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Assessment of Cytotoxic Potentials of *Styrax Officinalis* (Styracaceae) Extracts from Jordan against Human Cancer Cell Lines

Sarah I. Almulla [1,2], Noor Mukdam Diwood [3], Ahmed A. Alzbeede [4]

Abstract

Plants of *Styrax officinalis* are members of the family Styracaceae, which comprises small trees or shrubs. It is predominant in the Mediterranean region. The leaves of this plant exhibit active phytochemical compounds with antioxidant properties. This project aims to examine the cytotoxic effect of aqueous and methanolic extracts of the leaves of *Styrax officinalis* on two selected human cancer cell lines: mammary gland carcinoma (MCF7), human hepatocellular liver carcinoma (HepG2) in addition to the noncancerous cell line fibroblast. In vitro, the cytotoxic activity of aqueous and methanolic extracts of leaves of *S. officinalis* was investigated against three cell lines: MCF-7, HepG2, and fibroblast, and the outcomes of this study showed that the highest cytotoxic activity was revealed in methanolic extract of leaves against MCF-7 and HepG2 with IC_{50} values of 14.95µg/ml and 19.39µg/ml respectively.

Keywords: Biomarkers, Cell Survival, Mediterranean Diets, Plant Extracts, Traditional Medicine.

[1]Department of Biology, Al-Farabi University College, Baghdad, Iraq

[2]Department of Biological Sciences, College of Science, The University of Jordan, Amman, Jordan

[3]Department of Computer Engineering, Al-Farabi University College, Baghdad, Iraq

[4]Department of Medical Laboratory Science, College of Science, Komar University of Science and Technology, Sulaymaniyah, Kurdistan region, Iraq

Introduction

Medicinal plants are an indispensable source of chemical compounds used for pharmaceutical purposes, and a significant challenge is obtaining their potential natural products relatively easily (Forni et al., 2016; Mondal, et al., 2014).

Medicinal plants are considered a healthy source of human life because of their therapeutic capacities in treating different diseases (Jain, 1985). The therapeutic capacities of medicinal plants depend on the

content of active compounds such as tannins. lignans, flavones, catechins, anthocyanins, volatile oil, steroids, resin, and terpenoids (Rios, 2005). These active compounds can be found in different parts of medicinal plants, such as flowers, leaves, fruits, roots, and seeds (Jamshidi-Kia, et al., 2018; Khwaldeh, et al., 2024).

Plants of *styrax officinalis* are members of the family Styracaceae, which is constituted of small trees or shrubs (Feinbrun Dothan , 1978). It is found only in the Mediterranean region (Tayoub, et al., 2006). In Jordan, *Styrax officinalis* grows in forest formations in Irbid, Ajloun, Jerash, Salt, and Amman. It is flowering period: April -May (Al-Eisawi, 1998; Oran, 2014; Oran, 2015). *Styrax officinalis* is used in folkloric medicinal plants from Palestine and in treating some infections, diseases, and oxidative stress (Jaradat et al., 2018; Jaradat, 2020, Paşa, 2023). Multiple different applications have been reported throughout using plant extracts and their chemical compositions and active compounds from *Styrax officinalis* in medicinal, agricultural, biofuel, and industrial fields (Yesilyurt & Cesur 2020; Oni, et al., 2022; Balyen & Kiraz, 2024; Esen et al., 2022; Babahan-Bircan et al., 2022; Son et al., 2021).

Benzofuran is a chemical compound found in most plants, especially in the Styracaceae family, such as Styrax officinalis. It has a variety of biological activities, including insecticidal, fungicidal, antimicrobial, antidiabetic, cytotoxic, and cytotoxic effects (Bölükbaşı et al., 2016). The fruits of Styrax officinalis had been reported to be rich in saponins. Saponins are bioactive compounds, and many investigations have observed hemolytic activity resulting from their affinity for membrane sterols (Dib et al., 2016).

The methanolic extract was reported to have anti-microbial activity against gram-positive (Staphylococcus aureus) and gram-negative (Escherichia coli) bacteria. The active was compared with broad-spectrum antibiotics such as cefepime, amikacin, and Ceftriaxone (Mansour et al., 2016). Aqueous and organic extracts of *Styrax officinalis* have been found to have antioxidant and antimicrobial effects (Jaradat et al., 2018). They were also reported to have cytotoxic activity against multidrug-resistant human leukemia cells (Reiter et al., 2014).

The previous studies had mainly dealt with the content of benzofurans as egonol, lipids, or saponins of the seeds of *S. officinalis* (Tayoub et al., 2006). The resin of the species *S. officinalis*, known as storex, was used in traditional medicine in the Mediterranean basin for antiseptic purposes and against respiratory diseases (Oran & Al-Eisawi, 1998). Few studies have investigated the chemistry of the leaf, stem, or flower of *S. Officinalis*, particularly their essential compounds (Tayoub et al., 2006).

The aims of this project are to examine the cytotoxic effect of aqueous and methanolic extracts of the leaves of *Styrax officinalis* on two selected human cancer cell lines: mammary gland carcinoma (MCF7), human hepatocellular liver carcinoma (HepG2) and noncancerous cell line which is the fibroblast.

Materials and Method

Plant Materials

The leaves of *Styrax officinalis* were collected from different parts of Jordan. The collected plants are shown in Table 1.

Table 1: Description of the studied plant species

Plant species	Family	Site of collection	Voucher specimen	Collected time	Collected parts
Styrax officinalis L.	Styracaceae	Ajloun-Irbid	1	Any period of the year (2022)	Leaves

Plant extraction

The leaves were dried at room temperature (23-25 °C) for (30-40) days at a botany research lab at the Department of Biological Sciences at the University of Jordan; dried plants were powdered using an electrical blender. The powdered plant was then exposed to two types of extraction: aqueous and

methanolic. 10 gm of each powdered plant was dissolved in 100 ml and stirred using a hotplate magnetic stirrer. The solvents left for one and five days respectively then the extraction filtered by Whitman no.1 filtered paper. The filtrated solvents were then evaporated using a rotary evaporator at 60 °C.

Cell culture condition

Human cancer cell lines were used to evaluate the cytotoxic effect of plant extracts. Mammary gland carcinoma (MCF-7), human hepatocellular liver carcinoma (HepG2), and fibroblast (as control), cell lines were purchased from American Type Culture Collection (ATCC) company, and all cell lines cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 50 ml fetal bovine serum (FBS), 5 ml 2 mm L-glutamine and 5 ml 100U penicillin / 0.1 mg/ml streptomycin penicillin/streptomycin. Cells were maintained in the vented flask at 37 °C in a 5% CO_2 atmosphere with 95% humidity. All cell culturing work was carried out under sterile conditions in a laminar airflow cabinet.

Seeding of cell lines

After the cells had become confluent, the 25 cm^2 flask was washed first with 3ml of PBS, and then 1ml of trypsin/EDTA was added and the flask was incubated for 5-10 min at 37°C, then 3ml of fresh media was added to the flask with good mixing and transferred to sterile 15 ml centrifuge tube. The cell suspension was homogenized using a vortex mixer, and then 25 µL was transferred to an Eppendorf tube containing 25 µL trypan blue dye, which was loaded onto a hemocytometer and placed under a light microscope. The counting of the cells was in the four outer squares and the average number was calculated according to the following formula:

(Number of cells/ml = average number of cells × 2 × 10000)

From the cell suspension, a useful volume was withdrawn, and the volume was completed with fresh media to end up with final optimal density. Cells were seeded in different densities in triplicates. By using a multichannel pipette, 100 µL of cell suspension was seeded per well in 96-well plates and incubated overnight to allow for cell attachment. plates of 96 wells were prepared for studying the effects of the methanolic extracts of *styrax officinalis* L. leaves for 72 hours by using MTT assay

Extracts preparation for cytotoxic assay

The prepared aqueous and methanolic leaves of *styrax officinalis* were added to the cells in seven concentrations (200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml). After 24 hours of seeding the cells, a weight of 25 mg from each stock extract. Aqueous extracts were dissolved in 1 ml of PBS (25 mg/ml) and methanolic extracts in 1 ml of DMSO (25 mg/ml). Preparation of aqueous and methanolic extract concentrations started with 200 µg/ml concentration prepared by mixing 16 µl of the extract stock (25 mg/ml) with 984 µl media (the total volume will be 1 ml) hence the volume of each extract stock using the equation: C1*V1 = C2*V2. The serial dilution was then continued until reaching 3.125 µg/ml concentration and incubated for 72 hours until using MTT assay.

Cell viability assay (MTT)

The cytotoxic activity of *S. officinalis* extracts (aqueous and methanolic) was determined using an MTT assay (Van Meerloo, et al., 2011).

MTT (3-(4,5-dimethylthiazol-2w-yl)-2,5-diphenyl tetrazolium bromide) assay was used in order to estimate cell viability after incubation with various concentrations of plant extracts. MTT (3-(4,5-dimethylthiazol-2w-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS at 5mg/ml.

Control wells without treatment (cells with DMSO or PBS or media) and negative control wells containing only fresh media. After 72 hourss of incubation, 20 µL of MTT was added to every well and incubated for 3 hours. After that, MTT was withdrawn carefully, and 200 µL of DMSO was added to every well to dissolve the formed crystal. The absorbance at 570 nm was read on a spectrophotometer, and the difference between readings was used for the analysis of results. The percentage of survival cells

was calculated using the following formula:

% Survival= 100 - ([(AC- AT)/ AC] x 100)

The values of IC_{50} values were detected as concentrations that exhibited 50% inhibition of proliferation on any tested cell line. IC_{50} values were detected as the average of three replicates.

Statistical analysis

The percentage of the cell's viability treated with extract solution was calculated by taking the mean of the three wells absorbance of each used concentration divided by the mean of the absorbance of three negative control wells multiplied by 100. The percentage of toxicity was then calculated from the equation (% Toxicity = 100 - % viability).

The standard error of the mean was also calculated. GraphPad prism 5.0 was used to calculate IC_{50} values, and Excel sheets were used to draw a graph of extracts concentrations versus the plant's viability percentage.

Results and discussion

The cytotoxic activity of aqueous and methanolic extracts of leaves of *styrax officinalis* was studied against a panel of human cancer cell lines over 72 hours of exposure. Figure 1, figure 2 & figure 3 express the In vitro cytotoxic activity of methanolic and aqueous extracts of Styrax officinalis leaves on MCF-7, HepG2, and fibroblast cell lines, respectively. Table 2 displays the IC_{50} values cytotoxic activity using MTT assay against cancer cell lines, namely human hepatocellular liver carcinoma (HepG2) and mammary gland carcinoma (MCF-7). A noncancerous human fibroblast cell line was recruited to verify selective cytotoxicity.

All IC_{50} values listed are mean ± SD (standard deviation) (µg/ml). IC_{50} values (concentration at which 50% inhibition of cell proliferation took place in comparison to non-induced basal 72 hours incubations) were calculated within the treatment of different concentrations for all extracts and controls. In the U.S. NCI plant screening program, if the IC_{50} value (concentration causing a 50 percent cell kill) in carcinoma cells is less than 20 µg / mL for the crude extract after incubation between 48 and 72 hours, then it is mostly represented to have cytotoxic effect in vitro (Ackova, et al., 2016). Importantly, significant cytotoxic effectiveness of methanolic leaf extract (mainly displayed in both MCF-7 and HepG2) was shown with $IC_{50s} < 20$ µg/ml.

The evaluation of the cytotoxic activity of leaves of *S. officinalis* using two solvents with different polarities (aqueous and methanolic) of *S. officinalis* against MCF-7 and HepG2 cancer cell lines was conducted. Furthermore, the safety of the extracts of plants was determined by studying their activities against noncancerous fibroblasts. The MTT assay for the aqueous and methanolic extracts of *S. officinalis* leaves showed different results for the cytotoxic effect.

The essential oil from the leaves of *S. officinalis* revealed anti-cancer properties against all cancer cell lines, MCF-7, HCT-116, CacoII, and HepG2 (Almulla, 2020).

According to the U.S. National Cancer Institute guidelines (NCI), crude extract is considered a cytotoxic promising anticancer product for future bioguided studies only if it applies to IC_{50} value < 20 µg/ml (Ackova et al., 2016). Methanolic leaf extract showed significant cytotoxic activity on both MCF-7 and HepG2 with IC_{50} values of 14.95µg/ml and 19.39µg/ml, respectively. None of the aqueous extracts (leaves) was found to be cytotoxic against cell lines; this lack of activity can be attributed to low water solubility for the organic compounds, except for the aqueous leaves extract on HepG2 that showed medium cytotoxic activity with IC_{50} values of 41.94µg/ml. Moreover, extracts used in the study are considered safe against noncancerous cell line, which is fibroblast, because IC_{50} values determined for all used extracts were far away from those recorded against cancer cell lines under our study.

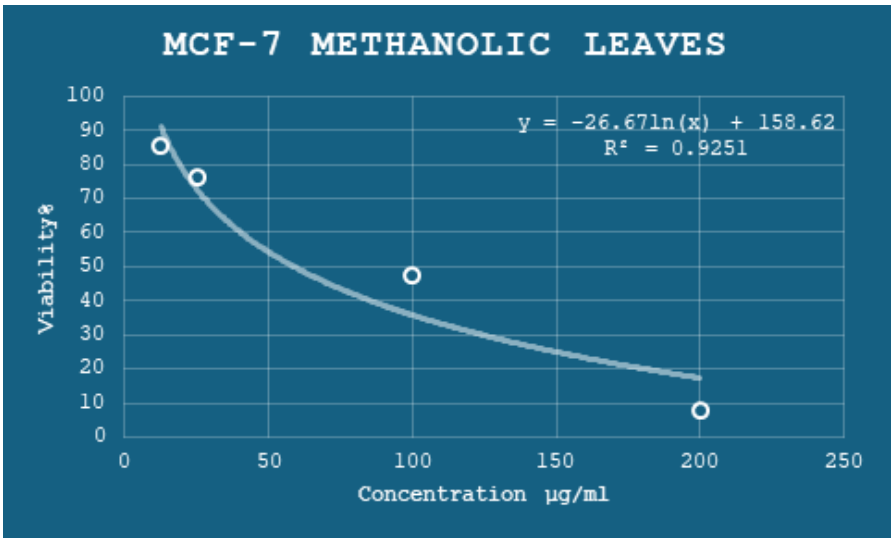
A study on an ethanolic crude extract of S. camporum used XTT assay showed significant cytotoxic activity on MCF-7 and HepG2 with IC_{50} values of 13.3µg/ml and 53.3µg/ml, respectively, after 72 hours

of incubation (De Oliveira et al., 2016). Another study showed significant cytotoxicity of stem bark extracted with methanol of S. japonica by Sulforhodamine B (SRB) assay against HCT-15 (colon adenocarcinoma) (Kim et al., 2004; Lee & Lim, 2010).

On the other hand, a study in Korea showed cytotoxic effect of aerial parts of S. obassia extracted with methanol by MTT method against MCF-7, Hela and HL-60 cancer cell lines (Cao et al., 2017). In China (Li et al., 2005), the seeds of S. perkinsiae extracted with ethanol showed significant cytotoxic activity against two breast cancer cell lines MCF-7 and MDA-MB-231 with IC_{50} values of 5.45µg/ml and 3.81µg/ml respectively by SRB method. The resin of S. tonkinensis extracted with ethanol against human leukemia cells HL-60 by used NBT reduction assay showed significant antiproliferative activity after 3 days incubation (Wang et al., 2006).

In parallel, a study in Brazil exhibited different significant activities against C6, Hep2 and HeLa cancer cell lines with IC_{50} values of 3.2µg/ml, 3.6µg/ml and 15.6µg/ml respectively by MTT assay of ethanolic extract from the stem of S. camporum (Teles et al., 2005).

In vitro cytotoxic activity of two extracts of *Styrax officinalis* leaves on MCF-7 cell line:



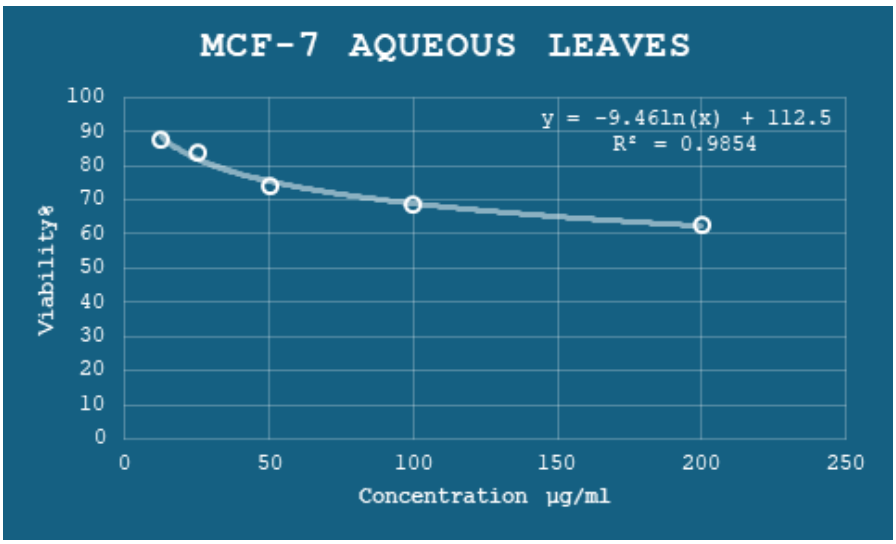
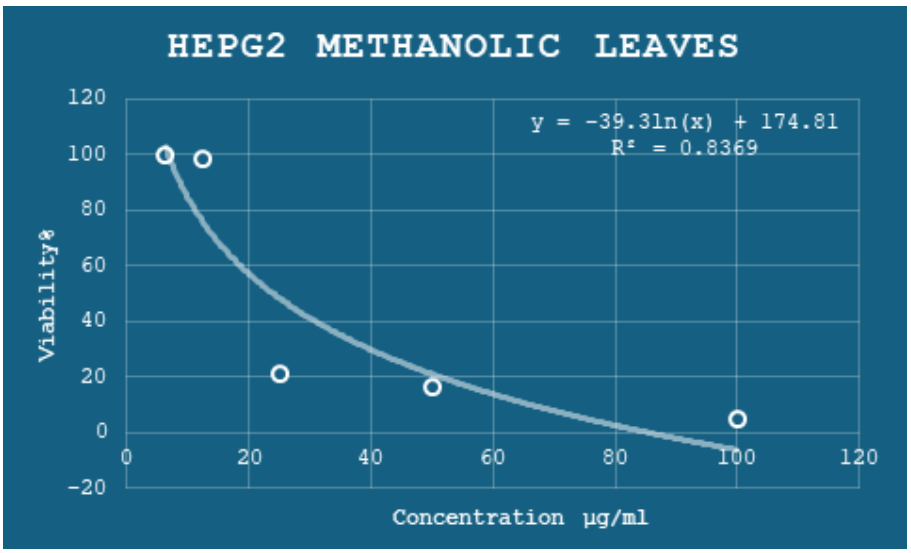


Figure 1: The cytotoxic activity of two extracts of *Styrax officinalis* leaves on MCF-7. Cells were plated onto 96 well plates and treated with or without (control) different concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.125µg/ mL) of the extracts for 72 h. Data are representative of at least three independent experiments.



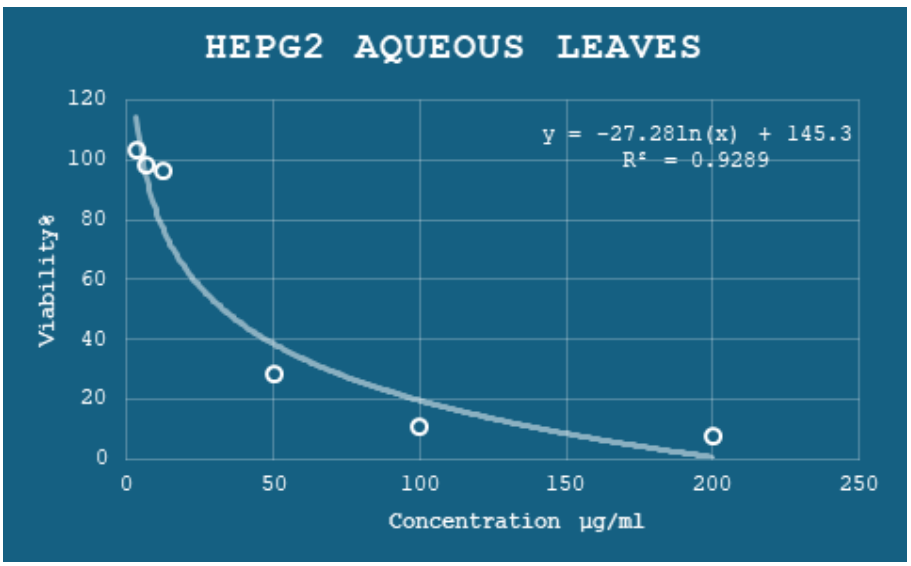
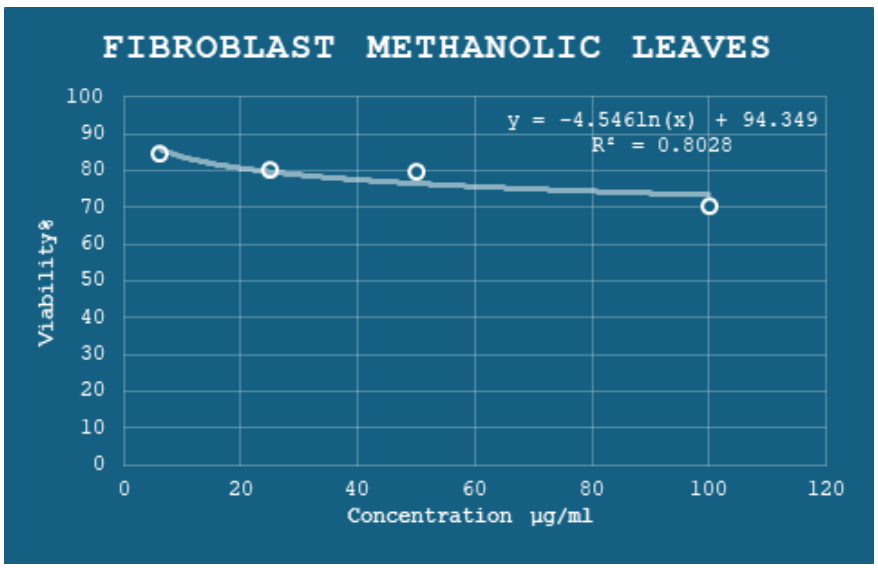


Figure 2: The cytotoxic activity of two extracts of *Styrax officinalis* leaves on HepG2. Cells were plated onto 96 well plates and treated with or without (control) different concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.125µg/ mL) of the extracts for 72 h. Data are representative of at least three independent experiments.



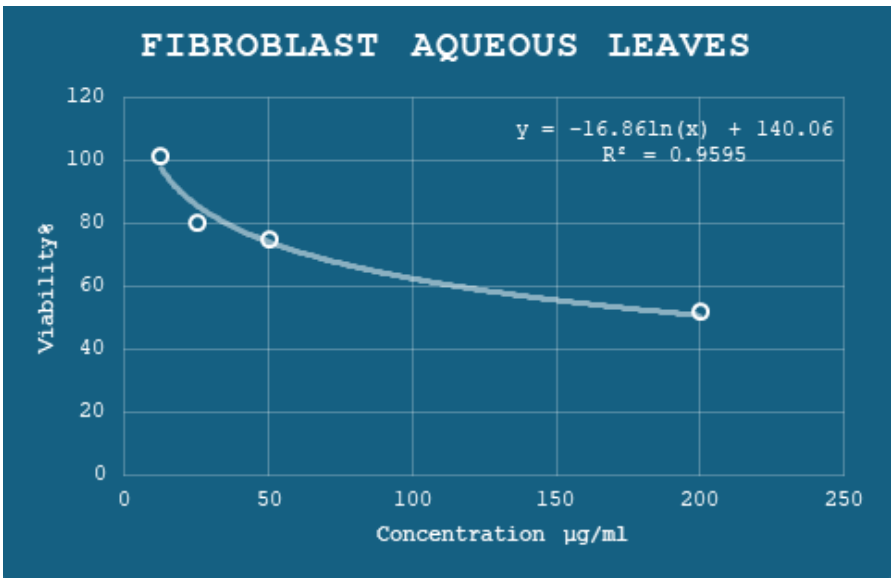


Figure 3: The cytotoxic activity of two extracts of *Styrax officinalis* leaves on Fibroblast Cells were plated onto 96 well plates and treated with or without (control) different concentrations (200, 100, 50, 25, 12.5, 6.25 and 3.125µg/ mL) of the extracts for 72 h. Data are representative of at least three independent experiments.

Table 2: IC_{50} values (µg/ml) for extracts of *Styrax officinalis* leaves against different cell lines after 72 hours of treatment.

Treatment	MCF-7	HepG2	Fibroblasts
Aqueous Leaves	219.5 ± 1.83	41.94 ± 4.65	589.2 ± 13.83
Methanolic Leaves	14.95 ± 3.87	19.39 ± 1.46	116.9 ± 4.52

IC_{50} values: mean ± SD (µg/ml)

Conclusion and Recommendations

Concerning the cytotoxicity results, *S. officinalis* showed cytotoxic effects on cancer cell lines used in the study. Additionally, almost all the tested extracts of the present study were found safe on normal human cells. Further studies are recommended to be applied to identify the specific phytochemical compounds of extracts that are responsible for the inhibition of cancer cell growth as well as in vivo assessment of the cytotoxicity against the detected cancer types.

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Conflicts of interest

The authors have stated that there are no conflicts of interest.

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