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Original article

Cytotoxic and protective DNA damage of three new diterpenoids from the brown alga *Dictyoyota dichotoma*

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ABSTRACT

Three new diterpenes Amijiol acetate (**3**), dolabellane, Dolabellatrienol (**4**), and dolastane, Amijiol-7, 10-diacetate **(9)** were isolated together with the previously described Pachydictyol A (**1**), Isopachydictyol A (**2**), 8β-hydroxypachydictyol A (**5**), Amijiol (**6**), Isodictyohemiacetal (**7**) and Dictyol C (**8**) from the Red Sea brown alga *Dictyota dichotoma* var. *implexa*. The structures and relative stereochemistry of the new diterpenoids were proposed on the basis of their spectral data. Compounds **3** and **9** have potent activity against DNA damage, cytotoxicity against WI-38, HepG2, and MCF-7 cell lines, and antioxidant using ABTS and erythrocytes hemolysis.

1. Introduction

Brown algae (Phaeophyceae) belonging to order Dictyotales, have emerged as an exceptionally rich source of terpenoids, which form part of a defensive strategy against herbivores in the marine environment [1,2]. Phytochemical studies have been undertaken on the family Dictyotaceae resulting in the isolation of more than 300 diterpenoids from at least 35 species collected all over the world. These Dictyotaceae produce a significant number of secondary metabolites, especially diterpenes. Generally, these diterpenes have three types of carbon skeletons: xenicanes; dolabellanes and extended sesquiterpenes. Many members of the family though, produce cyclic diterpenoids, unique in the structural variety of marine natural products. Biological studies have shown a significant number of dictyota secondary metabolites to possess cytotoxic, anti-bacterial, ichthyotoxic and anti-feedant activities [3–5].

In continuation of our search program for the isolation of bioactive natural products from marine organisms of the Red Sea [6–14], we collected *Dictyoyota dichotoma* at El-shuaiba 80 km south of Jeddah. Fractionation of the pet. ether:ether extract afforded three new diterpenoids as well as six known compounds. The chemotaxonomic implication of these findings is also discussed.

2. Results and discussion

2.1. Chemistry

The pet. ether–ether extract of the brown alga *Dictyota dichotoma* was fractionated on neutral aluminum oxide using a gradient of pet. ether–ether as eluant. The fractions were monitored by TLC using 50%-sulfuric acid in methanol as spray reagent, to afford, in order of elution nine compounds (Fig. 1). Structures of the known isolated compounds Pachydictyol A (**1**) [15], Isopachydictyol A (**2**) [4], 8β-Hydroxypachydictyol A (**5**) [16], Amijiol (**6**) [17], Isodictyohemiacetal (**7**) [18] and dictyol C **(8)** [19] were established by comparing their physical and spectral data with those in the literature.

Compound **3** was found by mass spectrometry and ^{13}C NMR spectroscopy to have the molecular formula $C_{22}H_{34}O_3$. The IR spectrum of **3** contained hydroxyl (3437 cm^{-1}) and carbonyl (1736 cm^{-1}) absorption bands. A three-proton singlet at δ 2.15 in

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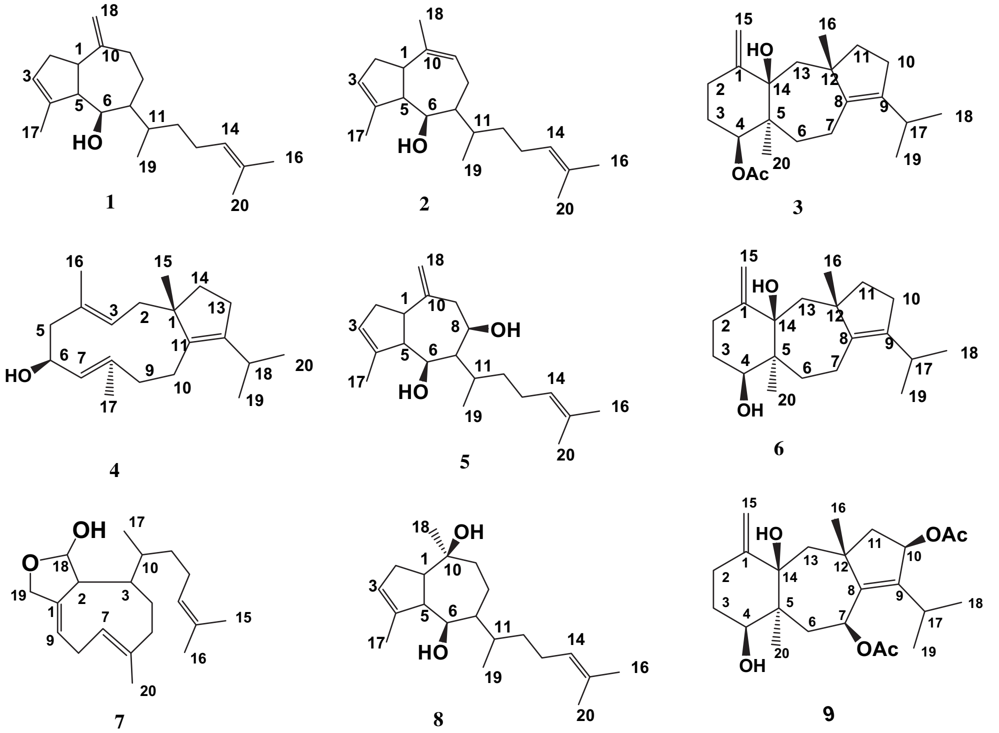
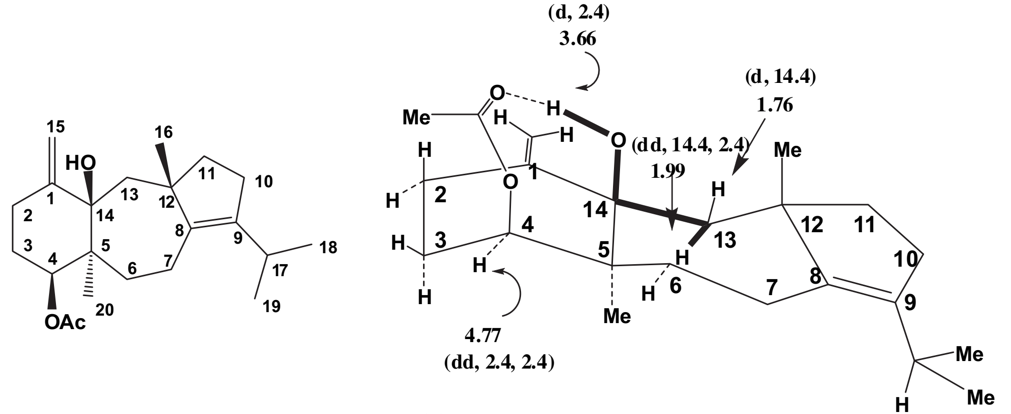


Fig. 1. Structure of compounds **1**–**9**.

the ^{1}H NMR spectrum, resonance at δ_c 169.6 and 21.5 in the ^{13}C NMR spectrum, and a significant ion at *m/z* 286 $[M^+ - CH_3CO_2H]$ in the mass spectrum, indicated the presence of an acetate ester in **3**. Other spectral similarities between **3** and the known Amijiol **6** [20] suggested that **3** is a monoacetate derivative of **6**. Similar to the differences seen between **3** and **6**, the ^{1}H NMR resonance for H (4) was deshielded from δ 3.39 in **6** to δ 4.77 in **3**, and the ^{13}C NMR resonance for C (4) from δ_c 80.6 in **6** to 82.8 in **3**. The signal of 14 β-OH proton appeared as a doublet at δ 3.66 ($J = 2.4$ Hz) by a long range coupling with 13 α-H. This unusual w-coupling across C–O–H bonding should be caused by the hydrogen bonding (Fig. 2) to the carbonyl oxygen of 4 β-OAc. Thus, **3** were deduced to be Amijiol acetate.

Compound **4** Mass spectrometry and NMR data revealed compound **4** to have a molecular formula of $C_{20}H_{32}O$. The presence of six sp^2 hybridised carbon atoms in the molecule, as deduced from the ^{13}C NMR and DEPT NMR spectra, being for three carbon–carbon double bonds and one carbon-oxygen bonds, indicated compound **4** to be bicyclic. The IR spectrum showed the presence of hydroxyl function (3404 cm^{-1}). ^{1}H and ^{13}C NMR spectra contained resonances for three double bonds at δ (4.83 (d), 4.75 (brs), 144.3, (s), 138.4 (s), 138.2 (s), 129.8 (d), 129.3 (s), 128.2 (d)), indicating two tri-substituted double bonds and one four substituted, and a secondary alcohol (69.2 d). Of five methyl groups exist in the molecule, two are associated with double bonds as indicated by their NMR chemical shifts, as well as their observed long range couplings with olefinic protons at δ (4.83 (br d), 1.42 (br s), 4.75 (br s), 1.56 (br s)), other two are part of an isopropyl group resonating at δ (0.94 d and 1.10 d), and the fifth one is tertiary (δ 0.90 s, 27.6 q) most likely located at a bride-head position. The structural type and substitution pattern of **4** was elucidated by means of 2D NMR correlation spectroscopy including HMQC, HMBC and ^{1}H–^{1}H COSY. Thus, the eleven-membered ring was deduced unambiguously by interpretation of ^{1}H–^{13}C long range and ^{1}H–^{1}H



(3)

Fig. 2. Hydrogen bonding of compound **3**.

couplings. The methyl protons at C-15 showed a heteronuclear long range coupling to C-2 (δ_c 40.9, t), while the protons at C-2 (δ 2.20, m) coupled to H-3 (δ 4.75, br s) which in turn coupled to H3-16 (δ 1.56, br s). The HMBC correlation of C-16 to H-5′and H-5 (δ 1.89 dd, 2.73, dd). Cross peaks in the ^{1}H–^{1}H COSY spectrum showed couplings between H-5 and H-6 (δ 4.44, td), from H-6 to H-7 (δ 4.83 br d) and from there to the methyl protons at C-17 (δ 1.42 br s). From the carbon C-9 (δ_c 21.4, t) heteronuclear long range couplings to H-7 (δ 4.83, br d) were observed. The 11-membered ring was completed by the HMBC correlation observed between C-11 (δ_c 138.4, s) and Me-15 (δ 0.90, s). HMBC correlation of C-12 (δ_c 138.2, s) with H-18 (δ 2.73, h), Me-19 (δ 1.10, d) and Me-20 (δ 0.94, d) clearly positioned the isopropyl group. At this point of the structure solution two intercoupling methylene groups were left to be considered, with one of them, C-14 (δ_c 34.3, t) showing a heteronuclear long range coupling to H-2 (δ 2.20, m). C-14 thus, had to be connected to C-l, and the remaining methylene group C-13 (δ 1.53, m, 2.18, m and δ_c 27.1, t) to the quaternary carbon C-12, forming a cyclopentene ring. Thus, **4** were deduced to be dolabellatrienol.

Compound **9** obtained as a white solid, had a molecular formula of $C_{24}H_{36}O_6$ (m/z 420.2501 [M]$^+$), as deduced from the HREIMS and ^{13}C NMR spectra. The fragment ion at m/z 402 [M − H_2O] in the mass spectrum, as well as the absorption band at 3330 cm^{-1} in the IR spectrum indicated the presence of at least one hydroxyl group. The ^{13}C NMR spectrum revealed 24 carbon signals, which as determined from DEPT experiments corresponded to eight quaternary, four methine, six methylene, and six methyl carbon atoms. The structural elements displayed in the ^{1}H and ^{13}C NMR spectra of **9** included four tertiary methyl groups ($\delta_{H/C}$ 0.72/17.8, 1.54/27.9, 2.05/21.6 and 2.06/21.5), two secondary methyl group ($\delta_{H/C}$ 0.98/19.6 and 1.04/22.5), one 1, 1-disubstituted double bond ($\delta_{H/C}$ 4.81, 4.92/110.1 and δ_C 151.1), one tetrasubstituted double bond (δ_C 145.4 and 144.3), three oxygenated methine ($\delta_{H/C}$ 3.48/80.0, 5.81/80.3 and 5.89/67.5) and one oxygenated quaternary carbon (δ_C 77.9), two carbonyl of acetate groups (δ_C 170.9 and 170.3). Since the two carbon–carbon double bond and two carbonyl accounted for four of the seven degrees of unsaturation, the molecular structure of **9** was determined as tricyclic. The coupling between H3-18 and H3-19 with H1-17 observed in the COSY spectrum indicated the presence of an isopropyl group, whereas the correlations of H-17 at δ 2.76 with C-9 at δ 144.3 and C-8 at δ 145.4 in the HMBC spectrum fixed its position. The methyl-proton H3-16 displayed additional HMBC correlations with C-8, C-10, C-11, and C-12. The COSY correlations of H-10 with H2-11 confirmed the assignment of the five-membered ring. The cross peaks of H2-6/H-7 and H-13/H-13, as well as the HMBC correlations of C-5 with H2-6 and H3-20, C-8 with H-7, C-12 with H-7, H2-13, H3-16 and C-14 with H2-13 and H3-20 concluded the assignment of the seven-membered ring. Finally, the correlations of C-1 with H2-2, H3-3, and H2-15, H-4 at δ 3.48with C-2, C-3, C-5, and C-20, and C-15 with H2-2 observed in the HMBC spectrum, in conjunction with the cross peaks of H2-2/H2-3 and H2-3/H-4 in the COSY spectrum identified the six-membered ring. A two methyl-proton singlet at δ 2.06, 2.05 in the ^{1}H NMR spectrum, resonance at δ_c 170.9, 170.3 and 21.6, 21.5 in the ^{13}C NMR spectrum, and a significant ion at m/z 360 [M$^+$ − CH_3CO_2H] and 300 [M$^+$ − 2(CH_3COOH)], in the mass spectrum, indicated the presence of a two acetate ester in **9**. The signal of 4 β-OH proton appeared as a doublet at δ 3.78 (J = 7.8 Hz) by *Trans*-coupling with 4 α-H. This unusual *Trans*-coupling across C–O–H bonding should be caused by the hydrogen bonding to the hydroxyl oxygen of 14 β-OH. Thus, **9** were deduced to be Amijiol-7, 10-diacetate (Fig. 3).

3. Pharmacology

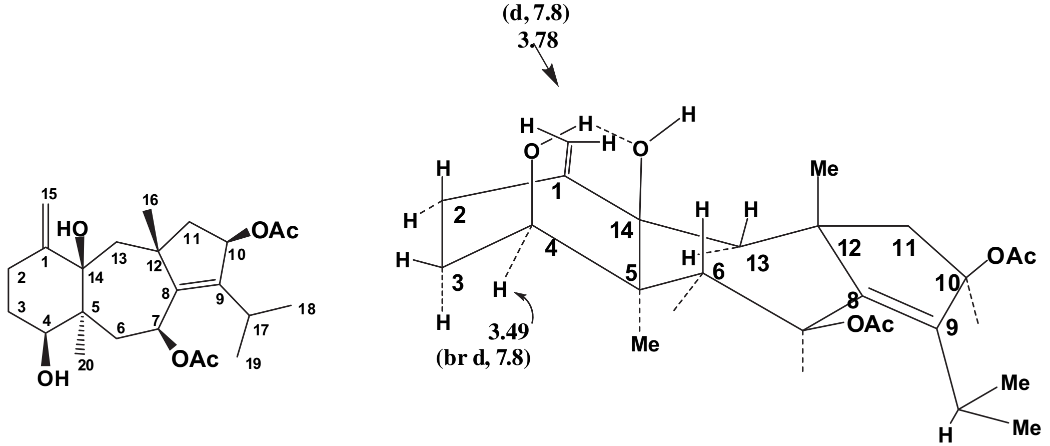
3.1. Bleomycin-dependent DNA damage

The bleomycins are a family of antitumor antibiotics, which are used routinely as antitumor agents. The bleomycin assay has been adopted for assessing the pro-oxidant effects of food antioxidants. The antitumor antibiotic bleomycin binds iron ions and DNA. The bleomycin–iron complex degrades DNA that, upon heating with thiobarbituric acid, yields a pink chromogen. Added suitable reducing agents “antioxidants” compete with DNA and diminish chromogen formation.

Among all tested compounds (Table 1), compounds **3** and **9** showed the highest protection activity against DNA damage induced by bleomycin–iron complex thus diminishing chromogen formation between the damaged DNA and TBA. However compounds 1, 2, 5, 6 and 7 showed a weak to moderate activity. In the meantime, compounds 4 and 8 exhibited a very low activity.

3.2. Cytotoxicity

The compounds were tested for cytotoxic activity (Table 2) against Vero African green monkey kidney cells, WI-38 (fibroblast cells), HepG2 (Hepatoma cells), MCF-7 (Cells from breast cancer). Compounds **3** and **9** showed the strong cytotoxic activity (Fig. 4).



(9)

Fig. 3. Hydrogen bonding of compound **9**.

Table 1
Results of bleomycin-dependent DNA damage assay of isolated compounds.

Compd. No.	Absorbance
1	0.23
2	0.24
3	0.006
4	0.18
5	0.28
6	0.50
7	0.45
8	0.05
9	0.004
Ascorbic acid	0.0038

Table 2
Cytotoxicity (IC50) of tested compounds on different cell lines.[a]

Compd. No.	(IC_{50}, (μg/ml)[a]			
	HepG2	WI-38	VERO	MCF-7
1	40.2	24.6	27.4	37.5
2	39.2	22.4	28.3	39.2
3	25.1	14.2	20.5	21.2
4	102.3	100.6	120.6	150.5
5	81.2	62.6	72.3	68.2
6	70.2	84.6	76.4	75.5
7	70.2	84.6	76.4	75.5
8	100.7	110.6	160.6	140.5
9	47.0	16.2	21.4	30.5
5-Fu	8.6	3.2	6.5	2.3

[a] IC_{50}, (μg/ml): 1–10 (very strong), 11–25 (strong), 26–50 (moderate), 51–100 (weak), 100–200 (very weak), above 200 (non-cytotoxic).

3.3. *Antitumor activity*

The isolated compounds were screened for their antitumor activity. The viability of the cells used in control experiments exceeded 95%. Compounds **3** and **9** proved to have the highest cytotoxic activity (Table 3) at 85.9% and 71.2%, respectively.

3.4. *Antioxidant activity for ABTS and erythrocytes hemolysis*

All compounds were tested for antioxidant activity as reflected in the ability to inhibit lipid peroxidation in rat brain and kidney homogenates and rate erythrocyte hemolysis. The pro-oxidant activities of the compounds were assayed for their antioxidant effects using ABTS assay. Compounds **3** and **9** proved to exhibit potent antioxidative activity. On the other hand, compounds **1**, **2**, **5**, **6**, and **7** showed a weak to moderate activity. In the meantime, compounds **4** and **8** exhibited a very low activity (Table 4).

4. Experimental

4.1. *Apparatus and material*

Chromatographic material: Aluminum oxide type 60–120 mesh was used for column chromatography. Thin layer chromatography silica-gel GF 254 was used; for TLC. Preparative thin layer chromatography (PTLC) was performed on aluminum oxide plates (20 cm × 20 cm) of 250 μm thickness.

Melting points were recorded on Gallenkamp electric melting point apparatus. The IR spectra (film) were recorded on a Perkin Elmer spectrometer model 100. Electron impact mass spectra were determined at 70 ev on a Kratos MS-25 instrument. 1D and 2D NMR

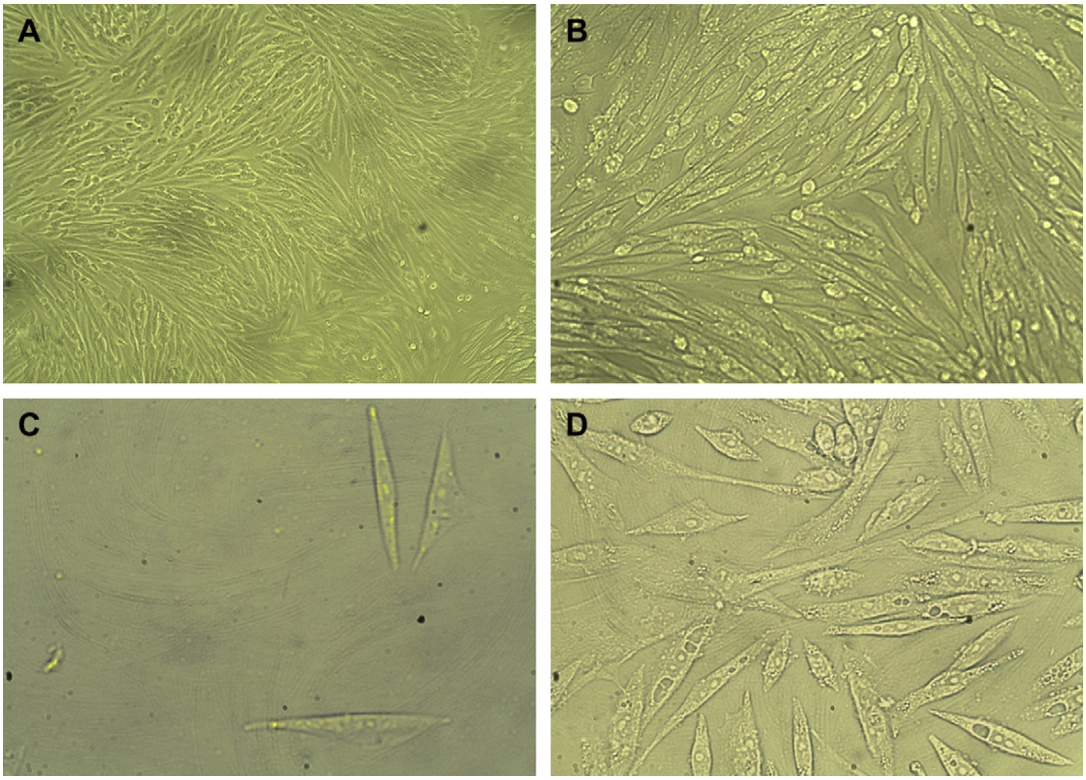


Fig. 4. Representative phase micrograph of WI-38 fibroblasts of the control group and of those treated with the three studied preparations. (A) Control cells, untreated WI-38-cells, (100% viability); (B) WI-38 cells treated with **3** at 10 μg/ml (65% viability); (C) WI-38 cells treated with **3** at 100 μg/ml (0% viability). (D) Culture treated with **3** at 50 μg/ml (12% viability).

Table 3
Ehrlich in vitro assay for isolated compounds.[a]

Compd. No.	Dead cells (%) 25 μl (1 mg/mL)
Control (no drug)	–
5-Florouracil	99.5
1	42.9
2	48.4
3	85.9
4	15.1
5	60.9
6	56.0
7	59.1
8	18.0
9	71.2

[a] Tested compounds were prepared 1 mg/mL in 100 μL DMSO and complete to 1 mL using RPMI-1640 medium. 5-Fluorouracil (25 μg/mL) in 100 μL DMSO and complete to 1 mL using RPMI-1640 medium.

spectra were recorded on Bruker AVANCE III WM 600 MHz spectrometers and ^{13}C NMR at 150 MHz. Chemical shifts are given in δ (ppm) relative to TMS as internal standard. The spray reagent used is: 50%-sulfuric acid in methanol as spraying reagent. The chromatoplate, was heated after spraying at 100–105 °C until the spots attained maximum color intensity.

The alga was described as *Dictyota dichotoma* (Family Dictyotaceae) was collected by hand from El-shuaiba 80 km south of Jeddah, Saudi Arabia, in the Red Sea, in May, 2008.

4.2. Extraction and isolation

The dried *D. dichotoma* 350 gm were extracted twice, 48 h for each time by soaking in a mixture of pet. ether:ether (1:1) at room temperature. The extract was concentrated under reduced pressure to obtain 17 gm residue. The residue was chromatographed on a column of Aluminum oxide using hexanes–diethyl ether as eluent. Fractions of ∼50 mL were collected. TLC using silica-gel chromatoplates, appropriate solvent system and 50%-sulfuric acid in methanol as spraying reagent The fractions containing a single compound were combined and further purified by prep. TLC of aluminum oxide plates (20 cm × 20 cm) of 250 μm thickness.

*4.2.1. Fraction A, pachydictyol A (1) and isopachydictyol A (**2**)*

The fraction A eluted by pet. ether–Ether (9.5:0.5) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexan–ether (8.5:1.5) the band observed as a red color with $R_f = 0.48$ (sulfuric acid–methanol) gave colorless oil (26 mg) as a mixture of compounds **1** and **2**.

Table 4
Antioxidant assay for the prepared new compounds.

Compd. No.	ABTS[a] (Inhibition (%))	Erythrocytes hemolysis (%)
Control	–	–
L-ascorbic acid[b]	88.61	0.85
1	50.23	2.45
2	59.90	1.40
3	83.35	0.70
4	24.36	5.75
5	61.65	1.10
6	59.20	0.50
7	57.40	0.75
8	57.72	1.10
9	80.24	1.40

[a] 50 μL of (2 mM) of tested compounds in spectroscopic grade MeOH/phosphate buffer reaction mixture (1 mL, 1:1v/v).
[b] 50 μL of (2 Mm) of L-ascorbic acid was used in spectroscopic grade MeOH/phosphate buffer reaction mixture (1 mL, 1:1v/v).

Pachydictyol A (**1**) (16 mg, 0.0046%), IR (film) 3498, 2922, 1637 cm^{-1}; EIMS (70 ev) *m/z* (rel.int) 288 (10) [M$^+$, $C_{20}H_{32}O$], 270 (4) [M$^+$ − H_2O], 255 (5) [M$^+$ − CH_3–H_2O], 159 (100), 145 (50), 119 (60), 107 (55), 105 (55), 69 (90), 55 (95); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.96 (d, 3H, $J = 6.0$ Hz, H-19), 1.60 (s, 3H, H-20), 1.68 (d, 3H, $J = 0.9$ Hz, H-16), 1.80 (td, 3H, $J = 2.0, 1.2$ Hz, H-17), 1–2.7 (14H), 3.92 (m, 1H, H-6), 4.74 (br s, 1H, H-18′), 4.75 (br s, 1H, H-18), 5.12 (br t, 1H, $J = 7.1$ Hz, H-14), 5.33 (br s, IH, H-3); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 152.6 (s, C-10), 141.4 (s, C-4), 131.6 (s, C-15), 124.6 (d, C-14), 124.0 (d, C-3), 107.1 (t, C-18), 75.0 (d, C-6), 60.4 (d, C-5), 47.8 (d, C-7), 46.9 (d, C-1), 40.7 (d, C-9), 35.4 (t, C-12), 35.0 (d, C-11), 34.7 (t, C-2), 25.8 (t, C-13), 25.6 (q, C-16), 23.5 (t, C-8), 17.7 (q, C-20), 17.5 (q, C-19), 15.9 (q, C-17).

Isopachydictyol A (**2**) (10 mg, 0.0029%), IR (film) 3498, 2922, 1637 cm^{-1}; EIMS (70 ev) *m/z* (rel.int) 288 (10) [M$^+$, $C_{20}H_{32}O$], 270 (8) [M$^+$ − H_2O], 255 (5) [M$^+$ − H_2O–CH_3], 159 (80), 145 (30), 120 (40), 107 (60), 105 (80), 82 (100), 69 (100), 55 (90); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.97 (d, 3H, $J = 6.6$ Hz, H-19), 1.61 (s, 3H, H-20), 1.69 (d, 3H, $J = 0.9$ Hz, H-16), 1.71 (d, 3H, $J = 1.5$ Hz, H-18), 1.84 (td, 3H, $J = 2.5, 1.5$ Hz, H-17), 3.92 (m, 1H, H-6), 5.12 (br t, 1H, $J = 7.1$ Hz, H-14), 5.36 (br s, IH, H-3), 5.50 (br d, 1H, $J = 9.0$ Hz, H-9); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 142.8 (s, C-4), 138.7 (s, C-10), 131.5 (s, C-15), 126.0 (d, C-9), 124.6 (d, C-14), 124.3 (d, C-3), 74.5 (d, C-6), 57.4 (d, C-5), 46.9 (d, C-7), 46.0 (d, C-1), 35.4 (t, C-2), 34.7 (t, C-12), 33.6 (d, C-11), 25.7 (q, C-16), 25.4 (t, C-13), 24.5 (t, C-8), 23.1 (q, C-18), 17.7 (q, C-20), 17.5 (q, C-19), 16.3 (q, C-17).

*4.2.2. Fraction B, Amijiol acetate (**3**)*

The fraction B eluted by pet. ether–Ether (9:1) was collected and purified by Prep-TLC chromatoplates of aluminum oxide using the solvent system Hexan–Ether (7.5:2.5) the band observed as a yellow color with $R_f = 0.46$ (sulfuric acid–methanol) give compound **3**.

Amijiol acetate (**3**) was obtained as a colorless crystal (6 mg, 0.0017%) m.p. 95–96 °C, IR (film) 3579, 2933, 1746, 1650 cm^{-1}; HREIMS calcd. for $C_{22}H_{34}O_3$ [M]$^+$ 346.2508 found 346.2498; EIMS (70 eV) *m/z* (rel.int) 346 (5) [M$^+$, $C_{22}H_{34}O_3$], 286 (10) [M$^+$ − CH_3COOH], 286 (10), 243 (20), 225 (17), 149 (100), 135 (25), 121 (35), 107 (40), 93 (35); ^{1}H NMR (600 MHz, $CDCl_3$), δ 0.84 (s, 3H, Me-20), 0.91 (d, 3H, $J = 6.6$ Hz, Me-19), 0.94 (d, 3H, $J = 6.6$ Hz, Me-18), 1.11 (ddd, 1H, $J = 15.0, 6.6, 1.2$ Hz, H-6′), 1.36 (s, 3H, Me-16), 1.56 (ddd, 1H, $J = 12, 9.0, 9.0$ Hz, H-11′), 1.67 (d, 1H, $J = 14.4$ Hz, H-13$_a$), 1.69 (m, 1H, H-11), 1.71 (dddd, 1H, $J = 12.0, 4.2, 3.0, 2.4$ Hz, H-3′), 1.85 (dddd, 1H, $J = 12.0, 12.0, 5.4, 3.0$ Hz, H-3), 1.88 (dd, 1H, $J = 14.4, 2.4$ Hz, H-13$_e$), 2.04 (ddd, 1H, $J = 12.6, 3.6, 1.2$ Hz, H-10′), 2.14 (m, 1H, H-7′), 2.15 (s, 3H, OAc), 2.17 (m, 1H, H-6), 2.19 (m, 1H, H-2′), 2.47 (ddd, 1H, $J = 16.8, 12.6, 6.6$ Hz, H-7), 2.61 (h, 1H, $J = 6.6$ Hz, H-17), 2.65 (m, 1H, H-10), 2.69 (m, 1H, H-2), 3.66 (d, $J = 2.4$ Hz, OH-14), 4.77 (t, 1H, $J = 2.4$ Hz, H-4), 4.86 (br s, 1H, H-15′), 4.89 (br s, IH, H-15); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C 169.6 (s, Ac), 151.2 (s, C-1), 139.4 (s, C-8), 138.3 (s, C-9), 109.5 (t, C-15), 82.8 (d, C-4), 79.3 (s, C-14), 50.8 (s, C-12), 45.9 (t, C-13), 44.2 (s, C-5), 42.9 (t, C-11), 27.6 (t, C-2), 27.4 (t, C-6), 27.3 (t, C-3), 26.9 (d, C-17), 26.8 (q, C-16), 26.7 (t, C-10), 21.9 (t, C-7), 21.5 (q, OAc), 21.3 (q, C-18), 20.3 (q, C-19), 17.6 (q, C-20).

*4.2.3. Fraction C, dolabellatrienol (**4**)*

The fraction **C** eluted by pet. ether–Ether (8.5:1.5) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexan–Ether (8:2) the band observed as brown color with $R_f = 0.23$ (sulfuric acid–methanol) give compound **4**.

Dolabellatrienol (**4**) was obtained as a yellow oil (11 mg, 0.0031%), IR (film) 3404, 2938, 1641, 909 cm^{-1}; HREIMS calcd. for $C_{20}H_{32}O$ [M]$^+$ 288.2453 found 288.2442 EIMS (70 eV) *m/z* (rel.int) 288 (3) [M$^+$, $C_{20}H_{32}O$], 270 (5) [M$^+$ − H_2O], 245 (12), 219 (15), 159 (30), 134 (100), 121 (90), 107 (40), 93(60); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.90 (s, 3H, Me-15), 0.94 (d, 3H, $J = 6.6$ Hz, Me-20), 1.10

(d, 3H, $J = 6.6$ Hz, Me-19), 1.42 (br s, 3H, Me-17), 1.48(m, 1H, H-14) 1.53(m, 1H, H-13) 1.56 (br s, 3H, Me-16), 1.68(m, 1H, H-14′), 1.89 (dd, 1H, $J = 12.0, 9.4$ Hz, H-5′), 2.0–1.5(4H, m), 2.18(m, 1H, H-13′), 2.2(m, 2H, H-2), 2.73(dd, 1H,$J = 12.0, 4.2$ Hz, H-5), 2.73 (h, 1H, $J = 6.6$ Hz, H-18), 4.44 (td, 1H, $J = 9.6, 4.2$ Hz, H-6), 4.75 (br s, 1H, H-3), 4.83 (br d, 1H, $J = 9.0$ Hz, H-7);^{13}C NMR (150 MHz, $CDCl_3$) δ_c 144.3 (s, C-8), 138.4 (s, C-11), 138.2 (s, C-12), 129.8 (d, C-7), 129.3 (s, C-4), 128.2 (d, C-3), 69.2 (d, C-6), 51.1 (s,C-1), 47.2 (t, C-5), 40.9 (t, C-2), 36.9 (t, C-10), 34.3 (t,C-14), 27.6 (q, C-15), 27.3 (d, C-18), 27.1 (t, C-13), 21.5 (q, C-17), 21.4 (t, C-9), 20.7 (q, C-19), 17.9 (q, C-20), 16.6 (q, C-16).

*4.2.4. Fraction D, 8β-hydroxypachydictyol A (**5**)*

The fraction **D** eluted by pet. ether–Ether (8:2) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexan–Ether (7:3) the band observed as a violet color with $R_f = 0.27$ (sulfuric acid–methanol) give compound **5**.

8β-Hydroxypachydictyol A (**5**) was obtained as a yellow oil (80 mg, 0.023%), IR (film) 3455, 2927, 1640, 1443, 1376 cm^{-1}; EIMS (70 eV) *m/z* (rel.int) 304 (4) [M^+, $C_{20}H_{32}O_2$], 286 (5) [$M^+ - H_2O$], 268 (3) [$M^+ - 2H_2O$], 173 (20), 145 (35), 121 (45), 105 (50), 82 (98), 69 (100), 55 (57); ^{1}H NMR (600 MHz, $CDCl_3$,) δ 1.13 (d, 3H, $J = 7.0$ Hz, Me-19), 1.34 (m, 1H, H-12), 1.53 (m, 1H, H-7), 1.61 (s, 3H, Me-20), 1.69 (s, 3H, Me-16), 1.68 (m, 1H, H-I2′), 1.79 (br s, 3H. Me-17), 1.94 (m, 1H, H-11), 1.95 (m, 1H, H-13), 2.11 (m, 1H, H-13′), 2.14 (m, 1H, H-2), 2.36 (dd, 1H, $J = 4.2, 15.0$ Hz, H-9), 2.48 (m, 1H, H-2′), 2.53 (m, 1H, H-5), 2.72 (m, 1H, H-l), 3.00 (br dd, 1H,$J = 3.3, 15.0$ Hz, H-9′), 4.07 (m, 2H, H-6), 4.10 (m, 1H, H-8), 4.86 (br s, lH, H-18), 4.96 (br s, 1H, H-l8`), 5.l2 (br t, 1H, $J = 7.4$ Hz, H-14), 5.34 (br s, 1H, H-3) ^{13}C NMR (150M Hz, $CDCl_3$) δ_c 145.2 (s, C-10), 139.5 (s, C-4), 129.4 (s, C-15), 122.2 (d, C-14), 121.6 (d, C-3), 109.7 (t, C-18), 70.1 (d, C-6), 66.5 (d, C-8), 55.8 (d, C-5), 49.5 (d, C-7), 44.9 (d, C-1), 42.9 (t, C-9), 33.3 (t, C-2), 33.1 (t, C-12), 31.5 (d, C-11), 24.2 (t, C-13), 23.3 (q, C-16), 16.0 (q, C-19), 15.3 (q, C-20), 12.4 (q, C-17).

*4.2.5. Fraction E, Amijiol (**6**)*

The fraction E eluted by pet. ether–Ether (8:2) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexan–Ether (6.5:3.5) the band observed as a brown color with $R_f = 0.29$ (sulfuric acid–methanol) give compound **6**.

Amijiol (**6**) was obtained as a colorless crystal (11 mg, 0.003%) m.p. 181–182 °C, IR (film) 3339, 2936, 1641, 908 cm^{-1}, EIMS (70 eV) *m/z* (rel.int) 304 (5) [M^+, $C_{20}H_{32}O_2$], 286 (10) [$M^+ - H_2O$], 243 (15) [$M^+ - H_2O + C_3H_7$], 149 (100) [$M^+ - C_{11}H_{17}$]; ^{1}H NMR (600 MHz, $CDCl_3$), δ 0.73 (s, 3H, Me-20), 0.92 (d, 3H, $J = 6.6$ Hz, Me-19), 0.95 (d, 3H, $J = 6.6$ Hz, Me-18), 1.36 (s, 3H, Me-16), 1.38 (m, 1H, H-6′), 1.51 (d, 1H, $J = 14.4$ Hz, H-13′), 1.56 (m, 1H, Hz, H-11′), 1.67 (d, 1H, $J = 14.4$ Hz, H-13), 1.69 (m, 1H, H-11), 1.73 (m, 1H, H-3′), 1.86 (dddd, 1H, $J = 14.4, 14.4, 4.8, 3.0$ Hz, H-3), 2.00 (m, 1H, H-2′), 2.18 (m, 1H, H-6), 2.20 (m, 1H, H-10′), 2.22 (m, 1H, H-7′), 2.50 (ddd, 1H, $J = 15.8, 13.2, 8.4$ Hz, H-7), 2.61 (s, OH-14), 2.63 (h, 1H, $J = 6.6$ Hz, H-17), 2.85 (ddd, 1H, $J = 14.4, 14.4, 5.4$ Hz, H-10), 2.92 (ddd, 1H, $J = 15.0, 12.6, 6.0$ Hz, H-2), 3.40 (ddd, 1H, $J = 8.4, 2.4, 1.8$ Hz, H-4), 3.88 (d, $J = 8.4$ Hz, OH-4), 4.83 (br s, IH, H-15),4.89 (br s, 1H, H-15′); ^{13}C NMR (150 MHz, $CDCl_3$) δ_c 152.3 (s, C-1), 139.3 (s, C-8), 138.4 (s, C-9), 109.4 (t, C-15), 81.2 (d, C-4), 80.6 (s, C-14), 50.8 (s, C-12), 47.8 (t, C-13), 44.2 (s, C-5), 42.9 (t, C-11), 30.6 (t, C-2), 28.3 (t, C-6), 27.3 (t, C-10), 26.8 (t, C-3), 26.7 (d, C-17), 26.6 (q, C-16), 22.1 (t, C-7), 21.3 (q, C-18), 20.3 (q, C-19), 17.6 (q, C-20).

4.2.6. Fraction F, Isodictyohemiacetal (7)

The fraction **F** eluted by pet. ether–Ether (7:3) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexane–Ether (6:4) the band observed as a brown color with $R_f = 0.40$ (sulfuric acid–methanol) give compound **7**.

Isodictyohemiacetal **(7)** was obtained as a colorless oil (4 mg, 0.0011%), IR (film) 3420, 1620 cm-^{1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.91 (d, 3H, $J = 6.6$ Hz, Me-17), 1.68 (s, 3H, Me-15), 1.60 (s, 3H, Me-16), 1.73 (s, 3H, Me-20), 2.4–1.0 (m, 11H), 2.60 (m, 1H, H-8′), 3.09 (td, 1H, $J = 14.4, 2.4$ Hz, H-8), 4.38 (brs, 2H, H-19), 5.09 (tt, 1H, $J = 7.2, 1.2$ Hz, H-13), 5.41 (dd, 1H, $J = 12.0, 3.0$ Hz, H-7), 5.62 (d, 1H, $J = 4.8$ Hz, H-18), 5.63 (dd, 1H, $J = 9.0,3.0$ Hz, H-9), ^{13}C NMR (150 MHz, $CDCl_3$) δ_c 17.2 (q, C-17), 17.6 (q, C-15), 17.7 (q, C-20), 25.7 (q-C-16), 26.2 (t, C-12), 28.2 (t, C-4), 29.9 (t, C-8), 31.6 (d, C-10), 37.9 (t, C-11), 40.6 (t, C-5), 45.7 (d, C-2), 53.9 (d, C-3), 71.4 (t, C-19), 100.7 (d, C-18), 120.3 (d, C-9), 124.7 (d, C-13), 125.9 (d, C-7), 131.3 (s, C-14), 134.8 (s, C-6), 145.4 (s, C-1), EIMS, *m/z* (rel. int.): 304 (8) [M^+ $C_{20}H_{32}O_2$]. 274(12), 244(5), 221(24), 193(30), 145 (40), 121 (75), I07 (85), 82 (100). HREIMS Obsd. $m/z = 304.2418$ [M^+, $C_{20}H_{32}O_2$] requires $m/z = 304.2402$.

4.2.7. Fraction G, Dictyol C (8) and Amijiol-7, 10-diacetate (9)

The fraction G eluted by pet. ether–Ether (6:4) was collected and purified by Prep. TLC chromatoplates of aluminum oxide using the solvent system Hexan–EtOAc (7.5:2.5) two band observed one of them as a brown color with $R_f = 0.16$ give compound **8** and another one as a red color with $R_f = 0.27$ give compound **9** (sulfuric acid–methanol).

Dictyol C (**8**) was obtained as a white solid (8 mg, 0.0023%) m.p. INS> 90–91 °C, IR(film) 3441, 2914, 1736, 1448 cm^{-1}, EIMS (70 eV) *m/z* (rel.int) 306 (5) [M^+, $C_{20}H_{34}O_2$], 288 (25) [$M^+ - H_2O$], 270 (8) [$M^+ - 2H_2O$], 159(87) [$M^+ - 2H_2O{-}C_8H_{15}$], 69 (100) [$M^+ - 2H_2O{-}C_{15}H\ _{21}$]; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.96 (d, 3H,$J = 6.6$ Hz, Me-19), 1.20 (s, 3H, Me – 18), 1.60 (s, 3H, Me-20), 1.69 (d, 3H, $J = 0.9$ Hz, Me-16), 1.80 (dd, 3H, $J = 2.0, 1.2$ Hz, Me-17), 2.74 (dd, 1H,$J = 7.8, 6.0$ Hz, H-5), 3.87 (dd, 1H,$J = 7.8, 3.6$ Hz, H-6), 5.12 (br t, 1H, $J = 7.1$ Hz, H-14), 5.27 (br s, 1H, H-3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 142.5 (s, C- 4), 131.6 (s, C-15), 124.7 (d, C-14), 123.4 (d, C-3), 74.4 (d, C-6), 72.4(s, C-10) 52.7 (d, C-5), 49.9 (d, C-7), 49.1 (d, C-1), 46.6 (t, C-9), 34.7 (t, C-12), 34.4 (d, C-11), 32.9 (t, C-2), 30.0 (q, C-18), 25.7 (q, C-16), 25.5 (t, C-13), 19.7 (t, C-8), 17.7 (q, C-20), 17.5 (q, C-19), 16.3 (q, C-17).

Amijiol-7, 10-diacetate **9** was obtained as a white residue (37 mg, 0.011%), IR (film) 3358, 2934, 1731, 1640 cm^{-1}; HREIMS calcd. for $C_{24}H_{36}O_6$ [M]$^+$ 420.2512 found 420.2501; EIMS (70 eV) *m/z* (rel.int) 420 (5) [M^+, $C_{24}H_{36}O_6$], 402 (8) [$M^+ - H_2O$], 360 (15) [$M^+ - CH_3COOH$], 300 (15) [$M^+ - 2(CH_3\ COOH)$], 286 (30), 243 (20), 225 (40), 149 (100), 135 (80), 121 (70), 107 (45), 93(55); ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.72 (s, 3H, Me-20), 0.98 (d, 3H, $J = 7.2$ Hz, Me-19), 1.04 (d, 3H, $J = 7.2$ Hz, Me-18), 1.54 (s, 3H, Me-16), 1.59 (dd, 1H, $J = 13.2, 5.4$ Hz, H-11), 1.73 (m, 1H, H-3), 1.75 (d, 1H, $J = 15.0$ Hz, H-13), 1.82 (d, 1H, $J = 15.0$ Hz, H-13′), 1.83 (dd, 1H, $J = 14.4, 7.8$ Hz, H-6), 1.86 (m, 1H, H-3″), 2.04 (ddd, 1H, $J = 15.6, 13.8, 3.6$ Hz, H-2), 2.05 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.31 (dd, 1H, $J = 13.2, 7.8$ Hz, H-11′), 2.76 (h, 1H, $J = 7.2$ Hz, H-17), 2.83 (d t, 1H, $J = 13.8, 4.8$ Hz, H-2′), 2.92 (dd, 1H, $J = 14.4, 11.4$ Hz, H-6′), 3.27 (s, OH-14), 3.48 (br d, 1H, $J = 7.8$ Hz, H-4), 3.79 (d, $J = 7.8$ Hz, OH-4), 4.81 (br s, 1H, H-15), 4.92 (br s, 1H, H-15′), 5.81 (dd, 1H, $J = 7.8, 5.4$ Hz, H-10), 5.89 (dd, 1H, $J = 11.4, 7.8$ Hz, H-7); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.9 (s, Ac), 170.3 (s, Ac), 151.1 (s, C-1), 145.4 (s, C-8), 144.3 (s, C-9), 110.1 (t, C-15), 80.3 (d, C-10), 80.0 (d, C-4), 77.9 (s, C-14), 67.5 (d, C-7), 50.9 (t, C-11), 49.1 (t, C-13), 48.9 (s, C-12), 42.6 (s, C-5), 33.9 (t, C-6), 30.2 (t, C-3), 27.9 (q, C-16), 26.8 (d, C-17), 26.3 (t, C-2), 22.5 (q, C-18), 21.6 (q, OAc), 21.5 (q, OAc), 19.6 (q, C-19), 17.8 (q, C-20).

4.3. Biological evaluation of the isolated compounds

Ehrlich cells (Ehrlich ascites Carcinoma, EAC) were derived from ascetic fluid from diseased mouse (the cells were purchased from National Cancer institute, Cairo, Egypt which is a certified institute by National Medical Research Ethics Committee). DNA (Calf

Thymus type1), bleomycin sulfate, butylated hydroxyanisole (BHA), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA) and ascorbic acid were obtained from sigma. 2,2′-azo-bis-(2-amidinopropane) dihydrochlorid (AAPH), 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) were purchased from Wako Co., USA.

4.3.1. *Bleomycin-dependent DNA damage assay*

The reaction mixtures contained in a final volume of 1.0 mL, the following reagents at the final concentrations stated: DNA (0.2 mg/ml), bleomycin (0.05 mg/ml), $FeCl_3$ (0.025 mM), magnesium chloride (5 mM), KH_2PO_4–KOH buffer pH 7.0 (30 mM) and ascorbic acid (0.24 mM) or the compounds tested in MeOH to give a concentration of (0.1 mg/ml). The reaction mixtures were incubated in a water-bath at 37 °C for 1 h. At the end of the incubation period, 0.1 mL of 0.1 M EDTA was added to stop the reaction (the iron–EDTA complex is unreactive in the bleomycin assay). DNA damage was assessed by adding 1 mL 1% (w/v) thiobarbituric acid (TBA) and 1 mL 25% (v/v) hydrochloric acid (HCl) followed by heating in a water-bath maintained at 80 °C for 15 min. The chromogen formed was extracted into butan-1-ol and the absorbance was measured at 532 nm [20,21].

4.3.2. *Cytotoxicity and antitumor assay*

Samples were prepared for assay by dissolving in 50 μl of DMSO and diluting aliquots into sterile culture medium at 0.4 mg/ml. These solutions were subdiluted to 0.02 mg/ml in sterile medium and the two solutions used as stocks to test samples at 100, 50, 20, 10, 5, 2, and 1 mg/ml in triplicate in the wells of microtiter plates.

The compounds were assayed in triplicate on monolayers grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) calf serum (HyClone Laboratories, Ogden, UT), 60 mg/ml Penicillin G and 100 mg/ml streptomycin sulfate maintained at 37 °C in a humidified atmosphere containing about 15% (v/v) CO_2 in air. All medium components were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Cells stocks were maintained at 34 °C in culture flasks filled with medium supplemented with 1% (v/v) calf serum. Subcultures of cells for screening were grown in the wells of microtiter trays (Falcon Microtest III 96-wells trays, Becton Dickinson Labware, Lincolin Park, NJ) by suspending cells in medium following trypsin–EDTA treatment, counting the suspension with a hemocytometer, diluting in medium containing 10% calf serum to 2×10^4 cells per 200 mL culture, aliquoting into each well of a tray and culturing until confluent.

Microtiter trays with confluent monolayer cultures of cells were inverted, the medium shaken out, and replaced with serial dilutions of sterile compounds in triplicate in 100 μl medium followed by titered virus in 100 μl medium containing 10% (v/v) calf serum in each well. In each tray, the last row of wells was reserved for controls that were not treated with compounds. The trays were cultured for 96 h. The trays were inverted onto a pad of paper towels, the remaining cells rinsed carefully with medium, and fixed with 3.7% (v/v) formaldehyde in saline for at least 20 min. The fixed cells were rinsed with water, and examined visually. The cytotoxic activity is identified as confluent, relatively unaltered monolayers of stained cells treated with compounds. Cytotoxicity was estimated as the concentration that caused approximately 50% loss of the monolayer. 5-Fluorouracil was used as a positive control [22].

4.3.3. *Antitumor activity using Ehrlich ascites in vitro assay*

Different concentrations of the tested compounds were prepared (100, 50 and 25 μl from 1 mg/mL in DMSO (<00.05%, v/v) and RPMI-1640 medium). Ehrlich cells (Ehrlich ascites Carcinoma, EAC) were derived from ascetic fluid from diseased mouse (purchased from National Cancer institute, Cairo, Egypt which is a certified institute by National Medical Research Ethics Committee). Ascites fluid from the peritoneal cavity of the diseased mouse (contains Ehrlich cells) was aseptically aspirated. The cells were grown partly floating and partly attached in a suspension culture in RPMI-1640 medium, supplemented with 10% fetal bovine serum. They were maintained at 37 °C in a humidified atmosphere with 5% CO_2 for 2 h [23]. The viability of the cells determined by the microscopical examination using a hemocytometer and using trypan blue stain (stains only the dead cells).

4.3.4. *Antioxidant activity screening assay for erythrocytes hemolysis*

The blood was obtained from rats by cardiac puncture and collected in heparinized tubes. Erythrocytes were separated from plasma and the buffy coat was washed three times with 10 volumes of 0.15 M NaCl. During the last wash, the erythrocytes were centrifuged at 2500 rpm for 10 min to obtain a constantly packed cell preparation. Erythrocyte hemolysis was mediated by peroxyl radicals in this assay system. A 10% suspension of erythrocytes in phosphate buffered saline pH 7.4 (PBS) was added to the same volume of 200 mM AAPH solution in PBS containing samples to be tested at different concentrations. The reaction mixture was shaken gently while being incubated at 37 °C for 2 h. The reaction mixture was then removed, diluted with eight volumes of PBS and centrifuged at 1500*g* for 10 min. The absorbance of the supernatant was read at 540 nm. Similarly, the reaction mixture was treated with 8 volumes of distilled water to achieve complete hemolysis, and the absorbance of the supernatant obtained after centrifugation was measured at 540 nm [24]. The data percentage hemolysis was expressed as mean ± standard deviation. L-ascorbic acid was used as a positive control.

4.3.5. *Antioxidant activity screening assay ABTS method*

For each of the investigated compounds (2 mL) of ABTS solution (60 μM) was added to 3 mL MnO_2 solution (25 mg/mL), all prepared in (5 mL) aqueous phosphate buffer solution (pH 7, 0.1 M). The mixture was shaken, centrifuged, filtered and the absorbance of the resulting green-blue solution (ABTS radical solution) at λ 734 nm was adjusted to approx. ca. 0.5. Then, 50 μL of (2 mM) solution of the tested compound in spectroscopic grade MeOH/phosphate buffer (1:1) was added. The absorbance was measured and the reduction in color intensity was expressed as inhibition percentage. L-ascorbic acid was used as standard antioxidant (positive control). Blank sample was run without ABTS and using MeOH/phosphate buffer (1:1) instead of tested compounds. Negative control was run with ABTS and MeOH/phosphate buffer (1:1) only [25].

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