Design and synthesis of novel anti-cancer curcumin derivatives: Investigation of anti-cancer properties against HepG2 cell line

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Abstract: The new anti-cancer and water-soluble curcumin derivatives have been synthesized solvent-freely by functionalizing the phenolic group of curcumin with various NH compounds to increase water solubility and cancerous properties. The biological applications of the new drugs have been investigated against the highly potent HepG2 carcinoma cell line. The studies showed that new curcumin derivatives could be used in low concentrations (μM/l<1) to avoid the anti-apoptotic properties against the cancerous cell. Also, the designed molecules with concentrations below 1 μM/l showed a good percentage of viability of 31.5, 45.3, and 66.7% against HepG2 cells. Furthermore, FTIR and 1H, and 13CNMR spectroscopy fully characterized the new curcumin derivatives.

Keywords: Curcumin; HepG2 cell line; MTT; NMR; Anti-cancer; Cytotoxicity.

1. Introduction

Curcumin is a hydrophobic polyphenol extracted from the Curcuma longa1 and is responsible for the yellow color of turmeric and curry spices [1,2]. Curcumin imparts several pharmacological effects such as anti-inflammatory, antioxidant, multidrug anti-cancer [3-11], anti-angiogenic [12], antimicrobial [13,14], anti-HIV [15], anti-diabetic, anti-alzheimer, wound healing, antiviral, anti-rheumatic, and antiparkinsonian [16]. Furthermore, curcumin has been used in food coloring, cosmetics, and photosensitizers in photodynamic therapy [17,18]. A wide range of therapeutic properties and many exciting pharmacological effects have been reported for curcumin, including anti-inflammatory, antioxidant, chemopreventive, and chemotherapeutic potential [19,20].

Although curcumin has potential therapeutic benefits through anti-inflammatory and antioxidant mechanisms, the clinical use of curcumin is limited by its low bioavailability, which results from low water solubility, low oral absorption, and rapid metabolic rate [21,22]. In addition, phenolic and methylene groups of curcumin are important for the diverse pharmacological activity of curcumin [23]. Still, it has a limiting pharmaceutical role due to its extremely low solubility in water, rapid metabolism, low systemic bioavailability, and alkaline pH degradation [24-26]. Several methods have been used to address these disadvantages, such as nanoencapsulation of curcumin. Use of additives that interfere with glucuronidation and structural changes [27].

Several methods have been used to address these disadvantages, such as nanoencapsulation of curcumin. Use of additives that interfere with glucuronidation and structural changes [11]. Given the importance of curcumin in treating diseases and the limited research on the synthesis of novel derivatives of curcumin, it is important to continue research into synthetic chemistry. New drug-like curcumins are being synthesized to increase curcumin derivatives' efficacy and therapeutic effects in different regions [28-31].

In the present work and based on our attempt to improve the synthetic methodology and synthesis of drug-like compounds [32-43], the synthesis of novel water-soluble curcumin derivatives is reported. To improve the water solubility of curcumin and its anti-cancer properties, especially in treating HepG2 hepatocellular carcinoma, the phenolic group of curcumin was functionalized with various NH compounds (Figure 1). The final structure of the new generation curcumin derivatives was determined by IR and NMR analysis. Finally, toxicity tests were performed on the synthesized derivatives to

demonstrate the therapeutic benefit of destroying   cancerous hepatocytes.
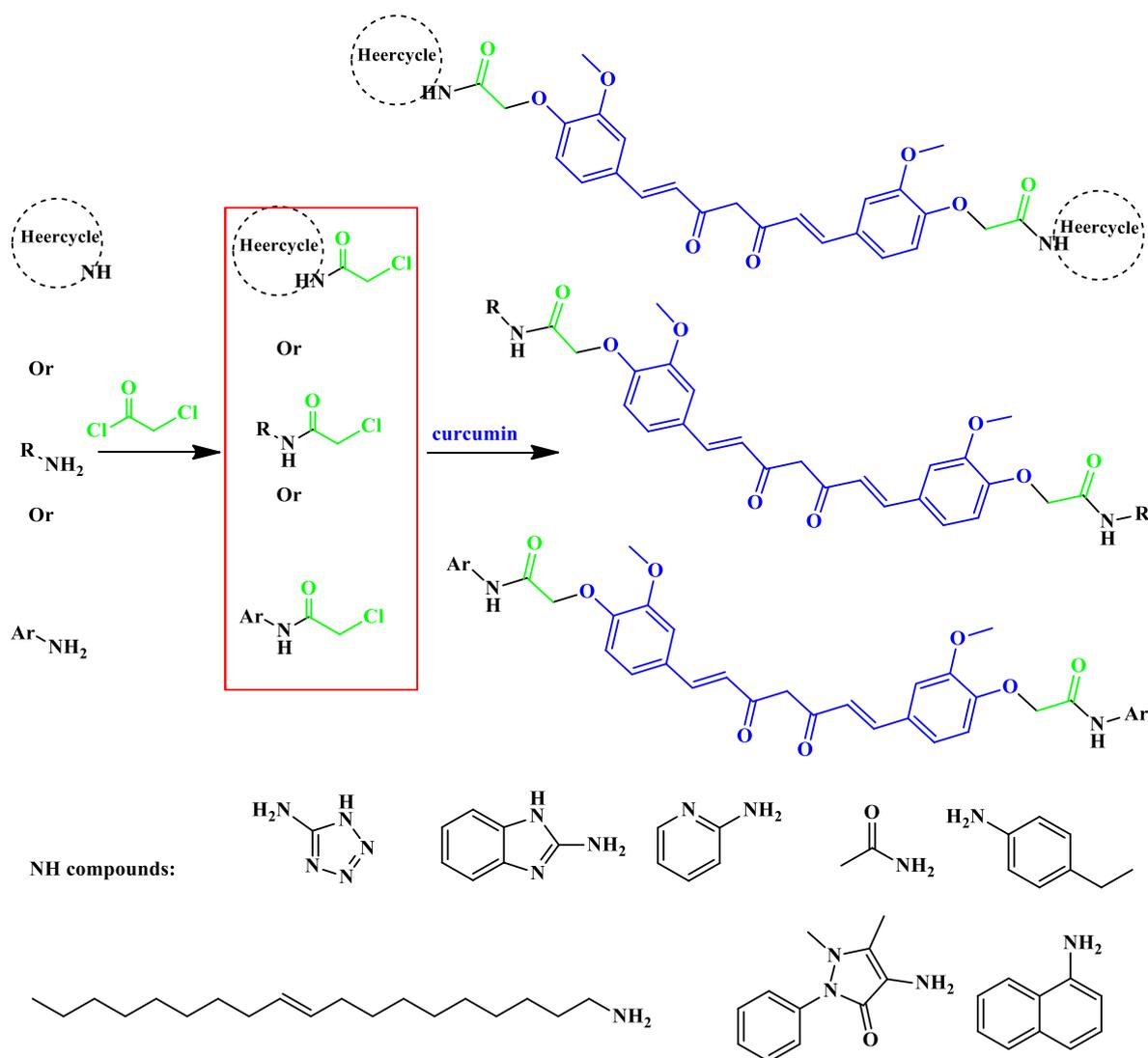
Figure 1. Schematic synthesis of curcumin derivatives

2. Results and Discussion

To generalize the synthesis, different NH compounds were reacted by 2-chloro-acetyl chloride. Then the acetamide intermediates were undergone a condensation reaction with the phenolic part of curcumin with a 2:1 equivalent ratio to produce the new drug, according to Table 1. Decreasing the reaction temperature reduces product yield and reaction rate. It was observed that the reaction did not occur at room temperature. In addition, a catalytic amount of K_2CO_3 is required for high reaction efficiency. Because in the absence of the catalyst, the reaction time is very long, and the output is low.

When a heterocyclic compound is used to condense by the phenolic group of curcumin, the solubility of the new derivative increases, and it becomes completely soluble in water. In addition, new products do not need to be distributed in water by nanoparticles, emulsifiers, nanocarriers, niosomes, etc.

The structure of synthesized derivatives using 13CNMR, 1HNMR, and FT-IR spectroscopy methods was identified and confirmed. The peak in the FT-IR spectrum of these compounds, in the region above 3200 cm^{-1}, indicates the NH group stretching vibrations. The amide group shows an absorption band of about 1650 to 1700 cm^{-1}. The presence of this absorption band confirms the synthesis of new derivatives with a 2-chloro-acetyl chloride linker. The 1HNMR spectra of the products show indicator peaks for the protons of the OCH_3, OCH_2, and $-CH_2$ groups in the 3-4 ppm regions. Also, peaks observed in the 5.5-6.5 ppm range indicate protons attached to aromatic carbon. In the 13CNMR spectra of products, peaks observed in the 0-80 ppm regions correspond to sp^3 carbons, and in the 80-155 regions to the sp, sp^2 carbons, and the carbonyl groups appear in the 158-220 ppm regions, which confirm the synthesis of the products.

Table 1. Synthesized new curcumin derivatives.

Entry	Compound	Time (h)	Yield (%)	MP (°C)
3a		8	67	180
3d		3	94	125
3e		2	68	122
3f		3	80	118
3h		3	97	210
3i		4	66	132
3m		3	70	130
3n		2	89	200
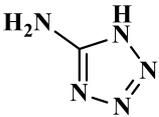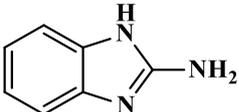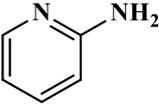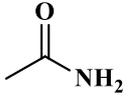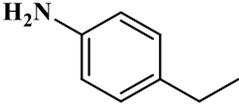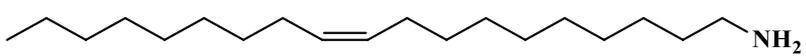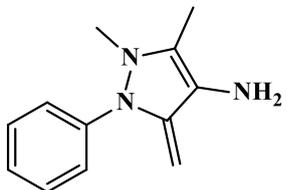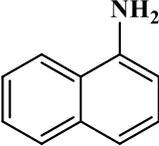
According to the proposed mechanism (Scheme 2), the NH compound is reacted with 2-chloro-acetyl chloride in a ratio of 1:1 to obtain the acetamide intermediate. The reaction of the NH$_2$ group with the acetyl chloride group is quick as the reaction is exothermic. Because curcumin has two functional groups, it was added to the reaction mixture in a ratio of 1:2. Then, the hydroxyl (OH) group of the curcumin compound attacks the carbon and destroys the Cl$^-$. The proposed synthesis of the curcumin derivatives in the presence of a K$_2$CO$_3$ catalyst is suggested as follows:
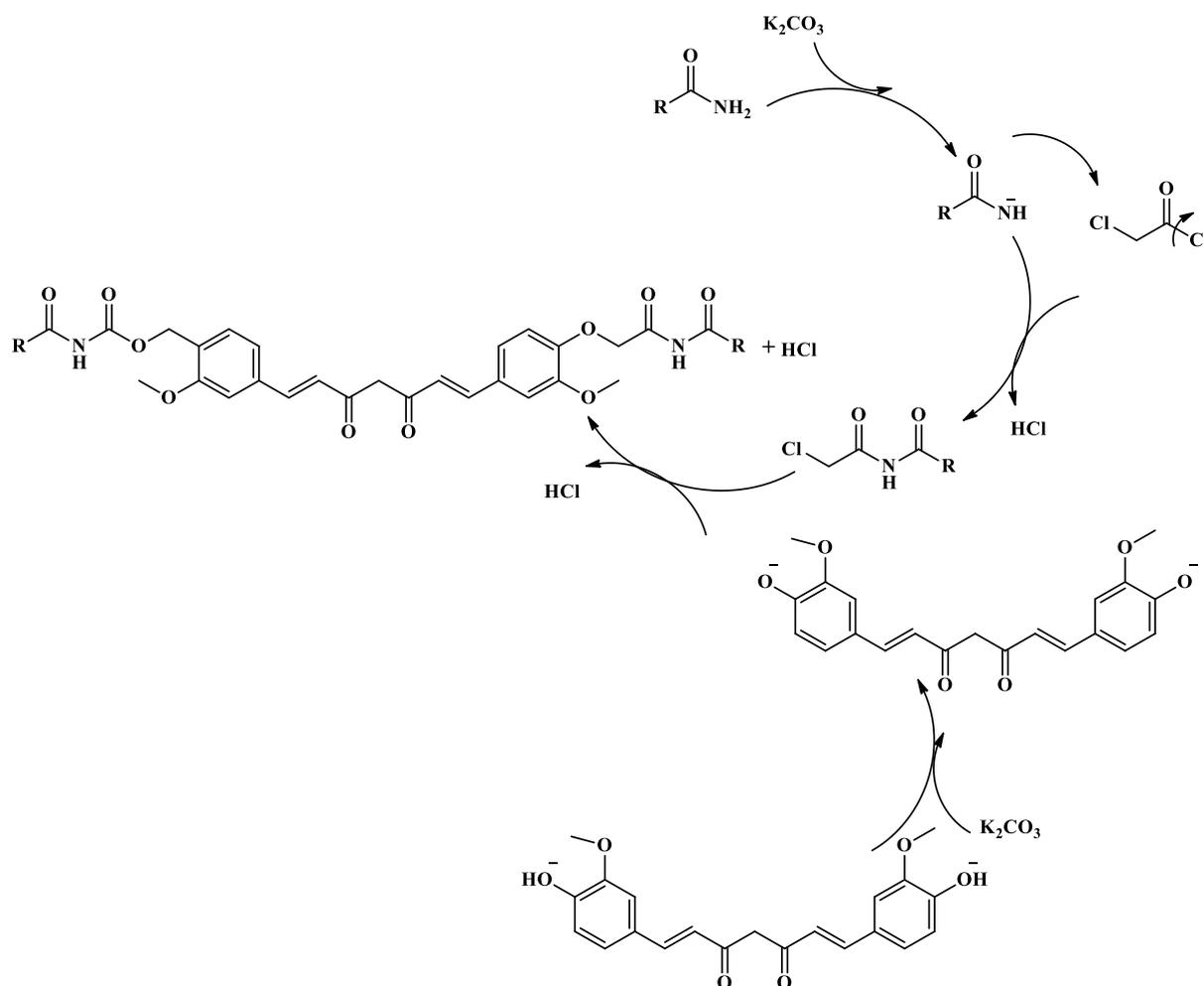
Figure 2. Schematic synthesis of curcumin derivatives in the presence of K_2CO_3 catalyst

Herein, the HepG2 cell line was used for cell experiments. Cell viability was assessed using the MTT assay, which was repeated three times. The basis for this test is the reduction of the yellow salt of tetrazolium with the enzyme succinate dehydrogenase from the mitochondria in living cells and the production of purple and water-insoluble formazan crystals. Formazan crystals dissolve in dimethyl sulfoxide isopropanol and absorb light at 570 nm. The greater number of living cells makes pigment production more intense. Absorption at 570 nm was achieved using Eliza rider. The variable percentage shown in Table 2 agrees that some curcumin derivatives have good to excellent anti-cancer properties against the HepG2 cell line. The **3m** product showed the highest antitumor properties with high efficacy and the highest mortality of 66.7% with perfect antitumor activity compared to pure curcumin. In addition, according to the results, the 3e sample with the highest proportion of HepG2 of 93.4% at 16 hours was obtained for the selected

formulation. Therefore, it is suggested that the **3e** biocompatible product could protect HepG2 cells from the cytotoxic effect of drugs.

The literature studies reported that 1 μM curcumin for 1 h before exposure to an antagonist reduced the cytotoxicity of HepG2 cells and possessed anti-apoptotic properties in high concentrations [21]. At the same time, the designed drugs in this paper, especially compounds **3a**, d, and m, with concentrations below 1 μM/l, showed a good percentage of viability of 31.5, 45.3, and 66.7%, respectively. These results show the new drugs could be used in low concentrations to avoid the anti-apoptotic properties against the cancerous cell.

Furthermore, we approved that the functionalization of the curcumin increases its dissolution in water and biological activity, especially against hepatocellular carcinoma HepG2 cell line in low concentration with high efficacy.

Table 2. MTT test results against HepG2 cell line.

	3a	3d	3e	3m	control
Concentration (μM/ml)	0.347	0.287	0.57	0.178	0.597
	0.404	0.304	0.499	0.227	0.601
	0.468	0.379	0.593	0.188	0.581
% Cell viability	68.5±1.7	54.7±3.8	93.0±3.8	33.3±0.8	100

Orange line: Curcumin derivaives, Blue line: Control
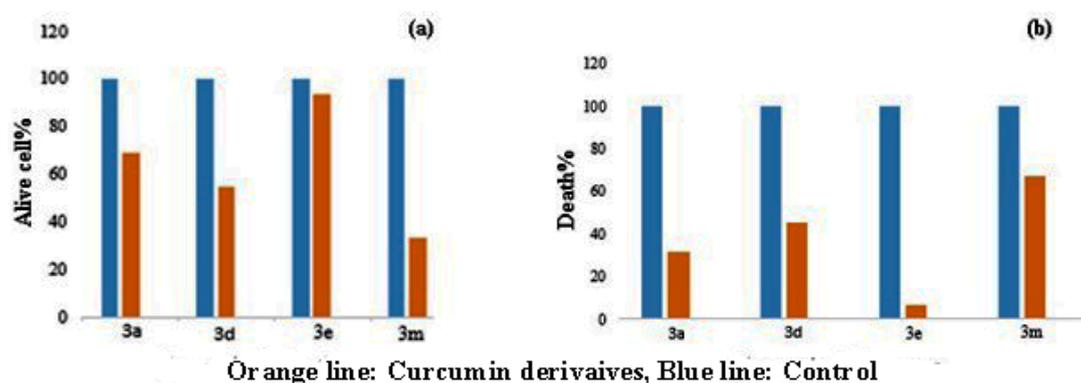
Figure 3. (a) Alive and (b) dead cell percentages of HepG2 were incubated with curcumin derivatives.

The cytotoxicity effect of some new curcumin derivatives on the HepG2 cell line was compared together in Table 3. As shown in Table 3, the novel curcumin derivatives presented in his research showed a high cytotoxicity effect in low concentration, while blank curcumin showed high cell viability.

Table 3. Cell viability of different curcumin derivatives on HepG2 cell line.

Entry	Compound	Concentration	Cell viability (%)	Reference
1	Curcumin	35 (μg/mL)	95	1
	CurcuEmulsomes	40 (μM)	55	2
3	Nanomicelle curcumin	80 (μM)	30	3
4	Curcumin on cisplatin	15.6 (μM)	40	4
5	Curcumin-doxazosin	25 (μM)	98.83	5
6	Curcumin-carvedilol	25 (μM)	115.9	5
7	Functionalized curcumin	0.188 (μM/mL)	33.3	Present work

3. Conclusion

This study presents new interesting and aqueous soluble curcumin derivatives by an efficient thermal solvent-free synthesis. Its final structure was determined using IR and NMR analysis. The toxicity test against the HepG2 cell line showed drug **3m** with the high-performance therapeutic benefits of destroying cancerous liver cells. Whereas sample **3e** is biocompatible and could be used as a drug carrier or nutritional supplement. Considering the importance of curcumin in treating disease and its low toxicity in the body, which was determined by the MTT test, as well as the medicinal properties of nitrogen heterocycles, biological studies such as anti-cancer properties are in progress by our research team.

4. Experimental

Chemical compounds, curcumin, 2-chloroacetyl chloride, 5-amino-1*H*-tetrazole, L-arginine, 2-amino benzimidazole, 2-aminopyridine, acetamide, ethyl aniline, oleyl amine, and 4-amino-2,3-dimethyl-1-phenyl-3-pyrazole-5-on, Naphthylamine, all purchased from Merck and used without any re-

purification. The solvents were used, including ethanol, acetone, n-hexane, dimethylformamide (DMF), and dichloromethane.

The IR spectrum of the products was prepared by the FT-IR spectrophotometer (Bruker Tensor II model, Germany). The melting point of the products was prepared by the melting point macro action. Completed completion of the reaction was performed by thin-layer chromatography (TLC) (Taba Teb, made in Iran). The oven (Memert model, Germany) was used to dry the samples. The raw materials were weighed by digital scale (Mettler AE 240, Switzerland). Elemental analyzer 2400 (Perkin Elmer Inc., Waltham, MA, USA) was used for elemental analysis.

4.1. Cell culture

The *HepG2* cell line was used for cellular experiments in this study. The steps of cell culture are as follows. First, a hot water bath is prepared at a temperature of 37°C. The vial containing the cells was removed from the water bath and immediately transferred to the bottom of the hood. Next, one mL of high glucose DMEM culture medium was supplemented with 10% fetal bovine serum (FBS) (GIBCO®EU 10270) was added and pipetted.

Cells and culture medium were centrifuged at 12,000 g for 5 min and slowly removed from the medium. This removed the DMSO from the center of the cell flask. Next, the resulting cell precipitate was suspended using 500 μL of DMEM culture medium. Finally, the prepared suspension was transferred to a culture flask containing a DMEM culture medium with a glucose concentration greater than 1%, penicillin, and streptomycin antibiotics and placed in a CO_2 incubator at 37°C and 95% humidity.

4.2. Cytotoxicity Test

The cytotoxic effects of the nanocarrier on HepG2 cells were examined using the MTT assay. The MTT method was first performed according to the protocol described by Mosmann. The assay was optimized for the cell line used in these experiments. Briefly, HepG2 cells (1x104) in 100 mL of medium alone or a formulation containing medium (1000 ppm). 96 wells were incubated for 24 hours at 37°C in a humid atmosphere with 5% CO_2. Then MTT (15 L, 4 mg/mL) was added to each well. After incubating the plate for more than 4 hours, DMSO (100 μl, 0.520 mmol) was added to each well to dissolve the pharmaceutical dye. Other cells were left untreated as a negative control. After 2 hours, the absorbance of the control and drug treatment wells was measured with an ELISA reader (Biotech, USA) at a wavelength of 570 nm.

4.3. Synthesis of new curcumin derivatives

2-Chloroacetyl chloride (2 mmol, 0.16 mL) and different NH compounds (2 mmol) were reacted with 1:1 eq in a round bottom flask at solvent-free and room temperature. After forming the intermediate, curcumin (1 mmol, 0.37 g) and a catalytic amount of K_2CO_3 (0.1 g) were stirred to the reaction mixture at 120°C.

Acetone and CH_2Cl_2 (2 mL, 1:1) were also used as solvents to homogenize the environment. TLC also carried out the process of reaction completion. After completion of the reaction, the crude product was purified by EtOH. The products were obtained in good to excellent yield of 66-94% (Table 1). The structure of the isolated products was determined by ^1H and 13CNMR spectroscopy and presented as follows:

Compound 3a: Color: brown;

(FT-IR, KBr, ν/cm^{-1}): 1027, 1279 (C-O stretch), 1515 (C=C stretch Ar), 1569 (C=O ketone), 1660 (C=O amid), 3107 (N-H stretch amid); 1HNMR (500.133 MHz, DMSO) δ: 3.8 (s, 6H, 2-OCH$_3$), 4.3 (s, 4H, 2-OCH$_2$), 6.6-7.6 (6H, 6C-H$_{Ar}$), 7.1 (2H, d, $^3J_{H-H}$ 2.5 Hz, C-H), 7.2 (2H, d, $^3J_{H-H}$ 2.5 Hz, C-H), 8.2 (H, N-H), 10.3 (H, N-H) ppm; Anal. calcd.: C, 51.47; H, 4.50; N, 26.11%; found: C, 51.51; H, 4.52; N, 26.13%.

Compound 3d: Color: dark brown; (FT-IR, KBr, ν/cm^{-1}): 1029, 1269 (C-O stretch), 1513 (C=C stretch$_{Ar}$), 1587 (C=O), 2938 (CH), 3500 (N-H stretch amid); 1HNMR (400.22 MHz, DMSO), δ: 3.4 (14H, -OCH$_2$, -OCH$_3$, -CH$_2$),7.2-7.8 (18H, 14Ar-H, 4 C-H) ppm; Anal. calcd.: C, 52.36; H, 10.47; N, 4.03%; found: C, 52.40; H, 10.45; N, 4.06%.

Compound 3e: Color: brown,

(FT-IR, KBr, ν/cm^{-1}): 1199, 1267 (C-O stretch), 1515 (C=C stretch$_{Ar}$), 1644 (C=O amid), 1760 (C=O ketone), 3082 (NH stretch amid), 2943 (CH); 1HNMR (400.223 MHz, DMSO) δ: 3.6 (s, 6H, 2-OCH$_3$), 3.9 (s, 4H, 2-OCH$_2$), 4.2 (s, 2H, -CH$_2$-), 7.07 (2H, d, $^3J_{H-H}$, 6 Hz, C-H), 7.9 (2H, d, $^3J_{H-H}$ 2 Hz, C-H), 6.6-7.9 (14H, 14-C-H$_{Ar}$,), 9.7 (H, N-H) ppm;13CNMR (100.05 MHz, DMSO), δ: 31.19 (CH$_2$), 42.02 (OCH$_3$), 55.65 (OCH$_2$), 111.1-154.6 (22C-H$_{Ar}$, 4C-H), 169 (C=O) ppm; Anal. calcd.: C, 54.68; H, 4.15; N, 7.73%; found: C, 54.69; H, 4.35; N, 7.69%.

Compound 3f: Color: dark brown,

(FT-IR, KBr, ν/cm^{-1}): 1032, 1267 (C-O stretch), 1512 (C=C stretch Ar), 1594 (C=O amid), 1659 (C=O ketone), 3417 (N-H stretch amid); 1HNMR (400.22 MHz, DMSO) δ: 2.9 (s, 6H, -CH$_3$), 3.07 (s, 6H, 2-OCH$_3$), 3.1 (s, 4H, -CH$_2$-), 3.7 (S, 4H, 2-OCH$_2$), 6.7 (2H, d, $^3J_{H-H}$ 4 Hz, C-H), 7.4 (2H, d, $^3J_{H-H}$, 4 Hz, C-H), 6.7-7.5 (6H, 6CH$_{Ar}$), 7.9 (H, N-H) ppm; 13CNMR (100.05 MHz, DMSO), δ: 29 (2CH$_3$), 31.25 (2OCH$_3$), 36.02 (2 OCH$_2$), 56.01 (CH$_2$-), 112.5-162.83 (12 CH$_{Ar}$, 4CH) ppm; Anal. calcd.: C, 45.86; H, 4.28; N, 4.28%; found: C, 45.60; H, 4.38; N, 4.28%.

Compound 3h: Color: brown,

(FT-IR, KBr, ν/cm^{-1}): 1032, 1271 (C-O stretch), 1448 (C=C stretch Ar), 1510 (C=O ketone), 1600 (C=O amide), 3183 (N-H stretch amid);

1HNMR (400.22 MHz, DMSO), δ: 1.1 (6H, t, CH$_3$), 2.1 (4H, t, CH$_2$), 3.4 (s, 6H, 2-OCH$_3$), 3.6-3.7 (s, 8H, -OCH$_2$, -CH$_2$), 6.7-7.3 (16H, Ar-H, 4CH), 8.8(2H, 2N-H) ppm;

13CNMR (100.05 MHz, DMSO) δ: 15.8 (2CH$_3$), 28.17 (2CH$_2$), 56.01 (OCH$_3$), 115.9-148.17 (24CH$_{Ar}$, 4CH) ppm;

Anal. calcd.: C, 57.87; H, 3.78; N, 3.65%; found: C, 57.91; H, 3.71; N, 3.74%.

Compound 3i: Color: dark brown,

(FT-IR, KBr, ν/cm^{-1}): 971, 1264 (C-O stretch), 1460 (C=C stretch Ar), 1663 (C=O ketone), 1732 (C=O amid), 3634 (N-H stretch amid);

1HNMR (500.133 MHz, DMSO), δ: 0.87 (6H, 2CH$_3$), 1.26-2.00 (28H, 14CH$_2$), 3.91 (s, 6H, 2OCH$_3$), 5.34 (4H, OCH$_2$), 6.8-7.2 (10H, CH$_{Ar}$, C-H) ppm;

Anal. calcd.: C, 64.47; H, 8.74; N, 2.55%; found: C, 64.41; H, 8.71; N, 2.61%.

Compound 3m: Color: brown;

(FT-IR, KBr, ν/cm^{-1}): 1036, 1282 (C-O stretch), 1375 (C=C stretch Ar), 1595 (C=O amid), 2930 (C-H), 3100 (N-H amid stretch);

1HNMR (400.22 MHz, DMSO), δ: 2.1 (-CH$_3$), 3.1 (-CH$_3$), 3.2 (s, 6H, OCH$_3$), 3.3 (s, 4H, -CH$_{2-}$), 3.4 (s, 4H, -OCH$_2$), 6.5-7.5 (20H, C-H $_{Ar}$, C-H) ppm;

13CNMR (100.05 MHz, DMSO) δ: 11.06 (CH$_3$), 35 (2CH$_3$), 55.96 (CH$_2$), 56.53 (2 OCH$_3$), 64.69 (2OCH$_2$), 146-160 (24C-H$_{Ar}$, 8C-H), 169 (C=O) ppm;

Anal. calcd.: C, 57.56; H, 5.12; N, 2.98%; found: C, 56.91; H, 5.16; N, 3.14%.

Compound 3n: Color: brown;

(FT-IR, KBr, ν/cm^{-1}): 1122, 1214 (C-O stretch), 1366 (C=C stretch Ar), 1667(C=O ketone), 1743 (C=O amid), 3166 (N-H amid stretch), 3337 (OH);

1HNMR (500.130 MHz, DMSO), δ: 1.5 (4H, m, -CH$_2$), 1.6 (4H, t, $^3J_{H-H}$ 4 Hz, C-H), 1.7 (4H, t, -CH$_2$) 3.8 (6H, s, -OCH$_3$), 3.9 (2H, t, -CH$_2$), 4.1 (4H, s, -CH$_{2-}$), 4.1 (4H, s, -OCH$_2$), 7.2-7.5 (10H, 6H$_{Ar}$, 4C-H), 8 (H, N-H) ppm;

Anal. calcd.: C, 59.84; H, 4.38; N, 3.41%; found: C, 59.83; H4.39; N, 3.62%.

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