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Synthesis of Some Indolyl Derivatives under Solvent Free Conditions, Their Cytotoxicity, and DNA Cleavage Studies

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Abstract—A one-pot, rapid, facile, green, and efficient method of synthesis of pyran fused indolyl and 1,3-dicarbonyl analogs has been carried out under the conventional and also solvent-free conditions involving MW irradiation. The structures of products have been confirmed by spectral data. All products have been tested for DNA cleavage and in vitro cytotoxicity against three tumor cell lines. Some products are characterized by high activity.

Keywords: indole, pyran, dimedone, thiobarbutiric and barbutiric acid, MW-assisted, green synthesis, anticancer activity, DNA cleavage

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INTRODUCTION

In antitumor therapy DNA cleavage agents are used as efficient drugs [1–3]. Among such compounds indolyl derivatives exhibited antitumor activity due to topoisomerase I- and II-mediated DNA cleavage [4]. Also indolyl analogs are used in medicinal chemistry as anticancer [5, 6], antioxidant [6–9], antirheumatoidal and anti-HIV [10, 11] agents. Fused pyrans demonstrate some pharmacological and biological properties, such as fungicidal, insecticidal [12], antiviral and antileishmanial [13, 14], and they inhibit non-peptide HIV protease [15–17].

As a development of our on-going research in green chemistry of bioactive indoles [6–8, 18–23], herein we report a rapid and clean routes to the synthesis of pyran fused indolyl analogs by conventional and MW-assisted (with and without a catalyst) method under solvent-free conditions (Scheme 1). The synthesized compounds were evaluated as potentially cytotoxic and DNA cleavage agents (Scheme 2).

RESULTS AND DISCUSSION

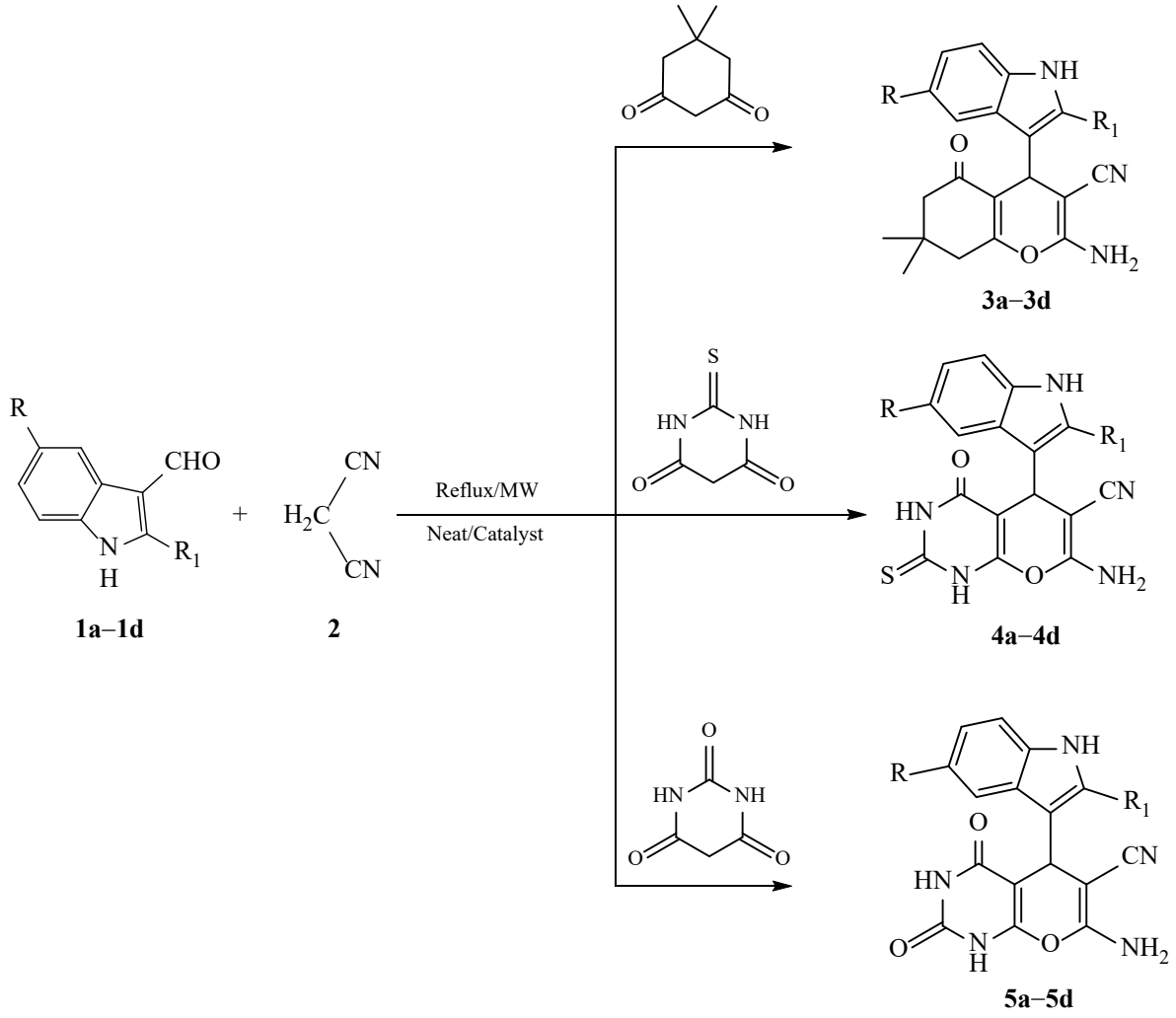
The conventional heating of equivalent amounts of a 2,5-disubstituted-1*H*-indole-3-carboxaldehyde **1a**–**1d**, malononitrile **2** and either dimedone, thiobarbutiric acid or barbutiric acid gave low yields (35–45%) of the corresponding products. The obvious disadvantages of the that approach were overcome by MW-assisted synthesis under solvent-free conditions. The products were isolated in 80–94% yield with high purity (Table 1). The process carried out without a solvent support or catalyst was lengthy and led to low yield of products, sometimes no reaction occurred. In the following experiments potassium sodium tartrate tetrahydrate (Rochelle salt = $KNaC_4H_4O_6{\cdot}4H_2O$) (10 mol %) as a catalyst under solvent-free conditions was used. Structures of the synthesized compounds were supported by IR, ^1H, and ^{13}C NMR, and mass spectral data.

In vitro anticancer activity. Evaluation of antitumor cytotoxicity of the synthesized compounds was tested against three tumor cell lines, A-549 (*Lung carcinoma*), HEp-2 (*Laryngeal carcinoma*) and HeLa (*Cervical carcinoma*) by the MTT assay [6] using Doxorubicin as a positive reference (Table 2, Fig. 1). Among the synthesized compounds **4a**, **4b**, and **5a** demonstrated higher activity against all cell lines. The products **3a**, **3b**, and **5b** were characterized by moderate cytotoxicity. The results clearly exhibited that indole analogs with Cl and CH_3 substituents at the position C_5 of the indolyl system containing dimedone, thiobarbituric and barbituric acids **4a**, **4b**, **5a**, and **5b**) were the most cytotoxic against all three cell lines.

The IC_{50} values were calculated by non-linear regression analysis data accumulated by three independent experiments as reported [6].

DNA cleavage activity. Gel electrophoresis method has been used for DNA cleavage studies [22]. Upon electrophoresis of the tested compounds with DNA tailing in the bands was recorded unlike the control DNA which demonstrated no cleavage. Compounds **3a**–**3c** and

Scheme 1. Synthesis of some indolyl analogs.



R = Cl, R_1 = Ph (**a**), R = CH_3, R_1 = Ph (**b**), R = H, R_1 = Ph (**c**), R = R_1 = H (**d**).

5b demonstrated the most intensive streaks. The results indicated the significance of indolyl analogs in DNA cleavage and their inhibiting activity in growth of the pathogenic organisms by cleaving the genome (Fig. 2).

EXPERIMENTAL

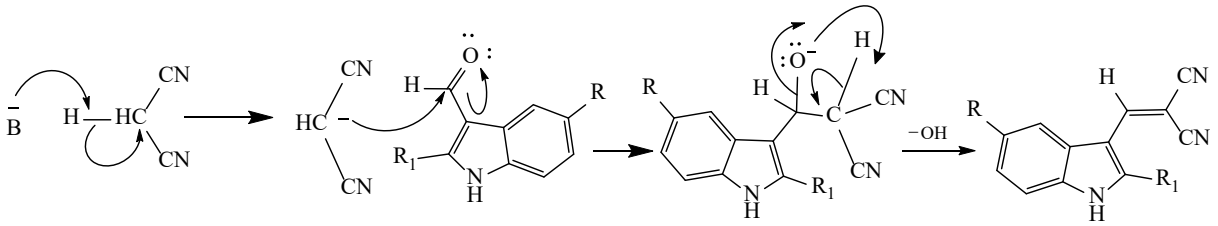
All chemicals used were purchased from Merck, Himedia and SD fine chemicals and used as received. Reaction progress was monitored by TLC (Merck Silica gel 60 F245 plates). The spots were visualized under UV light at 254 nm. Melting points were determined in open capillary tubes and are uncorrected. IR spectra were recorded on a Perkin Elmer FT-IR spectrophotometer for KBr discs. ^{1}H and ^{13}C NMR spectra were measured on a Bruker 400 MHz spectrometer in DMSO-d_6 using TMS as an internal standard. LCMS spectra were measured on a SHIMADZU, LCMS 2O1OA, mass spectrometer. MW-irradiation reactions were carried out in an ONIDA 20STP21 800W multimode microwave oven.

Synthesis of indolyl analogs. *Conventional method.* The mixture of equivalent amounts (1 mmol each) of 2,5-disubstituted indole-3-carboxaldehyde with malononitrile, dimedone/thiobarbutiric acid or barbutiric acid in ethanol (15 mL) was refluxed upon monitoring by TLC. Upon completion the reaction, the mixture was cooled down to room temperature and poured onto crushed ice upon constant stirring. A crude product was isolated and recrystallized from ethanol to yield the corresponding compounds (Table 1).

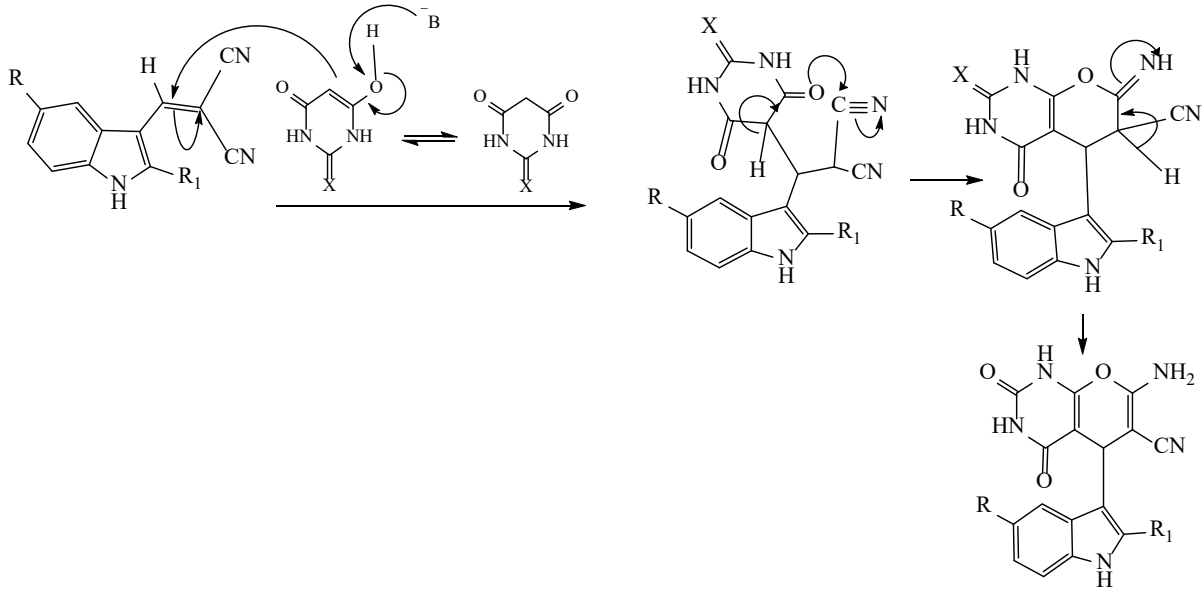
MW-assisted synthesis. (1) Neat reaction. A mixture of equivalent amounts of 2,5-disubstituted indole-3-carbox-

Scheme 2. Proposed mechanism for the formation of some indolyl analogs.

Step 1. Formation of carbanion and attack to indole aldehyde



Step 2. Cyclization with various 1,3-dicarbonyl compounds



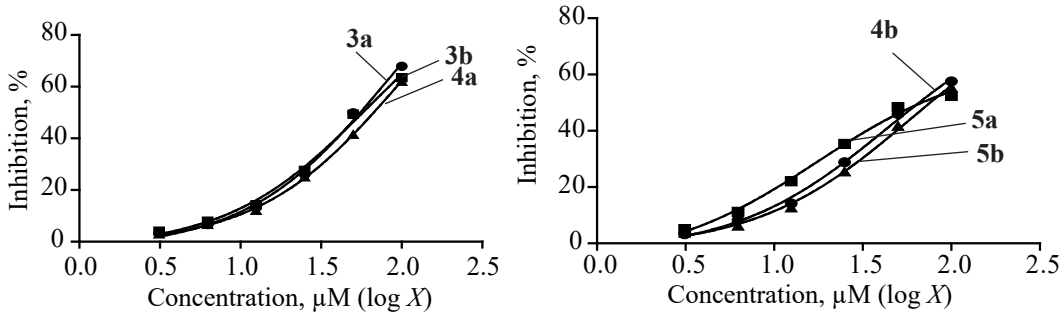
aldehyde with malononitrile, dimedone/thiobarbutiric acid or barbutiric acid (1 mmol each) was powdered and mixed with finely powdered 5 Å Molecular sieve (0.5–1.0 g) loaded into an open borosil glass vessel (to decrease internal pressure). The mixture was subjected to MW-irradiation at moderate power (350–450 W) for 8–10 min at 125–150°C followed by the procedure as reported earlier [6, 23] .

(2) Neat with $KNaC_4H_4O_6{\cdot}4H_2O$. A mixture of equivalent amounts of 2,5-disubstituted indole-3-carboxaldehyde with malononitrile, dimedone, thiobarbutiric acid or barbutiric acid (1 mmol each) and $KNaC_4H_4O_6{\cdot}4H_2O$ (10 mol %) was mixed with finely powdered 5 Å Molecular sieve (0.5–1.0 g) and loaded into an open borosil glass vessel. The mixture was subjected to MW-irradiation at power of 350–450 W for 8–10 min upon heating to 125–150°C followed by the procedure as reported earlier [6, 23].

After completion (TLC) the reaction mixture was cooled down to room temperature and poured onto crushed ice upon constant stirring. A crude product was isolated and recrystallized from ethanol to give the corresponding pure product in high yield (Table 1).

2-Amino-4-(5-chloro-2-phenyl-1*H*-indol-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (3a). Greenish powder, yield 94%, mp 248–250°C. IR spectrum, ν, cm^{-1}: 3490 (NH), 3341 (NH_2), 3050 (Ar-H), 2922 (CH_3), 2883 (CH), 2142 (CN), 1650 (C=O), 1350 (O), 711 (C–Cl). ^1H NMR spectrum, δ, ppm: 1.06 s (6H, CH_3), 2.49 s (2H, CH_2), 2.51 s (2H, CH_2), 3.41 s (1H, CH), 7.30–8.20 m (8H, Ar-H), 9.96 s (2H, NH_2), 12.62 s (1H, NH indole). MS: *m/z:* 443 $[M]^+$.

2-Amino-7,7-dimethyl-4-(5-methyl-2-phenyl-1*H*-indol-3-yl)-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (3b). Yellow powder, yield 92%, mp 258–259°C. IR spectrum, ν, cm^{-1}: 3572 (NH), 3333



IC_{50} = 79.92 (**3a**), 60.76 (**3b**), 94.13 (**4a**) IC_{50} = 44.03 (**4b**), 17.54 (**5a**), 58.04 (**5b**)

Fig. 1. The sample MTT assay using A549 cells.

(NH_2), 3000 (Ar-H), 2990 (CH_3), 2890 (CH), 2155 (CN), 1643 (C=O), 1320 (O). ^{1}H NMR spectrum, δ, ppm: 1.08 s (6H, CH_3), 2.43 s (3H, CH_3), 2.09 s (2H, CH_2), 2.51 s (2H, CH_2), 3.37 s (1H, CH), 7.10–8.04 m (8H, Ar-H), 9.95 s (2H, NH_2), 12.30 s (1H, NH indole). MS: *m/z:* 423 $[M]^+$.

2-Amino-7,7-dimethyl-5-oxo-4-(2-phenyl-1*H*-indol-3-yl)-5,6,7,8-tetrahydro-4*H*-chromene-3-carbonitrile (3c). Yellow powder, yield 85%, mp 249–251°C. IR spectrum, ν, cm^{-1}: 3442 (NH), 3300 (NH_2), 3020 (Ar-H), 2966 (CH_3), 2803 (CH), 2182 (CN), 1670 (C=O), 1300 (O). ^{1}H NMR spectrum, δ, ppm: 1.14 s (6H, CH_3), 2.49 s (2H, CH_2), 2.51 s (2H, CH_2), 3.41 s (1H, CH), 7.30–8.20 m (8H, Ar-H), 9.96 s (2H, NH_2), 12.62 s (1H, NH indole). MS: *m/z:* 443 $[M]^+$.

2-Amino-4-(1*H*-indol-3-yl)-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydro-4*H*-chromene-3-carbo nitrile (3d). Yellow shiny crystals, yield 80%, mp 209–210°C. IR spectrum, ν, cm^{-1}: 3422 (NH), 3303 (NH_2), 3011 (Ar-H), 2950 (CH_3), 2870 (CH), 2177 (CN), 1603 (C=O), 1320 (O). ^{1}H NMR spectrum, δ, ppm: 1.09 s (6H, CH_3), 2.11 s (2H, CH_2), 2.40 s (2H, CH_2), 3.88 s (1H, CH), 7.12–8.20 m (8H, Ar-H), 9.92 s (2H, NH_2), 11.82 s (1H, NH indole). MS: *m/z:* 423 $[M]^+$**.**

Table 1. Comparative data for the synthesis of novel indolyl analogs

Comp. no.	Reaction conditions and yield of products								mp,°C
	conventional		MW irradiation at 125–150°C						
	reflux–EtOH		neat			neat and $KNaC_4H_4O_6 \cdot 4H_2O$			
	t, h	yield, %	*t*, min	power, W	yield, %	*t*, min	power, W	yield, %	
3a	12	45	8	450	55	6	350	94	248–250
3b	12	40	8	450	52	6	350	92	258–259
3c	10	42	8	450	[a]	4	350	85	249–251
3d	10	35	10	450	[a]	6	350	80	209–210
4a	12	42	8	450	52	5	350	92	259–260
4b	12	40	8	450	55	5	350	90	250–252
4c	09	40	9	450	[a]	6	350	88	218–219
4d	09	35	10	450	[a]	6	350	80	220–221
5a	12	45	8	450	50	4	350	93	225–226
5b	12	45	8	450	50	4	350	90	238–239
5c	09	35	10	450	42	6	350	88	186–187
5d	09	35	10	450	[a]	6	350	82	210–211

[a] No reaction occurred.

Table 2. In vitro cytotoxicity of indolyl analogs

Comp. no.	IC_{50}, μM		
	A-549 (*Lung Carcinoma*)	HEp-2 (*Laryngeal Carcinoma*)	HeLa (*Cervical Carcinoma*)
3a	79.92	38.56	31.56
3b	60.76	50.53	30.24
3c	No active	No active	No active
3d	No active	No active	No active
4a	94.13	16.12	24.63
4b	44.03	26.50	43.92
4c	No active	No active	No active
4d	No active	No active	No active
5a	17.54	19.59	32.29
5b	58.04	No active	97.14
5c	No active	No active	No active
5d	No active	No active	No active
Doxorubicin	0.70	8.70	0.71

7-Amino-5-(5-chloro-2-phenyl-1*H*-indol-3-yl)-4-oxo-2-thioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]-pyrimidine-6-carbonitrile (4a). Greenish crystals, yield 92%, mp 259–260°C. IR spectrum, ν, cm^{-1}: 3450 (NH), 3368 (NH), 3310 (NH_2), 3010 (Ar-H), 2924 (CH), 2148 (CN), 1650 (C=O), 1318 (O), 1013 (C=S), 711 (C–Cl). ^{1}H NMR spectrum, δ, ppm: 4.50 s (1H, CH), 7.30–8.19 m (8H, Ar-H), 8.22 s (1H, NH), 9.96 s (2H, NH_2), 12.60 s (1H, NH indole). MS: *m/z:* 447 $[M]^+$.

7-Amino-5-(5-methyl-2-phenyl-1*H*-indol-3-yl)-4-oxo-2-thioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]-pyrimidine-6-carbonitrile (4b). Light yellow crystals, yield 90%, mp 250–252°C. IR spectrum, ν, cm^{-1}: 3542 (NH), 3332 (NH_2), 3020 (Ar-H), 2922 (CH_3), 2886 (CH), 2139 (CN), 1650 (C=O), 1318 (O), 1008 (C=S). ^{1}H NMR spectrum, δ, ppm: 2.43 s (3H, CH_3), 3.46 s (1H, CH), 7.10–7.93 m (8H, Ar-H), 8.24 s (1H, NH), 9.95 s (2H, NH_2), 12.38 s (1H, NH indole). MS: *m/z:* 427 $[M]^+$.

7-Amino-4-oxo-5-(2-phenyl-1*H*-indol-3-yl)-2-thioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]pyrimidine-6-carbonitrile (4c). Yellow crystals, yield 88%, mp 218–219°C. IR spectrum, ν, cm^{-1}: 3472 (NH), 3300 (NH_2), 3074 (Ar-H), 2822 (CH), 2188 (CN), 1660 (C=O), 1371 (O). ^{1}H NMR spectrum, δ, ppm: 3.41 s (1H, CH), 7.20–7.94 m (8H, Ar-H), 8.00 s (1H, NH), 9.95 s (2H, NH_2), 12.30 s (1H, NH indole). MS: *m/z:* 413 $[M]^+$.

7-Amino-5-(1*H*-indol-3-yl)-4-oxo-2-thioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]pyrimidine-6-carbonitrile (4d). Light brown crystals, yield 80%, mp 120–121°C. IR spectrum, ν, cm^{-1}: 3402 (NH), 3383 (NH_2), 3066 (Ar-H), 2840 (CH), 2145 (CN), 1653 (C=O), 1310 (O). ^{1}H NMR spectrum, δ, ppm: 3.47 s (1H, CH), 7.10–8.04 m (8H, Ar-H), 8.31 s (1H, NH), 9.95 s (2H, NH_2), 12.30 s (1H, NH indole). MS: *m/z:* 337 $[M]^+$.

7-Amino-5-(5-chloro-2-phenyl-1*H*-indol-3-yl)-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]pyrimidine-6-carbonitrile (5a). Yellow powder, yield 93%, mp 225–226°C. IR spectrum, ν, cm^{-1}: 3542 (NH), 3262 (NH_2), 3030 (Ar-H), 2886–2923 (CH), 2139 (CN), 1650 (C=O), 1640 (C=O), 1318 (O), 713 (C–Cl). ^{1}H NMR spectrum, δ, ppm: 3.36 s (1H, CH), 7.30–8.19 m (8H, Ar-H), 9.96 s (2H, NH_2), 12.60 s (1H, NH indole), 13. 21 s (1H, NH). MS: *m/z:* 431 $[M]^+$.

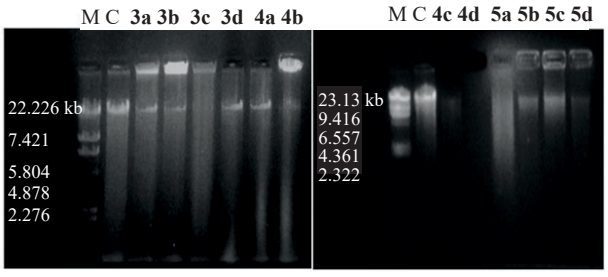


Fig. 2. DNA cleavage Image of Indolyl analogs.

7-Amino-5-(5-methyl-2-phenyl-1*H*-indol-3-yl)-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]-pyrimidine-6-carbonitrile (5b). Light green powder, yield 90%, mp 238–239°C. IR spectrum, ν, cm^{-1}: 3470 (NH), 3351 (NH_2), 3080 (Ar-H), 2921 (CH_3), 2850 (CH), 2138 (CN), 1650 (C=O), 1318 (O). ^1H NMR spectrum, δ, ppm: 2.43 s (3H, CH_3), 4.41 s (1H, CH), 7.11–7.96 m (8H, Ar-H), 9.94 s (2H, NH_2) 12.29 s (1H, NH indole), 12.95 s (1H, NH). MS: *m/z:* 411 $[M]^+$.

7-Amino-2,4-dioxo-5-(2-phenyl-1*H*-indol-3-yl)-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]pyrimidine-6-carbonitrile (5c). Yellow powder, yield 88%, mp 186–187°C. IR spectrum, ν, cm^{-1}: 3422 (NH), 3311 (NH_2), 3025 (Ar-H), 2810 (CH), 2159 (CN), 1613 (C=O), 1300 (O). ^1H NMR spectrum, δ, ppm: 3.37 s (1H, CH), 7.10–8.04 m (8H, Ar-H), 9.95 s (2H, NH_2), 12.30 s (1H, NH indole), 12.64 s (1H, NH). MS: *m/z:* 397 $[M]^+$.

7-Amino-5-(1*H*-indol-3-yl)-2,4-dioxo-2,3,4,5-tetrahydro-1*H*-pyrano[2,3-*d*]pyrimidine-6-carbo nitrile (5d). Yellow shining crystals, yield 82%, mp 210–211°C. IR spectrum, ν, cm^{-1}: 3400 (NH), 3310 (NH_2), 3100 (Ar-H), 2881 (CH), 2115 (CN), 1603 (C=O), 1355 (O). ^1H NMR spectrum, δ, ppm: 4.37 s (1H, CH), 7.10–8.04 m (8H, Ar-H), 9.91 s (2H, NH_2), 12.10 s (1H, NH indole), 12.31 s (1H, NH), MS: *m/z:* 321 $[M]^+$.

In vitro anticancer activity. A-549, HEp-2, and HeLa cell lines were procured from ATCC, stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/mL), streptomycin (100 μg/mL) in the humidified atmosphere of 5% CO_2 at 37°C until confluent. The cell was dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). Viability of the cells was tested, and then those were centrifuged. Further, 50 000 cells/well of Jurkat was seeded in a 96 well plate and incubated for 24 h at 37°C, 5% CO_2 atmosphere. After 24 h of incubation, MTT (10 μL) was added to the incubated cancer cells, and the cells were further incubated at 37°C for ca 4 h in 5% CO_2 atmosphere. Thereafter, the formazan crystals were dissolved in 200 μL of DMSO and the absorbance was monitored at 578 nm with the reference filter 630 nm. The percent of cytotoxicity was calculated.

MTT assay. Briefly, the test compounds were diluted in DMSO (0–100 mg/mL) and cytotoxic activity of the compounds against A-549, HEp-2, and HeLa cells (1×10^5 cells/well) was tested using the cell quantity MTT cell viability assay kit. The wells with the culture medium served as control and the graph was plotted with cell viability against the time period (h) at increasing concentrations of secondary metabolite phase (see figure). The IC_{50} values were calculated by non-linear regression analysis of three independent experiments [6].

DNA cleavage. DNA cleavage experiments were carried out according to the literature [22]. Nutrient broth (peptone 10, yeast extract 5, NaCl 10, in log/L) was used for culturing of *E. coli.* 50 mL Media was prepared, autoclaved for 15 min at 121°C under 15 lb pressures. The autoclaved media were inoculated for 24 h at 37°C.

Isolation of DNA. The fresh bacterial culture (1.5 mL) was centrifuged to obtain the pellet which was then dissolved in 0.5 mL of lysis buffer (100 mM tris pH 8.0, 50 mM EDTA, 10% SDS). To this, 0.5 mL of saturated phenol was added and incubated at 55°C for 10 min, then centrifuged at 10 000 rpm for 10 min, and to the supernatant, equal volume of chloroform, isoamyl alcohol (24 : 1) and 1/20 volume of 3 M sodium acetate (pH 4.8) was added, centrifuge at 10 000 rpm for 10 min, and to the supernatant three volumes of chilled absolute alcohol was added. The precipitated DNA was separated by centrifugation, the pellet was dried and dissolved in TAE buffer (10 mM tris pH 8.0, 1 mM EDTA) and stored under cold condition.

Agarose gel electrophoresis. Cleavage products were analyzed by agarose gel electrophoresis. Test samples were prepared in DMF. The samples were added to the isolated DNA of E. coli, incubated for 2 h at 37°C and then DNA sample (mixed with bromophenol blue dye at 1 : 1 ratio) was loaded carefully into the electrophoresis chamber wells along with standard DNA marker containing TAE buffer, and finally loaded on agarose gel, and the constant 50V of electric current was applied for 30 min. Upon removing the gel and staining it with 10.0 mg/ mL ethidium bromide for 10–15 min, the bands were observed under Vilber Lourmat gel documentation system and then photographed to determine the extent of DNA cleavage. The results were compared with the standard DNA marker.

CONCLUSIONS

We have applied efficient and ecologically friendly methods of synthesis of indolyl analogs under different reaction conditions: (1) conventional heating, and (2) one-pot, three-components solvent-free MW assisted process. The second method is characterized by operational simplicity, clean reaction conditions, high yields, easy work-up, and use of an inexpensive, nontoxic, and

efficient catalyst. The synthesized compounds demonstrate excellent to good in vitro cytotoxic activity against three cell lines A-549, HEp-2, and HeLa in comparison with the standard drug Doxorubicin. Compounds **4a** and **5a** exhibit potent growth inhibitory activity. This study also reveals some potential DNA cleavage activity of compounds **3a**–**3d**, **4a**, **4b**, and **5b**.

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CONFLICT OF INTEREST

No conflict of interests was declared by the authors.

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