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Assessment of the Cytotoxic Effects of Silymarin on the MCF7 Cell Line

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Abstract : Silymarin was isolated Using HPLC (high-performance liquid chromatography). The cytotoxicity of silymarin on breast cancer cells was examined using the MTT assay, followed by qRT-PCR to measure p53 expression. Silymarin exhibited dose-dependent cytotoxicity, significantly impeding the proliferation of breast cells at elevated concentrations ($P \leq 0.01$), with an IC50 value established at 12.1 ppm. Furthermore, it was shown that cells exposed to the IC50 of silymarin displayed a markedly elevated gene expression ($P \leq 0.05$) of p53, with a fold change of 4.5 relative to untreated cells. Silymarin derived from milk thistle exhibits antiproliferative properties on breast-cancer cells and enhances the expression of the p53 gene, suggesting its potential as a treatment for breast-cancer patients. The present investigation aims to evaluate the antiproliferative effects of milk thistle-derived silymarin on the MCF7 cell line and its impact on breast cancer cells' expression of the p53 gene.

Keywords: milk thisle, mcf7cell line, qRT-PCR, silimarin, cytotoxicity, gene regulation.

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Introduction

The p53 tumor suppressor gene, which is found on human chromosome 17p13, encodes the nuclear phosphoprotein p53. It is a transcription factor that responds to various stress signals by modifying the expression of target genes. The p53 protein is known as "the guardian of the genome" because it plays a role in cell cycle regulation, apoptosis, and DNA repair., and the preservation of genomic integrity (1). Cancer is one of the lethal diseases that contributes to a significant global mortality(2) rate. Breast cancer is a malignant tumor that originates in the breast cells. It generally arises from the epithelial cells of the mammary glands and is termed ductal carcinomas(3). Nearly 1.7 million new instances of breast cancer were reported globally in 2012, accounting for 25% of all cancer diagnoses. In addition, around 522,000 breast cancer deaths were documented worldwide in that year, with projections indicating that 13 million breast cancer fatalities will transpire over the next 25 years. In Iraq, breast cancer cases substantially increased, especially throughout the 1990s, constituting 14.3% of all cancer types in 1997. The rising incidence rate is associated with premenopausal women (4). Breast cancer has become a major threat to women's health in Iraq, ranking as the leading cause of death after cardiovascular diseases. Analyze the characteristics and manifestations of cancer in patients from diverse geographic locations, with a specific

focus on breast cancer (5). Natural antioxidants originating from plants, such as phenolics (flavonoids, phenolic acids), have become increasingly popular in recent years as preventative and medicinal medications. At present, about fifty per cent of available drug prescriptions originate from botanical sources. Milk thistle is one of the commonly recognized plants shown to possess therapeutic qualities (6). The plant, previously cultivated in Southern-Europe and Asia, is now internationally dispersed. In the United States, milk thistle is sold as a nutritional supplement. For almost 2,000 years, the milk thistle plant's fruit and seeds have been used to treat biliary and liver conditions. The medicinal advantages of milk thistle are tightly linked to the presence of a flavonoid (7). Silymarin, a compound of flavonoids that includes at least seven flavonolignans, is the main ingredient in milk thistle. The highest concentration of silymarin is found in the seed. The principal protective effect of silymarin is ascribed to its antioxidant and free radical scavenging capabilities. *Silybum marianum* is categorized as an annual, winter annual, and biennial herbaceous species. It is a perennial species in its indigenous habitat (8). This research seeks to assess the cytotoxic impact of silymarin on breast cancer cell lines.

Material and Method

Silymarin extraction

The Soxhlet apparatus was used to make thorough extractions using 50 g of powdered seeds. Accurately measured samples were placed into a paper protector. A 100 mL Soxhlet extractor was filled with a thimble. Extractions were conducted using a bifurcated approach. The initial stage of the process was defatting the botanical material for six hours with 300 mL of n-hexane. Silymarin was extracted for five hours using 300 mL of methanol in the second occurrence. Upon reaching room temperature, the extract was transferred to a 100-mL volumetric flask, which was then filled to the mark with methanol (9).

High performance liquid chromatography (HPLC)

In a 25 ml volumetric flask filled with distilled water, the silymarin standard and each test extract were diluted to a concentration of 0.01 mg. The samples were analyzed using a Shimadzu LC-6A high-performance liquid chromatography system fitted with 6A-UV spectrophotometers. The formula concentration (ppm) = (sample area/standard model area) x standard concentration (10) was used to compare the samples at 254 nm to extraction samples using peak area and retention time.

Maintenance of cell lines cultures

The RPMI-1640 medium supplemented with 10% fetal bovine serum (Capricorn, Germany), 100 µg/mL streptomycin, and 100 U/mL penicillin was used to cultivate and maintain the MCF7 breast cell line. The cell passage was then cultivated at 37 °C in 5% CO2 and reseeded every 2 weeks at 80% confluence using trypsin-EDTA (11).

Cytotoxicity assays of silimarin on MCF7

To assess the cytotoxic effects of silymarin on MCF7 cell lines, 96-well plates were used for the MTT cell viability experiment. The inoculation density of cell lines was 1 x 10^4 cells per well. Following day. A confluent monolayer or various doses of substances were introduced into the cells. To assess the viability of the cells, the medium was discarded after 72 hours of treatment, 28 µL of 2 mg/mL MTT solution was added, and the cells were incubated for 2.5 hours at

37 degrees Celsius. After removing the MTT solution, 130 µL of DMSO (dimethyl sulphoxide) was added to each well to dissolve any remaining crystals. The mixture was then agitated for 15 minutes at 37 °C (12). Transmission density was measured at 492 nm using a microplate reader, and the experiment was run in triplicate. The rate of inhibition of cellular proliferation was determined using the following calculation (the amount of cytotoxicity) (13). Assign the optical densities of the test and control as A and B, respectively. 96-well microtitration plates were filled with 200 µL of cell suspensions at a cell density of 1x10^4 mL^-1 in order to view the morphology of the cells under an inverted microscope. The plates were subsequently incubated for 48 hours at 37 °C. Following duration of twenty-four hours, the medium was substituted with oil at the IC50 concentration. After that, 50 µL of Crystal Violet was added to the plates, and they were incubated at 37 °C for 15 minutes. After that, tap water was used to wash away the discolouration. The cell was viewed under an inverted microscope at 100x magnification, and a digital camera was used to record and record the image (14).

Real-time quantitative PCR for p53 gene expression

The expression of the p53 tumor gene in silymarin-treated and untreated MCF7 breast cancer cells was assessed by quantitative real-time PCR, in accordance with the manufacturer's instructions using Trizol (Invitrogen, USA). The extracted RNA was transformed into cDNA using the High-Capacity RNA to cDNA kit (Applied Biosystems, USA).. For p53 gene expression, the forward primer 5-TTCCGTCTGGGCTTCTTG 3 and the reverse primer 5-TGCTGTGACTGCTTGTAGAT-3 were used. The forward 5-ACCACAGTCCATGCCATCAC-3 and reverse 5-TCCACCACCCTGTTGCTGTA-3 GAPDH primers were used for qPCR normalization (15). A total volume of 15 µl was calibrated using distilled water, and each reaction contained 7.5 µl of SYBR green (SYBR® Premix Ex Taq kit, USA), 1 µl of cDNA, and 0.3 µl of each corresponding primer. Utilizing the ABI PRISM 7500 sequence detection apparatus (Applied Biosystems, USA), the PCR reactions were carried out. Fold measured as 2^-ΔΔCT was used to assess p53 gene expression using the ΔΔCT approach.

Statistical Analysis

The Statistical Analysis Method software was used to determine how different factors affected the study parameters. The least significant difference utilized in this study for mean comparison was the T-test, also referred to as the LSD (Analysis of Variance, or ANOVA). A T-test was used to compare the levels of p53 gene expression in breast cancer cells that had received treatment to those that had not. $P < 0.05$ was the threshold for values to be deemed statistically significant (16).

Results and Discussion

HPLC stands for high-performance liquid chromatography.

High-performance liquid chromatography (HPLC) is a crucial analytical technique utilized in the stages of drug discovery, production, and research in the modern pharmaceutical industry. To find a peak position, silymarin components are first added to an HPLC system. A C18-ODS column (25 cm x 4.6 mm), a UV-Vis detector set at 288 nm, and a mobile phase consisting of acetonitrile and sodium citrate are utilized to determine

the retention and height of the standard peaks. Before the complex concentrations were assessed, the flavonoid sample from the milk thistle plant with the highest extraction yield was combined with the finished standard solution prepared under the same circumstances (Figure 1). After applying the mobile phase for 40 minutes under isocratic conditions using a 10/90 (v/v) mixture of phosphate buffer and methanol at a flow rate of 1.2 mL/min, the silymarin standard was detected at 3.50 minutes, showing a peak area of 58975.02 and a height of 480.14 (Figure 1). The flavonoid extract sample showed silymarin at 4.30 minutes (peak area = 155987.45, height = 820.11). The presence of silymarin flavonoid was detected in the crude extract sample at 5.88 minutes (peak area = 80142.65, height = 632.14) when the absorbance spectrum and retention time of the standard were compared (17)

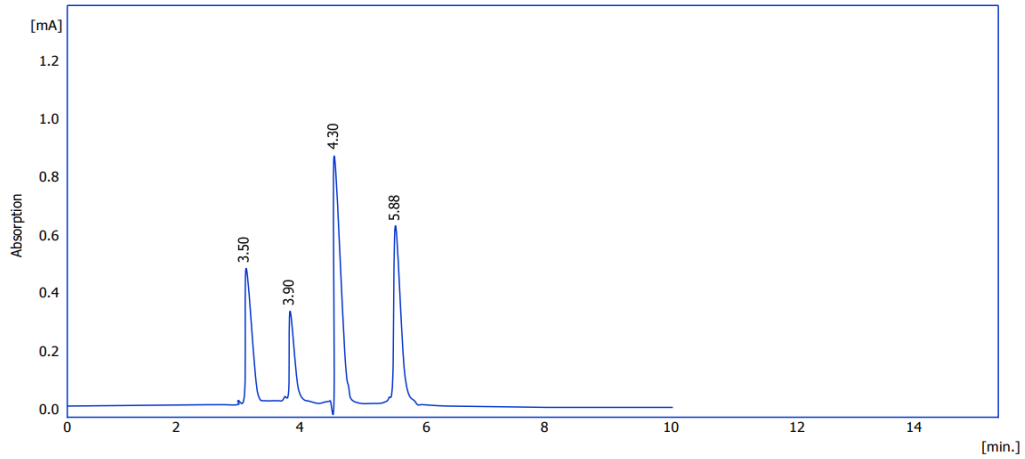


Figure (1): HPLC chromatogram for silymarin from milk thistle plant

Cytotoxic impact of silymarin on breast cell line

The data indicate that the percentage of inhibition on the breast cell line varied considerably ($P < 0.01$) according to the quantity of silymarin. Despite varying survival rates after 48 hours, the silymarin concentrations (0, 0.5, 2, 4, 6, 8, 10 mg/ml) were incubated at 37°C for a further 48 hours with the methanol extract. In the 48-hour incubation period, the extract at a concentration of 10 mg/ml exhibited viability rates of 57.463%. In the 48-hour incubation period, the 0.5 mg/ml concentration demonstrated a superior viability rate of 98.555% (Figure 2). The concentration of HFF-1 was 67.535% at 10 mg/ml. The methanol extract was utilized to co-culture MCF7 cancer cell lines, resulting in a growth inhibition concentration (IC) of 12.1 µg/ml over 48 hours. The cell lines employed are inverted in Figure 3, illustrating the inhibitory effect of Silymarin on the proliferation of MCF7(18) cells following 48 hours of therapy. qRT-PCR was used to examine how the expression of the p53 gene changed in MCF7 breast cancer cells treated with silymarin as opposed to those that were not. It is not surprising that the p53 was successfully expressed and measured in relation to the GAPDH gene, given that GAPDH is a frequently used housekeeping gene. supplement with information on gene expression.

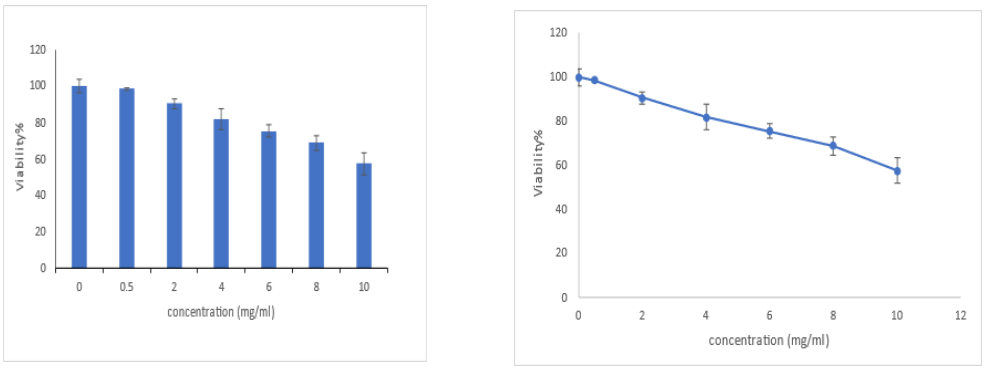


Figure (2): Cytotoxic effects of silymarin extract on MCF7 cells assessed via MTT test

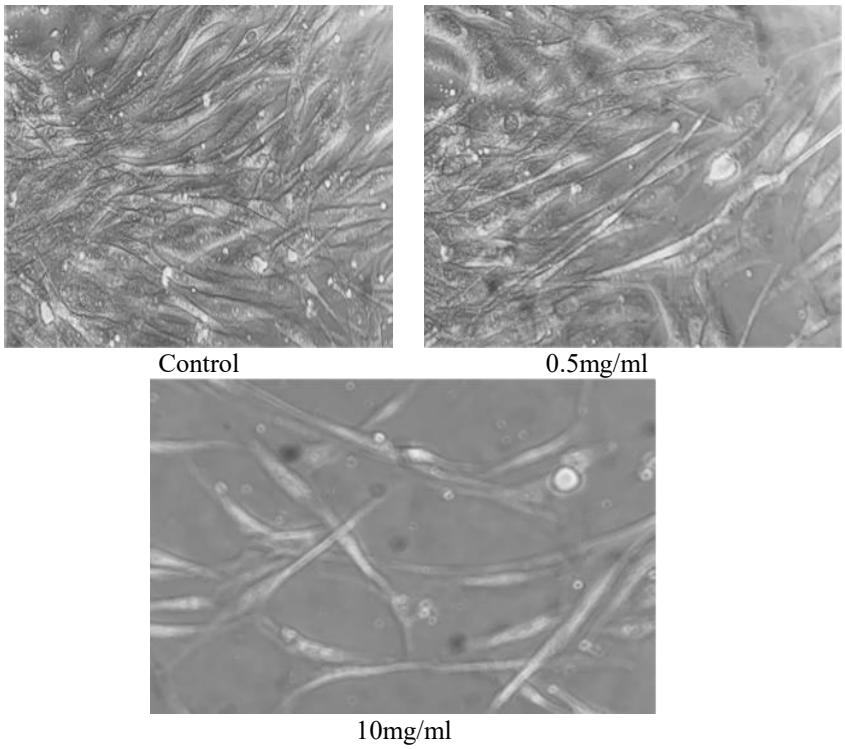


Figure (3): Microscopy at 100x magnification shows the effects of 48 hours of treatment of MCF7 cells with silymarin at doses of 0, 0.5, and 10 mg/ml.

Real-time quantitative PCR for p53 gene expression

An extremely reliable technique for analyzing the expression of 1,718 genes across 72 types of normal human tissue is to use GAPDH as an internal reference for RT-PCR normalization (19). In the early stages of the disease, real-time RT-PCR may be useful for assessing gene expression levels in cancer patients (20). The results demonstrated that treatment of cells with silymarin (highest dose, IC50, 12.1 mg/ml) led to a significantly elevated expression ($P \leq 0.05$) of p53 in comparison to untreated cells (control), with a fold change of 4.5 (Figure 4). The findings validated the antiproliferative impact of silymarin via the activation of p53, which promotes apoptosis. By activating caspase-8, the p53 protein stimulates the mitochondrial apoptosis pathway and causes the release of other apoptogenic components, including cytochrome-C (21) Figure (5).

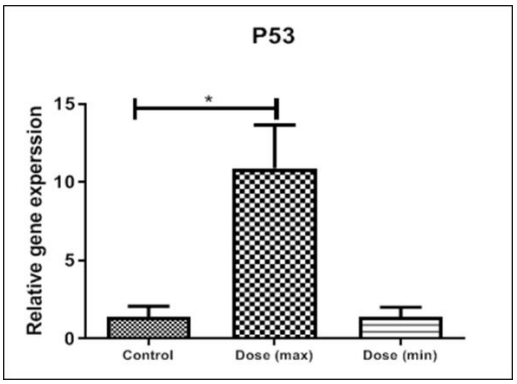
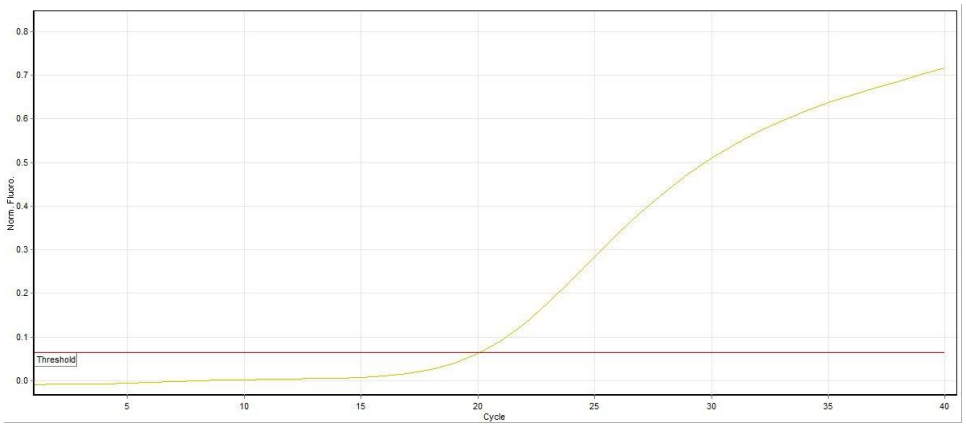
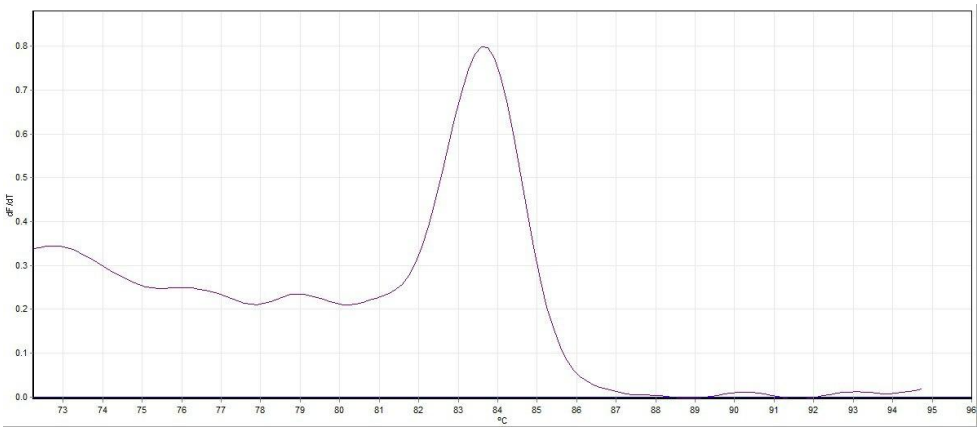


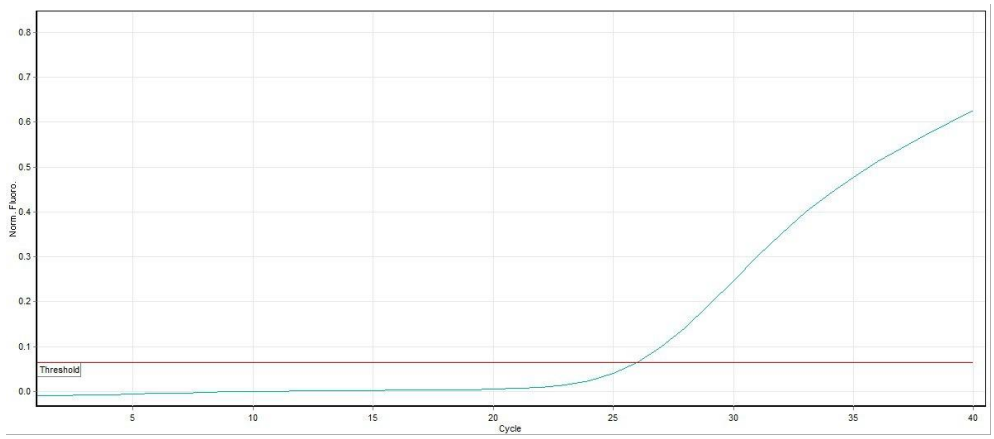
Figure (4): Silimarin enhances the expression of P53 in MCF7 breast cancer cells.



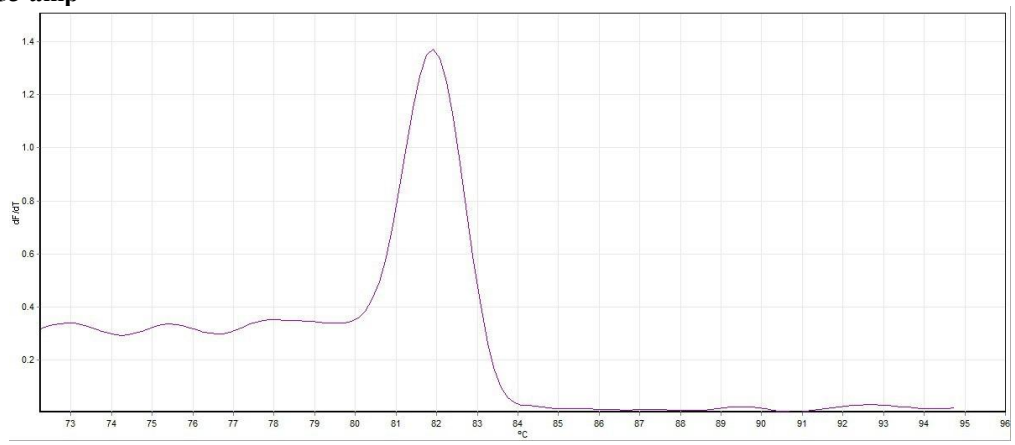
a. gap amp



b. gap melting



c. p53 amp



d. p53 melting

Figure (5): Melting curve of gene expression.

When upstream regulatory proteins, such as p53, are activated, downstream genes are subsequently activated. This process may lead to either cellular repair through cycle arrest or the removal of damaged cells through apoptosis. A variety of tactics are employed, including downregulating some genes, transactivating others, and transcription-independent methods. The initial data about the upregulation of p53 in breast cancer after silymarin treatment are presented in this paper. Impact of plant extract alternatives on p53 gene expression in many cancer cell lines. The extract of Euryale ferox showed apoptotic activity and promoted cell cycle arrest in lung cancer cells by upregulating p53 and downregulating Akt (22). It has been shown that the methanolic extracts of Albizia gummifera, Rhamnus staddo, and Senna didymobotrya boost p53 expression in DU145 prostate cancer cells by about 15.990, 16.066, and 15.985 fold, respectively (23). The assessment investigates the effectiveness of silymarin and doxorubicin in conjunction with the treatment of breast cancer (MCF7). Eight cell groups were created, and varying dosages of doxorubicin and silymarin were delivered. The findings indicate that silymarin improves chemotherapeutic efficacy, mitigates dose-limiting adverse effects, and amplifies the therapeutic potential of doxorubicin

Investigations have been undertaken on the prospective anticancer attributes of silymarin, a flavonoid derived from the milk thistle plant, particularly concerning its impact on human breast

cancer cells. The research demonstrates that silymarin augmented the generation of apoptotic bodies, triggered apoptosis, and reduced cell viability and proliferation. Moreover, it triggered apoptosis, reduced p-ERK1/2 levels, and suppressed the proliferation of MCF-7 tumours in mice. The findings suggest that silymarin may exhibit therapeutic or chemopreventive characteristics, perhaps functioning as a chemopreventive agent (24).

Conclusions

Research conducted in Iraq has proven the anticancer properties of milk thistle seed extract in vitro. The conclusion is that silymarin exerts a cytotoxic effect on MCF7 breast cancer cells and induces overexpression of the p53 gene, potentially serving as a promising treatment for breast cancer patients. The normal cell lines HFF-1 remained unaffected by this extract, yet the growth of the normal cells continued. Consequently, it regards this extract as an alternative to chemotherapy, which harms both healthy and cancerous cells. Consequently, the extract's impact on different cancer types may facilitate the creation of innovative therapies. Moreover, the enhanced study is recommended to ascertain the active compounds that directly influence subsequent cytotoxicity in both in vitro and in vivo contexts.

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