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RESEARCH ARTICLE

# Synthesis, Antioxidant, and Preliminary Antitumor Activities of New Curcumin Analogues

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## Abstract

Curcumin is a molecule gifted from nature to improve human health by its multiple biological activities. However, the application of curcumin in therapeutics is limited mainly due to its poor aqueous solubility, insignificant chemical stability, and low cellular uptake. To address some of these drawbacks, eight analogues of curcumin were synthesized starting from simple molecules. Characterization of the chemical structures of these analogues was documented by interpretation of their IR, $^{1}$H-NMR, and $^{13}$C-NMR spectra. Biological activities of the synthesized analogues were studied by comparing their activities with those of curcumin. This biological assessment included examining the antioxidant capacity via DPPH and hydroxyl radical scavenging activity tests, and screening the preliminary antitumor activity against MCF-7 and HeLa cancer cell lines by MTT test. Depending on the resulted data, it was found that these analogues have the comparable biological activities but with better stability and water solubility in comparison with curcumin. It is postulated that such analogues may be useful clues to improve the therapeutic applications of curcumin.

**Keywords:** *Curcumin, Synthesis, Analogues, Antioxidant, Antitumor.*

## Introduction

Since the dawn of humanity, natural products have been used in a traditional medicine to manage many illnesses affected on human health [1]. Recently, many of marketed drugs have been originated directly or indirectly from these products. Also, current drug research is actively screened the possible pharmacological activities of many Ayruvedic and traditional Indian medicinal therapies. Among those being investigated, curcumin attracts a growing interest [2].Curcumin (Fig. 1, CM) is the main natural polyphenol found in the rhizome of *Curcuma longa (turmeric)* [3]. This natural product has demonstrated a wide range of biological and pharmacological activities such as antimicrobial [4], anti-inflammatory [5], anticancer [6], anti-diabetic [7], anti-malarial [8], antiprotozoal [9] and antioxidant activities [10].

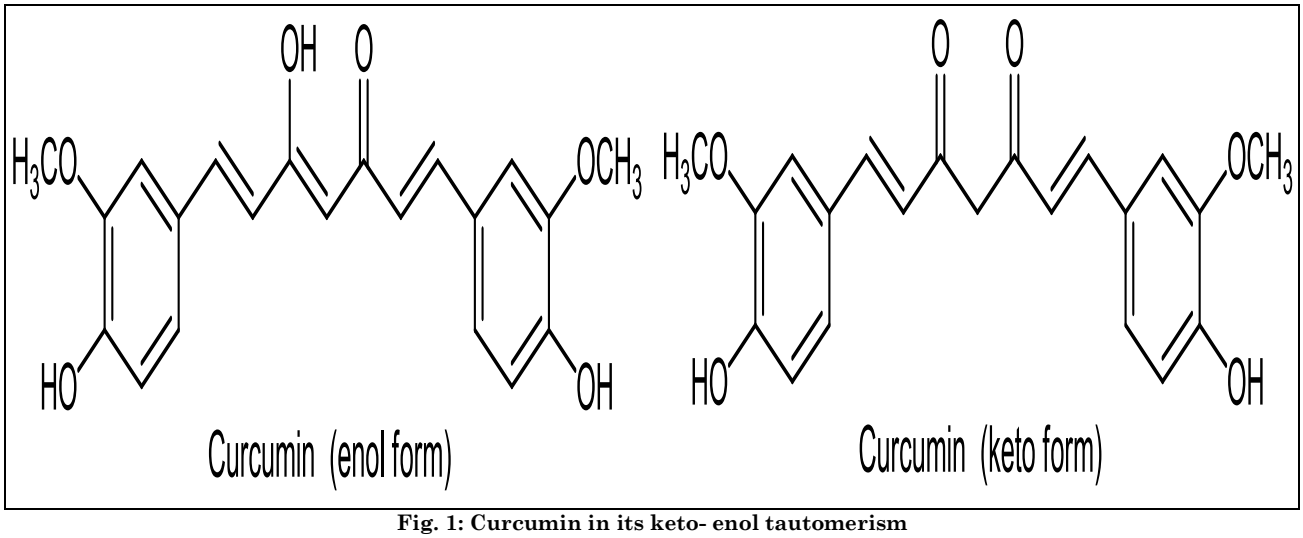



**Fig. 1: Curcumin in its keto- enol tautomerism**

In addition, the safety profile of CM reports that it is a well-tolerated even at very large dose without any toxicity. Therefore, CM has the potential to be a guide scaffold for the drug design and development. Unfortunately, the clinical applications of CM are restricted by its low bioavailability which results from poor aqueous solubility, low oral absorbability, and high metabolic rate [11]. To deal with that, numerous approaches have been undertaken such as use of technological strategies like Nano-encapsulation of CM, employment of adjuvant which interferes with glucuronidation of CM, and structural modification [12]. One the most important strategies which can be used to optimize drug-target interactions is ring variation. This structural modification may result in an improvement of drug activity and selectivity. There are great evidences indicate the utilization of heterocyclic compounds as a molecular template for the development of medicinally active agents with various potential activities [13]. One of the major classes of naturally occurring heterocyclic compounds, which attract many researchers in the biological and medicinal fields, is coumarin-containing compounds [14]. Coumarin nucleus (Fig. 2) can be found as a core structure in many medicinal agents reported to have different pharmacological activities such as anticoagulant, anticancer, antioxidant, anti-inflammatory, and anti-Alzheimer activity [15-17].

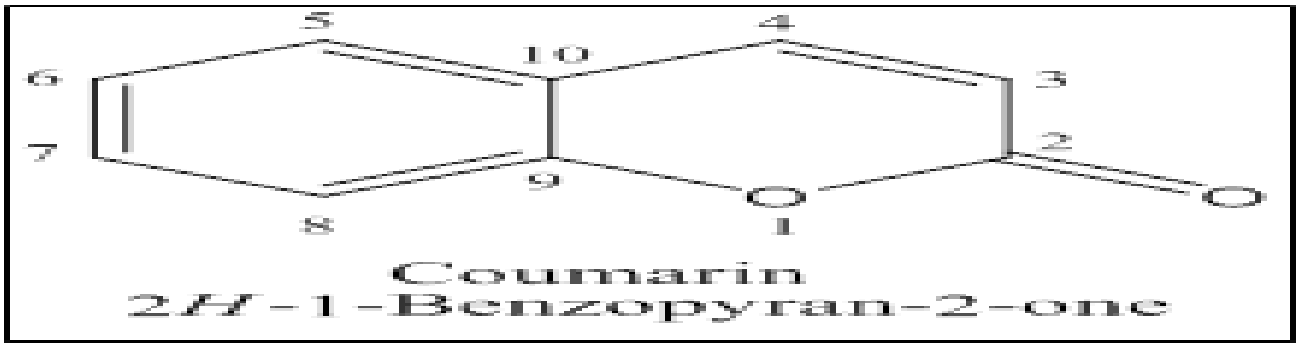



**Fig. 2: Chemical structure of coumarin**

The aim of the presented work is to synthesize and screen the antioxidant and antitumor activities of eight CM analogues. To accomplish this aim, several steps were done. The 2-methoxyphenol rings of CM were exchange with two series of halogenated coumarin derivatives affording CM analogues. These coumarins were synthesized through three steps started from *m*-aminophenol or *p*-aminophenol. The biological activities of the synthesized analogues were compared with CM and included the antioxidant capacity which examined via DPPH and hydroxyl radical scavenging activity tests, and preliminary antitumor activity which screened by MTT test against MCF-7 and HeLa cancer cell lines.

## Material and Methods

The chemicals, solvents and reagents used in the synthesis and assessment of biological activities were obtained from Sigma-Aldrich and Tokyo Chemical Industry except MTT dye (to test antitumor activity) which purchased from Bio-World.

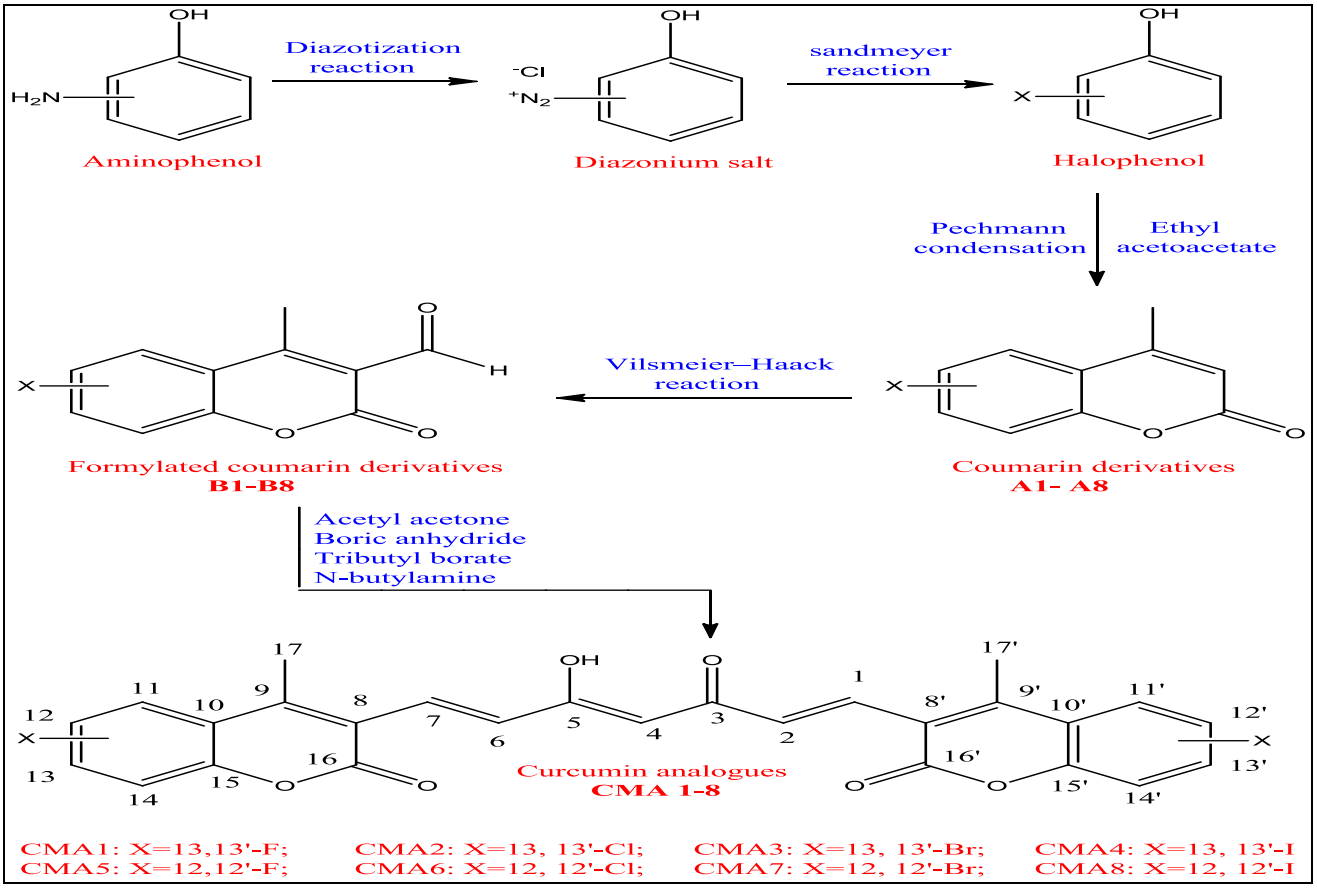
The apparatus used to measure the melting points of synthesized analogues was CIA 9300 by open capillary method. Bruker-Alpha ATR-FTIR and Varian spectrophotometers were used to detect the IR and UV spectra of the intermediates and target analogues, respectively. TLC sheets precoated with silica gel ($GF_{254}$ type 60, Merck) were applied to follow up reactions and to test the purity of compounds. $^{1}$H-NMR and $^{13}$C-NMR spectra were recorded on Bruker Analytische Messtechnik GmbH (400 MHz) using DMSO-*d6* as a solvent and TMS as internal standard.

## Chemical Synthesis:

### Synthesis of the Starting Intermediates

Diazotization of *m*-aminophenol and *p*-aminophenol was carried out based on Reference [18]. The resulted diazonium salts were subjected to Sandmeyer substitution reactions according to procedures reported by References [19-21] affording eight different halophenols. The synthetic pathway started from aminophenols and ended by the target analogues is illustrated in Scheme 1.




**Scheme 1: Synthetic route of CM analogues**

## Synthesis of Coumarin Derivatives

Halophenol (5 mmole) was dissolved in (5 mmole, 0.64 mL) of ethyl acetoacetate and the resulted solution was added dropwise to a conical flask containing a previously cooled $BiCl_3$ (2 mmole, 0.63g). The prepared mixture was stirred for 3 h at 50°C. The reaction mixture was kept at 40°C for 30 min with irradiation in an ultrasonic bath (40 kHz, 350 W, Power sonic 410, Korea).

Then 20 mL of 0.1N HCl was added to the reaction mixture to solubilize the catalyst and suppress the reaction. The solid was collected by filtration, washed with cold water, and recrystallized from EtOH [22]. Percentage of yield, melting point and other physical properties of the resulted halocoumarins are shown in Table 1.

## Synthesis of Formylated Coumarin Derivatives

To a conical flask immersed in an ice bath and contained DMF (8.2 mL), a previously cooled phosphorus oxychloride (30 mmole, 2.8 mL) was dropwise added. The resulted Vilsmeier-Haack reagent was kept at 0°C for 15 min with stirring and then dropwise added to precooled solution of the synthesized halocoumarin (10 mmole) in minimum amount of DMF. Then, the reaction mixture was stirred for 5 h at 60°C, 18 h at room temperature, and 1 h at 60°C. Into a bath of crushed ice, the mixture was poured and neutralized with aqueous sodium carbonate solution (10%). The solid was extracted with chloroform, dried over anhydrous $Na_2SO_4$, and recrystallized from aqueous EtOH [23]. Physical properties of the prepared compounds are demonstrated in Table 1.

## Synthesis of Target Analogues (CMA1-CMA8)

Under dry conditions, a solution of acetyl acetone (10 mmole, 1 mL) and boron oxide (3.5 mmole, 4.9 g) was prepared in 25 mL dry ethyl acetate and kept with stirring at 75°C for 1 h. Then, formylated coumarin derivative (20 mmole) and tributyl borate (20 mmole, 4.8 mL) were added in separated portions and the reaction mixture stirred at the same temperature for 1 h.

A solution of N-butylamine (5 mmole, 0.4 mL) in 5 mL dry ethyl acetate was made and added dropwise to that mixture. The reaction mixture was stirred at 50°C for 20 h and then diluted HCl (5 mL, 10%) was added; the mixture stirred for 2 h at the same temperature. The organic phase was dried over anhydrous $Na_2SO_4$, evaporated, and the solid was recrystallized from aqueous MeOH

(80%) [24]. Table 1 displays physical properties of the CM analogues while Table 2 represents their IR interpretation data.

**Table 1: Physical properties of the synthesized intermediates and analogues**

| Compound symbol | Compound name | m.p. (°C) | $R_f$ | $\lambda_{max}$EtOH (nm) | % yield |
|---|---|---|---|---|---|
| A1 | 7-Fluoro-4-methylcoumarin | 132-129 | 0.56 | 306 | 69 |
| A2 | 7-Chloro-4-methylcoumarin | 140-143 | 0.54 | 310 | 70 |
| A3 | 7-Bromo-4-methylcoumarin | 156-159 | 0.55 | 305 | 69 |
| A4 | 7-Iodo-4-methylcoumarin | 138–340 | 0.56 | 305 | 72 |
| A5 | 6-Fluoro-4-methylcoumarin | 169-166 | 0.48 | 319 | 55 |
| A6 | 6- Chloro-4-methylcoumarin | 181-183 | 0.46 | 312 | 56 |
| A7 | 6- Bromo-4-methylcoumarin | 166-169 | 0.54 | 314 | 57 |
| A8 | 6-Iodo-4-methylcoumarin | 148-151 | 0.50 | 312 | 55 |
| B1 | 7-Fluoro-4-methylcoumarin-3-carbaldehyde | 134-136 | 0.50 | 313 | 47 |
| B2 | 7-Chloro-4-methylcoumarin-3-carbaldehyde | 152-154 | 0.45 | 315 | 48 |
| B3 | 7-Bromo-4-methylcoumarin-3-carbaldehyde | 166-169 | 0.51 | 311 | 44 |
| B4 | 7-Iodo-4-methylcoumarin-3-carbaldehyde | 150-153 | 0.51 | 313 | 53 |
| B5 | 6-Fluoro-4-methylcoumarin-3-carbaldehyde | 177-180 | 0.44 | 324 | 51 |
| B6 | 6- Chloro-4-methylcoumarin-3-carbaldehyde | 190-193 | 0.41 | 317 | 46 |
| B7 | 6- Bromo-4-methylcoumarin-3-carbaldehyde | 180-183 | 0.46 | 318 | 48 |
| B8 | 6-Iodo-4-methylcoumarin-3-carbaldehyde | 160-163 | 0.43 | 319 | 54 |
| CMA1 | (1E, 6Z) 1,7-bis(13-fluoro-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 163-166 | 0.63 | 442 | 69 |
| CMA2 | (1E, 6Z) 1,7-bis(13-chloro-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 178-181 | 0.66 | 442 | 64 |
| CMA3 | (1E, 6Z) 1,7-bis(13-bromo-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 191-194 | 0.70 | 442 | 68 |
| CMA4 | (1E, 6Z) 1,7-bis(13-iodo-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 164-167 | 0.73 | 440 | 74 |
| CMA5 | (1E, 6Z) 1,7-bis(12-fluoro-9-ethylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 192-195 | 0.57 | 446 | 74 |
| CMA6 | (1E, 6Z) 1,7-bis(12-chloro-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 209-211 | 0.66 | 447 | 67 |
| CMA7 | (1E, 6Z) 1,7-bis(12-bromo-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 188-191 | 0.68 | 448 | 68 |
| CMA8 | (1E, 6Z) 1,7-bis(12-iodo-9-methylcoumarin-8-yl)-5-hydroxy-3-oxohepta-1,4,6-triene | 173-176 | 0.69 | 448 | 76 |
| CM | (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione | 182-185 | 0.84 | 416 | ----- |

**Table 2: IR interpretation data (v, stretching, $cm^{-1}$) of the synthesized intermediates and analogues.**

| Compound symbol | O-H (Alcohol) | C-H (alkene) | C-H (Alkane) | C=O (Ester) | C=O (Ketone) | C-X |
|---|---|---|---|---|---|---|
| CMA1 | 3236.55 | 3069.36 | 2876.98 | 1715.82 | 1633.67 | 1132.90 |
| CMA2 | 3209.12 | 3065.45 | 2914.35 | 1733.68 | 1656.02 | 1094.14 |
| CMA3 | 3200.95 | 3051.49 | 2923.82 | 1734.69 | 1648.62 | 1056.09 |
| CMA4 | 3212.85 | 3046.52 | 2917.82 | 1730.86 | 1645.65 | 929.05 |
| CMA5 | 3212.23 | 3064.50 | 2937.06 | 1700.13 | 1634.55 | 1133.08 |
| CMA6 | 3210.18 | 3065.89 | 2930.24 | 1732.41 | 1648.37 | 1091.48 |
| CMA7 | 3220.01 | 3053.78 | 2918.78 | 1736.87 | 1635.96 | 1058.42 |
| CMA8 | 3209.28 | 3045.93 | 2916.60 | 1716.75 | 1635.38 | 940.42 |

**CMA1:** **$^1$H-NMR** (DMSO-*d6*,400 MHz) δ ppm: 14.93 (s, 1H, C5-O**H**), 7.81 (d, 2H, C11-, C11'-**H**, *J*= 6 Hz), 7.69 (d,1H, C1-**H**, *J*= 16 Hz), 7.03 (d, 1H, C6-**H**, *J*= 8 Hz), 6.92 (d, 1H, C7-**H**, *J*= 8 Hz), 6.80 (d, 2H, C12-, C12'-**H**, *J*= 6 Hz), 6.77 (s, 2H, C14-, C14'-**H**),6.46 (d, 1H, C2-**H**, *J*= 16 Hz), 5.93 (s, 1H, C4-**H**), 1.84 (s, 6H, C17-, C17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*,100 MHz) δ ppm: 188.71 (**C**3), 180.66 (**C**5), 160.59 (**C**16, **C**16'), 159.75 (**C**13, **C**13'), 154.27 (**C**9, **C**9'), 152.98 (**C**15, **C**15'),148.81 (**C**1), 134.82 (**C**2), 125.81 (**C**7), 125.92 (**C**11, **C**11'), 122.54 (**C**6), 120.55 (**C**10, **C**10'), 120.24 (**C**8, **C**8'), 115.38 (**C**12, **C**12'), 110.58 (**C**14, **C**14'), 100.31 (**C**4), 17.53 (**C**17, **C**17').

**CMA2:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.97 (s, 1H, C5-O**H**), 7.77 (d, 2H, C11-, C11'-**H**, *J*= 6 Hz ), 7.74 (d, 1H, C1-**H**, *J*= 16 Hz), 7.02 (d, 1H, C6-**H**, *J*= 8 Hz), 6.90 (d, 1H, C7-**H**, *J*= 8 Hz), 6.81 (d, 2H, C12-, C12'-**H**, *J*= 6 Hz), 6.78 (s, 2H, C14-, C14'-**H**),6.44 (d, 1H, C2-**H**, *J*= 16 Hz), 5.98 (s, 1H, C4-**H**), 1.84 (s, 6H, C17-, C17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.70 (**C**3), 180.63 (**C**5), 160.58 (**C**16, **C**16'), 154.25 (**C**9, **C**9'), 153.25 (**C**15, **C**15'), 148.83 (**C**1), 134.73 (**C**2), 130.61 (**C**13, **C**13'),130.44 (**C**11, **C**11'), 127.12 (**C**12, **C**12'), 126.22 (**C**10, **C**10'), 125.83 (**C**7), 123.50 (**C**14, **C**14'), 122.52 (**C**6), 120.24 (**C**8, **C**8'), 100.31 (**C**4), 17.57 (**C**17, **C**17').

**CMA3:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.89 (s, 1H, C5-O**H**), 7.71 (d, 2H, C11-, C11'-**H**, *J*= 6 Hz ), 7.66 (d, 1H, C1-**H**, *J*= 16 Hz), 7.06 (d, 1H, C6-**H**, *J*= 8 Hz), 6.93 (d, 1H, C7-**H**, *J*= 8 Hz), 6.84 (d, 2H, C12-, C12'-**H**, *J*= 6 Hz), 6.81 (s, 2H, C14-, C14'-**H**),6.49 (d, 1H, C2-**H**, *J*= 16 Hz), 5.95 (s, 1H, C4-**H**), 1.85 (s, 6H, C17-, C17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.76 (**C**3), 180.61 (**C**5), 160.60 (**C**16, **C**16'), 154.23 (**C**9, **C**9'), 153.25 (**C**15, **C**15'), 148.82 (**C**1), 134.72 (**C**2), 127.94 (**C**11, **C**11'), 127.42 (**C**12, **C**12'), 126.58 (**C**14, **C**14'), 126.22 (**C**10, **C**10'),125.80 (**C**7), 122.50 (**C**6), 120.20 (**C**8, **C**8'), 119.36(**C**13, **C**13'),100.36 (**C**4), 17.56 (**C**17, **C**17').

**CMA4:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.91 (s, 1H, C5-O**H**), 7.68 (d, 1H, C1-**H**, *J*= 16 Hz), 7.60 (d, 2H, C11-, C11'-**H**, *J*= 6 Hz ), 7.00 (d, 1H, C6-**H**, *J*= 8 Hz), 6.89 (d, 1H, C7-**H**, *J*= 8 Hz), 6.42 (d, 1H, C2-H, *J*= 16 Hz), 6.13 (d, 2H, C12-, C12'-**H**, *J*= 6 Hz), 6.10 (s, 2H, C14-, C14'-**H**),5.97 (s, 1H, C4-**H**), 1.82 (s, 6H, C17-, C17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.66 (**C**3), 180.62 (**C**5), 160.60 (**C**16, **C**16'), 154.34 (**C**9, **C**9'), 153.35 (**C**15, **C**15'), 148.80 (**C**1), 135.62 (**C**2),134.77 (**C**14, **C**14'), 131.55 (**C**11, **C**11'), 130.26 (**C**12, **C**12'), 127.13 (**C**10, **C**10'),125.81 (**C**7), 122.57 (**C**6), 120.21 (**C**8, **C**8'), 101.61 (**C**4), 94.02 (**C**13, **C**13'), 17.59 (**C**17, **C**17').

**CMA5:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.90 (s, 1H, C5-O**H**), 7.70 (d, 1H, C1-**H**, *J*= 16 Hz), 7.53 (s, 2H, C11-, C11'-**H**), 7.05 (d, 2H, C13-, C13'-**H**, *J*= 6 Hz), 7.03 (d, 1H, C6-**H**, *J*= 8 Hz), 6.94 (d, 1H, C7-**H**, *J*= 8 Hz), 6.50(d, 2H, C14-, C14'-**H**, *J*= 6 Hz),6.45 (d, 1H, C2-**H**, *J*= 16 Hz), 5.95 (s, 1H, C4-**H**), 1.88(s, 6H, C17-, C17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.74 (**C**3), 180.70 (**C**5), 160.63 (**C**16, **C**16'), 156.10 (**C**12, **C**12'), 154.25 (**C**9, **C**9'), 148.83 (**C**1), 146.26 (**C**15, **C**15'), 134.84(**C**2), 126.20 (**C**10, **C**10'), 125.87 (**C**7), 124.62 (**C**14, **C**14'), 122.58 (**C**6), 120.26 (**C**8, **C**8'), 118.10 (**C**13, **C**13'), 110.69 (**C**11, **C**11'), 100.30 (**C**4), 17.47 (**C**17, **C**17').

**CMA6:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.94 (s, 1H, C5-O**H**), 7.84(s, 2H, C11-, C11'-**H**),7.76 (d, 1H, C1-**H**, *J*= 16 Hz), 7.06(d, 2H, C13-, C13'-**H**, *J*= 6 Hz), 7.02 (d, 1H, C6-**H**, *J*= 8 Hz), 6.89 (d, 1H, C7-**H**, *J*= 8 Hz), 6.85(d, 2H, C14-, C14'-**H**, *J*= 6 Hz),6.47 (d, 1H, C2-**H**, *J*= 16 Hz), 5.96 (s, 1H, C4-**H**), 1.83 (s, 6H, **C**17-, **C**17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.75 (**C**3), 180.62 (**C**5), 160.59 (**C**16, **C**16'), 154.23 (**C**9, **C**9'), 150.20 (**C**15, **C**15'),148.88 (**C**1), 134.71 (**C**2), 130.13 (**C**13, **C**13'), 129.63 (**C**10, **C**10'), 129.42 (**C**11, **C**11'), 127.61 (**C**12, **C**12'), 125.81 (**C**7), 124.50 (**C**14, **C**14'), 122.55 (**C**6), 120.27 (**C**8, **C**8'), 100.39 (**C**4), 17.51 (**C**17, **C**17').

**CMA7:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 15 (s, 1H, C5-O**H**), 7.99(s, 2H, C11-, C11'-**H**),7.64 (d, 1H, C1-**H**, *J*= 16 Hz), 7.05 (d, 2H, C13-, C13'-**H**, *J*= 6 Hz), 7.03 (d, 1H, C6-**H**, *J*= 8 Hz), 6.86 (d, 1H, C7-**H**, *J*= 8 Hz), 6.55(d, 2H, C14-, C14'-**H**, *J*= 6 Hz),6.44 (d, 1H, C2-**H**, *J*= 16 Hz), 5.86 (s, 1H, C4-**H**), 1.8 (s, 6H, **C**17-, **C**17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.73 (**C**3), 180.60 (**C**5), 160.57 (**C**16, **C**16'), 154.25 (**C**9, **C**9'), 151.08 (**C**15, **C**15'), 148.85 (**C**1), 134.74 (**C**2), 130.56 (**C**13, **C**13'), 129.36 (**C**10, **C**10'), 128.50 (**C**11, **C**11'), 125.84 (**C**7), 125.12 (**C**14, **C**14'), 122.50 (**C**6), 120.23 (**C**8, **C**8'), 116.13 (**C**12, **C**12'), 100.33 (**C**4), 17.52 (**C**17, **C**17').

**CMA8:** **$^1$H-NMR** (DMSO-*d6*, 400 MHz) δ ppm: 14.92(s, 1H, C5-O**H**), 8.21(s, 2H, C11-, C11'-**H**),7.70 (d, 1H, C1-**H**, *J*= 16 Hz), 7.04 (d, 1H, C6-**H**, *J*= 8 Hz), 6.96 (d, 1H, C7-**H**, *J*= 8 Hz), 6.46 (d, 1H, C2-**H**, *J*= 16 Hz), 6.30 (d, 2H, C13-, C13'-**H**, *J*= 6 Hz),5.96 (s, 1H, C4-**H**), 5.49(d, 2H, C14-, C14'-**H**, *J*= 6 Hz), 1.86 (s, 6H, **C**17-, **C**17'-**H**). **$^{13}$C-NMR** (DMSO-*d6*, 100 MHz) δ ppm: 188.66 (**C**3), 180.65(**C**5), 160.64 (**C**16, **C**16'), 154.37 (**C**9, **C**9'), 150.55 (**C**15, **C**15'), 148.78 (**C**1), 138.62 (**C**13, **C**13'), 137.53 (**C**11, **C**11'), 134.74 (**C**2), 129.83 (**C**10, **C**10'), 125.89 (**C**7), 124.20 (**C**14, **C**14'), 122.54 (**C**6), 120.23 (**C**8, **C**8'), 101.59 (**C**4), 91.42 (**C**12, **C**12'), 17.65 (**C**17, **C**17').

## Biological Studies

### Antioxidant Activity

The antioxidant activity of different concentrations of CM and its analogues was measured according to the methods described in Reference [25]. Five serial dilution (200, 100, 50, 25, 12.5μM) of CM and its analogues were used for testing their ability to scavenge DPPH (1,1-diphenyl-2-picryl-hydrazyl) and hydroxyl free radicals. The two methods were performed in triplicate for each detected concentration. The radical scavenging activity, expressed as %, was calculated using the following formula:

***Percentage of scavenging activity = (Ac - As)/ Ac × 100***

***Where Ac is the absorbance of positive control and as is the absorbance of sample***

The $SC_{50}$, the concentration required to scavenge 50% of the free radicals, of each of CM and its analogues was graphically measured by plotting the scavenging % versus the log concentration using a nonlinear regression [26].

**Hydroxyl Radicals Scavenging Assay**

To a test tube containing the sample (1.5 mL), the following additions were carried out: $FeCl_3$ (60 µL, 1 mM), 1, 10-phenanthroline (90 µL, 1mM), potassium phosphate buffer (2.4 mL, 200 mM, pH 7.8), and $H_2O_2$ (150µL, 170 mM). The test tube was incubated for 5 min at room temperature. Then, the absorbance at 560 nm was scanned using a positive control containing the same reaction mixture but in the absence of sample [25]. The results of this assay are recorded in Table 3.

**Dpph Radical Scavenging Assay**

Methanolic DPPH solution in a concentration of (0.1 mM) was prepared freshly. The sample (1.5 mL) and DPPH (0.5 mL) were placed in a labeled test tube, mixed thoroughly and kept at room temperature for 30 min with complete protection from light. After that, the absorbance of the positive control (0.5 mL of DPPH + 1.5 mL methanol) was measured at 517 nm [25]. The results of this assay are shown in Table 3.

**Preliminary Antitumor Activity**

Assessment of the preliminary antitumor activity of CM and its synthetic analogues was performed using two cancer cell lines, which are MCF-7 and HeLa. To achieve this target, MTT cell viability test was used. The specific cancer cell line was spread to reach 10,000 cells for each well of the 96-well plate. To each cell line in the plate and after 24 h, different concentrations (100, 50, 30, 20, 10 µg/mL) of the tested compounds were added. After 72 h of treatment, cell viability was tested by removing the medium, adding MTT solution (28 µL, 3.27 mM), and then incubating the cells at 37°C for 1.5 h. The absorbance of untreated well (Tc) and the absorbance of treated well (Ts) was detected by using the microplate reader which operated at 492 nm. This procedure was repeated in triplicate for every concentration of each tested compounds. The percentage of growth inhibition was calculated via the following formula [27]: Growth inhibition (%) = (Tc–Ts)/Tc ×100. The results of this assay are summarized in Table 3

**Results and Discussion**

**Chemical Synthesis**

In this work, eight CM analogues were designed and synthesized by substituting 2-methoxyphenol rings of CM with two series of halogenated coumarin derivatives. The synthetic plan as illustrated in Scheme 1 was started from the formation of diazonium salts by diazotization of *m*-aminophenol and *p*-aminophenol. By using Sandmeyer reaction, the resulted diazonium salts were replaced with halogens to yield 3- and 4-halophenols. Pechmann reaction was applied to condense these phenols with ethyl acetoacetate affording 7- and 6-halocoumarins in good yields.

Insertion of carbaldehyde group at position 3 of these coumarins resulted in formylated coumarin derivatives, which are joined with acetyl acetone forming the CM analogues. All these aforementioned analogues, formylated coumarins, and halocoumarins are novel except A1, A4, and A5. The chemical structure of CM is characterized by the presence of two 2-methoxyphenol rings, two carbon-carbon double bonds in the trans configuration, and keto-enol tautomerism [28].

In the other hand, the chemical structures of the CM analogues are characterized by the presence of two heterocycle rings, two carbon-carbon double bonds one of them in the trans form and the other in the cis form, and a predominant enol tautomer. These differences in the chemical skeleton of the compounds were confirmed by analyzing their IR, $^{1}$H-NMR, and $^{13}$C-NMR spectra.

## Biological Studies

The effects of structural variation of the CM analogues on *in vitro* biological activities were tested by the comparison the antioxidant and antitumor activities of CM as a standard with those of its analogues.

## Antioxidant Activity

Many reports and researches investigated the antioxidant activity of CM have attributed this effect to the ability of CM to act as a chain-breaking antioxidant by donating its phenolic hydrogen in a hydrogen atom transfer mechanism [29, 30].

The results of CM analogues, as expressed in Table 3, revealed that their $SC_{50}$ values are comparable to that of CM despite the lack of phenolic hydrogen in the chemical structures of these analogues. Therefore, it is proposed that the donation of enolic hydrogen could play a role in the antioxidant activity of CM analogues.

The presence of high conjugated system consisting of heptadiene and coumarin nuclei may facilitate the abstraction of the enolic proton [31]. As a consequence of such conjugation, a more stable enolate will be afforded than that found between the heptadiene and 2-methoxyphenol rings of CM. So, this finding proposed that the donation of phenolic hydrogen of CM may be equivalent to that of enolic hydrogen of the CM analogues.

## Antitumor Activity

CM has received great attention over the past two decades mainly as an anticancer agent with beneficial effects for preventing and managing cancer. However, the anticancer application of CM has been limited mainly due to its low water solubility which results in low cellular uptake. One of the most common approaches to address this limitation is structural modification to produce different CM derivatives and analogues [32].

The data reported in this work, as explained in Table 3, revealed that the $IC_{50}$ of CM analogues are related to that of CM against the tested cancer cell lines, MCF-7 and HeLa. The analogue CMA5 exhibits a superior activity against MCF-7 than CM and other analogues whereas CMA6 has the inferior effect. The results also indicated that CMA1 has the best activity against HeLa and CMA6 has the least effect. The presence of fluoride in the chemical structure of CMA1 and CMA5 analogues lead to conclude that it may enhance the antitumor activity of these analogues.

Fluoride has high electronegativity and small atomic size which may cause improvement of aqueous solubility and then the cellular uptake [33]. Consequently, the synthesized CM analogues may be superior to CM as they are more hydrophilic. This claim can be cleared by looking at the TLC results demonstrated in Table 1.

**Table 3: Data obtained from testing the antioxidant and antitumor activities of CM and its Analogues**

| Compound | Antioxidant activity $SC_{50}$ (µM) ± SD (*n=3*) | | Preliminary antitumor activity $IC_{50}$ (µg/mL) ± SD (*n=3*) | |
|---|---|---|---|---|
| | Scavenging of DPPH radicals | Scavenging of OH radicals | MCF-7 Cancer cell line | HeLa Cancer cell line |
| CM | 75.48 ± 0.0058 | 119.60 ± 0.0008 | 22.400 ± 0.671 | 20.367 ± 1.398 |
| CMA1 | 74.11 ± 0.0055 | 103.30 ± 0.0009 | 15.880 ± 0.791 | 14.423 ± 0.850 |
| CMA2 | 75.90 ± 0.0098 | 114.30 ± 0.0012 | 24.690 ± 0.911 | 23.157 ± 1.580 |
| CMA3 | 74.96 ± 0.0064 | 121.00 ± 0.0010 | 24.103 ±0.900 | 24.323 ±0.837 |
| CMA4 | 76.72 ± 0.0108 | 114.90 ± 0.0010 | 29.990 ±1.400 | 45.123 ±1.554 |
| CMA5 | 73.11 ± 0.0102 | 120.80 ± 0.0010 | 47.747 ±1.243 | 45.670 ± 1.076 |
| CMA6 | 76.87 ± 0.0090 | 118.80 ± 0.0010 | 24.657 ± 0.552 | 34.230 ± 0.954 |
| CMA7 | 76.87 ± 0.0091 | 110.10 ± 0.0012 | 27.950 ± 1.563 | 25.813 ± 1.479 |
| CMA8 | 76.05 ± 0.0093 | 116.30 ± 0.0007 | 33.340 ± 0.875 | 69.980 ± 1.086 |

## Conclusion

This work reported the synthesis of eight CM analogues from simple phenols through many serial reactions. According to the biological studies performed on these analogues, it is concluded that these analogues have improved antioxidant and antitumor activities compared with CM especially those with fluoride substitutions. This work supposes that such analogues with improved solubility, chemical stability, and biological activities may act as a template to design and synthesize new CM analogues which can serve better in therapeutics.

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