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Regio- and stereoselective synthesis of dispirooxindole-pyrrolocarbazole hybrids *via* 1,3-dipolar cycloaddition reactions: Cytotoxic activity and SAR studies

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Abstract

A library of novel dispiro compounds containing oxindole-pyrrolo-carbazole hybrid frame works has been synthesized in a fully regio- and stereoselective fashion by the three-component 1,3-dipolar cycloaddition of azomethineylides generated *in situ* from the condensation of isatins and benzylamine with 2-arylidene/heteroarylidene-2,3,4,9-tetrahydro-1*H*-carbazole-1-one. The structures of the compounds were established by FT-IR, ^{1}H NMR, ^{13}C NMR, X-ray diffraction and elemental analysis. The synthesized dispiro heterocycles have been screened for *in vitro* cytotoxic activity by MTT assay and displayed enviable growth inhibition on both the cancer cell lines i.e. breast cancer cell line MCF-7 and lung cancer cell line A-549. Morphological changes and apoptosis induction have been studied by inverted light microscopic, fluorescent microscopic techniques and by flow cytometry analyses. The preliminary structure activity relationships were also carried out. Data indicated that among dispiro-carbazole compounds,6-chloro-4'-(thiophen-2-yl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(*H*)-1,2''-dione **7e** could be exploited as a significant therapeutic drug against breast cancer as well as lung cancer cell proliferation.

Keywords

1,3-Dipolar cycloaddition

Azomethineylide

Anti-proliferative

Apoptosis

Flow cytometry

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1. Introduction

Breast cancer and lung cancer are the two most recurrently diagnosed malignant tumors worldwide. Variety of breast cancer drugs such as Tamoxife, Letrozole, Docetaxyl etc. are being prescribed to the patients as preventive and curative treatments [1,2]. However undue toxicity and side effects in these medicines spoil their efficacy [3-5]. Despite recent advances made in anti lung cancer drug development, the presence of resistance to chemotherapeutic agents is a major obstacle to the effective treatment of lung cancer [6]. Consequently, discovering novel, puissant molecular entities as potential anti cancer drugs with improved efficacy and resistance to complement the present chemotherapeutic strategies is highly desired. Interestingly natural products provide a healthy source for such compounds. The spirooxindole-pyrrolidine nucleus is often found in the molecular framework of many natural products, vizhorsfiline [7], coerulescine [8], elacomine [9], isopteropodine [10], formosanine [11], rychnophyilline [12] and spirotryprostatins A and B [13] etc. It possess a myriad of biological activities such as inhibition of the mammalian cell cycle at G2/Mphase [14], inhibition of microtubule assembly [15], modulation of the function of muscarinic serotonin receptors [16] and malignant glioma GAMG [17], and anticancer [18], antimicrobial [19] and local anesthetic [20] activities.

Carbazole and its derivatives represent an important class of bio-active heterocycles. Many natural products and drug molecules with the carbazole framework exhibit a broad range of biological and pharmacological activities [21-28]. In particular, carbazole derivatives show significant antitumor activity [29-31]. For example, glybomine B and C showed significant antitumor-promoting activity, which was confirmed by the inhibiting effect of these alkaloids in conjunction with the tumor promoter 12-*o*-tetradecanoylphorbol-13-acetate (TPA) [32], while heptaphylline and 7-methoxyheptaphylline displayed strong cytotoxicity against NCI-H187 and KB cell lines [33]. In addition carbazole arrests the tumor cell cycle at the M phase and induces apoptotic cell death by increasing expression of p-53 and promoting bcl-2 phosphorylation [34,35] (**Figure 1**).

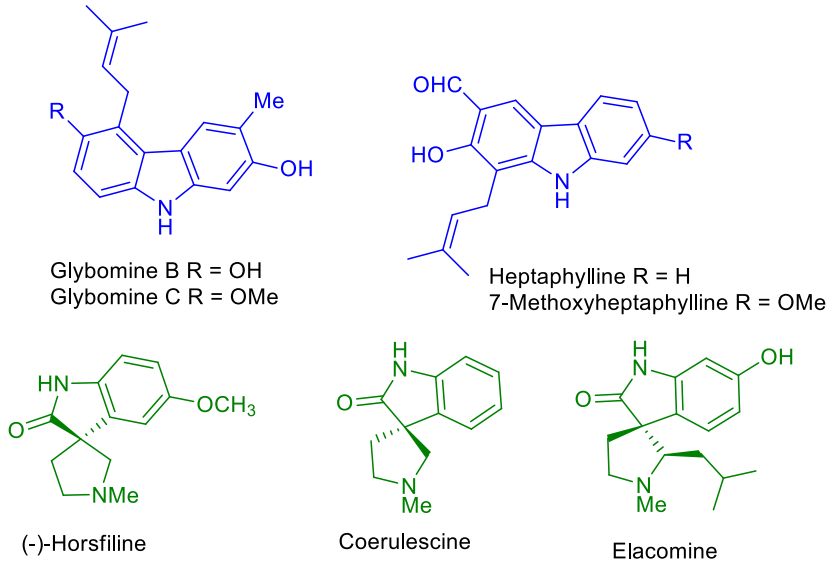


Figure 1. Biological relevance of the dispirooxindolpyrrolidine-carbazole hybrids to naturally occuring carbazoles and oxindolopyrrolidines

The dominance by these two pharmacophores (carbazole and spiro-pyrrolooxindole) in nature and their impact in medicinal chemistry inspired us to develop new molecular hybrids by incorporating both the frameworks for our anticancer studies. Mostly, design of the hybrid drugs aims to circumvent the drug resistance, minimize the risk of drug-drug interactions, counterbalance the known side effects associated with the other hybrid part and amplify the activity through the interaction with multiple targets as one single molecule [36,37]. Now, hybrid drug design has emerged as a premier tool for the discovery of innovative anticancer therapies that can potentially overcome most of the pharmacokinetic drawbacks encountered when using conventional anticancer drugs.

The 1,3-dipolar azomethineylide cycloaddition is a powerful tool for the construction of five-membered heterocycles and spiro-compounds [38] in a highly regio- and stereoselective manner [39]. Azomethineylides are reactive and versatile 1,3-dipoles, which readily react with diverse dipolarophiles with exocyclic double bonds to afford spiro-heterocycles [40].

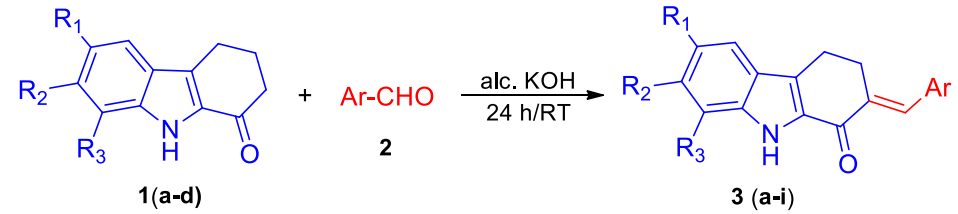
Herein, we report a regio- and stereoselective one-pot method for the synthesis of a novel class of polynuclear dispiroheterocyclic structures comprising spiro-pyrrolooxindole and carbazole moieties. The synthesis was accomplished by 1,3-dipolar cycloaddition of azomethine ylides generated *in situ* from isatins and benzylamine to 2-arylidene/heteroarylidene-2,3,4,9-tetrahydro-11*H*-carbazole-1-one. All the newly synthesized compounds were subjected to cytotoxic screening against breast cancer cell line MCF-7 and lung cancer cell line A-549.

Although there are reports available for the synthesis of substituted spiro-pyrrolooxindole, there seems to be no reports to the best of our knowledge for the synthesis of a rare class of dispiropyrrolooxindole having keto carbazole skeleton.

2. Results and discussion

2.1. Chemistry

The dipolarophiles **3** employed in the present work were synthesized by the reaction of 2,3,4,9-tetrahydro-1*H*-carbazole-1-one **1** with aryl/hetroaryl aldehydes **2** in the presence of alcoholic KOH in good yields at room temperature (**Scheme 1**).



1 & 3 a: $R_1 = CH_3$, $R_2 = H$, $R_3 = H$
b: $R_1 = Cl$, $R_2 = H$, $R_3 = H$
c: $R_1 = H$, $R_2 = H$, $R_3 = H$

Ar = C_6H_5-, 2-thienyl- & 4-OCH_3-C_6H_4-

Scheme1. Synthesis of 2-arylidene/heterolidene-2,3,4,9-tetrahydrocarbazol-1-one **3**

The structural elucidation of all the dipolarophiles **3** (**a-i**) was done with the help of spectroscopic (FT-IR, ^{1}H-NMR & ^{13}C-NMR) and elemental analysis data. The FT-IR spectrum of **3a** showed the characteristic absorptions of an indole NH at 3290 cm^{-1} and a carbonyl group at 1644 cm^{-1}. The ^{1}H NMR spectrum of **3a** showed a broad singlet at δ 11.64 ppm attributed to the indole NH proton and a singlet at δ 8.27 ppm due to the presence of C_5-H. Multiplet signals in the region of δ 7.68-7.34 ppm arise from the six aromatic protons, while a multiplet at δ 7.12-6.98 ppm was assigned to the C_7 and C_2 aromatic and olefinic protons. The methylene protons of C_3 and C_4 appeared as two multiplets centered at δ 3.04 and 3.01 ppm respectively. A singlet at δ 2.50 ppm accounts for three methyl protons at C_6 position. The ^{13}C NMR spectrum revealed the presence of 20 carbons. A signal resonating at δ 179.5 was attributable to the carbonyl carbon. The universal validity of the reaction was tested with other substituted derivatives **3** (**b-i**).

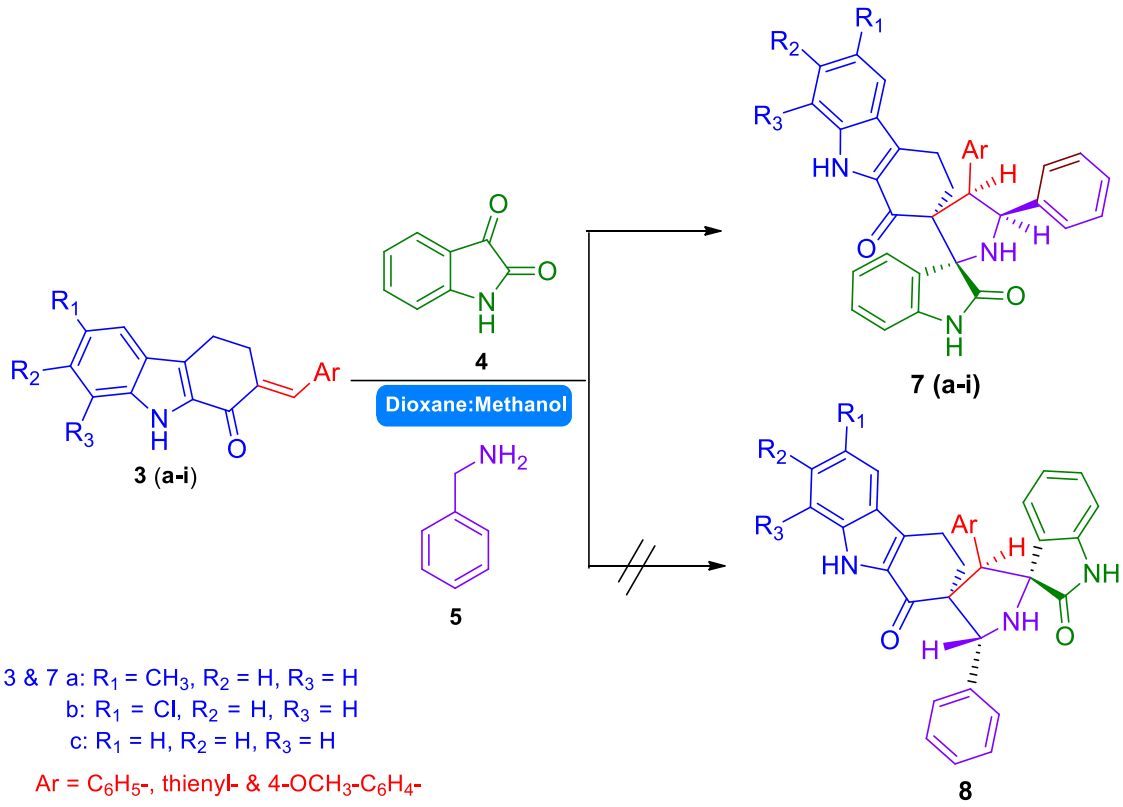
The choice of an appropriate reaction medium is of crucial importance for successful synthesis. Initially, the three component reaction of 2-arylidene/heteroarylidene-2,3,4,9-

tetrahydro-1*H*-carbazole-1-one **3a**, isatin **4** and benzylamine **5**, as a simple model substrate, was investigated to establish the feasibility of the strategy and optimize the reaction conditions. Various solvents such as EtOH, MeOH, 1,4-dioxane, acetonitrile, toluene, EtOH/dioxane and MeOH/dioxane were screened. MeOH/dioxane (**Table 1**, **entry 7**) emerged as the solvent of choice, producing the highest yield (87 %) of the target compound.

Table 1.Solvent screening for the Synthesis of dispiro compound **7a**

Entry	Solvent	Reaction time	Yield (%)
1.	EtOH	7	56
2.	MeOH	7	64
3.	1,4-Dioxane	8	70
4.	CH_3CN	10	52
5.	Toluene	10	38
6.	EtOH/1,4-Dioxane	7	73
7.	MeOH/1,4-Dioxane	5	87

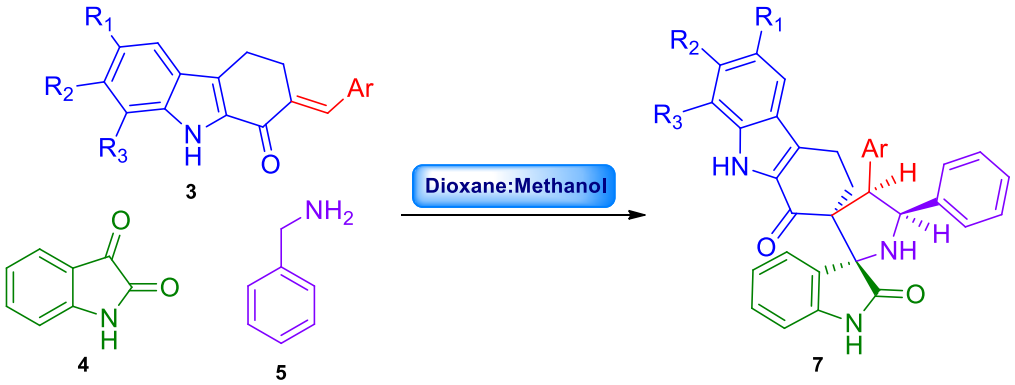
The one pot regioselective synthesis of compounds **7** (**a**–**i**) has been performed by taking 2-arylidene/heteroarylidene-2,3,4,9-tetrahydro-1*H*-carbazole-1-one **3** (**a**–**i**) (0.001 mol), isatin **4** (0.001 mol) and benzylamine **5** (0.001 mol) in dioxane and methanol under reflux conditions. After completion of the reaction as evident from TLC, the solvent was removed and the crude product was purified by column chromatography to obtain pure 4′-arylidene/heteroarylidene-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3"-indole]-9(*H*)-1,2"-dione **7**. The reaction proceeds through decarboxylative condensation of isatin **4** with benzyl amine **5** to generate unstabilize azomethineylide, which subsequently undergoes 1,3-dipolar cycloaddition with the dipolarophile **3** to afford novel cycloadduct **7** as a single regioisomer (**Scheme 2**).

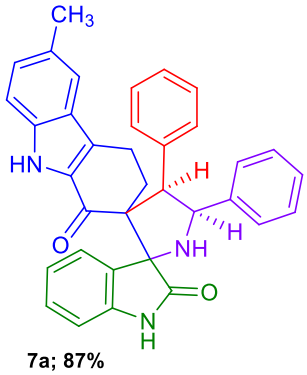


Scheme 2. Synthesis of dispiropyrrolidinyl oxindole- carbazole hybrids **7**(**a-i**)

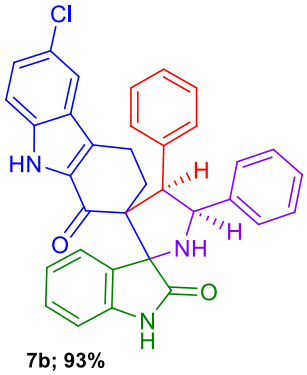
We next explored the scope and generality of this three component cycloaddition reaction with substrates having (i) aryl rings bearing electron-withdrawing and electron releasing substituents and (ii) heteroaryl rings at the arylidene side chain. Moreover, the carbazole core was either unsubstituted or substituted with electron-releasing (methyl) or electron-withdrawing (Cl) groups. These structurally and electronically varied starting materials reacted efficiently with isatin and benzylamine, affording the corresponding cycloadducts **7** in good yields under the same set of reaction conditions in all cases (**Table 2**).

Table2. Synthesis of a library of spiropyrrolidinyloxindole-carbazole hybrids via 1,3-dipolar cycloaddition reaction with variety of aromatic aldehydes were executed.

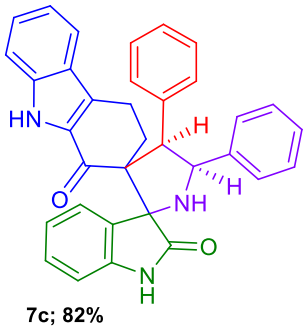




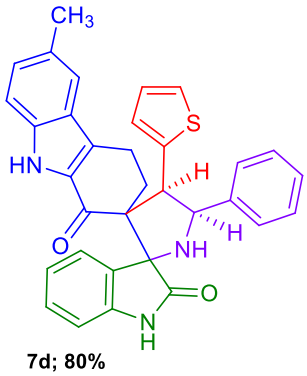
7a; 87%



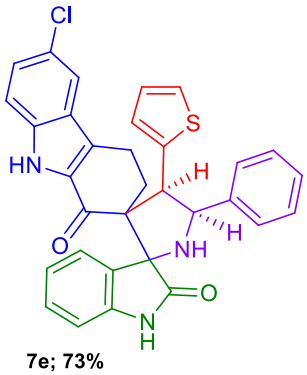
7b; 93%



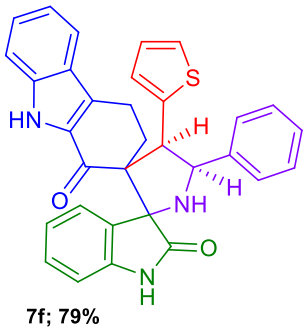
7c; 82%



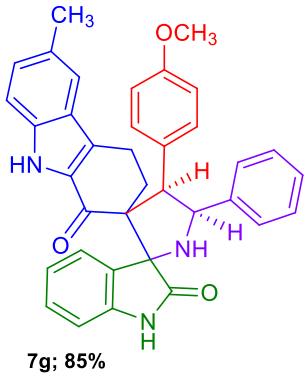
7d; 80%



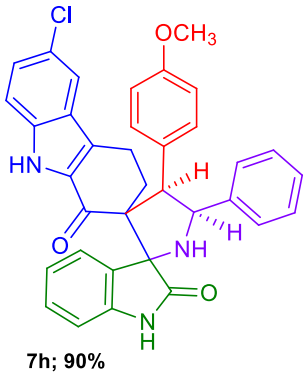
7e; 73%



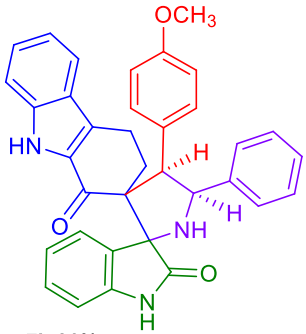
7f; 79%



7g; 85%



7h; 90%



7i; 86%

The products **7**(**a-i**) were characterized unambiguously by spectroscopic, crystallographic and analytical studies. The FT-IR spectrum of **7a** revealed prominent absorptions at 3419, 3322 and 3222 cm^{-1}due to indole NH, carbazole NH and pyrrolo NH stretchings respectively. The peak at 1696 cm^{-1} is due to the carbonyl of indole ring whilst a peak at 1617 cm^{-1} is assignable to the carbazole carbonyl group. In the ^{1}H NMR spectrum of **7a** a doublet appeared at δ 4.69 ppm (J_{cis} = 10.0 Hz) due to C_4' proton, which confirms the formation of cycloadduct. If the other possible regioisomer **8** had been formed then the ^{1}H NMR spectrum would have shown a singlet for C_4' proton instead. A pair of broad singlets appeared at δ 11.18 and δ 10.18 ppm corresponding to carbazole NH and oxindole NH respectively. A doublet at δ 7.50 ppm (J_o = 7.4 Hz) accounted for C_8 and C_7" aromatic protons and the aromatic protons on C_5"" and C_3"" resonated as a multiplet at δ 7.38 ppm. A multiplet in the region of δ 7.26-7.22 ppm accounted for the four aromatic protons on the substituted phenyl ring. The three aromatic protons in the C_6", C_6"" and C_2"" positions showed a multiplet in the region between δ 7.18-7.13 ppm. The C_5 proton appeared as a singlet at δ 7.09 ppm. Two doublets at δ 7.05 and δ 6.97 (J_o=7.4 Hz) ppm corresponding to C_4" and C_7 protons. Three triplets appeared at δ 6.84, 6.56 and 6.54 ppm (J = 7.4, 7.2 and 7.2 Hz) due to C_5", C_4"' and C_4"" protons respectively. The C_5' proton occurred as doublet of doublet at δ 5.45 ppm ($J_{1,2\text{-}cis}$ = 5.6 Hz & $J_{1,2\text{-}cis}$ = 10.0 Hz). The –NH proton of pyrrolo moiety resonated as a doublet at δ 3.61($J_{1,2\text{-}cis}$ = 5.6 Hz) ppm. Methylene protons of C_4 and C_3 appeared as multiplets centered at δ 2.57 and 2.47 ppm respectively. A singlet at δ 2.22 ppm assigned to the C_6-CH_3 protons. In ^{13}CNMR spectrum of **7a**, the peaks at δ 72.3 and δ 61.5 ppm corresponded to the two spiro carbons. The oxindole ring carbonyl resonated at δ 180.5 ppm, and benzoylcarbonyl resonated at δ 191.5 ppm. Scrutiny of all spectral data confirmed the formation of the dispiropyrrolo derivatives, 4′-arylidene/heteroarylidene-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3"-indole]-9(*H*)-1,2"-dione **7**.

In order to verify the structure of **7a**, a single crystal was obtained and analyzed by X-ray crystallography (**Figure 2**), which clearly indicated the formation of dispiro scaffold. Similarly, by straightforward considerations, the ^{1}H and ^{13}C chemical shifts of other derivatives **7**(**b-i**) were also assigned.

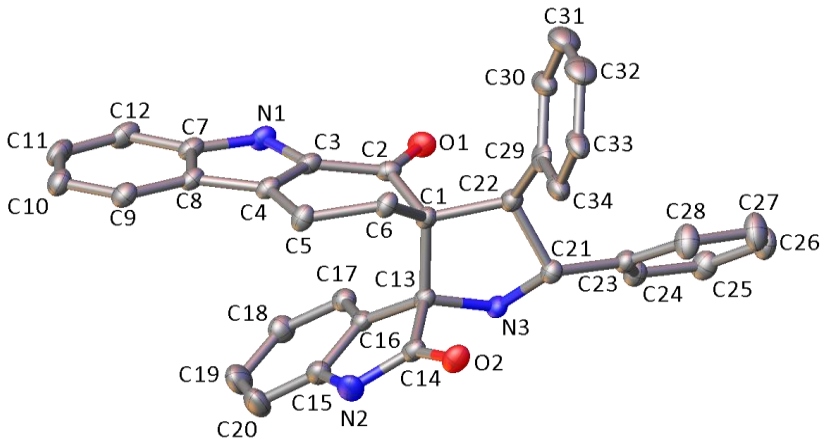
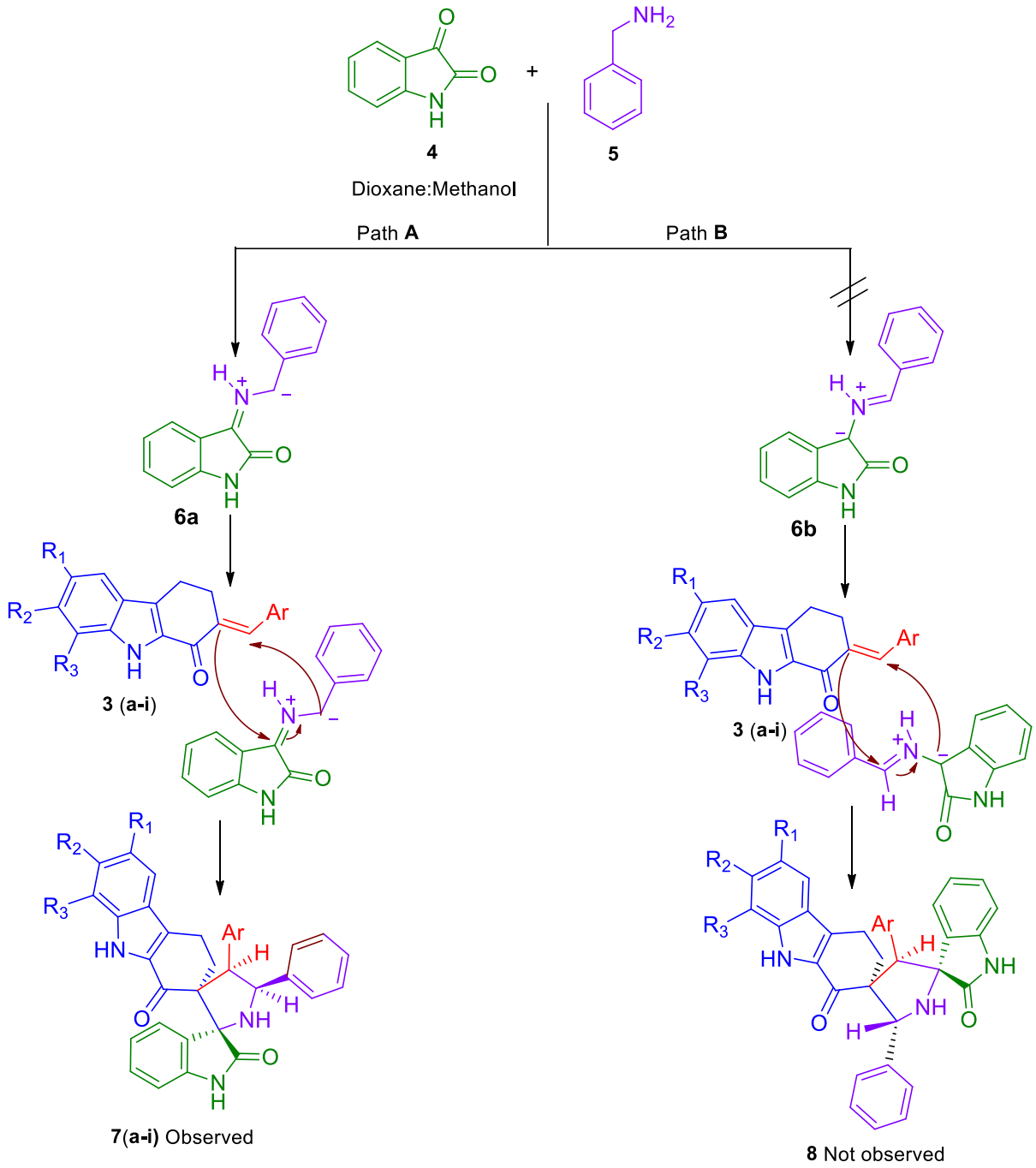


Figure 2. Structure of **7a** with the atomic numbering scheme shown. Hydrogen atoms omitted and only one position of disorder phenyl ring (C29-C34) shown for clarity. Thermal ellipsoids depicted at the 50% probability level.

A proposed mechanism for the regio- and stereoselective formation of the hybrid heterocycles **7(a-i)** is presented in **Scheme 3**. The azomethine ylide **6a** generated *in situ* from the reaction of isatin **4** with benzylamine **5,** has one potential nucleophilic carbon, which subsequently undergoes 1,3-dipolar cycloaddition with the electron deficient β-carbon of the dipolarophile **3** to afford novel cycloadduct **7** as a single regioisomer. Control of the relative stereochemistry at the spiro centre was observed. Accordingly the observed regioisomer **7** via path **A** is more favorable because of the secondary orbital interaction [41] which is not possible in path **B** for getting other isomer **8**.



Scheme 3. Plausible the machanism for the formation of **7**.

2.2. Biological evaluation

2.2.1. *In vitro* cytotoxic activity

Anti-proliferative activity of the newly synthesized dispiro compounds **7(a-i)** was examined in two human cancer cell lines, MCF-7 breast cancer and A-549 lung cancer using

MTT assay. Cisplatin is one of the most effective broad-spectrum anticancer drugs. Cisplatin enters cells via multiple pathways and forms different DNA–platinum adducts while initiating a cellular self-defense system by activating or silencing a variety of different genes [42]. Hence, we chose cisplatin as reference drug. Three independent experiments in triplicate were performed for the determination of sensitivity to each compound. The percentage cell viability was determined using the following formula, and the relationship between percentage cell viability and drug concentration was plotted to obtain the survival curve of the tested cancer cell lines. The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. The results are summarized in **Table 3**. The *in vitro* cytotoxic activity of the synthesized compounds (**7e** and **7d**) (10-100 µM concentrations) against both cancer cells has been presented in **Figure 3**. 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 3. *In vitro* cytotoxicity and IC_{50} (µM)

Compounds	MCF-7[a]	A-549[b]
7a	19±1.4	18±1.5
7b	18±0.8	15±1.4
7c	15±1.5	17±0.5
7d	**14±0.8**	**15±1.4**
7e	**13±1.6**	**14±1.2**
7f	**15±0.8**	**16±1.5**
7g	18±1.7	20±1.4
7h	19±0.8	18±1.4
7i	17±0.8	21±1.4
Cisplatin	9±1.0	10±1.5

Bold indicates more active compounds.
[a] breast cancer. [b] lung cancer.

As shown in **Table 3**, it was obvious that the synthesized dispiro-carbazole compounds displayed significant to modest growth inhibitory activity against the tested cancer cell lines. Investigations of the cytotoxic activity revealed that majority of the synthesized compounds exhibited potent anticancer activity against both the cancer cell lines, MCF-7 and A-549. Among the newly synthesized spiro compounds, compound **7e** was found to be the most promising derivative against MCF-7 with IC_{50} values 13±1.6 μM compared to the IC_{50} value of cisplatin (9±1.0 μM). The next most outstanding compounds are **7d** and **7f** which displayed stronger cytotoxicity against MCF-7 cell line with IC_{50} values 14±0.8 and 15±0.8 μM respectively. Furthermore, the screening against MCF-7 cell line, the compounds **7a**, **7b** and **7c** showed significant activity with IC_{50} value ranging from 15±1.5 to 19±1.4 μM. While compounds **7g**, **7h** and **7i** were moderately active with IC_{50} values of 18±1.7, 19±0.8 and 17±0.8 μM respectively. Subsequently, those compounds which showed good activity with MCF-7 were screened against A-549 cell line. Compounds **7e**, **7f** and **7d** were the most active analogs through this study with IC_{50} values of 14±1.2, 16±1.5 and 15±1.4 μM respectively. In addition, compounds **7a**, **7b** and **7c** with $1C_{50}$ values ranging from 15±1.4 to 18±1.5 μM displayed substantial activity against A-549 cell line whereas compounds **7g**, **7h** and **7i** were moderately active with IC_{50} values of 20±1.4, 18±1.4 and 21±1.4 μM respectively. In general, it was found that all the synthesized dispiro-carbazole derivatives displayed selective cytotoxicity against both the tested cell lines-A-549 and MCF-7 and finally, compound **7e** was emerged as the most promising anticancer agent against MCF-7 and A-549 cell lines with IC_{50} values of 13±1.6 and 14±1.2 μM respectively.

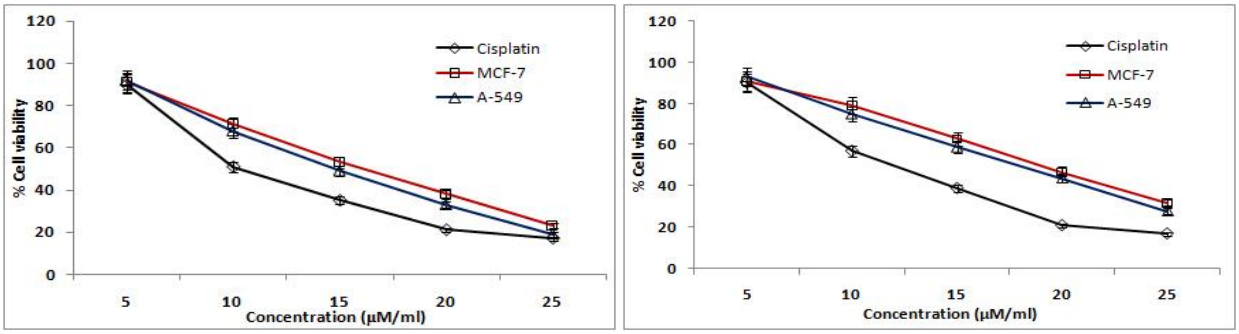
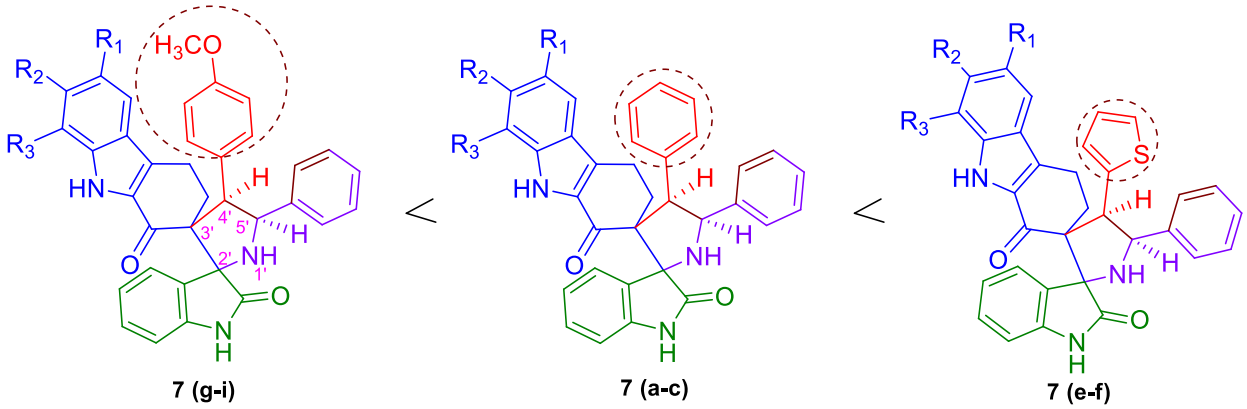


Figure 3. Cytotoxic effect of the compounds **7e** & **7d** on the viability of MCF-7 & A-549 cell lines

2.2.2. Structure activity relationship (SAR)

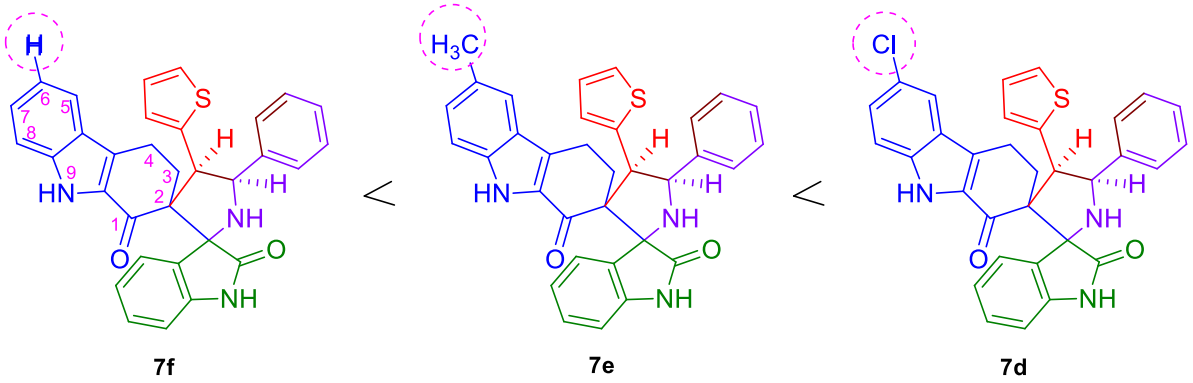
Based on the aforementioned biological data, the following assumptions could be deduced about the structural activity relationship (SAR):

- It is clear from the results summarized in **Table 3** that the substitution attached to the pyrrolo group oriented at the C_4'-position of the synthesized spiro heterocycle plays an important role in developing the observed anti-tumor properties. Among the synthesized compounds, the compound 6-chloro-4'-(thiophen-2-yl)-5'-phenyl-3,4-dihydrodispiro [carbazole-2,3'-pyrrolo-2',3"-indole]-9(*H*)-1,2"-dione **7e** showed higher cytotoxic efficacy with $1C_{50}$ value 13±1.6 μM for MCF-7 and 14±1.2 μM for A-549 cell line. Compounds **7d** and **7f** also exhibited stronger cytotoxic activity against both the tested cell lines. It was due to the presence of thiophene moiety [43,44] which boots the cytotoxic activity.
- On the other hand, replacing the thiophene group at the C_4'-position of the pyrrolo moiety by the electron donating methoxy group reduced the anticancer potency of the dispiro compounds. The compounds **7g**, **7h** and **7i** having methoxy substitution on the pyrrolo ring showed moderate anticancer activities (**Scheme. 4**).



Scheme 4. Role of C_4' substituents in increasing the efficancy of cytotoxicity.

- In general, it was observed that the substituent present in the carbazole ring plays a vital role in determining the anticancer potency. Among the dispiro-carbazole compounds, the compounds bearing electron withdrawing chloro group in the carbazole ring enhanced the cytotoxic activity more than the electron donating methyl group and unsubstituted group [45]. (**Scheme. 5**)



Scheme 5. Role of C_6- substituent in incresing the efficany of cytotooxicity

2.2.3. Apoptosis assay

Apoptosis, the process of programmed cell death (PCD), is an important therapy target for cancer chemotherapy. Apoptotic cell death is morphologically defined by chromatin condensation, nuclear fragmentation, shrinkage of cytoplasm and formation of apoptotic bodies. The apoptotic events were analyzed using various staining procedures to study the cytotoxicity and cell viability, extent of apoptosis, morphological changes, nuclear changes and DNA fragmentation, which occurs at the final stage of apoptosis. The development of human cancers is often mainly a consequence of deregulated cell cycle control and/or suppressed apoptosis.

2.2.3.1. Morphological changes of selected human cancer cell lines using Inverted Light Microscopic analysis.

The apoptogenic property of the active dispiro compounds was investigated through morphological changes in MCF-7 and A-549 human cancer cells. Apoptotic cells displayed typical common features such as cell shrinkage, nuclear condensation, membrane blebbing, chromatin cleavage, and formation of pyknotic bodies of condensed chromatin. These distinctive typical forms of morphological changes in apoptotic cells are widely used for the identification and quantification of apoptosis. Thus, determination of the morphological changes to define apoptosis was visualized using inverted light microscopic technique.

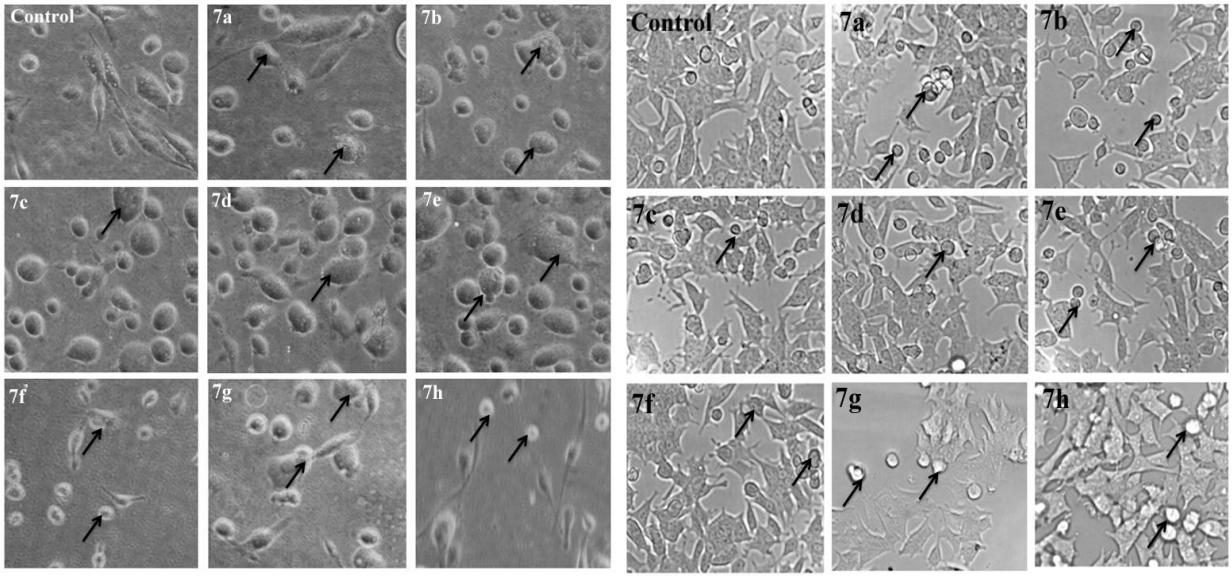


Figure 4. Morphometric analysis of treated MCF-7 & A-549 cells, the arrows indicates the formation of floating cells and appearance of membrane blebbing

Figure 4 shows 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 in comparison to control cells. Visualization of the control (untreated) cells showed that the cells maintained their original morphology form. Phase-contrast micrographs revealed that the compounds **7e** and **7d** induces increased cell shrinkage, membrane blebbing and forms floating cells, compared to compounds **7a, 7b, 7c, 7f, 7g** and **7h** in a dose-dependent manner.

2.2.3.2. Morphological observation of selected human cancer cell lines using Fluorescence Microscopic analysis.

Fluorescence light microscopy with differential uptake of fluorescent DNA binding dyes (such as EB/AO, DAPI staining) is a method of choice for its simplicity, rapidity, and accuracy. In such an assay, apoptotic index and cell membrane integrity can be assessed simultaneously and there is no cell fixation step, thus avoiding a number of potential artifacts. 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. 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.

2.2.3.2.1. 4',6-Diamidino-2-phenylindole (DAPI) staining method

DAPI detection provides a rapid and convenient assay for apoptosis based upon fluorescent detection. DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. Its high cell permeability allows efficient staining of nuclei. Once it overpasses cell membranes of normal cells, the blue fluorescence will be observed by fluorescent microscopy. With the process of apoptosis, the ability of permeability for dye is improved and the apoptotic cells will produce high blue fluorescence. Fluorescence microscopic images of breast cancer cells after 24 h stained with DAPI in the presence and absence of compounds are shown in **Figure 5**. The compounds **7e**, **7d, 7c** and **7f** displayed higher level of nuclear fragmentation and the untreated cells did not show any significant changes in the nuclear appearance, whereas the other compounds exhibited bright fetches when treated with MCF-7 cancer cells, which indicates the condensed chromatins and nuclear fragmentations in the cells. **Figure 5** showed fluorescent DAPI analysis of compounds treated A-549 lung cancer cells. Compounds **7d** and **7e** exhibit higher level of nuclear fragmentation in the treated A-549 cells, rather than the remaining compounds **7a**, **7b**, **7c**, **7f**, **7h** and **7g**. The apoptosis observed in compounds which are treated with MCF-7 breast cancer cells are higher than that observed in A-549 lung cancer cells.

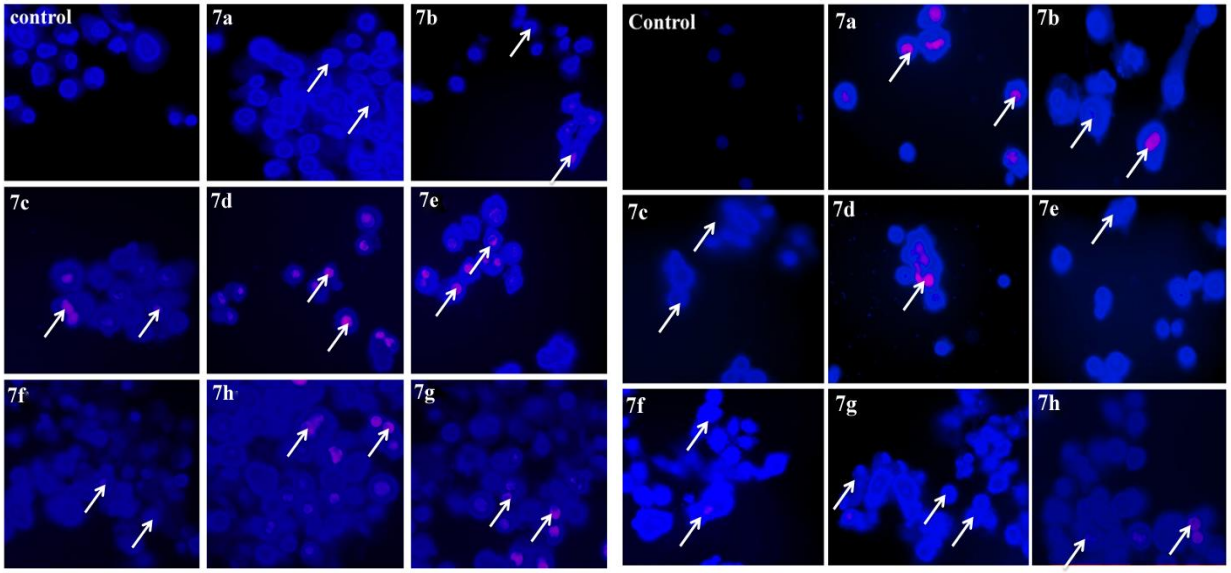


Figure 5. DAPI apoptotic analysis of treated MCF-7 & A-549 cells the arrows indicate apoptotic cells

2.2.3.2.2. 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 **Figure 6**. 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**7e** and **7d** 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 showed weak fluorescence intensity owing to the reduced level of induction of apoptosis in the MCF-7 cells. In the case of lung cancer cell, compounds **7d**, **7e** and **7f** exhibited significant apoptotic induction rather than the compounds **7a, 7b, 7c, 7g** and **7h.** The apoptotic induction values of the synthesized compounds were remarkable in MCF-7 cells compared to the lung cancer cells.

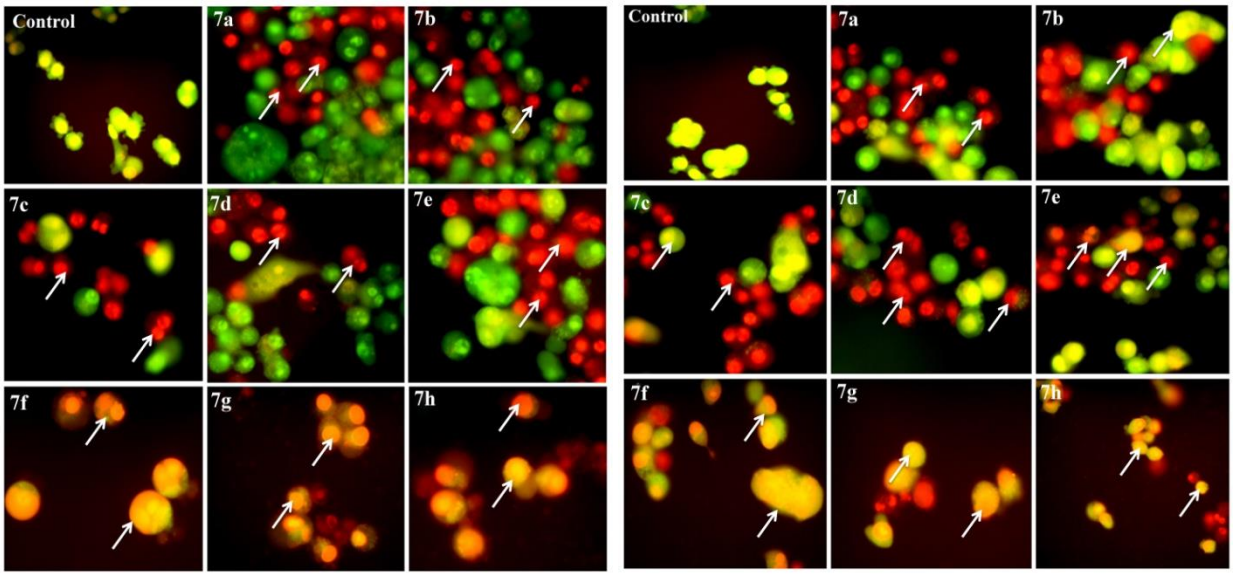


Figure 6. AO/EB apoptotic analysis of treated MCF-7 & A-549 cells the arrows indicate apoptotic cells

2.2.3.2.3. Apoptotic analysis of Human cancer cells by flow cytometry

To further confirm the dispiro hetrocycles-induced apoptosis, the apoptosis was detected by flow cytometric technique using the Annexin V method, with the aid of an Annexin V-FITC to perform double-staining with propidium iodide. Treatment with compound **7e** shows a significant population of annexin-V positive cells (pro and late apoptotic cells) in the right hand quadrants of flow cytometric graphs in a dose dependent manner (**Figure. 7**). The compound **7e** has the potential to stimulate the apoptotic signals and activate subsequent cell death mechanisms. Moreover the compound significantly active on MCF-7 cells rather than the A-549 cells (**Figure. 8**). This activity may be the marker receptor present on the cell surface and it could be more active on breast cancer cells. These results indicate that dispiro heterocyclic compound induced cell death is mediated by the induction of apoptotic pathways in selected human cancer cells.

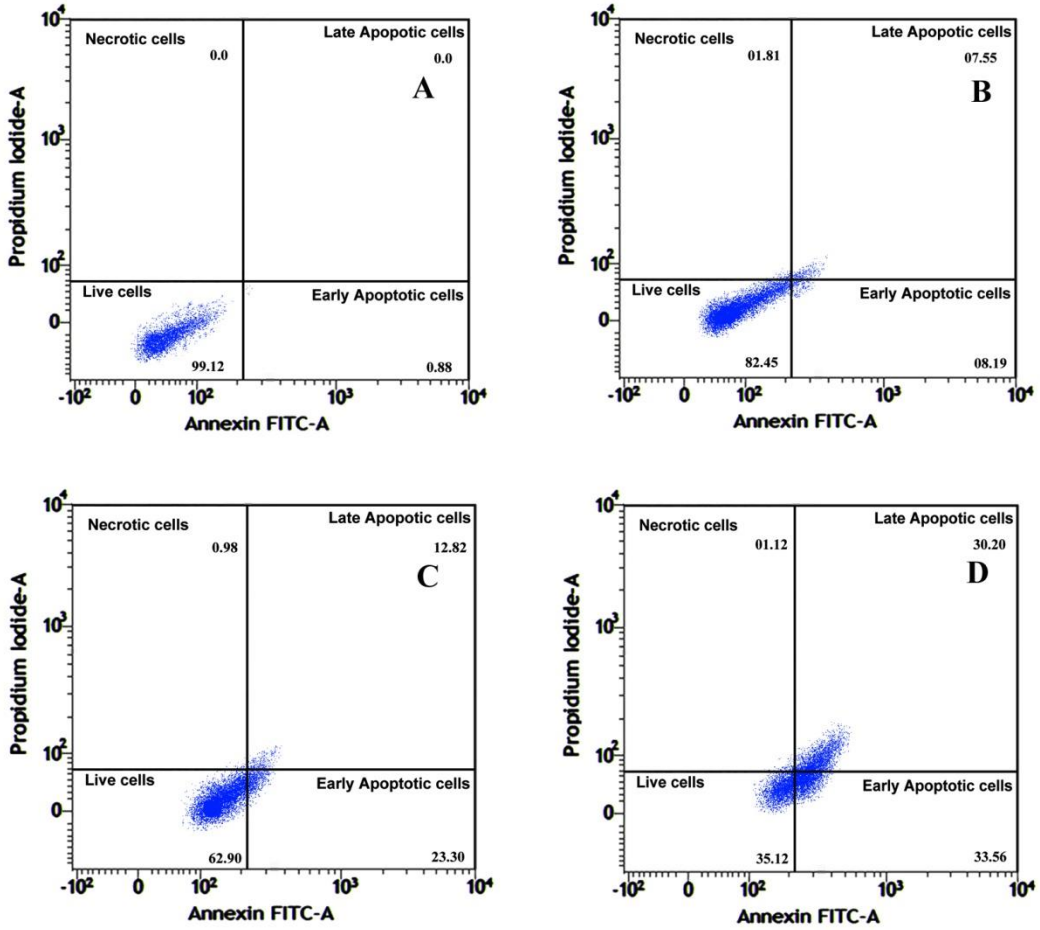


Figure 7. Flow cytometry analysis of breast cancer cells (MCF-7). Quadrant 4 represents necrotic cells (D-20µM/ml), Quadrant 3 represents late apoptotic cells (C-10µM/ml), Quadrant 2 represents proapoptotic cells (B-5µM/ml), and Quadrant 1 represents live cells (A-Control).

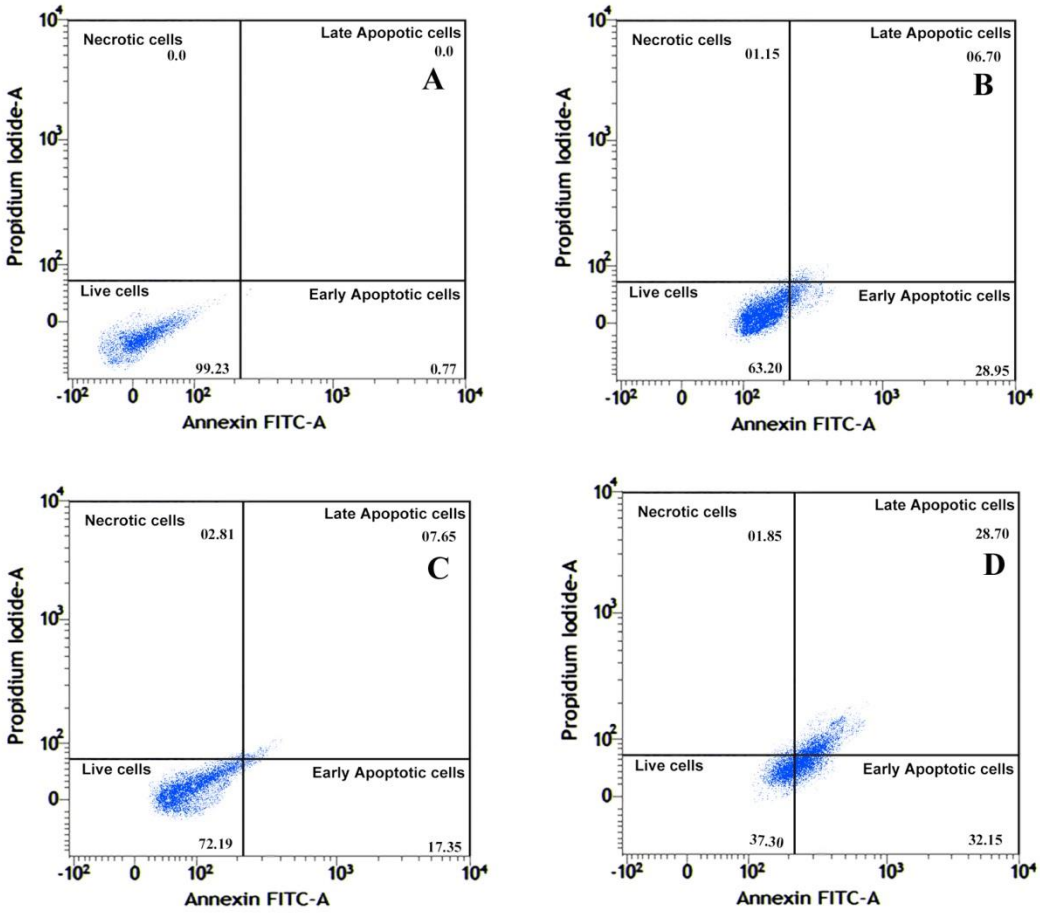


Figure 8. Flow cytometry analysis of Lung cancer cells (A-549). Quadrant 4 represents necrotic cells (D-20µM/ml), Quadrant 3 represents late apoptotic cells (C-10µM/ml), Quadrant 2 represents proapoptotic cells (B-5µM/ml), and Quadrant 1 represents live cells (A-Control).

3. Conclusions

The present investigation describes the synthesis of new and highly functionalized carbazole containing dispiropyrrolooxindole hybrid molecules obtained through regio- and stereoselective 1,3-dipolar cycloaddition reaction of azomethineylide with (E)-2-arylidine/heteroarylidine-2,3-4,9-tetrahydro-1*H*-carbazole-1-one as dipolarophiles. Interestingly, benzylamine played a critical role in the regioselectivity of this 1,3-cycloaddition to construct the 4'-arylidene/heteroarylidene-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3"-indole]-9(*H*)-1,2"-dione ring system and it was obtained as a single regioisomer. The structural assignments of the corresponding cycloadducts were confirmed by FT-IR, NMR spectroscopy and the structure of compound **7a** was further confirmed by X-ray crystallographic analysis. The cytotoxic efficacy of these compounds was assessed against the human cancer cell line MCF-7

and A-549 and all dispiro compounds were either equipotent or moderate to the positive control cisplatin. Among which, compounds **7e**, **7f** and **7g** having thiophene moiety showed potent *in vitro* anti-proliferative activity. Moreover, the preliminary structure activity relationships of these derivatives have been established. The mode of cell death assessed by inverted light microscopic, fluorescence microscopic (DAPI staining and AO/EB dual staining procedures) techniques and flow cytometry analyses revealed that the dispiro-carbzole compounds were able to trigger apoptosis in both MCF-7 and A-549 human cancer cells lines.

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.

4.1.2. Synthesis

*4.1.2.1. General procedure for the synthesis of 2-arylidene/heteroarylidene-2,3,4,9-tetrahydrocarbazol-1-one **3**.*

An equimolar mixture of the 2,3,4,9-tetrahydrocarbazol-1-one **1** (1.0 mmol) and aryl/heteroaryl aldehyde **2** (1.0 mmol) 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-arylidene/heteroarylidene-2,3,4,9-tetrahydrocarbazol-1-one **3**.

*4.1.2.1.1. 2-Benzylidene-6-methyl-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3a**).* Yellow solid; yield: 244 mg (85%); m.p. 228-230 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3290 (NH), 1644 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 11.64 (b s, 1H, N_9-H), 8.27 (s, 1H, C_5-H), 7.68-7.34 (m, 6H, C_8, C_6', C_5', C_4', C_3' & C_2'-H), 7.12-6.98 (m, 2H, C_7 & C_2-2H), 3.05-3.03 (m, 2H, C_3-2H), 3.02-3.00 (m, 2H, C_4-2H), 2.50 (s, 3H, C_6-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 179.5 (C=O), 138.4 (C_2), 136.9 (C_{8a}), 135.6 (C_{2a}), 133.6 (C_1'), 132.1 (C_{9a}), 129.5 (C_6), 128.4 (C_5' & C_3'), 128.1 (C_6' & C_2'), 127.7 (C_{4b}), 126.7 (C_4'), 124.9 (C_{4a}), 122.4 (C_7), 120.0 (C_5), 118.5 (C_8), 27.2 (C_3), 20.3 (C_4), 17.0 (CH_3); Anal. calcd. for $C_{20}H_{17}NO$: C, 83.59; H, 5.96; N, 4.87; Found: C, 83.50; H, 5.92; N, 4.93.

*4.1.2.1.2. 2-Benzylidene-6-chloro-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3b**).* Yellow solid; yield: 224 mg (73%); m.p. 231-233 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3324 (NH), 1652 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 9.43 (b s, 1H, N_9-H), 7.82 (s, 1H, C_5-H), 7.62 (s, 1H, C_2-H), 7.44-7.30 (m, 7H, C_8, C_7, C_6', C_5', C_4', C_3' & C_2'-H), 3.27-3.24 (m, 2H, C_3-2H), 3.03-3.00 (m, 2H, C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 180.1 (C=O), 138.7 (C_2), 137.3 (C_{8a}), 135.9 (C_{2a}), 134.1 (C_1'), 132.8 (C_{9a}), 129.7 (C_6), 128.7 (C_5' & C_3'), 128.5 (C_6' & C_2'), 128.2 (C_{4b}), 126.3 (C_4'), 125.4 (C_{4a}), 122.7 (C_7), 120.3 (C_5), 118.7 (C_8), 27.3 (C_3), 20.4 (C_4); Anal. calcd. for $C_{19}H_{14}ClNO$: C, 74.15; H, 4.58; N, 4.55; Found: C, 74.25; H, 4.54; N, 4.61 .

*4.1.2.1.3. 2-Benzylidene-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3c**).* Yellow solid; yield: 227 mg (83%); m.p. 232-234 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3262 (NH), 1647 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 9.51 (b s, 1H, N_9-H), 7.84 (s, 1H, C_2-H), 7.66 (d, 1H, C_5-H, J_o = 7.8 Hz), 7.49-7.33 (m, 7H, C_8, C_6, C_6', C_5', C_4', C_3' & C_2'-H), 7.15 (t, 1H, C_7-H, J = 7.8 Hz), 3.28-3.25

(m, 2H, C_3-2H), 3.09-3.06 (m, 2H, C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 179.3 (C=O), 138.2 (C_2), 136.7 (C_{8a}), 135.4 (C_{2a}), 133.8 (C_1'), 132.3 (C_{9a}), 129.2 (C_6), 128.6 (C_5' & C_3'), 128.5 (C_6' & C_2'), 127.4 (C_{4b}), 126.5 (C_4'), 124.8 (C_{4a}), 122.2 (C_7), 120.3 (C_5), 118.7 (C_8), 27.1 (C_3), 20.4 (C_4); Anal calcd. for $C_{19}H_{15}NO$: C, 83.49; H, 5.53; N, 5.12; Found: C, 83.41; H, 5.58; N, 5.5 .

*4.1.2.1.4. 6-Methyl-2-(thiophen-2-ylmethylene)-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3d**).*
Yellow solid; yield: 243 mg (83%); m.p. 221-223 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3242 (NH), 1631 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.84 (b s, 1H, N_9-NH), 7.96 (s, 1H, C_2'-H), 7.50 (d, 1H, C_4'-H, $J_{4',3'}$ = 5.2 Hz), 7.44 (s, 1H, C_5-H), 7.37 (d, 1H, C_2'-H, $J_{2',3'}$ = 4.0 Hz), 7.32 (d, 1H, C_8-H, J_o = 8.2 Hz), 7.21 (d, 1H, C_7-H, J_o = 8.2 Hz), 7.13 (d d, 1H, C_3'-H, $J_{3',2'}$ = 3.8 Hz & $J_{3',4'}$ = 4.8 Hz), 3.37-3.33 (m, 2H, C_3-2H), 3.15-3.13 (m, 2H, C_4-2H), 2.45 (s, 3H, C_6-CH_3); Anal. calcd. for $C_{18}H_{15}NOS$: C, 73.69; H, 5.15; N, 4.77; Found: C, 73.60; H, 5.19; N, 4.83 .

*4.1.2.1.5. 6-Chloro-2-(thiophen-2-ylmethylene)-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3e**).*
Yellow solid; yield: 223 mg (76%); m.p. 219-221 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3231 (NH), 1632 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.93 (b s, 1H, N_9-H), 7.97 (s, 1H, C_2-H), 7.64 (d, 1H, C_4'-H, $J_{4',3'}$ = 5.2 Hz), 7.52 (d, 1H, C_2'-H, $J_{2',3'}$ = 5.2 Hz), 7.42-7.28 (m, 2H, C_3' & C_5-H), 7.14-7.11 (m, 1H, C_8-H), 7.06-7.04 (m, 1H, C_7-H), 3.37-3.34 (m, 2H, C_3-2H), 3.13-3.10 (m, 2H, C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 179.4 (C=O), 144.5 (C_{2a}), 139.6 (C_{8a}), 137.0 (C_1'), 132.4 (C_2), 131.7 (C_4'), 130.7 (C_2'), 130.5 (C_{9a}), 127.3 (C_6), 126.6 (C_3'), 125.3 (C_{4b}), 124.1 (C_{4a}), 118.3 (C_7), 116.6 (C_5), 109.4 (C_8), 24.6 (C_3), 22.4 (C_4); Anal. calcd. for $C_{17}H_{12}ClNOS$: C, 65.07; H, 3.85; N, 4.46; Found: C, 65.16; H, 3.89; N, 4.40.

*4.1.2.1.6. 2-(thiophen-2-ylmethylene)-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3f**).* Yellow solid; yield: 223 mg (80%); m.p. 220--221 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3235 (NH), 1633 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.96 (b s, 1H, N_9-H), 7.97 (s, 1H, C_2-H), 7.69-7.63 (m, 1H, C_4'-H), 7.49-7.33 (m, 2H, C_2' & C_5-H), 7.18-7.10 (m, 3H, C_8, C_7 & C_6-H), 7.04 (t, 1H, C_3'-H, J = 4.4 Hz), 3.18-3.07 (m, 4H, C_3 & C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 180.2 (C=O), 145.4 (C_{2a}), 139.8 (C_{8a}), 136.5 (C1'), 130.0 (C_2), 127.9 (C_4'), 125.8 (C_{4b}), 124.0 (C_2'), 123.1

(C_{9a}), 122.7 ($C_{3'}$), 121.7 (C_{4a}), 116.6 (C_7), 116.4 (C_6), 115.7 (C_5), 107.6 (C_8), 24.9 (C_3), 22.7 (C_4); Anal. calcd. for $C_{17}H_{13}NOS$: C, 73.09; H, 4.69; N, 5.01; Found: C, 73.01; H, 4.73; N, 5.07.

*4.1.2.1.7. 2-(4'-methoxybenzylidene)-6-methyl-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3g**).* Yellow solid; yield: 265 mg (87%); m.p. 222-224 ℃; FT-IR (KBr, cm^{-1})ν_{max}: 3321 (NH), 1630 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.89 (b s, 1H, N_9-H), 7.74 (s, 1H, C_2-H), 7.43-7.41 (m, 3H, C_5, C_6' & C_5'-H), 7.33-7.27 (m, 1H, C_8-H), 7.21-7.18 (m, 1H, C_7-H), 6.95 (d, 2H, C_5' & C_3'-H, J_o = 8.8 Hz), 3.85 (s, 3H, C_4'-OCH_3), 3.27-3.24 (m, 2H, C_3-2H), 3.05-3.01 (m, 2H, C_4-2H), 2.44 (s, 3H, C_6-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 179.8 (C=O), 159.5 (C_4'), 138.7 (C_{8a}), 135.5 (C_2), 134.3 (C_{2a}), 132.2 (C_6' & C_2'), 131.7 (C_{4b}), 128.4 (C_{9a}), 127.5 (C_1'), 126.7 (C_{4a}), 125.6 (C_7), 121.5 (C_6), 120.1 (C_5), 114.0 (C_5' & C_3'), 112.4 (C_8), 55.5 (OCH_3), 27.7 (C_3), 24.8 (C_4), 18.7 (CH_3); Anal. calcd. for $C_{21}H_{19}NO_2$: C, 79.47; H, 6.03; N, 4.41; Found: C, 79.54; H, 6.00; N, 4.47.

*4.1.2.1.8. 2-(4'-methoxybenzylidene)-6-chloro-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3h**).* Yellow solid; yield: 253 mg (78%); m.p. 220-222 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3235 (NH), 1641 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 9.87 (b s, 1H, N_9-H), 7.81 (s, 1H, C_2-H), 7.47-7.45 (m, 3H, C_5, C_6' & C_5'-H), 7.37-7.33 (m, 1H, C_8-H), 7.26-7.22 (m, 1H, C_7-H), 7.05 (d, 2H, C_5' & C_3'-H, J_o = 8.8 Hz), 3.83 (s, 3H, C_4'-OCH_3), 3.25-3.21 (m, 2H, C_3-2H), 3.07-3.05 (m, 2H, C_4-2H); Anal. calcd. for $C_{20}H_{16}ClNO_2$: C, 71.11; H, 4.77; N, 4.15; Found: C, 71.11; H, 4.77; N, 4.15.

*4.1.2.1.9. 2-(4'-methoxybenzylidene)-2,3,4,9-tetrahydro-1H-carbazol-1-one (**3i**).* Yellow solid; yield: 241 mg (83%); m.p. 224-226 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3267 (NH), 1642 (C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 9.57 (b s, 1H, N_9-H), 7.81 (s, 1H, C_2-H), 7.65 (d, 2H, C_6' & C_2'-H, J_o = 8.4 Hz), 7.61-7.42 (m, 2H, C_8 & C_5-H), 7.38-7.34 (m, 1H, C_6-H), 7.16-7.12 (m, 1H, C_7-H), 6.98-6.94 (m, 2H, C_5' & C_3'-H,), 3.85 (s, 3H, C_4'-OCH_3), 3.30-3.26 (m, 2H, C_3-2H), 3.08-3.05 (m, 2H, C_4-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 181.0 (C=O), 159.7 (C_4'), 138.6 (C_{8a}), 135.2 (C_2), 134.5 (C_{2a}), 132.4 (C_6' & C_2'), 131.5 (C_{4b}), 128.0 (C_{9a}), 127.0 (C_1'), 126.9 (C_{4a}), 125.8 (C_7), 121.3 (C_6), 120.3 (C_5), 113.9 (C_5' & C_3'), 112.6 (C_8), 55.3 (OCH_3), 27.6 (C_3), 24.9 (C_4); Anal. calcd. for $C_{20}H_{17}NO_2$: C, 79.19; H, 5.65; N, 4.62; Found: C, 79.10; H, 5.69; N, 4.68.

4.1.2.2. General procedure for the synthesis of 4'-arylidene/heteroarylidene-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(*H*)-1,2''-dione **7**

An appropriate mixture of 2-arylidene/heterolidene-2,3,4,9-tetrahydrocarbazol-1-one **3** (1.0 mmol) isatin **4** (1.0 mmol) and bezylamine **5** (1.0 mmol) was refluxed in dioxane: methanol (1:1) for 5 h. After completion of the reaction, the solvent was removed in vacuum and crude product was subjected to silica gel column chromatography using petroleum ether: ethyl acetate (91:9) as eluant to yield the respective 4'-arylidene/heteroarylidene-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(*H*)-1,2''-dione **7**.

*4.1.2.2.1. 6-Methyl-4',5'-diphenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7a**).* White solid; yield: 455 mg (87%); m.p. 216-218 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3419 (Indole NH), 3322 (carbazole NH), 3222 (pyrrolo NH), 1696 (indole C=O), 1617 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 11.18 (b s, 1H, N_9-H), 10.18 (b s, 1H, N_1''-H), 7.50 (d, 2H, C_8 & C_7''-H, J_o = 7.4 Hz), 7.38 (m, 2H, C_5'''' & C_3''''-H), 7.26-7.22 (m, 4H, C_6''',C_5''', C_3''' & C_2'''-H), 7.18-7.13 (m, 3H, C_6'', C_6'''' & C_2''''-H), 7.09 (s, 1H, C_5-H), 7.05 (d, 1H, C_4''-H, J_o = 7.4 Hz), 6.97 (d, 1H, C_7-H, J_o = 7.4 Hz), 6.84 (t, 1H, C_5''-H, J = 7.4 Hz), 6.56 (t, 1H, C_4'''-H, J = 7.2Hz), 6.54 (t, 1H, C_4''''-H, J = 7.2 Hz), 5.45 (d d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 5.6 Hz & $J_{1,2\text{-}cis}$ = 10.0 Hz), 4.69 (d, 1H, C_4'-H, J_{cis} = 10.0 Hz), 3.61 (d, 1H, pyrrolo NH, $J_{1,2\text{-}cis}$ = 5.6 Hz), 2.58-2.56 (m, 2H, C_4-H), 2.48-2.45 (m, 2H, C_3-H) 2.22 (s, 3H, C_6-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 191.5 (C=O), 180.5 (C=O), 143.3 (C_{3a}''), 142.2 (C_{7a}''), 139.2 (C_1'''), 137.2 (C_{8a}), 131.9 (C_1''''), 129.6 (C_{9a}), 128.9 (C_5''), 128.9 (C_6), 128.5 (C_5''' & C_3'''), 128.4 (C_5'''' & C_3''''), 127.9 (C_6''' & C_2'''), 127.5 (C_6'''' & C_2''''), 127.0 (C_{4b}), 126.7 (C_6''), 126.1 (C_4''' & C_4''''), 125.1 (C_4''), 121.1 (C_{4a}), 120.5 (C_7), 112.7 (C_5 & C_8), 109.5 (C_7''), 72.3 (spiro indole carbon C_3''), 63.8 (C_5'), 61.5 (spiro carbazole carbon C_2), 58.3 (C_4'), 32.1 (C_4), 21.3 (CH_3), 18.5 (C_3); Anal. calcd. for $C_{35}H_{29}N_3O_2$: C, 80.28; H, 5.58; N, 8.02; Found: C, 80.36; H, 5.53; N, 8.08.

*4.1.2.2.2. 6-Chloro-4',5'-diphenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7b**).* White solid; yield: 504 mg (93%); m.p. 212-214 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3416 (Indol NH), 3322 (carbazole NH), 3220 (pyrrolo NH), 1695 (indole C=O), 1622 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 10.85 (b s, 1H, N_9-H), 10.02 (b s, 1H, N_1''-H), 7.49 (d, 2H, C_8 & C_7''-H, J_o = 7.4 Hz), 7.40 (d, 2H, C5'''' & C3''''-H, J_o = 6.0 Hz), 7.19-7.04 (m,

8H, C_5, C_5''', C_3''', C_4''', C_6'''', C_2'''', C_6'' & C_4'-H), 7.00 (d, 2H, C_6''' & C_2'''-H, J_o = 8.6 Hz), 6.76 (t, 1H, C_6'-H, J_o = 7.4 Hz), 6.54-6.46 (m, 2H, C_5'' & C_7-H), 5.47 (d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 8.0 Hz), 4.75 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 10.0 Hz), 261-2.50 (m, 2H, C_4-2H), 1.46-1.39 (m, 2H, C_3-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 192.3 (C=O), 179.8 (C=O), 141.9 (C_{3a}''), 141.7 (C_{7a}''), 138.6 (C_1'''), 136.9 (C_1''''), 132.6 (C_{8a}), 130.6 (C_5''), 128.9 (C_{9a}), 128.2 (C_6''' & C_2'''), 128.1 (C_6'''' & C_2''''), 127.6 (C_5''' & C_3'''), 127.3 (C_5'''' & C_3''''), 126.8 (C_4'''), 126.7 (C_4''''), 126.3 (C_{4b}), 125.3 (C_6), 124.8 (C_5 & C_7), 121.3 (C_6'' & C_4''), 119.9 (C_{4a}), 114.0 (C_7''), 109.6 (C_8), 78.0 (spiro indol carbon C_3''), 64.7 (C_5'), 62.2 (spiro carbazole carbon C_2), 58.5 (C_4'), 31.8 (C_4), 18.5 (C_3); Anal. calcd. for $C_{34}H_{26}ClN_3O_2$: C, 75.06; H, 4.82; N, 7.72; Found: C, 75.15; H, 4.78; N, 7.66.

*4.1.2.2.3. 4',5'-Diphenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7c**).* White solid; yield: 417 mg (82%); m.p. 214-216 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3374 (Indol NH), 3307 (carbazole NH), 3229 (pyrrolo NH), 1687 (indole C=O), 1636 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 10.50 (b s, 1H, N_9-H), 9.98 (b s, 1H, N_1''-H), 7.50 (t, 2H, C_5'''' & C_3''''-H, J = 7.2 Hz), 7.41 (d, 2H, C_8 & C_5-H, J_o = 7.2Hz), 7.24-7.04 (m, 11H, C_7'', C_5''', C_3''', C_6''', C_2''', C_6'''', C_2'''', C_4'', C_4''', C_4'''' & C_6''-H), 6.82 (t, 1H, C_5''-H, J = 7.2 Hz), 6.78-6.74 (m, 1H, C_6-H), 6.50-6.48 (m, 1H, C_7-H), 5.47 (d, 1H, C_5'-H, J_o = 10.2 Hz), 4.76 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 10.2 Hz), 2.60-2.46 (m, 2H, C_4-2H), 1.48-1.43 (m, 2H, C_3-2H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 197.0 (C=O), 184.6 (C=O), 146.7 (C_{3a}''), 146.5 (C_{7a}''), 143.5 (C_1'''), 143.4 (C_{8a}), 136.3 (C_1''''), 135.4 (C_5''), 133.6 (C_{9a}), 133.2 (C_5''' & C_3'''), 133.0 (C_5'''' & C_3''''), 132.8 (C_{4b}), 132.7 (C_6''' & C_2''') 132.4 (C_6'''' & C_2''''), 132.0 (C_4'''), 131.4 (C_4''''), 130.9 (C_6'' & C_4''), 130.1 (C_7), 129.7 (C_{4a}), 126.1 (C_6), 125.6 (C_5), 124.2 (C_6''), 117.4 (C_8), 114.3 (spiro indol carbon C_3''), 69.6 (C_5'), 67.1 (spiro carbazole carbon C_2), 63.6 (C_4'), 36.7 (C_4), 23.4 (C_3); Anal. calcd. for $C_{34}H_{27}N_3O_2$: C, 80.13; H, 5.34; N, 8.25; O, 6.28; Found: C, 80.06; H, 5.38; N, 8.18.

*4.1.2.2.4. 6-Methyl-4'-(thiophen-2-yl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]- 9(H)-1,2''-dione (**7d**).* White solid; yield: 423 mg (80%); m.p. 196-198 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3423 (Indole NH), 3392 (carbazole NH), 3263 (pyrrolo NH), 1697 (indol C=O), 1618 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.65 (b s, 1H, N_9-H), 7.79 (b s, 1H, N_1''-H), 7.67-7.65 (m, 2H, C_5'''' & C_3''''-H), 7.32-7.28 (m, 2H, C_4''' & C_7''), 7.24-7.21 (m, 1H, C_8-H), 7.18 (s, 1H, C_5-H), 7.16-7.07 (m, 5H, C_6'''',C_2'''', C_4'''', C_4''& C_6''-H), 6.93-6.89 (m, 2H, C_5'' &

C_7-H), 6.69-6.65 (m, 1H, C_3'''-H), 6.62 (d, 1H, C_2'''-H, J_o = 7.6 Hz), 5.48 (d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 9.0 Hz), 5.06 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 9.0 Hz), 2.70-2.68 (m,2H, C_4-H), 2.65-2.64 (m, 1H, C_{3b}-H), 2.33 (s, 3H, C_6-CH_3), 1.91-1.97 (m, 1H, C_{3a}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 191.7 (C=O), 179.6 (C=O), 141.3 (C_{3a}''), 141.1 (C_1'''), 140.2 (C_{7a}''), 136.7 (C_{8a}), 131.3 (C_1''''), 129.5 (C_5''), 129.2 (C_{9a}), 129.1 (C_6), 128.5 (C_5'''' & C_3''''), 128.3 (C_6'''' & C_2''''), 128.1 (C_{4b}), 127.7 (C_3'''), 127.6 (C_6''), 126.7 (C_4''''), 125.7 (C_4''' & C_2'''), 125.5 (C_4''), 124.5 (C_{4a}), 122.2 (C_7), 120.5 (C_5), 111.8 (C_7''), 109.3 (C_8), 73.2 (spiro indol carbon C_3'), 66.9 (C_5'), 62.1 (spiro carbazole carbon C_2), 53.9 (C_4'), 30.8 (C_4), 21.2 (C_3), 18.6 (CH_3); Anal. calcd. for $C_{33}H_{27}N_3O_2S$: C, 74.83; H, 5.14; N, 7.93; Found: C, 74.94; H, 5.10; N, 7.99.

*4.1.2.2.5. 6-Chloro-4'-(thiophen-2-yl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]- 9(H)-1,2''-dione (**7e**).* White solid; yield: 401 mg (73%); m.p. 194-196 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3427 (Indol NH), 3329 (carbazole NH), 3225 (pyrrolo NH), 1696 (indol C=O), 1620 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 10.51 (b s, 1H, N_9-H), 9.71 (b s, 1H, N_1''-H), 7.43 (d, 2H, C_9 & C_7''-H, J_o = 6.8 Hz), 7.12-6.98 (m, 5H, C_5, C_5'''',C_3'''', C_4''' & C_6''-H), 6.94-6.88 (3H, C_6'''', C_2'''' & C_4''''-H), 6.85 (d, 1H, C_4''-H, J_o = 7.4 Hz), 6.72-6.70 (m, 1H, C_7-H), 6.66 (t, 1H, C_5''-H, J = 7.4 Hz), 6.41 (d, 1H, C_2'''-H, J_o = 8.0 Hz), 6.35 (t, 1H, C_3'''-H, J = 8.0 Hz), 5.26 (d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 9.4 Hz), 4.80 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 9.4 Hz), 2.45-2.35 (m, 3H, C_{3a} & C_4-H), 1.63-1.54 (m, 1H, C_{3b}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 192.0 (C=O), 179.3 (C=O), 141.5 (C_{3a}''), 141.6 (C_1'''), 140.4 (C_{7a}''), 136.3 (C_{8a}), 131.8 (C_1''''), 129.7 (C_5''), 129.4 (C_{9a}), 129.2 (C_6), 128.7 (C_5'''' & C_3''''), 128.5 (C_6'''' & C_2''''), 128.0 (C_{4b}), 127.9 (C_3'''), 127.3 (C_6''), 126.4 (C_4''''), 125.9 (C_4''' & C_2'''), 125.3 (C_4''), 124.7 (C_{4a}), 122.5 (C_7), 120.1 (C_5), 112.0 (C_7''), 109.7 (C_8), 73.4 (spiro indole carbon C_3''), 67.1 (C_5'), 62.3 (spiro carbazole carbon C_2), 54.1 (C_4'), 31.0 (C_4), 21.3 (C_3); Anal .calcd. for $C_{32}H_{24}ClN_3O_2S$: C, 69.87; H, 4.40; N, 7.64; Found: C, 69.96; H, 4.44; N, 7.70.

*4.1.2.2.6. 4'-(Thiophen-2-yl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7f**).* White solid; yield: 406 mg (79%); m.p. 198-200 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3376 (Indole NH), 3314 (carbazole NH), 3275 (pyrrolo NH), 1685 (indol C=O), 1642 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.69 (b s, 1H, N_9-H), 7.78 (b s, 1H, N_1''-H), 7.67 (d, 2H, C_5'''' & C_3''''-H, J_o = 7.2 Hz), 7.37 (d, 1H, C_5-H), 7.33-7.22 (m, 4H, C_8, C_4''',

C_7" & C_4"-H), 7.18-7.12 (m, 4H, C_6"", C_2"", C_4"" & C_6"-H), 7.00 (t, 1H, C_5"-H, *J* = 7.2 Hz), 6.94-6.89 (m, 2H, C_7 & C_6-H), 6.67 (t, 1H, C_3"'-H, *J* = 7.6 Hz), 6.62 (d, 1H, C_2"'-H, J_o = 7.6 Hz), 5.50 (d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 9.4 Hz), 5.06 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 9.4 Hz), 2.73-2.63 (m, 3H, C_{3a} & C_4-H), 1.91-1.88 (m, 1H, C_{3b}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 191.3 (C=O), 179.9 (C=O), 141.4 (C_{3a}"), 141.8 (C_1"'), 140.4 (C_{7a}"), 135.2 (C_{8a}), 131.5 (C_1""), 129.3 (C_5"), 129.1 (C_{9a}), 129.0 (C_6), 128.7 (C_5"" & C_3""), 128.5 (C_6"" & C_2""), 128.3 (C_{4b}), 127.5 (C_3"'), 127.4 (C_6"), 126.9 (C_4""), 125.6 (C_4"' & C_2"'), 125.7 (C_4"), 124.3 (C_{4a}), 122.6 (C_7), 121.2 (C_5), 111.6 (C_7"), 109.5 (C_8), 73.6 (spiro indole carbon C_3'), 66.5 (C_5'), 62.3 (spiro carbazole carbon C_2), 53.7 (C_4'), 30.6 (C_4), 21.4 (C_3); Anal. calcd. for $C_{32}H_{25}N_3O_2S$: C, 74.54; H, 4.89; N, 8.15; Found: C, 74.53; H, 4.85; N, 8.21.

*4.1.2.2.7.6-Methyl-4'-(4'''-methoxyphenyl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7g**).* White solid; yield: 407 mg (85%); m.p. 200-202 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3420 (Indol NH), 3378 (carbazole NH), 3241 (pyrrolo NH), 1689 (indole C=O), 1643 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 8.78 (b s, 1H, N_9-H), 7.93 (b s, 1H, N_1"-H), 7.60 (d, 2H, C_6"' & C_2"'-H, J_o = 7.6 Hz), 7.43 (d, 2H, C_8 & C_7"-H, J_o = 7.6 Hz), 7.33-7.25 (m, 2H, C_6"" & C_2""-H), 7.20 (t, 3H, C_5"", C_3"" & C_4""-H, Jo =7.2 Hz), 7.11 (s, 1H, C_5-H), 7.07 (d, 1H, C_4"-H, J_o = 7.6 Hz), 6.90 (t, 1H, C_6"-H, *J* = 7.6 Hz), 6.80 (d, 2H, C_5"' & C_3"'-H, J_o = 8.4 Hz), 6.68 (t, 1H,C_5"-H, J=7.6 Hz), 6.61 (d, 1H, C_7-H, J_o = 7.6 Hz), 5.52 (d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 9.6 Hz), 4.80 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 9.6 Hz), 3.74 (s, 3H, OCH_3), 2.69-2.53 (m, 3H, C_{3a} & C_4-H), 2.31 (s, 3H, CH_3), 1.69-1.63 (m, 1H, C_{3b}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 192.3 (C=O), 180.0 (C=O), 158.4 (C_4"'), 141.6 (C_{3a}"), 140.2 (C_{8a}), 136.7 (C_{7a}"), 131.6 (C_1""), 131.4 (C_5"), 130.3 (C_{9a}), 129.4 (C_1"'), 129.1 (C_6"' & C_2"'), 128.9 (C_6), 128.5 (C_5"" & C_3""), 128.4 (C_6"" & C_2""), 128.3 (C_{4b}), 127.5 (C_6"), 127.3 (C_4""), 125.8 (C_4"), 125.5 (C_{4a}), 122.2 (C_7), 120.5 (C_5), 113.6 (C_7"), 111.8 (C_5"' & C_3"'), 109.3 (C_8), 73.1 (spiro indole carbon C_3"), 65.5 (C_5'), 62.4 (spiro carbazole carbon C_2), 58.1 (C_4'), 55.0 (OCH_3), 31.8 (C_4), 21.2 (CH_3), 18.7 (C_3); Anal. calcd. for $C_{36}H_{31}N_3O_3$: C, 78.10; H, 5.64; N, 7.59; O, 8.67; Found: C, 78.19; H, 5.59; N, 7.52.

*4.1.2.2.8. 6-Chloro-4'-(4'''-methoxyphenyl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7h**).* White solid; yield: 517 mg (90%); m.p. 201-203 ℃; FT-IR (KBr, cm^{-1}) ν_{max}: 3410 (Indole NH), 3371 (carbazole NH), 3315 (pyrrolo NH), 1686 (indole

C=O), 1651 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 11.51 (b s, 1H, N_9-H), 10.42 (b s, 1H, N_1''-H), 7.49 (d, 2H, C_7'' & C_8-H, J_o = 7.2 Hz), 7.41 (s, 1H, C_5-H), 7.30-7.24 (m, 5H, C_5'''', C_3'''', C_6''', C_2''' & C_4''''-H), 7.18-7.12 (m, 2H, C_6'''' & C_2''''-H), 7.00 (d, 1H, C_4''-H, Jo = 7.2 Hz), 6.85-6.80 (m, 3H, C_6'', C_5'' & C_7-H), 6.56-6.53 (m, 2H, C_5''' & C_3'''-H), 5.37 (d d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 5.6 Hz & $J_{1,2\ cis}$ = 9.6 Hz), 4.58 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 9.6 Hz), 3.66 (s, 3H, OCH_3), 3.63 (d, 1H, pyrrolo C_1'-NH, $J_{1,2\ cis}$ = 5.6 Hz), 2.65-2.54 (m, 3H, C_{3a} & C_4-H), 1.28-1.25 (m, 1H, C_{3b}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 193.0 (C=O), 183.1 (C=O), 158.6 (C_4'''), 141.5 (C_{3a}''), 140.7 (C_{8a}), 136.4 (C_{7a}''), 132.3 (C_1''''), 131.7 (C_5''), 131.0 (C_{9a}), 129.7 (C_1'''), 129.5 (C_6''' & C_2'''), 128.6 (C_6), 128.7 (C_5'''' & C_3''''), 128.2 (C_6'''' & C_2''''), 128.0 (C_{4b}), 127.3 (C_6''), 127.1 (C_4''''), 126.3 (C_4''), 125.7 (C_{4a}), 122.4 (C_7), 120.7 (C_5), 113.3 (C_7''), 112.1 (C_5''' & C_3'''), 109.7 (C_8), 73.4 (spiro indole carbon C_3''), 66.4 (C_5'), 62.8 (spiro carbazole carbon C_2), 58.3 (C_4'), 55.5 (OCH_3), 31.6 (C_4), 18.9 (C_3); Anal. calcd. for $C_{35}H_{28}ClN_3O_3$: C, 73.23; H, 4.92; N, 7.32; Found: C, 73.12; H, 4.96; N, 7.25.

*4.1.2.2.9.4'-(4'''-Methoxyphenyl)-5'-phenyl-3,4-dihydrodispiro[carbazole-2,3'-pyrrolo-2',3''-indole]-9(H)-1,2''-dione (**7i**).* White solid; yield: 463 mg (86%); m.p. 202-204 ℃; IR (KBr, cm^{-1})ν_{max}: 3439 (Indole NH), 3374 (carbazole NH), 3217 (pyrrolo NH), 1685 (indole C=O), 1643 (carbazole C=O); ^{1}H NMR (400 MHz, $CDCl_3$) (ppm) δ_H: 11.30 (b s, 1H, N_9-H), 10.41 (b s, 1H, N_1''-H), 7.50 (d, 2H, C_8 & C_5-H, J_o = 7.2 Hz), 7.34 (d, 1H, C_7''-H, Jo = 7.8 Hz), 7.27-7.23 (m, 5H, C_5'''', C_3'''', C_6''', C_2''' & C_4''''-H), 7.18-7.12 (m, 2H, C_6'''' & C_2''''-H), 7.04 (d, 1H, C_4''-H, J_o = 7.8 Hz), 6.88-6.79 (m, 4H, C_6'', C_5'' C_7 & C_6-H), 6.57-6.53 (m, 2H, C_5''' & C_3'''-H), 5.37 (d d, 1H, C_5'-H, $J_{1,2\text{-}cis}$ = 5.6 Hz & $J_{1,2\ cis}$ = 10.0 Hz), 4.61 (d, 1H, C_4'-H, $J_{1,2\text{-}cis}$ = 10.0 Hz), 3.65 (s, 3H, OCH_3), 3.60 (d, 1H, pyrrolo C_1'-NH, $J_{1,2\ cis}$ = 5.6 Hz), 2.61-2.57 (m, 3H, C_{3a} & C_4-H), 1.31-1.28 (m, 1H, C_{3b}-H); ^{13}C NMR (100 MHz, $CDCl_3$) (ppm) δ_C: 191.8 (C=O), 180.6 (C=O), 158.3 (C_4'''), 143.5 (C_{3a}''), 142.2 (C_{7a}''), 138.7 (C_{8a}), 131.8 (C_1''''), 131.6 (C_5''), 130.8 (C_{9a}), 129.6 (C_1'''), 128.9 (C_6''' & C_2'''), 128.4 (C_5'''' & C_3''''), 127.9 (C_{4b}), 127.4 (C_6'''' & C_2''''), 127.2 (C_6''), 126.4 (C_4''''), 126.1 (C_4''), 124.9 (C_{3a}), 121.3 (C_7), 121.1 (C_6), 119.7 (C_5), 113.9 (C_7''), 113.0 (C_5''' & C_3'''), 109.1 (C_8), 72.3 (spiro indole carbon C_3''), 64.0 (C_5'), 61.4 (spiro carbazole carbon C_2), 57.7 (C_4'), 55.2 (OCH_3), 32.5 (C_4), 18.5 (C_3); Anal. calcd. for $C_{35}H_{29}N_3O_3$: C, 77.90; H, 5.42; N, 7.79. Found: C, 77.99; H, 5.37; N, 7.74.

4.2. Biological evaluation

4.2.1. In vitro cytotoxic activity

4.2.1.1. 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.

4.2.1.2. 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).

4.2.2. 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-549selected cells were analyzed using Nikon (Japan) bright field inverted light microscopy at 40x magnification.

4.2.3. 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 24h. The fixed cells were permeabilised with 0.2% triton X-100 (50µl) for 10min at room temperature and incubated for 3min 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.

4.2.4. Flow cytometry analysis

The apoptotic effect of synthesized dispiro compound **7e** on MCF-7 and A-549 cells were determined by the annexin V-FITC and Propidium iodide double staining flow cytometric method. Initially cells ($1x10^5$ cells per ml) were treated with various concentrations and incubated for 6 h. The treated cells were harvested, washed with PBS, and then treated with trypsin/ EDTA solution. The suspended cells were centrifuged at 200xg for 10 min. To the cell pellet was added 100 ml of annexin V-FITC staining solution (Strong Biotech Co., Taipei, Taiwan) and the solution incubated for 10-15 min at 25^{o}C. The cells were then analyzed with a flow cytometry (FACS verse, BD Bioscience, USA).

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Supplementary data

CIF files for the compounds **7a** have been deposited with the Cambridge Crystallographic Data Centre as CCDC number1538472 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.

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