

Dose-Dependent Cytotoxicity of the *Origanum vulgare* and Carvacrol on Triple Negative Breast Cancer Cell Line [†]

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Abstract: Uncontrolled proliferation and death resistance are two hallmarks of cancer cells. Breast cancer (BC) has the highest incidence and mortality in women worldwide and national; subtype triple negative (TN) is the most aggressive type because it is not susceptible to conventional therapy. Since 2004, the World Health Organization (WHO) approved use of alternative treatments as adjuvants based on evidence of its benefits. Since then, different natural alternatives to treat cancer have been studied, including *Origanum vulgare* (*Ov*) and carvacrol (*Crv*), one of its main compounds. However, cytotoxic potential of these products has not been studied in this subtype of BC. The objective of this study was to evaluate the cytotoxic effect of *Ov* and *Crv* on TN BC cell line (HCC-70). Lethal dose 50 was determined on control cell line HaCaT through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays with *Ov* and *Crv* stimulus in different doses and concentrations; the found dose was used on the HCC-70 cell line. The results were evaluated by ANOVA, finding that *Ov* reduced the proliferation by $94.05\% \pm 0.11$ ($p < 0.001$) and *Crv* by $93.43\% \pm 0.21$ ($p < 0.001$) compared to untreated cells. This suggests that both *Ov* and *Crv* present a powerful cytotoxic effect against the HCC-70 cell line and are promising options that should receive further study.

Keywords: breast cancer; triple negative; cytotoxicity; *Origanum vulgare*; carvacrol; adjuvant treatment

1. Introduction

Cancer is defined by the WHO as a cellular uncontrolled process of growth and dissemination that can appear anywhere on the body [1]. Breast cancer (BC) is a type that originates in breast tissues, and it can be spread through blood and lymphatic vessels [2,3].

Perou proposed a molecular classification of breast cancer in 2000, identifying four subtypes based on expression or absence of a series of receptors necessary for the efficacy of conventional treatment: Luminal A is the most common and least aggressive (positive for progesterone receptor

and estrogen receptor). Luminal B has a higher proliferation index and a least response to conventional treatment (positive for progesterone receptor, estrogen receptor and Her2/neu). HER2/neu is the least common subtype, and highly aggressive due to increased expression of genes related with cellular proliferation (Her2/neu positive). The last basal subtype (triple negative, TN): does not express any of the receptors named above; this subtype is not susceptible to conventional treatments [4–6].

Regarding epidemiology, cancer is a great public health problem worldwide. The WHO suggests there are 10,000,000 cases of cancer per year worldwide, and 6,000,000 deaths because of cancer every year. It has also announced that the cancer population will increase up to 20 million cases/year in this year [7]. Talking about breast cancer, the last report of Global Cancer Observatory shows that worldwide incidence of BC is 2,088,849 cases, and in 2018 a total of 626,679 deaths were recorded because this type of cancer. Based on these data, BC leads in incidence and mortality in women worldwide [8]. Furthermore, cases diagnosed in late stages decrease the survival probabilities due to low effectiveness of conventional treatment in advanced stages of cancer. This diagnosis happens mainly in underdeveloped and developing countries where the costs for the patient can exceed their income [9,10].

Natural products have been used throughout human history in traditional medicine, and in later years, it has become evident that the development of novel treatments based on bioactive natural products would define the future of global healthcare, with natural products leading the way to the discovery of new therapeutic compounds for the treatment of human diseases [11–14]. Further, since 2004, the WHO approved the use of alternative treatments as adjuvants based on evidence of its benefits, showing a clear revival of interest in herbal medicine, and development of novel phytochemical anticancer agents have gained significant recognition in the field of cancer therapy [15,16]. In this field, medicinal plants represent one of the main sources of active compounds with antiproliferative activities, having been shown to be an excellent source of new drugs that, in addition to their effectiveness, do not have large side-effect consequences compared to synthetic drugs [16–18].

The infusions are products with various biological properties, and for centuries they have been used in traditional medicine. In recent decades, with the resurgence of interest in traditional medicine, herbalism has acquired great interest around the world due to the anticancer activity and the possible use as an adjuvant treatment of certain plants [19,20]. Here is highlighted *Origanum vulgare* (*Ov*); commonly called oregano, it is a perennial herbaceous plant (living more than two years), aromatic of the genus *Origanum* belonging to the *Lamiaceae* family. In addition to its uses in the kitchen, it is used in home remedies and as complementary medicine. Around 64 compounds have been described in this plant, and it has been found that carvacrol (*Crv*) is the one found in the highest proportion, a highly bioactive monoterpenoid phenol [19,21–27]. Both oregano and *Crv* have been studied for their cytotoxic potential in different subtypes of breast cancer; however, cytotoxic potential of these products has not been studied in this subtype of breast cancer.

The objective of this study was to evaluate the cytotoxic effect of the *Ov* and *Crv* on the TN BC cell line (HCC-70) and compare if all the compounds present in the *Ov* infusion can exert a synergistic effect that enhances the cytotoxic activity, or if *Crv* has the highest cytotoxic activity against this type of breast cancer.

2. Methods

2.1. Chemicals

Dulbecco's Modified Eagle Medium (DMEM) Roswell Park Memorial Institute (RPMI-1640), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), and *Crv* ($\geq 98\%$ purity) were purchased from Sigma Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS), extraction buffer (EB), trypan blue (TB), and Antibiotic-Antimycotic (AA) were purchased from Thermo Fischer Scientific™ (Waltham, MA, USA). All solvents and chemicals were of an analytical grade.

2.2. Plant Material

The *Ov* used in this study is an endemic species from the region of Guadalajara, Mexico.

2.3. Preparation of *Ov* Infusion

A total of 250 mg of dry leaves were ground to a fine powder using a porcelain mortar. The powder was suspended in 10 mL of sterile boiling water for 5 min. The resulting infusion was filtered with a 0.22 µm sterile filter into a laminar flow hood (Figure 1A).

2.4. Preparation of *Crv* Solution

A total of 20 µL of *Crv* was mixed with 80 µL of DMSO (allowing *Crv* to solubilize in an aqueous medium) and 900 µL of culture medium to obtain a 2% solution. Then the solution was mixed for 10 second in a vortex (Figure 1B).

2.5. Cell Lines and Cell Cultures

The cells lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Human keratinocyte transformed and immortalized HaCaT cells were maintained in RPMI supplemented with 10% of FBS and 1% of AA. This cell line was used as control for this study. Human TN BC cells HCC-70 were maintained in DMEM supplemented with 10% of FBS and 1% of AA. The cell cultures were kept in an incubator under physiological conditions (37 °C, 95% humidity and 5% CO₂ saturation). All cells were cultures and received the necessary culture media changes until reaching a confluence of 100%. Then cells were recovered by trypsinization technique (1–2 mL of trypsin in the culture flask without culture medium and incubate for 5 min), trypsin was inactivated with culture medium and the cells were centrifuged (1500 RPM for 5 min) to obtain a pellet. This was resuspended with 1 mL of culture medium.

2.6. Cell Viability Assay and Cell Count

The cell viability was evaluated through TB assay, and the cell count was performed with a Neubauer chamber. TB assay is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. A total of 1 µL of cells was mixed with 9 µL of TB to create a 1:10 dilution, then placed in the Neubauer chamber. Viable cells were counted when the chamber was analyzed in the microscope.

2.7. Cytotoxicity Test (MTT Test)

The MTT assay is based on the capability of viable cells to convert this yellow water-soluble tetrazolium salt into insoluble purple formazan crystals by cleavage of the tetrazolium ring by dehydrogenase enzymes. This water insoluble product can be solubilized using organic solvents and the resulting colored solutions are spectrophotometrically measured, getting an absorbance directly proportional to the percentage of viable cells [28]. Cells were seeded in triplicate in 96-well plates at a density of 1×10^4 cells/well. After 24 h of culture, cells were treated with or without increasing concentrations of *Ov* infusion and *Crv* solution (culture medium was used for untreated groups) and incubated for 24 h. Then MTT (5 mg/1 mL PBS) was added to the cells, incubating for 18–24 h. The formazan crystals were dissolved with EB. The absorbance was measured at a wavelength of 570 nm. The untreated cells were considered 100% viable. The concentration that reduces the cell viability to half is observed as IC₅₀, a measure of the effectiveness of a substance in inhibiting cell multiplication. The lethal dose 50 was found for both the *Ov* infusion and the *Crv* in the control cell line used in TN BC cells (HCC-70). The experiments were carried out in triplicate at independent times.

2.8. Statistical Analysis

Results were expressed as means ± SD. An ANOVA was performed using IBM SPSS Statistics 24, and a *p* value of <0.05 was considered statistically significant.

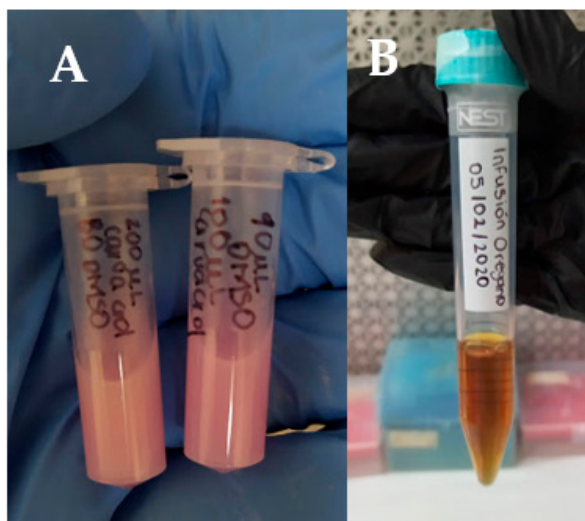


Figure 1. Photographs of the carvacrol (Crv) solution (A) and *Origanum vulgare* (Ov) infusion (B). Both stimuli were prepared the same day that were used.

3. Results and Discussion

3.1. Cell Viability

Figure 2A,B shows the difference in the proliferation of the HaCaT cell line culture. Figure 2C shows a culture with 90% confluence after the trypsinization process, indicating successful cell recovery. Percentages exceeding 80% were found in the cell viability tests, indicating the good state of the cell culture. An example of this assay in the HCC-70 cell line is shown in the Figure 2D.

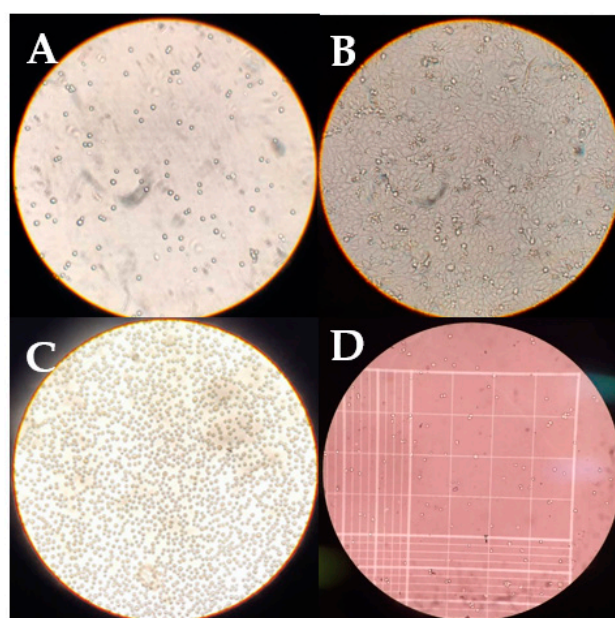


Figure 2. (A) HaCaT cell culture at day 1. (B) HaCaT cell culture upon reaching >90% confluence; in addition to cell proliferation it is appreciated how the cells are adhered to the base of the culture flask. (C) HaCaT cell culture after the recovery process by trypsinization; a more rounded shape is observed in cultured cells after the adherent cell recovery process. (D) Viable cells appear white and those that are not are stained blue. Practically no non-viable cells can be seen. Photographs of cell cultures were taken by observing them under an inverted microscope while the photograph of Neubauer's chamber was taken by observing it under a microscope.

3.2. Cytotoxicity

In the first trial, a dose response curve was performed in the HaCaT cell line, exposing it to four ascending doses of Crv (20 µL, 40 µL, 60 µL and 80 µL) to standardize the IC₅₀. The results showed that the proliferation indices decreased in a dose-dependent manner with significant reductions in proliferation index values from the second dose, which had a concentration of 0.028 M ($33.06\% \pm 5.35$), and it was further potentiated in the third ($10.28\% \pm 1.29$) and fourth doses ($9.95\% \pm 2.06$) with concentrations of 0.042 M and 0.056 M, respectively (Figure 3).

After that, the control cell line HaCaT was exposed to two ascending doses of the *Ov* infusion (20 µL and 40 µL) at a concentration of 250 g/10 mL, finding that the proliferation indices decreased to $18.62\% \pm 1.43$ with the first dose and $12.75\% \pm 1.41$ with the second (Figure 4).

Once the lethal dose 50 for the *Ov* infusion and Crv had been standardized, the cytotoxicity test was carried out in the TN BC cell line (HCC-70) which was exposed to dose 2 of Crv (0.028 M) and dose 2 of the *Ov* infusion (40 µL). The results showed that the *Ov* infusion reduced the proliferation index of these cells to $5.95\% \pm 0.11$ ($p < 0.0001$) while the Crv reduced it to 6.57 ± 0.21 ($p < 0.001$) (Figure 5).

Similar results on the cytotoxicity of carvacrol against different cancer cell lines have been reported. For example, Fahad Khan et al. in 2017 found in their study that carvacrol doses of 25, 50, 100, 250 and 500 µM reduce the in vitro cell viability of prostate cancer cells (DU145) by 79.42%, 59.48%, 46.85%, 15.63% and 11.70%, respectively, after a 24-hour stimulus exposure. The lethal dose 50 for this cell line was found with a concentration of approximately 84.39 µM [29]. Moreover, Hazem Elshafie et al. in 2017 analyzed the in vitro cytotoxic potential of the compounds present in a higher proportion in oregano against hepatocarcinoma cells (HepG2), reporting that carvacrol was the compound with the greatest potential to inhibit the proliferation of this cell line. The lethal dose 50 found in this study was 48 mg/L [19]. These results, among some other studies that have been carried out on this compound and its properties against cancer cells, are consistent with the fact that carvacrol has a potent in vitro cytotoxic effect against different cancer cell lines like MDA-231 and MCF-7 [29–35]. However, the present study provides the first evidence on the effectiveness of this compound against TN BC cells (HCC-70), demonstrating the potency of its cytotoxic activity against aggressive subtypes of BC.

Talking about *Ov*, studies of this plant have been carried out with different cancer cell lines, as in colon cancer (HT-29), melanoma (A375) and hepatocarcinoma (HepG2), where a significant reduction in cell viability has been found in a dose-dependent manner [19,36,37]. Specifically, about breast cancer, Makrane et al. reported in 2018 that MDA-MB-231 BC cells were more susceptible to the cytotoxic effect of *Ov* compared to colon cancer cells (HT-29), as it was observed that cell viability decreased drastically in BC cells as the dose of *Ov* increased, finding the IC₅₀ at a dose of 87.09 µg/mL [37].

Moreover, Katerina Spyridopoulou et al. reported that *Ov* showed anti-proliferative activity against MCF-7 (luminal A) BC cells in a dose-dependent manner, in their study, different cancer cell lines were subjected to *Ov* and the BC cell line was the third in which a stronger anti-proliferative effect was observed, with a lethal dose 50 of 10.0 ± 1.7 µg/mL [38]. Although there are few studies on the cytotoxicity of *Ov* in cancer cell lines the most relevant ones agree that it has a powerful cytotoxic potential. Specifically, in TN-BC cells, the effect of the infusion of *Ov* or Crv has not been studied, since most of the studies that have been published on the subject are in cell lines such as MCF-7 (luminal A) and MDA-MB-231 (breast adenocarcinoma) [22,23,31,33,38–42].

However, analyzing the results of studies on the cytotoxic effect of *Ov* and Crv on cancer cell lines, it can be inferred that both have a powerful cytotoxic activity against these cells. Some studies have even carried out tests in *in vivo* models with *Ov*, finding that it has the ability to significantly slow tumor growth of colon carcinoma in mice, among other effects [36]. Based on all the above and on the results of the present study, we can say that both the infusion of *Ov* and Crv show a powerful cytotoxic effect against TN BC cells (HCC-70), and these results represent the precedent for further studies to continue analyzing the efficacy of this effect because the results are promising so that in

the near future, alternatives of adjuvant treatment can be offered in the management of aggressive types of BC.

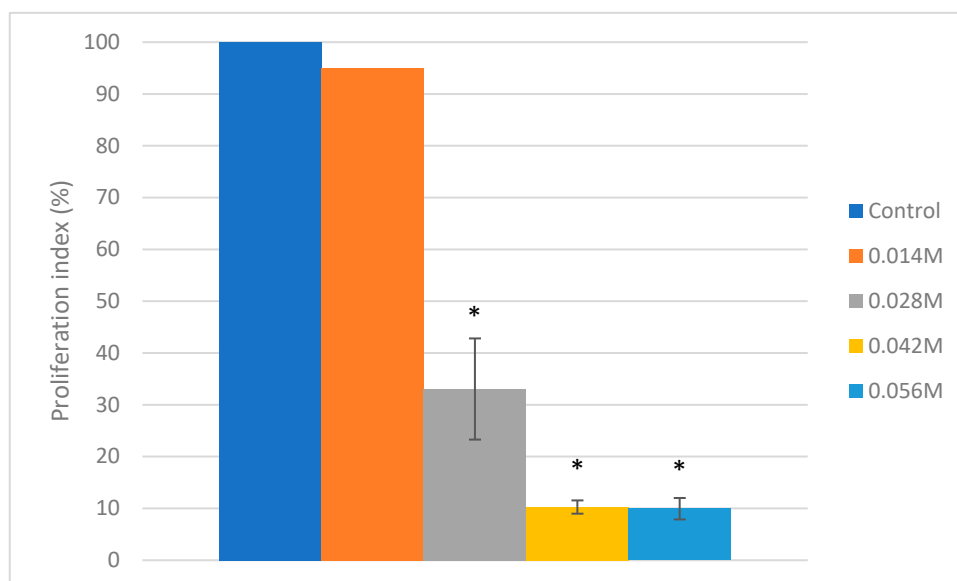


Figure 3. Graphic representation of the proliferation index of HaCaT cells exposed to Crv. Significance * $p < 0.0001$. Data are expressed as mean of three replicates.

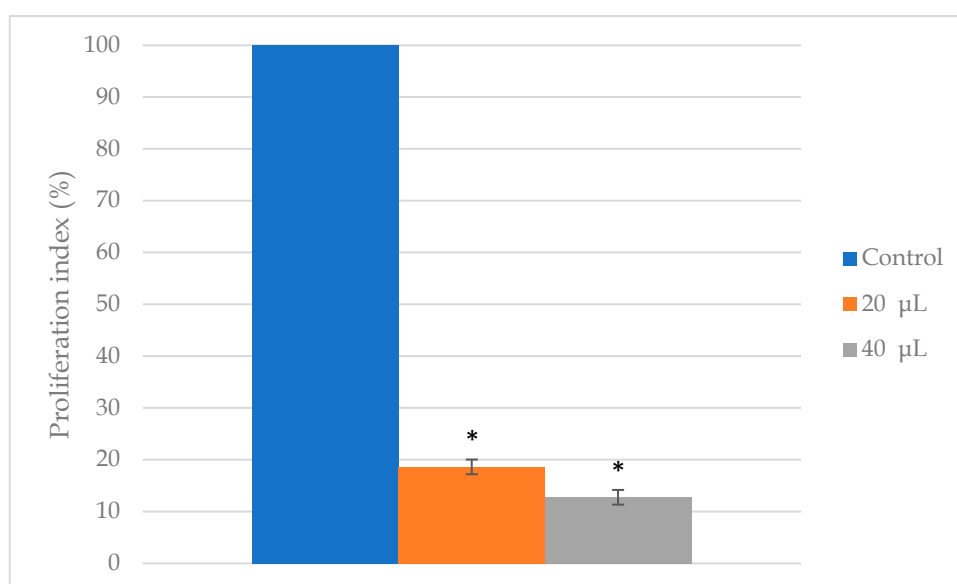


Figure 4. Graphic representation of the proliferation index of HaCaT cells exposed to the Ov infusion. Significance * $p < 0.0001$. Data are expressed as mean of three replicates.

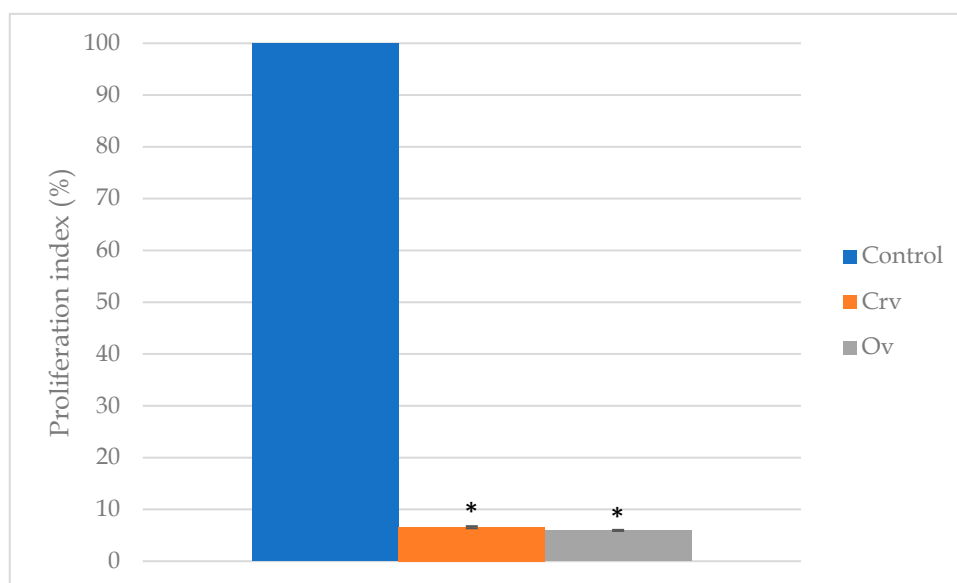


Figure 5. Graphic representation of the proliferation index of triple negative breast cancer (TN BC) cells exposed to *Ov* infusion and Crv. Significance * $p < 0.0001$ Data are expressed as mean of three replicates.

4. Conclusions

In conclusion, the present study provides insight into adjuvant treatments efficacy for breast cancer, especially the *Ov* infusion and Crv for treatment of aggressive BC subtypes like TN. Moreover, this information lays the groundwork so that in the near future effective and economical adjuvant treatments can be promoted, that are beneficial for the patient and society at different levels including patient health, and individual and collective economy, because *Ov* is a plant that is easily accessible to the general population. Furthermore, this study opens the way for future studies, with the aim of further analyzing the efficacy of *Ov* infusion and Crv in this BC subtype, performing *in vivo* preclinical tests showing the forcefulness of its use, with the sole purpose of evaluating the biosecurity of its use, and finally performing tests of its cytotoxic activity as adjuvant and support to the conventional treatments in patients with TN breast cancer.

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Conflicts of Interest: The authors declare no conflict of interest.

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