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In Vitro AnticancerPropertiesof Kefirand Kefir Products Produced by aNovel Method in Syria

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Abstract : *Kefir, which is a fermented milk product have many beneficial health effects. One of these health effects is its role as anticancer agent. In the current study we investigated the anticancer properties of kefir produced in Syria by a novel method, against human sarcoma cells in vitro. Moreover the exopolysaccharides (EPS), the kefir treated with sodium bicarbonate (alkaline kefir) (AK) and EPS treated with sodium bicarbonate (alkaline EPS) (AEPS), were investigated to determine their anticancer effects on the same cancer cell line (human sarcoma), and the alkaline solution was used as control at the same concentrations. Human sarcoma cell line was obtained from Al-Bairouni Hospital, Damascus-Syria and was preserved in liquid Nitrogen until it was used to determine anticancer effects of kefir and kefir products. Eight concentrations of kefir and AK were prepared: 250, 125, 60, 30, 15, 7.5, 3.75 and 2.5 µl.ml^{-1}. Similarly, eight concentrations of EPS and AEPS were prepared (250, 125, 60, 30, 15, 7.5, 3.75 and 2.5 µg.ml^{-1}).The anticancer effect was expressed as the percentage of cancer cell killing ratios. Results revealed the anticancer properties of kefir, AK and EPS in different concentration. The AK was superior to kefir and other kefir products, with the concentration of 3.75 µl.ml^{-1} was capable to kill all sarcoma cells. Kefir was capable to kill all sarcoma cells in concentration of 250 µl.ml^{-1}, and EPS could kill all sarcoma cells in a concentration of 30 µg.ml^{-1}, and 98.5% of the sarcoma cells in a concentration of 3.75 µg.ml^{-1}, while AEPS could kill all sarcoma cells in the concentration of 60 µg.ml^{-1}, and 75% of their in a concentration of 30 µg.ml^{-1}. while the alkaline solution was not effective against sarcoma cells.*

Keywords: *Alkaline exopolysaccharide (AEPS), Alkaline kefir (AK), Anticancer, Exopolysaccharide (EPS), Human sarcoma, Kefir.*

I. Introduction

Kefir is a fermented dairy beverage produced by the actions of the microflora encased in the ‘‘kefir grain’’ on the carbohydrates in the milk[1]. The name ‘‘kefir’’ is likely derived from the Turkish word ‘‘keyif’’ which means ‘‘good feeling’’ [2]. It was originated in central Asia between the Caucasus Mountains and Mongolia, and is very popular in many countries nowadays, such as Turkey, Russia, Poland, Czech Republic, Slovakia, Hungary, Bulgaria, Scandinavian countries, The United States, Brazil and Japan [3-9]. It has long been popular in Eastern Europe for its purported health benefits, where it is routinely administered to patients in hospitals and recommended for infants and the infirm [1]. Kefir is an acidic, viscous, somewhat effervescent, slightly alcoholic milk beverage produced by the actions of bacteria and yeast embedded in a resilient, insoluble protein and polysaccharide matrix [10-12]. Containing many bacterial species already known for their probiotic properties[1].Kefir differs from other fermented milk products in its unique starter culture, which is an aggregation of many different bacteria and yeast species bound together in an exopolysaccharide matrix produced by certain lactic acid bacteria. Farnsworth [13]describes kefir grains as a mass of bacteria, yeasts, polysaccharides, and other products of bacterial metabolism, together with curds of milk proteins. The starter cultures, termed “grains”, grow, propagate and pass their properties along to the following generations of grains [14].

The traditional method of producing kefir is achieved by directly adding kefir grains (2-10%) to milk that has been pasteurized and cooled to 20-25°C. After a period of fermentation lasting approximately 24 h, the grains are removed by filtration. The beverage, itself containing live microflora from the grains is then ready for consumption. The grains that grow in the process of kefir production are reused for subsequent fermentation. A second method, known as the "Russian method ", permits the production of kefir on a larger scale, and uses two fermentation steps. The first step is to prepare the cultures by incubating milk with grains (2-3%). The grains are then removed by filtration and the resulting mother culture is added to milk (1-3%), which is fermented for 12 to 18 h [15].

Cancer, abnormal division and reproduction of cells that can spread throughout the body [16], is a leading cause of death worldwide and the total number of cases is increasing globally [17]. A recent analysis estimated 13.2 million deaths and 20.3 million incident cases for cancer in 2030 [18]. Thus preventive strategies are critical in every cancer control plan [17]. Among factors responsible for cancer, it has been estimated that

diet accounts for about 20%–30% of all cases worldwide. Several studies support the idea that some dietary components, such as fruits and vegetables, are protective factors against cancer [19]. There is also evidence that a diet rich in fermented foods such as fermented milk products may have beneficial properties in reducing risk of some cancers [20-22].The definition of anti-carcinogenic is 'tending to inhibit or prevent the activity of a carcinogen or the development of carcinoma'. Tumors are classified as carcinomas or sarcomas. Sarcoma tumors are derived from supportive or connective tissues such as bone, fat, and cartilage [23].

Health aspects attributed to the consumption of kefir, as similar to other fermented dairy foods supplemented with probiotic bacteria, include, but are not limited to, improved lactose utilization, anticarcinogenic activity, control of intestinal infections and improved flavor and nutritional quality of the milk [24].

Kefir enjoys a rich tradition of beneficial claims. Consumption of kefir has been used in the former Soviet Union for the treatment of a variety of conditions including metabolic disorders, atherosclerosis, cancer, and gastrointestinal disorders [25]. Although there is lack of clinical trials or population based studies on kefir, in a case-control study done by Ronco et al [26] in Montevideo, Uruguay, consumption of ricotta cheese and skim yogurt were associated with a significant decreased risk of breast cancer. Low-fat and fermented products combined appeared to be the most protective dairy foods. Kefir contains a greater number of different bacteria than yogurt. Therefore consumption of kefir may have the similar effect. Encouraging results regarding the antitumor properties of kefir have been reported in animal studies [27, 28, 29, 30]. Components in fermented milk such as conjugated linoleic acid (CLA) [31], sphingolipids [32,33] polysaccharides [30], organic acids [34], and some proteins and peptides [35] have been shown to possess antimutagenic and antitumor effects.

Guven et al. [36] proposed an alternative suggestion as to how kefir may protect tissues. They found that mice exposed to carbon tetrachloride (a hepatotoxin to induce oxidative damage) and given kefir by gavage showed decreased levels of liver and kidney malondialdehyde, indicating that kefir was acting as an antioxidant. Their data also indicated that kefir was more effective than vitamin E (which is well known to have antioxidative properties) in combating oxidative damage. Many studies have shown evidence to warrant the use of probiotic foods like kefir in the treatment of gastrointestinal disturbances [37]. One example is diarrhea, which can be caused by a variety of conditions. Probiotics help in preventing diarrhea and in reducing its duration; they also alleviate conditions such as infant's diarrhea, irritable bowel syndrome, colitis, Crohn's disease, gastroenteritis, and traveler's diarrhea [38]. The consumption of kefir has shown good results in mitigating the symptoms of chronic constipation [39].

II. Materials And Methods

Sarcoma cell line source

Human sarcoma cell line were obtained from Al-Bairouni Hospital, Damascus-Syria. The cell line was preserved in liquid Nitrogen (-196° C) until it was used in the anticancer test.

Kefir grains preparation

Kefir grains were prepared by a novel method: goat colostrum was obtained from Damascus, Syria. Natural microflora was used to ferment colostrum to produce kefir grains, by incubating 500 ml of fresh colostrum at 37° C for 72 h, with stirring occasionally (every 4 h.). This process was repeated 3 times to ensure the constant properties of kefir grains produced. Kefir grains were obtained by filtration of colostrum including kefir grains by means of clean gauze.

EPS extraction

EPS was extracted by transferring 10 of filtrated kefir into 50 ml centrifuge plastic tubes, the tubes then heated in water bath at 100° C for 1 h. to release the EPS [40]. Then the tubes cooled to room temperature, and centrifuged at 8000 rpm at 4° C for 20 m, and the precipitate was discarded. Two volumes of ethanol 95% (v/v) were added to the supernatant and the solution was kept at 4° C for 24 h. The tubes and their contents were centrifuged at 8000 rpm at 4° C for 30 m and supernatant was discarded to obtain EPS.

Spectrophotometric determination of EPS

The resultant precipitate was dissolved in 10 ml of deionized water. Then the solution was centrifuged 8000 rpm at 4° C for 10 min, and the precipitate was discarded. The EPS amount was determined by phenol-sulfuric acid method: 1 ml of the supernatant was taken from the last centrifuge procedure and 1 ml of phenol solution (5% in deionized water w/v) was added, and finally 5 ml of concentrated sulfuric acid (density=1.84 g/cm^3) was added dropwise with gentle stirring. The absorbance of the solution was measured at 480 nm using spectrophotometer[41]. The amount of EPS was calculated by plotting the absorbance against standard curve of glucose (100, 50, 25 and 10 mg/l).

Preparation of kefir and EPS

The kefir solution was passed through 0.20 µm sterile filter after primary filtration using clean gauze to separate kefir grains. EPS solution was prepared by dissolving the precipitated EPS after final centrifuging with deionized water to obtain suitable concentrations, and finally filtered through 0.20 µm sterile filter.

Preparation of AK and AEPS

7.00 g of sodium bicarbonate was dissolved in 100 ml of deionized water, sterilized by filtration through 0.20 µm filter. The resultant solution was used to prepare the AK and AEPS in the concentrations of 250, 125, 60, 30, 15, 7.5, 3.75 and 2.5 $\mu g.ml^{-1}$.

The effect of kefir, AK, EPS and AEPS on human sarcoma cells in vitro

The human sarcoma cells line suspended in DMEM medium were distributed in a microtiter plate containing 96 well , where each ml of the medium contained 10,000 cell/ml. The suitable amounts of kefir and AK, were added to the microtiter plate wells to obtain concentrations of (250, 125, 60, 30, 15, 7.5, 3.75 and 2.5 $\mu l.ml^{-1}$**)**, while EPS and AEPS were added to the microtiter plate wells to obtain concentrations of (250, 125, 60, 30, 15, 7.5, 3.75 and 2.5 $\mu g.ml^{-1}$). All tests were repeated in triplicate. The microtiter plate then was transferred to humid incubator maintained at 37° C, and incubated in 5% CO_2atmosphere for 24 h. After the incubation time passed the trypsin was added and microtiter plate was reincubated at 37° C for 5 min. The cells centrifuged at 2000 rpm for 5 min and the precipitates were resuspended in trypan blue and the number of viable and dead cells were estimated using Thoma Zeiss counting cell. The control test was done at the same conditions, but without the addition of kefir, AK, EPS or AEPS. Another control test was done by adding the alkaline solution only.

III. Results

EPS determination

The standard curve using the glucose in the concentrations of 100, 50, 25 and 10 $mg.ml^{-1}$ in water when absorption measured at 480 nm , revealed that the equation was as follows:

$Y = 0.00043\ X\ (R^2 = 0.99)$

Where:Y = absorption, X = concentration

The kefir prepared by the novel method described in the "materials and methods" section contains a very high concentration of EPS 3.869 $g.l^{-1}$.

Anticancer properties of the kefir and kefir products

The anticancer properties of kefir and kefir products were estimated against human sarcoma cells. Eight concentrations of kefir and AK (expressed as $\mu l.ml^{-1}$), and eight concentrations of EPS and AEPS (expressed as $\mu g.ml^{-1}$) were prepared and were used to treat sarcoma cells in vitro. The effectiveness of kefir and kefir products was expressed as kill ratio after 24 h of treatment. Results of kill ratio after treatment with kefir, AK, EPS and AEPS are shown in Table 1.

Table 1 revealed that the AK was the most effective against human sarcoma cell when compared with kefir, it could kill all sarcoma cells in a concentration of 3.75 $\mu l.ml^{-1}$ of DMEM medium (Fig 1.D). Kefir could kill all sarcoma cells in a concentration of 250 $\mu l.ml^{-1}$, while it was able to kill only 50% of sarcoma cell when its concentration decreased into 125 $\mu l.ml^{-1}$ (Fig 1.C). EPS was more effective than AEPS, the former was able to kill all sarcoma cells in a concentration of 30 $\mu g.ml^{-1}$ and 98.5% of the cells at a very low concentration, 3.75 $\mu g.ml^{-1}$(Fig 1.E), while the later was able to kill all the cells at 60 $\mu g.ml^{-1}$, and only 25% when its concentration decreased into 30 $\mu g.ml^{-1}$ (Fig 1.F).

The evidence that the alkaline (sodium bicarbonate) has no effect on human sarcoma cells is that the alkaline treatment was not effective in killing sarcoma cells at the same concentrations.

All of the kefir products were passed through 0.20 µm sterile filters, that means they were free of live bacteria/yeasts (kefir and AK) or dead bacteria/yeasts (EPS and AEPS), this omit the emphasize that proposed the live lactic acid bacteria/yeasts have a direct role as anticancer agent (at least in the current research).

IV. Discussion

In the current study, it is revealed that kefir was able to kill sarcoma cells in vitro. This result is corresponding with two studies were identified on the antitumor effects of kefir in different types of sarcoma cells[27, 42]. Liuet al. [42] investigated the antitumor effects of oral administration of soy milk kefir, prepared by inoculation of soy milk with kefir grains, and milk kefir on female mice bearing sarcoma tumor cells. One week after tumorinoculation, with oral administration of 5mL/kg/day of differenttreatments for 30 days, tumor growth was inhibited 64.8% and 70.9% in the tumor-bearing group with milk and soy milk kefir respectively, compared with controls. Tumor volume was measured to assess antitumor activity of kefir.In another survey on

a different form of sarcoma cells in mice, the beneficial therapeutic effects of kefir to decrease tumor size were established by Cevikbas et al.[27]following 20 days of treatment with 0.5 mL kefir, compared with the saline administrated to the controls, reduction was seen in tumor size based on size oftumors after treatment. Furthermore, tumor disappearance was observed in those mice receiving kefir treatments.

The EPS prepared in the current study from kefir was effective in killing human sarcoma cells in vitro in low concentration (as little as 30 $\mu g.ml^{-1}$ of the medium to kill 100% of the sarcoma cells, and 3.75 $\mu g.ml^{-1}$ to kill almost all of the sarcoma cells). This result is not corresponding with that recorded by Shiomi et al.[30] studied the effect of a water-soluble polysaccharide (KGF-C) isolated from kefir of sarcoma tumor cells inoculated in male mice.It wasgiven to the mice atlibitum in drinking water in different concentrations.Oral administration of KGF-C, compared to the controls,inhibited the growth of both tumor cells which was measured bythe difference between tumor weight in the intervention and controlgroups. But, in vitro direct cytotoxicity of KGF-C on tumorcells was not as oral administration. There was no or little direct cytotoxicityagainst the tumor cell based on the ratio of the dead cells to thetotal cells.

V. Conclusion

In the current study, we have investigated the anticancer potential of kefir and some kefir products against a human sarcoma cell line in vitro. The kefir grains were made by a novel method in Syria, and were unique in microbial composition (results are not showed here). Results demonstrated the anticancer efficiency of all kefir/kefir products. Alkaline kefir (AK) was the most effective product against human sarcoma cells in vitro, followed by exopolysaccharides (EPS), Alkaline exopolysaccharides (AEPS), and finally kefir. As a result of the current study we suggest that AK can be used to treat human sarcomain vivo,because kefir is conventional drink in many countries, and the addition of sodium bicarbonate is safe as food additive.

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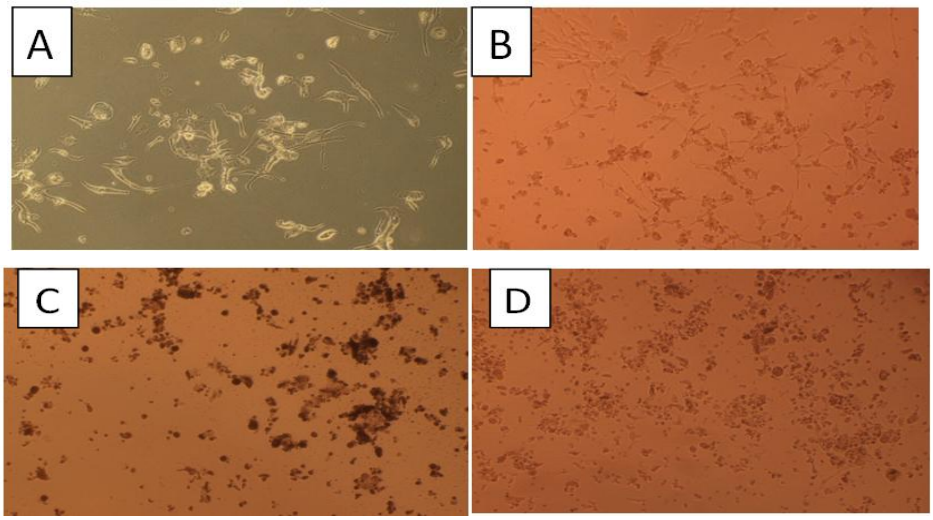
Table 1. Kill ratios of human sarcoma cells after treatment with: Kefir (K), alkaline kefir (AK), exopolysaccharide (EPS) and alkaline exopolysaccharide (AEPS) at different concentrations.

Treatment	Concentration (µl.ml^{-1} for K and AK, µg.ml^{-1} for EPS and AEPS)							
	250	125	60	30	15	7.5	3.75	2.5
K	100	50	0	0	0	0	0	0
AK	100	100	100	100	100	100	100	0
EPS	100	100	100	100	99	98.5	98.5	0
AEPS	100	100	100	25	0	0	0	0
Alkaline	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0



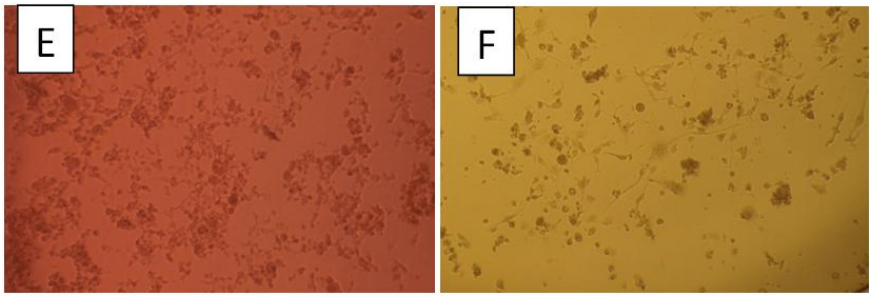


Fig 1. Human sarcoma cells after different treatments in vitro: A, untreated cells (control); B, cells treated with alkaline (sodium bicarbonate); C, cells treated with kefir; D, cells treated with AK; E, cells treated with EPS; F, cells treated with AEPS.