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ANTI-PROLIFERATIVE ACTIVITY OF INDOLES AND RELATED COMPOUNDS: A REVIEW

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Review Article

ABSTRACT

Indoles, imidazole, indazole and carbazole are one of the most important biologically active natural compounds in living system which are even can be synthesized in laboratory. They are known to play functional role in cellular metabolism, cell division, cell growth as well as cellular signaling. The aim of this review is to amalgamate various information already available in the area of study of bio-activity of indole derivatives, including their ability to antagonize uncontrolled cellular proliferation in cancerous growth. An array of such compounds having central indole moiety and variation in their side chains have been synthesized and their bio-activities have been studied by various authors. Most of them show anti-cancer and anti-proliferative activities and functions through caspases 3/7/9, procaspase-3, cytochrome c, intracellular and extracellular apoptosis pathways and modulation of transcription pathways. These compounds are reportedly capable of neutralizing flagellar movements in sperms as well as of leishmanial protists. Reports show such compounds having anti-flagellated activities also seem to have anti-proliferative and anti-cancer properties.

Keywords: Anticancer; anti-proliferative properties; biological activities; caspases; indoles.

1. INTRODUCTION

Heterocyclic organic compounds are some of the most valuable sources of novel agents with diverse biological activities, mainly because of the unique ability of the resulting compounds to mimic the structure of peptides and to bind reversibly to proteins [1]. Indole derivatives are such heterocyclic compounds that have been extensively used as source for the preparation of large number of biologically relevant heterocycles [2,3]. Indole and other related compounds like indazole, imidazole and carbazole compounds are experimentally proven to be preventive agents against the flagellated protist, *Leishmania sp*. They are, hence termed as anti-flagellated agents also. Generally this anti-flagellated property is correlated with anti-tumorigenic or anticancer activities of these compounds [4]. Beside this, they are also, a very important category of compounds that play a key role in cell physiology and

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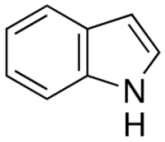
are probable intermediates for numerous biological reactions. Indole derivatives correspond to scores of important modules of therapeutic agents such as anti-cancer [5,6], anti-oxidant, anti-rheumatoidal, anti-HIV [7], anti-microbial [8,9], anti-inflamatory [10], analgesic, anti-pyretic [4], anti-convulsant, anti-helmintic cardiovascular [11], selective COX-2(cyclooxygenase-2) inhibitory activities (which is an enzyme accountable for inflammation and pain) [12] and DNA binding ability [13]. Cancer is a proliferative property of cellular metabolism having a variety of other properties like limitless number of cell divisions, promoting angiogenesis, invasion of tissue and formation of metastasis, avoidance of apoptosis etc. as well as alteration in DNA binding ability [13]. Cancer is also a cellular abnormality with the potential to spread or invade other parts of the body. Benign tumors do not spread to other parts of the body, while malignant tumors are cancerous. According to a recent report by the World Health Organization, there are now more than 10 million cases of cancer per year worldwide. Cancer results from a multistage, multi-mechanism carcinogenesis process that involves mutagenic, cell death and epigenetic mechanisms, during the three distinguishable but closely allied stages: initiation, promotion and progression [14]. A considerable account of the role of indole compounds in cancer progression has been discussed by Ahmad et al., Gurkan-Alp and Patel et al. [15,16,17]. Intricate mechanism of the action of indole as potential drug against cellular proliferation has been analyzed by Patil et al. [18]. diindolylmethane (DIM), Indole-3-Carbinol (I_3C), Indole-3-acetic acid (I3AA) are similar compounds having plant and animal origin, have also been found to be active against various kinds of cancerous growth as studied by Del et al., 2010; Fares, 2014; Jeong et al., 2010 and Tilton et al. [19,20,21,22]. Similarly analogous compounds like imidazole, indazole and carbazole play remarkable role in controlling neoplastic cell proliferation in animal tissues which was discussed by Abbassi et al., Elsayed et al., Finlay et al., Jones et al., Romero et al. and Salahuddin et al. [23,24,25,26,27, 28].

2. MATERIALS

2.1 Chemical Compounds under Study

2.1.1 Indoles

Indole was first isolated by treatment of the indigo dye with oleum [18]. It is an organic compound (C_8H_7N) found in coal tar and produced in the gut by the bacterial decomposition of tryptophan.



Indole moiety

Indole is an aromatic heterocycle, but exhibit very distinctive reactivity. Here are some general properties:

- The nitrogen is not basic (pKa -3.6)
- Indole can readily undergo aromatic electrophillic substitution.
- Highly ionic salts (e.g. Li+, K+) favours N substitution.
- When N is substituted, C-2 can be deprotonated [17].

Indole is an important heterocyclic system because it is built into proteins in the form of amino acid tryptophan, because it is the basis of drugs like indomethacin and because it provides the skeleton of indole alkaloids—biologically active compounds from plants including strychnine. The incorporation of indole nucleus, a biologically accepted pharmacophore in medicinal compound, has made it versatile heterocyclic possessing wide spectrum of biological activities. In the present study, we have also made an attempt to collect biological properties of imidazole nucleus reported in the new millennium [29]. One of the oldest and most reliable methods for synthesizing substituted indoles is the "Fischer Indole Synthesis", developed in 1883 by Emil Fischer (Fig. 1). This has also been accomplished in a one-pot synthesis using microwave irradiation [2].

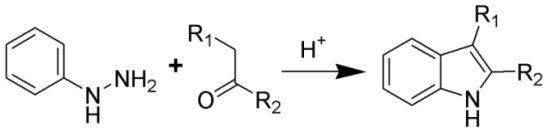
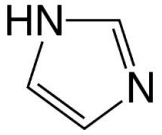


Fig. 1. Reaction mechanism of Fischer indole synthesis

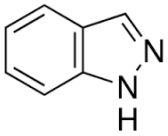
2.1.2 Imidazoles and Indazoles

Imidazole is an organic compound with the formula $C_3N_2H_4$. It is a white or colourless solid that is soluble in water, producing a mildly alkaline solution. In chemistry, it is an aromatic heterocycle, classified as a diazole, and having non-adjacent nitrogen atoms. Many natural products, especially alkaloids, contain the imidazole ring. This ring system is present in important biological building blocks, such as

histidine and the related hormone histamine. Many drugs contain an imidazole ring, such as certain antifungal drugs, the nitroimidazole series of antibiotics, and the sedative midazolam. Imidazole is a planar 5-membered ring. It exists in two equivalent tautomeric forms, because the positive charge can be located on either of the two nitrogen atoms. Imidazole is a highly polar compound, as evidenced by its electric dipole moment of 3.67D. It is highly soluble in water.



Imidazole moiety

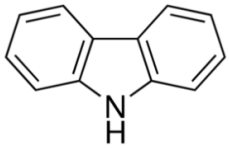


Indazole moiety

Indazole, also called isoindazole, is a heterocyclic aromatic organic compound. This bicyclic compound consists of the fusion of benzene and pyrazole. Indazoles are rare in nature. The alkaloids nigellicine, nigeglanine, and nigellidine are indazoles. Nigellicine was isolated from the widely distributed plant *Nigella sativa* L. (black cumin). Nigeglanine was isolated from extracts of *Nigella glandulifera*. Indazole derivatives display a broad variety of biological activities.

2.1.3 Carbazoles

Carbazole is an aromatic heterocyclic organic compound. It has a tricyclic structure, consisting of two six-membered benzene rings fused on either side of a five-membered nitrogen-containing ring. The compound's structure is based on the indole structure but in which a second benzene ring is fused onto the five-membered ring at the 2–3 position of indole (equivalent to the 9a–4a double bond in carbazole respectively).

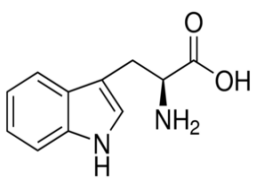


Carbazole moeity

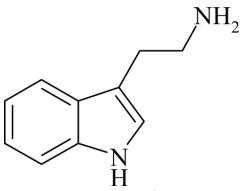
2.2 Biological and Anti-Proliferative Activities

2.2.1 Indoles

Indole derivatives are found to contain several biological activities those including antimicrobial, antibiotic, anti-inflammatory, analgesic, anticonvulsant, antimalarial, anticancer, antiulcer & anti-leishmanial, contraceptive, antioxidant etc. The derivatives are also found to have agonistic effects on several receptors such as Liver x receptor, 5- HT1D receptor etc (Fig. 2) [18]. Indoles are probably the most widely distributed heterocyclic compounds in nature having medicinal importance. Tryptophan is an essential amino acid and as such is a constituent of most proteins; it also serves as a biosynthetic precursor for a wide variety of tryptamine-indole, and 2,3-dihydroindole containing secondary metabolites. In animals, serotonin (5-hydroxytrytamine) is a very important neurotransmitter in the CNS, and also in the cardiovascular and gastrointestinal systems. The structurally similar hormone melatonin is thought to control the diurnal rhythm of physiological functions [1].



Tryptophan



Tryptamine

Fig. 2. Structures of naturally occurring indoles

Many novel indole retinoid derivatives act as anti-cancerous agents like 2-substituted quinoxaline, triazolo (4,3-a) quinoxaline, 2-chloro-3(1-substituted indol-3-yl) quinoxaline, etc. 1-benzoyl-3-bromoacetyl indole, 2-Phenyl-1H indole, (Tetrazol-5-yl) methylindole, 2- thienyl-3-substituted indole, novel 1,3-diheterocycles indole, indole-3-carbinol, 4-(2-(4-bromophenyl)-1H-indol-3-yl)-2-methoxy-6-(4-bromophenyl) nicotinonitrile, etc. are some of the indole dervatives with anti-cancerous activities. Bis-indole derivatives and benzopyridoindoles possess anti-tumorous activities.

Indoles are natural compounds that can be found in numerous types of plant. They are, however more predominantly found in cruciferous vegetables. Cruciferous vegetables comprise of cauliflower, cabbage, turnip, broccoli and brussels sprouts. Indoles fit in a class of phytonutrient compounds (plant compounds with health- protecting qualities) which have been systematically proven to profit the body in a number of imperative ways. Consuming of cruciferous vegetables has been associated with reduced of the risk of colon, breast and prostate cancers. These vegetables are a rich source of many hytochemicals, including indole derivatives, dithiolethiones and sothiocyanates. Cruciferous vegetables are full of glucobrassicin (GB) which throughout metabolism, produce indole-3-carbinol,3,3'-diindolylmethane (DIM) and ascorbigen (ASC) [14].

Table 1. Some indole ring containing drugs and their applications

Drugs	Applications
Vincristine	Anticancer
Bufotenidine	Toxin
Vincamine	Vasodilator
Reserpine	Antihypertensive
Oxypertine	Antipsychotic
Amedalin	Antidepressant
Panobinostat	Anti-leukamic
Yohimbine	Sexual Disorder
Bucindolol	β-Blockers
Mitragynine	Opioid agonist
Oglufanide	Immunomodulatory

Table 2. Anti-proliferative activities of some indole derivatives

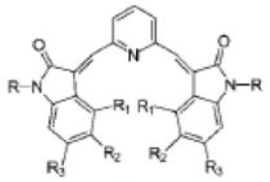
Name	Empirical formula	Anticancer activity	Other biological activities (if any)
Bis-indole derivatives	$C_{23}H_{15}N_3O_2$	Anti-tumor activity against several human cancer cell lines	Not reported yet
Indole-3-acetic acid (I3C)	$C_{10}H_9NO_2$	Apoptosis-inducing ligand in mammals	Induce cell elongation and cell division & acts as signaling molecule
2-substituted quinoxaline derivatives	$C_{23}H_{16}ClN_3$ [2-chloro-3-(1- benzyl indol-3- yl)quinoxaline]	Suppression of ovarian cancer in mice; tumor growth suppression in OVCAR3 and BG-1 cells	Antimicrobial activities
Indole-3-carbinol (I3C)	C_9H_9NO	Decreases tumor susceptibility in many cancer cell lines and demotes metastasis in liver	Antioxidant, and anti-atherogenic activities
Triazolo(4,3-a) quinoxaline derivatives	$C_{24}H_{17}N_5$ [4-(1-benzyl indol-3-yl)-(1,2,4)- triazolo(4,3- a) quinoxaline]	Tumor growth suppression in two human cancer cell lines- OVCAR3 and BG-1; and ovarian cancer cell line in mice	Antimicrobial activity
2-Phenyl-1H Indole	$C_{14}H_{11}N$	Anti-cancer activities in kidney, cervical and breast cancer cell lines	-
3,3'-Diindolylmethane (DIM)	$C_{17}H_{14}N_2$	Inhibit cell proliferation, cause cell cycle arrest at G1 phase and induce apoptosis in many cancer cell lines	Immuno-stimulant against human papilloma virus infection of the cervix
(Tetrazol-5-yl) methylindole	$C_{16}H_{13}N_5$ [1-[(2H- Tetrazol-5-yl)methyl]-2- phenyl-1H-indole]	Anti-cancer activity against human liver cancer cell line(HepG2)	Antimicrobial activity
2-thienyl-3-substituted indole	-	Antitumor activity against breast cancer cell line MCF-7	Anti-inflammatory, ulcerogenic and antimicrobial activity
Novel Indole Retinoid derivatives	-	Anti-proliferative activities in colon, breast and liver cancer cell lines.	-

2.2.2 Bis-indole derivative

Bis-indoles are produced in living organisms through dimerization of monomeric indole bases.

Derivatives like 3,3'-[pyridine-2,6-diylbis (methylene)] bis(1,3-dihydroindol-2-ones) and 3,6-Bis(2-oxo-1,2-dihydroindol-3-ylidene)piperazine-2,5-diones have anti-tumorous activity against a panel of many human tumor cell lines like bladder, colon, CNS, gastric, head-neck, lung, melanoma, ovarian, pancreas, breast, prostate, pleuramesothelioma, renal, uterus body. The pyridyl derivatives also have a high growth inhibitory effect against breast cancer cells and has a comparable

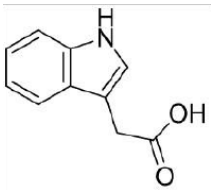
in vitro growth inhibitory activity to the known proteasome inhibitor MG-132 (Z-Leu-Leu-Leu-al) [4].



Bis-Indole Derivative

2.2.3 Indole-3-acetic acid or 3-IAA

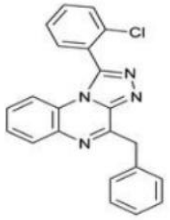
It is the most common, naturally-occurring, plant hormone of the auxin class. On a larger scale, IAA serves as signaling molecule necessary for development of plant organs and coordination of growth. IAA is an apoptosis-inducing ligand in mammals. IAA, along with horseradish peroxidase, induces apoptosis in TCCSUP human urinary bladder carcinoma cells via both death receptor-mediated and mitochondrial apoptotic pathways. IAA/HRP activates p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase(JNK) and also induces caspase-8 and caspase-9 activation, which results in caspase-3 activation and poly(ADP-ribose) polymerase (PARP) cleavage [21].



Indole-3-acetic acid

2.2.4 2-substituted quinoxaline derivatives

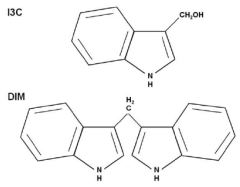
Conjugated indole-quinoxaline is vital for the antimicrobial activity and potential anti-cancer efficacy. Compounds like 3-(1- substituted indol-3-yl)quinoxalin-2(1H)ones, 2-(4-methyl piperazin-1-yl)-3-(1-substituted indol-3-yl) quinoxalines, 2-chloro3-(1-substituted indol-3-yl)quinoxalines, 2-(piperidin-1-yl)-3-(1- benzyl indol-3-yl)quinoxaline, 2-morpholino-3-(1-benzyl indol-3- yl)quinoxaline, etc. are derivatives of 2-substituted quinoxalines and have in vitro cytotoxic effect against OVCAR-3 and BG-1 cell lines in humans and also in vivo growth suppression of ovarian cancer xenografts in nude mice. Derivatives like 3-(1-substituted indol-3-yl)quinoxaline-2(1H)ones have anti-microbial activities against Gram-negative bacteria like *E. coli and P.aeruginosa*, 2-(4-methyl piperazin-1-yl)-3-(1-substituted indol-3-yl) quinoxalines show anti-microbial activity against Gram-negative bacteria *P. aeruginosa* and Gram-positive bacteria like *S. aureus* and *B. cereus*. Another derivative, 2-chloro-3-(1-substituted indol-3- yl)quinoxalines, show anti-microbial activity against a strain of fungi, *C. albicans* [30].



2-chloro-3-(1-substituted-indol-3-yl) quinoxalines

2.2.5 Indole-3-carbinol (I_3C) and 3,3'-Diindolylmethane (DIM)

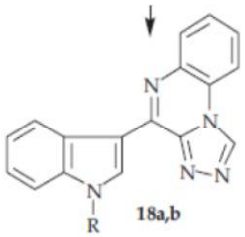
Indole-3-carbinol is produced by the breakdown of the glucosinolate glucobrassicin, whichcan be found at relatively high levels in cruciferous vegetables such as broccoli, cabbage, cauliflower, brussels sprouts, collard greens and kale. 3,3′-Diindolylmethane (DIM) is a compound derived from the digestion of indole-3-carbinol, found in cruciferous vegetables such as broccoli, Brussels sprouts, cabbage and kale. The diet-derived indole derivatives, I3C and DIM, exert anticancer effects mediated through the regulation of cell cycle, induction of apoptosis, transcription, cell signal transduction, inhibiting angiogenesis and suppressing cell invasion. The activation of the mitochondrial pathway through releasing of Cytochrome C and activation of caspases, together with inactivation of hormonal, PI3K/Akt, MAPK, Bcl-2 and NF-kB pathways represent the possible molecular mechanism of these indole derivatives in their anticancer activity. The anti-cancer activity of I3C and DIM has been detected in various target organs including breast, prostate, colon, liver, cervix, endometrium, melanoma and lung using human cancer cell lines or various animal models. Furthermore, these derivatives have been evaluated in clinical trials phase I and phase II as potential chemopreventive agents against breast, ovary and colon cancer [20]. Indole-3-carbinol has the ability to alter estrogen metabolism and other cellular effects. Indole-3-carbinol can shift estrogen metabolism towards less estrogenic metabolites. It promotes liver cancer in trout when it is combined with aflatoxin B1 and promotes metastasis [22]. 3,3'-diindolylmethane acts as an immunostimulant against human papilloma virus infection of the cervix, but not a statistically significant level [19].



Indole-3-carbinol and 3,3'-diindolylmethe

2.2.6 Triazolo(4,3-a) quinoxaline derivatives

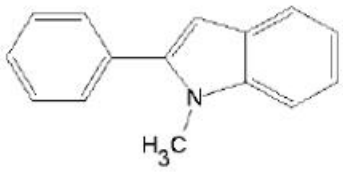
These compounds are 1-benzyl and 1-benzoyl-3-heterocyclic indole derivatives.4-(1-benzyl indol-3-l)-(1,2,4) - triazolo (4,3-a)quinoxaline, 4-(1-benzoyl indol-3-yl) -(1,2,4) -triazolo (4,3-a) quinoxaline, 1-methyl-4-(1- benzyl indol-3-yl)-(1,2,4)-triazolo(4,3-a)quinoxaline and 1-methyl-4-(1-benzoyl indol-3-yl)-(1,2,4) triazolo(4,3-a)quinoxalineare thederivatives of triazolo(4,3-a) quinoxalines, which are synthesized by treating 1-(2-(1-benzyl indol-3-yl)quioxalin-3-yl)hydrazine and 1-(2-(1-benzoyl indol-3-yl)quioxalin-3-yl)hydrazine with formic acid (25 mL) or acetic acid (25 mL) and allowed to stand at room temperature for 24 hours. These derivatives have *in vitro* cytotoxic effect against OVCAR-3 and BG-1 cell lines in humans and *in vivo* growth suppression of ovarian cancer xenografts in nude mice [30]. These indole derivatives also possess moderate level of antimicrobial activities.



Triazolo(4,3-a) quinoxaline, a:R=CH_2Ph, b:R=COPh

2.2.7 2-Phenyl-1H Indole

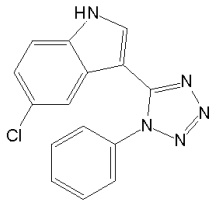
Derivatives of 2-Phenyl-1H indole like 4-(2-(4-bromophenyl)-1H-indole-3-yl)-2-methoxy-6-phenyl nicotinonitrile,4-(2-(4-bromophenyl)-1H-indole-3-yl)-2-methoxy-6-(4-minophenyl)nicotinonitrile,4-(2-(4-bromophenyl)-1H-indole-3-yl)-2-methoxy-6-(2-hydroxyphenyl) nicotinonitrile,4-(2-(4-bromo- phenyl -1H-indole-3-yl)-2-methoxy-6-(4-hydroxyphenyl) nicotinonitrile,4-(2-(4-romophenyl)-1H-indole-3-yl)-2-methoxy-6-(4-bromophenyl) nicotinonitrile, 4-(2-(4-bromophenyl)-1Hindole-3-yl)-2-methoxy-6-(3-hydroxyphenyl) nicotinonitrile and 4-(2-(4-bromophenyl)-1H-indole-3-yl)-2-methoxy-6-(4-methylphenyl) nicotinonitrile have potent *in-vitro* anti-cancerous activities against HEK293 (Human Epidermal Kidney Cell Line), HELA (Cervical Cancer Cell Line) and MDA MB 468 (Breast Cancer Cell Line) [17]. Substitution at phenyl ring on 6th position of pyridine ring gives good anticancer activity as in the order of Br>NH2>CH3>OH>H. Absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 550 and 600 nm according to the filters available for the ELISA reader, used . The percentage cell inhibition of the samples at different concentrations have been represented graphically below with the anticancer drug Methotrexate (MTX) as standard [31].



2-Phenyl-1H Indole

2.2.8 (Tetrazol-5-yl) methylindole

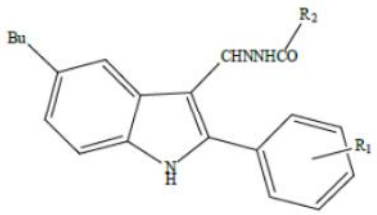
New (tetrazol-5-yl)methylindole derivatives are being synthesized from 2-phenylindole. Furthermore, the sugar acetyl hydrazones of the tetrazole derivatives as well as their derived acyclic *C*-nucleoside analogs show anticancer activity against human liver carcinoma cell line (HepG2). These indole derivatives reduce the expression of cyclooxygenase-2 in HepG2 cells and result in HepG2 cell death via apoptosis. These indole-containing compounds cause HepG2 cell cycle arrest at G0/G1 phase, thus preventing cell from entering S or G2/M phase and finally causing apoptosis. Sugar {5[(2-phenyl-1*H*-indol-1-yl)methyl]-2*H*tetrazol-2- yl} acetyl hydra- zones are the derivatives which are the most potent compounds and affect the cell viability in a dose dependent manner with IC_{50} values of 4.2 µg/mL [15]. These compounds also have potent antimicrobial activity against *Aspergillus Niger, Penicillium sp, Candida albican, Bacillus subtilis, Streptococcus lacti, Escherichia coli, Pseudomonas sp.*, and *Streptomyces sp*. [9].



5-chloro-3-(1-phenyl-1H-tetrazol-5-yl)-1H-indole

2.2.9 2-thienyl-3-substituted indole

Derivatives of 2-thienyl-3-substituted indoles are potent antitumor agents against breast cancer cell line (MCF-7) along with antiinflammatory and ulcerogenic activities. About 23 new compounds have been synthesized from the parent 2-thienyl-3-substituted indole compound like N'-((2- (Thiophen-2-yl)-1H-indol-3-yl) methylene) acetohydrazide, 3-(2-methylhydrazono) methyl)-2-(Thiophen-2-yl)-1Hindole, 2-(Thiophen-2-yl)-1H-indole-3- carbaldehyde Oxime, 2- (2- (Thiophen-2-yl)-1H-indol-3-yl) methylene) hydrazine Carboxamide, 5- (2- (Thiophen-2-yl)-1h-indol-3-yl) methylene) pyrimidine-2,4,6 (1H,3h,5h)- trione, 4-oxo-4- (2- ((2- (Thiophen-2-yl) -1H-indol-3-yl) methylene) Hydrazinyl) Butanoic Acid, etc. All these synthesized compounds show good to moderate anti-tumor activity against breast cancer cell line (MCF-7) using doxorubicin as a standard drug (IC50=2.97 µg/ml), with the most potent one being N-cyclohexyl-2-(2-(Thiophen-2-yl)-1H-indol-3- yl)methylene) Hydrazine carboxamide(IC50 2.6 µg/ml) and Methyl 2- (2- ((2-(Thiophen-2- yl)-1H-indol-3-yl) methylene) Hydrazinyl) Acetate (IC50: 38.4 µg/ml) having the weakest activity. Compounds 2-(thiophen-2- yl)-1H-indole-3-carbaldehyde, 2- (2- ((2- (Thiophen-2- yl)-1H-indol-3-yl) methylene) hydrazine Carbonyl) benzoic Acid show high antiinflammatory activity against carrageenan induced oedema in albino rats against indomethacin as a reference standard but less ulcerogenic effect. While compounds like 4-amino- n'- ((2- (Thiophen-2-yl) -1H-indol-3-yl) methylene) benzo Hydrazide, 4-oxo-4- (2- ((2-(Thiophen-2-yl) - 1H-indol-3-yl) methylene) Hydrazinyl) Butanoic Acid and 2- (2- ((2- (Thiophen-2-yl)-1H-indol-3-yl) methylene) hydrazine Carbonyl) phenyl) carbamic Acid show anti-inflammatory activity but no ulcerogenic effect [10]. These compounds also show moderate level of antimicrobial activity [31].



2-thienyl-3-substituted indole

2.2.10 Novel indole retinoid derivatives

Novel(E)-3-(5-substituted-1H-indol-3-yl)-1-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphtha len-2-yl)prop-2-en-1-one derivatives show *in vitro* anti-cancerous effects. Novel indole retinoid compounds like (E)-3-(1H-Indol-3-yl)-1-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one, (E)-3-(5-Methoxy-1H-indol-3-yl)-1-(5,5,8,8- tetramethyl-5,6,7,8-tetrahydrona phthalen-2-yl)prop-2-en-1-one, (E)-3-(5-Chloro-1H-indol-3-yl)-1-(5,5,8,8-tetramethyl -5,6,7,8-etrahydronaphthalen-2-yl)prop-2-en-1-one and (E)-3-(5-Bromo-1H-indol-3-yl)-1-(5,5,8,8-tetra-methyl-5,6,7,8- etrahydrona phthalen-2- yl)prop-2-en-1-one have anti-proliferative capacity in liver, breast and colon cancer cell lines. Compound (E)-3-(1H-Indol-3-yl)-1-(5,5,8,8-tetramethyl-5,6,7,8- tetrahy dronaphthalen-2-yl)prop-2-en-1-one has the lowest IC50 level in cytotoxicity assays in breast cancer cell line panel, which includes ER-positive and ER-negative cell lines. Furthermore, it is also less toxic in MCF-12A, which is a normal-like breast epithelial cell line and induces apoptosis as a cause of anti-proliferative effect [16].

3. MECHANISM OF ANTI-PROLIFERATIVE ACTIVITY

Indole compounds act on a number of cellular signaling pathways leading to their observed biological effects (Fig. 3). Cancer progression involves up-regulation of signaling pathways that favour proliferation, angiogenesis, and invasion. Mechanisms of apoptosis stimulation of indole derivatives include- a) down-regulation of anti-apoptotic gene products such as Bcl-2 (B-cell lymphoma) and Bcl-XL (B-cell leukemia-extra large) b) down-regulation of the inhibitor of apoptosis proteins, e.g. CIAPs, X-chromosome linked inhibitor of apoptose protein (XIAP) and survival, c) up-regulation of pro-apoptotic factors such as *Bax* gene d) liberation of mitochondrial cytochrome C in addition to stimulating of caspase-9 and caspase-3 and e) inhibition of the NF-kB signaling pathway.

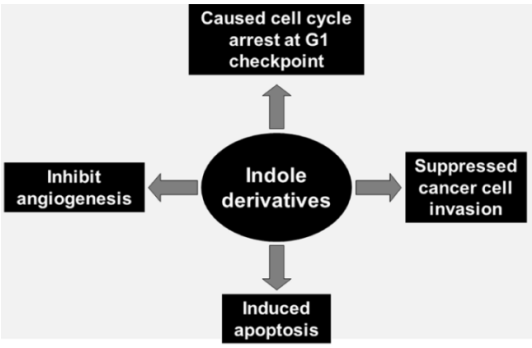


Fig. 3. Anti-proliferative activities performed by indole derivatives [20]

3.1 Mechanisms of Apoptosis Induction by Indoles Involve Two Pathways

3.1.1 Intrinsic and Extrinsic pathway

In the extrinsic pathway, signal molecules identified as ligands, which are released by the immune system's natural killer cells possess the Fas ligand (FasL) on their exterior to connect to transmembrane death receptors on the target cell (Fig. 4). After the binding of the death ligand to the death receptor the target cell triggers multiple receptors to aggregate together on the surface of the target cell. The aggregation of these receptors recruits an adaptor protein known as Fas-associated death domain protein (FADD) on the cytoplasmic side of the receptors. FADD, in turn, recruits Caspase-8. Caspase-8 will then be activated and will be now able to directly activate caspase-3 and caspase-7. The activation of caspase-3 will initiate the degradation of the cells. The intrinsic pathway is triggered by cellular strain, particularly mitochondrial stress caused by factors such as DNA damage from chemotherapy or UV exposure. Upon delivery of the stress signal, the pro-apoptotic proteins in the cytoplasm (Bcl-2-like protein 4 (BAX) and BAX-like Bcl-2 homology domain 3 protein (BID)) bind to the outer membrane of the mitochondria to signal the release of the internal content. The interaction between the pro-apoptotic (BAX and BID) and the antiapoptotic proteins (Bcl-2) on the surface of the mitochondria is thought to be important in the formation of the PT pores in the mitochondria, and hence, the release of cytochrome c and the intramembrane content from the mitochondria. Following the release, cytochrome c forms a multi protein complex [13].

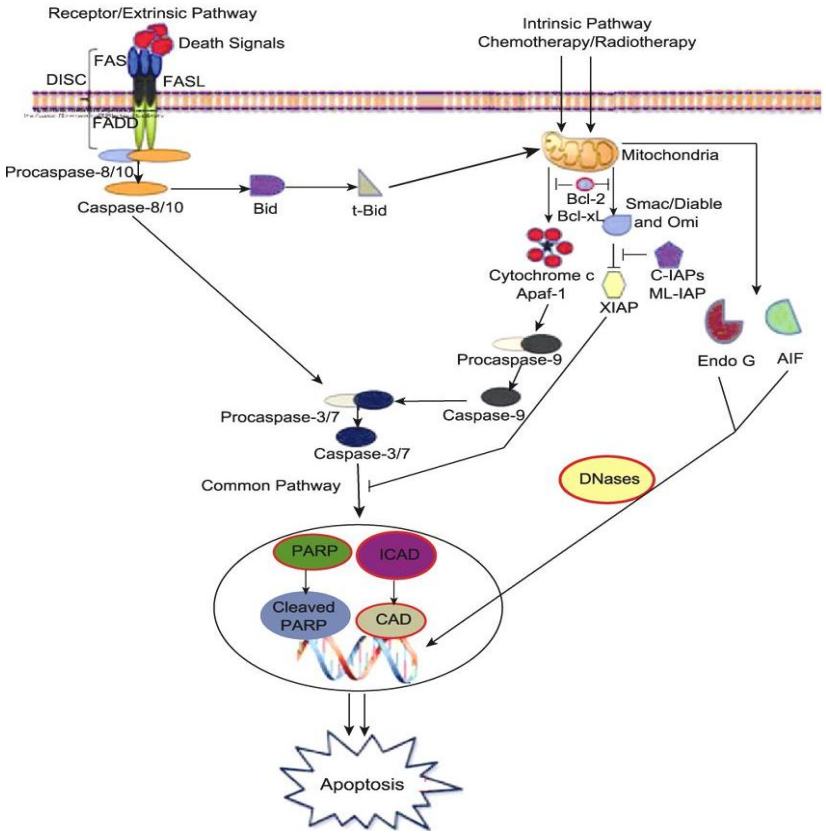


Fig. 4. Intrinsic and Extrinsic Pathways of Apoptosis activated by indoles [13] This is known as apoptosome which consists of cytochrome c, Apaf-1, procas-pase-9 and ATP. Following its formation, the complex will activate caspase-9. The activated caspase-9 will then turn the procaspase-3 and procaspase-7 into active caspase-3 and active caspase-7. These activated proteins initiate cell degradation or cell death. Besides the release of cytochrome C fromthe intramembrane space, the intramembrane also releases Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP). IAP is a protein family which consists of 8-human derivatives. Their function is to stop apoptotic cell death by binding to caspase-3, caspase-7 and caspase-9 and inhibit them [13]

Tumor angiogenesis starts with cancerous tumor cells releasing molecules that post signals to the neighboring host tissues. This signaling activates definite genes in the host tissue that, in turn, build proteins to support growth of new blood vessels.

Indole compounds restrain the invasion of cancer cells and the expansion of new blood vessels (angiogenesis). Indole compounds adapt the cellular signaling pathways through chemosensitization leading to apoptosis and thus conquer the chemo-plus immune-resistance of well-known chemotherapeutic drugs (Fig. 5). Emerging evidence also documents the ability of indoles to reverse the process of EMT via regulation of key miRNAs. An efficient induction of apoptosis and reversal of EMT not only ensures increased sensitivity to conventional drugs (chemosensitization) but also results in significantly reduced invasion and metastasis [13,15].

Cancer progression involves upregulation of signaling pathways that favours proliferation, angiogenesis and invasion. NF-kB is activated and translocated to nucleus leading to transcriptional up-regulation of genes that play important roles in these processes. As efficient anticancer agents, indole compounds target an array of cellular pathways causing a reversal of pro-survival and invasion pathways and an efficient induction of apoptosis. As an example, indoles inhibit the upstream pathway (PI3-K-Akt) that regulates NF-kB signaling as well as block NF-kB activation and translocation to nucleus, thus preventing the generation of transcription of multiple target genes (Fig. 6). Such a multistep regulation ensures a much increased efficacy and underlines the efficacy of these compounds as effective anticancer agents [15].

I_3C-induced P450-dependent estrogen metabolism is responsible for the chemopreventive action of I_3C. In a study to compare the mechanisms of the action of I_3C in estrogen-responsive MCF-7 breast cancer cells and the estrogen- nonresponsive MDA-MB-231 breast cancer cells, it has been reported that I_3C was able to inhibit the growth of only estrogen-responsive cells with little effect on estrogen-nonresponsive cells. It has been reasoned that the inhibitory effects of I_3C may involve elective induction of estradiol metabolism and the related cytochrome P450 s y s t e m that may be limited to estrogen-sensitive cells. However, it was later shown that I_3C can suppress the growth of breast cancer cells independent of estrogen receptor signaling. It was shown that a combination of I_3C and tamoxifen inhibited MCF-7 cell growth more stringently than either agent alone [15].

I_3C signaling was observed to induce the G1 cell cycle arrest of MCF-7 cells. Furthermore, I_3C-mediated cell cycle arrest was also observed in estrogen receptor-negative MDA-MB-231 cells under conditions in which the antiestrogen tamoxifen had no effect on cell growth, thus demonstrating a more versatile effect of I_3C, independent of estrogen receptor signaling . This study implicated cyclin-dependent kinase 6 as a target for cell cycle control in human breast cancer cells. The first clue for the involvement of NF-kB pathway in I_3C action came from a study where the effect of I_3C was compared using estrogen receptor-a-negative MDA-MB-468 cells versus immortalized non- tumorigenic HBL100 cells. 73 In this study, phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (PKB)/ Akt were identified as targets of I_3C. I_3C inhibits phosphory - lation and activation of PKB in MDA-MB-468 cells but not in the non- tumorigenic HBL100 cells. Because PKB can regulate NF-kB by the activation of IKK, resulting in increased phosphorylation of IkB and consequent release of NF-kB from the inhibitory complex, the effect of I_3C was tested on NF-kB and IKK. Despite inhibition of PKB, no decrease in IKK activity was observed in response to I_3C treatment. In support of this, nuclear levels of NF-kB (p65) were found to be unaltered. However, I_3C decreased NF-kB e DNA binding, as determined using electrophoretic mobility shift assay (EMSA). These results suggested that I_3C affected DNA binding of NF-kB protein family members, including p65 and p50, by a mechanism that does not involve the inhibition of IKK activity. I_3C is capable of inducing apoptotic cell death in MCF10A-derived cell lines with premalignant and malignant phenotypes but not in non-tumorigenic parental MCF10A cells. I_3C specifically inhibits Akt kinase activity and abrogates the epidermal growth factor (EGF)-induced activation of Akt in breast cancer cells. Transfection of Akt gene activates NF-kB directly, and such activation of NF- k B is completely abrogated by I_3C treatment. I_3C also directly inhibits the elastase-mediated proteolytic processing of CD40, which alters downstream signaling to disrupt NF-kB-induced cell survival and proliferative responses. DIM has similar activity against breast cancer cells as I_3C (Fig. 7). DIM can induce apoptosis processes in MCF10A-derived malignant cell lines but not in non-tumorigenic parental cells. DIM also specifically inhibits Akt kinase activity and abrogates the EGF-induced activation of Akt in breast cancer cells **(**Fig. 7), similar to those observed for I_3C. As a further mechanism, DIM can reduce the phosphorylation of IkBa, an inhibitor of NF-kB. Confocal studies revealed that DIM blocks the translocation of p65 subunit of NF-kB to the nucleus. Activation of NF-kB involves IkB kinase e-mediated kBa phosphorylation, which can be completely abrogated by DIM treatment [13,15,16,20].

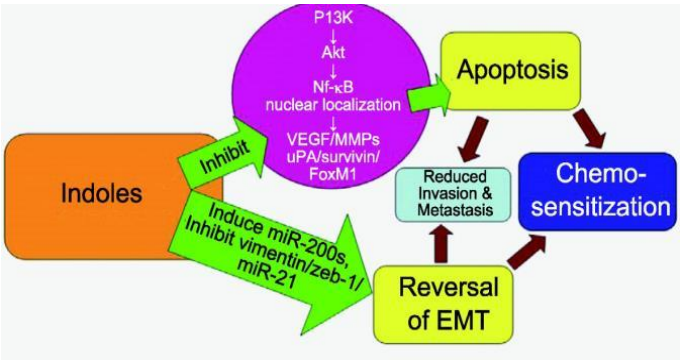


Fig. 5. Summary of mechanisms of anti-prliferative and chemosensitizing effects of indole compounds [13]

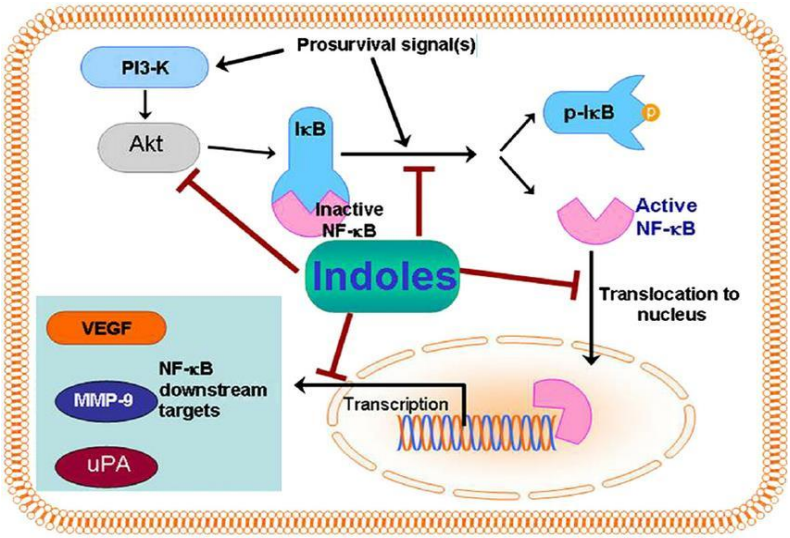


Fig. 6. Cellular effects of Indoles through regulation of NF-kB Signaling [15]

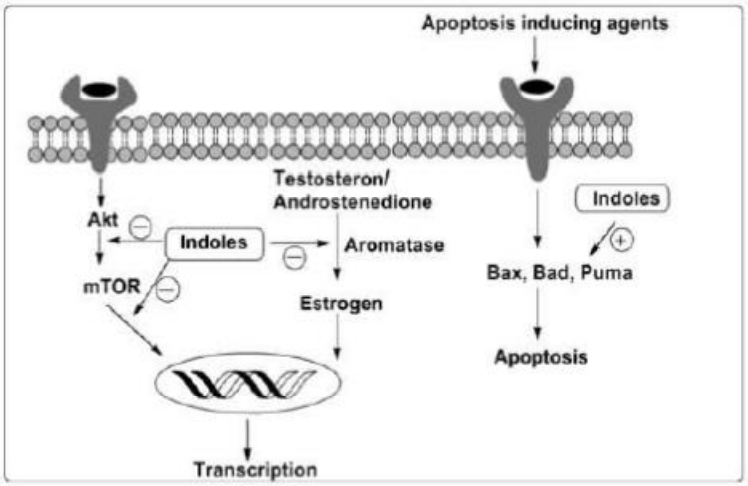
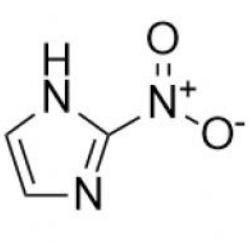


Fig. 7. I_3C-induced estrogen metabolism [20]

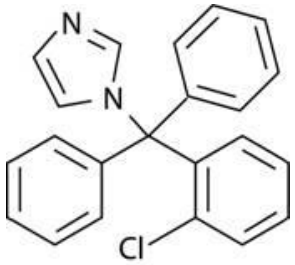
4. IMIDAZOLE AND INDAZOLE

Imidazole and its derivatives are reported to be physiologically and pharmacologically active and find applications in the treatment of several diseases. In the drug discovery the imidazole is the most important synthetic strategy. Many imidazoles are reported as pharmacological agents like Azomycine, Clotrimazole, Miconazole, Ergothionine, Clonidine and Moxonidine. One of the most important applications of imidazole derivatives is their usage as material for treatment of denture stomatities and in cancer [32].

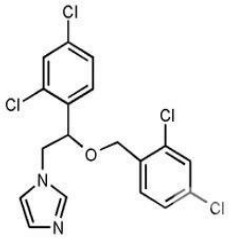
Imidazoles come under the category of antimetabolite, which have specific mechanism of action in cancer. Antimetabolite is a type of chemical that inhibits the use of a metabolite. They have toxic effects on cells, such as halting cell growth and cell division, thereby making these compounds of use as chemotherapy for cancer. Antimetabolites can be used in cancer treatment, as they interfere with DNA production and therefore cell division and the growth of tumors. These are the chemicals which become the building blocks of DNA. They prevent these substances becoming incorporated in to DNA during the S phase of the cell cycle, stopping normal development and division. They also affect RNA synthesis because thymidine is used in DNA but not in RNA where uracil is used instead of cytosine, inhibition of thymidine synthesis via thymidylate synthase selectively inhibits DNA synthesis over RNA synthesis [30,31,33].



Azomycin



Clotrimazole



Miconazole

Table 3. Anti-proliferative activities of some imidazole and indazole compounds

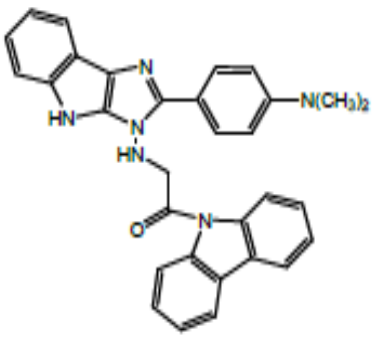
Name	Empirical Formula	Anti-proliferative Activity
2-thioxoimidazolidine derivatives	C20H17N3O2S [1-[(1-benzyl indol-3-yl) carbomethyl]-2- thioxoi-midazolidine-4-one]	Tumor growth suppression in two human cancer cell lines-OVCAR3 and BG-1; and ovarian cancer cell line in mice.
Imidazolidine-2,4 dione derivatives	C20H17N303 [1-[(1- benzyl indol-3-yl) carbomethyl]imidazolidine- 2,4-dione]	Tumor growth suppression in two human cancer cell lines-OVCAR3 and BG-1; and ovarian cancer cell line in mice.
2-substituted-*N*-[4(1-methyl-4,5-diphenyl-1*H*- imidazole -2-yl) phenyl] acetamide derivatives	-	Cytotoxic activity against colon carcinoma cell line
Imidazole piperazines	-	Inhibition of tumor growth when human SW620 xenografts are dosed orally in nude mice
Imidazole pyrimidine amides	-	Anti-proliferative activity against a range of cancer cell lines
Benzimidazoles	C7H6N2	Potent anti-cancer activity against many human cancer cell lines
Indazole pyrimidine-based derivatives	-	VEGFR-2 kinase inhibitors; with varying levels of anticancer activity against NCI-60 cancer cell line panel
2-alkyl-6-nitroindazole derivatives	-	Trigger apoptosis; arrest cells in G2/M phase of the cell cycle.

5. CARBAZOLE

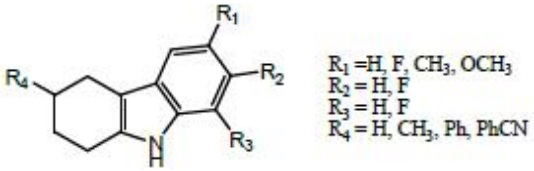
Carbazoles represent an important class of heterocycles. These have been reported toexhibitdiverse biological activities such as antimicrobial, antitumor, antiepileptic, antihistaminic, antioxidant, anti-inflammatory, antidiarrhoeal, analgesic, neuroprotective and pancreatic lipase inhibition properties.The carbazole derivatives have gained the attention of researchers due to their therapeutic potential against neurological disorders and cell proliferation. The biological profiles of these new generations of carbazole would represent a fruitful matrix for further development of carbazole nucleus, which can be a lead nucleus for future developments to get safer and effective anticancer therapeutic agents [34].

a) A number of new 1-substituted-6H-pyrido[4,3-b]carbazole derivatives have been synthesized by Beata Tylinska et al., 2013 and the compounds were subjected to preliminary *in vitro* cytostatic activity screening against murine leukemia (L1210), human lung cancer (A549) and human colon cancer (HT29) cell lines. One particular compound 6f exhibited over 20 times better activity against L1210 tumor cell line than the reference ellipticine [35].

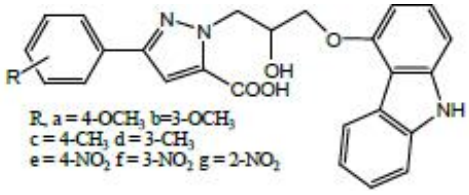
b) Kumar, Sharma and Pathak, 2013 worked on the microwave assisted and parallel synthesis of novel substituted carbazole derivatives. The synthesized compounds were evaluated for their antibacterial and anticancer activity. Some of the synthesized carbazole derivatives exhibited significant cytotoxic activity against Ehrlich's Ascites Carcinoma (EAC) and HEP2 cell lines [36].



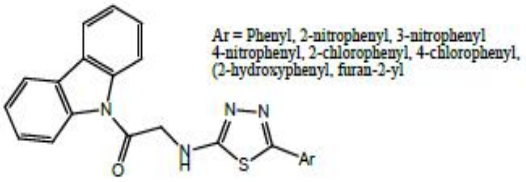
c) Kumar et al., 2014 synthesisized the 2,3-Dimethylindoles and Tetrahydrocarbazoles via Fisher Indole synthesis and evaluation of their anticancer properties. The differently substituted 2, 3-dimethylindoles and tetrahydrocarbazoles have reported to posses significant activity [37].



d) Nagarapu et al., 2010 carried out the Synthesis and cytotoxicity evaluation of 1-[3- (9H-carbazol-4-yloxy)-2-hydroxypropyl]-3-aryl-1H-pyrazole-5-carboxylic acid derivatives. The cytotoxicity of synthesized compounds was evaluated by a SRB (sulforhodamine B) assay against cancer cell such as SKeNeSH human neuroblastoma (NB), human A549 lung carcinoma and human breast cancer MCF-7 cell lines. The results showed that seven compounds can suppress SKeNeSH tumor cancer cell growth. Among them, compound 3d was the most effective small molecule in inhibiting SKeNeSH cell growth [38].

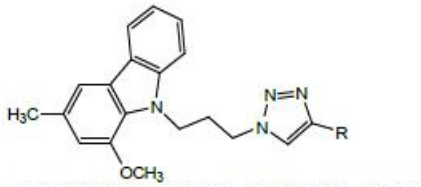


e) Shah et al., 2012 worked on the Design, Synthesis and Anticancer Evaluation of Carbazole Comprised With 1,3,4-Thiadiazole Derivative; All the synthesized compounds are evaluated for their anticancer activity by MTT assay and compared with standard drugs. The test compounds showed significant anticancer activity [39].



f) Haider et al., 2014 carried out the electrophilic substitution of Dimethyl 1- Methylcarbazole-2,3-dicarboxylate ie., synthesis of new b-Fused Carbazoles. Anti-proliferative activity of compounds was assessed using an XTT assay method [40].

g) Tran Thi Thu Thuy et al carried out the Synthesis of novel derivatives of murrayafoline. A and their inhibitory effect on LPS-stimulated production of proinflammatory cytokines in bone marrow-derived dendritic cells. Results indicated that murrayafoline, a derivative containing 1,2,3-triazole nucleus, potentially possessed anti-inflammatory action through inhibiting production of IL-6, IL-12 p40 and TNF-α [41].



R= -COOH, -CH_2-NHBoc, -CH_2-NH_2, -CH_2-OTBDPS, -CH_2OH

6. EXPERIMENTAL SCREENING OF ANTI-PROLIFERATIVE ACTIVITY

Some of the biological assays followed for the anticancer screening are as follows:-

6.1 Cell Culture Assay

A cell culture assay is any method which is used to assess the cytotoxicity of a material. This refers to the *in vitro* assessment of material to determine whether it releases toxic chemicals in sufficient quantities to kill cells either directly or indirectly through the inhibition of cell metabolic pathways. Cell culture assays are standardized by ASTM, ISO, and BSI (British Standards Institution). These assays can be performed in 3 methods- direct contact method, agar diffusion method and elution method. Most of the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 50 mg/ml penicillin/streptomycin [16].

6.2 Sulforhodamine B (SRB) Cytotoxicity Assay

The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The method is optimized for the toxicity screening of compounds to adherent cells (104cells/well) in a 96-well format. After an incubation period, cell monolayers are fixed with 10% (wt/vol) trichloroacetic acid (TCA) and stained for 30 min, after which the excess dye is removed by washing repeatedly with 1% (vol/vol) acetic acid. The protein-bound dye is dissolved in 10 mM Tris base solution for OD determination at 510 nm using a microplate reader. The results are linear over a 20-fold range of cell numbers and the sensitivity is comparable to those of fluorometric methods [16,19].

6.3 Hoechst Staining

Hoechst stains are part of a family of blue fluorescent dyes used to stain DNA. These Bis-benzimides were originally developed by Hoechst AG and have three Hoechst stains: Hoechst 33258, Hoechst 33342, and Hoechst 34580. The dyes Hoechst 33258 and Hoechst 33342 are the ones most commonly used and they have similar excitation/emission spectra. Cancer cells (50.000 cells/well) are seeded into six-well plates and 24 h later the agents are applied. Apoptotic cells need to be visualized under a fluorescent microscope at 40 objective [16,17].

6.4 Flow Cytometry Analysis

Cancer cells have to be seeded at 5.105 cells/well onto 75 mm^2 tissue culture plates and incubated in humidified incubators at 37°C, with 5% CO2. The next day, cells are treated with two different concentrations of the anti-cancer agent (1.8 mM and 3.6 mM). On day 2 and day 4, 1.106 cells are to be sampled and stained with FITC Annexin V Apoptosis detection Kit (BD Pharmingen, Cat: 556570) according to the manufacturer's instructions. Control groups include corresponding DMSO concentrations as negative controls and CPT (5 mM) and 1% v/v hydrogen peroxide, as positive controls. Stained cells have to be kept from light on ice andanalyzed immediately using Becton Dickinson FACScalibur Flow Cytometer. Flow cytometry results are analyzed using WinMDI 2.9 software for differentially stained percentage of cells over controls and results are plotted and analyzed using GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA) [16].

6.5 MTT Cytotoxicity Assay

The MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of mitochondrial reduction of the yellow tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple colour. Tetrazolium dye reduction is dependent on NAD(P)H- dependent oxidoreductase enzymes largely in the cytosolic compartment of the cell. Therefore, reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD(P)H flux. Cells with a low metabolism such as

thymocytes and splenocytes reduce very little MTT. In contrast, rapidly dividing cells exhibit high rates of MTT reduction. IC50 is calculated for the samples and negative control (cells with vehicle) by the probit analysis using a simple t-test (SPSS statistical analysis software package/version 11.0, SPSS Inc., (IL), Chicago, USA) [31].

7. DISCUSSION

Heterocyclic compounds like indoles and related group of molecules are well studied by various workers and their role in myriad biological activities have been revealed. It has been observed that bulky geometry and better topology of the compounds increase their biological activities. Indole derivatives are known to perform important function as anticancer [5,6], anti-oxidant, anti-rheumatoidal, anti-HIV [7], anti-microbial [8,9], anti-inflamatory [10], analgesic, anti-pyretic [4], anti-convulsant, anti-helmintic cardiovascular [11], selective COX-2(cyclooxygenase-2) inhibitory agents [12] and also have DNA binding ability [13]. The plethora of research in medicinal biology and their anti proliferative as well as anti carcinogenic properties show enhanced anticancer activity of these chemical derivatives as compared to the standard drugs. Many novel indole retinoid derivatives like 2-substituted quinoxaline, triazolo (4,3-a) quinoxaline, 2-chloro-3(1-substituted indol-3-yl) quinoxaline, etc. 1-benzoyl-3-bromoacetyl indole, 2-Phenyl-1H indole, (Tetrazol-5-yl) methylindole, 2- thienyl-3-substituted indole, novel 1,3-diheterocycles indole, indole-3-carbinol,4-(2-(4-bromophenyl)-1H-indol-3-yl)-2-methoxy-6-(4-bromophenyl)nicotinonitrile etc. are some of the indole dervatives with anti-cancerous activities. Bis-indole derivatives and benzopyridoindoles also possess antitumorous activities. They can also be extracted from plants and are hence known as natural indoles and primarily found in cruciferous vegetables like cauliflower, cabbage, turnip, broccoli and brussels sprouts. Consuming cruciferous vegetables which are rich in phytonutrients including glucobrassicin, indole-3-carbinol,3,3'-diindolylmethane, ascorbigen, dithiolethiones and sothiocyanates has been associated with reduced risk of colon, breast and prostate cancers [14,29]. Similarly, imidazoles and indazoles are related heterocyclic compounds having specific mechanism of action against proliferative tissues. Antimetabolites like imidazoles and indazoles can be used in cancer treatment, as they impede DNA production and cell division as well as the growth in uncontrolled fashion within tumours. These are the chemicals which become the building blocks of DNA. They prevent normal nucleic acid building substances becoming incorporated into DNA during the S phase of the cell cycle, disrupting normal development and division. They also affect RNA synthesis because thymidine is used in DNA but not in RNA where uracil is used, thus causing inhibition of thymidine synthesis via thymidylate synthase selectively inhibiting DNA synthesis over RNA synthesis [31,33]. Carbazole heterocycles have also been reported to exhibit antitumor and anti neoplastic activities. The carbazole derivatives have gained the attention of researchers due to their therapeutic potential against neurological disorders and cell proliferation. The biological profiles of these new generations of carbazole like 1-substituted-6H-pyrido[4,3-b]carbazole, 2,3-Dimethylindoles and tetrahydrocarbazoles, 1- [3- (9H-carbazol-4-yloxy) -2-hydroxypropyl] -3-aryl- 1H- pyrazole-5 carboxylic acid derivatives, carbazole with 1,3,4-thiadiazole derivative etc. are under experimental investigations in murine model as well as other cell lines regarding their roles in controlling cellular proliferation [34,35,38,40]. Function of indole compounds are targeted on a number of cellular signaling pathways leading to their observed biological effects like cellular proliferation, cell cycle progression, cellular growth and development**.** All these metabolic functions are closely related to cancer progression involving up-regulation of signaling pathways associated with proliferation, angiogenesis, and invasion [42,43]. Mechanisms of apoptosis stimulation by the indole derivatives include down-regulation of anti-apoptotic gene products such as Bcl-2 and Bcl-XL as well as down-regulation of the inhibitor of apoptosis proteins, e.g. CIAPs, X-chromosome linked inhibitor of apoptose protein (XIAP) and survival. Up-regulation of pro-apoptotic factors such as *Bax* gene, liberation of mitochondrial cytochrome C in addition to stimulation of caspase-9 and caspase-3 and inhibition of the NF-kB signaling pathway are also essential steps [15,16,17]. In this connection a number of techniques like cell culture assay, sulforhodamine B (SRB) cytotoxicity assay, Hoechst staining assay, flow cytometry and MTT assay are being followed for the experimental screening of anti-proliferative activity of these chemicals [16].

8. CONCLUSION

From this work it has been revealed that various types of derivatives of indoles and the associated aromatic heterocyclic compounds can serve as future therapeutic leads for the discovery of antiproliferative agents including anticancer drugs. Apart from anticancer activity they have scores of other important applications as anti-oxidant, anti-rheumatoidal, anti-HIV, anti-microbial anti-inflamatory, analgesic, anti-pyretic, anti-convulsant and anti COX-2 agents. Thus

these classes of compounds certainly hold great promises in medicinal biology also. A further study to acquire more information concerning pharmacological activities of these compounds is in progress. The biological profiles of these new generations of indoles and the associated ones represent much progress with regard to the older compounds. The cellular mechanism of action of these compounds may even indoctrinate many unexplored areas of knowledge in the field of cancer biology.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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