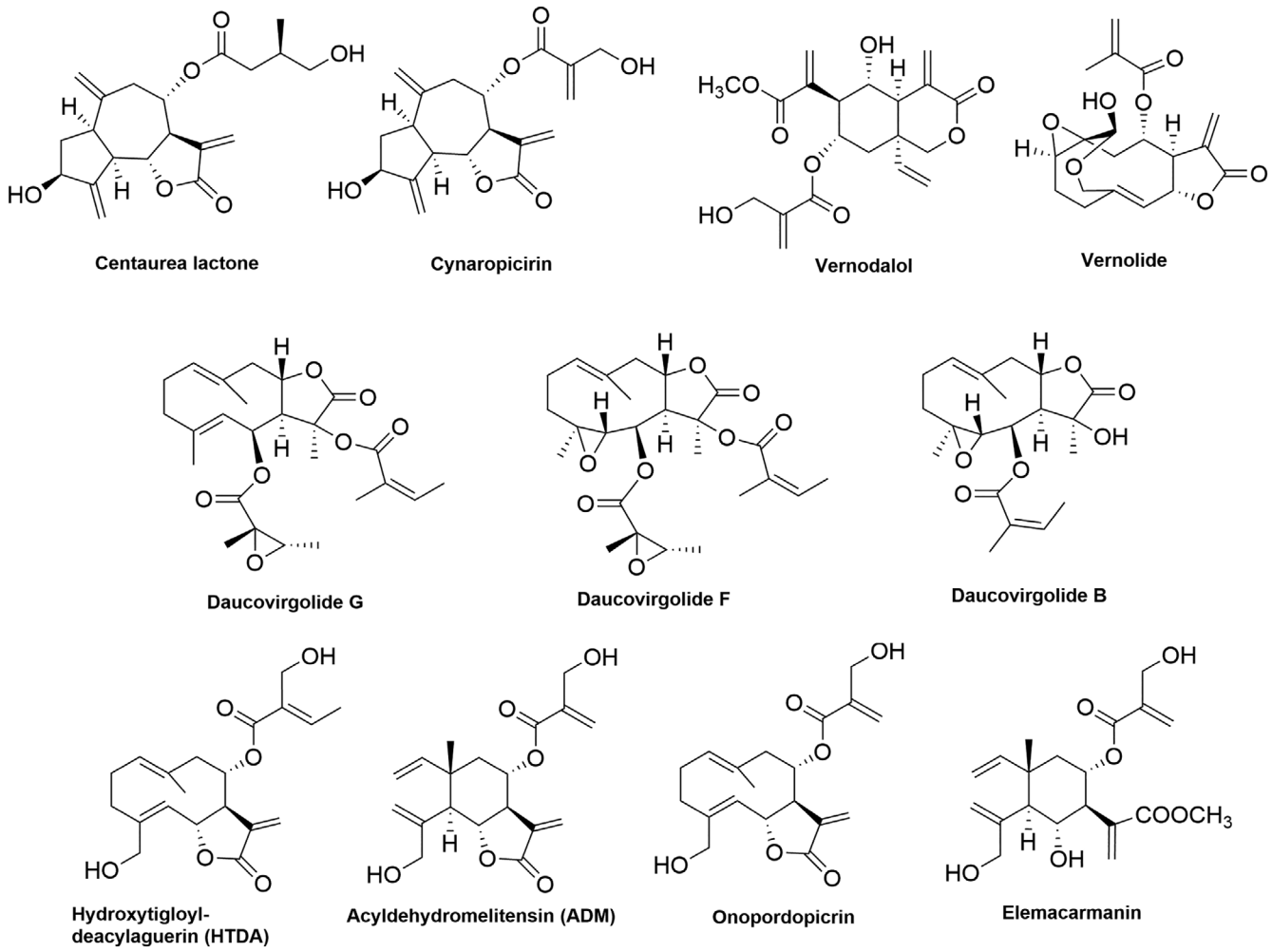

FIGURE 1 The library of sesquiterpene lactones evaluated for anticancer activity against melanoma cells

TABLE 1 Antiproliferative activity of sesquiterpene lactones against human melanoma cells

Compound	0 μM	3 μM	10 μM	30 μM	100 μM	IC_{50}(μM)
Centaurea lactone	0.765 ± 0.02	0.656 ± 0.02	0.653 ± 0.04	0.616 ± 0.02	0.251 ± 0.005	>100
Cynaropicrin	0.765 ± 0.02	0.651 ± 0.001*	0.536 ± 0.03***	0.165 ± 0.02***	0.123 ± 0.006***	25
Daucovirgolide G	0.866 ± 0.02	0.862 ± 0.02	0.834 ± 0.03	0.765 ± 0.03*	0.661 ± 0.07***	>100
Daucovirgolide F	0.866 ± 0.02	0.828 ± 0.01	0.781 ± 0.007*	0.741 ± 0.005**	0.671 ± 0.007***	>100
Daucovirgolide B	0.866 ± 0.02	0.865 ± 0.03	0.870 ± 0.01	0.879 ± 0.006	0.837 ± 0.005	Inactive
HTDA	0.783 ± 0.03	0.794 ± 0.05	0.789 ± 0.02	0.774 ± 0.02	0.741 ± 0.03	Inactive
ADM	0.783 ± 0.04	0.802 ± 0.03	0.819 ± 0.02	0.881 ± 0.04	0.725 ± 0.05	Inactive
Onopordopicrin	0.862 ± 0.02	0.815 ± 0.04	0.640 ± 0.02***	0.435 ± 0.03***	0.130 ± 0.002***	35
Elemacarmanin	0.866 ± 0.02	0.849 ± 0.03	0.814 ± 0.01	0.717 ± 0.009***	0.135 ± 0.001***	62
Vernodalol	0.919 ± 0.02	0.817 ± 0.03	0.460 ± 0.002***	0.248 ± 0.03***	0.202 ± 0.04***	39
Vernolide	0.765 ± 0.02	0.721 ± 0.03	0.521 ± 0.02	0.237 ± 0.01	0.192 ± 0.002	42

Note: A375 cells were treated with different concentration of tested compounds. Growth inhibition was measured at 72 hr using the MTT assay and is expressed as OD values ± SEM. 50% inhibitory concentration (IC_{50}) in μM is reported. Results were generated with three biological replicates each of them performed in technical quadruplicate (*p < .05; ***p < .001 vs. control [0 μM]).

TABLE 2 Effect of cynaropicrin on human melanoma cells proliferation

(A) 24 hr					
Cell line	**CTRL**	**Cyn 0.1 μM**	**Cyn 3 μM**	**Cyn 10 μM**	**Cyn 30 μM**
A375	0.459 ± 0.01	0.450 ± 0.02	0.432 ± 0.02**	0.362 ± 0.005***	0.274 ± 0.02***
SK-Mel-28	0.536 ± 0.008	0.516 ± 0.003	0.500 ± 0.001	0.488 ± 0.003***	0.475 ± 0.003***
WM983B	0.293 ± 0.01	0.285 ± 0.01	0.268 ± 0.004	0.234 ± 0.005***	0.200 ± 0.005***
WM3060	0.556 ± 0.007	0.532 ± 0.009	0.516 ± 0.002	0.475 ± 0.006***	0.431 ± 0.007***
NHEM	0.210 ± 0.003	0.222 ± 0.002	0.216 ± 0.002	0.198 ± 0.01	0.197 ± 0.005
(B) 48 hr					
Cell line	**CTRL**	**Cyn 0.1 μM**	**Cyn 3 μM**	**Cyn 10 μM**	**Cyn 30 μM**
A375	0.819 ± 0.03	0.736 ± 0.02	0.689 ± 0.007**	0.578 ± 0.008***	0.356 ± 0.03***
SK-Mel-28	0.758 ± 0.010	0.684 ± 0.020	0.630 ± 0.006***	0.615 ± 0.004***	0.540 ± 0.003***
WM983B	0.280 ± 0.008	0.275 ± 0.008	0.245 ± 0.006	0.169 ± 0.002***	0.141 ± 0.003***
WM3060	0.846 ± 0.01	0.853 ± 0.005	0.819 ± 0.002	0.793 ± 0.005	0.761 ± 0.01***
NHEM	0.256 ± 0.002	0.253 ± 0.003	0.229 ± 0.01	0.216 ± 0.003	0.221 ± 0.01

Note: Human melanoma cells (A375, Sk-Mel-28, WM983B, and WM3060) and normal melonocytes (NHEM) were incubated with different concentrations of cynaropicrin (cyn) (0.1–30 μM), and cell viability was determined after 24 and 48 hr by using the MTT assay. Data are expressed as mean ± SEM of OD values. Results were generated with three biological replicates each of them performed in technical quadruplicate (**$p < .01$; ***$p < .001$ vs. CTL).

melanoma cells, in vitro. Wound healing assay was used to determine whether sub-IC_{50} concentration of cynaropicrin could inhibit A375 cells motility. Treatment with cynaropicrin (3 and 10 μM) significantly and time-dependently reduced A375 cells migration as compared to untreated cells (Figure 3a,b).

A critical event in tumor invasion and metastasis is the ability of tumor cells to invade through the extracellular matrix, allowing tumor cells to move beyond the confines of primary tumor environment (Obenauf & Massague, 2015). To examine the effect of cynaropicrin on cell invasivity, Boyden chamber invasion assay was carried out. As shown in Figure 3c,d, treatment with cynaropicrin (3 and 10 μM) significantly reduced trans-well invasion of A375 cells across biological matrices. Furthermore, we found that cynaropicrin was able to reduce colony formation ability of A375 cells, which correlates with the capacity of tumor cells to produce progeny and form metastasis in vivo (Franken, Rodermond, Stap, Haveman, & van Bree, 2006). As shown in Figure 3e,f, sub-IC_{50} dose (3 μM) of cynaropicrin significantly reduces the number of colonies by 82% as well as focus diameter compared to untreated cells.

3.4 | Cynaropicrin inhibits the MAPK/ERK and NF-κB pathways in human melanoma cells

Among the various intracellular signalling pathways activated in melanoma cells, the NF-κB and the MAPK signaling pathways have been considered the crucial regulators in the initiation and development of cutaneous melanoma. Aberrant activation of the MAPK pathway is responsible for cell proliferation, survival, invasion, and motility. Therefore, we examined whether the ability of cynaropicrin to reduce the proliferation, migration, and invasion of melanoma cells was associated to the MAPK pathway activity. ERK, a downstream substrate of the MAPK signaling, is hyperactivated in about 90% of melanoma cells following BRAF mutation. Our results demonstrate that phosphorylation of ERK was significantly downregulated in A375 cells after treatment with cynaropicrin (30 μM) for 24 hr (Figure 3g). Mutational activation of BRAF has been also associated with an enhanced activity of NF-κB and increased survival of melanoma cells. NF-κB is the major antiapoptopic factor, inducing the transcription of several antiapoptotic proteins. Thus, constitutive induction of NF-κB lead to a consequent increase in proliferation rates and resistance to apoptosis of melanoma cells. In order to verify the effect of cynaropicrin on NF-κB activity, western blot analysis was carried out on nuclear extracts obtained from A375 cells. The p65/RelA protein, which is a component of NF-κB family normally overexpressed in melanoma cells, was reduced in the nuclei of cynaropicrin-treated A375 cells (Figure 3h).

3.5 | Cynaropicrin reduces ROS production and modulates the expression of antioxidant enzymes in human melanoma cells throughout the Nrf-2 pathway

Oxidative stress is associated with cancer development and progression by a direct mechanism involving DNA damage or indirectly by modulating cell signal transduction pathways. Therefore, we evaluated the ability of cynaropicrin to reduce oxidative stress in melanoma cells. The exposure of A375 melanoma cells to H_2O_2/Fe^{2+} (2 mM) produced a significant increase in ROS formation. Interestingly, pretreatment for 24 hr with cynaropicrin (10–30 μM) greatly reduced ROS formation as measured by the inhibition of DCF fluorescence intensity (Figure 4a). Since recent evidence suggests that phytochemicals' polyphenols content could induce endogenous antioxidant

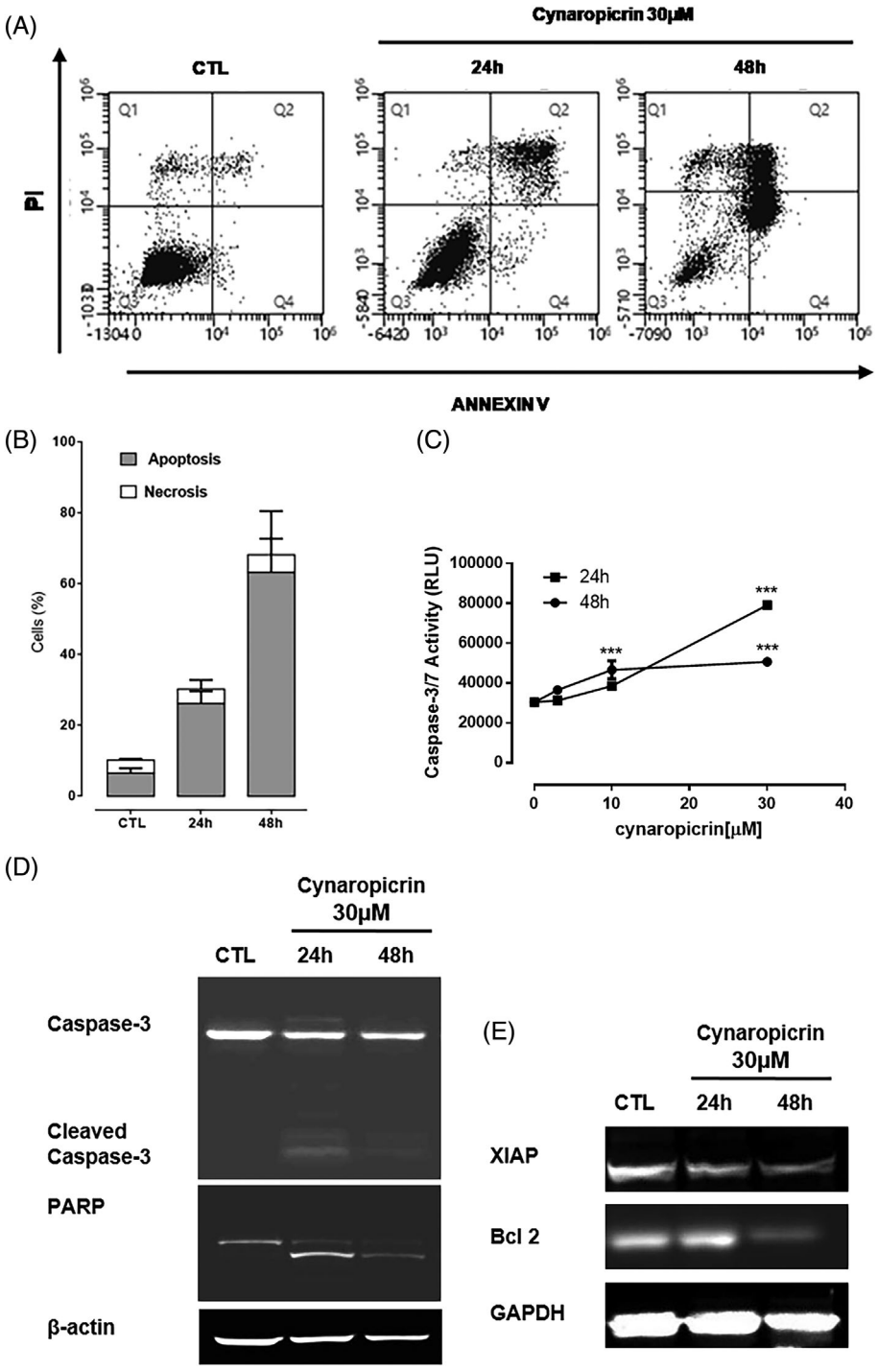

FIGURE 2 Cynaropicrin induces apoptosis in human melanoma cells. (a) Apoptosis was detected by Annexin V/propidium iodide (PI) staining in A375 cells treated with cynaropicrin (30 μM) for 24 and 48 hr by flow cytometric analysis. (b) Quantification of cell apoptosis at various time points. Data are expressed as percentage of apoptotic or necrotic cells. Experiments were performed in triplicate (n = 3). (c) Caspase 3/7 activity assay was performed in A375 cells treated with cynaropicrin (3–10-30 μM) for 24 and 48 hr. Results are expressed as relative luminescence unit (RLU). Experiments were performed in triplicate (n = 3). ***p < .001 versus cynaropicrin 0 μM. Western blot analysis of (d) caspase-3 and PARP and of (e) XIAP and Bcl-2 proteins expression carried out on A375 whole-cell lysates following the treatment with cynaropicrin (30 μM) for 24 and 48 hr. β-Actin and GAPDH were detected as loading control. The data shown are representative of three independent experiments (n = 3) with similar results

defense mechanisms by modulating transcription factors, such as the nuclear NF-E2-related factor 2 (Nrf2), we evaluated the total phenolic content (TPC) of cynaropicrin. The TPC of cynaropicrin, at our experimental condition (10-100 μM), was between 0.167 and 1.105 μmol GAE/ml (Figure S2). To investigate the cellular mechanism responsible for the ROS neutralizing activity of cynaropicrin in melanoma cells, we measured the effect of cynaropicrin on the expression of some antioxidant enzymes such as glutamate–cysteine ligase (GCL) and heme oxygenase-1 (HMOX-1). GCL, the rate-limiting enzyme involved in the de novo glutathione (GSH) synthesis, consists of catalytic (GCLC) and modulatory (GCLM) subunits. Treatment with cynaropicrin at 30 μM significantly enhanced the expression of both GCLC and GCLM mRNA in a time-dependent manner (Figure 4b,c). Similar results were obtained for HMOX-1 mRNA (Figure 4d). Most of the genes encoding phase II detoxifying and antioxidant enzymes are under the control of Nrf2, the major transcription factor involved in the cellular protection against oxidative stress. Therefore, we determine whether cynaropicrin could activate Nrf2 in A375 cells. As show in Figure 4e,f, cynaropicrin-treated cells displayed increased Nrf2 accumulation in the nuclear fraction.

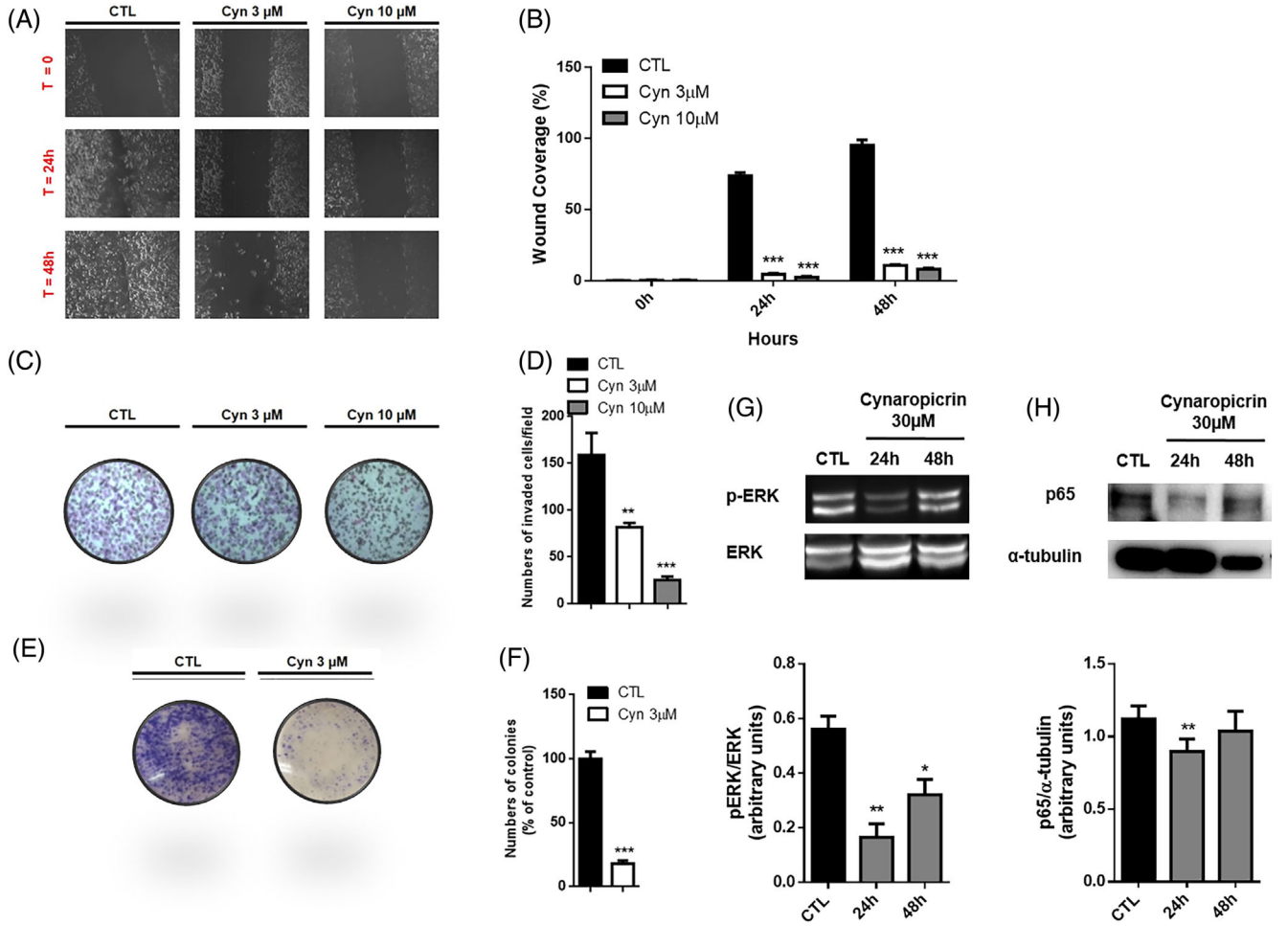

FIGURE 3 Cynaropicrin inhibits motility, invasion, and colony formation ability of A375 cells. (a) Representative photographs of migratory A375 cells treated with cynaropicrin (3–10 μM) at zero hour and at 24 and 48 hr later. (b) The scratched areas were quantified in three random fields in each treatment. Data are shown as mean ± SEM of three indipendent experiments (n = 3). ***p < .001 versus CTL. (c) Representative field of invasive A375 cells on the membrane after treatment with cynaropicrin (3–10 μM) for 16 hr. (d) Average number of invasive cells per field. Data are shown as mean ± SEM of three indipendent experiments (n = 3). ***p < .001 versus CTL. Representative photographs (e) and average number (f) of A375 stained colonies following the treatment with cynaropicrin (3 μM). Data are shown as mean ± SEM of three indipendent experiments (n = 3). **p < .01, ***p < .001 versus CTL. Representative western blot, and relative quantitative analysis, of phosphorilated ERK and total ERK (g) and nuclear p65 (h) proteins carried out on lysates obtained from A375 cells treated with cynaropicrin (30 μM) for 24 and 48 hr. α-tubulin was detected as loading control. Experiments were performed in triplicate (n = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

4 | DISCUSSION

Despite the increased knowledge of the biology and pathogenesis of melanoma reached in the last years, its incidence has grown considerably throughout the world, making the development of preventive measures particularly urgent. A great number of studies have been published recently investigating the beneficial role of natural dietary compounds in the prevention and adjuvant effect in the therapy of malignant melanoma. Because of their general safety, low toxicity, and antioxidant properties, fruit, vegetables, and other dietary elements (phytochemicals and minerals) have been identified as chemopreventive agents (Ombra et al., 2019).

In this study, we have screened a library of plant-derived sesquiterpene lactones in order to select compounds active in vitro against A375 melanoma cells. Obtained results indicated cynaropicrin, an abundant constituent of artichoke and of other numerous plants belonging to the Asteraceae (Compositae) family, as a promising hit. Moreover, a series of preliminary information could be drawn by the activities exhibited by the other compounds of the library. The presence of an α-hexomethylene-γ-lactone appears to be necessary for activity (the majority of bioactive compounds show this structural feature) but not sufficient. In this sense, very interesting appears the comparison between the activities of centaurea lactone and cynaropicrin: although the two compounds share the same sesquiterpene core, cynaropicrin showed a much higher activity, indicating that also the acylating groups play a key role in the modulation of bioactivity.

Cynaropicrin proved to reduce the proliferation of human metastatic melanoma cells in a time- and dose-dependent manner. The

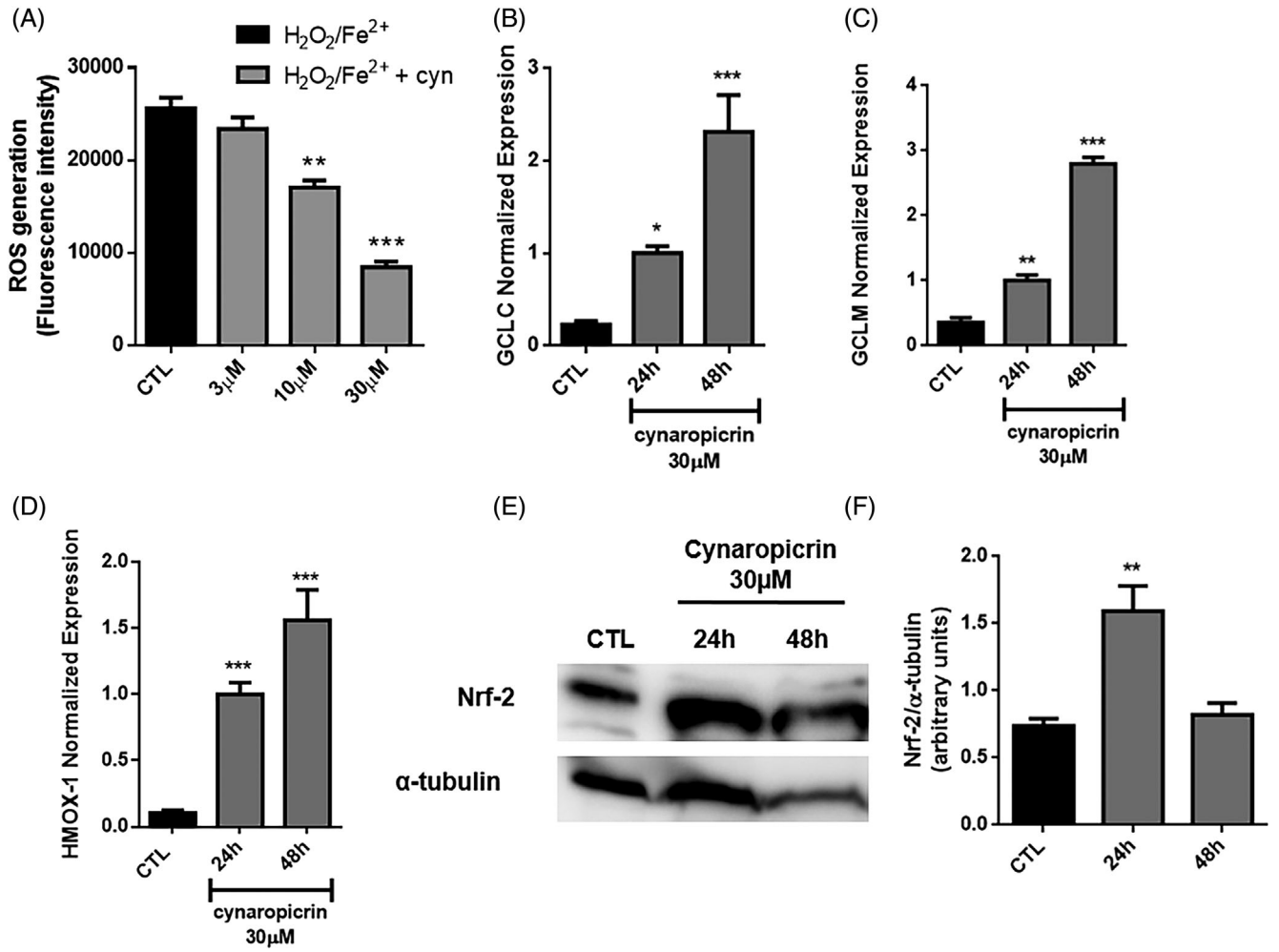

FIGURE 4 Cynaropicrin reduces ROS production and modulates the expression of antioxidant enzymes in human melanoma cells throughout the Nrf-2 pathway. (a) Intracellular ROS levels were measured with a fluorescent dye DCFH2-DA in A375 cells treated with Fenton's reagent (2 mM H_2O_2/Fe^{2+}) alone or in presence of cynaropicrin (3–30 μM). Values are expressed as fluorescence intensity normalized to viable cell numbers. Results are show as mean ± SEM of three indipendent experiments (n = 3). *p < .05, **p < .01 versus H_2O_2/Fe^{2+} alone. mRNA expression levels of GCLC (b), GCLM (c), and HMOX-1 (d) in A375 cells treated with cynaropicrin (30 μM) for 24 and 48 hr. Data are shown as mean ± SEM of three indipendent experiments (n = 3), each performed in technical quadruplicate. *p < .05, **p < .01, ***p < .001 versus CTL. Representative blot (e) and relative quantitative analysis (f) of Nrf-2 protein expression carried out on the nuclear extracts obtained from A375 cells treated with cynaropicrin (30 μM) for 24 and 48 hr. α-tubulin was detected as a loading control. Experiments (n = 3) were performed in triplicate

results of the MTT assay demonstrated that all BRAF mutant melanoma cells (Sk-Mel-28, A375, WM983B) were more sensitive to cynaropicrin treatment, with lower IC_{50}, compared to BRAF wild-type melanoma cells (WM3060). Interestingly, cynaropicrin demonstrated to be selective for cancer cells since it did not affect the proliferation of normal melanocytes. The antiproliferative effect of cynaropicrin was mediated by the induction of apoptosis in human melanoma cells A375 as demonstrated by the cytofluorimetric studies with Annexin V and PI. Caspase-3 is a frequently activated death protease which cleaves PARP, a DNA repair enzyme. Our results clearly demonstrate that cynaropicrin-induced apoptosis was mediated by caspase pathway activation as consequence of caspase-3 and PARP cleavage in cynaropicrin-treated A375 cells. In addition, the expression of anti-apoptotic proteins such as Bcl-2 and XIAP was also reduced in melanoma cells following the treatment with cynaropicrin. Collectively, these results suggest that cynaropicrin is an apoptosis-inducing natural product derivative. Cutaneous melanoma is an aggressive malignancy due to its high metastatic ability. Patients with metastatic melanoma show poor prognosis with 5-year survival rate less than 15% (Tas, 2012). Melanoma metastasis formation is related to the capacity of melanoma cells to acquire distinctive features such as: motility and invasion, plasticity, and ability to colonize secondary tissues (Welch & Hurst, 2019). Thus, chemopreventive agents should be also able to prevent or delay tumor promotion and progression. We found that cynaropicrin significantly reduced migration, invasion, and clonogenic ability of human melanoma cells. A growing body of evidence supports that constitutive activation of the Ras/Raf/MEK/ERK (MAPK) signaling pathway in melanoma is associated with increased

migratory and metastatic capacity (Testa, Castelli, & Pelosi, 2017). In addition, MAPK also promotes hyperactivation of the transcription factor NF-κB that regulates the expression of many antiapoptotic, proproliferative, and prometastatic genes (Liu et al., 2007). In melanoma, the NF-κB signaling pathway has been also found to be hyperactivated, and it plays an important role in cell survival, invasion, and metastasis (Ueda & Richmond, 2006). Thus, inhibition of MAPK and NF-κB pathways represents the most promising strategy to prevent melanoma invasion and metastasis. In the present study, we found that cynaropicrin greatly reduced the activation of ERK as well as the translocation of NF-κB p65 in to the nucleus suggesting the potential mechanism underlying its antiproliferative, antiinvasive, and antimigratory activity.

Oxidative stress also contributes to melanoma initiation and progression either by a direct mechanism involving DNA damage or indirectly by activating protumorigenic pathways (Cannavo et al., 2019). The importance of oxidative stress in melanoma is reinforced by the findings that mutations in several melanoma-associated genes, including the common somatic $BRAF^{V600E}$ mutation, result from ROS activity (Meierjohann, 2014). Secondarily, ROS promotes several signaling pathways, including MAPK, leading to cell proliferation and tumor initiation and promotion (Venza et al., 2015). Finally, elevated levels of ROS contribute to progression phase of carcinogenesis by generating additional genomic instability that increases the metastatic potential of tumor cells (Ferraro et al., 2006). Therefore, modulation of ROS emerges as a crucial mechanism for chemopreventive agents in order to stop or delay the occurrence of malignancy. For years, the dietary intake of foods that are naturally rich in antioxidants as well as antioxidant supplementation has been suggested to the public as a way to protect against cancer (Chikara et al., 2018). Nonetheless, the role of ROS and antioxidants in cancer is controversial because of their context-dependent ability to promote or suppress tumorigenesis. In particular, in melanoma context, it has been recently reported that ROS limit distant metastasis, while antioxidants promote disease progression (Piskounova et al., 2015). In our study, cynaropicrin demonstrated endogenous antioxidants properties as well as the abitity to reduce intracellular ROS generation in A375 cells in vitro, which may be associated with the proapoptotic effect. However, the antioxidant effect of cynaropicrin in metastasis development in vivo raises intriguing questions that remain to be address. An important role in the regulation of cellular redox balance in mammals is played by the transcription factor Nrf-2, which is considered as a master regulator of antioxidant response. Nrf-2 regulates the expression of protective antioxidant and phase II detoxificant enzymes including GCL, HMOX1, thioredoxin reductase 1 (Txnrd1), and NAD(P)H-quinone oxidoreductase 1 (NQO1) (Kansanen, Kuosmanen, Leinonen, & Levonen, 2013). These enzymes protect against the development of cancer by catalyzing reactions that convert highly reactive, carcinogenic chemicals to less reactive products. Thus, Nrf-2 represents an attractive target for cancer cells, and Nrf-2 inducers could be beneficial in cancer treatment approaches. Our results demonstrate that cynaropicrin increases Nrf-2 activity in melanoma cells and the expression of its antioxidant target gene, such as GCL and HMOX-1, suggesting that inactivation of ROS via the Nrf-2 pathway is one of the contributing factors toward the chemopreventive potential of cynaropicrin.

Natural products are a prolific source of cytotoxic compounds for cancer treatment, while less attention has been paid to natural compounds with antimigratory properties. In this light, the evidence shown in our study for a protective role of cynaropicrin in melanoma carcinogenesis, including cell proliferation inhibition, apoptosis induction, antimetastatic, and antioxidants potential, modulation of protumorigenic signaling pathways, assumes great relevance. Thus, cynaropicrin may serve as a promising agent in the development of therapeutic strategies to reduce the risk of melanoma development as well as to delay or inhibit melanoma progression.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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