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Original article

Synthesis and antioxidant activity of 1,3,4-oxadiazole tagged thieno[2,3-*d*] pyrimidine derivatives

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ABSTRACT

This study represents the synthesis of a new series of *N*-substituted phenyl-5-methyl-6-(5-(4-substituted phenyl)-1,3,4-oxadiazol-2-yl)thieno[2,3-*d*]pyrimidin-4-amine derivatives (**4a**–**l**) and substituted phenylamino-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid derivatives (**3a**–**d**). The newly synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, LC–MS and IR analyses. All these novel compounds were screened for their *in vitro* antioxidant activity by employing DPPH, hydrogen peroxide, and nitric oxide radical scavenging assays. Compounds **4k**, **4j**, **4d**, and **4e** showed significant radical scavenging due to the presence of electron donating substituent on both sides of the thienopyrimidine ring enhances the activity and electron withdrawing groups like nitro decrease.

1. Introduction

Several types of pyrimidines and thienopyrimidines have provoked much interest due to their valuable pharmacological properties such as antiviral [1], antioxidant [2] and antimalarial [3,4]. The antioxidants that scavenge reactive oxygen species may be of great value in preventing the onset and propagation of oxidative diseases like autoimmune diseases, cardiovascular diseases, neurovascular diseases [5] and neurodegenerative changes associated with aging [6]. The homeostatic balance between the reactive oxygen species (ROS) and endogenous antioxidants is important in maintaining healthy tissues. Excessive ROS states are important in diseases such as acute respiratory distress syndrome and idiopathic pulmonary fibrosis [7]. Most living organisms possess enzymatic and non enzymatic defense systems against excessive production of the reactive oxygen species. However, different external factors (smoke, diet, alcohol and some drugs) and aging decrease the efficiency of such protecting systems, resulting in disturbances of the redox equilibrium established under healthy conditions [8]. In addition, the pyrimidine based derivatives like thieno[2,3-*d*]pyrimidines have fascinated importance in medicinal chemistry, exhibiting pharmacological and therapeutic properties such as antidepressant [9], antibacterial [10–12], antifungal [13,14], anti-inflammatory [15,16], antiplatelet [17], antihypertensive [18], herbicidal [19] and plant growth regulatory [20] properties.

Furthermore, substituted 1,3,4-oxadiazole derivatives also have been reported to show broad spectrum of biological activities including anti-inflammatory, analgesic and ulcerogenicity [21] antioxidant agents [22]. Hence, the development of physiologically highly potent fused pyrimidines and 1,3,4-oxadiazole substituted fused pyrimidines has been of great interest in the synthesis as well as in the field of medicinal chemistry.

Over view of literature survey, the importance of thieno[2,3-*d*] pyrimidines and 1,3,4-oxadiazoles individually in the biological systems led to assimilate of these two moieties may show synergistic effect. In the present work we report the details of the synthesis of novel 1,3,4-oxadiazole tagged thieno[2,3-*d*]pyrimidine derivatives and *in vitro* antioxidant properties of them.

2. Results and discussion

2.1. Chemistry

Compound ethyl 5-amino-4-cyano-3-methylthiophene-2-carboxylate (**1**) was synthesized *via* single step process of ethyl acetoacetate, malononitrile, elemental sulfur in DMF and catalytic amount of imidazole by the reaction of Gewald method [23]. It was then converted into ethyl (halo substituted phenylamino)-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylate (**2a**–**d**) by cyclization with suitable triethyl orthoformate in the presence of few drops of acetic acid as a catalyst and appropriate substituted anilines to a good yield [24,25]. Compound **2** was treated with aq.

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NaOH and MeOH medium to give 4-(substitutedphenylamino)-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acid derivatives (**3a**–**d**). Further compound **3** on treatment with appropriate substituted arylacidhydrazides in the presence of the $POCl_3$ yielded the title compounds **4a**–**l** in good to excellent yields (Scheme 1). The structures of newly synthesized compounds **3a**–**d** & **4a**–**l** were confirmed on the basis of ^{1}H NMR, ^{13}C NMR, LC–MS and IR spectrometry analysis.

All the synthesized intermediates and titled chemical entities gave satisfactory analyses for the proposed structures on the basis of their spectral data. The formation of intermediates **2**(**a**–**d**) were confirmed by IR spectra which showed characteristic absorption bands in the range between 1551–1562 cm^{-1} and 3417–3428 cm^{-1} due to C=N and NH stretchings respectively and the ^{1}H NMR spectral data showed singlets in the range between at δ 7.39–8.38 ppm and δ 8.57–8.60 ppm for NH and CH groups respectively. Further, the appearance of the broad singlet in the range 13.54–13.58 ppm confirmed the transformation of ester **2**(**a**–**d**) to carboxylic acid **3**(**a**–**d**). The IR spectra of compounds **4a**–**l** revealed characteristic absorption bands at 1522–1556 cm^{-1} for C=N, 1602–1641 cm^{-1} for C=C, 2915–2986 cm^{-1} for thiophene–CH_3 and 3051–3486 cm^{-1} corresponding to NH stretching vibrations. In the ^{1}H NMR spectra displayed peaks in the range δ 3.05–3.28 ppm for –CH_3 of thiophene, δ 8.21–8.92 ppm for pyrimidine–NH, δ 8.60–9.00 ppm for C–H groups, and other prominent peaks also appeared in the aromatic region corresponding to both the phenyl rings which are attached to thienopyrimidine and oxadiazole rings. The ^{13}C NMR and LC–MS spectral data have provided further support for the confirmation of the structures of the synthesized compounds, which are presented in the Experimental section.

3. Pharmacological assay

3.1. Antioxidant testing

Compounds **4a**–**l** are tested for *in vitro* antioxidant property by 1,1-diphenylpicrylhydrazyl (DPPH) [26,27], nitric oxide (NO) [28,29] and hydrogen peroxide (H_2O_2) [30] methods which were summarized in Tables 1–3 respectively. Compounds **4k**, **4j**, **4d** and **4e** showed good radical scavenging activity in all three methods due to the presence of mild electron donating groups such as di-fluoro, fluoro and chloro-fluoro groups attached to the benzene rings when compared with the standard drug ascorbic acid. Compounds **4a**, **4b**, **4g** and **4h** showed moderate antioxidant activity whereas the other compounds **4c**, **4i** and **4f** displayed mild activity. In general, it was observed that halo substituted and unsubstituted compounds **4k**, **4j**, **4d** and **4e** exhibited greater activity when compared with the respective nitro substituted compounds. The IC_{50} value of the standard ascorbic acid in DPPH method was found to be 15.11 μg/ml at 25 μg/ml whereas the IC_{50} values of the compounds **4k**, **4j**, **4d** and **4e** were found to be 16.35, 16.91, 17.25 and 17.70 μg/ml, respectively. Further Tables 1–3 indicate that radical scavenging activity in DPPH, nitric oxide and hydrogen peroxide methods increases with concentration.

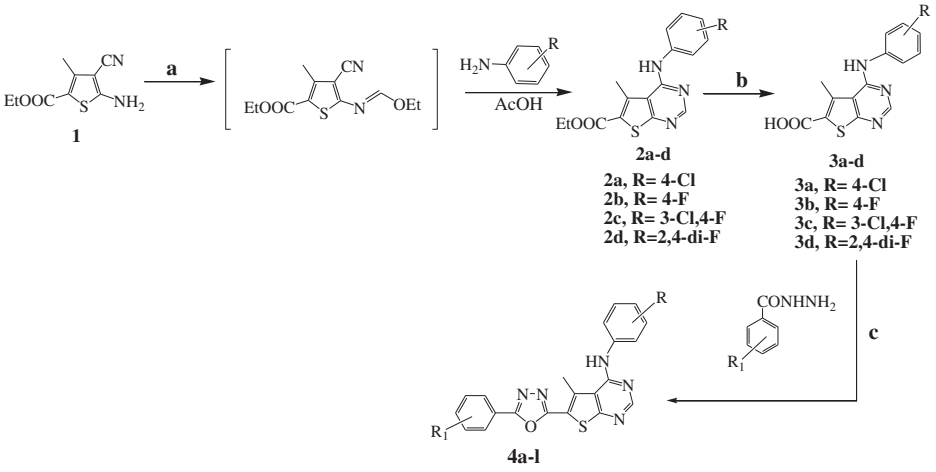
4. Conclusion

In conclusion, a new class of 1,3,4-oxadiazole tagged thieno[2,3-*d*]pyrimidines derivatives were prepared from simple starting material and substituted arylacidhydrazides in good yields and studied for their antioxidant activity. It was observed that the compounds having thieno[2,3-*d*]pyrimidines in combination with 1,3,4-oxadiazoles exhibited greater antioxidant activity. The investigation of antioxidant screening data reveals that among the 12 compounds screened, compounds **4k**, **4j**, **4d** and **4e** showed excellent, almost equivalent to that of standards the remaining compounds showed moderate to mild inhibition activity. The presence of electron donating substituent on both sides of the thienopyrimidine ring enhances the activity and electron withdrawing groups like nitro decrease.

5. Experimental section

5.1. Chemistry

Melting points were determined in open capillaries on a Mel-Temp apparatus and are uncorrected. All the reactions were



4	a	b	c	d	e	f	g	h	i	j	k	l
R	4–Cl	4–Cl	4–Cl	4–F	4–F	4–F	3–Cl, 4–F	3–Cl, 4–F	3–Cl, 4–F	2,4–diF	2,4–diF	2,4–diF
R_1	4–H	4–Cl	4–NO_2	4–H	4–Cl	4–NO_2	4–H	4–Cl	4–NO_2	4–H	4–Cl	4–NO_2

Scheme 1. *Reagents and conditions*: **a**: $HC(OEt)_3$, (5–10 equiv), reflux, 6 h, AcOH, halo substituted anilines, 4 h, reflux; **b**: NaOH, MeOH, RT, 16 h; **c**: $POCl_3$, substituted arylacidhydrazides.

Table 1
The *in vitro* antioxidant activity of **4a**–**l** in DPPH method.

Compound	Concentration (μg/ml)				
	25	50	75	100	IC_{50}
4a	69.32 ± 1.06	71.56 ± 0.87	76.84 ± 1.32	81.26 ± 1.05	18.03 ± 1.07
4b	64.70 ± 1.44	68.41 ± 1.21	73.84 ± 1.56	77.52 ± 0.95	19.31 ± 1.13
4c	52.74 ± 1.73	58.25 ± 1.02	61.37 ± 0.92	67.81 ± 1.60	23.70 ± 1.47
4d	72.46 ± 0.36	75.83 ± 0.60	78.36 ± 0.70	83.13 ± 0.86	17.25 ± 0.52
4e	70.59 ± 0.26	74.21 ± 0.43	77.85 ± 0.65	81.92 ± 0.70	17.70 ± 0.61
4f	59.73 ± 1.17	64.48 ± 1.24	68.94 ± 0.88	72.16 ± 0.95	20.92 ± 0.78
4g	68.49 ± 1.26	72.84 ± 1.41	74.93 ± 1.58	79.25 ± 1.04	18.25 ± 1.10
4h	63.87 ± 0.71	67.93 ± 1.32	71.48 ± 1.29	76.64 ± 1.34	19.57 ± 1.23
4i	48.65 ± 0.60	52.86 ± 1.24	56.63 ± 0.55	61.93 ± 0.82	25.69 ± 1.10
4j	73.89 ± 0.19	77.42 ± 0.45	79.63 ± 0.46	83.85 ± 0.70	16.91 ± 0.60
4k	76.43 ± 0.28	79.66 ± 0.49	83.29 ± 0.61	85.76 ± 0.78	16.35 ± 0.28
4l	64.61 ± 1.05	69.51 ± 1.40	73.92 ± 0.82	77.83 ± 1.63	19.34 ± 1.07
Ascorbic acid	82.68 ± 0.12	83.52 ± 0.38	85.52 ± 0.44	87.22 ± 0.53	15.11 ± 0.44
Blank	–	–	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

monitored by thin layer chromatography (TLC) on precoated silica gel 60 F254 (mesh); spots were visualized with UV light. Merck silica gel (60–120 mesh) was used for column chromatography. The IR spectra were recorded on a Perkin–Elmer BX1 FTIR Spectrophotometer as KBr pellets and the wave numbers were given in cm^{-1}. ^1H NMR (400 MHz), and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker AMX 400 MHz NMR spectrometer in $CDCl_3$/DMSO-d_6 solution using TMS as an internal standard. All chemical shifts are reported in δ (ppm) using TMS as an internal standard. The mass spectra were recorded on Agilent 1100 LC/MSD instrument with method API-ES at 70 eV. The microanalyses were performed on a PerkineElmer 240C elemental analyzer. The antioxidant property was carried out by using Shimadzu UV-2450 spectrophotometer.

*5.1.1. Synthesis of ethyl 5-amino-4-cyano-3-methylthiophene-2-carboxylate (**1**)*

The starting ethyl 5-amino-4-cyano-3-methylthiophene-2-carboxylate (**1**) was prepared according to Gewald synthetic procedure [23].

*5.1.2. General procedure for the synthesis of ethyl 4-(substituted phenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (**2a**–**d**)*

A mixture of ethyl 5-amino-4-cyano-3-methylthiophene-2-carboxylate (**1**) (2.0 g 9.5 mmol) was dissolved in dry toluene as a solvent to this added triethyl orthoformate (4.6 ml, 28.08 mmol) and catalytic amount of AcOH were refluxed for 4 h (the reaction was monitored by TLC). The reaction was then concentrated in vacuo. The obtained residue was dissolved in AcOH as a solvent to this added (13.47 mmol) of appropriate halo substituted anilines and refluxed for 4 h. After completion of the starting materials total reaction mixture was cooled to room temperature for 2 h then poured into ice cold water and stirred for 15 min, the product was separated by filtration and washed with water, dried well and recrystallized from chloroform and *n*-hexane to give compounds **2**(**a**–**d**) in good yield.

*5.1.2.1. Ethyl 4-(4-chlorophenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (**2a**).* Yield 81%; white crystalline solid m.p. = 174–176 °C; IR (KBr) ν (cm^{-1}): 3424 (NH), 2922 (thiophene–CH_3), 1708 (C=O), 1561 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.41 (t, 3H, CH_2–CH_3), 3.10 (s, 3H, thiophene–CH_3), 4.39 (q, 2H, –CH_2–CH_3), 7.36 (d, 2H, Ar–H, J = 8.0 Hz), 7.43 (s, 1H, N–H), 7.62 (d, 2H, Ar–H, J = 8.0 Hz), 8.57 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz); δ 14.26, 15.83, 61.48, 114.93, 117.32, 120.64, 125.86, 134.48, 139.94, 155.13, 157.45, 160.83, 162.25 and 166.18; LC–MS (negative ion mode): m/z 346 $(M - H)^-$ for $C_{16}H_{14}ClN_3O_2S$.

*5.1.2.2. Ethyl 4-(4-fluorophenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylate (**2b**).* Yield 78%; white solid m.p. = 186–188 °C; IR (KBr) ν (cm^{-1}): 3428 (NH), 2920 (thiophene–CH_3), 1706 (C=O), 1562 (C=N); ^1H NMR ($CDCl_3$, 400 MHz) δ 1.50 (t, 3H, CH_2–CH_3), 3.12 (s, 3H, thiophene–CH_3), 4.34 (q, 2H, –CH_2–CH_3), 7.32 (d, 2H, Ar–H, J = 8.0 Hz), 7.48 (s, 1H, N–H), 7.64 (d, 2H, Ar–H, J = 8.0 Hz),

Table 2
The *in vitro* antioxidant activity of **4a**–**l** in nitric oxide (NO) method.

Compound	Concentration (μg/ml)				
	25	50	75	100	IC_{50}
4a	71.26 ± 0.88	74.84 ± 1.07	77.14 ± 1.41	82.93 ± 1.27	17.54 ± 0.95
4b	68.39 ± 0.90	70.61 ± 1.39	74.18 ± 0.95	79.42 ± 1.21	18.27 ± 1.05
4c	60.98 ± 1.52	63.47 ± 1.21	67.56 ± 1.31	72.36 ± 0.71	20.49 ± 0.94
4d	73.06 ± 0.26	78.14 ± 0.45	82.44 ± 0.62	84.87 ± 0.79	17.10 ± 0.91
4e	71.84 ± 0.17	76.29 ± 0.35	79.65 ± 0.54	83.59 ± 0.69	17.39 ± 1.15
4f	62.04 ± 1.41	66.83 ± 1.56	70.92 ± 0.78	73.89 ± 1.07	20.14 ± 0.97
4g	72.90 ± 0.85	74.06 ± 0.94	78.25 ± 1.06	82.36 ± 1.41	17.14 ± 0.56
4h	64.37 ± 1.18	69.22 ± 1.57	74.69 ± 1.41	79.53 ± 0.71	19.41 ± 1.23
4i	53.64 ± 1.39	56.49 ± 1.24	60.03 ± 0.71	64.28 ± 1.08	23.30 ± 1.09
4j	75.23 ± 0.26	79.53 ± 0.37	82.64 ± 0.57	85.44 ± 0.72	16.61 ± 0.56
4k	78.19 ± 0.28	83.28 ± 0.47	84.62 ± 0.61	86.18 ± 0.80	15.98 ± 0.77
4l	63.57 ± 1.32	71.42 ± 1.24	76.22 ± 1.04	78.96 ± 0.79	19.66 ± 1.12
Ascorbic acid	84.72 ± 0.18	85.96 ± 0.36	88.32 ± 0.52	90.41 ± 0.69	14.75 ± 0.58
Blank	–	–	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

Table 3
The *in vitro* antioxidant activity of **4a–l** in hydrogen peroxide (H_2O_2) method.

Compound	Concentration (μg/ml)				
	25	50	75	100	IC_{50}
4a	61.06 ± 0.86	63.84 ± 1.58	68.22 ± 1.07	71.59 ± 1.37	20.47 ± 1.23
4b	58.29 ± 1.12	62.31 ± 1.17	65.74 ± 1.47	69.80 ± 1.55	21.22 ± 1.07
4c	51.37 ± 1.16	54.03 ± 0.86	58.12 ± 0.97	63.72 ± 0.63	24.33 ± 1.04
4d	66.28 ± 1.12	69.53 ± 1.31	71.68 ± 0.60	74.19 ± 0.71	18.85 ± 0.60
4e	64.12 ± 0.27	67.44 ± 0.64	69.81 ± 0.69	72.59 ± 0.80	19.49 ± 0.26
4f	53.08 ± 0.89	56.61 ± 1.39	59.95 ± 0.78	64.20 ± 1.05	23.54 ± 0.42
4g	60.26 ± 1.06	63.48 ± 1.27	67.84 ± 1.57	70.93 ± 0.83	20.74 ± 0.52
4h	61.94 ± 1.32	64.51 ± 1.18	68.22 ± 1.07	71.86 ± 1.57	20.18 ± 0.78
4i	45.02 ± 0.88	47.38 ± 1.17	50.44 ± 1.27	53.61 ± 1.39	27.76 ± 0.64
4j	63.87 ± 0.30	65.49 ± 0.46	68.97 ± 0.65	72.52 ± 0.78	19.57 ± 1.26
4k	68.42 ± 0.28	71.68 ± 0.45	74.51 ± 0.61	79.66 ± 0.82	18.26 ± 0.95
4l	54.38 ± 1.19	58.07 ± 0.88	62.75 ± 1.49	66.19 ± 1.00	22.98 ± 0.69
Ascorbic acid	76.41 ± 0.18	78.60 ± 0.33	82.39 ± 0.61	86.59 ± 0.71	16.35 ± 0.27
Blank	–	–	–	–	–

(–) Showed no scavenging activity. Values were the means of three replicates ± SD.

8.60 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.14, 16.76, 61.11, 103.54, 111.26, 119.58, 122.83, 124.64, 128.84, 141.97, 154.37, 158.15, 163.16 and 171.72; LC–MS (negative ion mode): *m/z* 330 $(M - H)^-$ for $C_{16}H_{14}FN_3O_2S$.

*5.1.2.3. Ethyl 4-(3-chloro-4-fluorophenylamino)-5-methylthieno [2,3-d]pyrimidine-6-carboxylate (**2c**).* Yield 72%; pale-yellow solid m.p. = 154–156 °C; IR (KBr) ν (cm^{-1}): 3417 (NH), 2923 (thiophene–CH_3), 1708 (C=O), 1557 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42 (t, 3H, $-CH_2-CH_3$), 3.11 (s, 3H, thiophene–CH_3), 4.40 (q, 2H, $-CH_2-CH_3$), 7.19 (t, 1H, Ar–H, *J* = 9.0 Hz), 7.39 (s, 1H, N–H), 7.45 (t, 1H, Ar–H, *J* = 8.0 Hz), 7.86 (d, 1H, Ar–H, *J* = 4.0 Hz), 8.58 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.29, 16.14, 60.89, 113.42, 116.31, 117.84, 119.62, 122.45, 140.79, 144.16, 149.49, 151.60, 154.71, 158.36, 159.24 and 169.86; LC–MS (negative ion mode): *m/z* 365 $(M - H)^-$ for $C_{16}H_{13}ClFN_3O_2S$.

*5.1.2.4. Ethyl 4-(2,4-difluorophenylamino)-5-methylthieno[2,3-d] pyrimidine-6-carboxylate (**2d**).* Yield 82%; white solid, m.p. = 192–194 °C; IR (KBr) ν (cm^{-1}): 3420 (NH), 2926 (thiophene–CH_3), 1710 (C=O), 1552 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.33 (t, 3H, $-CH_2-CH_3$), 3.00 (s, 3H, thiophene–CH_3), 4.32 (q, 2H, $-CH_2-CH_3$), 7.19 (m, 1H, Ar–H, *J* = 4.0 Hz), 7.36 (m, 1H, Ar–H, *J* = 8.0 Hz), 7.63 (q, 1H, Ar–H, *J* = 8.0 Hz), 8.38 (s, 1H, N–H), 8.59 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.16, 15.38, 61.07, 103.84, 106.46, 107.91, 114.66, 118.74, 130.89, 144.93, 148.68, 152.58, 154.16, 156.42, 158.73 and 162.58; LC–MS (negative ion mode): *m/z* 348 $(M - H)^-$ for $C_{16}H_{13}F_2N_3O_2S$.

*5.1.3. General procedure for the synthesis of 4-(substituted phenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylic acid (**3a–d**)*

Compounds **2**(**a–d**) was dissolved in MeOH/H_2O (8 ml:4 ml), and 15% v/v NaOH aq (2 ml) was added. Stirring was continued for 16 h at rt, then $CHCl_3$ was added. The aqueous layer was acidified with 1 N HCl, stirred for 15 min, the product was separated by vacuum filtration and washed with water, dried well and recrystallized from chloroform and methanol to give compounds **3**(**a–d**) in good yield.

*5.1.3.1. 4-(4-Chlorophenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylic acid (**3a**).* Yield 85%; off-white solid, m.p. = 316–318 °C; IR (KBr) ν (cm^{-1}): 3450 (NH), 2922 (thiophene–CH_3), 1700 (C=O), 1548 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.05 (s, 3H, thiophene–CH_3), 7.44 (d, 2H, Ar–H, *J* = 8.0 Hz), 7.69 (d, 2H, Ar–H, *J* = 8.0 Hz), 8.51 (s, 1H, C–H), 8.68 (s, 1H, N–H), 13.58 (br s, 1H, –COO*H*); ^{13}C NMR (DMSO-d_6,100 MHz): δ 16.73, 115.48, 119.87, 122.69, 124.53, 135.18, 140.06, 152.19, 153.34, 155.73, 157.47 and 164.96; LC–MS (positive ion mode): *m/z* 320 $(M + H)^+$ for $C_{14}H_{10}ClN_3O_2S$.

*5.1.3.2. 4-(4-Fluorophenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylic acid (**3b**).* Yield 88%; white solid m.p. = 260–262 °C; IR (KBr) ν (cm^{-1}): 3430 (NH), 2920 (thiophene–CH_3), 1713 (C=O), 1575 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.05 (s, 3H, thiophene–CH_3), 7.24 (t, 2H, Ar–H, *J* = 8.0 Hz), 7.64 (q, 2H, Ar–H, *J* = 4.0 Hz), 8.49 (s, 1H, N-H), 8.81 (s, 1H, C–H), 13.55 (br s, 1H, –COO*H*); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 15.26, 117.54, 123.70, 124.84, 128.38, 137.93, 138.86, 154.52, 156.48, 163.31, 166.93 and 169.26; LC–MS (positive ion mode): *m/z* 304 $(M + H)^+$ for $C_{14}H_{10}FN_3O_2S$.

*5.1.3.3. 4-(3-Chloro-4-fluorophenylamino)-5-methylthieno[2,3-d] pyrimidine-6-carboxylic acid (**3c**).* Yield 84%; white solid m.p. = 305–307 °C; IR (KBr) ν (cm^{-1}): 3430 (NH), 2927 (thiophene–CH_3), 1692 (C=O), 1562 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.04 (s, 3H, thiophene–CH_3), 7.44 (t, 1H, Ar–H, *J* = 8.0 Hz), 7.64 (t, 1H, Ar–H, *J* = 4.0 Hz), 7.92 (d, 1H, Ar–H, *J* = 4.0 Hz), 8.52 (s, 1H, N–H), 8.70 (s, 1H, C–H), 13.56 (br s, 1H, –COO*H*); ^{13}C NMR (DMSO-d_6,100 MHz): δ 16.18, 115.94, 117.28, 118.56, 121.72, 124.61, 139.83, 147.14, 148.69, 151.37, 154.48, 158.14, 161.62 and 164.81; LC–MS (positive ion mode): *m/z* 338 $(M + H)^+$ for $C_{14}H_9ClFN_3O_2S$.

*5.1.3.4. 4-(2,4-Difluorophenylamino)-5-methylthieno[2,3-d]pyrimidine-6-carboxylic acid (**3d**).* Yield 81%; white crystalline solid m.p. = 294–296 °C; IR (KBr) ν (cm^{-1}): 3417 (NH), 2926 (thiophene–CH_3), 1712 (C=O), 1565 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.00 (s, 3H, thiophene–CH_3), 7.15 (t, 1H, Ar–H, *J* = 8.0 Hz), 7.37 (t, 1H, Ar–H, *J* = 8.0 Hz), 7.65 (q, 1H, Ar–H, *J* = 8.0 Hz), 8.38 (s, 1H, N–H), 8.56 (s, 1H, C–H), 13.54 (br s, 1H, –COO*H*); ^{13}C NMR (DMSO-d_6,100 MHz): δ 14.47, 109.25, 115.62, 118.58, 126.93, 141.86, 147.72, 151.35, 153.48, 158.74, 159.28, 161.39, 164.18 and 169.06; LC–MS (positive ion mode): *m/z* 322 $(M + H)^+$ for $C_{14}H_9F_2N_3O_2S$.

*5.1.4. General procedure for the synthesis of (**4a–l**)*

An equimolar mixture of 4-(substituted phenylamino)-5-methylthieno[2,3-*d*]pyrimidine-6-carboxylic acids **3**(**a–d**) (0.01 mol) and appropriate substituted aromatic carboxylic acid hydrazide (0.01 mol) in phosphorus oxychloride (20 ml) was refluxed for 5 h. The reaction mixture was cooled to room temperature and then gradually

poured on to crushed ice with stirring. The mixture was allowed standing overnight and the solid separated out was filtered, treated with dilute sodium hydroxide solution and washed thoroughly with cold water, well dried and which was crystallized with chloroform and hexane to give the title compounds **4**(**a**–**l**) in good yield.

*5.1.4.1. N-(4-Chlorophenyl)-5-methyl-6-(5-phenyl-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4a**).* Brown solid (80%); m.p. = 248–250 °C; IR (KBr) ν (cm^{-1}): 3486 (NH), 2915 (thiophene–CH_3), 1605 (C=C), 1550 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.14 (s, 3H, thiophene–CH_3), 7.46 (d, 2H, Ar–H, J = 8.0 Hz), 7.64–7.72 (m, 5H, Ar–H), 8.10 (d, 2H, Ar–H, J = 8.0 Hz), 8.53 (s, 1H, N–H), 8.84 (bs, 1H, C–H); ^{13}C NMR (DMSO-d_6, 100 MHz); δ 14.03, 115.46, 116.28, 120.89, 123.06, 124.74, 125.87, 128.20, 129.46, 131.36, 135.99, 142.15, 147.57, 154.95, 158.26, 160.78 and 168.91; LC–MS (negative ion mode): m/z 418 $(M - H)^-$ for $C_{21}H_{14}ClN_5OS$.

*5.1.4.2. N-(4-Chlorophenyl)-6-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)-5-methylthieno[2,3-d]pyrimidin-4-amine (**4b**).* Pale yellow solid (85%); m.p. = 232–234 °C; IR (KBr) ν (cm^{-1}): 3256 (NH), 2922 (thiophene–CH_3), 1602 (C=C), 1556 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.25 (s, 3H, thiophene–CH_3), 7.40 (d, 2H, Ar–H, J = 8.0 Hz), 7.55 (d, 2H, Ar–H, J = 8.0 Hz), 7.65 (d, 2H, Ar–H, J = 8.0 Hz), 8.09 (d, 2H, Ar–H, J = 8.0 Hz), 8.60 (s, 1H, N–H), 8.92 (br s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.10, 116.84, 117.47, 121.80, 123.46, 128.40, 128.75, 129.26, 129.68, 130.31, 132.75, 136.20, 138.62, 155.02, 156.66, 161.02 and 163.38; LC–MS (negative ion mode): m/z 452 $(M - H)^-$ for $C_{21}H_{13}C_{l2}N_5OS$.

*5.1.4.3. N-(4-Chlorophenyl)-5-methyl-6-(5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4c**).* Yellow solid (78%); m.p. = 210–212 °C; IR (KBr) ν (cm^{-1}): 3221 (NH), 2926 (thiophene–CH_3), 1605 (C=C), 1528 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.28 (s, 3H, thiophene–CH_3), 7.41 (d, 2H, Ar–H, J = 8.0 Hz), 7.51 (d, 2H, Ar–H, J = 8.0 Hz), 7.65 (d, 2H, Ar–H, J = 8.0 Hz), 8.48 (dd, 2H, Ar–H, J = 8.0 Hz), 8.62 (s, 1H, N–H), 8.97 (br s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.14, 116.26, 117.68, 122.36, 124.16, 124.42, 128.75, 129.22, 129.68, 130.86, 135.42, 138.62, 146.64, 155.34, 156.12, 163.48, and 167.10; LC–MS (negative ion mode): m/z 463 $(M - H)^-$ for $C_{21}H_{14}ClN_5OS$.

*5.1.4.4. N-(4-Fluorophenyl)-5-methyl-6-(5-phenyl-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4d**).* Brown solid (72%); m.p. = 228–230 °C; IR (KBr) ν (cm^{-1}): 3224 (NH), 2925 (thiophene–CH_3), 1612 (C=C), 1522 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.08 (s, 3H, thiophene–CH_3), 7.36 (t, 2H, Ar–H, J = 8.0 Hz), 7.54 (q, 2H, Ar–H, J = 4.0 Hz), 7.61–7.74 (m, 5H, Ar–H), 8.21 (s, 1H, N–H), 8.60 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.70, 116.49, 117.28, 118.50, 126.98, 127.04, 128.54, 129.86, 132.39, 137.64, 139.58, 153.88, 155.41, 158.59, 158.71, 161.69 and 163.02; LC–MS (negative ion mode): m/z 402 $(M - H)^-$ for $C_{21}H_{14}FN_5OS$.

*5.1.4.5. 6-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-N-(4-fluorophenyl)-5-methylthieno[2,3-d]pyrimidin-4-amine (**4e**).* Brown solid (76%); m.p. = 246–248 °C; IR (KBr) ν (cm^{-1}): 3198 (NH), 2986 (thiophene–CH_3), 1610 (C=C), 1525 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.12 (s, 3H, thiophene–CH_3), 7.32 (t, 2H, Ar–H, J = 8.0 Hz),7.56 (q, 2H, Ar–H, J = 4.0 Hz), 7.64 (d, 2H, Ar–H, J = 8.0 Hz), 7.98 (d, 2H, Ar–H, J = 8.0 Hz), 8.24 (s, 1H, N–H), 8.78 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 15.91, 114.81, 116.63, 118.77, 123.93, 128.58, 129.54, 132.21, 135.01, 137.83, 138.68, 148.72, 152.28, 153.31, 157.09, 162.29 and 167.10; LC–MS (positive ion mode): m/z 438 $(M + H)^+$ for $C_{21}H_{13}ClFN_5OS$.

*5.1.4.6. N-(4-Fluorophenyl)-5-methyl-6-(5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)thieno-[2,3-d]pyrimidin-4-amine (**4f**).* Brown solid (80%); m.p. = 231–233 °C; IR (KBr) ν (cm^{-1}): 3254 (NH), 2916 (thiophene–CH_3), 1606 (C=C), 1554 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.36 (s, 3H, thiophene–CH_3), 7.41 (t, 2H, Ar–H, J = 8.0 Hz), 7.59 (q, 2H, Ar–H, J = 4.0 Hz), 7.71 (d, 2H, Ar–H, J = 8.0 Hz), 7.96 (dd, 2H, Ar–H, J = 8.0 Hz), 8.35 (s, 1H, N–H), 8.86 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.56, 116.42, 116.63, 117.49, 123.60, 127.66, 131.92, 135.48, 137.83, 137.64, 139.12, 149.08, 151.74, 153.63, 157.29, 162.48 and 166.27; LC–MS (positive ion mode): m/z 449 $(M + H)^+$ for $C_{21}H_{13}FN_6O_3S$.

*5.1.4.7. N-(3-Chloro-4-fluorophenyl)-5-methyl-6-(5-phenyl-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4g**).* Pale yellow solid (70%); m.p. = 214–216 °C; IR (KBr) ν (cm^{-1}): 3248 (NH), 2934 (thiophene–CH_3), 1614 (C=C), 1547 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.05 (s, 3H, thiophene–CH_3), 7.48 (t, 1H, Ar–H, J = 8.0 Hz), 7.54–7.68 (m, 5H, Ar–H), 7.70 (dd, 1H, Ar–H, J = 4.0 Hz), 7.96 (dd, 1H, Ar–H, J = 4.0 Hz), 8.57 (s, 1H, N–H), 8.75 (s, 1H, C–H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 17.69, 116.32, 116.55, 117.02, 118.96, 120.54, 126.71, 127.44, 128.51, 129.48, 132.16, 137.27, 141.84, 151.94, 152.61, 153.76, 157.23, 162.09 and 163.71; LC–MS (positive ion mode): m/z 438 $(M + H)^+$ for $C_{21}H_{13}ClFN_5OS$.

*5.1.4.8. N-(3-Chloro-4-fluorophenyl)-6-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)-5-methylthieno[2,3-d]pyrimidin-4-amine (**4h**).* Yellow solid (77%); m.p. = 254–256 °C; IR (KBr) ν (cm^{-1}): 3196 (NH), 2930 (thiophene–CH_3), 1641 (C=C), 1548 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.19 (s, 3H, thiophene–CH_3), 7.52 (t, 1H, Ar–H, J = 8.0 Hz), 7.72 (m, 1H, Ar–H), 7.76 (d, 2H, Ar–H, J = 8.0 Hz), 8.01 (dd, 1H, Ar–H, J = 4.0 Hz), 8.16 (d, 2H, Ar–H, J = 8.0 Hz), 8.60 (s, 1H, N–H), 8.90 (s, 1H, C–H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 16.16, 115.67, 116.64, 117.48, 118.78, 121.91, 123.96, 128.53, 129.66, 135.23, 135.87, 137.02, 142.68, 149.22, 152.71, 154.62, 156.52, 162.87 and 167.43; LC–MS (negative ion mode): m/z 470 $(M - H)^-$ for $C_{21}H_{12}Cl_2FN_5OS$.

*5.1.4.9. N-(3-Chloro-4-fluorophenyl)-5-methyl-6-(5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4i**).* Yellow solid (81%); m.p. = 239–241 °C; IR (KBr) ν (cm^{-1}): 3051 (NH), 2959 (thiophene–CH_3), 1614 (C=C), 1531 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.28 (s, 3H, thiophene–CH_3), 7.20 (t, 1H, Ar–H, J = 8.0 Hz), 7.50 (m, 1H, Ar–H), 7.89 (dd, 1H, Ar–H, J = 4.0 Hz), 8.49 (d, 2H, Ar–H, J = 8.0 Hz), 8.55 (dd, 2H, Ar–H, J = 8.0 Hz), 8.63 (s, 1H, N–H), 9.00 (s, 1H, C–H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 16.87, 115.38, 116.01, 117.59, 118.32, 122.02, 124.72, 128.37, 130.63, 132.54, 132.61, 139.81, 148.87, 149.52, 153.73, 15.21, 157.22, 162.19 and 163.37; LC–MS (negative ion mode): m/z 481 $(M - H)^-$ for $C_{21}H_{12}ClFN_6O_3S$.

*5.1.4.10. N-(2,4-Difluorophenyl)-5-methyl-6-(5-phenyl-1,3,4-oxadiazol-2-yl)thieno[2,3-d]pyrimidin-4-amine (**4j**).* Brown solid (76%); m.p. = 261–263 °C; IR (KBr) ν (cm^{-1}): 3251 (NH), 2926 (thiophene–CH_3), 1610 (C=C), 1535 (C=N); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.18 (s, 3H, thiophene–CH_3), 7.20 (m, 1H, Ar–H, J = 4.0 Hz), 7.38 (m, 1H, Ar–H, J = 4.0 Hz), 7.63 (q, 1H, Ar–H, J = 8.0 Hz), 7.84–7.91 (m, 5H, Ar–H), 8.76 (s, 1H, N–H), 8.92 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.15, 114.81, 116.63, 117.51, 118.97, 123.86, 126.72, 127.46, 128.51, 129.48, 132.21, 135.87, 147.35, 152.73, 154.54, 155.14, 156.46, 162.97 and 163.59; LC–MS (positive ion mode): m/z 422 $(M + H)^+$ for $C_{21}H_{13}F_2N_5OS$.

*5.1.4.11. 6-(5-(4-Chlorophenyl)-1,3,4-oxadiazol-2-yl)-N-(2,4-difluorophenyl)-5-methylthieno-[2,3-d]pyrimidin-4-amine (**4k**).* White solid (81%); m.p. = 248–250 °C; IR (KBr) ν (cm^{-1}): 3244 (NH), 2921 (thiophene–CH_3), 1608 (C=C), 1526 (C=N); ^{1}H NMR

($CDCl_3$, 400 MHz) δ 3.25 (s, 3H, thiophene–CH_3), 7.18 (m, 1H, Ar–H, J = 4.0 Hz), 7.28 (m, 1H, Ar–H, J = 4.0 Hz), 7.58 (q, 1H, Ar–H, J = 8.0 Hz), 7.68 (d, 2H, Ar–H, J = 8.0 Hz), 7.74 (d, 2H, Ar–H, J = 8.0 Hz), 8.48 (s, 1H, N–H), 8.69 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.70, 110.92, 115.04, 117.51, 119.26, 123.69, 124.72, 127.81, 129.47, 132.63, 135.94, 137.14, 149.08, 152.91, 155.32, 156.82, 160.10, 163.26 and 164.91; LC–MS (positive ion mode): m/z 456 $(M + H)^-$ for $C_{21}H_{12}ClF_2N_5OS$.

*5.1.4.12. N-(2,4-Difluorophenyl)-5-methyl-6-(5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)thieno-[2,3-d]pyrimidin-4-amine (**4l**).* Yellow solid (77%); m.p. = 226–228 °C; IR (KBr) ν (cm^{-1}): 3198 (NH), 2952 (thiophene–CH_3), 1620 (C=C), 1526 (C=N); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 3.28 (s, 3H, thiophene–CH_3), 7.24 (t, 1H, Ar–H, J = 8.0 Hz), 7.33 (m, 1H, Ar–H, J = 4.0 Hz), 7.64 (q, 1H, Ar–H, J = 8.0 Hz), 8.34 (d, 2H, Ar–H, J = 8.0 Hz), 8.42 (dd, 2H, Ar–H, J = 8.0 Hz), 8.71 (s, 1H, N–H), 8.96 (s, 1H, C–H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 17.53, 116.34, 116.65, 117.07, 118.77, 123.04, 128.58, 129.49, 131.94, 132.21, 139.61, 147.05, 149.58, 153.35, 153.80, 156.21, 162.10, 163.74 and 165.61; LC–MS (negative ion mode): m/z 465 $(M - H)^-$ for $C_{21}H_{12}F_2N_6O_3S$.

6. Pharmacological screening

6.1. Antioxidant screening (in vitro)

Compounds **4a**–**l** are tested for antioxidant property by DPPH, NO and H_2O_2 methods.

6.2. DPPH radical scavenging activity

The hydrogen atom or electron donation ability of the compounds was measured from the bleaching of the purple colored methanol solution of 1,1-diphenyl-1-picrylhydrazyl (DPPH). The spectrophotometric assay uses the stable radical DPPH as a reagent. 1 ml of various concentrations of the test compounds (25, 50, 75, 100 and 250 μg/ml) in methanol was added to 4 ml of 0.004% (w/v) methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. The percent of inhibition (I %) of free radical production from DPPH was calculated by the following equation

$$\%\ \text{of scavenging} = [(\text{A control} - \text{A sample})/\text{A blank}] \times 100 \quad (1)$$

where A control is the absorbance of the control reaction (containing all reagents except the test compound) and A sample is the absorbance of the test compound. Tests were carried at in triplicate.

6.3. Nitric oxide (NO) scavenging activity

Nitric oxide scavenging activity was measured by slightly modified methods of Green et al. and Marcocci et al. Nitric oxide radicals (NO) were generated from sodium nitroprusside. 1 ml of sodium nitroprusside (10 mM) and 1.5 ml of phosphate buffer saline (0.2 M, pH 7.4) were added to different concentrations (25, 50, 75 and 100 μg/ml) of the test compounds and incubated for 150 min at 25 °C and 1 ml of the reaction mixture was treated with 1 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbance of the chromatophore was measured at 546 nm. Nitric oxide scavenging activity was calculated using Eq. (1).

6.4. Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging ability of the test compound was determined according to the method of Ruch et al. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). 25, 50, 75 and 100 μg/ml concentrations of the test compounds in 3.4 ml phosphate buffer were added to H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. The percent of scavenging of H_2O_2 was calculated using Eq. (1).

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