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The Effects of Medicinal Plants on Cancer Cell Lines and Efficacy of Experimental Animal Model

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Abstract: The use of medicinal plants as an alternative treatment is a historical process and has been known for a long time so that classical treatment can be more effective in wound and cancer treatment. The purpose of this study was to investigate the effects of plant extracts used for therapeutic purposes in cancer cell lines in vitro wound model and in vivo experimental animal model in order to obtain this information. The plants, olive oil (*Oleocanthal*), mistletoe (*Viscum album*), Common Centaury (*Centaureum erythraea*), *Momordica charantia*, *Inula viscosa*, *Citrus aurantium*, Thyme oil (*Thymus vulgaris*) and algae (*Jania longifurca*), were used. MCF-7, MB-MDA-231, 67NR and 4T1 for breast, NB2a for neuron, L929 for fibroblast and normal somatic mesenchymal stem cell for comparison were selected for in vitro wound models. As an in vivo breast cancer model, female Balb/c mice were injected with 4T1 cells and skin wound healing in rats was investigated. The effects of medicinal plants were evaluated using MTT assay for viability and proliferation, immunocytochemistry staining NOS for oxidative stress and TGFbeta1 for wound healing. It was found that plant extracts reduced antioxidative damage and inhibited apoptosis. It was observed that oxidative stress and apoptosis were increased in cancer cells, but less effective in invasive cell lines. In vivo experiments showed that wound healing was accelerated and that these rates were achieved with antioxidative and antiapoptotic effects. It has been concluded that medicinal plants are beneficial for treatment of difficult diseases in which patient quality of life is very effective and they should be used as scientific-based medical applications.

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1. Introduction

Nowadays, breast cancer has become an increasingly important health problem and it is the second cause of cancer related deaths after lung cancer among women [1]. Early diagnosis and subsequent treatment are difficult and can result in death. Tumor cells with uncontrolled proliferation accumulate to form masses, furthermore tumors can squeeze normal tissues,

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infiltrate into them, or destroy them. If the cancer cells are separated from the tumor, they can go to other parts of the body through the blood or lymphatic circulation. In places where they grow, they form tumor colonies and continue to grow. The spread of cancer to other parts of the body in this way is called metastasis [2, 3].

Cancers are classified according to their appearance under the organ and the microscope. Different types of cancer grow at different rates, show different spreading patterns and respond to different treatments. For this reason, in the treatment of cancer patients, different types of treatment are applied according to the existing cancer type. Chemotherapy as well as alternative treatment options are also used in the treatment of cancer [4]. Especially in recent years plant extracts have been started to be used under the name of phytotherapy. In addition, there are plants that are widely used by the population and still subject to experimental and clinical trials such as olive oil (oleocanthal), mistletoe (*Viscum album*), *Centaurium erythraea*, *Momordica charantia*, *Inula viscosa*, *Citrus aurantium*, thyme oil (*Thymus vulgaris*) and algae (*Jania longifurca*) [5-9]. Because Oleocanthal is a phenylethanoid, a type of natural phenolic compound, has antiinflammatory and anticancer properties and several studies have demonstrated that it can promote apoptosis in tumor cells [10, 11]. On the other hand, it has been found that it can be used as an antioxidant. *V. album*, *C. erythraea*, *M. charantia*, *I. viscosa*, *C. aurantium*, Thyme oil (*T. vulgaris*) *J. longifurca* are very rare in the literature [12-14].

Additionally, the plants extracts are also used for wound healing. Wound healing is one of the unsolved problems of medicine nowadays and it needs to be improved especially in cases of poor recovery and salvation from diabetes. Herbal therapies are a popular product of our time and have been started to be applied to humans in current medical treatment.

Nitric oxide (NO) is catalyzed by the enzyme nitric oxide synthase (NOS) and has an important role in the cellular metabolism. There are three different NOS in mammals that synthesize NO: endothelial NOS, inducible NOS and neuronal NOS. The increase of NO levels into the cells, caused oxidative stress and cell death, also wound healing is delayed. Because of its short half-life, to detect NO is difficult, so levels of NOS are investigated [15, 16].

Transforming growth factor (TGFbeta) beta super family is involved in cell proliferation and differentiation. TGFbeta isoforms (TGFbeta1, 2, 3, 4 and 5) are critical for vascular diseases and wound healing process. Especially, TGFbeta 1 and 2 are released in response to the tissue injury and regulate cell proliferation, differentiation, and migration during wound healing [17].

The aims of our study were to detect the cytotoxic effects of oleocanthal, *V. album*, *C. erythraea*, *M. charantia*, *I. viscosa*, *C. aurantium*, Thyme oil (*T. vulgaris*), *J. longifurca* on MCF-7, MB-MDA-231, 67NR and 4T1 breast carcinoma cell lines, NB2a neuroblastoma cell line, L929 fibroblast cell line and to determine the effects of *V. album* extract on in vivo-breast cancer model and skin wound healing.

2. Material and Methods

67NR and 4T1 breast cancer cells were seeded in DMEM-F12, NB2a neuroblastoma and L929 fibroblast cell line were cultured in DMEM medium, MCF-7 and MB-MDA-231 breast cancer cells were maintained in RPMI-1640 medium at 37°C and 5% CO₂ incubator. Adipose tissue derived mesenchymal stem cells (MSC) were isolated from rat fat tissue and seeded in alpha-MEM medium. Also MSCs were differentiated into neurons (N-MSC) by administration of serum-free medium plus 20 µl combinations of epidermal growth factor (EGF) and fibroblast growth factor, neuronal differentiation was performed using a Mouse Rat Neural Stem cell Functional Identification Kit (SC013; RD Systems, Minneapolis, MN). For MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (M6494, Invitrogen) cytotoxicity analysis, all cells were seeded in 96-well plates (45.000 cells/well in 250 µL medium) for 24 hours and then plant extracts at different dilutions (0, 0.1, 1, 10, 100 µgr/ml) were applied for

24 hours. Subsequently, the medium containing extracts was removed and 100 μ L of fresh medium and 10 μ L MTT (5 mg/ml in distilled water) were added into each well and plate was incubated at 37°C for 4 hours. Later, medium containing MTT was decanted and the cells were exposed to the dimethyl sulphoxide (DMSO, A3672, AppliChem, Darmstadt, Germany) (100 μ L DMSO/well) for 10 min. The absorbance was measured at a wavelength of 570 nm using an UV visible spectrophotometer (ELx800UV, BioTek) and IC₅₀ dose for each plant extract was calculated using GraphPad InStat 3.0 statistical software [18].

The effects of all extracts were investigated at IC₅₀ dose in vitro wound model. For this purpose, all cell lines were seeded in 24 well plates (15.000 cells/well in 500 μ L medium) and a wound model was created by a pipette tip in (+) plus at the confluent phase [19]. The effect of extracts on wound healing at IC₅₀ dose was investigated by immunocytochemical analysis with eNOS, iNOS and TGFbeta1.

The effects of thyme oil (*T. vulgaris*) in the experimental skin wound healing model and the effect of *V. album* in the experimental breast cancer model were evaluated. The experimental skin wound healing was created by aseptic conditions in the Wistar albino rats where the back region was shaved to remove the skin 1.5x1.5 cm² and rat were divided in three groups, control (no application, sham (topical application of salin buffer) and thyme oil (topical application) [20]. The wound closure process was morphologically assessed by topical application of thyme oil daily. For experimental breast cancer, 1x10⁵ 4T1 breast cancer cells were injected orthotopically into the right breast in 50 μ L of medium to Balb / c female rats [21]. From the tenth day, intratumoral injection of 50 μ L of the extract of *V. album* (15 mg / kg) was injected every day for 15 days while tumor formation was initiated. During the experiment, the tumor size was measured using a ruler every two days by formula: Tumor size = (length x width²)/2 [22]. At the end of the experiment, the data about the size of tumor tissue were evaluated by one-way ANOVA analysis using GraphPad InStat 3.0 statistical software.

3. Results

All plant extracts were found to have toxic effects on cancer cells except for mesenchymal stem cells. Following MTT analysis, the % cell survival and IC₅₀ doses of all extracts were shown in Figure 1 and 2.

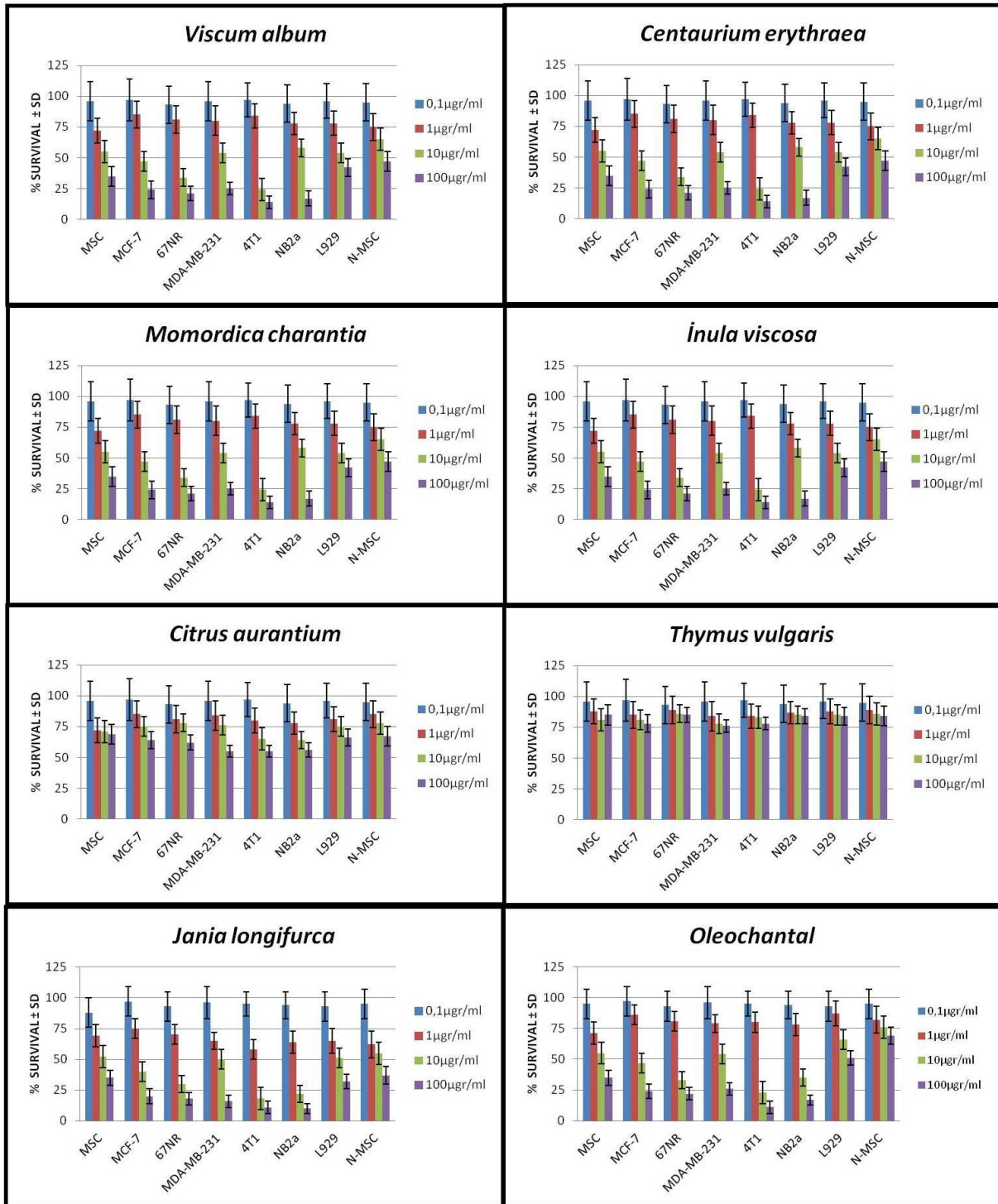


Figure 1. The % survival of all cells for each extracts.

IC ₅₀ Doses	MSC	MCF-7	67NR	MDA-MB-231	4T1	NB2a	L929
<i>Oleochanthal</i>	8.75 µgr/ml	7.25 µgr/ml	6.00 µgr/ml	8.00 µgr/ml	5.25 µgr/ml	6.00 µgr/ml	9.00 µgr/ml
<i>V. album</i>	8.50 µgr/ml	7.65 µgr/ml	6.25 µgr/ml	8.25 µgr/ml	6.00 µgr/ml	8.25 µgr/ml	8.00 µgr/ml
<i>C. erythraea</i>	8.75 µgr/ml	7.50 µgr/ml	6.00 µgr/ml	8.00 µgr/ml	5.75 µgr/ml	7.55 µgr/ml	8.25 µgr/ml
<i>M. charantia</i>	8.50 µgr/ml	7.25 µgr/ml	5.75 µgr/ml	7.50 µgr/ml	5.25 µgr/ml	7.50 µgr/ml	8.25 µgr/ml
<i>I. viscosa</i>	8.55 µgr/ml	7.15 µgr/ml	5.65 µgr/ml	6.65 µgr/ml	5.00 µgr/ml	7.25 µgr/ml	8.50 µgr/ml
<i>C. aurantium</i>	11.75 µgr/ml	10.55 µgr/ml	10.25 µgr/ml	10.00 µgr/ml	9.65 µgr/ml	9.25 µgr/ml	10.50 µgr/ml
<i>T. vulgaris</i>	12.25 µgr/ml	11.25 µgr/ml	10.75 µgr/ml	10.25 µgr/ml	10.25 µgr/ml	10.50 µgr/ml	11.00 µgr/ml

Figure 2. IC₅₀ doses of all extracts for each cells.

When the effects of extracts in the in vitro wound model were examined by inverted microscopy, it was found that all of the wound healing delayed the wound closure but the rate of wound healing was different in each cells. When NOS and TGFbeta1 staining were examined, there was an increase of NOS and TGFbeta1 stainings in cancer cells compared to mesenchymal stem cells (Figure 3).

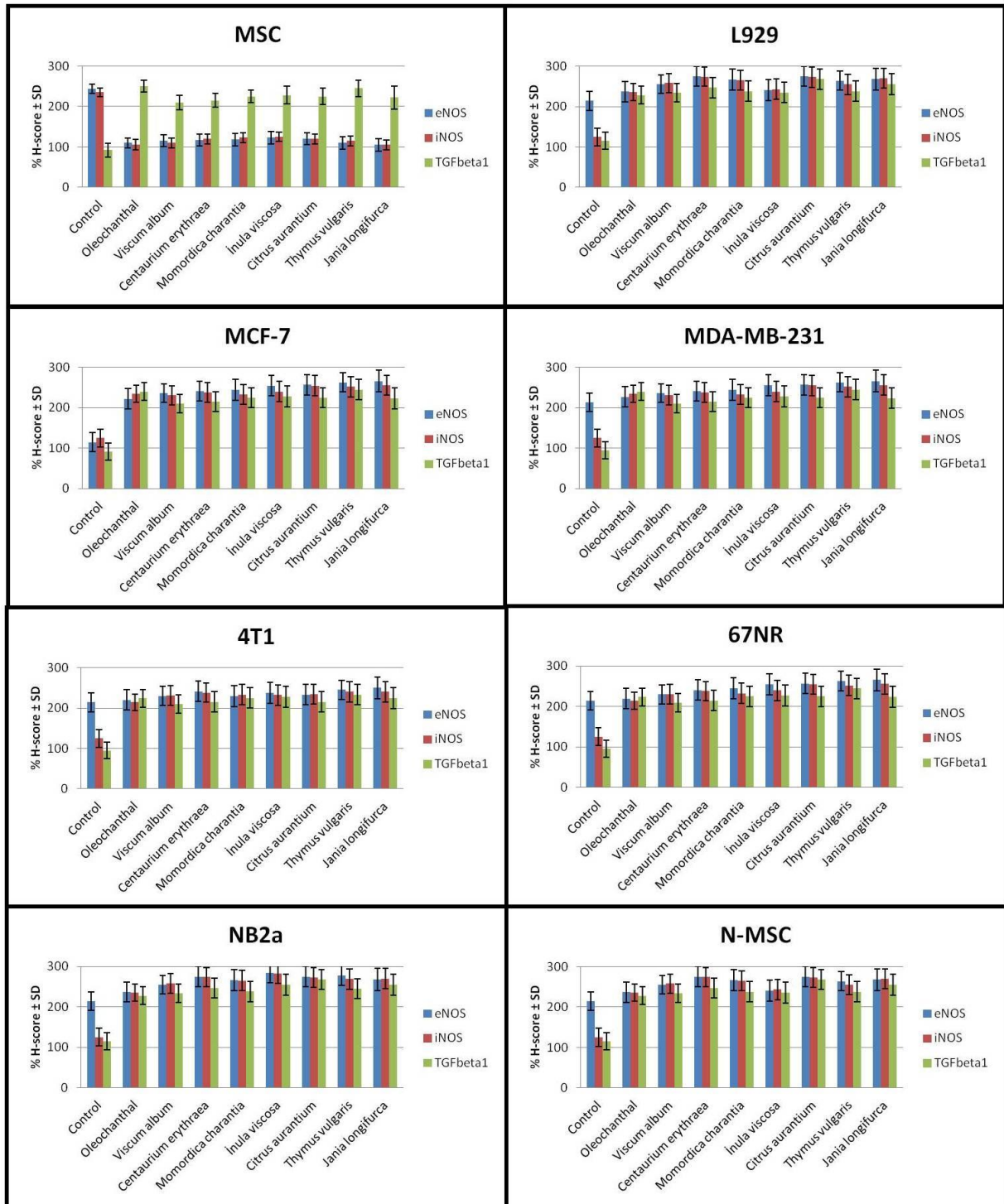


Figure 3. Immunocytochemical staining of eNOS, iNOS and TGFbeta1 in all cells.

In this study, to assess the effects of thyme oil in the experimental wound model, wounds were measured with a ruler on the 1st, 7th and 9th days. Analyses of the measurements revealed that healing in the wound area was achieved in all groups, and healing in the control groups was slower than in the application groups. The most effective and significant ($p < 0.05$) healing was found in the Thyme oil applied animals (Figure 4).

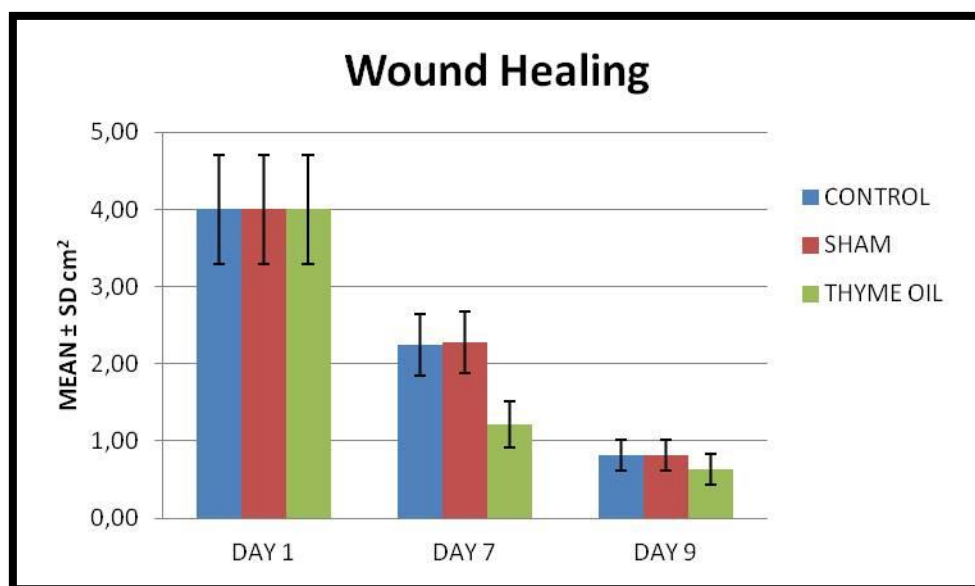


Figure 4. Wound healing with tyme oil in rat skin.

In the breast cancer model, increased oxidative stres markers, eNOS and iNOS, were observed in adenocarcinomas induced by 4T1 cells and the antiproliferative effected tumor growth rate slowed down. Only the neurotoxic effect of *J. longifurca* extract was shown and this was demonstrated in an experimental animal model in which it had serious clinical consequences (Figure 5).

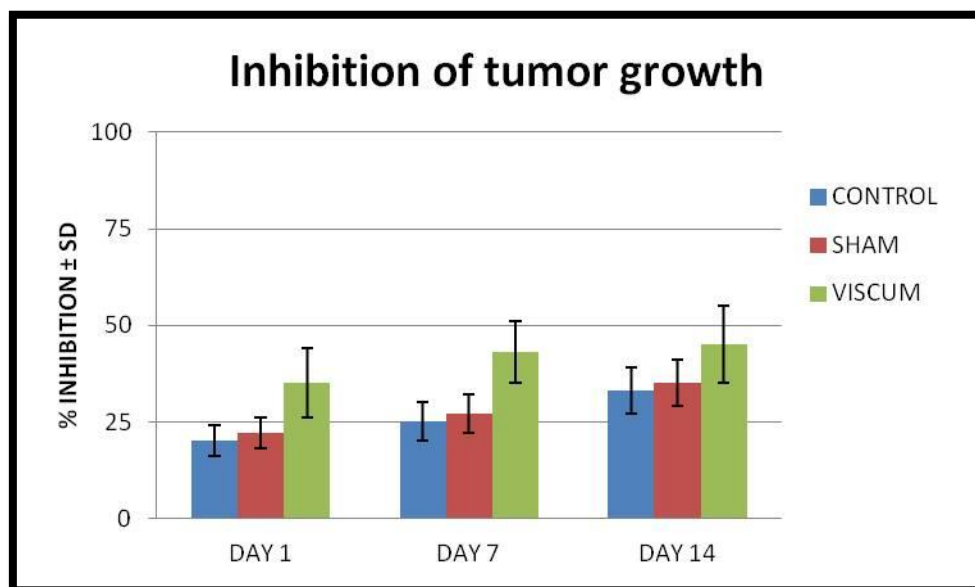


Figure 5. Inhibition of tumor growth after the application of *Viscum album* extract.

4. Discussion

In our study, the effects of oleocanthal, *Viscum album*, *Centaurium erythraea*, *Momordica charantia*, *Inula viscosa*, *Citrus aurantium*, Thyme oil (*Thymus vulgaris*) *Jania longifurca* on MCF-7, MB-MDA-231, 67NR and 4T1 breast carcinoma cell lines, NB2a neuroblastoma cell line, L929 fibroblast cell line were investigated. In addition, in vivo

experiments, anticancerogenic effects of *V. album* extract in breast cancer model and skin wound healing model were examined. All medical plants were found useful for both cancer cells and the cells in wound. They may induce oxidative stress and cell death for cancer cells in culture and they have antioxidant and antiapoptotic effects in wound healing.

Oleocanthal contains phenolic compounds and has anti-inflammatory properties. It has been reported that oleocanthal has cytotoxic effects on human melanoma cells. The cytotoxic effect was supported with the inhibition of c-Met activity, ERK1/2 and AKT phosphorylation and downregulation of Bcl-2 expression [23]. The pentacyclic triterpenes from *V. album* exhibit immunomodulatory, antitumor, and wound healing activity. Especially, compounds of lectins and viscotoxins of *V. album* has anticancer effects via apoptosis [24]. *C. erythraea* is used in traditional medicine because of antidiabetic, digestive, antipyretic and antifatulent effects. *C. erythraea* extract was a protective effect on diabetic rats by reducing oxidative stress and pancreatic β -cells' damage [12]. *M. charantia* is widely used in Southeast Asia and Indo-China. It has been stated that it has anticancer, antidepressant, antidiabetic, anti-inflammatory, antimicrobial, antiobesity, antioxidant, and antiulcer properties via phytochemical components include alkaloids, charantin, flavonoids, glycosides, phenolics, tannins, and terpenoids. It triggers AMP-activated protein kinase (AMPK) and CaMKK (Ca²⁺)/calmodulin-dependent protein kinase) and mTOR/p70S6K and/or the AKT/ERK/FOXO1 (Forkhead Box M1) signaling was suppressed in tumor cells. Also anticancer properties occurred by activation of apoptosis signaling of caspases and mitochondria [25]. These observations are similar to our results.

The genus *Inula* grows in the regions of Europe and Asia. This genus is used as herbal products expectorants, antitussives, diaphoretics, antiemetics, and bactericides. The extract of *I. viscosa* L. were studied and it has been shown that it inhibited the cell growth in HeLa and SiHa cells in a dose-dependent manner. The anticancer and antimicrobial properties were occurred in the presence of methylated quercetins isolated from *I. viscosa* [26]. Another anticancer agent is genus *Citrus* which contain flavonoids [27]. *C. aurantifolia* from genus *Citrus* is widely used because of its antibacterial, anticancer, antidiabetic, antifungal, anti-hypertensive, anti-inflammation, anti-lipidemia, and antioxidant properties. The anticancer effect of *C. aurantifolia* is occurs by inhibiting metastasis and angiogenesis signal pathways [28]. Nar isolated from *T. vulgaris* was shown to trigger apoptotic cell death via cell cycle arrest at S- and G2/M-phases in human colorectal and breast cancer cells. Also it caused the down-regulation of apoptosis and cell-cycle regulatory genes such as Cdk4, Cdk6, Cdk7, Bcl2, x-IAP and c-IAP-2. *T. vulgaris* inhibited the migration and invasion of HCT116 cells [29, 30]. In our experiments, the oil of *T. vulgaris* has antiproliferative effect on cancer cells by oxidative stress.

The research about the *J. longifurca* has shown that extracts of *J. longifurca* were toxic in MCF-7 cells and caused apoptosis and inhibition of proliferation [31]. In our study, we determined the toxic effect of *J. longifurca* in breast cancer cell lines, and also in neuroblastoma cells.

The use of medicinal herbs both in preventive medicine and in cases where treatment is difficult is an application that must be done as a medical procedure based on scientific data. Investigation of these effects using current techniques is very important to prevent harmful effects of herbal products after unconscious use.

5. Conclusion

In this study, the mechanisms by which the beneficial effects of the herbal plants used among the people are revealed and influenced. The neurotoxic algae extract proved to be very important in terms of showing the meaning of these scientific studies. The frequent use of algae, based on the beneficial effects of the vast majority of them, emphasized that this harmful effect

should be kept in mind. It was pointed out that herbal treatment practices may be meaningful in breast cancer treatment and should be studied in animal models, indicating that cancer patients may be an important alternative treatment product for the quality of life. It has been concluded that medicinal herbs are beneficial for treatment difficult diseases, where patient quality of life is highly affected, and they should be used as medicinal therapies.

Conflict of Interest

None declared.

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