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Synthesis, Structural Characterization and Biological Evaluation of 3-Amino-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid Derivatives

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1,4-Naphthoquinones are exceptional building blocks in organic synthesis and have been used to synthesize several well-known pharmaceutically active agents. Compounds containing oxygen, nitrogen or sulfur atoms inside the rings are attracting much attention and interest due to their biological importance. A series of 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid derivatives were synthesized by the Michael addition of 2,3-dichloronaphthalene-1,4-dione and 3,5-diaminobenzoic acid. All the synthesized compounds are screened for their bioactivity through molecular docking, cytotoxicity (against HeLa) and antioxidant activity. DPPH and ABTS evaluation procedures are employed to assess the antioxidant activity. Among the synthesized 3-amino-5-(3-chloro-1, 4-dioxo-1, 4-dihydronaphthalen-2-ylamino)benzoic acid derivatives (**1**, **2**, **3a-g**), compound **3d** exhibited the highest inhibition of 75 % and 83 % in the DPPH and ABTS antioxidant activity evaluation, respectively. Compound **3d** exhibited better glide energy and E model scores when docked with HDAC8 using GLIDE program. Cytotoxicity of the synthesized compounds was studied against cervical cancer cell line (HeLa) and compound **3d** showed the maximum inhibition and displayed a better activity than the standard drug.

Keywords: Michael addition, 2,3-Dichloronaphthalene-1,4-dione, Molecular docking, Cytotoxicity, Antioxidant activity.

INTRODUCTION

The essential and testing region of contemporary synthetic organic chemistry is to integrate heterocyclic mixes under ecologically amiable conditions. Oxygen, nitrogen and sulfur containing Heterocycles have been distinguished as powerful particles in numerous biological properties [1,2]. Tandem and multicomponent reactions are regularly used to synthesize Heterocycles [3,4]. To encourage the reaction plan and further expand the product diversity, organic molecules that contain various reactive centers were frequently employed [5]. Therefore, the rational structure and utilizations of bifunctional building squares are of extraordinary enthusiasm for organic synthesis [6,7]. Generally, there are loads of research exercises to grow new medications. In this situation, it is discovered that molecules with quinone moiety at the center display promising biological action because of their redox possibilities to create semiquinone radicals by bio-reduction and afterward this framework quickens intracellular hypoxic conditions in the cells [8]. 1,4-Naphthoquinone and its derivatives have pulled in expanding acknowledgment for their biological exercises [9]. The natural significance of these classes of quinones has prompted the advancement of new medications which has a core of 1,4-naphthoquinone moiety. An assortment of studies have been completed on these medication moieties, such as cytotoxic [10], antiviral [11], molluscidal [12], anti-inflammatory, antiplatelet, antiallergic [13], antimalarial [14], antileishmanial [15], antibacterial, antifungal [16,17] and antiproliferative studies [18].

An advancement has been made in malignant growth avoidance and its treatment but still the improvement of viable treatment remains challenging. One methodology with gigantic potential is chemoprevention, which is characterized as the utilization of regular, manufactured or natural specialists to invert, stifle or avert either the underlying periods of carcinogenesis or the movement of premalignant cells [19]. Due to the constraint of medical procedure, radiotherapy is used to

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fix malignant growth and chemotherapy has turned out to be progressively vital treatment process. Chemotherapy is the utilization of any medication to treat any sickness. Antioxidant and molecular docking studies are critical strategies to find new anticancer molecules. Antioxidants are atoms, characteristic or manufactured, equipped for collaborating with free radicals and ceasing their chain responses before fundamental indispensable particles are damaged [20]. The compounds having antioxidant and free radical scavenging properties are considered for use for the counteractive action or treatment of human diseases [21]. A few illnesses, for example, Alzheimer and Parkinson can be advanced by free radicals [22]. Antioxidants go about as a noteworthy protection against radical intervened poisonous quality by trapping the free radicals [23]. Molecular docking is an alluring framework to comprehend sedate bimolecular communications for the same medication plan and discovery [24]. In this article, synthesized compounds were docked with HDAC8 and SAHA was used as a standard. HDAC action is perpetually expanded in disease cells and there is a need to blend novel class of HDAC inhibitors. Suberoyl anilide hydroxamic acid (SAHA) is the medication which has been of late approved clinically and affirmed by FDA for the treatment of cutaneous T cell lymphoma [25]. Methyl-Gene′s isotype explicit HDAC inhibitor MGCD0103 is as of now clinically used for examining strong tumors and hematological malignancies [26]. The majority of HDAC inhibitors have three basic features, namely, metal restricting moiety, a carbon linker and a capping group. In HDAC inhibitors, capping group is dissolvable uncovered and associates with amino acids close to the passage of dynamic site, metal restricting moiety ties in the protein inside and edifices the metal particle engaged with catalysis. The linker helps for high- affinity interactions with proteins [27].

Heterocyclic amines functionalized 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid derivatives are synthesized. All the synthesized compounds were thoroughly analyzed and their structures are confirmed using FTIR, NMR (^{1}H and ^{13}C) and mass spectroscopy techniques. For all the synthesized compounds, molecular docking, antioxidant and cytotoxicity studies were carried out to evaluate their bioactivity.

EXPERIMENTAL

All the chemicals and reagents employed for the synthesis of 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid derivatives were purchased from Sigma-Aldrich. All the reagents and solvents were obtained from Aldrich and used without any further purification. Doxorubicin was purchased from Pfizer Pharma, India. ABTS was purchased from Nice Chemicals Ltd, India. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker FT-500 using tetramethylsilane (TMS) as an internal standard. The IR spectra were recorded on a Shimadzu FTIR spectrophotometer using KBr (4000-400 cm^{-1}). The compounds were purified by column chromatography using silica gel (100-200 mesh) and petroleum ether-ethyl acetate. TLC was performed using silica gel 60 F_{254} pre-coated on aluminum sheets, obtained from Merck. Visualization of spots on TLC plate was done with UV light (254 nm). *in vitro* Cytotoxicity of all the compounds was studied by cell viability assay method. Molecular docking studies of all the synthesized compounds were studied by GLIDE program (version 8.5, Schrodinger, LLC, New York, 2010).

Synthesis of 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid (1) : To a solution of 2,3-dichloronaphthalene-1,4-dione (2.27 g, 10 mmol) in ethanol (50 mL) at room temperature, 3,5-diaminobenzoic acid (1.52 g, 10 mmol) was added and the reaction mixture was stirred for 4 h. After completion of the reaction as indicated by the TLC, the reaction was stopped and purified by column chromatography using petroleum ether:ethyl acetate (8:2). Red solid; yield: 2.53 g, 74 %); IR (KBr, ν_{max}, cm^{-1}): 3728, 3269 (-NH), 2950 (OH), 1632 (C=O), 1572, 1512 (C=C), 810 (C-Cl); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 12.8 (1H, -NH), 9.3 (1H, -OH), 7.73-8.21 (4H, Ar-quinone), 6.51-7.33 (3H, Ar-benzene), 4.05 (2H, -NH_2); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 180.8, 177.4, 168.2 (3C, C=O), 149.3, 143.8, 139.3 (3C, -C-NH), 135.2, 133.6, 132.3, 114.7 (7C, Ar-quinone), 131.3, 113.7, 113.3, 111.7 (4C, Ar-benzene); MS (EI): *m/z* [M+H]$^+$: calcd. for $C_{17}H_{11}N_2O_4Cl$: 342.733; found:342.734.

Synthesis of 3-amino-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid (2): Compound **1** (3.42 g, 10 mmol) was added in 2-aminophenol (1.09 g, 10 mmol) in 95 % of ethyl alcohol (50 mL). A catalytic amount of anhydrous K_2CO_3 was added to the reaction mixture and it was refluxed for 4 h. After completion of the reaction as indicated by the TLC, the reaction was cooled at room temperature and poured into ice-water. The precipitate was filtered, dried and purified by column chromatography using petroleum ether:ethyl acetate (7:3). Reddish orange solid; yield: 2.69 g, 68 %; IR (KBr, ν_{max}, cm^{-1}): 3728, 3269 (-NH), 2950 (OH), 2200 (C=N), 1632 (C=O), 1572, 1512 (C=C), C-O (1300); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 12.8 (1H, -NH), 9.3 (1H, -OH), 7.70-8.20 (4H, Ar-quinone), 5.91-7.33 (7H, Ar-benzene), 4.05 (2H, -NH_2); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 183.6, 167.7 (2C, C=O), 145.1 (1C, C=N), 144.3, 143.6 (2C, -C-NH), 138.3 (1C, C-N), 138.2 (1C, C-O), 136.3, 135.7, 135.3, 134.8, 133.9, 133.4, 133.0, 132.7 (8C, Ar-quinone), 131.2, 130.8, 130.1, 129.0, 128.9, 128.8, 128.7 (8C, Ar-benzene); MS (EI): *m/z* [M+H]$^+$: calcd for $C_{23}H_{15}N_3O_4$: 397.382; found:397.384.

Synthesis of 3-amino-5-(5-oxo-5H-benzo[*a*]phenoxazin-6-ylamino)benzoic acid derivatives (3a-g): Acyl chlorides (1 mmol) were added to the solution of compound **2** (0.397 g, 1 mmol) in acetone (20 mL) and the reaction mixture was refluxed for 2h. After completion of the reaction as indicated by TLC, the reaction was stopped and purified by column chromatography using petroleum ether:ethyl acetate (7:3).

3-Acetamido-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid (3a): Reddish orange solid; yield: 0.280 g, 64 %); IR (KBr, ν_{max}, cm^{-1}): 3730, 3270 (-NH), 2955 (OH), 2210 (C=N), 1635 (C=O), 1575, 1518 (C=C), 1310 (C-O); ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm): 11.0 (1H, -NH), 9.3 (1H, -OH), 8.6 (1H, -NH), 7.72-8.25 (4H, Ar-quinone), 5.94-7.35 (7H, Ar-benzene), 2.01 (3H, -CH_3); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 183.2, 169.2, 167.8 (3C, C=O), 148.3 (1C, C=N), 144.2, 142.2 (2C, -C-NH), 138.2 (1C, C-N), 134.8 (1C, C-O), 132.8, 132.2, 130.3, 129.2, 127.8, 126.1, 125.5, 125.2

(8C, Ar-quinone), 122.6, 112.9, 112.3, 112.2, 104.8, 104.2 (8C, Ar-benzene), 26.9 (1C, $-CH_3$); MS (EI): *m/z* $[M+H]^+$: calcd for $C_{25}H_{17}N_3O_5$: 439.419; found:439.425.

3-Benzamido-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid (3b): Reddish orange solid; yield: 0.340 g, 68 %); IR (KBr, ν_{max}, cm^{-1}): 3735, 3280 (-NH), 2965 (OH), 2230 (C=N), 1650 (C=O), 1590, 1530 (C=C), 1340 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 10.6 (1H, -NH), 9.3 (1H, -OH), 8.8 (1H, -NH), 7.94-8.10 (3H, Ar-benzene), 7.72-7.85 (4H, Ar-quinone), 6.84-7.45 (9H, Ar-benzene); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 180.7, 170.3, 166.2 (3C, C=O), 147.8 (1C, C=N), 143.9, 140.7 (2C, -C-NH), 138.3 (1C, C-N), 134.2 (1C, C-O), 131.3, 131.2, 130.3, 129.2, 127.8, 126.1, 125.3, 125.2 (8C, Ar-quinone), 121.6, 111.9, 111.3, 111.2, 109.8, 100.3, 100.2, 100.0 (14C, Ar-benzene); MS (EI): *m/z* $[M+H]^+$: calcd for $C_{30}H_{19}N_3O_5$: 501.488; found: 501.496.

3-(2-Chlorobenzamido)-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid (3c): Reddish orange solid; (0.347 g, 65%); IR (KBr, ν_{max}, cm^{-1}): 3738, 3380 (-NH), 2985 (OH), 2231 (C=N), 1654 (C=O), 1595, 1530 (C=C), 1347 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 9.8 (1H, -NH), 9.2 (1H, -OH), 8.7 (1H, -NH), 7.84-8.10 (2H, Ar-benzene), 7.72-7.75 (4H, Ar-quinone), 6.84-7.45 (9H, Ar-benzene); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 179.8, 167.8, 166.1 (3C, C=O), 146.3 (1C, C=N), 145.2, 143.8 (2C, -C-NH), 139.3 (1C, C-N), 135.2 (1C, C-O), 132.3, 131.2, 131.3, 129.2, 127.8, 126.1, 125.3, 125.2 (8C, Ar-quinone), 122.6, 112.9, 112.3, 112.2, 109.8, 101.3, 101.2, 101.0 (14C, Ar-benzene); MS (EI): *m/z* $[M+H]^+$: calcd for $C_{30}H_{18}N_3O_5Cl$: 535.934; found:535.945.

3-(2-Nitrobenzamido)-5-(5-oxo-5*H*-benzo[a]phenoxazin-6-ylamino)benzoic acid (3d): Yellow solid; (0.387 g, 71 %); IR (KBr, ν_{max}, cm^{-1}): 3739, 3310 (-NH), 2955 (OH), 2241 (C=N), 1664 (C=O), 1515, 1540 (C=C), 1357 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 9.9 (1H, -NH), 9.4 (1H, -OH), 9.1 (1H, -NH), 8.24-8.30 (2H, Ar-benzene), 8.02-8.15 (4H, Ar-quinone), 7.64-7.95 (9H, Ar-benzene); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 185.1, 165.1, 163.1 (3C, C=O), 148.4 (1C, C=N), 148.0 (1C, $C-NO_2$), 146.4, 145.2 (2C, -C-NH), 145.1 (1C, C-N), 143.4 (1C, C-O), 133.4, 132.8, 132.7, 132.1, 131.0, 129.0, 128.4, 127.8 (8C, Ar-quinone), 126.8, 126.3, 126.1, 124.6, 123.5, 122.1 119.0, 118.1 (13C, Ar-benzene); MS (EI): *m/z* $[M+H]^+$: calcd. for $C_{30}H_{18}N_4O_7$: 546.486; found: 546.574.

3-(3,4-Dimethylbenzamido)-5-(5-oxo-5*H*-benzo[*a*]-phenoxazin-6-ylamino)benzoic acid (3e): Orange solid; yield: 0.333 g, 63 %; IR (KBr, ν_{max}, cm^{-1}): 3743, 3330 (-NH), 2975 (OH), 2251 (C=N), 1665 (C=O), 1535, 1520 (C=C), 1357 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 10.3 (1H, -NH), 9.3 (1H, -OH), 8.7 (1H, -NH), 8.04-8.10 (1H, Ar-benzene), 7.82-8.02 (4H, Ar-quinone), 6.84-7.75 (9H, Ar-benzene) 2.30 (6H, $-CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 181.6, 165.2, 161.8 (3C, C=O), 148.0 (1C, C=N), 144.6, 143.2 (2C, -C-NH), 142.8, 137.8 (2C, $C-CH_3$), 137.2 (1C, C-N), 136.0 (1C, C-O), 134.8, 133.9, 133.1, 130.3, 129.6, 129.4, 128.1, 126.1 (8C, Ar-quinone), 125.2, 122.6, 121.4, 120.7, 120.5, 117.2, 117.0, 115.8 (12C, Ar-benzene), 18.65 (2C, $-CH_3$); MS (EI): *m/z* $[M+H]^+$: calcd. for $C_{32}H_{23}N_3O_5$: 529.542; found:529.551.

3-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)-5-(3,4,5-trimethylbenzamido)benzoic acid (3f): Yellow solid; (0.331 g, 61 %); IR (KBr, ν_{max}, cm^{-1}): 3745, 3334 (-NH), 2985 (OH), 2261 (C=N), 1663 (C=O), 1538, 1540 (C=C), 1367 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 9.7 (1H, -NH), 9.3 (1H, -OH), 8.7 (1H, -NH), 8.04-8.10 (1H, Ar-benzene), 7.82-8.02 (4H, Ar-quinone), 6.84-7.75 (8H, Ar-benzene) 2.30 (9H, $-CH_3$); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 180.8, 166.8, 162.2 (3C, C=O), 147.0 (1C, C=N), 146.6, 144.2 (2C, -C-NH), 143.8, 136.8, 136.2 (3C, $C-CH_3$), 136.0 (1C, C-N), 134.8 (1C, C-O), 132.9, 132.1, 130.3, 129.6, 129.4, 128.1, 126.1, 125.2 (8C, Ar-quinone), 122.6, 121.4, 120.4, 120.1, 118.2, 118.0, 117.8 (11C, Ar-benzene), 21.45 (3C, $-CH_3$); MS (EI): *m/z* $[M+H]^+$: calcd. for $C_{33}H_{25}N_3O_5$: 543.568; found: 543.574.

3-(5-Oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)-5-(perfluorobenzamido)benzoic acid (3g): Yellow solid; yield: 0.384 g, 65 %); IR (KBr, ν_{max}, cm^{-1}): 3755, 3344 (-NH), 2983 (OH), 2260 (C=N), 1661 (C=O), 1536, 1541 (C=C), 1360 (C-O); ^1H NMR (500 MHz, DMSO-d_6, δ ppm): 11.0 (1H, -NH), 10.0 (1H, -OH), 8.5 (1H, -NH), 7.82-8.02 (4H, Ar-Quinone), 7.04-7.75 (7H, Ar-benzene); ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm): 183.6, 171.6, 167.7 (3C, C=O), 145.1 (1C, C=N), 144.3, 143.6 (2C, -C-NH), 138.3, 138.2, 136.3 (5C, C-F), 135.7 (1C, C-N), 135.3 (1C, C-O), 134.8, 133.9, 133.4, 133.0, 132.7, 131.2, 130.8, 130.1 (8C, Ar-quinone), 129.0, 128.9, 128.8, 128.7, 126.9, 126.7, 125.7, 125.4, 123.1 (9C, Ar-benzene); MS (EI): m/z $[M+H]^+$: calcd. for $C_{30}H_{14}N_3O_5F_5$: 591.441; found: 591.450.

***in vitro* Antioxidant activity:** The antioxidant properties of synthesized compounds were assessed by two different *in vitro* methods namely: DPPH radical scavenging activity and ABTS radical scavenging activity at different concentrations by dissolving the compounds in methanol.

DPPH radical scavenging activity: DPPH is a steady free radical with red shading (absorption at 517 nm). At the point when the free radicals are been scavenged, DPPH will produce a yellow shading. A solution of DPPH (0.1 mM, 0.000985 g) in methanol (25 mL) was prepared. A 1.0 mL of this solution was added to the sample solution (1:1) in methanol at various concentrations (0.5-5.0mm). After 30 min, the absorbance was estimated at 517 nm [28]. A blank was set up without including the sample solution. Lower the absorbance of reaction mixture, higher the free radical scavenging action. The inhibitory level of DPPH was determined according to the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$$

where, Abs (control) is the absorbance of DPPH solution and Abs (sample) is the absorbance of DPPH solution + sample (test samples). The IC_{50} values were calculated from the calibration curve.

ABTS radical scavenging activity: A solution of ABTS (7 mM, 0.0384 g) in distilled water (10 mL) was mixed with aqueous potassium persulphate (2.45 mM, 0.0066 g dissolved in 10 mL distilled water) [29]. The mixture was kept in dark at room temperature and left for overnight. Further, the mixture was diluted with methanol (20 mL) to give the absorbance 1.0 at 734 nm. Different concentrations of sample (0.5-5.0 mM) was prepared using methanol and mixed with ABTS mixture in the ratio of 9:1. This was kept in the dark for 30 min and then the absorbance was noted at 734 nm [30]. The inhibitory

percentage of ABTS was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$$

where, Abs (control) is the absorbance of ABTS mixture, and Abs (sample) is the absorbance of ABTS mixture + sample (test samples). Lower the absorbance of the reaction mixture, higher the free radical scavenging activity. The result of the radical scavenging also expressed in terms of half-inhibition concentration (IC_{50}) which denotes the concentration required to scavenge 50 % of ABTS radicals.

Molecular docking: To understand the interaction of all the synthesized molecules (**1**, **2**, **3a-g**) with HDAC8, the crystal structure of HDAC8 with SAHA [31] were downloaded from protein data bank and the molecular docking studies were performed using the GLIDE program [32]. To analyze the docking results and execute the protocol, the maestro user interface (version 8.5, Schrodinger, LLC, New York, 2010) was employed and the validation of the protocol was evaluated by redocking. SAHA (PDB ID: 1T69) is selected for docking studies as reference sample and was prepared for docking through a protein preparation wizard. Structures of synthesized molecules (**1**, **2**, **3a-g**) were sketched using ACD/chemsketch (Freeware version). The GLIDE grid generation wizard has been used to define the docking space. Docking was performed using XP (Extra Precision mode) docking protocol [33].

***in vitro* Cytotoxicity activity:** The *in vitro* cytotoxic activities of the synthesized compounds are evaluated by cell viability assay method (MTT assay) against a human cervical cancer cell line (HeLa). MTT is a quantitative colorimetric method for determining cell proliferation after treatment with the tested compounds. MTT, a tetrazolium compound (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) will reduce metabolically active cells to insoluble purple formazan dye crystals, which indicates the inhibition of cells. It is widely used to estimate the cytotoxic action of chemicals on different types of cells [34]. In this method, HeLa (cervical cancer cell line) were seeded (80 to 90 confluency) in a flat-bottomed 96-well tissue culture plates and incubated for 24 h. After incubation, the synthesized derivatives (**1**, **2**, **3a-g**) were added to each at 6, 12, 25, 55 and 85 µg/mL concentrations, respectively. The standard drug (doxorubicin) was also added as control. The test sample and standard were incubated in the 96-well tissue culture plates for 24 h and the MTT was added followed by 3 h of incubation. Detergent is then added to the wells solubilizing the crystals and the optical density values were noted spectrophotometrically at 570 nm [35,36]. The data was analyzed by plotting concentration of test samples *versus* absorbance, allowing the quantitation changes in cell proliferation. The rate of tetrazolium reduction is proportional to the rate of cell proliferation [37]. Data were collected for three replicates and the mean was calculated.

RESULTS AND DISCUSSION

In **Scheme-I**, 2,3-dichloronaphthalene-1,4-dione reacted with 3,5-diaminobenzoic acid to form 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid (**1**) in the presence of ethanol. A base free method was adopted for the synthesis of 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydro-

Ethanol, reflux 10 h

K_2CO_3, ethanol, reflux, 4 h

Acetone, reflux, 2 h

RCOCl

Where R = **3a** = Methyl
3b = Phenyl
3c = 2-Chloro phenyl
3d = 2-Nitro phenyl
3e = 3,4-Dimethyl phenyl
3f = 3,4,5-Trimethyl phenyl
3g = 2,3,4,5,6-Pentafluoro phenyl

Scheme-I: Synthesis of 3-amino-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid derivatives

naphthalen-2-ylamino)benzoic acid (**1**). Reaction optimization was carried out to select the suitable solvent and the results are presented in Table-1. It is clear that the reactions carried out in acetone medium resulted in good yields. After reaction optimization, 3-amino-5-(5-oxo-5*H*-benzo[*a*]phenoxazin-6-ylamino)benzoic acid was subjected to react with a variety of acyl chlorides (Table-2). For all the synthesized compound′s, molecular docking, antioxidant and cytotoxicity studies were carried out to evaluate their bioactivity. Structures of all the synthesized compounds (**1**, **2**, **3a-g**) have been confirmed using FT-IR, ^{1}H and ^{13}C NMR and mass spectral techniques.

***in vitro* Antioxidant activity:** Compounds (**1**, **2**, **3a-g**) was tested for *in vitro* antioxidant activity by DPPH and ABTS methods.

DPPH radical scavenging activity: All the synthesized derivatives (**1**, **2**, **3a-g**) exhibited expanded DPPH inhibitory percentage with the increase in concentration. Among all the tested antioxidants, compound **3d** exhibited the highest inhibition of 75 % and the other compounds showed inhibition in the range of 60-75 % at a concentration of 5 mm during a time duration of 90 min. The IC_{50} values of compounds (**1**, **2**, 3**a-g**) scavenging DPPH radical is presented in Table-3 and the results show that compound **3d** has better DPPH radical activity (IC_{50}: 0.67 mm) than the other synthesized compounds during a time duration of 90 min. From Table-3, it is also noticed that compounds **3c**, **3e**, **3f** and **3g** have better scavenging activity (IC_{50}) on the DPPH radical.

TABLE-1
SOLVENT SCREENING[a]

2 → (Solvent, Reflux, 2 h; 2-nitrobenzoyl chloride) → **3d**

Entry	Solvent	Yield[b] (%)
1	Dichloro methane	25
2	Chloroform	23
3	Tetrahydrofuran	42
4	Acetone	71
5	Methanol	52
6	Ethanol	50
7	Isopropyl alcohol	47
8	Toluene	40

[a]Reaction conditions: Compound **2** (1 mmol), acyl chloride (1 mmol) in solvent (20 mL) for 2 h refluxed. [b]Yield corresponding to the isolated product either by column chromatography or recrystallization.

TABLE-2
SYNTHESIS OF 3-AMINO-6,11-DIOXO-6,11-DIHYDRO-5*H*-BENZO[*b*]CARBAZOLE-1-CARBOXYLIC ACID DERIVATIVES

Starting material	Reagent	Product	Yield (%)
		1	74
		2	68
		3a	64

Reactant	Acyl chloride	Product	Yield (%)
COOH, NH, NH2, N, O	Cl, O	**3b** (COOH, N, O, NH, C=O)	68
COOH, NH, NH2, N, O	Cl, O, Cl	**3c** (COOH, N, O, NH, C=O, Cl)	65
COOH, NH, NH2, N, O	Cl, O, NO2	**3d** (COOH, N, O, NH, C=O, NO2)	71
COOH, NH, NH2, N, O	Cl, O	**3e** (COOH, N, O, NH, C=O)	63
COOH, NH, NH2, N, O	Cl, O	**3f** (COOH, N, O, NH, C=O)	61

TABLE-3
IC_{50} (mm) VALUES FOR EVALUATED ANTIOXIDANT ASSAY OF SYNTHESIZED COMPOUNDS (**1, 2, 3a-g**)

Compound	DPPH antioxidant				ABTS antioxidant			
	30 min	60 min	90 min	Mean	30 min	60 min	90 min	Mean
1	4.33	3.10	2.38	3.27	4.04	2.63	2.32	2.99
2	4.15	3.15	2.26	3.18	3.45	2.73	1.50	2.56
3a	3.17	2.43	1.71	2.43	3.18	2.57	2.00	2.58
3b	2.46	1.86	1.33	1.88	0.64	0.58	0.55	0.59
3c	2.16	1.63	0.73	1.50	0.91	0.69	0.59	0.73
3d	1.08	0.58	0.67	0.77	0.61	0.55	0.50	0.55
3e	2.76	2.29	0.84	1.96	0.86	0.68	0.53	0.69
3f	4.09	3.01	0.95	2.68	2.30	1.77	1.44	1.83
3g	2.26	0.73	0.76	1.25	0.83	0.59	0.54	0.65

ABTS radical scavenging activity: All the synthesized compounds were screened for their antioxidant potential. The antioxidant property of the tested samples was evaluated at different concentrations at 30 min difference. From the comparative bar chart (Fig. 1), it was understood that all the derivatives (**1**, **2**, **3a-g**) exhibited increased ABTS inhibitory percentage with the increase in concentration of standard antioxidants. Increase in concentration should be able to donate increased electrons to free radicals. Among all the tested compounds, compound **3d** exhibited the highest inhibition of 83 % and other compounds showed inhibition in the range of 69-82 % at a concentration of 5 mm during a time duration of 90 min. The IC_{50} values of compounds (**1**, **2**, **3a-g**) on scavenging ABTS radical is shown in Table-3 and the results showed that the compound **3d** has better ABTS radical activity (IC_{50}: 0.50 mm) than the other synthesized compounds in a time duration of 90 min. It is also observed that compounds **3b**, **3c**, **3e** and **3g** have better scavenging activity (IC_{50}) on the ABTS radical.

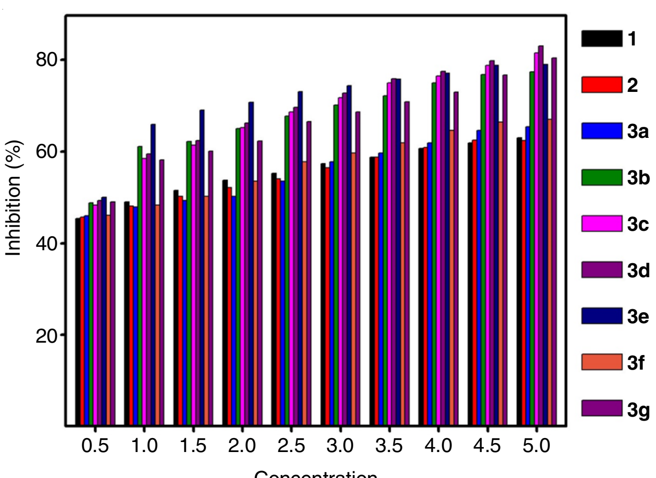

Fig. 1. ABTS inhibitory percentage of derivatives (**1, 2, 3a-g**) at different concentration in 90 min after the addition of antioxidants to the ABTS radicals

Molecular docking studies: The molecular docking studies were performed to understand the interaction of synthesized compounds **1**, **2**, **3a-g** with HDAC8 using the glide program. The crystal structures of HDAC8 with SAHA (PDB ID: 1T69) were downloaded from the protein data bank. Among the synthesized molecules docked, compound **2** showed the best glide score of -4.86. It exhibits two hydrogen bond with ASP A: 95 and ASP A: 101. All the docking results are presented in Table-4. Compound **3b** comes next with a glide score of -4.77 and it exhibited one hydrogen bond with GLY A: 200. Compound **3d** showed the best glide energy and E model score of -43.60 and -64.20, respectively and exhibited one hydrogen bond with ASP A: 95. Compound **3c** comes next with a glide energy and E model score of -42.10 and -50.41, respectively and exhibits one hydrogen bond one with ASP A: 95. Compound **3e** with a glide and an E model score of -4.31 and -55.05, respectively, exhibited one hydrogen bond with ASP A: 101.

Compound **1** had a low E model score of -17.75 and exhibited only one hydrogen bond with LYS A: 33. In all the synthesized compounds, nitro, methyl and fluoro functional groups in the aromatic carbonyl system (**3d**, **3e**, **3f** and **3g**) play a very important role in the interaction with HDAC8 and also exhibit a better E model values. The compound without any substitution in the aromatic carbonyl system also exhibited better glide scores (compound **3b**). The compound without the aromatic carbonyl system with building blocks functionalized also exhibited better glide values (compound **2**). The compound without the aromatic carbonyl system and building blocks exhibited low E model values (compound **1**).

TABLE-4
MOLECULAR DOCKING DATA OF SYNTHESIZED COMPOUNDS **1, 2, 3a-g** AGAINST HDAC8 PROTEIN RECEPTOR

Compounds	Molecular docking				
	Glide score (kcal/mol)	Glide energy (kcal/mol)	E model score	XP H bond (Å)	Number of hydrogen bonds interactions
1	-4.099	-20.937	-17.752	-0.49	1 (LYS A: 33)
2	-4.862	-33.834	-36.641	-0.696	2 (ASP A: 95, ASP A:101)
3a	-4.547	-34.030	-46.013	-0.494	1 (GLY A: 200)
3b	-4.778	-38.849	-54.473	-0.196	1 (GLY A: 200)
3c	-3.772	-42.105	-50.417	-0.207	1 (ASP A: 95)
3d	-3.368	-43.607	-64.203	-0.281	1 (ASP A: 95)
3e	-4.312	-38.903	-55.051	0	1 (ASP A: 101)
3f	-3.915	-36.976	-53.285	-0.354	1 (ASP A: 95)
3g	-3.762	-39.737	-52.985	-0.350	1 (ASP A: 95)
SAHA	-8.855	-81.152	-106.961	-0.165	1 (PHE 208)

***in vitro* Cytotoxicity activity:** Compounds (**1, 2, 3a-g**) were screened for *in vitro* cytotoxicity against human cervical cancer cell line (HeLa) using the MTT assay. Doxorubicin was used as the standard drug in this assay. All the synthesized compounds (**1**, **2**, **3a-g**) exhibited inhibition (cytotoxicity) activity on HeLa cells. At a concentration of 6 µg/mL, no cytotoxic effect was observed when tested against the cells (cells survical were more than 90 %); but at a concentration of 85 µg/mL the compounds were effective on Hela cells. Compound **3d** showed the maximum inhibition 91 % and its viability was 9 % as shown in Fig. 2. Thus, nitro group in aromatic carbonyl system shows higher antitumor activity than the other compounds. Doxorubicin exhibited an inhibition of 84 % and viability of 16 % at the same concentration of 85 µg/mL. The IC_{50} value of compound **3d** shows better results (IC_{50}: 24.1 µg/mL) than the other compounds. Compounds **3g** (IC_{50}: 25.0 µg/mL) and **3e** (IC_{50}: 25.9 µg/mL) also showed better IC_{50} values than the standard drug doxorubicin (IC_{50}: 26.4 µg/mL) as seen in Table-5. Biological activities of the compounds depend on the basic skeleton of the molecule as well as on the nature of substituents. Compound **3d** was observed to be the most active of all the tested compounds. The cytotoxicity of the compounds increased when the compound got acylated. From these results, the building blocks functionalized and acylated compounds have better activity than the parent compound.

TABLE-5
HALF-INHIBITION CONCENTRATION (IC_{50}) OF COMPOUNDS (**1, 2, 3a-g**) ON HELA CELLS

Compound	IC_{50} (µg/mL)	Compound	IC_{50} (µg/mL)
1	42.7	**3d**	24.1
2	40.8	**3e**	25.9
3a	33.6	**3f**	32.6
3b	27.6	**3g**	25.0
3c	34.9	Standard drug	26.4

Conclusion

A series of 3-amino-5-(3-chloro-1,4-dioxo-1,4-dihydronaphthalen-2-ylamino)benzoic acid derivatives have been synthesized in the base free conditions with appreciable yields. The synthesized compounds were screened for their *in vitro* antioxidant activity employing the DPPH and ABTS methods. Compound **3d** showed high inhibition efficiency of 75 % and 83 % in DPPH and ABTS antioxidant activity, respectively. Molecular docking of all the synthesized compounds was studied. Among the studied compounds, compound **3d** showed best glide energy and E model score of -43.60 and -64.20, respectively and exhibited one hydrogen bond with ASP A: 95. The entire set of compounds was also evaluated for their *in vitro* cytotoxicity against human cervical cancer cell line (HeLa). All the synthesized compounds exhibited inhibition (cytotoxicity) against HeLa cells and compound **3d** exhibited the maximum inhibition and its viability was 9 %.

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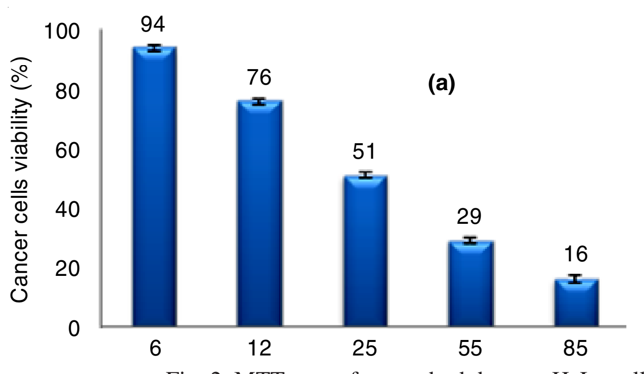
CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.



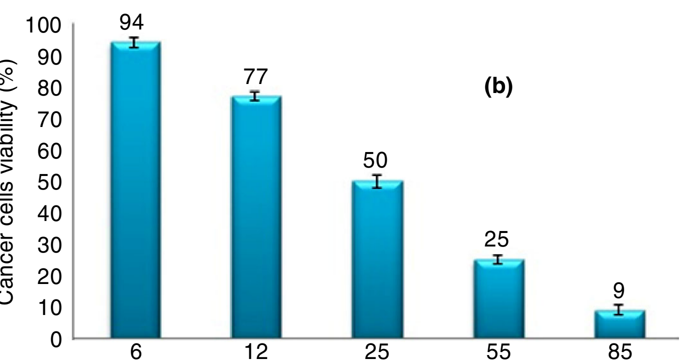


Fig. 2. MTT assay for standard drug on HeLa cell line and MTT assay for compound **3d** on HeLa cell line

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