Murali, K., Sparkes, H. A., & Rajendra Prasad, K. J. (2017). Synthesis of hetero annulated isoxazolo-, pyrido- and pyrimido carbazoles: Screened for *in vitro* antitumor activity and structure activity relationships, a novel 2-amino-4-(3'-bromo-4'-methoxyphenyl)-8-chloro-11H-pyrimido[4,5-a]carbazole as an antitumor agent. *European Journal of Medicinal Chemistry*, *128*, 319-331. https://doi.org/10.1016/j.ejmech.2017.02.009

Synthesis of hetero annulated isoxazolo-, pyrido- and pyrimido carbazoles: Screened for *in vitro* antitumor activity and structure activity relationships, A novel 2-amino-4-(3'-bromo-4'-methoxyphenyl)-8-chloro-11*H*-pyrimido[4,5-*a*]carbazole as an antitumor

Karunanidhi Murali [a], Hazel A. Sparkes [b], Karnam Jayarampillai Rajendra Prasad [a*]

[a*] Department of Chemistry, Bharathiar University, Coimbatore - 641046, India.

[b]Department of Chemistry, University of Bristol, Cantock's Close, Bristol, BS8 1TS, United Kingdom.

Abstract

Claisen-Schmidt condensation of 2,3,4,9-tetrahydro-1*H*-carbazol-1-one with 3-bromo-4-methoxy benzaldehyde afforded the 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydro-1*H*-carbazol-1-one **3**. Compound **3** was allowed to react with different organic reactants, hydroxylamine hydrochloride, malononitrile and guanidine nitrate through condensation cum cycloaddition reactions to afford a series of the respective novel hetero annulated carbazoles such as isoxazolo-, pyrido- and pyrimido carbazoles. The structures of the compounds were established by FT-IR, ^{1}H NMR, ^{13}C NMR, X-ray diffraction and elemental analysis. The compounds have been screened for *in vitro* anti-tumor activity by MTT assay and displayed enviable selective growth inhibition on MCF-7 cell line compared to A-549 cell line. Apoptotic morphological changes in MCF-7 and A-549 cells were visualized using fluorescent microscopic technique. The preliminary structure activity relationships were also carried out. Data pointed out that among pyrimido carbazole compounds, 2-amino-4-(3'-bromo-4'-methoxyphenyl)-8-chloro-11*H*-pyrimido[4,5-*a*]carbazole could be exploited as an excellent therapeutic drug against cancer cell proliferation.

Key words

Hetero annulated carbazoles

Pyrimidocarbazole

Structure-activity relationship

Cytotoxicity

Fluorescent microscopic analysis

---

*Corresponding author e-mail: prasad_125@yahoo.com, Tel: +91-422-2423763, Fax: +91-422-2422387

1. Introduction

Cancer is a genetic irregularity of immortal cells which exasperates. In the last few decades, cancer has become a second leading life-threatening disease accounting for high mortality rates after cardiovascular diseases [1,2]. Breast cancer and lung cancer are the most frequent causes of cancer related deaths. Breast cancer is a kind of malignant tumor for women [3]. In recent years, the mortality rate of breast cancer shows an increasing trend [4]. Lung cancer accounts for more than one million deaths a year worldwide with non-small cell lung cancer (NSCLC) [5] accounting for 80-85% of lung cancer and it remains a disease with poor prognosis and the primary cause of cancer related deaths in both men and women [6]. Chemotherapy is one of the most commonly used treatment options. The lack of selectivity of chemotherapeutic agents for cancer tissues is the main drawback of chemotherapy and which causes severe adverse effect. Therefore there is a strong need for the establishment of new chemopreventives and the discovery of new drugs to eradicate these cancer anomalies with less undesirable side effects. Among potential chemotherapeutic agents, heterocyclic compounds represent a remarkable type of anticancer drug candidates.

Carbazoles are probably the most widely spread nitrogen heterocycles in nature [7,8]. Carbazoles and hetero annulated carbazole derivatives have attracted substantial attention due to their wide range of biological activities such as antidiabetic, antimicrobial, antioxidant, anticancer, antitubercular and anticonvulsant activity [9-17]. Many carbazole derivatives especially those with chloro substituents are important in the synthesis of new anti-cancer agents [18], and the carbazole back bone has been chosen here because it possess better inhibition properties compared to other nitrogen containing alkaloids. The introduction of additional heterocyclic rings to the carbazole core tends to exert profound influence in increasing the compounds' anticancer activity owing to their larger π-conjugated system [19]. In the past few years, several studies have highlighted the importance of carbazole based compounds as promising chemotherapeutic agents. Beata Tylinska and co-workers [20] reported the synthesis of 1-substituted-6*H*-pyrido[4,3-*b*]carbazole derivatives, which exhibited over 20 times better activity against murine leukemia L1210 tumor cell line than the reference ellipticine. Kumar and co-workers [21] synthesized a novel substituted carbazole derivative, which showed significant cytotoxic activity against Ehrlich's Ascites Carcinoma (EAC) and HEP2 cell lines, while

Molatlhegi and co-workers [22] explored the anti breast cancer activity of a novel carbazole based compound against lung cancer cell line A-549.

The pyrimidine moiety is one of the most widespread heterocycles in biologically occurring compounds such as nucleic acids and vitamin B_1, and it is an important constituent of numerous drug molecules in many therapeutic areas [23-25]. Amino pyrimidine moieties are common in marketing drugs such as anti-atherosclerotic aronixil, anti-histaminic thonzylamine, anti-anxielyticbuspirone and other medicinally relevant compounds [26] (**Fig.1**). Pyridocarbazoles constitute an important class of therapeutic agents in medicinal chemistry. Olivacine and ellipticine are natural pyridocarbazole alkaloids, which have also been shown to have anticancer activity [27-32], DNA intercalation and inhibition of topoisomerase II. Moreover, compounds containing an isoxazole moiety exhibited anti-inflammatory, antidepressant, anticonvulsant and antibacterial activities. A number of drugs, including the COX-2 inhibitor valdecoxib possesses isoxazole as the core pharmacophore. Taking all the above findings into consideration and in searching for new heterocyclic compounds endowed with potent antitumor activity, we report herein the synthesis of some carbazoles bearing isoxazolo, pyrido and pyrimido moieties and evaluate their *in vitro* antitumor activities against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines and to explore a valuable SAR.

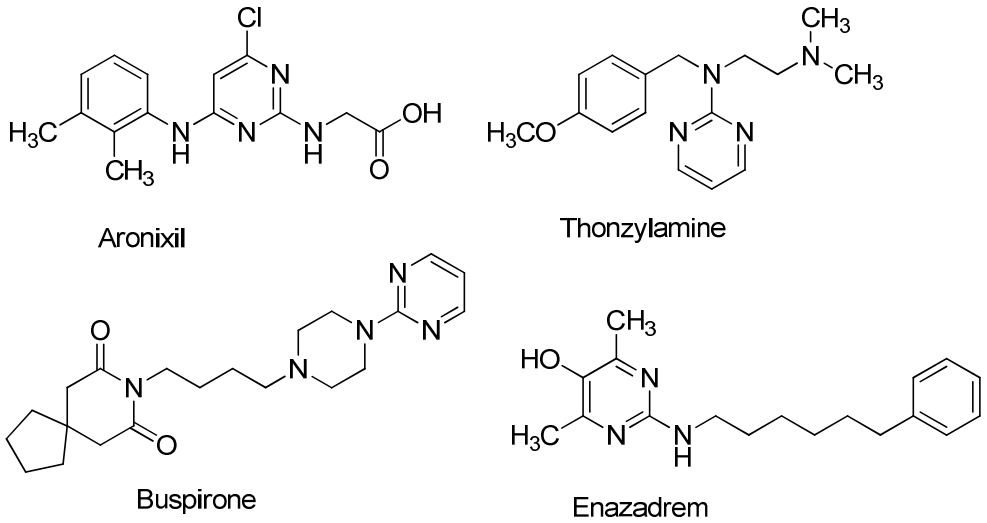
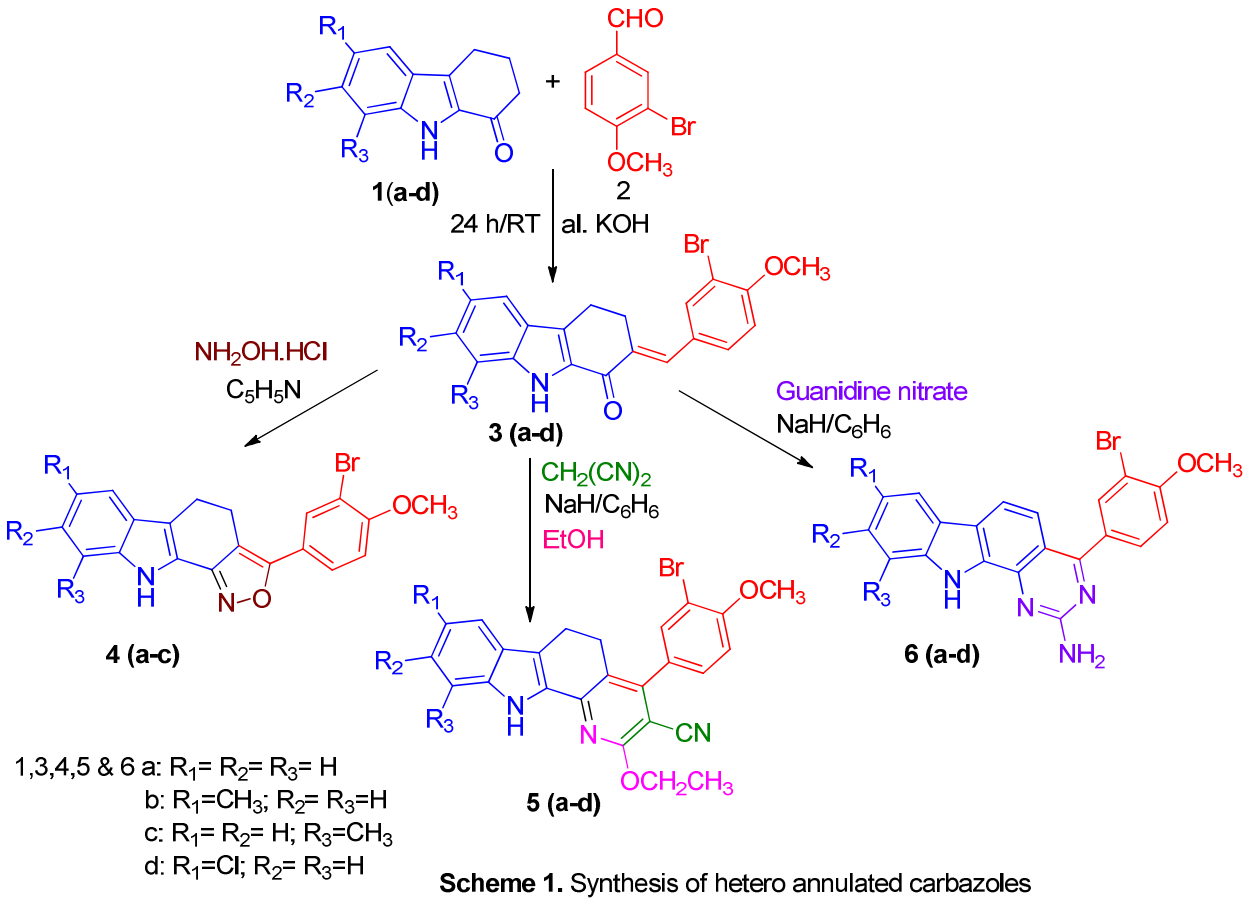


Fig.1. Biologically active aminopyrimidine derivatives

2. Results and Discussions

2.1. Chemistry

The synthetic pathways employed to synthesis the new targeted derivatives are depicted in **scheme. 1**.



Scheme 1. Synthesis of hetero annulated carbazoles

The synthon 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydro-1*H*-carbazol-1-one (**3a**) was derived by Claisen-Schmidt condensation reaction between an equimolar mixture of 2,3,4,9-tetrahydro-1*H*-carbazol-1-one (**1a**) and 3-bromo-4-methoxybenzaldehyde (**2**) in ethanolic KOH solution. The FT-IR spectrum of **3a** displayed a characteristic band for a carbazole carbonyl group at 1639 $cm^{-1}$and absorption at 3262 cm^{-1} ascribable to indole NH stretching. The ^{1}H NMR spectrum exhibited a broad singlet at δ 9.09 ppm due to the presence of the indole NH proton and multiplet signals in the region δ 7.68-7.36 ppm attributed to the seven aromatic protons. The proton at the C_8 position appeared as a doublet centered at δ 6.95 ppm (J_o = 8.0 Hz), and the three methoxy protons at the C'_4 position accounted for a singlet at δ 3.94 ppm. The

methylene protons of C_4 and C_3 appeared as two multiplets centered at δ 3.08 and 3.25 ppm respectively. The ^{13}C NMR spectrum revealed the presence of 20 carbons. A signal resonating at δ 56.3 ppm was attributed to the methoxy carbon. The universal validity of the reaction was tested with other substituted derivatives **1** (**b-d**) to afford the corresponding 2-arylidene carbazoles **3** (**b-d**).

The 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydro-1*H*-carbazol-1-one (**3a**) was reacted with hydroxyl amine hydrochloride in the presence of pyridine which lead to the formation of the cyclised product, 3-(3'-bromo-4'-methoxyphenyl)-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole (**4a**). Its FT-IR spectrum revealed prominent absorptions at 3222 and 1500 cm^{-1} due to the indole NH and C=N stretchings respectively. The ^1H NMR spectrum exhibited a broad singlet at δ 8.80 ppm due to the presence of the indole NH proton, and three doublets appeared at δ 8.21, 8.12 and 8.02 ppm (J = 2.0, 8.0 and 8.0 Hz) linked to the C_2', C'_6 and C_9 protons respectively. The C_7 proton signal occurred as a multiplet in the region δ 7.94-7.92 ppm while the C_8 proton accounted for a multiplet signal centered at δ 7.68 ppm. Two protons at the C_5' and C_6 positions appeared as two doublets at δ 7.61 ppm (J_o= 8.0 Hz), δ 7.56 ppm (J_o= 7.6 Hz) respectively. A singlet at δ 3.95 ppm assigned to the C_4'-OCH_3 protons, and methylene protons of C_5 and C_4 appeared as multiplets centered at δ 2.92 ppm. The ^{13}C NMR spectrum of **4a** displayed 20 resonances in agreement with the proposed structure. The methoxycarbon displayed a resonance signal at δ 56.3 ppm. The identities of the other compounds **4** (**b, c**) were established in a similar ways with all spectroscopic data provided in the supplementary information.

The intermediate **3a** was also reacted with malononitrile in the presence of sodium hydride to yield 4-(3'-bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-11*H*-pyrido[2,3-*a*]carbazole-3-carbonitrile **(5a)**. It is important to note that the condensation which occurred at the carbonyl carbon led to the formation of the C=N bond which resulted in the formation of **5a**. The FT-IR spectrum of **5a** showed absorption bands at 3335, 2218 and 1555 cm^{-1} assignable to the indole NH, cyano and C=N groups respectively. The ^1H NMR spectrum of **5a** showed a broad singlet at δ 8.76 ppm related to the indole NH proton, and the signals due to C_7, C_2', C_{10} and C_5' protons were visible as four doublets centered at δ 7.58, 7.52, 7.43 and 7.02 ppm (J = 7.6, 2.0, 8.0 and 8.4 Hz) respectively. The other aromatic protons at C_6' & C_9 appeared as a multiplet centered at δ 7.28 ppm whereas C_8-H appeared as a triplet at δ 7.24 ppm (J = 7.6 Hz). The two protons of the O**C**H_2CH_3 group appeared as a quartet centered at δ 4.61 (J = 7.2 Hz) and

the methoxy group at C'$_4$ position resonated as a singlet at 3.96 ppm. The methylene protons of C_6 and C_5 appeared as a multiplet at δ 2.92 ppm, and three protons of $OCH_2\mathbf{C}H_3$ group appeared as a triplet at δ 1.50 (J = 7.2 Hz). The ^{13}C NMR data afford further evidence of the structure and it shows the presence of 25 carbons. A resonance signal at δ 119.9 ppm corresponded to CN carbon. The resonance signals at δ 63.0 and 14.5 ppm were attributed to $O\mathbf{CH_2}CH_3$ and $OCH_2\mathbf{CH_3}$ carbons. The structure of the compound **5a** was further confirmed by X-ray diffraction (**Fig. 2**)**.** This was also the case for all the other compounds of the series **5** (**b-d**).

The intermediate **3a** was also reacted with guanidine nitrate in the presence of sodium hydride for about 18 h, which led to the formation of 2-amino-4-(3'-bromo-4'-methoxyphenyl)-11*H*-pyrimido[4,5-*a*]carbazole (**6a**).The appearance of C=N stretching at the expense of C=O and the disappearance of a benzylidene singlet indicates the formation of **6a**. The FT-IR spectrum of **6a** showed absorptions at 3405, 3328, 3212, 1573 and 1540 cm^{-1} which were assigned to the asym NH_2, sym NH_2, indole NH and two C=N groups respectively. The ^{1}H NMR spectrum of **6a** showed a broad indole NH singlet at δ 9.90 ppm. The disappearance of methylene protons in the aliphatic region and the appearance of further peaks in the aromatic region clearly indicated that the product was fully aromatized. The signals due to C_7, C_{10}, C_6, C_5, C_6', and C_5' protons were visible as six doublets centered at δ 8.11, 7.91, 7.75, 7.63, 7.58 and 7.06 (J_o = 8.0, 8.8, 8.4, 8.4, 8.0 and 7.2 Hz) respectively, and C_2'-H and C_4'-OCH_3 protons occurred as two singlets at δ 8.04 and 4.00 ppm. From a singlet at δ 5.57 the presence of an amino group in the substrate was inferred. The presence of 21 carbons as identified from its ^{13}C NMR spectrum and further confirmed by X-ray diffraction **Fig. 3**. A series of similar reactions were carried out with **3** (**b-d**) and similar results (formation **6** (**b-d**)), were obtained. All the compounds were obtained in moderate to good yield ranging from 65% to 75%.

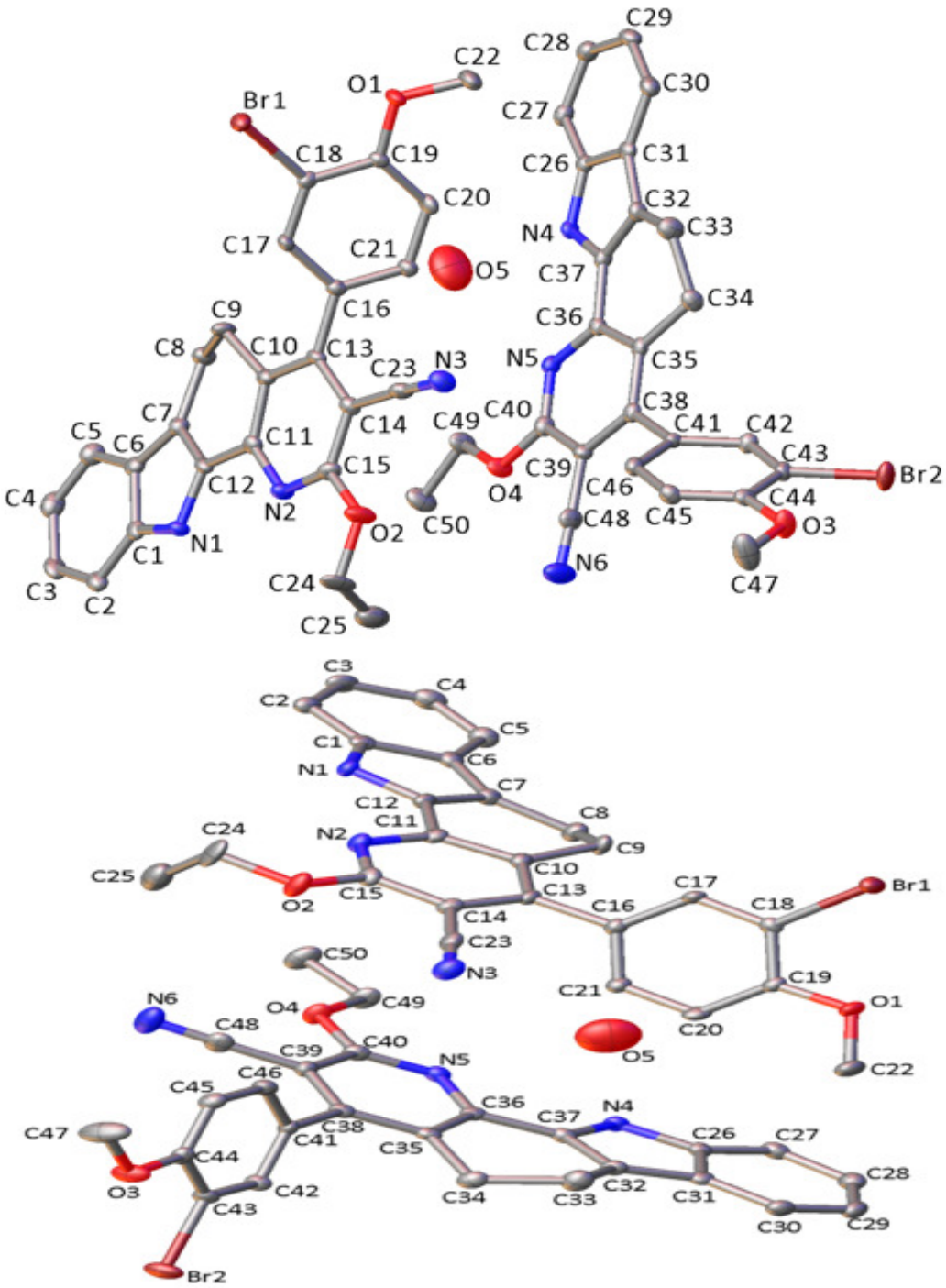


Fig.2. Structure of **5a** (hydrate) with ellipsoids depicted at the 50% probability level and atomic labeling shown. Hydrogen atoms are omitted and only one position of the disordered water oxygen O5 is shown for clarity.

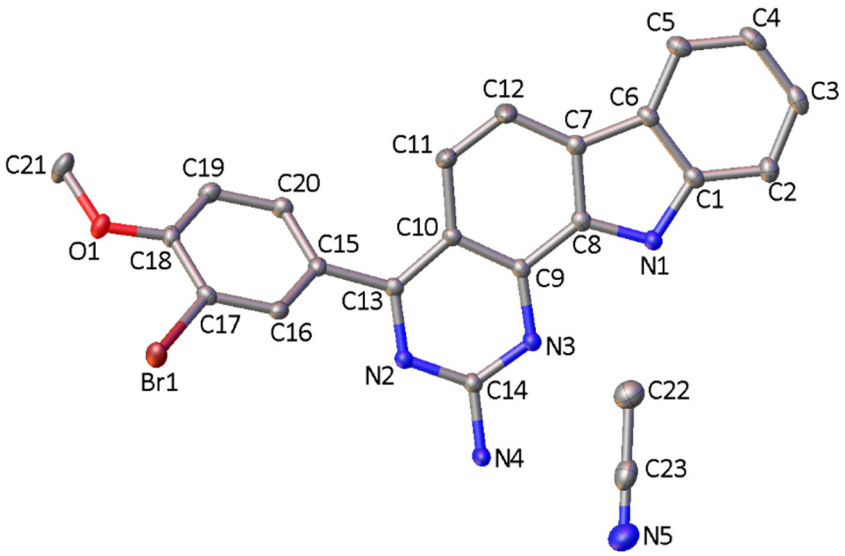
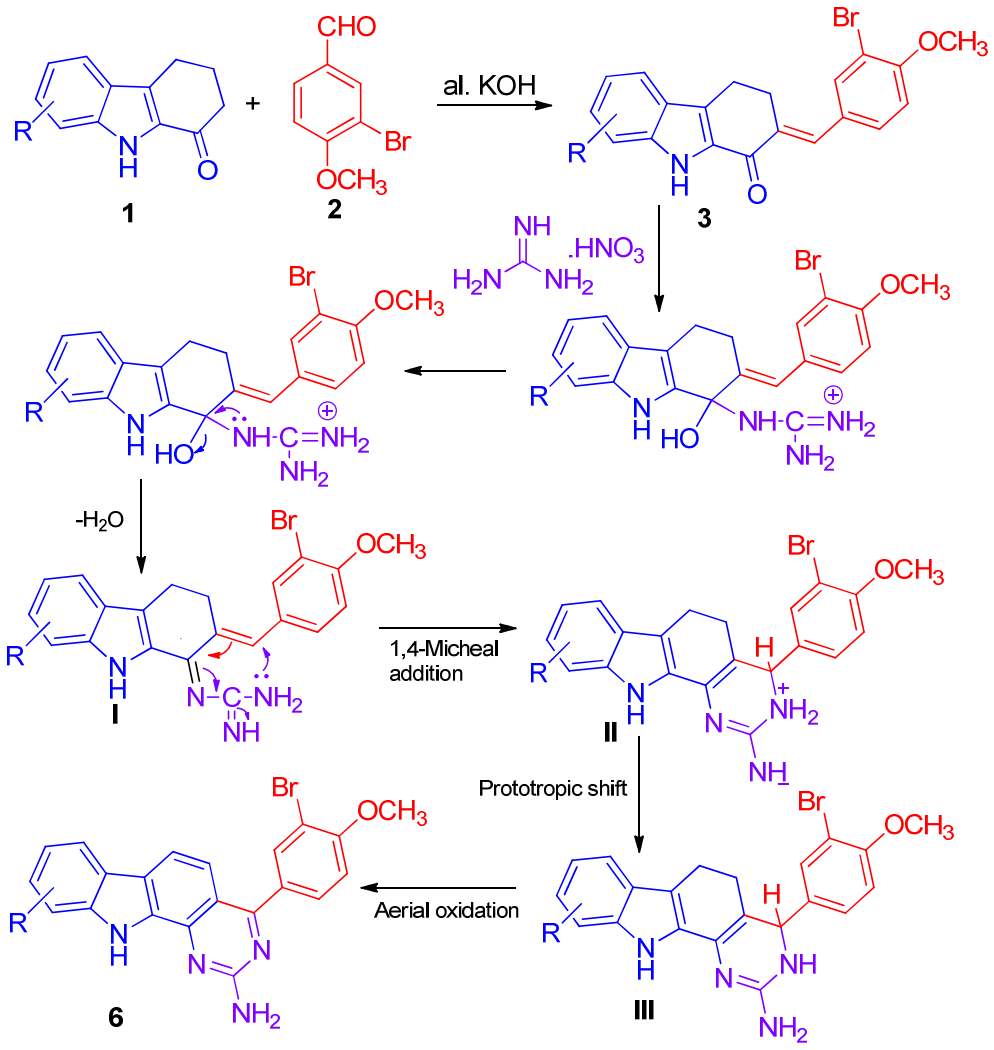


Fig.3. Structure of **6a** with ellipsoids depicted at the 50% probability level and atomic labeling shown. Hydrogen atoms are omitted for clarity.

2.1.1. Mechanism for the formation of pyrimido[4,5-*a*]carbazole

A plausible mechanism for the formation of product **6** is depicted in **Scheme 2**. The reaction occurs *via* initial formation of **3**, from the Claisen-Schmidt condensation of aromatic aldehyde and 2,3,4,9-tetrahydro-1*H*-carbazol-1-one. The synthon **3** reacts with the amino group of guanidine nitrate to afford the Schiff base intermediate **I**, which on intramolecular 1,4-Michael addition gives intermediate **II**. Subsequently, the cyclized intermediate **II** undergoes prototropic shift to yield intermediate **III**. Aerial oxidation of intermediate **III** gave the final stable aromatized product **6**.



Scheme 2. Plausible mechanism for the formation of pyrimido[4,5-*a*]carbazole **6**

2.2. Biological evaluation

2.2.1. *In vitro* cytotoxicity

To evaluate the cytotoxicity of the newly synthesized compounds, two different cancer cell lines were utilized: MCF-7 (breast cancer) and A-549 (lung cancer). The viability of the cells was assessed by the MTT (3-(4',5'-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [33] *in vitro*. Cisplatin was used as a positive control which showed IC_{50} values of 18±1.5µM for MCF-7 and 19±1.7µM for A-549 cancer cell lines. Three independent experiments in triplicate were performed for the determination of sensitivity to each compound. The anticancer potency of these compounds was expressed as growth inhibitory concentration (IC_{50}) values which represent the compound concentration that causes a 50% reduction of cell growth, the results are

summarized in **Table 1**. The percentage cell viability was determined using the following formula, and a graph was plotted between percentage cell viability and concentration, from this plot, the IC_{50} value was calculated. The *in vitro* cytotoxic activity of the synthesized compounds (**4b**, **6b** and **6d**) (10-100 µM concentrations) against both cancer cells has been presented in **Fig. 4-6**. The experimental results demonstrate that all the compounds have the ability to inhibit cell proliferation in a dose dependent manner.

$$\% \text{ of viability} = \frac{\text{OD value of experimental sample}}{\text{OD value of experimental control}} \times 100$$

Table 1. *In vitro* cytotoxicity and IC_{50} (µM/mL)

Compounds	MCF-7[a]	A-549[b]
4a	31±1.2	33±0.6
4b	**21±1.7**	**28±1.4**
4c	32±0.8	35±1.5
5a	35±1.6	37±1.7
5b	25±1.8	29±0.8
5c	36±0.5	39±1.3
5d	25±1.3	32±0.9
6a	26±0.9	29±1.2
6b	**22±0.**7	**24±1.5**
6c	28±1.4	31±1.7
6d	**20±1.1**	**25±0.8**
Cisplatin	18±1.5	19±1.7

[a] breast cancer.[b] lung cancer

As shown in **Table 1**, the synthesized compounds showed excellent to moderate anticancer activities against the two tested cancer cell lines. The cytotoxic activity results revealed that the majority of the synthesized compounds exhibited potent anticancer activity against MCF-7 cell line and moderate activity against A-549 cell line which is represented in **Table 1**. Among the synthesized compounds, compound **6d** was found to be the most potent derivative against MCF-7 with an IC_{50} value of 20±1.1 µM compared to the IC_{50} value of cisplatin (18±1.5 µM). The next most promising compound is **4b** which depicted stronger

cytotoxic activity against MCF-7 with an IC_{50} value of 21±1.7 µM, furthermore, compound **6b** displayed substantial activity (IC_{50} value of 22±0.7 µM) against MCF-7. In addition, compounds **6c, 5d, 5b, 4c, 6a** and **4a** with IC_{50} values of 28±1.4, 25±1.3, 25±1.8, 32±0.8, 26±0.9 and 31±1.2 µM respectively, showed good activity against MCF-7 cancer cell line. While compounds **5a** and **5c** were moderately active with IC_{50} values of 35±1.6 and 36±0.5 µM respectively.

Concerning activity against A-549 cell line, compounds **6b** and **6d** were found to be the most potent derivatives with IC_{50} values of 24±1.5 and 25±0.8 µM respectively, while compounds **4b**, **5b** and **6a** displayed significant IC_{50} values of 28±1.4, 29±0.8 and 29±1.2 µM respectively. Compounds **6c**, **5d**, **5c**, **5a**, **4c** and **4a** also showed moderate activity against A-549 with IC_{50} values of 31±1.7, 32±0.9, 39±1.3,37±1.7, 35±1.5 and 33±0.6 µM respectively. In general, it was found that all the synthesized compounds displayed selective cytotoxicity against MCF-7 compared to A-549 cell line.

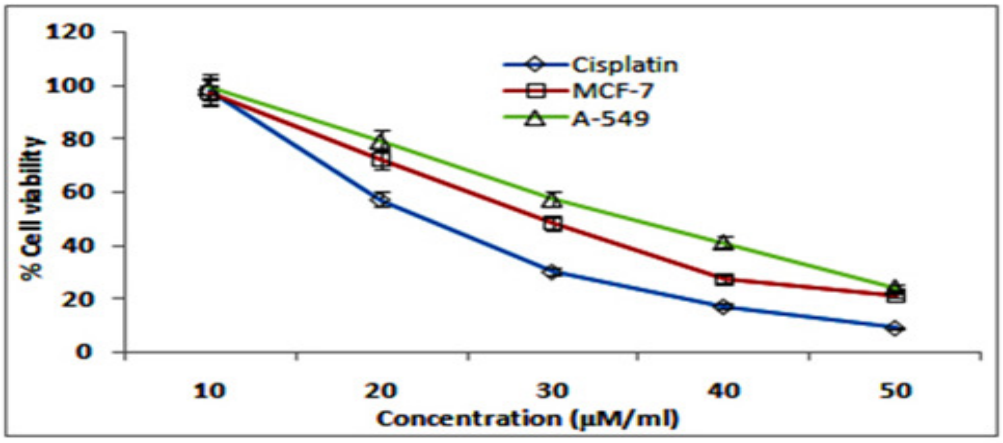


Fig.4. Cytotoxic effect of the compound **4b** on the viability of MCF-7 & A-549 cell lines

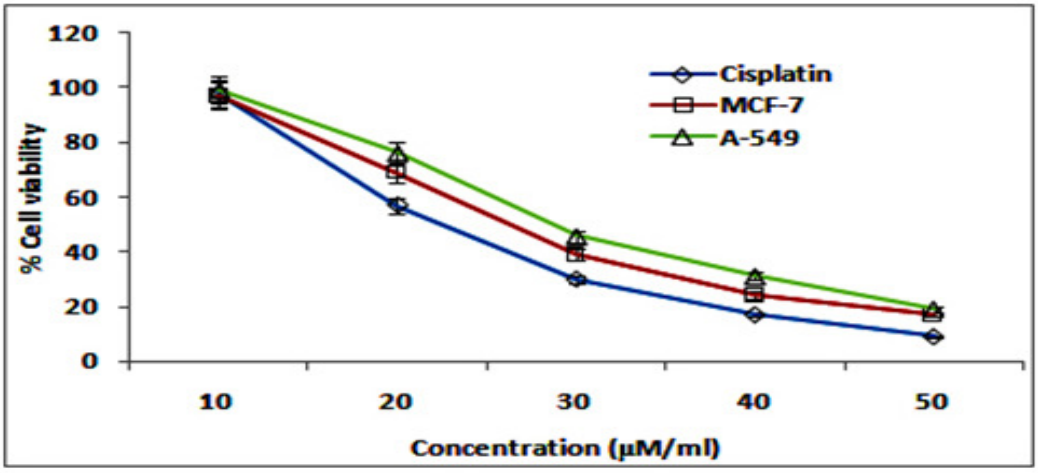


Fig.5. Cytotoxic effect of the compound **6b** on the viability of MCF-7 & A-549 cell lines

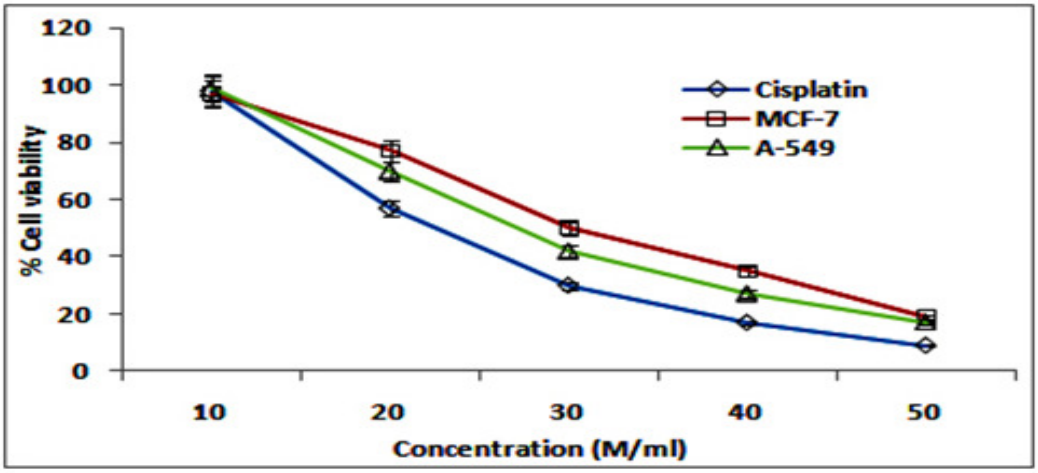


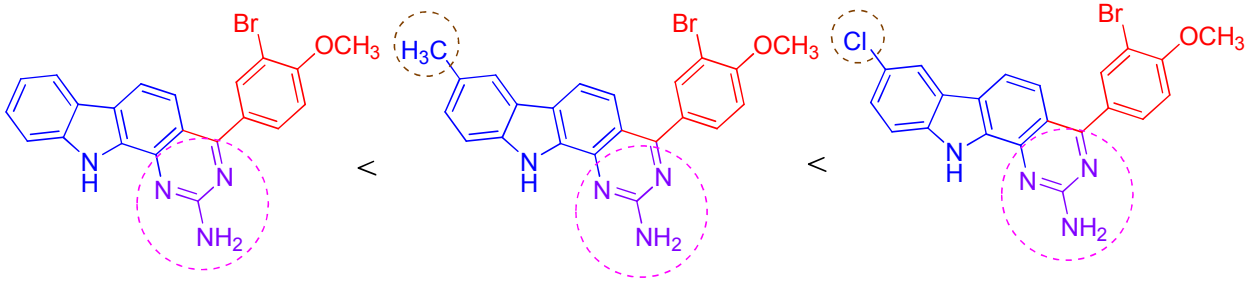
Fig.6. Cytotoxic effect of the compound **6d** on the viability of MCF-7 & A-549 cell lines

2.2.2. Structure activity relationship (SAR)

The results of the aforementioned anticancer activity lead to the following assumptions about the structural activity relationship (SAR):

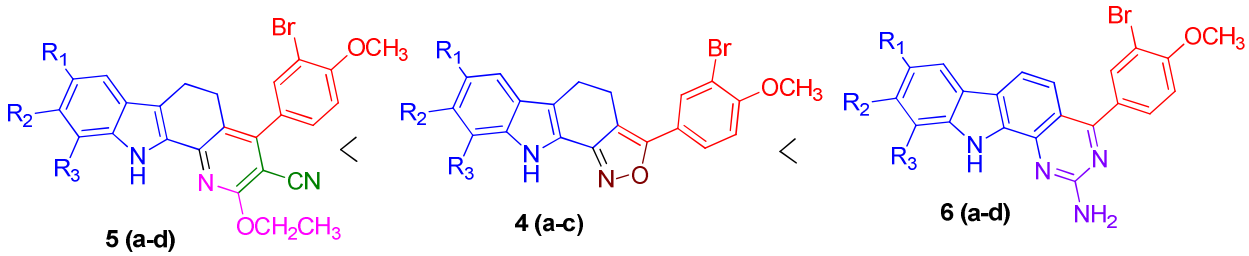
- It is clear from the results summarized in **Table 1** that, among the synthesized compounds, the compound **6d** showed highly improved cytoctoxic efficacy ($1C_{50}$; MCF-7=20±1.1 and A-549=25±0.8). Compounds **6a**, **6b** and **6c** also exhibited stronger cytotoxic activity against MCF-7 breast cancer cell line. This might be due to the presence of the pyrimido moiety and the amino group at the C_2 position which boosts the cytotoxic activity. The electron donating ability of the amino group increases the electron density of the parent molecule which rationally increases the potency of the compound towards anticancer activity. The role of NH_2 group at the C_2 position of pyrimido moiety in improving anticancer activities had been previously reported in the literature [34]. In addition combination of a pyrimidine group with a carbazole also increases its therapeutic value.
- The next most promising compounds were **4a**, **4b** and **4c** which exhibited potent cytotoxic activity. This might be due to the presence of isoxazolo moiety which enhanced the cytotoxic activity.
- Compounds **5a**, **5b**, **5c** and **5d** having pyrido group also showed moderate cytotoxic activities [35] with IC_{50} values <40 µM against MCF-7 cancer cell line.

❖ In general, it was observed that the substituent present in the carbazole ring plays a vital role in determining the anticancer potency. Among the hetero annulated carbazole compounds, the compounds bearing electron withdrawing chloro group in the pyrimidine annulated carbazole ring enhanced the cytotoxic activity more than the electron donating methyl group and unsubstituted group [36]. (**Scheme. 3**)



Scheme 3. Role of substituents in increasing the efficancy of cytotoxicity.

The detected patterns of cytotoxicity of annulated carbazoles are in the following order: **Scheme. 4**.



Scheme 4. Comparison cytotoxic activity of annulated hetrocyclics of carbazoles

1,3,4,5 & 6 a: R_1= R_2= R_3= H
b: R_1=CH_3; R_2= R_3=H
c: R_1= R_2= H; R_3=CH_3
d: R_1=Cl; R_2= R_3=H

2.2.3. Cell morphology analysis

To observe the effect of synthesized compounds on cell morphology, treated cancer cells were examined by inverted light microscopy and compared with untreated cells. Treated cells showed significant changes in comparison to the untreated cells. Cytological investigations elucidate the anticancer effect routed through membrane blebbing, membrane instability and disturbing the cytoskeleton of the cells by the compounds. **Fig.7** and **Fig.8** show the morphological changes in MCF-7 breast cancer and A-549 lung cancer cells after treatment with compounds with their respective inhibitory concentration for 24 h. Phase-contrast micrographs

revealed that the compounds **6b** and **6d** induce increased cell shrinkage, membrane blebbing and form floating cells, compared to compounds **5d**, **5b** and **4b** in a dose-dependent manner.

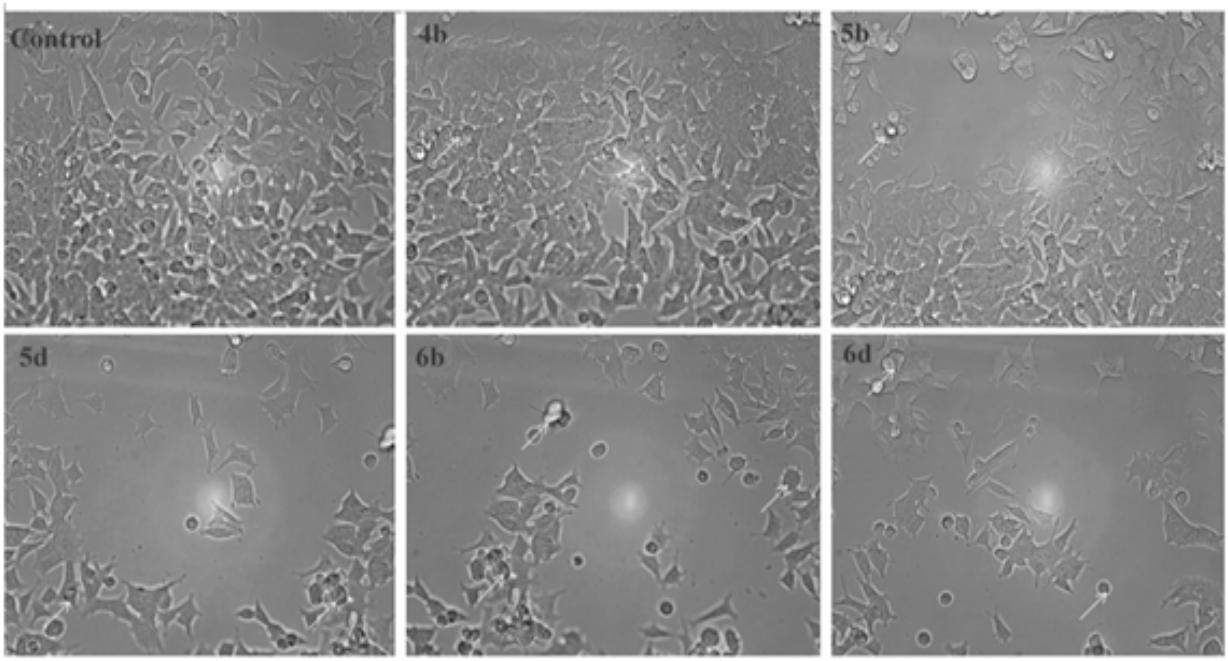


Fig.7. Morphometric analysis of treated MCF-7 cells the arrows indicates the formation of floating cells and appearance of membrane blebbing.

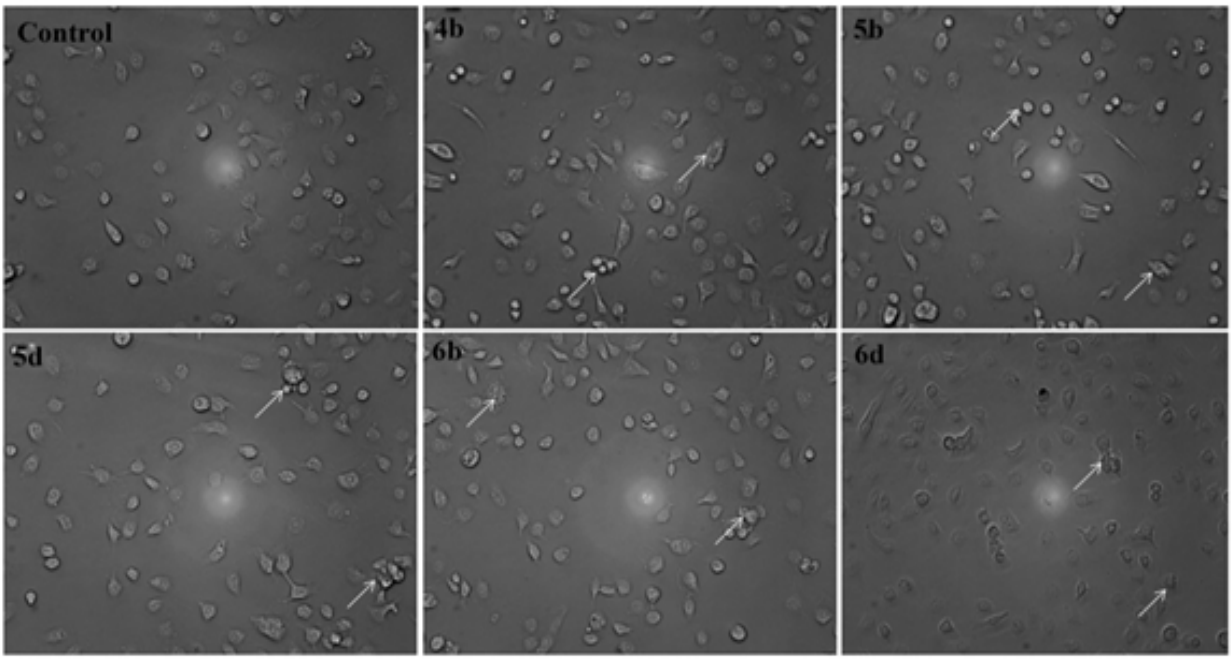


Fig.8. Morphometric analysis of treated A-549 cells the arrows indicates the formation of floating cells and appearance of membrane blebbing

2.2.4. Fluorescence microscopic analysis of cell death

Fluorescence microscopy analysis revealed the effects of synthesized compounds to induce apoptosis in MCF-7 breast cancer and A-549 lung cancer cells. Induction of apoptosis is the most important mechanism for many anticancer agents. In fact, an ideal anticancer agent mostly potentiates apoptotic effects in cancer cells [37]. Fluorescence microscopic analysis of cell death showed that treatment of cells with compounds induce more apoptotic cell death rather than necrotic death. Mechanism of cell death was studied by nuclear staining methods such as AO/EB and DAPI staining methods. Staining cells with fluorescent dye is used in evaluating the nuclear morphology of apoptotic cells. One of the characteristics of cells undergoing apoptosis is nuclear chromatin condensation. The DNA in condensed chromatin stains strongly with fluorescent dyes which allows for differentiation of apoptotic from non-apoptotic cells.

2.2.4.1. Acridine orange/ Ethidium bromide (AO/EB) staining method

To confirm the induction of apoptosis, treated cells were visualized by fluorescence microscopy following treatment with 1:1 ratio of AO/EB, which allow differentiation of dead and viable cells by staining DNA. Fluorescence microscopy images of MCF-7 and A-549 cancer cells in the absence of compounds (control) and in the presence of compounds are shown **Fig**.**9** and **Fig**.**10** respectively. The untreated MCF-7 cancer cells did not show any significant adverse effect compared to the compounds treated cancer cells. It can be observed that the addition of compounds **5d** and **6d** to the MCF-7 cancer cells, the fluorescence green colour of cells are changed to orange/red colour, which is due to induced apoptosis and nuclear condensation effect. The cells with intact membranes shows fluorescence green due to AO staining while EtBr stains cells with damaged membranes which exhibits orange/red fluorescence due to DNA intercalation of both stains. The rest of the compounds **4b**, **5b** and **6b** showed less fluorescence intensity leads to the reduced level of induction of apoptosis in the MCF-7 cells. In the case of lung cancer cell, compounds **4b**, **5b** and **6d** exhibited significant apoptotic induction rather than the compounds **5d** and **6b.** The apoptotic induction values of the synthesized compounds were remarkable in MCF-7 cells compared to the lung cancer cells.

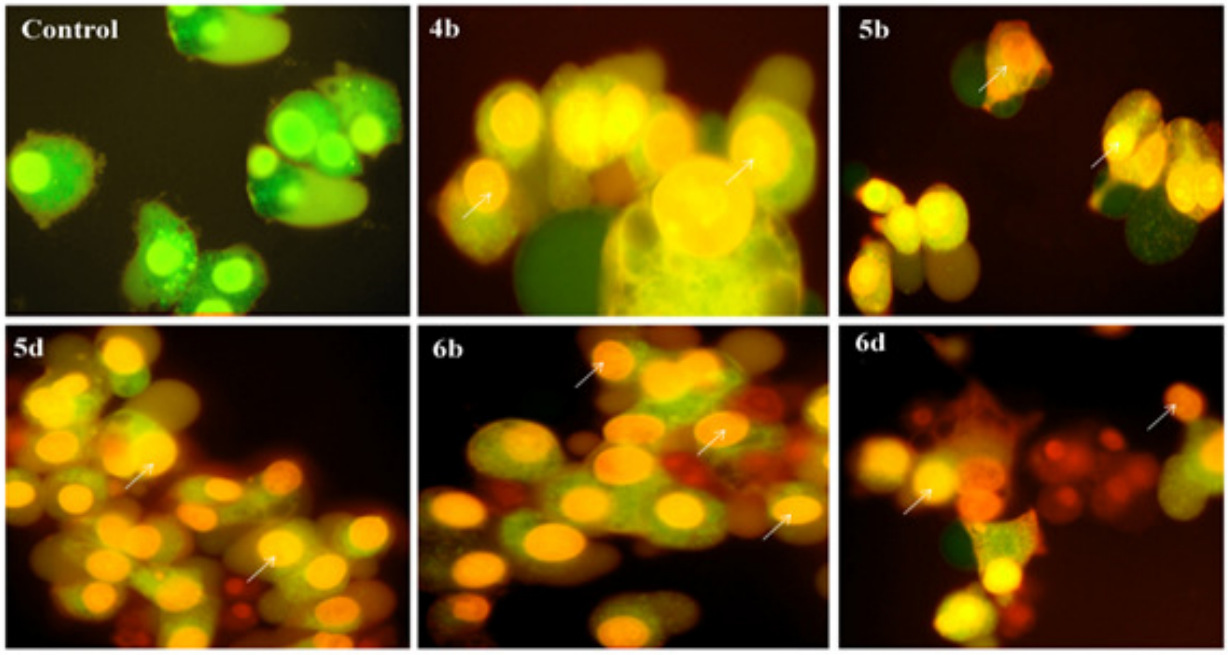


Fig.9. AO/EB apoptotic analysis of treated MCF-7 cells the arrows indicate apoptotic cells

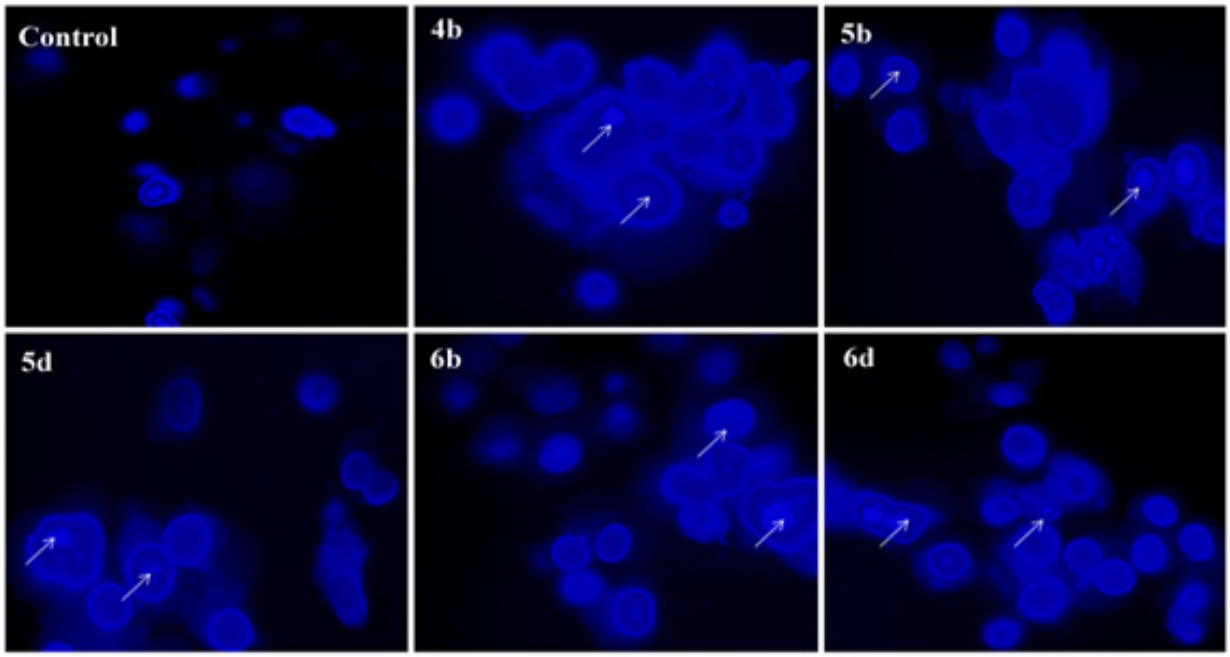


Fig.10. AO/EB apoptotic analysis of treated A-549 cells the arrows indicate apoptotic cells

2.2.4.2. DAPI staining method

In order to further confirm whether the synthesized compounds mediated cell death in MCF-7 breast cancer and A-549 lung cancer cells was due to apoptosis, the cells were stained with DAPI. DAPI (4',6-diamidino-2-phenylindole dihydrochloride) is a fluorescent nuclear dye that binds strongly to DNA. Fluorescence microscopic images of breast cancer cells after 24 h stained with DAPI in the presence and absence of compounds are shown in **Fig.11**. The compounds **4b**, **5b** and **6b** displayed higher level of nuclear fragmentation and the untreated cells did not show any significant changes in the nuclear appearance, whereas compounds **5d** and **6d** exhibited bright fetches when treated with MCF-7 cancer cells, which indicates the condensed chromatins and nuclear fragmentations in the cells. **Fig.12** showed fluorescent DAPI analysis of compounds treated A-549 lung cancer cells. Compounds **4b** and **6b** exhibit higher level of nuclear fragmentation in the treated A-549 cells, whereas, the compounds **5b**, **5d** and **6d** failed to show the same effect caused by compounds **4b** and **6b**. The bright fetch observed in compounds treated MCF-7 breast cancer cells are higher than that observed in A-549 lung cancer cells.

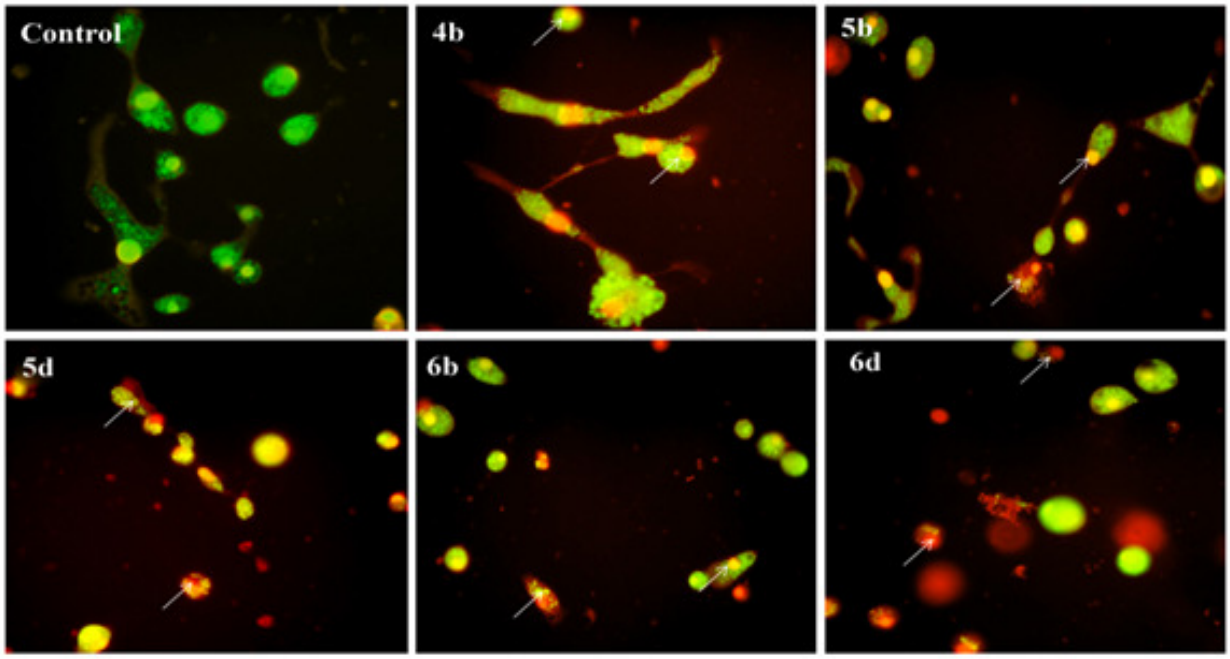


Fig.11. DAPI apoptotic analysis of treated MCF-7 cells the arrows indicate apoptotic cells

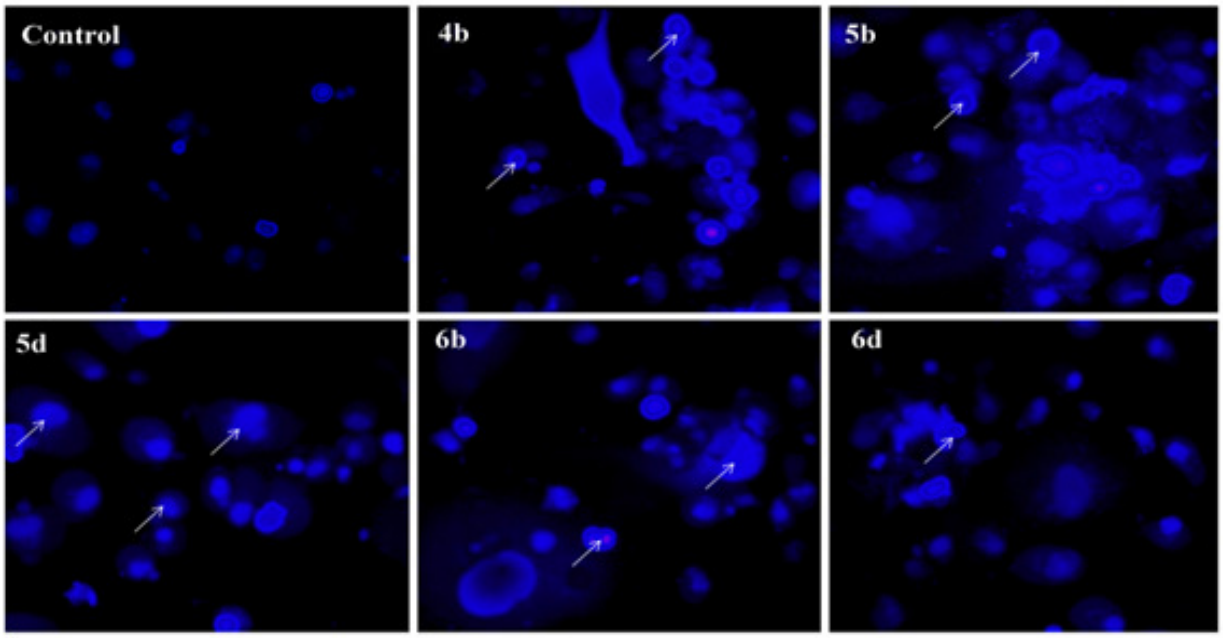


Fig.12. DAPI apoptotic analysis of treated A-549 cells the arrows indicate apoptotic cells

3. Conclusions

The newly synthesized heterocycles isoxazolo-, pyrido- and pyrimidocarbazoles were prepared from the easily accessible 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydro-1*H*-carbazol-1-one by cyclo condensation with appropriate reactants, hydroxylamine hydrochloride, malononitrile and guanidine nitrate. The structures of the compounds were characterized by spectroscopic and X-ray crystallographic methods. All the synthesized compounds were subjected to *in vitro* cytotoxicity against two human cancer cell lines: MCF-7 and A-549. Compound **6d** exhibited significant activity against MCF-7 and all other compounds showed moderate to potent activity and subsequent apoptotic cell death to be evidenced by AO/EB and DAPI of fluorescence microscopy analysis. The structure-activity relationship studies revealed that, the compound bearing the pyrimido moiety and the electron withdrawing chloro group in the carbazole displayed excellent cytoctoxic activity.

4. Experimental protocols

4.1. Chemistry

4.1.1. General

All the chemicals were bought from Sigma-Aldrich and Merck and were utilized for the process without further purification. Melting points (M.p.) were determined on a Mettler FP 51

apparatus (Mettler Instruments, Switzerland) and are uncorrected. They are expressed in degree centigrade (°C). FT-IR spectra were recorded on Avatar Model FT-IR(4000–400 cm^{-1}) spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Agilent- 400 MHz (^{1}H) and 100 MHz (^{13}C) spectrometers respectively in $CDCl_3$ using TMS (tetramethylsilane) as internal reference; chemical shifts are expressed in parts per million (ppm); coupling constants (*J*) are reported in hertz (Hz) and the terms J_o and J_m refer to ortho coupling constant and meta coupling constant. The signals were characterized as s (singlet), d (doublet), t (triplet), m (multipiet), bs (broad singlet) and dd (doublet, and doublet).Microanalyses were carried out using Vario EL III model CHNS analyzer (Vario, Germany). When known compounds had to be prepared according to literature procedures and pertinent references are given. The purity of the products was tested by TLC plates coated with silica gel-G using petroleum ether and ethyl acetate in the ratio of 1:1 as developing solvents.

4.2. Synthesis

*4.2.1. General procedure for the synthesis of 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one **3***

An equimolar mixture of the 2,3,4,9-tetrahydrocarbazol-1-one (**1**, 0.005 mol) and 3-bromo-4-methoxybenzaldehyde (**2**, 0.005 mol) was treated with 5 % ethanolic KOH (25 mL)solution and stirred for 24 h at room temperature. The completion of the reaction was monitored by TLC. After the completion of the reaction, the reaction mixture was cooled to room temperature and poured into ice cold water and neutralized with 1:1 HCl. The precipitated solid was filtered and purified by column chromatography over silica gel using petroleum ether: ethyl acetate (99:1) mixture as eluant to afford the respective product, 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one **3**.

4.2.1.1. 2-(3'-Bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one (3a). Yellow solid; m.p. 275-277°C; Yield: 83%; IR (KBr, cm^{-1}) υ_{max}: 3262 (NH), 1639 (C=O); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.09 (b s, 1H, N_9-H), 7.68-7.36 (m, 7H, C_7, C_6, C_5, C_2, C_6', C_5' & C_2'-H), 6.95 (d, 1H, C_8-H, J_o= 8.0 Hz), 3.94 (s, 3H, C_4'-OCH_3), 3.26-3.23 (m, 2H, C_3-2H), 3.09-3.06 (m, 2H, C_4-2H);^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc:180.4 (C_1), 155.8 (C_4'), 138.4 (C_{8a}), 135.6 (C_2), 134.5 (C_{2a}), 133.5 (C_2'), 132.2 (C_6'), 130.4 (C_{9a}), 130.0 (C_{4b}), 128.1 (C_1'), 127.2 (C_{4a}), 125.9

(C_7), 121.3 (C_6), 120.5 (C_5), 112.4 (C_5' & C_3'), 111.6 (C_8), 56.3 (C_4'-OCH_3), 27.5 (C_3), 20.7 (C_4); Anal.Calcd.for $C_{20}H_{16}BrNO_2$: C, 62.84; H, 4.22; N, 3.66. Found: C, 62.95; H, 4.27; N, 3.63 %.

4.2.1.2. 2-(3'-Bromo-4'-methoxybenzylidene)-6-methyl-2,3,4,9-tetrahydrocarbazol-1-one (3b). Yellow solid; m.p. 277-279°C; Yield: 75%; IR (KBr, cm^{-1}) υ_{max}: 3276 (NH), 1635 (C=O); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.04 (b s, 1H, N_9-H), 7.68-7.67 (m, 2H, C_2' & C_5-H), 7.43 (s, 1H, olefinic-H), 7.39 (d d, 1H, C_6'-H, J_m = 2.0 Hz & J = 8.4 Hz), 7.36 (d, 1H, C_8-H, J_o= 8.4 Hz), 7.22 (d d, 1H, C_7-H, J_m = 1.2 Hz & J_o = 8.4 Hz), 6.95 (d, 1H, C_5'-H, J_o = 8.4 Hz), 6.96-6.94 (d, 1H, C_8-H, J_o = 8.0 Hz) 3.95 (s, 3H, C'$_4$-OCH_3), 3.25-3.22 (m, 2H, C_3-2H), 3.07-3.04 (m, 2H, C_4-2H), 2.45 (s, 3H, C_6-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc:180.6 (C_1), 155.9 (C_4'), 137.1 (C_2), 135.9 (C_{8a}), 134.6 (C_{2a}), 133.5 (C_2'), 132.5 (C_6'), 130.6 (C_{9a}), 130.2 (C_6), 130.0 (C_1'), 129.4 (C_{4b}), 127.8 (C_{4a}), 126.2 (C_7), 120.7 (C_5), 112.3 (C_5'), 111.8 (C_3'), 111.8 (C_8), 56.4 (OCH_3), 27.7 (C_3), 21.5 (C_3), 20.9 (CH_3); Anal.Calcd.for $C_{21}H_{18}BrNO_2$: C, 63.65; H, 4.58; N, 3.53. Found: C, 63.74; H, 4.52; N, 3.56 %.

4.2.1.3. 2-(3'-Bromo-4'-methoxybenzylidene)-8-methyl-2,3,4,9-tetrahydrocarbazol-1-one (3c). Yellow solid; m.p. 276-278°C; Yield: 76%; IR (KBr, cm-1) υ_{max}: 3204 (NH), 1644 (C=O); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.81 (b s, 1H, N_9-H), 7.68-7.67 (m, 2H, olefinic & C_2'-H), 7.51(d, 1H, C_5-H, J_o = 7.6 Hz), 7.38 (d d, 1H, C_6'-H, J_o= 2.0 Hz & J_m = 8.0 Hz), 7.18 (d, 1H, C_7-H J_o = 6.4 Hz), 7.08 (t, 1H, C_6-H, J = 7.6 Hz), 6.95 (d, 1H, C_5'-H, J_o = 8.0 Hz), 3.94 (s, 3H, C_4'-OCH_3), 3.26-3.23 (m, 2H, C_3-2H), 3.09-3.05 (m, 2H, C_4-2H), 2.51 (s, 3H, C_8-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc:180.4 (C_1), 155.8 (C_4'), 138.2 (C_2), 135.6 (C_{8a}), 134.4 (C_{2a}), 133.5 (C_2'), 132.0 (C_6'), 130.4 (C_{9a}), 130.0 (C_6), 128.7 (C_1'), 127.4 (C_{4b}), 125.5 (C_{4a}), 121.7 (C_7), 120.8 (C_5), 118.9 (C_5'), 111.7 (C_3'), 111.7 (C_8), 56.3 (OCH_3), 27.5 (C_4), 20.8 (C_3), 16.6 (CH_3); Anal.Calcd.for $C_{21}H_{18}BrNO_2$: C, 63.65; H, 4.58; N, 3.53. Found: C, 63.71; H, 4.64; N, 3.49 %.

4.2.1.4. 2-(3'-Bromo-4'-methoxybenzylidene)-6-chloro-2,3,4,9-tetrahydrocarbazol-1-one (3d). Yellow solid; m.p. 279-281°C; Yield: 75%; IR (KBr, cm^{-1}) υ_{max}: 3249 (NH), 1648 (C=O); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.04 (b s, 1H, N_9-H),7.68-7.63 (m, 3H, olefinic, C_2' & C_6' -H), 7.39-7.30(m, 3H, C_8, C_7 & C_5-H), 6.95 (d, 1H, C_5'-H, J_o = 8.8 Hz), 3.94 (s, 3H, C_4'-OCH_3), 3.25-3.22 (m, 2H, C_3-2H), 3.04-3.01 (m, 2H, C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm)

δc:180.3 (C_1), 154.0 (C_4'), 137.5 (C_2), 136.0 (C_{8a}), 134.3 (C_{2a}), 133.7 (C_2'), 132.2 (C_6'), 131.1 (C_{9a}), 130.5 (C_6), 130.4 (C_1'), 129.7 (C_{4b}), 128.1 (C_{4a}), 126.7 (C_7), 121.5 (C_5), 112.7 (C_5'), 111.6 (C_3'), 111.4 (C_8), 56.3 (OCH_3), 27.4 (C_3), 21.7 (C_3); Anal.Calcd.for $C_{20}H_{15}BrClNO_2$: C, 57.65; H, 3.63; N, 3.36. Found: C, 57.77; H, 3.67; N, 3.32 %.

4.2.2. General procedure for the synthesis of 3-(3'-bromo-4'-methyoxyphenyl)-4,5-dihydro-10H-isoxazolo[3,4-a]carbazole 4.

The 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one (1, 0.001 mol) was refluxed with hydroxylamine hydrochloride (0.014 mol) in pyridine (5 mL) at 130 ºC for 8 h. The reaction was monitored by TLC. After completion of the reaction, the crude mixture was poured into ice-cold water and neutralized with 1:1 HCl, the resulting semi-solid that separated was extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulphate. It was then purified by column chromatography over silicagel using pet.ether: ethyl acetate (98:2) to yield the respective 3-(3'-bromo-4'-methyoxyphenyl)-4,5-dihydro-10*H*-isoxazolo[3,4-*a*]carbazole **4**.

4.2.2.1. 3-(3'-Bromo-4'-methyoxyphenyl)-4,5-dihydro-10H-isoxazolo[3,4-a]carbazole (4a).
Brown solid; m.p. 209-211 ºC; Yield: 70%; IR (KBr, cm^{-1}) υ_{max}: 3222 (NH), 1500 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.80 (b s, 1H, N_{10}-H), 8.21 (d, 1H, C_2'-H, J_m = 2.0 Hz), 8.12 (d, 1H, C_6'-H, J_o = 8.0 Hz), 8.02 (d, 1H, C_9-H, J_o = 8.4 Hz) 7.94-7.92 (m, 1H, C_7-H), 7.69-7.66 (m, 1H, C_8-H), 7.61 (d, 1H, C_5'-H, J_o = 8.0 Hz), 7.56 (d, 1H, C_6-H, J_o = 7.6 Hz), 3.95 (s, 3H, C_4'-OCH_3), 2.96-2.88 (m, 4H, C_5 & C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 163.6 (C_3), 155.8 (C_4'), 133.0 (C_{10b}), 133.0 (C_{9a}), 131.2 (C_{10a}), 128.5 (C_2'), 126.7 (C_6'), 124.1 (C_1'), 122.8 (C_{5b}), 120.6 (C_8), 120.2 (C_7), 119.4 (C_6), 117.1 (C_{5a}), 112.2 (C_3'), 112.1 (C_5'), 111.5 (C_9), 97.1 (C_{3a}), 56.3 (C_4'-OCH_3), 20.0 (C_5), 21.7 (C_4); Anal.Calcd.for $C_{20}H_{15}BrN_2O_2$: C, 60.78; H, 3.83; N, 7.09. Found: C, 60.69; H, 3.87; N, 7.04 %.

*4.2.2.2. 3-(3'-Bromo-4'-methyoxyphenyl)-4,5-dihydro-7-methyl-10H-isoxazolo[3,4-a]carbazole (4b).*Brown solid; m.p. 208-210 ºC; Yield: 63%; IR (KBr, cm^{-1}) υ_{max}: 3226 (NH), 1536 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.85 (b s, 1H, N_{10}-H), 7.38 (s, 1H, C_6-H), 7.32 (s, 1H, C_2'-H), 7.28 (d, 1H, C_8-H, J_o = 8.4 Hz), 7.19 (d, 1H, C_6'-H, J_o= 8.4 Hz), 7.11 (d, 1H, C_9, J_o = 8.4

Hz), 7.05 (d, 1H, C_5'-H, J_o = 8.4 Hz), 3.96 (s, 3H, C_4'-OCH_3), 2.89-2.80 (m, 4H, C_5 & C_4-2H), 2.44 (s, 3H, C_7-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 162.9 (C_3), 155.6 (C_4'), 133.3 (C_{10b}), 133.1 (C_{9a}), 130.9 (C_{10a}), 128.7 (C_2'), 125.6 (C_6'), 124.4 (C_1'), 123.0 (C_{5b}), 121.5 (C_8), 120.7 (C_7), 118.5 (C_6), 117.5 (C_{5a}), 112.7 (C_3'), 112.3 (C_5'), 111.8 (C_9), 96.4 (C_{3a}), 57.0 (OCH_3), 21.0 (C_5), 20.7 (C_4) 16.3 (CH_3); Anal.Calcd.for $C_{21}H_{17}BrN_2O_2$: C, 61.63; H, 4.19; N, 6.84. Found: C, 61.71; H, 4.15; N, 6.92 %.

4.2.2.3. 3-(3'-Bromo-4'-methyoxyphenyl)-4,5-dihydro-8-methyl-10H-isoxazolo[3,4-a]carbazole (4c). Brown solid; m.p. 215-217 ºC; Yield: 58 %; IR (KBr, cm^{-1}) υ_{max}: 3367 (NH), 1492 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.40 (b s, 1H, N_{10}-H), 7.56 (s, 1H, C_2'-H), 7.45 (d, 1H, C_6-H, J_o = 7.6 Hz), 7.40-7.22 (m, 3H, C_8, C_7 & C_5'-H), 6.90 (d, 1H, C_6'-H, J_o = 8.4 Hz), 3.92 (s, 3H, C_4'-OCH_3), 3.00-2.99 (m, 2H, C_4-2H), 2.94-2.91 (m, 2H, C_5-2H), 2.43 (s, 3H, C_8-H); Anal.Calcd.for $C_{21}H_{17}BrN_2O_2$: C, 61.63; H, 4.19; N, 6.84. Found: C, 61.69; H, 4.17; N, 6.89 %.

4.2.3. General procedure for the synthesis of 4-(3'-bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-11H-pyrido[2,3-a]carbazole-3-carbonitrile 5

The appropriate 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one (**3**,0.001 mol) in dry ethanol (20 mL) was added to an ice-cooled solution of 1.00g of NaH in dry benzene (10 mL). To this, malononitrile (0.001mol) was added and refluxed on an oil bath for 5 h. The reaction monitored by TLC indicated the formation of product. The mixture was poured into ice-cold water. The brown solid that separated was neutralized with 1:1 HCl, then filtered and dried. It was then purified by column chromatography over silica gel using pet.ether: ethyl acetate (98:2) as eluant to yield the respective 4-(3'-bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-11*H*-pyrido[2,3-*a*]carbazole-3-carbonitrile **5**.

4.2.3.1. 4-(3'-Bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-11H-pyrido[2,3-a]carbazole-3-carbonitrile (5a). Yellow solid; m.p. 265-267 °C; yield: 81%; IR (KBr, cm^{-1}) υ_{max}: 3335 (NH), 2218 (CN), 1555 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.76 (b s, 1H, N_{11}-H), 7.58 (d, 1H, C_7-H, J_o = 7.6 Hz), 7.52 (d, 1H, C_2'-H, J_m = 2.0 Hz), 7.43 (d, 1H, C_{10}-H, J_o = 8.0 Hz), 7.30-7.26 (m, 2H, C_6' & C_9-H), 7.14 (t, 1H, C_8-H, J = 7.6 Hz), 7.02 (d, 1H, C_5'-H, J_o = 8.4 Hz), 4.64-4.58 (q, 2H, O**CH_2**CH_3, J = 7.2 Hz), 3.96 (s, 3H, C_4'-OCH_3), 2.97-2.87 (m, 4H, C_6 & C_5-2H),

1.50 (t, 3H, $OCH_2C\mathbf{H_3}$, *J* = 7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 148.7 (C_2 & C_4'), 137.9 (C_{11b} & C_4), 133.2 (C_{10a}), 132.1 (C_1' & C_2'), 128.9 (C_6' & C_{11a}), 126.7 (C_{6b} & C_{4a}), 124.7 (C_9), 121.4 (C_8 & C_7), 120.3 (C_5' & C_3'), 119.9 (CN), 1119 (C_3 & C_{6a}), 111.7 (C_{10}), 63.0 ($O\mathbf{C}H_2CH_3$), 56.3 (C_4'-OCH_3), 25.2 (C_5), 19.4 (C_6), 14.5 ($OCH_2\mathbf{C}H_3$); Anal.Calcd.for $C_{25}H_{20}BrN_3O_2$: C, 63.30; H, 4.25; N, 8.86. Found: C, 63.39; H, 4.29; N, 8.79 %.

4.2.3.2. 4-(3'-Bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-8-methyl-11H-pyrido[2,3-a]carbazole-3-carbonitrile (5b). Yellow solid; mp 263-265 °C; yield: 79%; IR (KBr, cm^{-1}) υ_{max}: 3380 (NH), 2206 (CN), 1553 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.65 (b s, 1H, N_{11}-H), 7.52-7.09 (m, 5H, C_{10}, C_9, C_7, C_6' & C_2'-H), 7.03 (d, 1H, C_5'-H, J_o = 8.4 Hz), 4.63-4.57 (q, 2H, $OC\mathbf{H_2}CH_3$, *J* = 7.0 Hz), 3.96 (s, 3H, C_4'-OCH_3), 2.91-2.85 (m, 4H, C_6 & C_5-2H), 2.44 (s, 3H, C_8-CH_3) 1.49 (t, 3H, $OCH_2C\mathbf{H_3}$, *J* = 7.0 Hz); Anal.Calcd.for $C_{26}H_{22}BrN_3O_2$: C, 63.94; H, 4.54; N, 8.60. Found: C, 63.85; H, 4.49; N, 8.66 %.

4.2.3.3. 4-(3'-Bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-10-methyl-11H-pyrido[2,3-a]carbazole-3-carbonitrile (5c). Yellow solid; mp 264-256 °C; yield: 77%; IR (KBr, cm^{-1}) υ_{max}: 3367 (NH), 2215 (CN), 1552 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.58 (b s, 1H, N_{11}-H), 7.52 (d, 1H, C_2'-H, J_m = 2.0 Hz), 7.42 (d, 1H, C_7-H, J_o= 6.4 Hz), 7.30 (d d, 1H, C_6'-H, J_m = 2.0 Hz & J_o = 8.4 Hz), 7.10-7.03 (m, 2H, C_9 & C_8-H), 7.02 (d, 1H, C_5'-H, J_o = 8.4 Hz) 4.66-4.60 (q, 2H, $OC\mathbf{H_2}CH_3$, *J* = 6.8 Hz), 3.96 (s, 3H, C_4'-OCH_3), 2.93-2.83 (m, 4H, C_6 & C_5-2H), 2.57 (s, 3H, C_{10}-CH_3) 1.52-1.49 (t, 3H, $OCH_2C\mathbf{H_3}$, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 163.5 (C_2), 156.4 (C_{11b}), 152.3 (C_4'), 148.8 (C_4), 137.5 (C_{10a}), 133.2 (C_1'), 131.8 (C_{11a}), 129.0 (C_6' & C_2'), 126.3 (C_{6b} & C_{4a}), 125.2 (C_8), 121.5 (C_{10}), 120.9 (C_9), 120.5 (C_7), 119.7 (C_{6a}), 117.6 (CN), 115.7 (C_5' & C_3'), 111.9 (C_3), 63.1 ($O\mathbf{C}H_2CH_3$), 56.3 (OCH_3), 25.3 (C_5), 19.5 (C_6), 16.7 (CH_3), 14.5 ($OCH_2\mathbf{C}H_3$); Anal.Calcd.for $C_{26}H_{22}BrN_3O_2$: C, 63.94; H, 4.54; N, 8.60. Found: C, 63.87; H, 4.48; N, 8.65 %.

4.2.3.4. 4-(3'-Bromo-4'-methoxyphenyl)-2-ethoxy-5,6-dihydro-8-chloro-11H-pyrido[2,3-a]carbazole-3-carbonitrile (5d). Yellow solid; mp 269-270 °C; yield: 68%; IR (KBr, cm^{-1}) υ_{max}: 3316 (NH), 2219 (CN), 1556 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 8.79 (b s, 1H, N_{11}-H), 7.53-7.02 (m, 6H, C_{10}, C_9, C_7, C_6', C_5' & C_2'-H), 4.63-4.57 (q, 2H, $OC\mathbf{H_2}CH_3$, *J* = 7.0 Hz),

3.96 (s, 3H, C_4'-OCH_3), 2.92-2.85 (m, 4H, C_6 & C_5-2H), 1.51-1.48 (t, 3H, $OCH_2C\mathbf{H_3}$, *J* = 7.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc:158.2 (C_2 & C_{11b}), 156.0 (C_4' & C_4), 134.8 (C_{10}), 133.0 (C_{11a}), 131.2 (C_1'), 129.3 (C_2' & C_6'), 128.8 (C_{6b}), 126.5 (C_8), 120.8 (C_{4a}), 119.4 (C_9), 114.2 (C_7), 113.5 (CN), 112.3 (C_{6a}), 112.3 (C_{10}), 111.9 (C_5' & C_3'), 111.7 (C_3), 61.9 ($O\mathbf{C}H_2CH_3$), 56.4 (OCH_3), 27.3 (C_5), 20.7 (C_6), 14.7 ($OCH_2\mathbf{C}H_3$); Anal.Calcd.for $C_{25}H_{19}BrClN_3O_2$: C, 59.02; H, 3.76; N, 8.26. Found: C, 59.11; H, 3.72; N, 8.20 %.

4.2.4. General procedure for the synthesis of 2-amino-4-(3'-bromo-4'-methoxyphenyl)-11H-pyrimido[4,5-a]carbazole 6

A mixture of respective 2-(3'-bromo-4'-methoxybenzylidene)-2,3,4,9-tetrahydrocarbazol-1-one (**3**, 1 mmol), guanidine nitrate (0.10 mol) and benzene (10 mL) was refluxed in the presence of sodiumhydride (1.00 g) for 18 h. The reaction was monitored by TLC. After completion of the reaction, the excess of solvent was boiled off and the residue was poured into crushed ice. The mixture was then neutralized with 1:1 HCl and extracted with ethyl acetate. The organic layer was washed with water and dried over anhydrous sodium sulphate, upon removal of the solvent a brown mass was obtained.It was purified by column chromatography over silica gel using petroleum ether:ethyl acetate (85:15) to yield the respective 2-amino-4-(3'-bromo-4'-methoxyphenyl)-11*H*-pyrimido[4,5-*a*]carbazole **6**.

*4.2.4.1. 2-Amino-4-(3'-bromo-4'-methoxyphenyl)-11H-pyrimido[4,5-a]carbazole (6a).*Yellow solid; m.p. 292-294 °C; yield: 77%; IR (KBr, cm^{-1}) υ_{max}: 3405 (asym NH_2), 3328 (sym NH_2), 3212 (NH), 1573 (C=N), 1540 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.90 (b s, 1H, N_{11}-H), 8.11 (d, 1H, C_7-H, J_o = 8.0 Hz), 8.04 (s, 1H, C_2'-H), 7.91(d, 1H, C_{10}-H, J_o = 8.8 Hz), 7.75 (d, 1H, C_6-H, J_o = 8.4 Hz), 7.63 (d, 1H, C_5-H, J_o= 8.4 Hz), 7.58 (d, 1H, C_6'-H, J_o = 8.0 Hz), 7.48 (t, 1H, C_8-H, *J* = 7.2 Hz), 7.29 (t, 1H, C_9-H, *J* = 7.2 Hz), 7.06 (d, 1H, C_5'-H, J_o = 8.0 Hz), 5.57 (s, 2H, C_2-NH_2), 4.00 (s, 3H, C_4'-OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 139.5 (C_2 & C_4'), 134.7 (C_4 & C_{11b}), 130.4 (C_{10}), 126.7 (C_{11a} & C_2'), 123.2 (C_6' & C_1'), 120.7 (C_5 & C_{6b}), 120.1 (C_6 & C_8), 117.8 (C_7 & C_9), 116.7 (C_{6b} & C_{4a}), 111.8 (C_5' & C_3'), 111.4 (C_{10}), 56.4 (C_4'-OCH_3); Anal.Calcd.for $C_{21}H_{15}BrN_4O$: C, 60.16; H, 3.61; N, 13.36. Found: C, 60.25; H, 3.65; N, 13.29 %.

4.2.4.2. 2-Amino-4-(3'-bromo-4'-methoxyphenyl)-8-methyl-11H-pyrimido[4,5-a]carbazole (6b). Yellow solid; m.p. 295-297 °C; yield: 68%; IR (KBr, cm^{-1}) υ_{max}: 3414 (asymNH_2, sym NH_2& indole NH), 1575 (C=N), 1541 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.97 (b s, 1H, N_{11}- H), 8.04 (s, 1H, C_7-H), 7.88-7.74 (m, 3H, C_9, C_6' & C_2'-H), 7.61 (d, 1H, C_6-H, J_o = 8.4 Hz), 7.49 (d, 1H, C_5-H, J_o = 8.4 Hz), 7.33 (d, 1H, C_{10}-H, J_o = 8.4 Hz), 7.06 (d, 1H, C_5'-H, J_o = 8.0 Hz), 5.84 (s, 2H, C_2-NH_2), 4.00 (s, 3H, C_4'-OCH_3), 2.54 (s, 3H, C_8-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δc: 158.7 (C_2), 151.6 C_4'), 147.5 (C_4), 144.0 (C_{11b}), 131.5 (C_{10a}), 129.4 (C_2'), 128.5 (C_{6a}), 127.5 (C_8), 125.1 (C_{6b}), 124.9 (C_6'), 124.2 (C_1'), 123.5 (C_{11a}), 122.1 (C_5), 121.8 (C_7), 116.6 (C_6), 115.7 (C_9), 114.6 (C_{4a}), 113.8 (C_3'), 113.6 (C_5'), 112.6 (C_{10}), 51.6 (OCH_3), 20.5 (CH_3); Anal.Calcd.for $C_{22}H_{17}BrN_4O$:C, 60.98; H, 3.95; N, 12.93. Found:C, 60.89; H, 3.98; N, 12.97 %.

4.2.4.3. 2-Amino-4-(3'-bromo-4'-methoxyphenyl)-10-methyl-11H-pyrimido[4,5-a]carbazole (6c). Yellow solid; mp 296-278 °C; yield: 71%; IR (KBr, cm^{-1}) υ_{max}: 3391(asymNH_2), 3360 (sym NH_2& indole NH), 1544 (C=N), 1497 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 10.82 (b s, 1H, N_{11}- H), 7.80-6.98 (m, 8H, C_9, C_8, C_7, C_6, C_5, C_6', C_5' & C_2'-H), 5.66 (s, 2H, C_2-NH_2), 4.02 (s, 3H, C_4'-OCH_3), 2.39 (s, 3H, C_{10}-CH_3); Anal.Calcd.for $C_{22}H_{17}BrN_4O$:C, 60.98; H, 3.95; N, 12.93. Found: C, 60.91; H, 3.97; N, 12.98 %.

4.2.4.4. 2-Amino-4-(3'-bromo-4'-methoxyphenyl)-8-chloro-11H-pyrimido[4,5-a]carbazole (6d). Yellow solid; mp 299-281 °C; yield: 63%; IR (KBr, cm^{-1}) υ_{max}: 3401(symNH_2), 3387 (sym NH_2), 3204 (NH), 1545 (C=N), 1496 (C=N); ^{1}H-NMR (400 MHz,$CDCl_3$) (ppm) δ: 9.28 (b s, 1H, N_{11}- H), 7.79 (s, 1H, C_5-H), 7.53 (s, 1H, C_2'-H), 7.48-7.17 (m, 5H, C_{10}, C_9, C_7, C_6 & C_6'-H), 6.96 (d, 1H, C_5'-H, J_o = 8.8 Hz), 5.06 (s, 2H, C_2-NH_2), 3.94 (s, 3H, C_4'-OCH_3); Anal.Calcd.for $C_{21}H_{14}BrClN_4O$: C, 55.59; H, 3.11; N, 12.35. Found: C, 55.51; H, 3.15; N, 12.41 %.

4.3. Biological evaluation

4.3.1. In vitro cytotoxic activity

4.3.1.1. Cell line and cell culture

The Human lung and breast cancer cells were purchased from the National Center for Cell Sciences (NCCS), Pune, India. The cancer cells were maintained in Dulbecco's modified

eagles medium (DMEM) supplemented with 2mM l-glutamine and balanced salt solution (BSS) adjusted to contain 1.5 g/L Na_2CO_3, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, 1.5 g/L glucose, 10 mM (4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid) (HEPES) and 10% fetal bovine serum (GIBCO, USA). Penicillin and streptomycin (100 IU/100µg) were adjusted to 1mL/L. The cells were maintained at 37◦C with 5% CO_2 in a humidified CO_2 incubator.

4.3.1.2. In vitro assay

The inhibitory concentration (IC_{50}) value was evaluated using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Cancer cells were grown (1×10^4 cells/well) in a 96-well plate for 48 h in to 75% confluence. The medium was replaced with fresh medium containing serially diluted synthesized compounds, and the cells were further incubated for 48 h. The culture medium was removed, and 100µL of the MTT [3-(4,5-dimethylthiozol-2-yl)-3,5-diphenyl tetrazolium bromide] (Hi-Media) solution was added to each well and incubated at 37 ◦C for 4 h. After removal of the supernatant, 50 µL of DMSO was added to each of the wells and incubated for 10 min to solubilize the formazan crystals. The optical density was measured at 620 nm in an ELISA multiwell plate reader (ThermoMultiskan EX, USA).

4.3.2. Cell morphology analysis

The MCF-7 and A-549 cells that were grown on cover slips (1×10^5 cells/cover slip) incubated for 6-24 h with compounds at the IC_{50} concentration, and they were then fixed in an ethanol:acetic acid solution (3:1; v/v). The cover slips were gently mounted on glass slides for the morphometric analysis. Three monolayers per experimental group were photo micrographed. The morphological changes of the MCF-7 and A-549 selected cells were analyzed using Nikon (Japan) bright field inverted light microscopy at 40x magnification.

4.3.3. Fluorescence microscopic analysis of apoptotic cell death

Approximately 1µL of a dye mixture (100 mg/mL acridine orange (AO) and 100 mg/mL ethidium bromide (EtBr) in distilled water) was mixed with 9 mL of cell suspension (1×10^5 cells/mL) on clean microscope cover slips. The selected cancer cells were collected, washed with phosphate buffered saline (PBS) (pH 7.2) and stained with 1 mL of AO/EtBr. After incubation for 2 min, the cells were washed twice with PBS (5 min each) and visualized under a

fluorescence microscope (Nikon Eclipse, Inc, Japan) at 400× magnification with an excitation filter at 480 nm. Likewise the cells were plated on glass coverslip in a 24-well plate and treated with complex for 24 h. The fixed cells were permeabilised with 0.2% triton X-100 (50µl) for 10 min at room temperature and incubated for 3 min with 10µl of DAPI by placing a coverslip over the cells to enable uniform spreading of the stain. The cells were observed under (Nikon Eclipse, Inc, Japan) fluorescent microscope.

Acknowledgments

We would like to thank the University of Mysore, for NMR data. X-ray diffraction was collected at the University of Bristol, United Kingdom. Financial support from "UGC-Emeritus fellowship" for research, Dr. K. J. Rajendra Prasad gratefully acknowledged. We also thank UGC-RGNF, New Delhi for further financial assistance and successful completion of this work.

Supplementary data

CIF files for the compounds **5a** (anhydrous), **5a** (hydrate)and **6a** have been deposited with the Cambridge Crystallographic Data Centre as CCDC numbers 1410837, 1410840 and 1410844 respectively. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK. [Fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk.Spectral data of all the compounds are associated with this article will be available as supporting information.

References

[1] V. Kouznetsov, F. Rojas Ruiz, L.Vargas Mendez, M. Gupta, Letters in Drug Design & Discovery 9 (2012) 680–686.

[2] B. Bello, O. Fadahun, D. Kielkowski, G. Nelson, Trends in lung cancer mortality in South Africa. (1995–2006). Bio Med Central public health. 2011; 11:209. doi: 10.1186/1471-2458-11-209 PMID: 21463504.

[3] M.P. Leze, M. Le Borgne, P. Pinson, A. Palusczak, M. Duflos, G. Le Baut, R.W. Hartmann, Bioorg. Med. Chem. Lett. 16 (2006) 1134-1137.

[4] M.R. Yadav, M.A. Barmade, R.S. Tamboli, P.R. Murumkar, Eur. J. Med. Chem. 105 (2015) 1-38.

[5] (a) R.T. Greenlee, M.B. Hill-Marmon,T. Murray, P.A.Wingo, CA. Cancer. J. Clin. 51 (2001) 7; (b) R.T. Greenlee, T. Murray, S. Bolden, M. Thun, CA. Cancer. J. Clin. 51 (2001) 15-36.

[6] K. Kelly, L.P.J. Lovato, R.B. Livingston, J. Zangmeister, S.A. Taylor, D. Roychowdhury, J.J. Crowley, D.R.. Gandara, Clin. Cancer. Res. 7 (2001) 2325.

[7] B. Maryam, B. Afifa, S.I. Abdul, A.C. Bashir, Molecules 20 (2015) 13496-13517.

[8] K.N. Mounika, A.N. Jyothi, G.N. Raju, R..R. Nadendla, J. Pharm. Pharm. Sci. 4 (2015) 420-428.

[9] T. Indumathi, V.S. Jamal Ahamed, S.S. Moon, F.R. Fronczek, K.J. Rajendra Prased, Eur. J. Med. Chem. 46 (2011) 5580-5590.

[10] J. Balamani, R. Velmurugan, M. Dhandapani, M. Sekar, Indian J. Heterocycl. Chem. 20 (2010) 69-72.

[11] B.D. Kumar, A. Mitra, M. Mahadevappa, Int. J. Phytomedicine 2 (2010) 22-30.

[12] T. Thongthoom, P. Promsuwan, C. Yenjai, Eur. J. Med. Chem. 46 (2011) 3755-3761.

[13] C. Guillonneau, A. Nault, E. Raimbaud, S. Leonce, L.K. Berther, A. Pierre, S. Goldstein. Bioorg. Med. Chem. 13 (2005) 175-184.

[14] T. Taj, R.R. Kamble, T.M. Gireesh, R.K. Hunnur, S.B. Margankop. Eur. J. Med. Chem. 46 (2011) 4366-4373.

[15] G.M. Dumitriu, A. Ghinet, D. Belei, B. Rigo, P. Gautret, J. Dubois, E. Bicu, Lett. Drug Des. Discov. 12 (2012) 85-92.

[16] A.M.V. Zbancioc, G.N. Zbancioc, C. Tanase, A. Miron, C. Ursu, I.I. Mangalargiu, Lett. Drug Des. Discov. 7 (2007) 644-649.

[17] H. Kaur, S. Kumar, P. Vishwakarma, M. Sharma, K.K. Saxena, A. Kumar, Eur. J. Med. Chem. 45 (2010) 2777-2783.

[18] B.C. Seong, K. Yong, M. Young Yun, Z.A. Byung, Arch. Pharm. Res. 27 (2004) 485-494.

[19] B.C. Nandy, A.K. Gupta, A. Mittal, V. Vyas, J. Biomed. Pharm. Res. 3 (2014) 42–48.

[20] Beata Tylinska, Acta. Pol. Pharm. 68 (2011) 31-37.

[21] N. Kumar, G.K. Sharma, D. Pathak, International journal of pharmaceutical and chemical sciences Jan-Mar 2 (2013) 273-282.

[22] R..P. Molatlhegi, A. Phulukdaree, K. Anand, R..M. Gengan, C. Tiloke, A.A. Chuturgoon, Plos one | DOI:10.1371/journal.pone.0129874 July 2 (2015)

[23] Y. Awazu, A. Mizutani, Y. Nagase, H. Iwata, Y. Oguro, H. Miki, S. Imamura, A. Hori, Cancer Sci. 103 (2012) 939-944.

[24] I.M. Lagoja, Chem. Biodivers. 2 (2005) 1-50.

[25] (a) H. Kakiya, K. Yagi, H. Shinokubo. et al., J. Am. Chem. Soc. 124 (2002). 9032-9033; (b) M. Movassaghi, M.D. Hill, J. Am. Chem. Soc. 128 (2006) 14254-14255.

[26] G.L. Luo, L. Chen, G.S. Poindexter, Tetrahedron Lett. 43 (2002) 5739-5742.

[27] R.J. Howorko, M. Pelczynska, A. Nasulewicz, J. Wietrzyk, A. Opolski, Arch. Pharm. Chem. Life Sci. 338 (2005) 556-561.

[28] (a) A. Segall, H. Pappa, R. Causabon, G. Martin, R. Bergoc, M.T. Pizzorono, Eur. J. Med. Chem. 30 (1995) 165-169; (b) M.G. Ferlin, C. Marzano, V. Gandin, S.D. Acqua, L.D. Via, Chem. Med. Chem. 4 (2009) 363-377.

[29] V. Gaddam, R. Nagarajan, Tetrahedron Lett. 50 (2009) 1243-1248.

[30] M.G. Ferlin, C. Marzano, V. Gandin, S.D. Acqua, L.D. Via, Chem. Med. Chem. 4 (2009) 363-377.

[31] V. Gaddam, R. Nagarajan, Tetrahedron Lett. 50 (2009) 1243-1248.

[32] M. Dracinsky, J. Sejbal, B. Rygerova, M. Stiborova, Tetrahedron Lett. 48 (2007) 6893-6895.

[33] M. Blagosklonny, W.S. EI Diery, Int. J. Cancer, 67 (1996) 368.

[34] Fuchun Xie, Hongbing Zhao, Lizhi Zhao, Liguang Lou, Youhong Hu, Bioorg. Med. Chem. Lett. 19 (2009) 275-278.

[35] Christophe Labriere, Olivier Lozach, Melina Blairvacq, Laurent Meijer, Catherine Guillou, Eur. J. Med. Chem. 124 (2016) 920-934

[36] Xie Ming-Jin, Niu Yan-Fen, Yang Xiao-Da, Liu Wei-Ping, Li Ling, Gao Li-Hui, Yan Shi-Ping, Meng Zhao-Hui, Eur. J. Med. Chem, 45 (2010) 6077-6084.

[37] V.K. Vyas, K. Brahmbhatt, H. Bhatt, U. Parmar. Asian Pacific Journal of Tropical Biomedicine 3 (2013)156-162.