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Original article

Synthesis, analgesic, anti-inflammatory and antimicrobial studies of 2,4-dichloro-5-fluorophenyl containing thiazolotriazoles

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Dedicated to my beloved Professor B. Shivarama Holla on the occasion of his 62nd birthday.

Abstract

A series of 2,4-dichloro-5-fluorophenyl containing thiazolotriazoles (**1**) were synthesized starting from 3-(2,4-dichloro-5-fluorophenyl)-4*H*-1,2,4-triazole-3-thiol (**5**). The newly synthesized compounds were characterized by IR, ^{1}H NMR, mass and elemental analysis. Some of the synthesized compounds were tested for their anti-inflammatory, analgesic and antimicrobial activities. Compounds **8c**, **8d** and **8h** exhibited excellent anti-inflammatory activity whereas compounds **8d**, **8e** and **8h** showed excellent analgesic activity. Compounds **8a**, **8c–e**, and **8h** exhibited good antibacterial activity and compounds **8c**, **8e** and **8h** showed good antifungal activity.

Keywords: 3-(2,4-Dichloro-5-fluorophenyl)-4*H*-1,2,4-triazole-5-thiol; PPA cyclization; Thiazolotriazole; Biological activities

1. Introduction

In recent years active research has been initiated on halogen containing heterocycles, particularly fluorine and chlorine. 2,4-Dichloro-5-fluoroacetophenone is used in the synthesis of drugs like Ciprofloxacin and their analogues [1]. Incorporation of fluorine or CF_3 group into an organic molecule largely enhances the pharmacological properties [2–4] and also could lead to increased lipid solubility, thereby enhancing the rates of absorption and transport of drugs *in vivo*. The replacement of hydrogen or hydroxy group by fluorine is a strategy widely used in drug development to alter biological function [5].

Thiazoles are important class of heterocyclic compounds, found in many potent biologically active molecules such as Fentiazac, Meloxicam (anti-inflammatory agent) [6,7], Nizatidine (antiulcer agent) [8], Sulfatiazol (antimicrobial agent) [9], Ritonavir (antiretroviral drug) [10] and Bleomycine and Tiazofurin (antineoplastic agents) [11]. Thiazole ring is an important pharmacophore [12] and its coupling with other rings could furnish new biologically active compounds. Thiazole containing compounds exhibit a wide range of biological properties, such as anti-inflammatory, antimicrobial [13], antitumor [14], anticonvulsant [15], cardiotonic [16], IMP dehydrogenase inhibiton [17], antithermic, analgesic [18], anticancer [19], fungicidal and insecticidal [20].

1,2,4-Triazoles represent an overwhelming and rapid developing field in modern heterocyclic chemistry. From literature it is predictable that, 1,2,4-triazoles represent important pharmacophores, and play a vital role as medicinal agents. A degree of respectability has been bestowed for 1,2,4-triazole derivatives due to their wide range of biological activities such as antibacterial, antifungal [21], antitubercular [22] and anticancer [23]. Certain 1,2,4-triazoles also find applications in the preparation of photographic plates, polymers, and as analytical agents [24].

Fluorine incorporated heterocycles, thiazoles and triazoles displayed varied pharmacological properties. Since there have been only few reports on dichlorofluorophenyl containing

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thiazolotriazoles, it was contemplated to synthesize them and to pursue their anti-inflammatory, analgesic and antimicrobial activities. The corresponding results are presented in this paper.

2. Chemistry

2,4-Dichloro-5-fluorobenzoyl hydrazide (**3**) was obtained starting from 2,4-dichloro-5-fluoroacetophenone (**1**) according to Refs. [25,26]. 3-(2,4-Dichloro-5-fluorophenyl)-4*H*-1,2,4-triazole-5-thiol (**5**) was prepared according to Ref. [27] in good yield. Substituted phenacyl bromides were prepared according to Ref. [28]. When triazole (**5**) was treated with substituted phenacyl bromides (**6**) in the presence of base in ethanol medium yielded 2-[5-(2,4-dichloro-5-fluorophenyl)-4*H*-1,2,4-triazol-3-yl]thio-1-(substituted phenyl) ethanone (**7**) which on further cyclization in the presence of PPA yielded a series of 2,4-dichloro-5-fluorophenyl containing thiazolotriazoles (**8**). The reaction sequence is outlined in Scheme 1.

3. Results and discussion

In the IR spectrum of compound **8a**, the aromatic C–H stretching frequency was observed at 3098 cm^{-1}. The absorption band at 1618 cm^{-1} is due to the presence of –C=N– stretch of the triazole and thiazole ring system. The C–F stretching frequency was observed at 1098 cm^{-1} and another absorption band at 823 cm^{-1} due the C–Cl stretching.

The ^{1}H NMR spectrum of **8a**, a singlet at δ 3.96 was due to the O–CH_3 protons. The four aromatic protons of *p*-anisyl moiety resonated as two doublets at δ 6.93 and 7.11 ($J = 8.2$ Hz), respectively. The C_3 (H–F_{meta}) and C_6 (H–F_{ortho}) protons of 2,4-dichloro-5-fluorophenyl moiety resonated as a mutiplet between δ 7.91 and 8.08. A characteristic singlet was observed at δ 8.19 due to the proton of the thiazole ring. Thiazole proton resonated as a singlet between δ 8.02 and 8.30 for compounds **8b–h**.

Further evidence for the formation of thiazolotriazole (**8a**) was obtained by recording its mass spectrum. The mass spectrum of compound **8a** showed molecular ion peak at *m/z* 393, in conformity with the molecular formula $C_{17}H_{10}Cl_2FN_3OS$. A peak at *m/z* 189 was due to formation of dichlorofluorobenzonitrile cation. The characterization data of thiazolotriazoles (**8a–h**) are given in Table 1.

4. Pharmacological studies

4.1. Anti-inflammatory studies

All compounds were screened for their anti-inflammatory activity by carrageenan induced rat paw oedema (acute-inflammation model) as described by Winter et al. [29]. The anti-inflammatory results revealed that compounds **8c**, **8d** and **8h** exhibited excellent anti-inflammatory activity whereas compound **8a** showed good anti-inflammatory activity compared to that of the standard drug, Indomethacin. Compounds **8b**, **8e–g** showed lesser degree of activity.

4.2. Analgesic studies

All compounds were screened for analgesic activity by hot plate test according to Eddy and Leimbach [30]. The analgesic screening results revealed that compounds **8d**, **8e** and **8h** showed excellent analgesic activity whereas compounds **8a** and **8f** showed moderate to good analgesic activity compared with Pethidine. Compounds **8b**, **8c**, **8g** showed lesser degree of activity.

4.3. Antibacterial studies

The newly prepared compounds were screened for their antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes,* and *Klebsiella pneumoniae* (recultured) bacterial strains by the disc diffusion method [31,32]. Compounds **8a**, **8c–e**, and **8h** exhibited good antibacterial activity against all tested bacterial strains. Compounds **8b**, **8f** and **8g** showed lesser degree of activity.

4.4. Antifungal studies

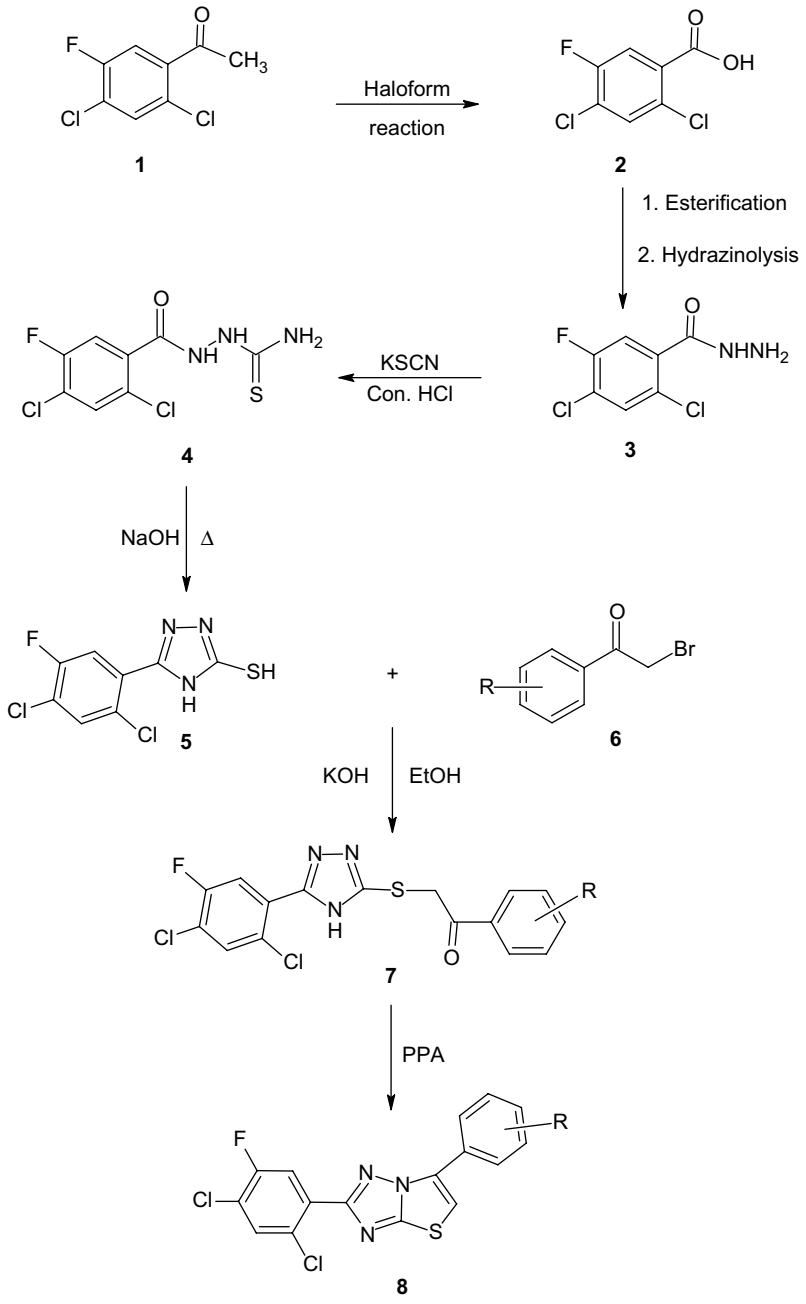
The newly prepared compounds were screened for their antifungal activity against *Aspergillus niger*, *Candida albicans*, *Aspergillus fumigatus*, *Penicillium marneffei*, and *Trichophyton mentagrophytes* (recultured) by agar diffusion method [33,34]. Compounds **8c**, **8e** and **8h** showed good activity against all tested fungal strains. Compounds **8a**, **8b**, **8d**, **8f** and **8g** showed lesser degree of activity.

5. Conclusion

Anti-inflammatory studies revealed that compounds with 4-fluorophenyl, 4-chlorophenyl and 2,4-dichloro-5-fluorophenyl at position 6 of the thiazolotriazole ring system showed excellent anti-inflammatory activity. Analgesic activity studies revealed that compounds with 4-chlorophenyl, 4-bromophenyl and 2,4-dichloro-5-fluorophenyl exhibited good analgesic activity. Compound bearing 4-fluorophenyl, 4-chlorophenyl and 2,4-dichloro-5-fluorophenyl at position 6 of the thiazolotriazole ring system displayed excellent antibacterial activity. Antifungal screening studies revealed that compounds with 4-chlorophenyl and 2,4-dichloro-5-fluorophenyl showed excellent antifungal activity. These inferences are based on preliminary tests, further study in these classes of compounds are in progress.

6. Experimental section

Melting points were determined by open capillary method. The IR spectra (in KBr pellets) were recorded on a Shimadzu FT-IR 157 spectrophotometer. ^{1}H NMR spectra were recorded (in $CDCl_3$/DMSO-d_6) on a Bruker 300/400 MHz NMR spectrometer using TMS as an internal standard. The mass spectra were recorded on a MASPEC/FAB mass spectrometer operating at 70 eV. The purity of



Scheme 1.

the compounds was checked by thin layer chromatography (TLC) on silica gel plates using a mixture of petroleum ether and ethyl acetate (5:5). Iodine was used as visualizing agent.

2,4-Dichloro-5-fluorobenzoic acid (**2**) was obtained from 2,4-dichloro-5-fluoroacetophenone (**1**) according to Ref. [25]. 2,4-Dichloro-5-fluorobenzoyl hydrazide (**3**) was prepared according to Ref. [26]. 5-(2,4-Dichloro-5-fluorophenyl)-4*H*-1,2,4-triazole-3-thiol (**5**) was prepared according to Ref. [27]. Substituted phenacyl bromides (**6**) were prepared according to Ref. [28].

*6.1. General procedure for the preparation of 2-{[5-(2,4-dichloro-5-fluorophenyl)-4H-1,2,4-triazol-3-yl]thio}-1-(substituted phenyl) ethanone (**7a–h**)*

To 0.01 mol of 5-(2,4-dichloro-5-fluorophenyl)-4*H*-1,2,4-triazole-3-thiol (**5**), 0.01 mol of substituted phenacyl bromides (**6**) were added in the presence of 0.015 mol of KOH in absolute ethanol and refluxed for 5 h. The reaction mixture was cooled and poured onto crushed ice. The resulting solid was filtered, dried and recrystallized from a mixture of ethanol

Table 1
Characterization data of thiazolotriazoles (**8a–h**)

Compd. No.	R	M.p. (°C)	Yield (%)	Analysis (%)		
				Found (Calculated)		
				C	H	N
8a	4-OCH_3	180–82	82	51.52 (51.78)	2.41 (2.54)	10.42 (10.66)
8b	4-CH_3	130–32	70	53.59 (53.97)	2.51 (2.65)	10.93 (11.11)
8c	4-F	207–09	68	49.92 (50.26)	1.68 (1.83)	10.80 (10.99)
8d	4-Cl	167–69	78	47.87 (48.18)	1.65 (1.76)	10.36 (10.54)
8e	4-Br	184–86	86	43.07 (43.34)	1.41 (1.58)	9.26 (9.48)
8f	4-NO_2	138–40	65	46.62 (46.94)	1.54 (1.71)	13.45 (13.69)
8g	2,4-Cl_2	189–91	70	44.08 (44.34)	1.14 (1.39)	9.52 (9.70)
8h	2,4-Cl_2-5-F	204–06	72	42.16 (42.57)	1.02 (1.11)	9.18 (9.31)

and dimethylformamide. The yields of compounds (**7a–h**) were between 65 and 90%.

6.2. General procedure for the preparation of 2-(2,4-dichloro-5-fluorophenyl)-6-(4-substituted phenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8a–h*)

To 0.01 mol of 2-{[5-(2,4-dichloro-5-fluorophenyl)-4*H*-1,2,4-triazol-3-yl]thio}-1-(substituted phenyl)ethanone (**7**), ~5 ml of PPA were added and refluxed at 120 °C for 6 h. The reaction mixture was cooled and poured onto crushed ice and neutralized by adding $NaHCO_3$. The resulting solid was filtered, dried and recrystallized from a mixture of ethanol and dimethylformamide.

6.2.1. 2-(2,4-Dichloro-5-fluorophenyl)-6-(4-methylphenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8b*)

$C_{17}H_{10}Cl_2FN_3S$; IR (KBr, ν, cm^{-1}): 3099 (Ar–H str), 2975 (C–H str), 1092 (C–F str), 832 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 2.31 (s, 3H, CH_3), 7.16 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.27 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.86 (d, 1H, dichlorofluorophenyl protons, $J = 6.5$ Hz), 7.98 (d, 1H, dichlorofluorophenyl protons, $J = 9.6$ Hz), 8.15 (s, 1H, thiazole proton); FABMS *m/z*: 378 (M + 1, 35%), 189 (dichlorofluorobenzonitrile cation, 25%).

6.2.2. 2-(2,4-Dichloro-5-fluorophenyl)-6-(4-fluorophenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8c*)

$C_{16}H_7Cl_2F_2N_3S$; IR (KBr, ν, cm^{-1}): 3092 (Ar–H str), 2972 (C–H str), 1112 (C–F str), 827 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 7.43–7.47 (m, 2H, Ar–H), 8.02–8.06 (m, 3H, Ar–H), 8.31–8.35 (m, 2H, dichlorofluorophenyl protons). FABMS *m/z*: 383 (M + 1, 25%).

6.2.3. 6-(4-Chlorophenyl)-2-(2,4-dichloro-5-fluorophenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8d*)

$C_{16}H_7Cl_3FN_3S$; IR (KBr, ν, cm^{-1}): 3090 (Ar–H str), 2965 (C–H str), 1098 (C–F str), 821 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 7.51 (d, 2H, Ar–H, $J = 8.5$ Hz), 7.56 (d, 1H, dichlorofluorophenyl protons, $J = 6.5$ Hz), 7.94 (d, 1H, dichlorofluorophenyl protons, $J = 9.6$ Hz), 8.15 (s, 1H, thiazole proton), 8.21 (d, 2H, Ar–H, $J = 8.5$ Hz); FABMS *m/z*: 399 (M + 1, 36%).

6.2.4. 6-(4-Bromophenyl)-2-(2,4-dichloro-5-fluorophenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8e*)

$C_{16}H_7BrCl_2FN_3S$; IR (KBr, ν, cm^{-1}): 3096 (Ar–H str), 2977(C–H str), 1092 (C–F str), 821 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 7.80 (d, 2H, Ar–H, $J = 8.6$ Hz), 8.01–8.05 (m, 2H, dichlorofluorophenyl protons), 8.12 (s, 1H, thiazole proton), 8.23 (d, 2H, Ar–H, $J = 8.6$ Hz); FABMS *m/z*: 444 (M + 1, 42%).

6.2.5. 2-(2,4-Dichloro-5-fluorophenyl)-6-(4-nitrophenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8f*)

$C_{16}H_7Cl_2FN_4O_2S$; IR (KBr, ν, cm^{-1}): 3089 (Ar–H str), 2965 (C–H str), 1110 (C–F str), 832 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 8.03 (d, 1H, dichlorofluorophenyl protons, $J = 6.8$ Hz), 8.07 (d, 1H, dichlorofluorophenyl protons, $J = 9.8$ Hz), 8.28 (s, 1H, thiazole proton), 8.43 (d, 2H, Ar–H, $J = 8.5$ Hz), 8.58 (d, 2H, Ar–H, $J = 8.5$ Hz); FABMS *m/z*: 410 (M + 1, 25%).

6.2.6. 2-(2,4-Dichloro-5-fluorophenyl)-6-(2,4-dichlorophenyl)-1,3-thiazolo[3,2-b]-1,2,4-triazole (*8g*)

$C_{16}H_6Cl_4FN_3S$; IR (KBr, ν, cm^{-1}): 3092 (Ar–H str), 2985 (C–H str), 1112 (C–F str), 827 (C–Cl str); ^1H NMR (400 MHz DMSO-d_6) δ: 7.94–8.04 (m, 2H, dichlorofluorophenyl and dichlorophenyl protons), 8.09 (s, 1H, thiazole proton), 8.12–8.14 (m, 3H, dichlorofluorophenyl and dichlorophenyl protons).

6.2.7. 2,6-Bis(2,4-dichloro-5-fluorophenyl)[1,3]thiazolo[3,2-b][1,2,4]triazole (*8h*)

$C_{16}H_5Cl_4F_2N_3S$; IR (KBr, ν, cm^{-1}): 3099 (Ar–H str), 2978 (C–H str), 1098 (C–F str), 821 (C–Cl str); ^1H NMR

Table 2
Anti-inflammatory activity data of thiazolotriazoles (**8a–h**)

Compd. No.	Dose (mg/kg, p.o.)	Increase in paw volume in ml	% Inhibition of paw oedema
8a	50	0.381 ± 0.0016	40.6
8b	50	0.653 ± 0.0016	0
8c	50	0.336 ± 0.0023	48.8
8d	50	0.37 ± 0.0031	42.2
8e	50	0.652 ± 0.0020	25
8f	50	0.578 ± 0.0026	10.9
8g	50	0.53 ± 0.0028	17.2
8h	50	0.26 ± 0.0020	59.4
Control	10 ml/kg	0.65 ± 0.0096	–
Standard[a]	2	0.24 ± 0.0014	62.9

[a] Indomethacin is used as the standard; $N = 6$ in each group.

Table 3
Analgesic activity data of thiazolotriazoles (**8a–h**)

Compd. No.	Dose (mg/kg)	Time of reaction to pain stimulus at time (h) [*s*] ± SEM		
		0	1	3
8a	25	7.8	11.1	11.96
8b	25	8.1	8.2	8.4
8c	25	8.4	8.3	8.2
8d	25	8.2	13.2	12.9
8e	25	9.1	14.8	13.9
8f	25	8.1	11.2	10.9
8g	25	8.9	8.96	9.6
8h	25	8.1	14.21	13.6
Control	10	8.25	8.2	8.8
Standard[a]	5	8.5	16.41	14.3

[a] Pethidine is used as the standard, $N = 6$ in each group.

(400 MHz DMSO-d_6) δ: 7.94 (d, 1H, dichlorofluorophenyl protons, $J = 9.8$ Hz), 8.01 (d, 1H, dichlorofluorophenyl protons, $J = 9.8$ Hz), 8.09 (s, 1H, thiazole proton), 8.11–8.14 (m, 2H, dichlorofluorophenyl protons); FABMS *m/z*: 452 (M + 1, 30%).

7. Pharmacological assay

7.1. Anti-inflammatory assay

Wister albino rats of either sex weighing 180–250 g were used for the experiment. They were housed in clean polypropylene cages and kept under room temperature (25 °C), relative humidity (60–70%) in 112 h light-dark cycle. The animals were given standard laboratory diet and water *ad libitum*. Food was withdrawn 12 h before and during experimental hours.

The animals were divided into 10 groups each group contained 6 animals. A mark was made on the hind paw (left) just beyond the tibio–tarsal junction, so that every time the paw was dipped in the mercury column up to fixed mark constant paw volume was ensured. The initial paw volume of each rat was noted by plethysmometrically (Ugo Basile, Italy). First group received normal saline and the second group received Indomethacin orally at a dose of 1.5 mg/kg. The 3rd to 10th groups were administered the test compounds (at a dose of 50 mg/kg suspended in 10 ml/kg of 2% gum acacia) orally.

After 30 min of treatment of test compounds, 0.1 ml of 1% (w/v) carrageenan was injected in the subplantar region of the left hind paw. The right paw served as a reference to non-inflammed paw for comparison. The initial paw volume was measured within 30 s of the injection. The relative increase in paw volume was measured in control, standard and test compounds at 1, 2 and 3 h after the carrageenan injection. The difference between the two readings was taken as the volume of oedema and the percentage inhibition by the drugs was calculated using the formula,

$$\text{Percentage of oedema inhibition} = 100 - (V_{\text{test}}/V_{\text{control}}) \times 100,$$

where V_{control} = volume of paw oedema in control group; V_{test} = volume of paw oedema in the test compounds in treated group. The results were expressed as % inhibition of oedema over the untreated control group. Experiments were approved by the Institutional ethics committee. The results of anti-inflammatory studies are given in Table 2.

7.2. Analgesic assay

Albino mice of either sex with weight between 20 and 25 g were used for analgesic study. The animals were divided in to 10 experimental groups each consisted of 6 animals. Gum Acacia (2%) was administered to group 1. Group 2 received Pethidine at a dose 5 mg/kg by intraperitoneal injection. Other groups were given the test compounds at a dose 25 mg/kg orally. The animals were housed and fed in laboratory kept at constant temperature of 22 °C under standard conditions (12:12 h light-dark cycle, standard pellet diet, tap water). In this test, reaction of mice to painful stimulus was measured. Mice were placed on the metal plate heated to 55 ± 0.4 °C and covered with a glass cylinder (25 cm high, 15 cm in diameter). The time(s) elapsing to the first pain response (licking or jumping) was determined by a stop watch and then recorded as response latency, prior to 60, 180 min following the p.o. administration of the investigated

Table 4
Antibacterial activity data of thiazolotriazoles (**8a–h**)

Compd. No.	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Klebsiella pneumoniae*	*Streptococcus pyogenes*
8a	21 (6.25)	25 (6.25)	30 (6.25)	19 (6.25)	23 (6.25)
8b	15 (6.25)	13 (12.5)	14 (6.25)	15 (6.25)	14 (12.5)
8c	18 (12.5)	23 (6.25)	28 (6.25)	20 (6.25)	21 (6.25)
8d	26 (6.25)	26 (6.25)	31 (6.25)	17 (6.25)	19 (6.25)
8e	21 (6.25)	24 (6.25)	29 (6.25)	19 (6.25)	25 (6.25)
8f	12 (12.5)	15 (6.25)	15 (6.25)	11 (12.5)	12 (12.5)
8g	14 (12.5)	11 (12.5)	13 (12.5)	13 (12.5)	15 (6.25)
8h	23 (6.25)	28 (6.25)	30 (6.25)	19 (6.25)	24 (6.25)
Standard[a]	23(6.25)	29 (6.25)	32 (6.25)	21 (6.25)	25 (6.25)

MIC values are given in brackets. MIC ($\mu g\,cm^{-3}$) = minimum inhibitory concentration, i.e. the lowest concentration to completely inhibit bacterial growth.
[a] Ciprofloxacin is used as the standard, diameter zones of inhibitions in mm.

Table 5
Antifungal activity of data of thiazolotriazoles (**8a–h**)

Compd. No.	*Candida albicans*	*Aspergillus Niger*	*Aspergillus fumigatus*	*Trichophyton mentagrophytes*	*Penicillium marneffei*
8a	17 (6.25)	20 (6.25)	–	14 (12.5)	17 (6.25)
8b	–	11 (12.5)	16 (6.25)	8 (25)	–
8c	19 (6.25)	21 (6.25)	29 (6.25)	17 (6.25)	21 (6.25)
8d	20 (6.25)	24 (6.25)	19 (6.25)	19 (6.25)	19 (6.25)
8e	14 12.5)	9 (25)	12 (12.5)	–	10 (25)
8f	16 (6.25)	–	17 (6.25)	10 (25)	18 (6.25)
8g	10 (25)	14 (12.5)	8 (25)	–	12 (12.5)
8h	18 (6.25)	22 (12.5)	20 (6.25)	18 (6.25)	23 (6.25)
Standard[a]	20 (6.25)	25 (6.25)	21 (6.25)	19 (6.25)	23 (6.25)

Diameter zones of inhibitions in mm. MIC values are given in brackets. MIC ($\mu g\ cm^{-3}$) = minimum inhibitory concentration, i.e. the lowest concentration to completely inhibit fungal growth.
[a] Amphotericin B is used as the standard; – indicates fungus is resistant to the compounds $>100\ \mu g\ cm^{-3}$.

compounds. Institutional ethics committee approved all the experiments. The analgesic screening results are given in Table 3.

7.3. Antibacterial assay

A standard inoculum ($1-2 \times 10^7$ cfu/cm^3 0.5 *McFarland* standards) was introduced onto the surface of sterile agar plates, and a sterile glass spreader was used for even distribution of the inoculum. The discs measuring 6.25 mm in diameter were prepared from Whatman no.1 filter paper and sterilized by dry heat at 140 °C for 1 h. The sterile disc previously soaked in a known concentration of the test compounds were placed in nutrient agar medium. Solvent and growth controls were kept. The plates were inverted and incubated for 24 h at 37 °C. The inhibition zones were measured and compared with the controls. Minimum inhibitory concentration (MIC) was determined by broth dilution technique. The Nutrient Broth, which contained logarithmic serially two-fold diluted amount of test compound and controls were inoculated with approximately 5×10^5 cfu of actively dividing bacteria cells. The cultures were incubated for 24 h at 37 °C and the growth was monitored visually and spectrophotometrically. The lowest concentration (highest dilution) required to arrest the growth of bacteria was regarded as minimum inhibitory concentration (MIC). Ciprofloxacin was used as a standard drug. The diameter of the zone of inhibition and minimum inhibitory concentration values are given in Table 4.

7.4. Antifungal assay

Sabourauds agar media was prepared by dissolving 1 g peptone, 4 g D-glucose, and 2 g agar in 100 cm^3 distilled water, and adjusting pH to 5.7 using buffer. Normal saline was used to make a suspension of spore of fungal strain for lawning. A loopful of particular fungal strain was transferred to 3 cm^3 saline to get a suspension of corresponding species. Agar media (20 cm^3) was poured into each Petri dish. Excess of suspension was decanted and the plates were dried by placing in an incubator at 37 °C for 1 h. Using an agar punch, wells were made and each well was labeled. A control was also prepared in triplicate and maintained at 37 °C for 3–4 days. The inhibition zones in diameter were measured and compared with the controls. The Nutrient Broth, which contained logarithmic serially two-fold diluted amount of test compound and controls was inoculated with approximately $1.6 \times 10^4-6 \times 10^4$ cfu cm^{-3}. The cultures were incubated for 48 h at 35 °C and the growth was monitored. The lowest concentration (highest dilution) required to arrest the growth of fungus was regarded as minimum inhibitory concentrations (MIC). Amphotericin B was used as the standard drug. The diameter of zone of inhibition and minimum inhibitory concentration values are given in Table 5.

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