Chapter 22

Immunotherapeutic and Preventive Role of Purified Extract Rich in Beta-Glucans Derived from D-Fraction of *Grifola frondosa* Mushroom in Experimental Mice Biomodel of Mammary Carcinogenesis

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Additional information is available at the end of the chapter

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Abstract

The overall vision of the modern science needs to change to a revalorization of the natural compounds and their beneficial effects on human diseases, such as cancer. Medicinal mushrooms have been used since thousands of years due to its healing properties. Maitake (*Grifola frondosa*) is presented as one of the most interesting medicinal mushrooms that have been studied. Until now, Maitake D-Fraction may have anticarcinogenic activity, preventing oncogenesis and metastasis in certain tumor types. However, the exact molecular mechanism by which D-Fraction acts are yet unknown. The results shown in this chapter suggest that Maitake D-Fraction Pro4X, administered intraperitoneally, prevents significantly the development of mammary tumorigenesis, increases survival, and reduces the process of angiogenesis in BALBc mice. Although yet to determine the active component of the extract and the molecular mechanism by which it operates in the breast carcinogenesis process. The socioeconomic impact of this research project could be important, considering that in Argentina similar studies using natural compounds derived from medicinal mushrooms for cancer therapy have not yet been performed. The beneficial effects of Maitake, if proven, could be useful for the treatment of cancer patients who are undergoing chemotherapy or radiation or for breast cancer prevention in high-risk population.

Keywords: breast cancer, prevention, Maitake D-Fraction

1. Introduction

Breast cancer now represents the second most common type of tumor pathology in the world and the highest incidence representing the leading cause of death in women in the world [1]. During cancer treatment, tumors often develop resistance mechanisms to chemotherapeutics, which occur in about 30% of patients treated with antineoplastic agents. For this reason, and for the many adverse effects of chemotherapy, more effective and less invasive therapeutic alternatives are sought. In the past century, with the development experienced in the chemical-pharmaceutical area, there was an increase in the production of synthetic and semisynthetic chemical drugs. This led to an increase in adverse reactions and negative side effects, in addition to the high cost of acquisition of these compounds. Therefore, there is a widespread tendency to use products derived from natural sources such as plants and edible mushrooms, consumed as dietary supplements in an increasing number of countries in the recent decades [2, 3]. These substances, which exhibit pharmacological properties in a broad spectrum of diseases, have shown their safety compared to drugs with chemical synthetic origin [4, 5].

An approach to the "ideal" anticancer drug could be derived from selective natural agents with low toxicity, such as fungal and extracts of medicinal plants, which possess significant antitumor and anticarcinogenic activities and avoid toxic side effects. Today, there is great interest in the study of natural extracts that meet these characteristics [6]. Plants and medicinal mushrooms are a source of obtaining active ingredients of marked importance in current research. Nature is a rich source of drugs. It is believed, for example, that only about 10% of the estimated 140,000 species of fungi on Earth are known. It is also estimated that only 5% of these species have been known to have pharmacological properties. The international scientific community has focused its efforts on the search for new sources of active ingredients from plants and fungi as potential anticancer drug [7, 8]. From natural products with anticancer activity, the best known are the vinca alkaloids (vincristine and vinblastine) isolated from the Madagascar periwinkle, *Catharanthus roseus*, *C. roseus* [9]. Probably the most important discovery and development is Paclitaxel (taxol) obtained from *Taxus brevifolia* tree [10, 11]. The new era of anticancer drug has been led by products such as taxol and docetaxol, among others. The discovery of penicillin from Filamentous fungus, *Penicillium notatum* and its therapeutic use, in the 1940s, became a new era of medicine and the "Golden Age" of antibiotics and thus promoted intense research in the nature as a source of new bioactive agents. Plants have a long history in the treatment of cancer, although they have often been observed with some skepticism by the own characteristics of the disease; but now, many people with cancer want to undergo known therapies as alternative products mainly from traditional usage, for example homeopathy and diet, among others, are widely used in oriental medicine. The traditional oriental medicine have been used for thousands of years as a medicinal mushrooms such as *Grifola frondosa* (Maitake) [12], Ganoderma lucidum (Reishi), Inonotus obliquus (Chaga), Lentinus edodes (Shiitake), among others. The production of biologically active fungal metabolites is a very broad field and is a promising study, which until now has been poorly studied [13–15]. Modulation of the immune system through stimulation

or suppression of it can contribute to maintaining good health. Numerous immune system stimulating substances have been isolated from higher plants and fungi, and open doors for the development of novel drugs. They are looming, thus, as an effective alternative for the treatment of various health conditions that alter the normal balance of the body's immune response. The use of mushrooms that activate host defense mechanisms (immunostimulatory or immunopotentiating) provides an additional therapeutic tool to conventional chemotherapy. Given the limitations of conventional therapies to reduce cancer mortality rate, many efforts are focused on cancer prevention. Within this context, the use of immunopotentiating and inmunostimulating agents as well as biological response modifiers (BRM), capable of stimulating the immune cells that can identify tumor cells as foreign, eliminate, and prevent carcinogenesis, have gained prominence [16–18]. An immunomodulatory polysaccharide obtained from higher fungi is the grifolano (GRN), derived from *Grifola frondosa*. Several studies suggest that the mechanism of the antitumor activity of GRN is strongly related to immunomodulation [19]. It has been shown that the active grifolano *in vitro* macrophages to produce the tumor necrosis factor-alpha (TNF-α) [20]. The β-glucans are one of the most abundant forms of polysaccharides found in the cell wall of bacteria and fungi, which exert effects on the immune system by stimulating phagocytic activity, activating leukocytes, and inducing the production of various cytokines, which could give them their antitumor activity. It has been shown that oral administration of β-glucans extracts derived from *Grifola frondosa* medicinal mushroom (Maitake) could stimulate hematopoiesis and recovery post-treatment with Paclitaxel in cancer patients [21]. Particularly β-glucans act on a variety of receptors related to immune system, particularly acting on Dectin-1 and CR3 receptors, which trigger a broad spectrum of immune responses [22]. The β-glucans targeting immune cells are macrophages, neutrophils, monocytes, NK cells, and dendrites cells. Immunomodulatory functions induced by β-glucans involve an innate and adaptive immune response. Maitake is an edible and a medicinal mushroom, whose extracts possess β-glucans with different degrees of purification and have antitumor properties [23]. The β-glucans are BRM that, unlike conventional chemotherapeutics, activate or reinforce the host immune system, helping to eliminate or inhibit tumor growth. It has been shown that fractions obtained from Maitake can fight cancer by slowing or stopping tumor growth; and preventing tumor metastasis [23]. On the other hand, could decrease the side effects of chemotherapy such as hair loss, pain, and nausea, and enhance its positive effects [23]. The immunomodulatory functions induced by β-glucans involve an innate response and adaptive immune response. However, the exact mechanisms of immune system activation mediated by β-glucans are still unknown and must be defined.

In this chapter, we present a summary of some experiments done in biomodels of mammary carcinogenesis. So far, we have demonstrated that the treatment with Maitake D-Fraction Pro4X prevents the development of mammary tumorigenesis, blocks tumor invasiveness, reduce tumor angiogenesis, increases overall survival in animals, and exhibits selective cytotoxicity [24–26]. Moreover, we also demonstrated that the use of Maitake D-Fraction Pro4X is safe and nontoxic as well.

2. Direct effect of Maitake D-Fraction compared with Chemotherapy on breast tumour death

2.1. Effect of Maitake vs. chemotherapy on breast tumor death

In order to demonstrate if Maitake D-Fraction is able to kill breast cancer cells in culture, we measured the number of murine breast tumor LM3 cells death after treatment. The effects of increasing concentrations of β-glucans contained in Maitake D-Fraction Pro4X (0.036, 0.091, 0.183, 0.367, and 0.734uM) on cell death were evaluated at 24, 48, and 72 hours of treatment. In parallel, we treated LM3 cells during the same time with chemotherapy drugs using a combination of doxorubicin and cyclophosphamide at increased concentrations from 5 to 40 μM and from 0.5 to 2.5 μM for each drug, respectively. Cell death was determined at 24, 48 and 72 hours of treatment according to the trypan blue exclusion stained method. **Figure 1** shows the cell death values depending on the concentration of the used chemotherapeutic drugs (doxorubicin and cyclophosphamide) for 24, 48, and 72 hours after treatment. In all treatments, cell death was significantly higher (Student's *t*-test, $p \leq 0.05$) relative to untreated controls. It is also observed that the highest percentage of cell death (96.70%) corresponded to the higher concentration of chemotherapy used drugs (40 mM doxorubicin + 2.5 mM cyclophosphamide). The optimal time of the maximal cytolysis was the longest treatment at 72 hours. Cell death values depending on the concentration of Maitake Pro4X Fraction D at 24, 48, and 72 hours of treatment, as shown in **Figure 2**. The percentage of cell death increased depending on both, concentration of Maitake Pro4X used and time of treatment. The highest percentage of cell death (61.52%) corresponded to treatment with the highest dose of Maitake (0.734 μM) for 72 hours, the longest treatment.

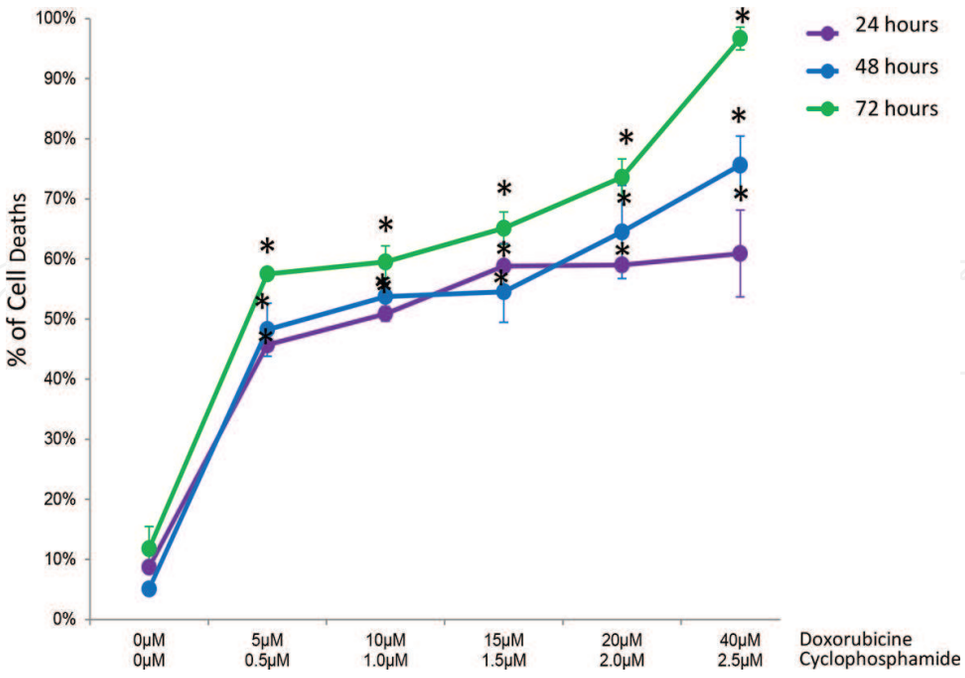

Figure 1. Cell death caused by different concentrations of doxorubicin + cyclophosphamide at 24, 48, and 72 hours of treatment. The values represented the mean ± correspond to SD($N = 2$). *$p < 0.05$ vs. control.

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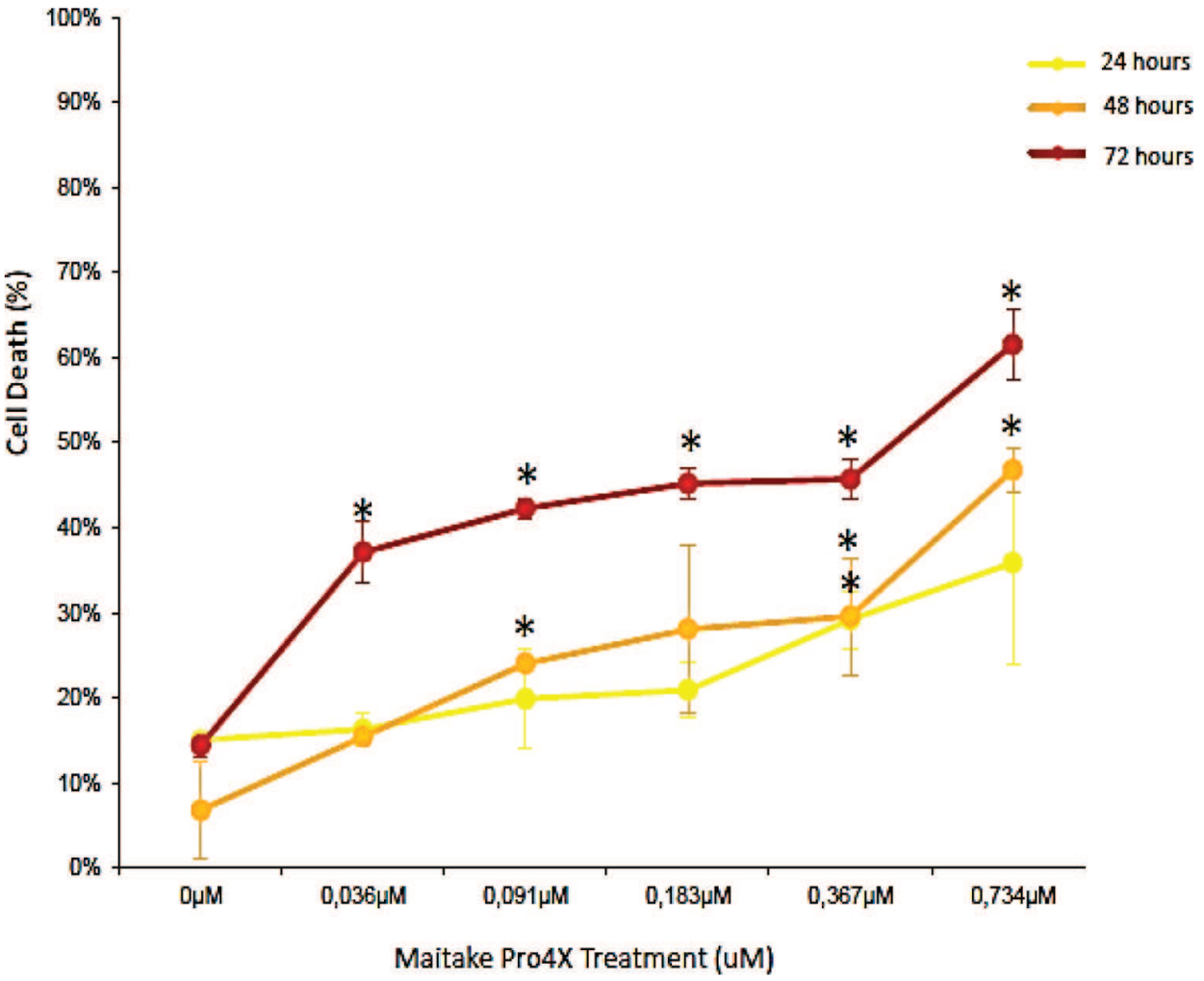

Figure 2. Cell death caused by different concentrations of Maitakeat 24, 48, and 72 hours of treatment. The values represented the mean ± correspond to SD ($n = 2$). *$p < 0.05$ vs. control.

In this work, we observed that both treatments, Maitake or chemotherapy, increased tumor cell death depending on the concentration and time of treatment. These results suggest that Maitake D-Fraction may have a chemotherapeutic effect by inducing a dose-dependent cell death. Here, we observed that the treatment with chemotherapeutic drugs increased mouse tumor cell death in a higher level compared to treatment with Maitake D-Fraction.

2.1.1. In vitro effect of Maitake on human breast tumour MCF-7 death cells

By another side, we performed the same experiments measuring the death in tumor human mammary cells (MCF-7). Using the time lapse microscope that takes pictures of the treated MCF-7 cell culture every 10 minutes during 1, 5, 10, and 24 hours (**Figure 3A**), it was found that Maitake D-Fraction increase the number of cell deaths significantly in a dose-dependent form, reaching the maximum deaths at the concentration of 367 μg/ml (equivalent to 0.367 μM) (**Figure 3B**). The treatment of tumoral MCF-7 breast cells at 24 hours with D-Fraction significantly increase ($p < 0.05$) the percentage of cell death in comparison with untreated controls.

2.1.2. Maitake D-fraction decreased MCF7 cell viability and increased apoptosis

These results made us to think about whether Maitake D-Fraction really exerted anticancer effects and induces cell death directly or is toxic for those cells and able to kill any kind of cell. To probe this, we measure the effect of this compound in MCF7 cells viability and examine if the cell death trigger mechanisms are related to apoptosis. The cell deaths were measured by examining using the MTS assay in MCF7 cell cultures incubated with five different concentrations of D-Fraction. A gradual decrease in the number of viable cells was observed with

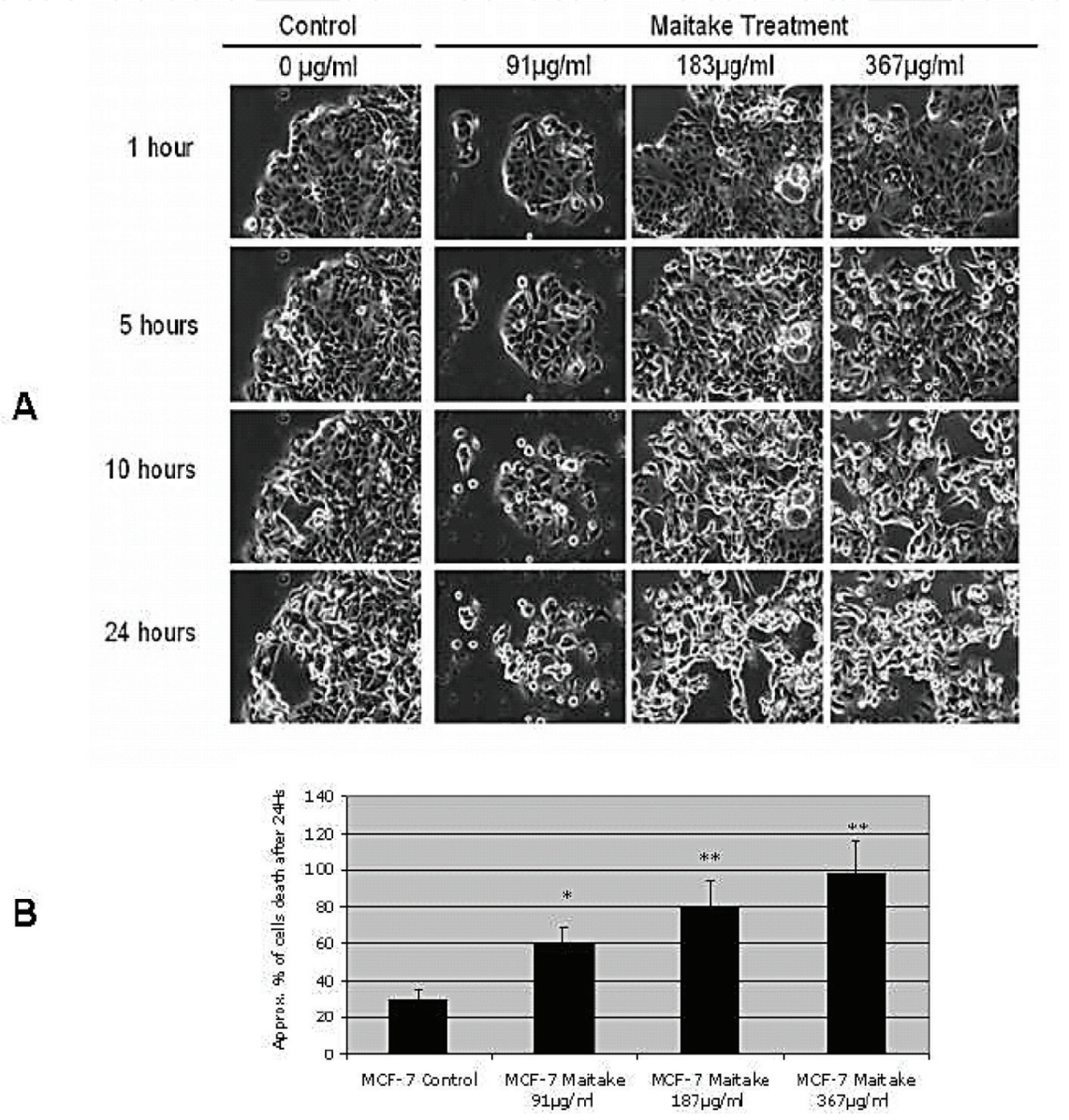

Figure 3. Analysis of cell death induced by Maitake in MCF-7 cells employing the time-lapse microscope. MCF-7 cells at 70% of confluence were treated with and without (control) increased concentrations of Maitake D-Fraction. The experiments were performed by triplicate. Cells were placed in the time-lapse microscope under CO_2 atmosphere, at room temperature during 24 hours. The camera was set up to take pictures every 10 minutes, using the 20× objective. Images and videos were analyzed employing specific software. (A) The representative image corresponding to the cell culture at 1, 5, 10, and 24 hours after Maitake treatment in the conditions indicated in the figure. (B) Corresponding to the approximate percentage of dead cells observed in each movie after 24 hours of Maitake incubation.

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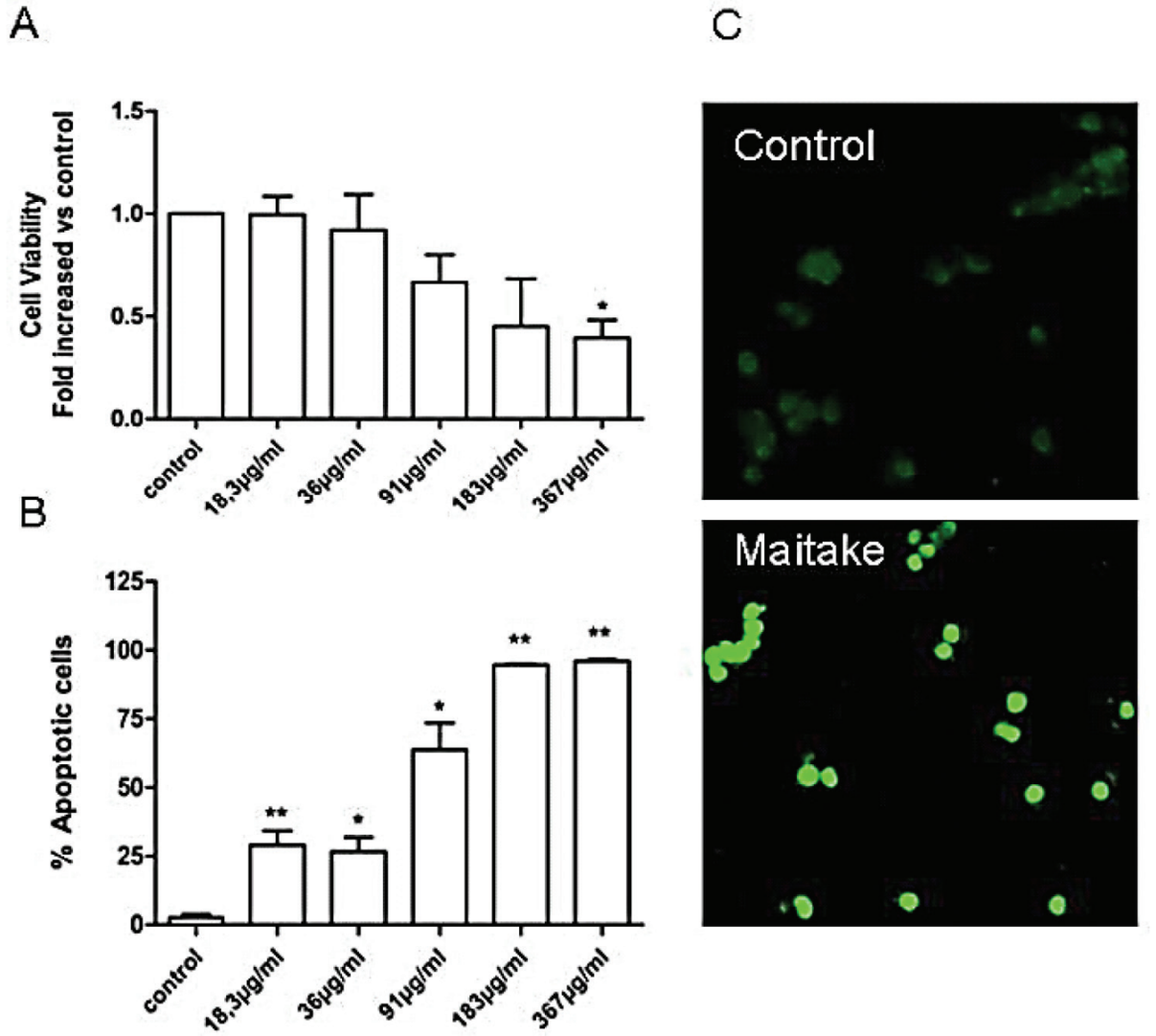

Figure 4. MCF-7 cell viability was evaluated after incubation with five concentrations of Maitake D-Fraction. (A) Cell viability was assessed by MTS assay. The results are expressed in absorbance values at 540 nm and are fold-increase relative to control cell cultures. Three independent experiments were performed in triplicate with identical results. *p = 0.05 vs. control (n = 9). (B) Apoptosis was increased at every incubation as evaluated by TUNEL assay, reaching statistical significance for every concentration of D-Fraction (*$p < 0.05$ vs. controls; **$p < 0.01$ vs. control). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. Experiments were repeated three times with identical results (n = 9). (C) Immunofluorescence for apoptotic cells (green). Note the increased number of apoptotic cells in 367 µg/ml Maitake-treated cells (Maitake) in comparison to untreated cells (control). Representative images are shown. Magnification ×200.

increasing concentrations of D-Fraction (**Figure 4A**). In fact, we observed that the highest concentration of D-Fraction resulted in a significant decrease in cell viability in comparison to control untreated (**Figure 4A**) (*$p < 0.05$ vs. control). To evaluate whether this decrease in cell viability was due to apoptosis, we employed the TUNEL assay. MCF7 (4×10^4) cells were incubated with increased concentrations of Maitake D-Fraction during 24 hours and the percentage of apoptotic cells was quantified. We observed that the treatment with this fraction, at any concentration, led to a significant increase in the number of apoptotic cells, in a dose-dependent manner (**Figure 4B**). Interestingly, nearly 95% of the cells became apoptotic whenever treated

with the highest concentration of Maitake D-Fraction (0.367 μM or 367 μg/ml) (**Figure 4B**). A representative microscope image of these findings is illustrated in **Figure 4C**. As observed, treatment with Maitake D-Fraction led to an effective increase in the number of apoptotic cells (green) as compared to the untreated culture. These findings indicated that Maitake D-Fraction was able to effectively induce apoptosis in human MCF-7 breast cancer cells.

2.2. Effect of Maitake D-Fraction on death of normal human breast cells MCF-10F

To investigate if Maitake D-Fraction is selective to cell death and only induces death on tumor cells not in normal cells, we performed studies using normal human breast cells MCF-10F. We operate at different times and increase concentrations of Maitake D-Fraction using an *in vitro* MCF-10F cell culture and measure cell death after treatment. Cells were incubated by triplicate with D-Fraction at 37°C in controlled atmosphere with 5% CO_2 in a serum-free medium. At the end of the treatment, cell deaths were determined by the technique of trypan blue exclusion assay, counting the percentage of dead and the percentage of live cells in Neubauer chamber. The assay were tested at increased concentrations of Maitake D-Fraction at 91, 183, and 367 μg/ml of culture medium during 24, 48, and 72 hours. All the experiments were performed by triplicate. Surprisingly, treatment of cells, normal mammary MCF-10F cells, with increased concentrations of D-Fraction did not cause significant increases in the percentage of cell death compared to control at the highest dose of 367 μg/ml equivalent to 0.367 μM at any time assayed (**Figure 5**). In conclusion from these experiments, we confirm that Maitake D-Fraction only induced *in vitro* cell death by apoptosis in breast tumor cells without affecting normal breast cells at any concentrations or time of treatment.

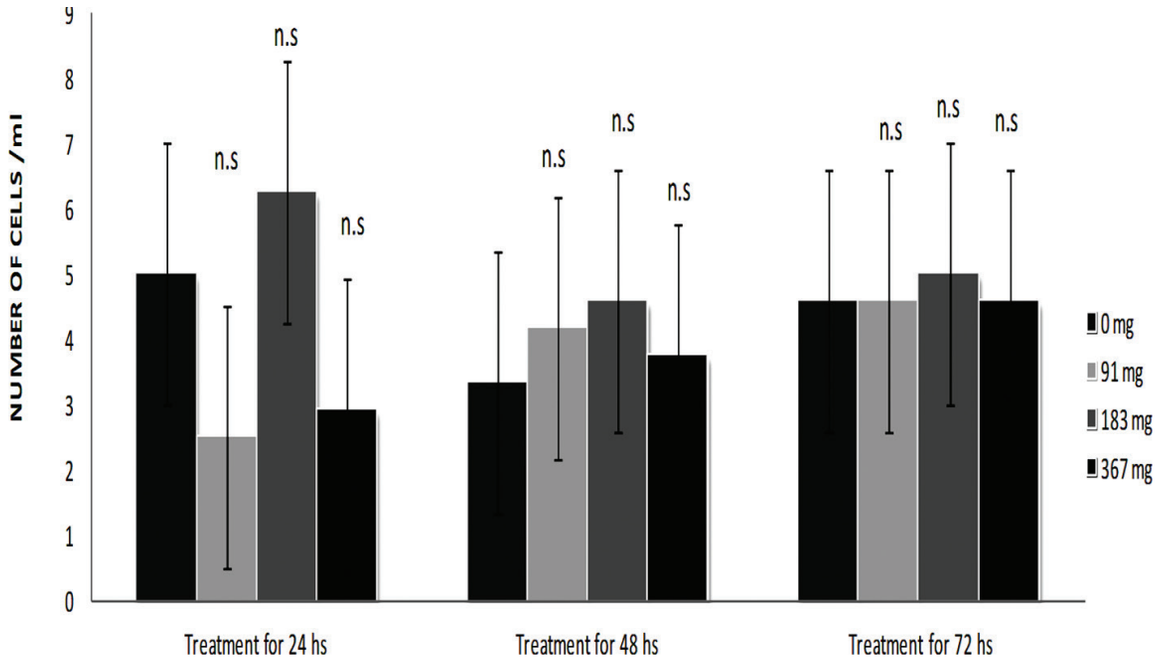

Figure 5. Cell death caused by different concentrations of Maitake D-Fraction on MCF-10F cells at 24, 48, and 72 hours of treatment. The values represented the mean ± correspond to SD ($n = 3$). *$p < 0.05$ vs. control.

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3. Breast cancer prevention studies

3.1. Studies of breast tumor prevention by Maitake Pro4X in BALBc mice

3.1.1. Effect of Maitake D-Fraction Pro4X on breast cancer prevention

In order to demonstrate whether the purified extract Maitake D-Fraction Pro4X (from Mushroom Wisdom Inc, NJ, USA) was related to breast cancer prevention or inhibited the mammary tumorigenesis process, three independent experiments were performed employing 20 female nulliparous BALBc mice. Two groups were separated with 10 animals each, control group and Maitake D-Fraction group (5 mg/Kg) that were treated daily during 15 days by intraperitoneal injection. After that, mammary tumorigenesis was induced using implant of 2×10^5 LM3 cells intraperitoneally. All animals were checked weekly for breast tumor development. **Figure 6** shows the picture of mice abdominal area (peritoneal mammary glands) from each condition after 30 days of tumor challenge. From this experiment, we observed that 100% of breast tumorigenesis was developed (10 out 10 animals) in the control group. However, only 3 out 10 animals (30% of tumorigenesis) developed mammary tumors in the condition treated with Maitake D-Fraction Pro4X (Pro4X) (**Figure 6**). The average from all the three independent experiments performed for prevention against breast tumorigenesis development in animals from the control group was 3.333 ± 5.774 (**Figure 7**), which was significantly different from the prevention generated by Maitake Pro4X (64.286 ± 23.862, $p < 0.01$).

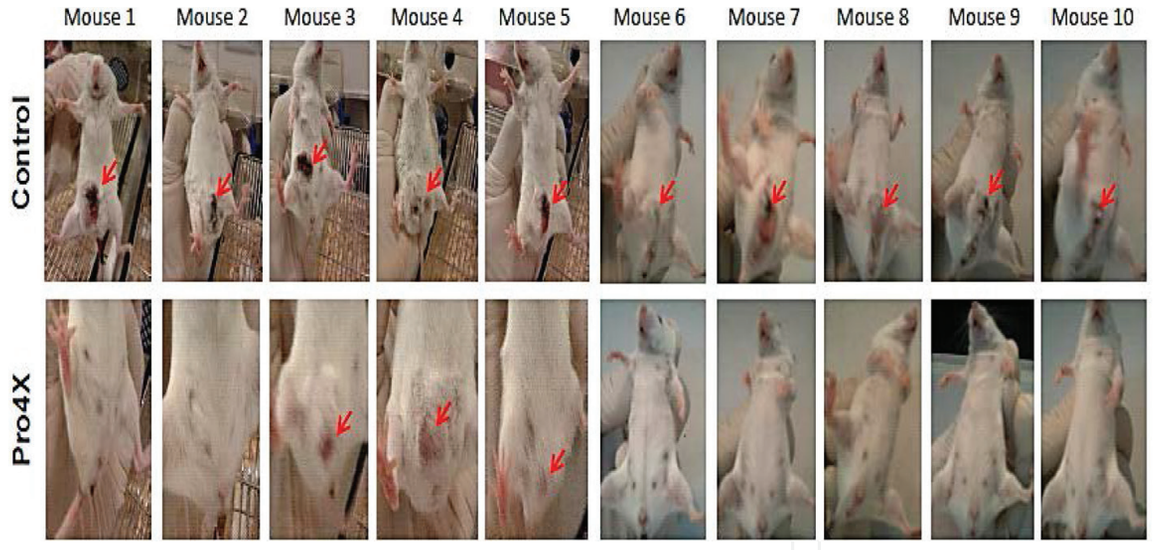

Figure 6. Effect of Maitake D-Fraction on breast cancer prevention in BALBc mice.

3.1.2. Effect of Maitake D-Fraction Pro4X in the tumor grows that escape to treatment

After analyzing the percentage of prevention in each treatment, now is important to study how the tumor grows in the animals that did not respond to the Maitake treatment and escapes its control. From **Figure 6**, 3 of 10 animals escaped the Maitake Pro4X prevention and developed breast tumors. We observed that breast tumor in the control group grew linearly 10–24 days

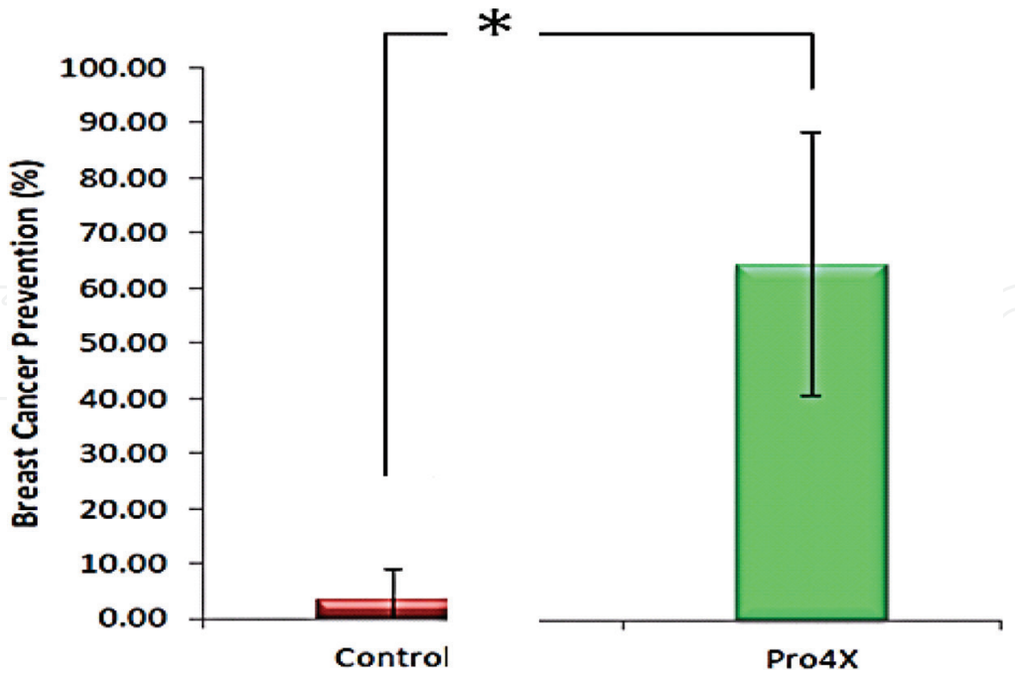

Figure 7. Percentage of breast cancer prevention induced by Maitake D-Fraction Pro4X in BALBc mice.

after tumor challenge; however, in the Maitake group, the breast tumor grew slowly at the same time and at 24 days achieved a similar size to compare the untreated control. At 46 days after Tumorigenesis (the end of experiment), the tumor area (cm^2) did not achieve a significant difference between the groups. The microscopy study of tissue paraffin sections shows that the untreated tumors from the control group were solid and have irregular edges; however, we were surprised to observe that tumors from the Maitake Pro4X group were almost the same size than the controls but full of liquid, not solid, with net tumor round edges, similar to benign tumors.

3.1.3. Effect of Maitake Pro4X on tumor necrosis

From the same experiments, we were interested in analyzing the necrosis area in the breast tumors. **Figure 8** shows the macroscopic aspect of a representative breast tumor at control and Maitake groups at the end of the experiment. After measuring the necrosis area (cm) from breast tumor in each animal group, it can be concluded that Maitake Pro4X reduce significantly ($*p < 0.01$) the area of necrosis in the surface of the breast tumors that escape to its control compared to the untreated group. Maitake D-Fraction Pro4X practically did not develop necrosis in their tumors (**Figure 8**).

3.1.4. Effect of Maitake Pro4X on metastasis in liver and lung tissues

The next question that we made is can Maitake Pro4X avoid the metastasis event in those animals with breast tumors. In order to verify that, lung and liver tissues were isolated from each tumor-bearing mice treated with or without Maitake Pro4X. Weight, macroscopic aspect, and sizes of lung and liver from those mice with breast tumors were checked. The lung and

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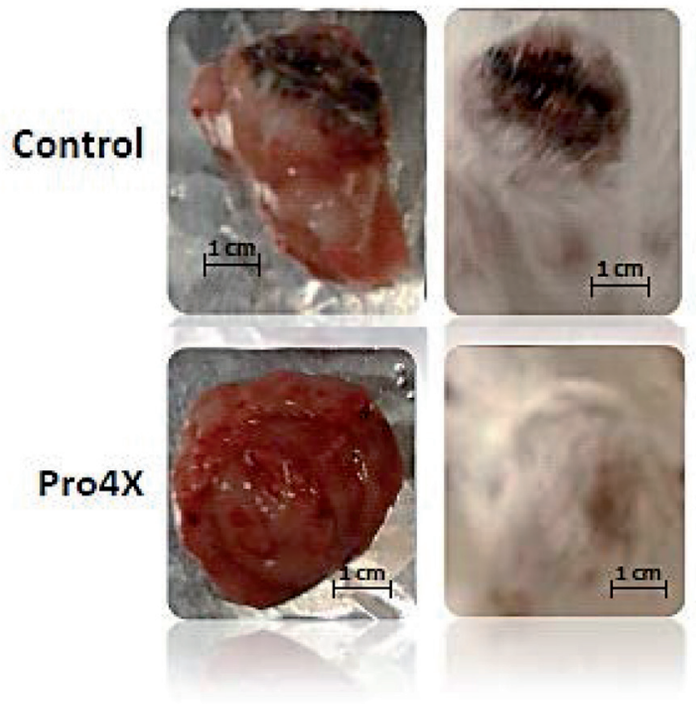

Figure 8. Maitake Pro4X treatment reduces tumor necrosis. The figure represents the pictures of breast tumors isolated (left) and in vivo (right) in both groups.

liver tissues' average area (cm^2) from each experimental group were analyzed. No significant differences in the size of the lung or the liver tissues from those animals in each experimental group were found. But nevertheless, macroscopically, liver tissues from the control group were completely different, colorless, and rigid, compared to those treated with Maitake Pro4X (**Figure 9A**). The histology of control's liver tissue shows and confirms cell proliferation and hyperplasia. However, liver tissues from Maitake Pro4X treated were darker, similar to nor-

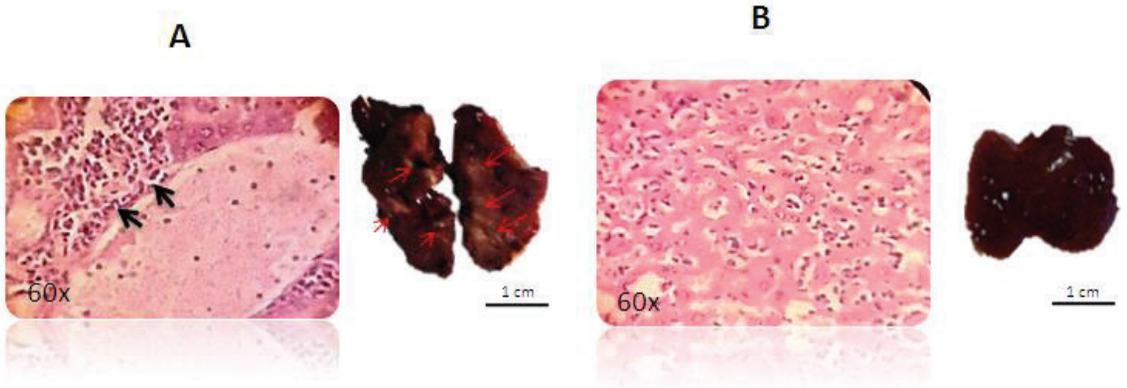

Figure 9. The liver tissues histology (left) of tumor-bearing mice from control (A) and Maitake Pro4X (B). Back arrows represent the cell proliferation area. Right pictures from each histology represent the liver tissue in each condition. Red arrows represent the metastasis area.

mal, with normal texture and aspect (**Figure 9B**). The histology studies from control's liver tissues indicated the presence of bigger blood vessels, with liver structure different than normal and some mitotic changes. Those experiments suggest that the treatment with Maitake Pro4X prevent the liver metastasis development.

The macroscopic study of the lung tissues revealed no morphological differences between tumor-bearing and nontumor-bearing mice. However, surprisingly, were observed higher mitosis percentage in the lung histology sections from control animals (7.50 ± 0.7) compared to Maitake Pro4x treatment (0.1 ± 0.02, $p < 0.001$ in the Pro4X). The percentage of mitosis found in the lung tissues pretreated with Maitake pro4X revealed no differences compared to normal lung tissue.

3.2. Comparison in Breast Tumorigenesis preventive potential: Maitake D-Fraction vs. Tamoxifen in experimental biomodel

In order to study whether Maitake D-Fraction can be adjuvant in breast cancer prevention with tamoxifen, we employed 20 BALBc female mice, 6–8 weeks old, separated into different groups: control group, D-Fraction group, tamoxifen group, and D-Fraction + tamoxifen group. The animals were inbred and kept in the Bioterio from BIOMED-UCA in compliance with National and International Standards of handling of laboratory animals with administration of water and food *ad libitum* kept on a 12-hour light/12-hour dark at room temperature, 22°C. Before performing the experiments, we got the approval of the ethical committee CICUAE from our Institution BIOMED-UCA for the animal use and manipulation in this study. Tumor induction in female mice was performed by exogenous implant of 2×10^5 murine tumor LM3 cells. To study the preventive effects of D-Fraction in mammary tumorigenesis, we have been working with 20 BALBc female mice divided in the following groups: control group treated orally with the dissolution vehicle; Maitake-treated group, with daily administration of 5 mg of β-glucans/kg; tamoxifen-treated group, daily treated with 20 mg of tamoxifen; and combined treatment of tamoxifen and D-Fraction at the indicated doses (tamoxifen + D-Fraction group). The treatments continued for 50 days (equivalent to 5 years in human), after that, mammary tumorigenesis were induced. The animals were observed until day 27 post tumorigenesis (sacrifice day). In this experiment, we observed that Maitake D-Fraction protects mammary tissue against tumor development in about 40% ($^*p < 0.05$) (**Figure 10** shows D-Fraction induce tumorigenesis in about 60%); however, we observed that tamoxifen treatment alone prevent only in about 25% (n.s., *p*-value not significant) against breast carcinogenesis. Surprisingly, the coadministration with tamoxifen + D-Fraction prevented mammary tumorigenesis in about 80% ($^{**}p < 0.01$) (**Figure 10** shows that combined treatment developed only 20% of tumorigenesis). To note, we observed that 100% of control animals developed breast tumor (**Figure 10**).

As for the post-tumorigenesis mortality, 20% of the animals treated with tamoxifen and 25% of control animals died after the tumor induction. Surprisingly, there was no mortality in Maitake and tamoxifen + Fraction D groups, 100% of the animals surviving at the end of the experiment. **Figure 11** show the overall survival rate of the animal from each condition at the end of the treatment.

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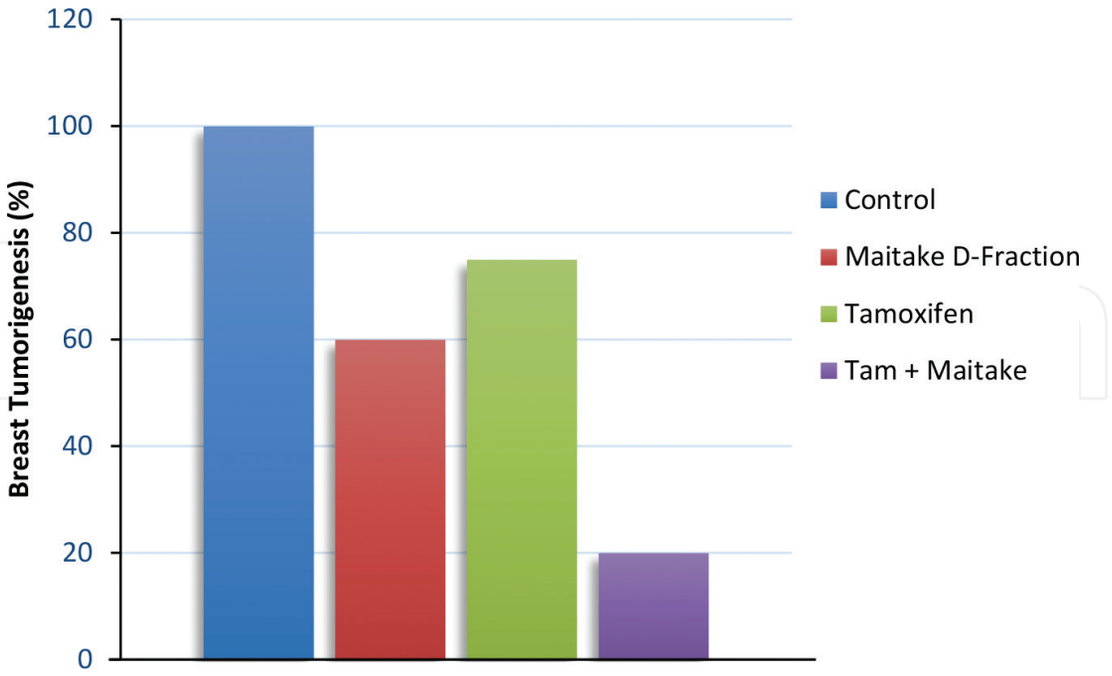

Figure 10. Breast tumorigenesis induced by Maitake or tamoxifen alone or in combination. Maitake D-Fraction was used in a concentration of 5 mg/kg and tamoxifen in concentration of 20 mg/animal. Treatment was performed during 50 days in female BALBc mice.

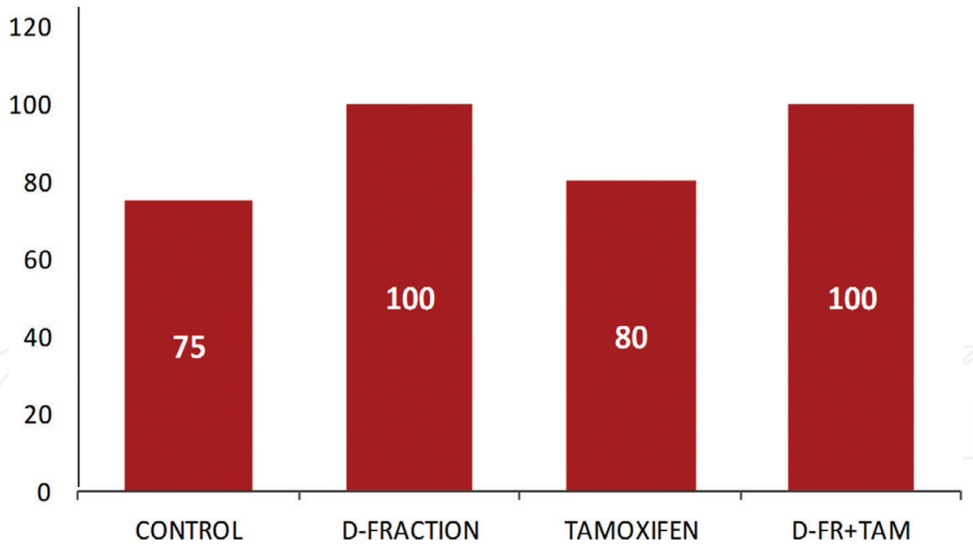

Figure 11. Overall survival rate after treatment with and without Maitake and tamoxifen alone or in combination.

Regarding adverse effects, tamoxifen-treated animals exhibit a remarkable intestinal jaundice, less evident in subjects treated with tamoxifen + Maitake, which was absent in the animals treated only with Maitake. All the treated groups showed significant increase in serum creatinine with $p < 0.05$ compared to control. These results suggest that D-Fraction has a higher preventive potential, compared with tamoxifen, in the development of mammary tumorigenesis.

Moreover, Maitake induce less or no side effects and the maximum overall survival rate in the mice. However, we observed from these experiments that tamoxifen and Maitake D-Fraction are able to achieve the maximal potential in breast cancer prevention when administered in combination.

4. Angiogenesis reduction

We estimate the angiogenic index in the tumoral breast tissues in order to establish if Maitake D-Fraction extracts are able to reduce or avoid the tumoral angiogenesis. **Figure 12A** shows the average blood vessels density in each group. **Figure 12B** shows the microscopy images (25×) of those breast tumors from both groups. We observe that the number of blood vessels/mm^2 in control's breast tumor tissues were significantly higher (0.637 ± 0.182, $p < 0.05$) than breast tumors treated with Maitake Pro4X (0.031 ± 0.028). We can also observe from the microscopy pictures that the area of control blood vessels are bigger compared to those in the breast tissue treated with Maitake Pro4x (**Figure 12B**).

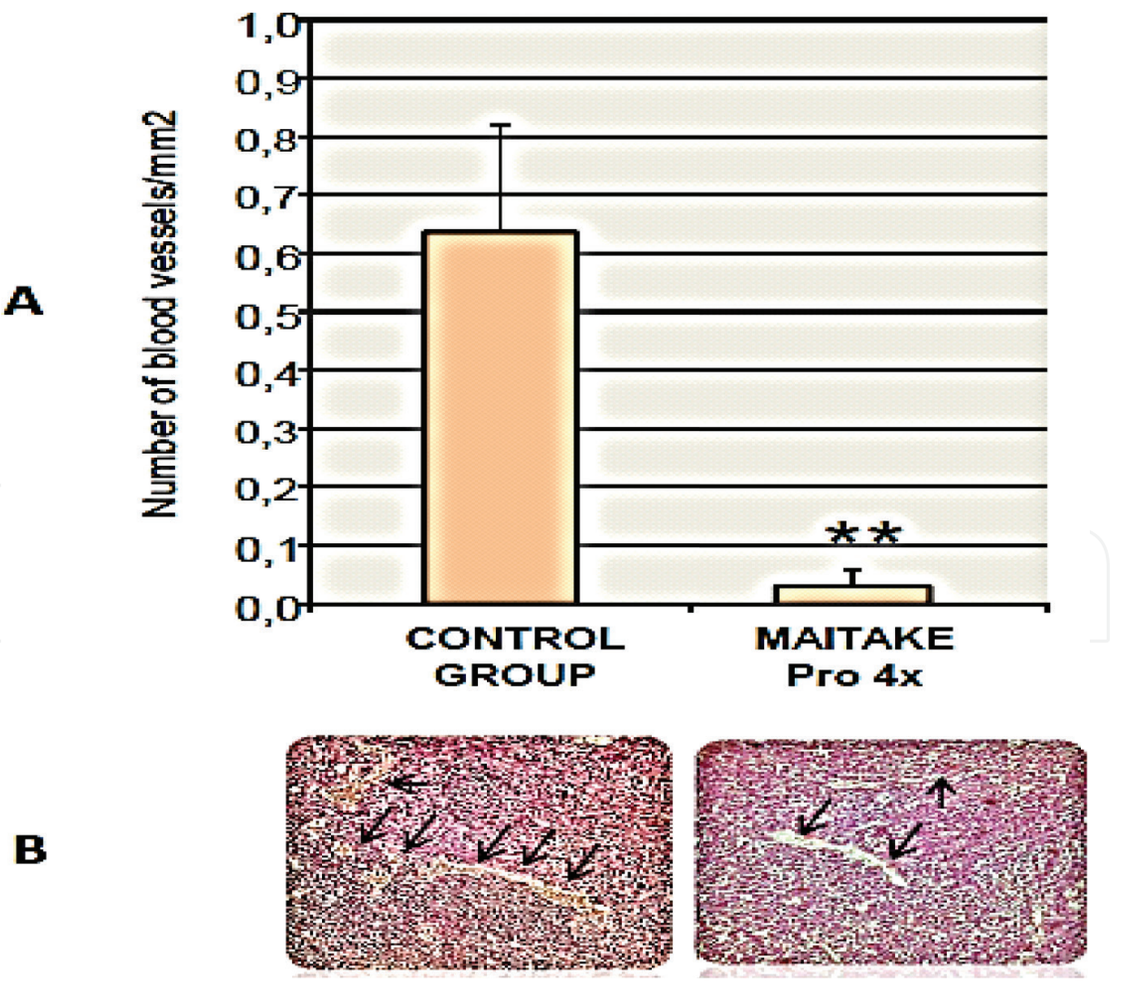

Figure 12. (A) The graphics of average of blood vessels density/mm^2 in each group. (B) The microscopy pictures (25×) of breast tumors analyzed. Black arrows indicated the size of blood vessels in each condition. **p<0.01.

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5. Survival increase

5.1. Effect of Maitake extract in the relative survival in BALBc mice

Another aspect in which we were interested was the overall survival of mice at the end of the experiment. **Figure 13** shows the percentage of overall relative survival at 46 days after tumorigenesis initiation when the experiment was terminated and the animals were sacrificed to analyze the results. Higher number of animals treated with Maitake D-Fraction lived until the end of the experiment. The overall survival in animals from Maitake Pro4X group at the end was 50% compared to control that was reduced to 10% (**Figure 13**). Here, we also analyzed other Maitake D-Fraction product called Maitake Standard with similar composition of Maitake Pro4X, but less concentrated. Both the Maitake compounds did induce a higher overall survival in BALBc mice at 46 days after tumorigenesis.

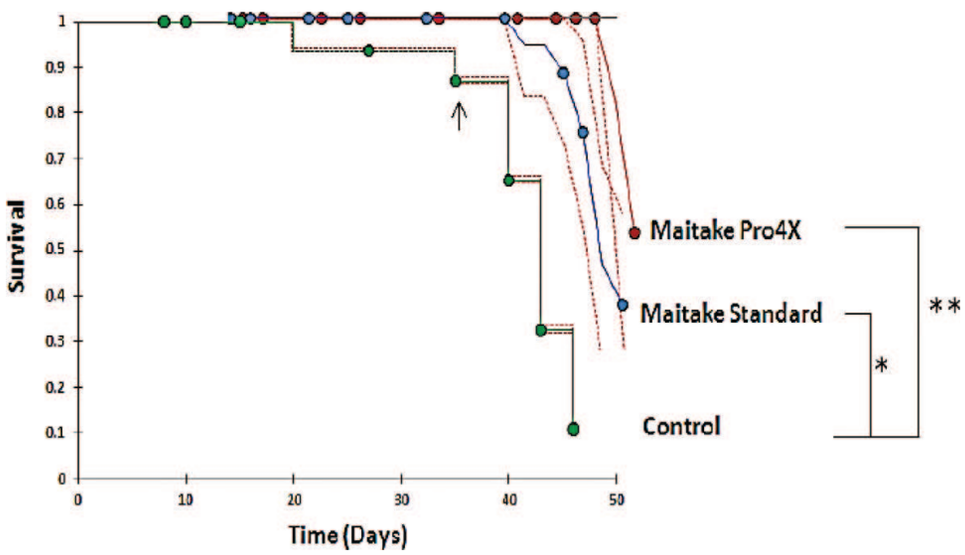

Figure 13. Kaplan-Meier overall survival curves. The graphic represents of overall relative survival from 7 to 50 days after tumorigenesis initiation. A green line represents the control group, a blue line represents the Maitake Standard treatment, and a red line indicates the survival after Maitake Pro4X treatment. *p<0.05; **p<0.01.

6. Effect of maitake pro4x on specific gene expression related to tumoral phenotype inhibition

With the objective to determine if Maitake D-Fraction PRO4X modifies the genomic expression of tumoral phenotype we isolated total RNA from tumor of all the experimental groups and mammary gland of nontumor-bearing mice. We choose genes such as ABCG2, CUL3, IGFBP5, PTEN, and SPACR, whose expressions were modified after Maitake treatment in MCF-7 cells previously published [25]. For this purpose, total RNA were isolated and after purification RT-PCR were performed in each breast tumor tissue from control, Maitake Standard, and Maitake Pro4X groups. We also isolated total RNA from normal breast tissue treated with Maitake Pro4X resistant to tumorigenesis. In **Figure 14A**, we shown the gene

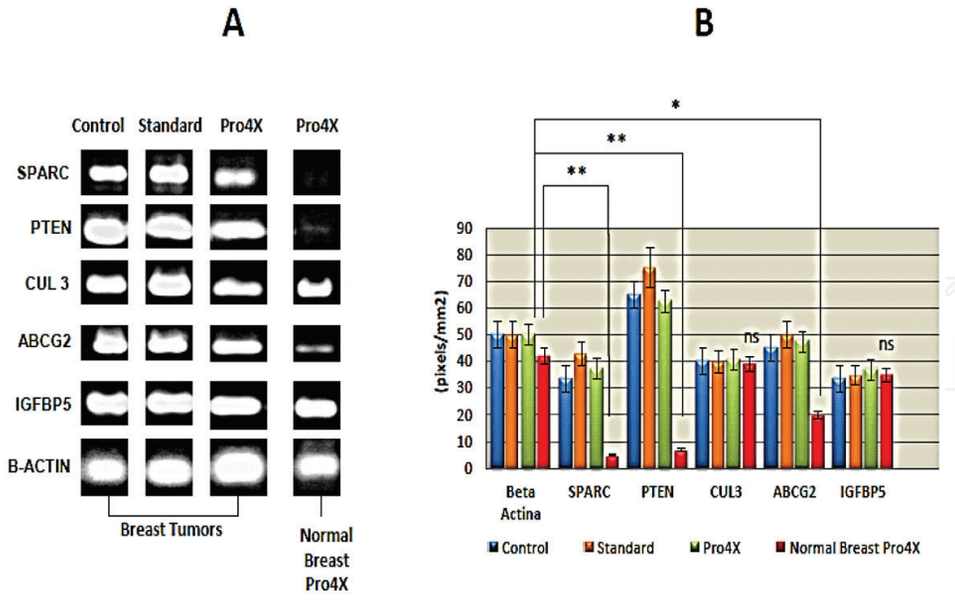

Figure 14. Gene expression analysis. (A) The gene expression at mRNA level in all the conditions. (B) Relative quantification of each RT-PCR reaction. *p<0.05 and **p<0.01.

expression in all the conditions assayed. We observed from this figure that SPARC gene is differentially expressed in all the conditions. SPARC gene expression were upmodulated in the breast tumor tissues treated with Maitake Pro4X (mouse 2 and mouse 3). On the other hand, we observed a downmodulation in the SPACR gene expression corresponding to mouse 4 treated with Maitake Pro4X. We did not observed PCR amplification of SPARC gene in the breast normal tissue without tumor corresponding to a mouse treated with Maitake Pro4X, who was resistant to carcinogenesis. We observed a similar pattern in mouse 2 treated with Maitake Standard. With respect to the gene expression of PTEN, in **Figure 14B**, we observed that are also differentially expressed in the assayed conditions. In the breast tissues of mouse 1 and mouse 4 treated with Maitake Pro4X we observed a downmodulation of this gene; however, we did not observe expression band in the mouse 2 treated with Maitake Standard or in the breast normal tissue resistant to carcinogenesis treated with Maitake Pro4X. ABCGs gene are also expressed differentially in all the conditions. No bands were observed in the tumoral tissue from mouse 2 treated neither with Maitake Standard, nor in the breast normal tissue resistant to carcinogenesis treated with Maitake Pro4X (**Figure 14**). Moreover, CUL3 and IGFBP5 genes were expressed in all conditions (**Figure 14A**). In order to see if there are differences in the level of expression we did quantify the PCR reaction with respect to β-actin amplifying all genes at 10, 20, 35, and 40 cycles. **Figure 14B** shows the quantification of each PCR reaction.

7. Toxicity studies

7.1. Acute Toxicity Studies in BALBc mice as biomodel

To investigate if Maitake D-Fraction Pro4X did not generate acute toxicity, we worked with a really high concentration of Maitake (2000 mg/kg). For this purpose, we employed 10 female

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and male BALBc mice, 6–8 weeks old. Control group animals received a single oral dose of 514 μl of BD (bi-distilled water) and the treat group animals received one oral dose of 514 μl of D-Fraction (corresponding to 2000 mg/kg of D-Fraction, dose equivalent at approx. 120 times highest compared with the therapeutic dose employed in previous experiments). The animals were observed daily until day 14 after treatment (day of sacrifice). The results show a significant increase in the body weight of the male mice from the control group (24.5 ± 1.49 gs) with respect to males treated with Maitake (22.57 ± 2.19 gs), $p < 0.05$ (**Figure 15**). No significant differences were observed in the body weight of female BALBc mice from both groups. We also observed that the Maitake acute treatment reduce significantly the value of hematocrite percentage from 33.00 ± 1.22 to 28.20 ± 4.44, with $p < 0.05$ in the control group. The percent of hemoglobin were also significantly reduced from 11.18 ± 0.41% to 9.52 ± 1.53%, with $p < 0.05$ in the control group. Toxicity tests revealed that the administration of a single dose of 2000 mg/kg D-Fraction does not cause mortality or any signs of toxicity in any subject. All the individuals survived treatment. Macroscopic examination and histological studies confirmed that breast, liver, lung, and kidney tissues from animals treated with a single dose of 2000 mg/kg of D-Fraction did not reveal histological alterations or significant differences in any tissue compared to controls. We need to study if the slight anemia induced by acute dose of Maitake D-Fraction is due to the reduction of Fe-absorption in duodenum.

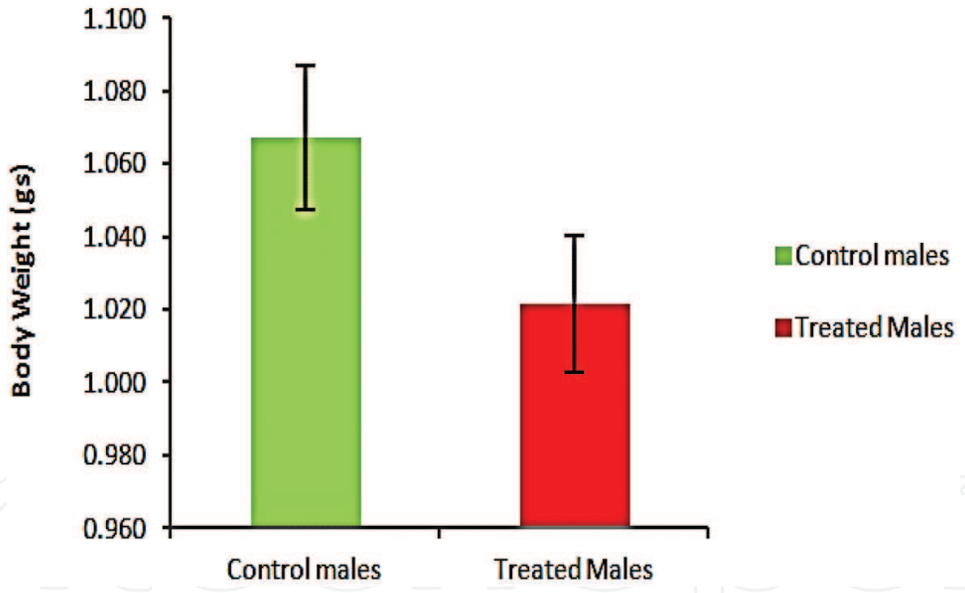

Figure 15. Body weight (grams) of male BALBc mice after treatment in the acute dose of Maitake D-Fraction.

7.2. Sub-Acute Toxicity Studies in biomodels mice BALBc

To determine whether the subacute dose of Maitake D-Fraction induce toxicity in BALBc mice, we worked with 10 female and male mice, 6–8 weeks old, divided into two groups: control group that received a daily volume of BD water and treated group daily treated orally with 5 mg/kg of D-Fraction during 28 days, after that they were proceeded to sacrifice. Toxicity tests revealed that the treatment for 28 days with 5 mg/kg of D-Fraction does not cause mortality or any signs of toxicity in any animal. All the individuals survived treat-

ment. Macroscopic examination and histological studies confirmed that breast, liver, lung, and kidney tissues from animals treated for 28 days with 5 mg/kg of D-Fraction did not reveal histological alterations or significant differences in any tissue compared to controls.

8. Conclusions

Our results demonstrate that Maitake D-Fraction Pro4X prevents mammary tumorigenesis and also increased the overall survival and reduced tumor angiogenesis in BALBc mice. It also protects from the adverse effects of chemotherapy and reduces the toxicity of tamoxifen. The LD50 value is above 2000 mg/kg of D-Fraction, proving to be a nontoxic and safe natural compound for the treatment of animals. It has selective cytotoxicity, causing significant cell deaths in tumor cells without affecting normal cells. Although still we needs to determine which is the active molecule from the Maitake Pro4X extract and which is the exact molecular mechanism utilized to acts as tumor preventive agent. Based upon these results we can postulate that Maitake D-Fraction Pro4X is a good candidate to be used as a preventive agent in breast carcinogenesis in a high-risk population.

All these results suggest that D-Fraction could be applied to the therapy of cancer patients under chemotherapy treatment or as preventive agent in individuals with family history and/or carriers of mutations in BRCA 1 or BRCA2 genes. The beneficial effects of *Grifola frondosa* extract demonstrated in this work could be useful in the near future to reduce the side effects of conventional chemotherapy or to use as a preventive agent against mammary tumorigenesis in high-risk Argentine population.

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