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Anticancer activity of "Trigno M", extract of *Prunus spinosa* drupes, against *in vitro* 3D and *in vivo* colon cancer models

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ABSTRACT

In 2018 there were over 1.8 million new cases worldwide of colorectal cancer and relapses after clinical treatments. Many studies ascribe the risk of the appearance of this cancer to the Western life style : a sedentary life, obesity, and low -fiber, high -fat diets can promote the onset of disease. Several studies have shown supplement phytochemicals to have an inhibiting effect on the growth of various cancers through the activation of apoptosis. Our goal was to prove the effectiveness of a natural compound in the combined therapy of colorectal cancer. Trigno M supplement was an optimal candidate as anticancer product for its high concentrations of phenolic acids, flavonoids and anthocyanins. Our work showed the antitumor activity of Trigno M, extract of *Prunus spinosa* drupes combined with the nutraceutical activator complex (NAC), in 2D, 3D and *in vivo* colorectal cancer models. The cellular model we used both *in vitro* and *in vivo* was the HCT116 cell line, particularly suitable for engraftment after inoculation in mice. Trigno M inhibited the growth and colony formation of HCT116 cells (35%) as compared to the chemotherapy treatment with 5-fluorouracil (80%) used in clinical therapy. The reduction of the morphological dimensions in the spheroid cells after Trigno M, was compared with 5-fluorouracil demonstrating the efficacy of the Trigno M compound also in 3D models. Flow cytometric analysis on 3D cells showed a significant increase in the apoptotic cell fraction after Trigno M treatment (44.8%) and a low level of necrotic fraction (6.7%) as compared with control cells. Trigno M and 5-fluorouracil induced the apoptosis in a comparable percentage. Monotherapy with Trigno M in severely immunodeficient mice, carrying colon rectal cancer xenografts, significantly reduced tumor growth. The histopatological analysis of the ectopic tumors showed a lower level of necrosis after Trigno M treatment compared with the control. We conclude that Trigno M is well tolerated by mice, delays colorectal cancer growth in these animals and should be weighed up for integration of the current multi-drug protocols in the treatment of colon carcinoma.

1. Introduction

Colon cancer is one of the most common malignancies and patients are often diagnosed in advanced stages of the disease. The overall 5-year survival rate of colorectal cancer is about 60%. After chemotherapy treatments, up to 40% of patients experience disease

Abbreviations: PsT, *Prunus Spinosa* Trigno; NAC, nutraceutical activator complex; ATCC, American Type Culture Collection; MTT, -(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5-FU, 5-Fluorouracil; STS, Staurosporine; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide; SEM, scanning electron microscopy; FBS, foetal bovine serum; CCD, charge coupled device; FITC, fluorescein isothiocyanate; PI, propidium iodide; SCID, severe combined immunodeficient; ANOVA, analysis of variance; CRC, colorectal cancer; P-gp, P-glycoprotein

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relapse. Colon cancer usually re-occurs as liver or lung metastases or as a locoregional recurrence in the pelvis or peritoneum [1]. Most relapses occur during the first 2 or 3 years after initial treatment [2]. First line treatments involve the use of targeted agents combined with standard chemotherapy such as the combination of cetuximab plus folinic acid, 5-fluorouracil, and oxaliplatin or irinotecan [3]. Unfortunately, a significant percentage of colonic cancers develop chemoresistance [4]. Moreover, this combined approach, despite its comparable efficacy, was not non cost-effective in some cohorts of colon cancer patients [5].

Research efforts are currently being directed towards the developing preclinical *in vitro* and *in vivo* models to speed up the evaluation of novel treatments for this neoplasm. Epidemiological analyses and clinical studies have highlighted a close relationship between small intestine, inflammation, intestinal microbiota and colon cancer [6]. The role between the constant intake of flavonoids and the induction of carcinogenesis is very important to understand their influence on the intestinal microbiota and the development of the tumor. Supplements with a high concentration of polyphenols have proved to be very effective and protective against the development of chronic degenerative diseases, especially in neoplasms where the pro-inflammatory context promotes carcinogenesis [7]. It has recently been hypothesized that compounds with high concentrations in polyphenols have a very important role, in particular, in the prevention of colorectal carcinoma and in sensitization to radio and chemotherapy [8]. This effect is probably due to the immunomodulatory properties of polyphenols towards cytokines, chemokines and the activation of immune cells. The immunomodulatory activity of natural polyphenols in Trigno M counteracts the inflammatory tumor microenvironment and increases cellular sensitivity to colon carcinoma therapies. It is all too evident that the complexity, heterogeneity, plasticity and diversity of the human tumor microenvironment can not be exhaustively explored in two-dimensional tumor cell line models. Spheroids are a reliable bridge between the cell monolayer and the *in vivo* model, as it offers a more reliable model of chemoresponse [9]. *Prunus Spinosa* Trigno ecotype (PsT) drupe extract combined with the nutraceutical activator complex (NAC) has shown an anticancer effect against several cancer cell lines, including colorectal, uterine, cervical, and bronchoalveolar cells [10]. The PsT plant extract contains many active molecules, in particular, it is rich in flavones, flavonols, phenolic acids and anthocyanins [10]. These molecules are considered the most important groups of secondary metabolites and bioactive compounds in plants with high antioxidant and antitumoral activity [11]. Many works show that the daily intake of flavonols, such as quercetin, flavones and anthocyanins, can reduce the colorectal cancer risk [12]. Unabsorbed flavonoids reach the colon in fairly high concentrations where they are metabolized by the intestinal microbiota to become small phenolic acids [13]. Intestinal microbiota can play an important role in the prevention of colorectal cancer [14]. Patients with different diets can modify the intestinal microbiota and its function by modulating and producing metabolites that regulate colon tumorigenesis through cellular metabolism and microbiota homeostasis. [15,16]. In our previous investigation, we described a novel supplemental diet product named Trigno M, corresponding to (PsT 10 mg/ml plus NAC)® [10]. This product displayed an antitumor effect ascribed to the reduction of mitochondrial activity in the tumor cells, which resulted in the induction of apoptosis. In the current study, we assessed the effects of Trigno M against colon cancer cell lines in monolayer, in spheroids and in an *in vivo* model, in order to gather evidence of its effectiveness and mechanisms of action in a thorough preclinical setting.

2. Materials and methods

2.1. Plant material

Blackthorn fruits of *Prunus spinosa* Trigno ecotype (PsT) were collected in the Molise region that is positioned on the Eastern side of the Apennines watershed, with the typical Mediterranean climate of the south-central Italy. The low human activity and the absence of industries make this territory healthy and clean from photochemical smog and fine particles.

The key morphological characteristics as described in Flora d'Italia were used for plant identification [17]. The fruits were kept in cooled bags and then stored in the deep-freezer at −20 °C for subsequent analysis. PsT was extracted by macerating the vegetable material in a 60° alcohol solution, obtaining a PsT 86 mg/ml solution (PsT 86 mg/ml).

2.2. 2D cell cultures

The established human colorectal carcinoma cell line (HCT116) was provided by the American Type Culture Collection (ATCC, Manassas, VA, USA) and used according to Meschini and colleagues [10]. The cell line was grown as a monolayer in RPMI medium (Gibco Life Technologies, Paisley, UK).

2.3. Cell treatments

Cells were treated with different solutions (1, 2, 5, or 10 mg/ml of *Prunus spinosa*) obtained after the progressive dilution of PsT 86 mg/ml solution with a complex blend of amino acids, vitamins and minerals, called nutraceutical activator complex (NAC), for 24 h [10].

The final product (PsT 2 mg/ml + NAC)® was obtained when PsT 86 mg/ml solution was diluted with NAC to obtain a concentration of *Prunus spinosa* equal to 2 mg/ml [10]. (PsT 5 mg/ml + NAC)® was obtained when PsT 86 mg/ml solution was diluted with NAC to obtain a concentration of *Prunus spinosa* equal to 5 mg/ml. Finally, (PsT 10 mg/ml + NAC)® was obtained when PsT 86 mg/ml solution was diluted with NAC to obtain a concentration of *Prunus spinosa* equal to 10 mg/ml. The latter concentration (PsT 10 mg/ml + NAC)® has been patented and named Trigno M in the paper.

As positive control of apoptosis induction, cells were treated with Stauropsorine (STS, 1 μM, Sigma-Aldrich, Saint Louis, MO, USA) for 24 h [18].

5-Fluorouracil (5-FU, Sigma) 300 μM, a fluoro-pyrimidine analogue widely employed for the treatment of colon cancer, was used to compare it to the cytotoxic effect of Trigno M [19].

2.4. MTT assay

Cell viability was assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma Aldrich, Saint Louis, MO, USA).

After removing the cell medium, untreated cells and cells treated with (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)®, Trigno M (PsT 10 mg/ml + NAC)®, 300 μM 5-FU and 1 μM STS were washed with phosphate buffered saline (PBS, Sigma) and incubated with 0.5 mg/ml MTT solution for 2 h at 37 °C. After removing the MTT solution, the samples were lysed by 100 μl dimethyl sulfoxide (DMSO), and analyzed with a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Cell viability (%) was calculated as follows: (absorbance mean value of the treated sample/absorbance mean value of the control sample) ×100 [20].

2.5. Clonogenic assay

Untreated and treated HCT116 cells were detached and plated (1×10^3) in a 60 mm tissue culture dish and allowed to grow in culture medium for 15 days. After growth, cell colonies were fixed with 95% ethanol for 15 min, and stained with a methylene blue solution in 80% ethanol for 2 h. Only colonies composed of more than 50 cells were evaluated. The cloning formation rate (%) was calculated by dividing the number of colonies of treated cells, and number of colonies of

untreated cells.

2.6. Scanning electron microscopy (SEM)

In order to evaluate the morphological characteristics and alterations after (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)®, Trigno M and 5-FU treatments for 24 h, SEM analysis was conducted on HCT116 monolayer cells. The cultures were fixed directly with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 15 min. After washing twice with the same buffer, the samples were post-fixed in 1% osmium tetroxide for 30 min at room temperature, dehydrated through graded ethanols, critical-point dried in CO_2 (CPD 030 Balzers device Baltec, Balzers) and gold coated by sputtering (SCD040 Balzers device, Baltec). The samples were examined with a Fei Quanta Inspect Feg, field emission scanning electron microscope (FEI Company, Eindhoven, The Netherlands).

2.7. Cell migration ability detected by wound scratch assay

The colon carcinoma HCT116 cells were seeded (1×10^6) on 35 mm Petri dishes. When the cell reached confluence, a vertical wound was performed, to evaluate its extent of recovery after 24 h treatment with (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)® and Trigno M compared to the control. PBS was used to wash the plate three times after making the wound and the RPMI plus 10% foetal bovine serum (FBS) was used for continuous cultivation. Inverted phase-contrast microscope (OLYMPUS BX51, Olympus Corporation of the Americas, Center Valley, PA) equipped with a charge coupled device (CCD) camera (Carl Zeiss, Jena, Germany) was used. This was repeated three times with image measurement.

2.8. Generation of spheroids and treatments

The method used was the 3D embedded assay modified [21]. To allow the formation of spheroids the growth factor reduced matrigel (BD Biosciences, San Jose, CA), once thawed at 4 °C was diluted with the complete medium (RPMI plus 10% FBS): 2/3 matrigel and 1/3 RPMI (matrigel coat). The bottom of the wells was covered with this composition. Once the matrigel coat solidified in the incubator, the cells were detached and deposited (2.5×10^3 HCT116 cells in each well) on the solidified matrigel and incubated at 37 °C to allow cell adhesion. Once adhesion had occurred, the cells were gently coated with an RPMI-matrigel mixture (10%) and the plates were placed in the incubator in a 5% CO_2 atmosphere. Once the spheroids of about 100 μm were obtained, they were treated with (PsT 5 mg/ml + NAC)®, Trigno M and with the chemotherapeutic drug 5-FU (300 μM) for 24 h.

Spheroids were photographed in an inverted phase contrast microscope (OLYMPUS BX51, Olympus Corporation of the Americas, Center Valley, PA) equipped with a CCD camera (Carl Zeiss, Jena, Germany). A micrometer scale was photographed at the same magnification, and spheroid size was determined.

2.9. Quantification of apoptosis on spheroids by AnnexinV-FITC labelling

Annexin V-fluorescein isothiocyanate (FITC)/ propidium iodide (PI) apoptosis detection kit (eBioscence, San Diego, CA, USA) was used on untreated and treated spheroids to investigate on cell death apoptotic induction. All spheroid samples were disaggregated into single cells by syringe. After washing with binding buffer solution (1X), cells were incubated with AnnexinV/FITC (5 μl in 100 μl of cell suspension) for 15 min and then with PI solution (5 μl in 200 μl of cell suspension). Samples were analyzed with a BDLSRII flow cytometer (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) equipped with a 5 mW, 488 nm, air-cooled argon ion laser and a Kimmon HeCd 325 nm laser. The fluorescence emissions were collected through a 530-nm band pass filter for FITC, a 575-nm band pass filter for PI. At least 10,000 events/sample were acquired in log mode for Annexin V-FITC/PI labeling. Percentages of apoptotic, necrotic and viable cells were calculated using the FACS Diva Software (Becton, Dickinson & Company).

2.10. Animal study

Female severe combined immunodeficient (SCID) mice (6–8 weeks old; weight 18–25 g) were obtained from Charles Rivers Laboratories (Milan, Italy). Mice were housed in individual cages, at a temperature of 22 °C and a photoperiod of 12 h of light and 12 h of darkness at the animal facility of the Regina Elena Cancer Institute of Rome for two weeks before each experiment. Animals had access to water and food *ad libitum*. Each experiment used 10 mice per treatment arm and was repeated twice for confirmation of the obtained data. All procedures were in accordance with institutional guidelines under the control of the Italian Ministry of Public Health (Italian Law D.lgs 26/2014) and with the Guide for the Care and Use of Laboratory Animals.

2.11. Colon carcinoma flank tumor model

HCT116 cells 1.5×10^6 in 0.2 ml of complete medium were injected in the flank of 30 mice. Ten animals were randomly allocated to each of the following groups: i) control (receiving saline solution gavage once a day for 5 days per week), ii) high-dose Trigno M (0.15 mg/ml) in mice with minimal disease (tumor palpable but not measurable) administered through gavage once a day for 5 days per week, iii) low-dose Trigno M (0.05 mg/ml) in mice with minimal disease (tumor palpable but not measurable) administered through gavage once a day for 5 days per week. Mice were weighed and callipered three times every week and were sacrificed when they met the established criteria so as to minimize pain and suffering. In detail, experimental endpoints were the following: tumor volume > 1 $cm^3 \pm 0.3\ cm^3$, tumor at risk of ulceration, weigh loss > 20%, inability to drink or feed independently or severe neurological symptoms. Tumor size was assessed by using the formula: (π x long axis x short axis x short axis)/6 as reported [22]. After one month the mice were sacrificed and histological exam performed on the internal organs.

An additional efficacy study to evaluate the preventive action of Trigno M was done by matching the efficacy of Trigno M (PsT 10 mg/ml + NAC)®, given at the time of tumor implant *versus* Trigno M administered in mice with measurable disease (0.2 mm^3). Both groups of 6 mice each were treated through gavage, as above described, and their response was matched against controls (receiving saline solution gavage) to evaluate the preventive action of Trigno M.

2.12. Histological analysis

Briefly, 3–5 micron sections of the xenografts, fixed with paraformaldehyde and embedded in paraffin, were mounted on glass slides and dried overnight at 37 °C. For histology, stainings with hematoxylin/eosin and hematoxylin/Van Gieson were used.

2.13. Statistical analysis

The distribution of each measurement of *in vitro* assays was examined for the assumption of normality with the Shapiro–Wilk test. One-way Analysis of Variance (ANOVA) was applied to detect differences between the control and treatments. Bonferroni post hoc analysis was applied to reveal differences between all treated samples in each cell line. The alpha level was set at $p < 0.05$.

Tumor volumes at a given time were analyzed by comparing the different groups in each experiment by using ANOVA with post-hoc testing (Wilkoxon test), when significant differences ($p < 0.05$) were found. The growth curves were designed considering the mean tumor volume at each time point. Cut off point for statistical significance

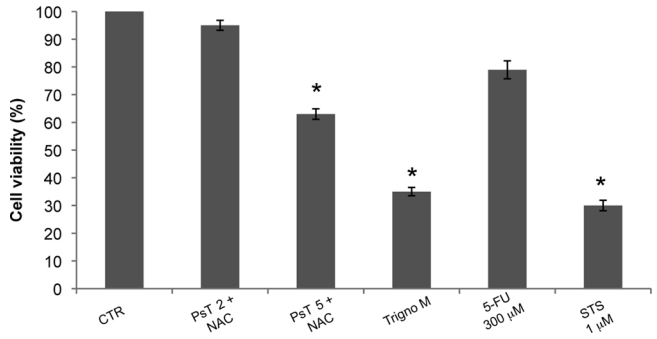

Fig. 1. The effect of *Prunus spinosa* plus NAC on HCT116 cell viability. *Prunus spinosa* plus NAC reduced HCT116 cell viability in a dose dependent manner. HCT116 cells were treated with (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)®, (PsT 10 mg/ml + NAC)® or Trigno M, and 5-FU (300 µM) for 24 h. As positive control for apoptosis induction, cells were treated with 1 µM STS for 24 h. Cell viability was assessed by MTT assay, performed for three independent experiments. One-way Analysis of Variance (ANOVA) was applied. * = significant differences compared to control cells ($p < 0.05$).

was < 0.05.

3. Results

3.1. Trigno M inhibited the growth and colony formation of colon carcinoma cells (HCT116) in a dose-dependent manner

Given the particular heterogeneity and variability of two-dimensional cell lines in the drugs response, we repeated and data confirmed of the cytotoxicity experiments on this line before producing 3D cells.

In vitro cytotoxicity of (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)®, and Trigno M (PsT 10 mg/ml + NAC)® was evaluated by MTT assay. As previously demonstrated, the MTT test is particularly suitable for the evaluation of cell viability inhibition because it is able to quantify the mitochondrial damage induced by a drug. In our previous work we had shown that after 24 h of treatment with Trigno M the HCT116 cancer cells modified their mitochondrial membrane potential with an increase of 20% depolarized mitochondria [10]. As shown in Fig. 1, Trigno M effectively inhibited the growth of HCT116 colon carcinoma cell line (65%) compared with control cells, while the reduction value of (PsT 2 mg/ml + NAC)® was not significant. Already a reduction in cell viability was observed at the concentration of (PsT 5 mg/ml + NAC)®; 5-FU (300 µM) for 24 h reduced the cell viability by only 15%.

Fig. 2 shows the typical colony formation images of HCT116 cells (A, control cells) treated with (PsT 2 mg/ml + NAC)® (B), (PsT 5 mg/ml + NAC)® (C) and Trigno M (D). The histogram (E) shows the quantification of the survival curves inhibition derived from clonogenic assay. Trigno M treatments led to reduction of the colony-forming ability compared with control cells. Staurosporine is used as apoptosis positive control.

3.2. Characterization and toxicity of Trigno M and 5-FU on HCT116 cells

SEM images show that treatments with (PsT 2 mg/ml + NAC)® (Fig. 3B), (PsT 5 mg/ml + NAC)® (Fig. 3C and D), and Trigno M (Fig. 3E) for 24 h have a clear dose-dependent cytotoxic effect compared to control cells (Fig. 3A). At greater magnification, signs of cellular retraction from the substrate, alteration of the microvilli morphology and smooth areas on the cell membrane, are highlighted by the arrow (Fig. 3D). Fig. 3E shows the effect of Trigno M. To understand the importance of the results obtained, as a positive control, we performed the treatment with the 5-FU, a drug used in the human colon cancer,. at a concentration of 300 µM for 24 h. After 5-FU treatment, cells were a regular cellular carpet, the microvilli were within normal size, no irregular extensions were revealed, and the cell membrane was intact (Fig. 3F). It should be noted that figures E and F have the same

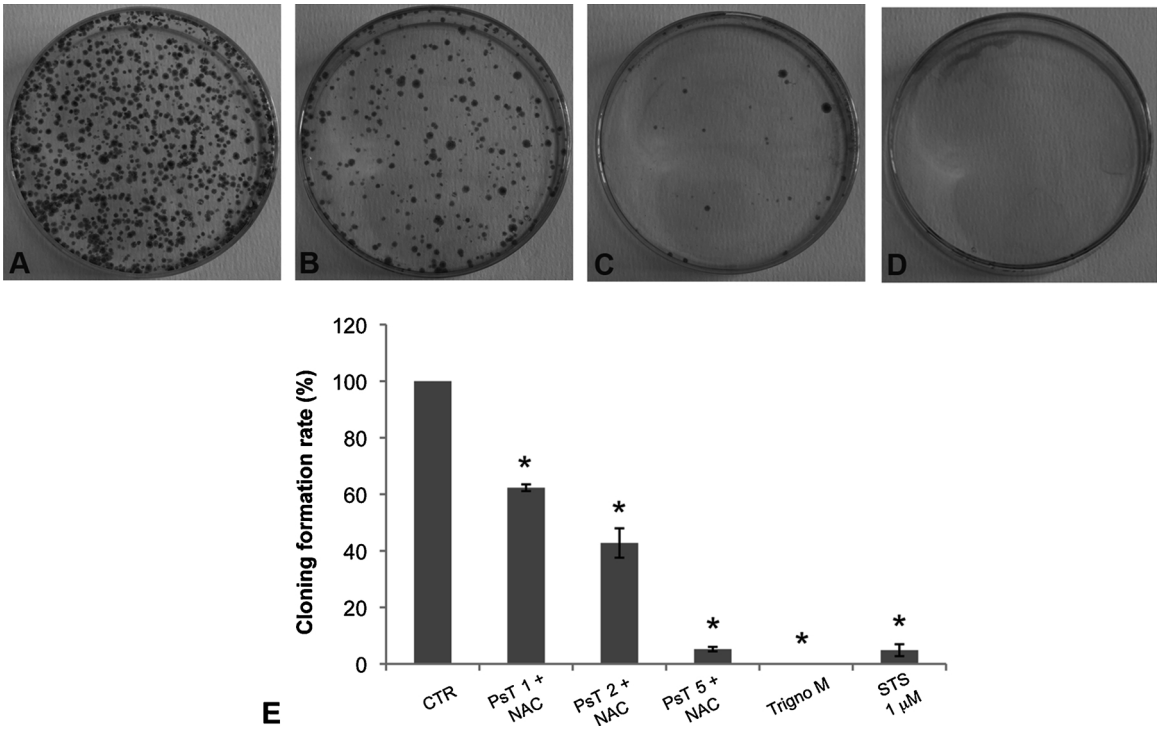

Fig. 2. The effect of *Prunus spinosa* plus NAC on HCT116 cell survival. *Prunus spinosa* plus NAC inhibit colony formation of HCT116 cells. Representative images of control HCT116 colonies (A), treated with (PsT 2 mg/ml + NAC)® (B), (PsT 5 mg/ml + NAC)® (C) and Trigno M (D). Quantitative analysis of clonogenic effect evaluated on HCT116 cells after treatment with different concentrations of (PsT + NAC)®. STS was positive control. Experiments were performed three times. One way ANOVA was applied, * = significant difference compared to control cells, $p < 0.001$.

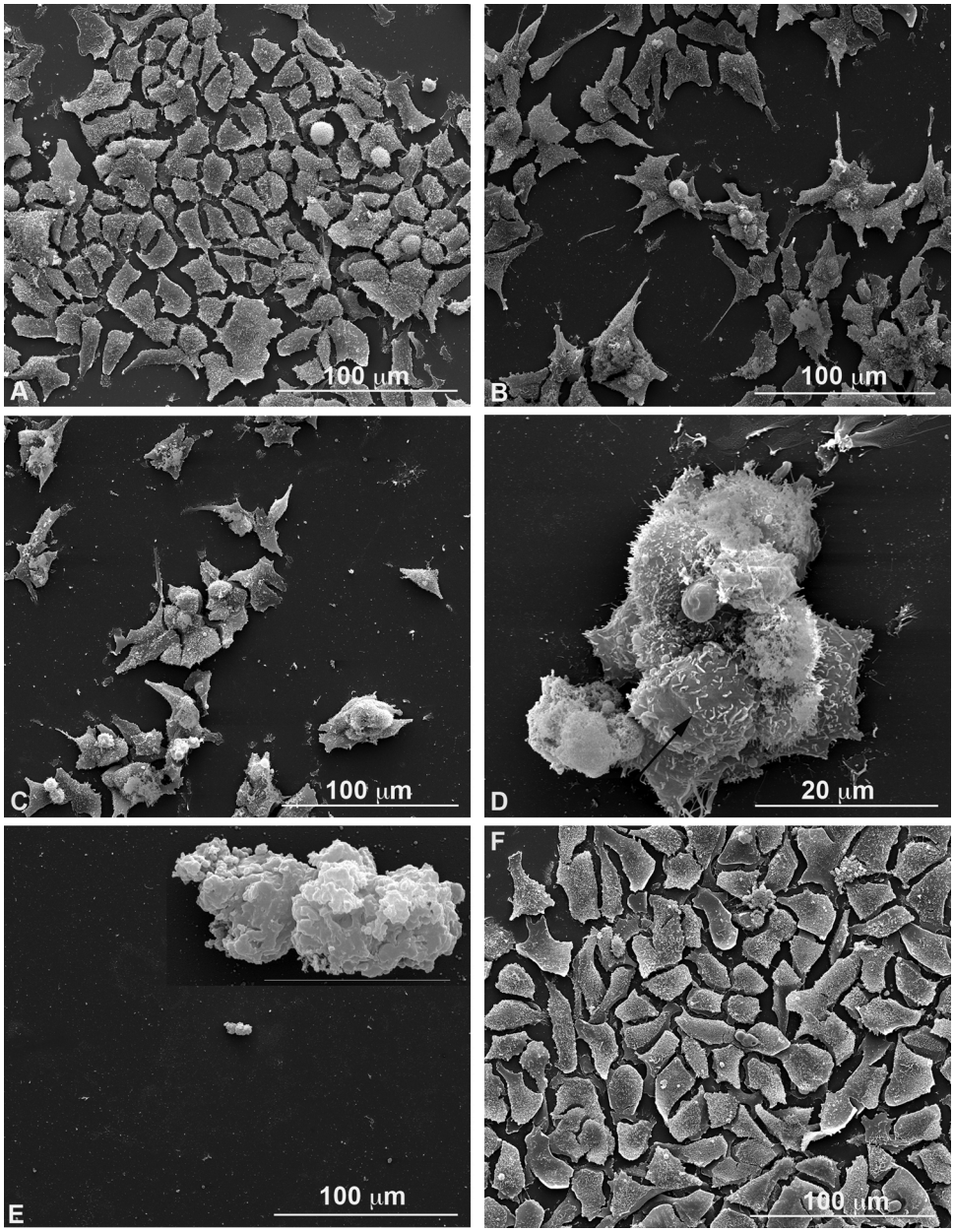

Fig. 3. The effect of *Prunus spinosa* plus NAC on HCT116 cell morphology. The alterations induced by different concentrations *Prunus spinosa* plus NAC on HCT116 cells. SEM images of cells treated with (PsT 2 mg/ml + NAC)® (B), (PsT 5 mg/ml + NAC)® (C and D), and Trigno M (E) for 24 h, showed a clear dose-dependent cytotoxic effect compared to control cells (A). No particular sign of cellular modification was observed after treatment with 5-FU (300 μM) (F).

magnification. With the Trigno M treatment all the cells were detached, the only cells remaining (in the enlargement) appeared as an accumulation of dead cells. As in the cloning efficiency image (Fig. 2D), Trigno M treated cells were no longer attached to the substrate as a result of a severe cytotoxic effect.

3.3. Cell migration ability of HCT116 cells in cell wound scratch assay

Fig. 4A is the zero time, namely, the time in which the wound was made on the cellular monolayer. After 24 h the cellular monolayer has reconstituted itself and two vertical continuous lines indicate the free space between the cells (Fig. 4B, control cells). Fig. 4C shows the HCT116 cells treated with a low concentration of (PsT 2 mg/ml + NAC)® for 24 h. Cell growth is very similar to zero time. At a higher concentration (PsT 5 mg/ml + NAC)®, cell growth is still similar to that at zero time, but now there are many rounded cells in the space delimited by the lines (Fig. 4D). When cells were treated with Trigno M, a complete detachment of the whole cellular monolayer was observed, indicating an irreversible cytotoxic damage (Fig. 4E). Fig. 4F shows treatment with 5-Fluorouracil 300 μM for 24 h. Many floating cells are observed indicating cytotoxicity but also a migratory growth front (arrow) is clearly visible. The evaluation of the migration distance of HCT116 cells treated with (PsT 2 mg/ml + NAC)®, (PsT 5 mg/ml + NAC)® and 5-FU for 24 h was significantly larger than that HCT116 cells of control group and the difference was statistically significant ($p < 0.05$) (Fig. 4G).

3.4. Morphological changes evaluated in HCT116 spheroids after (PsT 5 mg/ml + NAC)®, Trigno M and 5-FU treatments

In the phase contrast optical microscopy images the control spheroids (Fig. 5A), obtained with the colon carcinoma cell line HCT116 (the

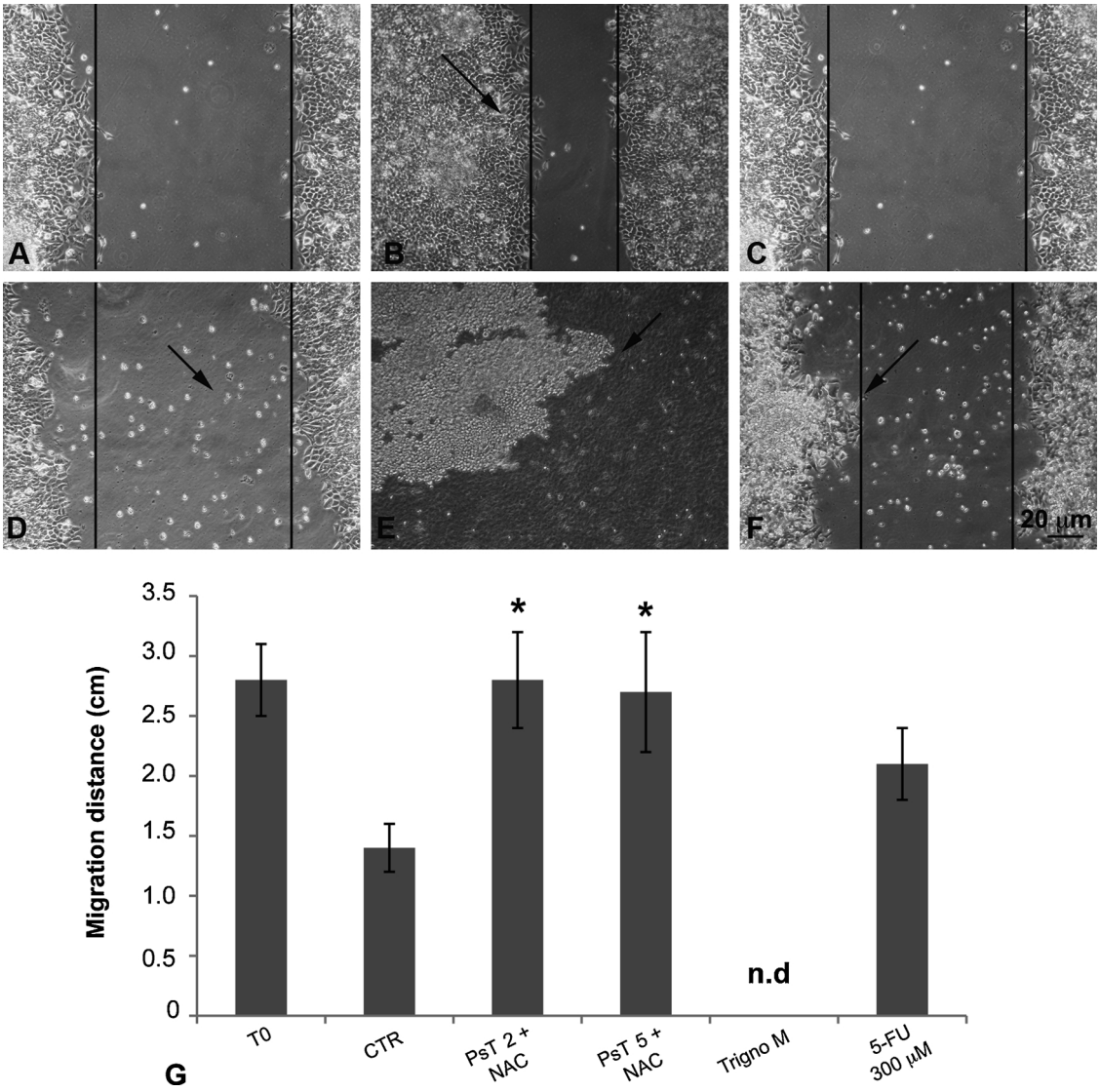

Fig. 4. The effect of *Prunus spinosa* plus NAC on HCT116 cell migration ability. Migration test was performed by wound scratch assay on HCT116 cells at zero time (A), on untreated cells (B), on cells treated with (PsT 2 mg/ml + NAC)® (C), (PsT 5 mg/ml + NAC)® (D), Trigno M (E), and 5-FU 300 µM (F) for 24 h. Images were captured at 0 and 24 h incubation with the drugs. (G) Migration ability of HCT116 cells in cell wound scratch assay. The migration distance of HCT116 cells treated with (PsT 2 mg/ml + NAC)® (PsT 5 mg/ml + NAC)® and 5-FU 300 µM was significantly larger than that control cells. The difference was statistically significant ($p < 0.05$). The migration distance of cells treated with Trigno M was not measurable because completly detached from the substrate (n.d., not detectable).

same cell line inoculated in the *in vivo* experiments), are compact, with a regular, continuous outer membrane. In Fig. 5B, spheroids treated with (PsT 5 mg/ml + NAC)® for 24 h, it is observed that the outer membrane is altered in several points, as evidenced by the up and laterally to the right arrows, the membrane is broken, the cellular material is leaking out and is beginning to observe an exfoliation of the spheroid. At the highest concentration of Trigno M for 24 h (Fig. 5C), cellular disintegration was observed in many spheroidal formations. At low magnification, little and disaggregated spheroids (arrows) was observed (Fig. 5D).

As shown in the 2D lines, 5-FU (300 µM for 24 h) was used to compare its effect on the HCT116 spheroids to Trigno M treatment. In the Fig. 6B the reduction and exfoliation at the cell membrane spheroids level treated with the Trigno M compared to the control (Fig. 6A) is noticeable. As can be seen also with the chemotherapeutic treatment (5-FU) (Fig. 6D), there is a reduction in the size of the spheroid as with treatment with Trigno M. In Fig. 6C, numerous disaggregated spheroids treated with Trigno M, at low magnification, are observed. Spheroids size reduction is related to induction of apoptosis, which is confirmed by Annexin V-FITC/PI flow cytometry (Fig. 7). These results showed the effectiveness of the natural compound also on 3D cellular systems, which are known to be more resistant to pharmacological treatments than two-dimensional lines.

3.5. Flow cytometric detection of apoptosis on 3D cells by Annexin V-FITC/PI staining

Flow cytometric analysis showed that Trigno M treatment induced a significant increase in apoptotic cell fraction in the HCT116 spheroid model (44.8% ± 1.8% compared to 24.9% ± 1.6% in the untreated control cells, 1.8% ± 0.2%) and a low increase in necrotic fraction (6.7%.8% ± 0.2% compared to the untreated control cells) (Fig. 7). The percentage of apoptotic cells in the control sample was due to the sample disintegration method adopted before Annexin V-FITC/PI staining. The necrotic fraction is significantly lower than the apoptotic fraction, thus the Trigno M effect is essentially due to induction of apoptosis, as the main mechanism of programmed cell death.

Upon comparison with the 5-FU treatment, a cytotoxic effect was

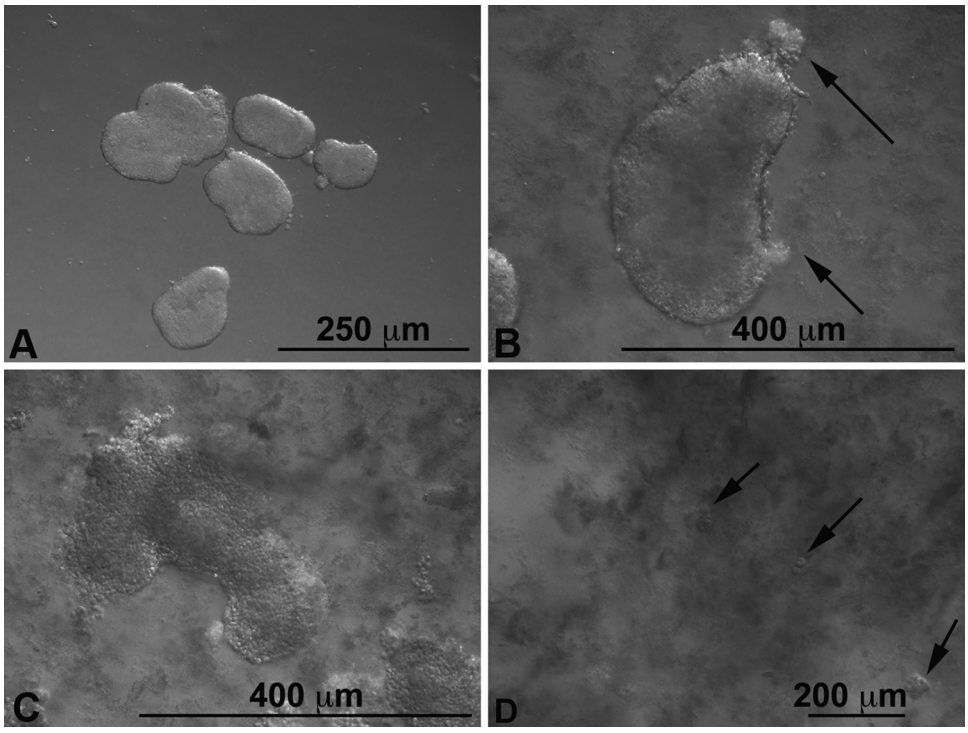

Fig. 5. The effect of *Prunus spinosa* plus NAC on spheroids. *Prunus spinosa* plus NAC disrupt HCT116 spheroids. Phase contrast optical microscopy observations showed representative images of control spheroids at six days of growth (A), spheroids treated with (PsT 5 mg/ml + NAC)® (B), and Trigno M (C, D) for 24 h. (D) Optical visualization at low magnification of spheroids (arrows) treated with Trigno M.

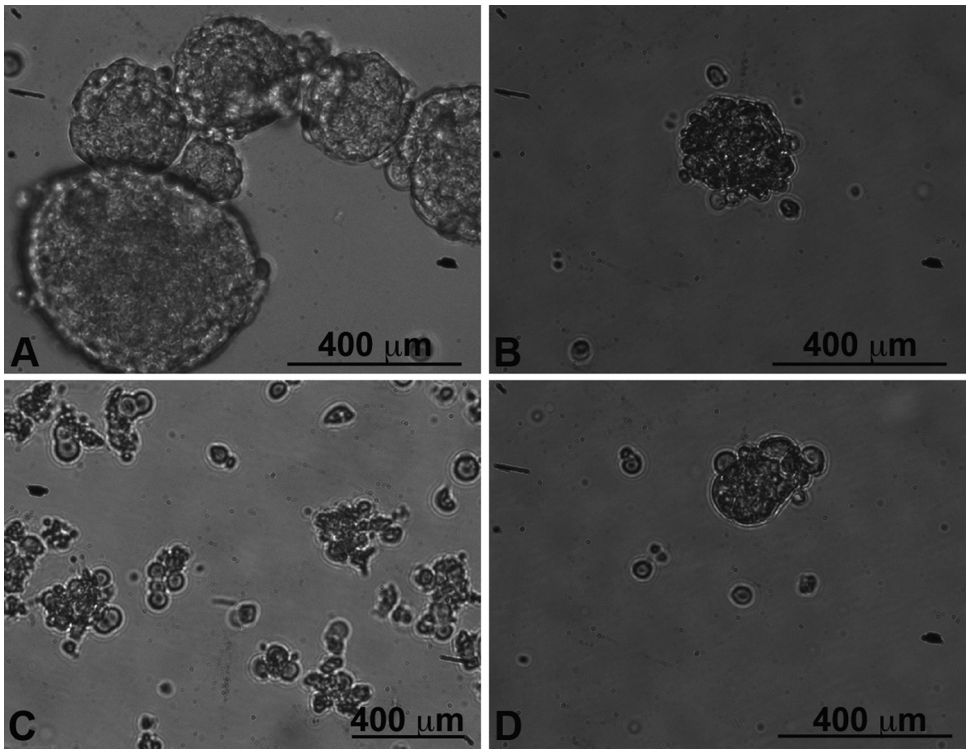

Fig. 6. The effect of Trigno M on spheroids. Trigno M disrupts HCT116 spheroids. Phase contrast optical microscopy observations showed representative images of control spheroids (A), spheroids treated with Trigno M (B), Trigno M image at low magnificantion (C) and 5-FU (D) for 24 h.

observed due to the apoptotic induction, a phenomenon similar to that obtained with Trigno M (Fig. 7). These quantitative analysis data on the 3D line confirm the results obtained from the morphological analysis (Fig. 5B and C), indicating that treatment with the antioxidant natural compound on colon carcinoma cells, may contribute to the cytotoxicity in a Trigno M combination with the chemotherapeutic agent.

3.6. Antitumor activity of Trigno M in mice carrying tumor xenografts

Fig. 8 illustrates the *in vivo* growth in volume of ectopic colon carcinoma induced by subcutaneous injection of 1.5×10^6 HCT116 cells in the flank of SCID mice. Tumor growth progressed in all experimental groups. In the control group, tumors reached volumes that led to euthanasia over a 25-day period ($1\ cm^3 \pm 0.4\ cm^3$), either because they

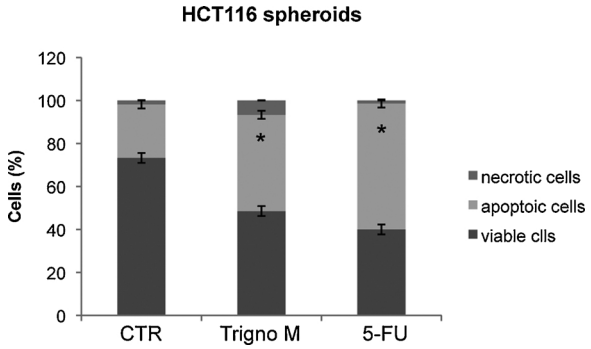

Fig. 7. Trigno M induce apoptosis on HCT116 spheroids. Cytofluorimetric analysis of apoptosis induction on HCT116 spheroids untreated (CTR) and treated with Trigno M and 5-FU (300 μM) for 24 h. * = significant difference compared to control cells, $p < 0.001$.

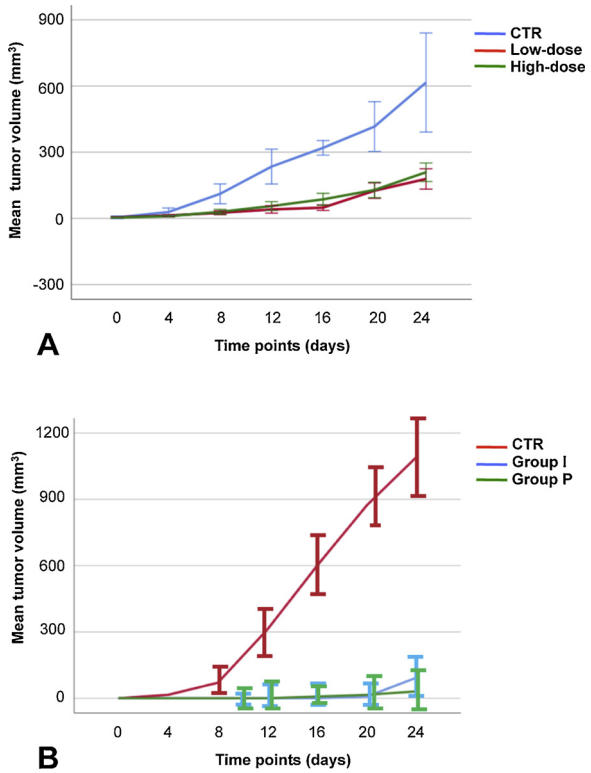

Fig. 8. The effect of Trigno M on colon tumor growth in SCID mouse model. (A) SCID mice were treated with low-dose (0.05 mg/ml) and with high-dose (0.15 mg/ml) of Trigno M. Trigno M significantly reduced tumor growth in SCID mouse model; n = 10, p = 0.001 *vs.* control group (CTR). (B) Trigno M was administered by gavage starting on day 2 after cell implantation (group I) or Trigno M was administered by gavage starting at the time of tumor detection by palpation (group P). Trigno M significantly reduced tumor growth of both treatment groups compared to control group (CTR); n = 6, ($p < 0.0001$).

reached the maximum tolerated volume of 1.4 cm^3 or because the tumors were prone to ulceration and resulted in earlier removal of the subject(s) from the experiment. Monotherapy with Trigno M slowed down tumor progression for both treatment groups as compared to controls (p = 0.001) (Fig. 8A). The comparison of low-dose *versus* high-dose, showed no statistical significance. By the end of the 25-day follow-up period, all the surviving mice of the two treatment groups were sacrificed according to the experimental design.

Aiming at further investigating the effect of concentrated Trigno M, we conducted a toxicology study in a group of 6 mice receiving whole extract Trigno M by oral gavage (0.2 mg daily, 5 days a week) for one month. The mice were weighted three times a week and observed for signs of toxicity. All the mice reached the experimental endpoint without showing signs of sufferance or weight loss. At the completion of the experiment, the mice were sacrificed and organs were analyzed for evidence of toxicity. Histological examination of the organs was negative for signs of pathology (not shown).

Fig. 8B illustrates the *in vivo* effect of "Trigno M" on the growth of colon ectopic carcinoma xenografts induced as above described. Mice were assigned to the following experimental groups: (CTR) Control; (group I, implantation) Trigno M administered by gavage starting on day 2 after cell implantation; (group P, palpation) Trigno M administered by gavage starting at the time of tumor detection by palpation. Tumor growth progressed in all experimental groups. Again, in the control group tumors reached volumes that led to euthanasia over a 25-day period (1 cm^3 ± 0.4 cm^3), as above described. Monotherapy with Trigno M slowed down tumor progression for both treatment groups as compared to controls ($p < 0.0001$). Interestingly, no statistically significant difference was observed between the two treatment groups.

Fig. 9A–C illustrate the histopathological characteristics of the tumors in the three experimental groups. The histological analysis revealed a significant lower level of necrosis in the tumors treated with Trigno M with respect to the control (Fig. 9A). The level of necrosis was lower in the animals treated with high-dose Trigno M (Fig. 9C), with respect to the animals treated with low-dose Trigno M (Fig. 9B). The extension of tumor necrosis was approximately 30% on the control group, while it decreased respectively to 10% and less to 10% in the tumors treated with low-dose and high-dose Trigno M. Trigno M did not induce modifications on normal colon tissue (Fig. 9D).

4. Discussion

Like other phytotherapic compounds [23,24], Trigno M, has shown *in vitro* antitumor efficacy, and some effectiveness at delaying tumor xenografts in mice. In our previous work we showed the peculiar and significant presence of functioning compounds with antioxidant activity and therapeutic properties in drupes of *Prunus spinosa* [10]. This plant is characteristic of a specific Italian Region, Molise, and its extracts contain a greater quantity of flavonoid, phenolic acid, and anthocyanins groups than plants of *Prunus spinosa* growing in other Italian areas. Trigno M supplement (Italian Patent No. RM2015A 000133) was obtained from the *Prunus spinosa* natural compound with the addition of B3, B6, B1, B2, B12 vitamins (less than 15% with respect to nutrient reference values) and amino acids. The presence of a significant amount of phenolic acids (39.95 mg/100 g), of flavone/ols (66.91 mg/100 g) and anthocyanins (100.81 μg/100 g) in Trigno M makes it particularly interesting.

Trigno M behaviour was evaluated in a complex colorectal cancer (CRC) 3D model. Drug resistance is known to develop in almost all patients with colon cancer and to decrease the therapeutic efficacy of anticancer agents [25]. Moreover, many human colon carcinoma cell lines intrinsically overexpress the P-glycoprotein (P-gp) [26], whose expression is also inducible by chemotherapeutic agents. HCT116 cell line has been shown to overexpress P-gp, so can be actually sensitive to drugs that can act as modulators of this membrane protein and promote the induction of cell death by apoptosis [27]. Several studies have shown that a high percentage of flavonoids inhibits cell proliferation, angiogenesis, causes cell cycle arrest, induces apoptosis and reverses multidrug resistance alone or in combination with other therapeutic drugs [28]. Furthermore, quercetin has been shown to block the MDR1 transcription thereby suppressing P-gp expression [29] and restoring daunorubicin chemosensitivity in different resistant cancer cell lines [30].

Before making CRC three-dimensional model we verified the effectiveness of Trigno M treatment on the same two-dimensional line (HCT116) that was to be inoculated in the mouse for *in vivo* experiments. Both the MTT and clonogenic tests (Figs. 1 and 2) showed

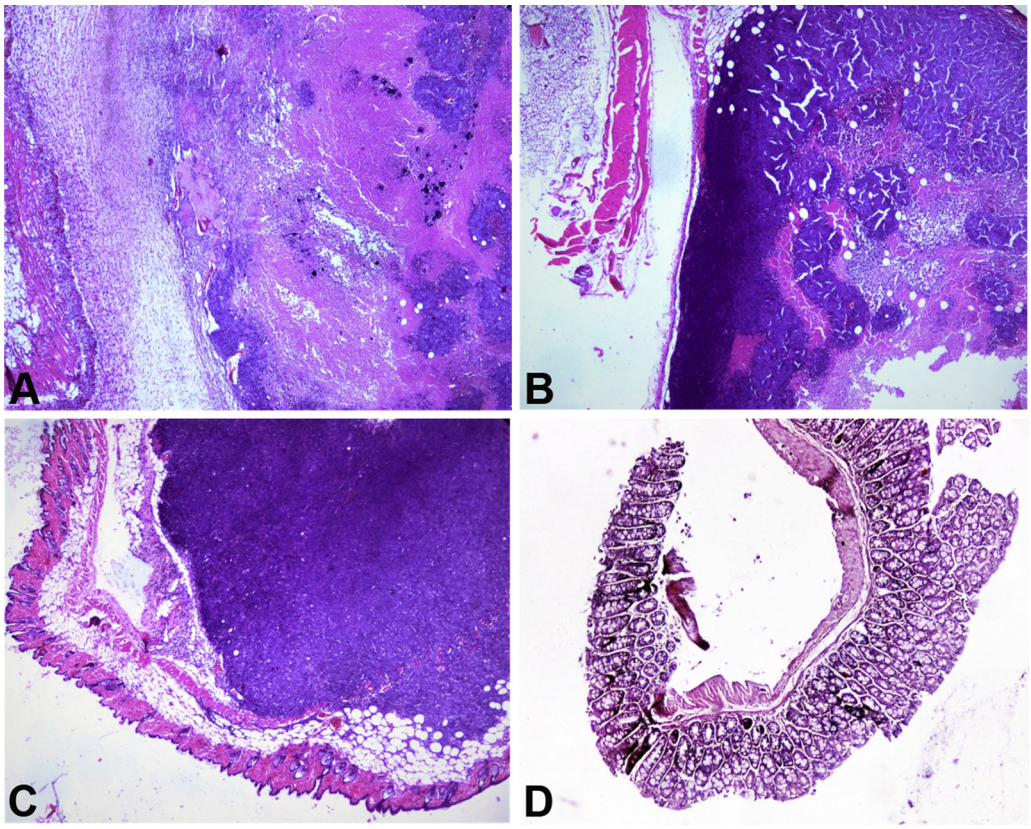

Fig. 9. Effect of Trigno M on histology of tumor and normal mouse colon (H&E staining). (A) Extensive necrosis is present in the control tumors (H&E, original magnification ×10); (B) the level of necrosis is significantly lower in the group of tumors treated with low-dose Trigno M (H&E, original magnification ×10); (C) the necrosis is almost absent in the tumors treated with high-dose Trigno M (H&E, original magnification ×10); (D) normal colon sample treated with Trigno M (H&E, original magnification ×10).

growth inhibition and complete remission of colony formation.

The cytotoxicity results of Trigno M on the HCT116 line were confirmed by scanning electron microscopy images (Fig. 3). 5-FU, a fluoro-pyrimidine analogue, widely used for the treatment of this type of cancer was chosen as chemotherapeutic to compare it with the cytotoxic effect of Trigno M [19]. Unfortunately, the mechanism of 5-FU resistance and induction of cell metastasis often occurred and resulted in poor outcome for the patients [31]. It is thus crucial to develop anticancer drugs to reduce tumor cell dissemination.

We explored the effect of Trigno M on the CRC anti-migratory capacity and observed that at the concentration of (PsT 5 mg/ml + NAC)® for 24 h, cell advancement was equal to that in the control but many cells were detached in the medium. Trigno M induced complete monolayer detachment, indicating a cytotoxic and inhibitory effect on the migratory potential of CRC cells. However, the understanding of this important process is largely limited and further studies are needed to explore the mechanism.

Three-dimensional tumor spheroids preserve many characteristics of avascular solid tumors and can be a useful *in vitro* model to investigate the aspects tumor biology. Spheroids cell-cell and cell-matrix interactions, growth characteristics, nutrient and oxygen diffusion dynamics and their microregions with heterogeneous features, surely reproduce *in vivo* solid tumors than in monolayer cultures [32,33]. Once spheroids were obtained with the HCT116 cell line, they were treated with Trigno M for 24 h, which resulted in an alteration of their morphology, an evident interruption of cell membrane continuity and cellular material extrusion (Fig. 6). The treatment on the same spheroids with the 5-FU resulted in the same size reduction rate as with Trigno M. Multicellular tumor spheroids are considered of intermediate complexity between *in vitro* monolayer cultures and *in vivo* tumors. 3D cultures are also a more resistance model not only because of the pharmacokinetic obstacles due to limitated penetration but also for the multicellular interactions leading to the altered expression of genes and proteins regulating drug response. To verify whether the mechanism of cell death observed in two-dimensional lines was the same in the 3D models, we verified and quantified the occurrence of this mechanism. Our experimental results show that the cytotoxicity induced by Trigno M both on 2D and 3D colon carcinoma lines is due to the triggering of an apoptotic program.

In vivo experiments showed that Trigno M exerted a delaying action on the growth of colon cancer xenografts, independently of concentration or treatment schedule. In detail, Trigno M was an effective growth delayer when administered in a preventive schedule or against palpable disease. The different concentrations worked in a similar fashion in terms of outcome. It must be pointed out, however, that different doses resulted in different percentages of tumor necrosis. Tumor necrosis in this model was caused by uncontrolled tumor cell growth that induced a low blood supply, mostly evident in the control group. This is consistent with other observations from phytoterapic agents used in cancer therapy, where the exact mechanism of action and antitumor contribution exerted by the different components of the extract(s) could not be fully quantified [34]. To note, pure extract of Trigno M is currently licensed to be used as a supplement for humans where it has displayed extremely high tolerability. Again, in our experimental settings, Trigno M showed no adverse effect in the treated laboratory animals. Our results suggest that the extract of *Prunus spinosa* is a supplement that could be evaluated as an integration to current multiagent protocols in individuals treated for colon carcinoma. Further studies are needed to identify the exact antitumor contribution of the different components of the *Prunus spinosa* extract and to assess its possible use as a chemosensitizer or immune modulator.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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