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Cytotoxic Effects of Hawthorn and Elderberry Extracts on 3T3 Fibroblast Cell Line

Alıç ve Mürver Ekstraktlarının 3T3 Fibroblast Hücre Hattı Üzerindeki Sitotoksik Etkileri

[1]Gizem Baysan, [1]R. Bugra Husemoglu, [2]Hasan Havitcioglu

[1]Dokuz Eylul University, Institute of Health Sciences, Department of Biomechanics, Izmir, Turkey
[2]Dokuz Eylul University, Faculty of Medicine, Department of Orthopedics and Traumatology, Izmir, Turkey

Abstract: Plants have been used in the medical remedies by countless people since the ancient times. Medicinal plants have multiple benefits for various cases. Nowadays, science is developing in correlation with these medicinal plants. Hawthorn (Crataegus oxyacantha) and elderberry (Sambucus nigra) is used in this study as a medicinal plant. Hawthorn has been claimed to be used in the treatment of heart and blood vessel diseases, chest pain, irregular heartbeat, digestive problems and cholesterol regulation. On the other hand, elderberry has been claimed to be used in inflammation, fever, joint and muscle pain, minor skin conditions, stress and even HIV. In this study, we investigated the cytotoxicity levels of the hawthorn and elderberry extracts with different concentrations on 3T3 murine fibroblast cell line. 1 × 104 cells/well were equally seeded in 96 well plate and incubated for 24 hours to form a half-confluent monolayer. Treatment medium containing various concentrations of extracts (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml) were added per well. The viability analysis was conducted after 48 hours by using the WST-1 colorimetric assay. In conclusion, each concentration used is non-cytotoxic and better proliferation activities were reached by using 0.05 mg/ml concentration for hawthorn and elderberry.
Keywords: Plant extracts, Hawthorn, Elderberry, Cytotoxicity, WST-1.

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Özet: Bitkiler, eski zamanlardan beri sayısız insan tarafından tıbbi ilaçlarda kullanılmaktadır. Şifalı bitkilerin çok çeşitli hastalıklar için birçok faydası vardır. Günümüzde, bilim ve tıbbi bitkiler birlikte ilerleyebilmektedir. Bu çalışmada, alıç (Crataegus oxyacantha) ve mürver (Sambucus nigra) bitkileri kullanılmaktadır. Kalp ve damar hastalıklarının tedavisinde, göğüs ağrısı, düzensiz kalp atışı, sindirim sorunları ve kolesterolün düzenlenmesinde alıç bitkisinden yararlanıldığı bildirilmektedir. Bunların yanısıra, iltihaplanma, ateş, eklem ve kas ağrısı, basit cilt problemleri, stres ve hatta HIV tedavisinde mürver bitkisinin kullanıldığı iddia edilmektedir. Bu çalışmada, 3T3 fare kökenli fibroblast hücre hattı kullanılarak farklı konsantrasyonlarda alıç ve mürver ekstraktlarının sitotoksisite araştırmaları gerçekleştirilmiştir. 1 x 104 hücre / kuyu miktarda hücre, 96 kuyucuklu plaka içine eşit olarak ekilerek yarı konfluent tek katman hücre oluşumu için 24 saat inkübe edilmiştir. Kuyucuk başına çeşitli konsantrasyonlarda alıç ve mürver ekstraktları (10, 5, 1, 0.5, 0.1, 0.05 ve 0.01 mg / ml) içeren ortam ilave edilerek inkübasyon süreci devam ettirilmiştir. Canlılık analizi, WST-1 kolorimetrik analiz kiti kullanılarak 48 saat sonra gerçekleştirilmiştir. Sonuç olarak, analizi gerçekleştirilen konsantrasyon oranlarının toksik özellik göstermediği ve iki ekstrakt için de 0.05 mg / ml konsantrasyon kullanılmasıyla daha iyi çoğalma olabileceği kanısına varılmıştır.
Anahtar Kelimeler: Bitki ekstraktları, Alıç, Mürver, Sitotoksisite, WST-1.

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ORCID ID of the authors: G.B 0000-0002-3195-9156, R.B.H 0000-0003-1979-160X, H.H 0000-0001-8169-3539

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Yazışma Adresi / Correspondence Address
Gizem BAYSAN
mail: gizemciner@gmail.com

1. Introduction

Western and Eastern herbs are both widely used in traditional medical therapies for centuries. Infact, healing with medicinal plants is as old as mankind itself. The search for drugs in nature is evidenced from various sources: written documents, preserved monuments, and even original plant medicines (1). In the beginning there was not sufficient information either concerning the reasons for the illnesses or concerning which plant and how it could be utilized as a cure, everything was based on experience. The reasons for the usage of specific medicinal plants for treatment of certain diseases were being discovered especially in the recent centuries (1) (2), however their full benefits are not clearly understood yet. In this study, we used two different plant extracts which are Hawthorn (*Crataegus oxyacantha*) and elderberry (*Sambucus nigra*).

Hawthorn is a common name of all plant species in the genus Crataegus and it has approximately 280 different species (3). It is a member of Rosaceae family which can be a thorny shrub or small tree that normally has bright green leaves, white flowers, and bright red berries. It is a widely used herb for prevention or treatment of cardiovascular diseases (4) (5) such as angina, hypertension, hyperlipidemia, arrhythmia. Also, it is suggested as an alternative therapy for poor blood circulation, diabetes, several cancer studies and dyspnea (6) (7) (8).

Black or common elder (Sambucus nigra L.) which is also called elderberry, grows on sunlight-exposed locations which is a deciduous shrub reaching up to 6 m in height, it has small, white hermaphrodite flowers in large corymbs in early summer. Umbels consist of dark purple individual berries, with a diameter up to 6 mm, which ripen in the late summer (9). Elderberry flavonoids have antioxidant, anti-diabetic, anticarcinogenic, immune-stimulating, antibacterial, antiallergic and antiviral properties (10) (11) (12).

In the present study, we aimed to investigate the cytotoxicity and proliferation activities of the hawthorn and elderberry extracts with different concentrations (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml) on 3T3 murine fibroblast cell line with the help of WST-1 assay. The obtained data is important for the further *in vitro* and *in vivo* studies with these extracts.

2. Materials and Methods

Materials

Hawthorn extract powder (100 g; %100 natural) was obtained from SYXLFDC store Store, China and Elderberry extract powder (100 g; %100 natural) was obtained from NatureHerb Store, China. Dulbecco's Modified Eagle Medium (DMEM), sterile filtered Phosphate buffered saline (PBS) and fetal bovine serum (FBS) were obtained from Cegrogen Biotech GmbH (Nordost, Germany), L-glutamine and Trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25%, sterile-filtered solution) were obtained from Sigma-Aldrich (Missouri, U.S.A), T-25 flasks, 96 well plates and falcon tubes were obtained from SPL Life Sciences Inc. (Pocheon, Korea), Penicillin-Streptomycin (10,000 U/mL) was obtained from Thermo Fisher Scientific (Massachusetts, U.S.A). 0.5% trypan blue solution (Biological Industries, Israel) used in cell counting and viability percentage calculations. Ready-to-use Cell Proliferation Colorimetric Reagent, WST-1 reagent (BioVision, Inc., San Francisco, U.S.A) was used in colorimetric viability analysis. 3T3 Swiss albino mouse fibroblasts (3T3, 950217, HÜKÜK, ŞAP Enstitüsü, Ankara, Turkey) was used in the present study. This research was conducted at Izmir Dokuz Eylül University, Department of Biomechanics and Bone - Cartilage Tissue Biotechnology Application and Research Center (KEKDOB) laboratories.

Cells

The viability tests were performed with 3T3 Swiss albino mouse fibroblasts on passage 18. The cells were grown in T-25 flasks as monolayer cultures and subcultured when they reached 80% confluency. The culture medium was DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin-streptomycin solution. The medium was changed every other day and incubated at 37°C in a humidifed

atmosphere containing 5% CO_2. Adherent cells were washed with PBS and detached with Trypsin-EDTA solution for 3–5 min incubation at 37°C and used observed by using an Inverted microscope (Olympus, CKX41SF, Tokyo, Japan). The cell suspension was centrifuged (Hettich, Universal 32, Germany) at 1200 rpm for 5 minutes. The cells were then resuspended in a culture medium.

Cell Seeding

The cells were counted by using hemocytometer and trypan blue solution before seeding on 96 well plates for further analysis. They were trypsinized with trypsin-EDTA solution as mentioned, previously. A cell suspension sample was taken by a micro pipette and diluted with PBS (1:10) before cell counting. The percentage viability of cells was calculated by a formula of (13) ;

$$\% \text{ Viability } = \frac{\text{Number of colorless cells}}{\text{Total number of cells}} \times 100$$

$1x10^4$ cells per well were seeded in each well with a culture medium and they were observed by an inverted microscope for homogeneous distribution. The cells were incubated at 37°C for 24 hours to form half-confluency.

Extract Preparation and Treatment of Cells

After 24 hours treatment, well plate was examined under an inverted microscope to identify cell seeding errors, growth characteristics, morphology and equal distribution. 50 mg/ml of both elderberry and hawthorn extracts were prepared as a stock solution prior to use. A complete medium was used to prepare this solution.

Hawthorn and elderberry extracts were separately weighted by a laboratory scale and solved in a fresh medium. The final solutions were filter-sterilized by using a syringe filters (0.2 µm, Minisart, Sartorius, Germany). Treatment medium containing various concentrations of extracts (10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml) were added per well in three replicates.

Viability Analysis

The viability was measured by using the WST-1 colorimetric assay after 48 hours, threating the cells by different extract concentrations medium. Each plate contained blanks, controls, and dilution series with three replicates. Cell viability was measured according to the kit instructions at 450 nm in a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT, USA). A 10 µl of WST-1 reagent (for 100 µl solution, 9:1 ratio) was added to each well and incubated at 37°C for 2 hours (14). The WST-1 assay is based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenases. Expansion in the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases, which in turn leads to increase in the amount of formazan dye formed. The dark yellow formazan dye produced by viable cells can be quantified by measuring the absorbance at 450 nm and is directly correlated to cell number (15) (16).

The optical densities (OD) were measured with a microplate reader and calculated the cell viability according to the following formula (17):

$$Cell\ viability\ (\%) = 100\ x\ (\frac{OD\ experiment}{OD\ negative\ control})$$

The percentage of cell viability over 90% indicated nontoxicity, while the amounts between 60% and 90%, 30-60% and <30%, indicated mild, moderate, and severe toxicity, respectively (17). OD negative control is the average absorbance with untreated cells control (18), and OD experiment is the average absorbance with various treatment groups.

Statistical Analysis

The data were analyzed with the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The mean and standard deviations were calculated. For statistical analysis, One-way ANOVA was applied and post-hoc Tukey's test was used for multiple comparisons. Also, level of significance was set at $p < 0.05$.

3. Results

In-vitro confirmation of the extracts toxicity on 3T3 murine fibroblast cell line. Percentage of viable cells were obtained by performing trypan blue dye exclusion technique by the formula given previously. The cell viability was measured nearly 97% and it claimed to be suitable to perform further cytotoxicity studies. The total cell numbers and percentage viability can be seen in Table 1. The cytotoxicity activity is carried out by using WST-1 assay. The equal distribution and half confluency of the fibroblasts can be seen in Figure 1. Cell line used in the present study was free from any kind of bacterial and fungal contamination. Hawthorn and elderberry extracts were used at concentrations of 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml. Each concentration set was replicated three times and all tests were performed after 48 hours of the treatment media.

Table 1. Cell line characterization and percentage cell viability.

Cell line	% Viability	Number of cells counted	Number of live cells counted	Passage number
3T3 murine fibroblast	96.83%	12.6×10^6	12.2×10^6	P18

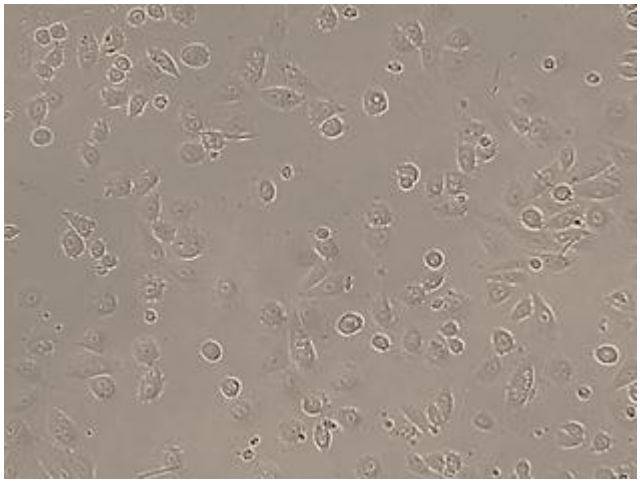

Figure 1. Relatively even cell distribution of 3T3 cell line by an inverted microscope, 20x magnification, after 24 hours from seeding, prior to the addition of treatment medium.

Viability Analysis

The viability and proliferation of extracts were evaluated by WST-1 assay (Figure 2). Our results claimed that in both extracts the applied concentrations were non-cytotoxic. Although, treated extracts caused proliferation of cells (Table 2 and Table 3 shows the percentage proliferation increase compared to the negative control group); but this increase was only significant in concentrations 0.1 mg / ml ($p = 0.045$) and 0.05 mg/ml ($p = 0.006$). Others were not statistically significant compared to the negative control group ($p > 0.05$). Moreover, for both hawthorn and elderberry the differences between the concentration of 0.05 mg / ml and 10 mg / ml were statistically significant as well ($p < 0.05$).

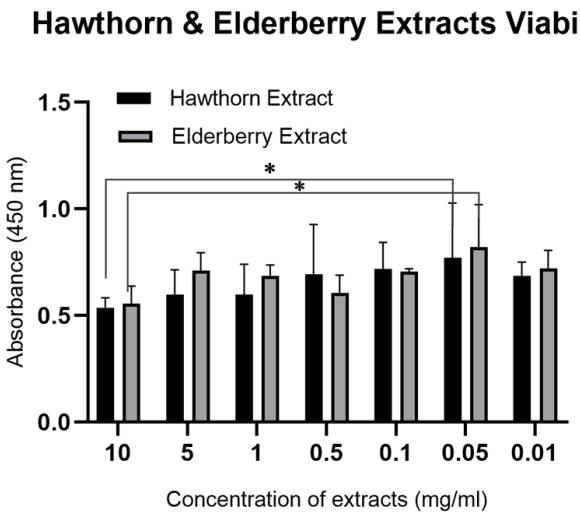

Figure 2. 3T3 murine fibroblast cell proliferation results by WST-1 assay after 48 hours.

Table 2. Increase in proliferation compared to the negative control group for hawthorn extract.

Extract Concentration (mg / ml)	WST-1 Average Density at 450 nm Absorbance	% Increase Compared to Negative Control Group
10	0.537	2.48
5	0.599	14.31
1	0.598	14.12
0.5	0.694	32.44
0.1	0.718	37.02
0.05	0.770	46.95
0.01	0.687	31.11

Table 3. Increase in proliferation compared to the negative control group for elderberry extract.

Extract Concentration (Mg / Ml)	WST-1 Average Density at 450 nm Absorbance	% Increase Compared to Negative Control Group
10	0.555	5.92
5	0.712	35.88
1	0.687	31.11
0.5	0.605	15.46
0.1	0.705	34.54
0.05	0.820	56.49
0.01	0.722	37.79

4. Discussion

In this study, we investigated the cytotoxic effects of hawthorn and elderberry extracts in terms of viability and proliferation assays on 3T3 murine fibroblasts. Our results showed that both extracts were non-cytotoxic and also they increased proliferation activity at the significant concentrations. According to WST-1 assay, the highest proliferation activity was determined at a concentration of 0.05 mg / ml for both hawthorn and elderberry extracts. Also, it can be claimed that elderberry extract treated group at that concentration had relatively higher proliferation activity, however the difference was not statistically significant. These results revealed that both extracts can be used for further studies.

We preferred the WST-1 assay, over MTT assay although which was claimed as a

standard method for the assessment of cytotoxicity of materials by ISO 10993. MTT assay determines cell viability by quantitative assessment of a metabolic product and indicates activity of mitochondria in living cells; which has a direct relationship with cell proliferation and longevity (17) (19). WST-1 works similarly to MTT by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. Also, WST-1 reagent produces a water soluble formazan and the reaction product can be quantified in 0.5 to 4 h without an additional solubilization step (14) (20) (21). Therefore, the WST-1 assay is more preferable in the recent years.

Hawthorn (*Crataegus oxyacantha*) and elderberry (*Sambucus nigra*), as a natural herbal medicine has long been used in the Middle East for treatment of various diseases as mentioned previously. However, to our knowledge, this is the first report that explains the effects of Hawthorn (*Crataegus oxyacantha*) and elderberry (*Sambucus nigra*) on viability and proliferation of 3T3 murine fibroblast cell line. Both extracts were used in several cancer studies. Such as hawthorn was investigated in human breast cancer MCF-7 (22) and MDA-MB-231 (23) cell lines. Also, elderberry was investigated in human breast cancer MCF-7 and adenocarcinoma (LoVo) (24) cell lines. Both extracts were showed promising results in these cell lines. Moreover, not only hawthorn but also elderberry is recommended in inflammation, diabetes (25) (26) and cardiovascular (27) (28) (29) diseases for a long time by herbal practitioners. By our research, we claimed that significant doses of hawthorn and elderberry can increase fibroblast tissue proliferation. The further studies should be carried out with these concentrations for detailed characterizations of these extracts both *in vitro* and *in vivo*.

5. Conclusion

In conclusion, the obtained data showed that the hawthorn and elderberry extracts at concentrations of 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/ml were considered as non-cytotoxic. Better proliferation activity was reached at 0.1 and 0.05 mg/ml concentrations when compared to negative control group. Also, it can be claimed that the optimal concentration is 0.05 mg/ml for both extracts, however there was no statistically significant difference among them.

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