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Antioxidant, Cytotoxic Activities, and Structure–Activity Relationship of Gallic Acid-based Indole Derivatives

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A new series of gallic hydrazones containing an indole moiety was synthesized through the reaction of gallic hydrazide and different indole carboxaldehydes. Their antioxidant activities were determined on DPPH radical scavenging and inhibition of lipid peroxidation. The *in-vitro* cytotoxic activities of the compounds were evaluated against HCT-116 (human colon cancer cell line) and MCF-7 (estrogen-dependent human breast cancer cell line) by the MTT method. An attempt to correlate the biological results with their structural characteristics has been done. A limited positive structure activity relationship was found between cytotoxic and antioxidant activities.

Keywords: Antioxidant activity / Cytotoxic activity / Gallic hydrazone / Indole

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Introduction

The generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) has been considered to be a possible mediator of cellular damage in many diseases [1]. Human bodies are constantly exposed to ROS and RNS generated from endogenous and some exogenous sources. Antioxidants, both enzymatic and non-enzymatic, prevent oxidative damage to biological molecules by various mechanisms. Polyphenols constitute an important class of chemopreventive agents because they can quench or prevent the formation of ROS and RNS [2]. The ability of phenolic compounds to quench free radicals arises because of both their acidity (ability to donate protons) and their delocalized π-electrons (ability to transfer electrons while remaining relatively stable) characteristic of benzene rings. Among polyphenols, gallic acid (3,4,5-trihydroxybenzoic acid) derivatives are a well-known group of naturally occurring compounds which have been found in many phytomedicines. In recent years, a number of their biological and pharmacological activities have been described resulting in much attention in the development of synthetic gallic acid derivatives [3–11]. These compounds have been proved to have various biological properties including neuroprotective, antioxidant [12] and anticancer activities [13]. Several studies have demonstrated that gallic acid and its derivatives can selectively induce cancer cell death by apoptosis without harming healthy cells [14–16].

Indole derivatives are biologically important chemicals with a wide range of therapeutic properties. Antibacterial [17], antifungal [18], antiviral [19], antimalarial [20], anti-HIV [21], anticancer [22–24], and antioxidant properties [25, 26] have been reported to be associated with the indolic nucleus. Indolic compounds are very efficient antioxidants, protecting both lipids and proteins from peroxidation, and it is known that the indole structure influences the antioxidant efficacy in biological systems [27].

In the design of new drugs, the development of hybrid molecules through the combination of different pharmacophores in one frame may lead to compounds with interesting pharmacological properties. The co-administration of the moieties, acting by different mechanisms may have a synergistic effect, resulting in a higher activity than each of the components. In the light of interesting biological activities associated with gallic acid derivatives and indolic compounds, it was considered worthwhile to synthesize some hybrid molecules incorporating both indole and 3,4,5-trihydroxy benzoyl nucleus. The synthesized compounds were investigated for their antioxidant and cytotoxic activities.

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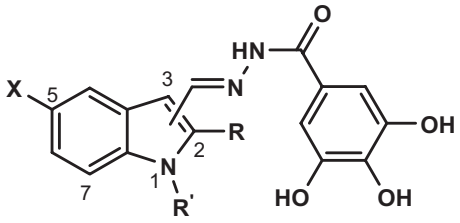
Table 1. Structure of the synthesized indole gallic hydrazones.



Compound	Position of the gallic hydrazide moiety	X	R	R′
2a	2	H	-	H
3a	3	H	H	H
3b	3	H	H	CH_3
3c	3	H	CH_3	H
3d	3	CH_3	H	H
3e	3	Cl	H	H
3f	3	Br	H	H
7a	7	H	H	H

Results

Chemistry

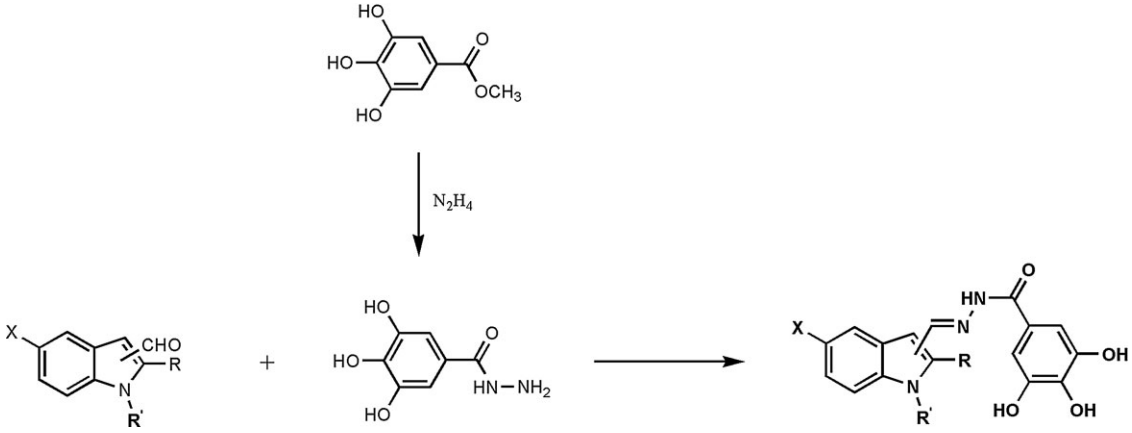
The structures of the synthesized compounds are presented in Table 1. The gallic hydrazide moiety is connected *via* an imine link on the indole nucleus, at different position (2, 3 or 7). Compounds differentiate also in substitution at position 1, 2 and 5 of the indole nucleus. Among those, the crystal structures of compounds **3a** [28], **3e** [29], and **3f** [30] have been reported previously by us. The compounds were synthesized upon the reaction of the appropriate indole carboxaldehydes with gallic hydrazide which had itself been prepared from the reaction between methyl 3,4,5-trihydroxybenzoate and hydrazine. The synthetic route is depicted in Scheme 1. All the compounds were characterized by elemental analysis and spectroscopic methods.

In vitro antioxidant activities

The synthesized compounds were subjected to DPPH radical scavenging and lipid peroxidation inhibitory assays to determine their antioxidant activity. The results were compared with Ascorbic acid and α-tocopherol. All the tested compounds showed noticeable DPPH radical scavenging activity, with IC_{50} values of 0.17–0.37mM, when compared to the reference compounds, ascorbic acid and α-tocopherol with IC_{50} values of 0.98 and 0.91 mM, respectively (Table 2). Among them, compounds **3e** and **3f** with a halogen group in position 5 of the indole ring showed the highest activity. All the compounds under study also possess inhibitory effect on lipid peroxidation. The effect of the test compounds and α-tocopherol (positive control) on their reaction with lipid is shown in Table 2. The results show a very similar trend to the one of DPPH radical scavenging assay. The halogenated compounds, **3e** and **3f**, showed strong inhibitory activity, with the lowest IC_{50} values, and comparable to α-tocopherol.

Cytotoxic activities

The synthesized compounds were tested for their cytotoxic activity on HCT-116 and MCF-7 cell lines by the MTT assay. As shown in Table 2, all compounds have comparable activities with the positive control, curcumin. The highest activities against MCF-7 cell line were found for the halogenated compounds **3e** and **3f** with IC_{50} values of 19.2 μM and 13.3 μM, respectively. A similar trend was observed with HCT-116 cell line, with the halogenated compounds **3e** and **3f** yielding the lowest IC_{50} values, 6.7 μM. Figure 1 displays the effect of increasing concentrations of the compounds on the cancer lines. It is apparent that the cell viability decreased almost linearly as a function of the log concentration of the compounds.



Scheme 1. Synthetic route for the preparation of compounds **2a–7a**.

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Table 2. DPPH radical scavenging, anti-lipid peroxidation (LP) and cytotoxic activities of **2a–7a**[1].

Compound	DPPH IC_{50} (μM)	LP IC_{50} (nM)	Cytotoxicity IC_{50} (μM)	
			MCF-7	HCT-116
2a	$357 \pm 12^{a,b}$	403 ± 8^{a}	$40.0 \pm 2.5^{a,b}$	29.4 ± 1.6^{a}
3a	$259 \pm 4^{c,d}$	344 ± 9^{b}	46.9 ± 3.1^{b}	29.2 ± 2.8^{a}
3b	$303 \pm 10^{a,d}$	621 ± 4^{c}	$35.1 \pm 2.8^{a,b}$	30.0 ± 3.7^{a}
3c	$216 \pm 9^{c,e}$	179 ± 4^{d}	$27.9 \pm 3.1^{a,c}$	$21.0 \pm 1.5^{a,b}$
3d	$358 \pm 10^{a,b}$	713 ± 18^{e}	$39.0 \pm 2.1^{a,b}$	29.4 ± 3.0^{a}
3e	189 ± 8^{e}	29 ± 3^{f}	$19.2 \pm 1.1^{c,d}$	6.7 ± 0.1^{c}
3f	172 ± 9^{e}	50 ± 3^{f}	13.3 ± 0.9^{d}	6.7 ± 0.2^{c}
7a	373 ± 12^{b}	657 ± 16^{c}	44.4 ± 1.6^{b}	29.9 ± 0.1^{a}
Ascorbic acid	981 ± 7^{f}	–	–	–
α-Tocopherol	911 ± 19^{g}	38 ± 6^{f}	–	–
Curcumin	–	–	$34.7 \pm 3.5^{a,b}$	$13.7 \pm 0.9^{b,c}$

[1] The values represent the mean ± S.E.M. of three determinations.[a–g] Values in a column with the same superscript letter are not significantly different, $P < 0.05$ (ANOVA and Tukey's test).

Discussion

The present study was conducted to investigate structure–activity relationship for the antioxidant and cytotoxic activities of hybrid molecules containing indole and gallic hydrazone moieties. Eight different indole gallic hydrazones were synthesized and tested for their antioxidant potency by well known *in-vitro* antioxidant assays, DPPH radical scavenging ability and lipid peroxidation inhibitory. It is well established that gallic acid derivatives possess antioxidant properties. The antioxidant activity of these phenolic compounds is mainly due to their redox properties, which can play an important role in the absorption and neutralization of free radicals, the quenching of singlet and triplet oxygen, or the decomposition of peroxides [31]. Likewise, indolic compounds are known to have antioxidant properties. The active redox center of indoles is the nitrogen atom. Replacement of the nitrogen by oxygen significantly reduces antioxidant activity [32]. Delocalization of the nitrogen electron pair over the aromatic system seems to be of great importance for antioxidant activity of indole derivatives. It is reasonable to conclude that multiple mechanisms regulate antioxidant action of the hybrid molecules, although they may contribute to the antioxidant activity to different degrees.

All the tested compounds showed significant DPPH radical scavenging activity and with a very similar trend, inhibitory effect on lipid peroxidation. Comparison of the activity of compounds **2a**, **3a**, and **7a** shows the importance of the attachment position of the imine link, while the activities of the other compounds reveal the effect of the substitution patterns of the halogen or methyl group. The higher antioxidant activity of compound **3a** compared to **2a** and **7a** can be explained by the fact that the attachment of the gallic hydrazone at the 3-position permits stabilization of the indolyl cation radical intermediate by the conjugated imine side chain without disruption of the benzene aromaticity. Introduction of a chlorine or bromine atom in position 5 of the indole moiety significantly enhances the antioxidant activity. Thus, compounds **3e** and **3f** show the highest activities among all the target compounds. This might be ascribed to the electron donating properties of the halogens by resonance, making the lone pair electron on the indole nitrogen atom more available to a plausible electron transfer. However, some other factors may be involved, as there are some reports which show different influences by halogens in the 5-position of indole [25, 33]. The effect of a methyl substituent depends on its attached position. Compound **3c**, with a methyl group in 2-position, has a higher activity than the unsubstituted compound **3a**, whereas introduction of a methyl group on the indole nitrogen or at the 5-position diminishes the antioxidant activity.

For further assessment, the compounds were subjected to cytotoxicity assay against HCT-116 and MCF-7 cell lines. In a previously reported study on cytotoxic and antioxidant activities of some phenolic systems (hydroxycinnamic acid derivatives), a positive structure-activity-property relationship between the activities was observed. It was suggested that the antitumor activity of phenolic derivatives is highly dependent upon their conformational characteristics, which, in turn determine their antioxidant properties [14]. The considerable antioxidant activities of our synthesized molecules intrigued us to know how relevant these results are to their cytotoxic activity. According to the *in-vitro* results, the target compounds have comparable activities with the positive control, curcumin. In excellent agreement with antioxidant efficiency, the halogenated compounds, **3e** and **3f**, showed the highest activities against both cell lines with IC_{50} values lower than curcumin. Furthermore, consistent with the anti-

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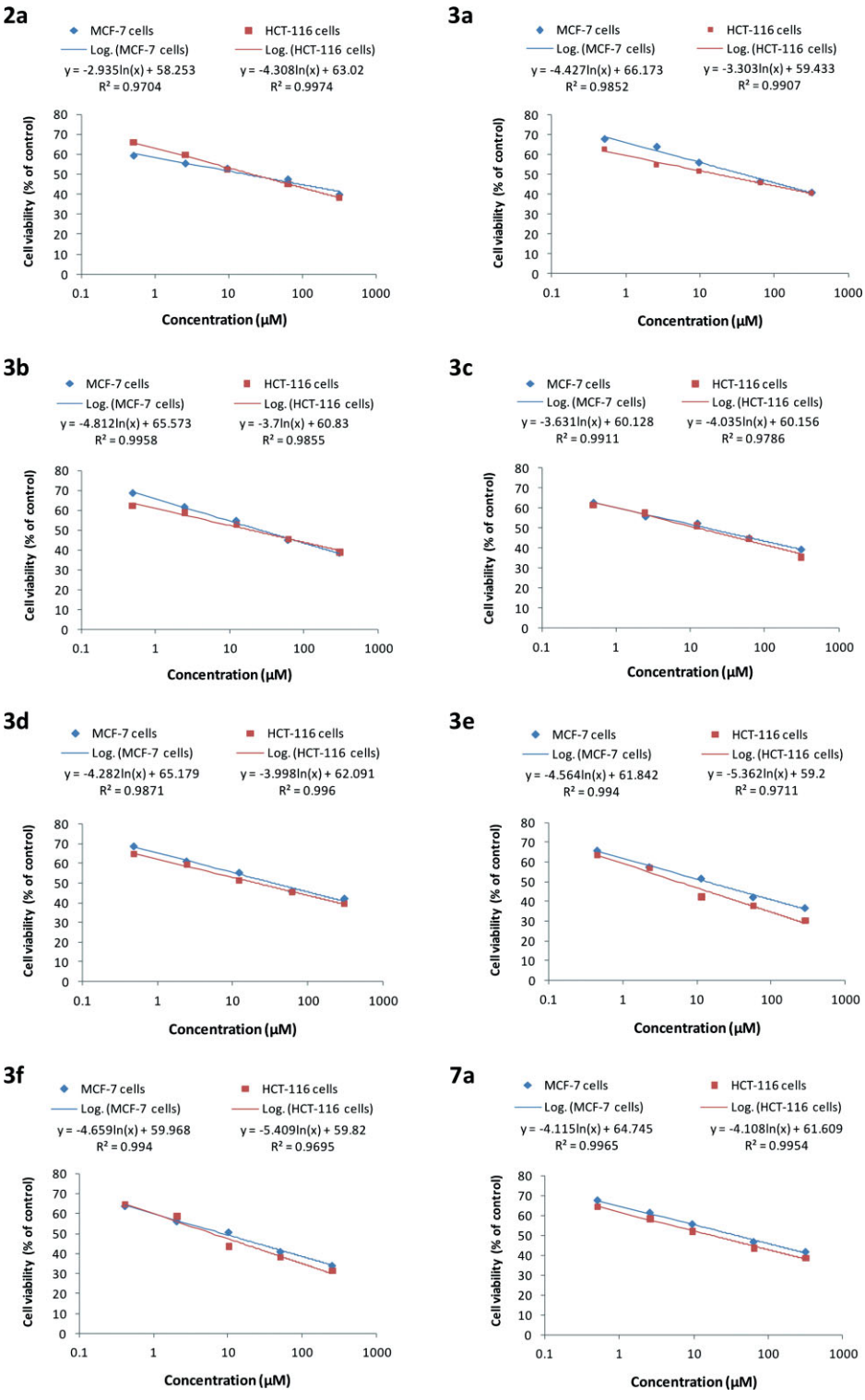

Figure 1. Dose-response curves of MCF-7 and HCT-116 cells to varying concentrations of compounds **2a–7a**.

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oxidant activity results, the replacement of the hydrogen in position 2 by a methyl group enhanced the cytotoxicity, thus, compound **3c** is more active than **3a**. To a lesser extent, introduction of a methyl group on the indole nitrogen (**3b**) or at position 5 of the indole moiety (**3d**) also increased the activity against MCF-7 cells, while it did not show any obvious effects on HCT-116 cell line (**3a** $\sim$ **3b** $\sim$ **3d**). A discrepancy was also observed with the effect of the position of the imine link on the indole system. While the 3-imineindole **3a** showed higher antioxidant activity compared to the 2-imineindole, **2a**, and the 7-imineindole, **7a**, the cytotoxicity effectiveness against MCF-7 cell line followed the order of **2a** $>$ **7a** $>$ **3a**. These three compounds showed almost the same effect on HCT-116 cells. Overall, the results obtained from this study showed a limited correlation between the antioxidant and cytotoxic properties of the synthesized compounds.

Conclusions

In conclusion, we have designed a series of hybrid molecules on the basis of the biological significance of indole and gallic acid. With a high degree of consistency, the compounds showed significant antioxidant activities in DPPH radical scavenging and inhibitory of lipid peroxidation assays. The compounds also exhibited noticeable cytotoxicity against HCT-116 and MCF-7 cell lines. A limited degree of agreement was observed between cytotoxic and antioxidant activities. The activities are dependent on the position of the imine link and different substituents on indole moiety. Among all, the halogenated compounds, **3e** and **3f**, are the most efficient compounds. Further study is required to understand the mechanisms of action of this class of compounds.

Experimental

Chemistry

Melting points were determined using a MEL-TEMP II melting point instrument and were not corrected. Microanalyses were carried out on a Perkin-Elmer 2400 elemental analyzer. ^{1}H-NMR and ^{13}C-NMR spectra were determined with a Lambda JEOL 400 MHz FT-NMR (^{1}H-NMR: 400 MHz and ^{13}C-NMR: 100.4 MHz) spectrometer. Chemical shifts are given in δ values (ppm) using TMS as the internal standard.

Synthesis of 3,4,5-trihydroxybenzoic hydrazide (gallic hydrazide)

A mixture of methyl 3,4,5-trihydroxybenzoate (9.2 g, 50 mmol) and hydrazine hydrate (45 mL) was stirred at room temperature for 30 min until the ester was dissolved completely. Ethanol (250 mL) was added to the mixture and it was stirred under reflux for 6 h and then at room temperature overnight. The white solid was filtered, washed with ethanol and dried over silica gel to give 5.13 g, 56% gallic hydrazide.

*General procedure for the synthesis of compounds **2a–7a***

An equimolar (5 mmol) mixture of indole carboxaldehyde and 3,4,5 trihydroxybenzoylhydrazine in the presence of acetic acid (1 mL) was heated in ethanol (70 mL) for 6 h. The solution was then cooled and filtered to remove the unreacted hydrazide. The hydrazide was washed with ethanol and the filtrates added together, evaporated partially, and poured to water (400 mL). The solid product formed was filtered off, washed with diethyl ether and dried over silica gel to give the related indole gallic hydrazone with 57–77% yield.

*3,4,5-Trihydroxy-N′-[(1H-indol-2-yl)methylidene]-benzohydrazide **(2a)***

Yield 68%, mp 261–263°C; ^{1}H-NMR (DMSO-d_6): δ 6.77 (s, 1H, Ar-*H*), 6.93 (s, 2H, Ar-*H*), 6.99 (t, 1H, Ar-*H*), 7.13 (t, 1H, Ar-*H*), 7.42 (d, 1H, Ar-*H*), 7.54 (d, 1H, Ar-*H*), 8.43 (s, 1H, C*H*N), 9.13 (br, 3H, O*H*), 11.50 (s, 1H, N*H*), 11.53 (s, 1H, N*H*); ^{13}C-NMR (DMSO-d_6): δ 106.26, 107.17, 111.92, 120.66, 123.16, 123.36, 127.68, 133.50, 136.96, 137.72, 139.34 (*C*HN), 145.57, 163.16 (*C*ONH); Anal. calcd. for $C_{16}H_{13}N_3O_4$: C, 61.73; H, 4.21; N, 13.50%. Found: C, 61.20; H, 4.98; N, 13.10%.

*3,4,5-Trihydroxy-N′-[(1H-indol-3-yl)methylidene]-benzohydrazide **(3a)***

Yield 73%, mp 238–240°C; ^{1}H-NMR (DMSO-d_6): δ 6.92 (s, 2H, Ar-*H*), 7.16 (t, 1H, Ar-*H*), 7.20 (t, 1H, Ar-*H*), 7.43 (d, 1H, Ar-*H*), 7.76 (s, 1H, Ar-*H*), 8.28 (d, 1H, Ar-*H*), 8.57 (s, 1H, Ar-*H*), 8.75 (s, 1H, O*H*), 9.13 (s, 2H, O*H*), 11.21 (s, 1H, indole N*H*), 11.53 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 107.45, 112.22, 112.33, 120.97, 122.41, 123.22, 124.34, 124.76, 130.39, 137.14, 137.41, 144.92 (*C*HN), 145.95, 163.57 (*C*ONH); Anal. calcd. for $C_{16}H_{13}N_3O_4$: C, 61.73; H, 4.21; N, 13.50%. Found: C, 61.44; H, 4.75; N, 13.17%.

*3,4,5-Trihydroxy-N′-[(1-methyl-1H-indol-3-yl)-methylidene]benzohydrazide **(3b)***

Yield 65%, mp 256–258°C; ^{1}H-NMR (DMSO-d_6): δ 3.81 (s, 3H, C*H*$_3$), 6.92 (s, 2H, Ar-*H*), 7.17 (t, 1H, Ar-*H*), 7.26 (t, 1H, Ar-*H*), 7.48 (d, 1H, Ar-*H*), 7.75 (s, 1H, Ar-*H*), 8.28 (d, 1H, Ar-*H*), 8.54 (s, 1H, Ar-*H*), 8.72 (s, 1H, O*H*), 9.10 (s, 2H, O*H*), 11.19 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 32.76 (*C*H$_3$), 107.04, 110.14, 110.99, 120.55, 122.18, 122.67, 124.11, 124.79, 133.48, 136.59, 137.54, 143.38 (*C*HN), 145.56, 162.70 (*C*ONH); Anal. calcd. for $C_{17}H_{15}N_3O_4$: C, 62.76; H, 4.65; N, 12.92%. Found: C, 63.01; H, 4.89; N, 12.57%.

*3,4,5-Trihydroxy-N′-[(2-methyl-1H-indol-3-yl)-methylidene]benzohydrazide **(3c)***

Yield 77%, mp 284–286°C; ^{1}H-NMR (DMSO-d_6): δ 2.50 (s, 3H, C*H*$_3$), 6.69 (s, 2H, Ar-*H*), 7.10 (m, 2H, Ar-*H*), 7.32 (d, 1H, Ar-*H*), 8.21 (d, 1H, Ar-*H*), 8.66 (s, 1H, Ar-*H*), 8.76 (s, 1H, O*H*), 9.12 (s, 2H, O*H*), 11.10 (s, 1H, indole N*H*), 11.41 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 11.50 (*C*H$_3$), 106.98, 107.78, 110.79, 120.13, 121.27, 121.75, 124.20, 125.47, 135.72, 136.53, 139.54, 143.57 (*C*HN), 145.59, 162.46 (*C*ONH); Anal. calcd. for $C_{17}H_{15}N_3O_4$: C, 62.76; H, 4.65; N, 12.92. Found: C, 62.51; H, 5.05; N, 13.00%.

*3,4,5-Trihydroxy-N′-[(5-methyl-1H-indol-3-yl)-methylidene]benzohydrazide **(3d)***

Yield 65%, mp 259–260°C; ^{1}H-NMR (DMSO-d_6): δ 2.41 (s, 3H, C*H*$_3$), 6.91 (s, 2H, Ar-*H*), 7.01 (d, 1H, Ar-*H*), 7.30 (d, 1H, Ar-*H*), 7.69 (s, 1H, Ar-*H*), 8.05 (s, 1H, Ar-*H*), 8.54 (s, 1H, Ar-*H*), 8.70–9.10 (br, 3H, O*H*),

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11.15 (s, 1H, indole N*H*), 11.41 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 21.58 (*C*H3), 107.28, 111.65, 111.76, 121.85, 124.29, 124.48, 124.86, 129.41, 130.33, 135.63, 136.90, 144.88 (*C*HN), 145.79, 163.18 (*C*ONH); Anal. calcd. for $C_{17}H_{15}N_3O_4$: C, 62.76; H, 4.65; N, 12.92. Found: C, 62.35; H, 4.98; N, 13.06%.

3,4,5-Trihydroxy-N′-[(5-chloro-1H-indol-3-yl)-methylidene]benzohydrazide ***(3e)***

Yield 67%, mp 274–276°C; ^{1}H-NMR (DMSO-d_6): δ 6.89 (s, 2H, Ar-*H*), 7.18 (dd, 1H, Ar-*H*), 7.42 (d, 1H, Ar-*H*), 7.82 (d, 1H, Ar-*H*), 8.28 (s, 1H, Ar-*H*), 8.52 (s, 1H, Ar-*H*), 8.77 (s, 1H, O*H*), 9.13 (s, 2H, O*H*), 11.26 (s, 1H, indole N*H*), 11.69 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 107.52, 112.22, 113.90, 121.71, 123.08, 124.44, 125.46, 125.87, 131.93, 136.02, 137.14, 143.84 (*C*HN), 146.05, 163.25 (*C*ONH); Anal. calcd. for $C_{16}H_{12}ClN_3O_4$: C, 55.58; H, 3.50; N, 12.15. Found: C, 55.76; H, 3.89; N, 11.96%.

3,4,5-Trihydroxy-N′-[(5-bromo-1H-indol-3-yl)-methylidene]benzohydrazide ***(3f)***

Yield 71%, mp 273–275°C; ^{1}H-NMR (DMSO-d_6): δ 6.89 (s, 2H, Ar-*H*), 7.29 (dd, 1H, Ar-*H*), 7.38 (d, 1H, Ar-*H*), 7.81 (d, 1H, Ar-*H*), 8.42 (s, 1H, Ar-*H*), 8.51 (s, 1H, Ar-*H*), 8.77 (s, 1H, O*H*), 9.13 (s, 2H, O*H*), 11.26 (s, 1H, indole N*H*), 11.70 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 107.51, 112.11, 113.51, 114.37, 124.43, 124.72, 125.63, 126.51, 131.79, 136.27, 137.14, 143.88 (*C*HN), 146.05, 163.45 (*C*ONH); Anal. calcd. for $C_{16}H_{12}BrN_3O_4$: C, 49.25; H, 3.10; N, 10.77. Found: C, 49.70; H, 3.43; N, 10.70%.

3,4,5-Trihydroxy-N′-[(1H-indol-7-yl)methylidene]-benzohydrazide ***(7a)***

Yield 57%, mp 237–240°C; ^{1}H-NMR (DMSO-d_6): δ 6.56 (m, 1H, Ar-*H*), 6.98 (s, 2H, Ar-*H*), 7.12 (t, 1H, Ar-*H*), 7.31 (d, 1H, Ar-*H*), 7.53 (s, 1H, Ar-*H*), 7.68 (d, 1H, Ar-*H*), 8.63 (s, 1H, C*H*N), 8.88 (s, 1H, O*H*), 9.22 (s, 2H, O*H*), 10.91 (s, 1H, indole N*H*), 11.81 (s, 1H, CON*H*); ^{13}C-NMR (DMSO-d_6): δ 102.3, 107.30, 118.10, 119.23, 122.73, 123.07, 124.04, 126.21, 128.23, 131.89, 137.26, 145.72, 147.55 (*C*HN), 163.44 (*C*ONH); Anal. calcd. for $C_{16}H_{13}N_3O_4$: C, 61.73; H, 4.21; N, 13.50%. Found: C, 62.2; H, 5.11; N, 13.77%.

DPPH free radical scavenging activity

Radical scavenging activity was determined by a spectrophotometric method based on the reduction of an ethanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) [34]. Briefly, to a solution of DPPH (200 μM) in absolute ethanol, the compound dissolved in DMSO was added at various concentrations. All the samples were incubated in dark for 30 min at room temperature. When DPPH reacts with an antioxidant, it will be reduced. The change in color (from purple to yellow) was measured spectrophotometrically at 517 nm for each sample after incubation. Ascorbic acid and α-tocopherol were used as positive controls. The free radical scavenging activity of the compounds was calculated as a percentage of radical reduction. Each experiment was carried out in triplicates and each experiment was performed three times. IC_{50} values were determined from a calibration curve for each compound. The radical scavenging activity was obtained from the equation:

$$\text{Percentage of scavenging activity } (\%) = [1-(A_s/A_c)] \times 100$$

where A_c is the absorbance of control (DPPH solution + DMSO) and A_s is the absorbance of compound/positive control.

Lipid Peroxidation Assay

The lipid peroxidation assay was carried out following the previously described method [35]. Fowl egg yolk, comprising mainly of phospholipids, triacyglycerols and proteins, was used as an alternative to rat liver microsomes and linoleic acid. The reactive mixture for inducing lipid peroxidation contained 1 mL egg yolk emulsified with phosphate buffer saline (0.1 M, pH 7.4), to obtain a final concentration of 12.5 g/L and 200 μL of 3000 μM $FeSO_4$. 40–43 mg (1×10^{-4} M) of each test compound were dissolved in 1 mL DMSO (100%). This stock solution was then diluted to final extraction concentration 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}, and 1×10^{-10} M. Each assay was carried out in triplicates. The mixture was incubated at 37°C for 1 h, after which it was treated with 0.5 mL of freshly prepared 15% TCA and 1.0 mL of 1% TBA. The reaction tubes were kept in boiling water bath for 10 min. Upon cooling, the tubes were centrifuged at $3500 \times g$ for 10 min to remove precipitated protein. LP was measured spectrometrically by estimation of thiobarbituric reactive substances (TBARS). The formation of TBARS was measured by removing 100 μL of supernatant and measuring the absorbance at 532 nm. α-Tocopherol ranging from 1×10^{-4} to 1×10^{-10} M was used as the standard reference. The control was buffered egg with $FeSO_4$ only. The percentage of inhibition was calculated from the following equation:

$$\%\,\text{Inhibition} = [1-(A_s/A_0)] \times 100$$

where A_0 refers to the absorbance of the control and A_s is the absorbance of the sample. To determine the concentration required to achieve 50% inhibition (IC_{50}) of phospholipid oxidation in egg yolk, the percentage of lipid peroxidation inhibition was plotted against extract concentration.

Cell culture

The human colon carcinoma HCT-116 and hormone-dependent breast carcinoma MCF7 were purchased from the American Type Culture Collection (ATCC, USA). HCT 116 and MCF 7 cells in RPMI 1640 Medium (Sigma), supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, PAA Lab, Austria), 100 μg/mL streptomycin and 100 U/ml penicillin (PAA Lab, Austria) and 50 μg/mL fungizone (PAA Lab, Austria). The media were filter sterilized using a 0.22 μm filter membrane (Minisart, Sartorius stedim). The cells were cultured in 5% CO_2 incubator at 37°C in a humidified atmosphere. The culture was subcultured every 2 or 3 days and routinely checked under an inverted microscope (Motic) for any contamination. Cells in the exponential growth phase were used for all experiments. The cells were harvested from culture flasks by accutase (Innovative Cell Technologies) and the viable cell count was determined using trypan blue exclusion assay with a hemocytometer.

MTT cytotoxicity assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was modified from Mossmann [36]. Cells were seeded at density of 5000 cells/well into a 96-well flat-bottomed culture plates (Orange Scientific) and allowed to adhere overnight. The experimental compounds were predissolved in dimethyl sulphoxide (DMSO) and diluted to the required concentration (five concentrations, 0.1–100 μg/mL), such that the total DMSO concentration did not exceed 0.5%. At this concentration, DMSO was

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found to be nontoxic to the cells tested. Vehicle DMSO was used as a control. Cells were incubated with the samples (three wells on a plate for each concentration) for 72 h. Treated and untreated cells were inspected qualitatively using an inverted light microscope (20 ×). Then, 20 μl of MTT (5 mg/mL) (Sigma) was added to each well and the plates were incubated at 37°C for 4 h. The media was then gently aspirated, and 150 μL DMSO was added to dissolve the formazan crystals. The amount of formazan product was measured spectrophotometrically at 570 nm and 650nm as a background using a microplate reader (Oasys UVM340).

The percentage of viability = (absorbance of treated cells/absorbance of untreated cells) × 100.

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