Full Paper

Synthesis and Antioxidant Activity of New Pyridines Containing Gallate Moieties

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Pyridines containing the galloyl moiety have been prepared utilizing 4-acetyl pyrogallol. In addition, fused pyridines were synthesized from the obtained pyridines via further chemical transformations. The results indicated that compound **4a** showed stronger DPPH scavenging activity than the other compounds, and the scavenging effect decreased in the following order **4a** > t-BHQ > **2a** > **2b** > **3a** > **3b** > **4b**. Accordingly, other antioxidant assays were conducted for **4a**. The results suggested that compound **4a** could be a good antioxidant candidate. The absence of mortality of rats receiving 5000 mg/kg body weight of **4a** as single oral dose may indicate that it could be a safe antioxidant and may be used for further studies.

Keywords: Acetylation / Antioxidant activity / Pyridines / Pyrogallol

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Introduction

Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage [1]. Against this background, the evaluation of the antioxidant properties of specific chemical scavengers is of particular value for their potential use in preventing or limiting the damage induced by free radicals [1]. Accordingly, various naturally occurring substances are receiving continuous attention from the viewpoint of antioxidation [2, 3]. Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers. Phenolic antioxidants function as free radical terminators and sometimes also metal chelators [4]. Meantime, several pyridines have been subject of many chemical and biological studies [5, 6]. Therefore, the objectives of this study were conducted to synthesize pyridine derivatives linked to a pyrogallol residue, and evaluate the potential antioxidant activity.

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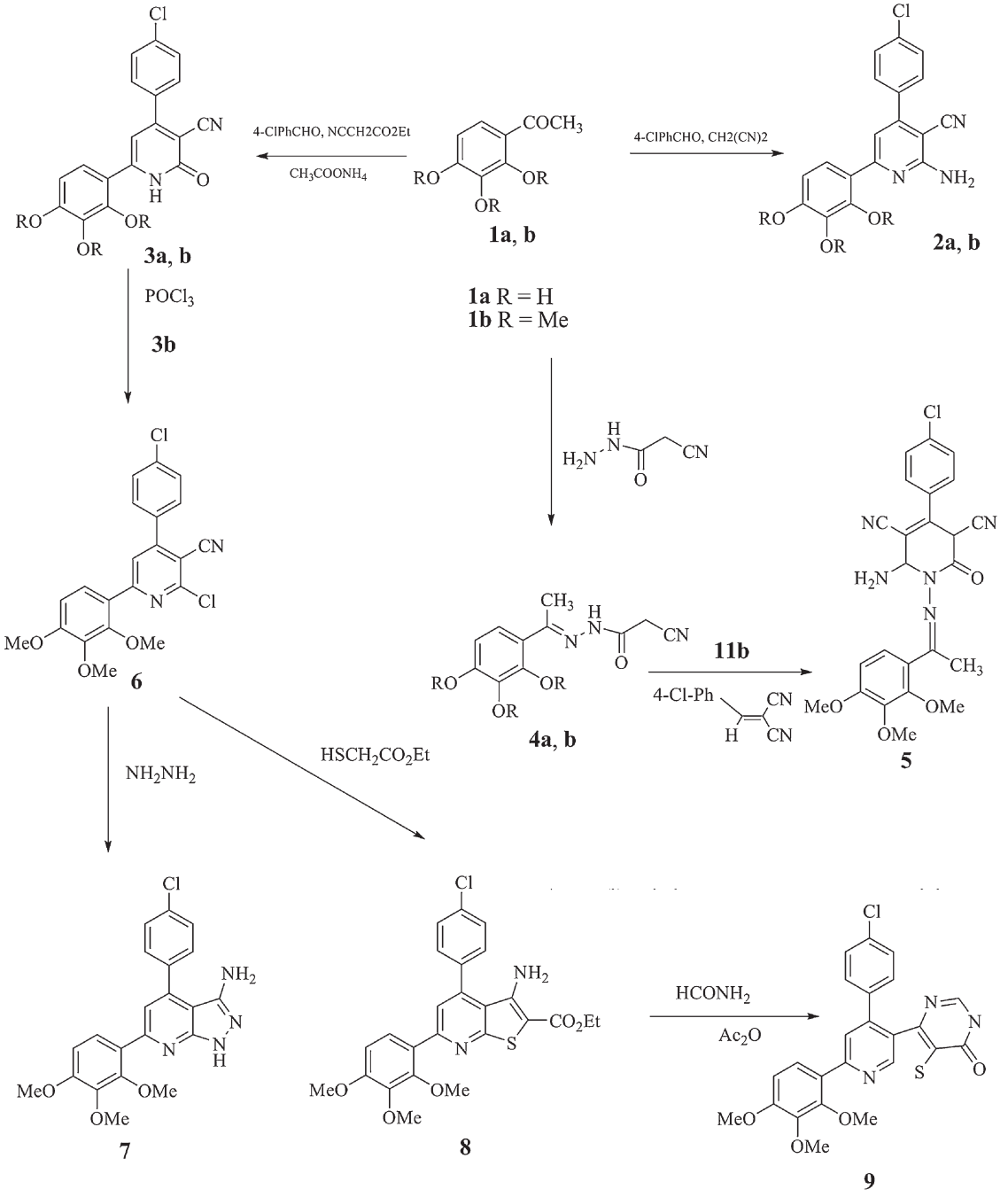
Abbreviations: *tert*-butylhydroquinone (t-BQH); 1,1-diphenyl-2-picrylhydrazyl (DPPH); lipid peroxidation (LPO); malondialdehyde (MDA); thiobarbituric acid reactive species (TBARS)

Results and discussion

Chemistry

The starting materials 2,3,4-trihydroxyacetophenone **1a** and 2,3,4-trimethoxyacetophenone **1b** were prepared according to the literature [7, 8]. **1a**, **b** were allowed to react with *p*-chlorobenzaldehyde and malononitrile in the presence of ammonium acetate to give the corresponding pyridine derivatives **2a**, **b** (Scheme 1). The IR spectra of compounds **2a**, **b** exemplified by **2a** showed NH_2 and CN groups in their expected locations at 3304, 3210, 2212 cm^{-1}, respectively. Moreover, the ^{1}H-NMR of compounds **2a**, **b** exemplified by **2a** showed a singlet (1H) at $\delta = 8.1$ ppm attributable to pyridine H-5, these along with the expected D_2O exchangeable protons. In a similar manner, **1a**, **b** were condensed with *p*-chlorobenzaldehyde and ethylcyanoacetate to afford the corresponding pyridine-2-one derivatives **3a**, **b** based on their elemental and spectral data (Scheme 1).

In continuation of our program aimed to synthesize heterocyclics with anticipated biological activity starting from precursors derived from waste [9], The acetophenone derivatives **1a**, **b** have been condensed with 2-cyanoacetohydrazide in ethanol in the presence of sulfuric acid to afford the corresponding hydrazone derivatives **4a**, **b** in good yield. The IR spectra of **4a**, **b** showed CN-absorption bands at $\nu = 2260$ and 2264 cm^{-1}, respectively and their ^{1}H-NMR spectra exemplified by **4a** revealed the methyl and the methylene groups as singlets at $\delta = 2.1$ and 4.1 ppm, respectively.



Scheme 1. Synthesis of compounds **2–9**.

Compound **4b** was allowed to react with 4-chlorobenzylidene malononitrile in the presence of a catalytic amount of piperidine, leading to the formation of the amino pyridine derivative **5**. Both, elemental and spectral data and a previous report [10] are consistent with the assigned structures. Thus, the IR spectrum revealed absorption bands at 3339, 3231, 2210, 1657 cm^{-1} corresponding to NH_2, CN, and CO, respectively, while the ^{1}H-NMR spectrum showed a broad singlet at $\delta = 8.17$ ppm corresponding to the D_2O-exchangeable protons of the NH_2 group. Moreover, its mass spectrum revealed a molecular ion peak at $m/z = 477$ corresponding to the molecular formula $C_{24}H_{20}ClN_5O_4$.

Compound **3b** underwent reaction with phosphorous oxychloride in the presence of a catalytic amount of dry pyridine

to give the chloropyridine derivative **6** in a fairly good yield. The IR spectrum of **6** revealed the absence of the CO and NH peaks which appeared in the parent compound **3b**, moreover, the mass spectrum of **6** showed a molecular ion peak at $m/z = 414$ which was in accordance with its molecular formula $C_{21}H_{16}Cl_2N_2O_3$.

In continuation of our investigation, compound **6** reacted with hydrazine hydrate to give the fused pyrazolopyridine derivative **7**. Structure **7** was established on the basis of its elemental and spectral data. Whereas the IR spectrum revealed absorption bands at $\nu = 3465$, 3285, and 3133 cm^{-1} attributed to NH and NH_2 groups and the absence of the CN absorption band of the parent compound **6**, its ^{1}H-NMR showed a singlet (1H) at $\delta = 7.3$ ppm attributable to the pyrazole pyridine H-5. The mass spectrum was also in accordance with its molecular formula ($m/z = 410$ [M^+]). It is assumed that the formations of **7** began via the N-nucleophilic substitution with subsequent self-cyclization involving the hydrazino amine and the pyridine cyano group.

In addition, **6** reacted with ethylthioglycolate in the presence of anhydrous potassium carbonate, affording the thienopyridine derivative **8** in moderate yield. The IR spectrum showed new peaks at $\nu = 3493$, 3352, and 1670 cm^{-1} attributed to NH_2 and CO (ester). Moreover, the ^{1}H-NMR showed signals at $\delta = 1.29$ and 4.27 ppm attributed to the ester group, and the mass spectrum was in accordance with its molecular formula $C_{25}H_{23}ClN_2O_5S$. The chemical behavior of **8** added further proofs to its structure: thus, when **8** was treated with formamide, the expected tricyclic product **9** was obtained in good yield [11].

In-vitro antioxidant activity

Free-radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. DPPH$^•$ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [12]. ESR results for the DPPH$^•$ radical are illustrated in Fig. 1, where it is documented that 0.28 μM of the compounds under study scavenged between 40.0 and 100.0% of the DPPH radicals. This increase in the % DPPH inhibition is caused by antioxidants – the reaction between the compounds under study (**2a**, **b**, **3a**, **b**, and **4a**, **b**) with DPPH radicals. Hence, DPPH$^•$ is an important substrate to evaluate antioxidant activity [13]. The results suggest that compound **4a** shows a stronger DPPH$^•$ scavenging activity than the other compounds and the scavenging effect decreases in the following order **4a** > t-BHQ > **2a** > **2b** > **3a** > **3b** > **4b**

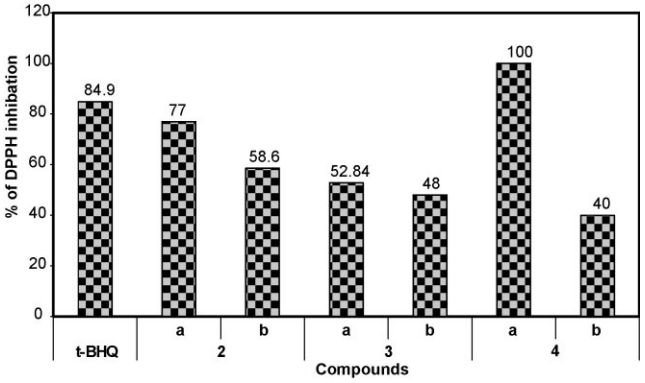
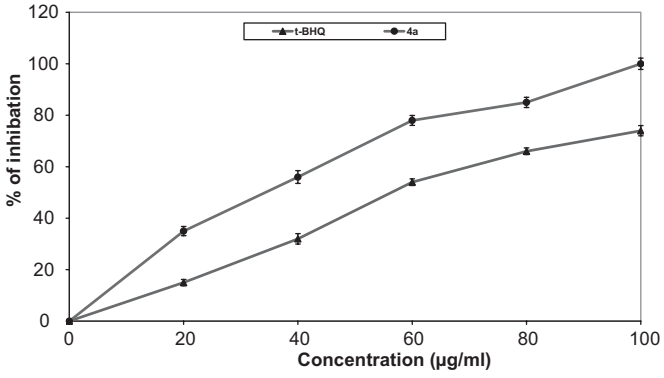


Figure 1. Scavenging effect of the compounds 2**a**, **b**, **3a**, **b** and **4a**, **b** on DPPH$^•$ radicals.

with 100, 84.9, 77, 58.6, 52.84, 48, and 40% of inhibition, respectively (Fig. 1). In this respect, the structure of compound **4a** is similar to the hydroquinoide structure of t-BHQ, and the imino group present in **4a** increases the acidity of the three hydroxyl groups at the benzene ring, which seems to enhance the antioxidant activity [14].

Hydrogen peroxide scavenging activity

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups [15]. H_2O_2 can cross cell membranes rapidly, and, once inside the cell, it can probably react with Fe^{2+} and possibly Cu^{2+} to form hydroxyl radicals, the latter may be the origin of many of its toxic effects [16]. Therefore, removing H_2O_2 is very important for the protection of biological systems. The scavenging ability of **4a** on hydrogen peroxide is shown in Fig. 2. Our results revealed that **4a** is capable of scavenging hydrogen peroxide in a concentration-dependent manner and **4a** had stronger hydrogen peroxide



Data are presented as mean ± SD ($n = 6$).

Figure 2. Hydrogen peroxide scavenging activity of **4a** and t-BHQ.

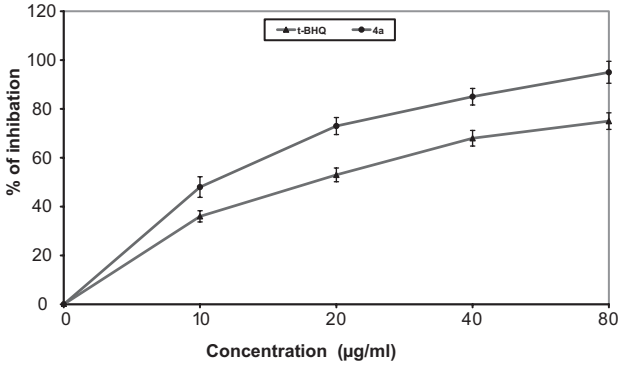
scavenging activity than t-BHQ; this difference was found to be statistically significant ($p < 0.05$). The percentage H_2O_2 scavenging effect by 20, 40, 60, 80, and 100 μg/mL of **4a** and t-BHQ was found to be 35, 56, 78, 85, and 100% and 15, 32, 54, 66, and 74%, respectively. IC_{50} values (concentration of **4a** and t-BHQ required to scavenge 50% of H_2O_2) of **4a** and t-BHQ were 36.95 μg/mL and 58.17 μg/mL, respectively. A lower IC_{50} value indicates a greater antioxidant activity.

Reduction power

The reducing power of **4a** was increased with increasing concentration (Fig. 3). At all the concentrations, **4a** showed higher activity than t-BHQ and this difference between **4a** and t-BHQ was found to be statistically significant ($p < 0.05$). It seems that the increase in the conversion of Fe^{3+} to Fe^{2+} in the presence of **4a** could be attributed to the availability of the nitrogen lone pair of electrons in **4a**. The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are the binding of transition metal ion, and, in this respect it seems that the tri-hydroxyl system in galloyl moiety plays a decisive role.

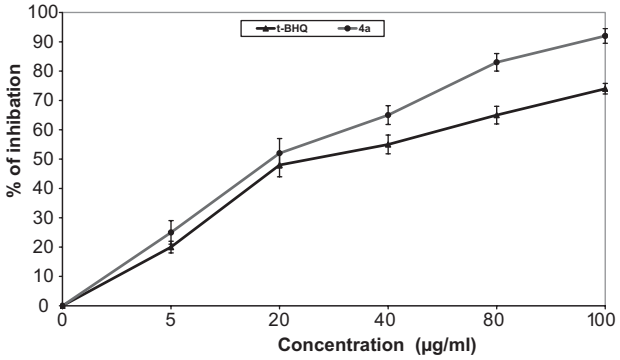
Inhibition effect on lipid peroxidation

Lipid peroxidation (LPO) mediated by free radicals is considered to be a primary mechanism of cell membrane destruction and cell damage [17]. The methods, known as thiobarbituric acid reactive species (TBARS) assay, concerns the spectrophotometric measurement of the pink color produced through the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA) and other secondary lipid peroxidation products. The evaluation of the absorbance at 532 nm gives a measure of the extent of lipid degradation. In the first two concentrations of compound **4a**, there was no significant different in the LPO inhibition compared to t-BHQ (Fig. 4). In contrast, as the concentration of **4a** increased, there was a significant ($p < 0.05$) increase in the % inhibition of LPO,



Data are presented as mean ± SD ($n = 6$).

Figure 3. Reducing power of **4a** and t-BHQ.



Data are presented as mean ± SD ($n = 6$).

Figure 4. Inhibition effect on lipid peroxidation of **4a** and t-BHQ.

compared to t-BHQ. It has been reported that the damage to lipids (by lipid peroxidation) occurs in three stages: initiation, propagation, and termination reactions. LPO may be induced by radical species, which are sufficiently reactive to abstract a hydrogen atom from the unsaturated fatty acids. This is the starting point for the lipid radical chain propagation reaction. The propagation cycle is broken by termination reactions (two radical species combine to form non-radical final products) which result in the destruction of free radicals. Results in the present study indicated that **4a** caused a significant inhibition of MDA. The presence of carbonyl, active methylen, and nitrile groups in **4a** afford a wide range of chemical activities, which could extend its reaction with free radicals and terminate lipid peroxidation.

Acute oral toxicity

In the present study, our results show no mortality in rats treated with **4a** at 500, 1000, 2000, and 5000 mg/kg b. wt. (body weight) in single oral dose. These data suggest that further studies should be continues with compound **4a** to consider it as an important antioxidant candidate.

Experimental

Chemistry

2-Thiobarbituric acid (2,6-dihydroxypyrimidine-2-thiol; TBA) was purchased from Merck (Germany) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) from Sigma. All reagents and solvents used were of reagent grades and obtained from the local scientific distributors in Egypt.

Melting points are uncorrected and were determined using an Electrothermal 9100 apparatus (Electrothermal, Essex, UK). Elemental microanalyses were performed at the Microanalytical Laboratory, National Research Centre, Dokki, Cairo, Egypt. IR spectra were recorded on a Beckman infrared spectrophotometer PU 7712 (Beckman Instruments, USA) using KBr. NMR spectra were recorded on Jeol EX-270 MHz and Jeol ECA 500 MHz spectrometer (Jeol, Tokyo, Japan) Varian Mercury VX 300 MHz and Varian

Gemini 200 MHz (Varian Inc., Palo Alto, CA, USA) in a suitable deuterated solvent using TMS as an internal standard. The mass spectra were recorded on GCMS-QP 1000Ex, Shimadzu spectrometer (Shimadzu, Japan) E.I. 70 eV at the Central Services Laboratory, Faculty of Science, Cairo University and National Research Centre, Cairo, Egypt. Samples were centrifuged using a Heraeus Labofuge 400R (Kendro Laboratory Products GmbH, Germany) and the spectrophotometric measurements were recorded using Shimadzu UV-VIS Recording 2401 PC spectrophotometer (Shimadzu).

*General procedure for the synthesis of compounds **2a, b***

To a solution of *p*-chlorobenzaldehyde (1.44 g, 0.01 mol), malononitrile (0.66 g, 0.01 mol), and ammonium acetate (6.16 g, 0.08 mol) in 25 ml ethanol (95%), compound **1a** and/or **1b** (0.01 mol) was added. The reaction mixture was refluxed for 4–5 h. A solid product precipitated after cooling it was filtered off, washed, and finally crystallized to afford **2a**, **b**.

*2-Amino-4-(p-chlorophenyl)-6-(2′,3′,4′-trihydroxyphenyl) pyridine-3-carbonitrile **2a***

Yield: 55%. M. p.: 270°C (decomposition) (ethanol); IR (film) ν: 3381(OH), 3304, 3210 (NH_2), 2212 (CN) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.1–3.7 (br s, 5H, 3 OH + H_2O), 6.9 (d, 1H, J = 8.55 Hz, H-6 trihydroxyphenyl), 7.36 (s, 2H, NH_2), 7.62–7.86 (m, 2H, *p*-chlorophenyl), 7.87 (d, 1H, J = 8.6 Hz, H-5 trihydroxyphenyl), 8.1 (s, 1H, H-5 pyridine), 8.4 (m, 2H, *p*-chlorophenyl) ppm; MS *m*/*z*: 353 [M^+] (3), 355 [$M^+ + 2$] (1). Anal. calcd. for $C_{18}H_{12}ClN_3O_3$ (353.75): C, 61.11; H, 3.42; Cl, 10.02; N, 11.88. Found: C, 60.90; H, 3.70; Cl, 9.93; N, 12.10.

*2-Amino-4-(p-chlorophenyl)-6-(2′,3′,4′-trimethoxyphenyl) pyridine-3-carbonitrile **2b***

Yield: 65%. M. p.: 186–188°C (ethanol); IR (film) ν: 3304, 3167 (NH_2); 2198 (CN) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.74, 3.77, 3.85 (3s, 9H, 3 OCH_3), 6.93 (d, 1H, J = 8.55 Hz, H-6 trimethoxyphenyl), 7.1 (s, 2H, NH_2), 7.53 (d, 1H, J = 8.85 Hz, H-5 trimethoxyphenyl), 7.59–7.66 (m, 5H, Ar-H and H-5 in pyridine); ^{13}C-NMR (DMSO-d_6) δ: 55.96, 60.52, 61.24 (3 OMe), 85.60 (CN), 107.98, 112.50, 116.96, 124.95, 125.36, 128.93, 130.12, 134.46, 135.94, 141.96, 152.17, 152.64, 154.76, 157.87, and 160.68 ppm (aromatic carbons). MS *m*/*z*: 395 [M^+] (100), 397 [$M^+ + 2$] (44.9). Anal. calcd. for $C_{21}H_{18}ClN_3O_3$: C, 63.72; H, 4.58; Cl, 8.96; N, 10.62. Found: C, 64.10; H, 4.80; Cl, 9.10; N, 10.80.

*General procedure for the synthesis of compounds **3a, b***

To a solution of a mixture of *p*-chlorobenzaldehyde (1.44 g, 0.01 mol), ethylcyanoacetate (1.13 g, 0.01 mol), and ammonium acetate (6.16 g, 0.08 mol) in ethanol (25 mL, 95%), compound **1a** and/or **1b** (0.01 mol) was added. The reaction mixture was refluxed for 2–6 h. The solid product which formed while hot was filtered off and crystallized to afford **3a**, **b**.

*3-Cyano-4-(p-chlorophenyl)-6-(2′,3′,4′-trihydroxyphenyl)-2(1H)-pyridone **3a***

Yield: 70%. M. p.: 280°C (decomposition) (ethanol); IR (film) ν: 3445 (OH), 3132 (NH), 2215 (CN), 1662 (CO) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.43–3.47 (br s, 5H, 3 OH + H_2O), 6.06 (d, 1H, J = 8.7 Hz, H-6 trihydroxyphenyl), 6.56 (s, 1H, H-5 pyridine), 7.05 (d, 1H, J = 8.7 Hz, H-5 trihydroxyphenyl), 7.55–7.65 (m, 4H, chlorophenyl); MS *m*/*z*: 354 [M^+] (100), 356 [$M^+ + 2$] (30). Anal. calcd. for $C_{18}H_{11}ClN_2O_4$: C, 60.94; H, 3.13; Cl, 9.99; N, 7.90. Found: C, 60.70; H, 3.30; Cl, 10.20; N, 8.10.

*3-Cyano-4-(p-chlorophenyl)-6-(2′,3′,4′-trimethoxyphenyl)-2(1H)-pyridone **3b***

Yield: 75%. M. p.: 259–260°C (ethanol); IR (film) ν: 3310 (NH), 2211 (CN) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.79, 3.86 (2s, 9H, 3 OCH_3), 6.54 (s, 1H, H-5 pyridine), 6.93 (d, 1H, J = 8.4 Hz, H-6 trimethoxyphenyl), 7.29 (d, 1H, J = 8.8 Hz, H-5 trimethoxyphenyl), 7.60–7.75 (m, 4H, chlorophenyl), 12.45 (s, 1H, NH); MS *m*/*z*: 398 [$M^+ - H$] (100), 396 [$M^+ + 2 - H$], (30). Anal. calcd. for $C_{21}H_{17}ClN_2O_4$: C, 63.56; H, 4.32; Cl, 8.93; N, 7.06. Found: C, 63.80; H, 4.50; Cl, 8.70; N, 7.30.

*General procedure for the synthesis of 2-cyano-N′-[1-ethylidene] acetohydrazide **4a, b***

A mixture of compound **1a** and/or **1b** (.0.01 mol) and 2-cyanoacetohydrazide (0.99 g, 0.01 mol) in absolute ethanol (50 mL) containing few drops of concentrated sulfuric acid was stirred for 12 h at room temperature. The solid that formed was filtered off, washed with water several times, air dried, and crystallized from the proper solvent to give the title compounds **11a**, **b**, respectively.

*2-Cyano-N′-[1-(2′,3′,4′-trimethoxyphenyl)ethylidene]acetohydrazide **4a***

Yield: 86%. M. p.: 239–240°C (methanol); IR (film) ν: 3755, 3451, 3395(OH), 3254 (NH), 2260 (CN) and 1684 (CO) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 2.26 (s, 3H, CH_3), 3.92 (s, 2H, CH_2), 6.32 (d, 1H, J = 7.5 Hz, H-6 phenyl), 6.93 (d, 1H, J = 7.5 Hz, H-5 phenyl), 8.40, 9.27, 13.27 (3s, 3H, 3 OH), 11.15 (s, 1H, NH); MS *m*/*z*: 249 [M^+] (25). Anal. calcd. for $C_{11}H_{11}N_3O_4$: C, 53.02; H, 4.44; N, 16.86. Found: C, 52.88; H, 4.70; N, 16.36.

*2-Cyano-N′-[1-(2′,3′,4′-trimethoxyphenyl)ethylidene]acetohydrazide **4b***

Yield: 87%. M. p.: 109–111°C (ethylacetate/*n*-hexane, 1:1); IR (film) ν: 3199 (NH), 2264 (CN), 1701 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 2.16 (s, 3H, CH_3), 3.73, 3.74, 3.77 (3s, 9H, 3 OCH_3), 4.07 (s, 2H, CH_2), 6.78 (d, H, J = 8.6 Hz, H-6 phenyl), 7.11 (d, J = 8.6 Hz, 1H, H-5 phenyl), 10.86 (s, 1H, NH); MS *m*/*z*: 291 [M^+] (39). Anal. calcd. for $C_{14}H_{17}N_3O_4$: C, 57.73; H, 5.88; N, 14.43. Found: C, 57.64; H, 6.18; N, 14.80.

*6-Amino-4-(p-chlorophenyl)-3,5-dicyano-1-{[1-(2′,3′,4′- trimethoxyphenyl)ethylidene]-amino}-2(1H)-pyridone **5***

A mixture of compound **4b** (2.91 g, 0.01 mol) and *p*-chlorobenzylidine malononitrile (1.9 g, 0.01 mol) in ethanol (30 mL, 95%) containing few drops of piperidine was refluxed for 15 min. The solid that formed while hot was filtered off and recrystallized to give **5** as colorless crystals (90% yield). M. p.: 278–280°C (methanol); IR (film) ν: 3339, 3231 (NH_2), 2210 (CN), 1657 (CO) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 2.22 (s, 3H, CH_3), 3.87, 3.88, 3.89 (3s, 9H, 3 OCH_3), 6.92 (d, 1H, J = 10.3 Hz, H-6 trimethoxyphenyl), 7.57–7.63

(m, 5H, *p*-chlorophenyl + H-5 trimethoxyphenyl), 8.17 (s, 2H, NH_2); MS *m/z*: 477 [M^+] (7), 479 [$M^+ + 2$] (3). Anal. calcd. for $C_{24}H_{20}ClN_5O_4$: C, 60.32; H, 4.22; Cl, 7.42; N, 14.65. Found: C, 60.45; H, 4.50; Cl, 7.70; N, 14.82.

2-Chloro-4-(p-chlorophenyl)-6-(2′,3′,4′-trimethoxyphenyl) pyridine-3-carbonitrile ***6***

Compound **3b** (1 g, 0.0025 mol) was refluxed with phosphorous oxychloride (5 mL) in the presence of dry pyridine (three drops) for 45 h. The reaction was allowed to cool to room temperature and then the reaction mixture was added dropwise to ice water with stirring. The solid formed was filtered off, air dried, and crystallized to afford **6** as colorless crystals (70% yield). M. p.: 167–168°C (ethanol); IR (film) ν: 2224 (CN) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.80, 3.81, 3.88 (3s, 9H, 3 OCH_3), 7.01 (d, 1H, $J = 9$ Hz, H-6 trimethoxyphenyl), 7.65 (d, 1H, $J = 9$ Hz, H-5 trimethoxyphenyl), 7.67–7.79 (m, 4H, chlorophenyl), 8.02 (s, 1H, H-5 pyridine); MS *m/z*: 414 [M^+] (100), 416 [$M^+ + 2$] (86), 418 [$M^+ + 4$] (20). Anal. calcd. for $C_{21}H_{16}Cl_2N_2O_3$: C, 60.74; H, 3.88; Cl, 17.07; N, 6.75. Found: C, 60.50; H, 4.10; Cl, 17.10; N, 6.50.

3-Amino-4-(p-chlorophenyl)-6-(2′,3′,4′-trimethoxyphenyl)-1H-pyrazolo[3,4-b]pyridine ***7***

Compound **4** (1 g, 0.0024 mol) was heated under reflux in hydrazine hydrate (80%, 5 mL) for 30 h. After cooling, the reaction mixture was poured into water (50 mL). The solid that formed was filtered off, washed with water, air dried, and crystallized to give compound **9** as colorless crystals (65% yield). M. p.: 219–220°C (petroleum ether (40-60)/ethylacetate, 2:1); IR ν: 3465 (NH), 3285 and 3133 (NH_2), 1638 (C=N) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.72, 3.80, 3.86 (3s, 9H, 3 OCH_3), 4.59 (s, 2H, NH_2), 6.95 (d, 1H, $J = 8.8$ Hz, H-6 trimethoxyphenyl), 7.33 (s, 1H, H-5 pyridine), 7.5 (d, 1H, $J = 8.6$ Hz, H-5 trimethoxyphenyl), 7.54–7.64 (m, 4H, chlorophenyl), 12.34 (s, 1H, NH); MS *m/z*: 410 [M^+] (100), 412 [$M^+ + 2$] (49.3). Anal. calcd. for $C_{21}H_{19}ClN_4O_3$: C, 61.39; H, 4.66; Cl, 8.62; N, 13.63. Found: C, 61.60; H, 4.70; Cl, 8.90; N, 13.70.

Ethyl 3-amino-4-(p-chlorophenyl)-6-(2′,3′,4′-trimethoxyphenyl)-thieno[2,3-b]pyridine-2-carboxylate ***8***

A mixture of compound **4** (4.15 g, 0.01 mol), ethyl thioglycolate (1.2 g, 0.01 mol), and anhydrous potassium carbonate (1.38 g, 0.01 mol) was heated under reflux and constant stirring for 6 h in absolute ethanol (50 mL). The solvent was evaporated under vacuum and the residue was solubilized with water, filtered off, air dried, and crystallized to give compound **8** as yellow solid (77% yield). M. p.: 199–200°C (ethylacetate); IR (film) ν: 3493 and 3352 (NH_2), 1670 (CO) cm^{-1}. ^{1}H-NMR (DMSO-d_6) δ: 1.29 (t, 3H, $J = 7.05$ Hz, CH_3), 3.73, 3.78, 3.86 (3s, 9H, 3 OCH_3), 4.27 (q, 2H, $J_1 = 7.05$, $J_2 = 12.8$ Hz, CH_2), 5.86 (s, 2H, NH_2), 6.97 (d, 1H, $J = 9$ Hz, H-6 trimethoxyphenyl), 7.58–7.66 (m, 6H, Ar-H). MS *m/z*: 498 [M^+] (100), 500 [$M^+ + 2$] (47.6). Anal. calcd. for $C_{25}H_{23}ClN_2O_5S$: C, 60.18; H, 4.65; Cl, 7.11; N, 5.61; S, 6.43. Found: C, 60.30; H, 4.81; Cl, 7.30; N, 5.90; S, 6.20.

7-(2′,3′,4′-Trimethoxyphenyl)-9-(p-chlorophenyl)-pyrido[3′,2′:4,5]-thieno-[3,2-d]pyrimidine-4(3H)-one ***9***

Compound **7** (4.98 g, 0.01 mol) was refluxed in formamide (50 mL) in the presence of acetic anhydride (3–4 drops) for 7 h. After cooling, the solid that formed was filtered off, washed with water, air dried, and crystallized to give compound **9** as pale yellow solid (60% yield). M. p.: 280°C (decomposition) (petroleum ether (40-60)/tetrahydrofuran, 5:1); IR (film) ν: 3165 (NH), 1666 (CO) cm^{-1}; ^{1}H-NMR (DMSO-d_6) δ: 3.82, 3.87, 3.88 (3s, 9H, 3 OCH_3), 7.00 (d, 1H, $J = 8.9$ Hz, H-6 trimethoxyphenyl), 7.55 (d, 1H, $J = 8.25$ Hz, H-5 trimethoxyphenyl), 7.66–7.69 (m, 4H, *p*-chlorophenyl), 7.85 (s, 1H, H-8), 8.1 (s, 1H, H-2); ^{13}C-NMR (DMSO-d_6) δ: 56.54, 61.09, and 61.79 (3 OMe), 108.92, 123.35, 123.35, 125.16, 126.14, 128.30, 132.23, 134.11, 136.21, 142.58, 147.21, 147.49, 151.85, 152.59, 155.46, 156.31, and 158.03 ppm (aromatic carbons) and 162.95 (CO); MS *m/z*: 479 [M^+] (62), 481 [$M^+ + 2$] 23%). Anal. calcd. for $C_{24}H_{18}ClN_3O_4S$: C, 60.06; H, 3.78; Cl, 7.39; N, 8.76, S, 6.68. Found: C, 59.80; H, 3.90; Cl, 7.60; N, 8.50; S, 6.90.

In-vitro **antioxidant activities**

Free radical scavenging activity using ESR-DPPH technique

The radical DPPH$^•$ scavenging activity of individual compounds and the standard antioxidant (*tert*-butylhydroquinone, t-BHQ) were determined by an electron spin resonance (ESR) spectrometry method, using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^•$) according to the method described by Ohtani *et al.* [18]. A DMF solution of 0.5 mL (0.28 μM/mL) of the individual compounds (or DMF itself as control) was added to 1 mL of DPPH$^•$ (1.3 μM/mL) in DMF solution to initiate the antioxidant-radical reaction. After mixing vigorously for 10 s, the solutions were transferred into a flat cell and fitted into the cavity of the electron spin resonance (ESR) spectrometer. ESR signals were recorded after 50 s following the start of the reaction. ESR analysis was conducted using ESR spectrometer Bruker-Flexsys 5000, operated at X-band (Bruker, USA). Frequency samples were measured in a pure silica liquid tube at room temperature in the Central Laboratory of the National Research Centre, Cairo, Egypt. The scavenging activity of each compound was estimated by comparing the DPPH$^•$ signals in the antioxidant-radical reaction mixture and the control reaction at the same reaction time, and expressed as percentage DPPH$^•$ inhibition. Percentage of DPPH$^•$ inhibition was calculated using the following formula:

$$\% \text{ DPPH inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

where: $A_{control}$ and A_{sample} are the peak height of the first line signals of the DPPH$^•$ of control and sample, respectively.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.* [19]. The principle of this method depended on the decrease in absorbance of H_2O_2 upon its oxidation. A solution of H_2O_2 (40 mM) was prepared in 0.1 M phosphate buffer (pH = 7.4). Then, 20–100 μg/mL of **4a** in 3.4 mL phosphate buffer were added to 0.6 mL H_2O_2 solution (40 mM). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2, and t-BHQ was used as standard.

The percentage of scavenged [H_2O_2]:

$$[H_2O_2] = [(A_c - A_t)/A_c] \times 100 \quad (2)$$

where A_c was the absorbance of the control and A_t was the absorbance in the presence of the standard sample or **4a**. The t-BHQ and **4a** concentrations providing 50% inhibition

(IC_{50}) were calculated from a graph plotting percentage inhibition against t-BHQ and **4a** concentrations.

Reduction power

The reduction power of **4a** was determined according to the method of Oyaizu [20]. The different concentrations of **4a** (10, 20, 40, 80 μg/mL) in 1 mL were mixed with phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$, 2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 × *g*. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. A higher absorbance of the reaction mixture indicated a greater reduction power.

Inhibition effect on lipid peroxidation

A modified thiobarbituric acid reactive species (TBARS) assay [21] with slight modifications was used to measure the potential antioxidant capacity using egg yolk homogenates as lipid-rich media. Briefly, 0.5 mL of 10% (w/v) tissue homogenate and 0.1 mL of **4a** (5, 10, 20, 40, 80, and 100 μg/mL), were added to a test tube and made up to 1.0 mL with distilled water. 50 μL of ferrous chloride ($FeCl_2$, 10 μM) in water were added to induce lipid peroxidation. Samples were incubated with different concentrations of compound **4a** and with a standard in a water-bath at 37°C for 30 min. 1.5 mL of 20% acetic acid (pH = 3.5) and 1.5 mL 0.5% (w/v) thiobarbituric acid in 1.1% (w/v) sodium dodecyl sulfate (SDS) solution were added and the resulting mixture vortexed and then heated at 95°C for 60 min. After cooling, 5.0 mL of *n*-butanol were added to each tube, then extensively vortexed and centrifuged at 1200 × *g* for 10 min. The absorbance of the organic upper layer was measured at 532 nm. All the values were based on the percentage antioxidant index (A_I%):

$$A_I\% = [1 - (A_t/A_c)] \times 100 \quad (3)$$

where A_c was the absorbance of the control and A_t was the absorbance in the presence of **4a** or t-BHQ.

Acute oral toxicity

Experimental animals

The healthy male albino rats of the Wistar strain *Rattus norvegicus*, weighing 150 to 160 g, (Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt) were used for the acute oral toxicity study. The animals were housed in clean plastic cages in the laboratory animal room (23 ± 2°C) on a standard pellet diet and tap water given *ad libitum*, a minimum relative humidity of 40% and a 12 h dark/light cycle. Rats were allowed to acclimate to laboratory conditions for at least one week before treatment. All the experimental procedures were conducted according to the NRC Guidelines of the Care and Use of Laboratory animals [22], and approved by the Animal Care & Experimental Committee, National Research Centre, Cairo, Egypt.

*Acute oral toxicity of compound **4a***

Compound **4a** was dissolved in DMSO and administered by gavage at a fixed volume of 0.5 mL/rat. Five groups of eight rats each were used and four for different single doses of the test compound (500, 1000, 2000, and 5000 mg/kg b. wt.), and one served as control (0.5 mL DMSO/rat). The mortality of the treated rats was recorded after 24 h.

Statistical analysis

The data were analyzed by using SPSS (version 14.0) for Windows and expressed as means ± S.D. Paired samples *t*-test was used to compare between the data of compound **4a** and those of t-BHQ.

The authors have declared no conflict of interest.

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