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Article

Synthesis and Antiproliferative Activity of Diethylamine Mannich Base of Asymmetrical Mono-Carbonyl Curcumin Analogs against HeLa Cell Lines

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Abstract: A series of diethylamine Mannich base of asymmetrical mono-carbonyl analogs of curcumin (AMACs) were synthesized and evaluated for cytotoxic activity against Hela Cell lines. The structures of the synthesized compounds were confirmed on the basis of FTIR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. Preliminary cytotoxic test using BSLT showed that all the synthesized compounds exhibited more potent cytotoxic activity than that of curcumin. While results of MTT assay showed that all the synthesized compounds exhibited more potent antiproliferative activity against HeLa cell lines than that of cisplatin. Compound **2b** exhibited as the most potent compound of the series. Compound 2**a**, **2b**, **2c**, and **2f** had IC_{50} (μM) less than that of compound **1a**, **1b**, **1c** and **1f** indicating that the addition of diethylamine Mannich base improves the antiproliferative activity of the parent compound.

Keywords: diethylamine Mannich base; asymmetrical mono-carbonyl analogs of curcumin; AMACs; synthesis; cytotoxicity; antiproliferative activity; Hela cell lines

1. Introduction

The mono-carbonyl analogs of curcumin (MACs) exhibit the potency of 10-30 times for cell lines and cellular proteins compared to curcumin [1-4]. MACs pharmacokinetic profile will be much more stable than curcumin, resulting in greater tumor regression [1,5]. The introduction an aminoalkyl side chain by Mannich reaction in phenolic compounds increases significantly the biological activity of the compounds [6]. Some of the Mannich bases of symmetrical mono-carbonyl analogs of curcumin (MACs), heterocyclic chalcone analogs, pterostilbene, and other phenolic compounds showed significant improvement in cytotoxic activity [7-10].

Nowadays, some of the asymmetrical MACs (AMACs) with different constituents on the two phenyl rings have been developed and reported to show antioxidant, anti-inflammatory, antimicrobial [11-15] and antitumor properties [16]. However, the study of the Mannich bases of AMACs as an anti-cancer agent has never been reported. As a continuation of our study in Mannich bases compound derivatives, herein we report the synthesis and anti-proliferative activity of AMACs and their diethylamine Mannich base derivatives against HeLa Cells (**Scheme 1**).

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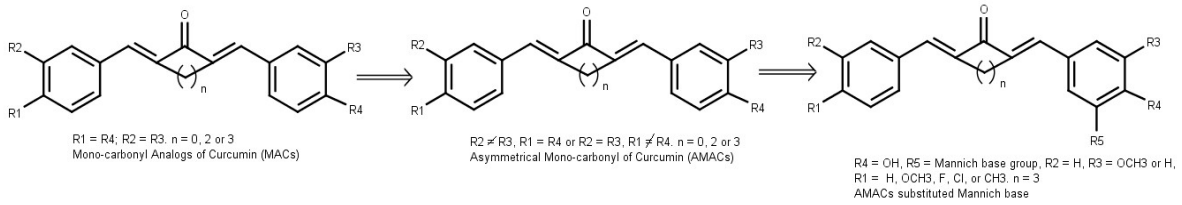

Scheme 1. The design of the title compounds

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 by the TLC method on silica gel 60 F254 plates (Merck). Melting points were determined in the capillary tube using melting point apparatus (Stuart Scientific) and are uncorrected. Infrared (IR) spectra were recorded on an FTIR 8400S spectrophotometer (Shimadzu), ^{1}H-NMR and ^{13}C-NMR spectra were recorded on NMR spectrometer (Agilent) at 500 MHz for 1H and 125 MHz for 13C using TMS as internal standard, and high-resolution mass spectra (HRMS) were measured with a Waters LCT Premier XE (ESI-TOF) system in negative mode.

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

The synthesis was performed according to the method of the synthesis of 2-benzylidene acetone by replacing acetone with cyclohexanone [17]. A mixture of aromatic aldehyde (0.32 mol) and cyclohexanone (0.88 mol) was added a solution of NaOH (10%) dropwise while stirring for 2 hours. The mixture was neutralized with dilute HCl to pH 7, separated the organic layer and extracted the water layer 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 rotary vacuum evaporator to give the crude product. The crude product was used as starting material for the next step without further purification.

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

The synthesis the compounds were performed by the aldol condensation of (2E)-2-(phenylmethylidene)cyclohexan-1-one or it's analogs and vanillin or p-hydroxybenzaldehyde under acidic condition, respectively. The mixture of (2E)-2-(phenylmethylidene)cyclohexan-1-one or it's analogs (0.005 mol) and vanillin or p-hydroxybenzaldehyde (0.01 mol) in ethanol (10 ml) was heated under reflux condition until dissolved and added a drop of diluted HCl/ethanol (1:1), and stirred for 30 mins. The progress of the reaction was monitored by thin layer chromatographic method. Upon completion, the solvent was evaporated, 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-one (**1a**).* The compound was a bright yellow powder, in a 50.0 % yield, mp: 149-151 °C and Rf = 0.8 (ethyl 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-C$\underline{H}_2$-C$_{cyclohexanone}$); 2.93 (t, 4H, =C-C$\underline{H}_2$-C$_{cyclohexanone}$); 3.91 (s, 3H, OCH_3); 5.89 (s, 1H, OH); 6.96 (d, 1H, *J*=8, H$_{Ar}$); 7.00 (s, 1H, H$_{Ar}$; 7.09 (d, 1H, *J*=8 Hz, H$_{Ar}$); 7.33 (t, 1H, *J*= 7, H$_{Ar}$); 7.39 (t, 2H, *J*=7 Hz, H$_{Ar}$); 7.46 (d, 2H, *J*= 7.2 Hz, H$_{Ar}$); 7.75 and 7.79 (s, 1H Ar-C$\underline{H}$=C and 1H, C=C$\underline{H}$-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23 (1C, C-$\underline{C}H_2$-C$_{cyclohexanone}$), 29 (2C, =C-$\underline{C}H_2$-C$_{cyclohexanone}$), 56 (O-$\underline{C}H_3$), 113, 114, 124, 128, 130,

134 (10C, C_{Ar}), 136, 137 (4C, -C=C-) 146 (2C, C_{Ar}-O), 190 (1C, C=O); HRESIMS (*m/z)* found 319.1346 ([M-H]$^-$), calculated masses of $C_{21}H_{19}O_3$: 319.1334

(2E, 6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-methoxyphenyl)methylidene] cyclohexan-1-one (***1b***). The compound was a yellow powder, in a 2.7 % yield, 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-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.92 (m, 4H, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 3.84 (s, 3H, CH_3-O); 3,91 (s, 3H, CH_3-O); 5.86 (s, 1H, OH); 6.96 (t, 3H, *J*=8, H_{Ar}); 6.99 (s, 1H, H_{Ar}); 7.08 (d, 1H, *J*=8 Hz, H_{Ar}); 7.46 (d, 2H, *J*=8, Hz, H_{Ar}); 7.73, 7.76 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23.18 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 28 (2C, =C-$\underline{C}H_2$-$C_{cyclohexanone}$), 55, 56 (2C, OCH_3), 113, 114, 124, 128, 132 (9C, C_{Ar}), 134, 137 (4C, -C=C-), 146, 160 (3C, C_{Ar}-O) 190 (1C, C=O). HRESIMS (*m/z)* found 349.1432 ([M-H]$^-$), calculated masses of $C_{22}H_{21}O_4$: 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, in a 47.3 % yield, 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-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.89 (t, 2H, *J*=7 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.93 (t, 2H, *J*=6 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 3.93 (s, 3H, CH_3-O); 5.90 (s, 1H, -OH); 6.96 (d, 1H, *J*=9 Hz, H_{Ar}); 7.00 (s, 1H, H_{Ar}); 7.10 (t, 3H, *J*=8.2 Hz, H_{Ar}); 7.44 (dd, 2H, *J*=5 Hz, H_{Ar}); 7.75 (s, 2H, Ar-CH=C). ^{13}C NMR (100 MHz, $CDCl_3$) δ/ppm: 23 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 28 (2C, =C-$\underline{C}H_2$-$C_{cyclohexanone}$), 56 (1C, OCH_3), 113, 114, 115, 116, 125, 129, 132, 134 (9C, C_{Ar}), 135, 136, 138, 146 (4C, C=C), 147 (C_{Ar}-F), 161, 164 (2C, C_{Ar}-O), 190 (1C, C=O);); HRESIMS (*m/z)* found 337.1270 ([M-H]$^-$), calculated masses of $C_{21}H_{18}FO_3$: 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, in 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-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.87 (t, 2H, *J*=7.3 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.93 (t, 2H, *J*=7,35 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 3.92 (s, 3H, CH_3-O); 5.86 (s, 1H, OH); 6.96 (d, 1H, *J*=8 Hz, H_{Ar}); 6,99 (s, 1H, H_{Ar}); 7.08 (d, 1H, *J*=8 Hz, H_{Ar}); 7.37 (d, 2H, *J*=9 Hz, H_{Ar}); 7,39 (d, 2H, *J*=9 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 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 29 (2C, =C-$\underline{C}H_2$-$C_{cyclohexanone}$), 56 (1C, OCH_3), 113, 114, 125, , 128.51 (C15), 129, 132, 134 (9C, C_{Ar}), 134, 135, 137, 137 (4C, -C=C-), 138 (C_{Ar}-Cl), 146 (2C, C_{Ar}-O), 190 (1C, C=O). HRESIMS (*m/z)* found 353.0947 ([M-H]$^-$), calculated masses of $C_{21}H_{19}ClO_3$: 353.0945.

(2E, 6E)-2-[(4-hydroxy-3-methoxyphenyl)methylidene]-6-[(4-methylphenyl)methylidene] cyclohexan-1-one (***1e***). The compound was a yellow powder, in 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-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.38 (s, 3H, C$\underline{H}_3$-Ar), 2.91 (t, 2H, *J*=7 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.93 (t, 2H, *J*= 7.15 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 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*=8 Hz, H_{Ar}); 7.37 (d, 2H, *J*=8 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: 21.54 (CH_3-Ar), 23 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 29 (1C, =C-$\underline{C}H_2$-$C_{cyclohexanone}$), 56 (CH_3-O), 113.40 (C18), 114.56 (C16), 124,60 (C17), 128.66 (C15), 129.26 (C2, C4), 130.58 (C1,C5), 133.36 (C8), 134.39 (10C, C_{Ar}), 136, 137, 137, 139 (4C, -C=C-), 146 (2C, C_{Ar}-O), 190.38 (1C, C=O); HRESIMS (*m/z)* found 333.1492 ([M-H]$^-$), calculated masses of $C_{22}H_{21}O_3$: 333.1492

(2E, 6E)-2-[(4-hydroxyphenyl)methylidene]-6-(phenylmethylidene)cyclohexan-1-one (***1f***). The compound was a yellow powder, in a 55,5 % yield, mp: 214-216 °C and Rf = 0.81 (ethyl acetate : n-hexane = 1:2).

FTIR (KBr) υmax cm-1: 3292 (OH), 3059 (CH aromatic), 2933 (CH aliphatic), 1653 (C=O), 1587 (C=C), 1506 and 1431 (C=C aromatik), 1161 (C-O). ^{1}H-NMR (500 MHz, $CDCl_3$), δ/ppm: 1.80 (m, 2H, C-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.93 (t, 4H, *J*=6 Hz =C-C$\underline{H}_2$-$C_{cyclohexanone}$); 5.1 (s, 1H, OH); 6.88 (d, 2H, *J*=8, H_{Ar}); 7.33 (t, 1H, *J*=7 Hz, H_{Ar}); 7.42 (m, 4H, H_{Ar}), 7.46 (d, 2H, *J*=7 Hz, H_{Ar}); 7.75 and 7.79 (s, 1H, s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 23 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 28 (2C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 115, 128, 129, 130, 133, 134 (11C, C_{Ar}), 136, 137 (4C, -C=C-), 156 (1C, C_{Ar}-O), 190 (1C, C=O); HRESIMS (*m/z)* found 289.1232 ([M-H]$^-$), calculated masses of $C_{20}H_{17}O_2$: 289.1229

*2.1.4. Synthesis of Diethylamine Mannich Base of AMACs (**2a-2f**)*

The synthesis were performed according to the method 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 reported previously [18, 22]. Compound **1a-1f** (2 mmol) was dissolved in ethanol, cooled in an ice bath, and added diethylamine (5 - 7 mmol) and formaldehyde solution 37% (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-(phenylmethylidene)cyclohexan-1-one (**2a**)*. The compound was a caramel-like solid, in 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_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 Hz, CH_3-C$\underline{H}_2$-N), 2.89 and 2.94 (t, 4H, *J*=7 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$), 3.85 (s, 3H, CH_3-O), 3.95 (s, 2H, Ar-CH_2-N), 4.86 (s, 1H, -OH), 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_3OD) δ/ppm: 11 (2C, $\underline{C}H_3$-CH_2) 24 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 29 and 30 (2C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 57 (2C, CH_3-$\underline{C}H_2$-N-), 56 (1C, Ar-$\underline{C}H_2$-N), 57 (1C, $\underline{C}H_3$-O), 115, 122, 126, 126. 129, 130, 131, 134 (10C, C_{Ar}), 137, 138, 139 (4C, -C=C-), 150 and 153 (2C, C_{Ar}-O), 192 (1C, C=O); HRESIMS (*m/z)* found 404.2285 ([M-H]$^-$), calculated masses of $C_{26}H_{30}NO_3$: 404.2227

*(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-methoxyphenyl)methylidene]cyclohexan-1-one (**2b**)*. The compound was an orange sticky powder, in 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_3OD), δ/ppm: 1.17 (t, 6H, *J*=7, C$\underline{H}_3$-CH_2-), 1.78 (m, 2H, C-C$\underline{H}_2$-$C_{cyclohexanone}$); 2.77 (q, 4H, *J*=7 Hz, CH_3-C$\underline{H}_2$-N), 2.88 and 2.92 (t, 2H, *J*=5 Hz, =C-C$\underline{H}_2$-$C_{cyclohexanone}$ and t, 2H, *J*=5 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), 4.86 (s, 1H, -OH), 6.89 (s, 1H, H_{Ar}), 6.96 (d, 2H, *J*= 9, H_{Ar}), 7.01 (s, 1H, H_{Ar}); 7.44 (d, 2H, *J*=9 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_3OD) δ/ppm: 11 (2C, $\underline{C}H_3$-CH_2) 24 (1C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 29 and 30 (2C, C-$\underline{C}H_2$-$C_{cyclohexanone}$), 47 (2C, CH_3-$\underline{C}H_2$-N-), 56 (1C, $\underline{C}H_3$-O), 56 (1C, $\underline{C}H_3$-O), 57 (1C, Ar-$\underline{C}H_2$-N), 114, 115, 123, 126, 127, 130, 133 (9C, C_{Ar}), 134, 136, 138, 139 (4C, -C=C-), 150, 153 and 162 (3C, C_{Ar}-O), 192 (1C, C=O); HRESIMS (*m/z)* found 434.2101 ([M-H]$^-$), calculated masses of $C_{27}H_{32}NO_4$: 434.2332

*(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxy-5-methoxyphenyl}methylidene)-6-[(4-fluorophenyl)methylidene]cyclohexan-1-one (**2c**)*. The compound was an orange powder, in a 33,01 % yield, mp: 79-81 °C and Rf = 0.48 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax cm-1: 3041 (CH aromatic), 2937 (CH

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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 Hz, CH_3-CH_2-), 1.77 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.77 (q, 4H, *J*=7 Hz, CH_3-CH_2-N), 2.87 and 2.94 (t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$ and t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.93 (s, 2H, Ar-CH_2-N), 4.86 (s, 1H, -OH), 6.92 (s, 1H, H_{Ar}), 7.03 (s, 1H, H_{Ar}); 7.14 (d-d, 2H, *J*=9 Hz, H_{Ar}), 7,49 (d-d, 2H, *J*=6 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: 11 (2C, CH_3-CH_2) 24 (1C, C-CH_2-$C_{cyclohexanone}$), 29 and 30 (2C, C-CH_2-$C_{cyclohexanone}$), 47 (2C, CH_3-CH_2-N-), 55 (1C, CH_3-O), 57 (1C, Ar-CH_2-N), 114, 116, 117, 122, 126, 133, 134 (9C, C_{Ar}), 136, 138, 140, 153 (4C, -C=C-), 169 and 163 (2C, C_{Ar}-O), 165 (1C, C_{Ar}-F), 192 (1C, C=O); HRESIMS (*m/z*) found 422.2178 ([M-H]$^-$), calculated masses of $C_{26}H_{29}FNO_3$: 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, in 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 Hz, CH_3-CH_2-), 1.78 (m, 2H, C-CH_2-$C_{cyclohexanone}$); 2.79 (q, 4H, *J*= 7 Hz, CH_3-CH_2-N), 2.87 and 2.95 (t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$ and t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.94 (s, 2H, Ar-CH_2-N), 4.86 (s, 1H, -OH), 6.93 (s, 1H, H_{Ar}), 7.04 (s, 1H, H_{Ar}); 7.42 (d, 2H, *J*=9 Hz, H_{Ar}), 7.44 (d, 2H, *J*=9 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 (2C, CH_3-CH_2), 24 (1C, C-CH_2-$C_{cyclohexanone}$), 29 and 30 (2C, C-CH_2-$C_{cyclohexanone}$), 48 (2C, CH_3-CH_2-N), 55 (1C, CH_3-O), 57 (1C, Ar-CH_2-N), 115, 122, 126, 130, 133, 134 (9C, C_{Ar}), 135, 136, 139 (4C, -C=C-), 153 and 150 (2C, C_{Ar}-O), 153.33 (1C, C_{Ar}-Cl), 191.54 (1C, C=O); HRESIMS (*m/z*) found 438.1881 ([M-H]$^-$), calculated masses of $C_{26}H_{29}ClNO_3$: 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, in 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 Hz, CH_3-CH_2-), 1.77 (m, 2H, C-CH_2-$C_{cyclohexanone}$), 2.35 (s, 3H, CH_3-Ar), 2.80 (q, 4H, *J*= 7 Hz, CH_3-CH_2-N), 2.90 and 2.94 (t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$ and t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$); 3.85 (s, 3H, CH_3-O), 3.94 (s, 2H, Ar-CH_2-N), 4.86 (s, 1H, -OH), 6.92 (s, 1H, H_{Ar}), 7.04 (s, 1H, H_{Ar}); 7.23 (d, 2H, *J*=7.85 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: 11 (2C, CH_3-CH_2), 21 (CH_3-Ar), 24 (1C, C-CH_2-$C_{cyclohexanone}$), 29 and 30 (2C, C-CH_2-$C_{cyclohexanone}$), 48 (2C, CH_3-CH_2-N), 56 (1C, CH_3-O), 57 (1C, Ar-CH_2-N), 115, 122, 126, 127, 130, 132, 134 (10C, C_{Ar}), 137, 138, 139, 140 (4C, -C=C-), 150 and 153 (2C, C_{Ar}-O), 192 (1C, C=O); HRESIMS (*m/z*) found 419.1925 ([M-H]$^-$), calculated masses of $C_{27}H_{32}NO_4$: 419.2461.

(2E,6E)-2-({3-[(diethylamino)methyl]-4-hydroxyphenyl}methylidene)-6-(phenylmethylidene)cyclohexan-1-one (***2f***). The compound was an yellow sticky powder, in a 56,0 % yield, mp: 62-65 °C and Rf = 0.5 (ethyl acetate: ethanol = 1:1). FTIR (KBr) υmax cm-1: 3049 (CH aromatic), 2970 (CH aliphatic), 1734 (C=O), 1662 (C=C), 1610 and 1494 (C=C aromatic), 1298 (C-N), 1155 (C-O). ^{1}H-NMR (500 MHz, CD_3OD), δ/ppm: 1.16 (t, 6H, *J*= 7,15 Hz, CH_3-CH_2-), 1.79 (m, 2H, C-CH_2-$C_{cyclohexanone}$), 2.75 (q, 4H, *J*= 7.2 Hz, CH_3-CH_2-N), 2.94 (m, 4H, =C-CH_2-$C_{cyclohexanone}$), 3.89 (s, 2H, Ar-CH_2-N), 4.86 (s, 1H, -OH), 6.77 (s, 1H, H_{Ar}), 7.26 (s, 1H, H_{Ar}); 7.35 (t, 1H, *J*=7.15 Hz, H_{Ar}), 7.39 (t, 1H, *J*=7.7 Hz, H_{Ar}), 7,43 (d, 2H, *J*=7.35 Hz, H_{Ar}), 7.47 (d, 2H, *J*= 9 Hz, H_{Ar}), 7.70 and 7.71 (s, 1H, Ar-CH=C and s, 1H, C=CH-Ar). ^{13}C NMR (100 MHz, CD_3OD) δ/ppm: 11 (2C, CH_3-CH_2), 24 (1C, C-CH_2-$C_{cyclohexanone}$), 29 and 30 (t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$ and t, 2H, *J*=5 Hz, =C-CH_2-$C_{cyclohexanone}$), 48 (2C, CH_3-CH_2-N), 57 (1C, Ar-CH_2-N), 118, 123, 127, 129, 130, 131, 133, 133, 134 (11C, C_{Ar}), 137, 138, 140, 139 (4C, -C=C-), 162 (1C, C_{Ar}-O), 192 (1C, C=O); HRESIMS (*m/z*) found 374.2303 ([M-H]$^-$), calculated masses of $C_{27}H_{32}NO_4$: 374.2121.

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3.2. Cytotoxicity Test

3.2.1. Brine Shrimp Lethality Test

The assay was carried out according to the principle and protocol previously described by Meyer [19-20], with slight modification. Artemia salina L. eggs were inserted in a box that containing seawater, the box was placed under UV lamp, after 48 hours the eggs hatched into larvae and ready for the test. The compound (1a-1f, 2a-2f) were diluted in 10 mL seawater that contains 10 larvae (1% DMSO (v/v)) until concentration 20, 200, 500, and 1000 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 LC_{50} values for a given compound was obtained by calculating according to the linear regression formula: y = a + b x; y = % mortality, x = log C.

3.2.2. MTT Proliferation Assay

The method refers to MTT proliferation assay protocol by American Type Culture Collection [21]. HeLa cells line were seeded into 96-well plates at a density of 1000-10000 cells per well, replenished with 5% heat-inactivated serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in 5% CO2 for 24 h. Various concentrations of tested compounds (100 μL) were added to each well of the plate and incubated for 48 h. After that, a fresh solution of methyl thiazolyl tetrazolium (MTT) reagent (10 μL) was added to each well and then the plate was incubated in a CO2 incubator for 3 h. After a purple precipitate was obtained, the cells were dissolved in DMSO (100 μL) and were recorded their optical density at 515 nm. Percent growth inhibition was calculated by the following formula :

$$\% \text{ Proliferation cells inhibition} = 100-\{(At-Ab)/(Ac- Ab)\}x100\%$$

At = absorption of test compound, Ab = absorption of blank, Ac = absorption of control.

IC_{50} values were calculated for a given compound was obtained by calculating according to the linear regression formula: y = a + b x; y = % proliferation cells inhibition, x = log C.

3. Results and Discussion

3.1.Chemistry

The title compounds were synthesized stepwise by the method summarized in **Schemes 2**. The intermediate compounds, (2E)-2-(phenylmethylidene)cyclohexan-1-one and analogs, was 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 [17]. The aldol condensation of the intermediate compounds obtained with vanilin or p-hydroxybenzaldehyde in addition of diluted HCl/ethanol (1:1) under reflux conditions for 30 min gave asymmetrical mono-carbonyl of curcumin (AMACs) (**1a-1f**). Finally, the Mannich reaction of **1a-1f** with diethylamine and formaldehyde at reflux condition in ethanol for 7-11 h (TLC monitoring) afforded the title compounds **2a-2f** [18].

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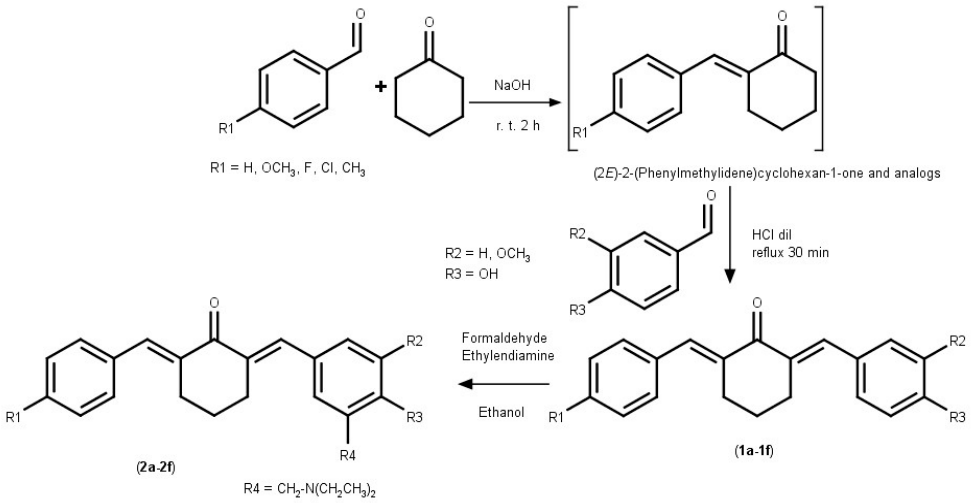

Scheme 2. Synthesis of the title compounds (**2a-2f**)

The IR spectra of compounds **1a-1f** 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 C-O-C ether, while the α,β-unsaturated carbonyl groups of the AMACs are observed as strong bands at about 1,600 cm-1. In the 1H-NMR spectra, the protons of the OH group appear as a broad singlet at δ 5.10 or 5.85-8.90 ppm, while the two protons of the ethenyl chain of the compounds appeared as two singlet at 7.72-7.79 ppm (2H, respectively) indicate the asymmetrical compound. The IR spectra of compounds **2a-2f** showed the disappearance of OH phenolic. The bands at 1,151–1,271 cm-1 correspond to C-O-C and C-N, while the α,β-unsaturated carbonyl groups of the compounds are observed as strong bands at 1,734 cm-1. In the 1H-NMR spectra, the protons of the OH group appear as a broad singlet at δ 4.86 ppm, and the two protons of the ethenyl chain of the compounds are observed as two singlets at a range of 7.63-7.71 ppm (1H, respectively). The protons of the diethylamine groups are observed at 1.16-1.18 as doublet and 2.75-2.80 ppm as a quintet, while the protons of methylene adjacent N to phenyl ring are observed as a singlet at 3.90-3.95 ppm. The structures were further supported by 13C-NMR and HR-ESI-MS of the compounds which showed the complete agreement with the assigned molecular structures.

3.2. Cytotoxicity Activity

3.2.1. Brine shrimp lethality test (BSLT)

The brine shrimp lethality test (BSLT) was used as a preliminary bioassay to predict the toxicity level of the synthesized compounds. It is a rapid, inexpensive, and simple method, and need not special equipment or training. The results of BSLT bioassay of the synthesized compounds are shown in Table 1. All the synthesized compounds (**1a-1f** and **2a-2f**) exhibited LC_{50} < 1000 μg/mL, indicating to have cytotoxic activity. Compound 1e, the AMAC which has methyl group and compound 2d, the diethylamine Mannich base of AMAC which has Cl group in the phenyl ring exhibited $LC_{50} \leq 30$ μg/mL, indicating to have very active as cytotoxic compound [19-20]. Except for compounds 2a and 2e, all the synthesized compounds is more active than that of curcumin having the value of LC_{50} = 210.30 μg/mL, and only compounds 1b, **1f**, **2a** and **2e** having cytotoxic activity lower than the common drug used for cervical cancer treatment, cisplatin, having the value of LC_{50} = 106.71 μg/mL [19]. Except compound **2a** and **2d**, the diethylamine Manncih base of AMACs are generally more active as the cytotoxic agent than that of the parent AMACs.

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2.2.2. *Anti-proliferative activity*

The MTT cell viability assay was used to measure the anti-proliferative activity of the synthesized compounds against HeLa Cell lines using cisplatin as a positive control. The method has been widely accepted as a reliable cell proliferation test. In the method, yellow tetrazolium was reduced by metabolically active cells, partially by dehydrogenase enzymes, to promote equivalent reductions such as NADH and NADPH. The intracellular purple formazan obtained was dissolved and quantified by means of spectrophotometry. When metabolic events cause apoptosis or necrosis, the cell viability will be reduced [21]. Almost all the synthesized compounds (**1a-1f** and **2a-2d,** and **2f**) exhibited more potent antiproliferative activity against HeLa cell lines than that of cisplatin (**Table 1, Figure 1**). Compound **2b**, which has an OCH_3 group in the R1 position and diethylamine Mannich base in the R4 position exhibited as the most potent compound of the series. The IC_{50} (μM) of compound **2a, 2b, 2c** and **2f** had less than that of compound **1a, 1b, 1c** and **1f** indicating that addition of diethylamine Mannich base improves the antiproliferative activity of the parent compound. The improvement of the activity may be due to the additional number of molecular sites for electrophilic attack by cellular constituents compared to the parent compounds [6-7].

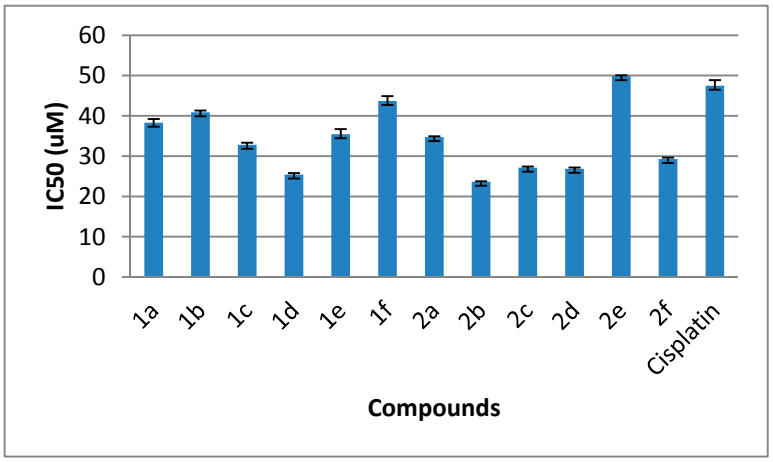
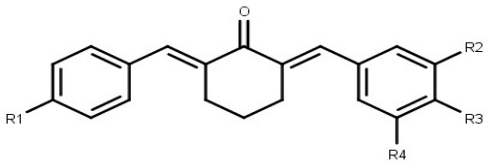


Figure 1. Antiproliferative activity of the synthesized compounds **(1a-1f** and **2a-2f)**

Tabel 1. Cytotoxicity activity of the title compound



No	Compound	Substituents				BSLT LC_{50} (μg/mL)	MTT [1)] IC_{50} (μM) [2)]
		R1	R2	R3	R4		
1	**1a**	H	OCH_3	OH	H	62.95	38.30 ± 0.91
2	**1b**	OCH_3	OCH_3	OH	H	112.97	40.90 ± 0.43
3	**1c**	F	OCH_3	OH	H	59.97	32.83 ± 0.52
4	**1d**	Cl	OCH_3	OH	H	72.27	25.43 ± 0.37
5	**1e**	CH_3	OCH_3	OH	H	26.30	35.44 ± 1.29
6	**1f**	H	H	OH	H	146.21	43.69 ± 1.21
7	**2a**	H	OCH_3	OH	$CH_2N(CH_2CH_3)_2$	373.25	34.72 ± 0.22

8	**2b**	OCH_3	OCH_3	OH	$CH_2N(CH_2CH_3)_2$	34.91	23.64 ± 0.14
9	**2c**	F	OCH_3	OH	$CH_2N(CH_2CH_3)_2$	37.41	27.14 ± 0.26
10	**2d**	Cl	OCH_3	OH	$CH_2N(CH_2CH_3)_2$	13.06	26.86 ± 0.30
11	**2e**	CH_3	OCH_3	OH	$CH_2N(CH_2CH_3)_2$	714.49	49.85 ± 0.19
12	**2f**	H	H	OH	$CH_2N(CH_2CH_3)_2$	95.49	29.30 ± 0.35
13	Curcumin					210.30 [19]	n.t[3)]
14	Cisplatin					106.71[19]	47.48 ± 1.37

[1)]Againts HeLa cell lines; [2)]values are the mean ± SD (n = 3); [3)]n.t = not tested

5. Conclusions

A series of diethylamine Mannich base of asymmetrical mono-carbonyl analogs of curcumin (AMACs) were successfully synthesized. Almost all the synthesized compounds exhibited more potent antiproliferation against HeLa cell lines than that of cisplatin. Generally, the IC_{50} of the diethylamine Mannich base of AMACs compounds were less than that of the parent compound, indicated that addition of diethylamine Mannich base improves the antiproliferative activity of the parent compound. This result indicates that the AMACs compounds and their diethylamine Mannich base derivatives might serve as a potential agent for the treatment of cervical 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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