Evaluation of *In Vitro* Antimicrobial and Antioxidant Activities of 4-Substituted-1,2,4-Triazolidine-3,5-Dione Derivatives

Hadi Adibi\(^a\), Ramin Abiri\(^b\), Shadpour Mallakpour\(^c\), Mohammad Ali Zolfigol\(^d\), Mohammad Bagher Majnoonie\(^e\)*

\(^a\) Novel Drug Delivery Research Center, Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran.
\(^b\) Department of Microbiology, Faculty of Medicine, Kermanshah University of Medical Sciences, Kermanshah, Iran.
\(^c\) Organic Polymer Chemistry Research Laboratory, Department of Chemistry, Isfahan University of Technology, Isfahan, Iran.
\(^d\) Faculty of Chemistry, Bu-Ali Sina University, Hamedan, Iran.
\(^e\) Student Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran and Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.

**ABSTRACT**

In a wide search program towards new and efficient antimicrobial agents, a series of 4-substituted-1,2,4-triazolidine-3,5-dione derivatives namely urazoles holding an alkyl moiety at position 4 have been synthesized and tested for their in vitro antibacterial and antifungal activities. The structures of these compounds have been investigated by spectral data. The synthesized compounds were tested in vitro against two Gram-positive, six Gram-negative bacteria, and the yeast *Candida albicans* (clinical isolated) in comparison with several control drugs. The antioxidant activity of the tested compounds was also assessed by 2 tests highly documented in the literature: capability to prevent lipid peroxidation, and direct scavenging effect on a stable free radical, 1,1-diphenyl-2-picryl-hydrazide (DPPH).
**Introduction**

Urazole derivatives (1,2,4-triazolidine-3,5-diones) are very interesting five-membered heterocyclic compounds, which at position 4 can provide a wide variety of aliphatic as well as aromatic constituents. There are only few commercially available urazoles. Urazoles are unusual, because they possess two pyramidal nitrogen atoms (sp$^3$) and a planar nitrogen atom (sp$^3$) in the same molecule. They are also unusual since the two pyramidal nitrogen atoms are bonded to one another. Since optically active forms of urazole do not appear to exist, it has always been assumed that nitrogen inversion takes place in urazole that is similar to nitrogen inversions in other amines [1].

Recently urazole and substituted urazoles have become an important structure in biological systems [2]. They have been shown to exhibit some anticonvulsant [3], agricultural fungicide [4], anti-inflammatory, antihyperlipidemic, antineoplastic, antidepressant activities [5] as well as catalytic activity in radical polymerization [6]. In biomedical research, urazole has been employed as a template for the syntheses of triazaprostaglandin analogues. These analogues were shown to have a bronchodilatory effect of a similar magnitude compared to the natural prostaglandins [7]. Antioxidants are of great interest because of their involvement in important biological and industrial processes. In general, compounds with antioxidant activity have also been found to have anticancer, ant心血管, anti-inflammatory, and many other activities [8-11]. Reactive oxygen species (ROS) and free radicals are considered to be implicated in a variety of pathological events, such as cancer and aging [12]. ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical, are thought to be generated subsequent to the reduction of molecular oxygen in aerobic organisms [13,14]. Under normal conditions, cells and tissues are protected against ROS by an array of enzyme defense systems, such as superoxide dismutase, catalase, and glutathione peroxidases, in addition to numerous non-enzymatic small molecules distributed widely in the biological system and capable of scavenging free radicals. These molecules include glutathione, α-tocopherol (vitamin E), vitamin C, β-carotene, and selenium [15]. In general, the cell is able to maintain an appropriate balance between oxidants and antioxidants under normal conditions. In the present study, some urazoles holding alkyl groups at position 4 were prepared and evaluated for their antimicrobial and antioxidants activities (Scheme 1).

![Scheme 1. Synthetic routes of urazoles](image-url)

**Materials and Methods**

**Chemistry**

All chemicals were purchased from Merck and Fluka chemical companies. The products were characterized by comparing the physical data with those of known samples or by their spectral data. Infrared spectra were recorded on Nicollet (impact 400D model) FTIR spectrophotometer. Spectra of solids were carried out using KBr pellets. Vibrational transition frequencies are reported in wavenumber (cm$^{-1}$). Band intensities are assigned as weak (w), medium (m), shoulder (sh), strong (s), and broad (br). $^1$HNMR spectra were recorded on Bruker DRX 300 Avance spectrophotometer in appropriate deuterated solvent and TMS as internal standard. All yields refer to isolated yield. Structural assignments of the products are based on their IR, $^1$HNMR, and also by comparison of their melting point with those of known compounds. Urazoles were synthesized according to reported literature [11]. **Caution:** Alkylisocyanates are lachrymator (tear producer). Thus, all operations involving alkylisocyanates must be conducted in an efficient hood.

**Typical procedure for the synthesis of 4-ethylurazole (1a)**

1-Ethoxycarbonyl-4-ethylsemicarbazide (35.00 g, 0.20 mol) was placed in 500-mL Erlenmeyer flask. The suspension was warmed on a hot plate and stirred by a magnetic stirrer for 1.5 h. Then 80 ml of 4 M KOH was added to ensure that the reaction had taken place to a large extent. The hot solution was filtered by suction filtration. The filtrate was cooled.
in an ice bath and then acidified with concentrated hydrochloric acid (about 50 mL). A white solid precipitated, filtered, and then dried in vacuum desiccators at room temperature. The yield was 25.80 g (100%) of product. Recrystallization from hot water (about 300 mL) yielded a white crystalline compound. IR (KBr, cm\(^{-1}\)): \(\nu\) 3250-3300 (m), 2920-2970 (s), 1650 (s), 1440-1460 (m), 1330 (m); \(^1\)HNMR (DMSO-\(d_6\), 400 MHz): \(\delta\) 10.02 (s, 2H, NH amide), 3.32 (q, \(J = 7.5\) Hz, CH\(_2\)), 1.07 (t, \(J = 7.5\) Hz, CH\(_3\)) ppm\(^1\).

Typical procedure for the synthesis of 4-n-propylurazole sodium salt (1c)

In a 1000-mL three-necked round-bottom flask, which was equipped with water cooled condenser, a constant pressure-dropping funnel, and a mechanical stirrer, 0.1690 mol of sodium metal (3.88 g) was placed. To this metal, absolute ethanol (300 mL) was added dropwise over a period of 60 min. Although being stirred magnetically, at the end of addition a clear solution was obtained (to obtain EtONa/EtOH, and for H-urazole, use 4 M KOH/H\(_2\)O as a base).

1-Ethoxycarbonyl-4-n-propyl semicarbazide 0.1690 mol (32.00 g) was added to the resulting clear solution under reflux conditions. Upon addition, an orange and then a red solution were obtained. After 2 h, slurry was formed that was refluxed for 54 h. The hot reaction mixture was filtered, and the desired solid compound was isolated as a white solid (17.82 g). Concentration of the filtered to about 30 mL afforded more material 6.12 g; yield: 23.94 g, 85.5%, mp 230 \(^\circ\)C. IR (KBr, cm\(^{-1}\)): \(\nu\) 3480 (m, sh), 3400 (s), 3200 (s), 2990 (m), 2920 (m), 2880 (m), 2870-2950 (m), 1690 (s), 1620 (s), 1590 (s), 1460 (s), 1420 (m), 1370 (m), 1260 (m), 1050 (m), 910 (w), 807 (s), 770 (w), 720 (w), 640 (m, br); \(^1\)HNMR (D\(_2\)O, 400 MHz): \(\delta\) 0.85 (t, \(J = 9.0\) Hz, CH\(_2\)), 1.63 (sextet, CH\(_2\)), 9.72 (s, 2H, NH amide) ppm\(^1\). The other urazoles were synthesized according to same procedure\(^1\).

4-Cyclohexylurazole (1d):

IR (KBr, cm\(^{-1}\)): \(\nu\) 3175-3250 (w), 2800-2925 (s), 2870-2950 (m), 1600 (s), 1475 (s), 1370-1450 (m); \(^1\)HNMR (DMSO-\(d_6\), 400 MHz): \(\delta\) 0.79 (3H,CH\(_3\)), 1.44 (2H, CH\(_2\)), 3.18 (2H, N-CH\(_2\)) ppm\(^1\).

1,6-Hexamethylene-bis-urazole (1e):

IR (KBr, cm\(^{-1}\)): \(\nu\) 3350-3425 (w), 2860-2920 (s), 1590 (s), 1090 (m), 1475 (m); \(^1\)HNMR (DMSO-\(d_6\), 400 MHz): \(\delta\)

Microbial strains

The synthesized compounds were evaluated for their antimicrobial activity against Staphylococcus aureus ATCC 25922, Pseudomonas aeroginosa ATCC 27853, and clinically isolated microorganisms including Staphylococcus aureus, Entercococcus coli, Entrococcus faecium, Sigella dysenteriae, Salmonella typhi and the yeast Candida albicans were provided by Department of Microbiology (Faculty of Medicine, Kermanshah University Medicinal Sciences, Kermanshah, Iran).

Determination of Minimum Inhibitory Concentrations (MIC)

Micro dilution broth susceptibility assay was used, as recommended by National Committee for Clinical Laboratory Standards\(^{16}\). Stock solutions of the test compounds were prepared in 10% DMSO and then serial dilutions of the compounds were made in a concentration range from 2 to 1000 \(\mu\)g/ml. The 96-well plates were prepared by dispensing into each well 95 \(\mu\)l of MHB, 100 \(\mu\)l of the oil (dissolved in 10% DMSO) and 5 \(\mu\)l of the inoculum. The inoculum of microorganisms was prepared using 24 h cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The final volume in each well was 200 \(\mu\)l. A positive control (containing 5 \(\mu\)l inculcums and 195 \(\mu\)l MHB) and negative control (containing 100 \(\mu\)l of essential oil dissolved in 10% DMSO, 100 \(\mu\)l MHB without inoculum) were included on each microplate. The contents of the wells were mixed and the microplates were incubated at 37 \(^\circ\)C for 24 h. The MIC was defined as the lowest concentration of the compounds to inhibit the growth of microorganisms. The standard antibiotics (gentamicin, ciprofloxacin, ceftriaxone and fluconazole) were also diluted in the same manner. Data of MICs of the test compounds and control drugs are shown in Table 1.
Table 1. The MICs (in µg/mL) values of urazoles and bisurazole against bacteria and fungus

<table>
<thead>
<tr>
<th>Compound</th>
<th>E. coli (clinical isolated)</th>
<th>shigella dysenteriae</th>
<th>E. faecium (clinical isolated)</th>
<th>K. pneumoniae (clinical isolated)</th>
<th>Salmonella typhi</th>
<th>S. aureus ATCC 25922</th>
<th>P. aeruginosa ATCC 27853</th>
<th>Candida albicans (clinical isolated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>32</td>
<td>256</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>1b</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>32</td>
<td>512</td>
<td>16</td>
</tr>
<tr>
<td>1c</td>
<td>512</td>
<td>64</td>
<td>256</td>
<td>512</td>
<td>64</td>
<td>64</td>
<td>512</td>
<td>32</td>
</tr>
<tr>
<td>1d</td>
<td>128</td>
<td>512</td>
<td>64</td>
<td>128</td>
<td>16</td>
<td>16</td>
<td>256</td>
<td>16</td>
</tr>
<tr>
<td>1e</td>
<td>128</td>
<td>128</td>
<td>16</td>
<td>128</td>
<td>16</td>
<td>32</td>
<td>128</td>
<td>16</td>
</tr>
<tr>
<td>1f</td>
<td>512</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>512</td>
<td>16</td>
</tr>
</tbody>
</table>

**Fluconazole**
- - - - - - 4

**Ciprofloxacin**
4 16 4 8 2 4 4 -

**Gentamicin**
4 8 8 2 8 4 4 -

**Ceftriaxone**
32 8 8 16 8 4 16 -

**Assays of Lipid Peroxidation Using Rat Brain Homogenates**

For the in vitro studies, the brains of normal rats were dissected and homogenized with a Polytron (speed setting 7-8) in ice-cold Tris-HCl buffer (20 µM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 14,000 rpm for 15 min. 1 mL aliquots of the supernatant were incubated with the 1 mL test samples dissolved in DMSO in the presence of 10 µM FeSO₄ and 0.1 mL of 0.1 mM L-ascorbic acid at 37 °C for 1 h. The reaction was stopped by addition of 1.0 mL trichloroacetic acid (TCA, 28% w/v) and 1.5 ml thiobarbituric acid (TBA, 1% w/v) in succession and the solution were then heated at 100 °C for 15 min. After centrifugation to remove precipitated protein, the color of the malondialdehyde (MDA)-TBA complex was detected at OD 532 nm using a UV–VIS spectrophotometer. BHA (butylated hydroxyl anisole) was used as a positive control. The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = (A-A₀)/A × 100, where A was the absorbance of the control, and
At was the absorbance of the test sample. The inhibition of lipid peroxidation of sample was expressed as 50% inhibition concentration (IC$_{50}$) which represented the concentration of sample having 50% inhibition effect on lipid peroxidation of the rat brain tissue.$^{[17]}$

**DPPH Radical Scavenging Activity Assay**

The compounds were dissolved in appropriate solvent mixed with 1 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) in ethanol, and final volume was adjusted to 2 mL. Mixtures were variously shaken and left for 30 min in the dark. Absorbance was measured at 517 nm using a UV–VIS spectrophotometer (Agilent). 1 mL of 0.2 mM DPPH diluted in 1 mL of ethanol was used as control. Neutralization of DPPH radical was calculated using the equation: $$S(\%) = 100 \frac{(A_o-As)}{A_o},$$ where $A_o$ is the absorbance of the control (containing all reagents except the test compound) and $A_s$ is the absorbance of the test sample. Results were compared with the activity of L-ascorbic acid. The IC$_{50}$ value represented the concentration of the test compounds that caused 50% inhibition.$^{[18]}$

**Results and discussion**

**Antimicrobial activity**

Microbiological results showed that the synthesized urazoles 1a-f holding an alkyl moiety at position 4 (ethyl 1a, methyl 1b, n-propyl sodium salt 1c, cyclohexyl 1d, n-butyl 1f) and 1,6-hexamethylene bisurazole 1e possessed a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative microorganisms (Table 1). All the urazoles 1a-f indicated significant antibacterial and antifungal activities having MIC values of 16-512 μg/mL, but showing less potency than the control drugs (Ciprofloxacin, Fluconazole, Gentamicin, and Ceftriaxone)$^{[16]}$. Among the urazoles tested, ethyl 1a, n-butyl 1f, methyl 1b urazole and 1,6-hexamethylene bisurazole 1e have exhibited good antibacterial property against *Enterococcus faesium* and *Salmonella typhi* having MIC value of 16 μg/mL (Table 1). Moreover, the synthesized compounds also possessed antimycotic activity against the yeast *Candida albicans* showing MIC value of 16 μg/mL (Table 1) except n-propylurazole sodium salt 1c having MIC value of 32 μg/mL. On the other hand, n-propylurazole sodium salt 1c exhibited lowest antibacterial and antifungal potency than the other urazoles with MIC values of 32-512 μg/mL. Perhaps the reason is that n-propylurazole sodium salt 1c has one hydrogen whereas other urazoles have two hydrogen linked to pyramidal nitrogen atoms. This shows the necessity of hydrogen linked to pyramidal nitrogen atoms for having better results. Also, the similarity of results (MIC) shows that substitution of electron-donating alkyl groups did not affect antimicrobial activity.

**Antioxidant activity**

On the basis of the IC$_{50}$ values determined, all the tested compounds inhibited brain lipid peroxidation (as measured by malondialdehyde formation)$^{[17]}$, and scavenged free radical DPPH$^{[18]}$ (Table 2). According to DPPH radical scavenging assay, cyclohexyl urazole 1d was found as the most active compound showing IC$_{50}$ value of 0.15 ± 1.36 μM and 1,6-hexamethylene bisurazole 1e was found as the least active one showing IC$_{50}$ value of 4.8 ± 0.4 μM. Unlike, according to lipid peroxidation assay n-butyl urazole 1f shows the most effect having IC$_{50}$ value of 0.1 ± 2.09 μM whereas the control drug 1h has IC$_{50}$ 2.8 ± 0.8 μM (Table 2). The obtained results show that urazoles and bisurazole have higher potency than the compared control drugs (Ascorbic acid and BHA) and seem to be unsafe agents for industrials purposes.

Antioxidant activity of urazoles by DPPH method proceeds according to the stoichiometry of Scheme 2. The possible mechanism is proposed according to a radical pathway upon homolytic cleavage of N–H bond in urazole I. The radical R initiates the oxidation reaction by abstracting hydrogen on the nitrogen of urazole I to give the radical specie II and RH. This radical II which can convert to the resonance form III loses the second hydrogen to form diradical intermediate IV and RH, subsequently resulting in the aromatized diradical(s) V.
Table 2. The IC₅₀ (in µM) values of urazoles and bisurazole according to DPPH radical scavenging and lipid peroxidation assays

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>IC₅₀</th>
<th>DPPH</th>
<th>Lipid peroxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td></td>
<td></td>
<td>1.7 ± 1.2</td>
<td>0.4 ± 0.01</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td></td>
<td>0.4 ± 2.9</td>
<td>0.2 ± 0.14</td>
</tr>
<tr>
<td>1c</td>
<td></td>
<td></td>
<td>1 ± 0.7</td>
<td>0.18 ± 0.16</td>
</tr>
<tr>
<td>1d</td>
<td></td>
<td></td>
<td>0.15 ± 1.36</td>
<td>0.23 ± 1.02</td>
</tr>
<tr>
<td>1e</td>
<td></td>
<td></td>
<td>4.8 ± 0.4</td>
<td>0.26 ± 0.087</td>
</tr>
<tr>
<td>1f</td>
<td></td>
<td></td>
<td>0.46 ± 1.8</td>
<td>0.1 ± 2.09</td>
</tr>
<tr>
<td>1g</td>
<td>L-Ascorbic acid</td>
<td>5 ± 1.7</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1h</td>
<td>BHA</td>
<td></td>
<td>-</td>
<td>2.8 ± 0.8</td>
</tr>
</tbody>
</table>

Conclusion

In summary, all of the synthesized urazoles and bisurazoles showed some antimicrobial activity. A strong antioxidant activity was obtained against lipid peroxidation and DPPH radical scavenging, showing more potent than the compared control drugs ascorbic acid and BHA. We believe that urazoles and bis-urazoles will find an important application to fulfill the needs of academia as well as pharmaceutical industries and also can act as best candidates for arteriosclerosis disorders.

Conflict of interest

Authors certify that no actual or potential conflict of interest in relation to this article exists.

Acknowledgments

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