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Investigation of Anti-proliferative and Anti-migratory Properties of Recombinant J2-C2 Against Tumor Cells

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ABSTRACT: Cancer is a big public health problem in many parts of the world. A novel anti-tumor protein (J2-C2) was previously isolated from *Arca inflata* and it was reported that this protein had anti-proliferative effect on some human tumor cell lines such as A549, HepG2 and SPC-A-1. In this study, firstly, J2-C2 was produced by recombinant techniques in the *Escherichia coli* strain BL21 (DE3) pLysE and this protein was purified by Ni-NTA agarose affinity chromatography. Expressed recombinant J2-C2 was analyzed with SDS-PAGE. 75.5 mg ml^{-1} of J2-C2 was achieved from a 600 mL culture. Then using HT-29, MCF7 and PC3 cancer cell lines, we showed the effect of recombinant of J2-C2 on cell proliferation, migration and apoptosis in a cell specific manner. Cell viability was measured using MTT assay. Additionally, real-time-qPCR was applied to analyze the transcript levels of apoptosis related genes such as Bcl-2, Bax and p53. The $2^{-\Delta\Delta Ct}$ method was performed to determine the relative changes in gene transcription. Moreover, scratch wound healing assay was performed to evaluate the effect of J2-C2 on cancer cell migration. Consequently, we found that recombinant J2-C2 did not have a significant effect on cell viabilities of MCF7, PC3 and HT29 in concentration-dependent manner. Furthermore, our results showed that recombinant J2-C2 declined HT29, MCF7 cell migration. However, we did not observe the same results for PC3 cancer cell line.

Keywords: Recombinant J2-C2, therapeutic protein, cell proliferation, cell migration, apoptosis.

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INTRODUCTION

Cancer is one of the biggest health problems in the world today. In addition, there are deficiencies in the category of antimetastatic drugs in chemotherapy. However, metastasis is one of the main causes of treatment failure and cancer-related deaths in cancer patients (Qian et al.,2017). Therefore, the development of possible therapeutic agents that can suppress metastasis is very important for cancer treatment (Li et. al., 2019).

Chemotherapy is one of the most effective methods for cancer treatment. Toxic side effects of chemotherapy and acquired chemotherapy resistance are still important problems to be solved (Cheok, 2012; Fan et. al., 2019).

Therapeutic proteins are a critical component of the pharmaceutical industry and are actively used in the treatment of many diseases, including cancer (Lagassé et. al., 2017). In the past, the main way to obtain a specific therapeutic protein was to isolate it from a natural source. Generally, this method was inefficient and time consuming. Recently, therapeutic proteins can be produced recombinantly with high efficiency with the developing recombinant DNA technology.

Recombinant therapeutic proteins are produced in different prokaryotic and eukaryotic systems such as mammalian, insect cells, bacteria and yeast. The majority of therapeutic proteins are produced in in mammalian cell lines because they have ability to produce high-quality proteins which are similar to the naturally occurring ones (Andersen et. al., 2002; Assenberg et. al., 2013). However, many recombinant therapeutic proteins are produced in *Escherichia coli* because of its inexpensive and fast high-density cultivation, the well-characterized genetics, and high-yield production (Swiech et. al., 2012; Rosano et. al., 2014).

In a previous research, a novel *in vitro* anti-tumor protein J2-C2 with a molecular weight of 27.153 kDa was purified from the *Arca inflata* (Zhu et. al., 2017). *Arca inflata*, a member of the family Arcidae which is a commercially important bivalve species in Asian countries (Li et. al., 2008). J2-C2 is a monomeric and a non-glycoprotein. As stated in the article published in 2017, Zhu and his friends investigated the anti-tumor property of J2-C2 against some human tumor cell lines (lung and liver) and also reported that the IC_{50} values of this isolated protein were 42.38, 45.64 and 48.73 μM for A549 (lung), HepG2 (liver) and SPC-A-1(lung) cell lines, respectively.

In the current study, J2-C2 was first recombinantly produced to obtain a high purity and desired amount of protein for use in biological activity experiments. And then we tested the anticancer potential of recombinant J2-C2 in MCF7 (breast), HT29 (colon) and PC3 (prostate) cancer cell lines with MTT assay. Additionally, the effect of J2-C2 on cancer cell migration and Bcl-2, Bax, p53 gene expression were analyzed.

MATERIALS AND METHODS

Gene Cloning and Expression

Recombinant protein expression using bacterial and other eukaryotic host organisms is a fundamental technology for protein production. The basic step in recombinant protein expression is codon optimization where a coding sequence for a protein of interest is designed by synonymous substitution aiming to enhance its expression level. For example, a conventional approach is to substitute rare codons by frequent codons according to the genomic codon usage in a host organism (Saito et.al., 2019). In this context, we performed a codon optimization study for *J2-C2* gene sequence from *A.inflata* (Zhu et. al., 2017) on the basis of *Escherichia coli*. The optimized synthetic gene was synthesized by Biomatik Corporation. *Escherichia coli* strain BL21 (DE3) pLysE (Novagen, Inc.) was used as the host

for recombinant J2-C2 expression. This strain was transformed by Pet30a-J2-C2 plasmid. Transformed cells were spread on several Luria-Bertani (LB) agar plates containing both 50 μg mL^{-1} kanamycine and chloramphenicol (34 μg mL^{-1}). One colony of BL21 (DE3) pLysE recombinant was cultivated in 4mL of selective LB medium and was grown at 37°C with orbital shaking (240 rpm) for 16h. This overnight culture was inoculated into 600 mL of selective LB and incubated at same conditions until the OD_{600} was 0.6. The expression of recombinant protein was induced by adding IPTG to a final concentration of 0.001M.

Purification of J2-C2

Following additional three hours of incubation, the culture was harvested by centrifugation and the cell pellets were resuspended in 100 mM Tris-HCl buffer (pH 7.0) containing 1 mg mL^{-1} lysozyme, 1 mM PMSF and 1mM benzamidine. The cells were lysed by a sonicator (Sonics VCX 130) and the insoluble fraction was collected by high speed centrifugation (Vision VS-30000i) at 30.000 rpm, 4°C for 1 h. Ni-NTA metal-affinity chromatographic procedure (Qiagen) was used to purification of recombinant protein carrying N-terminal 6x histidine (containing six histidine residues). Purity of recombinant J2-C2 was determined by 12% SDS-PAGE and visualized by commasie brillant blue (Weber et. al., 1972). The protein concentration was determined using UV spectroscopy by measuring the absorbance of the pure protein at 280 nm (Cabrita et. al., 2004). The purified proteins were collected and dialyzed against PBS (phosphate buffered saline). The dialyzed protein was stored 4ºC until using for cancer cell proliferation and migration analyses.

MTT Cell Viability Assay

Antiproliferative effects of recombinant J2-C2 was investigated on cancer cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Serva, Germany) cell proliferation assay. This was performed according to the method described in our previous studies (Ispir et.al.,2019; Inan et al., 2018). HT-29, PC3 and MCF7 cancer cells were cultured in multiwell plates and in media (RPMI 1640 and DMEM, respectively) with 10% FBS. These cell lines were treated with recombinant J2-C2 at eight different doses (240-1,875 μg ml^{-1}) for 24 and 48 hours. 5-Fu (5-fluorouracil) was used as a positive control reagent.

Cell Migration Assay

Tumor cell migration was examined *in vitro* by scratch wound closure model. Tumor cells (HT29, PC3, MCF7) were seeded (5×10^4 cells $well^{-1}$) in 6-well plates in appropriate culture media. When cells reached more than 80% confluency, cell monolayer was wounded by a steril 200-μl pipette tip, the medium and cell debris were aspirated and all the wells were washed with sterile phosphate buffer saline. Then cells were treated with 240 μg ml^{-1} of J2-C2 which was determined by MTT cell viability assay in the previous experimental stage. Images were captured with an inverted light microscope (OLYMPUS, Japan) at different time points (0h, 24h and 48h) post- J2-C2 administration. Percentage of cell migration rate was calculated with the following formula= (remaining wound area/ wound area at 0h) x100. These wound areas were measured by image analysis software of OLYMPUS inverted light microscope. The calculated values were compared with negative control and evaluated whether the substance was effective on cancer cell migration.

RNA Extraction, cDNA Synthesis and Quantitative PCR Analysis

For quantitative RT-PCR analysis of p53, Bax and Bcl-2 mRNA expression levels, confluent cancer cells (HT-29, PC3, MCF7) were treated with 240 μg ml^{-1} of compound for 48h. Controls were treated with the same amount of medium as used in the corresponding experiments. After the incubation

period, total RNA was extracted from the cultured cell using TRIzol reagent (Ambion, USA) according to the manufacturer's protocol. RNA purity and concentration were assessed by spectrophotometry using the 260nm/280nm ratio. Then cDNA was obtained using the manual which was described by the manufacturer of reverse transcriptase enzyme (Thermo Scientific, USA).

Real-time quantitative PCR (qRT-PCR) was conducted with qTOWER3G Real-Time PCR Thermocycler using EvaGreen. The primer pairs for genes were presented in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. All experiments were performed in triplicates. Target cDNA was amplified as follows: 3 min at 94ºC then 40 cycles of amplification (denaturation at 94ºC for 30 s, annealing at 56ºC for 30 s and elongation at 72ºC for 1 min per cycle). The increase in fluorescence was measured and recorded in real-time during the elongation step. The $2^{-\Delta(\Delta CT)}$ method (Priego, et.al., 2008; Rao et. al., 2013) was used for relative quantitation of Bax, Bcl-2, p53 mRNA expression.

Table 1. Oligonükleotides utilized for qRT-PCR amplification

Gene	Primer Sequences
P53	5'-GCCCAACAACACCAGCTCCT-3'
	5'-CCTGGGCATCCTTGAGTTCC-3'
Bcl-2	5'- ATCGCCCTGTGGATGACTGAG-3'
	5'-CAGCCAGGAGAAATCAAACAGAGG-3'
Bax	5'-GGACGAACTGGACAGTAACATGG-3'
	5'-GCAAAGTAGAAAAGGGCGACAAC-3'
GAPDH	5'-GGATTTGGTCGTATTGGG-3'
	5'-GGAAGATGGTGATGGGATT-3'

Statistic Analysis

Statistic analysis was performed using Student's t-test on all data. A statistically significant difference was considered with a p value < 0.05.

RESULTS AND DISCUSSION

Gene Cloning and Expression

Cell cultures were harvested 3 h after IPTG induction, sonicated, separated into soluble and insoluble fractions and recombinant J2-C2 was purified by Ni-NTA affinity chromatography as detailed in materials and methods. The molecular weight and molar absorption coefficient of recombinant protein were respectively calculated as 28631.43 Dalton and 38180 $M^{-1}cm^{-1}$ by using "ExPASy ProtParam tool". Subsequently the total yield of purified recombinant J2-C2 protein was determined as 75.5 mg ml^{-1} by measuring absorbance at 280 nm on UV spectrophotometer. The recombinant J2-C2 expression was confirmed by SDS-PAGE. The SDS-PAGE analysis showed that the purified protein around 28 kDa which corresponds with a theoretical molecular weight of 28631.43 Da (Figure 1).

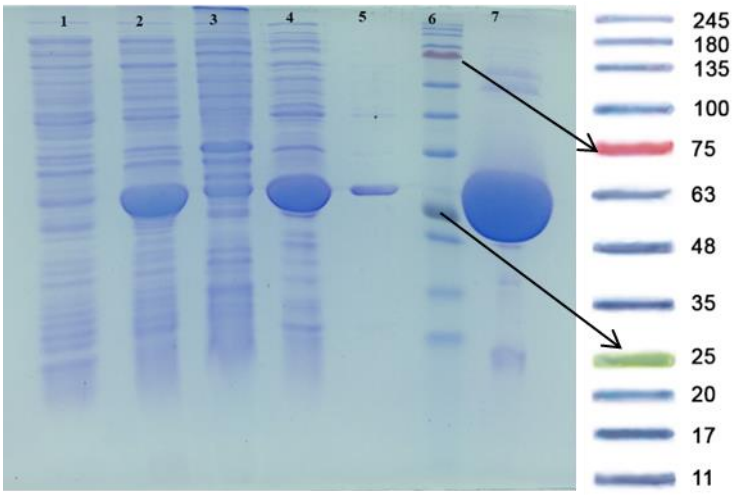

Figure 1. Purification of J2-C2 was confirmed with SDS-PAGE (%12). 1. Bacterial cell lysates before IPTG addition, 2. Bacterial cell lysates after IPTG addition 3. Collected pellet after centrifugation of the lysate 4. Collected supernatant after centrifugation of the lysate, 5. Wash with 0.1 M Tris-HCl buffer containing 10 mM imidazole.,6.NZYColour Protein Marker II, 7. The eluate collected from Ni-NTA agarose affinity column.

MTT Cell Viability Assay

We performed MTT cell viability experiments and determined that the recombinant J2-C2 did not significantly affect cell viability and proliferation of MCF7, PC3 and HT29 (Figure 2).

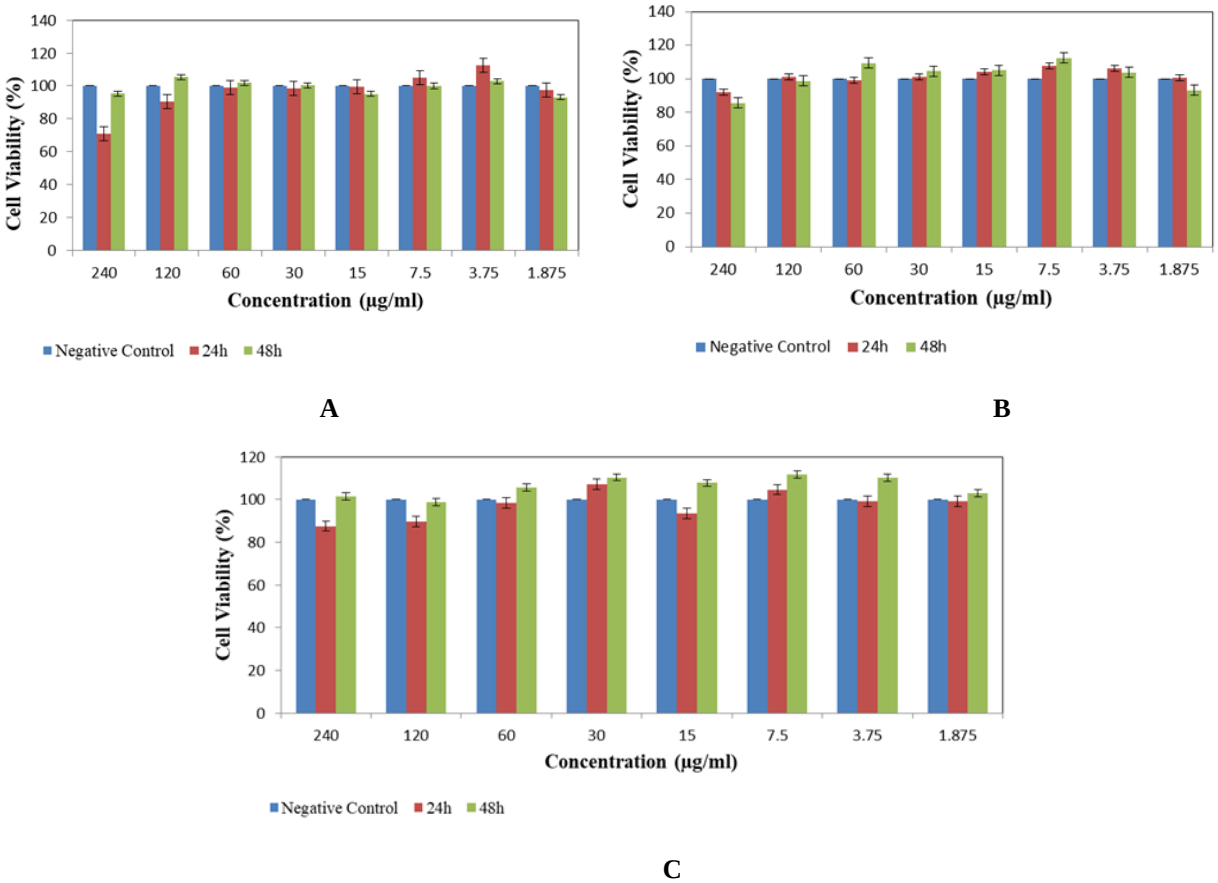

Figure2. The effect of different concentrations of J2-C2 on the viability of **A.** MCF7 cells **B.** PC3 cells **C.** HT29 cells

In studies conducted by Jian Xu et al. (2013), it was reported that native J2-C2 isolated and purified from *Arca inflata* showed antiproliferative effect against A549, SPC-A-1 and HepG2 cell lines (Zhu et. al., 2017). However in our study recombinant J2-C2 didn't displayed remarkable anti-proliferation activity against HT29, MCF7 and PC3 cell lines.

RNA Extraction, cDNA Synthesis and Quantitative PCR Analysis

The effects of recombinant J2-C2 on mRNA expression of Bax, Bcl-2, p53 were shown in Fig. 3. The experiments were repeated three times. Results were normalized according to the the housekeeping gene (GAPDH) in the samples. Values represent the degree of changes in mRNA for recombinant J2-C2 treated PC3, MCF7, HT29 relative to untreated ones.

Figure 3 shows the values of $2^{-\Delta\Delta Ct}$ reflecting the fold change in Bcl2, Bax and p53 gene transcription level in cells treated with 240 µg ml^{-1} recombinant J2-C2 for 48 h, calculated relative to the level of GAPDH expression and the ΔΔCt value ranges. The value of $2^{-\Delta\Delta Ct} > 1$ reflects increased expression of the target gene, the value of $2^{-\Delta\Delta Ct} < 1$ points to a decrease in the gene expression.

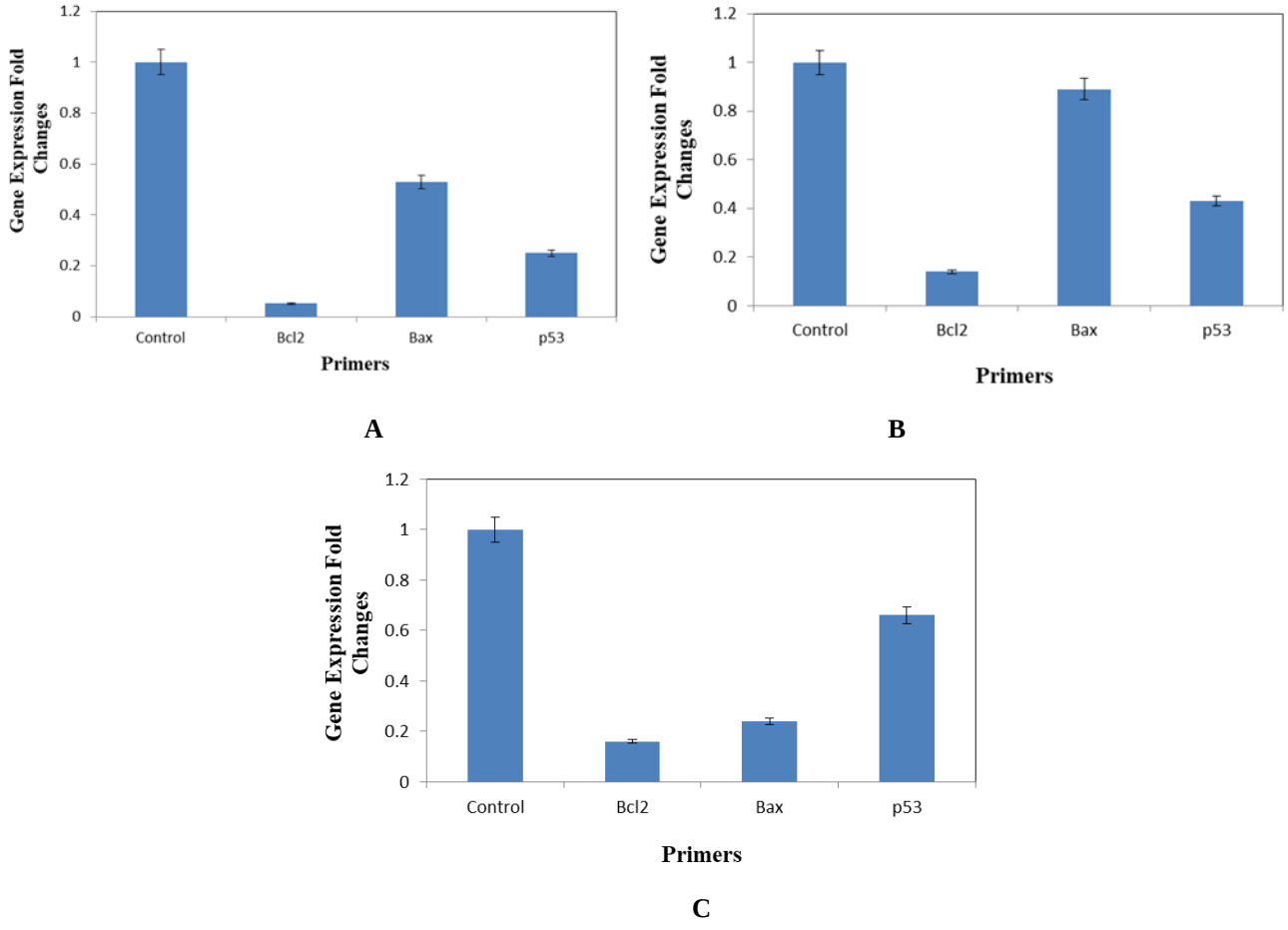

Figure 3. J2-C2 effect on mRNA levels of PC3 (A), MCF7 (B) and HT29 (C) cell lines

In all cell cultures treated with 240 µg ml^{-1} recombinant J2-C2 for 48 h, Bcl2, Bax and p53 gene transcription tended to decrease below the transcription of these genes in the non-treated control cells (Table 2). There was no significant decrease in Bcl2 / Bax ratio in PC3, MCF7 and HT29 cell lines treated with 240 µg ml^{-1} recombinant J2-C2 for 48 hours. These results are compatible with the results of the cell viability assay (p<0.005).

Table 2. Down regulated genes in treated compared with untreated cell lines

Symbol	Description	Gene name	Fold change (log 10 down regulation)		
			PC3	MCF7	HT29
Bcl2	Anti-apoptosis	B-cell CLL/lymphoma 2	-1.28	-0.85	-0.80
Bax	Induction of apoptosis	Bcl2-associated X protein	-0.27	-0.051	-0.62
p53	Tumor suppressor gene	Tumor protein p53	-0.60	-0.37	-0.18

Cell Migration Assessment

To explore whether J2-C2 could inhibit breast, prostate, colon cancer cell migration, we carried out *in vitro* scratch assay which is used conveniently by cell scientists. Representative images and quantitative analysis are demonstrated in Figure 4 and 5.

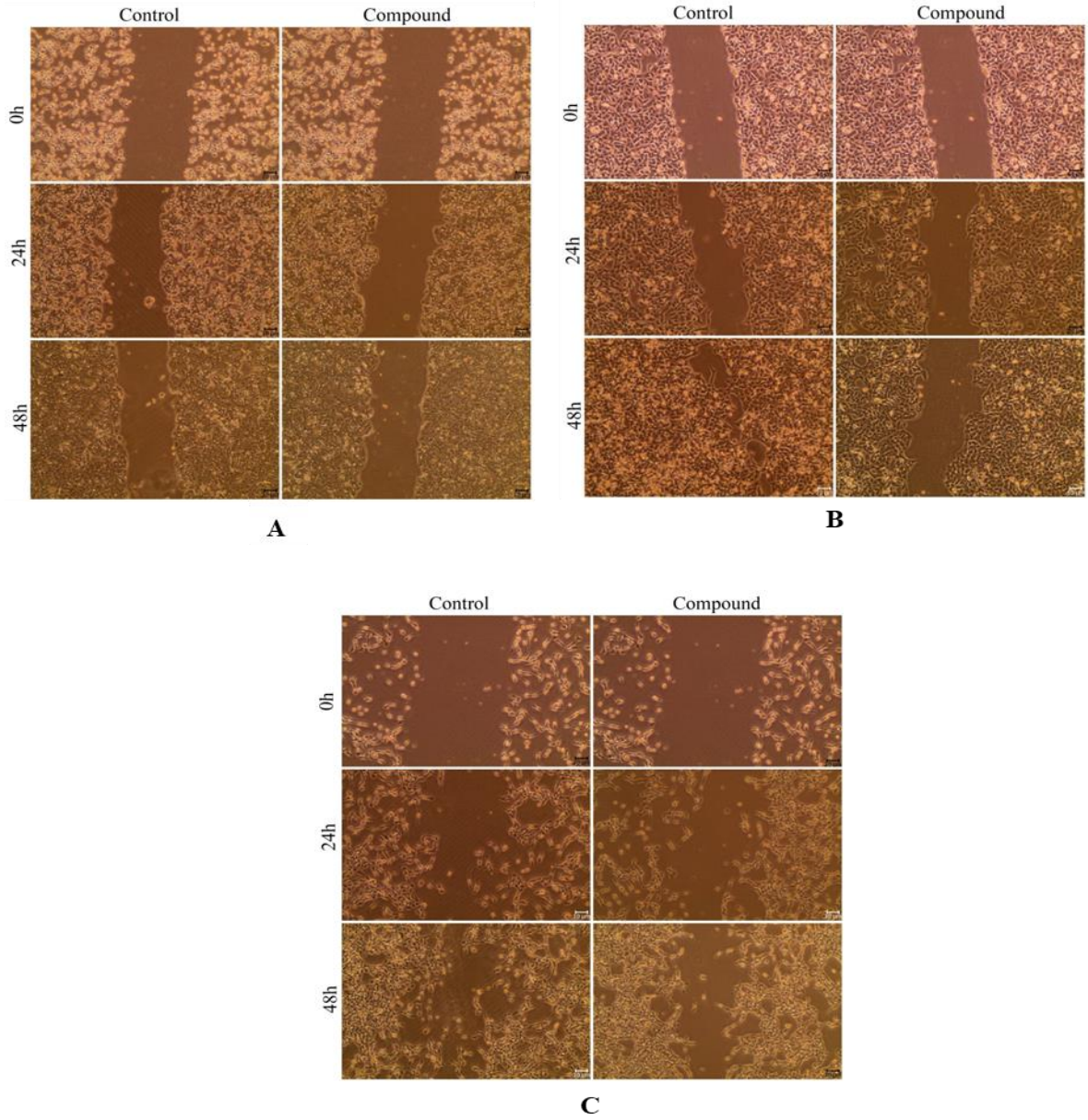

Figure 4. Efect of J2-C2 on migration of cancer cells in wound healing assay. The wounded monolayers which were created by scratching (A. HT29, B. MCF7, C. PC3) incubated for 24h and 48h alone (control) and in the precence of J2-C2 (240 µg/ml). The scale bars indicated 100 µm for x4 magnification

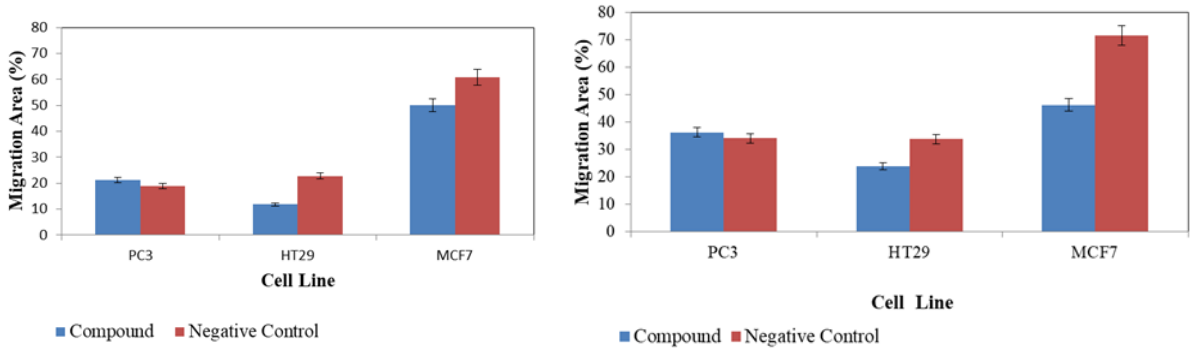

Figure 5. Effect of J2-C2 on migration of PC3, MCF7, HT29 cancer cells. Wound healing assay showing inhibiton of cell migration after 24h (A) and 48h (B) of exposure to J2-C2 (240 µg ml^{-1})

According to data that we obtained from wound-healing assay and MTT cell viability assay, we observed that J2-C2 protein reppressed cell migration of HT29 and MCF7 cells.

We did not find any significant direct effect of recombinant J2-C2 on PC3 cancer migration. However, this protein significantly inhibited the migration of HT29 and MCF7 cell lines. As a result, recombinant J2-C2 was more effective in blocking cell migration than cell viability inhibition.

Cancer metastasis, rather than primary tumors, is reported to account for over 90% of all cancer deaths, including colon, prostate and breast (Yang et. al., 2017; Ortega et. al.,2003). Research has shown that most colon cancer patients with cancer metastasis have low survival rates (Zhu et. al.,2016). Various chemotherapy drugs are used in clinical practice to inhibit colon cancer metastasis. Nevertheless, specific antimetastatic drugs are not sufficient for the treatment of solid cancer. Therefore, considering the importance of metastasis in cancer treatment and the need of oncologists for antimetastatic drugs, researches to develop drug candidates to prevent metastasis appear to be very valuable. The results show that recombinant J2-C2 can be considered as a drug candidate in this regard.

CONCLUSION

The anti-migrative effect of recombinant J2-C2 on PC3, MCF7 and HT29 were determined by investigating viability and migration of these cells treated with recombinant protein. The results demonstrated that recombinant J2-C2 inhibited migration of MCF7 and HT29 cancer cells. The effects of J2-C2 inhibition on migration were not a result of reduced viability. Because, MTT assay indicated that this recombinant protein did not demonstrate any anti-proliferative effects on these cells. In conclusion, our findings regarding the inhibitory effects of J2-C2 on cell migration suggest that recombinant J2-C2 may be useful as a therapy agent against breast and colon cancers.

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