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NOVEL 6-ARYL-7-ALKYL/ARYL-[1,2,4]TRIAZOLO[4,3-*a*][1,3,5]TRIAZINE-5(6*H*)-THIONES, PROCESSES FOR THEIR PREPARATION, CHARACTERIZATION AND EVALUATION OF THEIR *IN VITRO* ANTIOXIDANT ACTIVITY

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ABSTRACT. A series of nine new 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thiones (**2a-i**) were synthesized by a reaction of *N*-triazol-3-yl imidates (**1**) with three different isothiocyanate derivatives (RNCS) in refluxing toluene. The structures of the final heterocyclic compounds were confirmed by ^{1}H-NMR, ^{13}C-NMR, FT-IR, elemental analysis, and mass spectral analysis. The target compounds (**2a-i**) were in vitro screened for their activity as antioxidants using DPPH (2,2′-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing/antioxidant power) methods. The results revealed that some triazolotriazine-5-(6*H*)thiones exhibited antioxidant activity ranging from moderate to high. The obtained findings revealed that the triazolotriazine-5-(6*H*)thiones (**2g**, **2h**, and **2i**) have superiority among all compounds, It is obvious that the presence of a hydroxyl group in the structure is essential for the antioxidant properties and should be taken into consideration in further design of structures with potential antioxidant properties.

KEY WORDS: Imidates, Isothiocyanates, Antioxidant, Triazole, DPPH, FRAP

INTRODUCTION

Extensive research in the organic-medicinal chemistry field has led to the discovery of different classes of bioactive substances, most of being sulfur and nitrogen-containing heterocycles [1–4]. Heterocyclic compounds are present in various drugs, several natural products, some vitamins, biomolecules, and biologically active compounds such as anti-inflammatory [5], antitumour [6], antimalarial [7], antidepressant [8], anti-HIV [9], and antimicrobial [10] agents. Antioxidants (natural or synthetic) are the molecules, which are able to neutralize free radicals by acting at various stages like interception, prevention and repair [11–13]. It is, therefore, necessary to develop therapeutic agents with improved potential for treating broad spectrum of oxidant infections. In this work, we focused on the design, synthesis, and characterization of new triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thiones (**2a-i**). The target heterocyclic compounds (**2a-i**) were evaluated for their antioxidant activity using different assays.

RESULTS AND DISCUSION

Chemistry

The 3-amino[1,2,4]triazole was reacted with orthoesters in the presence of acetic acid to afford the imidates **(1)** which has been described [14]. The reaction between *N*-triazol-3-yl imidates (**1**) with appropriate isothiocyanate under reflux of toluene leads to 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thione (**2a-i**) (Scheme 1, Table 1).

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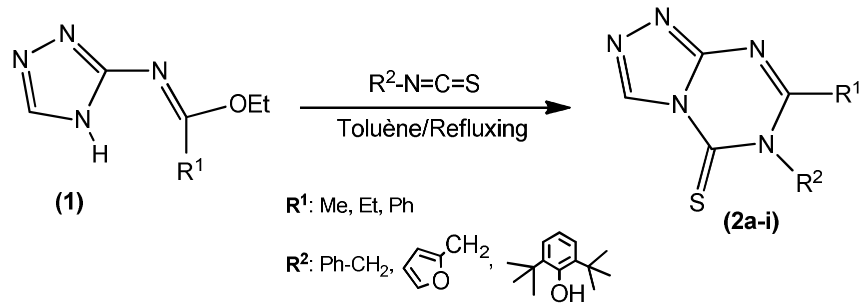
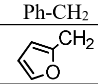
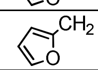
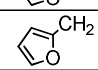
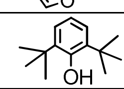
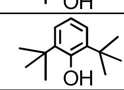
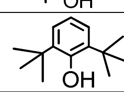


Scheme 1. Synthesis reaction of 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2a-i**).

Table 1. The results of synthesis of 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6*H*)-thione (**2a-i**).

Entry	Products	R^1	R^2	Chemical Formula	Time (hours)
1	(**2a**)	Me	Ph-CH_2	$C_{12}H_{11}N_5S$	36
2	(**2b**)	Et	Ph-CH_2	$C_{13}H_{13}N_5S$	48
3	(**2c**)	Ph	Ph-CH_2	$C_{17}H_{13}N_5S$	24
4	(**2d**)	Me		$C_{10}H_9N_5OS$	72
5	(**2e**)	Et		$C_{11}H_{11}N_5OS$	72
6	(**2f**)	Ph		$C_{15}H_{11}N_5OS$	48
7	(**2g**)	Me		$C_{19}H_{25}N_5OS$	36
8	(**2h**)	Et		$C_{20}H_{27}N_5OS$	24
9	(**2i**)	Ph		$C_{24}H_{27}N_5OS$	24

Et = CH_3-CH_2, Me = CH_3, Ph = C_6H_5.

The structure of the products (**2a-i**) was established with help of the spectral data. The IR spectra of compounds (**2a-i**) revealed the absorption bands corresponding to C=N and C=S in the region of 1615-1612 and 1272-1270 cm^{-1}, respectively, and revealed the absence of the absorption band of the NH group. The IR spectra of (**2g-i**) showed a band at around 3580 cm^{-1} which was assigned to the new hydroxyl (OH) band in the 2,6-di-tert-butylphenol motif (**R^2**). The ^{1}H- NMR spectrum of heterocyclic compounds (**2a-i**) revealed the disappearance of the signals of NH and the ethoxy (OEt) groups. The presence of hydroxyl motif in molecules (**2g-i**) was confirmed by the presence of D_2O-exchangeable signals at δ 10.25 (**2g**), 10.68 (**2h**), or 10.47 ppm (**2i**) assigned to the 2,6-di-tert-butylphenol moiety introduced by isothiocyante further confirmed the cyclization.^{13}C-NMR spectra of (**2a-i**) exhibit a signal at around δ 180 ppm corresponding to the carbon of C=S motif and display the characteristic signals of all carbons (see experimental part).

Antioxidant evaluation

In this paper, the in vitro antioxidant properties of the newly synthesized compounds (**2a-i**) at different concentrations (25; 50 and 100 µg/mL) on DPPH (Figure 1) and FRAP (Figure 2) were examined. It was found from Table 2 that newly synthesized heterocyclic compounds showed various antioxidant activities relative to BHT. In fact, among the analysed structures, highest DPPH radical scavenging activity was demonstrated (**2i**) compound (IC_{50} = 159 µg/mL) followed by (**2h**) (IC_{50} = 210 µg/mL) and (**2g**) (IC_{50} = 252 µg/mL). Additionally, compounds (**2a-c**) showed moderate DPPH radical scavenging activity at all tested concentrations (Table 2). However, no such inhibitory on DPPH were seen with compounds (**2d-f**). In this study, the obtained IC_{50} values of all triazolotriazine-5-(6*H*)thione were lower than that of BHT (IC_{50} = 26.5 µg/mL).

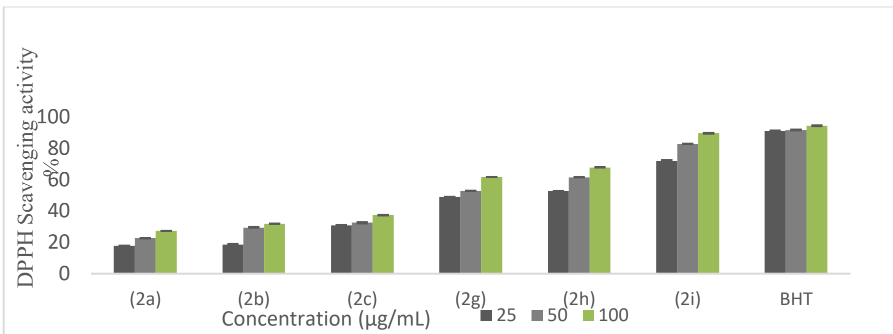


Figure 1. DPPH radical scavenging activity of studied triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thiones derivates (**2a**, **2b**, **2c**, **2g**, **2h**, **2i**) and BHT. Each value is expressed as mean ± SD, n = 3. Significant difference was calculated against BHT; $p < 0.0001$.

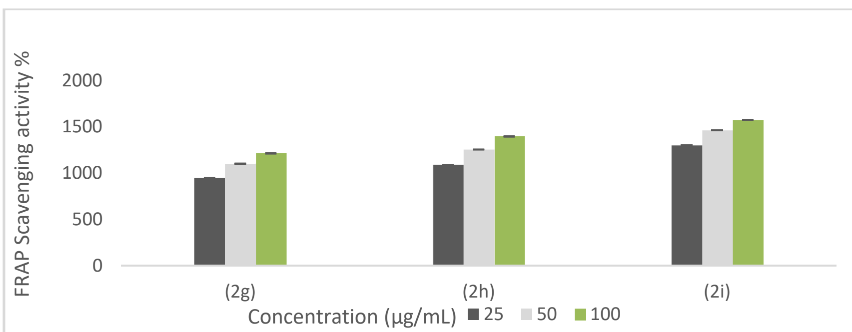
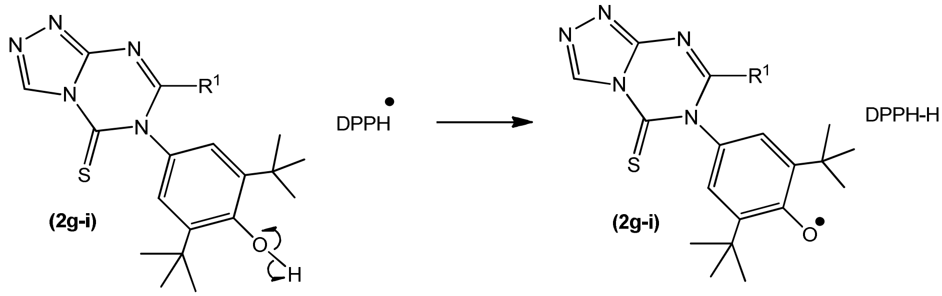


Figure 2. Ferric reducing antioxidant power (FRAP) of studied triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thiones derivates (**2g**, **2h**, **2i**). Each value is expressed as mean ± SD, n = 3.

Table 2. DPPH-radical scavenging of studied compounds (**2a-i**).

Compounds	DPPH IC_{50} (µg/mL)
(**2g**)	$252 \pm 3.1^{***}$
(**2h**)	$210 \pm 2.0^{***}$
(**2i**)	$159 \pm 0.2^{***}$
BHT	26.5 ± 0.3

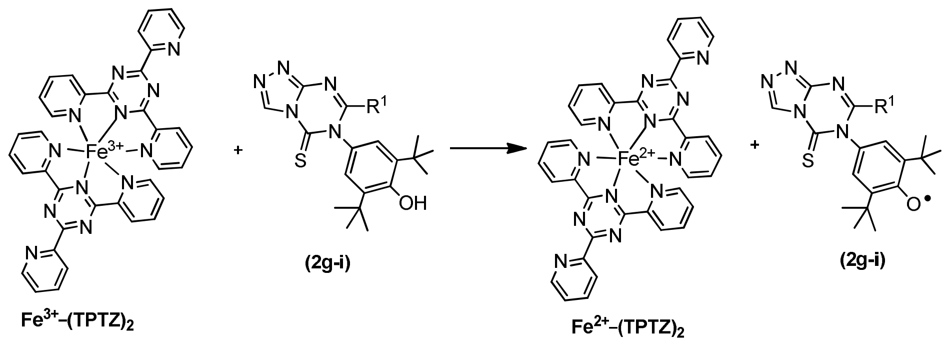
Data expressed as mean ± SD, n = 3; significant difference was calculated against control; ***$p < 0.0001$.



Scheme 2. The probable mechanism for the reaction of compounds (**2g-i**) with DPPH radical.

In accordance with other reported antioxidant results, the triazolotriazine-5-(6*H*)thione is expected to undergo a sequential proton loss electron transfer (SPLET) mechanism as illustrated in Scheme 2.

In the performed FRAP assay, as depicted in Figure 2, among the synthesized compounds only (**2g**, **2h** and **2i**) showed activity in the FRAP method, this result is due to the presence of hydroxyl group manifesting some activity in the FRAP method. While compound (**2i**) demonstrated the highest activity (1573 ± 0.33 µmol Trolox/100 g), followed by (**2h**) (1393± 0.55 µmol Trolox/100 g) and (**2g**) (1210 ± 0.33 µmol Trolox/100 g), at same concentration close to 100 µg/mL. These effects are probably due to the possibility of the analytes breaking up the free radical chain by donating a hydrogen atom (Scheme 3).



Scheme 3. The probable mechanism for the ferric reducing antioxidant power (FRAP) of compounds (**2g-i**).

As seen from the presented results for the two discussed methods used for evaluation of the free radicals scavenging activity and FRAP of the newly synthesized structures, the highest antioxidant activity was demonstrated by compound (**2i**). We believe that this result is due to the presence of 2,6-di-tert-butylphenol group (**R**^{2}) and phenyl ring (**R**^{1}) in the structure of this product.

CONCLUSION

In this work, a total of nine new 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-*a*][1,3,5]triazine-5(6*H*)-thione derivatives (**2a-i**) were successfully prepared by the reaction of *N*-triazol-3-yl imidates (**1**) and appropriate isothiocyanate as the reactants. Based on the obtained result, in all two assays used, the (**2i**) products have been found to possess promising antioxidant activity. Moreover, depth study on the free radicals and antioxidants area, to understand their mechanisms and characteristics, should be accelerated as they are of valuable points in preventing different diseases and displayed a favorable treatment approach.

EXPERIMENTAL

Chemicals

IR spectra were recorded with a Fourier Transform Infrared Spectrometer (Nicolet IR 200 FT-IR, USA). ^{1}H and ^{13}C-NMR spectra were recorded with dimethyl sulfoxide-d$_6$ (DMSO-d$_6$) solvent containing tetramethylsilane (TMS) on a Bruker 300 spectrometer (USA) (^{1}H: 300 MHz, ^{13}C: 75.47 MHz). The chemical shifts were reported in δ values relative to TMS (internal reference) for ^{1}H and ^{13}C. For the ^{1}H-NMR, the multiplicities of signals are indicated by the following abbreviations: s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet, dd: doublet of doublets. Melting point (mp) was determined on an Electrothermal 9100 melting point apparatus (Weiss-Gallenkamp, Loughborough, UK). Elemental microanalysis was performed on a Perkin-Elmer analyzer apparatus (model 2400, series II-CHN, USA). The electron spray ionization (ESI) positive MS spectra were recorded on a Brüker Daltonics LC-MS spectrometer (USA). All chemicals, DPPH, butylated hydroxyl toluene (BHT), 2,4,6-tripyridyl-s-triazine (TPTZ), potassium ferricyanide, Trolox, ferrous chloride ($FeCl_2$), and ferric chloride ($FeCl_3$) reagents and solvents were obtained from Sigma-Aldrich Company and were used without any purification. The completion of the reaction was monitored by TLC.

*Synthesis of 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2a-i**).* A mixture of *N*-triazol-3-yl imidates (**1**) (0.001 mol) and the appropriate isothiocyanate (0.001 mol) in dry toluene was refluxed for 24-72 hours. The solid material obtained on cooling was filtered off and recrystallized from ethanol.

*6-Benzyl-7-methyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2a**).* (102.8 mg, 40%); a beige solid; mp 182-184 °C; IR (FT-IR 200, ν (cm^{-1})): 1272 (C=S), 1612 (C=N); ^{1}H NMR (300 MHz, DMSO-*d$_6$*): δ 2.48 (s, 3H, C$\underline{H}_3$-C(N)=N), 4.71 (s, 2H, Ph-C$\underline{H}_2$-N), 7,32-7,46 (m, 5Ar-$\underline{H}$), 8.89 (s, 1H, N=C$\underline{H}$-N-C=S); ^{13}C NMR (75.47 MHz, DMSO-*d$_6$*): δ 21.9 ($\underline{C}H_3$-C(N)=N), 50.8 (Ph-$\underline{C}H_2$-N), 126.2 (1$\underline{C}$, Ar), 126.5 (2$\underline{C}$, Ar), 127.8 (2C, Ar), 136.2 (1$\underline{C}$, Ar), 153.7 (N=$\underline{C}$(N)-N), 154.9 (N=$\underline{C}$(CH$_3$)-N), 155.8 (N=$\underline{C}$H-N), 180.2 (N-$\underline{C}$(N)=S), ESI-MS [M+H]$^+$: *m/z* = 258. Anal. calcd. for $C_{12}H_{11}N_5S$ (%): C, 56.01; H, 4.31; N, 27.22. Found: C, 55.98; H, 4.32; N, 27.20.

*6-Benzyl-7-ethyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2b**).* (122.0 mg, 45%); a beige solid; mp 190-192 °C; IR (FT-IR 200, ν (cm^{-1})): 1270 (C=S), 1612 (C=N); ^{1}H NMR (300 MHz, DMSO-*d$_6$*): δ 1.22 (t, 3H, $^3J_{HH}$ = 9.0 Hz, C$\underline{H}_3$-CH$_2$-C(N)=N), 2.82 (q, 2H, $^3J_{HH}$ = 9.0 Hz, CH$_3$-C$\underline{H}_2$-C(N)=N), 4.75 (s, 2H, Ph-C$\underline{H}_2$-N), 7.32-7.48 (m, 5Ar-$\underline{H}$), 8.90 (s, 1H, N=C$\underline{H}$-N); ^{13}C NMR (75.47 MHz, DMSO-*d$_6$*): δ 12.3 ($\underline{C}H_3$-CH$_2$-C(N)=N), 25.8 (CH$_3$-$\underline{C}H_2$-C(N)=N), 50.1 (Ph-$\underline{C}H_2$-N), 126.3 (1$\underline{C}$, Ar), 126.6 (2$\underline{C}$, Ar), 127.2 (2$\underline{C}$, Ar), 136.5 (1$\underline{C}$, Ar), 153.8 (N=$\underline{C}$(N)-N), 154.6 (N=$\underline{C}$(CH$_3$)-N), 155.7 (N=$\underline{C}$H-N), 180.4 (N-$\underline{C}$(N)=S); ESI-MS [M+H]$^+$: *m/z* = 272. Anal. calcd. for $C_{13}H_{13}N_5S$ (%): C, 57.54; H, 4.83; N, 25.81. Found: C, 57.55; H, 4.85; N, 25.82.

*6-Benzyl-7-phenyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2c**).* (178.6 mg, 56%); a yellowish solid; mp 232-234 °C; IR (FT-IR 200, ν (cm^{-1})): 1271 (C=S), 1612 (C=N); ^{1}H NMR (300 MHz, DMSO-*d$_6$*): δ 4.78 (s, 2H, Ph-C$\underline{H}_2$-N), 7.28-7.45 (m, 10Ar-$\underline{H}$), 8.85 (s, 1H, N=C$\underline{H}$-N-C=S); ^{13}C NMR (75.47 MHz, DMSO-*d$_6$*): δ 50.5 (Ph-$\underline{C}H_2$-N), 126.1 (2$\underline{C}$, Ar), 126.8 (1$\underline{C}$, Ar), 127.2 (2$\underline{C}$, Ar), 128.1 (2$\underline{C}$, Ar), 128,6 (2$\underline{C}$, Ar), 129.8 (1$\underline{C}$, Ar), 130.2 (1C, Ar), 135.8 (1$\underline{C}$, Ar), 153.8 (N=$\underline{C}$(N)-N), 154.7 (N=$\underline{C}$(Ph)-N), 155.6 (N=$\underline{C}$H-N), 180.8 (N-$\underline{C}$(N)=S); ESI-MS [M+H]$^+$:

m/z = 320. Anal. calcd. for $C_{17}H_{13}N_5S$ (%): C, 63.93; H, 4.10; N, 21.93. Found: C, 63.91; H, 4.12; N, 21.95.

6-(Furan-2-ylmethyl)-7-methyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione ***(2d)***. (126.11 mg, 51%); a yellowish needles solid; mp 221-223 °C; IR (FT-IR 200, ν (cm^{-1})): 1270 (C=S), 1614 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.64 (s, 3H, C$\underline{H}_3$-C(N)=N), 3.94 (s, 2H, CH=C(O)-C$\underline{H}_2$-N), 6.78 (dd, 1H, $^3J_{HH}$ = 3.6 Hz, $^3J_{HH}$ = 1.64 Hz, CH=C$\underline{H}$-CH=C(O)-CH_2-N), 7.61 (d, 1H, $^3J_{HH}$ = 3.4 Hz, C$\underline{H}$=C(O)-CH_2-N), 8.05 (d, 1H, $^3J_{HH}$ = 1.64 Hz, C$\underline{H}$=CH-CH=C(O)-CH_2-N), 8.80 (s, 1H, N=C$\underline{H}$-N-C=S); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 20.8 ($\underline{C}H_3$-C(N)=N), 52.3 (CH=C(O)-$\underline{C}H_2$-N), 111.2 (CH=CH-$\underline{C}$H=C(O)-CH_2-N), 111.8 (CH=$\underline{C}$H-CH=C(O)-CH_2-N), 143.6 ($\underline{C}$H=CH-CH=C(O)-CH_2-N), 149.5 (CH=CH-CH=$\underline{C}$(O)-CH_2-N), 153.8 (N=$\underline{C}$(N)-N), 154.2 (N=$\underline{C}$(CH_3)-N), 155.6 (N=$\underline{C}$H-N), 180.4 (N-$\underline{C}$(N)=S); ESI-MS $[M+H]^+$: *m/z* = 248. Anal. calcd. for $C_{10}H_9N_5OS$ (%): C, 48.57; H, 3.67; N, 28.32. Found: C, 48.58; H, 3.69; N, 28.30.

7-Ethyl-6-(furan-2-ylmethyl)-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione ***(2e)***. (112.35 mg, 43%); a dark yellow solid; mp: 261-263 °C; IR (FT-IR 200, ν (cm^{-1})): 1272 (C=S), 1615 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.25 (t, 3H, $^3J_{HH}$ = 8.7 Hz, C$\underline{H}_3$-CH_2-C(N)=N), 2.78 (q, 2H, $^3J_{HH}$ = 9.0 Hz, CH_3-C$\underline{H}_2$-C(N)=N), 3.92 (s, 2H, CH=C(O)-C$\underline{H}_2$-N), 6.76 (dd, 1H, $^3J_{HH}$ = 3.6 Hz, $^3J_{HH}$ = 1.64 Hz, CH=C$\underline{H}$-CH=C(O)-CH_2-N), 7.65 (d, 1H, $^3J_{HH}$ = 3.4 Hz, C$\underline{H}$=C(O)-CH_2-N), 8.08 (d, 1H, $^3J_{HH}$ = 1.66 Hz, C$\underline{H}$=CH-CH=C(O)-CH_2-N), 8.83 (s, 1H, N=C$\underline{H}$-N-C=S); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 11.8 ($\underline{C}H_3$-CH_2-C(N)=N), 26.1 (CH_3-$\underline{C}H_2$-C(N)=N), 52.8 (CH=C(O)-$\underline{C}H_2$-N), 112.2 (CH=CH-$\underline{C}$H=C(O)-CH_2-N), 112.5 (CH=$\underline{C}$H-CH=C(O)-CH_2-N), 141.9 ($\underline{C}$H=CH-CH=C(O)-CH_2-N), 149.4 (CH=CH-CH=$\underline{C}$(O)-CH_2-N), 153.4 (N=$\underline{C}$(N)-N), 154.3 (N=$\underline{C}$(CH_3)-N), 155.7 (N=$\underline{C}$H-N), 180.6 (N-$\underline{C}$(N)=S); ESI-MS $[M+H]^+$: *m/z* = 262. Anal. calcd. for $C_{11}H_{11}N_5OS$ (%): C, 50.56; H, 4.24; N, 26.80. Found: C, 50.58; H, 4.25; N, 26.81.

6-(Furan-2-ylmethyl)-7-phenyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione ***(2f)***. (179.22 mg, 58%); a dark yellow solid; mp: 205-207 °C; IR (FT-IR 200, ν (cm^{-1})): 1270 (C=S), 1612 (C=N); ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.96 (s, 2H, CH=C(O)-C$\underline{H}_2$-N), 6.72 (dd, 1H, $^3J_{HH}$ = 3.6 Hz, $^3J_{HH}$ = 1.66 Hz, CH=C$\underline{H}$-CH=C(O)-CH_2-N), 7.30-7.46 (m, 5Ar-$\underline{H}$), 7.63 (d, 1H, $^3J_{HH}$ = 3.5 Hz, C$\underline{H}$=C(O)-CH_2-N), 8.09 (d, 1H, $^3J_{HH}$ = 1.66 Hz, C$\underline{H}$=CH-CH=C(O)-CH_2-N), 8.83 (s, 1H, N=C$\underline{H}$-N-C=S); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 20.8 ($\underline{C}H_3$-C(N)=N), 52.3 (CH=C(O)-$\underline{C}H_2$-N), 112.2 (CH=CH-$\underline{C}$H=C(O)-CH_2-N), 112.7 (CH=$\underline{C}$H-CH=C(O)-CH_2-N), 128.3 (1C, Ar), 129.3 (2$\underline{C}$, Ar), 129.6 (2$\underline{C}$, Ar), 131.1 (1$\underline{C}$, Ar), 144.1 ($\underline{C}$H=CH-CH=C(O)-CH_2-N), 150.1 (CH=CH-CH=$\underline{C}$(O)-CH_2-N), 153.1 (N=$\underline{C}$(N)-N), 154.0 (N=$\underline{C}$(CH_3)-N), 155.7 (N=$\underline{C}$H-N), 180.9 (N-$\underline{C}$(N)=S); ESI-MS $[M+H]^+$: *m/z* = 310. Anal. calcd. for $C_{15}H_{11}N_5OS$ (%): C, 58.24; H, 3.58; N, 22.64. Found: C, 58.26; H, 3.59; N, 22.62.

6-(3,5-Di-tert-butyl-4-hydroxyphenyl)-7-methyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione ***(2g)***. (192.4 mg, 52%); a Yellowish needles solid; mp: 283-285 °C; IR (FT-IR 200, ν (cm^{-1})) : 1270 (C=S), 1612 (C=N), 3580 (OH); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.36 (s, 18H, 2-C(C$\underline{H}_3$)$_3$), 2.51 (s, 3H, C$\underline{H}_3$-C(N)=N), 7.24 (s, 2H, Ar-$\underline{H}$), 8.86 (s, 1H, N=C$\underline{H}$-N-C=S), 10.25 (br.s, 1H, Ar-O$\underline{H}$, D_2O exchangeable); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 22.8 ($\underline{C}H_3$-C(N)=N), 31.2 (6C, -C_{Ar}-C($\underline{C}H_3$)$_3$), 34.8 (2C, -C_{Ar}-$\underline{C}$(CH_3)$_3$), 122.2 (2$\underline{C}$, Ar), 126.4 (-N-$\underline{C}_{Ar}$), 138.1 (2C, -$\underline{C}_{Ar}$-C(CH_3)$_3$), 150.4 (1 $\underline{C}_{Ar}$-OH), 153.1 (N=$\underline{C}$(N)-N), 154.6 (N=$\underline{C}$(CH_3)-N), 155.7 (N=$\underline{C}$H-N), 180.9 (N-$\underline{C}$(N)=S); ESI-MS $[M+H]^+$: *m/z* = 372. Anal. calcd. for $C_{19}H_{25}N_5OS$ (%): C, 61.43; H, 6.78; N, 18.85. Found: C, 61.45; H, 6.79; N, 18.83.

6-(3,5-Di-tert-butyl-4-hydroxyphenyl)-7-ethyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione ***(2h)***. (205.2 mg, 54%); a yellowish needles solid; mp 270-272 °C; IR (FT-IR 200, ν (cm^{-1})): 1270 (C=S), 1612 (C=N), 3580 (OH); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.23 (t, 3H, $^3J_{HH}$ = 8.7

Hz, C<u>H</u>$_3$-CH$_2$-C(N)=N), 1.37 (s, 18H, 2 -C(C<u>H</u>$_3$)$_3$), 2.62 (q, 3H, $^3J_{HH}$ = 8.9 Hz, CH$_3$-C<u>H</u>$_2$-C(N)=N), 7,23 (s, 2H, Ar-<u>H</u>), 8.86 (s, 1H, N=C<u>H</u>-N-C=S), 10.68 (br.s, 1H, Ar-O<u>H</u>, D$_2$O exchangeable); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 11.8 (<u>C</u>H$_3$-CH$_2$-C(N)=N), 26.1 (CH$_3$-<u>C</u>H$_2$-C(N)=N), 31.1 (6C, -C$_{Ar}$-C(<u>C</u>H$_3$)$_3$), 34.5 (2C, -C$_{Ar}$-<u>C</u>(CH$_3$)$_3$), 121.8 (2<u>C</u>, Ar), 153.1 (N=<u>C</u>(N)-N), 126.2 (-N-<u>C</u>$_{Ar}$), 138.3 (2C, -<u>C</u>$_{Ar}$-C(CH$_3$)$_3$), 150.2 (1 <u>C</u>$_{Ar}$-OH), 154.7 (N=<u>C</u>(CH$_3$)-N), 155.4 (N=<u>C</u>H-N), 180.8 (N-<u>C</u>(N)=S); ESI-MS [M+H]$^+$: *m/z* = 386. Anal. calcd. for $C_{20}H_{27}N_5OS$ (%): C, 62.31; H, 7.06; N, 18.17. Found: C, 62.32; H, 7.04; N, 18.18.

*6-(3,5-Di-tert-butyl-4-hydroxyphenyl)-7-phenyl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (**2i**).* (219.3 mg, 51%); a dark Yellow solid; mp 284-286 °C; IR (FT-IR 200, ν (cm^{-1})): 1271(C=S), 1613 (C=N), 3581 (OH); ^{1}H NMR (300 MHz, DMSO-d_6): 1.39 (s, 18H, 2 -C(C<u>H</u>$_3$)$_3$), 7.23 (s, 2H, Ar-<u>H</u>,), 7.42-7.78 (m, 5H, Ar-<u>H</u>), 8.85 (s, 1H, N=C<u>H</u>-N-C=S),10.47 (br.s, 1H, Ar-O<u>H</u>, D$_2$O exchangeable); ^{13}C NMR (75.47 MHz, DMSO-d_6): δ 31.2 (6C, -C$_{Ar}$-C(<u>C</u>H$_3$)$_3$), 34.3 (2C, -C$_{Ar}$-<u>C</u>(CH$_3$)$_3$), 121.8 (2<u>C</u>, Ar), 126.2 (-N-<u>C</u>$_{Ar}$), 128.1 (2<u>C</u>, Ar), 128.5 (1<u>C</u>, Ar), 129.1 (2<u>C</u>, Ar), 131.2 (1<u>C</u>, Ar), 138.1 (2C, -<u>C</u>$_{Ar}$-C(CH$_3$)$_3$), 150.6 (1 <u>C</u>$_{Ar}$-OH), 152.9 (N=<u>C</u>(N)-N), 154.1 (N=<u>C</u>(CH$_3$)-N), 155.2 (N=<u>C</u>H-N), 180.6 (N-<u>C</u>(N)=S); ESI-MS [M+H]$^+$: *m/z* = 434. Anal. calcd. for $C_{24}H_{27}N_5OS$ (%): C, 66.49; H, 6.28; N, 16.15. Found: C, 66.50; H, 6.29; N, 16.17.

Antioxidant evaluation

DPPH radical scavenging activity. The antioxidant activity of triazolotriazine-5-(6*H*)thiones (**2a-i**) was performed using DPPH free radical scavenging [15]. The prepared methanol solution of DPPH (20 μg/mL) was stored at 10 °C in the dark. The heterocyclic compounds (**2a-i**) were dissolved in methanol. (0.5 mL) of different concentrations (25; 50; 100 μg/mL) of the tested compounds was added to DPPH solution (1.0 mL). Then, the plate was incubated in dark for 30 min at room temperature. At a wavelength of 517 nm, the process of occurrence of discoloration was recorded 5 min after the reaction and was compared with a blank control. Butylated hydroxyl toluene (BHT) was taken as standard. The free radical scavenging ability of tested compounds expressed as %inhibition was calculated using the following equation [16]:

$$\% \text{ I} = [(A_c - A_S / Ac)] \times 100$$

where A_c means absorbance of control; A_s means absorbance of the sample.

Ferric reducing antioxidant power assay (FRAP). Benzie and Strain method with some modifications were conducted in the ferric reducing antioxidant power (FRAP) assay [17]. The stock solutions of 300 mM of the acetate buffer, pH 3.6; 20 mM $FeCl_3 \cdot 6H_2O$ solution and 10 mM TPTZ solution in 40-mM HCl were previously prepared. Three reagents were prepared; acetate buffer (300 mM, pH = 3.6), 20 mM $FeCl_3 \cdot 6H_2O$ solution and 10 mM TPTZ in 40 mM HCl. The freshly mixed solution was prepared by mixing $FeCl_3 \cdot 6H_2O$, acetate buffer, and TPTZ in the ratio of 2.5: 25: 2.5 (v/v/v), respectively. The mixture was warmed at 37°C. In a dark condition, FRAP solution (2850 μL) was allowed to react with triazolotriazine-5-(6*H*)thiones (**2a-i**) (150 μL) for 30 min.. Measurement of the absorbance readings of the colored product (ferrous tripyridyltriazine complex) at 593 nm. The results are represented in μmol Trolox/100 g dry matter. When the measured FRAP value exceeded the linear range of the standard curve, an extra additional dilution was applied to lower the measurement with consideration of the dilution factor.

Statistical analysis

Statview v.5.0.1 software (SAS Institute, Cary, NC) was used for all statistical analyses. Experimental data were presented as mean standard deviation. One-way analysis of variance was used to determine the statistical significance. Differences were considered statistically significant if $p < 0.0001$.

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