Bioorganic Chemistry 54 (2014) 12–20

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Solvent-free synthesis of heteroannulated carbazoles: A novel class of anti-tumor agents

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ARTICLE INFO

Article history:
Received 21 October 2013
Available online 12 March 2014

Keywords:
Carbazole
Cytotoxicity
Crystal structure
IC_{50}

ABSTRACT

A series of novel carbazole analogues that hold pyrido, isoxazolo, pyrimido and pyrazolo templates were designed and synthesised in good yield by grinding conditions. All the synthesised compounds were screened for their anti-tumor activity and displayed enviable selective growth inhibition on HeLa cell line compared to AGS cell line. Among these compounds, compound 2-(3′,4′-diethoxy-benzylidene)-6-methyl-2,3,4,9-tetrahydro-carbazol-1-one,6-chloro-2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1one, 2-(3′,4′-diethoxy-benzylidene)-8-methyl-2,3,4,9-tetrahydro-carbazol-1-one, 3-(3′,4′-diethoxyphenyl)-7-methyl-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole, 7-chloro-3-(3′,4′-diethoxyphenyl)-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole, 4-(3′,4′-diethoxyphenyl)-2-ethoxy-8-methyl-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile, 8-chloro-4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile, 4-(3′,4′-diethoxyphenyl)-2-ethoxy-10-methyl-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile were found to have promising anti-tumor properities with reference to the standard ellipticine against the HeLa cancer cell line. All these intermediates showed IC_{50} outranged the standard ellipticine. The same compounds showed moderate activity against AGS cancer cell lines. The efforts were undertaken to optimize potency and selectivity of this class of compounds.

1. Introduction

In the last few decades, reports on carcinoma cases have shown a drastic increase, although there have been significant advancements in cancer treatment. Changes in lifestyle and environment are regarded as the leading causes, resulting in over 10 million reported cases globally in year 2001 [1]. Two of the most common type of cancer in females are cervical and breast cancer, with the mortality rate for cervical cancer in the United States in 2006 being 37%. So there is a strong need for the establishment of new chemo preventives and the discovery of new drugs to combat these carcinomas [1,2]. Carbazoles constitute an important class of alkaloids displaying a wide variety of biological activities [3] and their analogues are also widely used as building blocks for new organic materials [4]. Accordingly, syntheses of simple carbazoles and modified carbazoles have been extensively studied [5,6]. Benzodihydro[*a*]carbazoles (BDHC) and pyrido carbazoles have been reported as starting compounds for the synthesis of various drugs and possess important biological, pharmacological and medicinal activities [7–14]. They are associated with anticancer, antimicrobial and antifungal activities [13,15,16]. Many carbazole derivatives especially that bearing chloro groups are important for the creation of promising new anti-tumor agents [17]. The combination of pharmacopores may provide a synergisitic effect to improve the activity and reducing the risk of side effect. The carbazole back bone has been chosen because it possess better inhibition properties among the other nitrogen containing alkaloids. This adds considerable support in favor of carbazole as core moiety for new potential anti-tumor compounds. Based on the previous findings and in continuation of our interest in the synthesis of bioactive heterocycles, including a promising anti-tumor agents, the present study includes the green synthesis of some carbazoles that bearing, isoxazolo, pyrimido, pyrazolo and pyrido moiety and to evaluate their *in vitro* antitumor activities against two human tumor cell-lines HeLa and AGS.

2. Results and discussion

2.1. Chemistry

A central underpinning aim of our research was to develop an alternative solvent free green synthetic route for the synthesis of cyclised hetero annulated carbazoles with potential improvements

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http://dx.doi.org/10.1016/j.bioorg.2014.03.003
0045-2068/

in yield. In this paper, we describe the synthesis of benzylidene derivatives of carbazole having ethoxy groups at C_3' & C_4' by solvent free conditions. The higher yields, mild reaction conditions, easy isolation and purification make the eco-friendly procedure an attractive alternative to the existing methods for the synthesis of carbazole analogues. Nowadays, organic chemists are vigorously taking on the challenge of developing green synthetic methodologies to meet the criteria of sustainable, environmentally conscious development. Green chemistry has emerged as an important area of chemistry and has achieved outstanding progress towards the development of green reaction processes [18]. As a result, solvent-free synthetic methods have attracted much interest not only for laboratory synthesis but also in chemical industry, because of reduced pollution, lower costs, mild conditions, and ease of purification. Recently, practical procedures in the absence of solvents have been accomplished for greener and cleaner syntheses [18–23]. As the typical representative of solvent-free reactions, the grinding technique has been widely used in organic synthesis [24–30].

In earlier work from our laboratory, we have reported the synthesis of hetero annulated carbazoles [31–35]. In order to show the accessibility of the present work, we compared this method (*grinding technique*) with the results obtained using solvent (Table 1), which showed that grinding is the most efficient technique with respect to the reaction time, temperature and exhibited broad applicability in terms of yields. The reason for the efficiency of the current procedure might be due to an enhanced second-order reaction rate resulting from ultimately high concentration of reactants in the absence of solvent. We are able to obtain the cyclised product in a single step using this method. The yield of the products obtained using solvent conditions were low when compared to the yield obtained by grinding conditions. The yield of the product obtained, using solvent and without solvent conditions were compared in Table 1.

Here we have synthesized 3′,4′-diethoxy-benzylidene derivatives by mixed aldol condensation of 2,3,4,9-tetrahydro-1*H* carbazol-1-ones with 3,4-diethoxy benzaldehyde by using solvent and solvent free conditions and the yields of the product were compared which was given in Table 1. Consequently, the formation of diethoxy-benzylidene derivatives was identified as a key precursor in affording the corresponding panel of isoxazolo, pyrido, pyrimido and pyrazolocarbazoles Scheme 2. Further the3′,4′-diethoxy-benzylidene derivatives were reacted with hydroxylamine hydrochloride, malononitrile, hydrazine hydrate, and guanidine nitrate to give the corresponding cyclised heteroannulated isoxazolo, pyrido, pyrazolo and pyrimido carbazoles respectively. The synthetic routes were shown in the Scheme 1. The same procedure was repeated for other derivatives also (see Fig. 1).

2.2. Biology

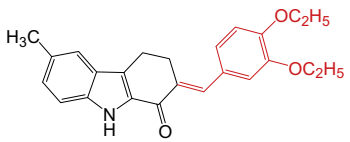
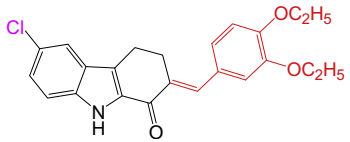
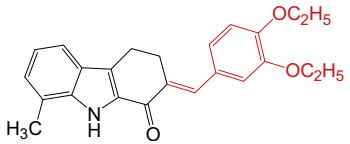
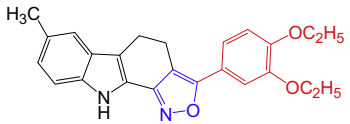
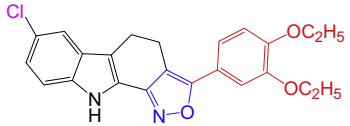
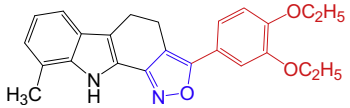
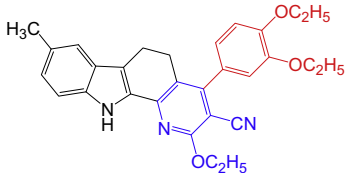
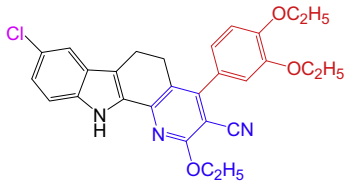
The ability of the fifteen newly synthesized hetero annulated carbazoles to inhibit cell growth was evaluated by means of *in vitro* assay performed against two human tumor cell lines namely HeLa (cervix adeno carcinoma) and AGS cancer cell lines (human stomach cancer). The ellipticine was used as a standard which showed IC_{50} value 4.12 μM for Hela and IC_{50} value 7.33 μM for AGS cancer cell lines. The obtained results (Table 2) revealed that the compounds exhibited variable degrees of inhibitory activity toward the two tested human tumor cell lines. The cytotoxic activity results revealed that majority of the synthesized compounds exhibited potent anticancer activity against HeLa cell-line and moderate activity against AGS cancer cell lines which is represented in Table 2. These results indicate that all the synthesized carbazole analogues depicted selective inhibition against HeLa cancer cell line. All the compounds exhibited varying inhibitory concentrations depending on the kind of substituent present. Furthermore, the inhibitory activity was governed by the nature and position of the substituent. The cytotoxicity of all the three intermediates showed IC_{50} outranged the standard ellipticine (IC_{50} value 4.12 μM) against HeLa. The 3′,4′-diethoxy-benzylidene derivatives intermediates showed selective toxicity against HeLa cancer cell line. Among the intermediates, as for activity against HeLa cell line, the very highest cytotoxic activity was displayed by compound 6-chloro-2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1-one which showed the percentage viability IC_{50} at 0.37 μM compared with the corresponding cyclised analog was due to the presence of an electron withdrawing chloro group at C_6 position, whereas, the next highest cytotoxic activity was displayed by compound 2-(3′,4′-diethoxy-benzylidene)-6-methyl-2,3,4,9-tetrahydro-carbazol-1-one which showed the percentage viability IC_{50} at 0.80 μM and moderate inhibitory activity was also demonstrated by compound 2-(3′,4′-diethoxy-benzylidene)-8-methyl-2,3,4,9-tetrahydro-carbazol-1-one.

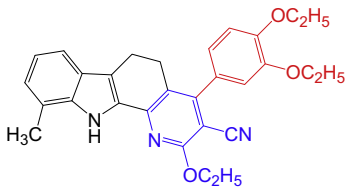
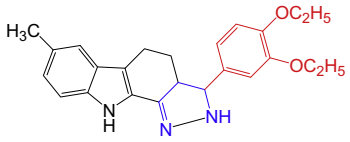
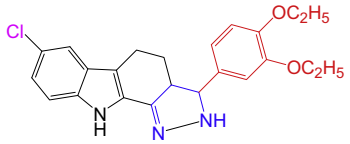
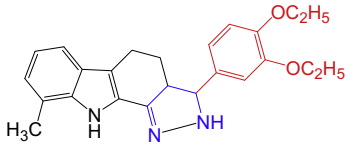
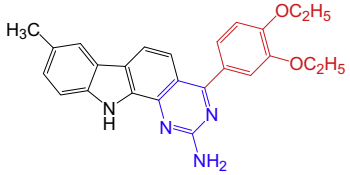
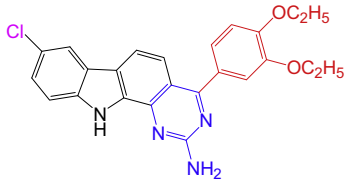
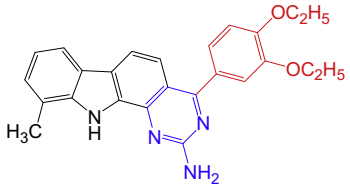
Out of the products formed, the pyrido, pyrazolo, isoxazolo, pyrimido substituted carbazoles that bearing chloro group at the 7 and 8th position [**7-chloro-**3-(3′,4′-diethoxyphenyl)-4,5-dihydro-10*H***-**isoxazolo[3,4-*a*]carbazole**, 8-chloro**-4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile**,** 7-chloro-3-(3′,4′-diethoxyphenyl)-2,4,5,10-tetrahydropyrazolo-5*H*-[3,4-*a*]carbazole & **8-chloro**-4-(3′,4′-diethoxyphenyl)-10-methyl-11*H*-pyrimido-[4,5-*a*]carbazole-2-amine] showed a preferential growth inhibitory activity against the two cell lines than the corresponding methyl substituted analogues. Among these, the compound **8-chloro**-4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5*H*-**pyrido**[2,3-*a*]carbazole-3-nitrile which holds the pyrido substituted moiety showed IC_{50} value 1.02 μM against HeLa was found to exhibit significant activity. This may be due to the presence of electron withdrawing cyano group at C_3 position. In this series, the next highest toxicity was shown by **7-chloro**-3-(3′,4′-diethoxyphenyl)-4,5-dihydro-10*H*-**isoxazolo**[3,4-*a*]carbazole which carries the isoxazolo group was endowed with IC_{50} value 1.30 μM against HeLa. The next most active compound was the one which holds the pyrimido group at the C_1 position (**8-chloro**-4-(3′,4′-diethoxyphenyl)-11*H*-**pyrimido**-[4,5-*a*]carbazole-2-amine) with the IC_{50} value 4.51 μM against HeLa. In this series, the least activity was shown by the one which carries the pyrazolo moiety (**7-chloro**-3-(3′,4′-diethoxyphenyl)-2,4,5,10-tetrahydro**pyrazolo**-5*H*-[3,4-*a*]carbazole) with the IC_{50} value 10.05 μM against HeLa cancer cell line.

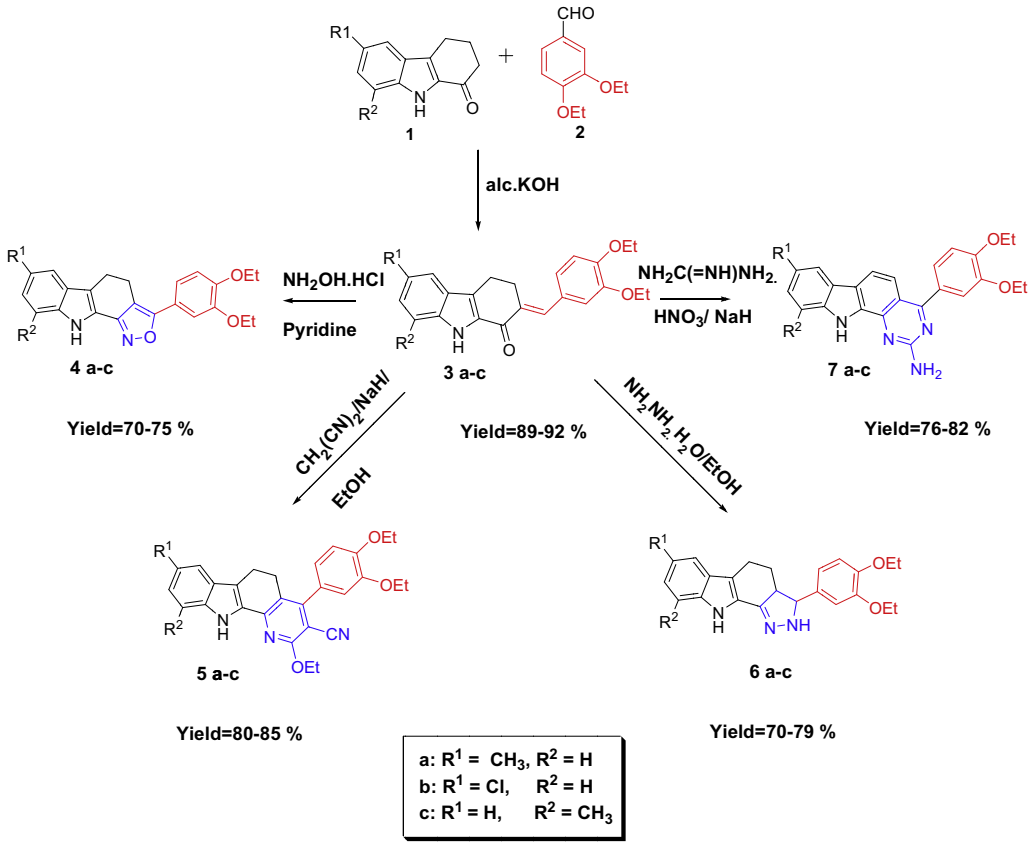
Between the cyclised products, that bearing methyl group at the 7 and 8th position (3-(3′,4′-diethoxyphenyl)-**7-methyl**-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole**,** 4-(3′,4′-diethoxyphenyl)-2-ethoxy-**8-methyl**-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile, 3-(3′,4′-diethoxyphenyl)-**7-methyl**-2,4,5,10-tetrahydropyrazolo-5*H*-[3,4-*a*]carbazole & 4-(3′,4′-diethoxyphenyl)-**8-methyl**-11*H*-pyrimido-[4,5-*a*]carbazole-2-amine), the highest toxicity was obtained with the pyrido substituted moiety, 4-(3′,4′-diethoxyphenyl)-2-ethoxy-**8-methyl**-6,11-dihydro-5*H*-**pyrido**[2,3-*a*]-carbazole-3-nitrile with IC_{50} value 1.50 μM against HeLa. In this series, the second highest toxicity was shown by the compound 3-(3′,4′-diethoxyphenyl)-**7-methyl**-4,5-dihydro-10*H*-**isoxazolo**-[3,4-*a*]carbazole with the IC_{50} value 2.73 μM against HeLa cancer cell line. The compound substituted with the pyimido group at the C_1 position (4-(3′,4′-diethoxyphenyl)-**8-methyl**-11*H*-**pyrimido**-[4,5-*a*]carbazole-2-amine) was found to be next highly active compound with the IC_{50} value 5.08 μM against HeLa. In this series, the least activity was shown by the intermediate that is substituted with the pyrazolo moiety (3-(3′,4′-diethoxyphenyl)-**7-methyl**-2,4,5,10-tetrahydro**pyrazolo**-5*H*-[3,4-*a*]carbazole) with the IC_{50} value 18.97 μM against HeLa.

The compounds that bearing methyl group at the 9 and 10th position (3-(3′,4′-diethoxyphenyl)-**9-methyl**-4,5-dihydro-10*H*-

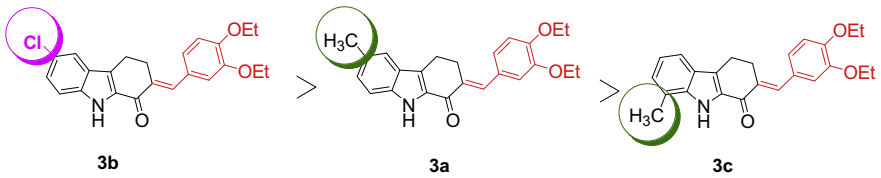
Table 1
Comparison of yields and physical parameters of compunds **3a-7c**.

Entry	Structure	Melting poiint (°C)	Conventional method (Using solvent)				Grinding technique (without solvent)	
			Tempera ture (°C)	Solvent	Reaction time (h)	Yield (%)	Reaction time (min)	Yield (%)
3a		180–182	RT	KOH/EtOH (25 mL)	24	85	10	92
3b		189–193	RT	KOH/EtOH (25 mL)	24	82	10	90
3c		184–186	RT	KOH/EtOH (25 mL)	24	79	10	89
4a		200–202	130	Pyridine (5 mL)	8	67	15	75
4b		220–223	130	Pyridine (5 mL)	8	66	15	74
4c		210–214	130	Pyridine (5 mL)	8	61	15	70
5a		187–190	80	NaH (1.00 g)/EtOH (20 mL)/benzene (10 mL)	5	77	15	83
5b		198–202	80	NaH (1.00 g)/EtOH (20 mL)/benzene (10 mL)	5	73	15	85

5c		182–185	80	NaH (1.00 g)/EtOH (20 mL)/benzene (10 mL)	5	68	15	80
6a		192–195	80	EtOH (25 mL)	6	69	10	77
6b		205–208	80	EtOH (25 mL)	6	65	10	79
6c		198–202	80	EtOH (25 mL)	6	62	10	70
7a		182–184	80	NaH (1.00 g)/benzene (10 mL)	18	71	20	82
7b		198–200	80	NaH (1.00 g)/benzene (10 mL)	18	72	20	81
7c		189–192	80	NaH (1.00 g)/benzene (10 mL)	18	57	20	76



Scheme 1. Solvent free synthesis of hetero annulated carbazoles.



Scheme 2. Comparison of cytotoxic activity of the intermediates.

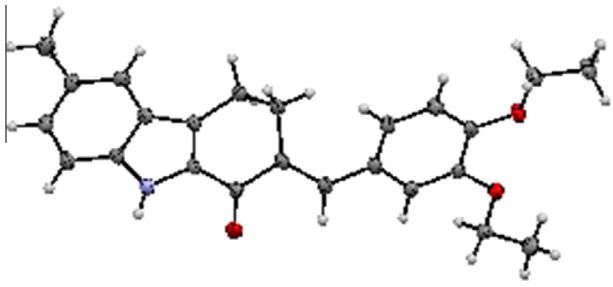


Fig. 1. X-ray crystal structure and atom numbering of compound 2-(3′,4′-diethoxy-benzylidene)-6-methyl-2,3,4,9-tetrahydro-carbazol-1-one as thermal ellipsoids at 50% probability level.

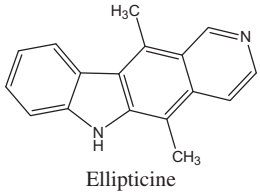
isoxazolo[3,4-*a*]carbazole, 4-(3′,4′-diethoxyphenyl)-2-ethoxy-**10-methyl**-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile**,** 3-(3′,4′-diethoxyphenyl)-**9-methyl**-2,4,5,10-tetrahydropyrazolo-5*H*-[3,4-*a*]carbazole & 4-(3′,4′-diethoxyphenyl)-**10-methyl**-11*H*-pyrimido-[4,5-*a*]carbazole-2-amine), the remarkable potency was shown by the pyrido substituted moiety 4-(3′,4′-diethoxyphenyl)-2-ethoxy-**10-methyl**-6,11-dihydro-5*H*-**pyrido**[2,3-*a*]carbazole-3-nitrile with IC_{50} value 4.51 μM against HeLa cancer cell line. In this series, the second highest toxicity was shown by the compound 3-(3′,4′-diethoxyphenyl)-**9-methyl**-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole with the IC_{50} value 5.30 μM against HeLa cancer cell line. The compound substituted with the pyimido group at the C_1 position (4-(3′,4′-diethoxyphenyl)-**10-methyl**-11*H*-**pyrimido**-[4,5-*a*]carbazole-2-amine) was found to be next highly active compound with the IC_{50} value 7.12 μM against HeLa. In this series, the least activity was shown by the compound that is substituted with the pyrazolo moiety (3-(3′,4′-diethoxyphenyl)-**9-methyl**-2,4,5,10-tetrahydropyrazolo-5*H*-[3,4-*a*]carbazole) with the IC_{50} value 24.41 μM against HeLa cancer cell line.

2.3. Structure–Activity relationship

From our present work, the following structure activity relationship can be derived.

- It is clear from the results summarized in Table 2 that, substituent present at C_6 position plays a vital role in determining the anti cancer potency of 3′,4′-diethoxy-benzylidene intermediates (**3a-c**). Out of these, substitution of electronegative chloro atom

Table 2
Invitro cytotoxicity and IC_{50}(μM).

Compound no.	HeLa[a]	AGS[b]
3a	0.80	46.05
3b	2.73	45.09
3c	1.50	44.32
4a	18.97	71.05
4b	5.08	50.05
4c	0.37	15.12
5a	1.30	30.50
5b	1.02.	25.90
5c	10.05	76.05
6a	4.51	38.05
6b	2.90	35.09.
6c	5.30	46.64.
7a	3.41	43.34
7b	24.41	75.52
7c	7.12	58.09
 Ellipticine	4.12	7.33

[a] Human cervical cancer cell line.
[b] Stomach cancer cell line.

at 6th position (6-chloro-3,4-diethoxy-benzylidene intermediate) gave the remarkable potency against the HeLa and AGS cancer cell lines compared with the substitution of electron donating methyl group at the 6th position. This may be due to the electron withdrawing nature of chloro group that enhances the anti cancer potency [36]. In general, for the intermediates, orders of activities were as follows which was given in Scheme 2.

Chloro substituted analogue > **para** methyl subtituted analogue > **ortho** methyl substituted analogue.

- From the data illustrated in Table 2 it was found that out of the products, the most successful combinations for anticancer activity were found out to be chloro group at the 8th position and pyrido moiety at the 1st position of the carbazole nucleus, i.e. pyrido substituted carbazole was found to be promising than the isoxazolo, pyrazolo and pyrimido substituted carbazoles. It may be due to the strong electron withdrawing nature of the cyano group which is presented in the pyrido moiety [37–39]. From the literaure survey, it could be clear that the cyano substituted hetero aryls showed prominent cytotoxicity toward HeLa tumor cells by induction of severe apoptosis through the inhibition of tublin polymerization [40]. The SAR analysis also confirms that the cyano group is essential for the induction of apoptosis and extreme cytotoxicity.

For the products which were derived from 3′,4′-diethoxy-benzylidene intermediates, the orders of activities were as follows which was given in Scheme 3.

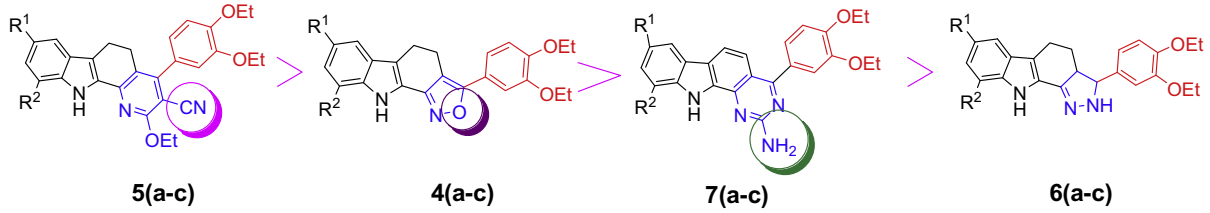
pyrido substituted carbazole moiety > **isoxazolo** substituted carbazole moiety > **pyrimido** substituted carbazole moiety > **pyrazolo** substituted carbazole moiety.

In addition to its high over all activity, all the synthesized compounds showed selective activity against HeLa cancer cell line. Biological results suggest that the presence of chloro group at the 6th position was found to be more advantageous for the anti proliferative activity among the tested compound. Further substitution (methyl group) resulted in a slight activity drop which leads to slightly less active compounds. These results suggest that all of these derivatives may have antitumor properties. In order to evaluate their therapeutic utility, investigation of these compounds on other tumor cell lines and lower-dose, longer-time treatment are currently under investigation.

By considering the further modification, the potent compound 6-chloro-2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1-one was selected as the bench mark agent for subsequent optimization. In general, it was observed that the activity of the intermediate was superior to their products. This provides a platform for further development of these compounds into promising anti tumor agents.

3. Conclusion

In summary, the present work demonstrated the design, synthesis and quantitative evaluation of a series 1-oxo-2-benzylidene carbazole derivatives and their cyclised analogues as efficient antitumor agents. Moreover, one novel and efficient synthetic approach to the core structure provides a quick method for the target compounds. This article reports the shorter and greener route available for the transformation of 1-oxo-2-benzylidene carbazoles into their pyrido, pyrimido, isoxazolo and pyrazolo derivatives. The first step was the transformation of intermediate by the mixed aldol condensation. Enhanced antitumor activity arising from this pharmacophore will be discussed in the context of this transformation and further the intermediate was subjected to react with hydroxylamine hydrochloride, hydrazine hydrate, malononitrile and guanidine nitrate to give the desirable products in a good global yield when it was carried out by solvent free conditions. The pure products were obtained simply by aqueous washing and extraction with ethyl acetate. The higher yields, mild reaction conditions, easy isolation and purification make the eco-friendly procedure an attractive alternative to the existing methods for the synthesis of carbazole analogues The cytotoxic actvity of the newly synthesised benzylidene derivatives was studied against HeLa and AGS cancer cell lines revealing that all the three intermediates displayed significant cytotoxic activities particularly for the 3′,4′-diethoxy benzylidene derivative that bearing chloro group at the para position which outranged the standard ellipticine. The final products namely pyrido, pyrazolo, pyrimido and isoxazolo moieties that carries chloro group at the 8th position



Scheme 3. Comparison of cytotoxic activity of the products.

displayed potent cytotoxic activity superior to their corresponding methyl substituted analogous. Furthermore, the screening for anti proliferative properties showed that all products may have a good future as new potent antitumor agent.

To the best of our knowledge, this is the first report on the bioactive potential of carbazole analogues against the HeLa and AGS cancer cell lines with IC_{50}value 0.37. This result could be used as a basis for the development of a structure oriented drug discovery program and further study is underway.

4. Experimental

4.1. Chemistry

4.1.1. General procedure for the synthesis of 2-(3′,4′-diethoxybenzylidene)-2,3,4,9-tetrahydro-carbazol-1-ones (3a-c).

An equimolar mixture of the-2,3,4,9-tetrahydro-carbazol-1-ones (1, 0.005 mol) and 3,4-diethoxy benzaldehyde, (0.005 mol) was grinded in the presence of KOH for 10 min. The reaction was monitored by TLC. The reaction mixture was poured into ice cold water and neutrralised with 1:1 HCl. The precipated product was recrystallised from ethanol to yield the respective 2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1-one (**3**).

4.1.2. 2-(3′,4′-Diethoxy-benzylidene)-6-methyl-2,3,4,9-tetrahydro-carbazol-1-one (3a)

Yield: 92%, m.p. 180–190 °C IR:$_{max}$ (cm^{-1}) 3414, (N—H), 1725 (C═O), H NMR ($CDCl_3$) δ: 9.85 (s, 1H, N_9—H), 7.60 (s, 1H, olefinic-H), 7.20–7.50 (m, 6H, C_5,C_7, C_8, C_2', C_5', C_6'—H), 4.16–4.32 (m, 4H, —C_3', C_4'—O**CH_2**CH_3). 3.06–3.08 (m, 4H, C_3—2H, C_4—2H), δ 2.47 (s, 3H, C_6—CH_3), δ 1.50–1.70, (m, 6H, C_3', C_4'—OCH_2**CH_3**), MS: *m/z* 375 (100%), Anal. calcd for $C_{24}H_{25}NO_3$: C, 76.77; H, 6.71; N, 3.73. Found: C, 76.73; H, 6.73; N, 3.71%.

4.1.3. 6-Chloro-2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1-one (3b)

Yield: 90%, m.p. 189–195 °C IR:$_{max}$ (cm^{-1}) 3440, (N—H), 1732 (C═O), H NMR ($CDCl_3$) δ: 9.51 (s, 1H, N_9—H), 7.90 (s, 1H, olefinic-H), 7.32–7.49 (m, 6H, C_5, C_7, C_8, C_2', C_5', C_6'—H), 4.25–4.39 (m, 4H, —C_3', C_4'—O**CH_2**CH_3), 3.29–3.40 (m, 4H, C_3—2H, C_4—2H), 1.45–1.75,(m, 6H, C_3', C_4'—OCH_2**CH_3**), MS: *m/z* 395 (100%), Anal. calcd for $C_{23}H_{22}NCl\ O_3$: C,69.78; H, 5.60; N, 3.54. Found: C, 69.76; H, 5.62; N, 3.56% Cl.

4.1.4. 2-(3′,4′-Diethoxy-benzylidene)-8-methyl-2,3,4,9-tetrahydro-carbazol-1-one (3c)

Yield: 89%, m.p. 184–197 °C IR:$_{max}$ (cm^{-1}) 3435, (N—H), 1765 (C═O), H NMR ($CDCl_3$), δ: 9.41 (s, 1H, N_9—H), 7.80 (s, 1H, olefinic—H), 7.12–7.45 (m, 6H, C_5,C_6, C_7, C_2', C_5', C_6'—H), 4.16–4.32 (m, 4H, —C_3', C_4'—O**CH_2**CH_3), 3.24–3.36 (m, 4H, C_3—2H, C_4—2H), 2.51 (s, 3H, C_8—CH_3), 1.30–1.60,(m, 6H, C_3',C_4'—OCH_2**CH_3**), MS: *m/z* 375 (100%), Anal. calcd for $C_{24}H_{25}NO_3$: C, 73.29.; H, 6.31; N, 3.38. Found: C, 73.31; H, 6.29; N, 3.40%.

4.1.5. General procedure for the preparation of 3-(3′,4′-diethoxyphenyl)-4,5-dihydro-10H-isoxazolo[3′,4′-a]carbazoles(4a-c)

The 2-(3′,4′-diethoxy-benzylidene)- 2,3,4,9-tetrahydro-carbazol-1-ones (1, 0.001 mol) was grinded with hydroxylamine hydrochloride (0.01 mol) and pyridine (1 mL) for 15 min. The reaction monitored by TLC, indicated the formation of product and the mixture was poured into ice–water. The solid separated was then neutralized with 1:1 HCl, filtered and dried over anhydrous sodium sulphate. It was then purified by column chromatography over silica gel using pet.ether: ethylacetate (95:5) as eluent to yield respective the 3-(3′,4′-diethoxyphenyl)-5,10-dihydro-4*H*-isoxazolo[3,4-*a*]carbazole (**4a-c**).

4.1.6. 3-(3′,4′-Diethoxyphenyl)-7-methyl-4,5-dihydro-10H-isoxazolo[3,4-a]carbazole(4a)

Yield: 75%, m.p. 200–207 °C. IR:$_{max}$ (cm^{-1}) 3305 cm^{-1} (N—H). ^{1}H NMR ($CDCl_3$) δ: 8.95 (s, 1H, N_{10}—H), 7.30–7.80 (m, 6 H, C_6, C_8, C_9, C_2', C_5', C_6'—H), 4.05–4.20 (m, 4H, C_3', C_4'—O**CH_2**CH_3), 3.20–3.60 (m, 4H, C_4—2H, C_5—2H), 2.20 (s, 3H, C_7—CH_3), 1.05–1.08 (m, 6H, C_3', C_4'—OCH_2**CH_3**). MS: *m/z* 388 (100%) Anal.calcd for $C_{24}H_{24}N_2O_3$: C, 74.23; H, 6.18.; N, 7.22. Found: C, 74.25; H, 6.17; N, 7.27%.

4.1.7. 7-Chloro-3-(3′,4′-diethoxyphenyl)-4,5-dihydro-10H-isoxazolo[3,4-a]carbazole(4b)

Yield: 74%, m.p. 220–235 °C. IR:$_{max}$ (cm^{-1}) 3334 cm^{-1} (N—H) 1682 (C═O). ^{1}H NMR ($CDCl_3$) δ: 9.19 (s, 1H, N_{10}—H), 7.21–7.69 (m, 6 H, C_6, C_8, C_9, C_2', C_5', C_6'—H), 4.05–4.20 (m, 4H, C_3',C_4'—O**CH_2**-CH_3), 3.30–3.54 (m, 4H, C_4—2H, C_5—2H), 1.05–1.08 (m, 6H, C_3', C_4'—OCH_2**CH_3**). MS: *m/z* 408 (100%) Anal.calcd for $C_{23}H_{21}N_2\ Cl\ O_3$: C, 74.34; H, 6.10.; N, 7.18. Found: C, 74.33; H, 6.13; N, 7.16%.

4.1.8. 3-(3′,4′-Diethoxyphenyl)-9-methyl-4,5-dihydro-10H-isoxazolo[3,4-a]carbazole(4c)

Yield: 70%, m.p. 210–220 °C. IR:$_{max}$ (cm^{-1}) 3325 cm^{-1} (N—H), 1672 (C═O). ^{1}H NMR ($CDCl_3$) δ: 9.10 (s, 1H, N_{10}—H), δ 7.21–7.69 (m, 6H, C_6, C_7, C_8, C_2', C_5', C_6'—H), δ 4.05–4.20 (m, 4H, C_3', C_4'—O**CH_2**-CH_3), δ 3.20–3.60 (m, 4H, C_4—2H, C_5—2H), δ 2.20 (s, 3H, C_9—CH_3), δ 1.05–1.08 (m, 6H, C_3', C_4'—OCH_2**CH_3**). MS: *m/z* 388 (100%) Anal.calcd for $C_{24}H_{24}N_2O_3$: C, 74.27; H, 6.20.; N, 7.22. Found: C, 74.30; H, 6.18; N, 7.25%.

4.1.9. General procedure for the preparation of 4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5H-pyrido[2,3-a]carbazole-3-nitrile.(5a-c)

The 2-(3′,4′-diethoxybenzylidene)-2,3,4,9-tetrahydro-carbazol-1-one (**3,** 0.001 mol) was grinded with malononitrile (0.001 mol), sodium hydride (1.00 g) and ethanol (1 mL). The reaction monitored by TLC indicated the formation of product. After the completion of the reaction (15 min), the mixture was poured into ice–water and neutralized with 1:1 HCl The brown solid separated was then filtered and dried over anhydrous sodium sulphate ate. It was then purified by column chromatography over silica gel using pet.ether: ethylacetate (98:2) as eluant to yield respective 4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5*H*-pyrido[2,3-*a*]carbazole-3-nitrile **(5)**.

4.1.10. 4-(3′,4′-Diethoxyphenyl)-2-ethoxy-8-methyl-6,11-dihydro-5H-pyrido[2,3-a]carbazole-3-nitrile (5a)

Yield: −83%, m.p. 187–196 °C. IR:$_{max}$(cm^{-1}), 3263 (N—H), 2231 (C≡N). ^{1}H NMR ($CDCl_3$) δ: 9.31 (s, 1H, N_{11}—H), 6.94–7.77 (m, 6 H, C_7, C_9, C_{10}, C_2', C_5', C_6'—H), 4.13–4.17 (m, 6H, C_2, C_3', C_4'—O**CH_2**CH_3,), 3.20–3.60 (m, 4H, C_5, C_6–H), 2.73 (s, 3H, C_8—CH_3), 1.46–1.52 (m, 9H, C_2, C_3', C_4'—OCH_2**CH_3**) MS: *m/z* 455 (100%) Anal.calcd for $C_{28}H_{29}N_3O_3$: C, 73.66; H, 6.39; N, 3.58. Found: C, 73.63; H, 6.36; N, 3.60%.

4.1.11. 8-Chloro-4-(3′,4′-diethoxyphenyl)-2-ethoxy-6,11-dihydro-5H-pyrido[2,3-a]carbazole-3-nitrile (5b)

Yield: −85%, m.p. 198–215 °C. IR:$_{max}$ (cm^{-1}) 3256 (N—H) 2221 (C≡N) ^{1}H NMR ($CDCl_3$) δ: 9.43 (s, 1H, N_{11}—H), 6.45–7.83 (m, 6H, C_7, C_9, C_{10}, C_2', C_5', C_6'—H), δ 4.13–4.17 (m, 6H, C_2, C_3', C_4'— O**CH_2**-CH_3), 3.16–3.49 (m, 4H, C_5, C_6 –H), 1.46–1.52 (m, 9H C_2, C_3, 'C_4' OCH_2**CH_3**–) MS: *m/z* 475 (100%) Anal.calcd for $C_{27}H_{26}N_3\ Cl\ O_3$: C, 73.29; H, 6.35; N, 3.54. Found: C, 73.31; H, 6.33; N, 3.55%.

4.1.12. 4-(3′,4′-Diethoxyphenyl)-2-ethoxy-10-methyl-6,11-dihydro-5H-pyrido[2,3-a]carbazole-3-nitrile (5c)

Yield: −80%, m.p. 182–191 °C. IR:$_{max}$(cm^{-1}) 3245 (N—H) 2229 (C≡N) ^{1}H NMR ($CDCl_3$) δ: 9.11 (s, 1H, N_{11}—H), δ 6.85–7.89(m, 6H, C_7, C_8, C_9, C_2', C_5', C_6'—H) δ, 4.13–4.17 (m, 6H, C_2, C_3', C_4'—O**CH$_2$**-CH_3), δ 2.45 (s, 3H, C_{10}—CH_3), δ 1.46–1.52 (m, 9H C_2, C_3', C_4' OCH_2-**CH$_3$**-) MS: *m/z* 455 (100%) Anal.calcd for $C_{28}H_{29}N_3O_3$: C, 73.41; H, 6.27; N, 3.45. Found: C, 73.43; H, 6.25; N, 3.48%.

4.1.13. General procedure for the preparation of 3-(3′,4′-diethoxyphenyl)-2,4,5,10-tetrahydro pyrazolo-5H-[3,4-a]carbazoles (6a-c)

The 2-(3′,4′-diethoxybenzylidene)-2,3,4,9-tetrahydro-carbazol-1-one, (**3**, 0.001 mol) was grinded with hydrazine hydrate (0.001 mol). After the completion of reaction (10 min), it was poured into crushed ice with stirring. The solid thus separated was filtered, dried over anhydrous sodium sulphate and purified by column chromatography over silica-gel using petroleum ether and ethylacetate mixture (90:10) as a solvent system to yield the respective 3-(3,4-diethoxyphenyl)-2,4,5,10-tetrahydro pyrazolo-5*H*-[3,4-*a*]carbazole (**6**).

4.1.14. 3-(3′,4′-Diethoxyphenyl)-7-methyl-2,4,5,10-tetrahydropyrazolo-5H-[3,4-a]carbazole (6a)

Yield: −77%, m.p. 192–205 °C. IR:$_{max}$ (cm^{-1}) 3383 (N—H), ^{1}H NMR ($CDCl_3$) δ: 10.31 (s, 1H, pyrozolo NH), 9.01 (s, 1H N_{10}—H), 6.35–7.15 (m, 6H, C_6, C_8, C_9, C_2', C_5', C_6'—H), δ 4.19–4.22 (m, 4H, C_3', C_4'—O**CH$_2$**CH_3), δ 3.03–4.17 (m, 6H C_3, C_{3a}, C_4, C_5—H) δ: 2.73 (s, 3H, C_7—CH_3), δ 1.46–1.52 (m, 6H C_3', C_4'—OCH_2**CH$_3$**) MS: *m/z* 389 (100%) Anal.calcd for $C_{24}H_{27}N_3O_2$: C, 73.35; H, 6.39; N, 3.32. Found: C, 73.33; H, 6.37; N, 3.31%.

4.1.15. 7-Chloro-3-(3′,4′-diethoxyphenyl)-2,4,5,10-tetrahydropyrazolo-5H-[3,4-a]carbazole (6b)

Yield: −79%, m.p. 205–215 °C. IR:$_{max}$ (cm^{-1}) 3345 (N—H), ^{1}H NMR ($CDCl_3$) δ: 10.19 (s, 1H, pyrozolo NH), 9.23 (s, 1H N_{10}—H), 6.39–7.65 (m, 6H, C_6, C_8, C_9, C_2', C_5', C_6'—H), 4.12–4.28 (m, 4H, C_3', C_4'—O**CH$_2$**CH_3), 3.54–4.67 (m, 6H C_3, C_{3a}, C_4, C_5—H), δ 1.34–1.76 (m, 6H C_3', C_4'—OCH_2**CH$_3$**) MS: *m/z* 409 (100%) Anal.calcd for $C_{23}H_{24}N_3ClO_2$: C, 73.35; H, 6.39; N, 3.32. Found: C, 73.33; H, 6.37; N, 3.31%.

4.1.16. 3-(3′,4′-Diethoxyphenyl)-9-methyl-2,4,5,10-tetrahydropyrazolo-5H-[3,4-a]carbazole (6c)

Yield: −70%, m.p. 198–210 °C. IR:$_{max}$ (cm^{-1}) 3378 (N—H), ^{1}H NMR ($CDCl_3$) δ: 10.25 (s, 1H, pyrozolo NH), 9.10 (s, 1H N_{10}—H), δ 6.36–7.29 (m, 6H, C_6, C_7, C_8, C_2', C_5', C_6'—H), δ 4.17–4.32 (m, 4H, C_3', C_4'—O**CH$_2$**CH_3), δ 3.28–4.45 (m, 6H C_3, C_{3a}, C_4, C_5—H) δ: 2.57 (s, 3H, C_9—CH_3), δ 1.43–1.59 (m, 6H C_3', C_4'—OCH_2**CH$_3$**) MS: *m/z* 300(100%) Anal.calcd for $C_{24}H_{27}N_3O_2$: C, 73.35; H, 6.39; N, 3.32. Found: C, 73.33; H, 6.37; N, 3.31%.

4.1.17. General procedure for the preparation of 4-(3,4-diethoxyphenyl)-11H-pyrimido-[4,5-a]carbazole-2-amine (7a-c)

The respective 2-(3′,4′-diethoxy-benzylidene)-2,3,4,9-tetrahydro-carbazol-1-one (**3,** 1 mmol) was grinded with guanidine nitrate (10 mmol) in the presence of sodium hydride (1.00 g) for 20 min. The reaction was monitored by TLC. The residue was poured into crushed ice and then neutralized with 1:1 HCl and extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulphate. Removal of the solvent yielded brown solid mass. It was purified by column chromatography over silica gel using petroleum ether: ethylacetate(85:15) to yield the respective 4-(3′,4′-diethoxyphenyl)-11*H*-pyrimido-[4,5-*a*]carbazole-2-amine (**7**).

4.1.18. 4-(3′,4′-Diethoxyphenyl)-8-methyl-11H-pyrimido-[4,5-a]carbazole-2-amine (7a)

Yield: 82%, m.p. 182–191 °C. IR:$_{max}$ (cm^{-1}), 3351 (N—H), 3289, 3190 (NH_2) ^{1}H NMR ($CDCl_3$) δ: 9.89 (s, 1H, N_{11}—H), 6.75–7.89 (m, 8 H, C_5, C_6, C_7, C_9, C_{10}, C_2', C_5', C_6'—H), 5.23 (b s, 2H, NH_2), 4.13–4.17 (m, 4H, C_3', C_4'—O**CH$_2$**CH_3), 2.63 (s, 3H, C_8—CH_3), 1.40–1.52 (m, 6H, C_3', C_4'—OCH_2**CH$_3$**) MS: *m/z* 412 (100%) Anal.calcd for $C_{25}H_{24}N_4O_2$: C, 73.66; H, 6.39; N, 3.58. Found: C, 73.64; H, 6.41; N, 3.56%.

4.1.19. 8-Chloro-4-(3′,4′-diethoxyphenyl)-10-methyl-11H-pyrimido-[4,5-a]carbazole-2-amine (7b)

Yield: 81%, m.p. 198–218 °C. IR:$_{max}$ (cm^{-1}), 3322 (N—H), 3267, 3176 (NH_2) H NMR ($CDCl_3$) δ: 9.98 (s, 1H, N_{11}—H), 7.25–7.86 (m, 8 H, C_5, C_6, C_7, C_9, C_{10}, C_2', C_5', C_6'—H), 5.54 (b s, 2H, NH_2), 4.30–4.41 (m, 4H, C_3', C_4'—O**CH$_2$**CH_3), 1.53–1.65 (m, 6H, C_3', C_4'—OCH_2-**CH$_3$**) MS: *m/z* 432 (100%) Anal.calcd for $C_{24}H_{21}N_4ClO_2$: C, 73.10; H, 6.33; N, 3.50. Found: C, 73.12; H, 6.31; N, 3.49%.

4.1.20. 4-(3′,4′-Diethoxyphenyl)-10-methyl-11H-pyrimido-[4,5-a]carbazole-2-amine (7c)

Yield: 76%, m.p. 189–195 °C. IR:$_{max}$(cm^{-1}), 3342 (N—H), 3275, 3196 (NH_2) ^{1}H NMR ($CDCl_3$) δ: 9.78 (s, 1H, N_{11}—H), 7.15–7.92(m, 8H, C_5, C_6, C_7, C_8, C_9, C_2', C_5', C_6'—H), 5.59 (b s, 2H, NH_2), 4.34–4.39 (m, 4H, C_3',C_4'—CH_2), 1.53–1.65 (m, 6H, C_3', C_4'—OCH_2**CH$_3$**) MS: *m/z* 412 (100%)Anal.calcd for $C_{25}H_{24}N_4O_2$: C, 73.10; H, 6.33; N, 3.50. Found: C, 73.12; H, 6.31; N, 3.49%.

4.2. Biology

4.2.1. Cell lines and cell culture by MTT assay method

Cytotoxicity studies of the compounds along with ellipticine were carried out on human cervical cancer cells (HeLa) human stomach cancer cells (AGS) and which were obtained from National Centre for Cell Science, Pune, India. Cell viability was carried out using the MTT assay method [41]. The HeLa and AGS cells were grown in Eagles minimum essential medium containing 10% fetal bovine serum (FBS). For the screening experiment, the cells were seeded into 96-well plates in 100 μL of the respective medium containing 10% FBS, at a plating density of 10,000 cells/well, and incubated at 37 °C, under conditions of 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to the addition of compounds. The compounds were dissolved in DMSO and diluted in the respective medium containing 1% FBS. After 24 h, the medium was replaced with the respective medium with 1% FBS containing the compounds at various concentrations and incubated at 37 °C under conditions of 5% CO_2, 95% air, and 100% relative humidity for 48 h. After 48 h, 10 μL of MTT (5 μg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off, and the formed formazan crystals were dissolved in 100 μL of DMSO. The absorbance was then measured at 570 nm using a microplate reader. The percentage of cell inhibition was determined using the following formula, and a graph was plotted with the percentage of cell inhibition versus concentration. From this, the IC_{50} value was calculated: % inhibition = [mean OD of untreated cells (control)/mean OD of treated cells (control)] × 100. The results were expressed as the concentration at which there was 50% inhibition (IC_{50}).

Acknowledgments

We thank the IIT-Madras, Chennai, and IICT-Hyderabad India for their support in spectral studies. We acknowledge Dr. Aathirajan, Assistant Professor, Cell Culture Laboratory, KMCH College of Pharmacy, Coimbatore for evaluating cytotoxicty.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.03.003.

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