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

Azhar Hajri*, Dhouha Alimi, Kaies Rtibi and Hichem Sebai

Laboratory of Functional Physiology and Valorization of Bio-resources (UR17ES27), Higher Institute of Biotechnology of Beja, Habib Bourguiba Street, Box 382, 9000 Beja, Jendouba University, Tunisia

(Received March 1, 2021; Revised December 23, 2021; Accepted December 24, 2021)

ABSTRACT. A series of nine new 6-aryl-7-alkyl/aryl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-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 1H-NMR, 13C-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(6H)thiones exhibited antioxidant activity ranging from moderate to high. The obtained findings revealed that the triazolotriazine-5(6H)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(6H)-thiones (2a-i). The target heterocyclic compounds (2a-i) were evaluated for their antioxidant activity using different assays.

RESULTS AND DISCUSSION

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(6H)-thione (2a-i) (Scheme 1, Table 1).

*Corresponding author. E-mail: lazhharhajri.fsb@gmail.com

This work is licensed under the Creative Commons Attribution 4.0 International License
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(6H)-thione (2a-i).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Products</th>
<th>R¹</th>
<th>R²</th>
<th>Chemical Formula</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2a)</td>
<td>Me</td>
<td>Ph-C₂H₅</td>
<td>C₂H₁₀N₅S</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>(2b)</td>
<td>Et</td>
<td>Ph-C₂H₅</td>
<td>C₂H₁₀N₅S</td>
<td>48</td>
</tr>
<tr>
<td>3</td>
<td>(2c)</td>
<td>Ph</td>
<td>Ph-C₂H₅</td>
<td>C₂H₁₀N₅S</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>(2d)</td>
<td>Me</td>
<td></td>
<td>C₆H₁₂N₄OS</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>(2e)</td>
<td>Et</td>
<td></td>
<td>C₆H₁₂N₄OS</td>
<td>72</td>
</tr>
<tr>
<td>6</td>
<td>(2f)</td>
<td>Ph</td>
<td></td>
<td>C₆H₁₂N₄OS</td>
<td>48</td>
</tr>
<tr>
<td>7</td>
<td>(2g)</td>
<td>Me</td>
<td></td>
<td>C₆H₁₂N₄OS</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>(2h)</td>
<td>Et</td>
<td></td>
<td>C₅H₁₀N₄OS</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>(2i)</td>
<td>Ph</td>
<td></td>
<td>C₅H₁₀N₄OS</td>
<td>24</td>
</tr>
</tbody>
</table>

Et = CH₃-C₂H₅, Me = CH₃, Ph = C₆H₅.

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 (R²). The ¹H- 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 D₂O-exchangeable signals at δ 10.25 (2g), 10.68 (2h), or 10.47 ppm (2i) assigned to the 2,6-di-tert-butylphenol motif introduced by isothiocyante further confirmed the cyclization. ¹³C-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).
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-(6H)-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(6H)-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(6H)-thione derivates (2g, 2h, 2i). Each value is expressed as mean ± SD, n = 3.

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

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DPPH IC_{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2g)</td>
<td>252 ± 1.1^{***}</td>
</tr>
<tr>
<td>(2h)</td>
<td>210 ± 2.0^{***}</td>
</tr>
<tr>
<td>(2i)</td>
<td>159 ± 0.2^{***}</td>
</tr>
<tr>
<td>BHT</td>
<td>26.5 ± 0.3</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD, n = 3; significant difference was calculated against control; ***p < 0.0001.
In accordance with other reported antioxidant results, the triazolotriazine-5-(6H)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).

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 (R1) and phenyl ring (R2) 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(6H)-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). ¹H and ¹³C-NMR spectra were recorded with dimethyl sulfoxide-d₆ (DMSO-d₆) solvent containing tetramethylsilane (TMS) on a Bruker 300 spectrometer (USA) (¹H: 300 MHz, ¹³C: 75.47 MHz). The chemical shifts were reported in δ values relative to TMS (internal reference) for ¹H and ¹³C. For the ¹H-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.

Synthesis of 6-aryl-7-alkylaryl-[1,2,4]triazolo[4,3-a][1,3,5]triazine-5(6H)-thione (2a-c). A mixture of N-triazol-3-yl imidates (I) (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 ºC; IR (FT-IR 200, v (cm⁻¹)): 1272 (C-S), 1612 (C=N); ¹H NMR (300 MHz, DMSO-d₆): δ 2.48 (s, 3H, CH₃-C(N)=N); 7.32-7.46 (m, 5Ar-H), 8.89 (s, 1H, N=C=N-C=S); ¹³C NMR (75.47 MHz, DMSO-d₆): δ 21.9 (CH₃-C(N)=N), 50.8 (Ph-CH₂-N), 126.2 (1C, Ar), 126.5 (2C, Ar), 136.2 (1C, Ar), 154.7 (N=C=N), 155.8 (N=CH-N), 180.2 (N=C(N)=S), ESI-MS [M+H⁺]: m/z = 258. Anal. calcd. for C₁₁H₁₁N₃S (%): 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 ºC; IR (FT-IR 200, v (cm⁻¹)): 1270 (C=S), 1612 (C=N); ¹H NMR (300 MHz, DMSO-d₆): δ 1.22 (t, 3H, J₉₀₁ = 9.0 Hz, CH₃-CH₂-C(N)=N), 2.82 (q, 2H, J₉₀₁ = 9.0 Hz, CH₃-CH₂-C(N)=N), 7.32-7.48 (m, 5Ar-H), 8.90 (s, 1H, N=C=N), ¹³C NMR (75.47 MHz, DMSO-d₆): δ 12.3 (CH₃-CH₂-C(N)=N), 25.8 (CH₃-CH₂-C(N)=N), 50.1 (Ph-CH₂-N), 126.3 (1C, Ar), 126.6 (2C, Ar), 127.2 (2C, Ar), 136.5 (1C, Ar), 153.8 (N=C(N)=S), 154.6 (N=C(H)=N), 155.7 (N=CH-N), 180.4 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 272. Anal. calcd. for C₁₁H₁₃N₃S (%): 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 ºC; IR (FT-IR 200, v (cm⁻¹)): 1271 (C=S), 1612 (C=N); ¹H NMR (300 MHz, DMSO-d₆): δ 4.78 (s, 2H, Ph-CH₂-N), 7.28-7.45 (m, 10Ar-H), 8.85 (s, 1H, N=CH-N=C=S); ¹³C NMR (75.47 MHz, DMSO-d₆): δ 50.5 (Ph-CH₂-N), 126.1 (2C, Ar), 126.8 (1C, Ar), 127.2 (2C, Ar), 128.1 (2C, Ar), 128.6 (2C, Ar), 129.8 (1C, Ar), 130.2 (1C, Ar), 135.8 (1C, Ar), 153.8 (N=C(N)=N), 154.7 (N=C(Ph)=N), 155.6 (N=CH-N), 180.8 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 272. Bull. Chem. Soc. Ethiop. 2021, 35(3)
Azhar Hajri et al.

570

\[ m/z = 320. \text{ Anal. calcd. for C}_{17}H_{12}N_{5}S (\%): } C, 63.93; H, 4.10; N, 21.93. \text{ Found: C, 63.91; H, 4.12; N, 21.95.} \]

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); 1H NMR (300 MHz, DMSO-d₆): δ 2.64 (s, 3H, CH₃-C(N)=N), 3.94 (s, 2H, CH=CH-C(O)-CH₂-N), 6.78 (dd, 1H, 1J_H = 3.6 Hz, 1J_H = 1.64 Hz, CH=CH-C(O)-CH₂-N), 7.61 (d, 1H, 1J_H = 3.4 Hz, CH=C(O)-CH₂-N), 8.05 (d, 1H, 1J_H = 1.64 Hz, CH=CH-CH=C(O)-CH₂-N), 8.80 (s, 1H, N=C₄H₃-N=C=S); 13C NMR (75.47 MHz, DMSO-d₆): δ 20.8 (CH₃-C(N)=N), 52.3 (CH=CH-C(O)-CH₂-N), 111.2 (CH=CH-C(O)-CH₂-N), 111.8 (CH=CH-C(O)-CH₂-N), 143.6 (CH=CH-C(O)-CH₂-N), 149.5 (CH=CH-CH=C(O)-CH₂-N), 153.8 (N=C(N)=N), 154.2 (N=C(CH₃)=N), 155.6 (N=C(CH₃)=N), 180.4 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 248. Anal. calcd. for C₁₇H₁₂N₅S (\%): 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); 1H NMR (300 MHz, DMSO-d₆): δ 1.25 (t, 3H, 1J_H = 8.7 Hz, CH₃-C(CH₃)=N), 2.78 (q, 2H, 1J_H = 9.0 Hz, CH₂-CH₂-C(CH₃)=N), 3.92 (s, 2H, CH=CH-C(N)=N), 6.76 (dd, 1H, 1J_H = 3.6 Hz, 1J_H = 1.64 Hz, CH=CH-CH=C(O)-CH₂-N), 7.65 (d, 1H, 1J_H = 3.4 Hz, CH=CH-C(O)-CH₂-N), 8.08 (d, 1H, 1J_H = 1.66 Hz, CH=CH-CH=C(O)-CH₂-N), 8.83 (s, 1H, N=C₃H₃-N=C=S); 13C NMR (75.47 MHz, DMSO-d₆): δ 11.8 (CH₃-C(CH₃)=N), 26.1 (CH₃-C(CH₃)=N), 52.8 (CH=CH-C(O)-CH₂-N), 112.2 (CH=CH-CH=C(O)-CH₂-N), 112.5 (CH=CH-CH=C(O)-CH₂-N), 141.9 (CH=CH-CH=C(O)-CH₂-N), 153.4 (N=C(N)=N), 154.3 (N=C(CH₃)=N), 155.7 (N=C(CH₃)=N), 180.6 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 262. Anal. calcd. for C₁₇H₁₂N₅S (\%): 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); 1H NMR (300 MHz, DMSO-d₆): δ 3.96 (s, 2H, CH=CH-CH₂-N), 6.72 (dd, 1H, 1J_H = 3.6 Hz, 1J_H = 1.66 Hz, CH=CH-CH=CH-C(O)-CH₂-N), 7.30-7.46 (m, 5Ar-H), 7.63 (d, 1H, 1J_H = 3.5 Hz, CH=CH-C(O)-CH₂-N), 8.09 (d, 1H, 1J_H = 1.66 Hz, CH=CH=CH=C(O)-CH₂-N), 8.83 (s, 1H, N=C₃H₄-N=C=S); 13C NMR (75.47 MHz, DMSO-d₆): δ 20.8 (CH₃-C(N)=N), 52.3 (CH=CH-C(O)-CH₂-N), 112.2 (CH=CH-CH=C(O)-CH₂-N), 112.5 (CH=CH-CH=C(O)-CH₂-N), 128.3 (1C, Ar), 129.3 (2C, Ar), 129.6 (2C, Ar), 131.1 (1C, Ar), 144.1 (CH=CH-CH=C(O)-CH₂-N), 150.1 (CH=CH-CH=C(O)-CH₂-N), 151.3 (N=C(N)=N), 154.0 (N=C(CH₃)=N), 155.7 (N=C(CH₃)=N), 180.9 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 310. Anal. calcd. for C₁₆H₁₂N₅OS (\%): C, 58.24; H, 3.58; N, 32.26. Found: C, 58.26; H, 3.59; N, 32.22.

6-(3,5-Di-t-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≡N), 1612 (C≡N), 3580 (OH); 1H NMR (300 MHz, DMSO-d₆): δ 1.36 (s, 18H, 2C(CH₃)₃), 2.51 (s, 3H, CH₃-C(N)=N), 7.24 (s, 2H, Ar-H), 8.86 (s, 1H, N=C₃H₄-N=C=S), 10.25 (br.s, 1H, Ar-OH, D₂O exchangeable); 13C NMR (75.47 MHz, DMSO-d₆): δ 22.8 (CH₃-C(N)=N), 31.2 (6C, -C₆H₃-C(CH₃)₃), 34.8 (2C, -C₆H₃-C(CH₃)₃), 122.2 (2C, Ar), 126.4 (-N=C₆H₃), 138.1 (2C, -C₆H₃-C(CH₃)₃), 150.4 (1C, -O-CH₂), 153.1 (N=C(N)=N), 154.6 (N=C(CH₃)=N), 155.7 (N=C(CH₃)=N), 180.9 (N=C(N)=S); ESI-MS [M+H⁺]: m/z = 372. Anal. calcd. for C₁₆H₁₂N₅OS (\%): C, 61.43; H, 6.78; N, 18.85. Found: C, 61.45; H, 6.79; N, 18.83.

6-(3,5-Di-t-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≡N), 1612 (C≡N), 3580 (OH); 1H NMR (300 MHz, DMSO-d₆): δ 1.23 (t, 3H, 1J_H = 8.7 Hz 2g).
Experimental data were presented as mean ± standard deviation. One-way analysis of variance was considered statistically significant if \( p < 0.0001 \).

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 \( p < 0.0001 \).

DPPH radical scavenging activity. The antioxidant activity of triazolotriazine-5-(6H)-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 hydroxytoluene (BHT) was taken as standard. The free radical scavenging ability of tested compounds expressed as %inhibition was calculated using the following equation [16]:

\[
\% \text{I} = \left( \frac{A_c - A_s}{A_c} \right) \times 100
\]

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 acetic buffer, pH 3.6; 20 mM FeCl\(_3\)·6H\(_2\)O solution and 10 mM TPTZ solution in 40-mM HCl were prepared. Three reagents were prepared; acetic buffer (300 mM, pH = 3.6), 20 mM FeCl\(_3\)·6H\(_2\)O solution and 10 mM TPTZ in 40 mM HCl. The freshly mixed solution was prepared by mixing FeCl\(_3\)·6H\(_2\)O, acetic 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, the mixture was allowed to react with triazolotriazine-5-(6H)-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.
REFERENCES


