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Article

Synthesis and Cytotoxicity Evaluation of Novel Asymmetrical Mono-carbonyl Analogs of Curcumin (AMACs) against Vero, HeLa, and MCF7 Cell lines

Pekik Wiji Prasetyaningrum [1], Anton Bahtiar [1] and Hayun Hayun [1,*]

[1] Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia, 16424; pekikwiji@gmail.com (P.W.P.); anton.bahtiar@gmail.com (A.B.); hayun.ms@ui.ac.id (H.H

* Correspondence: hayun.ms@ui.ac.id; Tel.: +62-21-727-0031

Abstract: A series of novel asymmetrical mono-carbonyl analogs of curcumin (AMACs) were synthesized and evaluated for cytotoxic activity using the brine shrimp lethality test (BSLT) and the methyl thiazolyl tetrazolium assay against Vero, HeLa, and MCF7 cell lines. The structures of the synthesized compounds were confirmed by Fourier transform infrared spectrophotometry (FTIR), ^{1}H-nuclear magnetic resonance (NMR), ^{13}C-NMR, and mass spectral data. The results of the cytotoxicity evaluation showed that the synthesized compounds exhibited moderate to very high toxic activity in BSLT, requiring a concentration of 13.06–714.49 μg/mL to kill half the population. Most of the compound exhibited cytotoxic activity against HeLa cell lines, comparable to the activity of cisplatin with a concentration of the synthesized compounds required to inhibit 50% of the growth of the cell lines (IC_{50}) value of 40.65–95.55 μM, and most of the compounds tested against MCF7 cell lines exhibited moderate to very high cytotoxic activity (IC_{50} value 7.86–35.88 μM). However, the selectivity index of the compounds was low, less than 1–1.96. Among the synthesized compounds, compound **1b** showed the highest cytotoxicity and selectivity against MCF7 cell lines. Compound **1b** could be considered for further development to obtain more active and selective chemotherapeutic agents against breast cancer.

Keywords: asymmetrical mono-carbonyl analogs of curcumin (AMACs); synthesis; cytotoxicity, Vero; HeLa; MCF7; cell lines

1. Introduction

Cancer is one of the main causes of death worldwide, especially in developing countries. Breast and cervix uterine cancer have the highest cancer incidence in Indonesian female populations with 48,998 and 20,928 cases, respectively. The reported mortality profile caused by the two cancers are 21.4 and 10.3% [1]. Some chemotherapeutic agents had been developed and used to treat cancer. Unfortunately, no drug shows good selectivity for cancer cells. Many chemotherapeutic drugs produce serious chronic and delayed toxicities that may be irreversible, particularly in the heart, lung, and kidneys [2]. Curcumin (diferuloylmethane) has demonstrated various biological activities, such as growth suppression in a wide variety of tumor cells, as well as chemopreventive effects on certain types of cancers, with low toxicity [3]. Nevertheless, curcumin has not yet been accepted as a therapeutic compound because of its low chemical stability, low solubility, poor absorption, and rapid metabolism, resulting in low bioavailability and weak in vivo biological activity [4–7]. Many curcumin analogs have been synthesized and investigated, such as mono-carbonyl analogs of curcumin (MACs), to improve the bioactivity, stability, and bioavailability.

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The MACs have exhibited potency for cell lines and cellular proteins 10–30 times that of curcumin [8–11]. Some MAC compounds, using acetone and cyclohexanone as a linker between the two phenyl rings, inhibited the growth of leukemia, colon, renal, melanoma, ovarian, central nervous system (CNS), and prostate cancer cells better than cisplatin [12]. The MAC pharmacokinetic profile is much more stable than curcumin, resulting in higher tumor regression [8,12]. Some of the asymmetrical mono-carbonyl analogs of curcumin (AMACs) with different constituents on the two phenyl rings have been developed and reported to have antioxidant, anti-inflammatory, antimicrobial [13–17], and antitumor properties [18]. However, reports on studies of AMACs compounds as anti-cancer agents are still limited. To further explore AMACs as anticancer compounds, we report the synthesis and in vitro cytotoxicity evaluation of novel AMACs (**1a–1e** and **2a–2e**, Scheme 1) against Vero, HeLa, and MCF7 Cell lines.

2. Materials and Methods

2.1. Chemistry

2.1.1. General Procedures

All solvents, chemicals, and reagents were obtained commercially and used without purification. Purity tests of the products were performed using thing layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Darmstadt, Germany). Melting points were determined in a capillary tube using melting point apparatus (Stuart Scientific) and were uncorrected. Infrared (IR) spectra were recorded on a Fourier transform infrared (FTIR) 8400S spectrophotometer (Shimadzu). ^{1}H-nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were recorded on an NMR spectrometer (Agilent) at 500 MHz for ^{1}H and 125 MHz for ^{13}C using tetramethylsilane (TMS) as the internal standard. High-resolution mass spectra (HRMS) were measured with a Waters LCT Premier XE Electrospray ionisation time-of-flight mass spectrometry (ESI-TOF) (Waters Corp., Milford, MA, USA) system in negative mode.

2.1.2. Synthesis of (2E)-2-(phenylmethylidene)cyclohexan-1-one and analogs

The syntheses were performed according to the synthesis method used for 2-benzylidene acetone by replacing acetone with cyclohexanone [19]. A mixture of aromatic aldehyde (0.32 mol) and cyclohexanone (0.88 mol) was added to a solution of 10% sodium hydroxide (NaOH) dropwise while stirring for 2 hours. The mixture was neutralized with dilute hydrochloric acid (HCl) to pH 7, the organic layer was separated, and the water layer was extracted with 16 mL of toluene. The toluene layer was mixed with the organic layer, washed with 16 mL of water, dried with anhydrous sodium sulfate, and evaporated using a rotary vacuum evaporator to create the crude product. The crude product was used as the starting material for the next step without further purification.

*2.1.3. Synthesis of Asymmetrical Mono-carbonyl Analogs of Curcumin (AMACs)(**1a–1e**)*

The synthesis of the compounds was performed by aldol condensation of (2E)-2-(phenylmethylidene)cyclohexan-1-one or its analogs and vanillin under acidic condition. The mixture of (2E)-2-(phenylmethylidene)cyclohexan-1-one or its analogs (0.005 mol) and vanillin (0.01 mol) in ethanol (10 mL) was heated under reflux conditions until dissolved, then a drop of diluted HCl/ethanol (1 drop:1 mL) was added and stirred for 30 mins. The progress of the reaction was monitored by TLC. Upon completion, the solvent was evaporated, then the solid material obtained was triturated with a cold mixture of glacial acetic acid/water (1:1) and filtered using Buchner funnel. The solid product obtained was washed with a cold mixture of glacial acetic acid/water (1:1), dried, and purified by column chromatography with a mixture of the appropriate ratio of n-hexane and ethyl acetate.

(2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-(phenylmethylidene)cyclohexan-1-on e (**1a**). The compound was a bright yellow powder, yield 50.0%, mp: 149–151 °C and Rf = 0.8 (ethyl

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acetate : n-hexane = 1:2). FTIR (KBr), υmax, cm^{-1}: 3211 (OH), 2999 (CH aromatic), 2837 (CH aliphatic), 1647 (C=O), 1587, 1531, 1448 (C=C), 1174 (C-O). ^{1}H-NMR (500 MHz, $CDCl_3$), δ/ppm: 1.80 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.93 (m, 4H, =C-CH_2-$C_{cyclohexanone}$); 3.91 (s, 3H, OCH_3); 5.89 (s, 1H, OH); 6.95 (d, 1H, *J* = 8.5, H_{Ar}); 7.00 (s, 1H, H_{Ar}; 7.09 (d, 1H, *J* = 8.3 Hz, H_{Ar}); 7.33 (t, 1H, *J* = 7.4, H_{Ar}); 7.39 (t, 2H, *J* = 7.3 Hz, H_{Ar}); 7.46 (d, 2H, *J*= 7.2 Hz, H_{Ar}); 7.75 and 7.79 (s, 1H Ar-CH=C and 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23.1 (1C, C-CH_2-$C_{cyclohexanone}$), 28.5 (1C, =C-CH_2-$C_{cyclohexanone}$), 28.7 (1C, =C-CH_2-$C_{cyclohexanone}$), 56.0 (O-CH_3), 113.4, 114.5, 124.6, 128.6, 130.4, 134.2 (10C, C_{Ar}), 136.1, 137.5 (4C, -C=C-) 146.4 (2C, C_{Ar}-O), 190.3 (1C, C=O) [20]. HRESIMS (*m/z)* found 319.1346 ([M-H]$^-$), calculated masses for $C_{21}H_{19}O_3$: 319.1334.

(2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-methoxyphenyl)methylidene] cyclohexan-1-one (**1b**). The compound was a yellow powder, yeild 2.7%, mp: 133–136 °C and Rf = 0.55 (ethyl acetate : n-hexane = 1:2). FTIR (KBr) υmax cm^{-1}: 3431 (OH), 3003 (CH aromatic), 2935 (CH aliphatic), 1734 (C=O), 1656, 1593 and 1512 (C=C), 1161 (C-O). ^{1}H NMR (500 MHz, $CDCl_3$), δ/ppm: 1.80 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.92 (m, 4H, =C-CH_2-$C_{cyclohexanone}$); 3.84 (s, 3H, CH_3-O); 3,91 (s, 3H, CH_3-O); 5.86 (s, 1H, OH); 6.92 (d, 1H, *J* = 8.5, H_{Ar}); 6.99 (s, 1H, H_{Ar}); 7.08 (d, 1H, *J* = 8.3 Hz, H_{Ar}); 7.46 (d, 2H, *J* = 8.3, Hz, H_{Ar}); 7.73 and 7.76 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23.1 (1C, C-CH_2-$C_{cyclohexanone}$), 28.5 (2C, =C-CH_2-$C_{cyclohexanone}$), 56.0 (1C, OCH_3), 55.4 (1C, OCH_3), 113.3, 114.4, 124.5, 128.5, 132.3 (9C, C_{Ar}), 134.4, 137.0 (4C, -C=C-), 146.4,146.5,160.0 (3C, C_{Ar}-O) 190.1 (1C, C=O) [20]. HRESIMS (*m/z)* found 349.1432 ([M-H]$^-$), calculated masses for $C_{22}H_{21}O_4$: 349.1440.

(2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-fluorophenyl)methylidene] cyclohexan-1-one (**1c**). The compound was a light yellow powder, yield 47.3%, mp: 129–131 °C and Rf = 0.52 (ethyl acetate : n-hexane = 1:2). FTIR (KBr) υmax cm^{-1}: 3313 (OH), 3003 (CH aromatic), 2939 (CH aliphatic), 1734 (C=O), 1656, 1604 and 1514 (C=C), 1220 (C-F), 1155 (C-O). ^{1}H-NMR (500 MHz, $CDCl_3$), δ/ppm: 1.82 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.89 (t, 2H, *J* = 7.1 Hz, =C-CH_2-$C_{cyclohexanone}$); 2.93 (t, 2H, *J* = 6.1 Hz, =C-CH_2-$C_{cyclohexanone}$); 3.93 (s, 3H, CH_3-O); 5.90 (s, 1H, -OH); 6.96 (d, 1H, *J* = 9.3 Hz, H_{Ar}); 7.00 (s, 1H, H_{Ar}); 7.11 (d, 3H, *J* = 8.2 Hz, H_{Ar}); 7.44 (dd, 2H, *J* = 5.5 Hz, H_{Ar}); 7.75 (s, 2H, Ar-CH=C). ^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm: 23.1 (1C, C-CH_2-$C_{cyclohexanone}$), 28.7 (2C, =C-CH_2-$C_{cyclohexanone}$), 56.0 (1C, OCH_3), 113.4, 114.5, 115.5, 115.6, 124.6, 128.5, 132.3, 134.1 (9C, C_{Ar}), 135.5, 136.0, 137.8, 146.4 (4C, C=C), 163.7 (C_{Ar}-F), 146.7, 160.7 (2C, C_{Ar}-O), 190.1 (1C, C=O) [20]. HRESIMS (*m/z)* found 337.1270 ([M-H]$^-$), calculated masses for $C_{21}H_{18}FO_3$: 337.1240.

(2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-chlorophenyl)methylidene] cyclohexan-1-one (**1d**). The compound was a light yellow powder, with a 6.1% yield, mp: 150–154 °C and Rf = 0.75 (ethyl acetate : n-hexane = 1:2). FTIR (KBr) υmax cm^{-1}: 3296 (OH), 3003 (CH aromatic), 2939 (CH aliphatic), 1734 (C=O), 1658, 1604 and 1514 (C=C), 1163 (C-O), 833 (C-Cl). ^{1}H-NMR (500 MHz, $CDCl_3$), δ/ppm: 1.80 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.87 (t, 2H, *J* = 7.3 Hz, =C-CH_2-$C_{cyclohexanone}$); 2.93 (t, 2H, *J* = 7,3 Hz, =C-CH_2-$C_{cyclohexanone}$); 3.92 (s, 3H, CH_3-O); 5.86 (s, 1H, OH); 6.96 (d, 1H, *J*=8 2 Hz, H_{Ar}); 6,99 (s, 1H, H_{Ar}); 7.08 (d, 1H, *J* = 8.2 Hz, H_{Ar}); 7.37 (d, 2H, *J* = 8.8 Hz, H_{Ar}); 7,39 (d, 2H, *J*=9.3 Hz, H_{Ar}), 7.72, 7.74 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23.0 (1C, C-CH_2-$C_{cyclohexanone}$), 28.5, 28.6 (2C, =C-CH_2-$C_{cyclohexanone}$), 56.1 (1C, OCH_3), 113.4, 114.5, 124.7, 128.5 (C15), 129.2, 131.6., 134.1 (9C, C_{Ar}), 134.6, 135.2, 136.8, 137.8 (4C, -C=C-), 134.0. (C_{Ar}-Cl), 146.6, 146.7 (2C, C_{Ar}-O), 196.1 (1C, C=O) [20]. HRESIMS (*m/z)* found 353.0947 ([M-H]$^-$), calculated masses for $C_{21}H_{18}ClO_3$: 353.0945.

(2E,6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-methylphenyl)methylidene] cyclohexan-1-one (**1e**). The compound was a yellow powder, with a 13.2% yield, mp: 130–131 °C and Rf = 0.75 (ethyl acetate : n-hexane = 1:2). FTIR (KBr) υmax cm^{-1}: 3323 (OH), 3007 (CH aromatic), 2939 (CH aliphatic), 1734 (C=O), 1653, 1593 and 1462 (C=C), 1161 (C-O). ^{1}H-NMR (500 MHz, $CDCl_3$), δ/ppm: 1.79 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.38 (s, 3H, CH_3-Ar), 2.91 (t, 2H, *J* = 5.0 Hz,

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=C-C$\underline{H}_2$-C$_{cyclohexanone}$); 2.93 (t, 2H, *J* = 5.0 Hz, =C-C$\underline{H}_2$-C$_{cyclohexanone}$), 3.92 (s, 3H, CH$_3$-O); 5.86 (s, 1H, -OH); 6.96 (t, 3H, *J* = 8.2 Hz, H$_{Ar}$); 6.99 (s, 1H, H$_{Ar}$); 7.08 (d, 1H, *J* = 8.3 Hz, C15=CH-C18)); 7.22 (d, 2H, *J* = 9.0 Hz, H$_{Ar}$); 7.37 (d, 2H, *J* = 9.0 Hz, H$_{Ar}$), 7,74 and 7.77 (s, 1H, s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, CDCl$_3$) δ/ppm: 21.5 (1C, CH$_3$-Ar), 23.1 (1C, C-C$\underline{H}_2$-C$_{cyclohexanone}$), 28.6, 28.7 (2C, =C-C$\underline{H}_2$-C$_{cyclohexanone}$), 56.0 (CH$_3$-O), 113.4, 114.5, 124.6, 128.6, 129.2, 130.5, 133.3, 134.3 (10C, C$_{Ar}$), 134.6, 135.2, 136.8, 137.8 (4C, -C=C-), 146.4, 146.5 (2C, C$_{Ar}$-O), 190.3 (1C, C=O) [20]. HRESIMS (*m/z)* found 333.1492 ([M-H]$^-$), calculated masses for C$_{22}$H$_{21}$O$_3$: 333.1491.

*2.1.4. Synthesis of Diethylamine Mannich Base of AMACs (**2a–2e**)*

The syntheses were performed according to the method used for the synthesis of di-Mannich bases of curcumin and the synthesis of 2-[(2,6-dimethylmorpholin-4-yl)methyl]-4-[(E)-2-{3[(E)-2-{3-[(2,6-dimethylmorpholin-4-yl)methyl]-4-hydroxy-5-methoxyphenyl}ethenyl]-1H-pyrazol-5yl}etheyl]-6-methoxyphenol as reported previously [21-22]. Compounds **1a–1f** (2 mmol) were dissolved in ethanol, cooled in an ice bath, and slowly added to diethylamine (5–7 mmol) and 37% formaldehyde solution (5–7 mmol) slowly. The mixture was stirred for 30 min at room temperature and then refluxed for 7–11 h. The progress of the reaction was monitored by TLC. After the reaction was completed, the solvent was evaporated to obtain the solid residue. The residue was dissolved in methanol (40 mL) and evaporated to a residue. The residue was then dissolved in methanol (50 mL), warmed, and poured slowly with constant stirring into about 400 mL of cold distilled water. The solvent was decanted and the precipitate obtained was filtered off, washed with cold distilled water, dried at room temperature, and then purified by column chromatography.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-(phenylmet hylidene)cyclohexan-1-one (**2a**). The compound was a caramel-like solid, with a 65.5% yield, mp: 79–80 °C and Rf = 0.51 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax cm^{-1}: 3053 (CH aromatic), 2972 (C-H), 1660 (C=O), 1599 (C=C), 1269 (C-N), 1157 (C-O). ^{1}H-NMR (500 MHz, CD$_3$OD), δ/ppm: 1.18 (t, 6H, *J* = 7.2 Hz, C$\underline{H}_3$-CH$_2$-), 1.77 (m, 2H, C-C$\underline{H}_2$-C$_{cyclohexanone}$); 2.77 (q, 4H, *J* = 7.0 Hz, CH$_3$-C$\underline{H}_2$-N), 2.89 and 2.94 (t, 4H, *J* = 7.0 Hz, =C-C$\underline{H}_2$-C$_{cyclohexanone}$), 3.85 (s, 3H, CH$_3$-O), 3.95 (s, 2H, Ar-CH$_2$-N), 6.92 (s, 1H, H$_{Ar}$), 7.04 (s, 1H, H$_{Ar}$); 7.33 (t, 1H, *J*=8,7 Hz, H$_{Ar}$); 7.40 (t, 2H, *J* = 7.4 Hz H$_{Ar}$), 7.45 (d, 2H, *J* = 10.9 Hz, H$_{Ar}$), 7.66 and 7.68 (s, 1H, s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, CD$_3$OD) δ/ppm: 10.9 (2C, C$\underline{H}_3$-CH$_2$) 24.1 (1C, C-C$\underline{H}_2$-C$_{cyclohexanone}$), 29.3 and 29.9 (2C, C-C$\underline{H}_2$-C$_{cyclohexanone}$), 47.5 (1C, CH$_3$-C$\underline{H}_2$-N-), 56.9 (1C, Ar-C$\underline{H}_2$-N), 56.4 (1C, C$\underline{H}_3$-O), 114.9, 122.2, 126.6, 126.4. 129.5, 129.7, 131.3, 133.9 (10C, C$_{Ar}$), 137.2, 137.3, 137.9, 139.7 (4C, -C=C-), 149,7 and 153.0 (2C, C$_{Ar}$-O), 191.8 (1C, C=O) [20] HRESIMS (*m/z)* found 404.2285 ([M-H]$^-$), calculated masses for C$_{26}$H$_{30}$NO$_3$: 404.2226.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-methoxy phenyl)methylidene]cyclohexan-1-one (**2b**). The compound was an orange sticky powder, with a 46.8% yield, mp: 98–99 °C and Rf = 0.51 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax cm^{-1}: 3059 (CH aromatic), 2970 (CH aliphatic), 1734 (C=O), 1556 (C=C), 1595 and 1510 (C=C aromatic) 1271 (C-N), 1155 (C-O). ^{1}H NMR (500 MHz, CD$_3$OD), δ/ppm: 1.17 (t, 6H, *J* = 7.1, C$\underline{H}_3$-CH$_2$-), 1.78 (m, 2H, C-C$\underline{H}_2$-C$_{cyclohexanone}$); 2.77 (q, 4H, *J* = 7.1 Hz, CH$_3$-C$\underline{H}_2$-N), 2.88 and 2.92 (t, 2H, *J* = 5.4 Hz, =C-C$\underline{H}_2$-C$_{cyclohexanone}$ and t, 2H, *J*=5.4 Hz, =C-C$\underline{H}_2$-C$_{cyclohexanone}$), 3.81 (s, 3H, CH$_3$-O-), 3.85 (s, 3H, CH$_3$-O-), 3.91 (s, 2H, Ar-CH$_2$-N), 6.89 (s, 1H, H$_{Ar}$), 6.96 (d, 2H, *J* = 8.2, H$_{Ar}$), 7.01 (s, 1H, H$_{Ar}$); 7.44 (d, 2H, *J* = 8.8 Hz, H$_{Ar}$), 7.64 and 7.65 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C NMR (100 MHz, CD$_3$OD) δ/ppm: 11.0 (2C, C$\underline{H}_3$-CH$_2$) 24.1 (1C, C-C$\underline{H}_2$-C$_{cyclohexanone}$), 29.4 and 29.6 (2C, C-C$\underline{H}_2$-C$_{cyclohexanone}$), 47.4 (2C, CH$_3$-C$\underline{H}_2$-N-), 56.3 (1C, C$\underline{H}_3$-O), 55.8 (1C, C$\underline{H}_3$-O), 57.0 (1C, Ar-C$\underline{H}_2$-N), 114.8, 122.8, 126.7, 126.2, 126.7, 133.4 (9C, C$_{Ar}$), 129.7, 135.6, 137.7, 139.3 (4C, -C=C-), 149.6, 152.8 and 161.7 (3C, C$_{Ar}$-O), 191.9 (1C, C=O) [20]. HRESIMS (*m/z)* found 434.2101 ([M-H]$^-$), calculated masses for C$_{27}$H$_{32}$NO$_4$: 434.2332.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-fluoroph enyl)methylidene]cyclohexan-1-one (**2c**). The compound was an orange powder, with a 33.01%

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yield, mp: 79–81 °C and Rf = 0.48 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax cm^{-1}: 3041 (CH aromatic), 2937 (CH aliphatic), 1734 (C=O), 1656 (C=C), 1595 and 1492 (C=C aromatic) 1271 (C-N),1224 (C-F), 1157 (C-O). ^{1}H-NMR (500 MHz, CD_3OD), δ/ppm: 1.18 (t, 6H, *J*=7.2 Hz, $C\underline{H}_3$-CH_2-), 1.77 (m, 2H, C-$C\underline{H}_2$-$C_{cyclohexanone}$); 2.77 (q, 4H, *J*=7.2 Hz, CH_3-$C\underline{H}_2$-N), 2.87 and 2.94 (t, 2H, *J*=5.5 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$ and t, 2H, *J*=5.2 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.93 (s, 2H, Ar-CH_2-N), 6.92 (s, 1H, H_{Ar}), 7.03 (s, 1H, H_{Ar}); 7.14 (d-d, 2H, *J* = 8.8 Hz, H_{Ar}), 7,49 (d-d, 2H, *J*=5.5 Hz, H_{Ar}), 7.65 and 7.66 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, CD_3OD) δ/ppm: 10.9 (2C, $C\underline{H}_3$-CH_2) 24.0 (1C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 29.4 and 29.6 (2C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 47.4 (2C, CH_3-$C\underline{H}_2$-N-), 56.3 (1C, $C\underline{H}_3$-O), 57.0 (1C, Ar-$C\underline{H}_2$-N), 114.9, 116.3, 116.5, 122.3, 126.4, 133.5, 133.9 (9C, C_{Ar}), 136.1, 137.7, 139.3, 139.8 (4C, -C=C-), 153.2 and 163.0 (2C, C_{Ar}-O), 165.0 (1C, C_{Ar}-F), 191.6 (1C, C=O) [20]. HRESIMS (*m/z)* found 422.2178 ($[M-H]^-$), calculated masses for $C_{26}H_{29}FNO_3$: 422.2132.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-chlorophenyl)methylidene]cyclohexan-1-one (**2d**). The compound was an orange powder, with a 76.93 yield, mp: 95–97 °C and Rf = 0.45 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax, cm^{-1}: 3032 (CH aromatic), 2972 (CH aliphatic), 1734 (C=O), 1656 (C=C), 1597 and 1491 (C=C aromatic) 1271 (C-N), 1157 (C-O), 839 (C-Cl). ^{1}H-NMR (500 MHz, CD_3OD), δ/ppm: 1.18 (t, 6H, *J* = 7.2 Hz, $C\underline{H}_3$-CH_2-), 1.78 (m, 2H, C-$C\underline{H}_2$-$C_{cyclohexanone}$); 2.79 (q, 4H, *J* = 7.2 Hz, CH_3-$C\underline{H}_2$-N), 2.87 and 2.95 (t, 2H, *J* = 5.1 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$ and t, 2H, *J* = 7.3 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.94 (s, 2H, Ar-CH_2-N), 6.93 (s, 1H, H_{Ar}), 7.04 (s, 1H, H_{Ar}); 7.42 (d, 2H, *J* = 8.4 Hz, H_{Ar}), 7.44 (d, 2H, *J* = 8.7 Hz, H_{Ar}), 7.63 and 7.66 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, CD_3OD) δ/ppm: 11.0 (2C, $C\underline{H}_3$-CH_2), 24.1 (1C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 29.2 and 29.6 (2C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 47.4 (2C, CH_3-$C\underline{H}_2$-N), 56.3 (1C, $C\underline{H}_3$-O), 56.9 (1C, Ar-$C\underline{H}_2$-N), 114.9, 122.5, 126.5, 129.6, 133.7, 132.8 (9C, C_{Ar}), 136.1, 137.7, 138.5, 139.8 (4C, -C=C-), 153.2 and 149.7 (2C, C_{Ar}-O), 139.9 (1C, C_{Ar}-Cl), 191.6 (1C, C=O) [20]. HRESIMS (*m/z)* found 438.1881 ($[M-H]^-$), calculated masses for $C_{26}H_{29}ClNO_3$: 438.1837.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-methylphenyl)methylidene]cyclohexan-1-one (**2e**). The compound was an orange sticky powder, with a 76.9% yield, mp: 86–89 °C and Rf = 0.48 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax, cm^{-1}: 3032 (CH aromatic), 2974 (CH aliphatic), 1734 (C=O), 1664 (C=C), 1599 and 1498 (C=C aromatic) 1269 (C-N), 1157 (C-O). ^{1}H-NMR (500 MHz, CD_3OD), δ/ppm: 1.18 (t, 6H, *J* = 7.2 Hz, $C\underline{H}_3$-CH_2-), 1.77 (m, 2H, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 2.35 (s, 3H, CH_3-Ar), 2.80 (q, 4H, *J* = 7.2 Hz, CH_3-$C\underline{H}_2$-N), 2.90 and 2.94 (t, 2H, *J* = 7.3 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$ and t, 2H, *J* = 5.4 Hz, =C-$C\underline{H}_2$-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.94 (s, 2H, Ar-CH_2-N), 6.92 (s, 1H, H_{Ar}), 7.04 (s, 1H, H_{Ar}); 7.23 (d, 2H, *J* = 7.8 Hz, H_{Ar}), 7.37 (d, 2H, *J* = 7,9 Hz, H_{Ar}), 7.65 and 7.67 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, CD_3OD) δ/ppm: 10.9 (2C, $C\underline{H}_3$-CH_2), 21.4 (1C, $C\underline{H}_3$-Ar), 24.1 (1C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 29.3 and 29.6 (2C, C-$C\underline{H}_2$-$C_{cyclohexanone}$), 47.5 (2C, CH_3-$C\underline{H}_2$-N), 54 (1C, $C\underline{H}_3$-O), 56.9 (1C, Ar-$C\underline{H}_2$-N), 114.9, 122.2, 126.3, 126.7, 130.2, 131.5, 134.1 (10C, C_{Ar}), 134.3, 137.0, 137.6, 139.5 (4C, -C=C-), 149.7 and 152.9 (2C, C_{Ar}-O), 191.6 (1C, C=O) [20]. HRESIMS (*m/z)* found 419.1925 ($[M-H]^-$), calculated masses for $C_{27}H_{32}NO_3$: 419.2384.

3.2. Cytotoxicity Test

3.2.1. Brine Shrimp Lethality Test

The assay was performed according to the principle and protocol previously described by Meyer [23], with slight modifications. *Artemia salina* L. eggs were inserted into a box containing seawater, the box was placed under an ultraviolet (UV) lamp, and after 48 hours, the eggs hatched into larvae that were ready for the test. The compounds (**1a–1e, 2a–2e**) were diluted in 10 mL seawater containing 10 larvae (1% DMSO (v/v)) at compound concentrations of 20, 200, 500, and 1,000 ppm. After 24 hours, the live and dead shrimp were counted. The mortality rate (%) was obtained by comparing the number of total dead larvae and the total number of larvae. The experiment was conducted in triplicate. The concentration dose-response (% mortality) data were transformed into a straight line using logit transformation, and the concentration required to kill

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50% of the population (LC_{50} value) was derived from the best fit line obtained by linear regression analysis.

3.2.2. MTT Proliferation Assay

The cytotoxic activity of the synthesized compounds was evaluated against human cervix carcinoma (HeLa and ATCC CCL-2) cell lines, human estrogen-dependent breast carcinoma (MCF7 and ATCC HTB-22) cell lines, and the kidney of an African green monkey (Vero, ATCC CCL-81) as a normal cell lines using the methyl thiazolyl tetrazolium (MTT) method conducted according to the MTT assay protocol published by the American Type Culture Collection (ATCC) [24]. Cisplatin was used as a reference drug. Curcumin was also evaluated as a comparative compound on the tests against Vero and MCF7 cell lines, and doxorubicin was evaluated as an additional comparator on the test against MCF7 cell lines. The assay detects the reduction of yellow tetrazolium (MTT) by metabolically active cells to purple formazan measured using spectrophotometry [24].

The cell lines were seeded into 96-well plates at a density of 5,000 cells per well, replenished with growth media consisting of Dulbecco's Modified Eagle's medium (D-MEM) for Vero or Roswell Park Memorial Institute (RPMI) 1640 medium for MCF7 and HeLa, 5% Fetal Bovine Serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37 °C in 5% CO_2 for 24 h. Then, a series of concentrations of the tested compounds (1.5, 3.1, 6.2, 12.5, 25.0, 50.0, 100.0, and 200.0 µg/mL) were added to each well of the plate and incubated for 48 h. Afterward, 10 µL fresh solution of MTT reagent was added to each well, and the plate was incubated in a CO_2 incubator at 37 °C for four hours. After the purple precipitate was obtained, the cells were dissolved in ethanol and their optical density was recorded at 595 nm. The experiment was performed in triplicate. Percent proliferation inhibition was calculated using the following formula:

$$\text{Viability cells inhibition (\%)} = 100 - \left[\frac{(At - Ab)}{(Ac - Ab)}\right] x\ 100\%$$

where *At* is the absorption of test compound, *Ab* is the absorption of blank, and *Ac* is the absorption of control.

The concentration of the synthesized compounds required to inhibit 50% of the growth of the cell lines (IC_{50} value) was calculated by analyzing the relationship between concentration and percent inhibition using GraphPad Prism 7 version 7.00 for Windows (GraphPad Software, La Jolla, CA, U.S., www.graphpad.com) [25].

3. Results and Discussion

3.1. Chemistry

The novel asymmetrical mono-carbonyl analogs of curcumin (AMACs) (**1a–1e**) and its diethylamine Mannich base derivatives (**2a–2e**) compounds were synthesized to further explore as anticancer compounds. The synthetic route of the compounds is shown in Scheme 1. The intermediate compounds, (2E)-2-(phenylmethylidene)cyclohexan-1-one and its analogs, were synthesized by the Claisen-Schmidt reaction between benzaldehyde or its analogs with cyclohexanone in the presence of aqueous alkali according to the preparation method of 4-phenylbut-3-en-2-one [19]. The aldol condensation of the intermediate compounds obtained with vanilin, with the addition of diluted HCl/ethanol under reflux conditions for 30 min, produced AMACs (**1a–1e**). Finally, the Mannich reaction of **1a–1e** with diethylamine and formaldehyde under reflux condition in ethanol for 7–11 h under TLC monitoring produced the diethylamine Mannich bases of the AMACs compounds (**2a–2e**).

The FTIR spectra of compounds **1a–1e** showed absorption bands at 3,200–3,500 cm^{-1} due to the presence of the OH group. The bands at about 1,100 cm^{-1} correspond to C-O-C ether, whereas the α,β-unsaturated carbonyl groups of the AMACs were observed as strong bands at about 1,600 cm^{-1}. In the ^{1}H-NMR spectra, protons of OH phenolic and OCH_3 group appear as a singlet at δ 5.86–5.90 ppm (1H) and as a singlet at δ 3.91–3.93 ppm (3H), respectively. The two protons of the ethenyl chain of the compounds appeared as two singlets at 7.72–7.79 ppm (2H), indicating an asymmetrical

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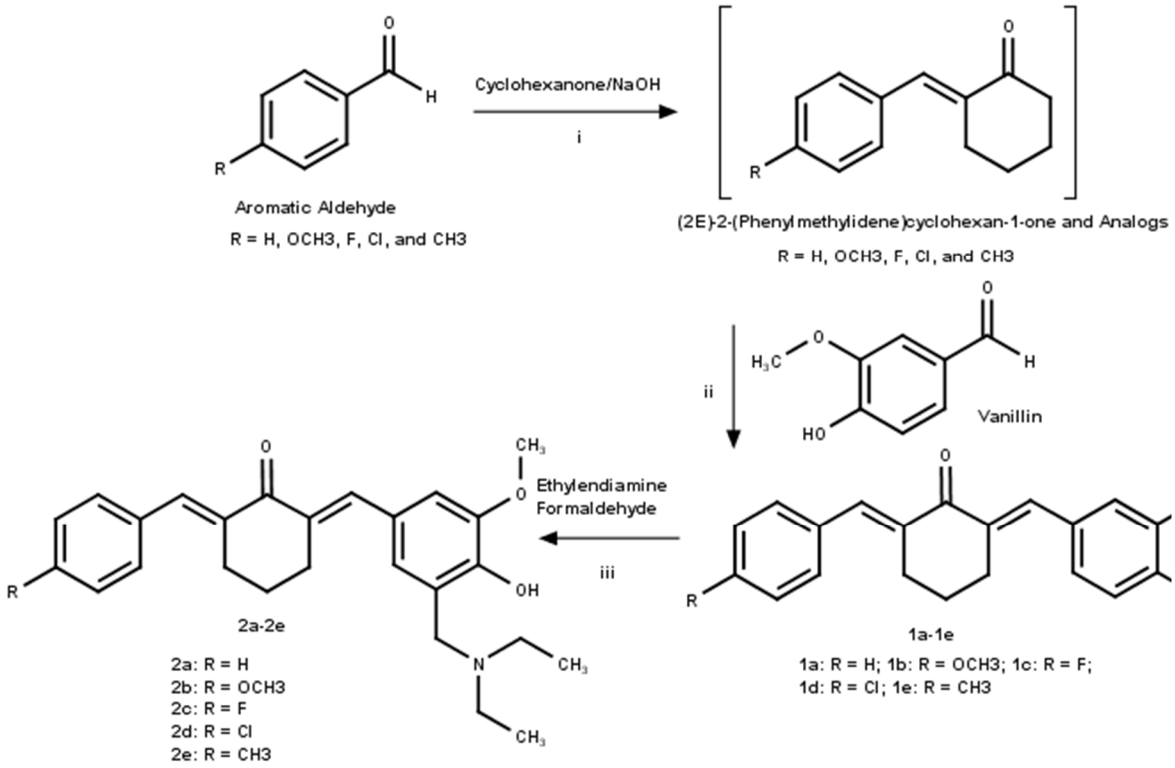
compound. The FTIR spectra of compounds **2a–2e** showed the disappearance of the OH phenolic caused by intermolecular hydrogen bonding formation between the OH and the N atom of the Mannich base formed [21]. The bands at 1,151–1,271 cm^{-1} correspond to C-O-C and C-N, whereas the α,β-unsaturated carbonyl groups of the compounds were observed as strong bands at 1,734 cm^{-1}. In the ^{1}H-NMR spectra, protons of the OCH_3 group appeared as a singlet at δ 3.85 ppm (3H). The two protons of the ethenyl chain of the compounds were observed as two singlets in the range of 7.63 to 7.71 ppm (1H). The protons of diethylamine groups were observed at 1.16–1.18 as a triplet (6H) and 2.75–2.80 ppm as a quintet (4H), and the protons of methylene-adjacent N to the phenyl ring were observed as a singlet (2H) at 3.90–3.95 ppm. The proton signal of OH phenolic disappeared because of exchangea with deuterium from CD_3OD used as a solvent in the experiment [20]. Furthermore, the structures of the compounds were supported by ^{13}C-NMR and high-resolution mass specctrometry (HR-MS), which showed complete agreement with the expected molecular structures.



Scheme 1. Synthesis of the title compounds (**1a–1e** and **2a–2e**). Conditions: (i) r.t.: 2 h.; (ii) ethanol, reflux, diluted HCl/ethanol, 30 min.; and (iii) ethanol, reflux, 7–11 h.

3.2. Cytotoxic Activity

The cytotoxic activity of the compounds was evaluated first using a brine shrimp lethality test (BSLT) method as a preliminary test. All the synthesized compounds exhibited toxic activity with LC_{50} values in the range of 29.80 to 1704.23 μM (17.06–714.49 μg/mL) (Table 1). Compounds **1b**, **2a**, and **2e** had moderate toxicity (LC_{50} value > 100–1000 μg/mL), compounds **1a, 1c, 1d, 2b**, and **2c** had high toxic activity (LC_{50} > 30–100 μg/mL), and **1e** and **2d** had very high toxic activity (LC_{50} < 30 μg/mL) [26,27]. The BSLT is a rapid, inexpensive, and simple method to predict the toxicity level of the compounds. However, the method is not specific for antitumor activity. However, a positive correlation was found between BSLT toxicity and cytotoxicity toward some cell lines [27]. Therefore, in the present study, all the synthesized compounds were then evaluated for their potential as anti-cancer agents.

The cytotoxic activity of the compounds were evaluated against HeLa and Vero cell lines and for certain selected compounds against MCF7 cell lines. The IC_{50} values and selectivity index (SI)

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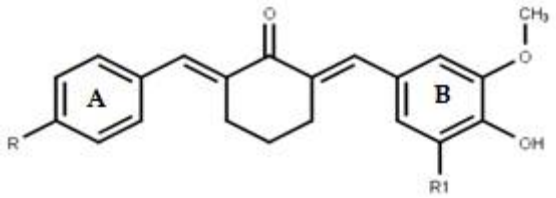
obtained from the MTT assay are presented in Table 1 and Figure 1. Most of the synthesized compounds (**1b**–**1e** and **2a**–**2e**) exhibited cytotoxic activity against HeLa cell lines with IC_{50} values in the range of 40.65 to 95.55 μM. In our experiment, cisplatin exhibited an IC_{50} value of 67.59 μM, but an earlier study [28] reported a much lower value (12.3 μM). The reason for this difference could be due to differences in the conditions of the assay [26]. Based on the experimental data, the cytotoxicity of the compound was comparable with that of cisplatin. Unfortunately, all the synthesized compounds exhibited higher cytotoxic activity on Vero cell lines compared to HeLa cell lines (IC_{50} value 3.94–16.15 μM). As a result, the SI of the synthesized compounds was less than 1, indicating the synthesized compounds were more toxic to a normal cell than to cervix carcinoma cells. Their SI values were lower than that of cisplatin, with a SI value of 1.26. The results also showed that most of the diethylamine Mannich base derivatives of AMACs (**2a**–**2e**) exhibited slightly higher cytotoxic activity against Hela cell lines than the parent compounds (**1a**–**1e**). This result is in line with a previous study that showed that the introduction of Mannich bases enhanced the biological activity of the compounds [29–33]. However, the increasing cytotoxicity was not selective because the effect was also observed with Vero cell lines.

The IC_{50} values of compounds **2a**–**2d** to Vero cell lines were in the range of 3.94 to 7.28 μM (1.73–3.17 μg/mL). Based on the cytotoxicity criteria of a pure compound (IC_{50} < 4 μg/mL or < 10 μM) [34,35], the compounds were considered highly toxic to the normal cell. Therefore, the cytotoxicity of **2a**–**2d** against MCF7 cell lines was not evaluated. The results of the MTT assay against MCF7 cell lines showed that compound **1a** exhibited noncytotoxic (IC_{50} > 100 μM), whereas compounds **1b**–**1e** and **2e** exhibited cytotoxic activity with IC_{50} values in the range of 7.86 to 35.88 μM. Based on the data obtained, the cytotoxic activity of the synthesized compound is more selective to MCF7 cells rather than to HeLa cell lines. Among the synthesized compounds evaluated, compound **1b** was the most cytotoxic and selective against MCF7 cell lines with an IC_{50} value of 7.86 μM (2.75 μg/mL) and a SI value of 1.96. The compound exhibited slightly higher cytotoxic activity, with an IC_{50} value of 7.86 μM, than curcumin or cisplatin (IC_{50} values of 10.47 and 12.85 μM, respectively). However, the cytotoxicity was much lower when compared with doxorubicin, which exhibits an IC_{50} value of less than 2.94 μM (Table 1 and Figure 1). Moreover, the selectivity index (SI) of the compounds to Vero and MCF7 was lower than that of curcumin and cisplatin, with SI values of 1.96, 3.00, and 6.61, respectively (Table 1). The greater the SI value, the safer the compound.

The cytotoxicity of compound **1b**, containing a 4-OCH_3 group at the phenyl ring A (Table 1), was higher than **1a**, **1d**, **1c**, and **1e** containing 4-H, 4-Cl, 4-F, and 4-CH_3, respectively. The results were in line with earlier reported findings that substituent on the four-position of the phenyl ring of the AMACs or MACs significantly influenced the cytotoxicity of the compounds. In addition, a weak electron-donating substitution in the four-position was reported to be the most favorable to the cytotoxic activity of a compound [12,18]. Our data indicate that the electron-withdrawing substitution at the four-position reduced the cytotoxic activity. The effect differs with the electron-withdrawing substitution at the two-position, which enhances the cytotoxic activity [12].

As a standard used earlier to further evaluate compounds as chemotherapeutic agents in preclinical studies using an animal model [26 35], a pure compound should have potency of 10 μM (4 μg/mL) or less in cell culture studies and a SI value less than 2. Compound **1b** could be considered as a new lead compound for further development to produce more active and selective chemotherapeutic agents against breast cancer.

Tabel 1. Cytotoxicity of the synthesized compounds against brine shrimp and Vero, Hela, and MCF7 cell lines.



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No.	Compound	Substuents		LC_{50} (μM)	IC_{50} (μM)[1)]			SI[2)]	
		R_1	R_2	BSLT	Vero	Hela	MCF7	Hela	MCF7
1	**1a**	H	H	196.63	13.98 ± 0.04	>100	>100	<1	<1
2	**1b**	OCH_3	H	322.63	15.43 ± 0.34	95.55 ± 7.19	7.86 ± 1.05	<1	1.96
3	**1c**	F	H	177.36	13.39 ± 0.39	49.15 ± 1.17	10.94 ± 0.79	<1	1.28
4	**1d**	Cl	H	204.09	14.06 ± 0.18	55.60 ± 1.49	35.88 ± 4.57	<1	<1
5	**1e**	CH_3	H	78.71	16.15 ± 0.18	61.19 ± 2.86	10.39 ± 0.36	<1	1.55
6	**2a**	H	X	921.08	4.14 ± 0.21	46.61 ± 1.54	nt	<1	-
7	**2b**	OCH_3	X	80.21	7.29 ± 0.12	69.29 ± 3.17	nt	<1	-
8	**2c**	F	X	88.37	4.23 ± 0.32	41.10 ± 0.16	nt	<1	-
9	**2d**	Cl	X	29.80	3.94 ± 0.07	40.65 ± 0.98	nt	<1	-
10	**2e**	CH_3	X	1704.23	15.02 ± 0.14	76.61 ± 4.27	14.55 ± 1.96	<1	1.03
11	Curcumin	-	-	nt	31.41 ± 0.41	nt	10.47 ± 1.10	-	3.00
12	Cisplatin	-	-	nt	84.66 ± 2.09	67.59 ± 2.04	12.85 ± 1.35	1.26	6.61
13	Doxorubicin	-	-	nt	nt	nt	< 2.94	-	-

Note: [1)] values are the mean ± SD (n = 3); nt = not tested. [2)] SI = Selectivity Index = IC_{50} value normal ell/IC_{50} value cancer cell. X = CH_2-N(CH_2-CH_3)$_2$

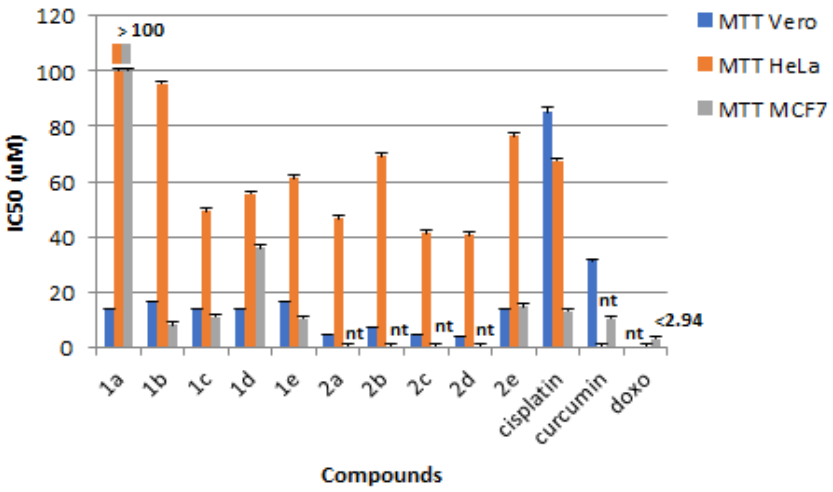

Figure 1. Cytotoxicity of the synthesized compounds against Vero, HeLa, and MCF7 Cell lines. nt = not tested

Figures 2a–2h depict the morphological analysis of untreated MCF7 cells (Figure 2a) and Vero cells (Figure 2b) versus treated MCF7 and Vero cells with respect to compound **1b** (7.88 μM) (Figures 2c and 2d), curcumin (8.48 μM) (Figures 2e and 2f) and cisplatin (12.59 μM) (Figures 2g and 2h). The figures compare the cytotoxicity of the compounds at the same concentration against human breast cancer cells MCF7 and normal cell Vero.

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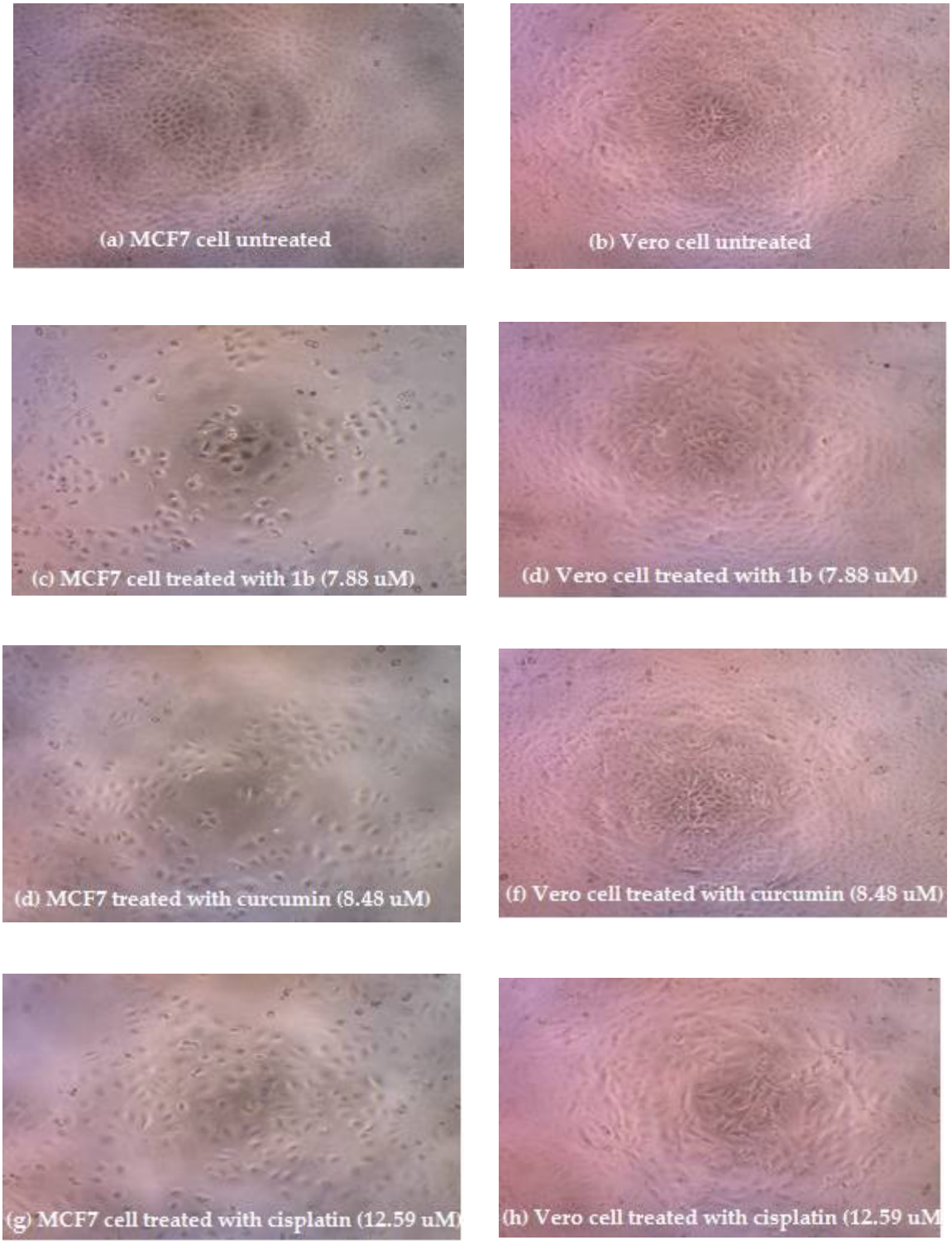

Figure 2. Morphological assessment of MCF7 cells (left) and Vero cells (right) using the methyl thiazolyl tetrazolium (MTT) assay: (**a**,**b**) untreated cells, (**c**,**d**) cells treated with compound **1b** at 7.88 μM, (**e**,**f**) cells treated with curcumin at 8.48 μM, and (**g**,**h**) cells treated with cisplatin at 12.59 μM.

5. Conclusions

A series of asymmetrical mono-carbonyl analogs of curcumin (AMACs) were successfully synthesized. All the synthesized compounds exhibited moderate to very high toxicity based on

BSLT, most of them exhibited comparable cytotoxic activity with cisplatin against HeLa cell lines, and the selected compound exhibited moderate to very high cytotoxic activity against MCF7 cell lines. However, all compounds had a low SI, less than 1–1.96. Among the synthesized compounds, compound **1b** showed the highest cytotoxic and selective activity against MCF7 cell lines. This compound could be considered for further development to find more active and selective chemotherapeutic agents against breast cancer.

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Author Contributions: H.H. and A.B. conceived, and designed the experiments; P.W. performed the experiments; H.H. and P.W. analyzed the data; H.H. and P.W. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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