Prophylactic Effects of Dairy *Kluyveromyces marxianus* YAS through Overexpression of BAX, CASP 3, CASP 8 and CASP 9 on Human Colon Cancer Cell Lines

Amir Saber Gharamaleki, Beitollah Alipour, Zeinab Faghfoori, Ahmad YariKhosroushahi

Abstract—Colorectal cancer (CRC) is one of the most prevalent cancers and intestinal microbial community plays an important role in colorectal tumorigenesis. Probiotics have recently been assessed as effective anti-proliferative agents and thus this study was performed to examine whether CRC undergo apoptosis by treating with isolated Iranian native dairy yeast, *Kluyveromyces marxianus* YAS, secretion metabolites. The cytotoxicity assessments on cells (HT-29, Caco-2) were accomplished through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as well as qualitative DAPI (4',6-diamidino-2-phenylindole staining) and quantitative (flow cytometry assessments) evaluations of apoptosis. To evaluate the main mechanism of apoptosis, Real time PCR method was applied. *Kluyveromyces marxianus* YAS secretions (IC50) showed significant cytotoxicity against HT-29 and Caco-2 cancer cell lines (66.57 % and 66.34 % apoptosis) similar to 5-Fluorouracil (5-FU) while apoptosis only was developed in 27.57 % of KDR normal cells. The prophylactic effects of *Kluyveromyces marxianus* (PTCC 5195), as a reference yeast, was not similar to *Kluyveromyces marxianus* YAS indicating strain dependency of bioactivities on CRC disease prevention. Based on real time PCR results, the main cytotoxicity is related to apoptosis phenomenon and the core related mechanism is depended on the overexpression of BAX, CASP 9, CASP 8 and CASP 3 inducing apoptosis genes. However, several investigations should be conducted to precisely determine the effective compounds to be used as anticancer therapeutics in the future.

Keywords—Anticancer, anti-proliferative, apoptosis, cytotoxicity, yeast.

I. INTRODUCTION

CANCER, one of the most prevalent non communicable diseases, is a serious health related problem worldwide

A. Saber Gharamaleki is with Department of Biochemistry and Diet Therapy, Nutrition Research Center, Faculty of Nutrition, Student Research Committee, Tabriz University of Medical Sciences, Daneshgah Street, Tabriz, Iran (phone: +98-914-332-2602; fax: +98-41-3334-0634; e-mail: amirsbr@yahoo.com).

B. Alipour is with Faculty of Nutrition, Tabriz University of Medical Sciences, Daneshgah Street, Tabriz, Iran (phone: +98-41-3337-6228; fax: +98-41-3334-0634; email: alipourb@tbzmed.ac.ir).

Z. Faghfoori is with Biotechnology Research Center, Student Research Committee, Faculty of Nutrition, Tabriz University of Medical Sciences, Tabriz, Iran (phone: +98-914-306-1994; fax: +98-41-3334-0634; email: zfaghfoori@gmail.com).

A. YariKhosroushahi is with Faculty of Pharmacy, Tabriz University of Medical Sciences, Daneshgah Street, Tabriz, Iran (phone: +98 41 33372250-1; P.O. Box 51664-14766; Fax: +98 41 33344798; e-mail: Yarikhosroushahia@tbzmed.ac.ir).

and its incidence shows a growing trend [1], [2]. Among the other types of cancer, CRC is the most common malignancy of the gastrointestinal tract and the third most frequent cancer where more than 1 million people are globally diagnosed with CRC each year leading to about 0.5 million deaths [3], [4]. Approximately 70% of CRCs begin with unknown etiology [5]. The large and complex microbial community hosted by the large intestine and known as "gut microbiota" plays evidently an important role in the physiology of the host and also colorectal tumorigenesis [6], [7]. The human intestinal microbiota contains about 1014 organisms, and their collective genome is approximately 150 times more than the human genome [8]. The intestinal microbiota can influence the cellular proliferation, differentiation and expression of genes in intestine epithelial cells [5], [7]. Regarding to the involvement of the endogenous microflora in the onset and development of CRC, all changing in the intestinal microflora may be able to affect tumor development/suppression. Therefore, much attention has recently been focused on the strategies that can influence the gut microflora particularly in the direction of increasing the probiotic counts via direct administration or using prebiotics [9]. Probiotics are live microorganisms when administered in adequate amounts confer a health benefit on the host [10]. The most common types of yeasts, has been used as probiotics and some of them shows cancer preventing properties. Different studies proved that gut microflora is involved in the etiology of cancer [11]. The quantitative and qualitative alterations along the length of the GI tract is reflected in xenobiotic biotransformation, carcinogen synthesis and activation [12]. According to investigations probiotics are able to modify the gastrointestinal enzymes activity and inhibit carcinogenic factors and pre-cancerous lesions [13]. In this regard, the modification of the microbiota due to dietary amendment may change the process of carcinogenesis. Thus, different probiotics has attracted much attention toward the improvement of intestinal flora.

Although, the lactic acid producing bacteria are the famous probiotic microorganisms in human microbiota, but some benign yeast strains that exist in dairy products are classified as probiotics. Among the multiple yeast families, *Saccharomyces cerevisiae*, *Saccharomyces boulardii* and some strains of *Candida*, *Pichia*, and *Kluyveromyces* are well known probiotics that possesses beneficial effects on health [11]. Numerous investigations have revealed the ability of

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

yeasts to induce apoptosis in some cancerous cell lines such as, breast, tongue and colon [14]-[17]. Moreover, *Saccharomyces boulardii* downregulated extracellular signal-regulated kinase (Erk) 1/2 and mitogen activated protein kinase (MAPK) pathway *in vitro* and *in vivo* [18], [19]. ERKs are associated with cell proliferation, transformation, and metastasis development [20], whereas the Akt pathway regulates cell survival processes [21]. Different studies showed that activation of EGFR signaling pathway can stimulate proliferation, angiogenesis and metastasis, while suppresses apoptosis [22]-[25]. However, the suppression of these pathways via downregulation of regulator genes or by interruption of proliferation mechanism in malignant cells resulted in apoptosis. Usually, apoptosis can be triggered by development and aging and act as a homeostatic mechanism to keep cell populations in tissues. But, different physiological and pathological situations such as; disease, exposure to irradiation, chemical and physical harmful agents [26], oxidative stress [27], the removal of growth factors [28] and chemotherapy resulted in apoptosis through intrinsic or extrinsic pathways [29]. However, the induction of apoptosis is the main pathway of various anticancer drugs [30]. Experimental studies showed that the common anticancer drugs besides inducing of apoptosis in malignant cells possess indiscriminative killing in normal cells [31]. By considering these side effects, investigators are trying to find new compounds that induce apoptosis in cancerous cells without or with negligible side effects on normal cells. Yeasts ease apoptosis process through upregulation of pro-apoptotic protein, Bax, as well as downregulation of anti-apoptotic protein Bcl-2 in the MDA-MB-231 cells [32]. These imbalances toward expression of Bax protein may be responsible for the yeast-induced apoptosis. In this regard, induction of apoptosis by safe and natural compounds may be expedient approach in order to destroying tumor cells [33]. Previous investigations verified beneficial effects of common probiotic yeasts on different cancers, but the most of researchers have been focused on β-glucan (polysaccharide derived from the intermediate layer of the cell wall of yeasts) or live and killed yeasts [34]-[36]. However, other important yeasts with probiotic properties and their secretory compound(s) may be more effective than common yeasts and should not be ignored. In the present study, to determine which pathway possesses the effective functionality in apoptosis related signaling, we investigated the intrinsic and extrinsic apoptosis pathway crucial genes such as BAX (crucial gene in extrinsic IL-3 mediated apoptosis pathway), CASP 3 (main executioner as it can be activated through both extrinsic and intrinsic signaling pathway), CASP 9 (starter gene in intrinsic apoptosis pathway), and CASP 8 (starter gene in TNF-α apoptosis pathway). Therefore, this study aimed to assess the anti-proliferative effect of *Kluyveromyces marxianus* YAS on human colon cancer cell carcinoma by focusing on the apoptotic cellular/molecular mechanisms.

II. MATERIALS AND METHODS

A. Sampling, Isolation and Identification of Yeast

Ten grams of the traditional yogurt in east Azerbaijan, northwest state in Iran sample was suspended in 2% (w/v) sodium citrate solution (Sigma, St. Louis, MO, USA), and gently homogenized in stomacher (Seward Laboratory Systems Inc., USA) for 5 min. Then, 1 ml of the homogenized sample was added to 14 ml of yeast malt broth (YMB) (Sigma, St. Louis, MO, USA) and were incubated at 37ºC for overnight. After incubating time, yeast colony was isolated on yeast malt agar (YMA) (Sigma, St. Louis, MO, USA) using streak plated method at similar previous condition [37]. The isolated yeast cells were grown over nightly in 25 ml of YMB medium at 37ºC in aerobic condition. Two ml of overnight culture was transferred into the sterile Eppendorf micro tube and was centrifuged at 3500 rpm for 15 min at 4ºC then the supernatant was removed. The achieved cell pellet was subjected to 500 µl of Harju-Buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) for lysing cells. The cell lysate was used to isolate the genomic DNA according to Harju S et al method [38]. The PCR amplification was conducted in a thermal cycler PTC 200 (MJC research, Waltham, USA). The amplification sets of primers forward: 5/-CGATGCGAGAGCCAAGAGAT -3/ and reverse: 5/-GTCGTGCTGGGGATAGAGCATT -3/ were used for amplifying the ribosomal DNA fragments including; the internal transcribed spacer 1 (ITS 1), 5.8S rDNA, and internal transcribed spacer 2 (ITS 2). PCR amplification was performed under the following conditions: denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 59°C for 40 sec, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were checked and visualized via 1% agarose gel electrophoresis and staining with ethidium bromide, respectively. Subsequently, the gel monitoring apparatus (Biometra, Gottingen, Germany) was used to evaluate the quality of the products [39]. The gel recovered PCR products were sequenced at Sinaclone Corporation, Tehran, Iran.

The sequences were then analyzed using the BLAST program of the National Center for Biotechnology Information [40].

B. Cell Free Supernatant Preparation

The isolated and identified yeast and *Kluyveromyces marxianus* PTCC 5195 as reference yeast were over nightly cultured into 50 ml culture tube in yeast malt broth medium (Sigma, St. Louis, MO, USA) at aerobic condition, 37ºC. To prepare cell free supernatant, the declared yeast culture was centrifuged at 4500 rpm for 10 min at 4ºC and supernatant's pH was adjusted to 7.2 before sterling with 0.22 µm membrane filter (Millipore, Eschborn, Germany). The sterile supernatant was used for the treatment of human colon cancer cells (HT-29, Caco-2) and human normal epithelial (KDR) cell lines.

C. Cell Culture

The human colon cancer cell lines caco-2 and HT-29 and

the human epithelial normal cell line KDR were purchased from Pasteur Institute, National Cell Bank of Iran. The Cells were cultured in 25 cm^2 culture T-flasks and were incubated under standard conditions at 37˚C in a humidified atmosphere containing 5% CO2 with medium renewing every 3 days. The caco-2 and KDR cells were grown in a high glucose concentration (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum, 8 mM L-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100 µg/ml). The culture media for HT-29 cells was Roswell Park Memorial Institute medium (RPMI 1640, Sigma, Poole, United Kingdom) supplemented similar to above-mentioned DMEM medium.

D. MTT Assay

The HT-29, Caco-2 and KDR cell lines with cell seeding density 1.2 × 10^4 (cell/ well) were seeded in 96 well microplate and were overnight incubated similar to growth condition then were subjected to treatment with sterile yeast's cell free supernatant. The IC50 of supernatant were determined using MTT [3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide] (Sigma, St. Louis, MO, USA) assay and the based on finding, the determined amount of supernatant (60 µl) and 5- fluorouracil (5-FU) (7 µg/µl) as positive control were used for treatment of all normal KDR and cancerous HT-29, Caco-2 cell lines. All untreated controls and treated groups with IC50 of supernatant were incubated for 24 h similar to the growth condition. After incubation time, the medium of each well was carefully removed and was replaced with fresh medium containing 50 µl of MTT solution (2 mg/ml in phosphate buffer saline 0.01 M, pH 7.2) and the microplates were incubated again for 4 h at 37ºC. The MTT containing medium of each well was replaced with 200 µl of dimethyl sulfoxide (DMSO) plus 25 µl of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5) and continued incubation for 15 min at room temperature. The microplate absorbance was measured by employing a microplate reader (Biotek, ELx 800, USA) at 570 nm.

E. DAPI Staining

To detect apoptotic cells, sterile cover slip slides were placed into each well of a six-well culture plate. Then, 3 mL of HT-29, Caco-2 and KDR cells (1.2×10^4 cells/ml) were added to each well and then incubated at growth condition. After reaching 50% confluence, untreated control and treated with 900 µl of the yeast's sterile supernatant and 5-Fluorouracil (7 µg/µl) as positive control groups were incubated for 24 h similar to growth condition. The treated cells, 24 h after seeding, were washed with pre-warmed tissue culture media and were carefully replaced with freshly prepared fixative solution (pre-warmed RPMI containing 4% formaldehyde). For cell fixation, the plates were incubated for 5 min at 37°C, the fixed cells were washed twice with PBS, and then permeabilized with PBS containing 0.1% Triton X-100 for 5 min at 37°C. The permeabilized cells were then stained with 100 µl per well of DAPI (4',6-diamidino-2-phenylindole) (250 ng/ml for each well) for 3 min of incubation at room temperature. Finally, the slides were washed with PBS and then assessed by using fluorescent microscopy (Olympus BX64, Olympus, Japan) equipped with a U-MWU2 fluorescence filter (excitation filter BP 330e385, dichromatic mirror DM 400, and emission filter LP 420).

F. Flow Cytometry

The fraction of apoptotic cells was quantitatively measured via flow cytometry using the Annexin V-FITC apoptosis detection kit (eBioscience, San Diego, CA). HT-29, Caco-2 and KDR cell lines (1.2×10^5 cells/well) were seeded into a six-well culture plate and the treatment of cells were similar to DAPI staining. After treatment time point, the treated/untreated control cells were detached by trypsin, the supernatant was discarded after centrifugation at 1100 rpm for 7 min at 28°C, and the cell pellet was resuspended in 500 µl of 1X binding buffer and transferred into a new 5 ml tube. The tubes were centrifuged again and the supernatants were replaced with 100 µl binding buffer (1X). Afterward, the tubes were added with 5 µl of FITC-conjugated Annexin V then were incubated for 15 min at room temperature under dark conditions. The incubated cells were centrifuged and the cell plates were resuspended in 500 µl of binding buffer (1X). Finally, 5 µl of propidium iodide solution was added to the cells, and quadrant settings were fixed with untreated, single-stained controls and copied to dot plots of the treated cells. Quadrant statistic calculations were performed using CELL Quest Pro software (BD Biosciences, San Jose, CA, USA). The experiment was repeated two times with triplicate samples for each experiment. Analyses were accomplished using 10000 cells at a rate of 450 cells/s. FL-1 and FL-2 were represented in dot plots illustrating the viable, apoptotic, and necrotic cells.

G. Real-time PCR Amplification

For RNA analysis, 24 h post treatment or untreated control monolayer cells were lysed using TRI Reagent® (2 ml per 25 cm^2 T-flask) (Sigma Chemical Co., Poole, United Kingdom) according to manufacture guidelines and the cell lysates were transferred to RNAse/DNAse-free microtubes. Chloroform (0.2 ml per each ml of TRI Reagent™ used for lysing) was added to each sample tube, and the mixture was gently vortexed. After 5 min at room temperature, the samples were centrifuged at 12000 × g, 4°C for 10 min and the colorless upper aqueous phase was carefully separated and mixed with ice-cold iso-propanolol (0.5 ml per each ml of TRI Reagent® used initially) then was centrifuged at 12000 × g, 4°C for 10 min, yielding total RNA pellet that was washed thrice with 75% ethanol. The samples were again centrifuged at 7500 × g for 8 min and the washed RNA plates were air dried then were dissolved in diethyl pyrocarbonate (DEPC) treated water and were tested qualitatively and quantitatively prior to its use for RT-PCR experiments.

The isolated RNA was reverse transcribed to cDNA using MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD). For RT reaction, 1 µl RNA

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

(1 µg/µl) was mixed with master mix [DEPC treated water 13 µl, dNTP's (10 µM) 2 µl, MMLV buffer with DTT 2 µl, random hexamer primer (pdN6; 400 ng/µl) 0.5 µl], and denatured at 95°C subsequently was cooled down to 4°C for 5 min. Then 1 µl MMLV (200 U/µl) and 0.5 µl RNas in (40 U/µl) were added to each sample and the mixture was incubated using following thermos cycling program: 10 min at 25°C, 42 min at 42°C, and 5 min at 95°C. The prepared cDNA templates were used for real time PCR experiments. Primers were designed from published Gene Bank sequences using Beacon Designer 5.01 [41] and listed in Table I. All amplification reactions were performed in a total volume of 20 µl using ABI-step I plus (Applied Biosystems, Forster City, CA, USA) instrument using thermal cycling condition as follows: 95°C for 5 min followed by 95°C for 20 sec, 60°C for 35 sec, and at 72°C for 10 sec. Interpretation of the result was performed using the Pfaffle method and the threshold cycle (Ct) values were normalized to the expression rate of GAPDH as a housekeeping gene [42]. All reactions were performed in triplicate and negative controls were included in each experiment.

TABLE I
PRIMERS SEQUENCES FOR RT-PCR AMPLIFICATION

Gene name and symbol	Sequence (5' → 3')	Amplicon size (bp)	TM	
			F	R
GAPDH	F:5'-AAGCTCATTTCCTGGTATGACAACG-3' R:5'-TCTTCCTCTTGTGCTCTTGCTGG-3'	126	60.3	61.1
BAX	F: 5'-CCCGAGAGGTCTTTTTCCGAG-3' R: 5'- CCAGCCCATGATGGTTCTGAT-3'	155	62.1	61.9
CASPASE- 3	F:5'-TGCCTGTAACTTGAGAGTAGATGG-3' R:5'-CTTCACTTTCTTACTTGGCGATGG-3'	172	58.7	58.0
CASPASE- 8	F: 5'-GACAGAGCTTCTTCGAGACAC-3' R: 5'-GCTCGGGCATACAGGCAAAT-3'	116	60.1	63
CASPASE- 9	F: 5'-CTCAGACCAGAGATTCGCAAAC-3' R: 5'-GCATTTCCCCTCAAACTCTCAA-3'	116	60.9	60.5

H. Statistical Analysis

The statistical analysis was performed by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). The normal distribution of data was tested by Kolmogorov-Smirnov test. ANOVA and Tukey's post hoc test were used for analyzing data and multiple mean comparisons, respectively. Statistical significance was considered as a value of $P \leq 0.05$ and quantitative data were reported as mean ± SD.

III. RESULTS

A. Molecular Identification

Based on ITS1- 5.8s- ITS2 region identification results by blasting and considering the threshold values of taxonomical studies (97%) [43], the isolated yeast was identified to *Kluyveromyces marxianus* YAS strain with 99% to 100% homology.

B. Toxicity Assay

MTT assay was performed to determine the cytotoxicity effects of the metabolites secreted by *Kluyveromyces marxianus* YAS on human colon cancer cell lines (HT-29, Caco-2) and human epithelial normal cell line KDR. The cytotoxicity potential of the metabolites produced by *Kluyveromyces marxianus* YAS and reference yeast on malignant cells was determined (Fig. 1). The anti-proliferative effect of the metabolites (60 µl/ml) on colon cancer cells significantly differed from that of the un-treated and reference strain-treated groups. The effect of the metabolites on KDR normal cells was also examined (Fig. 1). *Kluyveromyces marxianus* YAS secretions exhibited no toxic effect on normal cells; more than 81% of the cells grew well. These results indicated that *Kluyveromyces marxianus* YAS is a potential candidate for cancer treatment.

C. Morphological Change Observations

To observe any nuclear morphological changes induced by yeasts' cell free-supernatants, all treated/untreated control HT-29 and Caco-2/KDR cell groups were stained with DAPI and analyzed through fluorescent microscopy to analyze the effect of *Kluyveromyces marxianus* YAS secretions on cells viability. The intact viable cells displayed completely healthy nuclei (Fig. 2); by contrast, the apoptotic cells were characterized by shrunk cells with condensed (early apoptosis) or fragmented (late apoptosis) nuclei. Other morphological and apoptotic changes, such as membrane blebbing and apoptotic body formation, were observed in the treated cancer cells but not in normal cells. Based on these findings, apoptosis can be considered as the main cytotoxic mechanism of yeast metabolites (Fig. 2). Therefore, the metabolites produced by *Kluyveromyces marxianus* YAS strain may be used as an alternative nutraceutical with promising therapeutic index because these metabolites are non-cytotoxic to normal mammalian cells.
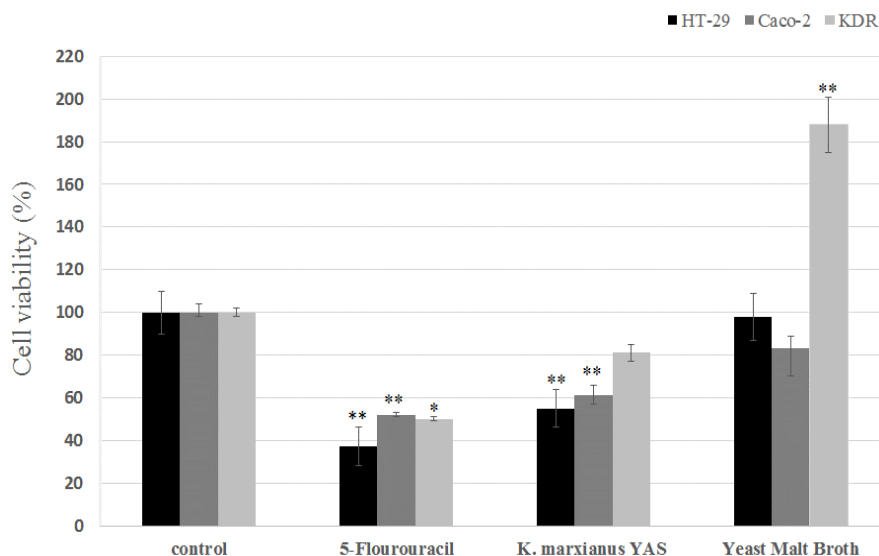
World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

Fig. 1 Effect of *Kluyveromyces marxianus* YAS secretion metabolites on the viability of HT-29 and Caco-2 cancer cells and KDR normal cell by 60 µl for 24 hour incubation. Data are expressed as mean viability ± S.D (error bars). Asterisks denote statistically significant differences (**p≤0.01, *p≤0.05). Untreated cells were used as negative control and 5- fluorouracil was used as positive control. Yeast malt broth as yeast culture medium was used to determine its effect on cell viability

D. Flow Cytometry

Compared with the control cells that exhibited natural cell death (Fig. 3), the HT-29 and Caco-2 cells treated with 60 µl/ml of filtered *Kluyveromyces marxianus* YAS demonstrated significant amounts of Annexin V+/PI− (early apoptotic cells) and Annexin V+/PI+ cells (late apoptotic cells) after incubating for 24 h (Fig. 3). In the treated HT-29 cells, 62.3%, 4.27% and Caco-2 cells 61.2%, 5.14% as well as in KDR normal cells 23.6% and 3.97% were in early apoptosis and late apoptosis respectively. Based on the flow cytometry findings, *Kluyveromyces marxianus* YAS can inhibit the proliferation of cancer cells similar to 5-FU as common approved anticancer drug. In addition, the metabolites produced by *Kluyveromyces marxianus* YAS strain may be used as an alternative nutraceutical/pharmaceutical with promising therapeutic activity because these metabolites are low-cytotoxic to normal mammalian cells.

E. The Expression Levels of Genes

Fig. 4 demonstrates the expression level of BAX, CASP 3, CASP 8 and CASP 9 genes in colon cancer and normal cell lines after treatment with the supernatant of *Kluyveromyces marxianus* YAS isolated from traditional fermented dairy products and 5-FU (as positive control), compared to untreated control groups.

The expression of CASP 8 (starter gene in TNF-α apoptosis pathway) CASP 3 (the chief executioner of programmed cell death in extrinsic and intrinsic signaling pathway), CASP 9 (starter gene in intrinsic apoptosis pathway) and BAX (crucial gene in extrinsic IL-3 mediated apoptosis pathway) genes upregulated significantly in all treatments in colon cancer cells. The cancerous cells treated with 5-FU and *Kluyveromyces marxianus* YAS showed significant increasing of intended gene expression in HT-29 and Caco-2 cells

compared to untreated control group (Fig. 4). The upregulation in the mentioned genes by *Kluyveromyces marxianus* YAS in all treated groups was similar to anticancer drug, 5-FU, indicating same inducing pathways of apoptosis.

IV. DISCUSSION

There is an overall opinion that microbes are harmful. This idea comes from the morbidity and mortality due to pathogenic bacteria, yeast, and viruses. Nonetheless, a lot of evidences currently indicate that the certain microbes, called probiotics, can contribute to human health [44]. Thus, there is a great interest in finding probiotics and especially yeast strains with probiotic potential [45]. Considering that, sometimes a certain strain of probiotics creates different response in special disease treatment at different population, designing of population-specific probiotics with respect to the native microbiomes may finally help in standardizing utilization of certain probiotic strains in alleviation of certain diseases in each population [46]. Traditional dairy products like cheese and yogurt are good sources for the genus of yeast as probiotics according to native flora [47], [48].

Some of the yeast probiotic strains have been reported to influence malignancies [32], [49]. However, a lot of studies showed that probiotic yeasts may prevent the cancer initiation or its development thorough antitoxin effects against different toxins, antimicrobial activity, production of biological substances like short chain fatty acids (SCFA), trophic effects on enterocytes, inactivation of carcinogenic compounds and improvement of intestinal barrier function [50]-[52]. Besides, other mechanisms are involved in the cancer prevention including the enhancement of the host's immune response, binding and degrading potential carcinogens, alterations in the intestinal microflora incriminated in producing putative carcinogens (e.g., bile acid-degrading bacteria), producing

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

antitumorogenic or anti-mutagenic compounds, improvement in the metabolic activities of intestinal microflora, alteration of physicochemical conditions in the colon, effects on physiology of the host, anti-oxidant function, and finally stimulating the apoptosis and anti-proliferative effects [53]. These beneficial effects suggest the use of some probiotic yeast strains as adjuvants in cancer treatment [51], [54], [55]. However, Saccharomyces *boulardii* makes its anticancer effects by different potential mechanisms including the neutralization of bacterial virulence factors [56], interference with bacterial adhesion [57], strengthening of enterocyte tight junctions [58], enhancement of the mucosal immune response [59], alteration in immune cell redistribution [60], and modulation of cell proliferation signaling pathways of the host [49]. Additionally, intratumorally (IT) injection of killed *S. cerevisiae* causes the tumor degeneration, apoptosis, and ischemic (coagulative) and liquefactive necrosis in Swiss albino mice possessing Ehrlich Ascites Carcinoma (EAC) cells. Despite the fact that numerous studies have been done in this field, the exact mechanisms of probiotic yeasts in cancer are still unknown. Based on numerous research results, the probiotic yeasts can be beneficial in cancer therapy by inducing apoptosis pathways in cancerous cells and modulating the host's immune system. Thus, some of the yeast strains, as safe probiotic, could be candidates for therapeutic implications.

In this study, the effect of secretion metabolites which were produced by *Kluyveromyces marxianus* YAS had been investigated by molecular and cellular methods. The effect of different concentrations of yeast secreted metabolites on the colon cancer cell lines (HT-29, Caco-2) was evaluated using MTT assay to study the anti-proliferative effects. 60 µl of cell free supernatant was selected as the IC50 concentration of *Kluyveromyces marxianus* YAS on colon cancer cell lines (the effective dose resulted from prescreening MTT tests). Studying the effects of metabolites on the normal and cancer cell lines using DAPI staining revealed that *Kluyveromyces marxianus* YAS secretory metabolites had noxious effect on colon cancer cell lines which is seen as cellular shrinking and condensation of cell nuclear whereas there was not significant difference in the apoptosis between the normal (KDR) and control cell lines (Fig. 2). However, isolated yeast secreted metabolites induced the apoptotic pathways more specifically in cancer cells rather than normal cells. The inhibition of cell growth by the metabolites of *Kluyveromyces marxianus* YAS from the DAPI staining test was evaluated using flow cytometry as a quantitative method.

Dot plot analysis of flow cytometry which were obtained from the treated and control groups including healthy (KDR) and cancer (HT-29, Caco-2) cell lines showed that yeast secretory metabolites of *Kluyveromyces marxianus* YAS lead to develop the apoptosis in HT-29 (66.57 %) and Caco-2 (66.34%) while apoptosis only develops in 27.57% of normal KDR cells. These finding suggested that apoptosis is induced in colon cancer cells more than normal cells by the yeast secretory metabolites. Additionally, the negligible incidence of necrosis by *Kluyveromyces marxianus* YAS showed the

lower necrotic cytotoxicity in colon cancer and KDR cells. These findings proved the MTT assay results which suggested that *Kluyveromyces marxianus* YAS secretion metabolites have anti proliferative effects on CRC cells especially via apoptosis inducing mechanisms. The over expression of BAX, CASP 3, CASP 8 and CASP 9 genes in colon cancer treated cells with *Kluyveromyces marxianus* YAS secretions (Fig. 4) is an important mediator of the cell growth inhibition, lowering the cell survivability and increasing apoptosis. The release of cytochrome c from the mitochondria together with the adaptor APAF1, forming an "apoptosome", activates the initiator intrinsic apoptosis gene (CASP 9) which is responsible for destroying the cell critical components resulting in apoptosis [61]. We also examined the expression of caspase-3, the chief executioner of programmed cell death. It is proved that caspase-8 and -3 are direct activators of apoptosis. Rogalska et al showed a strong correlation between the activity of caspase-8 and -3 and a direct relationship between the activity of caspase-8 and -3 in A 549 and MCF-7 cells treated with aclarubicin (ACL) [62]. In this study we have also noticed upregulation of caspase-3 and -8 at the same time. There are different mechanisms that tumor cells used to suppress apoptosis. Tumor cells can resist to apoptosis by the expression of antiapoptotic proteins such as Bcl-2 or by the downregulation or mutation of pro-apoptotic proteins such as BAX [33]. Bcl-2 family are localized in the mitochondria and have either pro-apoptotic (Bax, Bak, Bid, and Bim) or anti-apoptotic (Bcl-2, Bcl-xL, and Bcl-W) functions [63]-[65]. These proteins form either homodimers (such as Bcl-2/Bcl-2) or heterodimers (e.g., Bcl-2/Bax) depending on the levels present of each component. Excess level of homodimers can either inhibit (e.g., Bcl-2/Bcl-2) or induce (e.g., Bax/Bax) apoptosis [66]. In this study, upregulation of Bax gene via *Kluyveromyces marxianus* YAS secretions facilitate apoptosis phenomena through intrinsic pathway. Evidence in human studies has shown that the consumption of fermented milk, yogurt or other dairy products is causally related to prevention of cancer development [67]. Mechanisms of this microorganisms (yeast or probiotic bacteria) involve increasing a production of inflammatory cytokines in the host, changing enzyme activities, reducing the mutagenicity by inhibition the uptake of potential carcinogens or production of anti-proliferative and anti-tumorgenic compounds [68]. Similarly, Mee et al. showed that Chinese red yeast releases microbial products including lovastatin (LV) that decreased cellular proliferation and induced apoptosis in HCT-116 and HT-29 human colon cancer cells [69]. By considering these results, *Kluyveromyces marxianus* YAS may possibly produce compounds that can inhibit the cancerous cell growth like CRC which proved by MTT assay, DAPI staining, flow cytometry, and real-time PCR results. Moreover, based on this findings, *Kluyveromyces marxianus* YAS secretions induce apoptosis in KDR human epithelial normal cells significantly lower than HT-29 and Caco-2 cancerous cells indicating the relative safety of mentioned metabolites for future individual consumptions as nutriceutical/pharmaceutical ingredients as a chemo preventive therapy.

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

V. CONCLUSION

The anticancer activity through the induction of apoptosis of cancer cells seems to be a promising approach for using some probiotic strains as a support therapy or disease prevention. There is a great interest in finding yeast strains with probiotic potential. The identified *Kluyveromyces marxianus* YAS isolated from yogurt was used to evaluate its prophylactic effects on HT-29 and Caco-2 human colon cancer cell lines. The cytotoxic findings showed that *Kluyveromyces marxianus* YAS secretions exhibited acceptable anticancer activity on colon cancer cell lines. In addition, *Kluyveromyces marxianus* YAS secretions showed similar anticancer activity of 5-flourouracil on the colon cancer cells. Also, *Kluyveromyces marxianus* YAS secretions have not significant cytotoxic effects on the KDR epithelial normal cell line. In conclusion, the physicochemical, structural, and functional properties of effective anticancer substances (probably exopolysaccharides) for use as anticancer therapeutics should be investigated thoroughly.
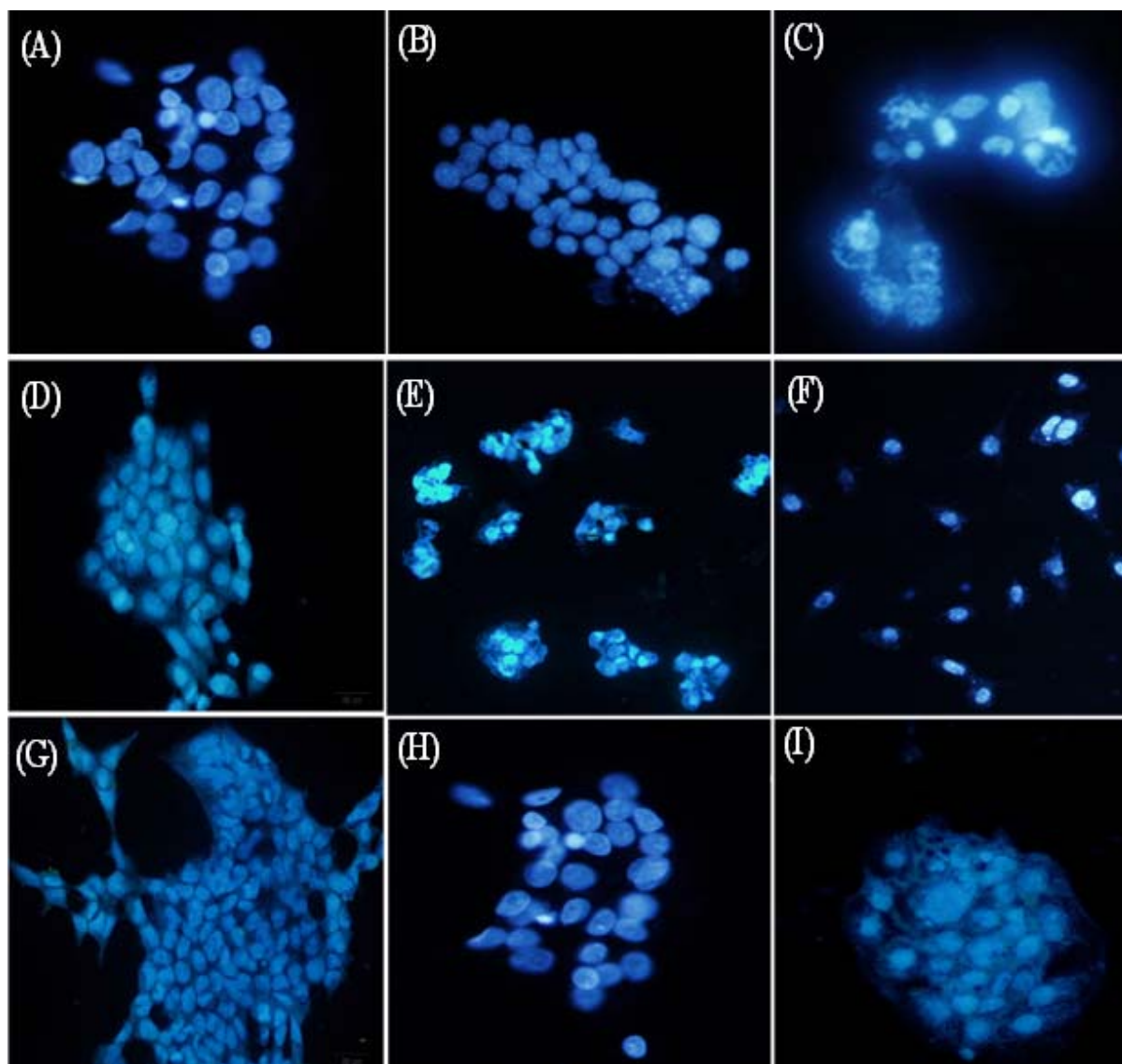

Fig. 2 DAPI staining of treated/untreated HT-29, Caco-2 and KDR cells. Panels (A, B, and C) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at HT-29 cells and panels (D, E, and F) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at Caco-2 cells and panels (G, H, and I) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at KDR cells respectively for 24 h incubation

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
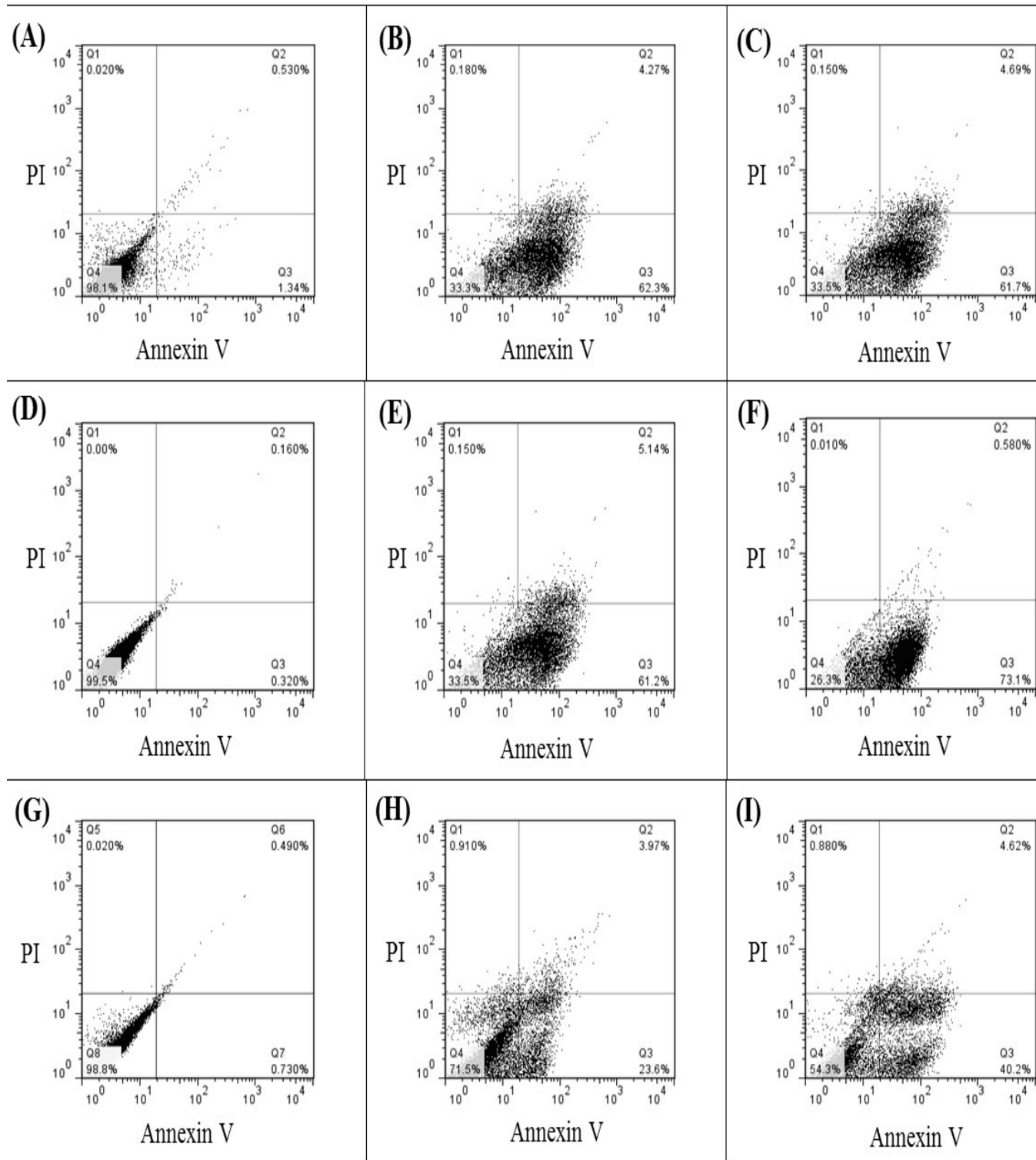Vol:10, No:3, 2016

Fig. 3 Flow cytometric analysis of treated/untreated HT-29, Caco-2 and KDR cells. Panels (A, B, and C) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at HT-29 cells, panels (D, E, and F) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at Caco-2 cells and panels (G, H, and I) represent untreated, treated with cell free supernatant (60 µl/ml), and treated with 5- fluorouracil (IC50) at KDR cells respectively for 24 h incubation. Dots with Annexin V−/PI+ (Q1), Annexin V+/PI+ (Q2), Annexin V+/PI− (Q3), and Annexin V−/PI− (Q4) and feature represent necrotic, late apoptotic, early apoptotic, and viable intact cells respectively

World Academy of Science, Engineering and Technology
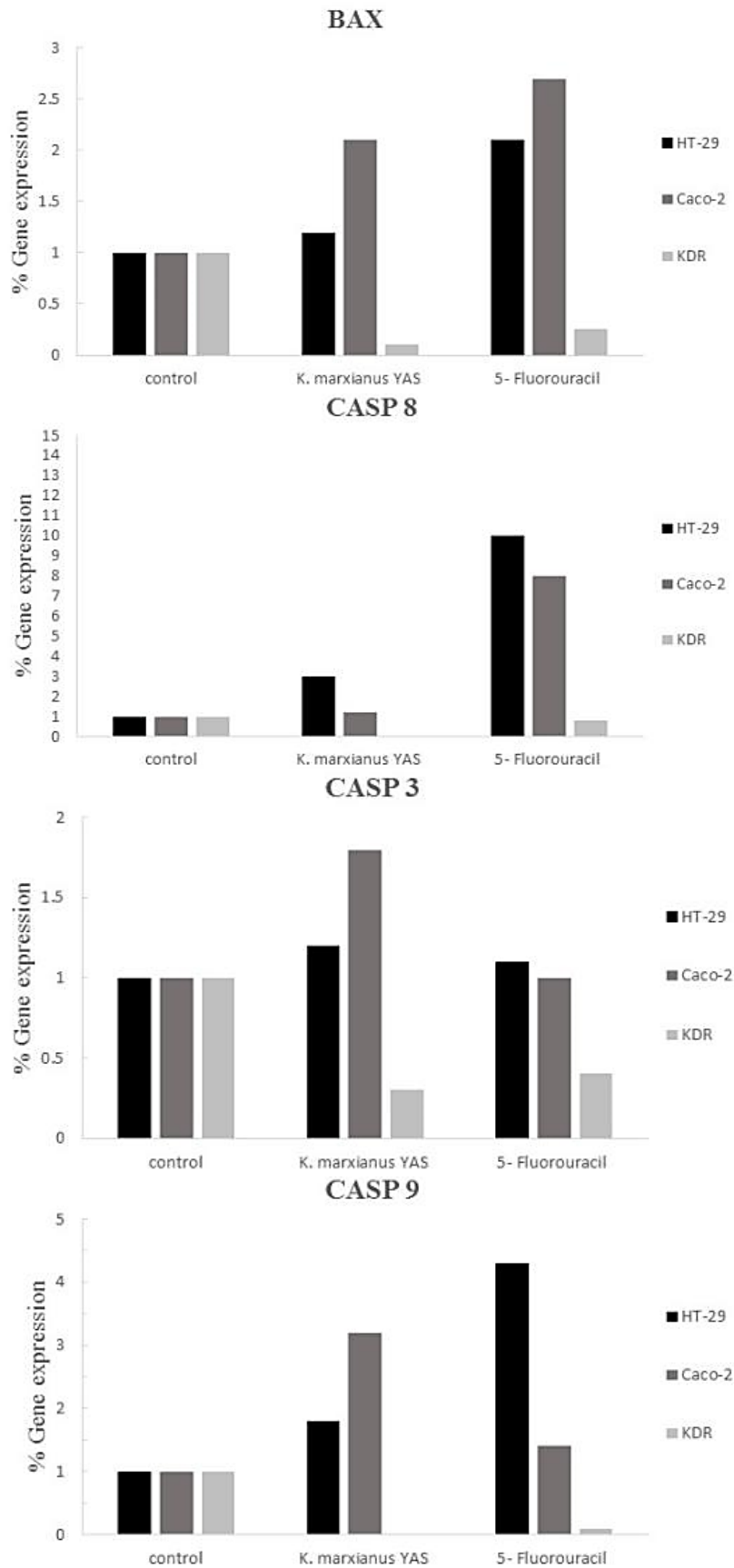International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

Fig. 4 Apoptosis related gene expression ratio in treated (60 µl *Kluyveromyces marxianus* YAS secretion metabolites) and untreated control HT-29, Caco-2 and KDR cells for 24 h

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

REFERENCES

[1] Liboredo JC, Anastácio LR, Pelúzio MdCG, Valente FX, Penido LCP, Nicoli JR, et al. Effect of probiotics on the development of dimethylhydrazine-induced preneoplastic lesions in the mice colon. Acta Cirurgica Brasileira. 2013;28(5):367-72.

[2] McCormack V, Boffetta P. Today's lifestyles, tomorrow's cancers: trends in lifestyle risk factors for cancer in low-and middle-income countries. Annals of Oncology. 2011;mdq763.

[3] Arvelo F, Sojo F, Cotte C. Biology of colorectal cancer. ecancermedicalscience. 2015;9.

[4] Yao Y-L, Shao J, Zhang C, Wu J-H, Zhang Q-H, Wang J-J, et al. Proliferation of colorectal cancer is promoted by two signaling transduction expression patterns: ErbB2/ErbB3/AKT and MET/ErbB3/MAPK. 2013.

[5] Ciernikova S, Mego M, Hainova K, Adamcikova Z, Stevurkova V, Zajac V. Modification of microflora imbalance: future directions for prevention and treatment of colorectal cancer? Neoplasma. 2014;62(3):345-52.

[6] Zhong L, Zhang X, Covasa M. Emerging roles of lactic acid bacteria in protection against colorectal cancer. World J Gastroenterol. 2014;20(24):7878-86.

[7] Gao Z, Guo B, Gao R, Zhu Q, Qin H. Microbiota disbiosis is associated with colorectal cancer. Frontiers in microbiology. 2015;6.

[8] Zhu Y, Luo TM, Jobin C, Young HA. Gut microbiota and probiotics in colon tumorigenesis. Cancer letters. 2011;309(2):119-27.

[9] Rafter J. Lactic acid bacteria and cancer: mechanistic perspective. British Journal of Nutrition. 2002;88(S1):S89-S94.

[10] Mizock BA. Probiotics. Disease-a-month: DM. 2015;61(7):259-90.

[11] Burns A, Rowland I. Anti-carcinogenicity of probiotics and prebiotics. Current issues in intestinal microbiology. 2000;1(1):13-24.

[12] Rowland IR, Gangolli SD. Role of gastrointestinal flora in the metabolic and toxicological activities of xenobiotics. General and Applied Toxicology. 2nd Edition ed. Macmillan Publishers Ltd, London, p1999. p. 561-76.

[13] Cousin FJ, Jouan-Lanhouet S, Dimanche-Boitrel M-T, Corcos L, Jan G. Milk fermented by Propionibacterium freudenreichii induces apoptosis of HGT-1 human gastric cancer cells. PLoS One. 2012;7(3):e31892.

[14] Ghoneum M, Hamilton J, Brown J, Gollapudi S. Human squamous cell carcinoma of the tongue and colon undergoes apoptosis upon phagocytosis of Saccharomyces cerevisiae, the baker's yeast, in vitro. Anticancer research. 2005;25(2a):981-9.

[15] Ghoneum M, Gollapudi S. Modified arabinoxylan rice bran (MGN-3/Biobran) enhances yeast-induced apoptosis in human breast cancer cells in vitro. Anticancer research. 2005;25(2a):859-70.

[16] Ghoneum M, Wang L, Agrawal S, Gollapudi S. Yeast therapy for the treatment of breast cancer: a nude mice model study. In vivo (Athens, Greece). 2007;21(2):251-8.

[17] Badr El-Din NK, Noaman E, Ghoneum M. In Vivo Tumor Inhibitory Effects of Nutritional Rice Bran Supplement MGN-3/Biobran on Ehrlich Carcinoma-Bearing Mice*. Nutrition and cancer. 2008;60(2):235-44.

[18] Dahan S, Dalmasso G, Imbert V, Peyron JF, Rampal P, Czerucka D. Saccharomyces boulardii interferes with enterohemorrhagic Escherichia coli-induced signaling pathways in T84 cells. Infect Immun. 2003;71(2):766-73.

[19] Chen X, Kokkotou EG, Mustafa N, Bhaskar KR, Sougioultzis S, O'Brien M, et al. Saccharomyces boulardii inhibits ERK1/2 mitogen-activated protein kinase activation both in vitro and in vivo and protects against Clostridium difficile toxin A-induced enteritis. Journal of Biological Chemistry. 2006;281(33):24449-54.

[20] Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Adv Cancer Res. 1998;74:49-139.

[21] Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002;296(5573):1655-7.

[22] Giordano A, Rustum YM, Wenner CE. Cell cycle: molecular targets for diagnosis and therapy: tumor suppressor genes and cell cycle progression in cancer. Journal of cellular biochemistry. 1998;70(1):1-7.

[23] De Jong JS, van Diest PJ, van der Valk P, Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol. 1998;184(1):53-7.

[24] Wells A. Tumor invasion: role of growth factor-induced cell motility. Adv Cancer Res. 2000;78:31-101.

[25] Gibson EM, Henson ES, Haney N, Villanueva J, Gibson SB. Epidermal growth factor protects epithelial-derived cells from tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by inhibiting cytochrome c release. Cancer research. 2002;62(2):488-96.

[26] Moshnikova AB, Moshnikov SA, Afanasyev VN, Krotova KE, Sadovnikov VB, Beletsky IP. Cell death induced by chemical homobifunctional cross-linkers. Cross-linker induced apoptosis. The international journal of biochemistry & cell biology. 2001;33(12):1160-71.

[27] Liu Y, Song XD, Liu W, Zhang TY, Zuo J. Glucose deprivation induces mitochondrial dysfunction and oxidative stress in PC12 cell line. Journal of cellular and molecular medicine. 2003;7(1):49-56.

[28] Katayama K, Dobashi Y, Kitagawa M, Kawai M, Kadoya Y, Kameya T. Cdk4/cyclin D1 kinase, a universal and critical regulator of apoptosis. Anticancer research. 2003;23(1a):235-43.

[29] Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology. 2007;35(4):495-516.

[30] Hickman JA. Apoptosis induced by anticancer drugs. Cancer and Metastasis Reviews. 1992;11(2):121-39.

[31] Apontes P, Leontieva OV, Demidenko ZN, Li F, Blagosklonny MV. Exploring long-term protection of normal human fibroblasts and epithelial cells from chemotherapy in cell culture. Oncotarget. 2011;2(3):222.

[32] Ghoneum M, Matsuura M, Braga M, Gollapudi S. S. cerevisiae induces apoptosis in human metastatic breast cancer cells by altering intracellular Ca2+ and the ratio of Bax and Bcl-2. International journal of oncology. 2008;33(3):533-9.

[33] Hassan M, Watari H, AbuAlmaaty A, Ohba Y, Sakuragi N. Apoptosis and Molecular Targeting Therapy in Cancer. BioMed Research International. 2014;2014:150845.

[34] Oliveira RJ, Salles MJ, da Silva AF, Kanno TY, Lourenco AC, Leite Vda S, et al. In vivo evaluation of the antimutagenic and antigenotoxic effects of beta-glucan extracted from Saccharomyces cerevisiae in acute treatment with multiple doses. Genet Mol Biol. 2013;36(3):413-24.

[35] Ghoneum M, Gollapudi S. Induction of apoptosis in breast cancer cells by Saccharomyces cerevisiae, the baker's yeast, in vitro. Anticancer research. 2004;24(3A):1455-64.

[36] Ghoneum M, El-Din NKB, Noaman E, Tolentino L. Saccharomyces cerevisiae, the Baker's Yeast, suppresses the growth of Ehrlich carcinoma-bearing mice. Cancer Immunology, Immunotherapy. 2008;57(4):581-92.

[37] Garofalo C, Osimani A, Milanovic V, Aquilanti L, De Filippis F, Stellato G, et al. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. Food microbiology. 2015;49:123-33.

[38] Harju S, Fedosyuk H, Peterson KR. Rapid isolation of yeast genomic DNA: Bust n' Grab. BMC Biotechnol. 2004;4:8.

[39] Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular ecology. 1993;2(2):113-8.

[40] National Center for Biotechnology Information (NCBI). Available from: http://www.ncbi.nlm.nih.gov/BLAST.

[41] PREMIER Biosoft. Retrieved January 23, 2016, from http://www.premierbiosoft.com/

[42] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research. 2001;29(9):e45-e.

[43] Deng W, Xi D, Mao H, Wanapat M. The use of molecular techniques based on ribosomal RNA and DNA for rumen microbial ecosystem studies: A review. Molecular Biology Report. 2008;35:265-74.

[44] Douglas LC, Sanders ME. Probiotics and prebiotics in dietetics practice. Journal of the American dietetic association. 2008;108(3):510-21.

[45] Kumar H, Salminen S, Verhagen H, Rowland I, Heimbach J, Bañares S, et al. Novel probiotics and prebiotics: road to the market. Current opinion in biotechnology. 2015;32:99-103.

[46] Barzegari A, Saei AA. Designing probiotics with respect to the native microbiome. Future microbiology. 2012;7(5):571-5.

[47] Lopandic K, Zelger S, Banszky LK, Eliskases-Lechner F, Prillinger H. Identification of yeasts associated with milk products using traditional and molecular techniques. Food Microbiol. 2006;23(4):341-50.

[48] Kumura H, Tanoue Y, M T, Tanaka T, Shimazaki K. Screening of dairy yeast strains for probiotic applications. J Dairy Science. 2004;87:4050-6.

World Academy of Science, Engineering and Technology
International Journal of Nutrition and Food Engineering
Vol:10, No:3, 2016

[49] Chen X, Fruehauf J, Goldsmith JD, Xu H, Katchar KK, Koon HW, et al. Saccharomyces boulardii inhibits EGF receptor signaling and intestinal tumor growth in apcmin mice. Gastroenterology. 2009;137(3):914-23.

[50] Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther. 2008;27(2):104-19.

[51] Fleet G, Balia R. The public health and probiotic significance of yeasts in foods and beverages. Yeasts in Food and Beverages: Springer; 2006. p. 381-97.

[52] Boirivant M, Strober W. The mechanism of action of probiotics. Curr Opin Gastroenterol. 2007;23(6):679-92.

[53] Hirayama K, Rafter J. The role of probiotic bacteria in cancer prevention. Microbes Infect. 2000; 2(6):681-6.

[54] McFarland LV. Systematic review and meta-analysis of Saccharomyces boulardii in adult patients. World journal of gastroenterology: WJG. 2010;16(18):2202.

[55] Marteau P, Boutron-Ruault M. Nutritional advantages of probiotics and prebiotics. British Journal of Nutrition. 2002;87(S2):S153-S7.

[56] Castagliuolo I, LaMont JT, Nikulasson ST, Pothoulakis C. Saccharomyces boulardii protease inhibits Clostridium difficile toxin A effects in the rat ileum. Infection and immunity. 1996;64(12):5225-32.

[57] Gedek B. Adherence of Escherichia coli serogroup 0 157 and the Salmonella Typhimurium mutant DT 104 to the surface of Saccharomyces boulardii Adhärenz von Escherichia coli Serogruppe 0 157 und von der Salmonella Typhimurium-Mutante DT 104 an der Oberfläche. mycoses. 1999;42:261-4.

[58] Philpott DJ, McKay DM, Sherman PM, Perdue MH. Infection of T84 cells with enteropathogenic Escherichia coli alters barrier and transport functions. Am J Physiol. 1996;270(4 Pt 1):G634-45.

[59] Qamar A, Aboudola S, Warny M, Michetti P, Pothoulakis C, LaMont JT, et al. Saccharomyces boulardii Stimulates Intestinal Immunoglobulin A Immune Response to Clostridium difficileToxin A in Mice. Infection and immunity. 2001;69(4):2762-5.

[60] Dalmasso G, Cottrez F, Imbert V, Lagadec P, Peyron J-F, Rampal P, et al. Saccharomyces boulardii inhibits inflammatory bowel disease by trapping T cells in mesenteric lymph nodes. Gastroenterology. 2006;131(6):1812-25.

[61] Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat Rev Cancer. 2002;2(6):420-30.

[62] Rogalska A, Szwed M, Jóźwiak Z. Aclarubicin-induced apoptosis and necrosis in cells derived from human solid tumours. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2010;700(1):1-10.

[63] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100(1):57-70.

[64] Farrow SN, Brown R. New members of the Bcl-2 family and their protein partners. Current Opinion in Genetics & Development. 1996;6(1):45-9.

[65] Agarwal R, Kaye SB. Ovarian cancer: strategies for overcoming resistance to chemotherapy. Nature reviews Cancer. 2003;3(7):502-16.

[66] Ashkenazi A, Pai RC, Fong S. Safety and antitumor activity of recombinant soluble Apo2 ligand. J Clin Invest 1999;104:155-62.

[67] Yang B, McCullough ML, Gapstur SM, Jacobs EJ, Bostick RM, Fedirko V, et al. Calcium, vitamin D, dairy products, and mortality among colorectal cancer survivors: the Cancer Prevention Study-II Nutrition Cohort. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32(22):2335-43.

[68] Habil N, Abate W, Beal J, Foey AD. Heat-killed probiotic bacteria differentially regulate colonic epithelial cell production of human beta-defensin-2: dependence on inflammatory cytokines. Beneficial microbes. 2014;5(4):483-95.

[69] Mee YH, Navindra PS, Yanjun Z, David H. Anticancer effects of Chinese red yeast rice versus monacolin K alone on colon cancer cells Journal of Nutritional Biochemistry. 2008;19 448-58.