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1-Acylthiosemicarbazides, 1,2,4-triazole-5(4*H*)-thiones, 1,3,4-thiadiazoles and hydrazones containing 5-methyl-2-benzoxazolinones: Synthesis, analgesic-anti-inflammatory and antimicrobial activities

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Abstract—Acetic acid hydrazide containing 5-methyl-2-benzoxazolinone (**4**) was synthesized by the condensation of 2-(5-methyl-2-benzoxazolinone-3-yl)acetate with hydrazine hydrate. Thiosemicarbazide derivatives (**5a**–**5d**) were afforded by the reaction of corresponding compound **4** with substituted isothiocyanates. The cyclization of compounds **5a**–**5d** in the presence of triethylamine resulted in the formation of compounds **6a**–**6d** containing 1,2,4-triazole ring. On the other hand, the treatment of compounds **5a**–**5d** with orthophosphoric acid caused the conversion of side chain of compounds **5a**–**5d** into 1,3,4-thiadiazole ring: thus, compounds **7a**–**7c** were obtained. The treatment of compound **4** with aromatic aldehydes resulted in the formation of arylidene hydrazides as *cis–trans* conformers (**8a**–**8e**). The structures of the compounds were elucidated by spectral and elemental analysis. While most compounds were exhibiting high activity in the analgesic-anti-inflammatory field, most of them were found to be inactive against bacteria and fungi.

1. Introduction

Rheumatic diseases are the most prevalent causes of disability in Western countries, and non-steroidal anti-inflammatory drugs (NSAIDs) are stilll the most commonly used remedies. NSAIDs cause several serious adverse effects, the most important one is gastric injury that might later cause gastric ulceration and renal injury.[1] Attempts to develop non-steroidal anti-inflammatory drugs that are devoid of classical NSAID toxicity, especially gastrointestinal injury, follow several strategies. One of which is selective cyclooxygenase-2 inhibition (COX-2).[2,3] Although agents that inhibit COX-2 while sparing COX-1 represented a new attractive therapeutic development, they also gave rise to some of the side effects seen with traditional dual COX inhibitors (NSAIDs), namely, effects on the kidney that might manifest as an increased incidence of hypertension, oedema and associated clinical states.[4–6] Therefore, selective COX-2 inhibitors may not be the proper strategy to overcome the damaging effects of conventional NSAIDs which are used for the chronic inflammatory diseases in a long term basis. The other strategy is the inhibition of inducible nitric oxide synthase (iNOS), which contributes to acute and chronic inflammation.[7–9]

In addition, it is known that bacterial infections often produce pain and inflammation. In normal practice, two groups of agents (chemotherapeutic, analgesic and anti-inflammatory) are prescribed simultaneously. Unfortunately, none of the drugs possesses these three activities in a single component. Therefore, our aim is

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to find a compound having dual effect both analgesic-anti-inflammatory and antimicrobial activities. While searching for such a compound, we have found that 2-benzoxazolinone ring is one of the moieties on which studies have been concentrated. In our laboratory, we have designed and synthesized some 2-benzoxazolinone derivatives in the search for new non-steroidal anti-inflammatory agents (Fig. 1).[10–19] A considerable number of the prepared compounds have been found to have analgesic-anti-inflammatory activity comparable to or higher than that of indomethacine. 2-Benzoxazolinone (I) is also a cyclic isostere of coumarin (II) whose antimicrobial activities have been extensively investigated and performed.[20,21] All of these have made us think that 2-benzoxazolinones are promising compounds for finding a drug which has analgesic-anti-inflammatory and antimicrobial action.

1,2,4-Triazoles, 1,3,4-thiadiazoles and their condensed derivatives constitute an important class of organic compounds with analgesic-anti-inflammatory[22–32] and antimicrobial activities.[33–43] In addition, hydrazone derivatives also exhibit potent antimicrobial[44–48] and analgesic-anti-inflammatory[49–52] activities. Prompted by these findings and in continuation of our efforts in synthesizing various bioactive molecules, we have combined 2-benzoxazolinone with 1,2,4-triazole, 1,3,4-thiadiazoles and investigated the eventual role of the triazole, thiadiazole moieties and the *para*-phenyl substituent of the hydrazone subunit on the analgesic-anti-inflammatory and antimicrobial activities.

2. Results and discussion

2.1. Chemistry

One series of novel derivatives of 5-methyl-2-benzoxazolinones has been synthesized in an attempt to find new

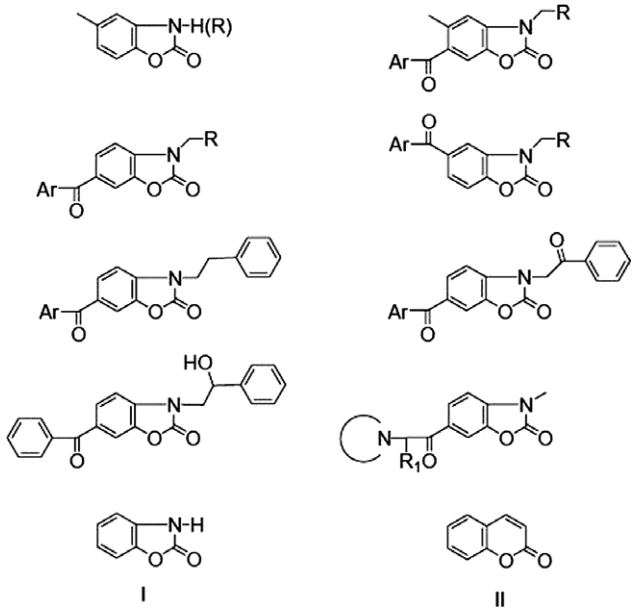


Figure 1. 2-Benzoxazolinone derivatives having analgesic-anti-iflammatory inhibitory activity.

derivatives having both analgesic/-anti-inflammatory and antimicrobial activities. One of the most interesting characteristics of these novel compounds is their basic nature, which differentiates them from the classical, acidic nonsteroidal anti-inflammatory agents (NSAIDs). It is of interest, therefore to study analgesic-anti-inflammatory properties of these novel compounds.

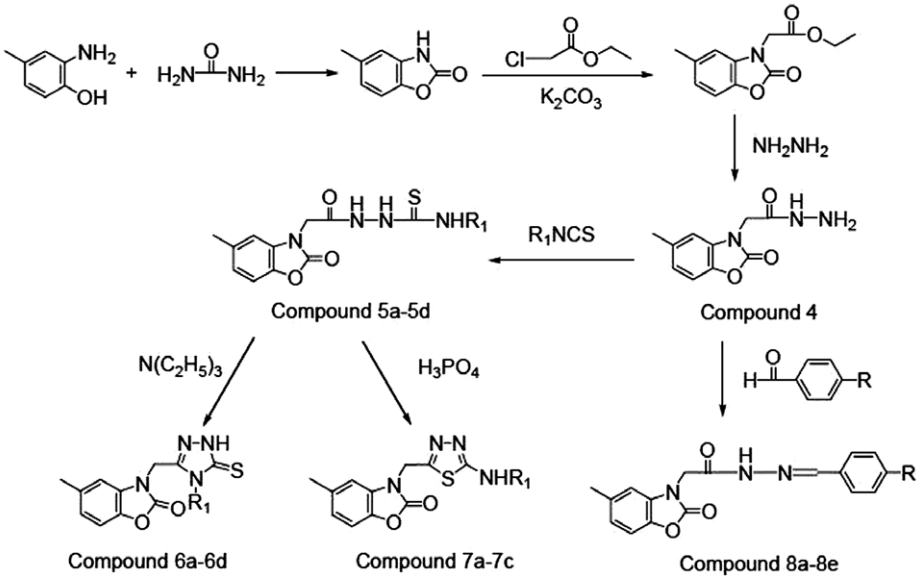
The synthesis pathway leading to the title compounds is given in Scheme 1. 5-Methyl-2-benzoxazolinone, starting material **2**, was synthesized according to the literature method using 5-methyl-2-hydroxyaniline and urea.[53] Treatment of 5-methyl-2-benzoxazolinone with ethyl chloroacetate in K_2CO_3/Acetone gave the *N*-alkylated product (5-methyl-2-benzoxazolinone-3-yl)-acetic acid ethyl ester **3**.[54] The acid hydrazide **4** was prepared by the reaction of ethyl 2-benzoxazolinone-3-yl acetate and hydrazine hydrate in ethanol, which produced a 89% yield. The reaction of hydrazide **4** with various alkyl/aryl isothiocyanate gave compounds **5a–5d**. On heating with triethyl amine in ethanol the thiosemicarbazides **5a–5d** underwent smooth cyclization through dehydration to afford the 3-[(5-methyl-2-benzoxazolinone-3-yl)methyl]-4-substituted-1*H*-1,2,4-triazole-5(4*H*)-thione **6a–6d**.

2-Substituted amino-5-[(5-methyl-2-benzoxazolinone-3-yl)methyl]-1,3,4-thiadiazoles **7a–7c** were obtained by cyclization of **5a–5d** by treating with orthophosphoric acid. The preference formation of the thiadiazole ring under such acidic conditions can be due to the loss of nucleophilicity of N-4 as a result of its protonation leading to a comparable increase in the nucleophilicity of the sulfur atom towards the attack of the carbonyl carbon. On the other hand, when the cyclization of **6a–6d** was carried out under alkaline conditions, the nucleophilicity of N-4 was enhanced and led to cyclization with the carbonyl carbon atom to give the 1,2,4-triazole-5(4*H*)-thiones **6a–6d**. The carbohydrazide **4** was condensed with different aromatic aldehydes in ethanol to give the corresponding Schiff's bases **8a–8e** in very good yields.

The purity of the synthesized compounds was checked by elemental analyses. The structures of the various synthesized compounds were determined on the basis of spectral data analysis; such as UV, IR, ^{1}H NMR, ^{13}C NMR and MS. The structure of compound **6b** was also elucidated by the X-ray diffraction.

The IR spectra of acid hydrazide **4** showed a peak at 1702 cm^{-1} due to exocyclic carbonyl function derived from hydrazide structure beside the endocyclic carbonyl peak (1766 cm^{-1}) at position 2 of 5-methyl-2-benzoxazolinone ring. The IR spectra of compounds **5a–5d** exhibited a characteristic strong absorption at 1343 cm^{-1} attributable to the C=S of the thiosemicarbazide residue.

In the ^{1}H NMR spectra of compound **4**, singlet signals derived from hydrazide structure appeared between 4.23 ppm (–NHN$\underline{H}_2$) and 9.54 ppm (–N$\underline{H}$NH$_2$) showing the integration for two protons and one proton, respectively. Methylene protons resonated as a singlet at



5a: R_1=CH_3, **5b**: R_1=C_2H_5, **5c**: R_1=C_3H_7, **5d**: R_1=C_6H_5, **6a**: R_1=CH_3, **6b**: R_1=C_2H_5, **6c**: R_1=C_3H_7, **6d**: R_1=C_6H_5, **7a**: R_1=CH_3, **7b**: R_1=C_2H_5, **7c**: R_1=C_6H_5, **8a**: R=H, **8b**: R=Br, **8c**: R=Cl, **8d**: R=CH_3, **8e**: R=OCH_3.

Scheme 1. Synthetic pathway of compounds.

4.86 ppm. The ^{1}H NMR spectra of compounds **5a**–**5d** displayed three singlets due to three different –NH groups around 8.1, 9.4 and 10.30 ppm (exchangeable with D_2O) each showing the integration for one proton. In the ^{1}H NMR spectra of compounds **6a**–**6d**, signals of the NH of the 1,2,4-triazole-5(4H)-thione derivatives were observed in the δ 13.7–13.98 ppm range. X-ray analysis confirmed that compound **6b** exists in the thione form (Fig. 2). The ^{1}H NMR spectra of 1,3,4-thiadiazole derivatives **7a**–**7c** showed the proton signals typical of the NH group in the δ range 7.72–10.39 ppm.

According to the literature, the hydrazones may exist as *Z*/*E* geometrical isomers about C=N double bonds and *cis*/*trans* amide conformers. Besides hydrazones derived from aldehydes and substituted hydrazides are present in solution in the *E* form. It has been reported that when hydrazones are dissolved in dimethyl-d_6 sulfoxide solution, the *E* geometrical isomers of these compounds undergo a rapid *cis*/*trans* amide equilibrium, in which the *c*is conformer predominates.[55,56]

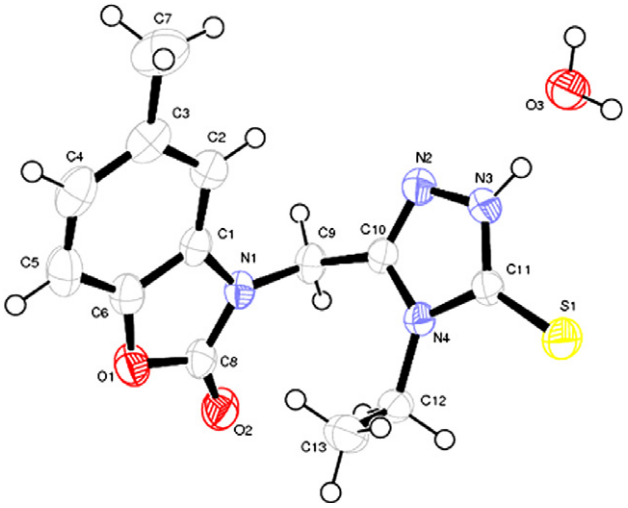


Figure 2. The structure of **6b**, showing 50% probability displacement ellipsoids and the atom numbering scheme.

The investigation of ^{1}H NMR spectra of **8a**–**8e** demonstrated that these hydrazones behaved similarly in this solution and no signal belonging to *Z* isomer was observed. This assignment was further confirmed by a molecular minimization energy study using Molecular Operating Environment (MOE, version 2005.06)) for **8a**. This study indicated that the compound bearing *E* configuration (*E*/65.6856 Kcal mol^{-1}) has lower energy than the isomeric compound having *Z* configuration (*Z*/74.0366 Kcal mol^{-1}) (Fig. 3).

In the ^{1}H NMR of the compounds **8a**–**8e**, the signals belonging to benzylidene group were observed at aromatic region while the signals belonging to –$NHNH_2$ disappeared. Two sets of signals each belonging to the CH_2 and =CH group of *cis* and *trans* conformers were observed between 4.60–4.85/8.18–8.25 and 5.02–5.05/8.00–8.07 ppm. The upfield lines of =CH protons were assigned to *cis*-conformer of the amide structure and downfield lines of the protons of the same group were assigned to *trans*-conformer of the amide structure.[55]

Additional support for the structures of the synthesized compounds was provided by ^{13}C NMR spectra. In the ^{13}C NMR spectra of the compounds **5a**, **8c**, lactam and hydrazide C=O gave two peaks at around 154 and 166 ppm. The peak resonated at 168 ppm in the ^{13}C NMR spectrum of compound **6c**, assigned for C=S, confirming thione form of the triazole ring.

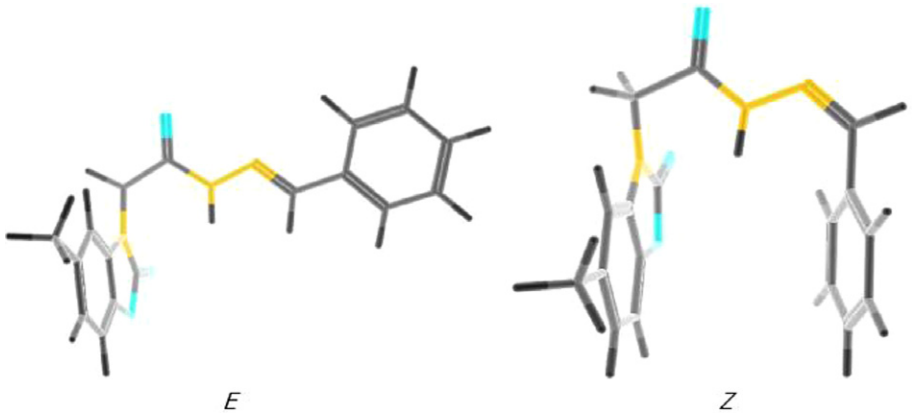


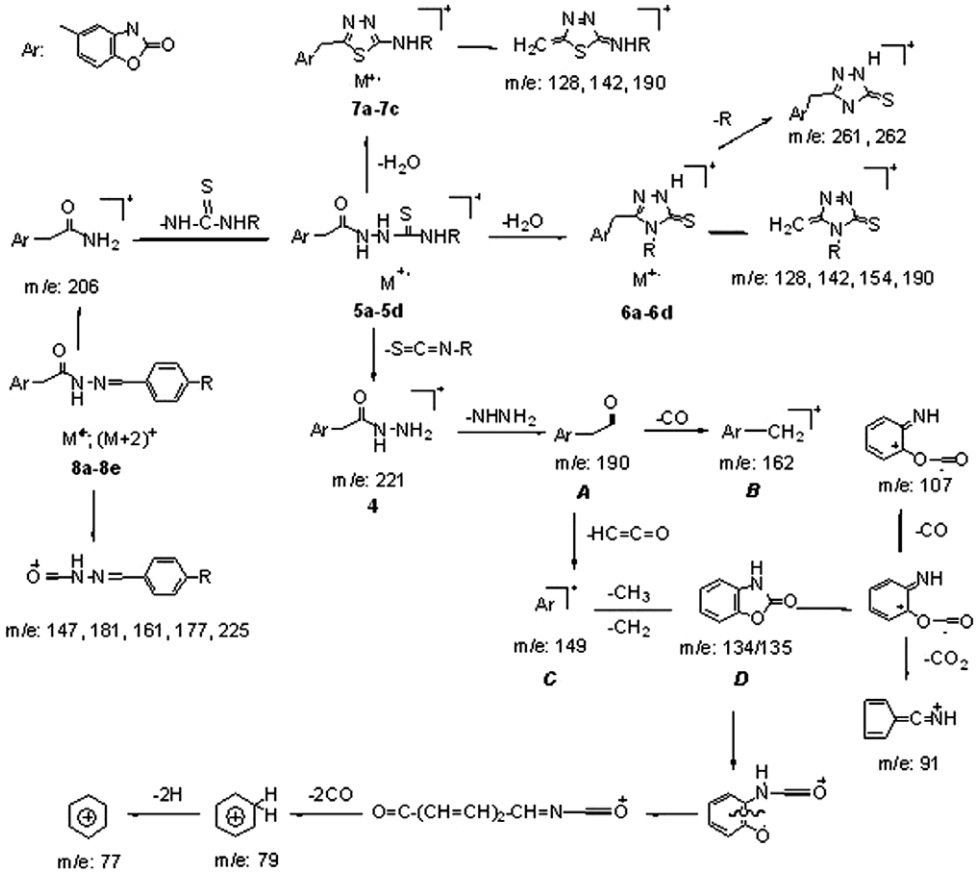
Figure 3. Representation of *E* and *Z* configuration of compound **8a**.

The mass spectra of all the synthesized compounds were studied under electron ionization. The molecular ion peak was present in all the compounds, although their relative intensities varied from 1 to 100% (Scheme 2).

Further spectroscopic details of these compounds are presented in the experimental part. In addition, it was shown by X-ray crystallographic analysis that compound **6b** and compound **7a** contain 1 mol H_20 and 1 mol HPO_4[57] with X-ray crystallographic analysis, respectively.

2.2. X-ray crystal analysis of 6b

The molecular structure of compound **6b** was determined by X-ray crystallographic analysis. The compound crystallizes in the monoclinic system, space group $P2_1/c$ with $a = 11.9854(11)$ Å $b = 7.2021(4)$ Å $c = 17.8274(14)$ Å, $\beta = 107.363(7)$ Å. Ortep depiction with atom numbering of compound **6b** is shown in Figure 2, respectively. Compound **6b** contains essentially planar benzoxazolinone ring system, within which the C–N bond distances and angles do not differ significantly from our related structures, 3-[4-(4-fluorophenyl)piperazine-1-ylmethyl]-



Scheme 2. Fragmentation pattern of the compounds.

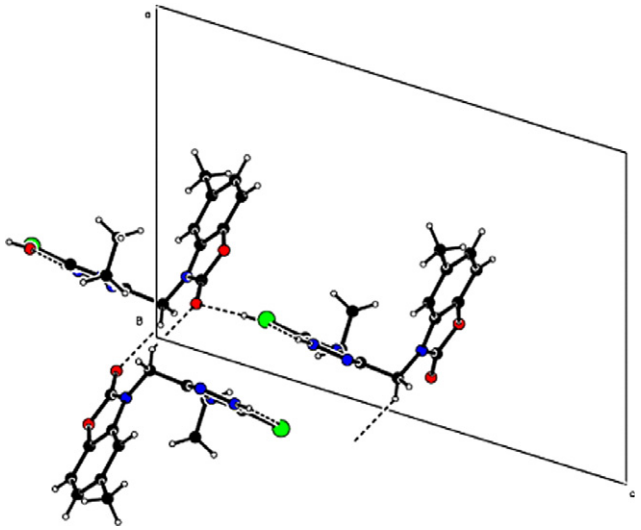


Figure 4. Crystal packing of **6b** projected onto ac plane. The dashed lines indicates the intermolecular hydrogen bonds.

5-methyl-1,3-benzoxazol-2(3H)-one and 3-[4-(2-fluorophenyl)piperazine-1-ylmethyl]-5-methyl-1,3-benzoxazol-M2(3H)-one.[58,59] The maximum deviations from the plane of the nine-membered ring system is 0.013(2) Å for atom C15. The benzoxazolinone and substituent triazole groups are almost perpendicular to each other with a dihedral angle of 82.25(4)°. The structure of the compound **6b** contains intermolecular, O–H···O [for O3–H3A···O2^{i}, donor and acceptor distance is 2.8359(17) Å, O3–H3A = 0.82(3) Å, O3··· O2^{i} = 2.03(3) Å, O3–H3A···O2^{i} = 169(2)° i) $x,-y+1/2,+z-1/2$], O–H···S [for O3–H3B··· S1^{ii}, donor and acceptor distance is 3.2929(14) Å, O3–H3B = 0.82(2) Å, O3··· S1^{ii} = 2.47(2) Å, O3–H3B··· S1^{ii} = 175(2)°, (ii) x,+y+1,+z] and intramolecular N–H···O [for N3–H3···O3, donor and acceptor distance is 2.7793(17) Å, N3-H3 = 0.88(2) Å, N3···O3 = 1.91(2)Å, N3–H3···O3 = 171.1(18)°] type hydrogen bonds. Crystal packings of **6b** are shown in Figure 4. The H atoms of the coordinated and uncoordinated water molecules are engaged in hydrogen bonding with O2 and S1 atoms of the ring systems, resulting in the formation of extended chains along the c axis. There are also π–π stacking interactions between the parallel triazole systems. The closest perpendicular separation is 2.948 Å between the ring system at (x,y,z) and that at $(-x,-1/2+y,-1/2-z)$.

2.3. Pharmacology

Continuing our studies on 2-benzoxazolinones that are attractive candidates as antinociceptive agents, we designed a new series of functionalized 2-benzoxazolinone derivatives. The rational design of these new derivatives was planned by molecular hybridization of previously described analgesic compounds benzoxazolinone, triazole and thiadiazole. In the pharmacological study, we investigated anti-inflammatory and analgesic activity as well as the ulcerogenic risk of both thiosemicarbazide intermediates (**5a**–**5d**) and corresponding triazole, thiadiazole and benzylidene hydrazine derivatives (**6a**–**6d**, **7a**–**7c**, **8a**–**8e**).

2.3.1. Analgesic activity. The analgesic activity of the compounds was studied by using both the acetic acid-induced writhing test[60] and hot plate test [61] in mice. The animals were first administered at 100 mg/kg (body weight) dose of the test drugs in two of the screening tests. These compounds presented an important analgesic profile measured by the classical acetic acid induced constrictions. From the obtained results of acetic acid-induced writhing test, it was noticed that all compounds possess analgesic activity at 100 mg/kg dose comparable with or higher than that of aspirin (ASA) (Table 1). However, hot plate test results showed a relative decrease in the analgesic activity of the compounds (except **7a**, **8c**, **8d**). The analgesic effects of **7a** (54.3%, 73.8%), **8c** (50.7%, 62.5%) and **8d** (52.9%, 77.8%) were higher than those of both morphine and aspirin (50.7%, 65.7%) (Fig. 5).

Table 1. Effects of the compounds on acetic acid induced abdominal writhing and hot plate tests in mice

Compound	Dose (mg/kg)	Hind paw lick ± SEM (Inhibition %) (A)	Writhing reflex ± SEM (Inhibition %) (B)
Control		13.8 ± 0.2	24.8 ± 0.9
Morphine	10	6.8 ± 0.5** (**50.7**)	—
ASA	200	—	8.5 ± 0.6** (**65.7**)
4	100	8.0 ± 0.6** (**42.0**)	6.5 ± 0.6** (**73.8**)
5a	100	10.5 ± 0.3** (**23.9**)	9.5 ± 0.6** (**61.7**)
5b	100	11.3 ± 0.3** (**18.1**)	13.8 ± 1.1** (**44.4**)
5c	100	11.3 ± 0.3** (**18.1**)	10.0 ± 1.1** (**59.7**)
5d	100	10.5 ± 0.6** (**23.9**)	11.0 ± 0.9** (**55.6**)
6a	100	13.5 ± 0.3$^{n.s.}$ (**2.2**)	12.5 ± 1.2** (**49.6**)
6b	100	8.8 ± 0.5** (**36.2**)	14.0 ± 0.4** (**43.5**)
6c	100	9.5 ± 0.6** (**31.2**)	8.3 ± 0.5** (**66.5**)
6d	100	12.0 ± 0.4* (**13.0**)	12.5 ± 0.6** (**49.6**)
7a	100	6.3 ± 0.3** (**54.3**)	6.5 ± 0.6** (**73.8**)
7b	100	10.3 ± 0.3** (**25.4**)	7.8 ± 0.6** (**68.5**)
7c	100	9.5 ± 0.3** (**31.2**)	8.3 ± 0.6** (**66.5**)
8a	100	10.5 ± 0.3** (**23.9**)	9.3 ± 0.9** (**62.5**)
8b	100	11.0 ± 0.4** (**20.3**)	6.8 ± 0.6** (**72.6**)
8c	100	6.8 ± 0.3** (**50.7**)	9.3 ± 0.8** (**62.5**)
8d	100	6.5 ± 0.3** (**52.9**)	5.5 ± 0.6** (**77.8**)
8e	100	10.0 ± 0.4** (**27.5**)	8.5 ± 0.6** (**65.7**)

*$p < 0.01$; **$p < 0.001$ Significant from control ($n = 5$); n.s., not significant.

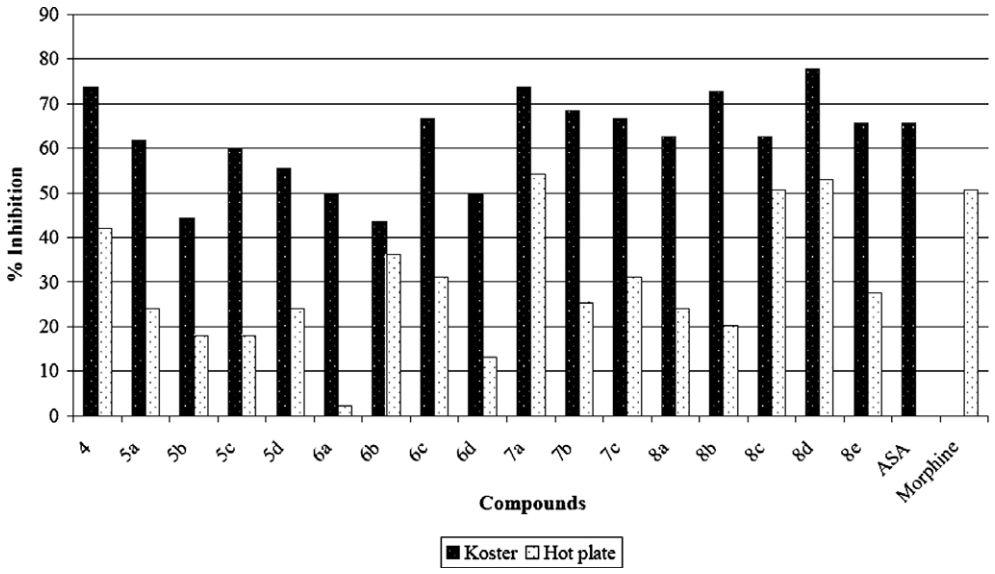


Figure 5. Analgesic effect of compounds on acetic acid induced abdominal writhing and hot plate tests in mice.

Additionally, these studies showed that the most potent analgesic agent **8d** carries methyl substituent at the phenyl ring of the hydrazone group. As shown in Table 1, compound **6a** has analgesic activity in acetic acid-induced writhing test, although it was almost inactive in the hot-plate test.

However, the mechanism underlying their antinociceptive activity remains to some degree unknown. Thus further studies are essential to ascertain the mechanisms involved in the analgesic properties of the ring system.

2.3.2. Anti-inflammatory activity. In order to screen the anti-inflammatory profile of the synthesized compounds, carrageenan-induced hind paw oedema model in mice was used.[62] At first, the anti-inflammatory activity of the synthesized compounds was studied at 100 mg/kg dose. Test compounds which possessed more than 20% effect, even some of them are not significant statistically, were considered for further evaluation and the experiments were repeated for additional two different dose levels (50 and 200 mg/kg) (Table 2). The anti-inflammatoy activity decreased dramatically when compounds were administered at a half dose (50 mg/kg, p.o.). The anti-inflammatory effects of the compounds at 100 mg/kg dosage reached significant values after 90 min (Fig. 6). We also screened all compounds for ulcerogenic adverse effect at 200 mg/kg dose level. After microscopic examination, no ulceration risk was seen in compounds **6a**–**6d** which have triazole moiety in their structure.

In order to probe structural requirements for optimal anti-inflammatory activity in this series, the substituents attached to the nitrogen atom, size of these substituents and cyclization of thiosemicarbazide unit were examined. According to the results of in vivo experiments, we can conclude that the cyclization of the thiosemicarbazide moiety to thiadiazole produced more active compounds at 200 mg/kg (compare **7c/5d**; **7b/5b**; **7a/5a**) (Fig. 7). When compared the effect of substituents on the phenyl ring of hydrazone derivatives (compounds **8a**–**8e**), methoxy substituent (**8e**) resulted in significant anti-inflammatory activity. Whereas, this derivative gave also rise to the ulcer risk. As shown in Table 2, substituent size did not produce noticeable differences in activity of the compounds. However an increase in anti-inflammatory activity was observed with replacement of alkyl chain to phenyl ring (compounds **5d**, **6d**, **7c**). Among these compounds, compound **6d** which has *N*-phenyltriazole exhibited the highest anti-inflammatory activity. Measurements were done in every 90 min along 6 h at 100 mg/kg dose level and percentage inhibition values were 79.8%, 63.3% and 31.8%, 27.4%. This compound was also safe in ulcer incidence.

Since our findings are preliminary results; further studies need to be carried out to investigate the other specifications, such as in vitro assays, toxicological studies or side effect-activity profiles of these compounds.

2.4. Microbiology

2.4.1. In vitro antimicrobial activity. To develop new antimicrobial agents, we have synthesized benzoxazolinone derivatives having thiosemicarbazide ring (**5a**–**5d**), 1,2,4-triazole (**6a**–**6d**) and 1,3,4-thiadiazole moiety (**7a**–**7c**) and hydrazone derivatives (**8a**–**8e**). The assessment of the antimicrobial activities of the synthesized compounds was performed using the broth microdilution test in Mueller-Hinton Broth and RPMI 1640 (with L-glutamine without sodium bicarbonate) medium for the determination of antibacterial and antifungal activity, respectively.[63,64] The results revealed that most of the newly synthesized compounds exhibited poor antibacterial activities whereas promising antifungal activities (Table 3). It is worth mentioning that compounds **4**, **5a**, **6d**, **8a**, **8d**, **8e** (MIC 128 μg/mL) showed moderate inhibitory activities against *Candida krusei*, *Candida albicans* and *Candida parapsilosis*.

Table 2. Anti-inflammatory effect of compounds against carrageenan-induced hind paw oedema model in mice and ratio of ulceration ($n = 5–6$)

Compound	Ratio of ulceration	Dose (mg/kg)	Swelling in thickness ($\times 10^{-2}$ mm) ± SEM (Inhibition %)			
			90 min	180 min	270 min	360 min
Control	0/5		66.0 ± 1.0	79.0 ± 2.0	90.0 ± 3.4	80.0 ± 4.6
4		100	10.0 ± 0.2*** (**84.8**)	23.3 ± 1.7*** (**70.5**)	54.5 ± 3.4*** (**39.4**)	58.1 ± 2.6* (**27.4**)
		50	92.10 ± 2.5	88.7 ± 3.6	108.4 ± 2.5	101.1 ± 1.1
	3/5	200	23.4 ± 1.3*** (**65.1**)	22.4 ± 1.4*** (**71.6**)	40.8 ± 1.6*** (**54.7**)	38.7 ± 1.7*** (**51.6**)
5a		100	23.3 ± 3.3*** (**64.7**)	52.3 ± 5.8** (**33.8**)	90.3 ± 4.5$^{n.s.}$	85.8 ± 1.3$^{n.s.}$
		50	118.2 ± 1.8	125.2 ± 5.9	187.2 ± 1.2	188.9 ± 5.0
	0/5	200	27.7 ± 3.1*** (**58.0**)	35.9 ± 2.0*** (**54.6**)	58.3 ± 2.2*** (**35.2**)	58.0 ± 2.5** (**27.5**)
5b		100	28.3 ± 1.7*** (**57.1**)	50.3 ± 7.8** (**36.3**)	58.0 ± 3.4*** (**35.6**)	56.6 ± 0.2** (**29.3**)
		50	116.7 ± 5.7	121.5 ± 2.7	139.1 ± 3.8	127.8 ± 4.7
	0/5	200	53.2 ± 2.3*** (19.4)	62.8 ± 2.4*** (**20.5**)	65.0 ± 2.5*** (**27.8**)	42.2 ± 2.2*** (**47.2**)
5c		100	13.3 ± 1.7*** (**79.8**)	42.6 ± 2.0*** (**46.1**)	78.3 ± 1.7* (13.0)	80.6 ± 1.3$^{n.s.}$
		50	147.3 ± 6.1	147.1 ± 2.9	188.2 ± 1.9	176.7 ± 2.1
	2/5	200	46.8 ± 2.0*** (**29.1**)	59.2 ± 1.7*** (**25.1**)	80.0 ± 1.6* (11.1)	74.7 ± 1.4 (**6.6**)$^{n.s.}$
5d		100	13.3 ± 3.3*** (**79.8**)	54.3 ± 3.9*** (**31.3**)	92.0 ± 10.2$^{n.s.}$	79.2 ± 0.2$^{n.s.}$
		50	118.2 ± 1.8	140.9 ± 4.9	162.6 ± 2.5	153.3 ± 4.1
	0/5	200	39.4 ± 2.1*** (**40.3**)	36.8 ± 2.9*** (**53.4**)	62.5 ± 1.8*** (**30.5**)	52.7 ± 2.4*** (**34.1**)
6a		100	13.3 ± 1.7*** (**79.8**)	69.7 ± 5.8$^{n.s.}$ (11.8)	76.7 ± 3.0* (14.8)	58.1 ± 2.6* (**27.4**)
		50	67.5 ± 2.9	85.1 ± 2.7	120.7 ± 2.1	97.8 ± 1.3
	0/5	200	38.3 ± 2.0*** (**42.0**)	48.5 ± 2.6*** (**38.6**)	75.8 ± 2.7* (15.8)	79.1 ± 1.9$^{n.s.}$
6b		100	28.3 ± 1.7*** (**57.1**)	95.9 ± 2.9	117.6 ± 3.0	87.1 ± 2.1
		50	82.9 ± 6.1	91.1 ± 5.5	147.3 ± 2.9	146.7 ± 5.4
	0/5	200	28.7 ± 2.8*** (**56.5**)	53.9 ± 2.0*** (**31.8**)	66.7 ± 1.8*** (**25.9**)	64.2 ± 2.3* (19.7)
6c		100	11.7 ± 1.7*** (**82.3**)	60.0 ± 7.7* (**24.1**)	78.4 ± 1.7* (12.9)	60.8 ± 1.3* (**24.0**)
		50	58.3 ± 3.1* (11.7)	120.3 ± 4.9	164.7 ± 2.6	160.0 ± 5.9
	0/5	200	38.3 ± 3.1*** (**41.9**)	44.0 ± 2.6*** (**44.3**)	65.0 ± 3.1*** (**27.8**)	62.4 ± 3.2* (**22.0**)
6d		100	13.3 ± 1.7*** (**79.8**)	29.0 ± 3.4*** (**63.3**)	61.4 ± 3.0** (**31.8**)	58.1 ± 1.3* (**27.4**)
		50	122.8 ± 0.3	115.5 ± 2.4	157.3 ± 2.5	147.2 ± 1.6
	0/5	200	30.9 ± 2.0*** (**53.2**)	41.3 ± 3.6*** (**47.7**)	75.0 ± 2.9** (16.7)	75.6 ± 2.5$^{n.s.}$ (5.5)
7a		100	24.6 ± 3.7*** (**62.7**)	62.1 ± 2.3** (**21.4**)	110.4 ± 3.1	106.7 ± 2.7
		50	66.0 ± 2.5	101.3 ± 2.1	106.8 ± 3.7	96.0 ± 4.0
	2/5	200	24.1 ± 3.5*** (**63.5**)	41.9 ± 4.2*** (**46.9**)	60.0 ± 2.7*** (**33.3**)	68.4 ± 2.3$^{n.s.}$ (14.5)
7b		100	10.0 ± 0.2*** (**84.8**)	52.3 ± 1.7*** (**33.8**)	83.6 ± 1.7$^{n.s.}$ (7.1)	80.6 ± 1.3$^{n.s.}$
		50	60.8 ± 1.6* (7.9)	90.1 ± 2.8	102.8 ± 5.0	94.0 ± 2.5
	4/5	200	9.7 ± 1.7*** (**85.3**)	22.7 ± 2.2*** (**71.3**)	49.1 ± 1.7*** (**45.4**)	71.1 ± 3.1$^{n.s.}$ (11.1)
7c		100	10.0 ± 0.2*** (**84.8**)	54.3 ± 3.9*** (**31.3**)	95.4 ± 3.4	81.8 ± 2.6
		50	53.1 ± 2.5** (19.5)	73.4 ± 2.8$^{n.s.}$ (7.1)	93.9 ± 4.1	110.0 ± 3.2
	4/5	200	8.0 ± 2.5*** (**87.9**)	47.9 ± 5.6*** (**39.4**)	63.6 ± 3.2*** (**29.3**)	87.1 ± 3.0
8a		100	13.3 ± 3.3*** (**79.8**)	56.1 ± 3.9** (**30.0**)	95.4 ± 1.7	84.5 ± 2.6
		50	98.3 ± 3.2	100.1 ± 3.6	108.8 ± 4.5	115.0 ± 1.6
	2/5	200	24.1 ± 2.5*** (**63.5**)	49.1 ± 4.4*** (**37.8**)	65.5 ± 2.3*** (**27.2**)	81.8 ± 3.0
8b		100	36.7 ± 3.3*** (**44.4**)	44.5 ± 3.9*** (**43.7**)	92.0 ± 0	64.7 ± 1.3* (19.1)
		50	64.7 ± 2.1$^{n.s.}$ (1.9)	94.6 ± 1.8	95.9 ± 2.9	83.0 ± 2.0
	5/5	200	33.8 ± 3.1*** (**48.8**)	41.9 ± 1.9*** (**46.9**)	80.0 ± 1.8* (11.1)	84.4 ± 3.9
8c		100	23.3 ± 3.3*** (**64.7**)	75.0 ± 0.2$^{n.s.}$ (5.1)	85.2 ± 3.4$^{n.s.}$ (5.3)	76.6 ± 2.6$^{n.s.}$ (4.3)
		50	72.5 ± 2.5	89.0 ± 3.0	111.8 ± 4.5	107.0 ± 3.0
	2/5	200	54.7 ± 3.1** (17.1)	61.0 ± 4.4** (**22.8**)	74.5 ± 2.3** (17.2)	73.8 ± 3.9$^{n.s.}$ (7.7)
8d		100	20.0 ± 0.2*** (**69.7**)	71.6 ± 3.9$^{n.s.}$ (9.4)	92.0 ± 0.2	69.9 ± 2.6$^{n.s.}$ (12.6)
		50	73.8 ± 1.6	86.8 ± 1.3	103.8 ± 1.6	98.0 ± 3.4
	3/5	200	54.7 ± 3.1** (17.1)	52.7 ± 3.5*** (**33.3**)	89.1 ± 3.1$^{n.s.}$	83.6 ± 1.7
8e		100	11.6 ± 1.7*** (**82.4**)	100.8 ± 7.8	95.4 ± 3.4	66.0 ± 2.6$^{n.s.}$ (17.5)
		50	53.1 ± 2.5** (19.5)	82.3 ± 2.8	108.8 ± 1.6	87.2 ± 3.0
	5/5	200	8.0 ± 2.5*** (**87.9**)	27.5 ± 3.1*** (**65.2**)	55.5 ± 3.3*** (**38.3**)	86.2 ± 3.9
Indomethacin		10	38.3 ± 1.7*** (**42.0**)	45.0 ± 2.9*** (**43.0**)	48.3 ± 1.7*** (**46.3**)	46.7 ± 1.7*** (**41.6**)

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Significant from control ($n = 5$); n.s., not significant.

3. Conclusion

Various substituted thiosemicarbazides, triazoles, thiadiazoles and benzylidene hydrazine derivatives were synthesized and screened for analgesic-anti-inflammatory and antimicrobial activities. Most compounds exhibited high analgesic-anti-inflammatory activity. Compounds **7a**, **8c**, **8d** which have thiadiazole moiety and benzylidene hydrazine showed strong analgesia in both tests. Among the synthesized compounds, compounds **4** and **6d** at 100 mg/kg dose and compounds **4**, **7b and 8e** at 200 mg/kg dose possessed the most prominent and consistent antiinflammatory activity. Therefore, we can conclude that compound **4** possessed considerably high amount of analgesic activity and high anti-inflammatory activity. These activities might be

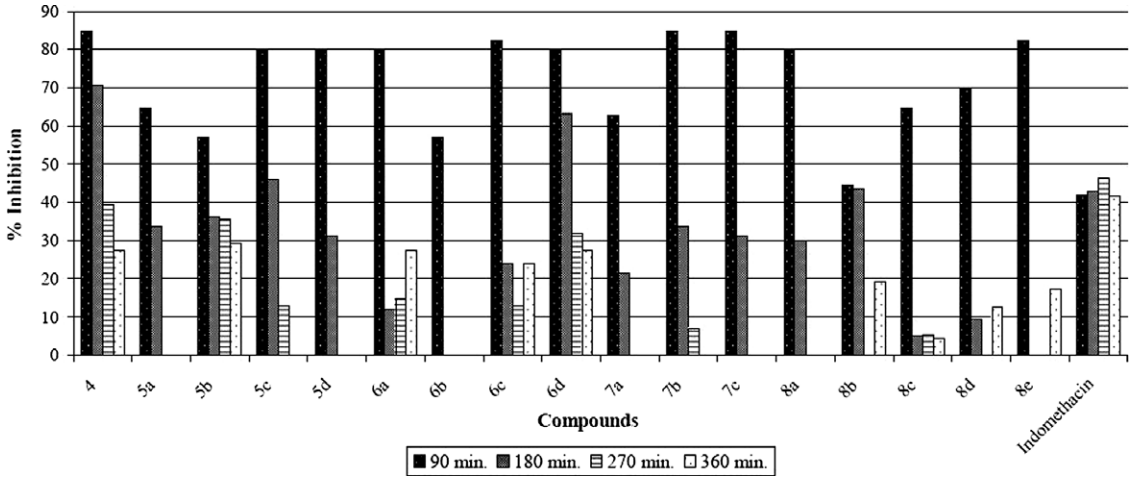


Figure 6. Effect of compounds against carrageenan-induced hind paw edema model at 100 mg/kg dose.

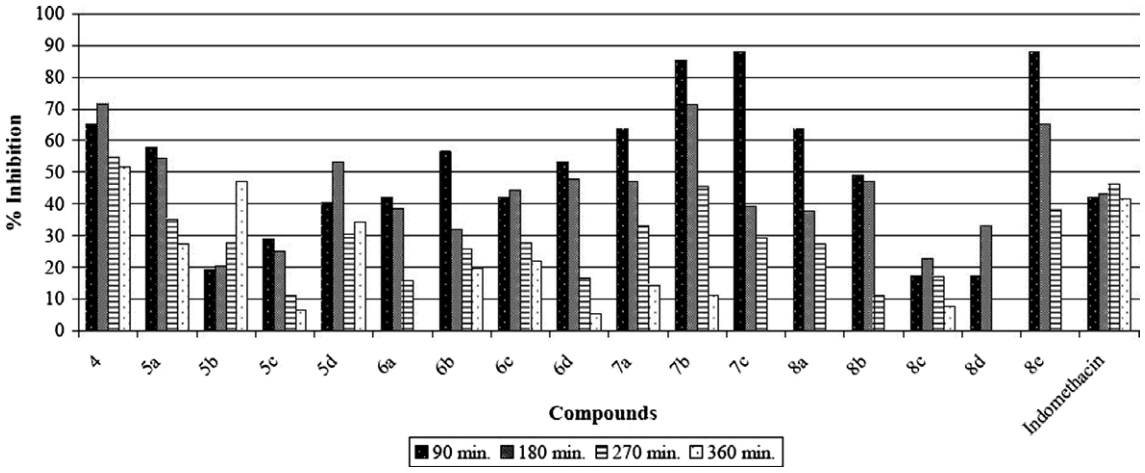


Figure 7. Effect of compounds against carrageenan-induced hind paw edema model at 200 mg/kg dose.

resulting from degradation of compound **4** to the corresponding acid by hydrolysis during metabolism of it. Most of the compounds were inactive against bacteria but they showed promising antifungal activity. Furthermore, in order to understand the structure activity relationship and the mechanism of inhibition, the research on modification of the title compounds is underway.

4. Experimental

4.1. Chemistry

All chemicals were obtained from Aldrich Chemical Co. (Steinheim, Germany). Melting points were determined through a Thomas Hoover capillary melting point apparatus and are uncorrected. Ultraviolet (UV) spectra were taken with a Agilent 8453 UV–visible spectra spectrometer in methanol at approximately 2×10^{-5} M concentration. Infrared (IR) spectra were obtained with a Bruker Vector 22 IR (Opus Spectroscopic Software Version 2.0) spectrometer using potassium bromide plates and the results were expressed in wave number (cm^{-1}). Nuclear magnetic resonance (^1H NMR and ^{13}C NMR) spectra were scanned on a Bruker 400 MHz UltraShield spectrometer using dimethylsulfoxide (DMSO-d_6) as solvent. Chemical shifts are expressed in δ (parts per million) relative to tetramethylsilane. Splitting patterns are as follows: s, singlet; d, doublet; m, multiplet; b, broad; dd (doublet in doublet). The mass spectra were obtained with electron impact technique using a Direct Insertion Probe and Agilent 5973-Network Mass Selective Dedector at 70 eV. Elemental analyses (C, H, N) were performed on Leco CHNS 932 analyzer.

4.1.1. Synthesis of 5-methyl-2-benzoxazolinones. 5-Methyl-2-benzoxazolinone was synthesized as a result of the reaction of 5-methyl-2-hydroxyaniline with urea in oil bath according to the method reported earlier.[53]

4.1.2. Synthesis of ethyl 2-(5-methyl-2-benzoxazolinone-3-yl)acetate. Ethyl 2-(5-methyl-2-benzoxazolinone-3-yl)acetate was synthesized as a result of the reaction of 5-methyl-2-benzoxazolinone and ethyl chloroacetate in acetone under basic conditions as published before.[54]

4.1.3. Synthesis of 2-(5-methyl-2-benzoxazolinone-3-yl)acetylhydrazine (4). About 1.46 mL (30 mmol) Hydrazine hydrate (100%) was added to a 50 mL ethanolic solution of 3.525 g (15 mmol) ethyl 2-(5-methyl-2-benzoxazolinone-3-yl)acetate and refluxed for 2 h. The reaction mixture was then cooled and the solid precipi-

Table 3. In vitro antibacterial and antifungal activity of compounds **4**, **5a–5d**, **6a–6d**, **7a–7c**, and **8a–8e** (MIC μg/ml)

Compound	MIC values (lμg/ml)						
	A	B	C	D	E	F	G
4	256	512	256	256	128	128	128
5a	512	256	256	128	128	128	128
5b	256	512	256	512	256	256	256
5c	256	512	256	512	256	256	256
5d	256	512	256	256	256	256	128
6a	>512	>512	512	512	512	512	256
6b	512	512	512	256	512	256	256
6c	256	512	256	256	256	256	128
6d	256	256	256	256	128	128	128
7a	512	512	512	512	256	256	256
7b	512	>512	512	512	512	512	256
7c	512	512	>512	512	256	256	256
8a	256	512	256	256	128	128	128
8b	>512	>512	>512	>512	64	256	128
8c	>512	>512	>512	>512	128	256	256
8d	512	256	128	256	128	128	128
8e	256	128	512	256	128	128	512
Ampicillin	1	8	2	—	—	—	—
Fluconazole	—	—	—	—	1	64	8

A, *Staphylococcus aureus*; B, *Enterococcus faecalis*; C, *Escherichia coli*; D, *Pseudomonas aeruginosa*; E, *Candida albicans*; F, *Candida crusei*; G, *Candida parapsilosis*.

tated was recrystallized from ethanol–water (3/1) to give 89% of **4** as a cream solid. Mp 215–217 °C. UV (CH_3OH) nm: 202 (log ε: 4.3), 284 (log ε: 3.28); IR (KBr) cm^{-1}: 3337, 3222, 2923, 1766, 1702; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.21 (s, 3H, –C*H*$_3$), 4.23 (s, 2H, –N*H*$_2$), 4.86 (s, 2H, –N–C*H*$_2$–CO), 6.82 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 6.98 (dd, 1H, 2-benz.-*H*$_6$, *J* 8, *J* 2), 7.08 (d, 1H, 2-benz.-*H*$_4$, *J* 2), 9.54 (bs, 1H, –CO–N*H*); MS (70 eV, EI): *m*/*z* (%): 221 ($M^{+\cdot}$, 76%), 190 (M^+–$NHNH_2$, 15%), 162 (M^+–$CONHNH_2$, 83%), 149 (M^+–$CH_2CONHNH_2$, 40%), 134 (2-benz., 100%), 107 ($C_7H_5NO_2$, 16%), 91 (C_6H_5N, 30%) and 77 (C_6H_5, 28%). Anal. Calcd for $C_{10}H_{11}N_3O_3$: C, 54.29; H, 5.01; N, 19.00. Found: C, 53.88; H, 5.11; N, 18.71.

4.1.4. General procedure for the preparation of thiosemicarbazide derivatives (5a–5d). About 1.547 g (7 mmol) of **4** and 7 mmol appropriate isothiocyanate derivative were refluxed for 4 h in 5 mL DMF and 25 mL ethanol mixture. The reaction mixture was then cooled and poured into ice water. The solid precipitated was filtered off and recrystallized from appropriate solvents.

4.1.4.1. 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-methylthiosemicarbazide (5a). Derivative **5a** was obtained by the reaction of **4** with methylisothiocynate as a white solid substance with a yield of 63% and recrystallized from methanol–ether (4/1). Mp 206–207 °C. UV (CH_3OH) nm: 203 (log ε: 4.67), 241 (log ε: 4.12) and 278 (log ε: 3.58); IR (KBr) cm^{-1}: 3320, 3154, 2940, 1760, 1681, 1344; ^{1} H NMR (300 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.3 (s, 3H, –C*H*$_3$), 2.9 (s, 3H, –NH–C*H*$_3$), 4.5 (s, 2H, –N–C*H*$_2$–CO), 6.9 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 6.99 (s, 1H, 2-benz.-*H*$_4$), 7.2 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 8.1 (s, 1H, –CS–N*H*–CH_3), 9.4 (s, 1H, –NH–N*H*–CS), 10.2 (bs, 1H, –CO–N*H*–NH); ^{13}C NMR (400 mHz, DMSO-d_6) δ (ppm); 21.5 (*C*H_3), 31.3 (NH–*C*H_3), 43.4 (N–*C*H_2), 109.7, 110.5, 123.1, 131.6, 133.8, 140.4 (Arom-C.), 154.7 (*C*=O), 166.3 (*C*=S), 182.7 (CH_2–*C*O–NH); MS (70 eV, EI): *m*/*z* (%): 294 ($M^{+\cdot}$, 74%), 276 (M^+-H_2O, 29%), 221 (M^+-S=$CNHCH_3$, 33%), 206 (M^+–$NHCSNHCH_3$, 7%), 190 (M^+–$NHNHCSNHCH_3$, 9%), 162 (M^+–$CONHNHCSNHCH_3$, 100%), 149 (5-methyl-2-benz., 46%), 134 (2-benz., 28%), 107 ($C_7H_5NO_2$, 9%), 91 (C_6H_5N, 51%), 77 (C_6H_5, 16%) and 74 (S=$CNHCH_3$, 37%). Calculated for $C_{12}H_{14}N_4O_3S$: C, 48.97; H, 4.79; N, 19.04. Found: C, 48.82; H, 4.88; N, 18.53.

4.1.4.2. 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-ethylthiosemicarbazide (5b). Derivative **5a** was obtained by the reaction of **4** with ethylisothiocynate as a white solid substance with a yield of 60% and recrystallized from acetone–water (4/1). Mp 200–202 °C. UV (CH_3OH) nm: 203 (log ε: 4.73), 245 (log ε: 4.2) and 278 (log ε: 3.73); IR (KBr) cm^{-1}; 3307, 3171, 2982, 2933, 1763, 1683, 1343; ^{1}H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 1.1 (t, 3H, –CH_2–C*H*$_3$), 2.3 (s, 3H, –C*H*$_3$), 3.5 (q, 2H, –NH–C*H*$_2$–CH_3), 4.6 (s, 2H, –N–C*H*$_2$–CO), 6.96 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.02 (s, 1H, 2-benz.-*H*$_4$), 7.23 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 8.0 (t, 1H, –CS–N*H*–CH_2), 9.3 (s, 1H, –NH–N*H*–CS), 10.2 (bs, 1H, –CO–N*H*–NH); MS (70 eV, EI): *m*/*z* (%): 308 ($M^{+\cdot}$, 32%), 290 (M^+-H_2O, 72%), 221 (M^+-S=$CNHCH_2CH_3$, 28%), 206 (M^+–$NHCSNHCH_2CH_3$, 17%), 190 (M^+–$NHNHCSNHCH_2CH_3$, 8%), 162 (M^+–$CONHNHCSNHCH_2CH_3$, 97%), 149 (5-methyl-2-benz., 97%), 142 (M^+–$C_8H_8NO_3$, 100%), 134 (2-benz., 26%), 107 ($C_7H_5NO_2$, 10%), 91 (C_6H_5N, 67%), 88 (S=$CNHCH_2CH_3$, 22%), 77 (C_6H_5, 22%) and 44 ($NHCH_2CH_3$, 31%). Anal. Calcd for $C_{13}H_{16}N_4O_3S$: C, 50.64; H, 5.23; N, 18.17. Found: C, 50.17; H, 5.20; N, 17.99.

4.1.4.3. 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-allylthiosemicarbazide (5c). Derivative **5c** was obtained by the reaction of **4** with allylisothiocynate as a white solid substance with a yield of 36% and recrystallized from ethanol–water (3/1). Mp 193–194 °C. UV (CH_3OH) nm: 203 (log ε: 4.66), 246 (log ε: 4.06) and 278 (log ε: 3.57); IR (KBr) cm^{-1}: 3305, 3162, 3015, 2989, 2925, 1759, 1681, 1343; ^{1}H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.34 (s, 3H, –C*H*$_3$), 4.15 (t, 2H, –NH–C*H*$_2$–CH=), 4.57 (s, 2H, –N–C*H*$_2$–CO), 5.06 (d, 1H, –CH_X=C*H*$_A$H$_B$, H$_A$, J_{AX}: 10), 5.13 (d, 1H, –CH_X=CH_A*H*$_B$, H$_B$, J_{BX}: 17), 5.8–5.94 (m, 1H, –CH_2–C*H*$_X$=CH_AH$_B$), 6.96 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.0 (s, 1H, 2-benz.-*H*$_4$), 7.23 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 8.2 (t, 1H, –CS–N*H*–CH_2), 9.4 (s, 1H, –NH–N*H*–CS), 10.25 (bs, 1H, –CO–N*H*–NH); MS (70 eV, EI): *m*/*z* (%): 320 ($M^{+\cdot}$, 0.9%), 302 (M^+-H_2O, 17%), 221 (M^+-S=$CNHCH_2CH$=CH_2, 38%), 206 (M^+–NHCSNHCH$_2$CH=CH_2, 35%), 190 (M^+–$NHNHCSNHCH_2CH$=CH_2, 10%), 162 (M^+–$CONHNHCSNHCH_2CH$=CH_2, 100%), 149 (5-methyl-2-benz., 43%), 134 (2-benz., 61%), 107 ($C_7H_5NO_2$, 12%), 99 (S=C=NCH_2CH=CH_2, 20%), 91 (C_6H_5N, 53%), 77 (C_6H_5, 22%), 56 ($NHCH_2CH$=CH_2, 27%) and 41 (CH_2CH=CH_2, 46%). Anal. Calcd for $C_{14}H_{16}N_4O_3S$: C, 52.49; H, 5.03; N, 17.49. Found: C, 52.32; H, 5.21; N, 17.28.

4.1.4.4. 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-phenylthiosemicarbazide (5d). Derivative **5d** was obtained by the reaction of **4** with phenylisothiocynate as a white solid substance with a yield of 59% and recrystallized from acetonitrile–water (4/1). Mp 201–202 °C.UV (CH_3OH) nm: 203 (log ε: 4.82) and 273 (log ε: 4.22); IR (KBr) cm^{-1}: 3260, 3162, 3018, 2970, 1756, 1679, 1343; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.3 (s, 3H, –C*H*$_3$), 4.6 (s, 2H, –N–C*H*$_2$–CO), 6.96 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.06 (s, 1H, 2-benz.-*H*$_4$), 7.2–7.5 (m, 6H, 2-benz.-*H*$_7$ and arom-H), 9.7 (s, 2H, –NH–N*H*–CS–N*H*), 10.5 (bs, 1H, –CO–N*H*–NH); **MS** (70 eV, EI): *m/z* (%): 356 ($M^{+\cdot}$, 0.9%), 338 (M^+-H_2O, 3%), 221 (M^+-S=CNHC_6H_5, 55%), 206 (M^+–NHCSNHC_6H_5, 4%), 190 (M^+–NHNHCSNHC_6H_5, 13%), 162 (M^+–CONHNHCSNHC_6H_5, 93%), 149 (5-methyl-2-benz., 36%), 135 (S=C=NC_6H_5, 100%), 134 (2-benz., 21%), 107 ($C_7H_5NO_2$, 5%), 91 (C_6H_5N, 61%) and 77 (C_6H_5, 73%). Anal. Calcd for $C_{17}H_{16}N_4O_3S$: C, 57.29; H, 4.52; N, 15.72. Found C, 57.72; H, 4.73; N, 15.65.

4.1.5. General procedure for the preparation of 1,2,4-triazole derivatives (6a–6d). A 15 mL ethanolic solution of 1 mmol 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-substituted thiosemicarbazide was refluxed with 15 drops of triethylamine for 6 h. The solvent was evaporated and the precipitated product was recrystallized from appropriate solvents.

4.1.5.1. 3-[(5-Methyl-2-benzoxazolinone-3-yl)methyl]-4-methyl-1H-1,2,4-triazole- 5(4*H*)-thione (6a). Derivative **6a** was obtained by the reaction of **5a** as a yellow solid substance with a yield of 82% and recrystallized from acetone–water (3/1). Mp 222–224 °C. UV (CH_3OH) nm: 202 (log ε: 4.55), 231 (log ε: 4.16) and 254 (log ε: 3.85); IR (KBr) cm^{-1}: 3106, 3050, 2927, 1744, 1330; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.3 (s, 3H, –C*H*$_3$), 3.5 (s, 3H, N–C*H*$_3$), 5.2 (s, 2H, –N–C*H*$_2$–CO), 6.97 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.1 (s, 1H, 2-benz.-*H*$_4$), 7.25 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 13.7 (bs, 1H, =N–N*H*–CS); **MS** (70 eV, EI): *m/z* (%): 276 ($M^{+\cdot}$, 73%), 162 (M^+-4-methyl-1*H*-1,2,4-triazole, 4%), 149 (5-methyl-2-benz., 100%), 134 (2-benz., 4%), 128 (M^+-5-methyl-2-benz., 54%), 91 (C_6H_5N, 10%) and 74 (S=CNHCH_3, 15%). Calculated for $C_{12}H_{12}N_4O_2S.H_2O$: C, 48.97; H, 4.79; N, 19.04. Found: C, 49.37; H, 4.54; N, 19.46.

4.1.5.2. 3-[(5-Methyl-2-benzoxazolinone-3-yl)methyl]-4-ethyl-1H-1,2,4-triazole-5(4*H*)-thione (6b). Derivative **6b** was obtained by the reaction of **5b** as a white solid substance with a yield of 97% and recrystallized from acetone–water (3/1). Mp 215–216 °C. UV (CH_3OH) nm: 202 (log ε: 4.54) and 256 (log ε: 4.17); IR (KBr) cm^{-1}: 3483, 3383, 2937, 1740, 1350; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 1.19 (t, 3H, –CH$_2$–C*H*$_3$), 2.33 (s, 3H, –C*H*$_3$), 4.05 (q, 2H, N–C*H*$_2$–CH$_3$), 5.24 (s, 2H, –N–C*H*$_2$–CO), 6.97 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.1 (s, 1H, 2-benz.-*H*$_4$), 7.26 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 13.75 (bs, 1H, =N–N*H*–CS); MS (70 eV, EI): *m/z* (%): 290 ($M^{+\cdot}$, 100%), 262 (M^+–C_2H_5, 12%), 162 (M^+-4-ethyl-1*H*-1,2,4-triazole, 5%), 149 (5-methyl-2-benz., 88%), 142 (M^+-5-methyl-2-benz., 36%), 134 (2-benz., 8%), 91 (C_6H_5N, 10%) and 77 (C_6H_5, 7%). Calculated for $C_{13}H_{14}N_4O_2S.H_2O$: C, 50.64; H, 5.23; N, 18.17. Found: C, 50.21; H, 5.38; N, 18.05.

4.1.5.3. 3-[(5-Methyl-2-benzoxazolinone-3-yl)methyl]-4-allyl-1H-1,2,4-triazole-5(4*H*)-thione (6c). Derivative **6c** was obtained by the reaction of **5c** as a white solid substance with a yield of 88% and recrystallized from acetone–water (3/1). Mp 202–204 °C. UV (CH_3OH) nm: 202 (log ε : 4.58) and 257 (log ε : 4.21); IR (KBr) cm^{-1}: 3087, 3036, 2943, 1787, 1351; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 4.70 (d, 2H, –N–C*H*$_2$–CH=; *J* 4.88), 4.81 (dd, 1H, –CH$_X$=CH$_A$*H*$_B$; H$_B$; J_{AB}: 1, J_{BX}: 18), 5.06 (dd, 1H, –CH$_X$=C*H*$_A$H$_B$; H$_A$; J_{AB}: 1, J_{AX}: 10), 5.15 (s, 2H, –N–C*H*$_2$–CO), 5.79–5.88 (m, 1H, –CH$_2$–C*H*$_X$=CH$_A$H$_B$), 6.96 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.03 (s, 1H, 2-benz.-*H*$_4$), 7.24 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 13.85 (bs, 1H, =N–N*H*–CS); ^{13} C NMR (400 mHz, DMSO-d_6) δ (ppm); 21.5 (*C*H$_3$), 37.4 (*C*H$_2$–CH=CH$_2$), 45.6 (N–*C*H$_2$-), 117.2 (–CH=*C*H$_2$), 130.8 (–CH$_2$–*C*H=CH$_2$), 109.9, 110.3, 123.5, 131.7, 134.0, 140.4 (Arom-C.), 147.5 (*C*H in triazole ring), 154.2 (*C*=O), 168.3 (*C*=S); MS (70 eV, EI): *m/z* (%): 302 ($M^{+\cdot}$, 100%), 287 (M^+–CH_3, 58%), 261 (M^+–$CH_2CH=CH_2$, 6%), 162 (M^+-4-allyl-1*H*-1,2,4-triazole, 12%), 154 (M^+-5-methyl-2-benz., 20%), 149 (5-methyl-2-benz., 43%), 134 (2-benz., 12%) 91 (C_6H_5N, 13%), 77 (C_6H_5, 9%) and 41 ($CH_2CH=CH_2$, 15%). Anal. Calcd for $C_{14}H_{14}N_4O_2S$: C, 55.61; H, 4.67; N, 18.53. Found C, 55.49; H, 4.95; N, 18.47.

4.1.5.4. 3-[(5-Methyl-2-benzoxazolinone-3-yl)methyl]-4-phenyl-1H-1,2,4-triazole- 5(4*H*)-thione (6d). Derivative **6d** was obtained by the reaction of **5d** as a white solid substance with a yield of 93% and recrystallized from acetone–water (3/1). Mp 263–265 °C. UV (CH_3OH) nm: 203 (log ε: 4.64) and 263 (log ε: 4.08); IR (KBr) cm^{-1}: 3196, 3058, 2957, 2923, 1764, 1348; ^1H NMR (400 mHz, DMSO-d_6) (ppm) (*J* in Hz): 2.31 (s, 3H, –C*H*$_3$), 4.96 (s, 2H, –N–C*H*$_2$–CO), 6.86–7.51 (m, 8H, Arom-H.), 13.98 (bs, 1H, =N–N*H*–CS); MS (70 eV, EI): *m/z* (%): 338 ($M^{+\cdot}$, 17%), 311 (M^+-HCN, 43%), 268 (M^+–CNCS, 40%), 190 (M^+-5-methyl-2-benz., 24%), 162 (M^+-4-phenyl-1*H*-1,2,4-triazole, 88%), 149 (5-methyl-2-benz., 48%), 134 (2-benz., 100%), 91 (C_6H_5N, 34%) and 77 (C_6H_5, 43%). Anal. Calcd for $C_{17}H_{14}N_4O_2S$: C, 60.34; H, 4.17; N, 16.56. Found C, 60.59; H, 4.44; N, 16.68.

4.1.6. General procedure for the preparation of 1,3,4-thiadiazole derivatives (7a–7c). About 1.25 mmol 1-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-substitutedthiosemicarbazide derivative was added to 4 mL orthophosphoric acid at 95–100 °C by mixing for 30 min. After addition is finished, the reaction mixture was heated for 45 min and later poured into ice water. The solid precipitated was filtered off and recrystallized from appropriate solvents.

4.1.6.1. 2-Methylamino-5-[(5-methyl-2-benzoxazolinone-3-yl)methyl]-1,3,4-thiadiazole (7a). Derivative **7a** was obtained by the reaction of **5a** as a yellow solid substance with a yield of 47% and recrystallized from ethanol–water

(3/1). Mp 193–195 °C. UV (CH_3OH) nm: 202 (log ε: 4.49) and 275 (log ε: 3.89); IR (KBr) cm^{-1}: 2816, 1776; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.34 (s, 3H, –C*H*$_3$), 2.84 (s, 3H, NH–C*H*$_3$), 5.23 (s, 2H, –N–C*H*$_2$–CO), 6.97 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.11 (s, 1H, 2-benz.-*H*$_4$), 7.25 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 7.72 (bs, 1H, –N*H*–CH_3); MS (70 eV, EI): *m*/*z* (%): 276 ($M^{+\cdot}$, 79%), 162 (M^+-2-methylamino-1,3,4-thiadiazole, 7%), 149 (5-methyl-2-benz., 22%), 128 (M^+-5-methyl-2-benz., 100%), 91 (C_6H_5N, 11%) and 74 (S=CNHCH$_3$, 24%). Anal. Calcd for $C_{12}H_{12}N_4O_2S.HPO_4$: C, 38.71; H, 3.52; N, 15.05. Found: C, 38.58; H, 4.27; N, 14.93.

4.1.6.2. 2-Ethylamino-5-[(5-methyl-2-benzoxazolinone-3-yl)methyl]-1,3,4-thiadiazole (7b). Derivative **7b** was obtained by the reaction of **5b** as a white solid substance with a yield of 74% and recrystallized from acetone–water (3/1). Mp 188–189 °C. UV (CH_3OH) nm: 202 (log ε: 4.48) and 275 (log ε: 3.92; **IR** (KBr) cm^{-1}: 3195, 2976, 2883, 1781; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 1.14 (t, 3H, –CH_2–C*H*$_3$), 2.34 (s, 3H, –C*H*$_3$), 3.26 (2H; q; –NH–C*H*$_2$–CH_3), 5.23 (s, 2H, –N–C*H*$_2$–CO), 6.97 (d, 1H, 2-benz.-*H*$_6$, *J* 8), 7.12 (s, 1H, 2-benz.-*H*$_4$), 7.25 (d, 1H, 2-benz.-*H*$_7$, *J* 8), 7.91 (bs, 1H, –N*H*–CH_2–CH_3); ^{13}C NMR (400 mHz, DMSO-d_6) δ (ppm); 14.6 (CH_2–*C*H$_3$), 21.5 (*C*H$_3$), 109.9, 110.3, 123.5, 130.7, 134.0, 140.5 (Arom-C.), 151.5 (CH_2–*C*=N in thiadiaozle ring), 154.1 (*C*=O), 169.9 (N=*C*–NH); MS (70 eV, EI): *m*/*z* (%): 290 ($M^{+\cdot}$, 98%), 162 (M^+-2-ethylamino-1,3,4-thiadiazole, 12%), 149 (5-methyl-2-benz., 7%), 142 (M^+-5-methyl-2-benz., 100%), 91 (C_6H_5N, 18%), 88 (S=CNHCH$_2$CH$_3$, 24%) and 77 (C_6H_5, 9%). Anal. Calcd for $C_{13}H_{14}N_4O_2S$: C, 53.78; H, 4.86; N, 19.30. Found: C, 53.00; H, 4.99; N, 19.19.

4.1.6.3. 2-Phenylamino-5-[(5-methyl-2-benzoxazolinone-3-yl)methyl]-1,3,4-thiadiazole (7c). Derivative **7c** was obtained by the reaction of **5d** as a white solid substance with a yield of 93% and recrystallized from acetone–water (3/1). Mp 195–197 °C. UV (CH_3OH) nm: 203 (log ε: 4.62), 230 (log ε: 4.21) and 285 (log ε: 3.94); IR (KBr) cm^{-1}: 3200, 3050, 2924, 2828, 1769; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.34 (s, 3H, –C*H*$_3$), 5.35 (s, 2H, –N–C*H*$_2$–CO), 6.96–7.59 (m, 8H, Arom-H.), 10.39 (bs, 1H, –N*H*–C_6H_5); MS (70 eV, EI): *m*/*z* (%): 338 ($M^{+\cdot}$, 32%), 190 (M^+-5-methyl-2-benz., 100%), 162 (M^+-2-phenylamino-1,3,4-thiadiazole, 50%), 149 (5-methyl-2-benz., 19%), 136 (S=CNHC$_6$H$_5$, 24%), 134 (2-benz., 14%), 91 (C_6H_5N, 63%) and 77 (C_6H_5, 55%). Anal. Calcd for $C_{17}H_{14}N_4O_2S$: C, 60.34; H, 4.17; N, 16.56. Found C, 59.57; H, 4.39; N, 16.54.

4.1.7. General procedure for the preparation of benzylidenehydrazine derivatives (8a–8e). A 40 mL ethanolic solution of 1 mmol **1** was refluxed with 1 mmol of substituted benzaldehyde for 30 h. The reaction mixture was then cooled and the solid precipitated was recrystallized from appropriate solvents.

4.1.7.1. 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-benzylidenehydrazine (8a). Derivative **8a** was obtained by the reaction of **4** and benzaldehyde as a white solid substance with a yield of 80% and recrystallized from acetone–water (3/1). Mp 241–243 °C. UV (CH_3OH) nm: 203 (log ε: 4.62), 217 (log ε: 4.38) and 281 (log ε: 4.35; IR (KBr) cm^{-1}: 3337, 3086, 2982, 1774, 1682; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 4.85 and 5.05 (s, 2H, –N–C*H*$_2$–CO), 6.81–7.77 (m, 8H, Arom-H.), 8.07 and 8.25 (s, 1H, N=C*H*), 11.80 (bs, 1H, –CO–N*H*–N=); MS (70 eV, EI): *m*/*z* (%): 309 ($M^{+\cdot}$, 68%), 206 (M^+–N≡C–C_6H_5, 18%), 189 (M^+–NHN=CH–C_6H_5, 10%), 162 (M^+-O≡CNHN=CH–C_6H_5, 50%), 161 (CH_2CONHN=CH–C_6H_5, 23%), 147 (O≡C–NHN=CH–C_6H_5, 100%), 134 (2-benz., 32%), 91 (C_6H_5N, 60%) and 77 (C_6H_5, 23%). Anal. Calcd for $C_{17}H_{15}N_3O_3$: C, 66.01; H, 4.89; N, 13.58. Found: C, 65.76; H, 4.80; N, 13.58.

4.1.7.2. 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-bromobenzylidenehydrazine (8b). Derivative **8b** was obtained by the reaction of **4** and 4-bromobenzaldehyde as a white solid substance with a yield of 80% and recrystallized from acetone–water (3/1). Mp 261–262 °C (dec.). UV (CH_3OH) nm: 202 (log ε: 4.60), 220 (log ε: 4.44) and 286 (log ε: 4.30); IR (KBr) cm^{-1}: 3095, 2980, 2948, 1774, 1683; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 4.62 and 5.05 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.73 (m, 7H, Arom-H.), 8.04 and 8.22 (s, 1H, N=C*H*), 11.87 (bs, 1H, –CO–N*H*–N=); ^{13}C NMR (400 mHz, DMSO-d_6) δ (ppm); 21.5 (*C*H$_3$), 43.5 (*C*H$_2$), 109.7, 110.5, 123.0, 123.8, 129.4, 132.0, 132.3, 133.7, 133.9, 140.5 (Arom-C.), 143.7 (N=*C*H), 154.9 (*C*=O), 167.9 (CH_2–*C*O–NH); MS (70 eV, EI): *m*/*z* (%): 387 ($M^{+\cdot}$, 10%), 389 (M+2, 11%), 225 (O≡CNHN=CH–C_6H_4Br, 18%), 221 (M^+–CH–C_6H_4Br, 70%), 206 (M^+–N≡C–C_6H_4Br, 9%), 190 (M^+–NHN=CH–C_6H_4Br, 13%), 162 (M^+-O≡CNHN=CH–C_6H_4Br, 88%), 149 (5-methyl-2-benz., 39%), 134 (2-benz., 100%), 107 ($C_7H_5NO_2$, 17%), 91 (C_6H_5N, 43%) and 77 (C_6H_5, 24%). Anal. Calcd for $C_{17}H_{14}N_3O_3Br$: C, 52.60; H, 3.63; N, 10.82. Found C, 52.62; H, 3.93; N, 10.97.

4.1.7.3. 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-chlorobenzylidenehydrazine (8c). Derivative **8c** was obtained by the reaction of **4** and 4-chlorobenzaldehyde as a white solid substance with a yield of 82% and recrystallized from acetone–water (3/1). Mp 264–265 °C (dec). UV (CH_3OH) nm: 202 (log ε: 4.64), 220 (log ε: 4.49) and 285 (log ε: 4.37); IR (KBr) cm^{-1}: 3184, 3096, 2980, 1774, 1683; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 4.63 and 5.05 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.80 (m, 7H, Arom-H.), 8.06 and 8.24 (s, 1H, N=C*H*), 11.87 (bs, 1H, –CO–N*H*–N=); MS (70 eV, EI): *m*/*z* (%): 343 ($M^{+\cdot}$, 27%), 345 (M+2, 9%), 221 (M^+–CH–C_6H_4Cl, 38%), 206 (M^+–N≡C–C_6H_4Cl, 10%), 190 (M^+–NHN=CH–C_6H_4Cl, 9%), 181 (O≡CNHN=CH–C_6H_4Cl, 31%), 162 (M^+-O≡CNHN=CH–C_6H_4Cl, 44%), 149 (5-methyl-2-benz., 28%), 134 (2-benz., 100%), 107 ($C_7H_5NO_2$, 15%), 91 (C_6H_5N, 20%) and 77 (C_6H_5, 18%). Anal. Calcd for $C_{17}H_{14}N_3O_3Cl$: C, 59.40; H, 4.10; N, 12.22. Found C, 58.98; H, 3.83; N, 12.33.

4.1.7.4. 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-methylbenzylidenehydrazine (8d). Derivative **8d** was obtained by the reaction of **4** and 4-methylbenzaldehyde

as a white solid substance with a yield of 84% and recrystallized from acetone–water (3/1). Mp 265–266 °C (dec.). UV (CH_3OH) nm: 202 (log ε: 4.63), 220 (log ε: 4.43) and 285 (log ε: 4.39); IR (KBr) cm^{-1}: 3192, 3092, 2981, 1773, 1681; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 4.60 and 5.03 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.66 (m, 7H, Arom-H.), 8.03 and 8.20 (s, 1H, N=C*H*), 11.73 (bs, 1H, –CO–N*H*–N=); MS (70 eV, EI): *m/z* (%): 323 ($M^{+\cdot}$, 40%), 206 (M^+–N≡C–$C_6H_4CH_3$, 15%), 162 (M^+-O≡CNHN=CH–$C_6H_4CH_3$, 39%), 161 (O≡CNHN=CH–$C_6H_4CH_3$, 100%), 134 (2-benz., 22%), 118 (N=CH–$C_6H_4CH_3$, 18%), 91 (C_6H_5N, 44%) and 77 (C_6H_5, 14%). Anal. Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.41; H, 4.99; N, 13.11.

4.1.7.5. 2-[2-(5-Methyl-2-benzoxazolinone-3-yl)acetyl]-4-methoxybenzylidenehydrazine (8e). Derivative **8e** was obtained by the reaction of **4** and 4-methoxybenzaldehyde as a white solid substance with a yield of 85% and recrystallized from acetone–water (3/1). Mp 254–256 °C (dec.). UV (CH_3OH) nm: 202 (log ε: 4.55), 222 (log ε: 4.34) and 287 (log ε: 4.27); IR (KBr) cm^{-1}: 3194, 3097, 2982, 1773, 1683; ^1H NMR (400 mHz, DMSO-d_6) δ (ppm) (*J* in Hz): 2.33 (s, 3H, –C*H*$_3$), 3.82 (s, 3H, -OC*H*$_3$), 4.60 and 5.02 (s, 2H, –N–C*H*$_2$–CO), 6.94–7.71 (m, 7H, Arom-H.), 8.00 and 8.18 (s, 1H, N=C*H*), 11.67 (bs, 1H, –CO–N*H*–N=); MS (70 eV, EI): *m/z* (%): 339 ($M^{+\cdot}$, 29%), 206 (M^+–N≡C–$C_6H_4OCH_3$, 12%), 177 (O≡CNHN=CH–$C_6H_4OCH_3$, 100%), 162 (M^+-O≡CNHN=CH–$C_6H_4OCH_3$, 24%), 149 (5-methyl-2-benz., 8%), 134 (2-benz., 29%), 91 (C_6H_5N, 39%) and 77 (C_6H_5, 17%). Anal. Calcd for $C_{18}H_{17}N_3O_4$: C, 63.71; H, 5.05; N, 12.38. Found C, 63.36; H, 5.05; N, 12.39.

4.2. Single crystal X-ray crystallographic data of compound 6b

The data collection was performed on a STOE IPDS 2 diffractometer employing graphite-monochromated MoK_α radiation (λ = 0.71073 Å). H atoms were located geometrically and treated using a riding model, with C-H distances of 0.93 Å (aromatic), 0.97 Å (CH_2) and 0.96 Å (CH_3). *Data collection:* X-AREA[65]; cell refinement: X-AREA; data reduction: X-RED32[65]; program(s) used to solve structure: SHELXS97[66]; program(s) used to refine structure: SHELXL97[67]; software used to prepare material for publication: WinGX[68] and PARST.[69] Crystallographic data (excluding structure factors) for the title compound reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 635224. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223-336033 or e-mail: deposit@ccdc.cam.ac.uk).

4.3. Pharmacology

4.3.1. Animals. Before treatments, the animals were acclimatized to animal lab conditions for two days and were fed on standard pellet diet and water ad libitum. On the day before the treatments, the food was withdrawn, but the animals were allowed free access to water. A minimum of five animals was used in each group, otherwise it is described in the procedure. Mice used were cared in accordance with the directory of Refiksaydam Hıfzıssıhha Institute's Animal Care Unit, where the Guidelines of National Institutes of Health on Laboratory Animal Welfare were applied.

4.3.2. Preparation of test samples for bioassay. After suspending in 0.5% sodium carboxymethyl cellulose (CMC) and distilled water, test samples were given to test animals orally. The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the reference drug, either indomethacin (10 mg/kg), or acetyl salicylic acid (ASA) (200 mg/kg).

4.3.3. Analgesic activity

4.3.3.1. Koster test.[60] One hour after the oral administration of test sample, each mouse was injected with 3% (w/v) acetic acid solution (0.1 ml/10 g body weight) intraperitoneally. Starting 5 min after the acetic acid injection, the number of muscular contractions on mice was counted for a period of 10 min. A significant reduction in the number of writhings by any treatment as compared to control animals was considered as a positive analgesic response. The antinociceptive activity was expressed as percentage change from writhing controls. Percent inhibitory effects were estimated according to the following equation, where *n* was the average difference in thickness between the left and right hind paw of control group and *n*′ was that of test group of animals.

$$\text{Inhibition}(\%) = [(n - n')/n] \times 100$$

4.3.3.2. Constant temperature hot-plate test.[61] An HTC Inc. Mod. 35-D analgesiameter was set to give a plate temperature of 54 ± 0.5 °C. One hour after oral administration of the compounds (100 mg/kg), the animals were placed on the hot-plate and confined by a lidded perspex box in a compartment measuring 13.8 x 13.8 cm, and the latency to the first hind-paw lick was recorded. If no hind-paw lick occurred, the test was terminated after 30 s. Morphine was used as a reference analgesic and administered i.p. at 10 mg/kg.

4.3.4. Anti-inflammatory activity

4.3.4.1. Carrageenan induced oedema.[62] For the determination of the effects of samples on carrageenan-induced paw oedema, the modified method of Kasahara et al. was employed.[40] One hour after the oral administration of either test sample or the control, the subplantar tissue of the right hind paw of each mouse was injected with freshly prepared (0.5 mg/25 μl) suspension of carrageenan (Sigma, St. Louis, Missouri, USA.) in physiological saline (154 mM NaCl). As controls, 25 μl saline solutions were used. After induction of inflammation, paw oedema was measured in every 90 min during 6 h period. Afterwards,

the difference in footpad thickness between the right and left foot of each mouse was measured with a pair of dial thickness gauge callipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods.

4.3.5. Gastric–ulcerogenic effect. Six hours after the treatments of the mice with synthesized compounds at a dose of 200 mg/kg b.w. for anti-inflammatory activity tests they were killed under deep ether anaesthesia and their stomachs were removed. Then the stomach of each mouse was opened through great curvature and examined for lesions or bleedings under dissecting microscope.

4.3.6. Statistical analysis of data. Data obtained from animal experiments were expressed as means ± standard error (SEM). Statistical differences between the treatment and the control group of animals were evaluated by two tailed Student's *t* test.

4.4. Microbiology

4.4.1. In vitro antimicrobial activity. Minimal inhibitory concentrations (MICs, ug/ml) were determined on different microorganisms using broth microdilution procedure according to the recommendations of the National Committee for Clinical Laboratory Standards.[63,64] Minimum inhibitory concentrations (MIC) were defined as the lowest concentration of the compounds that inhibited visible growth of microorganisms after incubation at 35 °C for 24 h for bacteria or 48 h for yeast like fungi. Gram-positive (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis ATCC 29212*) and Gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) bacteria were used as quality control strains. For testing antifungal activities of the compounds, following reference strains were tested: *Candida albicans* ATCC 90028, *Candida crusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019. Bacterial strains were grown in Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA). The inoculum densities were 5×10^5 colony forming units (cfu) in 1 mL for bacteria and $0.5–2.5 \times 10^3$ cfu/mL for fungi. Ampicillin anhydride and fluconazole were used as standard antibiotic powders. Each of the test compounds was dissolved in DMSO and further dilutions were prepared in sterile distilled water. Ampicillin anhydride and Fluconazole were diluted in sterile distilled water. Two fold dilutions of the compounds and standards were prepared as 512–0.5 μg/mL and 64–0.0625 μg/mL concentrations, respectively. After dilution was completed, microorganism suspensions were inoculated into each well of the row. Minimum inhibitory concentrations (MIC) were defined as the lowest concentration of the compounds that inhibited visible growth of microorganisms after incubation at 35 °C for 24 h for bacteria or 48 h for yeast like fungi. MIC values were given as μg/mL. It was determined that the solvent had no antimicrobial activity against any of the test microorganisms.

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