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Original article

Synthesis and biological evaluation of some hydrazone derivatives as new anticandidal and anticancer agents

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ABSTRACT

New hydrazone derivatives were synthesized via the nucleophilic addition–elimination reaction of 2-[(1-methyl-1*H*-tetrazol-5-yl)thio)]acetohydrazide with aromatic aldehydes/ketones. The compounds were tested *in vitro* against various *Candida* species and compared with ketoconazole. Genotoxicity of the most effective anticandidal compounds was evaluated by umuC and Ames assays. All compounds were also investigated for their cytotoxic effects on NIH3T3 and A549 cell lines. Compound **8** was the most effective antifungal derivative against *C. albicans* (ATCC-90028) with a MIC value of 0.05 mg/mL. Compound **5** can be identified as the most promising anticancer agent against A549 cancer cell lines due to its inhibitory effect on A549 cell lines and low toxicity to NIH3T3 cells.

1. Introduction

The last two decades have witnessed the greatly increased incidence of invasive fungal infections, particularly those caused by *Candida* species throughout the world. Although new antifungal agents have become available to treat these infections, the treatment of invasive candidiasis remains a major challenge due to the adverse effects and resistance accompanying the widespread use of these drugs [1–4].

Triazole antifungal agents, play a leading role in the treatment of systemic fungal infections owing to their broad spectrum and improved safety profile. Fluconazole, itraconazole, voriconazole, and posaconazole are widely used antifungal drugs bearing triazole ring for the treatment of systemic fungal infections [5,6]. In a study to find potent antifungal agents, the synthesis and antifungal evaluation of tetrazole-based triazole derivatives were described and the study demonstrated that some compounds exhibited good antifungal activity against the different fungal cultures such as *Candida* species, *C. neoformans* and *Aspergillus* species [7].

Tetrazoles have received considerable attention due to their broad spectrum and metabolic profile. Medicinal chemists have studied tetrazoles extensively due to the fact that new effective compounds can be obtained by the bioisosteric replacement of the carboxylic acid group with tetrazole ring. A considerable amount of research has confirmed that tetrazoles are also metabolically more stable than carboxylic acid group. Tetrazole derivatives have been reported to exhibit a wide spectrum of biological effects including antifungal and anticancer effects [7–19].

Among tetrazole derivatives, 1-substituted-1*H*-tetrazol-5-thiol derivatives have gained great importance in the synthesis of pharmacologically active drugs. Some synthetic β-lactam antibiotics possess 5-thio-1-methyl-1*H*-tetrazole moiety as the side chain [10,14].

Medicinal chemists have also carried out considerable research for novel antimicrobial and anticancer agents bearing hydrazone moiety. Some studies have confirmed that hydrazone derivatives exhibit antifungal and anticancer activities [20–25].

Some researchers have reported anticancer effects of some antifungal agents and carried out considerable research for deciphering the underlying mechanisms of antitumor activity [26–28].

In antifungal and anticancer drug design, the lack of selectivity of conventional chemotherapeutic agents and the acquisition of

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multiple-drug resistance are two major challenging problems. As a consequence of this situation, the search for new effective chemotherapeutic agents has attracted a great deal of interest [29–31].

On the basis of these findings, we became interested in biological evaluation of hydrazone derivatives as anticandidal and anticancer agents. Herein, we described the synthesis of a novel series of hydrazone derivatives bearing 5-thio-1-methyl-1*H*-tetrazole moiety and focused on their potential anticandidal effects. Among these derivatives, the most effective anticandidal compounds were evaluated for their genotoxicity using umuC and Ames assays. Furthermore, all compounds were evaluated for their cytotoxic effects against A549 cancer cell lines and NIH3T3 cell lines.

2. Chemistry

The synthesis of hydrazone derivatives (**1**–**33**) was carried out according to the steps shown in Scheme 1. In the initial step, ethyl 2-[(1-methyl-1*H*-tetrazol-5-yl)thio]acetate (**A**) was synthesized via the reaction of 5-mercapto-1-methyl-1*H*-tetrazole with ethyl chloroacetate in the presence of potassium carbonate. The treatment of the ester (**A**) with hydrazine hydrate afforded the corresponding hydrazide (**B**) [32]. The nucleophilic addition–elimination reaction of the hydrazide (**B**) with aromatic aldehydes or ketones gave the target compounds (**1**–**33**). Some properties of the compounds were given in Table 1.

3. Results and discussion

The structures of all compounds (**1**–**33**) were confirmed by ^{1}H NMR, ^{13}C NMR, mass spectral data and elemental analysis.

In the ^{1}H NMR spectra of the compounds (**1**–**33**), the signal due to the hydrazone proton appeared in the region 10.5–12.5 ppm. The signal due to the $-S-CH_2-$ protons was observed in the region 4.0–5.0 ppm. In the ^{1}H NMR spectra of the compounds, N–H and $-S-CH_2-$ protons gave rise to two singlet peaks in accordance with the presence of the *E* and *Z* isomers [33,34]. The signal due to the methyl protons attached to tetrazole ring was observed in the region 3.9–4.0 ppm as a singlet peak. Other aromatic and aliphatic protons were observed at expected regions.

In their ^{13}C NMR spectra, the signal due to the $-S-CH_2-$ carbon appeared at 35–40 ppm. The signal due to the hydrazone carbon was observed at 165–170 ppm. The signal due to the methyl carbon attached to tetrazole ring was observed in the region 34.0–35.0 ppm. Other aromatic and aliphatic carbons were observed at expected regions.

In the mass spectra of all compounds (**1**–**33**), the M + 1 peak was observed in agreement with their molecular formula. All compounds gave satisfactory elemental analysis.

The compounds were tested *in vitro* against three *C. albicans* strains (ATCC-10231, ATCC-90028 and a clinical isolate), *C. utilis*, *C. krusei*, *C. parapsilosis* and two clinically isolated *C. glabrata* strains. Among these compounds, compounds **8**, **10**, **11**, **12**, and **14** exhibited more significant activity than other derivatives as described in Table 2.

Among *Candida* species, *C. albicans* (ATCC-90028) was the most susceptible yeast to compound **8**. Compound **8** exhibited the inhibitory activity against *C. albicans* (ATCC-90028) with a MIC value of 0.05 mg/mL, whereas ketoconazole exhibited the inhibitory activity with a MIC value of 0.031 mg/mL. Cytotoxicity assay indicated that the effective dose of the compound against *C. albicans* (ATCC-90028) was lower than its cytotoxic dose.

Compound **8** was also found to be the most potent derivative against *C. albicans* (ATCC-10231) and *C. krusei*. It is apparent that there is a positive correlation between anticandidal activity and *p*-dimethylaminophenyl moiety.

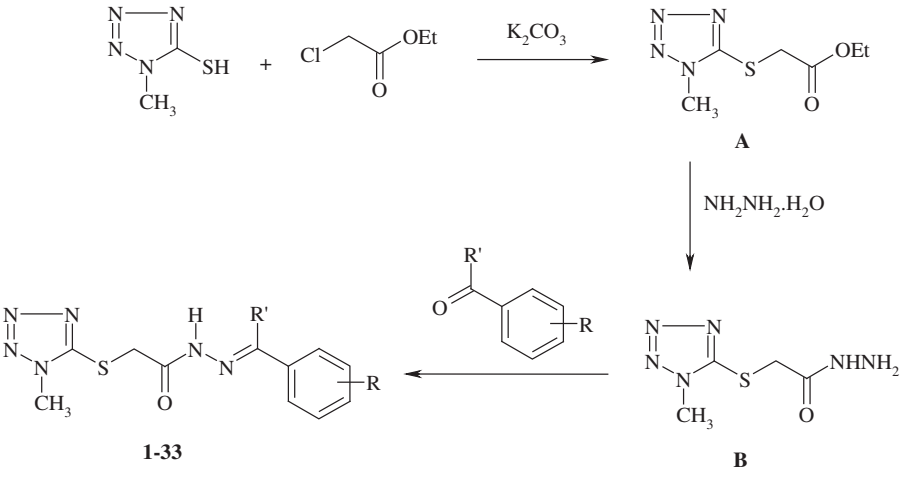
Compounds **8** and **14** exhibited the highest inhibitory activity against *C. albicans* (Clinical Isolate) and *C. utilis* with a MIC value of 0.125 mg/mL. Compound **8** was the most effective derivative against *C. glabrata* (Clinical Isolate, Anadolu University), whilst compound **14** was the most effective derivative against *C. glabrata* (Clinical Isolate, Osmangazi University).

Compound **11** bearing *p*-methoxyphenyl moiety showed the highest antifungal activity against *C. parapsilosis*.

Genotoxicity of the most effective anticandidal compounds was evaluated by umuC and Ames assays.

In umuC test procedure, the whole test is considered valid if the positive controls reach an induction ratio IR of $\geq$2. The average OD600 of the Negative Controls of the second plate should increase by a factor of $\geq$2 during 2 h incubation (growth control). Our experiments provided these validity criteria. By umuC results, a sample dilution is considered genotoxic if the Induction ratio IR $\geq$ 1.5 and the Growth factor $G \geq 0.5$ and also to call a test compound genotoxic in the umuC Easy CS test it is recommended that a dose response should be observed. Our results were presented in Table 3.

Compounds **8**, **10**, **11** and **14** did not show an Induction ratio IR $\geq$ 1.5, while the Growth factors were $G \geq 0.5$. Only compound **12** showed an Induction ratio IR $\geq$ 1.5 and Growth factors were $G \geq 0.5$. In this case, compound **12** showed a genotoxic potential by these results. Furthermore, a partially dose-response was observed (Fig. 1).



Scheme 1. The synthesis of the compounds (**1**–**33**).

Table 1
Some properties of the compounds (**1**–**33**).

Compound	R	R′	Yield (%)	M.p. (°C)	Molecular formula	Molecular weight
1	H	H	82	166–167	$C_{11}H_{12}N_6OS$	276
2	p–NO_2	H	90	210–212	$C_{11}H_{11}N_7O_3S$	321
3	p–CH_3	H	79	175	$C_{12}H_{14}N_6OS$	290
4	p–F	H	83	181	$C_{11}H_{11}FN_6OS$	294
5	p–Cl	H	87	178–180	$C_{11}H_{11}ClN_6OS$	310.5
6	p–Br	H	89	204–205	$C_{11}H_{11}BrN_6OS$	355
7	p–$CH(CH_3)_2$	H	77	150–151	$C_{14}H_{18}N_6OS$	318
8	p–$N(CH_3)_2$	H	84	199–200	$C_{13}H_{17}N_7OS$	319
9	p–CF_3	H	81	141–142	$C_{12}H_{11}F_3N_6OS$	344
10	p–OH	H	76	236–240	$C_{11}H_{12}N_6O_2S$	292
11	p–OCH_3	H	78	169–171	$C_{12}H_{14}N_6O_2S$	306
12	o–NO_2	H	87	162–164	$C_{11}H_{11}N_7O_3S$	321
13	m–NO_2	H	88	189–190	$C_{11}H_{11}N_7O_3S$	321
14	o–F	H	79	158–159	$C_{11}H_{11}FN_6OS$	294
15	m–F	H	81	178–182	$C_{11}H_{11}FN_6OS$	294
16	o–Cl	H	84	162	$C_{11}H_{11}ClN_6OS$	310.5
17	m–Cl	H	86	160–162	$C_{11}H_{11}ClN_6OS$	310.5
18	o–Br	H	85	165–167	$C_{11}H_{11}BrN_6OS$	355
19	m–Br	H	87	191–193	$C_{11}H_{11}BrN_6OS$	355
20	o–OH	H	73	211–212	$C_{11}H_{12}N_6O_2S$	292
21	m–OH	H	75	229–232	$C_{11}H_{12}N_6O_2S$	292
22	o–OCH_3	H	74	192–194	$C_{12}H_{14}N_6O_2S$	306
23	m–OCH_3	H	76	148–150	$C_{12}H_{14}N_6O_2S$	306
24	o–CH_3	H	77	185–188	$C_{12}H_{14}N_6OS$	290
25	m–CH_3	H	78	152–154	$C_{12}H_{14}N_6OS$	290
26	2,3-Di(Cl)	H	81	185–186	$C_{11}H_{10}Cl_2N_6OS$	345
27	2,4-Di(Cl)	H	84	163	$C_{11}H_{10}Cl_2N_6OS$	345
28	2,6-Di(Cl)	H	80	158	$C_{11}H_{10}Cl_2N_6OS$	345
29	o–Cl	CH_3	83	110–111	$C_{12}H_{13}ClN_6OS$	324.5
30	m–Cl	CH_3	84	165–166	$C_{12}H_{13}ClN_6OS$	324.5
31	p–Cl	CH_3	85	201–203	$C_{12}H_{13}ClN_6OS$	324.5
32	2,4-Di(Cl)	CH_3	80	156–157	$C_{12}H_{12}Cl_2N_6OS$	359
33	2,5-Di(Cl)	CH_3	83	134–136	$C_{12}H_{12}Cl_2N_6OS$	359

In Ames MPF assay, more than 25 positive wells were observed with 2-aminoanthracene in the presence of S9 mix with TA98 and TA100, while 4-nitro-o-phenylenediamine showed more than 25 positive wells in the absence of S9 with TA98 and TA100. This complied with the requirements in the Ames MPF assay manual. Negative controls showed less than five positive wells in the presence and the absence of S9 with TA98 and TA100. Our results were presented in Table 4.

Compound **8** did not show more than 5 positive wells in the presence of S9 mix with TA100 and TA 98. It showed more than 5 positive wells without S9 mix with TA100. But, fold induction over the negative control was less than 3, while fold induction over baseline was less than 2. Furthermore, the significant different results obtained did not show a dose-response. So, the results of the Ames test indicate the nonmutagenic potential of compound **8**.

Compounds **10** and **11** showed more than 5 positive wells in the presence of S9 mix with TA100. In the other experiments performed, the positive wells were not more than 5. Fold induction over the negative control was also less than 3, while fold induction over baseline was less than 2 and there were no significant different results obtained. So, the results of the Ames test indicate the nonmutagenic potential of compounds **10** and **11**.

Compound **12** showed more than 5 positive wells with/without S9 mix, with TA100 and TA98. Fold induction over baseline was more than 2 in the experiments with S9 mix TA98/100 and also without S9 mix TA98. Significant different results were obtained and the compound showed a dose-response. According to Ames MPF test, the compound can be classified as a weak mutagen (Fig. 2).

Compound **14** showed more than 5 positive wells only in the presence of S9 mix with TA98/100. But, fold induction over the negative control was less than 3, while fold induction over baseline was less than 2. Furthermore, the significant different results obtained did not show a dose-response. So, the results of the Ames test indicate the non-mutagenic potential of compound **14**.

The genotoxic potential of compound **12** which was shown by the results of AMES MPF was supported by umuC Easy assay, while the other compounds can be accepted as non-genotoxic agents according to these assays.

The IC_{50} values for all compounds were presented in Table 5 for NIH3T3 cells. According to our results, compounds **8**, **18**, **19** and **31** were mildly toxic on NIH3T3 cells. Mitochondrial activity was not affected by other compounds.

The IC_{50} values for all compounds were presented in Table 6 for A549 cells. According to our results, compounds **5**, **7**, **8** and **27** were mildly toxic on A549 cells. Mitochondrial activity was not affected by other compounds.

Small differences in IC_{50} like a factor of 2 or even 4 are probably meaningless, but larger differences could be due to some specific cytotoxic mechanisms. In this case, compounds **2**, **5** and **28** which are more active in A549 cell line and have less activity on NIH3T3 cells may go for further investigation. Since, significant differences between the IC_{50} values of different cell lines could be due to some specific cytotoxicity mechanisms.

Table 2
Anticandidal activities of the compounds (**1**–**33**) as MIC values (mg/mL).

Microorganism	8	10	11	12	14	Ketoconazole
A	0.125	0.5	0.5	0.5	0.125	0.003
B	0.125	0.5	0.5	0.5	0.25	0.015
C	0.5	1.0	0.5	0.5	0.25	0.015
D	0.125	0.5	0.25	0.25	0.125	0.015
E	0.0625	0.5	0.125	0.125	0.5	0.003
F	0.5	1.0	0.25	0.5	1.0	0.003
G	0.05	1.0	0.5	0.0625	0.5	0.031
H	0.05	0.5	0.125	0.25	0.25	0.001

MIC values of other derivatives were >8 mg/mL.
A: *C. albicans* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskişehir, Turkey), **B**: *C. albicans* (ATCC 10231), **C**: *C. glabrata* (Clinical Isolate, Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskişehir, Turkey), **D**: *C. utilis* (NRRL Y-900), **E**: *C. krusei* (NRRL Y-7179), **F**: *C. parapsilosis* (NRRL Y-12696), **G**: *C. albicans* (ATCC 90028), **H**: *C. glabrata* (Clinical Isolate, Anadolu University, Faculty of Science, Department of Biology, Eskişehir, Turkey).

Table 3
Results of compounds **8**, **10**, **11**, **12**, and **14** in the umuC Easy CS assay.

Compound	Concentration (mg/mL)	Growth factor		Induction ratio	
		S9+	S9−	S9+	S9−
8	5	2.820	2.781	0.379	0.565
	2.5	1.992	1.743	0.598	0.880
	1.25	1.538	1.191	0.670	1.134
	0.63	1.268	0.962	0.704	1.360
10	5	2.139	2.049	0.419	0.661
	2.5	1.663	1.397	0.657	0.979
	1.25	1.277	1.098	0.699	0.990
	0.63	1.130	1.009	0.797	1.181
11	5	2.261	1.990	0.568	0.763
	2.5	1.830	1.166	0.681	1.045
	1.25	1.294	1.137	0.769	1.119
	0.63	1.117	1.028	0.814	1.357
12	5	0.913	1.281	1.194	1.429
	2.5	0.922	1.030	1.014	1.630[a]
	1.25	0.879	1.022	1.182	1.515[a]
	0.63	0.969	1.068	1.229	1.509[a]
14	5	2.579	3.877	0.284	0.321
	2.5	1.699	1.559	0.352	0.779
	1.25	1.163	1.084	0.548	1.022
	0.63	0.935	1.045	0.936	1.312

[a] A sample dilution is considered genotoxic if the induction ratio IR $\geq$ 1.5 and the growth factor $G \geq 0.5$.

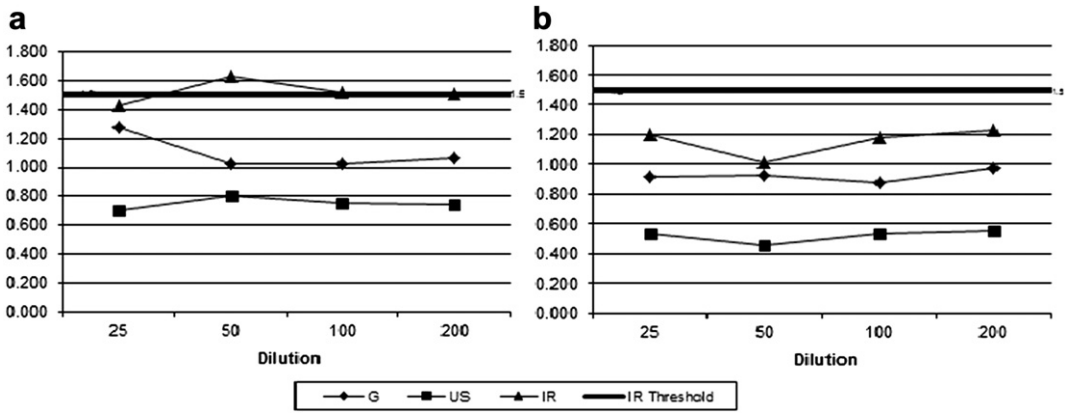


Fig. 1. Dose response curve of compound **12** with TA1535/pSK1002 in the presence of S9 mix and in the absence of S9 mix.

4. Conclusion

In the present paper, we synthesized a new series of hydrazone derivatives and investigated their anticandidal activity. umuC and Ames assays were carried out to determine genotoxicity of the most effective anticandidal compounds. All compounds were also investigated for their cytotoxic effects on NIH3T3 and A549 cell lines.

In particular, compound **8** was the most promising antifungal derivative against *C. albicans* (ATCC-90028) with a MIC value of 0.05 mg/mL. In addition, the cytotoxic dose (IC_{50} = 0.316 mM) of compound **8** was higher than its effective dose.

Among these compounds, compound **5** can be considered as the most promising anticancer agent for further investigation owing to its inhibitory effect on A549 cancer cell lines and low toxicity to NIH3T3 cells.

Table 4
Results of compounds **8**, **10**, **11**, **12**, and **14** in the Ames MPF™ 98/100 assay.

Compound	Concentration (mg/mL)	Revertants fold increase (over baseline)			
		TA 98		TA100	
		S9+	S9−	S9+	S9−
8	0.156	0.86	0.32	0.33	1.90*
	0.3125	0.57	1.27	0.47	1.19
	0.625	1.00	1.59*	0.18	1.38*
	1.25	0.86	0.64	0.26	1.00
	2.5	1.14*	0.00	0.15	1.14*
	5	0.86	0.00	0.26	0.81
10	0.156	0.32	0.52	0.63	0.66
	0.3125	0.53	0.13	0.63	0.41
	0.625	0.32	0.13	0.70	0.66
	1.25	0.63	0.13	0.59	0.91
	2.5	0.53	0.13	0.63	0.33
	5	0.63	0.26	0.82	0.00
11	0.156	0.42	0.16	0.46	0.70
	0.3125	0.84	0.16	0.77	0.40
	0.625	0.32	0.65	0.89	0.30
	1.25	0.53	0.33	0.81	0.20
	2.5	0.53	0.33	0.62	0.60
	5	0.84	0,33	0.50	0.50
12	0.156	1.00	1.63*	1.35*	1.67*
	0.3125	1.22*	1.63*	1.08	1.67*
	0.625	1.78*	1.47*	1.28*	0.83
	1.25	2.00*	2.78**	1.93*	1.33
	2.5	2.22**	1.96*	2.20**	0.67
	5	1.11	1.14	1.70*	1.33
14	0.156	0.29	0.64	0.66	0.23
	0.3125	0.71	1.59*	0.51	0.06
	0.625	1.00	0.64	0.66	0.23
	1.25	1.28*	0.32	0.88	0.29
	2.5	1.57*	0.95	0.95	0.17
	5	1.14	1.59*	0.58	0.17

*t test p value (unpaired 1-sided) < 0,05.
**Fold increase over baseline ≥ 2.

5. Experimental

5.1. Chemistry

All reagents were purchased from commercial suppliers and were used without further purification. Melting points were determined on an Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK) and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 500 MHz and 125 MHz spectrometer (Bruker, Billerica, USA), respectively. Mass spectra were recorded on a VG Quattro Mass spectrometer (Agilent, Minnesota, USA). Elemental analyses were performed on a Perkin Elmer EAL 240 elemental analyser (Perkin–Elmer, Norwalk, USA).

5.1.1. General procedure for the synthesis of the compounds

*5.1.1.1. Ethyl 2-[(1-methyl-1H-tetrazol-5-yl)thio]acetate (**A**).* A mixture of 5-mercapto-1-methyl-1*H*-tetrazole (0.05 mol) and ethyl chloroacetate (0.05 mol) in the presence of potassium carbonate (0.05 mol) in acetone was refluxed for 10 h. The reaction mixture was cooled, filtered and the crude product was solved in water and then extracted with ether [32].

*5.1.1.2. 2-[(1-Methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**B**).* A mixture of the ester (**A**) (0.05 mol) and hydrazine hydrate (0.1 mol) in ethanol was stirred at room temperature for 3 h and then filtered [32].

*5.1.1.3. 2-[(1-Methyl-1H-tetrazol-5-yl)thio]-N′-[(aryl)methylidene/ethylidene]acetohydrazide derivatives (**1**–**33**).* A mixture of the hydrazide (**B**) (0.01 mol) and aldehydes/ketones (0.01 mol) was refluxed in ethanol for 5 h, filtered and crystallized from ethanol.

*5.1.1.3.1. N′-Benzylidene-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**1**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.19 and 4.62 (2H, 2s), 7.45–7.70 (5H, m), 8.04 and 8.20 (1H, 2s), 11.76 and 11.81 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.15 (CH_3), 36.10 (CH_2), 128.80 (2CH), 129.20 (2CH), 131.00 (CH), 133.70 (C), 144.68 (CH), 153.88 (C), 168.39 (C).

For $C_{11}H_{12}N_6OS$ calculated: C, 47.81; H, 4.38; N, 30.41; found: C, 47.80; H, 4.40; N, 30.39.

MS (FAB) $[M + 1]^+$: m/z 277.

*5.1.1.3.2. N′-(4-Nitrobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**2**).* ^{1}H NMR (500 MHz, DMSO-d_6): 4.00 (3H, s), 4.24 and 4.67 (2H, 2s), 7.98 (2H, d, J = 9 Hz), 8.14 (1H, s), 8.31 (2H, d, J = 8.5 Hz), 12.06 and 12.12 (1H, 2s).

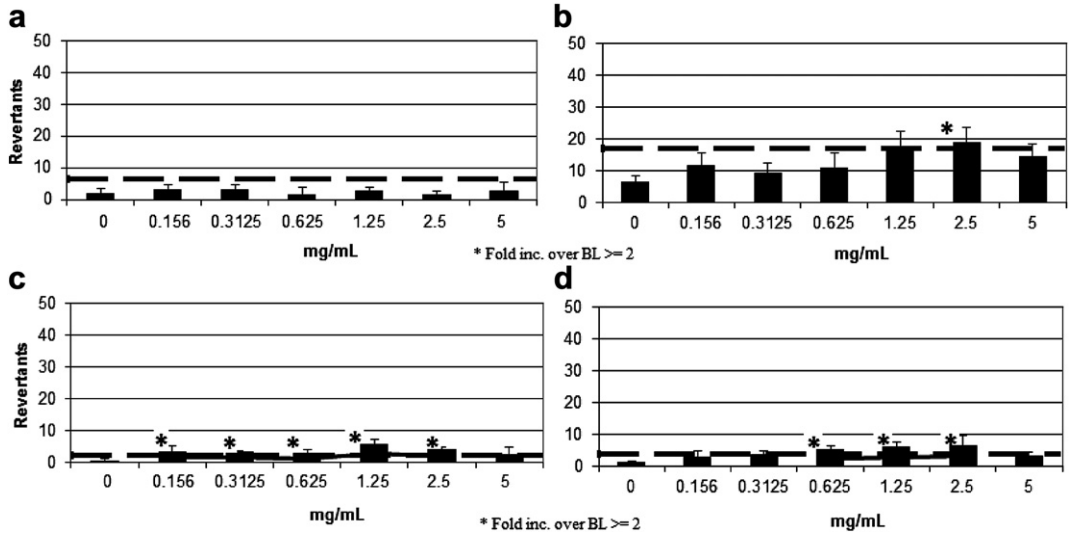


Fig. 2. Dose response curve of compound **12** with TA98 and TA100 in the presence of S9 mix and in the absence of S9 mix.

^{13}C NMR (125 MHz, DMSO-d_6): 34.15 (CH_3), 36.10 (CH_2), 124.54 (2CH), 128.39 (CH), 128.60 (CH), 140.61 (C), 142.28 (CH), 148.31 (C), 153.88 (C), 168.96 (C). For $C_{11}H_{11}N_7O_3S$ calculated: C, 41.12; H, 3.45; N, 30.51; found: C, 41.11; H, 3.44; N, 30.50.

MS (FAB) $[M + 1]^+$: *m*/*z* 322.

*5.1.1.3.3. N′-(4-Methylbenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl) thio]acetohydrazide (**3**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.35 (3H, s), 3.99 (3H, s), 4.18 and 4.61 (2H, 2s), 7.27 (2H, d, $J = 8$ Hz), 7.59 (2H, d, $J = 8$ Hz), 7.99 and 8.15 (1H, 2s), 11.70 and 11.75 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 21.51 (CH_3), 34.12 (CH_3), 36.25 (CH_2), 127.39 (CH), 127.63 (CH), 129.92 (2CH), 131.63 (C), 140.38 (C), 144.68 (CH), 153.88 (C), 168.39 (C). For $C_{12}H_{14}N_6OS$ calculated: C, 49.64; H, 4.86; N, 28.95; found: C, 49.63; H, 4.86; N, 28.96.

MS (FAB) $[M + 1]^+$: *m*/*z* 291.

*5.1.1.3.4. N′-(4-Fluorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl) thio]acetohydrazide (**4**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.19 and 4.62 (2H, 2s), 7.29–7.32 (2H, m), 7.76–7.79 (2H, m), 8.03 and 8.19 (1H, 2s), 11.78 and 11.82 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.12 (CH_3), 36.22 (CH_2), 116.48 (2CH), 129.58 (C), 131.09 (2CH), 143.44 (CH), 153.88 (C), 162.52 (C), 168.39 (C). For $C_{11}H_{11}FN_6OS$ calculated: C, 44.89; H, 3.77; N, 28.56; found: C, 44.90; H, 3.75; N, 28.57.

MS (FAB) $[M + 1]^+$: *m*/*z* 295.

*5.1.1.3.5. N′-(4-Chlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl) thio]acetohydrazide (**5**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.62 (2H, 2s), 7.53 (2H, d, $J = 8.5$ Hz), 7.74 (2H, d, $J = 8.5$ Hz), 8.03 and 8.19 (1H, 2s), 11.82 and 11.87 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.13 (CH_3), 36.21 (CH_2), 129.07 (2CH), 129.41 (2CH), 133.29 (C), 134.97 (C), 143.31 (CH), 153.93 (C), 168.61 (C). For $C_{11}H_{11}ClN_6OS$ calculated: C, 42.51; H, 3.57; N, 27.04; found: C, 42.49; H, 3.58; N, 27.04.

MS (FAB) $[M + 1]^+$: *m*/*z* 311.

*5.1.1.3.6. N′-(4-Bromobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl) thio]acetohydrazide (**6**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.19 and 4.62 (2H, 2s), 7.66 (4H, s), 8.01 and 8.17 (1H, 2s), 11.83 and 11.88 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.13 (CH_3), 36.20 (CH_2), 123.77 (C), 129.29 (2CH), 132.32 (2CH), 133.63 (C), 143.42 (CH), 153.94 (C), 168.61 (C). For $C_{11}H_{11}BrN_6OS$ calculated: C, 37.19; H, 3.12; N, 23.66; found: C, 37.21; H, 3.11; N, 23.65.

MS (FAB) $[M + 1]^+$: *m*/*z* 356.

*5.1.1.3.7. N′-(4-Isopropylbenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**7**).* ^{1}H NMR (500 MHz, DMSO-d_6): 1.22 (6H, d, $J = 7$ Hz), 2.91–2.94 (1H, m), 3.99 (3H, s), 4.19 and 4.61 (2H, 2s), 7.33 (2H, d, $J = 8$ Hz), 7.61–7.64 (2H, m), 8.01 and 8.15 (1H, 2s), 11.71 and 11.75 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 24.13 ($2CH_3$), 33.84 (CH), 34.12 (CH_3), 36.23 (CH_2), 127.29 (2CH), 127.74 (2CH), 129.90 (C), 143.42 (CH), 149.50 (C), 153.94 (C), 168.61 (C). For $C_{14}H_{18}N_6OS$ calculated: C, 52.81; H, 5.70; N, 26.39; found: C, 52.80; H, 5.69; N, 26.41.

MS (FAB) $[M + 1]^+$: *m*/*z* 319.

*5.1.1.3.8. N′-(4-(Dimethylamino)benzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**8**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.97 (6H, s), 3.99 (3H, s), 4.15 and 4.58 (2H, 2s), 6.75 (2H, d, $J = 8.5$ Hz), 7.49–7.52 (2H, m), 7.89 and 8.03 (1H, 2s), 11.48 and 11.50 (1H, 2s).

Table 5
Cytotoxic activity of the compounds against NIH3T3 cell line.

Compound	IC_{50} (mM)	Compound	IC_{50} (mM)	Compound	IC_{50} (mM)
1	9.7	**12**	2.02	**23**	0.6
2	9.3	**13**	2	**24**	6.32
3	3.16	**14**	2	**25**	6.25
4	10	**15**	6.32	**26**	0.52
5	2.1	**16**	>10	**27**	2.14
6	10	**17**	7.5	**28**	>10
7	1.2	**18**	0.02	**29**	6
8	0.316	**19**	0.316	**30**	3.4
9	>10	**20**	>10	**31**	0.1
10	0.632	**21**	3.16	**32**	1
11	>10	**22**	3.16	**33**	1

Table 6
Cytotoxic activity of the compounds against A549 cell line.

Compound	IC_{50} (mM)	Compound	IC_{50} (mM)	Compound	IC_{50} (mM)
1	3.16	**12**	1.6	**23**	>10
2	0.632	**13**	>10	**24**	>10
3	3.16	**14**	9	**25**	2
4	3.16	**15**	6.32	**26**	0.632
5	0.1	**16**	6.32	**27**	0.5
6	>10	**17**	7.5	**28**	1
7	0.4	**18**	2	**29**	6.32
8	0.1	**19**	0.6	**30**	>10
9	6.32	**20**	>10	**31**	6
10	>10	**21**	4.5	**32**	0.8
11	>10	**22**	7	**33**	1.5
Cis-platin	0.0316	–	–	–	–

^{13}C NMR (125 MHz, DMSO-d_6): 34.10 (CH_3), 36.32 (CH_2), 36.37 ($2CH_3$), 112.21 (2CH), 121.63 (C), 128.70 (2CH), 145.40 (CH), 151.95 (C), 154.08 (C), 167.86 (C). For $C_{13}H_{17}N_7OS$ calculated: C, 48.89; H, 5.36; N, 30.70; found: C, 48.90; H, 5.35; N, 30.68.

MS (FAB) $[M + 1]^+$: *m*/*z* 320.

*5.1.1.3.9. N′-(4-(Trifluoromethyl)benzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**9**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.22 and 4.65 (2H, 2s), 7.82 (2H, d, $J = 8.5$ Hz), 7.93 (2H, d, $J = 8$ Hz), 8.11 and 8.27 (1H, 2s), 11.96 (1H, s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.15 (CH_2), 124.10 (C), 126.19 (2CH), 128.02 (2CH), 133.30 (C), 137.00 (C), 142.94 (CH), 153.91 (C), 168.82 (C). For $C_{12}H_{11}F_3N_6OS$ calculated: C, 41.86; H, 3.22; N, 24.41; found: C, 41.85; H, 3.20; N, 24.43.

MS (FAB) $[M + 1]^+$: *m*/*z* 345.

*5.1.1.3.10. N′-(4-Hydroxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**10**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.16 and 4.58 (2H, 2s), 6.82 (2H, s), 7.52 (2H, s), 7.93 and 8.07 (1H, 2s), 9.96 (1H, s), 11.57 and 11.60 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.11 (CH_3), 36.32 (CH_2), 116.17 (2CH), 125.33 (C), 129.14 (2CH), 144.89 (CH), 154.02 (C), 159.85 (C), 168.12 (C). For $C_{11}H_{12}N_6O_2S$ calculated: C, 45.20; H, 4.14; N, 28.75; found: C, 45.19; H, 4.15; N, 28.76.

MS (FAB) $[M + 1]^+$: *m*/*z* 293.

*5.1.1.3.11. N′-(4-Methoxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**11**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.81 (3H, s), 3.99 (3H, s), 4.17 and 4.60 (2H, 2s), 7.02 (2H, d, $J = 8.5$ Hz), 7.65 (2H, d, $J = 9$ Hz), 7.98 and 8.13 (1H, 2s), 11.64 and 11.68 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.11 (CH_3), 36.29 (CH_2), 55.78 (CH_3), 114.81 (2CH), 126.91 (C), 129.01 (2CH), 144.46 (CH), 154.01 (C), 161.27 (C), 168.25 (C). For $C_{12}H_{14}N_6O_2S$ calculated: C, 47.05; H, 4.61; N, 27.43; found: C, 47.06; H, 4.59; N, 27.44.

MS (FAB) $[M + 1]^+$: *m*/*z* 307.

*5.1.1.3.12. N′-(2-Nitrobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**12**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.21 and 4.62 (2H, 2s), 7.67–8.11 (4H, m), 8.43 and 8.60 (1H, 2s), 12.03 and 12.16 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.11 (CH_2), 125.10 (CH), 128.73 (C), 131.16 (CH), 131.30 (CH), 134.14 (CH), 143.54 (CH), 148.54 (C), 154.01 (C), 168.85 (C). $C_{11}H_{11}N_7O_3S$ calculated: C, 41.12; H, 3.45; N, 30.51; found: C, 41.10; H, 3.45; N, 30.49.

MS (FAB) $[M + 1]^+$: *m*/*z* 322.

*5.1.1.3.13. N′-(3-Nitrobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**13**).* ^{1}H NMR (500 MHz, DMSO-d_6): 4.00 (3H, s), 4.23 and 4.67 (2H, 2s), 7.74–8.55 (5H, m), 11.99 and 12.07 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.13 (CH_2), 121.68 (CH), 124.77 (CH), 130.92 (CH), 133.46 (CH), 136.20 (C), 142.39 (CH), 148.73 (C), 153.92 (C), 168.83 (C). For $C_{11}H_{11}N_7O_3S$ calculated: C, 41.12; H, 3.45; N, 30.51; found: C, 41.12; H, 3.44; N, 30.52.

MS (FAB) $[M + 1]^+$: *m*/*z* 322.

*5.1.1.3.14. N′-(2-Fluorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**14**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.19 and 4.63 (2H, 2s), 7.25–7.94 (4H, m), 8.24 and 8.41 (1H, 2s), 11.88 and 11.96 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.13 (CH_3), 36.11 (CH_2), 116.44 (CH), 121.84 (C), 125.45 (CH), 132.47 (CH), 137.43 (CH), 142.39 (CH), 153.92 (C), 160.16 (C), 168.65 (C). For $C_{11}H_{11}FN_6OS$ calculated: C, 44.89; H, 3.77; N, 28.56; found: C, 44.88; H, 3.76; N, 28.56.

MS (FAB) $[M + 1]^+$: *m*/*z* 295.

*5.1.1.3.15. N′-(3-Fluorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**15**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.63 (2H, 2s), 7.27–7.57 (4H, m), 8.03 and 8.20 (1H, 2s), 11.86 and 11.93 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.11 (CH_2), 113.28 (CH), 117.36 (CH), 123.91 (CH), 131.36 (CH), 136.89 (C), 143.16 (CH), 153.93 (C), 163.55 (C), 168.74 (C). For $C_{11}H_{11}FN_6OS$ calculated: C, 44.89; H, 3.77; N, 28.56; found: C, 44.90; H, 3.76; N, 28.57.

MS (FAB) $[M + 1]^+$: *m*/*z* 295.

*5.1.1.3.16. N′-(2-Chlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**16**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.64 (2H, 2s), 7.43–8.00 (4H, m), 8.43 and 8.59 (1H, 2s), 11.93 and 12.05 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.17 (CH_2), 127.36 (CH), 128.13 (CH), 130.42 (CH), 131.60 (CH), 131.99 (C), 133.51 (C), 140.61 (CH), 153.93 (C), 168.72 (C). For $C_{11}H_{11}ClN_6OS$ calculated: C, 42.51; H, 3.57; N, 27.04; found: C, 42.50; H, 3.59; N, 27.03.

MS (FAB) $[M + 1]^+$: *m*/*z* 311.

*5.1.1.3.17. N′-(3-Chlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**17**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.63 (2H, 2s), 7.46–7.79 (4H, m), 8.02 and 8.17 (1H, 2s), 11.86 and 11.96 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.13 (CH_3), 36.12 (CH_2), 126.24 (CH), 126.62 (CH), 130.19 (CH), 131.22 (CH), 134.15 (C), 136.57 (C), 142.98 (CH), 153.93 (C), 168.72 (C). For $C_{11}H_{11}ClN_6OS$ calculated: C, 42.51; H, 3.57; N, 27.04; found: C, 42.51; H, 3.58; N, 27.03.

MS (FAB) $[M + 1]^+$: *m*/*z* 311.

*5.1.1.3.18. N′-(2-Bromobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**18**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.64 (2H, 2s), 7.39–7.97 (4H, m), 8.39 and 8.54 (1H, 2s), 11.96 and 12.08 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.19 (CH_2), 123.84 (C), 127.74 (CH), 128.62 (CH), 132.23 (CH), 133.09 (CH), 133.65 (C), 142.96 (CH), 153.90 (C), 168.71 (C). For $C_{11}H_{11}BrN_6OS$ calculated: C, 37.19; H, 3.12; N, 23.66; found: C, 37.20; H, 3.11; N, 23.65.

MS (FAB) $[M + 1]^+$: *m*/*z* 356.

*5.1.1.3.19. N′-(3-Bromobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**19**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.64 (2H, 2s), 7.41–7.93 (4H, m), 8.01 and 8.17 (1H, 2s), 11.86 and 11.93 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.15 (CH_2), 122.69 (C), 126.59 (CH), 129.54 (CH), 131.46 (CH), 133.07 (CH), 136.81 (C), 142.89 (CH), 153.93 (C), 168.72 (C). For $C_{11}H_{11}BrN_6OS$ calculated: C, 37.19; H, 3.12; N, 23.66; found: C, 37.18; H, 3.10; N, 23.67.

MS (FAB) $[M + 1]^+$: *m*/*z* 356.

*5.1.1.3.20. N′-(2-Hydroxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**20**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.20 and 4.61 (2H, 2s), 6.86–7.71 (4H, m), 8.34 and 8.42 (1H, 2s), 10.08 and 10.91 (1H, 2s), 11.69 and 12.00 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.11 (CH_3), 36.36 (CH_2), 116.82 (CH), 119.09 (C), 120.45 (CH), 126.67 (CH), 132.05 (CH), 141.94 (CH), 153.99 (C), 156.92 (C), 168.19 (C). For $C_{11}H_{12}N_6O_2S$ calculated: C, 45.20; H, 4.14; N, 28.75; found: C, 45.21; H, 4.13; N, 28.74.

MS (FAB) $[M + 1]^+$: *m*/*z* 293.

*5.1.1.3.21. N′-(3-Hydroxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**21**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.18 and 4.62 (2H, 2s), 6.83–7.27 (4H, m), 7.95 and 8.10 (1H, 2s), 9.64 and 9.67 (1H, 2s), 11.71 and 11.75 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.11 (CH_3), 36.36 (CH_2), 113.03 (CH), 117.91 (CH), 119.05 (CH), 130.38 (CH), 135.55 (C), 144.84 (CH), 154.00 (C), 158.12 (C), 168.41 (C). For $C_{11}H_{12}N_6O_2S$ calculated: C, 45.20; H, 4.14; N, 28.75; found: C, 45.20; H, 4.12; N, 28.77.

MS (FAB) $[M+1]^+$: *m*/*z* 293.

*5.1.1.3.22. N′-(2-Methoxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**22**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.86 (3H, s), 3.99 (3H, s), 4.16 and 4.61 (2H, 2s), 7.00–7.83 (4H, m), 8.37 and 8.53 (1H, 2s), 11.72 and 11.80 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.12 (CH_3), 36.31 (CH_2), 56.18 (CH_3), 112.33 (CH), 121.21 (C), 125.90 (CH), 132.07 (2CH), 140.23 (CH), 153.98 (C), 158.15 (C), 168.40 (C). For $C_{12}H_{14}N_6O_2S$ calculated: C, 47.05; H, 4.61; N, 27.43; found: C, 47.05; H, 4.60; N, 27.45.

MS (FAB) $[M + 1]^+$: *m/z* 307.

*5.1.1.3.23. N′-(3-Methoxybenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**23**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.81 (3H, s), 3.99 (3H, s), 4.20 and 4.62 (2H, 2s), 7.01–7.39 (4H, m), 8.00 and 8.16 (1H, 2s), 11.78 and 11.82 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.12 (CH_3), 36.11 (CH_2), 55.66 (CH_3), 112.19 (CH), 116.40 (CH), 120.01 (CH), 130.44 (CH), 135.75 (C), 144.38 (CH), 153.99 (C), 160.01 (C), 168.58 (C). For $C_{12}H_{14}N_6O_2S$ calculated: C, 47.05; H, 4.61; N, 27.43; found: C, 47.03; H, 4.62; N, 27.43.

MS (FAB) $[M + 1]^+$: *m/z* 307.

*5.1.1.3.24. N′-(2-Methylbenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**24**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.43 (3H, s), 3.99 (3H, s), 4.19 and 4.62 (2H, 2s), 7.25–7.78 (4H, m), 8.31 and 8.45 (1H, 2s), 11.69 and 11.80 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 19.87 (CH_3), 34.11 (CH_3), 36.31 (CH_2), 126.70 (CH), 126.86 (C), 130.19 (CH), 131.45 (CH), 132.29 (CH), 137.20 (C), 143.75 (CH), 153.99 (C), 168.40 (C). For $C_{12}H_{14}N_6OS$ calculated: C, 49.64; H, 4.86; N, 28.95; found: C, 49.65; H, 4.85; N, 28.94.

MS (FAB) $[M + 1]^+$: *m/z* 291.

*5.1.1.3.25. N′-(3-Methylbenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**25**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.35 (3H, s), 3.99 (3H, s), 4.19 and 4.62 (2H, 2s), 7.25–7.53 (4H, m), 8.00 and 8.15 (1H, 2s), 11.73 and 11.79 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 21.33 (CH_3), 34.12 (CH_3), 36.25 (CH_2), 124.72 (CH), 127.79 (CH), 129.22 (CH), 131.28 (CH), 134.26 (C), 138.58 (C), 144.77 (CH), 153.99 (C), 168.46 (C). For $C_{12}H_{14}N_6OS$ calculated: C, 49.64; H, 4.86; N, 28.95; found: C, 49.66; H, 4.87; N, 28.94.

MS (FAB) $[M + 1]^+$: *m/z* 291.

*5.1.1.3.26. N′-(2,3-Dichlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**26**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.21 and 4.64 (2H, 2s), 7.43–7.98 (3H, m), 8.44 and 8.60 (1H, 2s), 12.01 and 12.14 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.11 (CH_2), 125.98 (CH), 128.98 (CH), 131.37 (C), 132.84 (CH), 134.06 (C), 140.36 (CH), 143.52 (C), 153.86 (C), 168.82 (C). For $C_{11}H_{10}Cl_2N_6OS$ calculated: C, 38.27; H, 2.92; N, 24.34; found: C, 38.28; H, 2.93; N, 24.32.

MS (FAB) $[M + 1]^+$: *m/z* 346.

*5.1.1.3.27. N′-(2,4-Dichlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**27**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3*H*, s), 4.20 and 4.63 (2H, 2s), 7.51–7.74 (2H, m), 7.94 and 7.99 (1H, 2d, $J_{1,2} = 8.5$ Hz), 8.36 and 8.53 (1H, 2s), 11.97 and 12.10 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.14 (CH_3), 36.10 (CH_2), 128.49 (CH), 128.57 (C), 129.88 (2CH), 130.71 (C), 134.21 (C), 139.60 (CH), 153.86 (C), 168.76 (C). For $C_{11}H_{10}Cl_2N_6OS$ calculated: C, 38.27; H, 2.92; N, 24.34; found: C, 38.25; H, 2.93; N, 24.33.

MS (FAB) $[M + 1]^+$: *m/z* 346.

*5.1.1.3.28. N′-(2,6-Dichlorobenzylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**28**).* ^{1}H NMR (500 MHz, DMSO-d_6): 3.99 (3H, s), 4.22 and 4.57 (2H, 2s), 7.43–7.59 (3H, m), 8.28 and 8.38 (1H, 2s), 12.02 and 12.10 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 34.09 (CH_3), 36.19 (CH_2), 129.52 (2CH), 129.90 (CH), 131.68 (C), 134.40 (2C), 139.61 (CH), 153.86 (C), 168.85 (C). For $C_{11}H_{10}Cl_2N_6OS$ calculated: C, 38.27; H, 2.92; N, 24.34; found: C, 38.26; H, 2.94; N, 24.32.

MS (FAB) $[M + 1]^+$: *m/z* 346.

*5.1.1.3.29. N′-(1-(2-Chlorophenyl)ethylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**29**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.25 and 2.26 (3H, 2s), 3.99 (3H, s), 4.34 and 4.56 (2H, 2s), 7.31–7.58 (4H, m), 10.85 and 11.10 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 18.58 (CH_3), 34.14 (CH_3), 35.45 (CH_2), 127.81 (C), 130.33 (2CH), 130.79 (CH), 132.40 (CH), 137.20 (C), 153.69 (C), 163.95 (C), 166.02 (C). For $C_{12}H_{13}ClN_6OS$ calculated: C, 44.38; H, 4.03; N, 25.88; found: C, 44.39; H, 4.01; N, 25.87.

MS (FAB) $[M + 1]^+$: *m/z* 325.

*5.1.1.3.30. N′-(1-(3-Chlorophenyl)ethylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**30**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.28 and 2.30 (3H, 2s), 3.99 (3H, s), 4.33 and 4.67 (2H, 2s), 7.47–7.84 (4H, m), 10.87 and 11.09 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 14.29 (CH_3), 34.11 (CH_3), 36.87 (CH_2), 125.36 (CH), 126.32 (CH), 129.49 (CH), 130.77 (CH), 133.84 (C), 140.50 (C), 147.89 (C), 154.05 (C), 169.53 (C). For $C_{12}H_{13}ClN_6OS$ calculated: C, 44.38; H, 4.03; N, 25.88; found: C, 44.40; H, 4.03; N, 25.86.

MS (FAB) $[M + 1]^+$: *m/z* 325.

*5.1.1.3.31. N′-(1-(4-Chlorophenyl)ethylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**31**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.27 and 2.29 (3H, 2s), 3.99 (3H, s), 4.32 and 4.66 (2H, 2s), 7.48–7.84 (4H, m), 10.83 and 11.06 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 14.20 (CH_3), 34.11 (CH_3), 36.87 (CH_2), 128.42 (2CH), 128.91 (2CH), 134.45 (C), 137.16 (C), 148.16 (C), 154.04 (C), 169.44 (C). For $C_{12}H_{13}ClN_6OS$ calculated: C, 44.38; H, 4.03; N, 25.88; found: C, 44.38; H, 4.01; N, 25.89.

MS (FAB) $[M + 1]^+$: *m/z* 325.

*5.1.1.3.32. N′-(1-(2,4-Dichlorophenyl)ethylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**32**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.24 and 2.26 (3H, 2s), 3.99 (3H, s), 4.33 and 4.55 (2H, 2s), 7.41–7.71 (3H, m), 10.89 and 11.14 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 18.39 (CH_3), 34.17 (CH_3), 36.98 (CH_2), 128.03 (C), 129.85 (C), 132.14 (CH), 132.67 (CH), 134.46 (CH), 137.97 (C), 153.95 (C), 164.14 (C), 169.51 (C). For $C_{12}H_{12}Cl_2N_6OS$ calculated: C, 40.12; H, 3.37; N, 23.39; found: C, 40.10; H, 3.37; N, 23.41.

MS (FAB) $[M + 1]^+$: *m/z* 360.

*5.1.1.3.33. N′-(1-(2,5-Dichlorophenyl)ethylidene)-2-[(1-methyl-1H-tetrazol-5-yl)thio]acetohydrazide (**33**).* ^{1}H NMR (500 MHz, DMSO-d_6): 2.24 and 2.26 (3H, 2s), 3.99 (3H, s), 4.34 and 4.57 (2H, 2s), 7.46–7.61 (3H, m), 10.92 and 11.15 (1H, 2s).

^{13}C NMR (125 MHz, DMSO-d_6): 18.31 (CH_3), 34.17 (CH_3), 36.98 (CH_2), 130.28 (C), 130.39 (CH), 130.47 (CH), 132.05 (CH), 132.35 (C), 140.66 (C), 153.95 (C), 164.22 (C), 169.57 (C). For $C_{12}H_{12}Cl_2N_6OS$ calculated: C, 40.12; H, 3.37; N, 23.39; found: C, 40.11; H, 3.38; N, 23.39.

MS (FAB) $[M + 1]^+$: *m/z* 360.

5.2. Microbiology

5.2.1. Microorganisms

Microorganisms were stored at −85 °C in sterile 50% glycerol solution. Cultures were refreshed in Mueller Hinton Broth (MHB-Merck) at 35–37 °C and inoculated on Mueller Hinton Agar plates (MHA-Merck) for checking purity. Three strains of *C. albicans* (ATCC-10231, ATCC 90028 and a clinical isolate-Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskisehir, Turkey), *C. utilis* (NRRL Y-900), *C. krusei* (NRRL Y-7179), *C. parapsilosis* (NRRL Y-12696) and two clinical isolate of *C. glabrata* (Clinical isolate-Anadolu University, Faculty of Science, Department of Biology, Eskişehir, Turkey and Osmangazi University, Faculty of Medicine, Department of Microbiology, Eskişehir, Turkey) were used as test microorganisms in the assay.

5.2.2. Determination of anticandidal activity

Microdilution susceptibility assay was used for the antifungal evaluation of the samples [35,36]. Stock solutions were prepared in dimethyl sulfoxide (DMSO, Carlo-Erba, France). Dilution series were prepared in sterile distilled MHB in 96-well microtiter plates. Overnight grown microorganism suspensions in Mueller-Hinton

Broth were standardized to 10^6 CFU/mL) by using suspension turbidity detector (Biosan, Latvia) adjusted to McFarland No: 0.5 standard. 100 μL of each culture suspension was then added into the wells. The last row without microorganism was used as sterility control. Microorganism and the MHB medium served as a positive growth control in a different row. After incubation at 37 °C for 24 h the first well without turbidity was determined as the minimal inhibitory concentration (MIC). For the best visualization 20 μL of Tetrazolium Violet 1% (w/v, EtOH) (2,5-diphenyl-3-[α-naphthyl]) tetrazolium chloride, TTC, Sigma) reagent was transferred to plates and incubated at 37 °C for 3 h. Ketoconazole (Sigma) was used as standard antifungal agent.

5.3. Genotoxicity test

5.3.1. umuC assay

Genotoxicity of the compounds was evaluated by umuC assay using umuC Easy CS Kit (Xenometrix AG, Gewerbertrasse 25, Switzerland). *Salmonella typhimurium* strain TA1535/pSK1002 was used for the assay. TG medium (200 μL) was added to the vial to obtain homogenous suspensions of *Salmonella* strain. 10 μL of ampicillin (50 mg/mL) was added to 10 mL TG medium (=TGA medium) in 50 mL culture tubes. 50 μL of *Salmonella* suspension was mixed with 10 mL TGA medium. Negative control was devoid of *Salmonella* TA1535/pSK1002. The culture tubes were loosely capped, to allow aeration, and incubated in a shaker (SI-600, Jeio Tech, Korea) at 37 °C, 250 rpm for 14–16 h. The 'overnight grown' cultures were diluted 10 times with TG medium and the absorbance was measured at 600 nm. Positive controls were prepared with S9 (2-aminoanthracene) and without S9 (4-nitroquinolone). TGA medium and S9 fraction were added to each wells of plate. Then, test compounds were added to wells at 5, 2.5, 1.25 and 0.63 mg/mL concentrations. The plates were incubated 37 °C, 120–150 rpm for 2 h. During the 2 h, a second plate was prepared with TG medium with freshly added ampicillin to all wells (for 1 plate: 28 μL ampicillin stock (50 mg/mL) to 28 mL TG medium). After 2 h, 30 μL of the contents of the first plate was transferred to the second plate. The second plate was read the OD600. Then, the second plate was incubated 37 °C, 120–150 rpm for 2 h. During the 2 h, a third plate was prepared with 150 μL B-buffer/ONPG (*o*-nitrophenyl-β-D-galactopyranoside) mixture (for 1 plate: 15 mL of B buffer, 40.5 μL 2-mercaptoethanol, 1 mL ONPG solution) and pre-warmed to 28 °C. At the end of the 2 h incubation, the second plate was mixed and read the OD600. Then, 30 μL of each wells of the second plate was transferred to the third plate. The third plate was incubated at 28 °C, 120–150 rpm for 30 min. After 30 min, 120 μL of stop reagent was added to each well. The plate was mixed and read the OD420. The results were determined with umuC Easy CS Excel Programme.

5.3.2. Ames MPF™

Mutagenicity of the compounds was evaluated by Ames assay using Ames MPF™ 98/100 mutagenicity assay sample kit (Xenometrix AG, Gewerbertrasse 25, Switzerland). *S. typhimurium* strains, TA98 (frameshift mutation) and TA100 (base-pair substitution), were thawed for 5 min. Growth medium (200 μL) was added to each of the vials to obtain homogenous suspensions of *Salmonella* strains (TA98 and TA100). The suspension (25 μL) was added to a mixture of 10 mL growth medium and 10 μL ampicillin (50 mg/mL) in 50 mL culture tubes. Negative control was devoid of *Salmonella* strains. The culture tubes were loosely capped, to allow aeration, and incubated in a shaker (SI-600, Jeio Tech, Korea) at 37 °C, 250 rpm for 14–16 h. The 'overnight grown' cultures were diluted 10 times with growth medium and the absorbance was measured at 600 nm. The absorbance for the 'overnight grown' culture and negative control should be 0.25 and 0.005, respectively. The 'overnight grown' cultures (1 mL) were added to 3 mL growth medium in 50 mL culture tubes and reincubated in the environmental shaker at 37 °C, 250 rpm for 90 min. The absorbances of the 'reincubated' cultures were measured at 600 nm. Absorbances of reincubated cultures should be 1.5–1.9. Compounds **8**, **10**, **11**, **12** and **14** were prepared in six different concentrations (5, 2.5, 1.25, 0.625, 0.3125, 0.156 mg/mL) in DMSO. Mutagenic potential of the compounds was assessed in absence and presence of S9 mix in sterile medium. The final concentration of S9 mix in the assay was 4.5% v/v. 2-nitrofluorene (2 μg/mL) and 4-nitro-o-phenylenediamine (0.1 μg/mL) and 2-aminoanthracene (5 mg/mL) were used as positive controls in absence of S9 mix. In presence of S9 mix with TA 98 and TA100, 2-aminoantracene was used as positive control in the concentrations of 1 μg/mL and 2.5 μg/mL, respectively. DMSO was used as negative control. Briefly, exposure medium and 'reincubated' culture were added to each compound's solution (5, 2.5, 1.25, 0.625, 0.3125, 0.156 mg/mL) and transferred to each well of 24-well plates. For the experiments with S9 mix, S9 mix was added in the same manner. The 24-well plates were incubated in the environmental shaker at 37 °C, 250 rpm for 90 min. Each sample was analyzed in triplicate. At the end of 90 min, 2.8 mL of indicator medium was added to each well of the 24-well plates. This mixture from each well was distributed into 48 wells of a 384-well microtitre plate and was incubated at 37 °C in a dry incubator for 48 h. Catabolic activity of revertant cells would drop the pH of solution resulting in color change from purple to yellow. The number of positive (yellow) wells out of 48 wells in triplicate were counted and compared with the negative control. Fold induction over the negative control and fold induction over the baseline were calculated. Fold induction over the negative control is the ratio of the mean number of positive wells for the dose concentration divided by the mean number of positive wells for the zero dose (negative) control. Fold induction over the baseline is the ratio of the mean number of positive wells for the dose concentration divided by zero dose baseline. The zero dose baseline is obtained by adding one standard deviation to the mean number of positive wells of the zero dose control.

5.4. Cytotoxicity

NIH3T3 and A549 cells were used for cytotoxicity tests. NIH3T3 cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum (Hyclone, Thermo Scientific, USA), 100 IU/mL penicillin (Hyclone, Thermo Scientific, USA) and 100 mg/mL streptomycin (Hyclone, Thermo Scientific, USA) and 7.5% $NaHCO_3$ at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. A549 cells were incubated in RPMI 1640 (Hyclone, Thermo Scientific, USA) supplemented with 10% fetal calf serum (Hyclone, Thermo Scientific, USA), 100 IU/mL penicillin (Hyclone, Thermo Scientific, USA) and 100 mg/mL streptomycin (Hyclone, Thermo Scientific, USA) and 7.5% $NaHCO_3$ at 37 °C, 5% CO_2. A549 and NIH3T3 cells were seeded at 20,000 cells into each well of 96-well plates. After 24 h of incubating period, the culture mediums were removed and compounds were added to culture medium at 0.00316 μM–10 mM doses. After 24 h of incubation, cytotoxicity test was performed using the In Cytotox-XTT 1 Parameter Cytotoxicity Kit (Xenometrix AG, Gewerbertrasse 25, Switzerland), which measures mitochondrial activity (tetrazolium hydroxide (XTT)) in NIH3T3 and A549 cell cultures. Firstly, the cells were washed with phosphate buffer saline (PBS) and were added 200 μL/well of fresh culture medium. XTTI and XTTII solution were mixed at 1:100 ratio. Then, 50 μL of this mixture was added to all wells. The plate was incubated for 3 h at 37 °C, 5% CO_2. After 3 h, the content of the well was mixed by pipetting up and down. Then, OD of the plate was read at 480 nm with a reference wave length

at 680 nm % inhibition was calculated each concentration of compounds. IC_{50} values were estimated by non-linear regression analysis.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.10.011.

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