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Design, synthesis and antitumor evaluation of novel thalidomide dithiocarbamate and dithioate analogs against Ehrlich ascites carcinoma-induced solid tumor in Swiss albino mice

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ABSTRACT

A series of 16 novel thalidomide sulfur analogs containing one and two sulfur atoms **2** and **4–18**, respectively, were designed and synthesized. These compounds were screened for in vitro antitumor activity against Ehrlich ascites carcinoma (EAC) cell line and exhibited potent cytotoxic activity. On the bases of the obtained results for in vitro cytotoxic activity, thalidomide sulfur analogs containing two sulfur atoms **8**, **9**, **13** and **14** were selected and tested in vivo against EAC-induced solid tumor in female mice compared to thalidomide **1** as well as its analog **2** and exhibited a highly significant reduction in tumor volume (TV). Results illustrated the antioxidative activity of these compounds as the level of hepatic lipid peroxidation decreased and levels of antioxidant enzymes like superoxide dismutase (SOD) and catalase were elevated. The histopathological investigations revealed that thalidomide sulfur analogs **2, 8, 9, 13** and **14** have antimitotic, apoptotic and necrotic activities against solid tumor. These compounds lead to increase of Fas-L expression. The immunohistochemical studies showed a decrease in Ki67 and vascular endothelial growth factor (VEGF) staining in tumor cells from treated-animals when compared with non-treated groups, which suggests an inhibition of tumor proliferation rate and angiogenic process associated with tumor growth. Compounds **9** and **13** were the most potent compounds in tumor necrosis without liver necrosis. At the same time, treatment with compound **9** resulted in liver degeneration.

1. Introduction

Thalidomide [(*R*,*S*)-2-(2,6-dioxo-3-piperidinyl)-1*H*-isoindole-1,3(2H)-dione **1** was administrated in 1950s as a treatment for insomnia and as an antiemetic agent. The racemic compound **1** was assumed to be non-toxic compared to the other existing sedative drugs, the barbiturates. Later investigations found that while the *R*-enantiomer of thalidomide was responsible for the sedative effect the *S*-enantiomer had teratogenic properties.[1–3] This apparent lack of toxicity led to its use in the treatment of morning sickness in pregnant women.[4] Due to its side effects like teratogenicity[5,6] and peripheral neuropathy,[7,8] thalidomide was withdrawn from the worldwide market long back in 1961.[9] Sheskin, in 1965, first reported clinical efficacy of thalidomide in the treatment of erythema nodosum leprosum (ENL), an acute inflammatory state occurring in lepromatous leprosy.[10] This led to the renewal of interest in the clinical development of thalidomide and discovery novel analogs with improved activities and decreased side effects. Nearly three decades after this, in 1998, US Food and Drug Administration (FDA) approved thalidomide in the treatment of ENL.[11]

Thalidomide, once discarded as the worst teratogen, is again regaining status as a drug in the treatment of various hematological malignancies[12] as well as variety of inflammatory and autoimmune diseases[13] and in the treatment of multiple myeloma and other solid tumors.[14] The unique and broad physiological properties discovered during recent years, prompted a reevaluation of its therapeutic potential.[15–17] Thus, thalidomide is currently applied for treatment of painful inflammations associated with leprosy,[18] rheumatoid arthritis[19] and graft-versus host disease (GVHD).[20] Furthermore, promising results in the case of treatment of AIDS,[21] Crohn's disease,[22] behcet's syndrome[23] and cancer related pathologic angiogenesis[24–27] have been disclosed.

Thalidomide teratogenic effect was partially explained by its anti-angiogenic activity.[28] This property appeared to be particularly interesting in the treatment of solid tumors such as brain or prostate cancers, and clinical trials were realized.[21,29]

On the other hand, dithiocarbamates have received considerable attention due to their numerous biological activities[30] and have found recently application in the treatment of cancer.[31–36]

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In view of the fact that, cancer is another fatal condition which is continuing to be one of the largest causes of death in both men and women, claiming over 6 million lives each year in the world. In the last decade, basic cancer research has produced remarkable advances in understanding of cancer biology and cancer genetics.[37] To date; many anticancer drugs have been developed and applied by physicians. However, the resistance to anticancer drugs and side effects were discovered. Therefore, the research and development of new and safe drugs have become necessary by the pharmaceutical industry.[38] Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection against infection and degenerative diseases.[39]

From these viewpoints and in continuation of our interest searching for antitumor active compounds,[40–43] the present study was carried out to evaluate the antitumor activity, lipid peroxidation, antioxidant, apoptotic, necrotic, antimitotic, antiproliferative and anti-angiogenic effects of thalidomide and its sulfur analogs against EAC in Swiss albino mice.

2. Results and discussion

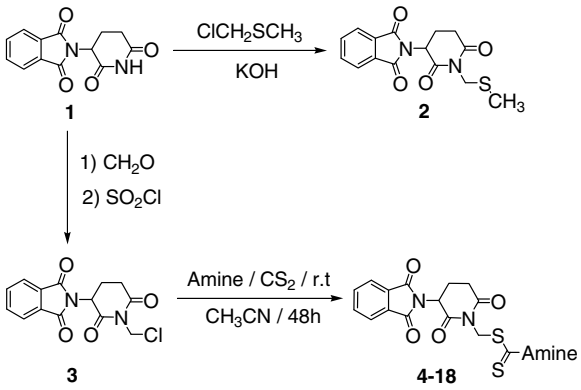
2.1. Chemistry

In a previous investigation on the synthesis of different class of heterocyclic compounds with promising antitumor activities, Obvious and clear antitumor activities were recognized for different N-substituted thalidomide derivatives which were synthesized by our group through the alkylation of thalidomide at the nitrogen of the piperidin-dione moiety with different alkylating agents containing oxygen, nitrogen and sulfur. Most of these alkylated thalidomide analogs were screened in vitro, investigated in vivo and exhibited higher antitumor activity than thalidomide itself. The more potent and active analog was that contain sulfur atom. Meanwhile of this investigation, a Chinese patent was published[44] including some of our synthesized N-alkylated thalidomide derivatives and the published results in the patent were in a complete accordance with the our data.[45]

One of our new N-alkylated thalidomide derivatives, to our knowledge does not reported yet, was methylthiomethylthalidomide **2** which showed higher antitumor activity compared to the other synthesized N-alkylated thalidomide derivatives. By studying this observation, it was found that this activity might be referred to the presence of sulfur atom.

On the basis of this recognition and the understanding for the reason of the activity of methylthiomethylthalidomide **2**, it was contemplated to design and synthesize a novel series of thalidomide dithiocarbamate and dithioate analogs **4–18** which contain two sulfur atoms in order to investigate the effect of increasing the sulfur content in the thalidomide analogs. Thus, on the bases of the previously mentioned thalidomide activities, the fact that dithiocarbamate compounds acting as anticancer agents and the enhanced antitumor activity of methylthiomethylthalidomide **2**, we present in this context a novel synthesis of a new thalidomide dithiocarbamate and dithioate analogs **4–18** through the introduction of two sulfur atoms in the side chain at the nitrogen of the piperdin-dione moiety which might enhance the anticancer activity (Scheme 1).

General methods for the synthesis of dithiocarbamate derivatives involve the reaction of an amine with costly and toxic reagents, such as thiophosgene and/or an isothiocyanate.[46] Furthermore, a one-pot reaction of amines with carbonyl sulfide and alkyl halides in the presence of a catalyst also has been reported.[47] However, there are several disadvantages for these methods; many isothiocyanates are hazardous, tedious to prepare and display poor long-term stability with the formation of side products such as urethane in alcoholic media. These intermediates also require high reaction temperature, gave low or moderate yield of products and usually entail multistep procedures. These reactions require very toxic reagents and harmful organic solvent such as DMF and DMSO in the presence of a catalyst. Due to these disadvantages, a simple reaction condition without catalyst or harmful organic solvent was established.



Scheme 1. Synthesis of thalidomide sulfur analogs **2** and **4**–**18**.

Chloromethylthalidomide **3**, carbonyl sulfide and the appropriate amine (Table 1) were dissolved in acetonitrile and stirred for 48 h at room temperature to afford thalidomide dithiocarbamate and dithioate analogs **4–18** in good to excellent yields (Table 1).

The amines applied in all entries **A–O** in this reaction protocol were selected from various classes of amines such as aliphatic, alcyclic and aromatic amines (Table 1). In case of using phenylhydrazine (Entry **M**), the reaction finished after 6 h stirring at room temperature. Surprisingly, the thalidomide dithiocarbamate **6** showed the introduction of two molecules of the amino ethanol. The structure of the dithiocarbamate **6** was elucidated on the basis of IR, MS, ^{1}H NMR, and elemental analysis. Moreover, the single-crystal and molecular structure of dithiocarbamate **6** was established by X-ray crystallographic analysis[48] (Fig. 1).

During the course of adjusting an applicable reaction condition, several trials had been done using different solvents as chloroform, dichloromethane and diethyl ether. But unfortunately these reactions were failed to proceed properly. In other highly polar solvents such as dimethylformamide (DMF) and tetrahydrofuran (THF), a mixture of the desired product beside other byproducts was obtained. Applying 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or triethylamine as bases to catalyze the reaction afforded hydrolyzed product of 1-chloromethylthalidomide **3**.

The chemical structure of all the synthesized compounds was elucidated on the basis of their spectral data IR, ^{1}H NMR and electron impact (EI) mass spectrometry beside the elemental analysis. The IR spectra of thalidomide sulfur analogs **2** and **4–18** supported the expected structures and showed absorption bands in the region 1609–1785 cm^{-1} resulting from the C=O function. The IR spectra of thalidomide dithiocarbamate and dithioate **4–18** supported the expected structures and showed absorption bands in the 1108–1183 cm^{-1} region resulting from C=S function. The ^{1}H NMR spectra of methylthiomethylthalidomide **2** showed the appearance of the methylene protons (N–CH$_2$–S) as multiplet at δ 5.28–5.34 ppm. The ^{1}H NMR spectra of thalidomide dithiocarbamate and dithioate analogs **4–18** showed the appearance of each proton of the methylene protons (N–CH$_2$–S) as doublet at δ 4.96 and 5.10 ppm. The ^{1}H NMR spectra of compound **2** and **4–18** display signals at δ 5.21–5.31 (m, H-3′), δ 2.10–2.15, 2.83–2.88 (m, H-4′) and δ 2.53–2.63, 3.01–3.12 ppm (m, H-5′) were associated with the piperidone ring. EI mass spectra of thalidomide sulfur analogs **2**

Table 1
Appropriate amine entries (**A**–**O**) and the corresponding thalidomide sulfur analogs **4**–**18**

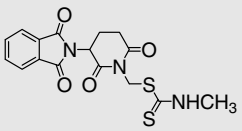
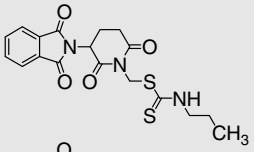
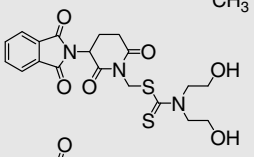
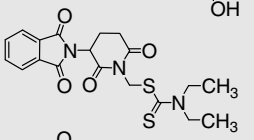
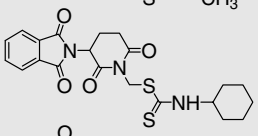
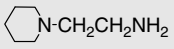
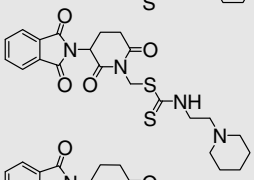
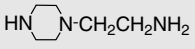
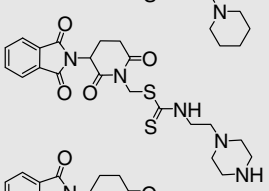
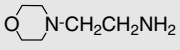
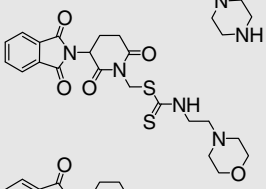
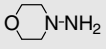
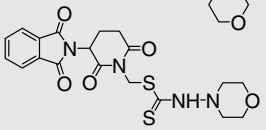
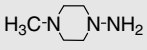
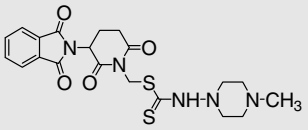
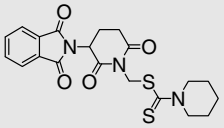
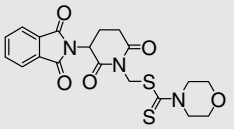
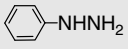
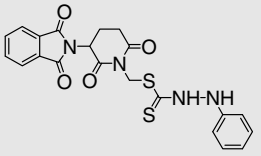
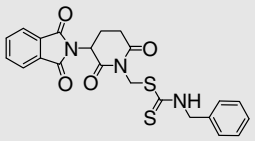
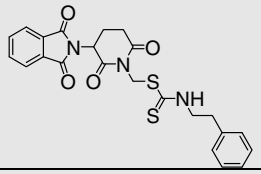
Entry	Amine	Product	Structure	Yield (%)/(mp°C)
A	CH_3NH_2	**4**		89/(140–142)
B	$CH_3CH_2CH_2NH_2$	**5**		73/(146–148)
C	$HOCH_2CH_2NH_2$	**6**		83/(175–177)
D	$(CH_3CH_2)_2NH$	**7**		58/(140–142)
E		**8**		72/(175–177)
F		**9**		57/(170–172)
G		**10**		88/(160–162)
H		**11**		58/(115–117)
I		**12**		58/(120–122)
J		**13**		91/(110–112)
K		**14**		81/(167–169)
L		**15**		71/(248–250)
M		**16**		67/(165–167)

Table 1 (*continued*)

Entry	Amine	Product	Structure	Yield (%)/(mp°C)
N	CH_2NH_2	**17**		75/(110–112)
O	$CH_2CH_2NH_2$	**18**		91/(120–122)

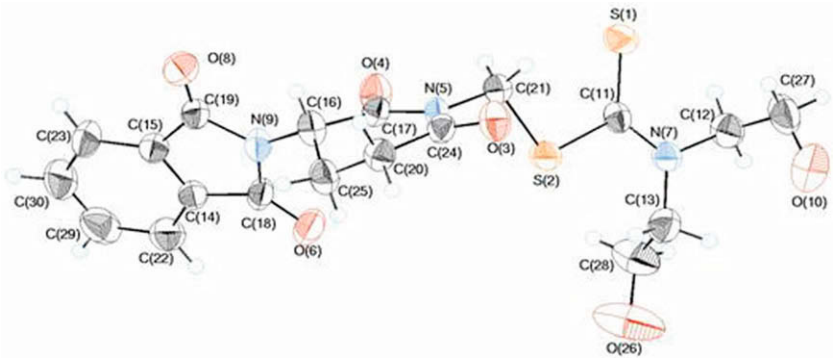


Figure 1. X-ray crystal structure of derivative **6**.

and **4–18** displayed molecular ions (M^+) which confirmed their molecular weights. The microanalysis data confirmed the structure of thalidomide sulfur analogs **2** and **4–18**. All the analytical data were in complete accordance with the proposed structures. Reagents and analytical data are presented in Section 4.

2.2. Antitumor and antioxidative examination

The present study evaluated the antitumor activity of new sensitized derivatives of well-known drug thalidomide. Screening for antitumor activity included measurement of% cell death of EAC by Trypan blue exclusion assay.[49] Three serial concentrations of thalidomide and its derivatives were tested in vitro cytotoxicity assay. Results shown in Table 2 indicated that thalidomide sulfur analogs **2** and **4–18** exhibit dose-dependent significant increases in cytotoxicity when compared to those of thalidomide **1**. The maximal cytotoxic activity of tested compounds was observed at dose 30 μL. Meanwhile, it is noted that most of compounds containing two sulfur atoms **4–18** have higher cytototoxic effect rather than that of compound **2** which contains one sulfur atom. Furthermore, results illustrated that compounds **8, 9, 13** and **14** exhibited the highest antiproliferative activities against EAC cells in vitro (95.2 ± 3.3%, 100 ± 0.0%, 100 ± 0.0% and 95.3 ± 2.7%, respectively), and can therefore be candidates for further stages of screening in vivo.

In Table 3, the in vivo results presented that treatment of solid-tumor bearing mice with thalidomide analogs containing one sulfur atom **2** as well as with two sulfur atom **8, 9, 13** and **14** resulted in a highly significant reduction in TV ($p < 0.001$) as compared with that of tumor control group of animals. Compounds **9** and **13** exhibited the most potent inhibitory action on the TV (99.6% and 98.9%, respectively) as compared to that of the compound **2** that contains one sulfur atom. Meanwhile, compound **2** exhibited consequently higher potent inhibitory action on TV (90.6%) rather than thalidomide **1** (80.6%). From these findings, we can conclude that

Table 2
In vitro cytotoxic activities of thalidomide **1** and its sulfur analogs **2** and **4–18**

Compound	% Cytotoxicity		
	10 μL	20 μL	30 μL
Control media	0.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.2
1	20.5 ± 2.5	20.5 ± 1.8	56.4 ± 2.1
2	62.5 ± 6.5	71.5 ± 7.8	82.8 ± 3.2
4	76.5 ± 3.4	80.0 ± 8.6	85.7 ± 3.2
5	10.6 ± 2.1	75.5 ± 7.6	88.6 ± 2.5
6	20.6 ± 3.2	30.6 ± 7.5	87.8 ± 2.7
7	10.2 ± 5.6	20.7 ± 3.5	90.6 ± 1.8
8	20.6 ± 3.6	60.8 ± 7.1	95.2 ± 3.3
9	80.5 ± 7.6	80.8 ± 5.6	100 ± 0.0
10	45.5 ± 4.3	60.6 ± 8.6	68.8 ± 1.6
11	20.6 ± 2.1	75.7 ± 6.0	94.7 ± 4.3
12	30.5 ± 3.2	70.6 ± 2.3	91.3 ± 2.1
13	80.4 ± 4.6	80.7 ± 5.1	100 ± 0.0
14	10.3 ± 2.1	80.4 ± 3.6	95.3 ± 2.7
15	80.0 ± 6.5	80.5 ± 5.4	89.7 ± 1.6
16	80.0 ± 5.3	81.6 ± 4.1	92.0 ± 1.7
17	56.7 ± 1.5	59.0 ± 1.0	61.3 ± 0.6
18	45.0 ± 2.6	47.3 ± 2.1	52.0 ± 1.0

Table 3
Effect of thalidomide and its sulfur analogs on the volume of solid tumor

Groups	TV × 10^{-3} (mm^3)	Inhibition %
Control	188.6 ± 2.5	—
1	36.4 ± 0.8*	80.6
2	17.7 ± 0.7*	90.6
8	13.6 ± 0.1*	92.7
9	0.7 ± 0.1*	99.6
13	2.0 ± 0.1*	98.9
14	7.7 ± 0.1*	95.9

* Statistical significant reduction of tumor volume as compared with a tumor control group ($p < 0.001$).

the degree of inhibitory activity may be attributed to the presence of sulfur atoms in the compound in the order of 2S > 1S > 0.

The level of malondialdehyde (MDA) as the end product of lipid peroxidation and antioxidant enzymes activities in solid-tumor bearing mice after treatment with 1.25 mM/kg body weights of thalidomide and its sulfur analogs **2**, **8**, **9**, **13** and **14** for five constitutive days was demonstrated (Table 4). Results showed a significant decrease ($p < 0.001$) in the level of hepatic lipid peroxidation in the tumor-bearing mice treated with these compounds as compared with that of tumor control group. On the other hand, the activities of SOD and catalase measured in the liver homogenate were significantly elevated ($p < 0.001$) in tumor bearing mice after treatment with thalidomide sulfur analogs **2**, **8**, **9**, **13** and **14** as compared with that of tumor control group. In Table 4, treatment of solid tumor with thalidomide sulfur analogs **9** and **13** resulted in reasonable significant changes in lipid peroxidation and catalase that were closed to the normal control group as compared to that of the rest of compounds **1**, **2**, **8** and **14**. Similarly, level of SOD activity was the most significant value which close to the normal control after treatment of solid-tumor bearing mice with compound **13** compared to that of the rest of compounds **1**, **2**, **8**, **9** and **14**. Taken together, these findings suggest that these thalidomide sulfur analogs **2**, **8**, **9**, **13** and **14** might exert its antitumor activity through the scavenging of free radicals resulting in a reduction of lipid oxidation and augmentation the level of antioxidant enzymes.

2.3. The histopathological and immunohistochemical examinations

In addition to the antitumor and antioxidative activities observed for thalidomide sulfur analogs **1, 2, 8, 9, 13** and **14**, the histopathological and immunohistochemical examinations of these compounds were investigated to explore more details on the mechanism of their action. Data presented in Table 5 illustrated a massive necrosis of the solid tumor after treatment with compound **9** as compared with that of compounds **1**, **2**, **8**, **13** and **14**

Table 4
Effect of thalidomide and its sulfur analogs on the level of lipid peroxidation and antioxidant enzymes activity in solid-tumor bearing mice

Groups	Lipid peroxidation (nmol MDA/g tissue)	Catalase (kU/mg tissue)	SOD (U/g tissue)
Normal control	246.6 ± 5.4	6.9 ± 1.4	156.2 ± 6.8
Tumor control	405.2 ± 8.7	5.1 ± 1.0	100.4 ± 8.3
1	330.2 ± 8.1*	5.8 ± 1.5	108.4 ± 10.2
2	289.6 ± 6.3*	6.7 ± 0.9*	139.8 ± 5.9*
8	257.8 ± 10.0*	6.4 ± 1.4*	128.6 ± 10.5*
9	247.6 ± 12.4*	7.2 ± 2.0*	145.8 ± 8.6*
13	248.4 ± 11.9*	8.2 ± 1.0*	162.2 ± 8.8*
14	276.2 ± 10.8*	7.2 ± 0.9*	152.4 ± 8.0*

* Statistical significant change as compared with a tumor control group ($p < 0.001$).

Table 5
Effect of thalidomide and its sulfur analogs on the grade, percentage of tumor necrosis and its H score

Groups	Tumor necrosis grade	Tumor necrosis %	Tumor necrosis H score
Control	—	—	—
1	2	44.0 ± 4.2	69.0 ± 20.1
2	2	49.0 ± 4.2	87.0 ± 18.6
8	2	41.0 ± 4.2	82.0 ± 8.4
9	3	100.0 ± 0.0*	300.0 ± 0.0*
13	3	61.0 ± 4.2*	110.0 ± 23.5*
14	2	50.0 ± 3.5	109.0 ± 24.6*

* Statistical significant change as compared with thalidomide group ($p < 0.001$).

Table 6
Effect of thalidomide and its sulfur analogs on liver

Groups	Liver degeneration	Liver necrosis
Normal control	0	0
Tumor control	0	0
1	1	1
2	0	0
8	1	0
9	2	0
13	1	0
14	1	0

($p < 0.001$). On the other hand, thalidomide analogs **9** and **13** showed the highest statistical significance in necrosis percentage and H score compared with that of the rest compounds **1**, **2**, **8** and **14**.

The thalidomide sulfur analogs **2**, **8**, **9**, **13** and **14** had no necrotic effect on the liver, while thalidomide **1** exhibited toxic effect on the liver of treated mice (Table 6). As regard with the statistical significance, Tables 5 and 6 revealed that compound **9** is the most potent compound in tumor necrosis without liver necrosis as compared with the other compounds (Fig. 2A). Moreover, liver degeneration has been remarkably observed after treatment with compound **9** (Fig. 2H).

Apoptosis is one of the most important mechanisms in the antitumor drugs. Fas-L belongs to the tumor necrosis factor (TNF) family. Fas (also known as APO-1 or CD95) are the receptor for Fas-L, a member of TNF receptors nerve growth factor receptor superfamily. Fas-L and Fas pathway is one of the famous extrinsic apoptotic pathways.[50] The histopathological examination by using H & E staining showed a statistically significant increase in apoptotic index (AI) after treatment of tumor-bearing mice with thalidomide **1** and its sulfur analogs **2, 8, 9, 13** and **14** as compared with that of the tumor control group. As shown in Figure 2B and Table 7, treatment with compound **13** exhibited the most potent apoptotic activity ($p < 0.001$) rather than the rest of the compounds **1, 2, 8, 9** and **14**. On the other hand, the mitotic index (MI) was significantly decreased ($p < 0.001$) in the studied analogs **1, 2, 8, 9, 13** and **14** as compared to that of the tumor control group (Table 7). Compound **13** exhibited the most potent antimitotic activity ($p < 0.001$).

Thus, immunohistochemical staining for Fas-L, as the one of the famous extrinsic apoptotic pathways, showed a highly statistical significant increase ($p < 0.001$) in the intensity, percentage and H score of Fas-L after treatment with thalidomide **1** and its sulfur analogs **2**, **8**, **9**, **13** and **14** as compared to those of tumor control group (Table 8 and Fig. 2C). These data showed that compound **14** which contain two sulfur atoms exhibited the most potent stimulatory effect on the Fas-L expression (120.0 ± 5.0%) as compared to that of the rest of compounds **1**, **2**, **8**, **9** and **13**. These results were in agreement with the previous findings reported that thalidomide has a potent apoptotic effect in cancer cells by enhancement of cell sensitivity to Fas-induced apoptosis.[51,52]

Angiogenesis is the process of generating new capillary blood vessels which plays an important role in the proliferation, invasion and metastasis of malignant tumor. Blocking tumor induced angiogenesis continues to be an attractive strategy for cancer therapy.[53] Also, VEGF is the prototypical pro-angiogenic molecule that has been implicated in several steps throughout the angiogenic process because it disrupts the endothelial barrier function that might contribute to tumor cell extravasations and metastasis.[54] The effect of these compounds on the angiogenesis process by immunohistochemical staining for VEGF was examined. Figure 2D and E shows strong and weak expressions of VEGF in tumor control and after treatment with compound **13**, respectively. As

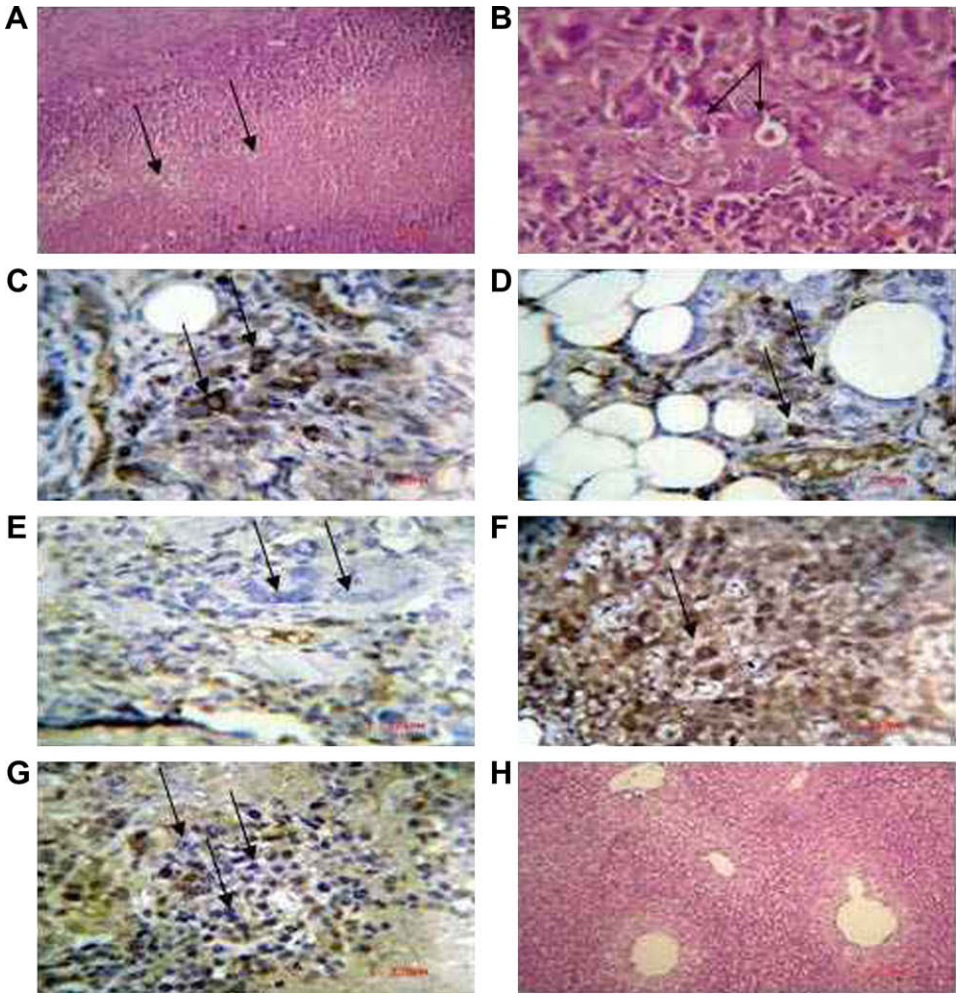


Figure 2. Histopathological and immunohistochemical examination of the study (A) showed extensive necrosis in solid-tumor bearing mice treated with compound **9**; (B) showed apoptotic bodies (arrows) in tumor after treatment with compound **13**; (C) showed strong immunoreactivity staining for Fas-L in the membrano-cytoplasmic of the cells of compound **14**; (D and E) showed strong and weak expression of VEGF in tumor control and after treatment with compound **13**, respectively; (F and G) showed strong nuclear positivity for Ki67 staining in tumor control and negative nuclear staining in treated mice with compound **2**, respectively and (H) illustrated the H & E stained section from the liver of treated mice with compound **9** showing a diffuse degeneration (grade +++).

Table 7
Effect of thalidomide and its sulfur analogs on apoptotic and mitotic indices (AI and MI)

Groups	AI	MI
Control	0.001 ± 0.001	0.018 ± 0.0
1	0.003 ± 0.002*	0.006 ± 0.0*
2	0.002 ± 0.001	0.004 ± 0.0*
8	0.002 ± 0.001	0.005 ± 0.001*
9	0.004 ± 0.002*	0.004 ± 0.002*
13	0.005 ± 0.001*	0.002 ± 0.0*
14	0.002 ± 0.001	0.004 ± 0.0*

* Statistical significant change as compared with tumor control group ($p < 0.001$).

Table 8
Intensity, H score and percentage of Fas-L expression to thalidomide and its sulfur analogs

Groups	Fas-L intensity			Fas-L%	Fas-L (H score)
	1	2	3		
Control	5	0	0	16.0 ± 4.2	16.0 ± 4.2
1	0	3	2	66.0 ± 4.2*	159.0 ± 40.1*
2	0	1	4	45.0 ± 5.0*	125.0 ± 18.7*
8	0	1	4	44.0 ± 4.2*	116.0 ± 33.4*
9	0	1	2	55.0 ± 5.0*	129.0 ± 17.7*
13	0	4	1	60.0 ± 5.0*	131.0 ± 20.7*
14	0	2	3	120.0 ± 5.0*	337.0 ± 61.9*

* Statistical significant change as compared with tumor control group ($p < 0.001$). Test of significance χ^2 (Chi square) = 88.83.

shown in Table 9, a highly statistical significant decrease ($p < 0.001$) in the intensity, percentage and H score of VEGF for the treated tumor-bearing mice with thalidomide **1** and its sulfur analogs **2**, **8**, **9**, **13** and **14** compared to that of untreated group. These data showed that compound **13** which contain two sulfur atoms exhibited the most potent inhibitory effect on the VEGF expression (2.0 ± 0.0%).

This finding was compatible with the previous report which concluded that thalidomide exhibit potent anti-angiogenic properties that were thought to contribute to the teratogenic effects of limb bud malformations observed in offspring of thalidomide exposed gravid mothers.[28] Other studies displayed that thalidomide has been shown to suppress the induction of VEGF in co-cultures of multiple myeloma cell lines and bone marrow stromal cells.[55]

Proliferation index is an important prognostic factor in cancer. The Ki67 protein is expressed in all phases of the cell cycle except G0 and serves as a good marker for proliferation.[56] The proliferation index was investigated by immunohistochemical staining for Ki67 on solid tumors removed from the untreated and treated ani-

Table 9
Intensity, H score and percentage of VEGF expression to thalidomide and its sulfur analogs

Groups	VEGF Intensity			VEGF%	VEGF (H score)
	1	2	3		
Control	1	4	0	20.0 ± 5.0	55.0 ± 4.5
1	1	4	0	8.0 ± 5.0*	25.0 ± 18.7*
2	0	1	4	3.4 ± 1.5*	9.2 ± 3.7*
8	0	2	3	7.0 ± 2.7*	16.0 ± 8.6*
9	0	1	2	3.2 ± 1.0*	8.3 ± 2.7*
13	0	2	3	2.0 ± 0.0*	5.2 ± 1.9*
14	0	4	1	7.0 ± 4.2*	20.0 ± 12.5*

* Statistical significant change as compared with tumor control group ($p < 0.001$). Test of significance χ^2 (Chi square) = 76.65.

Table 10
Intensity, percentage and H score of Ki67 expression to thalidomide and its sulfur analogs

Groups	Ki67 Intensity			Ki67%	Ki67 (H score)
	1	2	3		
Control	0	0	5	78 ± 5.7	234 ± 17.1
1	0	3	2	7.4 ± 2.5*	16.8 ± 3.0*
2	3	2	0	3.2 ± 1.6*	4.6 ± 3.3*
8	3	1	1	7.2 ± 2.6*	8.2 ± 2.5*
9	3	1	1	3.4 ± 1.4*	4.2 ± 1.1*
13	3	2	0	3.4 ± 1.5*	4.4 ± 1.5*
14	4	1	0	24 ± 4.2*	72 ± 12.6*

* Statistical significant change as compared with tumor control group ($p < 0.001$). Test of significance χ^2 = 81.2.

mals with thalidomide **1** and its sulfur analogs **2**, **8**, **9**, **13** and **14**. Nuclear staining and good preservation of morphological details were observed in tumor section immunostained with Ki67 antibody. Figure 2F and G shows the amount of Ki67 negative and positive cells in analyzed slides, respectively. In Table 10, the relative number of Ki67 positive tumor cells was substantially smaller in tumors from mice treated thalidomide and its sulfur analogs ($p < 0.001$). These data showed that compound **2** which contain one sulfur atom exhibited the most potent inhibitory effect on the Ki67 expression (3.2 ± 1.6%) as compared to that of the rest of compounds **1**, **8**, **9**, **13** and **14**. These findings were in accordance with the previous report which concluded that thalidomide has a direct anti-myeloma activity mediated through inhibition of DNA synthesis resulting in cell proliferation arrest in the G1 phase.[57]

Further investigations are now under consideration on thalidomide **1** and its sulfur analogs **9** and **13** to assess their antitumor activity on female albino mice-bearing solid tumor by studying the differential display of the expressed proteins in healthy, cancerous and treated tissues as well as the determination of NO concentration in homogenate liver tissues. Moreover, following up the biochemical analysis of serum in treated and untreated mice and studying the level of methylation in the genomic DNA during the development of cancerous tissues in treated and healthy mice. Finally, using the RT-PCR technique to study two of transcribed genes involved in treated and untreated cancerous tissues.

3. Conclusion

A series of novel thalidomide dithiocarbamate and dithioate analogs were designed and synthesized as potential antitumor agents. Compounds containing two sulfur atoms **8**, **9**, **13** and **14** displayed the most potent antitumor activity higher than compound **2** that contain one sulfur atom.

Compound **9** and **13** were commonly exhibited the most potent tumor necrotic activity with no toxicity to the liver. At the same time, treatment with compound **9** resulted in liver degeneration. Moreover, this study shed light on the mechanism of action of these newly sensitized compounds. As obtained from the results, it can be concluded that thalidomide sulfur analogs may exert their anticancer activity via antioxidative pathway resulting in diminishing of free radicals and augmentation of antioxidant enzymes. These compounds have the ability to interfere with the expression of VEGF and Ki67 resulting in their significant reduction and subsequently tumor growth inhibition. Induction of Fas-L expression was significantly shown with the treatment of thalidomide sulfur analogs suggesting the apoptotic activity of these compounds.

4. Experimental

4.1. General information

The synthesized product and each reaction were monitored on Merck silica gel 60 F254 (type E; Merck) plates and spots were located by UV light. All ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz Spectrometer, Cairo University. Chemical shifts are reported in parts per million (ppm) relative to the respective solvent or tetramethylsilane (TMS) and standard abbreviations were used (a = apparent; b = broad; s = singlet; d = doublet; t = triplet; q = quartet; m = multiple). Coupling constant are given in Hz. Elemental analyses were determined on a Yanaca CHN Corder MT-3 elemental analyzer in microanalysis laboratory at Cairo University. IR spectra were recorded (KBr) on a Pye-Unicam Sp-883 Perkins- Elmer spectrophotometer, Microanalytical Laboratory, Faculty of Science, Cairo University. MS spectra were run on GC MS-QP 1000 EX (SHIMADZU) Mass Spectrometer, Microanalytical Laboratory, Faculty of Science, Cairo University. Melting points were recorded on Stuart scientific melting point apparatus. All diagrams and calculations were performed using maXus (Bruker Nonius, Delft & MacScience, Japan). Scoring was carried out using an Olympus CH_2 light microscope, Tokyo, Japan with wide angle (field size of 0.274 mm^2, and field diameter of 0.59 mm). The starting material, 2-(1-Chloromethyl-2,6-dioxo-piperidine-3-yl)-1,3-dihydro-2*H*-isoindole-1,3-dione **3** was prepared according to the reported procedure.[58] All chemicals and solvents were purchased from E. Merck (Darmstadt, Germany) and Sigma–Aldrich.

4.2. Chemistry

4.2.1. The procedure for the synthesis of methylthiomethyl thalidomide (2)

Powdered KOH (15 mmol) and tris[2-(2-methoxy)ethoxy)ethyl]amine (TDA-1, 0.31 mmol) were added to a solution of thalidomide **1** (6 mmol) in anhydrous acetonitrile (50 mL). The mixture was stirred for 1 h at room temperature. After that, $ClCH_2SCH_3$ (6.3 mmol) was added and stirring was continued for 48 h. Insoluble materials were filtered off and the filtrate was evaporated under reduced pressure. The residue was purified by preparative TLC with chloroform.

4.2.1.1. 2-{1-[(Methylthio)methyl]-2,6-dioxopiperidin-3-yl}-1H-isoindole-1,3(2H)-dione (2). White powder; IR (KBr) 3039, 2977, 2932, 2876, 1787, 1772, 1690, 1680, 1513 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.10–2.13 (m, 1H, H-4′), 2.14 (s, 3H, CH_3), 2.48–2.59 (m, 1H, H-5′), 2.84–2.87 (m, 1H, H-4′), 2.95–3.11 (m, 1H, H-5′CO), 4.81–4.86 (m, 1H, H-3′), 5.28–5.34 (m, 2H, NCH_2S), 7.90–7.97 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 15.82 (CH_3), 21.11 (C_4′), 26.59 (C_5′), 41.37 (NCH_2S), 50.11 (C_3′), 123.12 (C_4, C_7), 130.12 (C_{3a}, C_{7a}), 132.44 (C_5, C_6), 167.14, 167.92, 169.02 (C_1, C_3, C_2′, C_6′); EIMS m/z = 318 (M^+). Anal. Calcd for $C_{15}H_{14}N_2O_4S$: C, 56.59; H, 4.43; N, 8.80; S, 10.07. Found: C, 56.34; H, 4.22; N, 8.68; S, 10.10.

4.2.2. General procedure for the synthesis of thalidomide ditiocarbamate and dithioate analogs (4–18)

A mixture of chloromethylthalidomide **3** (1 mmol), CS_2 (2 mmol) and the appropriate amine (2 mmol) (Table 1) in acetonitrile (50 mL) was stirred for 48 h at room temperature. The solvent was removed in vacuo; the residue was coevaported two times with dichloromethane and crystallized from ethanol to afford thalidomide dithiocarbamates and dithioate analogs **4–18**.

4.2.2.1. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl] methyl methyldithiocarbamate (4). White powder; IR (KBr) 3235, 3046, 2977, 2948, 2923, 1768, 1728, 1710, 1669, 1549, 1389, 1161 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.11–2.17 (m, 1H, H-4′), 2.48–2.59 (m, 1H, H-5′), 2.84–2.87 (m, 1H, H-4′), 2.95–3.11 (m, 1H, H-5′CO), 3.01 (d, 3H, CH_3), 4.90–4.92 (m, 1H, NH), 5.28-5.32 (m, 1H, H-3′), 5.34 (d, J = 12.6 Hz, 1H, NCH_2S), 5.54 (d, J = 12.6 Hz, 1H, NCH_2S), 7.89–7.96 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.34 ($C_{4'}$), 29.13 ($C_{5'}$), 30.12 (CH_3), 42.18 (NCH_2S), 50.17 ($C_{3'}$), 123.60 (C_4, C_7), 131.15 (C_{3a}, C_{7a}), 133.15 (C_5, C_6), 167.22, 168.11, 169.22 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.61 (C=S); EIMS m/z = 377 (M^+). Anal. Calcd for $C_{16}H_{15}N_3O_4S_2$: C, 50.91; H, 4.01; N, 11.13; S, 16.99. Found: C, 51.12; H, 3.90; N, 10.96; S, 17.10.

4.2.2.2. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl propyldithiocarbamate (5). White crystals; IR (KBr) 3267, 3055, 2965, 2932, 2876, 1786, 1770, 1716, 1679, 1529, 1393, 1156 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.05 (t, 3H, CH_3), 1.50–1.66 (m, 2H, CH_2), 2.08–2.14 (m, 1H, H-4′), 2.55–2.65 (m, 1H, H-5′), 2.79–2.88 (m, 1H, H-4′), 2.97–3.04 (m, 1H, H-5′CO), 3.61 (t, 2H, NCH_2), 4.91–4.92 (m, 1H, NH), 5.27–5.34 (m, 1H, H-3′), 5.33 (d, J = 12.9 Hz, 1H, NCH_2S), 5.52 (d, J = 12.9 Hz, 1H, NCH_2S), 7.88–7.95 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 11.22 (CH_3), 21.33 ($C_{4'}$), 22.43 (CH_2), 29.10 ($C_{5'}$), 42.15 (NCH_2S), 46.44 ($NHCH_2$), 50.14 ($C_{3'}$), 123.58 (C_4, C_7), 131.12 (C_{3a}, C_{7a}), 133.14 (C_5, C_6), 167.19, 168.11, 169.11 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.11 (C = S); EIMS m/z = 405 (M^+). Anal. Calcd for $C_{18}H_{19}N_3O_4S_2$: C, 53.32; H, 4.72; N, 10.36; S, 15.82. Found: C, 52.87; H, 5.32; N, 9.96; S, 15.80.

4.2.2.3. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl bis(2-hydroxyethyl)dithiocarbamate (6). White crystals; IR (KBr) 3520, 3038, 2957, 2923, 2885, 1769, 1736, 1713, 1689, 1513, 1485, 1145 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.11–2.17 (m, 1H, H-4′), 2.57–2.64 (m, 1H, H-5′), 2.84–2.86 (m, 1H, H-4′), 3.01–3.02 (m, 1H, H-5′CO), 3.64–3.69 (m, 4H, CH_2NCH_2), 3.82–3.86 (m, 2H, CH_2OH), 4.04–4.08 (m, 2H, CH_2OH), 4.86 (t, 1H, OH), 5.02 (t, 1H, OH), 5.28–5.32 (m, 1H, H-3′), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 5.47 (d, J = 11.7 Hz, 1H, NCH_2S), 7.88–7.96 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.40 ($C_{4'}$), 29.15 ($C_{5'}$), 42.43 (NCH_2S), 50.18 ($C_{3'}$), 58.13 (CH_2OH), 59.72 (CH_2N), 123.61 (C_4, C_7), 131.24 (C_{3a}, C_{7a}), 133.16 (C_5, C_6), 167.23, 168.12, 169.40 (C_1, C_3, $C_{2'}$, $C_{6'}$), 196.31 (C=S); EIMS m/z = 451 (M^+). Anal. Calcd for $C_{19}H_{21}N_3O_6S_2$: C, 50.54; H, 4.69; N, 9.31; S, 14.20. Found: C, 50.30; H, 4.44; N, 9.54; S, 14.21.

4.2.2.4. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl diethyldithiocarbamate (7). colorless crystals; IR (KBr) 3072, 2977, 2959, 2930, 2871, 1783, 1769, 1716, 1693, 1554, 1488, 1137 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.14 (m, 3H, CH_3), 2.11–2.17 (m, 1H, H-4′), 2.57–2.62 (m, 1H, H-5′), 2.82–2.89 (m, 1H, H-4′), 3.01–3.04 (m, 1H, H-5′CO), 3.69 (q, 2H, CH_2), 3.94 (q, 2H, CH_2), 5.28–5.33 (m, 1H, H-3′), 5.34 (d, J = 11.7 Hz, 1H, NCH_2S), 5.49 (d, J = 11.7 Hz, 1H, NCH_2S), 7.88–7.94 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 12.13 (CH_3), 21.31 ($C_{4'}$), 29.09 ($C_{5'}$), 42.11 (NCH_2S), 48.60 (CH_2), 50.11 ($C_{3'}$), 123.57 (C_4, C_7), 131.12 (C_{3a}, C_{7a}), 133.11 (C_5, C_6), 167.09, 168.09, 169.11 (C_1, C_3, $C_{2'}$, $C_{6'}$), 196.31 (C=S); EIMS m/z = 419 (M^+). Anal. Calcd for $C_{19}H_{21}N_3O_4S_2$: C, 54.40; H, 5.05; N, 10.02; S, 15.29. Found: C, 54.33; H, 5.20; N, 9.90; S, 15.30.

4.2.2.5. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl cyclohexyldithiocarbamate (8). White powder; IR (KBr) 3219, 2939, 2882, 2859, 2835, 2781, 1719, 1685, 1676, 1619, 1507, 1391, 1126 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.09–1.92 (m, 10H, cyclohex. $C_{2,3,4,5,6}$-H), 2.11–2.16 (m, 1H, H-4′), 2.56–2.60 (m, 1H, H-5′), 2.84–2.88 (m, 1H, H-4′), 2.90–2.91 (m, 1H, cyclohex. C_1-H), 3.01–3.04 (m, 1H, H-5′CO), 4.91–4.92 (m, 1H, NH), 5.28–5.32 (m, 1H, H-3′), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 5.47 (d, J = 11.7 Hz, 1H, NCH_2S), 7.89–8.06 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.29 ($C_{4'}$), 24.82 (cyclohex. C_3, C_5), 25.71 (cyclohex. C_4), 29.06 ($C_{5'}$), 31.64 (cyclohex. C_2, C_6), 42.10 (NCH_2S), 50.09 ($C_{3'}$), 51.31 (cyclohex. C_1), 123.55 (C_4, C_7), 131.06 (C_{3a}, C_{7a}), 133.10 (C_5, C_6), 167.06, 168.11, 169.02 (C_1, C_3, $C_{2'}$, $C_{6'}$), 199.71 (C=S); EIMS m/z = 445 (M^+). Anal. Calcd for $C_{21}H_{23}N_3O_4S_2$: C, 56.61; H, 5.20; N, 9.43; S, 14.39. Found: C, 56.32; H, 4.99; N, 9.50; S, 14.40.

4.2.2.6. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl 2-piperidin-1-ylethyldithiocarbamate (9). White powder; IR (KBr) 3429, 3028, 2955, 2920, 1785, 1770, 1717, 1691, 1467, 1390, 1153 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.53–1.57 (m, 6H, pip. $C_{3,4,5}$-H), 2.10–2.14 (m, 1H, H-4′), 2.57–2.61 (m, 1H, H-5′), 2.63-2.69 (m, 6H, CH_2–N–$C_{2,6}$–H pip.), 2.82–2.91 (m, 1H, H-4′), 3.04–3.08 (m, 1H, H-5′CO), 3.25–3.38 (m, 2H, –HN–CH_2–), 4.90–4.92 (m, 1H, NH), 5.08–5.22 (m, 1H, H-3′), 5.24 (d, J = 11.7 Hz, 1H, NCH_2S), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 7.88–7.95 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.63 ($C_{4'}$), 24.50 (pip. C_4), 25.94 (pip. C_3, C_5), 29.19 ($C_{5'}$), 41.31 ($NHCH_2$), 44.98 (NCH_2S), 50.74 ($C_{3'}$), 50.88 (CH_2N), 54.31 (pip. C_2, C_6), 123.70 (C_4, C_7), 131.65 (C_{3a}, C_{7a}), 133.30 (C_5, C_6), 167.48, 168.37, 170.12 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.10 (C=S); EIMS m/z = 474 (M^+). Anal. Calcd for $C_{22}H_{26}N_4O_4S_2$: C, 55.68; H, 5.52; N, 11.80; S, 13.51. Found: C, 55.44; H, 5.33; N, 11.96; S, 13.50.

4.2.2.7. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl 2-piperazin-1-ylethyldithiocarbamate (10). White powder; IR (KBr) 3418, 2944, 2831,1780, 1770, 1716, 1621, 1514, 1318, 1108 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.10–2.14 (m, 1H, H-4′), 2.40–2.41 (m, 4H, pip. $C_{2,6}$–H), 2.47–2.50 (m, 2H, CH_2–N–), 2.52–2.61 (m, 1H, H-5′), 2.63–2.67 (m, 4H, pip. $C_{3,5}$–H), 2.87–2.91 (m, 1H, H-4′), 3.04–3.07 (m, 1H, H-5′CO), 3.61–3.75 (m, 2H, –NH–CH_2–), 4.90–4.92 (m, 1H, NH), 5.28–5.32 (m, 1H, H-3′), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 5.47 (d, J = 11.7 Hz, 1H, NCH_2S), 7.62–5.92 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.65 ($C_{4'}$), 29.21 ($C_{5'}$), 41.33 ($NHCH_2$), 45.12 (NCH_2S), 46.24 (pip. C_3, C_5), 50.76 ($C_{3'}$), 53.10 (CH_2N), 57.32 (pip. C_2, C_6), 123.71 (C_4, C_7), 131.69 (C_{3a}, C_{7a}), 133.32 (C_5, C_6), 167.51, 168.40, 170.45 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.12 (C=S); EIMS m/z = 475 (M^+). Anal. Calcd for $C_{21}H_{25}N_5O_4S_2$: C, 53.03; H, 5.30; N, 14.73; S, 13.48. Found: C, 52.96; H, 5.44; N, 14.66; S, 13.50.

4.2.2.8. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl 2-morpholin-4-ylethyldithiocarbamate (11). White powder; IR (KBr) 3472, 3028, 2955, 2917, 1785, 1770, 1717, 1690, 1467, 1390, 1151 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.10–2.14 (m, 1H, H-4′), 2.57–2.61 (m, 1H, H-5′), 2.63–2.69 (m, 6H, –CH_2–N–$C_{3,5}$–H morph.), 2.82–2.87 (m, 1H, H-4′), 3.00–3.04 (m, 1H, H-5′CO), 3.24–3.39 (m, 2H, –HN–CH_2–), 3.56–3.60 (m, 4H, morph. $C_{2,6}$–H), 4.90–4.92 (m, 1H, NH), 5.13–5.18 (m, 1H, H-3′), 5.24 (d, J = 11.7 Hz, 1H, NCH_2S), 5.34 (d, J = 11.7 Hz, 1H, NCH_2S), 7.88–7.96 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.69 ($C_{4'}$), 29.22 ($C_{5'}$), 41.34 ($NHCH_2$), 45.40 (NCH_2S), 50.77 ($C_{3'}$), 53.35 (CH_2N), 55.81 (morph. C_3, C_5), 66.76 (morph. C_2, C_6), 123.73 (C_4, C_7), 131.70 (C_{3a}, C_{7a}), 133.34 (C_5, C_6), 167.52, 168.42, 170.55 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.16 (C=S); EIMS m/z = 476 (M^+). Anal. Calcd for $C_{21}H_{24}N_4O_5S_2$: C, 52.92; H, 5.08; N, 11.76; S, 13.46. Found: C, 52.80; H, 4.91; N, 11.82; S, 13.50.

4.2.2.9. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl morpholin-4-yldithiocarbamate (12). White powder; IR (KBr) 3430, 2959, 2917, 2853, 1785, 1771, 1717, 1691, 1518, 1330, 1151 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.10–2.14 (m, 1H, H-4′), 2.52–2.61 (m, 1H, H-5′), 2.79–2.84 (m, 1H, H-4′), 3.01–3.04 (m, 1H, H-5′CO), 3.15–3.36 (m, 4H, morph. $C_{3,5}$–H), 3.65–3.69 (m, 4H, morph. $C_{2,6}$–H), 4.90–4.92 (m, 1H, NH), 5.11–5.22 (m, 1H, H-3′), 5.24 (d, J = 11.7 Hz, 1H, NCH_2S), 5.34 (d, J = 11.7 Hz, 1H, NCH_2S), 7.89–7.96 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.90 ($C_{4'}$), 29.27 ($C_{5'}$), 45.55 (NCH_2S), 50.88 ($C_{3'}$), 55.11 (morph. C_3, C_5), 65.22 (morph. C_2, C_6), 123.79 (C_4, C_7), 131.76 (C_{3a}, C_{7a}), 133.70 (C_5, C_6), 167.61, 168.52, 171.12 (C_1, C_3, $C_{2'}$, $C_{6'}$), 203.16 (C═S); EIMS m/z = 448 (M^+). Anal. Calcd for $C_{19}H_{20}N_4O_5S_2$: C, 50.88; H, 4.49; N, 12.49; S, 14.30. Found: C, 51.10; H, 4.32; N, 12.25; S, 14.31.

4.2.2.10. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl 4-methylpiperazin-1-yldithiocarbamate (13). White powder; IR (KBr) 3418, 2941, 2831, 2772, 2686, 1773, 1735, 1700, 1654, 1534, 1280, 1183 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.11–2.16 (m, 1H, H-4′), 2.22 (s, 3H, CH_3), 2.34–2.40 (m, 4H, pip. $C_{3,5}$–H), 2.49–2.63 (m, 1H, H-5′), 2.75–2.85 (m, 1H, H-4′), 2.97 (br s, 4H, pip. $C_{2,6}$–H), 3.01–3.22 (m, 1H, H-5′CO), 4.90 (m, 1H, NH), 5.28–5.32 (m, 1H, H-3′), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 5.47 (d, J = 11.7 Hz, 1H, NCH_2S), 7.91–7.92 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.82 ($C_{4'}$), 29.26 ($C_{5'}$), 45.51 (CH_3), 45.55 (NCH_2S), 50.84 ($C_{3'}$), 52.22 (pip. C_3, C_5), 56.22 (pip. C_2, C_6), 123.78 (C_4, C_7), 131.75 (C_{3a}, C_{7a}), 133.46 (C_5, C_6), 167.59, 168.48, 171.10 (C_1, C_3, $C_{2'}$, $C_{6'}$), 203.11 (C═S); EIMS m/z = 461 (M^+). Anal. Calcd for $C_{20}H_{23}N_5O_4S_2$: C, 52.04; H, 5.02; N, 15.17; S, 13.89. Found: C, 51.96; H, 5.12; N, 15.10; S, 13.90.

4.2.2.11. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl piperidin-1-carbodithioate (14). White powder; IR (KBr) 2948, 2843, 2808, 2762, 2737, 1785, 1772, 1717, 1695, 1592, 1469, 1141 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.54–1.69 (m, 6H, pip. $C_{3,4,5}$–H), 2.11–2.14 (m, 1H, H-4′), 2.51–2.69 (m, 1H, H-5′), 2.85–2.91 (m, 1H, H-4′), 3.00–3.11 (m, 1H, H-5′CO), 3.65–3.85 (m, 4H, pip. $C_{2,6}$–H), 4.15–4.22 (m, 4H, pip. $C_{2,6}$–H), 5.27–5.33 (m, 1H, H-3′), 5.34 (d, J = 11.7 Hz, 1H, NCH_2S), 5.49 (d, J = 11.7 Hz, 1H, NCH_2S), 7.88–7.95 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.70 ($C_{4'}$), 24.24 (pip. C_4), 24.71 (pip. C_3, C_5), 29.24 ($C_{5'}$), 45.43 (NCH_2S), 48.63 (pip. C_2, C_6), 50.79 ($C_{3'}$), 123.75 (C_4, C_7), 131.72 (C_{3a}, C_{7a}), 133.40 (C_5, C_6), 167.55, 168.44, 170.66 (C_1, C_3, $C_{2'}$, $C_{6'}$), 201.71 (C═S); EIMS m/z = 431 (M^+). Anal. Calcd for $C_{20}H_{21}N_3O_4S_2$: C, 55.67; H, 4.91; N, 9.74; S, 14.86. Found: C, 55.95; H, 4.77; N, 9.60; S, 14.89.

4.2.2.12. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl morpholin-4-carbodithioate (15). White powder; IR (KBr) 3061, 2946, 2893, 2852, 2759, 1784, 1770, 1714, 1690, 1586, 1469, 1110 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.13–2.16 (m, 1H, H-4′), 2.49–2.61 (m, 1H, H-5′), 2.85–2.86 (m, 1H, H-4′), 2.97–3.13 (m, 1H, H-5′CO), 3.49–3.52 (m, 4H, morph. $C_{2,6}$–H), 3.63–3.66 (m, 4H, morph. $C_{2,6}$–H), 3.75–3.79 (m, 4H, morph. $C_{3,5}$–H), 4.27–4.30 (m, 4H, morph. $C_{3,5}$–H), 5.27–5.34 (m, 1H, H-3′), 5.39 (d, J = 11.7 Hz, 1H, NCH_2S), 5.54 (d, J = 11.7 Hz, 1H, NCH_2S), 7.90–7.94 (m, 4H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.77 ($C_{4'}$), 29.24 ($C_{5'}$), 45.48 (NCH_2S), 49.44 (morph. C_3, C_5), 50.81 ($C_{3'}$), 65.51 (morph. C_2, C_6), 123.76 (C_4, C_7), 131.74 (C_{3a}, C_{7a}), 133.41 (C_5, C_6), 167.56, 168.46, 170.98 (C_1, C_3, $C_{2'}$, $C_{6'}$), 201.79 (C═S); EIMS m/z = 433 (M^+). Anal. Calcd for $C_{19}H_{19}N_3O_5S_2$: C, 52.64; H, 4.42; N, 9.69; S, 14.79. Found: C, 52.51; H, 4.39; N, 9.88; S, 14.80.

4.2.2.13. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl 2-phenylhydrazinecarbodithioate (16). White powder; IR (KBr) 3430, 3004, 2942, 2847, 2697, 1769, 1716, 1691, 1609, 1499, 1343, 1167 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.11–2.16 (m, 1H, H-4′), 2.51–2.69 (m, 1H, H-5′), 2.88–2.91 (m, 1H, H-4′), 3.01–3.04 (m, 1H, H-5′CO), 4.90–4.92 (m, 1H, NH), 5.27–5.32 (m, 1H, H-3′), 5.33 (d, J = 11.7 Hz, 1H, NCH_2S), 5.47 (d, J = 11.7 Hz, 1H, NCH_2S), 6.90–7.55 (m, 9H, H_{arom}), 10.31 (s, 1H, NH aromatic); ^{13}C NMR (DMSO-d_6) δ 21.55 ($C_{4'}$), 29.16 ($C_{5'}$), 44.88 (NCH_2S), 50.38 ($C_{3'}$), 113.22 (ph. C_2, C_6), 122.81 (ph. C_4), 123.79 (C_4, C_7), 129.23 (ph. C_3, C_5), 131.55 (C_{3a}, C_{7a}), 133.27 (C_5, C_6), 149.10 (ph. C_1), 167.45, 168.26, 169.98 (C_1, C_3, $C_{2'}$, $C_{6'}$), 203.10 (C═S); EIMS m/z = 454 (M^+). Anal. Calcd for $C_{21}H_{18}N_4O_4S_2$: C, 55.49; H, 3.99; N, 12.33; S, 14.11. Found: C, 55.30; H, 4.12; N, 12.20; S, 14.12.

4.2.2.14. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl benzyldithiocarbamate (17). White powder; IR (KBr) 3219, 3004, 2965, 2932, 2893, 1784, 1770, 1714, 1690, 1586, 1343, 1124 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.10–2.14 (m, 1H, H-4′), 2.51–2.61 (m, 1H, H-5′), 2.84–2.88 (m, 1H, H-4′), 3.04–3.31 (m, 1H, H-5′CO), 4.82 (d, 2H, –NH–CH_2–), 4.91–4.93 (m, 1H, NH), 5.08–5.34 (m, 1H, H-3′), 5.31 (d, J = 12.6 Hz, 1H, NCH_2S), 5.57 (d, J = 12.6 Hz, 1H, NCH_2S), 7.28–7.96 (m, 9H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.50 ($C_{4'}$), 29.14 ($C_{5'}$), 44.43 (NCH_2S), 50.34 ($C_{3'}$), 52.34 ($NHCH_2$), 123.66 (C_4, C_7), 126.72 (ph. C_4), 126.91 (ph. C_2, C_6), 128.56 (ph. C_3, C_5), 131.54 (C_{3a}, C_{7a}), 133.23 (C_5, C_6), 137.93 (ph. C_1), 167.44, 168.23, 169.66 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.62 (C═S); EIMS m/z = 453 (M^+). Anal. Calcd for $C_{22}H_{19}N_3O_4S_2$: C, 58.26; H, 4.22; N, 9.27; S, 14.14. Found: C, 58.10; H, 4.30; N, 9.14; S, 14.12.

4.2.2.15. [3-(1,3-Dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-2,6-dioxopiperidin-1-yl]methyl phenethyldithiocarbamate (18). White powder; IR (KBr) 3267, 3004, 2942, 2876, 2697, 1786, 1769, 1716, 1679, 1554, 1390, 1151 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.09–2.14 (m, 1H, H-4′), 2.55–2.65 (m, 1H, H-5′), 2.80–2.90 (m, 1H, H-4′), 2.93–3.10 (m, 1H, H-5′CO), 3.72–3.79 (m, 2H, –CH_2-phenyl), 3.88–3.93 (t, 2H, –NH–CH_2–), 4.90–4.92 (m, 1H, NH), 5.15–5.36 (m, 1H, H-3′), 5.33 (d, J = 12.6 Hz, 1H, NCH_2S), 5.55 (d, J = 12.6 Hz, 1H, NCH_2S), 7.20–7.96 (m, 9H, H_{arom}); ^{13}C NMR (DMSO-d_6) δ 21.44 ($C_{4'}$), 29.11 ($C_{5'}$), 34.43 (CH_2-phenyl), 44.14 (NCH_2S), 44.52 ($NHCH_2$), 50.31 ($C_{3'}$), 123.65 (C_4, C_7), 125.92 (ph. C_4), 127.70 (ph. C_2, C_6), 128.64 (ph. C_3, C_5), 131.52 (C_{3a}, C_{7a}), 133.20 (C_5, C_6), 139.40 (ph. C_1), 167.40, 168.21, 169.59 (C_1, C_3, $C_{2'}$, $C_{6'}$), 200.14 (C═S); EIMS m/z = 467 (M^+). Anal. Calcd for $C_{23}H_{21}N_3O_4S_2$: C, 59.08; H, 4.53; N, 8.99; S, 13.72. Found: C, 58.94; H, 4.44; N, 9.14; S, 13.73.

4.3. Cytotoxic activity

4.3.1. Tumor cells

EAC cells were supplied by National Cancer institute, Cairo, Egypt. The cells were maintained in vivo in Swiss albino female mice by intraperitoneal transplantation (0.2 mL of 2×10^6 cells/mL). EAC cells were aspirated from the peritoneal cavity of mice, washed with saline and given intraperitoneally to develop ascites tumor.

4.3.2. Preparation of suspensions and solutions and methodology of the in vitro cancer screen

For the cytotoxicity assay against the EAC cell line, thalidomide and its sulfur analogs were dissolved in DMSO/H_2O (0.7:0.3) to obtain the concentration of 10 mmol. These stock solutions were stored at 4 °C until further use.

Cytotoxic activity of these compounds was assayed by determining the percentage viability of EAC cells using the Trypan blue dye exclusion technique.[59] Briefly, EAC cells were aspirated aseptically from the peritoneal cavity of the mice and washed with Hank's balanced salt solution (HBSS) and centrifuged for 15 min at 1500 rpm in a cooling centrifuge. The pellet was re-suspended with HBSS and the process was repeated three times. Finally, the

cells were suspended in known volume of RPMI1640, supplemented with 10% fatal bovine serum, 10 μg/mL streptomycin and 100 U/mL penicillin, and the cell count was adjusted to 2×10^6 cells/mL. Then, 0.2 mL of this diluted cell suspension was titrated in 96 flat-bottomed tissue culture plates. Thirty microliters of each tested compound was added in triplicate and incubated at 37 °C for 24 h in 5% CO_2 atmosphere. The Trypan blue dye exclusion test was performed to determine the percentage viability and cytotoxicity were calculated against control EAC cells.[49]

4.4. Antitumor activity

The antitumor activity of thalidomide and its sulfur analogs was evaluated in vivo on mice-bearing solid tumor. Solid tumor was induced in Swiss albino female mice by implementation of 0.2 mL of EAC cells (2×10^6 cell/mL) subcutaneously (S.C) between thighs of the lower limb.[60] Animals were divided into 8 groups ($n = 8$). Seven days post-tumor induction; all groups were treated with 1.25 mM/kg of thalidomide derivatives by subcutaneous daily for 5 constitutive days except positive control group which treated with DMSO/H_2O. Mice were sacrificed, dissected and change in TV was assessed on day 12 after implantation of EAC cells. The following formula was used to calculate the volume of the developed tumor mass[61]:

$$\text{Tumor volume } (\text{mm}^3) = 0.52 \times \text{length} \times (\text{width})^2.$$

4.5. Estimation of catalase, SOD and lipid peroxidation levels in liver homogenate

The livers were excised, rinsed in ice-cold normal saline followed by cold 0.15 mol/L Tris–HCl buffer (pH 7.4), blotted dry, and weighed. A 10% w/v homogenate was prepared in 0.15 mol/L Tris–HCl buffer and a portion utilized for the estimation of lipid peroxidation according to the method of the remaining homogenate was centrifuged at 1500 rpm for 15 min at 4 °C.[62] The supernatant obtained was used for the estimation of SOD,[63] catalase.[64]

4.6. Histopathologic examination

Both tumor specimen and liver specimen for each mouse were preserved in 10% formalin solution and dehydrated in a graded alcohol series. After xylene treatment, the specimens were embedded in paraffin blocks. Five-micron thick sections were cut and stained with hematoxylin and eosin (H & E). H & E stained sections were examined and investigated for the following purposes:

(1) Liver specimens were used to grade the severity of degeneration and necrosis as: 0, no hepatocyte necrosis or degeneration; grade 1, focal necrosis or degeneration (mild); grade 2, multifocal necrosis or degeneration of hepatocytes (moderate); grade 3, locally extensive or diffuse necrosis or degeneration of hepatocytes (sever).[65]

(2) H&E stained slides of tumor specimens were used to assess. (a) Grade the severity as well as percentage of necrosis according to the previous reported[65] and calculate H score according to the previous reported[66]. (b) Mitosis counting: mitotic tumor cells were counted in 10 randomly selected microscopic fields (corresponding to a total of at least 1000 tumor cells) under high magnification 400×[67]. (c) Apoptotic tumor cell counting: they were counted in 10 randomly selected microscopic fields under the same magnification.[68–70] The MI and AI were expressed as percentage by dividing the total number of mitotic cells as well as apoptotic bodies by total number of intact tumor cells.[70,71]

4.7. Immunohistochemistry (IHC) examination specimens

IHC staining was performed on formalin fixed, paraffin embedded material that were sectioned at 5 μm thickness and placed onto positive charged slides. Fas-L, VEGF and Ki67 IHC staining were performed using the Universal Dako cytomation Labelled stretavidin–Biotin®2 system, Horseradish Peroxidase (LSAB®2 System, HRP Kit, Cat. No. k0679). All slides were de-paraffinized using xylene and then dehydrated in decreasing concentrations of ethanol. Antigen retrieval using microwave heating (20 min; 10 mmol/citrate buffer, pH 6.0) after inhibition of endogenous peroxidase activity (0.3 hydrogen peroxidase for 15 min) were used. The primary antibodies were applied to the slides. The slides were incubated overnight with the primary antibody at room temperature, and washed by using phosphate buffered solution (PBS) then incubated with secondary antibody for 15 min followed by PBS wash. Finally the detection of bound antibody was accomplished using a modified labeled avidin–biotin (LAB) reagent for 20 min then PBS wash. A 0.1% solution of diaminobenzidine (DAB) was used for 5 min as a chromogen. Slides were counterstained with Mayer's hematoxylin for 5–10 min Fas-L, VEGF and Ki67 immunoreactivities were evaluated using a light microscope by three methods for each marker (intensity, % and H score).[66,72–75] Unintentional bias was prevented by coding tissue samples so that, IHC study was done without knowledge of the used component characteristics.

4.8. Statistical analysis

Data are presented as means ± SD. The differences between experimental groups were compared by ANOVA followed by *t*-Student test of significance ($p < 0.05$).

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