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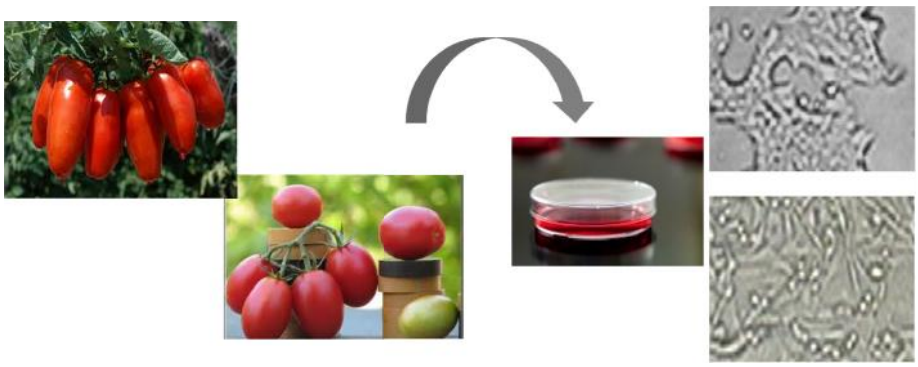
BIOLOGICAL EFFECTS OF TWO TOMATOES LIPOPHILIC EXTRACTS ON BREAST CANCER CELL LINES AND RELATED MOLECULAR PATHWAY INVOLVED



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Ringraziamenti e dediche

A chi sfida la sorte e vince

A chi ha coraggio ad affrontare l'ignoto

A chi crede in se stesso

A chi non si è mai arreso

A chi dimostra il proprio valore

A chi lavora con passione

A chi è umile

A chi è paziente

A chi spera nel futuro

Riassunto

Il cacnro della mammella è il tipo di cancro più frequente nelle donne. Dati recenti suggeriscono che lo stile di vita e la dieta giocano un ruolo fondamentale nello sviluppo e nella prevenzione del cancro mammario (1). Le attuali conoscenze confermano che una dieta salutare, come la dieta Mediterranea, caratterizzata da un'eleveta assunzione di frutta, verdura, fibre, pesce e oli insaturi, in particolare acidi grassi n-3, ha un effetto protettivo sul cancro al seno, mentre una dieta tipicamente occidentale caratterizzata da un'alta assunzione di grassi saturi, zuccheri raffinati, carne rossa e uno scarso apporto di fibre aumenta il rischio di sviluppo di tale neoplasia.

I pomodori sono elementi chiave della Dieta Mediterranea. Sono economici e facilmente conservabili, e in più sono un'eccellente fonte di composti fitochimici con forti proprietà antiossidanti.Finora il pomodoro non è mai stato valutato come alimento con potenziali proprietà antitumorali perché ci si è focalizzati sui singoli composti antiossidanti in esso contenuti come il licopene, β-carotene, ecc.

In questo studio abbiamo analizzato un possibile ruolo antineoplastico dei pomodori. In particolare, abbiamo utilizzato gli estratti lipofili totali di due cultivar del Sud Italia, il San Marzano e il Corbarino, che meglio mimano l'assunzione di pomodoro attraverso la dieta, senza isolare i singoli composti antiossidanti, o le vitamine, come spesso è riportato in letteratura. Abbiamo scelto modelli *in vitro* di cancro mammario, perché la maggior parte del licopene si accumula nella mammella essendo un organo costituito principalmente da tessuto adiposo.

Summary

Breast cancer is the most frequent type of cancer in women. Recent data suggest that lifestyle factors including dietary factors play a significant role in breast cancerdevelopment and survival(1).Current knowledge suggests that a healthy/Mediterranean-like diet characterized by high intake of fruit, vegetables, fiber, fish and unsaturated oils, particularly n-3 fatty acids, has a protective effect on breast cancer, whereas a typical Western diet characterized by high intake of total/saturated fat, refined carbohydrates, processed and red meat and low fiber intake is associated with poorer outcome.

Tomatoes are of specific interest because they are key elements of the Mediterranean diet. Moreover, they are relatively cheap and easy to store, and also because they are the main source of phytochemicals with strong antioxidant properties. Whole tomato as a food with potential anticancer properties has not been extensively evaluated so far, because greater attention has been given to single antioxidant compounds, such as lycopene, β-carotene, etc.

Here we presented our results on a possible antineoplastic role of tomatoes without significantly affecting non-tumorcells(primaryhuman skin fibroblasts). We used total lipophilc tomato extracts of two Southern Italy cultivars, San Marzano and Corbarino, which better mimics tomato intake by diet, without isolating antioxidants, or vitamins, as often reported in the literature. We chose*in vitro* models of breast cancer, because much of lycopene accumulates in breast, an organ consists mainly of adipose tissue.

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1. Introduction

1.1 Breast cancer: epidemiology and etiology

Breast cancer is the most frequently diagnosed cancer in women and one of the leading causes of cancer death for women. Worldwide, over 1.3 million cases of invasive breast cancer are diagnosed, and more than 450,000 women die from breast cancer annually (2). According to GLOBOCAN 2012, breast cancer is the second most common cancer in the world and so far, the most frequent cancer among women with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). It is the most common cancer in women both in more and less developed regions, with slightly more cases in less developed (883,000 cases) than in more developed (794,000) countries. Incidence rates vary nearly four-fold across the world regions, with rates ranging from 27 per 100,000 in Middle Africa and Eastern Asia to 92 in Northern America (Fig. 1). Breast cancer ranks as the fifth cause of death from cancer overall (522,000 deaths) and it is the most frequent cause of cancer death in women in the Southern emisphere (324,000 deaths, 14.3% of total), it is the second cause of cancer death in the Northern one (198,000 deaths, 15.4%) after lung cancer. The range in mortality rates between world regions is less than that for incidence because of the more favorable survival of breast cancer in (high-incidence) developed regions, with rates ranging from 6 per 100,000 in Eastern Asia to 20 per 100,000 in Western Africa (Fig. 2).

The disease is caused by multiple genetic defects that can be due to infectious and non-infectious factors, environmental and lifestyle factors, e.g. diet, physical inactivity, obesity, alcohol consumption, tobacco smoking (3). It is suggested that about 90% of cancers is linked to the environmental exposure (4, 5).

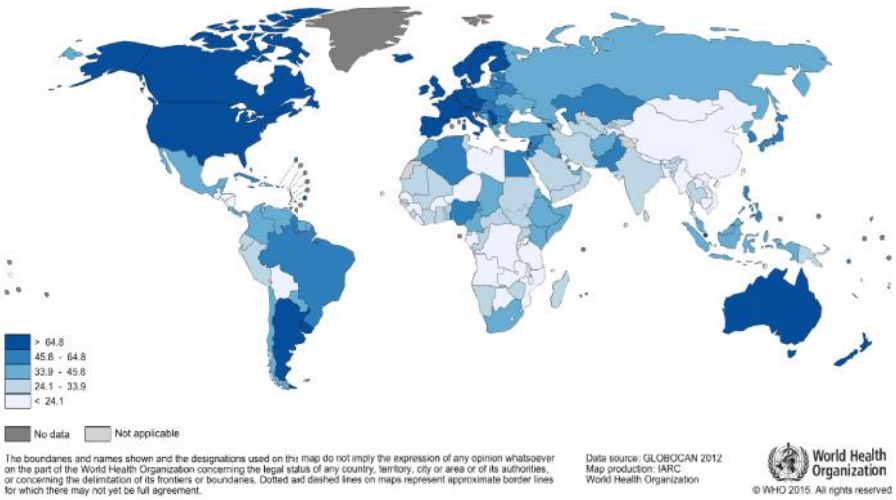


Figure 1

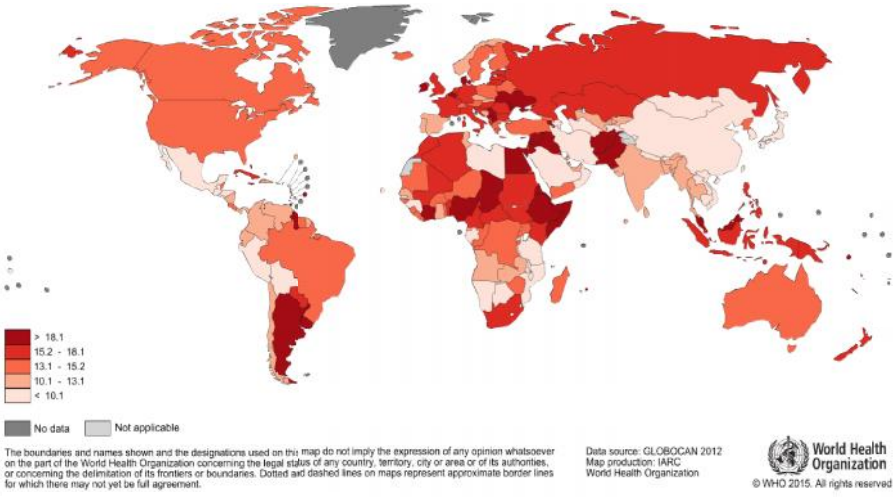


Figure 2

The main strategy for a control of breast cancer would be through primary prevention, followed by theidentification of risk factors for cancer. There is a large amount of evidence that the strongest factors that increase breast cancer risk in women are: age above 65 years, genetic mutations in *BRCA1* and/ or *BRCA2* genes (breast cancer susceptibility 1 and 2), high mammographic breast density, atypical hyperplasia (relative risk, PR>4); exposure to endogenous sex hormones, exposure to high-dose of ionizing radiation (RR=2.1-4.0); age at menarche <12 years, menopause above 55 years, no full-term pregnancies, postmenopausal obesity, personal history of colon or other gynecological cancers, long-term use of hormones containing estrogen and progestin (RR=1.2-2.0) (6). The first basic factor increasing breast cancer risk is patient's age at the moment of diagnosis of neoplastic disease. As it was mentioned above, breast cancer is most frequently found in women around menopause. It is significantly less frequent in women below 45 years of age, although recently, several cases of breast cancer in under-forty women are documented (7). The analysis of morbidity coefficients for the Polish population has indicated a linear increase in the group of women aged between 40 and 59 years, then it reaches a plateau with a slight decreasing tendency in women aged 70 and older. A very interesting correlation can be observed between the age when neoplastic disease is diagnosed and the expression of the estrogen receptor found in the examined tumor tissue. Neoplasms showing estrogen receptor overexpression ER (+) are characterized by a frequency increasing with age as opposed to ER (–) tumors, which occur more frequently up to 50 years of age and then reach a plateau. This phenomenon explains an increased percentage of ER (+) tumors diagnosed in women after menopause (8).

Another intrinsic factor conditioning the occurrence of breast cancer is familial susceptibility to this type of neoplasm. Intensive studies have been conducted in the recent decades, which led to identification of genes whose function disorder is associated with an increased risk of occurrence of malignant

breast or ovarian cancer. The most important are genes *BRCA1* and *BRCA2* fulfilling the function of tumor suppressor genes in a cell. The occurrence of changes in the coding sequence may lead to the development of hereditary syndromes called HBC-SS (Hereditary Breast Cancer Site Specific) or HBOC (Hereditary Breast Ovarian Cancer) syndrome, which manifest themselves in the form of breast and/or ovarian cancer. The correlation of epidemiologic and population studies has allowed for the estimation of the number of familial breast and/or ovarian cancer cases. They constitute about 10% of all newly diagnosed neoplasms of these organs (9). Identification of mutations in *BRCA1* or *BRCA2* genes is associated with an increased risk of occurrence of breast and/or ovarian cancer in 65% or 45% of mutation carriers, respectively, depending on the mutation type (9).

A high endogenous estrogen level is a well-defined risk factor contributing to a higher incidence of breast cancer. The analysis of prospective studies confirmed a strong association between increasing concentrations of sex hormones (total estradiol, free estradiol, estrone, estrone sulfate, androstenedione, dehydroepiandrosterone, dehydroepiandrosterone sulfate and testosterone) in postmenopausal women and a higher breast cancer risk (10).

The relationship between oral contraception and the risk of breast cancer still remains controversial.Some studies have suggested that using oral hormonalcontraceptives increases the risk of breast cancer by24% compared to women who have never used them (8), and the largest incidence increase is observed in thecourse of using the contraception. On the other hand,the reanalysis of epidemiological studies showed a littleor even no association between the risk of breast cancer and oral contraception (11).

Another factor increasing the risk of developing breast cancer is exposure to ionizing radiation, especially at a young age (numerous X-rays, radiation therapy in the treatment of dermatological lesions, radiation therapy in the treatment of

cancer) (12).

A clear risk factor for endometrial cancer and breast cancer in postmenopausal women is overweight. Epidemiological studies suggest that there are links between fat intake and breast cancer. It was hypothesized that the high-fat or high-calorie diets, leading to an increase in the fat content in the body, can affect the development of breast cancer. This is done by raising the circulation levels of certain hormones, prolactin and estrogen, which may facilitate the development of breast cancer. It is believed that food fat, or fat contained in the body adversely affects the immune system, which becomes less efficient in combating emerging cancer cells (13).

Studies show a link between obesity and risk of breast cancer in postmenopausal women. Most cases of breast cancer in postmenopausal women are sensitive to estrogen, and estrogen produced in adipose tissue is conducive to the formation of a tumor (14). Obesity causes secretion of inflammatory factors that stimulate aromatase enzyme responsible for the steroid hormone biosynthesis (15). This affects the conversion of androgens into estrogen in adipose tissue. Neuhouser*et al*. (16) also confirm that obesity is closely linked to the growth of invasive breast cancer in postmenopausal women compared to women of normal weight.

1.2 Classification of breast cancer

Breast cancer is a heterogeneous disease, including several clinicopathological subtypes with different biological behavior, clinical risk factor, natural histories, response to individualized therapy and prognosis (17). In the past, management of breast cancer was essentially based on histopathological grade and TNM stage, which had achieved some consensus (17).

Breast cancer is classified into five subtypes using the expression of four markers: estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2

(HER2 and Ki-67). These markers allow classification of breast cancer tumors as: luminal A (ER-and/or PR-positive/HER2-negative/low Ki-67), luminal B (ER- and/or PR-positive/HER2-negative/high Ki-67), HER2-positive luminal B (ER- and/or PR-positive/HER2 overexpression/any Ki-67), non-luminal HER2-positive (ER and PR absent/HER2 overexpression), and triple negative breast cancer -TNBC- (ER and PR absent/HER2-negative) (17). Here, we will show results obtained in two breast cancer cell lines representing *in vitro* models of triple positive breast cancer (MCF-7) and TNBC (MDA-MA-231). Generally, hormone receptor expressing breast cancers have a more favorable prognosis than either those with HER2 amplification or those that are triple negative (18). While all breast tumor types may be treated with chemotherapy, therapeutic options in both early and late stage breast cancer are affected significantly by the expression of these three markers. Tumors that express ER and PR are treated with agents that interfere with hormone production or action. Tumors that have amplified HER2 are treated with agents that inhibit HER2. These targeted therapies are the mainstay of the successful outcomes seen in hormone receptor positive and HER2 amplified tumors. Both early stage and advanced TNBC tumors are treated with predominantly chemotherapy (19). The discovery of several clinicopathological subtypes of breast carcinoma has led to a better understanding of molecular biology and has produced an effect on the risk assessment of recurrence and clinical treatment of breast cancer.

1.3 Diet and breast cancer

Due to international variance in cancer rates, geography-related dietary habits, importance of diet in the development of cancers including BC, the association between dietary pattern and BC risk has been suggested and hundreds of studies have examined this association during last 35 years. According to previous research, dietary factors were thought to be responsible for about 30% of all

cancers in developed countries and for 20% in developing countries (20, 21). It is commonly accepted that a diet of the Western developed countries is high in animal products, fat and sugar. In contrast, a diet of developing countries is more “healthy” basing on starchy staple foods with low consumption of animal products, fat and sugar (22). The diet may impact all stages of breast cancer carcinogenesis. For example, chemical carcinogens, such as heterocyclic amines, polycyclic aromatic hydrocarbons, and nitrites that may be produced in a high temperature processed meat can damage DNA and initiate carcinogenesis. In addition, the compounds present in an unhealthy diet may stimulate formation of IGF-1, increase concentrations of circulating endogenous sex hormones, thus influence the initiation, promotion and progression of tumor stages. In turn, red meat is rich in heme iron which is necessary for production of hydroxyl radical via the Fenton reaction-the most biologically toxic oxygen species involved in oxidative stress. Thus, iron ions may indirectly influence on cytoplasmic and nuclear signal transduction pathways (23, 24). In contrast, the healthy diet (rich in antioxidants) may influence on DNA repair, metabolic detoxification and decrease of estrogens (25).

1.4 Mediterranean diet

Mediterranean diet, which is a traditional dietary pattern of the inhabitants of the Mediterranean countries, is considered to be one of the healthiest diet rich in many nutrients. This is not only due to its taste, but also a varied menu based on a large amount of fresh vegetables and fruits, fish, legumes, whole grains, olive oil and herbs. However, the main cause of the promotion of the Mediterranean diet is its health-related properties.

Fundamentals of the Mediterranean diet have been formulated on the basis of eating habits of people living in the Mediterranean countries. The following common features have been found in their diet:

- high consumption of fruits, vegetables, legumes, nuts, whole grains,
- high consumption of olive oil as the main source of fat,
- high consumption of spices such as oregano, garlic, basil, thyme, rosemary, sage,
- moderate consumption of fish and seafood,
- moderate consumption of milk and dairy products (mainly cheese and yoghurt),
- moderate consumption of wine, mainly for meals,
- low consumption of meat and meat products,
- consumption of local, seasonal fresh produce.

Health properties of a traditional Mediterranean diet result from consumption of large quantities of vegetable products that are a source of bioactive components. These components have anti-cancer properties that is, carotenoids, antioxidant vitamins (vitamin C, E, A), lycopene, resveratrol, flavonoids, polyphenols and dietary fiber. Mediterranean diet is characterized by a favorable ratio of polyunsaturated fatty acids of omega-6 family to omega-3, which is about 2 : 1. In other European countries and the United States, this ratio is 10 : 1 and 20 : 1, respectively.

The traditional Mediterranean diet was popularized since 1995 using the world famous pyramid representation that graphically highlights the food groups to be consumed daily, weekly, or less frequently (26), yet, a new Mediterranean diet pyramid was recently proposed. The new pyramid, effectively, reflects the changing process that the Mediterranean diet is undergoingwithin the Mediterranean societies (Fig. 3). The recommendations target the healthy adult population (18-65 years old) and it should be adapted to the special needs of children, pregnant women and those suffering from health conditions.

Over the past three decades numerous studies have documented that products of Mediterranean diet play a critical role against cancer (27, 28, 29). For example, Alegre *et al*. (29) reported that

olive oil has potential not only to decrease breast cancer risk but also aggressiveness of this disease. In addition, Berrino (30) conducted a PREDIMED study to evaluate the effect of 2 interventions with Mediterranean diet (Mediterranean diet supplemented with extra-virgin olive oil, a Mediterranean diet supplemented with mixed nuts) vs the advice to follow a low-fat diet (control) on breast cancer incidence.After a median follow-up of 4.8 years, the author identified 35 confirmed incident cases of breast cancer. Observed rates (per 1000 person-years) were 1.1 for the Mediterranean diet with extra-virgin olive oil group, 1.8 for the Mediterranean diet with nuts group, and 2.9 for the control group. These results suggest a beneficial effect of a Mediterranean diet supplemented with extra-virgin olive oil in the primary prevention of breast cancer.

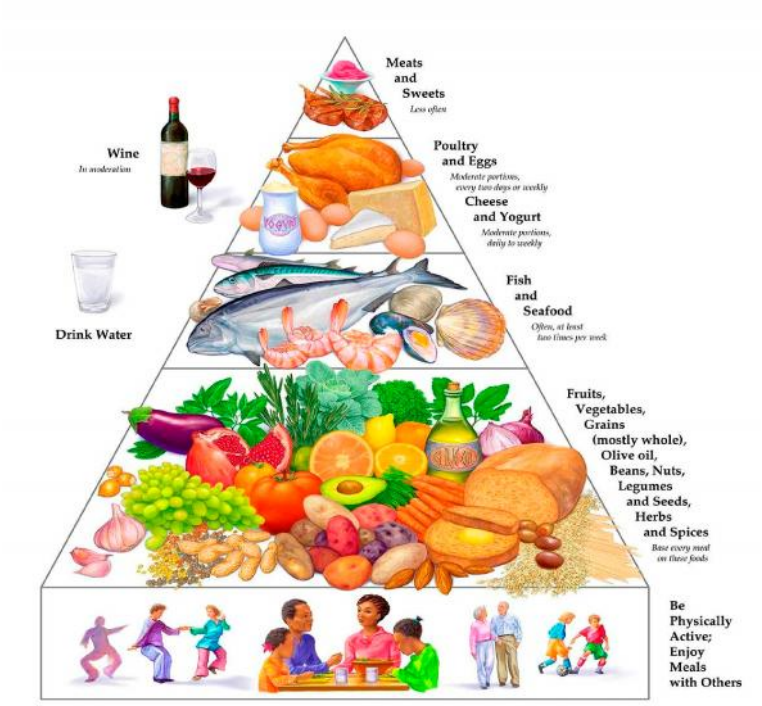


Figure 3

1.5 Tomato and breast cancer

Tomatoes may be included among the most representative elements of the Mediterranean diet. They are relatively cheap and easy to store and they are the main source of micronutrients such as potassium, folate and the vitamins A, C and E, if compared with other widespread vegetables. In addition to their micronutrient benefits, tomatoes also contain important phytochemicals, like polyphenols and carotenoids, especially lycopene, with strong antioxidant properties (31) (Table 1). The latter are not essential nutrients and are not required by the human body for sustaining life, but they have protective, or disease preventive properties.

Various epidemiologic studies have correlated risk of cancer at various body sites, with intake of tomatoes and tomato-based products. Evidence for beneficial effects was the strongest for cancers of the prostate, lung and digestive tract. A consistent pattern of protection by high intake of tomatoeswas also seen in cervix, pancreas, colorectum, esophagus, oral cavity, pharynx and breast cancer (32, 33). In particular, tomato plays a fundamental chemopreventive role among postmenopausal women at high risk for developing breast cancer, through the insulin-like growth factor-I (IGF-I) and IGF-binding protein-3 (IGFBP-3) signaling pathway (34). IGF-I is a mitogenic and antiapoptotic peptide hormone that is involved in cell regulation and tumorigenesis. The effects of IGF-I are regulated by binding to IGFBP-3. Animal and human studies show that increased levels of both IGF-I and IGFBP-3 are associated with breast cancer risk (35-37). Possible benefits from tomatoes are thought to be derived from lycopene: human studies indicate that lycopene interacts with IGF-I and IGFBP-3 and can eliminate free oxygen radicals-thereby preventing DNA mutation, cell-cycle alteration, and apoptotic disruption (34).In 2006, Nahum *et al*. (38) showed that lycopene and all-trans retinoic acid (atRA) inhibit IGF-I induced proliferation of MCF-7 breast cancer cells through reduction in cyclin D1 levels. Dietary

lycopene and others carotenoids have also been shown to inhibit estrogen signaling of 17β-estradiol that may attenuate the effects of hormone-dependent malignancies (39). *In vitro*, carotenoids (lycopene, phytoene, phytofluene, and beta-carotene) inhibit breast cancer cell (T47D and MCF-7) proliferation induced by either 17β-estradiol, or genistein by slow-down of cell-cycle progression from G1 to S phase. Moreover, carotenoids inhibit estrogen-induced transactivation of ERE mediated by both estrogen receptors (ERs) ERalpha and ERbeta.

Considering incidence and relevance of breast cancer, relatively few studies examined its relationship to tomato, or lycopene intake. FDA identified four case-control studies (40-43) that examined the association between tomato intake and risk of breast cancer. Two of these studies (40, 41) were not considered for further review because they had scientific deficiencies. On the basis of the others two studies, FDA concluded that there was no credible evidence supporting an association between tomato or tomato-based food consumption and breast cancer risk (42, 43).

Principle	Nutrient Value	Percentage of RDA
Energy	18 Kcal	1%
Carbohydrates	3.9 g	3%
Protein	0.9 g	1.6%
Total Fat	0.2 g	0.7%
Cholesterol	0 mg	0%
Dietary Fiber	1.2 g	3%
Vitamins		
Folates	15 µg	4%
Niacin	0.594 mg	4%
Pyridoxine	0.080 mg	6%
Thiamin	0.037 mg	3%
Vitamin A	833 IU	28%
Vitamin C	13 mg	21.5%
Vitamin E	0.54 mg	4%
Vitamin K	7.9 µg	6.5%
Electrolytes		
Sodium	5 mg	>1%
Potassium	237 mg	5%
Minerals		
Calcium	10 mg	1%
Iron	0.3 mg	4%
Magnesium	11 mg	3%
Manganese	0.15 mg	6.5%
Phosphorus	24 mg	3%
Zinc	0.17 mg	1.5%
Phyto-nutrients		
Carotene-ß	449 µg	--
Carotene-α	101 µg	--
Lutein-zeaxanthin	123 µg	--
Lycopene	2573 µg	--

Table 1

1.6 Scientific hypothesis and aim of the work

Whole tomato as a food with potential anticancer properties has not been extensively evaluated so far, because greater attention has been given to single antioxidant compounds, such as lycopene, β-carotene, etc**.**These bioactive natural compounds have been long used for healing purposes. Although various*in vitro* and *in vivo* studies focused on the possible anti-carcinogenic properties of food-derived principles, in order to identify novel drugs for cancer chemoprevention and/or therapy, they usually investigate the effects of pure phytochemicals. Far fewer studies, in fact, focus upon interventions with whole foods, or processed food products. Here, we aimed to evaluate the biological and molecular effects of treatment with total lipophilic extracts from the two Italian tomato cultivars, San Marzano and Corbarino, using as biological system two in *vitro* model of breast cancer.

2. Materials and Methods

2.1 Cell culture

Two breast cancer (MCF-7 and MDA-MB-231) and a primary human skin fibroblasts (HSF) cell lines were used. MCF-7 cell line was isolated in 1970 from a 69 year sold Caucasian woman. MCF-7 cells are useful for in vitro breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm. This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line (44). MDA-MB-231 was established in 1970 from primary tumor of a 51 years old Caucasian woman. MDA-MB-231 is a triple negative breast cancer cell line because it do not express ER, progesteron receptor (PR), and do not have HER-2/Neu amplification (45). Primary human skin fibroblasts were kindly provided by Michele Fimiani, Giancarlo Mariotti, and Stefania Mei (University of Siena, Italy) (46). MCF-7 and HSF cell lines were cultured and maintained in DMEM while MDA-MB-231 cell line in RPMI supplemented with 10% fetal bovine serum, 100U/mL of penicillin and 0.1mg/mL of streptomycin. All the cell lines were incubated at 37°C in a 5% CO_2 incubator.

2.2 Cell growth curve analysis

1.0×10^5 cells were plated in tissue culture dishes (60 mm) containing culture medium with 10% FBS. MCF-7 and MDA-MB-231 cell lines were treated with 15, 30 and 60 µg/mL of total lipophilic extracts of San Marzano (SM) and Corbarino (COR) cultivars and 1.5, 3 and 6 µL/mL of DimethylSulfoxide (DMSO) as control. After 24, 48 and 72 hours (h) culture medium was removed, adherent cells were trypsinized and the

total number of adherent cells in each well was quantified using a Burker chamber. The cell counts for 3 wells/time-point were averaged for each group and data were used to draw growth curves. HSF cell line was treated with 30 µg/mL of total lipophilic extracts of tomato and counted after 24, 48 and 72 h following the same procedure.

2.3 Soft Agar colony formation assay

Soft agar colony formation assay was performed to evaluated anchorage-independent cell growth. Briefly, a base layer of agar (0.5%) in cell culture media was plated in twenty-four-well plates. MCF-7 and MDA-MB-231cells were mixed in top layer agar solution (0.3%) plus 30 µg/mL of total lipophilic extracts of SM and COR cultivars and 3 µL/mL of DMSO, as control, and then plated at a density of 3000 and 8000 cells per well, respectively. Each treatment group was plated in triplicates. Plates were incubated at 37°C in a 5% CO_2-containing humidified incubator. After 14 days of colony growth, cells were stained with 0.5 mg/mL nitrobluetetrazolium, and colonies were visualized and counted with Image Pro-Plus Software (Media Cybernetics).

2.4 Scratch test

Cells were plated in tissue culture dishes (60 mm) and grown to ~100% confluency before scratching with a sterile P10 pipette tip, across the monolayer. Cell debris were removed by washing in PBS 1x and cells were cultured in DMEM and 10% FBS supplemented with 30 µg/mL of tomato extracts and 3 µL/mL of DMSO, as control. Area of the scratch was measured at 0, 24, 48 and 72 h and quantification was performed by measuring the area of cell migration at different time points compared to the

scratch area at 0 h. Each experiment was repeated 3 times.

2.5 Cell Cycle analysis

Cells, after 72h of incubation with 30 µg/mL of tomatoes extracts and 3 µl/ml of DMSO, were trypsinized and $1x10^6$ cells were fixed with 70% ethanol at −20°C overnight. Cells were washed and stained with 5 µg/mL propidium iodide and 20 µg/mL RNAase at 4°C overnight. DNA content and cell cycle distribution were analyzed by FACS.

2.6 Western Blotting

Cell extracts containing 50γ protein were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were then blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 containing 5% nonfat dry milk for 1 hour at room temperature. Subsequently, the membranes were incubated with specific primary antibodies at 4°C overnight: mouse anti-pRb/p105 (1:250), mouse anti-pRb2/p130 (1:250), rabbit anti-p107 (1:100) and -p27Kip1 (1:250), and rabbit anti-GAPDH (1:1000). After washing, the membranes were incubated with secondary antibodies at the appropriate dilutions for 1 hour at room temperature and detected using enhanced chemiluminescence ECL substrate Kit. Protein expression levels were normalized to GAPDH.

2.7 Statistical analysis

Statistical analyses were performed using the GraphPad Software, version 5.01 for Windows. Statistically significant differences between the means of multiple matched groups were

evaluated by one-way repeated measures Anova, with either Dunnett post-test, to compare all data versus control (growth curve analysis). $P<0.05$ was considered to indicate a statistically significant difference.

2.8 San Marzano and Corbarino tomato extracts, composition and antioxidant activity

Tomato extracts were provided under lyophilized form by the research group coordinated by Dr. Barbara Nicolaus from Council of National Research, Institute of Biomolecular Chemistry, Pozzuoli, Italy. The aforementioned group also performed the analysis of the antioxidant activity and lycopene composition of our extracts, as previously described (47).

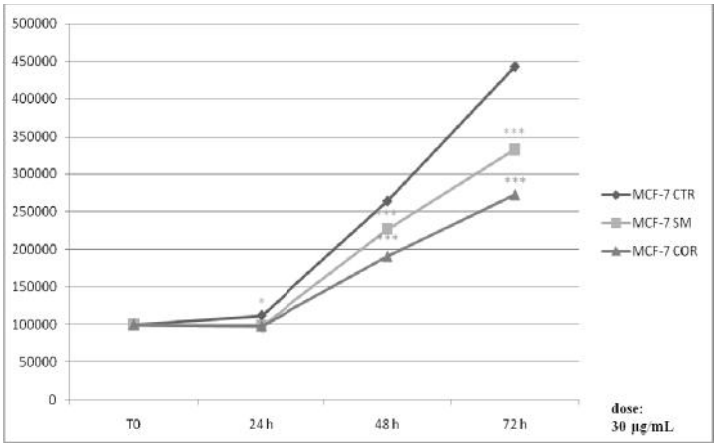
3. Results

3.1 Dose response analysis of tomato lipophilic extracts

Primarily, we analyzed possible biological effects of tomatoes lipophilic extracts on cell growth. We treated cells with 15, 30 and 60 µg/mL of tomato extracts and analyzed proliferation at 24, 48 and 72 h. Results in Fig.4 showed that 30 µg/mL of SM represent the most effective concentration in inhibiting MCF-7 cell growth, although COR extract also exerted a good inhibitory effect. MDA-MB-231 were impaired in their growth by 30 µg/mL of COR and by a lesser extend by SM. In both cell lines, the highest cell growth inhibitory effect was corresponding to 30 µg/mL of extract. Overall, we did not observe a dose-response anti-proliferative effect of the whole tomato extracts (Fig. 5, 6) and focused for further experiments on the 30 µg/mL which showed a good anti-proliferative efficacy in both cell lines. The anti-proliferative effect of tomato extracts was particularly consistent after 72 hours of incubation. No toxic effects were observed on HSF cell line after treatment with 30 µg/mL of both lipophilic extracts (Fig. 7).

3.2 Inhibition of cell growth in semisolid cell culture medium

We investigated the effect of tomato extracts on anchorage-independent growth of cancer cells in a typical soft-agar assay. After a 14 days incubation in soft agar, control colonies of MCF-7 and MDA-MB-231 cells were found more numerous and larger than COR treated cells, whereas no differences were observed between control cells and SM extract treated cells (Fig. 8).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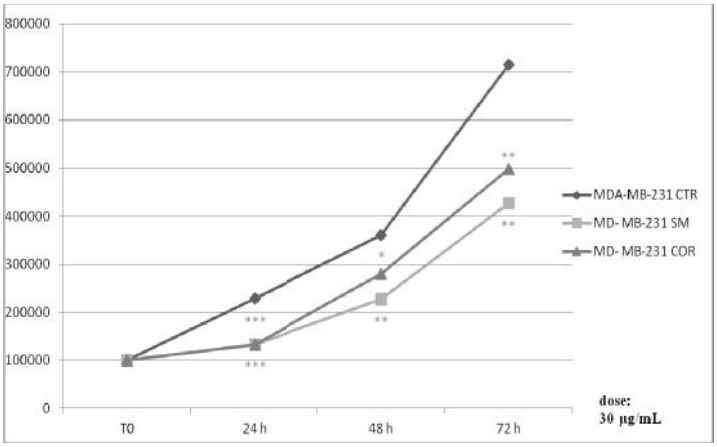
24 h
48 h
72 h
MCF-7 CTR
MCF-7 SM
MCF-7 COR
dose:
30 µg/mL

a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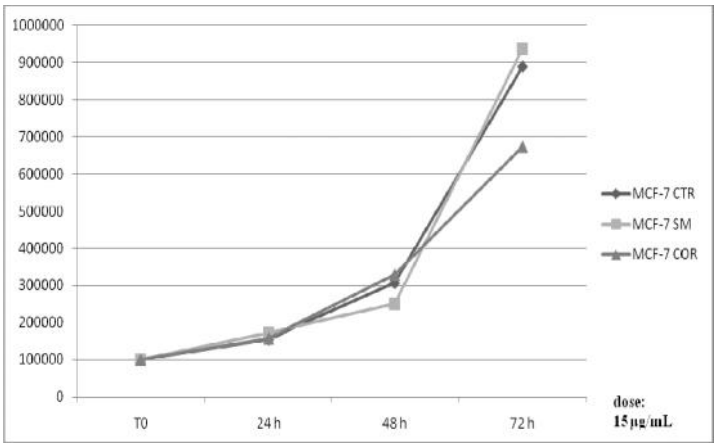
MDA-MB-231 CTR
MD- MB-231 SM
MD- MB-231 COR
dose:
30 µg/mL

b

Figure 4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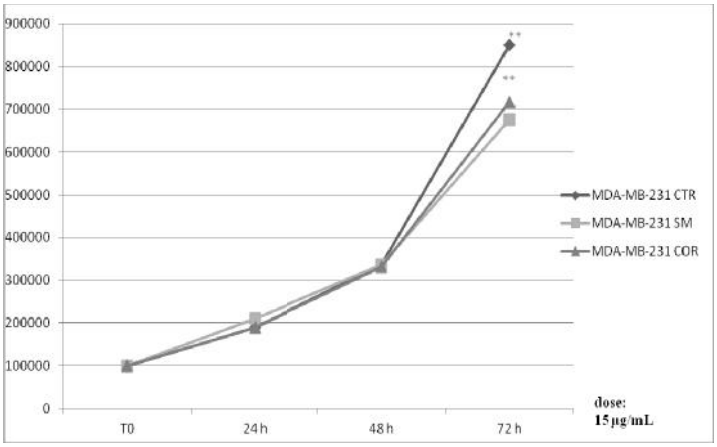
MCF-7 CTR
MCF-7 SM
MCF-7 COR
dose:
15 µg/mL

a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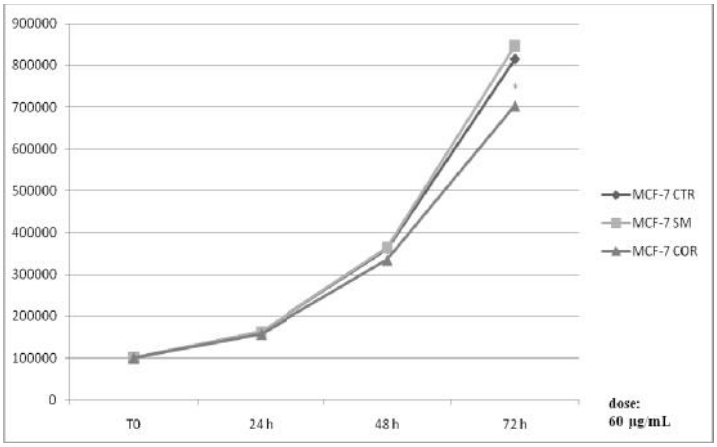
**
MDA-MB-231 CTR
MDA-MB-231 SM
MDA-MB-231 COR
dose:
15 µg/mL

b

Figure 5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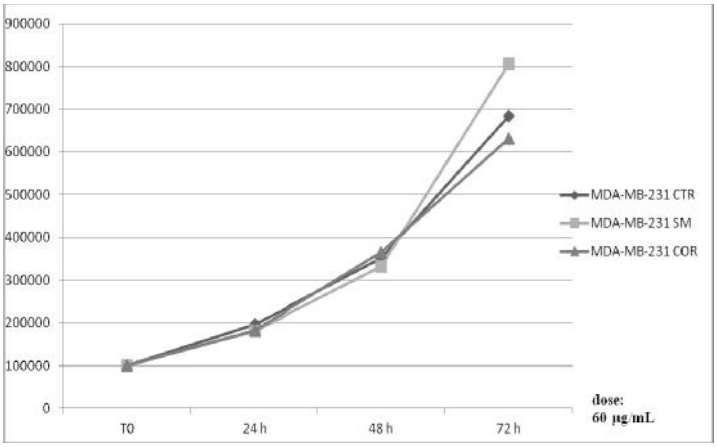
0
T0
24 h
48 h
72 h
MCF-7 CTR
MCF-7 SM
MCF-7 COR
dose:
60 µg/mL

a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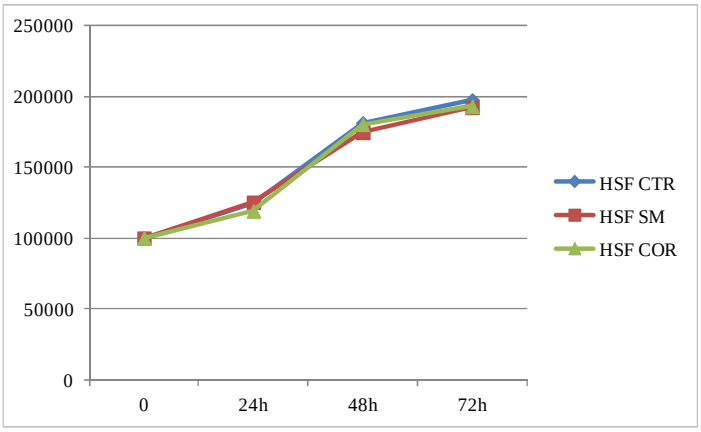
24 h
48 h
72 h
MDA-MB-231 CTR
MDA-MB-231 SM
MDA-MB-231 COR
dose:
60 µg/mL

b

Figure 6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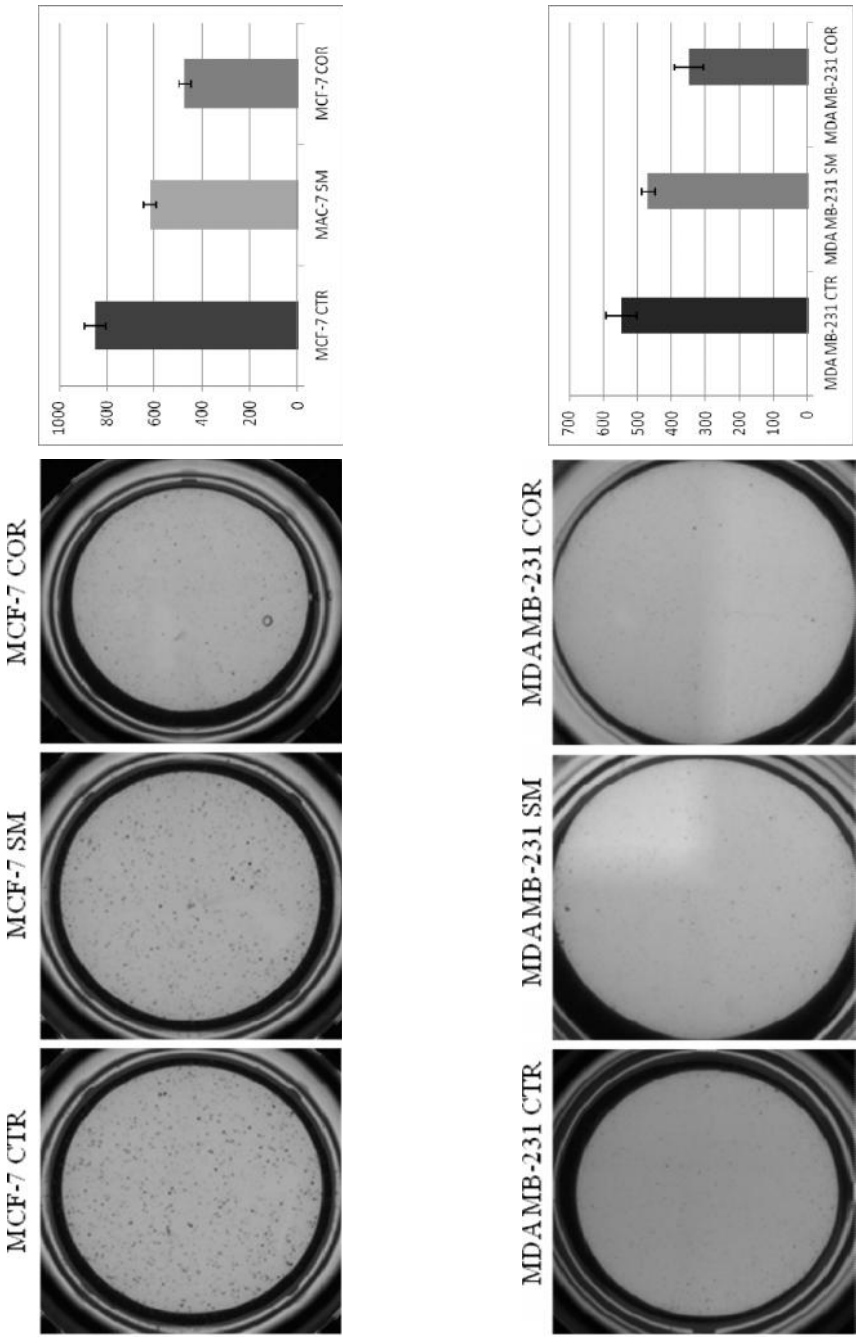
72h
HSF CTR
HSF SM
HSF COR

Figure 7


MCF-7 CTR
MCF-7 SM
MCF-7 COR
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800
600
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MCF-7 CTR
MAC-7 SM
MCF-7 COR
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MDA MB-231 CTR
MDA MB-231 SM
MDA MB-231 COR
700
600
500
400
300
200
100
0
MDA MB-231 CTR
MDA MB-231 SM
MDA MB-231 COR
b

Figure 8

3.3 Inhibition of cell migration

We performed a scratch test assay to investigate possible tomatoes effects on cancer cell migration, in order to analyze another biological effect typical of neoplastic growth. All tomato extracts significantly inhibited migration in both cell lines. After 24 hours of incubation, MDA-MB-231 control cells showed a faster rate of injury repair compared with treated cells, whereas, MCF-7 cell line exhibited the same effect after 72 hours (Fig. 9).

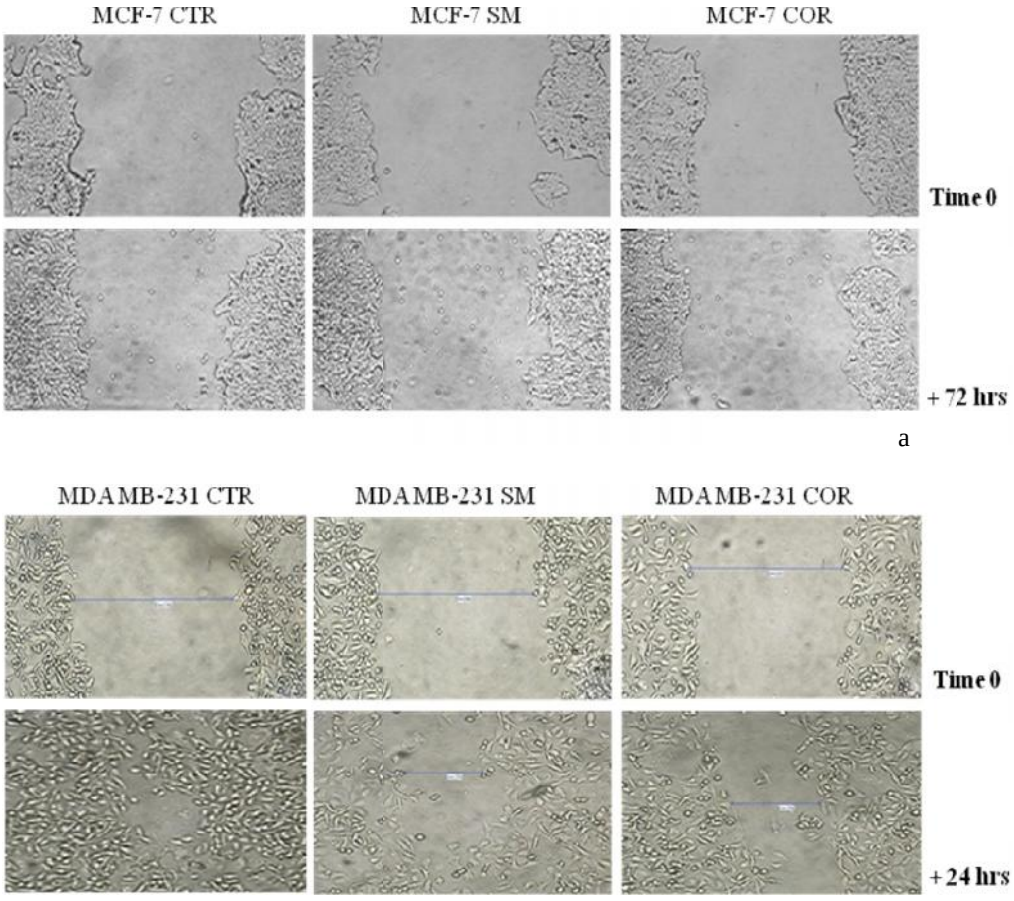


Figure 9

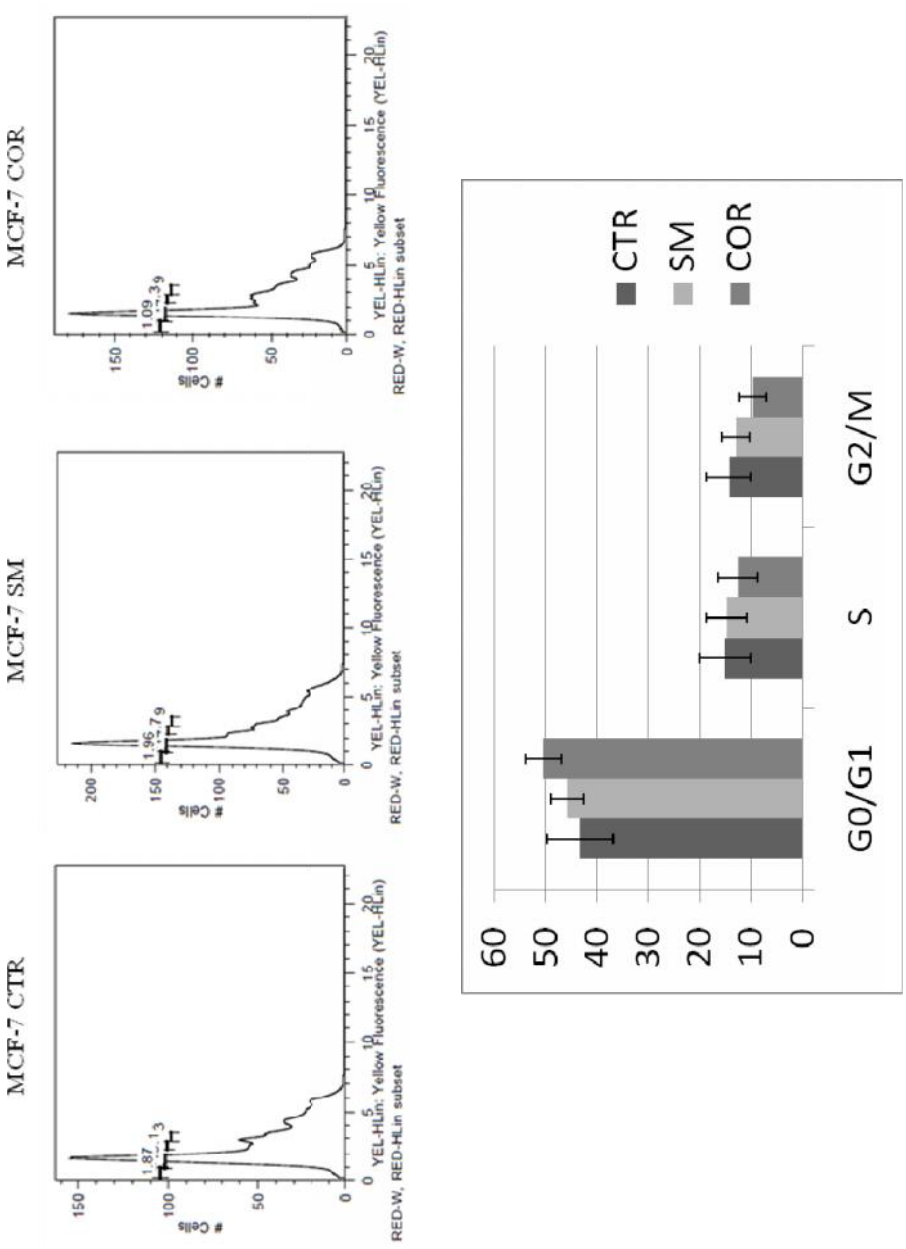
3.4 Tomato extracts influenced cell cycle

To investigate whether the inhibitory effects of tomato extracts were due to an arrest of cell cycle, we performed the analysis of cell cycle by staining with PI and reading by FACS. In both cell lines, we saw a slowdown of cell cycle, in particular a gain in G0/G1 phase and a significant decrease in S phase (Fig. 10).

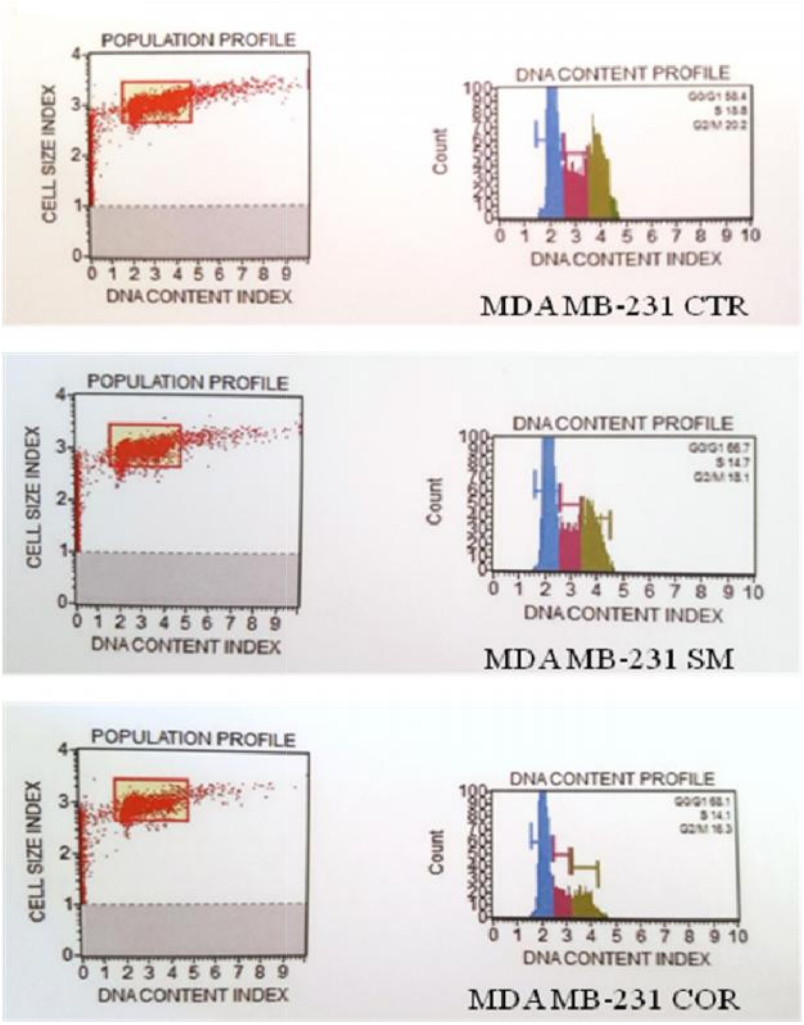
3.5 Tomato extracts regulate pocket proteins expression

We hypothesized that the inhibition of cancer cell growth and migration induced by tomato extracts could be mediated by pocket proteins, key regulators of cell cycle. Hence, expression levels of the pRb/p105, pRb2/p130 and p107, were detected by Western Blotting in order to investigate possible molecular pathways involved in the previously observed biological phenomena. In both cell lines, tomato extracts induced a strong increase of pRb2/p130 expression levels, especially upon treatment with COR, that compensate the reduction of pRb/p105 expression levels (only in MCF-7). In MCF-7 cell line, we also observed a higher expression level of p107 in treated cells than in control, whereas in MDA-MB-231 no particular differences were observed between control and treated cells. Finally, MCF-7 showed upregulation of p27Kip1, a cell cycle inhibitor, as consequence of COR treatment (Fig. 11). In conclusion, these observation at the molecular level seem to explain the biological effects observed following treatment with tomato extracts, and supported the hypothesis that tomatoes may hinder neoplastic features, by influencing cell cycle, one of the most important events of cells number control.

a


MCF-7 CTR
MCF-7 SM
MCF-7 COR
Cells
YEL-HLin: Yellow Fluorescence (YEL-ALin)
RED-W, RED-HLin subset
1.87
13
1.96
7.9
1.09
3.9
CTR
SM
COR
G0/G1
S
G2/M
0
10
20
30
40
50
60

b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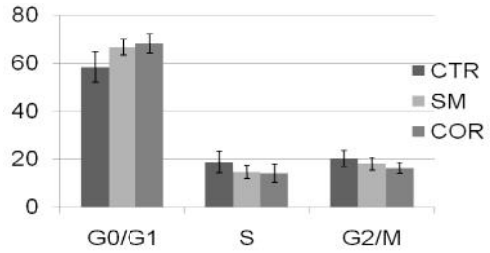
Count
DNA CONTENT INDEX
MDAMB-231 COR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CTR
SM
COR
G0/G1
S
G2/M

Figure 10

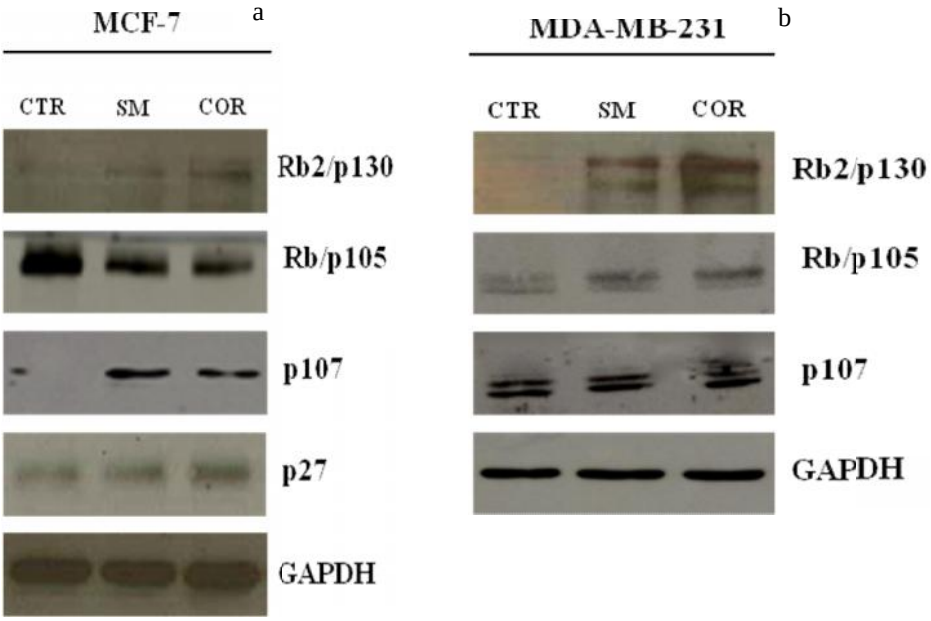

Figure 11

3.6 Tomato antioxidant activity and their lycopene content

Antioxidant activity of lipophilic extracts evaluated by ABTS method is reported in the Figure 12. We observed that the SM variety showed a greater antioxidant power, if compared to COR.Lipophilic extract from each sample was analyzed by reversed-phase High-Performance-Liquid-Chromatography (HPLC), in order to evaluate lycopene contents. Data shown in Table 1, displayed that SM content of lycopene is almost double, if compared to COR (7,50 mg /100 g of fresh product versus 4,20 mg/100 g of fresh sample). These data were kindly provided by a research group of the Council of National Research, Institute of Biomolecular Chemistry, Pozzuoli, Italy, with whom we collaborate.

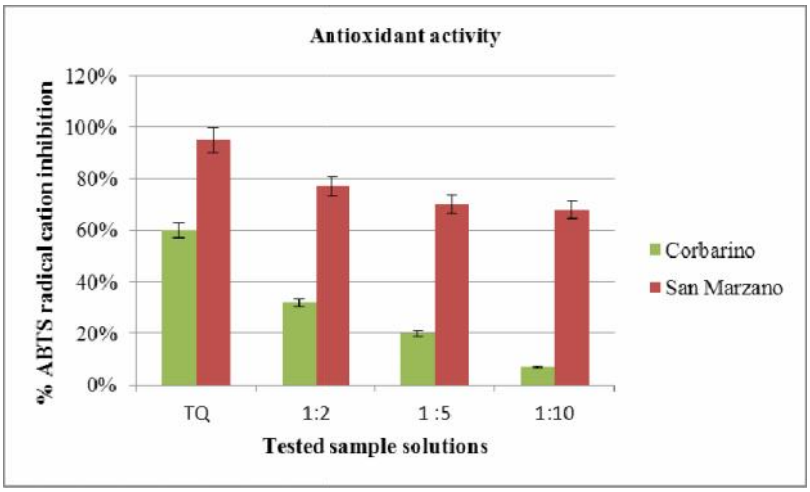

Figure 12

SAMPLE	VOLUME OF HOMOGENATE (mL)	VOLUME OF IDROPHILIC PHASE (mL)	LYCOPENE CONTENT IN 100 g OF FRESH SAMPLE (mg)
COR	100 mL	53 mL	**4,20 mg**
SM	92 mL	56 mL	**7,50 mg**

Table 2

4. Discussion/Conclusions

In the last years, growing interest about food plants and their derivatives arose in scientific community because of a powerful role in neoplasms prevention. Fresh and carved tomatoes are typical of Italian diet, which represents a sort of subtype of Mediterranean diet, whose healthiness is worldwide approved. Their consumption is also diffuse because of their cheapness and easiness of storage. Beneficial effects of tomato consumption on human health are generally attributed to carotenoids, particularly to lycopene, although they are also an excellent source of vitamins (A, C, and E) and flavonoids. Epidemiological studies showed a consistent inverse relationship between tomato consumption and the risk of various neoplasms. So far, most studies analyzed the effects of individual compounds, especially lycopene, whereas our study is focused on assessing, *in vitro*, the effects of lipophilic tomato extracts on breast cancer cell lines, hence considering the food not only its bioactive compounds. We firstly investigated possible biological effects, such as cell growth and migration impairment. Actually, we found that both tomato cultivars were effective in inhibiting both cell growth, and migration, also in semisolid cell culture medium in MCF-7 and MDA-MB-231 cell lines. The effects induced by COR treatment were relatively surprising because of its lower amount of lycopene and, consequently, a lower antioxidant capability. However, this might be in accordance to previous observations suggesting that antioxidants anticancer effects are less effective in reducing more aggressive neoplastic behavior.Watson, reported various papers showing that in advanced stage neoplasms, antioxidants compounds become pro-tumor development, because they contribute to protect cancer cells by damages induced by ROS (48). Hence, compounds that may help in preventing cell transformation, in the fourth stage tumors are metabolized more efficaciously by cancer cells, which can take advantages of their

properties. Watson highlighted, then, the failing of strategies also based on vitamin supplements in advanced stage tumors.So, although our experiments were focused on testing the effects of whole vegetables, our results were in accordance with the hypothesis that when a cancer is progressed, considering antioxidant compounds, the less is more. In our study we highlighted how tomato extracts affect different molecular pathways. Upregulation of pocket protein togheter with the increase of the cell cycle inhibitor p27Kip1, controlled cell cycle causing cell block in G0/G1 phase. Hence, observed biological effects are supported by molecular mechanisms among which we considered only cell cycle regulation, so further investigations will be necessary to better analyze other pathways involved.

In conclusion our data pave the way for future investigations aimed at assessing whether diet implementation with continuous consumption of tomatoes and its processed forms might be effective for cancer prevention and supporting therapy, considering that dietary intake of tomatoes is generally judged safe (49). In particular, our data show that tomatoes *in toto*act as potent anticarcinogenic agents, which may help to support breast cancer therapy strategies.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

Novel findings about management of gastric cancer: A summary from 10th IGCC

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Abstract

The Tenth International Gastric Cancer Congress (IGCC) was held in Verona, Italy, from June 19 to 22, 2013. The meeting enclosed various aspects of stomach tumor management, including both tightly clinical approaches, and topics more related to basic research. Moreover, an overview on gastrointestinal stromal tumors was provided too, although here not discussed. Here we will discuss some topics related to molecular biology of gastric cancer (GC), inherent to prognostic, diagnostic and therapeutic tools shown at the conference. Results about well known subjects, such as E-cadherin loss of expression/function, were presented. They revealed that other mutations of the gene were identified, showing a continuous research to improve diagnosis and prognosis of stomach tumor. Simultaneously, new possible molecular markers with an established role for other neoplasms, were discussed, such as mesothelin, stomatin-like protein 2 and Notch-1. Hence, a wide overview including both old and new diagnostic/prognostic tools was offered. Great attention was also dedicated to possible drugs to be used against GC. They included monoclonal antibodies, such as MS57-2.1, drugs used in other pathologies, such as maraviroc, and natural extracts from plants such as biflorin. We would like to contribute to summarize the most impressive studies presented at the IGCC, concerning novel findings about molecular biology of gastric cancer. Although further investigations will be necessary, it can be inferred that more and more tools were developed, so as to better face stomach neoplasms.

Key words: Gastric cancer; Prognostic tools; Markers; Therapy

Core tip: Gastric cancer (GC) is one of the most common tumors in the world, although scientists' knowledge about this neoplasm grew in the last years. In June, an international meeting (10th International Gastric Cancer Congress), focused on GC management, was held in Verona (Italy). It gave an overview about the state-of-the-art stomach tumor treatments, including chemotherapy, surgical therapies and nutritional support. Moreover, several new possible prognostic markers were shown. Here we report a summary of novel findings taken from some molecular biology sessions, focused on prognosis and treatment of GC.

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ORIGINAL RESEARCH ARTICLE

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pRb2/p130 Localizes to the Cytoplasm in Diffuse Gastric Cancer

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pRb2/p130 is a key tumor suppressor, whose oncosuppressive activity has mainly been attributed to its ability to negatively regulate cell cycle by interacting with the E2F4 and E2F5 transcription factors. Indeed, pRb2/p130 has been found altered in various cancer types in which it functions as a valuable prognostic marker. Here, we analyzed pRb2/p130 expression in gastric cancer tissue samples of diffuse histotype, in comparison with their normal counterparts. We found a cytoplasmic localization of pRb2/p130 in cancer tissue samples, whereas, in normal counterparts, we observed the expected nuclear localization. pRb2/p130 cytoplasmic delocalization can lead to cell cycle deregulation, but considering the emerging involvement of pRb2/p130 in other key cellular processes, it could contribute to gastric tumorigenesis also through other mechanisms. Our data support the necessity of further investigations to verify the possibility of using pRb2/p130 as a biomarker or potential therapeutic target for diffuse gastric cancer.
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Gastric cancer is still one of the most frequent causes of cancer death among women and men (Pinheiro et al., 2014). The most frequently used gastric cancer classification, which is based on Lauren's criteria, distinguishes two main groups that differ not only in morphology but also clinically and epidemiologically: the intestinal and the diffuse histotypes (Lauren, 1965; Vauhkonen et al., 2006). The first has a glandular morphology and is characterized by a stepwise progression, whereas gastric tumors of the diffuse histotype are characterized by an undifferentiated morphology, the lack of precursor lesions, occur most commonly in young patients and generally have a worse prognosis (Nardone, 2003; Vauhkonen et al., 2006; Chiaravalli et al., 2012; Corso et al., 2012). In the clinical practice however, regardless of histotype, the clinical stage seems to be the most important single and independent factor affecting survival (Vauhkonen et al., 2006). Although various markers for early diagnosis have been discovered (Guilford et al., 1998; Shafaghi et al., 2013; Liu et al., 2014), the lack of remarkable early symptoms still results in a late stage diagnosis. Therefore, it is a priority to identify new tools for an early diagnosis.

pRb2/p130 is a member of the Rb family of tumor suppressors (Giordano et al., 1991; Yeung et al., 1993; Indovina et al., 2013), whose altered expression and delocalization was found in various cancers. In particular, reduced expression of

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