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An Investigation of the Complex Effects of a *Saccharomyces cerevisiae* Cytoplasmic Extract on Apoptosis in K562 Cells

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Abstract

Background: *Saccharomyces cerevisiae*, a common probiotic, can induce in vitro apoptosis in human cancer cells, which could explain its antitumor activity.
Objectives: The present study was conducted to investigate the in vitro effects of a cytoplasmic extract from *S. cerevisiae* on the proliferation and viability of a K562 (chronic myeloid leukemia) cell line.
Methods: *S. cerevisiae* was cultured and then disrupted by sonication. After centrifugation, the harvested supernatant was considered to be a cytoplasmic extract. The protein concentration was determined by the Biuret method and the extract was diluted to concentrations of 500, 1000, and 2000 μg protein/ml. The frequencies of apoptosis and necrosis were assessed in extract-treated K562 cells by electrophoresis to show DNA segmentation and by flow cytometry.
Results: The cytoplasmic extract exhibited a time-dependent antitumor activity. DNA electrophoresis did not reveal apoptosis and necrosis in the treated cells, but the DNA bands were weak. The flow cytometry results indicated the induction of apoptosis as well as necrosis in the K562 cell line and the intensity of apoptosis increased with time.
Conclusions: The cytoplasmic extract of *S. cerevisiae* investigated here may inhibit cell growth and induce apoptosis of chronic myeloid leukemia cells.

Keywords: Apoptosis, Cytoplasmic Extract, K562 Cell Line, *Saccharomyces cerevisiae*

1. Background

Cancer is caused by the cumulative effects of genetic defects (1). The incidence of consecutive mutations leads to the strengthening and extension of the growth potential of cells, which results in the formation of a tumor mass from several cell types with different properties. Therefore, most tumors in the advanced stages consist of heterogeneous structures (2). Cancer cells are unable to perform their normal duties in the body and they also harm adjacent cells through their abnormal growth.

Cancers of the hematopoietic tissue are considered the most common body malignancies. A recent world health organization (WHO) report indicated that 8.2 million people died of various cancers in 2012. A total of 60% of the world's new annual cancer cases now occur in Africa, Asia, and central and south America. Of these, 30% could possibly be prevented.

Based on the categories assigned by the French, American, British, and World health organization groups (3), myeloid as well as lymphoid cancers are divided into separate groups. Both of these types of leukemia are also divided into acute and chronic categories. Therefore, four types of leukemias are recognized: acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML). Unlike the other types of cancers, leukemia is not a packed solid tumor. The bone marrow is the source of this problem; consequently, the treatment of leukemia is much more complex than for solid tumors (4).

K562 cells are derived from myeloid leukemia and are categorized as a form of erythroleukemia. They were first isolated from a 53-year-old woman suffering from chronic myeloid leukemia. These cells are non adherent and round and show a displacement of the *bcr* and *ab1* genes (5).

Apoptosis is a genetically programmed cell death which plays important roles in many physiologic and pathologic conditions. Cell death is often divided into two categories of programmed (apoptosis) and accidental death (necrosis), but another classification recognizes about 11 types of cell death, including apoptosis, necrosis, autophagy, oncosis, and pyroptosis (6).

Probiotics are live microorganisms that, if consumed by humans or animals, are capable of creating a balance in

the intestinal microbial flora, thereby providing beneficial effects on the health of the host. Most probiotics belong to a large group of the major bacteria of the microflora in the human intestine, where they live harmlessly (7). The consumption of probiotics may have anticancer properties through neutralization of the factors in the intestines that can lead to genetic damage (8). One line of thought is that probiotics could play an effective role in disease prevention through reductions in the enzyme concentrations in digesta, in bile salt levels, and in the absorption of harmful mutagens that are mediators of colon cancer. For example, one intestinal microorganism, *Bifidobacterium infantis*, has the ability to suppress tumors by stimulating the host immune system. In addition, in animal models, *B. longum* shows the ability to produce antimutagenic compounds in the colon (9).

The oral consumption of another type of probiotic, the Saccharomyces yeasts, can prevent gastrointestinal diseases and can be used for the prevention or treatment of different types of diarrhea or other intestinal diseases, such as colitis and intestinal ulcers (10). Compounds isolated from *S. cerevisiae* show antimicrobial activities and increase the immune potential by boosting the population of monocytes and neutrophils (11).

In recent years, scientists have paid more attention to preventive food sources; thus, the use of probiotics, prebiotics, and synbiotics has gained importance in the prevention or treatment of cancers. The Lactobacilli, another class of probiotics, stimulate immune reactions in the gastrointestinal tract and mesenteric lymphatic system, and can reduce the development of cancers, infectious diseases, and inflammatory intestinal diseases, as well as prevent allergies in experimental human and animal models (12). Recent investigations have indicated that some lactic acid bacteria, such as *Lactobacillus acidophilus*, *L. casei*, *L. rhamnosus*, and *B. longum* can inhibit the growth of induced (chemical binding) tumor cells in rodents (13). In this regard, the first enzyme to be industrially produced was an amylase from a fungal source, and it was used as a pharmaceutical drug for the treatment of digestive disorders (14).

Previous studies have shown that heat-killed *Saccharomyces cerevisiae* can induce apoptosis and cell death in breast cancer cell lines (MCF-7, ZR-75-1 and HCC70) (15). Breast cancer is a major problem in human populations and requires different therapeutic measures, such as chemotherapy, radiotherapy, and other methods that are all invasive; thus, in all instances, the cancer treatment damages living healthy cells while destroying cancer cells. The use of probiotics such as *Saccharomyces cerevisiae* through an oral route as a food additive could therefore be relevant as a replacement for more invasive cancer treatment methods. This method of treatment was investigated here for the first time.

2. Objectives

The aim of this study was to investigate apoptosis induction in a chronic myeloid leukemia cell line by a complex cytoplasmic extract from *Saccharomyces cerevisiae*.

3. Materials and Methods

3.1. Yeast Preparation and Culture

Saccharomyces cerevisiae (Code 5269) was provided by the Persian type culture collection (PTCC) and cultured in a yeast medium containing yeast extract (Merck, Germany), 5% glucose (Sigma, United States), and 1% K2HPO4 (Sigma, United States) for 72 hours at 28 °C in a shaker incubator (Biotek, South Korea) at 130 rpm. After growth, the medium was washed twice with sterile physiological saline and centrifuged at 3000 rpm for 10 minutes at 4°C (16).

3.2. The preparation of a Cytoplasmic Extract from Saccharomyces cerevisiae

A sonicator (Tomy Co., 7-9-10 Tateishi, Katsushika-ku, Tokyo 124-8511, Japan) was used to break the yeast cells by suspending the cells in cold sodium phosphate buffer (0.1 M, pH = 7.2) and sonicating at a 60% full frequency for 2 minutes. The machine was turned off for 4 minutes to avoid a temperature increase in the solution and sonicator probe. This procedure was repeated several times and the degree of breakage of the yeast cells was evaluated each time by light microscopy (17). The final cell extract was centrifuged at 500 × g and 4°C for 1 minute to pellet the cell debris and the supernatant was considered to be the cytoplasmic extract. The protein content of this cytoplasmic extract was measured by the Biuret method (Protein Assay Kit, Pars Azmoon, Gisha - khash -No. 13, Tehran, Iran) and the extract was then stored at - 80°C until use (18).

3.3. Preparation of Yeast Lysis Buffer (Sodium Phosphate 0.1 M, pH = 7.2)

In first step, 0.0716 g of $Na_2HPO_4.7\ H_2O$ (Sigma, United States) and 0.0285 g of $NaH_2PO_4.2\ H_2O$ (Sigma, United States) were weighed, dissolved in 1 liter distilled water, and sterilized by autoclaving (at 121°C, 15 lb, for 15 minutes) (18).

3.4. Preparation of Different Yeast Cytoplasmic Extract Concentrations

Different serial concentrations of the yeast cytoplasmic extract (500, 1000, and 2000 μg protein/mL) were prepared using the culture medium of the Roswell Park Memorial Institute (RPMI1640) (Gibco, 315 Halliwell Road, Bolton, Lancashire BL1 3PF Greater Manchester, North-West England) (19).

3.5. The Procurement and Culture of Cancer Cell Line

The K562 cell line was procured from the national cell bank of Pasteur institute of Iran (C122) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, England), 100 IU/mL penicillin (Sigma, United States), and 100 μg/mL streptomycin (Sigma, United States) at 37°C in a humidified atmosphere of 5% CO_2 (20).

3.6. Gel electrophoresis and DNA Fragmentation Assays

The differential analysis of apoptosis or necrosis was investigated using the DNA fragmentation test. The K562 cells were treated with increasing concentrations (500, 1000, and 2000 μg protein/ml) of *S. cerevisiae* cytoplasmic extract and then were lysed in lysis buffer (25 mM EDTA (Sinagene, Iran), 100 mM, NaCl 10 mM Tris, 1% sodium dodecyl sulfate (SDS, Merck, Germany), pH = 8). After centrifugation, DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated in absolute ethanol overnight. The precipitated DNA was then dissolved in TE buffer (Tris-HCl, 10 EDTA 10 mM) and subjected to horizontal electrophoresis for 1 hour at 80 V/cm in a 2% agarose gel. The separated DNA was visualized after electrophoresis by ethidium bromide staining (Sinagene, Iran) (21).

3.7. Flow Cytometry

The percentages of apoptotic, necrotic, and viable cells were assessed using acridine orange (AO, Sigma, United States) and propidium iodide (PI, Sigma, United States) stains (22) and flow cytometry analysis according to previously published protocols, with minor modifications (23, 24). Briefly, 5×10^5 K562 cells were suspended in flat bottom wells containing 1000 μL of RPMI medium supplemented with 10% fetal bovine serum (FBS, Gibco, England). The cells were then treated with increasing concentrations (500, 1000, and 2000 μg protein/mL) of the *S. cerevisiae* cytoplasmic extract. After incubation for 24, 48, or 72 hours, the cells were washed with phosphate buffered saline (PBS) and then stained with acridine orange. After 30 minute incubation in darkness, the cells were washed again, stained with PI, and immediately harvested and subjected to flow cytometry analysis. Flow cytometry was performed using a PAS flow cytometer (Partec GmbH, Otto-Hahn-Str. 32, and 48161 Munster, Germany). At least 15000 events for each sample were acquired. Flowmax software was used for data analysis (25).

3.8. The assessment Cell Morphologic Changes

The K562 cells were examined with an inverted microscope (Ziess, Konigsallee 9-21, 37081 Göttingen, Germany). The K562 cells treated with different levels of cytoplasmic extract for 24, 48, or 72 hours were stained with trypan blue (Merck, Germany) to assess any changes in cell morphology. The morphologic changes in nuclei were observed by light microscopy ($\times$ 1000) (26).

4. Results

4.1. Gel Electrophoresis and DNA Fragmentation Assays

The results of gel electrophoresis for K562 cells treated with cytoplasmic extract at 500, 1000, and 2000 μg protein/mL revealed weak DNA bands (Figure 1). An increase in the dose of the cytoplasmic extract resulted in increased destruction of the DNA with time, as indicated by the appearance of increasingly paler bands on the gel; therefore, the effect was both dose and time dependent. The most prominent and the weakest bands of DNA were seen in cells treated with 500 μg/mL and 2000 μg/mL cytoplasmic extract, respectively (Figure 1).

4.2. Flow Cytometry

Flow cytometry revealed the induction of both apoptosis and necrosis in the K562 cells treated with the *S. cerevisiae* cytoplasmic extract. Apoptosis increases were time dependent (Figure 2, Table 1), with levels of 3.49, 12.36, and 34.34% at 24, 48, and 72 hours, respectively, following treatment with 500 μg/mL cytoplasmic extract. The lowest and highest apoptosis levels following treatment with 1000 μg/mL cytoplasmic extract were 12.17% at 24 hours and 44.4% at 72 hours and 6.17% at 24 hours and 36.07% at 72 hours following treatment with 2000 μg/mL cytoplasmic extract. The levels of apoptosis were higher in all text groups when compared to the negative control (live untreated cells).

4.3. The Assessment of Cell Morphologic Changes

Morphologic inspection of the K562 cell line under an inverted microscope ($\times$ 400) confirmed a reduction in living cells (transparent cells in Figure 3) in the groups treated with *S. cerevisiae* cytoplasmic extract, and an increase in the numbers of dead and wizened cells (Figure 3).

Table 1. Cytotoxic Effects of Various Concentrations (μg Protein/mL) of *Saccharomyces cerevisiae* Cytoplasmic Extract on K562 Cells (%)

Time, h	Concentration of Cytoplasmic Extract, μg Protein/mL						Live Cells (Negative Control)	
	500		1000		2000			
	Apoptosis	Necrosis	Apoptosis	Necrosis	Apoptosis	Necrosis	Apoptosis	Necrosis
24	3.49	17.09	12.17	6.58	6.17	22.3	3.02	1.08
48	12.36	4.85	31.76	0.65	43.72	0.32	10.05	0.17
72	34.34	0.72	44.4	39.13	36.07	53.22	14.23	0.66

Light microscopy examinations of apoptotic K562 cells with trypan blue staining ($\times$1000) indicated changes typical of early apoptosis, such as membrane blebbing and chromatin condensation and fragmentation (Figure 3).

The cytoplasmic extract of *S. cerevisiae* clearly caused *in vitro* growth inhibition of the K562 cell line by the induction of apoptosis.

5. Discussion

At present, cancer is one of the most important causes of human mortality in different societies; thus, considerable research efforts are being expended to treat or prevent its complications. Many experiments have used the K562 cell line for *in vitro* study the inhibition of cancer cell growth. For example, Zhong et al. (27) studied the effects of various concentrations of griseofulvin (an antifungal drug) on *in vitro* growth inhibition of K562 cells and found that a 24 hour exposure to this drug dose-dependently reduced cell proliferation. The same authors also confirmed that the griseofulvin effect on K562 cells occurred by an arrest of the cell cycle in the G2/M stage and led ultimately to programmed cell death (apoptosis). Other natural compounds having dose-dependent antitumor activity against K562 cells include gamma linoleic acid (28), curcumin (29), deguelin (30), and emodin (31). The mechanisms underlying the antitumor action of these compounds are likely to involve the induction of apoptosis on K562.

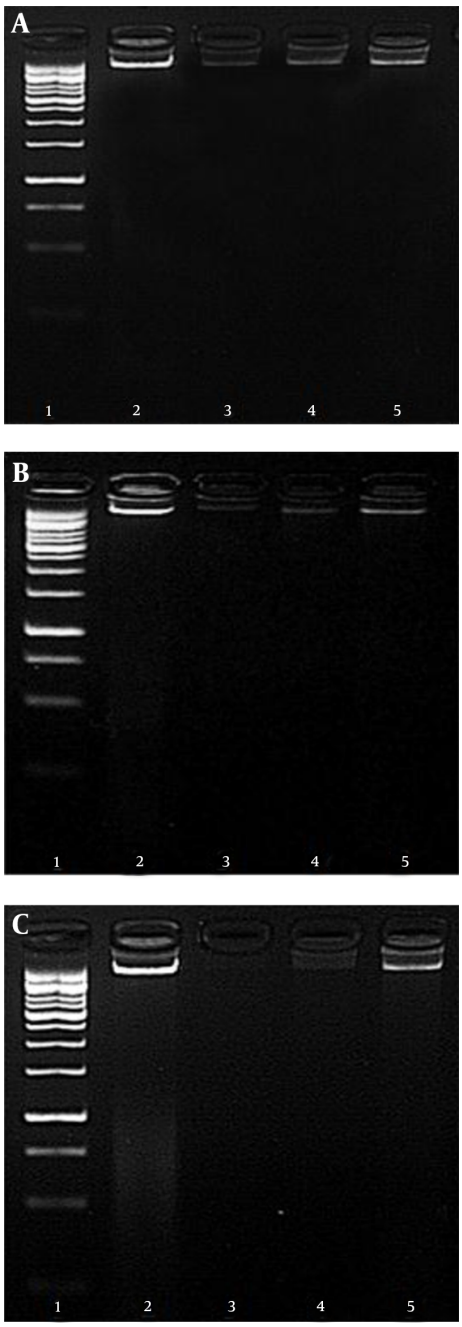
The increased understanding of the action of probiotics in preventing disease and improving health conditions is now leading to the use of these microorganisms to promote patient safety (9). A study conducted by Lee et al. (13) determined that the cytoplasmic extract of *L. casei* and B. animalis had a direct impact on the growth inhibition of different types of tumor cells. According to this report, these probiotics at a 50 μg/mL concentration inhibited the growth of approximately 50% of the tumor cells. A study conducted by Kim et al. (32) that examined the effect of 10 probiotics on the 11 cell lines, including K562, showed inhibition of growth of the K562 cell line by a cytoplasmic extract, whereas addition of a peptidoglycan resulted in no inhibition of K562 cell growth. Addition of whole probiotic cells inhibited the growth of a small percentage of the K562 cells.

Another study examined the anticancer effect of several species of heat killed Lactobacillus and their soluble components (e.g., polysaccharide) by examining DNA fragments of colon cancer cells treated with *L. acidophilus* using polysaccharide gels. Smears of DNA fragments were detected, which were indicative of necrotic cells in the cell population, whereas the PI staining and fluorescence microscopy examination indicated that the soluble polysaccharide fraction induced apoptosis, rather than necrosis, in the cancer cells (33).

Yeasts are probiotics with known anti cancer properties. The results of the present investigation of the anticancer properties of *S. cerevisiae* in the K562 cell line are supported by previous studies. For example, Ghoneum and Gollapudi (34) showed that *S. cerevisiae* could inhibit the growth of most types of human cancer cells including breast, tongue, intestine, and blood cells. Our results show that fixed doses of yeast cytoplasmic extract (500, 1000, and 2000 μg/mL) promoted a time-dependent increase in apoptosis and the destruction of DNA (35). Two of our previous studies confirmed that the cytoplasmic extract of *S. cerevisiae* is dose-dependently and time-dependently cytotoxic to the K562 cell line, as confirmed by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assays and gel electrophoresis (36). Kabiri et al. (19) showed that a cytoplasmic extract of Lactobacillus spp. could inhibit the growth of K562 cells in a dose dependent manner. A recent study by Abedigheshlaghi et al. (37) showed that ethanol and n-butanol extracts could induce cytotoxicity in the K562 cell line in a dose-dependent manner (37). Our findings are consistent with these previous reports.

In our previous study (36), we investigated apoptosis and necrosis in the K562 cell line using qualitative methods and higher extract doses (2000, 4000, and 8000 μg protein/ml) than in the current study, but we found similar results (weak DNA bands). The reason for this similarity is not yet clear; however, according to previous reports, the existence of nucleases in the cytoplasmic extracts of *S. cerevisiae* can lead to digestion and elimination of DNA in K562 cells. For instance, the Apn1 and Apn2 enzymes that have 3'-

Figure 1. K562 Cell Death Following Treatment With a Cytoplasmic Extract From *Saccharomyces cerevisiae* .



A; 24, B; 48; C, 72 hours after treatment; band 1, 1Kb marker; band 2, negative control; band 3, cells treated with 2000 μg protein/mL cytoplasmic extract; band 4, cells treated with 1000 μg protein/mL cytoplasmic extract; Band 5, cells treated with 500 μg protein/mL cytoplasmic extract.

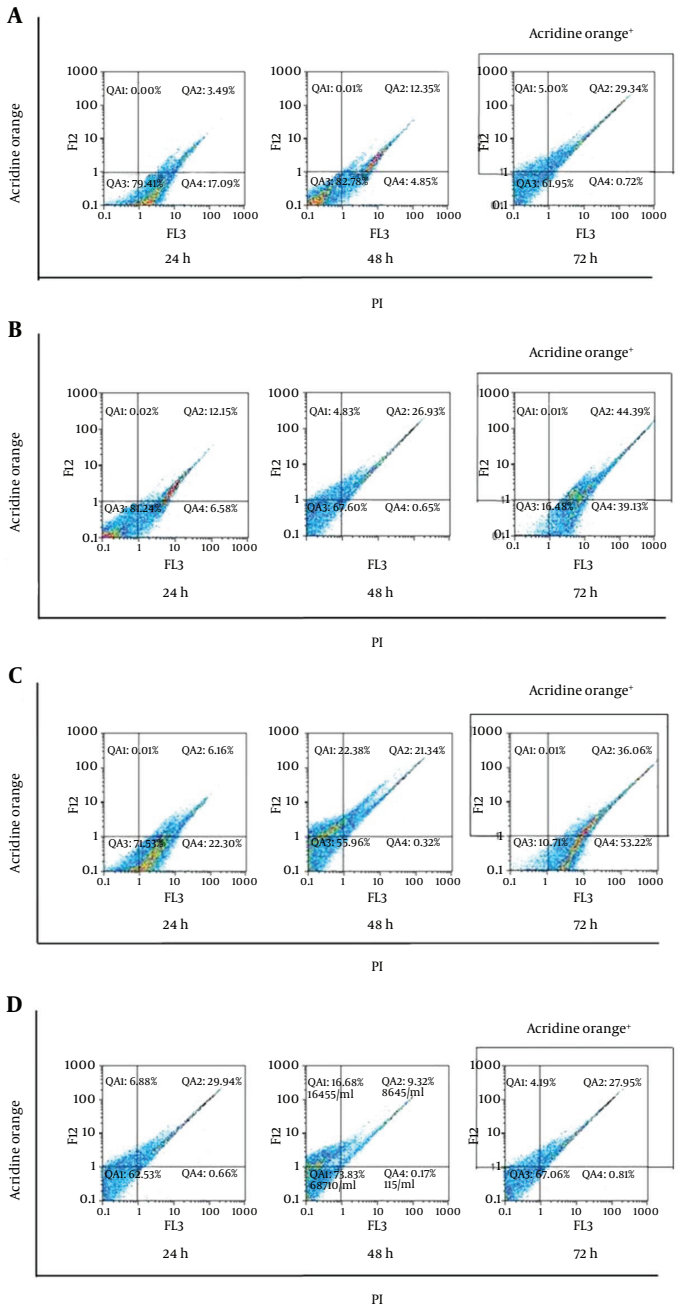
phosphodiesterase activity are present in *S. cerevisiae*. Apn1 is reported to be responsible for more than 90% and Apn2 (which also has 5'-phosphodiesterase activity) is responsible for 10% of this activity (38). In addition, some nucleases in yeast are involved in transcription and repairing of DNA damage and continually perform activities as chromosomal endonucleases and exonucleases (39).

In the present study, flow cytometry was used to obtain quantitative data on apoptosis and necrosis in the K562 cells. The cytoplasmic extract from *S. cerevisiae* clearly resulted in a time-dependent induction of apoptosis in the K562 cell line. Notably, apoptosis was increased with time by all concentrations of the cytoplasmic extract, when compared to negative control cells. The results of a previous study indicated that the metabolites produced by a vaginal *Enterococcus faecalis* strain used as a probiotic could represent an alternative to pharmaceutical compounds. The metabolites showed promising therapeutic indices because they were not cytotoxic to normal mammalian cells (40). Jafari et al. (2013) stated that lovastatin can induce apoptosis in HT29 human colon cells (41). The existing literature does not yet provide a reason for the destructive effect of *S. cerevisiae* on tumor cells; however, the mechanism underlying the antitumor properties of yeast probably involves the compounds present in them that stimulate innate immunity and subsequently increase the activity of macrophages, neutrophils, and natural killer cells involve in the synthesis and secretion of cytokines (42).

The details of probiotic action remain obscure. Possible mechanisms that regulate the recognition between ingested probiotic cells and the host have been partially elucidated. An animal study revealed that Lactobacillus phagocytosed by macrophages in the Peyer's patch could be observed by histological examination (43). Murine macrophages stimulated with Lactobacillus also produced IL-12 (Interleukin-12) *in vitro* (44). These observations suggest that probiotics may be degraded in gut-associated lymphoid tissue, and their signal from immune competent cells then leads to a systemic effect. Therefore, in order for a probiotic to exert its systemic effects, the migration of viable probiotic cells from the gut to the peripheral circulation may not be required (43).

Delineating the precise mechanism of probiotic action will provide insights into the development of rational approaches to cancer control. The results of the present study indicate that a cytoplasmic extract of *S. cerevisiae* has antitumor properties, as indicated by the cytotoxic effects on K562 exerted through inhibition of cell growth and induction of apoptosis. Therefore, probiotics would appear to have significant potential for use as therapeutic agents in patients with chronic myeloid leukemia.

Figure 2. Dot Plots Showing Representative Data From Three Separate Experiments



All acridine orange + cells (including acridine orange + PI - cells (upper right quadrant) and acridine + PI + cells (upper left quadrants)) were identified as apoptotic cells. A, frequency of K562 cell apoptosis and necrosis at 24, 48, and 72 hours following treatment with a cytoplasmic extract (500 μg/mL) from *Saccharomyces cerevisiae*; B, Frequency of K562 cell apoptosis and necrosis at 24, 48, and 72 hours following treatment with cytoplasmic extract (1000 μg/mL); C, frequency of K562 cell apoptosis and necrosis at 24, 48, and 72 hours following treatment with cytoplasmic extract (2000 μg/mL); D, frequency of K562 cell apoptosis and necrosis at 24, 48, and 72 hours in control untreated cells.

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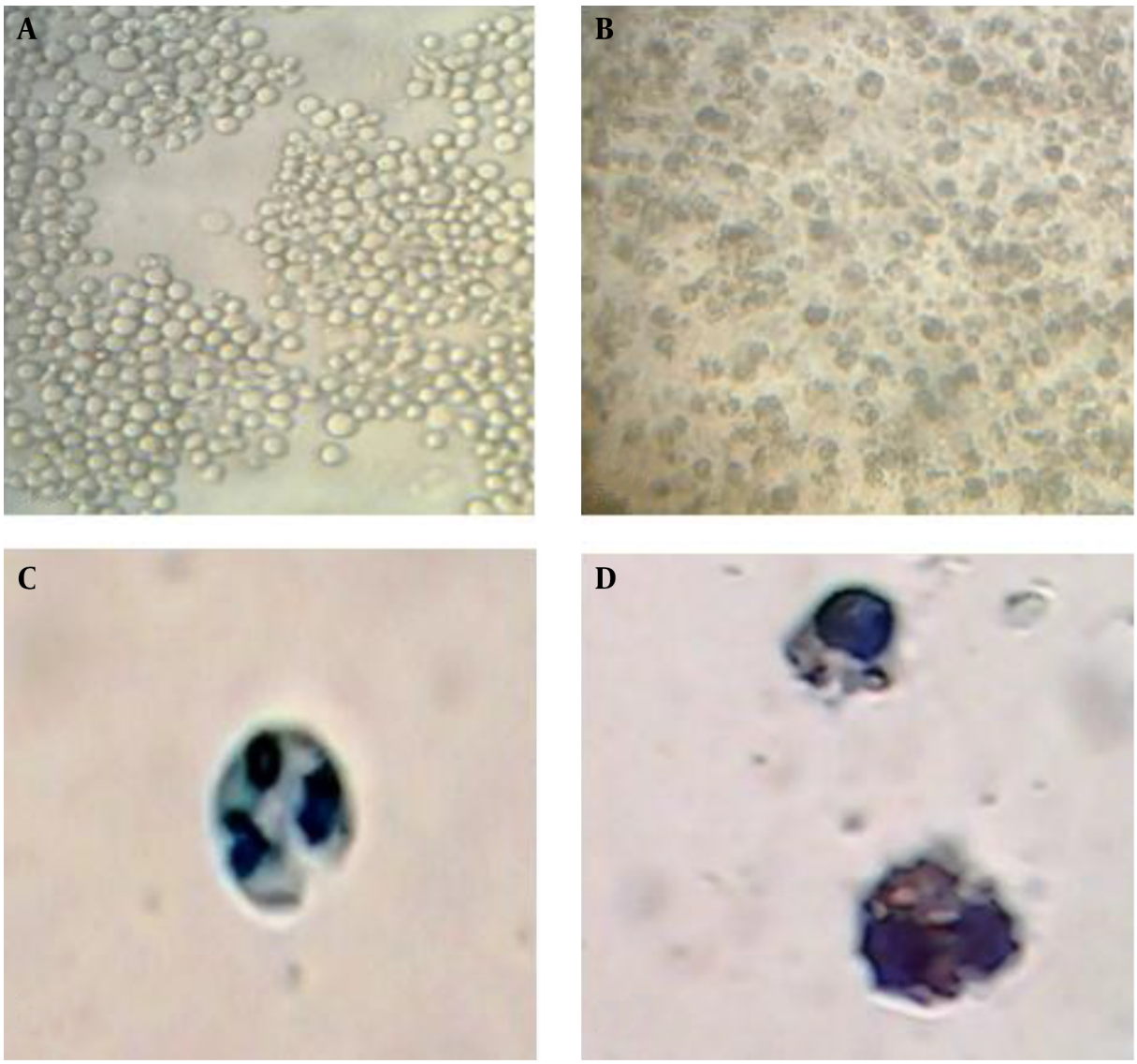

Figure 3. A, Photomicrograph of Live K562 Cells (× 400); B, Photomicrograph of Apoptotic K562 Cells (× 400); C, Apoptotic K562 Cells Showing Chromatin Fragmentation; D, Apoptotic K562 Cells Showing Intense Chromatin Condensation and Fragmentation; Trypan Blue Stain, × 1000

Footnote

Authors' Contribution: Study concept and design: Farzaneh Bonyadi, Amir Tukmechi, and Vahid Nejati; acquisition of data: Farzaneh Bonyadi, Amir Tukmechi, and Aram Mokarizadeh; analysis and interpretation of data: Farzaneh Bonyadi and Aram Mokarizadeh; drafting of the manuscript: Farzaneh Bonyadi, Shapoor Hasanzadeh, and Amir Tukmechi; critical revision of the manuscript for important intellectual content: Farzaneh Bonyadi and Shapoor Hasanzadeh; statistical analysis: Farzaneh Bonyadi, Aram Mokarizadeh, and Amir Tukmechi; administrative, technical, and material support: Amir Tukmechi, Vahid Nejati, and Aram Mokarizadeh; study supervision: Shapoor Hasanzadeh, Farzaneh Bonyadi, Vahid Nejati, Aram Mokarizadeh, and Amir Tukmechi.

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