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In vitro Cytotoxic Study of Benzylthio-triazol-5-haloisatin Scaffolds and their Evaluation on Supernatants Activities and Levels of MMP-2 and MMP-9 in MCF-7 Cell Line

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ABSTRACT

Angiogenesis is the formation of new blood capillaries, and it is important for physiological processes such as growth and development and pathological conditions such as tumor growth and metastasis. Angiogenesis inhibition is considered very useful in the prevention and treatment of cancer. Matrix metalloproteinases (MMPs) are a family approximately consists of 28 zinc-dependent content endopeptidase which degrade the extracellular matrix (ECM) and play an important role on angiogenesis development and tumor metastasis. Therefore, inhibition of MMPs prevents the angiogenesis. The expression and levels of MMP-2 and MMP-9 is increased in many human tumors, including ovarian, breast and prostate tumors. In this study, cytotoxic effects of (*Z*)-3-((5-(benzylthio)-4*H*-1,2,4-triazol-3-yl)imino)-5-haloindolin-2-one derivatives **1a-1l** were evaluated in MCF-7 (human breast adenocarcinoma) cell line by MTT assay. Then, the potency of the tested of some synthesized compounds was evaluated on supernatants activities and levels of MMP-2 and MMP-9 by gelatin zymography and ELISA methods. Among the tested compounds, **1j**, **1l** and **1k** had the greatest cytotoxicity against MCF-7 cell line compared to the positive (sunitinib) and negative (DMSO) controls. Moreover, our observations indicated that the compounds **1j**, **1l** and **1k** decreased the supernatants activities of MMP-2 and MMP-9 more than others and all of the tested compounds considerably decreased the supernatants levels of MMP-9. Finally, our findings suggest that the tested derivatives are probably able to inhibit macromolecules like MMPs which have essential role in angiogenesis pathway.

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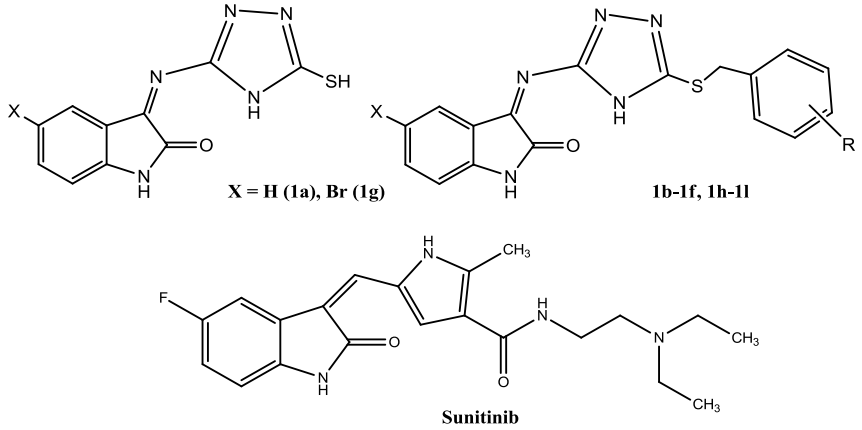
Introduction

Cancer is a class of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. There are more than one-hundred different types of cancer. Therefore, according the variety of cancer, anti-cancer drugs act with different mechanisms, new and more powerful anticancer agents are needed to fight different cancer diseases [1, 4].

Angiogenesis is physiological process through which new blood vessels formed from pre-existing vessels. It is a necessary process in growth and development and also an essential step in the change of tumors from a benign state to a malignant. Angiogenesis is performed by various growth factors, such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and matrix metalloproteinase (MMP). MMPs help degrade the extracellular matrix (as remodeling of ECM) that keep the vessel walls solid and so facilitate the growth tumor. Angiogenesis inhibition is considered very useful in the management and treatment as well as in the prevention of cancer [5].

Matrix metalloproteinases (MMPs) are a family approximately consists of twenty eight zinc-dependent endopeptidases. Members of the MMP family were recognized by descriptive names that were assigned based on their preferred substrate specificities, e.g., collagenases, gelatinases, stromelysins, and matrilysins [6, 7]. MMPs play an important role on many physiological processes such as embryonic development, nerve growth, tissue repair, angiogenesis development and they involve in pathological conditions including artheritis rheumatoid, Alzheimer, myelin degeneration in nervous disease, tumor metastasis and multiple sclerosis [8- 11]. MMP-2 and MMP-9 are concepts thought to be important in metastasis [12]. MMP-2 and MMP-9 degrade of type IV collagen and gelatin in ECM. Type IV collagen is a main component of ECM and basement membrane. The expression and levels of MMP-2 and MMP-9 is increased in many human tumors, including ovarian, breast, prostate tumors and melanoma [13- 15]. A significant correlation has been showed between tumor metastasis and increased levels of MMP-2 and MMP-9 in many experimental and clinical studies [16-21]. Increased secretion of MMPs has been related with different steps of tumor growth such as promotion, progression, invasion, angiogenesis, and metastasis [22].

Isatin was found in mammalian body fluids and tissues in the nineteenth century which has many biological effects such as antibacterial, antifungal, anticonvulsant, antiviral, antiproliferative and antiangiogenesis activity by inhibition of VEGF [23-24]. Azza T. Taher *et al.* in 2011 reported that isatin-thiazoline and isatin-benzimidazole conjugates have antiproliferative effects against MCF-7 [23]. In 2011 Murty et al. showed that some of S-alkylated-3-mercapto-1, 2, 4-triazole derivatives has suitable cytotoxic effects against U937 and HL60 cell lines [25]. Mitropoulou TN et al. in 2003 showed that letrozole is a potent *in vitro* inhibitor of cell proliferation and of MMP-2 and MMP-9 expressed by ER-positive MCF-7 cells and may be of value for suppressing breast tumor growth and invasiveness. Letrozole is one of the derivatives with 1*H*-1, 2, 4-triazole ring [26]. Kreituss I et al. in 2013 showed that a series of C (2)-monosubstituted aziridine-1, 2, 3-triazole conjugates were the selective inhibitors of MMP-2 [27]. Hoffmann S *et al.* in 2005 showed that carboxyamido-triazole inhibited MMP-2 secretion of choroidal endothelial cells with the concentration of 10 microM [28]. Recently, Sjøli et al. reported that the isatin derivatives could have little effect on the activity of MMPs [29]. As a part of our try to find new chemotherapeutic agents as potential anticancer agent [31- 35], we have examined a series of benzylthio-triazol-isatin derivatives **1a-1l** (which were synthesized in our laboratory and will be reported elsewhere) and tested *in vitro* cytotoxic evaluation of them against MCF-7 (human breast adenocarcinoma) (Scheme 1). Taking into account the above facts about the role of MMPs in angiogenesis development and tumor metastasis, the other purpose of this study focuses on the effects of the tested compounds on the activities and levels of MMP-2 and MMP-9.



Scheme 1. Structure of the tested compounds and Sunitinib as control compound.

Materials and method

Cell culture conditions

MCF-7 (human breast adenocarcinoma) was obtained from Pasture Institute of Tehran-Iran. Cells were cultured in RPMI1640 (Roswell Park Memorial Institute) supplemented with 10% (V/V) heat inactivated fetal bovine serum (FBS), glutamine (200 mM) and antibiotic (1 µl/ml gentamicin) in 95% humidified incubator at 37 ^{o}C with 5% CO_2 atmosphere. All solutions were then sterilized using 0.22 µ microfilters and stored at 4 °C before use. The compounds tested were dissolved in dimethylsulfoxide (DMSO), and concentration of stock solutions was 40 mg/mL (It must be noted that the concentration of DMSO was less than 0.5% in cell culture).

MTT cytotoxicity assay

Cellular toxicities of the tested compounds **1a-1l** were studied against MCF-7 (human breast adenocarcinoma) by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and compared to DMSO as negative control and Sunitinib as positive one. Cells were seeded onto 96-well plate at a number of 1.0×10^4 cells in each well and in a volume of 200 µl of growth medium. The tested compounds **1a-1l** was dissolved in dimethylsulfoxide (DMSO) and the stock solutions prepared with a specific concentration. The last concentration of the DMSO in the medium was 0.33%. After 24 h of seeding, 2 µl of the solutions of the tested compounds **1a-1l** at different concentrations were added to in each set of three wells. After 24 hours of incubation, the MTT solution was prepared with a concentration of 5 mg/mL and then fresh media (130 µl) and MTT solution (20 µl) were added to each well and the plates put in a 37 °C incubator for 4 hr. After this time, the supernatants were discarded slowly and 100 µl of dimethylsulfoxide was added to each well and the plate was shaken by shaker incubator for 30 min to soluble the purple formazan crystals. Finally, absorbance was measured by ELISA plate reader (Biotek, H1M.) at a 570 nm and 630 nm for reference wavelength.

Supernatants collection

After 24 hours of seeding the cell onto 96-well plate, the tested compounds at different concentrations were added to each well and supernatants were collected, labeled and kept frozen at −80 °C until laboratory measurements.

Measuring Supernatants concentration of MMPs

MMP-2, MMP-9, Quantikine® ELISA kits (R&D systems, Minneapolis, USA) were used to measure supernatants MMPs.

Protein assay

Protein content of the supernatant medium from MCF-7 cell was treated with the tested compounds for 24 hours was measured by the Bradford method and the same amount of protein for normalizing the zymography was loaded in gels.

Gelatin zymography

Effect of the tested compounds on the enzymatic activity of MMP-2 and MMP-9 was assessed using gelatin zymography. Gelatin zymography was performed according to the method of La Rocca G *et al.* [36] as follows: stacking gel (SDS–PAGE, 4%) with resolution gel (SDS–PAGE, 8%) and copolymerizing with gelatin (1 mg/ml) (Merck). For each sample, the same amount of protein was loaded. Electrophoresis was performed using the mini gel slab apparatus vertical slab model VS 3000 (Akhtariyan-Iran) by gradient program as follows: step one – 30 min with voltage of 50 V, second step - with voltage of 150 V, until the dye reached the bottom of the gel. Following electrophoresis, gels were washed with 100 ml renaturation buffer comprising of 2.5% Triton X-100 in 50 mM Tris–HCl (pH 7.5). Then every 20 min the buffer was renewed, which was repeated three times in an orbital shaker. Subsequently the gels were incubated for 18 h at 37 ^{0}C in incubation buffer (0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris–HCl (pH 7.5). Gels were then stained with Coomassie blue and destained with 7% methanol and 5% acetic acid. The gelatinolytic activities of MMPs were detected as clear transparent bands against the background of Coomassie blue stained gelatin.

Quantitative activity of MMPs

Areas of bands were digested to be quantified by Image J software, using a high resolution digitally image of gel against the amount of MMPs standard in each gels.

Statistical analysis

Analysis of all data was performed with the SPSS software version 16.0. Data were presented as Mean±SEM. Comparison of MMPs groups was performed using the One-way analysis of variance (ANOVA) and Tukey's test for quantitative and normal data. *p*-Value of less than 0.05 was considered as statistically significant.

Results and Discussion

Cytotoxic effects of the tested compounds on MCF-7 cell line

All the tested compounds (**1a-1l**) were evaluated against MCF-7 and cytotoxic assay estimated using MTT and the value of IC_{50} for each the tested compounds has been shown in Table 1. IC_{50} were calculated by plotting the $\log_{10}$ of the percentage of viability versus drug concentration (Figures 1 and 2). The tested compounds having 5-Br substituent on isatin ring (**1g-1h**) showed more cytotoxic activity against MCF-7 cell line than the other derivatives without the bromine substituent (**1a-1f**). Among of the compounds (**1a-1l**), **1j** (IC_{50} = 15 µM), **1l** (IC_{50} = 17 µM), and **1k** (IC_{50} = 21 µM) with *p*-nitro, *p*-fluorine, and *o*-fluorine substituents respectively on phenyl ring have shown the greatest cytotoxicity, and **1a, 1b** and **1c** (IC_{50} = 146 µM, 153 µM, 145 µM respectively) without bromine substituent have the lowest cytotoxicity (Table 1).

Table 1. Cytotoxicity activity (IC_{50}, µM) of the tested compounds against MCF-7

Compound	Molecular formula	X	R	IC_{50} (µM)
1a	$C_{10}H_7N_5OS$	H	-	146
1b	$C_{17}H_{12}N_6O_3S$	H	2-NO_2	153
1c	$C_{17}H_{12}N_6O_3S$	H	3-NO_2	145
1d	$C_{17}H_{12}N_6O_3S$	H	4-NO_2	118
1e	$C_{17}H_{12}FN_5OS$	H	2-F	144
1f	$C_{17}H_{12}FN_5OS$	H	4-F	80
1g	$C_{10}H_6BrN_5OS$	Br	-	38
1h	$C_{17}H_{11}BrN_6O_3S$	Br	2-NO_2	32
1i	$C_{17}H_{11}BrN_6O_3S$	Br	3-NO_2	30
1j	$C_{17}H_{11}BrN_6O_3S$	Br	4-NO_2	15
1k	$C_{17}H_{11}BrFN_5OS$	Br	2-F	21
1l	$C_{17}H_{11}BrFN_5OS$	Br	4-F	17
Sunitinib	$C_{22}H_{27}FN_4O_2$	-	-	10

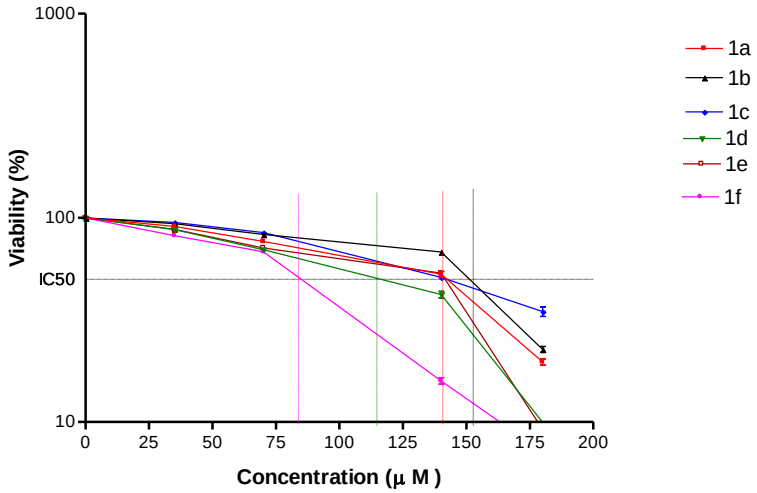


Fig. 1. Cell viability of MCF-7 cells after exposure to **1a-1f** compounds. Cells were treated with different concentrations of derivatives (35-180 µM) for24 hr. The cell viability was determined by MTT assay.

Evaluation the structure-activity relationship (SAR) in cell culture experiments

Our study shows that substitution of NO_2, F, and Br groups at different positions of the main structure can change the efficacy of the tested compounds against MCF-7 cell line according to the data presented in Table 1. *p*-Nitro compounds with and without bromine substituent **1j** (IC_{50} = 15 µM) and **1d** (IC_{50} = 118 µM) respectively had more cytotoxicity than *m*-nitro compounds, and *m*-nitro compounds with and without bromine substituent **1i** (IC_{50} = 30 µM) and **1c** (IC_{50} = 145 µM) respectively had more cytotoxicity than *o*-nitro compounds with or without bromine substituent (**1h,** IC_{50} = 32 µM; **1b,** IC_{50} = 153 µM respectively). In the compounds with bromine substituent on isatin ring, putting electron-withdrawing substituent like nitro on phenyl ring also leads to an enhancement in the cytotoxicity of derivatives. When the nitro group was placed in *para* position on phenyl ring, the cytotoxicity of compounds significantly increased compared to *ortho* and *meta* positions (Table 1). *p*-Fluorine

compounds with and without bromine substituent **1l** (IC_{50} = 17 μM) and **1f** (IC_{50} = 80 μM) respectively had more cytotoxicity than *o*-fluorine compounds with and without bromine substituent **1k** (IC_{50} = 21 μM) and **1e** (IC_{50} = 144 μM) respectively. The results indicated that putting electron-withdrawing substituent of fluorine in *para* position on phenyl ring increases the cytotoxicity in comparison to *ortho* position (Table 1). In addition, the compound **1g** (IC_{50} = 38 μM) with 5-Br had more cytotoxicity than the **1a** (IC_{50} = 146 μM) without the bromine (Table 1).

Levels and activities of MMP-2 and MMP-9

Our results show that the tested compounds (**1g-1l**) decreased the levels and activities of MMP-2 and MMP-9 in supernatant of culture medium relative to control cells in which only solvent was added to their culture medium. According to the structure (Scheme 1), putting electron-withdrawing substituent like nitro and fluorine on phenyl ring and changing the situation (*ortho*, *meta* and *para*) can affect the supernatants activities and levels of MMP-2 and MMP-9 (Table 2). The synergism of 5-Br substituent and *p*-nitro group in compound (**1j**) showed that it has the most potency to decrease the activities of MMP-2 and MMP-9 compared to the other compounds (Figures 3-5). The results obtained from the tested compounds (**1g-1l**) considerably decrease levels of MMP-9 compared to the control group (Figure 6). Secretion of MMP-2 in MCF-7 cell lines was very little which we could not measure supernatants concentration of MMP-2 by ELISA method in comparison to MMP-9. According to the studies by Roomi et al. expression and secretion of MMPs is extremely regulated by cytokines and signal transduction pathways, including those activated by phorbol 12-myristate 13-acetate (PMA) in cancer cell lines. Gelatinase zymogram of MCF-7 cell line showed absence of MMP-2 expression in induced and uninduced media by PMA and only a single band corresponding to the molecular weight of MMP-9 (92 kDa) in induced media by PMA [12]. Previous studies showed that the complex of MMP-3 and plasminogen activate the MMP-9, and then MMP-9 and tissue inhibitors of MMPs enzyme (TIMPs) inhibit the other MMPs [10, 37]. The results of Tukey's test and the One-way analysis of variance (ANOVA) indicated significant decrease in the supernatants activity of MMP-2 (*p*-value = 0.0001) and MMP-9 (*p*-value = 0.0001) and the supernatants level of MMP-9 (*p*-value = 0.0001) between each of the tested compounds (**1g-1l**) and control group. In ANOVA and Tukey's tests *p*-value was obtained less than 0.05 (*p*-value = 0.000 for each of test) which showed meaningful correlation between each of the tested compounds (**1g-1l**) and control group in decreasing levels and activities of MMP-2 and MMP-9 in supernatants of MCF-7 cell line (see Table 2 for *p*-values).

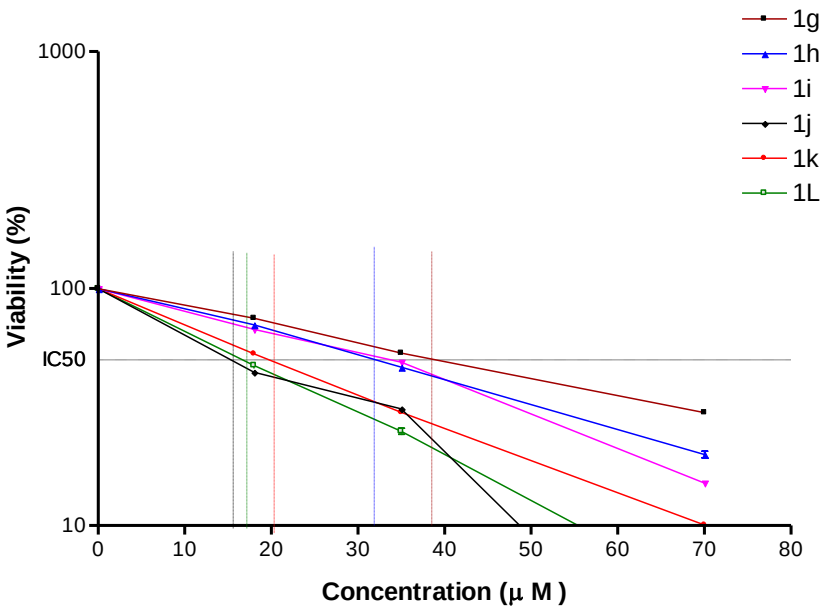


Fig. 2. Cell viability of MCF-7 cells after exposure to **1g-1l** compounds. Cells were treated with different concentrations of derivatives (10-70 μM) for 24 hr. The cell viability was determined by MTT assay.

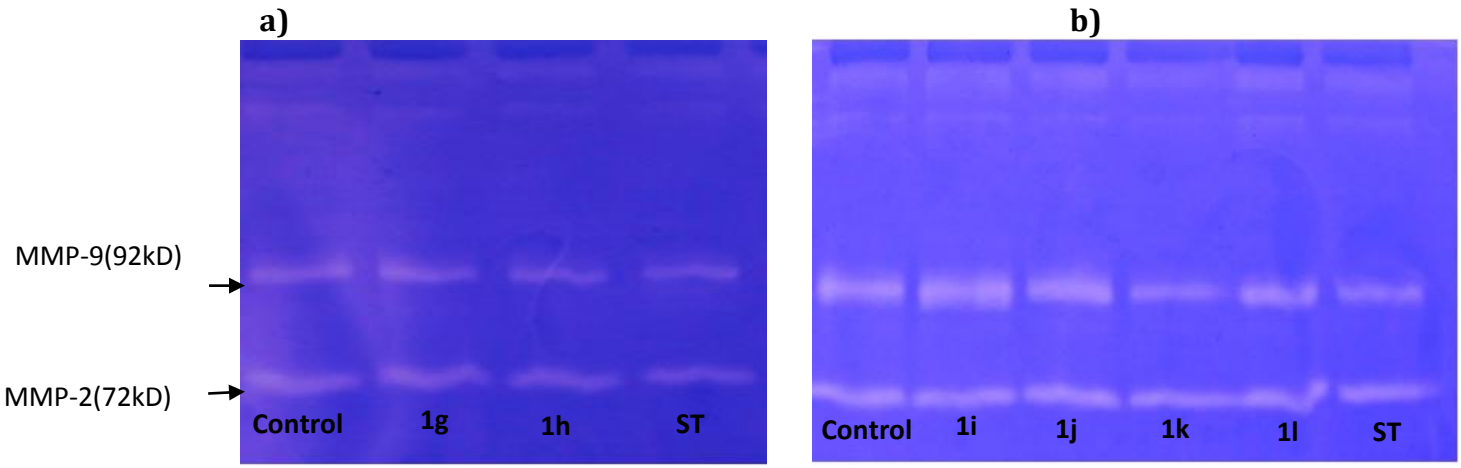


Fig. 3. Gelatinase zymogram of MCF-7 cell line show the effect of **1g-1l** compounds on MMP-2 and MMP-9 activities (**a:** Standards of MMP-2 and MMP-9(ST), **Control**, **1g,** and **1h** respectively, **b:** Standards of MMP-2 and MMP-9(ST), **Control**, **1i**, **1j**, **1k** and **1l** respectively.)

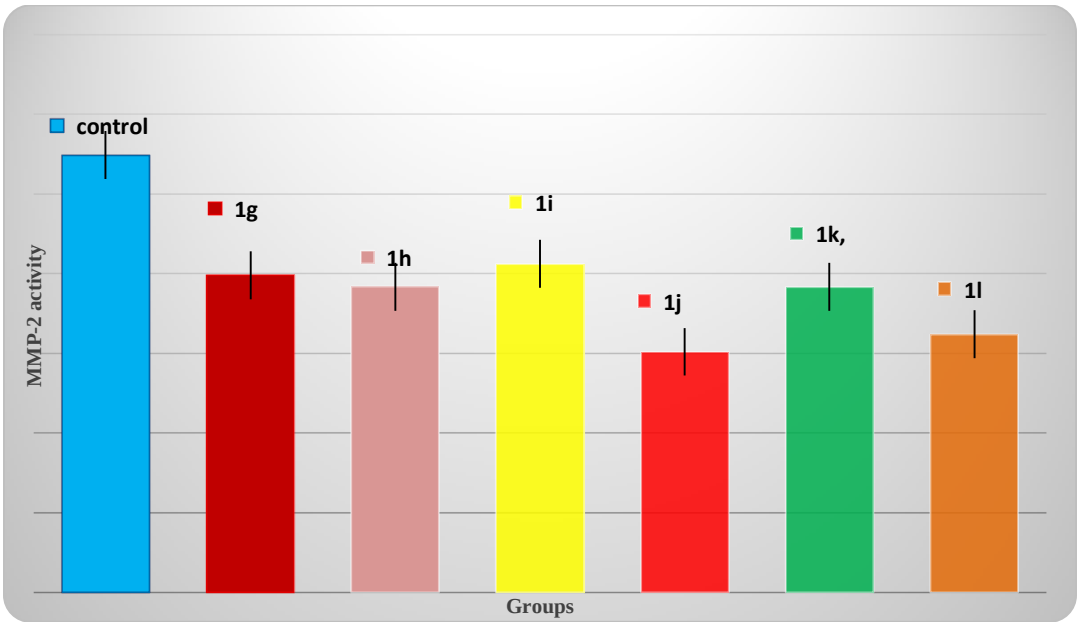


Fig. 4. The effect of the compounds **1g-1l** on MMP-2 activity in MCF-7cell line. Cells pretreated with IC_{50} concentrations of **1g-1l**. MMP-2 activity was measured by gelatin zymography and quantified by Image J software. Data are expressed as the mean±SEM of three separate experiments.

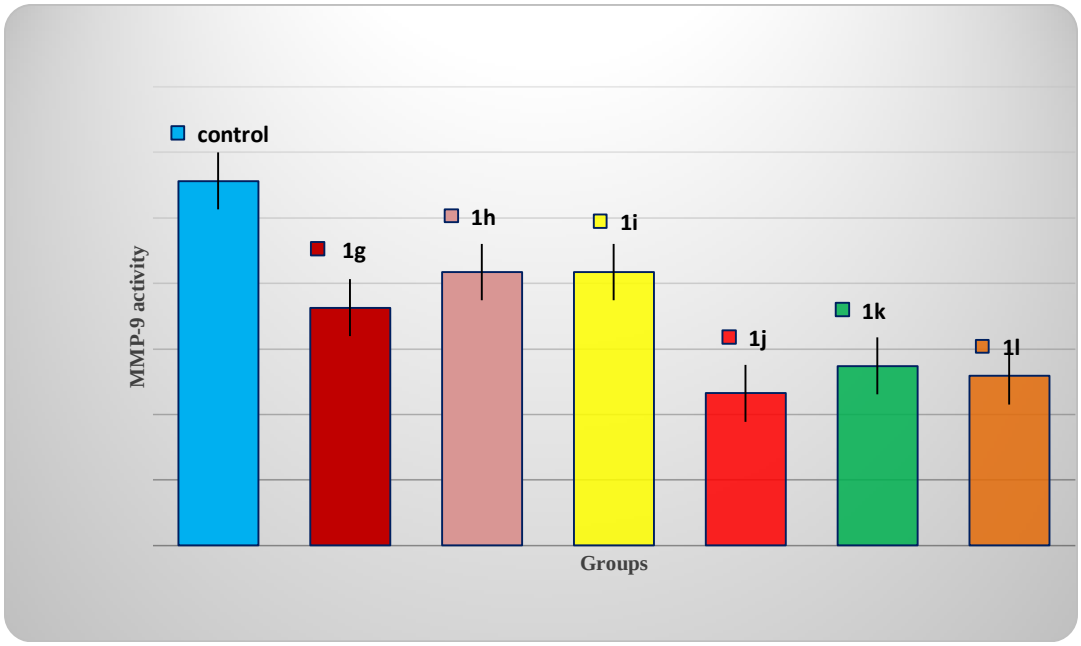


Fig. 5. The effect of the compounds **1g-1l** on MMP-9 activity in MCF-7cell line. Cells pretreated with IC_{50} concentrations of **1g-1l**. MMP-9 activity was measured by gelatin zymography and quantified by Image J software. Data are expressed as the mean±SEM of three separate experiments.

Table 2. Comparison of the effect of **1g-1l** on the supernatants activities and levels of MMP-2 and MMP-9 relative to control group. Depicted data are Mean±SEM with the mentioned *p*-value.

Compound	Control	1g	1h	1i	1j	1k	1l
Number	3	3	3	3	3	3	3
MMP2 activity	54.873 ±0.159	39.7986±0.1 0982	38.364±0.1 1884	41.2365±0.0 2138	30.1981±0.1 0211	38.3396±0.1 3123	32.3782±0.1 0516
p-value	96	0.000	0.000	0.000	0.000	0.000	0.000
MMP9 activity	1112.7 ±3.607	725.69±4.11 033	834.5±0.92 399	834.66±2.15 137	464.74±2.43 674	548.28±1.70 062	517.69±0.83 282
p-value	59	0.000	0.000	0.000	0.000	0.000	0.000
MMP9 (ng/ml)	1298.6 ±45.9	139.76±10..9 4318	168.64±26. 2702	111.98±1.11 111	100.2±10.71 517	118.64±8.01 234	120.87±5.09 175
p-value		0.000	0.000	0.000	0.000	0.000	0.000

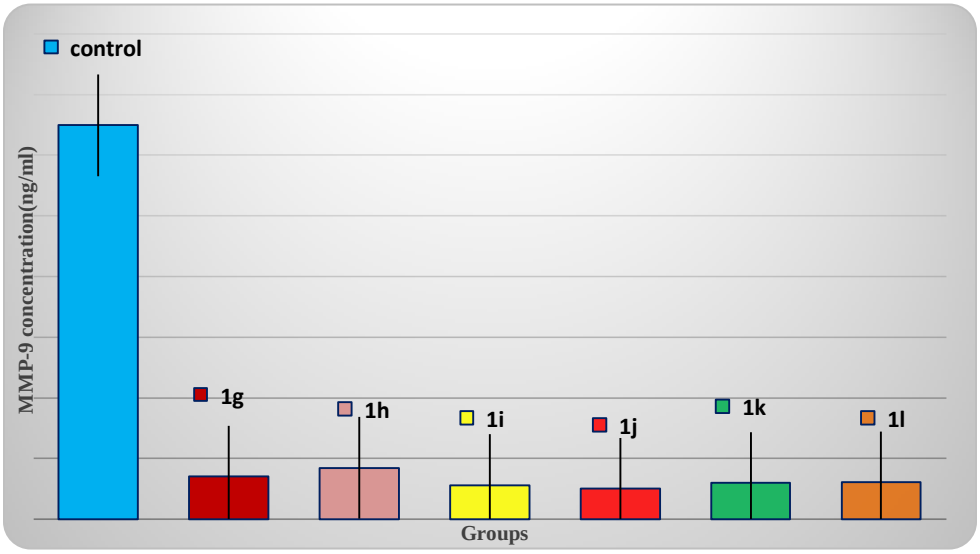


Fig. 6. The effect of the compounds **1g-1l** on MMP-9 levels in MCF-7cell line. Cells pretreated with IC_{50} concentrations of **1g-1l**. MMP-9 levels was measured by Quantikine® ELISA kits. Data are expressed as the mean±SEM of three separate experiments.

Conclusion

Our findings show that, among the tested compounds **1a-1l**, the compound **1j** with two substituent consisting of 5-Br on isatin ring and nitro with *para* situation on phenyl ring is the most cytotoxic derivative against MCF-7 cell line. The compound **1j** has resulted in more than the others to decrease the supernatants levels and

activity of MMP-9 and MMP-2. Our findings show that the tested compounds decrease levels and activities of MMP-2 and MMP-9 in supernatants of MCF-7 (human breast adenocarcinoma) cell line in which level of MMPs is high. Thus levels and activities of MMP-2 and MMP-9 can be considered as valuable biomarkers for devising therapies to prevent angiogenesis and tumor metastasis.

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Conflict of Interests

Authors certify that there is no actual or potential conflict of interest in relation to this article.

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