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Antioxidant and antiproliferative potential of ethanolic extracts from *Moringa oleifera*, *Tropaeolum tuberosum* and *Annona cherimola* in colorrectal cancer cells

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ABSTRACT

Moringa oleifera, *Tropaeolum tuberosum* and *Annona cherimola* are medicinal plants traditionally used in Ecuador. However, their therapeutic properties are not completely known. We analyzed chromatographically ethanolic extracts of the seeds of *M. oleifera*, *A. cherimola* and the tubers of *T. tuberosum*; all presented a high content of polyphenols. The extract of *A. cherimola* showed the highest antioxidant activity and *M. oleifera* had the highest capacity to enhance the activity of detoxifying enzymes such as glutathione S-transferase and quinone oxidoreductase. The antitumor effect of these extracts was evaluated in vitro with colorectal cancer (CRC) cell lines T84, HCT-15, SW480 and HT-29, as well as with cancer stem cells (CSCs). *A. cherimola* and *M. oleifera* extracts presented the lowest IC_{50} in T-84 and HCT-15 (resistant) cells, respectively, as well as the highest level of inhibition of proliferation in multicellular tumor spheroids of HCT-15 cells. The inhibitory effect on CSCs is noteworthy because in vivo, these cells are often responsible for cancer recurrences and resistance to chemotherapy. Moreover, all extracts showed a synergistic activity with 5-Fu. The antiproliferative mechanism of the extracts was related to overexpression of caspases 9, 8 and 3 and increased production of reactive oxygen species. In addition, we observed cell death by autophagy in *M. oleifera* and *T. tuberosum* extracts. Therefore, these ethanolic extracts are excellent candidates for future molecular analysis of the presence of bioactive compounds and in vivo studies which could improve colon cancer therapy.

1. Introduction

After breast and lung cancer, colorectal cancer (CRC) is the third most common tumor type worldwide, with 1.9 million new cases diagnosed in 2020, representing 10% of all neoplasms. In addition, after lung cancer it was the second leading cause of cancer deaths (a total of 935,173 deaths in 2020) accounting for 9.4% of cancer mortality [1]. The etiology of CRC involves multiple factors, including a history of colon polyps, inflammatory bowel diseases, diabetes mellitus, the gut microbiome, and lifestyle factors such as inappropriate dietary patterns, obesity, physical inactivity, and tobacco and alcohol use, among others [2]. As a result of these factors, cells at the base of colon crypts form polyps that progress to adenomas and eventually become cancerous [3]. Even though surgical treatment in non-metastatic CRC patients shows acceptable results, chemotherapy is still required in metastatic cases. Of note, the severe side effects and low and non-selective antitumor efficacy of the latter are associated with a poor patient prognosis.

In this context, new strategies are needed to improve CRC therapies, including research into the activity of various plant extracts or derivatives that have been widely demonstrated to show antioxidant and antitumor properties and have thus, garnered great interest in recent years [4]. In addition, because CRC is a multifactorial disease, it may be

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best managed with a polypharmacological therapeutic approach. These natural extracts or their bioactive components could be applied to address multiple targets and to enhance the therapeutic effect of chemotherapeutic agents such as 5-fluorouracil (5-FU), oxaliplatin, or cisplatin. This would reduce the concentrations of chemotherapy drugs required to achieve the same effect and would thereby limit adverse effects and minimize the destruction of healthy tissue [5].

Plants traditionally grown in Ecuador such as *Tropaeolum tuberosum*, *Annona cherimola*, and *Moringa oleifera* have already exhibited therapeutic properties which suggest their possible application in cancer treatments [6–8]. In fact, plants in the Tropaeolaceae family have been widely used in the treatment of lung, skin, venereal, renal, and prostate diseases, among others. This family contains bioactive metabolites such as alkaloids, flavonoids, anthocyanins, tannins, hydroxybenzoic acids, isothiocyanates, flaks, and phytosterols [9] some of which exhibit antitumor effects. In fact, two alkaloids isolated from the black tubers of *T. tuberosum* were recently shown to exhibit cytotoxic activity against prostate, renal, urinary bladder, and lung cancer cell lines (PC-3, Caki-1, T24, and A549, respectively) by inducing apoptosis via the mitochondrial pathway [10]. In addition, N-benzyl linoleamide analogues compound derived from this plant have shown anti-inflammatory properties evaluated in brain cell lines (C8-D1 A, Neuro-2a, and EOC 13.31) by activating the NF-kB pathway [11].

Similarly, *A. cherimola*, a member of the Annonaceae family, has long been used as a traditional product to treat parasitation diseases, diabetes, peptic ulcers, and cancer [12]. Specifically, the annonaceous acetogenin metabolites (ACG) present in these plants seem to be responsible to their antitumor activity against lung (A-459), breast (MCF-7), colon (HT-29), prostate (PC-3), pancreatic (MIA PaCa-2), and kidney (A-498) cancer cells [13]. Likewise, *M. oleifera*, which is widely used in traditional phytomedicine due to its antibacterial, antioxidant, and anti-inflammatory activities [14], contains bioactive molecules (alkaloids, polyphenols, and terpenes) that have been related to significant antitumor activity [15]. Recently, an aqueous extract of *M. oleifera* leaves showed in vitro and in vivo antiproliferative activity in a murine model of Ehrlich ascites carcinoma, which was attributed to the presence of bioactive compounds such as quinic acid, palmitic acid, and ɣ-sitosterol [16]. Similarly, the ethanolic extract of *M. oleifera* fruits inhibited the proliferation of HepG2 liver cancer tumor cells [17]. Furthermore, an aqueous and methanolic extract of this plant showed synergistic activity with the agent 5-Fu against HCC 1395 (breast), DU145 (prostate) and Hela (cervical) cancer cell lines [18].

Thus, the objective of this research was to determine the antioxidant and antitumor potential of ethanolic extracts from the seeds of *M. oleifera*, *T. tuberosum,* and *A. cherimola* species against CRC cell lines and to analyze the molecular mechanisms of their activity. In addition, we analyzed the therapeutic benefit of associating the use of these ethanolic extracts with 5-FU, one of the antitumor drugs of choice in current CRC therapies.

2. Materials and methods

2.1. Chemicals and reagents

5-Fluorouracil, hydrogen peroxide solution, trizma® base, gallic acid, (glutathione (GSH, reduced form), 1-chloro-2,4-dinitrobenzene (CDNB), β-nicotinamide adenine dinucleotide (NAD, reduced disodium salt hydrated), flavin adenine dinucleotide disodium (FAD, salt hydrated), 2.6-dichloroindophenol (2.6-DCIP, sodium salt hydrate), DL-Sulforaphane (SFN), N-Acetyl-L-cysteine were purchased from Sigma-Aldrich (Madrid, Spain).

2.2. Cell culture

Human CRC cells T84, HCT-15 (resistant to chemotherapy), SW480, HT-29 and CCD-18 (human colon epithelial cell line) cells were obtained from American Type Culture (ATCC) and Scientific Instrumentation Center (CIC, Granada University, Granada, Spain). All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Madrid, Spain) and antibiotics (gentamicin/amphotericin-B + penicillin/streptomycin) (Sigma Aldrich, Madrid, Spain) at 1% and maintained in an incubator at 37 C and 5% CO2 humidified atmosphere.

2.3. Preparation the extract

The tuber of *T. tuberosum* was first lyophilized prior the extraction of its components. The dried seeds of *M. oleifera*, *A. cherimola* and tuber of *T. tuberosum* were ground to a fine flour with a particle size of 100–150 μM and preserved at −80 °C. Flour (5 g) was extracted with 15 mL of an extraction solution (50:50:0.25p ethanol: water: 12 N HCL) at 4 °C, pH 2, in a nitrogen atmosphere for 30 min using a magnetic stirrer. After 30 min stirring, the extract was centrifuged at 3500 rpm, 4 °C for 5 min. The supernatant was collected and stored and the pellet re-extracted with 10 mL of extraction solution under the same conditions above. Finally, the two supernatants obtained were mixed, aliquoted (1 mL), and stored at −80 °C. For the treatment of cell lines with the extracts, ethanol was evaporated using a Savant DNA 120 evaporation system (Thermo Scientific) to avoid toxic effects on cells. To assess the concentration of the extract, three ethanol-evaporated aliquots derived from 1 mL of ethanol extract each were lyophilized (TESLSTAR Cryodos-50) for 24 h.

2.4. Characterization of antioxidant capacity

Total polyphenol content of the ethanolic extracts was measured by the Folin-Ciocalteu methodology using gallic acid as calibration curve samples (0–500 μg/mL) as described by Kapravelou et al. [19]. The results were expressed as μg gallic acid equivalents (GAE) per mg of sample. ABTS assay was used to determine the total antioxidant capacity of the extracts according to the methodology described by described by Cabeza et al. [20]. A standard curve of GA concentrations ranging from 0 to 60 μg/mL was used in the analysis. The results were expressed as μg of gallic acid equivalent (GAE) per mg of sample.

2.5. Antioxidant capacity assay in cell culture

To determine the antioxidant capacity of ethanolic extracts in cell culture, HT-29 cells were seeded in 96-well plates (5×10^4 cells/well). After 24 h incubation, the media was replaced by serum-free medium. One day later, ethanolic extracts were added using a non-cytotoxic dilution and incubated for another 24 h. The supernatant containing the ethanolic extracts was then discarded and the oxidizing agent H_2O_2 was added using two concentrations (2 mM and 3 mM), incubated for 6 h and subsequently replaced by serum-free medium. The cells were then incubated for an additional period of 12 h and cellular viability was assessed using MTT (3-(4,5-Dimethylthiazol-2-yl)−2,5-Diphenyltetrazolium Bromide) Sigma-Aldrich (Madrid, Spain). Briefly, 30 μL of MTT was added per well for 4 h in culture conditions. Then, the medium was discarded, and 200 μL of dimethyl sulfoxide (DMSO) Sigma-Aldrich (Madrid, Spain) plus 25 μL of Sorensen's glycine buffer (glycine 0.1 M, NaCl 0.1 M, pH 10.5 with 0.1 NaOH) were added per well to dissolve formazan crystals. After 5 min of incubation at room temperature, the optical density of the wells was measured at 570 nm and a reference wavelength of 690 nm (Titertek multiscan Colorimeter, Flow, Irvine) to determine the relative proliferation (%RP) of treated cells. by the MTT assay. The results of this test were expressed as Antioxidant Activity Units (AAU), which is defined as the value of 10% units (10%) recovery of cell viability with respect to the corresponding control treated with hydrogen peroxide (H_2O_2).

2.6. Detoxifying enzyme induction capacity

2.6.1. Treatment and Purification of the Cytosolic Fraction

HT29 colon adenocarcinoma cells were seeded in T25 culture flask (1×10^6) and incubated for 24 h. Then, cells were exposed to the ethanolic extracts of the seeds using non-cytotoxic doses for 48 h. Sulforaphane was used as positive control at two concentrations (5 μM and 10 μM). After this incubation period of 48 h, the medium was removed and the cells washed with PBS and trypsinized. Trypsin activity was neutralized with 2 mL of DMEM and cells were transferred to 1.5 mL eppendorf tubes and centrifuged at 10000g, 4 °C for 5 min. The supernatant was discarded and the pellet was re-suspended into 500 μL of PBS and centrifuged under the same conditions. PBS was discarded and the cells were re-suspended into 500 μL of 25 mM Tris-HCl, pH-6.4. The cells were then lysed by sonication for 10 s to 40% frequency in ice and centrifuged at 10000g for 5 min at 4 °C. The cytosolic supernatant was used to determine the enzymatic activity of GST and QR.

2.6.2. Glutathione S-transferase (GST) assay

The GST enzyme catalyzes the nucleophilic addition of glutathione to an electrophilic center found in xenobiotics, deactivating cytotoxic and genotoxic compounds. This enzyme does not usually operate at its maximal capacity, but can be induced by a variety of natural compounds, thereby exerting efficient protection against carcinogenesis. The GST assay is measured by observing the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) (molar extinction 9.6 mM^{-1} cm^{-1}) with reduced glutathione (GSH). The reaction mix contained 980 μL of 100 mM phosphate buffer (pH 6.5), 10 μL of 100 mM CDNB, 10 μL of 100 mM reduced glutathione (GSH). 100 μL of each sample (cytosolic supernatant) was added to a cuvette containing 1 mL of the reaction mix and the absorbance was measured at 340 nm each minute for 5 min. To the blank cuvette 100 μL of PBS was added to the reaction mix. The GST activity was calculated as the increase in absorbance per min per mg total protein of the sample.

2.6.3. NAD(P)H: quinone oxidoreductase (QR) assay

Quinone oxidoreductase is a cytosolic flavoprotein that prevents the toxicity of quinones and quinoneimines by reducing them to their corresponding hydroquinones using both NADH and NADPH as donors, avoiding the generation of semi-quinonic intermediaries, which have a high tendency to react with oxygen resulting in superoxide. The QR assay is measured by observing the reduction of 2.6-dichloroindophenol (2.6-DCPIP) (molar extinction 0.0205 μM-1/cm) by QR. The reaction mix contained 881.5 μL of 25 mM Tris-HCl (pH-6.5), 60 μL of BSA (1 mg/mL), 2.5 μL of Tween (20%), 5 μL of 10 μM FAD, 10 μL of 20 mM NADH, and 16 μL of 5 mM DCPIP. 25 μL of each sample (cytosolic supernatant) was added to a cuvette containing 1 mL of the reaction mix and the absorbance was measured at 600 nm each minute for 5 min. For the blank cuvette 25 μL of Tris-HCl was added to the reaction mix. The QR activity was calculated as the decrease in absorbance per min per mg total protein of the sample.

2.7. Chromatographic analyses

The analysis of constituents with biological activity present in the ethanolic extracts of seed flours was analyzed by Ultra Performance Liquid Chromatography (UPLC) coupled with a Quadrupole Time of Flight (QTOF) Mass Spectrometer (Synap G2, Waters, Milford, MA, USA). The polyphenols were separated analytically by an Acquity HSS T33 analytical column (100 mm × 2.1 mm internal diameter, Waters, Milford, MA, USA). The mobile phase of the column consisted of a gradient formed by solvent A (deionized water with 0.5% acetic acid), and solvent B (acetonitrile with 0.5% acetic acid). The flow rate of the mobile phase was 0–4 mL/min. High-resolution mass spectrometry analysis was carried out in negative electro spray ionization (ESI-eve) and spectra recorded over a 50–1200 mass/charge (m/z) range. The chromatograms were analyzed using the MassLynx V4.1 program and the compounds were validated by analyzing at least 3 sub-fragments obtained from the CHEMnetBase and Chemspider database.

2.8. Cell viability assay

To investigate the effect of ethanolic extracts on CRC cell proliferation, T-84 (4×10^3 cells/well), HCT-15 (5×10^3 cells/well), SW480 (5×10^3 cells/well) and CCD18 (4×10^3 cells/well) were seeded in 48-well plates and incubated overnight. After 24 h, cell cultures were exposed to the ethanolic extracts dissolved in DMEM. Previously, extracts were evaporated to remove ethanol toxicity. Then, cell cultures were exposed to increasing concentrations of the evaporated ethanolic extracts for 72 h. In addition, combined therapy using ethanolic extracts (*M. oleifera, T. tuberosum* and *A. cherimola*) associated to 5-Fu (1.5–5 μM) was tested. After treatment exposure (72 h), cell viability was determined by sulforhodamine B. Cells were fixed with 10% trichloroacetic acid (TCA) (20 min at 4 C). Once dried, the plates were stained with 0.4% sulforhodamine B (SRB) in 1% acetic acid (20 min, in agitation). After three washes with 1% acetic acid, SRB was solubilized with Trizma® (10 mM, pH 10.5). Finally, the optical density (OD) at 492 nm was measured in a spectrophotometer EX-Thermo Multiskan. Cell survival (%) was calculated according to the following equation: Cell survival (%) = Treated cells OD – blank/Control OD − blank × 100. In addition, half maximal Inhibitory Concentration (IC_{50}) was calculated (GraphPad Prism 6 Software, La Jolla, CA, USA). For the combination effect the combination index (CI) was calculated using the Compusyn software (Chou and Martin, 2005), where a CI> 1 indicates antagonism, where a CI level of < 1 indicates synergy and a CI level equal to 1 indicates additivity.

2.9. Cell viability in MTSs cultures

We selected HCT-15 cell to generate multicellular tumor spheroids (MTS) and to investigate the effect of ethanolic extracts and combined therapy (ethanolic extracts +5-Fu) in an experimental system that mimics the primary tumor in vivo. HCT-15 cells (1.5×10^4 cells/well) were seeded in 96-well plates containing an agarose surface (50 μL). The plate was centrifuged at 900g for 15 min and incubated during 3 days. Then, MTSs were treated with ethanolic extracts ($1.5 \times IC_{50}$, $2 \times IC_{50}$, and $4 \times IC_{50}$) alone or in combination to 5-Fu (2 μM). At the end of the exposure time cell proliferation was tested with the Cell Counting Kit-8 (CCK-8). (Dojindo Molecular Technologies, Inc). Briefly, CCK-8 was added to each well to reach a final concentration of 10%. After 4 h of incubation, optical density of the wells was measured at 450 nm and a reference wavelength of 620 nm (Titertek multiscan Colorimeter, Flow, Irvine) to determine the relative proliferation (%RP) of MTS.

2.10. Cell cycle analysis

HCT-15 Cells were seeded in 6-well plates (1.5×10^4 cells). After 24 h, the culture medium was removed, and a serum-free culture medium was added to arrest the cell cycle. Then, the culture medium was replaced by DMEM with ethanolic extracts (IC25 and IC_{50}) and combined therapy (ethanolic extracts (IC10 and IC20) + 5-Fu (2 μM) for 48 h. Then, cells were trypsinized, fixed with 70% ethanol in agitation at 4 C (1 h) and washed twice with PBS. Finally, cells were processed using the PI/RNASE Solution Kit (Immunostep, Salamanca, Spain) to quantify the total content of cellular DNA by FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using FlowJo software (Treestar, Ashland, OR, USA), determining the phase of the predominant cell cycle.

2.11. Western blot analysis

HCT-15 cells exposed to the ethanolic extracts (2 ×IC50 during 12 h and 24 h) were collected and centrifuged and total proteins were extracted using Radio-Immunoprecipitation Assay (RIPA) lysis buffer

(Thermo Fisher Scientific, Waltham, MA, USA). Protein concentration was determined by Bradford and aliquots of the lysated (40 ug protein) were heated at 95 °C for 5 min and separated in a 12% SDS-PAGE gel using a Mini Protean II cell (Bio-Rad, Hercules, CA). Proteins were transferred to a nitrocellulose membrane with a 45 μm pore size (200 V at room temperature for 1 h) (Millipore) and treated with blocking solution (Phosphate-Buffered Saline (PBS)− 0.1% Tween-20 + 5% (w/v) milk powder) for 1 h. After washing three times with PBS-0.1% Tween-20, membranes were incubated with the primary antibody overnight at 4 °C (mouse polyclonal Immunoglobulin G (IgG) anti-caspase-3 (sc-271759; 1:500 dilution), anti-caspase-8 (sc-166320; 1:1000 dilution), anti-caspase-9 (sc-133109; 1:1000 dilution) and anti-MAP LC3β (1:1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After three washes, the membranes were incubated for 1 h at room temperature with the secondary antibody peroxidase conjugate (1:5000 dilution) (Goat anti-mouse IgG-HRP, Santa Cruz Biotechnology, CA, USA). In addition, anti-actin IgG (A3854, Sigma Aldrich, Madrid, Spain) (1:10,000 dilution) was used as an internal control. Signals were detected by an ECLTM Western blot detection reagent (Enhanced Chemiluminescence; Bonnus, Amersham, Little Chalfont, UK). Once the Western blot was performed, the bands obtained in the gels were analyzed using Quantity One analytical software (Bio-Rad, Hercules, CA, USA).

2.12. Lysotracker labeling

To determine apoptosis by autophagy, HCT-15 cells (1.5 $\times 10^4$ cells) were seeded in 8 well Culture Slides (Corning, USA), exposed to ethanolic extracts (IC_{50}) during 24 h and stained and loaded with Lysotracker (50 nM) for 30 min at 37 C (LysoTracker® Red DND-99, Thermo Fisher Scientific, Waltham, MA, USA). Cells were washed with PBS and stained with DAPI (1:1000). Finally, autophagy vesicles were observed under fluorescence microscopy.

2.13. Determination of cellular reactive oxygen species

Reactive oxygen species (ROS) were assessed using flow cytometry and DCFDA/H2DCFDA cellular ROS Assay Kit (Abcam, Spain) according to the manufacturer's instruction. Briefly, T84 cells seeded in 6-well plates (1.5 $\times 10^4$ cells/well) were exposed to ethanolic extracts (IC_{25} and IC_{50}) and combined therapy (ethanolic extracts +5-Fu) for 48 h. Then, cells were collected, stained with 20 μM DCFDA for 30 min at 37 °C and immediately analyzed by FACScan flow cytometer (Becton Dickinson, San Jose, USA). To determinate whether ROS production influences cytotoxicity of the extracts in T84 cells, cells were seeded in 48-well plates (3 $\times 10^3$ cell/well) for 24 h, and the, media was changed by DMEN without FBS and cells were pretreated with 100 μM N-acetyl-L-cysteine (NAC) for 2 h. Then, ethanolic extracts were added and incubated for 72 h to assess the cell proliferation. T84 cells without NAC pretreatment were used as control. NAC is commonly used to identify ROS inducers due to it inhibits their production.

2.14. Real time PCR analysis of cancer stem cells

Antitumor activity of the ethanolic extracts against colon CSCs was analyzed following our protocol and experience using T84 cells (Mesas et al., 2021). Cells were exposed (72 h) to ethanolic extract (IC_{50}), washed with PBS and then cultured. Total RNA was extracted using Trizol Reagent (RNeasy Mini Kit, Qiagen, MD, USA), quantified with NanoDrop 2000 (Thermo Fisher, Waltham, MA, USA), and converted (1 μg of RNA) into cDNA using a retro-transcriptase kit (Promega, Madison, WI, USA) following the manufacturer's instructions. CD24, CD44, SOX2, OCT4 and NANOG genes expression was analyzed using RT-PCR and SYBR green supermix (Taq Universal SYBR Green Supermix; Bio-Rad Laboratories, Hercules, CA). The quantitative RT-PCR primers and annealing temperatures (Tm) used are listed in Table S1 (Supplementary material). GAPDH was used to gene expression normalization. All quantitative RT-PCR assays were performed in an ABI 7900 system (ABI), and the 2-ΔΔCt method was applied to calculate relative expression levels.

2.15. Statistical analysis

Statistical analysis was performed by IBM SPSS Statistics 26.0 and GraphPad Prism 8. All the data were presented as the mean value with standard deviation (SD). All experiments were performed in triplicate. After the homogeneity test of variance, *t*-test was performed to compare the differences between groups with equal variance, while F-test was used for groups with uneven variance. Significance values were denoted by (*) $p < 0.05$ significant; (**) $p \leq 0.01$ highly significant, (***), $p \leq 0.001$ very highly significant.

3. Results

3.1. Antioxidant activity

Antioxidant activity was assessed using different assays such as total polyphenol content, ABTS, and in vitro antioxidant activity in HT-29 cells. All the ethanolic extracts exhibited antioxidant properties (Table 1), with the extract from *A. cherimola* showing the highest activity, followed by *T. tuberosum* and *M. oleifera*.

3.2. Mass spectrometry analysis

Ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was conducted to assess the presence of bioactive compounds present in the different ethanolic extracts. Chromatograms of the extracts and validated compounds are presented in Fig. S1 (Supplementary material) and Tables 2–4. The tentative compounds identified showed both antioxidant and antitumor activity, were polar, and corresponded to secondary plant metabolites such as phenolic compounds, terpenoids, glycosides, saponins, and polyketides. Within the group of phenolic compounds, flavonoids (flavones, flavanones, and flavanols), phenolic acids, and phenylpropanoids stood out. In addition, we identified acetogenins, a class of polyketides belonging to the Annonaceae family, in the *A. cherimola* ethanolic extract.

Table 1
Quantification of total polyphenol content and antioxidant activity of ethanolic extracts.

	Extraction yield (mg/g flour)	Total polyphenols (μg GAE/mg extract)	ABTS (μg GAE/mg extract)	*In vitro* antioxidant activity (UAA/mg extract)	
				2.5 mM	3 mM
M. oleifera	170.1 ± 0.57^b	9.71 ± 1.78^a	1.68 ± 0.18^a	20.9 ± 1.33^b	24.9 ± 2.11^b
T. tuberosum	280.6 ± 1.79^c	18.27 ± 0.83^b	2.18 ± 0.25^b	18.8 ± 2.45^a	20.3 ± 2.17^a
A. cherimola	131.7 ± 0.47^a	27.7 ± 1.03^c	2.50 ± 0.21^b	41.6 ± 3.68^c	47.6 ± 1.52^c

Data are reported as mean ± SD of experiments performed in triplicate. GAE: gallic acid equivalent. Activity Units (AAU), which is defined as the value of 10% units (10%) recovery of cell viability with respect to the corresponding control treated with hydrogen peroxide (H2O2). Anova analysis and HSD Tukey test are indicated by superscript. The values of a, b and c correspond to the groups in the homogeneous subsets. Means within a column with different superscripts are significantly different ($P < 0.05$).

Table 2
Identification of bioactive compounds in the ethanolic extract of *M. oleifera*.

Compound and activity	MF	[M-H]-	TR	PPM	% Conf	MS Fragments			Ref.
Phenylpropanoids									
3-O-caffeoyl-5-O-malonylquinic acid[1]	$C_{19}H_{20}O_{12}$	439.0857	0.961	-4.6	98.11	293.0602	239.0855	226.9868	[21]
Polyphenolic acids									
Secalonic acid D[2]	$C_{32}H_{30}O_{14}$	637.1563	4.249	0.9	99.73	370.1013	324.0951	272.1134	[22]
Flavonoids									
Vicenin-2[2]	$C_{27}H_{30}O_{15}$	593.1526	3.617	3.4	87.31	173.0702	172.0379	167.0394	[23]
Vitexin[2]	$C_{21}H_{20}O_{10}$	431.0996	4.283	4.2	99.99	324.1230	243.0340	198.0094	[24]
Isovitexin[2]	$C_{21}H_{20}O_{10}$	431.1013	4.249	8.1	99.93	241.0167	225.0247	209.0264	[25]
Isorhamnetin 3-glucoside[2]	$C_{22}H_{22}O_{12}$	477.101	4.948	-4.8	83.37	324.1212	243.0298	239.0885	[26]
Pancibiflavonol[2]	$C_{30}H_{20}O_{12}$	571.0916	0.995	6.8	77.3	370.1054	348.0979	328.0919	[27]
Abiesinol A[2]	$C_{30}H_{22}O_{12}$	573.1038	1.66	0.9	99.79	294.0162	255.0672	189.0529	[28]
Callistephin[2]	$C_{21}H_{21}O_{10}$	432.102	4.249	-8.3	87.12	402.0869	393.1151	363.0781	[29]
Dihydrokaempferol[2]	$C_{15}H_{12}O_6$	287.0556	5.473	3.8	99.97	221.0791	207.0600	201.0170	[30]
Glycosides									
Diphyllin[2]	$C_{21}H_{16}O_7$	379.0818	0.927	-5.8	99.82	380.0959	226.0583	216.0362	[31]
Forsythoside E[2]	$C_{20}H_{30}O_{12}$	461.1708	3.409	10.6	99.77	461.1745	339.1331	338.1213	[32]
Neesiinoside B[1]	$C_{33}H_{36}O_{17}$	703.1874	0.82	-0.4	98.58	539.1399	456.1078	455.1072	[33]
Cleistanthin B[2]	$C_{27}H_{26}O_{12}$	541.1322	0.82	-4.4	93.55	457.1229	456.1078	455.1072	[34]
Sesquiterpenoid									
Artemisinin[2]	$C_{15}H_{22}O_{15}$	441.0872	0.927	-1.8	97.52	225.0664	222.0822	221.0739	[35]

MF: molecular formula; [M-H]-: mass; TR: retention time; PPM: error; % Conf: reliability percentage.
[a] antioxidant activity.
[b] antitumoral activity.

Table 3
Identification of bioactive compounds in the ethanolic extract of *T. tuberosum*.

Compound and activity	MF	[M-H]-	TR	PPM	% Conf	MS Fragments			Ref.
Phenylpropanoids									
3-O-caffeoyl-5-O-malonylquinic acid[1]	$C_{19}H_{20}O_{12}$	439.0831	0.927	-10.5	88.65	395.1028	377.0927	323.0671	[21]
Flavonoids									
Gallocatechin[2]	$C_{15}H_{14}O_7$	305.0678	2.776	5.6	99.89	275.0494	254.0492	248.0617	[36]
Rutin[1]	$C_{27}H_{30}O_{16}$	609.1439	4.106	-2.8	99.31	432.0998	339.1267	293.0536	[37]
Cynarotrioside	$C_{33}H_{40}O_{20}$	755.205	4.699	2	85.99	432.0785	392.1186	327.1155	[38]
Glycoside									
Sasanquin	$C_{21}H_{30}O_{11}$	457.1719	5.121	2	100	368.1500	338.1278	295.1510	[39]
Kelampayoside A[1]	$C_{20}H_{30}O_{13}$	477.1619	3.582	2.3	99.97	392.1106	338.1295	324.114	[40]
Zizybeoside II[2]	$C_{25}H_{38}O_{16}$	593.2072	2.674	-1.7	99.21	338.1290	324.1156	281.0872	[41]
Manglieside B[1]	$C_{20}H_{28}O_{11}$	433.1587	4.699	7.7	99.7	392.1186	339.1281	327.1155	[42]
Sesquiterpenoid									
Glucozaluzanin C[1]	$C_{21}H_{28}O_8$	407.1734	2.602	6.9	99.58	338.1290	324.1156	292.1751	[43]
Terpene									
Plantarenaloside[1]	$C_{16}H_{24}O_9$	359.1368	3.582	7.2	88.72	324.1114	267.1150	265.0703	[44]
Triterpenoid									
Ganoderic acid H[1]	$C_{32}H_{44}O_9$	571.2888	14.909	-3.3	94.6	556.2717	339.1367	338.1315	[45]
Polyketide									
Amphidinin B[1]	$C_{25}H_{42}O_7$	453.2831	11.831	-4.6	89.62	354.2745	345.2077	313.2462	[46]

MF: molecular formula; [M-H]-: mass; TR: retention time; PPM: error; % Conf: reliability percentage.
[1] antitumoral activity.
[2] antioxidant activity.

3.3. Ethanolic extracts enhanced detoxifying enzyme activity

The chemopreventive action of the ethanolic extracts was assessed based on their induction of detoxifying enzymes in HT-29 cells. To induce the activity of detoxifying enzymes, HT-29 cells were exposed to ethanolic extracts from *M. oleifera*, *T. tuberosum,* and *A. cherimola.* As shown in Table 5, the activity of the drug metabolizing enzymes GST and QR were induced by all the extracts in greater magnitudes compared to positive control (sulforaphane). The *M. oleifera* extract showed the highest capacity to induce GST activity, followed by *T. tuberosum* and *A. Cherimola*, with no significant differences between the latter. The extract from *A. Cherimola* showed the greatest induction of QR, followed by *T. tuberosum* and *M. oleifera*. Of special note, the GST induction potential exhibited by the extracts surpassed that of a well-known inductor-sulforaphane although this effect was not observed for QR.

3.4. Antiproliferative activity in cultured cells

The antiproliferative effect the ethanolic extracts had on different CRC cell lines is described in Table 6. CRC cell viability was inhibited in a dose-dependent manner by treatment with the ethanolic extracts from *M. oleifera*, *T. tuberosum,* and *A. cherimola*. The *A. cherimola* ethanolic extract showed the lowest IC_{50} values in T84 cells, whereas the *M. oleifera* extract showed the lowest IC_{50} values in HCT-15 and SW480 cells. The *T. tuberosum* extract exhibited the highest IC_{50} values in all three CRC cell lines tested (T84, HCT15, and SW480). Inhibition of proliferation by the extracts in all the CRC cell lines was higher compared to the non-tumor cell line CCD18. In addition, the ethanolic extracts also caused dose-dependent inhibition of the proliferation of HCT15 cell multicellular tumor spheroids (MTSs; Fig. 1; $p < 0.001$). The *A. cherimola* ethanolic extract showed the highest inhibition levels, followed by *M. oleifera* and *T. tuberosum*. In fact, the *A. cherimola* extract reduced the proliferation of HCT15 MTS to 34% and 10% using 1.5

Table 4
Identification of bioactive compounds in the ethanolic extract of *A. Cherimola*.

Compound and activity	MF	[M-H]-	TR	PPM	% Conf	MS Fragments			Ref.
Phenylpropanoids									
3-O-caffeoyl-5-O-malonylquinic acid[a]	$C_{19}H_{20}O_{12}$	439.0877	0.926	-6.1	88.6	395.1389	395.0916	380.1014	[21]
Flavonoids									
Procyanidin B1[a]	$C_{30}H_{26}O_{12}$	577.1326	3.199	-3.5	83.66	255.0349	241.0181	207.0597	[47]
Procyanidin B2[a]	$C_{30}H_{26}O_{12}$	577.1331	3.231	-2.6	99.74	221.0785	212.0748	209.0731	[48]
Quercetin-3-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside[a]	$C_{27}H_{30}O_{16}$	609.143	4.173	-2.6	96.72	432.0931	311.1356	302.0753	[49]
Rutin[a]	$C_{27}H_{30}O_{16}$	609.1475	4.139	3.1	96.28	326.1186	313.1008	311.1356	[50]
Triterpenoids									
Salannin[a]	$C_{34}H_{44}O_9$	595.2877	13.788	-5	91.87	339.1322	327.1168	324.1174	[51]
Linearifolianoid A[a]	$C_{34}H_{42}O_9$	593.2732	12.006	-3.2	89.53	415.2292	339.1310	327.1133	[52]
Trichagmalin D[a]	$C_{36}H_{44}O_{13}$	683.2697	5.82	-1	98.9	645.2592	636.2661	629.3030	[53]
Terpene									
Dulcioic acid[b]	$C_7H_{12}O_8$	223.0455	0.926	0.4	93.79	207.0594	187.0321	181.0741	[54]
Acetogenins									
Annohexocin[a]	$C_{35}H_{64}O_9$	627.4454	13.788	-2.9	86.42	389.2912	339.1435	315.2602	[55]
Annopentocin A[a]	$C_{35}H_{64}O_8$	611.4494	13.613	-4.7	83.66	321.1305	301.2411	287.2318	[56]
(2,4-cis)-isoannonacin[a]	$C_{35}H_{64}O_7$	595.4561	15.012	-2.2	89.62	499.3634	411.2801	382.2663	[57]
Annocatalin[a]	$C_{35}H_{64}O_7$	595.4561	15.012	-2.2	89.62	417.3553	413.3029	411.2801	[56]
Glycosides									
Diphyllin[a]	$C_{21}H_{16}O_7$	379.0779	0.926	-10.3	88.9	335.0846	293.0541	216.0387	[31]
Saponins									
Filiasparoside C[a]	$C_{44}H_{72}O_{16}$	855.4698	7.252	-5.1	99.96	639.3006	637.2774	617.2721	[58]

MF: molecular formula; [M-H]-: mass; TR: retention time; PPM: error; % Conf: reliability percentage.
[a] antitumoral activity.
[b] antioxidant activity.

Table 5
GST and QR induction activity on HT-29 cells after treatment with ethanolic extracts.

		GST			QR		
	Concentration of the extract	U/mL	U/mg	Induction Rate (treated/control)	U/mL	U/mg	Induction Rate (treated/control)
Control	–	72.9 ± 0.22	20.6 ± 0.13	1.00 ± 0.00	229.4 ± 0.17	591.8 ± 0.10	1.00 ± 0.00
M. oleifera	2.5 μg/mL	224.9 ± 1.01	48.4 ± 0.42	2.34 ± 0.02***	5603.6 ± 0.45	1033.9 ± 0.25	1.73 ± 0.02***
T. tuberosum	2.0 μg/mL	208.1 ± 0.71	37.1 ± 0.28	1.78 ± 0.02***	6941.3 ± 0.51	1200.9 ± 0.31	2.01 ± 0.02***
A. cherimola	1.5 μg/mL	229.9 ± 1.20	34.8 ± 0.54	1.67 ± 0.03***	6752.4 ± 0.59	1490.6 ± 0.34	2.51 ± 0.02***
Sulforaphane	5 μM	66.3 ± 1.99	22.3 ± 1.21	1.07 ± 0.04*	5852.4 ± 0.12	1336.1 ± 0.34	2.28 ± 0.02***
Sulforaphane	10 μM	116.2 ± 0.41	26.5 ± 0.19	1.27 ± 0.01***	4947.9 ± 0.64	1660.4 ± 0.12	2.81 ± 0.01***

Induction results expressed as a mean of ratio of GST, QR activity of treated vs. control samples (non-treated). Significant values are denoted by (*) $p < 0.05$ *significant*; (**) $p \leq 0.01$ highly significant, (***), $p \leq 0.001$ very highly significant.

Table 6
Antiproliferative activity of ethanolic extracts from *M. oleifera*, *T. tuberosum*, and *A. cherimola* against CRC cell lines.

	IC_{50} (μg/mL)			
Ethanolic Extract	T84	HCT-15	SW480	CCD18
M. oleifera	33.3 ± 2.70^b	24.6 ± 2.16^a	19.8 ± 2.68^a	98.9 ± 1.85^a
T. tuberosum	84.4 ± 0.84^c	41.4 ± 0.98^c	43.7 ± 0.25^c	189.8 ± 0.66^c
A. cherimola	23.2 ± 2.75^a	30.9 ± 0.32^b	33.0 ± 1.52^b	176.3 ± 0.06^b

Data are reported as mean ± SD with experiments performed in triplicate. Anova analysis and HSD Tukey test are indicated by superscript. The values of a, b and c correspond to the groups in the homogeneous subsets. Means within a column with different superscripts are significantly different ($P < 0.05$).

$\times IC_{50}$ and 4 $\times IC_{50}$, respectively. Furthermore, the ethanolic extracts of *M. oleifera* reduced the proliferation of HCT15 MTS to 43% (1.5 $\times IC_{50}$) and 24% (4 $\times IC_{50}$) and those from *T. tuberosum* reduced it to 46% (1.5 $\times IC_{50}$) and 24% (4 $\times IC_{50}$). Importantly, the highest level of inhibition was recorded for 4 $\times IC_{50}$ ($p < 0.001$) for all the extracts.

3.5. Ethanolic extracts enhanced the 5-FU antiproliferative effect in HCT-15 cells

As shown in Fig. 2, ethanolic extracts and 5-FU both inhibited the proliferation of HCT-15 cells, and their combined treatment (ethanolic extracts + 5-Fu) showed synergistic effects compared to monotherapies (CI < 1). This synergism was most evident for all the titrations of the *A. cherimola* ethanolic extract combined with 5-FU (CI < 1). In addition, 5-FU (2 μM) combined with ethanolic extracts from *A. cherimola*, *M. oleifera,* and *T. tuberosum* at 2.5, 5, and 10 μg/mL, respectively produced the highest synergistic effect, with inhibition close to 50% (CI < 1). These results were also confirmed in MTSs where the highest synergistic activity was attained with the combination of the *A. cherimola* ethanolic extract and 5-FU (Fig. 3). In addition, combinations of the ethanolic extracts from *A. cherimola* and *M. oleifera* (5 and 20 μg/mL, respectively) with 5-FU (2 μM) produced more than 50% inhibition (CI < 1). In contrast, the combination of the *T. tuberosum* ethanolic extract (40 μg/mL) with of 5-FU (2 μM) only gave rise to 40% inhibition (CI < 1).

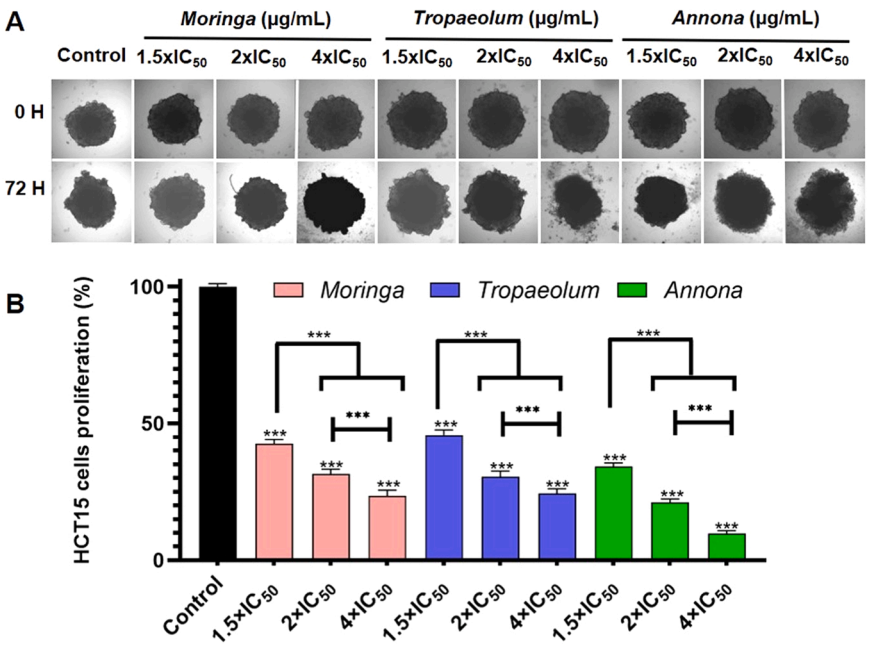

Fig. 1. Multicellular tumor spheroids (MTS) from HCT15 treated with the ethanolic extracts of *M. oleifera, T. tuberosum, and A. cherimola*. (A) Representative image of MTS treated with the ethanolic extracts. The images were taken with light microscopy images (10 ×magnification). (B) Proliferation assay with CCK8 on MTS treated for 72 h with the ethanolic extracts at different doses (1.5 × IC_{50}, 2 × IC_{50} and 4 × IC_{50}. The pink bars represent the ethanolic extract of *M. oleifera*, the blue bars represent the ethanolic extract of *T. tuberosum* and the green bars represent the ethanolic extract of *A. cherimola*. Significant differences vs control are denoted by (*) $p < 0.05$ significant; (**) $p \leq 0.01$ highly significant, (***), $p \leq 0.001$ very highly significant.

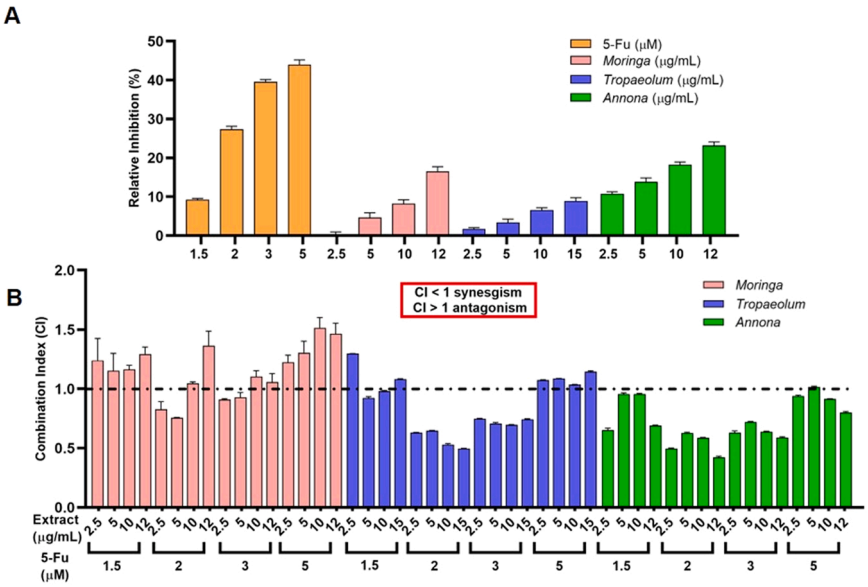

Fig. 2. Antiproliferative effect of the combined treatment (ethanolic extracts and 5-Fu) on HCT-15 cells. A. *M. oleifera* and 5-Fu. B. *T. tuberosum* and 5-Fu. C. *A. cherimola* and 5-Fu. Combination index (CI) values of ethanolic extracts and 5-Fu are shown above the bars. CI< 1, = 1 and > 1 indicate synergism, addition and antagonism, respectively.

3.6. Effect of the ethanolic extracts on the cell cycle

As shown in Fig. 4, all the ethanolic extracts induced HCT-15 cell cycle modulation. In fact, the three species, *M. oleifera*, *A. cherimola*, and *T. tuberosum*, induced an increase in the SubG1 phase, with an S-phase increase was also being observed with the use of *M. oleifera* and *A. cherimola* extracts. A significant decrease in G2/M-phase cells was only seen with the *A. cherimola* and *T. tuberosum* extracts. In addition, HCT-15 cells were treated with combinations ethanolic extracts + 5-FU. As shown in Fig. 5, the use of *A. cherimola* ethanolic extracts and 5-FU resulted in a lower percentage of cells in G0/G1 and an increase in those in S-phase, indicating that this extract enhanced the effect of 5-FU ($p < 0.001$). No cell cycle modulation was observed for the combinations with the other extracts (data not shown).

3.7. Molecular analysis of cell death induction by ethanolic extracts

Western blot analysis was conducted to determine the antitumoral molecular mechanisms of the ethanolic extracts. As shown in Fig. 6A and B, the *M. oleifera* ethanolic extract increased the expression of cleaved caspases (8, 9, and 3) by more than 1.13, 2.18, and 1.83-fold in HCT15 cells at 24 h compared to the untreated cells (Fig. 6A and B). *T. tuberosum* ethanolic extract also increased the expression of cleaved caspases (8, 9, and 3) by more than 6.97, 1.85, and 1.30-fold in HCT15 cells at 12 h. However, the highest expression of cleaved caspase 9 was obtained at 24 h with a 3.06-fold increase in expression in HCT15 cells compared to the untreated control (Fig. 6A and B). Finally, *A. cherimola* ethanolic extract induced the highest increase in cleaved caspase (8, 9, and 3) expression, by over 1.12, 3.63, and 2.22-fold in HCT15 cells at

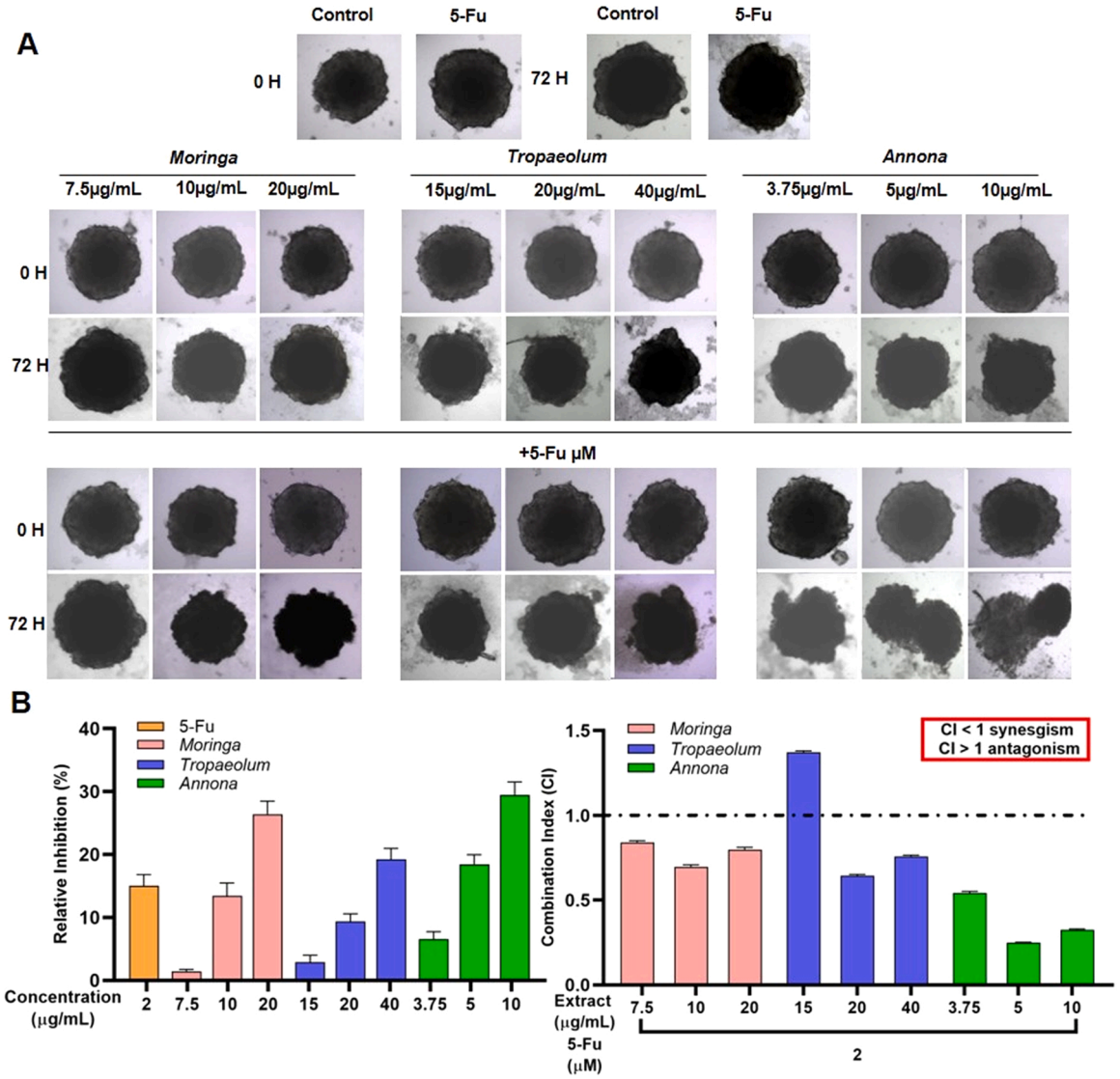

Fig. 3. Antiproliferative effect of the combined treatment (ethanolic extracts and 5-Fu) on HCT-15 MTSs. (A) Representative image of MTS treated with the combined treatment ethanolic extracts (different concentrations in µg/mL) and 5-Fu (2 µM). The images were taken with light microscopy images (10 ×magnification). (B) Relative Inhibition assay with CCK8 on MTS treated for 72 h with the ethanolic extracts at different of the combined treatment (ethanolic extracts and 5-Fu). Combination index (CI) values of ethanolic extracts and 5-Fu are shown above the bars. CI< 1, = 1 and > 1 indicate synergism, addition and antagonism, respectively.

24 h (Fig. 6A and B).

Of interest, we used Lysotracker to analyze which mechanisms by which autophagy contributed to cell death, which revealed that ethanolic extracts formed autophagic vesicles in HCT-15 cells (Fig. 6C). To verify this, we used the autophagosome-associated microtubule-associated protein light chain 3 (MAP-LC3) to study the expression of the LC3-type II by western blot. This analysis indicated that the ethanolic extracts from *M. oleifera* and *T. tuberosum* significantly increased the expression of MAP-LC3 ($p < 0.001$; Fig. 6D and E).

3.8. Induction of reactive oxygen species by ethanolic extracts

As shown in Fig. 7A, compared to the control, ethanolic extracts significantly increased intracellular ROS production after 48 h of treatment. This effect was especially evident after treatment with *T. tuberosum* ethanolic extract (IC_{75} dose) which increased ROS production by about 21-fold, while *M. oleifera* and *A. cherimola* extracts increased ROS production by about 2 and 9-fold, respectively compared to the control (Fig. 7B). NAC pretreatment was added to decrease ROS production and, as shown in Fig. 7C, NAC suppressed ROS production, causing a very significant increase in cell proliferation ($p < 0.001$).

3.9. Effect of ethanolic extracts on cancer stem cell markers

To determinate the modulation of CRC stem cell marker expression after treatment with ethanolic extracts, we performed RT-qPCR analysis. As shown in Fig. 8, ethanolic extracts from *M. oleifera*, *T. tuberosum,* and *A. cherimola* decreased the expression of CSC markers, indicating a reduction in the number of CSCs in the culture. The *M. oleifera* ethanolic extract showed the strongest effect on the markers CD133, CD24, SOX2, and NANOG. In contrast, ethanolic extracts from *T. tuberosum* and *A. cherimola* only showed significant effects for the OCT4 and CD44 markers, respectively.

4. Discussion

The treatment of CRC, especially in its more advanced or metastatic stages, frequently fails because of the limitations of chemotherapy and

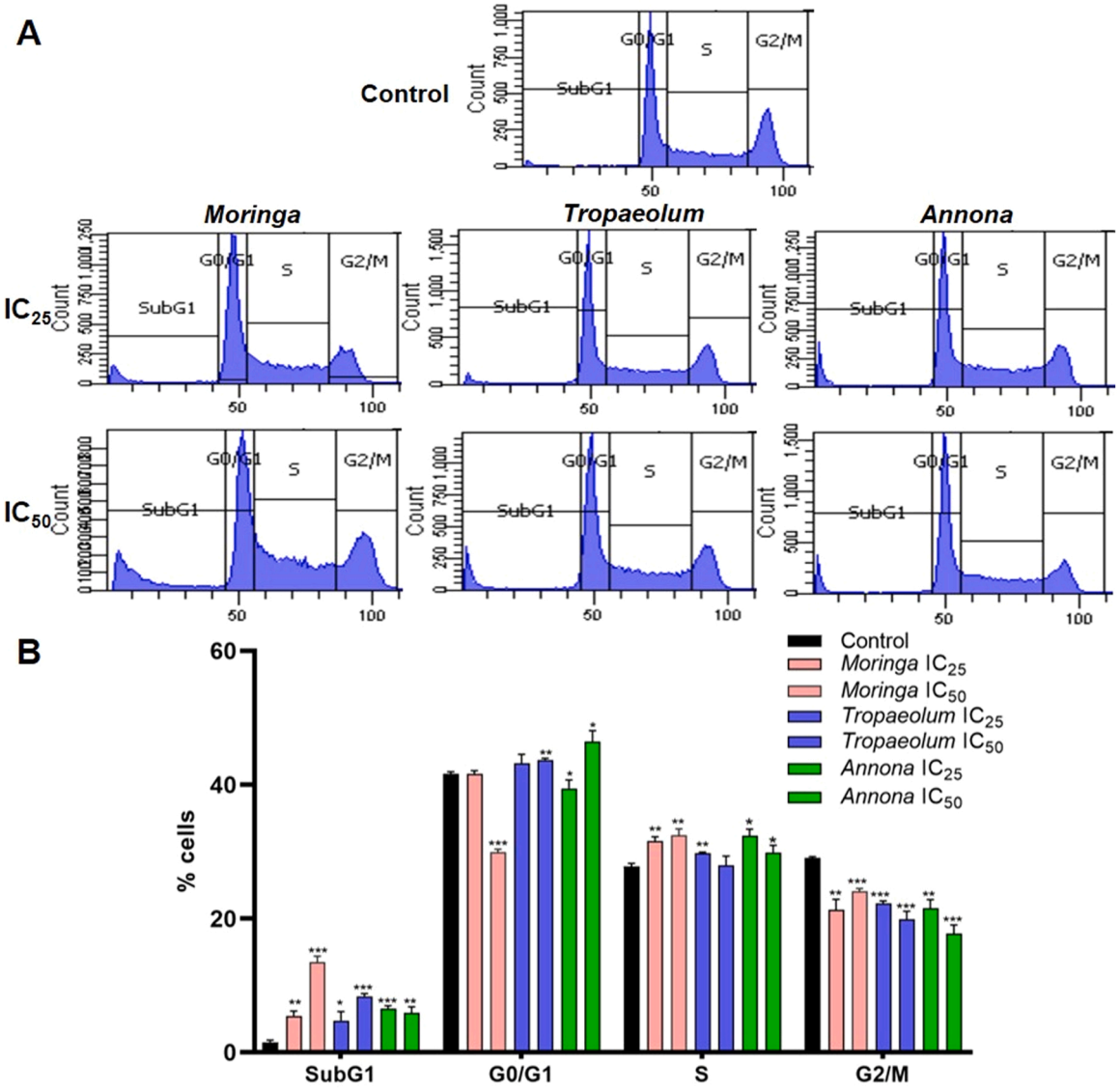

Fig. 4. Cell cycle analysis of HCT-15 cells treated with ethanolic extracts. (A) Images of the FACScan flow cytometry results from HCT-15 cells exposed to PI/RNAse. (B) Graphic representation of percentage of labeled cells in each cell cycle phase. Significant differences vs control are denoted by (*) $p < 0.05$ *significant*; (**) $p \leq 0.01$ highly significant, (***), $p \leq 0.001$ very highly significant.

its adverse effects and toxicity in non-cancerous tissues, as well as the development of chemoresistance [59]. The use of plant extracts, either alone or in association with other therapeutic agents, has great potential in the field of cancer because of their safety, efficacy, reduced toxicity, and low propensity for the development of resistance [60,61]. In this context, Ecuadorian indigenous communities have used plants for many years for their multiple health-related benefits [62] although their effect in CCR remains unknown. Three of these species, *M. oleifera*, *T. tuberosum,* and *A. cherimola*, were selected because of their anti-tumor activity in CRC cells as well as their mechanisms of action.

The use of adequate solvents to prepare functional plant extracts is essential for optimal extraction of the bioactive compounds [63]. Most bioactive molecules from plants with antiproliferative activity are poorly soluble in water because of the presence of phenolic groups or hydrophobic residues. Organic solvents are classically used to obtain these antiproliferative molecules, however, the toxicity of these solvents is a serious limitation [64]. In 2019, Truong et al., evaluated the antioxidant and anti-inflammatory activity of *Severinia buxifolia* branch extracts using different solvents and showed that hydroalcoholic solvents presented higher yields, recovered a large proportion of bioactive compounds, and had higher antioxidant and anti-inflammatory activity compared to organic solvents [65]. In fact, a similar procedure was used by Khalil et al., to obtain an ethanolic extract of the aerial parts of *Thymbra spicata* L. which showed a high antiproliferative activity against MCF-7 human breast cancer cells [66]. We recently used a similar procedure to obtain bioactive compounds from the defatted seeds of *Euphorbia lathyris* which showed antiproliferative activity against human CRC cell lines [67].

Thus, in this current work we used hydroalcoholic extraction to recover a sizable number of bioactive components from the seeds and tubers of *M. oleifera*, *T. tuberosum,* and *A. cherimola*. Our results showed that the ethanolic extract from *M. Oleifera* seeds exhibited the highest antiproliferative activity against CRC cell lines compared to the rest of the extracts. In fact, this extract showed an IC_{50} in HCT-15 and SW480 CRC cells of 24.6 ± 2.16 and 19.8 ± 2.68 μg/mL, respectively. In addition, ethanolic extract from *M. oleifera* seeds also showed moderate antioxidant activity in HT-29 colon adenocarcinoma cells. These results support those of Xu et al., who demonstrated that ethanolic extracts of leaves, roots and seeds showed high antioxidant and anti-inflammatory activity as a result of their different concentration of flavonoids[68].

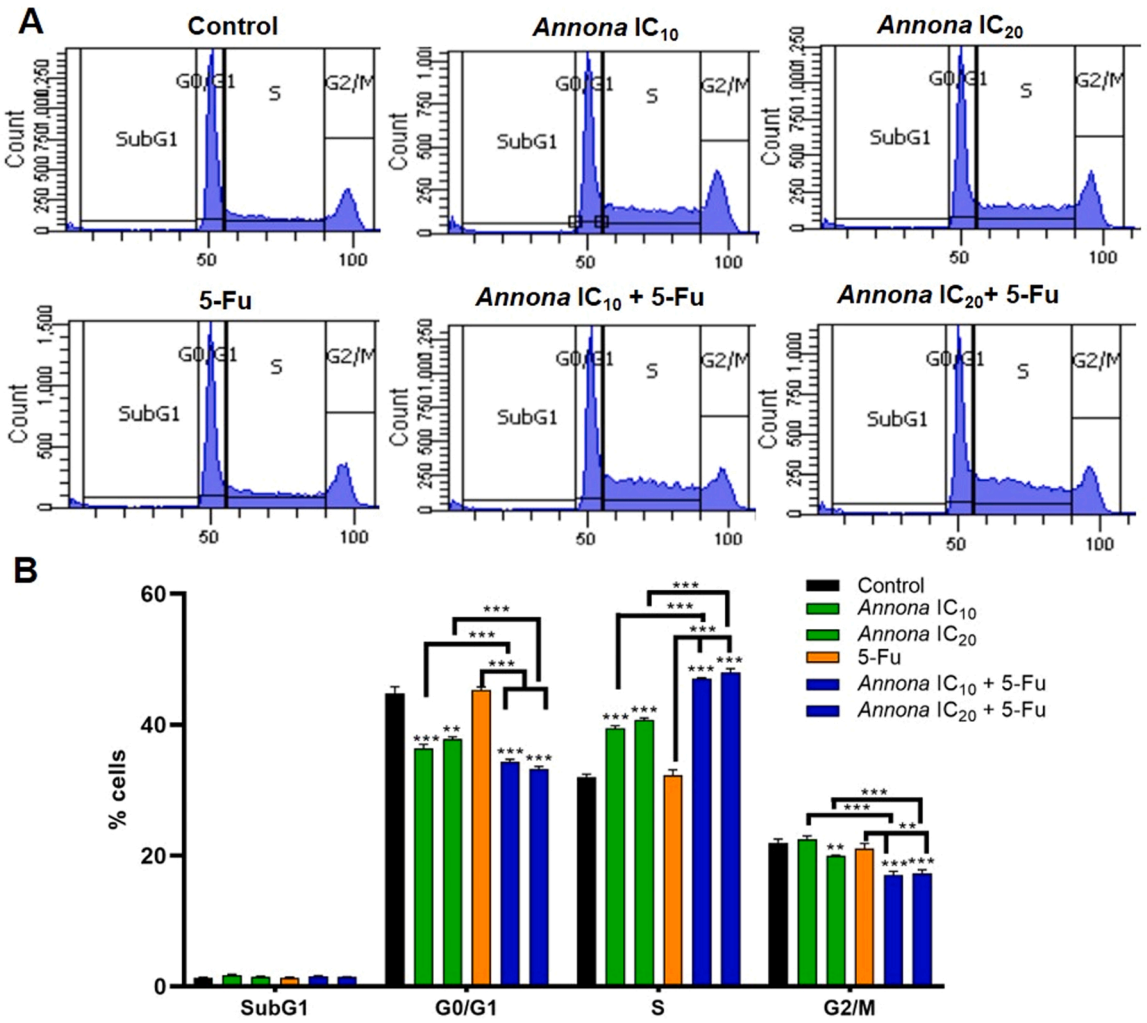

Fig. 5. Cell cycle analysis of HCT-15 cells treated with *A. cherimola* ethanolic extracts (IC10, IC20) and 5-Fu (2 μM). (A) Images of the FACScan of flow cytometry results of HCT-15 cells exposed to PI/RNAse. (B) Graphic representation of percentage of labeled cells in each cell cycle phase. Significant differences vs control are denoted by (*) $p < 0.05$ *significant*; (**) $p \leq 0.01$ highly significant, (***), $p \leq 0.001$ very highly significant.

Meanwhile, Xu et al., previously evaluated the antioxidant activity of the ethanolic extract of leaves of the *M. oleifera* and attributed a strong antioxidant activity to the presence of phenolic compounds such as kaempferol 3-O-rutinoside, quercetin 3-O-(6″-malonyl-glucoside), kaempferol 3-O-glucoside, and quercetin derivative [69].

Regarding its antiproliferative activity, Mohd Fisall, et al., showed that dichloromethane fraction of *M. oleifera* leaf methanolic extract inhibited proliferation of breast cancer cells MCF7 and they attributed this effect to presence of compounds such as benzeneacetonitrile, 4-hydroxy- and benzeneacetic acid, 4-hydroxy-, methyl ester among others founded in this fraction [70]. Likewise, *M. oleifera* alkaloid extract inhibited the proliferation and migration of A549 lung cancer cells by inhibiting JAK2/STAT3 pathway activation [71]. On the other hand, in order to investigate the bioaccessibility of these compounds, Bhadresha et al. [72], subjected the *M. oleifera* extract to an in vitro digestion process and demonstrated its protective effect on bone metastasis by inhibiting the proliferation of PC3 tumor cells.

In this context, we conducted a chromatographic analysis of the ethanolic extract of *M. oleifera* seeds which showed the presence of a high proportion of polyphenolic compounds such as vitexin, isovitexin, and isorhamnetin-3-O-β-D-glucopyranoside. Similar compounds were also detected in *M. oleifera* leaves by Ye et al., [73]. Moreover, Liu et al., recently demonstrated that vitexin induces a significant decrease in proliferation in A549 lung cancer cells, inducing apoptosis through a mitochondrial pathway and the PI3K/Akt/mTOR signaling pathway [24]. In addition, Cao et al., demonstrated that isovitexin inhibited the carcinogenicity of hepatic carcinoma stem cell-like cells (HSCLCs), in so downregulating FoxM1 and manganese superoxide dismutase (MnSOD) expression [25]. Finally, isorhamnetin-3-O-β-D-glucopyranoside exhibited antiproliferative action in the MCF-7 breast cancer line by inducing ROS-dependent apoptosis [74]. The presence of these compounds could explain the significant antiproliferative effect of this extract against CRC cells.

In contrast, the *T. tuberosum* tuber ethanolic extract showed moderate antioxidant activity in both the ABTS assay and the in vitro study with HT-29 cells. Interestingly, this ethanolic extract showed a higher and selective antiproliferative effect against the T84, HCT-15, and SW480 CRC cells (84.4 ± 0.84; 41.4 ± 0.98; and 43.7 ± 0.25 μg/mL, respectively) than against the CCD-18 normal epithelial colon line ($IC_{50} = 189.8 \pm 0.66$ μg/mL). Previous work showed that the raw extracts from four colored *T. tuberosum* genotypes had significant antioxidant activity, as shown by their inhibitory effect on APPH or Cu-induced oxidation of polyunsaturated fatty acids, as well as on APPH-induced erythrocyte hemolysis [75]. These protective effects and the antioxidant activity was attributed to the high content of phenolic compounds, anthocyanins, and flavonoids in the extract. In addition, similar extracts showed antiproliferative activity against the A549, Caki-1, T24, and PC-3 tumor lines [10].

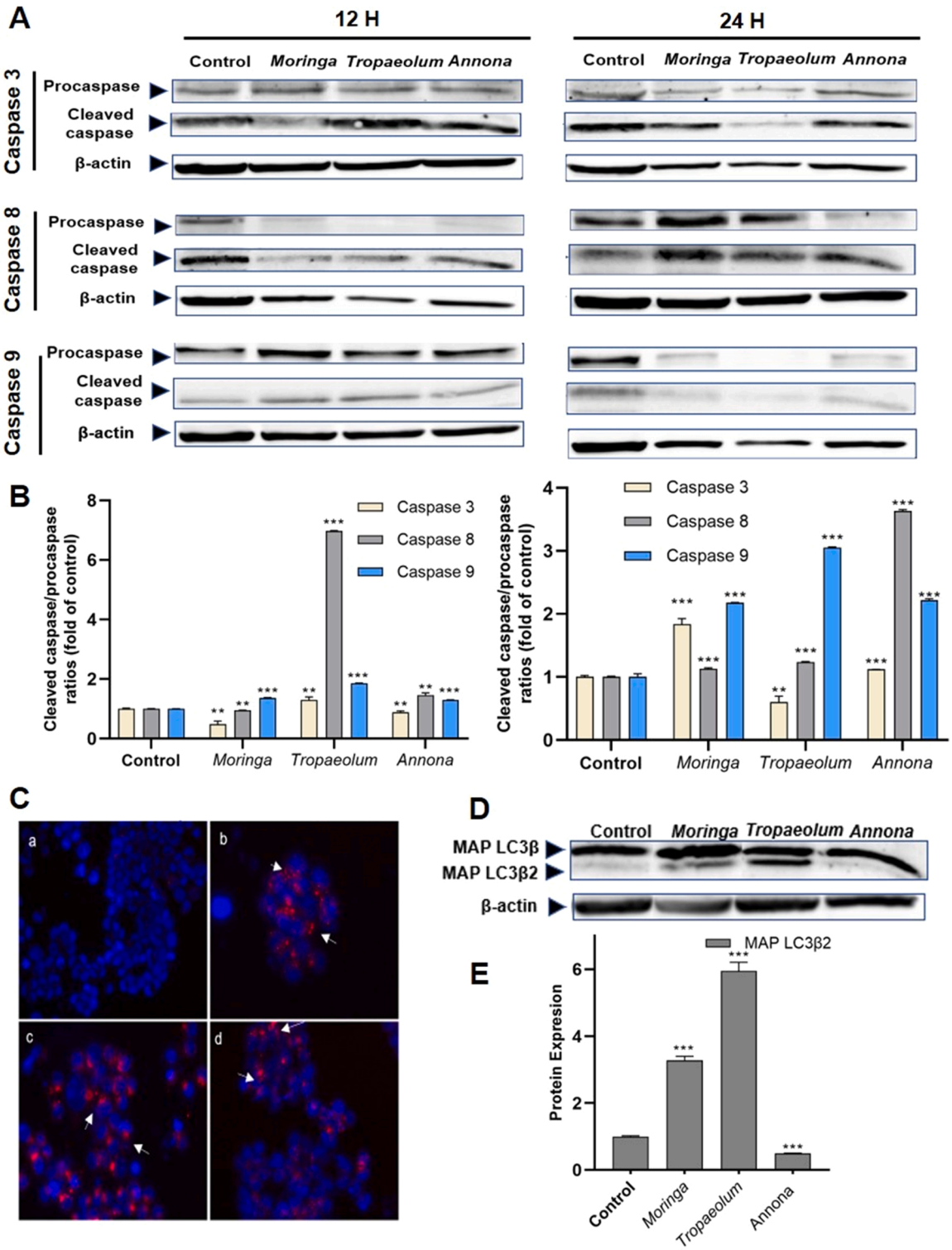

Fig. 6. Mechanisms of cell death induction by ethanolic extracts. (A) Western blot analysis of the procaspase and cleaved caspase 3, 8 and 9; (B) Graphic representation of the densitometric analysis of the caspase cleaved caspases. (C) LisoTracker staining revealing the formation of autophagy vesicles after ethanolic extract treatment. (E) Western blot analysis of MAP LC3β2 expression, (E) Graphic representation of the densitometric analysis of the MAP LC3β2 bands. Significant differences vs control are denoted by (*) $p < 0.05$ *significant*, (**) $p < 0.01$ highly significant, (***), $p < 0.001$ very highly significant.

Finally, the ethanolic extract from *A. cherimola* showed the highest antioxidant activity in vitro (HT29 cells) and in the ABTS test (2.50 ± 0.21 μg GAE/mg extract) compared to the other extracts. Nonetheless, although its antitumor activity was also significant, this activity was lower than the extracts described previously, showing a more intense antiproliferative capacity against T-84 cells (IC_{50} 23.2 ± 2.75 μg/mL). Haykal et al., attributed antioxidant activity of a leaf ethanolic extract of this plant to its phenolic content and its antiproliferative activity against HeLa and HepG2 cell lines to the presence of phytosterols such as β-sitosterol, β-stigmasterol, and acetogenin compounds [76]. Chromatographic analysis showed the presence of compounds such as catechin and quercetin 3-O-rutinoside and others

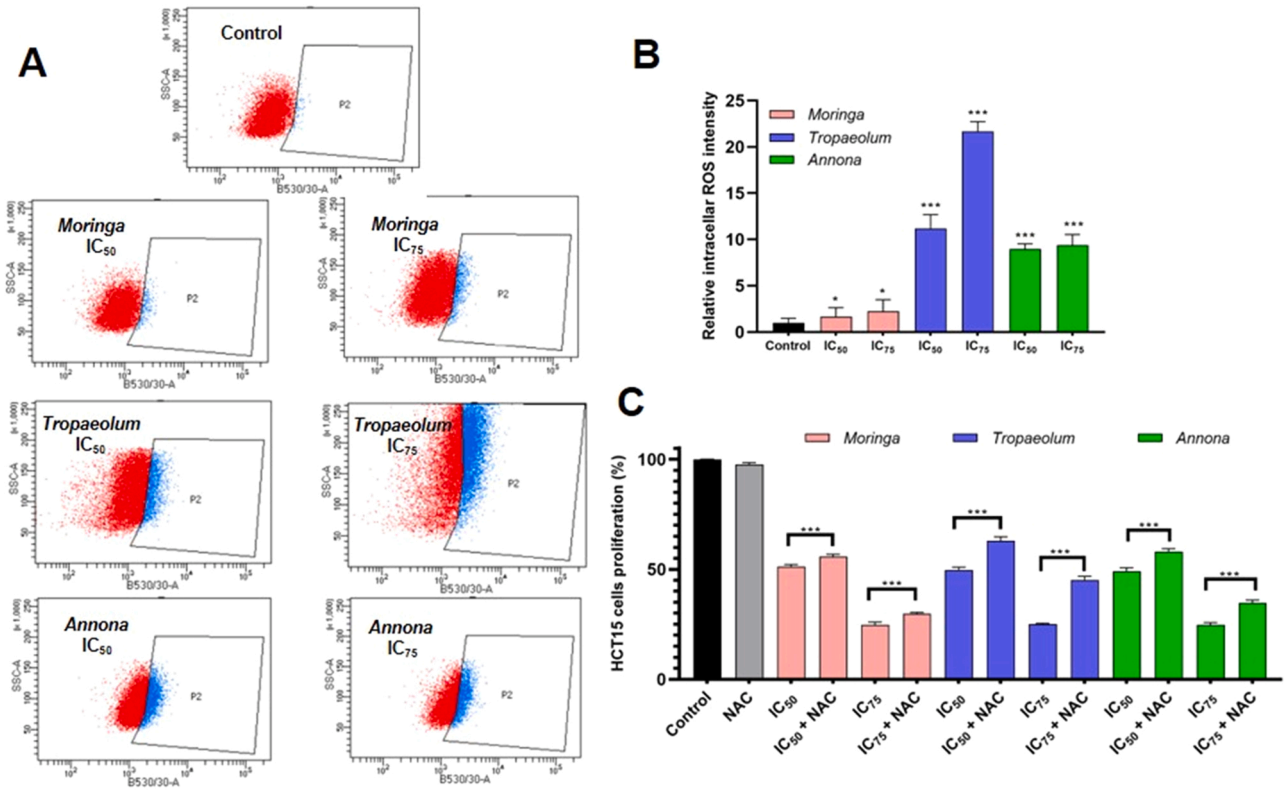

Fig. 7. Effect of ethanolic extracts on the induction of ROS accumulation. (A) Images of the FACScan of flow cytometry results of T-84 cells exposed to DCFDA (Fluorescein, FITC). (B) Graphic representation of ROS production by ethanolic extracts; (C) Analysis of ROS production after NAC pretreatment (100 μM). Significant differences vs control are denoted by (*) $p < 0.05$ significant, (**) $p < 0.01$ highly significant, (***), $p < 0.001$ very highly significant.

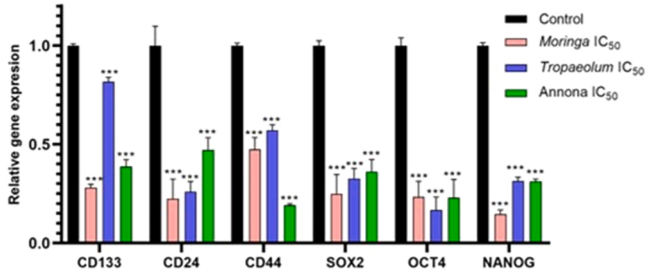

Fig. 8. RT-qPCR analysis of cancer stem cells markers. Significant differences vs control are denoted by (*) $p < 0.05$ significant, (**) $p < 0.01$ highly significant, (***), $p < 0.001$ very highly significant.

that had previously been detected in the genus *Annonaceae* such as a procyanidin B2 [77], rutin [78], acetogenins compounds [68], and procyanidin B1 [79].

Interestingly, when ethanolic extracts were associated with 5-FU treatment (the drug of choice for CCR treatment), we detected a significant increase in its antitumor activity. In fact, the ethanolic extract from *M. oleifera* (5 μg/mL) combined with 5-FU enhanced the antitumor activity of the latter to reach approximately 50% inhibition of HCT15 CRC cell proliferation. A similar proliferation rate inhibition (53%) was detected by combining the ethanolic extract from *M. oleifera* (20 μg/mL) and 5-FU in HCT15 MTS, an in vitro system that mimics primary CRC tumors. Nur et al., also showed that the *M. oleifera* ethanolic extract increased the sensitivity of WiDr CRC cells to 5-FU [80]. In addition, the ethanolic extract of *M. oleifera* leaves also showed synergistic activity with chemotherapeutic agents such as cisplatin in the Panc-1 pancreatic cancer line [81].

The association of both *T. tuberosum* and *A. cherimola* extracts with 5-FU also improved the antitumor activity of 5-FU. In fact, a synergistic effect of the genus *Tropaeolum* (species, *Tropaeolum majus* L) was reported after combining its extracts with 5-FU in MCF-7 breast cancer cells [82]. Our results showed that combining *T. tuberosum* extract either at 15 μg/mL or 40 μg/mL with 5-FU significantly inhibited the proliferation of HCT-15 CRC cells (50%) and their MTS (40%). Similarly, the ethanolic extract of *A. cherimola* showed synergistic activity with 5-FU for all the combinations, although the greatest effect was achieved with 12 μg/mL of the extract (55% proliferation inhibition in HCT15 cells) and 5 μg/mL 5-FU (64% proliferation inhibition in HCT15 MTS). In this context, ethanolic and chloroform extract of another species of the Annonaceae family, *Annona muricata*, showed a synergistic effect with 5-Fu against SKG esophageal carcinoma cells [83]. Moreover, the ethanolic extract of *A. muricata* leaves together with doxorubicin produced a greater cytotoxic effect against 4T1 murine breast cancer cells [84]. Lastly, *A. muricata* bark extract enhanced the activity of docetaxel by 50% against DU-145 human prostate cancer cells [85]. These results suggest the applicability of the combined use of chemotherapy with our extracts to enhance the antitumor effects. The possible causes to justify this synergy with 5-Fu could be related to the extract effect on various 5-Fu resistance mechanisms such as increased ROS production (Fig. 7) that it has been demonstrated that increase 5-Fu apoptosis [86,87], the clear decrease in colon CSCs (Fig. 8), a cell population more resistant to 5-Fu [86,87] and, finally, the possible inhibition of P-glycoprotein (P-gp), a major efflux pump associated to the development of multi-drug resistance including 5-Fu. In fact, extracts of the same species from our plants were able to block resistance mediated by P-gp [88–90].

Finally, the ethanolic extracts from *M. oleifera*, *T. tuberosum,* and *A. cherimola* were able to induce GST and QR activity which both constitute defense mechanisms against chemical carcinogenesis [91]. Of note, the ethanolic extract of *M. oleifera* caused greater induction of enzyme activity (induction rate = 2.34 ± 0.002) compared to the other extracts. Recently, Cuellar-Núñez et al., reported the beneficial effect of

Moringa oleifera leaf consumption in a murine model of colorectal cancer-associated colitis by reducing inflammation and elevating the enzymatic activities of the liver and colonic enzymes GST and NQO1 [92]. Likewise, Famurewa et al., showed that seed oil of from *M. oleifera* reduced the in vivo nephrotoxicity and hepatoxicity of 5-FU by modulating both the redox imbalance and the iNOS/NF-κB/caspase-3 signaling pathway, as well as by increasing GSH expression [93].

The ethanolic extract of *A. cherimola* produced higher QR expression (induction rate = 2.51 ± 0.02) compared to the sulforaphane control and the other extracts. Interestingly, Ramos et al., previously reported that crude extract and polyphenol-rich fraction of the *A. crassiflora fruit* reduced hepatic oxidative damage in Triton WR-1339-induced hyperlipidemic mice by elevating the levels of detoxifying enzymes such as glutathione [94]. Although more studies will be necessary, our results suggest that ethanolic extracts could play an important role in the prevention of chemotherapy toxicity in cancer patients.

5. Conclusion

We developed and analyzed ethanolic extracts from *M. oleifera*, *T. tuberosum.* and *A. cherimola* and showed that they contain a high proportion of bioactive compounds, especially phenols. In vitro assays revealed that all the ethanolic extracts possessed moderate antioxidant capacity and a high antiproliferative capacity against CRC cell cultures as monolayers and MTSs. Interestingly, they had a significant inhibitory effect on colon CSCs as a model for cells that, in vivo, are responsible for cancer recurrences and resistance to chemotherapy. Furthermore, the ethanolic extracts showed synergistic activity with the chemotherapeutic agent 5-FU. Finally, our ethanolic extracts induced the detoxifying activity of GST and QR enzymes, suggesting that they can be used to reduce the toxicity and side effects of some chemotherapeutic agents. Therefore, although in vivo testing will be necessary, ethanolic extracts from *M. oleifera*, *T. tuberosum,* and *A. cherimola* could become new therapeutic options, either alone or in combination with other antitumor agents, for the prevention and treatment of CRC.

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CRediT authorship contributions statement

José Prados: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. Jesús M. Porres: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Consolación Melguizo:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Data curation, Validation, Visualization. **Marco Fuel:** Data curation, Investigation, Methodology, Writing - original draft. **Cristina Mesas:** Data curation, Investigation, Methodology, Validation, Visualization, Conceptualization, Data curation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing. **Rosario Martínez:** Data curation, Investigation, Methodology, Validation, Visualization. **Raul Ortiz:** Formal analysis, Investigation, Methodology, Software. **Francisco Quiñonero:** Formal analysis, Investigation, Methodology, Software. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

All authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112248.

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