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Secretion metabolites of probiotic yeast, *Pichia kudriavzevii* AS-12, induces apoptosis pathways in human colorectal cancer cell lines

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ABSTRACT

There is a common agreement on the important role of the gastrointestinal microbiota in the etiology of cancer. Benign probiotic yeast strains are able to ameliorate intestinal microbiota and regulate the host metabolism, physiology, and immune system through anti-inflammatory, antiproliferative, and anticancer effects. We hypothesized that *Pichia kudriavzevii* AS-12 secretion metabolites possess anticancer activity on human colorectal cancer cells (HT-29, Caco-2) via inhibiting growth and inducing apoptosis. This study aimed to assess the anticancer effect of *P. kudriavzevii* AS-12 secretion metabolites and the underlying mechanisms. The cytotoxicity evaluations were performed via 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay; 4′,6-diamidino-2-phenylindole staining; and FACS-flow cytometry tests. Also, the effects of *P. kudriavzevii* AS-12 secretion metabolites on the expression level of 6 important genes (BAD, Bcl-2, Caspase-3, Caspase-8, Caspase-9 and Fas-R) involved in the extrinsic and intrinsic apoptosis pathways were studied by real-time polymerase chain reaction method. *P. kudriavzevii* AS-12 secretion metabolites showed significant ($P < .0001$) cytotoxic effects on HT-29 cells (57.5%) and Caco-2 (32.5%) compared to KDR/293 normal cells (25%). Moreover, the cytotoxic effects of examined yeast supernatant on HT-29 cells were comparable with 5-fluorouracil, as a positive control (57.5% versus 62.2% respectively). Flow cytometric results showed that the induction of apoptosis is the main mechanism of the anticancer effects.

Abbreviations: 5-FU, 5-fluorouracil; cDNA, complementary DNA; CFS, cell-free supernatant; DAPI, 4′,6-diamidino-2-phenylindole; DMEM, Dulbecco modified Eagle medium; IC50, half-maximal inhibitory concentration; MTT, tetrazolium dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide); PBS, phosphate-buffered saline; PI, propidium iodide; RPMI, Roswell Park Memorial Institute medium; YMB, yeast malt broth.

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Also, according to the reverse transcriptase polymerase chain reaction results, the expression level of proapoptotic genes (BAD, Caspase-3, Caspase-8, Caspase-9, and Fas-R) in treated HT-29 and Caco-2 cells was higher than untreated and normal cells, whereas the antiapoptotic gene (Bcl-2) was downregulated. *P. kudriavzevii* AS-12 secretion metabolites exert its anticancer effects by inhibiting cell proliferation and inducing intrinsic and extrinsic apoptosis in colon cancer cells.

1. Introduction

Cancer placed among the main causes of morbidity and mortality worldwide [1]. Colorectal cancer (CRC) is the third most common type of cancers in developed countries [2,3] and the fourth leading cause of cancer-related death with 1.4 million new cases in the world [4-6]. The number of new cases is rising quickly because of the increase of exposure to different risk factors such as inflammatory bowel disease and lifestyle changes [7]. Both hereditary and environmental agents are the most predisposing factors [6], and the hereditary factors such as familial polyposis, hereditary nonpolyposis colon cancer, and ulcerative colitis cannot be totally under control; therefore, numerous studies focused on the controllable environmental risk factors such as physical inactivity; obesity; and high-meat, fat-rich, fiber-deficient diets [8,9]. By considering difference in gut microbiome composition between CRC patients and healthy controls, numerous investigators confirmed the significant role of the gastrointestinal (GI) microbiota in the health status and prevention of some malignancies [10,11]. The colonic microbiota can be a protective or predisposing factor in the etiology of CRC via controlling epithelial proliferation and differentiation, regulation of host immune system, and protection against pathogens [7,12].

However, dietary modification, particularly increasing intake of prebiotics and consumption of probiotics as adjuvant therapy, may be the beneficial approach in prevention or treatment of CRC [9]. Probiotics, as viable live microorganisms, when administered in sufficient amounts affect the host health via its beneficial activities on the intestinal tract [13]. Dysbiosis, imbalances in the intestinal microbiota, can stimulate acute and chronic clinical disorders and produce carcinogenic metabolites leading to different malignancies [10]. Besides, probiotics, by balancing intestinal microbiota and modifying the carcinogenic factors and related enzymes' activity, reduces the risk of developing of some neoplasia and tumor growth and inhibits the formation of precancerous lesions [14-16]. Although the most famous probiotic microorganisms belong to the lactic acid–producing bacteria, some yeast species such as *Debaryomyces hansenii*, *Torulaspora delbrueckii*, *Kluyveromyces lactis*, *K marxianus*, *Pichia fermentase*, *Saccharomyces cerevisiae*, *S boulardii*, and *Candida krusei* (*Issatchenkia orientalis*) have shown probiotic properties (tolerance to acid and bile) and their benefits on the host [17-20] through prevention and inhibition of the GI disorders, production of short-chain fatty acids, and particularly reduction of the incidence of CRC [21-23]. Probiotic yeasts exert their anticancer activities in different ways like degradation of carcinogenic compounds [24-26], alterations in the intestinal microbiota, producing antitumorogenic or antimutagenic compounds [27], stimulating the apoptosis and antiproliferative effects [28-30], and modulation of cell proliferation signaling pathways of the host [31]. *S cerevisiae* induces apoptosis through increasing Bax protein expression vs Bcl-2 in the MDA-MB-231 cells [32] and degeneration/liquefactive necrosis in vivo [33]. Other the probiotic yeasts are able to induce apoptosis pathways in different cancer cells [12,32,34,35]; thus, they can be used as anticarcinogenic and antitumorigenic agents in prevention and inhibition of different cancers, particularly CRC.

Pichia as anamorphs of some *Candida* species is a genus of yeasts in the family *Saccharomycetaceae* with spherical, elliptical, or oblong acuminate cells with more than 100 known species. Some *Pichia* species such as *P anomala*, *P membranifaciens*, *P fermentans*, and *P. kudriavzevii* can be found in dairy products [36,37].

Also, *P. kudriavzevii* as a potential probiotic yeast strain and anamorph of *C krusei* has been isolated from fruit juices, berries, tea beer, and some African fermented dairy products [38-41]. It has been reported that *P. kudriavzevii* plays a leading role in flavor improvement with proteolytic activity and, because of these properties, is used as a starter culture in dairy products [42].

P. kudriavzevii possesses appropriate probiotic characteristics and increases folate content of foods by folate production and high phytase activity [28,39,40,43]. Thus, it seems that *P. kudriavzevii* has the significant favorable ability to improve the nutritional quality of foods through natural fermentation as well as by possessing probiotic beneficial effects on human health [40]. Preceding studies verified beneficial effects of common probiotic yeasts on different cancers [12,34,35]. However, other unfamiliar probiotic yeasts like *P. kudriavzevii* and their components such as secretory metabolites may be more effective than common yeasts and should not be ignored. Despite numerous studies in this field, the exact mechanisms of some probiotic yeasts on CRC are unclear.

Therefore, we hypothesized that *P. kudriavzevii* AS-12 secretion metabolites might possess anticancer activity against human colorectal cancer cell lines (HT-29 and Caco-2). To test this hypothesis, we pursued the following objectives: (*a*) culture *P. kudriavzevii* AS-12 and isolate its cell-free supernatant (CFS); (*b*) prepare dried methanolic extract from isolated supernatant; (*c*) determine the half-maximal inhibitory concentration (IC50) for HT-29 and Caco-2 cells; (*d*) examine the antiproliferative effects; and (*f*) determine the expression levels of apoptosis-related genes such as BAD, Bcl-2, Caspase-3, Caspase-8, and Caspase-9. Hence, the present study aimed to assess the apoptotic effects of *P. kudriavzevii* AS-12 supernatant on HT-29 and Caco-2 cell lines compared with the normal epithelial cell (KDR/293) by focusing on the induced apoptosis mechanisms.

2. Methods and materials

2.1. Materials

Yeast malt broth (YMB) broth, methanol, L-glutamine, penicillin-streptomycin, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glycine, NaCl, formaldehyde, phosphate-buffered saline (PBS), 4′,6-diamidino-2-phenylindole (DAPI), trypsin-EDTA, and Roswell Park Memorial Institute medium (RPMI) 1640 were purchased from Sigma (Sigma, St Louis, MO, USA). Dulbecco modified Eagle medium (DMEM) (Gibco, Grand Island, NY, USA), 0.22-μm Millipore filter (Milli-Q, Millipore, Germany), fetal bovine serum (HyClone, Logan, UT, USA), dimethyl sulfoxide, Triton X-100, DEPC water (Merck, Germany), Annexin V-FITC/propidium iodide (PI) apoptosis kit (eBioscience, San Diego, CA, USA), RNX-plus solution (Sina Clone, Iran), Prime Script RT Reagent kit, and SYBR Green polymerase chain reaction (PCR) master mix (Takara Bio Inc, Tokyo, Japan) were used in this study.

2.2. *P. kudriavzevii* AS-12 CFS preparation

To prepare sterile CFS, *P. kudriavzevii* AS-12 was cultured in 50 mL of YMB at the aerobic condition for 72 hours at 37°C. Then, the culture was centrifuged at 4500 rpm for 10 minutes at 4°C, and 50 mL of supernatant was mixed with 75-mL volume of methanol and gently agitated for 24 hours. After 24 hours, the methanolic extract was dried by lyophilization, and remaining dried materials (95 mg) were dissolved in different amounts (5, 10, 15, 20, 25, or 30 mg/mL) in each used cell culture medium, DMEM and RPMI 1640. Finally, the prepared samples were adjusted to pH 7.2 and filtrated through 0.22-μm Millipore filter before using in the anticancer activity assessments. There was no significant difference in solubility of the methanolic extract in the 2 different media.

2.3. Cell lines and culture medium

Human colorectal adenocarcinoma cell lines (Caco-2, HT-29) and human embryonic kidney normal cell line (KDR/293) were purchased from the same vendor, Pasteur Institute, National Cell Bank of Iran, Tehran, 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% CO_2 with medium renewed every 3 days. The KDR/293 cells were grown in a high–glucose concentration (4.5 g/L) DMEM, whereas Caco-2 and HT-29 cells were grown in RPMI 1640 supplemented with 10% (v/v) inactivated fetal bovine serum, 8 mmol/L L-glutamine, 1% of mixture penicillin (100 IU/mL)/streptomycin (100 μg/mL).

2.4. MTT assay

Cell viability in treated and untreated colorectal cancer cell lines was determined by the MTT assay. Cell viability assessment was performed with methanolic extract of *P. kudriavzevii* AS-12 supernatant and 5-fluorouracil (5-FU) (7 μL/well of 96-well plate) as the positive control. At first, the IC50 for HT-29 and Caco-2 cells was determined by prescreening MTT tests (in the range of 10 to 100 μg/mL) at 24 and 48 hours (Fig. 1). Briefly, 1.2×10^4 cells/well were seeded into 96-well microplate containing 200 μL of growth medium for 24 hours. Then, the medium of each well was carefully removed and replaced with 200 μL of growth medium containing determined effective dose dried materials of the supernatant. Afterward, the treated cells were incubated similarly to growth condition. After determined time point, the medium of each well was replaced with 150 μL of fresh growth medium and 50 μL of MTT solution (3 mg/mL in PBS 0.01 mol/L, pH 7.2) and were incubated similar to the growth condition for 4 hours. Then, the medium of each well was carefully removed, and subsequently 200 μL of of dimethyl sulfoxide and 25 μL of Sorenson buffer (0.1 mol/L glycine, 0.1 mol/L NaCl, pH 10.5) were poured to each well. Then, the plates were incubated for 15 minutes at room temperature. The absorbance was measured using enzyme-linked immunosorbent assay plate reader (ELx 800; Biotek, Winooski, VT, USA) at 570 nm. The growth inhibitory effects of supernatant were calculated according to the following formula: the growth inhibition ratio = [(the absorbance of blank control group – the absorbance of experimental group)/the absorbance of blank control group] × 100% [44,45].

2.5. Morphological analysis of the cancerous (Caco-2 and HT-29)/normal (KDR/293) cells

Morphological changes due to treatment by methanolic extract of yeast secretion metabolites were investigated by DAPI staining. For this purpose, sterile coverslip slides were placed into each well of a 6-well culture plate. Then, 3 mL of growth medium including 3×10^5 cells was cultured in each 6-well plate and incubated at growth condition for 24 hours. Subsequently, untreated control and treated with 3 mL of the sterile growth medium containing determined dried materials of supernatant and 5-FU (105 μL/well of 6-well plate) as the positive control group was incubated similarly to growth condition. After 48 hours, the cells were washed with prewarmed tissue culture media and were carefully replaced with a freshly prepared fixative solution (prewarmed RPMI containing 4% formaldehyde). With the aim of cell fixation, the plates were incubated for 5 minutes at 37°C, and the fixed cells were washed twice with PBS and then permeabilized with PBS containing 0.1% Triton X-100 for 5 minutes at 37°C. The permeabilized cells were then stained with 100 μL per well of DAPI (250 ng/mL for each well) for 3 minutes of incubation at room temperature. Finally, the slides were washed with PBS, and morphological changes of cell nucleus were 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) [46,47].

2.6. Flow cytometry

Colon cancer cells including HT-29, Caco-2, and normal KDR/293 cells were seeded into 6-well culture plates (1.2×10^5 cells/well) and treated similarly to DAPI staining method. After treatment time point (48 hours), the treated/untreated control cells were detached by trypsin-EDTA, and supernatants were discarded after centrifugation at 900 rpm for 10 minutes

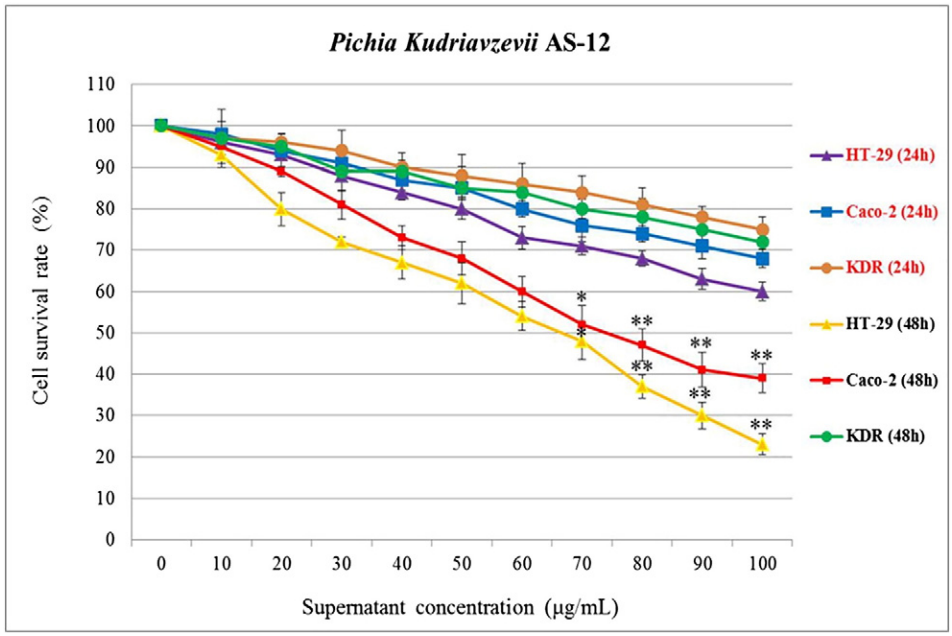

Fig. 1 – MTT assays were used to determine IC50 in HT-29 and Caco-2 cells after treatment with different concentrations of secretion metabolites of *P. kudriavzevii* AS-12 (in the range of 10 to 100 μg/mL dried materials from the methanolic extract) for 24 and 48 hours. The effective dose was determined to be about 65 μg/mL for HT-29 and 75 μg/mL for Caco-2 cells at 48 hours, which significantly decreased survival rates, whereas KDR/293 normal cells remained intact. Data were obtained from 3 independent experiments for each test and were normalized to naive cells and presented as percent viable cells (means ± SD). Asterisks indicate a significant increase in cell death (*$P \leq .05$, **$P \leq .01$).

at 28°C. According to the Annexin V-FITC/PI apoptosis kit instructions, the cell pellets were washed once in PBS and once in 1× binding buffer (1 mL 10× binding buffer + 9 mL dH_2O) and centrifuged and were disposed supernatant in each stage. Afterward, the cells were resuspended in 100 μL of 1× binding buffer and transferred into a new 5-mL tube. Then, 5 μL of FITC-conjugated Annexin V was added to 100 μL of the cell suspension and incubated for 15 minutes at room temperature under dark condition. After incubation time, the cells were washed with 1× binding buffer and resuspended in 200 μL of 1× binding buffer again. Finally, 5 μL of PI staining solution was added to the cells and analyzed by flow cytometry within 4 hours [48]. Quadrant settings were fixed with untreated, single-stained controls and copied to dot plots of the treated cells. Data analysis was conducted using CELL Quest Pro software (BD Biosciences, San Jose, CA, USA). The experiment was repeated 2 times with triplicate samples for each experiment [49]. Analyses were performed using 150 000 cells at a rate of 900 cells/s.

2.7. RNA isolation, complementary DNA synthesis, quantitative real-time PCR analysis

After treatment, all treated/untreated cells were washed 3 times with sterile PBS (pH 7.2). Total RNA was isolated from cells by direct lysis using RNX-plus solution according to manufacturer's instruments. The obtained total RNA pellet was dissolved in 50 μL DEPC-treated water, and then quantity and quality of total RNA were assessed by UV spectrophotometry and agarose gel electrophoresis, respectively. One microgram of isolated RNA was used for synthesis of complementary DNA (cDNA) using Prime Script RT Reagent kit according to manufacturer's recommendations for cDNA synthesis.

To amplify intended genes, particular primers, listed in the Table, were designed for each gene. All amplification reactions were performed in triplicate for each sample, and every experiment mixture (20 μL), containing 10 μL SYBR Green PCR master mix, 1 μL cDNA (1 μg/μL), 1 μL primer (forward and reverse), and 0.8 μL 6-carboxy-X-rhodamine (ROX as reference dye), was subjected to ABI-step I plus (Applied Biosystems, Foster City, CA, USA) instrument [50,51]. Thermal cycling condition was as follows: 1 cycle at 95°C for 5 minutes followed by 40 cycles at 95°C for 20 seconds, 60°C for 35 seconds, and 72°C for 10 seconds.

Interpretation of the results was performed using the Pfaffle method, and the threshold cycle values were normalized to the expression rate of GAPDH as a housekeeping gene [52,53]. All reactions were performed in triplicate, and negative controls were included in each experiment.

2.8. Statistical analyses

All data were obtained from at least 3 independent experiments and are expressed as means ± standard deviations (SDs). All the experimental data were analyzed by using the 1-way analysis of variance, following Tukey post hoc using the Statistical Package for the Social Sciences (version 16.0; SPSS Inc, Chicago, IL, USA). Statistical significance was set at $P \leq .05$.

Table – Primers sequences for reverse transcriptase PCR amplification

Gene name and symbol	Sequence (5′ ➔ 3′)	Amplicon size (base pair)	TM	
			F	R
Bcl-2	F:5′-GGTGGGGTCATGTGTGTGG-3′ R:5′-CGGTTCAGGTACTCAGTCATCC-3′	89	60.6	60.1
BAD	F:5′-TGGACTCCTTTAAGAAGGGAC-3′ R:5′-CAAGTTCCGATCCCACCAG-3′	113	56.6	57.8
Fas-R	F: 5′-AGCGCTGAAGAGCCAACATA-3′ R: 5′-TGGGTACTTAGCATGCCACT-3′	126	59.7	58.7
Caspase-3	F:5′-TGCCTGTAACTTGAGAGTAGATGG-3′ R:5′-CTTCACTTTCTTACTTGGCGATGG-3′	172	59.8	60.1
Caspase-8	F: 5-ACATGGACTGCTTCATCTGC-3′ R:5′-AAGGGCACTTCAAACCAGTG-3′	123	58.2	58.6
Caspase-9	F:5′-TGCTGCGTGGTGGTCATTCTC-3′ R:5′-CCGACACAGGGCATCCATCTG-3′	94	63.2	63.1
GAPDH	F:5′-AAGCTCATTTCCTGGTATGACAACG-3′ R:5′-TCTTCCTCTTGTGCTCTTGCTGG-3′	126	61.6	62.6

TM, melting temperature of primers; F, forward primer; R, reverse primer.

3. Results

3.1. *Cytotoxicity assessment*

The IC50s after treatment by prepared secretion metabolites of *P. kudriavzevii* AS-12 (explained in Section 2.2) were determined to be about 65 μg/mL and 75 μg/mL for HT-29 and Caco-2 cells at 48 hours, respectively (Fig. 1). Also, the KDR/293 normal cells as control group were treated with the highest determined concentration (75 μg/mL). As seen in Fig. 2, because of the treatment by secretion metabolites of *P. kudriavzevii* AS-12, the survival rates of studied colon cancer cells were significantly decreased compared with control groups. The survival rates after treatment of HT-29 and Caco-2 cells were 42.5% and 67.5% in comparison with KDR/293 normal cells (75% cell viability), respectively. Besides, the survival rates of 5-FU as a positive control in HT-29 and Caco-2 cells were 37.8% and 52.7%, respectively. Moreover, treatment with an equal volume of YMB (as yeast culture medium) methanolic extract showed a significant proliferative effect on the KDR/293 cells (1.9-fold), whereas it did not indicate a significant antiproliferative impact on the all epithelial cancer cell lines (Fig. 2).

3.2. *Qualitative apoptosis evaluation (DAPI staining)*

The morphological changes like nuclear fragmentation, chromatin condensation, and membrane blebbing associated with

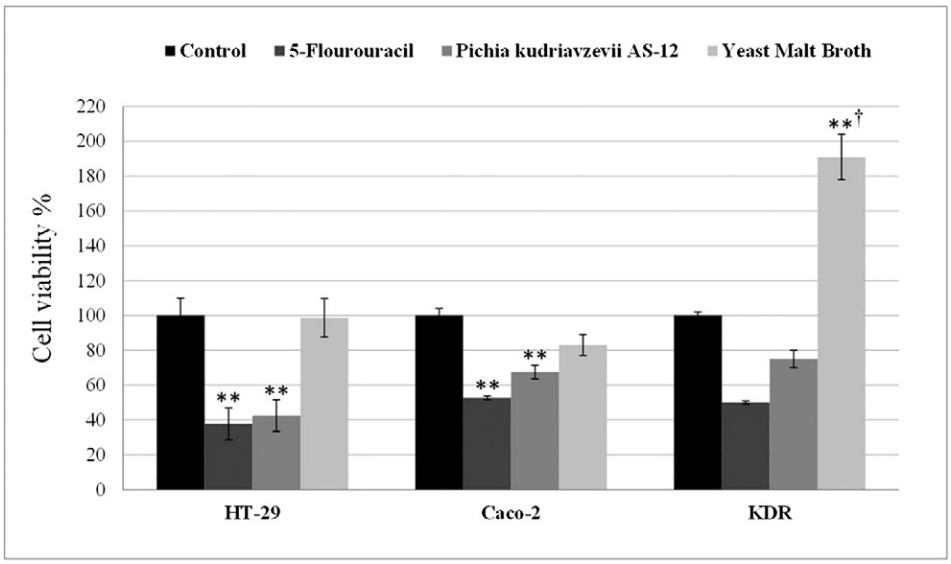

Fig. 2 – Effects of secretion metabolites of *P. kudriavzevii* AS-12 containing indicated concentrations of dried materials from the methanolic extract (65 μg/mL for HT-29 cells and 75 μg/mL for Caco-2 cells) on the viability of HT-29, Caco-2 cancer cells, and KDR/293 (75 μg/mL) normal cells for 48-hour incubation. Untreated cells were used as negative control, and 5-FU (7 μL/well of 96-well plate) was used as positive control. YMB as yeast culture medium was used to determine its effect on cell viability. All the experiments were performed in triplicate (n = 3), and the data were presented as means ± SD. *P ≤ .05 and **P ≤ .01 indicate significant and highly significant vs the control group.†YMB significantly increased cell proliferation in KDR/293 cell lines.

induction of apoptosis were observed by DAPI staining. As shown in Fig. 3, the HT-29 and Caco-2 cells treated with 5-FU (105 μL/well of 6-well plate) and 65 μg/mL and 75 μg/mL of methanolic extract of secretion metabolites of *P. kudriavzevii* AS-12, respectively, showed the cell volume shrinkage, and condensed (early apoptosis) or fragmented (late apoptosis) nuclei, whereas whole control cells were in a normal state with intact nuclei and membrane. Also, other apoptotic symptoms such as membrane blebbing and apoptotic body formation were observed in the treated cancer cells but not in the normal cells (Fig. 3).

3.3. *Quantitative apoptosis examination (flow cytometry)*

Flow cytometry was applied to detect quantitatively apoptotic rates through Annexin V-FITC/PI staining. Treatment of HT-29 and Caco-2 cells with 65 and 75 μg/mL of methanolic extract of *P. kudriavzevii* AS-12 secretion metabolites for 48 hours increased the percentage of cells in early (Annexin V+/PI–) and late (Annexin V+/PI+) apoptosis compared with the untreated control and normal KDR/293 cells treated with the highest determined dose (75 μg/mL). After treatment with secretion metabolites of *P. kudriavzevii* AS-12 for 48 hours, total induced apoptosis ratio (total early and late apoptosis) in HT-29, Caco-2, and normal KDR/293 cells was found to be 78.32%, 54.1%, and 8.8%, respectively (Fig. 4). Our findings suggested that methanolic extract of secretion metabolites of *P. kudriavzevii* AS-12 is able to trigger apoptosis pathways in colon cancer cells similar to 5-FU as approved anticancer drug.

3.4. *Detection of gene expression levels*

Fig. 5 depicts the expression level of proapoptotic and antiapoptotic key genes in colon cancer and normal cell lines after treatment with a methanolic extract of *P. kudriavzevii* AS-12 supernatant and 5-FU compared with untreated control cells. The expression level of the proapoptotic genes BAD, CASP-3, CASP-8, CASP-9, and Fas-R was increased significantly, whereas the antiapoptotic gene Bcl-2 was decreased, in the treated cancer cells. Interestingly, the expression level of proapoptotic genes Fas-R, CASP-3, CASP-8 ,and CASP-9 in the HT-29 and Caco-2 cancer cells approximately increased 2-fold and in some cases were even higher than 5-FU as a positive control group. Based on the findings, secretion metabolites of *P. kudriavzevii* AS-12 can trigger the intrinsic and extrinsic apoptosis pathways in colon cancer cells through simultaneous downregulation of Bcl-2 and upregulation of BAD and CASP-9 genes and also upregulation of Fas-R, CASP-3, and CASP-8, respectively. As well, results did not show unfavorable significant effects on apoptotic critical genes' (intrinsic or extrinsic signaling pathway genes) expression in studied normal epithelial cells (KDR/293).

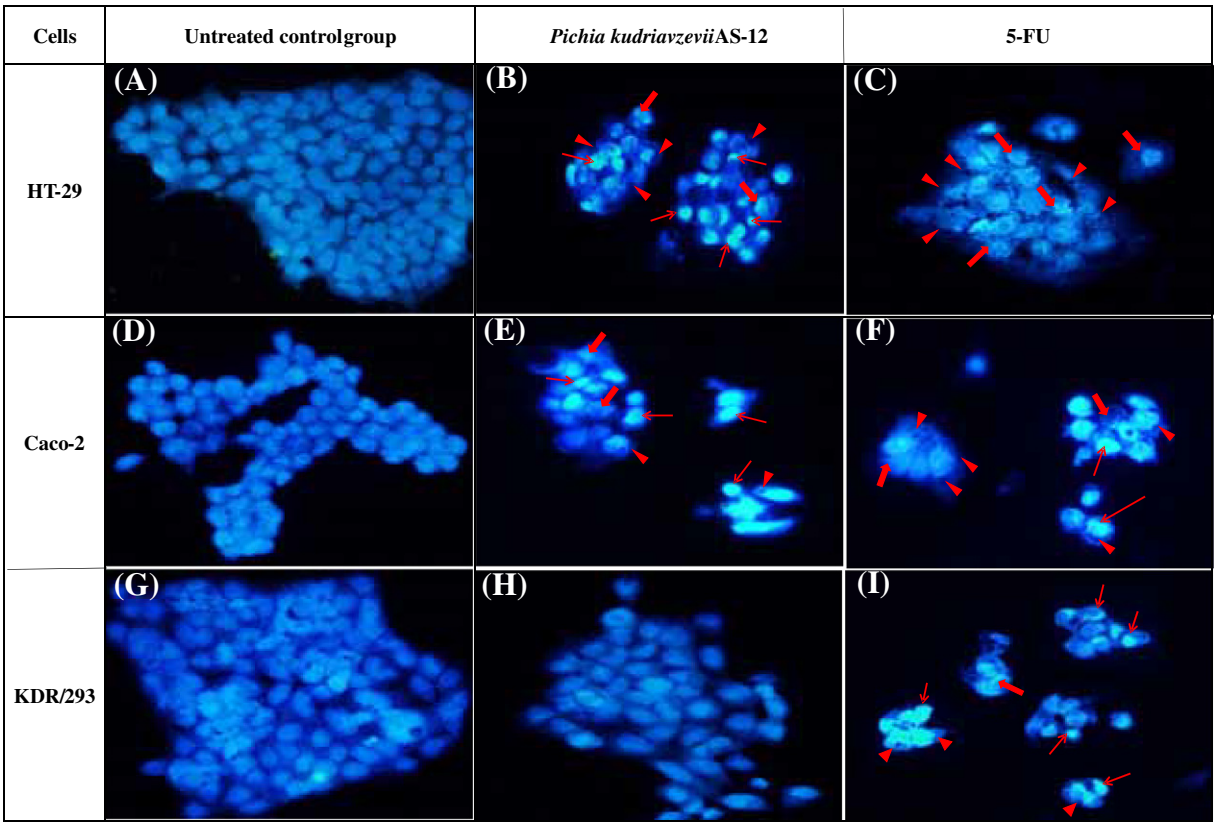

Fig. 3 – DAPI staining of treated/untreated HT-29, Caco-2, and KDR/293 cells. Panels represent untreated control groups, treated with a sterile extract of *P. kudriavzevii* AS-12 secretion metabolites and treated with 5-FU (105 μL/well of 6 well plate) as a positive control group for 48-hour incubation. Arrows depict chromatin condensation (thin arrows), fragmented nuclei (thick arrows), and membrane blebbing (arrowheads).

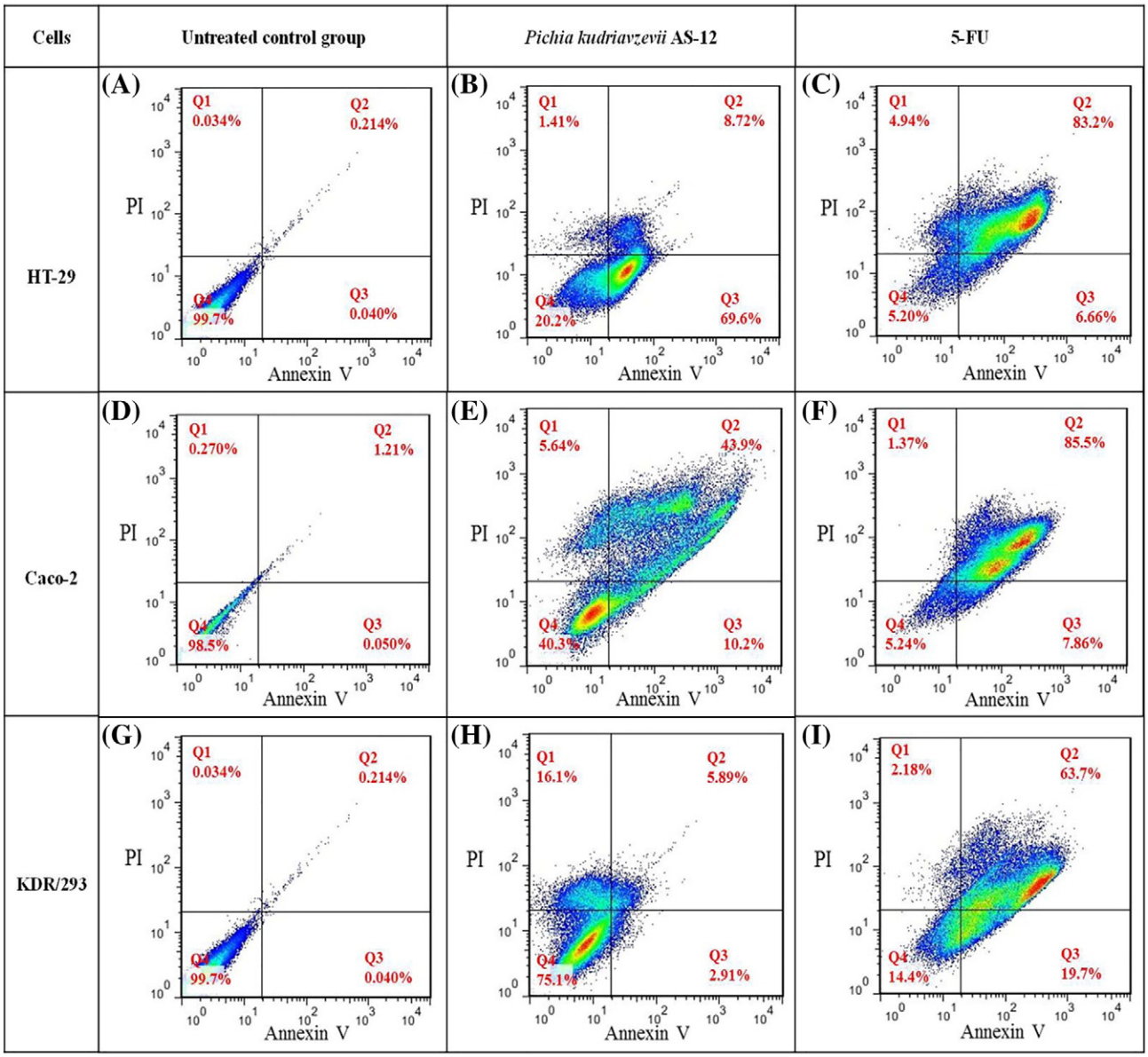

Fig. 4 – Flow cytometric analysis of treated/untreated cancerous and normal cells. Cells were treated with FITC-Annexin V in combination with PI to detect apoptosis and necrosis before being subjected to analysis by flow cytometry by a method described in Section 2.6. Panels illustrate untreated control groups, treated with a sterile extract of *P. kudriavzevii* AS-12 secretion metabolites and treated with 5-FU (105 μL/well of 6-well plate) as a positive control group for 48-hour incubation. Cells stained with Annexin V-FITC and PI were classified as necrotic cells (the upper left quadrant [Q1]; Annexin –/PI +), late apoptotic cells (the upper right quadrant [Q2]; Annexin +/PI +), early apoptotic cells (the lower right quadrant [Q3]; Annexin +/PI –), or intact cells (the lower left quadrant [Q4]; Annexin –/PI –).

4. Discussion

Different therapeutic strategies are being used for CRC treatment such as surgery, chemotherapy, and targeted therapy [54-56]. Apoptosis, as a precise gene-regulated mechanism to control cell proliferation/death [57], has been suggested as one of the targeted therapy strategies [58]. In the present study, treatment with secretion metabolites of *P. kudriavzevii* AS-12 induces intrinsic and extrinsic apoptosis pathways in human colorectal cancer cells (HT-29 and Caco-2). Moreover, yeast supernatant increased the expression level of proapoptotic genes (BAD, CASP-3, CASP-8, CASP-9, and Fas-R), while it decreased the expression level of the antiapoptotic gene (Bcl-2) simultaneously. Numerous investigations reported the anticancer effects of probiotic yeasts in vivo and in vitro [32,35]. *S boulardii* supernatant has been reported to trigger apoptosis and possesses prophylactic effects on cancer colony formation in C57BL/6 J Min/+ (Apc^{Min}) mice [59]. Also, a nontoxic glycosylated compound of supernatant inhibits NF-κB–mediated IL-8 gene expression [60]. The methanolic extract of *P. kudriavzevii* AS-12 supernatant induced apoptosis in colon cancer cells while showing less apoptotic effects on KDR/293 normal cells. Similarly,

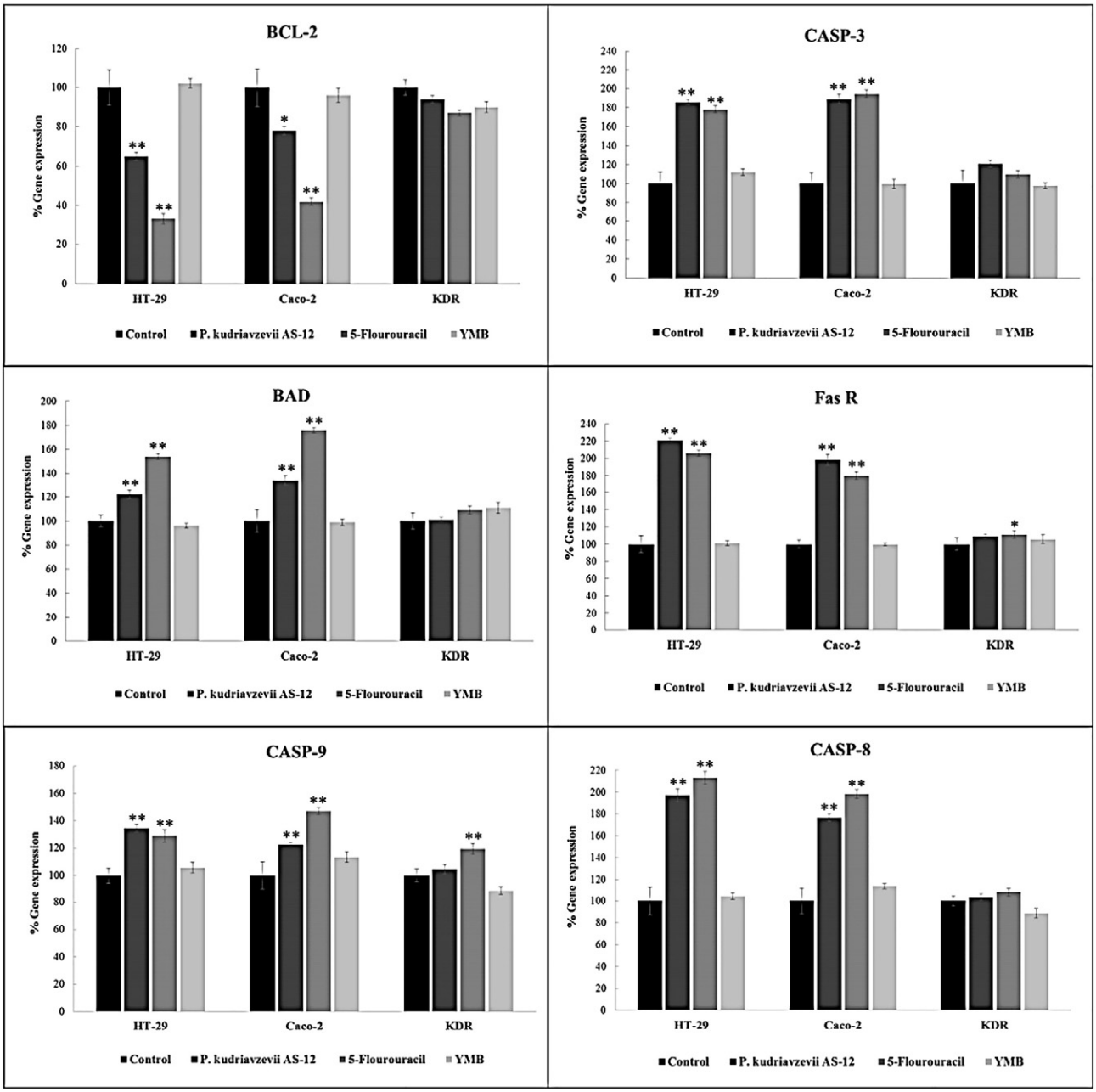

Fig. 5 – Main intrinsic and extrinsic apoptosis pathway gene expression ratio in the HT-29, Caco-2, and KDR/293 normal cells treated with sterile extract of *P. kudriavzevii* AS-12 secretion metabolites containing 65 and 75 μg/mL of dried materials, and treated with 5-FU (105 μL/well of 6-well plate) as positive control group compared with untreated group for 48 hours, respectively. Target genes were normalized to GAPDH as housekeeping control gene. All the experiments were performed in triplicate (n = 3), and values are means ± SD. *P ≤ .05 and **P ≤ .01 indicate significant and highly significant vs the control group.

Ghoneum and Gollapudi [34] showed that metastatic human breast cancer (MCF-7 and ZR-75-1) and colon cancer (Caco-2) cell lines undergo apoptosis following treatment by *S cerevisiae* with apoptosis rates of 30.3% and 76%, respectively. Apoptosis is morphologically described by cell shrinkage, membrane blebbing, and condensed (early apoptosis) or fragmented (late apoptosis) nuclei [61]. Qualitative apoptosis evaluation indicated that because of the treatment by heat-killed *S cerevisiae*, cancer cells underwent apoptosis [34,62]. Probiotics fermentation technology induced apoptosis through a significant decrease in expression of Bcl-2 and mitochondrial membrane potential and an increase in activation of the executioner CASP-3 in human multidrug-resistant myeloid leukemia compared with untreated cells [63,64]. By considering different cell types showing different responses to same yeast treatment [32,34,35,62], our findings demonstrated that supernatant of *P. kudriavzevii* AS-12 affected HT-29 cells more than Caco-2 cells, and related survival rates of them were 42.5% and 67.5%, respectively. Generally, chemotherapeutic synthetic agents induce apoptosis in an untargeted manner and affect normal cells through toxic, immunosuppressive,

mutagenic, and carcinogenic adverse effects [65-69]. Hence, a lot of investigators are trying to find natural anticancer compounds that induce apoptosis in cancer cells without adverse effects [59,62]. Imbalances among proapoptotic and antiapoptotic proteins can trigger or decline malignant cell survival [70]. As well, *S cerevisiae* causes a significant alteration in Bax to Bcl-2 ratio and triggers apoptosis in human metastatic breast cancer cells [32,34]. BAD, as a proapoptotic protein, in response to death signals dephosphorylated and binds to antiapoptotic molecules like Bcl-2 and Bcl-XL and neutralizes their antiapoptotic functions [71]. Based on this study findings, *P. kudriavzevii* AS-12 supernatant decreased Bcl-2 gene expression and simultaneously increased BAD and CASP-9 gene expression that proved the induction of apoptosis through intrinsic pathway in HT-29 and Caco-2 cell lines, whereas it affected normal KDR/293 cells less.

Probiotic yeasts showed various beneficial properties such as inhibition of pathogen adhesion, neutralization of bacterial toxins [72-74], enhancement of the mucosal immune response [75], and modulation of cell proliferation signaling pathways of the host [59]. In this way, *S boulardii* produces a soluble anti-inflammatory factor that inhibits NF-κB–mediated IL-8 gene expression [60]. Several studies showed that activated NF-κB upregulates 4 genes (TNF receptor-associated factor 1 and 2, and cellular inhibitor of apoptosis protein 1 and 2) at mRNA level that are responsible for suppression of TNF-α and its effect on activation of CASP-8 [76]. Cytotoxic drugs applied in chemotherapy treatments caused induction of apoptosis in cancer cells through upregulation of Fas ligand and NF-κB–regulated gene products that suppress TNF-α–induced apoptosis and following Fas-induced apoptosis [77]. Some investigations suggested that inhibition of NF-κB by different chemotherapeutic compounds increased apoptosis in response to various stimuli [76].

Coincubation of stimulated Caco-2 cells (by lipopolysaccharide) with lactic yeast *K marxianus* B0399 modulated the immune responses and decreased the proinflammatory cytokines such as IP-10, IL-8, IL-12, and IFN-γ [78]. Unlikely, Dalmasso et al revealed that *S boulardii* blocks mitochondrial and extracellular death receptors via reduction of TNF-α secretion and production and prevention of CASP-9 and -8 activities in EHEC-infected T84 human colonic cell line [79]. By considering various studies' findings, probiotic yeasts perform their anticancer effects by immunomodulatory activities via downregulation or upregulation of anti- or proapoptotic cytokines like NF-κB and TNF-α [12,60].

The fermentation process in colonic microbiota by probiotic yeasts increased production of short-chain fatty acids especially butyrate (having anticarcinogenic, anti-inflammatory, and antioxidative activities) from undigested dietary carbohydrates [78,80]. Also, butyrate showed inhibitory and preventive effects on colon cancer through different mechanisms [81,82]. Numerous investigations showed that treatment of human colon cancer cells by butyrate induced apoptosis via downregulation of cyclin B1 (CB1); upregulation of the p21 cell-cycle inhibitor proapoptotic protein BAK; and increase the CASP-8, -9, and -3 activities [83,84].

In this study, supernatant of *P. kudriavzevii* AS-12 inhibited the proliferation and induced the apoptosis in colorectal cancer cell lines. The induction of apoptosis through yeast supernatant was mediated by changes in the expression levels of apoptosis-related genes. Therefore, the effective inhibition of HT-29 and Caco-2 cells in vitro suggests that the secretion metabolites of *P. kudriavzevii* AS-12 can be a potentially promising anticancer agent for effective treatment of colorectal cancer cells.

There are some limitations and weakness in this study: (1) the cell culture study, (2) the examination of anticancer effects on colorectal cancer cells, (3) it is not specified whether *P. kudriavzevii* AS-12 supernatant can cause anticancer effects on other types of cancer cell lines, and (4) the secretion metabolites of *P. kudriavzevii* AS-12 consisted of several compounds and the main effective compound(s) was(were) not characterized.

In conclusion, the results of the current study are the first to indicate that methanolic extract of *P. kudriavzevii* AS-12 supernatant significantly inhibits proliferation and induces apoptosis through an increase or decrease in expression level of pro/antiapoptotic genes in the human colon cancer cells (HT-29 and Caco-2). As well, different assessments supported the proapoptotic effects of secretion metabolites of *P. kudriavzevii* AS-12 on colon cancer cells with less adverse effects on normal epithelial cells. Interestingly, our findings represented lower cytotoxic effects due to supernatant treatment than common chemotherapy drug, 5-FU, on normal KDR/293 cells. Therefore, we accept the hypothesis that *P. kudriavzevii* AS-12 secretion metabolites possess anticancer activity against human colorectal cancer cell lines (HT-29 and Caco-2). However, additional investigations should be conducted to clarify the exact mechanisms of yeasts and their effective compound(s) in human colon cancer.

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