

Investigation of the cytotoxic effects of Thymbra spicata polysaccharides on MCF-7 breast cancer cell proliferation and migration in vitro conditions

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Research Article

Keywords: breast cancer, cell migration, cytotoxicity, proliferation, Thymbra spicata polysaccharides

Posted Date: March 18th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4093817/v1>

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Additional Declarations: No competing interests reported.

Abstract

Purpose

Thymbra spicata species is a widely used plant, especially in the eastern Mediterranean region, and is known to have many health benefits. However, the effects of its polysaccharides on tumor cells have not been searched. We aimed to evaluate the biological effects of *Thymbra spicata* polysaccharides in MCF-7 breast cancer cells.

Methods

MTT test was performed to determine the cytotoxicity levels of polysaccharides and doxorubicin in MCF-7 and L929 fibroblast cells. The expression levels of VEGF and GSK-3 β were examined immunocytochemically. For the *in vitro* wound healing assay, the scratch wound model was created in the shape of plus (+), and the closure percentage was calculated.

Results

Thymbra spicata polysaccharides and doxorubicin had a cytotoxic effect on MCF-7 cells depending on the dose increase. The percentage of wound closure also decreased in correlation with the MTT results. In L929 cells, there was no significant difference in VEGF and GSK-3 β immunoreactivity after polysaccharides and doxorubicin treatments, but a significant decrease in VEGF and GSK-3 β expression was observed in MCF-7 cells. We demonstrated that polysaccharides exert toxic effects by suppressing VEGF and GSK-3 β molecules. In addition, the polysaccharides inhibited cell proliferation and migration, so *in vitro* wound healing was delayed at high concentrations.

Introduction

Breast cancer in women is an important health problem due to its high mortality and morbidity, and therefore, intensive research is needed for the treatment of this cancer type [1, 2]. Breast cancer cell lines are widely used in both *in vitro* and *in vivo* breast cancer studies. Michigan Cancer Foundation-10A Cells (MCF-10A), Michigan Cancer Foundation-7 Cells (MCF-7), SK-BR-3 human breast cancer cell line, MD Anderson-Metastatic Breast-231 Cells (MDA-MB-231), MD Anderson-Metastatic Breast-436 Cells (MDA-MB-436) and 4T1 mouse mammary carcinoma line are some of them. These cell lines are adenocarcinomas originating from breast tissue and differ in terms of estrogen receptors (ERs) and progesterone receptors (PRs). MCF-7 was first isolated in 1970 from the pleural effusion of a female patient whose breast cancer had metastasized to the lung [3]. MCF-7 cells are positive for ERs and PRs and, therefore useful for *in vitro* breast cancer studies [3, 4]. At the same time, other molecular pathways such as vascular endothelial growth factor (VEGF), phosphatidylinositol 3-kinase (PI3K), protein kinase B (AKT), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), mechanistic

target of rapamycin (mTOR), nuclear factor-kappaB (NF-kappaB), glycogen synthase kinase-3beta (GSK-3β) are the factors that determine the aggressive and metastatic behavior of tumor cells [5–10]. Cancer treatment involves the use of agents (synthetic or herbal products) that target these molecules [11–13].

VEGF is a factor involved in vasculogenesis and angiogenesis in embryonic and adult periods. It has a dual role, while it triggers the formation of blood vessels in healthy tissues, it also facilitates the spread of the tumor cells. Due to this feature, it is one of the targeted molecules in cancer therapies [5, 14, 15]. In addition to regulating the glycogen metabolism, GSK-3β, together with other molecules such as β-catenin, NF-κB, AKT, phosphatase and tensin homolog (PTEN), has been determined to have a role in tumor cell proliferation and drug resistance [16, 17].

Many chemotherapeutic agents and herbal products with anti-tumorigenic properties are used in *in vitro* and *in vivo* studies. The demonstration of the anticarcinogenic effects of herbal products or extracts will pave the way for their use as supportive agents in cancer treatment. *Thymbra spicata* belongs to the Lamiaceae family. It grows in countries in the eastern Mediterranean region and is used as a spice and herbal tea by the local people for different purposes, such as antitussive, carminative, etc. In studies, the antioxidant, antiseptic, antihypercholesterolaemic, anti-inflammatory, antifungal, antiviral, antimicrobial, and cytotoxic properties of *Thymbra spicata* of Thymbra genus, their extracts, essential oils or components, have been demonstrated [18–32]. Nevertheless, there was no data about the *Thymbra spicata* polysaccharides' cytotoxicity on cancer cells.

So, this study, it was aimed to investigate the possible cytotoxic effects of *Thymbra spicata* polysaccharides on MCF-7 breast cancer cells and their effects on cell migration, distributions of VEGF, and GSK-3beta.

Materials and methods

Preparation of agents

This study was approved by the Niğde Ömer Halisdemir University Clinical Research Ethics Committee (approval number: 06.09.2019-2019/22). After the *Thymbra spicata* plant was commercially obtained, polysaccharide isolation was performed by hot water extraction and ethanol precipitation, using the method specified by Lv et al [33]. Also, doxorubicin was commercially available (Saba, Turkey). Both polysaccharides and doxorubicin were dissolved in cell culture media to apply to cells.

Cell culture

In the study, the MCF-7 breast cancer cell line (Cat. No: 00092502) was used as tumor cells, and L929 fibroblast cells (Cat. No: 92123004) were used as control. Both cells were obtained from Şap Institute, Ankara, Turkey. MCF-7 cells were cultured in Roswell Park Institute-1640 (RPMI-1640) media (Wisent, Canada) containing 10% fetal bovine serum (FBS, Biowest, France), 2 mM L-glutamine (Biosera, France), 100 UI/ml penicillin/streptomycin (Capricorn Scientific GmbH, Germany), whereas L929 fibroblast cells

were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% fetal calf serum, 50 µg/ml gentamicin (Gibco, USA), 100 UI/ml penicillin, 100 UI/ml streptomycin at 37°C and 5% CO₂ condition [34].

MTT assay

To detect the cytotoxic effects of *Thymbra spicata* polysaccharides and doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Serva Electrophoresis GmbH, Germany) assay was performed. Cells were cultured in 96-well plates (45x10³ cells/well) until they were monolayer (70–80% confluency), and they were treated with *Thymbra spicata* polysaccharides (0 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, 100 µg/ml and 1000 µg/ml) and doxorubicin (0 µM, 0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM) for 24, 48 and 72 h. The media containing polysaccharides and doxorubicin was discharged, and 100 µl of fresh culture medium with 10 µl of 12 mM MTT stock solution was added to each well. After 4 hours of incubation at 37°C, the medium containing MTT was removed by pipetting, and dimethyl sulfoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) was put into each well. The absorbance values were determined at a wavelength of 570 nm using a UV visible microplate reader (EPOCH2, BioTek, USA). The cytotoxicity levels and the half-maximal inhibitory concentration (IC₅₀) doses of two agents were identified for each cell. The protocol was performed thrice [35].

In vitro wound healing assay

The *in vitro* wound healing, known scratch wound model, experiment was performed to determine the effects of *Thymbra spicata* polysaccharides on cell migration. MCF-7 and L929 cells were seeded in the 6-well plate (25x10⁴ cells/well), and 90–95% confluency was reached. The *in vitro* wound was created using a 200 µl pipette tip in the shape of plus (+). Then *Thymbra spicata* polysaccharides (0 µg/ml, 0.1 µg/ml, 10 µg/ml, 1000 µg/ml) and doxorubicin (0 µM, 0.1 µM, 10 µM, 1000 µM) for 48 h were applied to both cells. At hours 0 and 48, images were taken under the camera-attached inverted microscope (Zeiss, Germany), and the closure percentage was measured via ImageJ.1.47 software for each concentration [34].

Immunocytochemistry

The distributions of VEGF and GSK-3β were defined by immunocytochemical staining. Both cells were cultured in a 24-well plate (2.5x10⁵ cells/well) until confluency. Cells were exposed to three concentrations of *Thymbra spicata* polysaccharides (0 µg/ml, 0.1 µg/ml, 10 µg/ml, 1000 µg/ml) and doxorubicin (0 µM, 0.1 µM, 10 µM, 1000 µM) for 48 h. After fixation in 4% paraformaldehyde, 0.1% Triton X-100 (AppliChem, Darmstadt, Germany) was used for permeabilization. Following washing with PBS, 3% hydrogen peroxide (Merck, Darmstadt, Germany) inhibited the endogenous peroxidase activity. The primary antibodies, anti-VEGF (NB100-664, Novus, USA) and anti-GSK-3beta (NBP1-47470, Novus, USA) were treated in cells for 18 h. For negative control, a primary antibody was not applied to the cells. After the stage of secondary antibodies, biotinylated secondary antibodies, and peroxidase-conjugated streptavidin (Histostain kit, 85- 9043, Zymed, Carlsbad, USA), the immunoreactivity was made visible

using diaminobenzidine/hydrogen peroxide (DAB, Invitrogen, CA, USA). And the counterstaining was carried out using Mayer's hematoxylin (ScyTek, UT, USA). Samples were mounted with an aqueous medium (DBS, Pleasanton, USA). They were evaluated under a light microscope with a camera attachment microscope (IX71 inverted-fluorescence phase microscope, Olympus, Japan). The results of staining were given as H-score; firstly five different areas were chosen in the images, and immunoreactivities were determined as weak (+), moderate (++), and strong (+++) respectively. The H-score was calculated using the formula: $\sum P_i$ (intensity of staining + 1). P_i means the percentage of immunopositive cells for each intensity (from 0–100%) [35].

Statistical analysis

The results of the experiments were analyzed by repeated measures of the Analysis Of Variance (ANOVA) test using GraphPad software (San Diego, CA). The differences between the groups were revealed by the Tukey-Kramer multiple comparisons test. The data was given as mean $\pm$ standard deviation, and the $p < 0.05$ was accepted as statistically significant [35].

Results

MTT assay

It was determined that *Thymbra spicata* polysaccharides and doxorubicin had cytotoxic effects on MCF-7 cells depending on the dose increase (Fig. 1). The IC₅₀ doses of *Thymbra spicata* polysaccharides were found to be 14.75 $\mu\text{g/ml}$ for MCF-7 breast cancer cells and 65.04 $\mu\text{g/ml}$ for L929 fibroblast cells, while the IC₅₀ doses of doxorubicin were 12.50 μM for MCF-7 breast cancer cells and 25.45 μM for L929 fibroblast cells (Fig. 1).

In vitro wound healing

In our study, we found the statistically significant inhibitory effect of *Thymbra spicata* polysaccharides and Doxorubicin on cell migration at 48 h (Fig. 2, 3, 4). The cells were treated with two agents for 48 h (*Thymbra spicata* polysaccharides; 0 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$, 10 $\mu\text{g/ml}$, 1000 $\mu\text{g/ml}$ and doxorubicin; 0 μM , 0.1 μM , 10 μM , 1000 μM). In control groups, closure rates were 90.5% for L929 and 85.0% for MCF-7 cells at 48 h. After 10 and 1000 $\mu\text{g/ml}$ *Thymbra spicata* polysaccharides applications, closure rates of MCF-7 were diminished compared to the control group, 55.4% and 42.3%, respectively ($***p < 0.001$) (Fig. 3). In L929 cells, these rates were 80.4% and 73.0%. So, there was no significant difference between groups in L929 cells. Wound closure rates as a result of exposure to 10 and 1000 μM doxorubicin were 50.1% and 25.1% in MCF-7 breast cancer cells, whereas they were 78.1% and 55.3% in L929 fibroblast cells. As a result of the experiments, it was determined that cell proliferation and thus wound closure were suppressed depending on the dose increase.

Immunocytochemistry results

After applications of *Thymbra spicata* polysaccharides and doxorubicin, the distributions of VEGF and GSK-3 β were determined by immunocytochemical staining in L929 and MCF-7 cells at 48 h. The evaluation of immunoreactivities was made using the H-score method (Fig. 5). In L929 cells, the VEGF and GSK-3 β immunoreactivities did not change after the applications of *Thymbra spicata* polysaccharides and doxorubicin. So, there was no statistically significant difference between the groups ($p > 0.05$) (Fig. 5a).

In MCF-7 cells, the decreased expression levels of those two molecules were detected in 10 and 1000 $\mu\text{g/ml}$ *Thymbra spicata* polysaccharides, 10 and 1000 μM doxorubicin applications (Fig. 5, 6, 7). The expressions of VEGF and GSK-3 β were close to each other in control, 0.1 $\mu\text{g/ml}$ *Thymbra spicata* polysaccharides and 0.1 μM doxorubicin groups. There was not a significant difference between these groups ($p > 0.05$). In a dose of 10 $\mu\text{g/ml}$ polysaccharides, immunoreactivities of VEGF and GSK-3 β were decreased significantly, 245.22 ± 17.35 and 235.45 ± 15.70 , respectively ($***p < 0.001$). Their decrease at 1000 $\mu\text{g/ml}$ was more pronounced compared to control and 0.1 $\mu\text{g/ml}$ groups, the VEGF rate was 121.65 ± 16.00 , and GSK-3 β was 126.15 ± 15.00 ($***p < 0.001$). In the 10 μM doxorubicin group, rates of VEGF and GSK-3 β were 255.25 ± 18.45 and 243.00 ± 17.00 .

Discussion

With this study, we revealed that the *Thymbra spicata* polysaccharides have a significant cytotoxic effect on the MCF-7 breast cancer cell line depending on the dose increase. And we also used an antineoplastic drug, Doxorubicin, to compare the cytotoxic effect of *Thymbra spicata* polysaccharides. The antiproliferative effect of this agent was detected in the *in vitro* wound healing assay. The decrease in VEGF and GSK-3 β molecules, which play a role in tumor progression, also supported its inhibitory effect in MCF-7 cells.

Studies are showing that different species belonging to the genus *Thymbra* or *Thymbra spicata* species have antioxidant, antibacterial, or cytotoxic properties *in vivo* and *in vitro* conditions. The ethanolic extract of *Thymbra spicata* has been shown to have a cytotoxic effect in MCF-7 cells via the production of reactive oxygen species (ROS) and apoptosis, and its half-maximal inhibitory concentration (IC₅₀) has been reported as $80 \pm 5.6 \mu\text{g/ml}$. According to the wound healing assay, 25 $\mu\text{g/ml}$ ethanolic extract inhibited the proliferation of MCF-7 cells, thus cell migration was also suppressed. It has been stated that it reduces the phosphorylation of signal transducer and activator of transcription 3 (STAT3) and NF κ B molecules, which play a crucial role in tumor formation and metastasis. In the same study, the ethanolic extract of *Thymbra spicata* also showed cytotoxic effects on other tumor cell lines, such as human breast epithelial cell spontaneously immortalized (MCF-10A), non-small-cell lung cancer cells (A549), human cervix carcinoma cells (HeLa), acute T cell leukemia cells (Jurkat), and human prostate adenocarcinoma cells (PC3) [36]. In another study, thymol was isolated from *Thymbra spicata*, and its antiproliferative effects were searched in olfactory ensheathing cells (OECs) derived from rat pups at *in vitro* conditions. When its effects on these cells in normal and high glucose environment was examined, it was reported that it protected the cells from high glucose. At the same time, it has been determined that the thymol in

the extract of *Thymbra spicata* has a similar effect to thymol. After applications of two agents, decreased reactive oxygen species (ROS) and nitric oxide (NO) levels were observed, and Western blot analysis revealed a reduction in VEGF and an increase in brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) levels in OECs under high glucose condition. Despite the protective effects of both agents against high glucose, it has been reported to have a cytotoxic effect at high doses in normal glucose levels, lethal concentration 50% (LC50) doses were $121 \pm 5.3 \mu\text{M}$ thymol and $125 \pm 5.8 \mu\text{M}$ thymol in extract [27]. Unlike these studies, we used the *Thymbra spicata* polysaccharides and ascertained their effects on the MCF-7 breast cancer cell line in our study. We detected its IC50 dose as $14.75 \mu\text{g/ml}$ for MCF-7 cells at normal glucose levels. We found that it has a cytotoxic effect on MCF-7 cells with a decrease in VEGF and GSK-3 β molecule levels and inhibited cell proliferation and migration.

In another study with proven cytotoxic effect of *Thymbra spicata* extract, extracts prepared with water and ethanol were compared, and it was observed that the aqueous solution did not show any toxicity in three cancer cell lines, MDA-MB-231 breast adenocarcinoma cell line, A375 melanoma cell line and HCT116 colorectal carcinoma cell line. However, the ethanolic extract was found to be significantly toxic in these cell lines by MTT analysis. Its IC50 doses were $58.447 \mu\text{g/ml}$ for MDA-MB-231 cells, $110.238 \mu\text{g/ml}$ for HCT116 cells, and $31.443 \mu\text{g/ml}$ for A375 cells at 24 h. Also, the ethanolic extract was subjected to enzymatic processes (α -salivary amylase, pepsin, and pancreatin with bile salts) in vitro conditions, then its toxic effects were compared with the ethanolic extract that did not undergo any treatment. In that comparative experiment, it was revealed by the increase in IC50 as a result of MTT analysis that enzymatically treated ethanolic extract lost some of its toxic effects. Besides, carvacrol, a known toxic agent, was used as a positive control [29]. In our experiment, we used the polysaccharides, which obtained a powder form as a result of isolation, by dissolving it into cell culture media. Therewithal, we did not apply any enzymatic treatment to the polysaccharides. Therefore, it eliminated the toxic effect of ethanol or similar solvents, as well as any treatment. And, an antineoplastic drug, doxorubicin, was used as a positive control in our experiments.

In recent years, studies in which plant products are loaded into nanoparticles (NPs) have become widespread. Erci et al. investigated the cytotoxic effect of *Thymbra spicata* extract loaded into silver NPs at different dilutions (1 ml and 5 ml plant extract loaded). They found their toxic effect at $50 \mu\text{g/ml}$ for 1 ml extract-loaded NPs and $150 \mu\text{g/ml}$ for 5 ml extract-loaded NPs [25]. Again the same researchers carried out a similar study by loading *Thymbra spicata* extract (40 and 80 ml aqueous form) into the copper oxide NPs and reported that its extract had a cytotoxic effect at high concentrations [26]. In both studies, they demonstrated the cytotoxic effect of *Thymbra spicata* extract at high concentrations only in L929 fibroblast cells. In the current study, we aimed to determine the antitumorogenic effect of *Thymbra spicata* polysaccharides, and for this purpose, we used the MCF-7 breast cancer cell line, and L929 cells as were control group for our experiment. According to our results, the polysaccharides were more toxic in MCF-7 cells than in L929 cells.

Conclusion

According to the literature survey, the cytotoxic effects of both aqueous and ethanolic forms of *Thymbra spicata* extract were mostly investigated by MTT assay. Studies showing the molecular mechanisms of cytotoxicity are limited and only a few experiments have been found in this regard. Unlike other studies, we isolated the *Thymbra spicata* polysaccharides and demonstrated their cytotoxicity by MTT assay, and changes in VEGF and GSK-3 β molecules by immunocytochemically and cell migration analysis. For the products of *Thymbra spicata* to be used in cancer treatment, it is necessary to determine the molecular signaling pathways that determine the cytotoxic effect via *in vitro* or *in vivo* studies by advanced molecular techniques.

Declarations

Conflicts of interest

There are no conflicts of interest.

Author Contribution

Author A.I., Ö.C. made substantial contributions to the conception or design of the work. Author Ö.C., A.I., Ö.O, Ç.E. made substantial contributions to the the acquisition, analysis, and interpretation of data. Author A.I., and Ç.E. wrote the main manuscript text and A.I. and Ö.C. prepared all figures. Author A.I., Ö.C., Ö.O. drafted the work and revised it critically for important intellectual content. All authors approved the version to be published. All authors reviewed the manuscript.

Acknowledgments

This study was supported by the Scientific Research Committee of Niğde Ömer Halisdemir University (SAT 2020/3-BAGEP).

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Figures

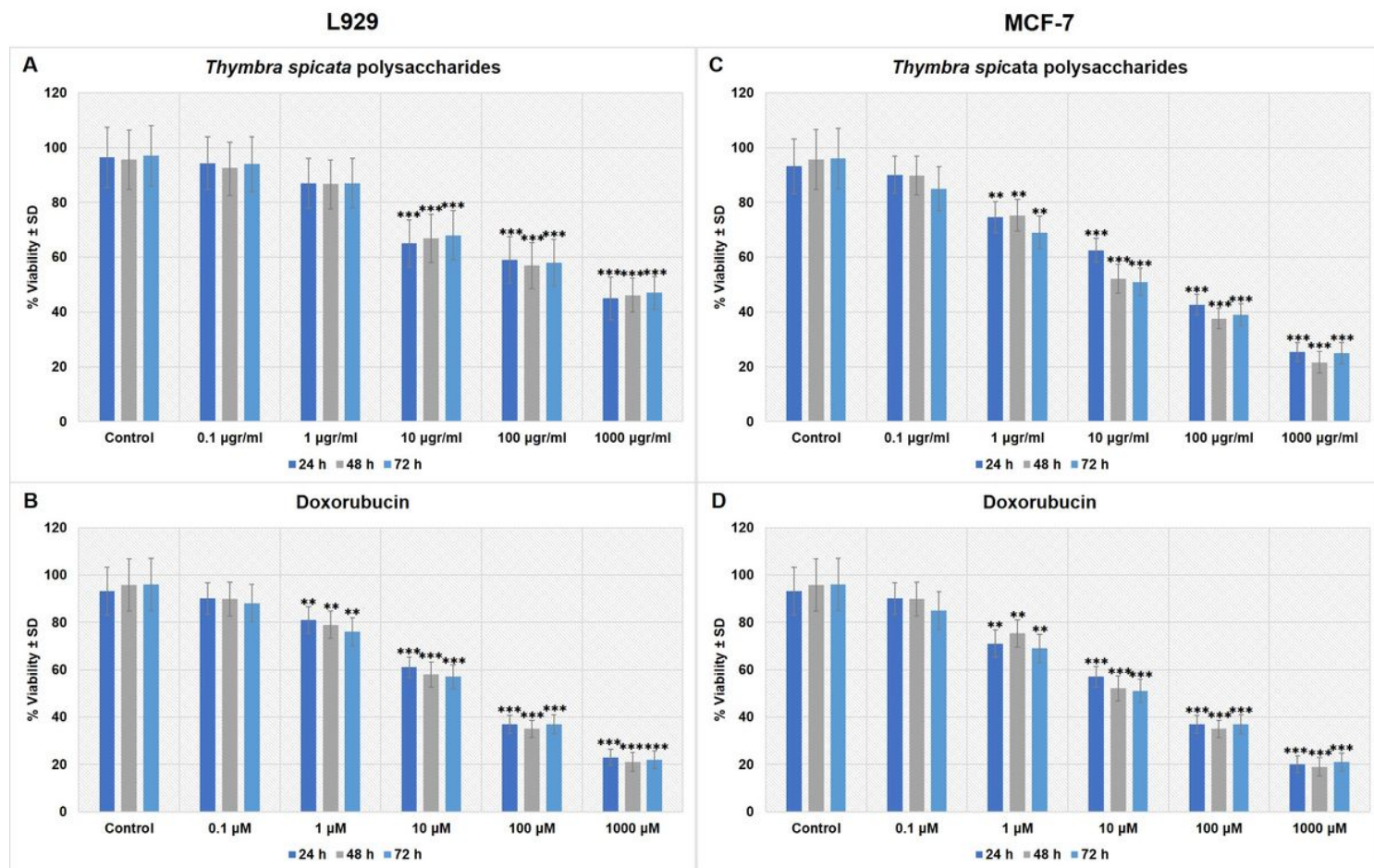


Figure 1

Cytotoxicity levels of *Thymbra spicata* polysaccharides and doxorubicin on L929 fibroblast cells (a and b) and MCF-7 (c and d) cells. **P<0.01, and ***P<0.001.

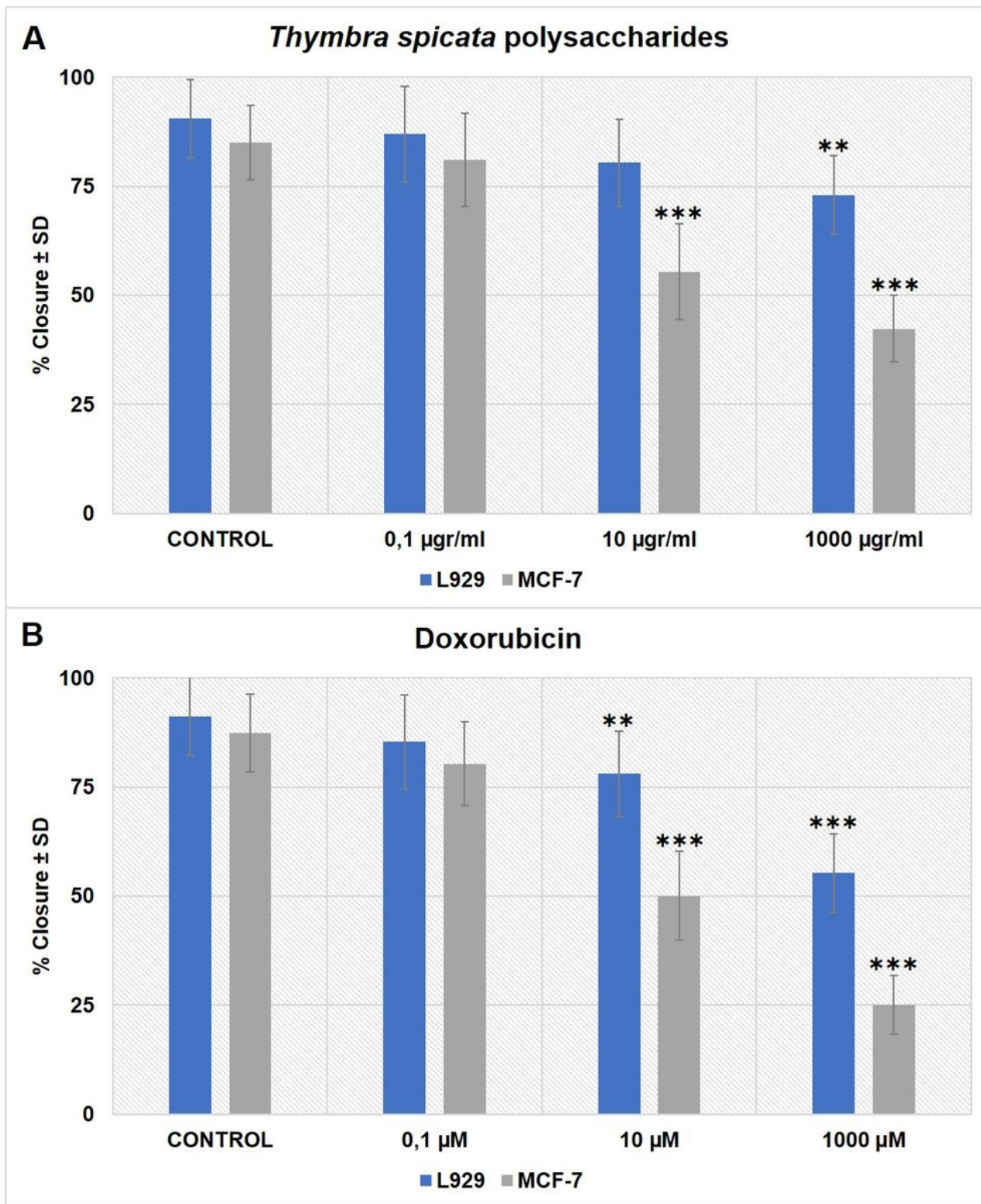


Figure 2

The closure rates of in vitro wound healing after the applications of *Thymbra spicata* polysaccharides (a) and doxorubicin (b) on MCF-7 and L929 fibroblast cells for 48 h. **P<0.01, and ***P<0.001.

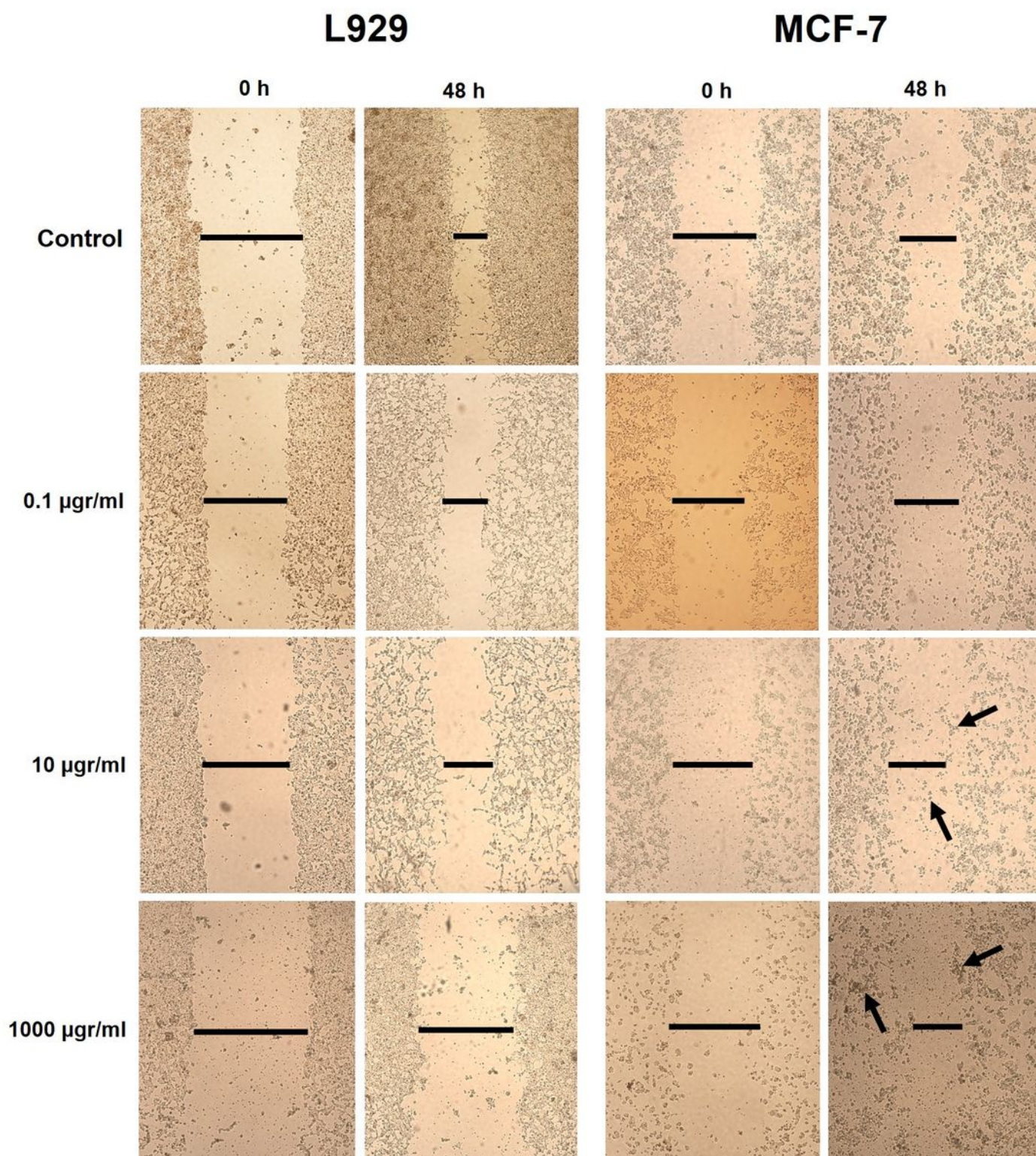


Figure 3

The images of *in vitro* wound healing after the applications of *Thymbra spicata* polysaccharides on L929 fibroblast cells and MCF-7 cells for 0 and 48 h. Arrows: Dead cells. Magnification: x100.

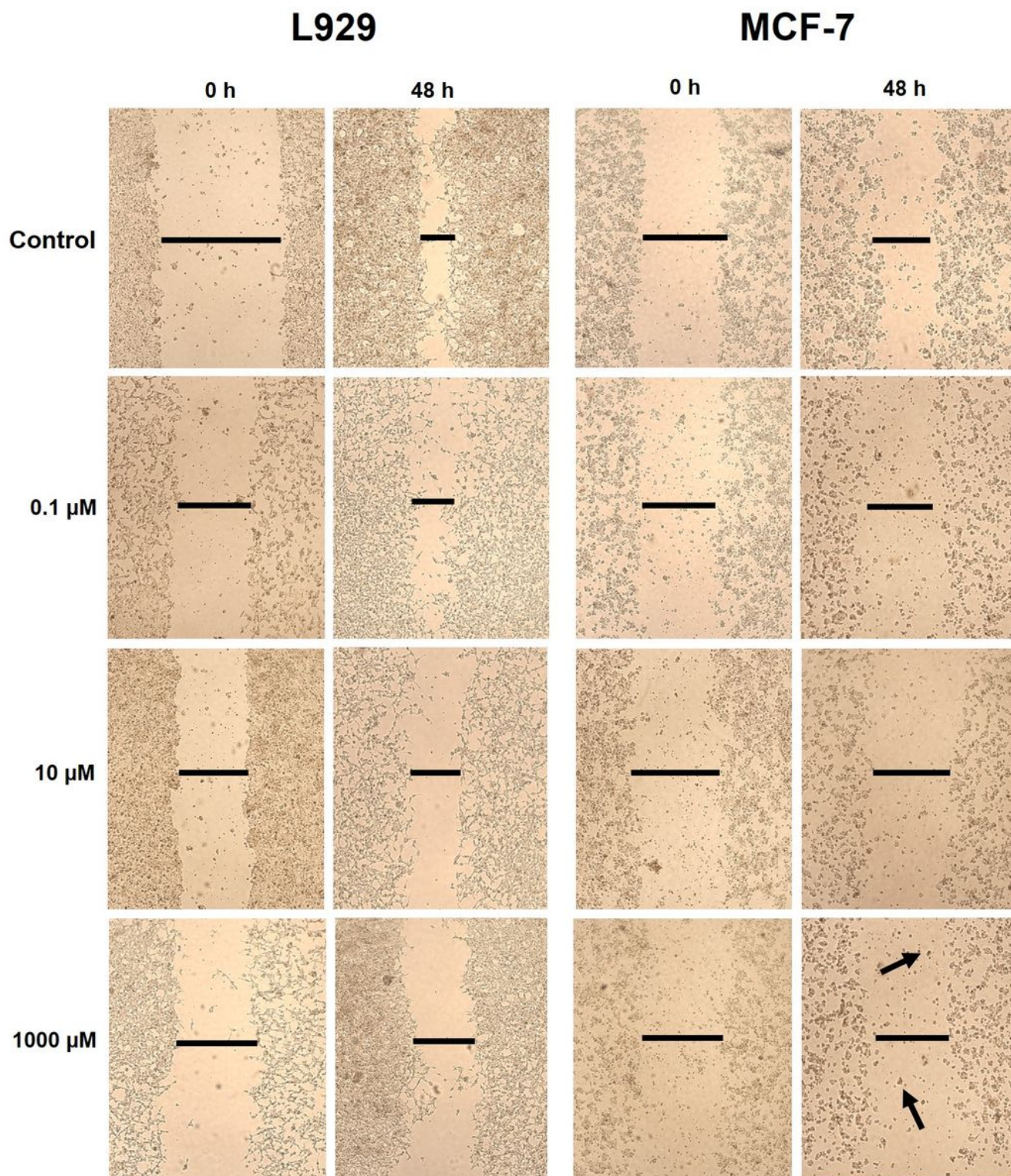


Figure 4

The images of *in vitro* wound healing after the applications of Doxorubicin on L929 fibroblast cells and MCF-7 cells for 0 and 48 h. Arrows: Dead cells. Magnification: x100.

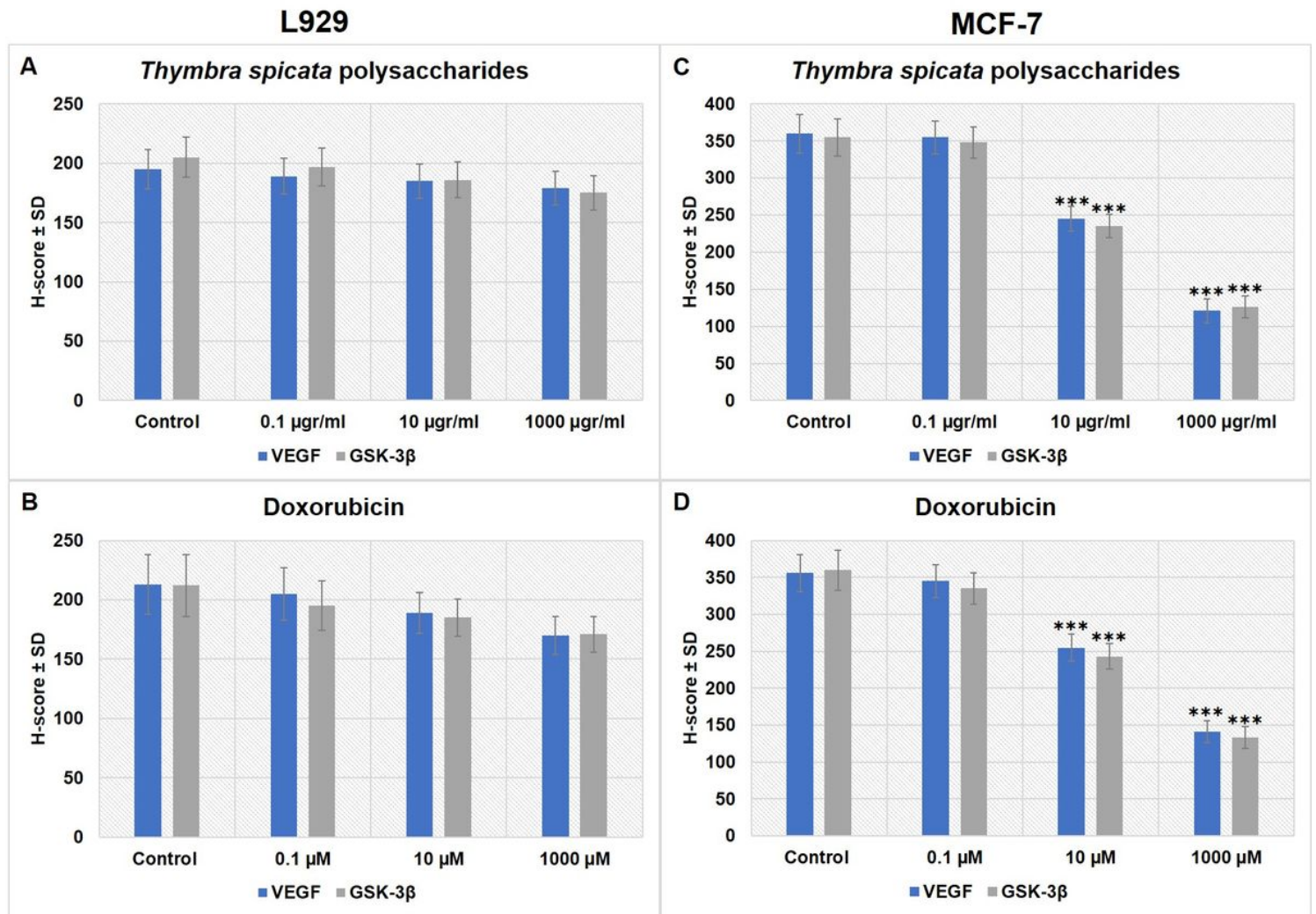


Figure 5

The H-Score rates of immunocytochemical staining of VEGF and GSK-3β in L929 fibroblast cells (a and b) and MCF-7 (c and d) cells after applications of *Thymbra spicata* polysaccharides and Doxorubicin for 48 h. ***P < 0.001.

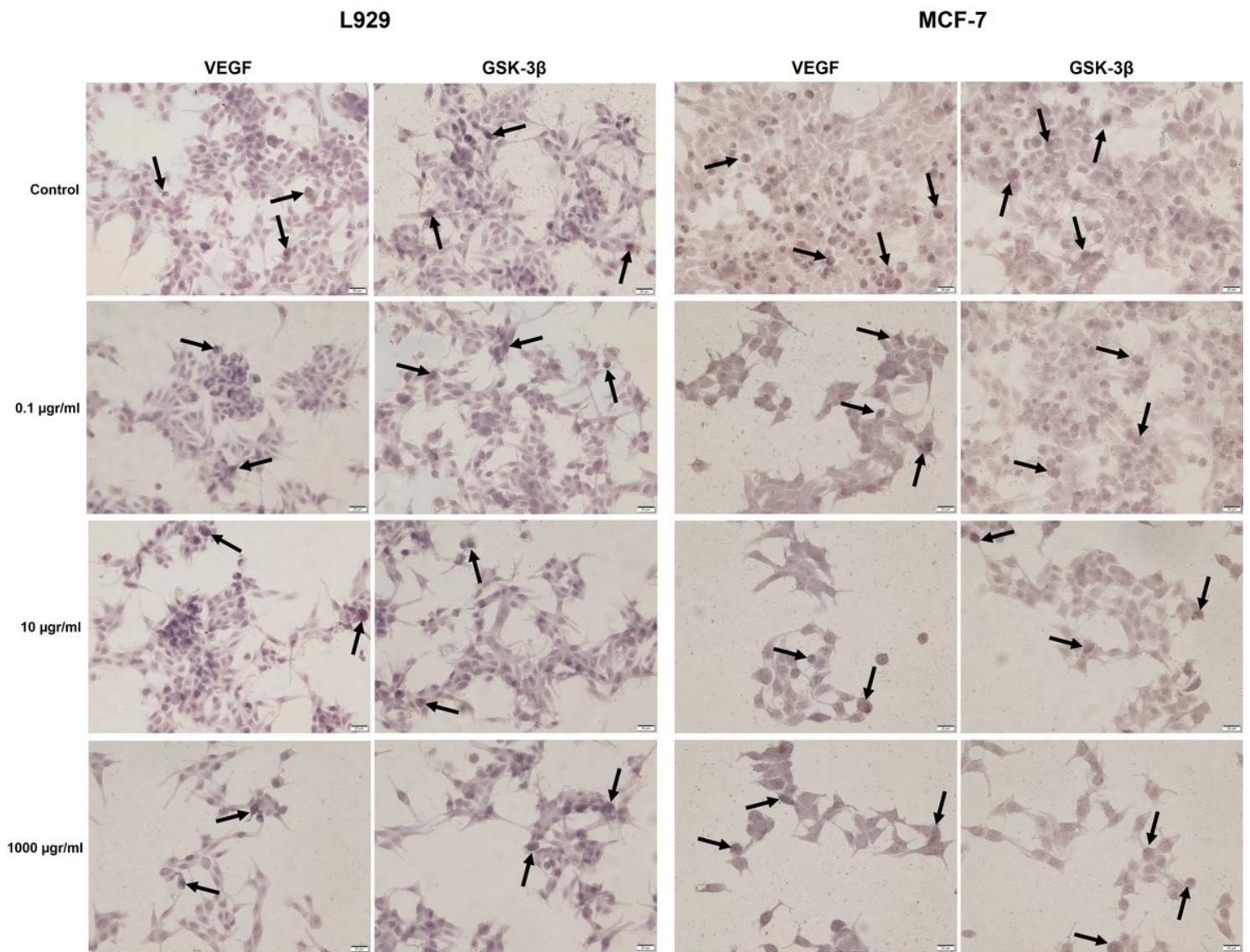


Figure 6

The immunocytochemical images of VEGF and GSK-3 β after the applications of *Thymbra spicata* polysaccharides in L929 fibroblast cells and MCF-7 cells at 48. h. Arrows: Immunopositive cells. Scale bars: 20 μ m.

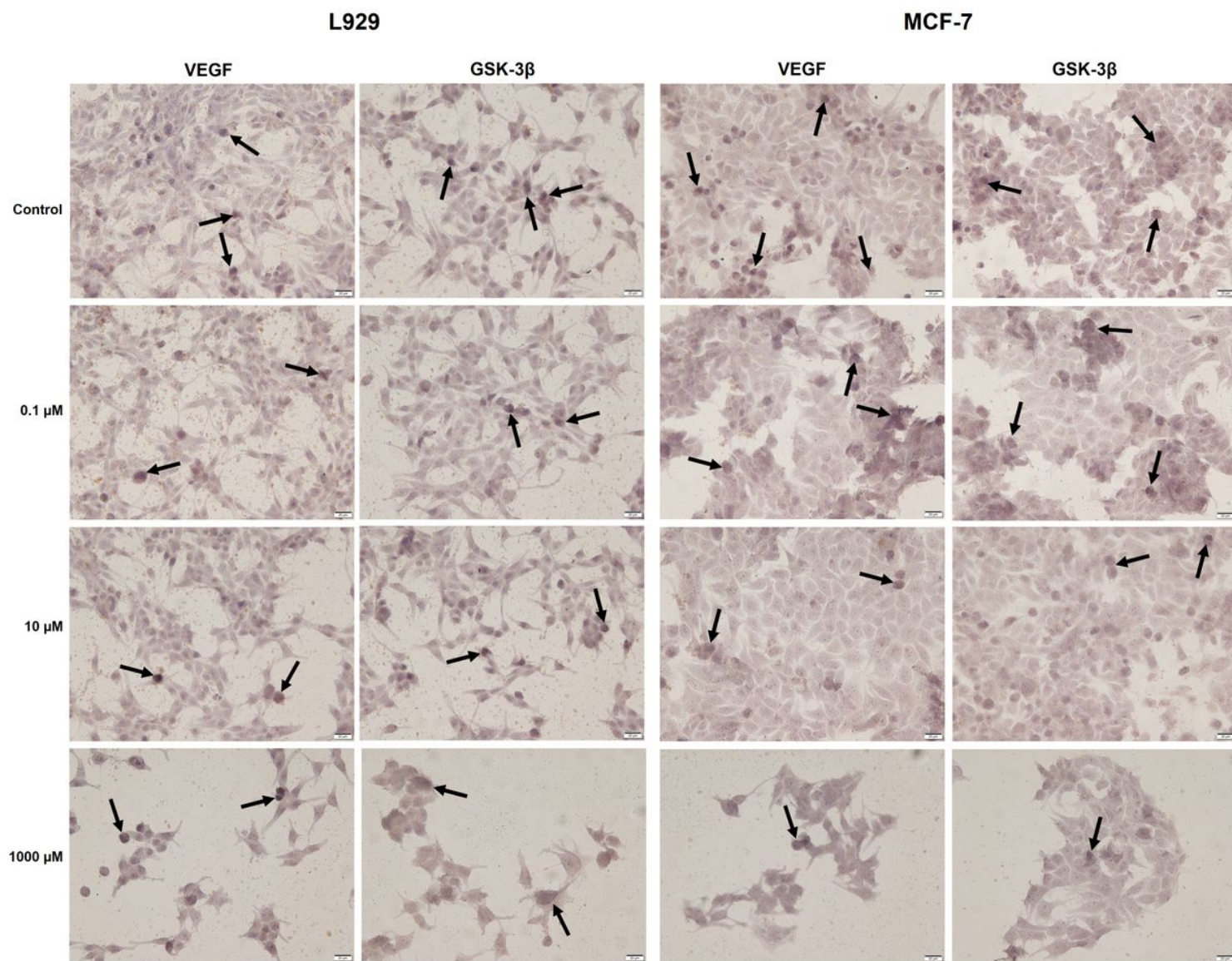


Figure 7

The immunocytochemical images of VEGF and GSK-3 β after the applications of Doxorubicin in L929 fibroblast cells and MCF-7 cells at 48. h. Arrows: Immunopositive cells. Scale bars: 20 μ m.