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Research

Methodology Development To Synthesis, Characterisation Of Flavone Derivatives And Their Anticancer Activity

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Abstract

Flavone derivatives have garnered significant interest in medicinal chemistry due to their promising anticancer properties. This study focuses on the development of an efficient and reproducible methodology for the synthesis and characterization of flavone derivatives. A systematic approach was employed, integrating modern synthetic techniques to achieve high yield and purity of the derivatives. Comprehensive characterization was performed using spectroscopic methods, including NMR, FTIR, and Mass Spectrometry, to confirm structural integrity and molecular composition. The anticancer activity of the synthesized compounds was evaluated through in vitro cytotoxicity assays on various cancer cell lines, highlighting their potential as chemotherapeutic agents. Notable findings include specific derivatives exhibiting significant cytotoxicity with IC50 values comparable to standard anticancer drugs. The structure-activity relationship (SAR) analysis revealed key functional groups contributing to enhanced efficacy. This research lays the groundwork for future studies on flavone derivatives as novel anticancer agents and their translation into clinical applications.

Keywords: Flavone derivatives, synthesis methodology, characterization techniques, anticancer activity,

Introduction

Flavonoids are secondary polyphenolic metabolites occurring commonly in many fungi and plants. To the family of flavonoids belong several classes of compounds including anthoxanthins, in turn subdivided into two subgroups: flavones and flavonols;flavanones; flavanonols; flavans, in turn subdivided into three subgroups: flavan-3-ols, flavan-4-ols and flavan-3,4-diols; antocyanidins and isoflavonoids. The chemical structure of flavonoids is a 15-carbon skeleton which consist of two phenyl rings named A and B linked via a heterocyclic 4H-pyrane ring named C.
Examples of known flavonoids are: Apigenin 1 and Quercetin 2 (antoxanthins: flavone and flavonol respectively); Naringenin 3 (flavanone); Taxifolin 4 (flavanonol); Catechin 5, Apiforol 6 and Leucocyanidin 7 (flavans: flavan-3-ol, flavan-4-ol and flavan-3,4-

diol respectively); Malvidin 8 (anthocianidin) and Genistenin 9 (isoflavon). The biosynthesis of flavonoids starts from the phenylalanine, which in turn comes from shikimic acid pathway.

As reported in Fig. 4, the precursor phenylalanine leads to p-coumaril-CoA which reacts with 3 molecules of malonyl-CoA, coming from the fatty acid biosynthesis, to give the 4,20 ,40 ,60 -Tetrahydroxychalcone (Naringenin chalcone) and 4,40 ,60 -Trihydroxychalcone (Isoliquiritigenin) [1e4]. The 4, 20, 40 ,60 -tetrahydroxychalcone is the precursor to obtain the flavanones which represent the precursor for all the other flavonoids (Fig. 4). Isoflavonoids can be obtained by two different biosynthetic pathways: from 4,40, 60 -trihydroxychalcone or from flavanones (Fig. 4). This class of compounds is important because both they are essential components to the humans and animal diet (flavonoids cannot be synthesized by humans and animals) and possess a therapeutic potential. They are present in almost all type of plants occurring virtually in all plant parts. The versatile health benefit of flavonoids is well known. Antioxidant activity, weight management, cardiovascular disease protection, allergy, vascular fragility, viral and bacterial infection, antinflammatory activity.

History of Cancer[1]

The word cancer came from the father of medicine, Hippocrates, a Greek physician. He used the Greek words, carcinos and carcinoma to describe tumors, thus calling cancer "karkinos". The Greek terms actually were words to describe a crab, which Hippocrates thought a tumor resembled. Although Hippocrates may have named "Cancer", he was certainly not the first to discover the disease. At present one of the six deaths in united states is caused by cancer. One in four Americans now living may contract cancer, and two-thirds who do so will die of it at present cure rates. In England and wales, one fifth of all deaths is due to cancer.

Terminology[2]

The medical term for “cancer” or “tumor” is neoplasm, which means “a relatively autonomous growth of tissue”. “Tumor” is a general term indicating any abnormal mass or growth of tissue, not necessarily life threatening. A cancerous tumor is a malignant neoplasm with potential danger. The difference between benign and malignant neoplasm is that benign tumors do not metastasize, whereas malignant tumors do. A metastasis is a secondary growth originating from the primary tumor and growing elsewhere in the body. There is no system of nomenclature for tumors is accepted universally. Some tumors are named after the individual who first described the condition, such as Ewing’s tumor of bone, Paget’s disease, and Hodgkin’s disease. Some are named after the tissue of origin, such as papillary, cystic, or follicular tumors. The suffix – oma literally means tumor, and the words with this suffix refer to neoplasms.

Cancer Cell Cycle

The cycle is divided into four main parts , the G 1or gap 1 phase is the period when a newly created cell is born, the period of time a cell remains in the G1 phase depends on the tissue type and whether it is a normal cell or a tumor cell. If the cell is a proliferating cell, it will quickly move into the S or synthesis phase. It is during this period the nuclear DNA is replicated, and at the end of the S phase two copies of DNA are present in the cell. The next phase is the G2 or Gap2 period and this phase is largely a time during which preparations are made for the final cell cycle phase, the M phase or mitosis. There are two major control points in the cell cycle one of these is at G1 / S phase when cells commit to replicate. The second is at G2 / M phase when the cells commit to divide. Of these two major points in the cell cycle synthesis, the G1 / S is of major importance in understanding cancer and cancer treatment. During the G1 phase a cell can take one of the ‘3’ routes. First the cell may enter the ‘S’ Phase. Second the cell in the G1 phase may enter into the Fifth phase called G 0 / Gap0.Third the cell may terminally differentiate and die. Cancer can be defined broadly as a disease in which there is an uncontrolled / abnormal multiplication of cells and spread of these abnormal cells within the body. Cancer cells manifest four characteristics that distinguish them from normal cells.

I. Uncontrolled proliferation
II. Loss of function
III. Metastasis
IV. Invasiveness

1.1. THE GENESIS OF A CANCER CELL[1]

A normal cell can turn into a cancer cell because of one or more mutations in its DNA, which can be inherited or acquired. The development of cancer is a complex multistage process, involving not only more than one genetic change but usually also other epigenetic factors (hormonal action, co-carcinogen and tumor promoter effects, etc.) that are not themselves cancer producing but which increase the likelihood of the genetic mutation.

The initiation of the cancer process can occur due to

1) The activation of proto-onocogenes to oncogenes.

Proto-oncogenes are genes that normally control cell division, apoptosis and differentiation but which can be converted to oncogens by viral or carcinogen action.

2) Due to the inactivation of tumor suppressor genes.

Normal cells contain genes that have ability to suppress malignant changes are termed tumor suppressor genes (anti-oncogens). The loss of function of these cells leads to cancer.

Theses changes may occur due to point mutations, gene amplification or chromosomal translocation. In general these changes occur due to the action of certain viruses or chemical carcinogens.

The terms cancer, malignant neoplasm and malignant tumor are synonymous; they are distinguished from benign tumors by the properties of dedifferentiation invasiveness and the ability to metastasize (spread to other parts of the body).
Most of the cancers fall into following categories based on tissue of origin[1].
1. Carcinoma: In this case the tumor originates from epithelial cells.
2. Sarcoma: If the tumor originates from muscle or connective tissue then it is called as sarcoma.
3. Leukemia's or lymphomas: In this case the tumor originates from lymphatic or hematological origin.
4. Glioma: in this case the tumor originates from the neural origin.

Cancer therapy[1]
In general there are three approaches for treating cancer. They are :
(a) Surgical Excision
(b) Irradiation
(c) Chemotherapy
Role of each these type depends on the type of the tumor and the stage of its development.

Before the 1940s the principal non surgical treatment of neoplasm was radiograph and radium therapy, although certain arsenicals and urethanes were also in use. Radioisotopes, nitrogen mustards, antifolic acid agents and sex hormones for the treatment of certain types of neoplasm's and of adrenal corticoids and Adreno corticotropic hormone (ACTH) for the treatment of leukemia also developed considerably during these years.

Much excitement was generated by these early development in anti neoplastic therapy, but it was later tempered by the realization, not only that the drugs were not curative but also for the most part of life expectancy was a negligibly increased by the drugs, being mainly palliative subsequently there has been a great proliferation in both the number and the classes of anti cancer drugs therapy of cell kinetic and cell proliferation statistics, so that with the consequently improved collections and regimens, long term disease free remissions are achievable with several neoplasms, and even a few carcinomas can be cured .

Anyhow, chemotherapy of cancer, as compared with that of bacterial disease, presents a difficult problem. In biochemical terms, microorganisms are both quantitatively and qualitatively different from human cells. However cancer cells and normal cells are so similar in many respects that it is more difficult to find general exploitable, biochemical differences between them. So almost all anti-neoplastic drugs have draw backs of severe side-effects.

Common/Toxic side effects associated with antineoplastic drugs:
- Bone marrow toxicity
- Impaired wound healing
- Loss of hair
- Damage to gastrointestinal epithelium
- Depression of growth in children
- Sterility and teratogenicity.
- They can also be carcinogenic, sometimes with extensive purine catabolism, urates may precipitate in the renal tubules and cause kidney damage.

CHEMOTHERAPY
Apart from the irradiation and surgical exission cancer can also treated with various chemotherapeutic agents.

CLASSIFCATION OF ANTICANCER DRUGS
Antineoplastic agents are classified based on chemical structure and mechanism of action of drugs.

I. Alkylating agents
- Nitrogen mustards- e.g. : Mechlorethamine, Melphalan, Cyclophosphamide, Chlorambucil, ifosamide
- Ethylenimines- e.g. :Hexamethylmelamine, Thiotepa
- Alkyl Sulfonates- e.g: Busulfan
- Nitrosourea- e.g. :Carmustine, Streptozocin
- Triazenes – e.g: Dacarbazine, Temozolamide

II. Anti metabolites
- Folic acid analogs – e.g. : Methotrexate
- Pyrimidine analogs – e.g : 5-Fluorouracil, Floxuridine
- Purine analogs – e.g. : 6-Merceptopurine
- Purine antagonist – e.g : 6-Thioguanine, pentostatin

III. Natural Products
- Vinca alkaloids – e.g :Vincristine, Vinblastine, Vindesine,
- Taxanes – e.g : Paclitaxel, Doclitaxel

- Epipodophyllotoxins – e.g : Etoposide, Teniposide
- Camptothecines – e.g : Topotecan, Irinotecan

IV. Antibiotics
e.g : Dactinomycin, Daunorubicin, Mitomycin, Bleomycin, Doxorubicin

V. Enzymes and Immunomodulators
e.g: L-Asparaginase, Interferon, α-Interleukin

VI. Hormones and antagonists
- Estrogens and antiestrogens – e.g: Diethylstilbestrol, Tamoxifen, Anastrazole, Ethinyl estradiol
- Androgens and antagonists – e.g : Testosterone, Flutamide
- Adrenocorticosteroids - e.g : Prednisone

(a)Alkylating agents
These agents and related compounds contain chemical groups that can form covalent bonds with particular nucleophilic substances in cell. Most have two alkylating groups and can cross-link two nucleophilic sites as the N7 of guanine in DNA. Cross linking can cause defective replication through pairing of alkylguanine and thiamine leading to substitution of AT for GC, or it can cause excision of guanine and chain breakage. Ex: cyclophosphamide, chlorambucil.

(b) Antimetabolites
Antimetabolites including folate antagonists, pyrimidine analogues, purine analogues. Mainly these agents are acting through blocking or subverting pathways in DNA synthesis.

Folate pathway enzymes[3]
Tetrahydrofolate cofactors are essential for the synthesis of purines, certain aminoacids andthymidines. Compounds that interfere with this pathway are called antifolate agents and these are generally used as antibacterials, antimalarials, and anticancer agents. Many enzymes are involved in the biosynthesis pf tetrahydrofolate but inhibitors of any enzyme involved in biosynthesis of tetrahydrofolicacid fall under the term antifolates because majority of antifolates are inhibitors of DHFR, a key enzyme in folate utilization. Of all the enzymes involved in the biosynthetic pathways, the following are some of the enzymes that have been targeted for discovery of new drugs.

1. Dihydrofolate reductase (DHFR)
2. Thymidylate synthetase (TS)
3. Serine hydroxyl methyl transferase (SHMT)
4. Folyl polyglutamyl synthetase (FPGS) etc.,

AIM AND OBJECTIVE
Objectives
1. To synthesize new chromen derivatives based on literature review and purify the synthesized compounds by recrystallization from suitable solvents or column chromatographic techniques.
2. To characterize the synthesized compounds by physical and spectral analysis (IR, ^{1}H &^{13}C NMR, Mass Spectra).
3. To evaluate the synthesized compounds for their anti-diabetic activities.
4. To evaluate SAR based on pharmacological screening.

Synthetic Scheme
- Infrared spectra are recorded on Perkin Elmer model 283B and nicolet 740 FT-IR
- Instruments and values are given in cm-1
- Proton Nuclear Magnetic Resonance spectra are recorded on varianGemini-200, varianunity-200 and advance-400MHZ Bruker UX-NMRinstrument.The samples are made in chloroform-d(1:1) or/and DMSO-d6 using tetramethyl silane (MeSi) as the internal standard and are given in the d scale.
- ESI Mass spectra were recorded on a Micro mass Quattro LC using ESI+ software with capillary voltage 3.98 kV and ESI mode positive ion trap detector.
- High-resolution mass spectra (HRMS) were recorded on a QSTAR XL Hybrid MS– MS mass spectrometer.
- Elemental analysis is carried out on VARFIO EL, se Elementor.
- Analytical Thin-layer Chromatography (TLC) is performed on pre coated silica-gel-60 F
- All extracts are extracted with ethyl acetoacetate and water and concentrated at reduced pressure on Buchi-R-3000 rotary evaporated below 50°C.yields reported are isolated yields of materials judged homogenous by TLC and NMR spectroscopy.
- Employing TLC techniques using appropriate solvent system for development monitored all the reasons. Moisture sensitive reactions are carried out by standard syringe-septum techniques.Dry ether, dry toluene is made by distilling them from sodium benzophenone ketyl and dry methanol is prepared by using potassium hydroxide.
- 254(0.5mm) glass plates. Visualisation of the sports on TLC plates is achieved either to iodine vapour or UV light.
- Melting Points were recorded on Melter Fp-51 instrument and were uncorrected.

EXPERIMENTAL METHODS

General procedure for preparation of 5,6,7-trimethoxy-2- arylquinoline-4-carboxylic acid

A solution of appropriate benzaldehyde (9.45 mmol) and pyruvic acid (1.26 g, 14.3 mmol) in ethanol or acetic acid (5 ml) was heated for 30 min then 3,4,5-trimethoxyaniline (9.45 mmol) was added to the solution and refluxed overnight. After cooling, the produced precipitate was filtered and washed with ethanol and hexane and recrystallized in ethanol.

5,6,7-trimethoxy-2-(4-methoxy phenyl)quinoline-4- carboxylic acid (5a)

Yield: 16%; yellow crystalline powder; mp ¼ 203e205 C; IR (KBr): n (cm1) 3378 (OH), 1652 (C]O), 1 H NMR (500 MHz-DMSOd6): d (ppm) 3.79 (s, 3H, OCH3), 3.83 (s, 3H, OCH3), 3.85 (s, 3H, OCH3), 3.96 (s, 3H, OCH3), 7.02e7.04 (d, 2H, 4-methoxy phenyl H3& H5, J ¼ 8.64 Hz), 7.29 (s, 1H, quinoline H8), 7.81 (s, 1H, quinoline H3), 8.19e8.21 (d, 2H, 4-methoxy phenyl H2& H6, J ¼ 8.64 Hz), 13.2 (s, 1H, COOH), 13C NMR (DMSO, 75 MHz): d 55.76, 56.57, 61.17, 61.65, 105.13, 113.32, 114.18, 114.67, 128.97, 130.89, 140.10, 141.27, 146.16, 146.90, 155.23, 156.64, 161.19, 170.47, LC-MS (ESI): 370.1 (Mþ1)þ.

2-(3-hydroxy-4-methoxyphenyl)-5,6,7-trimethoxyquinoline4-carboxylic acid (5b)

Yield: 52%; yellow crystalline powder; mp ¼ 282e284 C; IR (KBr): n (cm1) 3416 (OH), 1618 (C]O), 1 H NMR (500 MHz-DMSOd6): d (ppm) 3.80 (s, 3H, OCH3), 3.83 (s, 3H, OCH3), 3.84 (s, 3H, OCH3), 3.96 (s, 3H, OCH3), 6.98e7.00 (d, 1H, 3-hydroxy-4-methoxy phenyl H5, J ¼ 8.53 Hz), 7.25 (s, 1H, quinoline H8), 7.62e7.65 (dd, 1H, 3-hydroxy-4-methoxy phenyl H6, J ¼ 8.53 Hz, J ¼ 2.19 Hz), 7.72 (s, 1H, quinoline H3), 7.73e7.74 (d, 1H, 3-hydroxy-4-methoxy phenyl H2, J ¼ 2.18 Hz), 9.14 (s, 1H, OH), 13.29 (s, 1H, COOH), 13C NMR (DMSO, 75 MHz): d 56.10, 56.55, 61.16, 61.65, 105.06, 112.48, 112.98, 113.38, 114.42, 119.03, 131.27, 139.96, 141.24, 146.13, 146.92, 197.25, 149.92, 155.42, 156.61, 170.48, LC-MS (ESI): 386.1 (Mþ1)þ.

-(4-hydroxy-3-methoxyphenyl)-5,6,7-trimethoxyquinoline4-carboxylic acid (5c)

Yield: 25%; yellow crystalline powder; mp ¼ 266e268 C; IR (KBr): n (cm1) 3430 (OH), 1614 (C]O), 1 H NMR (500 MHz-DMSOFig. 6. The 2D representation of the interaction between compound 6e in the crystal structure of tubulin (PDB ID: 1SA0) using LigX in MOE. N. Shobeiri et al. / European Journal of Medicinal Chemistry 114 (2016) 14e23 21 d6): d (ppm) 3.83 (s, 3H, OCH3), 3.85 (s, 3H, OCH3), 3.87 (s, 3H, OCH3), 3.96 (s, 3H, OCH3), 6.85e6.87 (d, 1H, 3-methoxy-4- hydroxy phenyl H6, J ¼ 8.28 Hz), 7.27 (s, 1H, quinoline H8), 7.70e7.72 (dd, 1H, 3-methoxy-4- hydroxyphenyl H6, J ¼ 8.28 Hz, J ¼ 2.01 Hz), 7.81e7.82 (d, 1H, 3-methoxy-4- hydroxyphenyl H2, J ¼ 2.01 Hz), 7.82 (s, 1H, quinoline H3) 9.40 (s, 1H, OH), 13.28 (s, 1H, COOH), 13C NMR (DMSO, 75 MHz): d 56.25, 56.57, 61.16, 61.64, 105.12, 111.18, 112.87, 113.38, 116.11, 120.83, 129.79, 139.97, 141.12, 146.08, 146.91, 148.41, 149.09, 155.54, 156.54, 170.53, LC-MS (ESI): 386.1 (Mþ1)þ.

2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxyquinoline-4- carboxylic acid (5d)

Yield: 30%; yellow crystalline powder; mp ¼ 143e145 C; IR (KBr): n (cm1) 3428 (OH), 1643 (C]O), 1 H NMR (500 MHz-DMSOd6): d (ppm) 3.79 (s, 3H, OCH3), 3.83 (s, 3H, OCH3), 3.85 (s, 3H, OCH3), 3.86 (s, 3H, OCH3), 3.96 (s, 3H, OCH3), 7.03e7.05 (d, 1H, 3,4- dimethoxy phenyl H5, J ¼ 9.07 Hz), 7.29 (s, 1H, quinoline H8), 7.82e7.83 (m, 2H, 3,4-dimethoxy phenyl H2& H6) 7.88 (s, 1H, quinoline H3), 13.30 (s, 1H, COOH),13C NMR (DMSO, 75 MHz): d 56.07, 56.14, 56.61, 61.18, 61.66, 105.18, 110.61, 112.17, 113.04, 113.50, 120.51, 131.07, 140.06, 141.27, 146.07, 146.88, 149.49, 150.95, 155.27, 156.61, 170.47, LC-MS (ESI): 400.1 (Mþ1)þ.

5,6,7-trimethoxy-2-(3,4,5-trimethoxyphenyl)quinoline-4- carboxylic acid (5e)

Yield: 52%; yellow crystalline powder; mp ¼ 178e179 C; IR (KBr): n (cm1) 3366 (OH), 1652 (C]O),1 H NMR (500 MHz-DMSOd6): d (ppm) 3.70 (s, 3H, OCH3), 3.84 (s, 3H, OCH3), 3.85 (s, 3H, OCH3), 3.88 (s, 6H, 2OCH3), 3.97 (s, 3H, OCH3), 7.31 (s, 1H, quinoline H8) 7.55 (s, 2H, 3,4,5-trimethoxy phenyl H2 & H6), 7.98 (s, 1H, quinoline H3), 13.20 (s, 1H, COOH), 13C NMR (DMSO, 75 MHz): d 56.56, 56.63, 60.59, 61.19, 61.68, 104.87, 105.25, 113.31, 113.90, 133.89, 139.59, 140.03, 141.49, 145.96, 146.81, 153.70, 155.13, 156.67, 170.36, LC-MS (ESI): 430.1 (Mþ1)þ.

2-(4-hydroxyphenyl)-5,6,7-trimethoxyquinoline-4-carboxylic acid (5f)

Yield: 53%; yellow crystalline powder; mp ¼ 263e265 C; IR (KBr): n (cm1) 3389 (OH), 1649 (C]O, 1 H NMR (500 MHz-DMSOd6): d (ppm) 3.82 (s, 3H, OCH3), 3.84 (s, 3H, OCH3), 3.95 (s, 3H, OCH3), 6.84e6.86 (d, 2H, 4-hydroxyphenyl H3 & H5, J ¼ 8.72 Hz), 7.26 (s, 1H, quinoline H8), 7.73 (s, 1H, quinoline H3), 8.08e8.10 (d, 2H, 4-hydroxyphenyl H2 & H6, J ¼ 8.72 Hz), 9.82 (s, 1H, OH), 13.1 (s, 1H, COOH),13C NMR (DMSO, 75 MHz): d 56.54, 61.15, 61.63, 105.06, 112.82, 113.18, 116.05, 129.05, 139.97, 141.11, 146.16, 146.91, 153.70, 155.56, 156.57, 159.61, 170.47), LC-MS (ESI): 356.1 (Mþ1)þ.

Biological Activity

PHARMACOLOGICAL EVALUATION

Introduction

Cell proliferation/viability assay methods

A variety of methods have been devised that measures the viability or proliferation ofcells*in vitro* and *in vivo.* These can be subdivided into four groups:

a) Reproductive assays can be used to determine the number of cells in a culture that arecapable of forming colonies *in vitro.* In these types of experiments, cells are plated at lowdensities and the number of colonies is scored after a growth period. These

clonogenicassays are the most reliable methods for assessing viable cell number. These methodshowever are very time consuming and become impractical when many samples have tobeanalyzed.

b) Permeability assays involve staining damage (leaky) cells that exclude the dye (exampletrypan blue). Counts can be performed manually using hemocytometer. This method isquick inexpensive and requires only a small fraction of total cells from a cell population.Therefore this method is generally used to determine the cell concentration (cell numberper ml) in batch cell culture. This is helpful in ensuring that cell cultures have reached theoptimal level of growth and cell density before routine sub-culture, freezing, or anyexperiment. Counts can also be performed mechanically using for example flow cytometer and propidium iodide. Alternatively, membrane integrity can be assayed byquantifying the release of substances from cells when membrane integrity is lost.Example: Lactate dehydrogenase or 51Cr.

c) Metabolic activity can be measured by adding tetrazolium salt to cells. Viable cellsconvert this salt to colored formazan dye which is measured spectrophotometrically.

d) Direct proliferation assays use DNA synthesis as an indicator of cell growth. Theseassays are performed using either radioactive or nonradioactive nucleotide analogs. Theirincorporation into DNA is then measured.

Traditionally, counting cells determine cell proliferation *in vitro* directly, by thedetermination of mitotic index or, in the case of hemopoeitic cells, by performing a clonogenicassay. All these assays are labor-intensive and therefore not practical for evaluating large numberof samples. Alternatively, as an indirect measure of variable cell number, the overall activity of acell population may be determined.

Tetrazolium salts like MTT (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyltetrazoliumbromide) are metabolized by NAD-dependent dehydrogenase activity to form a coloredreactionproduct. In these assays the amount of dye formed directly correlates the number of viable cells(Allely MC., 1998). These assays are performed in 96-well micro titre plate (MTP) and the resultsare easily quantified with a standard ELISA reader, allowing the processing of large number ofsamples. However such assays, which measure the metabolically active cells, would fail when, for example, a small number of proliferating cells are masked by overwhelming majority ofnonproliferating cells (Example: Antigen-specific stimulation of lymphocytes) or when DNAsynthesis is induced in an arrested cell population without any change in cell number or cellviability (Example: short term measurement of growth factor activity on 3T3 or AKR-2B cells).

Since cellular proliferation requires the replication of cellular DNA, the monitoring ofDNA synthesis is another indirect parameter of cell proliferation as well as being suitable for thestudy of the regulation of DNA synthesis itself. DNA synthesis has been the most commonmeasure of mitosis and cell proliferation, and [3H]-thymidine has traditionally been used to labelthe DNA of mitotically active cells. Disadvantages of [3H]-thymidine incorporation assays are: necessity of radioisotopes, the inherent handling and disposal problems, the requirement ofspecialized and expensive equipment like cell harvester and scintillation counter and the hazardcaused by the handling of toxic scintillation fluids. These problems have led to the pursuit ofnon-radioactive replacements for this assay[61].

An important development is the replacement of [3H]-thymidine by 5-bromo-2-deoxyuridine (BrdU). This technique is based on the incorporation of the pyrimidine analogueBrdU instead of thymidine into the DNA of proliferating cells. After its incorporation into DNA,BrdU is detected by immunoassay. Several monoclonal immunohistochemical detection of cells during the S-phase and quantification of cell proliferation have been done by microscopic orflow cytometric analysis of the cell samples. Although very informative, these techniques do notallow a high throughput in routine cell proliferation analysis.

Important assays for proliferation studies

Viable cell count by dye exclusion method

Trypan blue is one of the several dyes (Erytrosin-B [acid red 51], Nigrosin acid black 2) recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells actively pump out the dye by efflux mechanism, whereas dead (non-viable) cells do not. So in this assay white transparent cells are viable cells and blue cells are dead cells. This assay is particularly recommended to measure cell viability for suspension cultures.

MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazoliumbromide) method

MTT measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a micro titer plate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction produces water-insouble formazan salt that must be soluilized. Procedure involves culturing the cells in a 96-well micro titer plate, and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water insoluble formazan dye[62]. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number. This can be applicable for adherent cells cultured in MTP.

Sulforhodamine-B method

Sulforhodamine-B is a dye, which binds to the basic amino acids of the proteins. The total cellular protein of the cells is fixed to the tissue culture plates by cold trichloroacetic acid and stained with sulforhodamine-B. The dye is solubilized in Tris buffer and quantified by colorimetric readings. The amount of dye indirectly represents the amount of bound protein. This can be applicable for adherent cells cultured in MTP.

MTT ASSAY METHOD

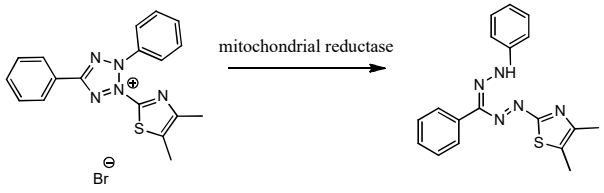
Materials

Cell culture:HeLa- Human cervical carcinoma cell lines.

Cell number for subculture: one million cells for flask (30ml capacity).
Cell loading into plate: 1000-2000 cells per well (96-well plate).
Drug solutions: 1 µg/ml to 100 µg/ml.

Introduction and Principle

MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) measures the metabolic activity of the viable cells. The assay is non-radioactive and can be performed entirely in a micro titer plate (MTP). It is suitable for measuring cell proliferation, cell viability or cytotoxicity. The reaction between MTT and 'mitochondrial dehydrogenase' produces water-insolule formazan salt. Procedure involves culturing the cells in a 96-well micro titer plate and then incubating them with the MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water-insoluble formazan dye. The formazan dye in the MTP is solubilized and quantified with an ELISA plate reader. The absorbance directly correlates with the cell number. This is applicable for adherent cells cultured in MTP.



MTT Formazan

Procedure

1. The adherent cells were trypsinized according to protocol and were resuspended infresh medium after centrifugation. Cell suspension was mixed thoroughly by pippettingseveral times to get a uniform single cell suspension.
2. Different dilutions of drug solutions were made in media with final DMSO concentrationin the well to be less than 1%.
3. 100µl of cell suspension was transferred aseptically to each well of a 96 well plate and toit 100µl of 1% media/ drug solution (in triplicate) in media was added.
4. The plate was then incubated at 37^0C for 72 hours in CO_2 incubator.
5. After 72 hours of incubation, 20µl of MTT was added to each well. The plate was againincubated for 2 hours.
6. 80µl of lysis buffer was added to each well the plate was wrapped in aluminum foil toprevent the oxidation of the dye and the plate was placed on a shaker for overnight.
7. The absorbances were recorded on the ELISA reader at 562nm wavelength. The
8. absorbance of the test was compared with that of DMSO control to get the %inhibition.

RESULTS & DISCUSSION

Calculation of IC_{50} values

Calculation of IC_{50} values for molecules (IIIa-IIIj) and standard drug (Cisplatin) on HeLa cell lines using MTT-assay are as follows.

Table 1: CODE-IIIa

Concentration (µg/ml)	% Inhibition
1	26.55
3	35.25
10	46.66
30	55.36
100	70.26

IC_{50} value of IIIa is 37.48 µg/ml.

Fig. 1:

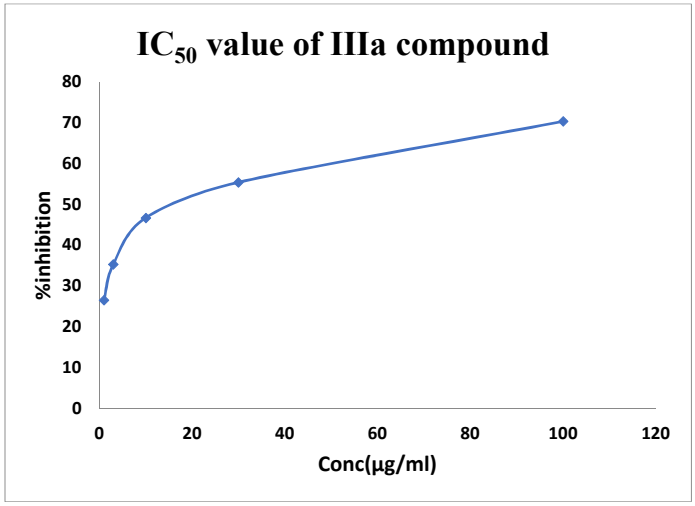


Table 2: CODE-IIIb

Concentration(µg/ml)	% Inhibition
1	21.08
3	24.73
10	56.86
30	66.97
100	95.66

IC_{50} value of IIIb is 24.23µg/ml.

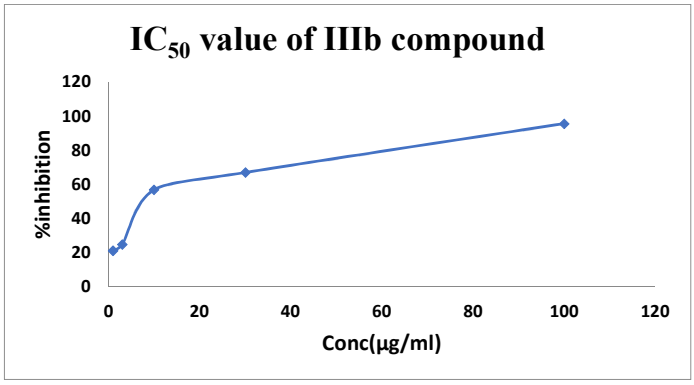


Table 3: CODE-IIIc

Concentration(µg/ml)	% Inhibition
1	21.84
3	34.39
10	56.58
30	63.65
100	95.55

IC_{50} value of IIIc is 21.84µg/ml.

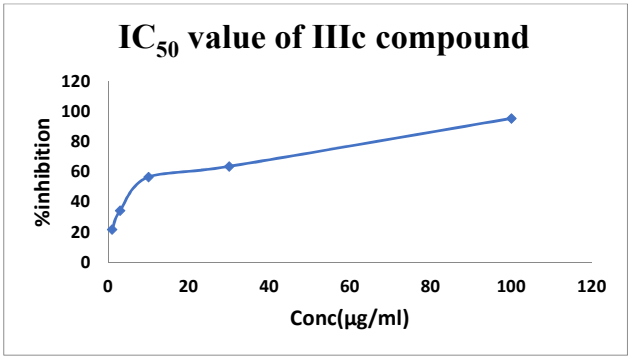


Table 4: CODE-IIId

Concentration(µg/ml)	% Inhibition
1	29.54
3	39.66
10	45.32
30	61.47
100	89.87

IC_{50} value of IIId is 23.50µg/ml.

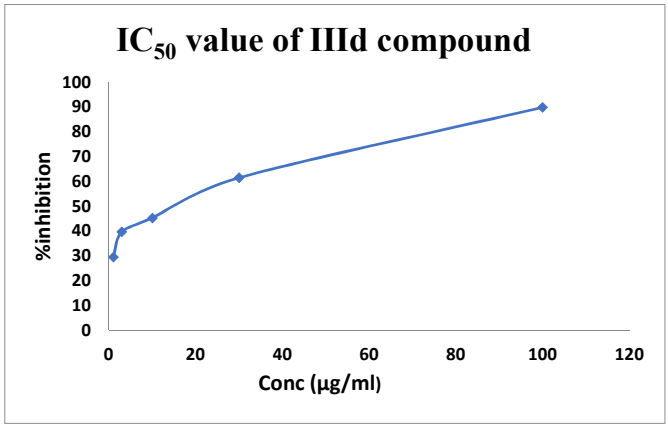


Table 5: -IIIe

Concentration(µg/ml)	% Inhibition
1	29.25
3	33.26
10	53.58
30	65.32
100	84.66

IC_{50} value of IIIe is 22.38µg/ml.

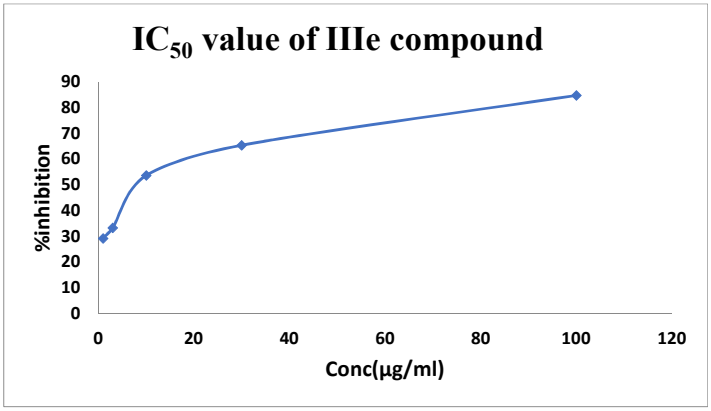


Table 6: CODE-IIIf

Concentration(µg/ml)	% Inhibition
1	21.81
3	35.38
10	51.81
30	69.58
100	90.87

IC_{50} value of IIIf is 22.25µg/ml.

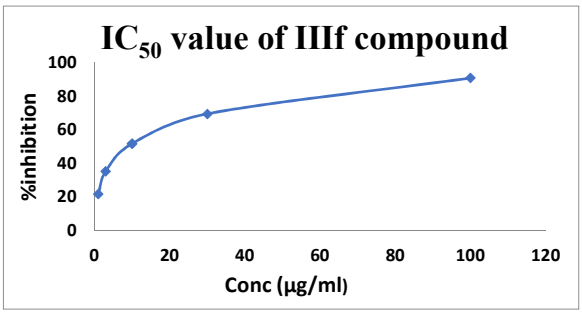


Table 7: CODE-IIIg

Concentration(µg/ml)	% Inhibition
1	28.65
3	34.11
10	42.09
30	67.13
100	91.47

IC_{50} value of IIIg is 24.34µg/ml.

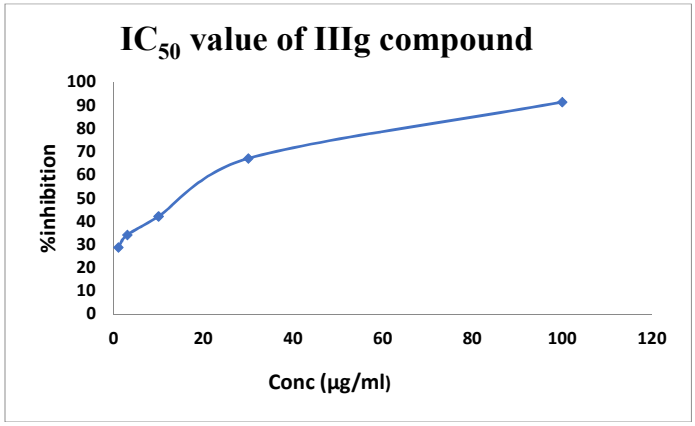


Table 8: CODE-IIIh

Concentration(µg/ml)	% Inhibition
1	27.64
3	35.69
10	49.38
30	61.58
100	90.11

IC_{50} value of IIIh is 23.70µg/ml.

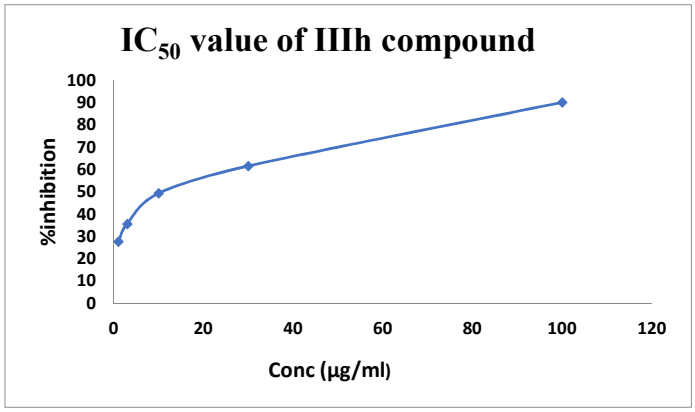


Table 9: CODE-IIIi

Concentration(µg/ml)	% Inhibition
1	21.12
3	38.98
10	51.60
30	71.65
100	87.45

IC_{50} value of IIIi is 21.20µg/ml.

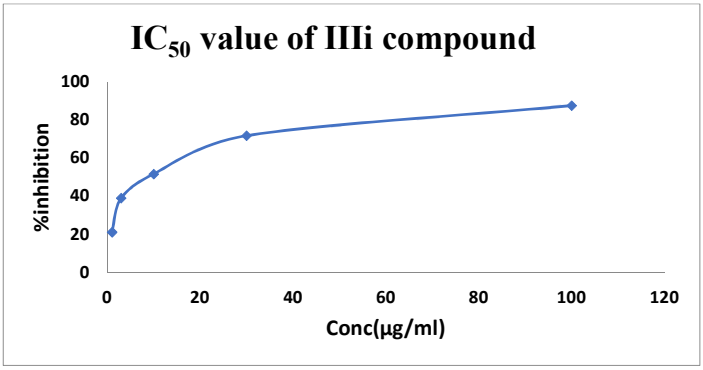


Table 10: CODE-IIIj

Concentration(µg/ml)	% Inhibition
1	25.58
3	31.58
10	49.87
30	68.61
100	86.54

IC_{50} value of IIIj is 24.41µg/ml.

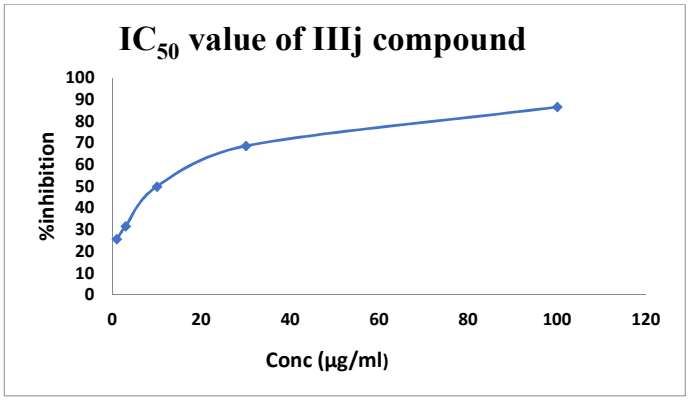
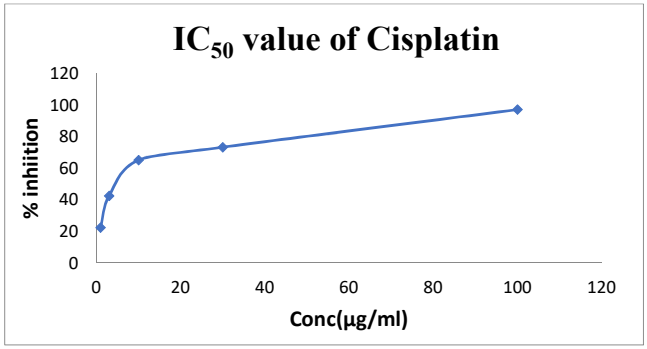


Table 11: IC $_{50}$ value of Cisplatin

Concentration(µg/ml)	% Inhibition
1	22.52
3	42.56
10	65.14
30	73.31
100	97.12

IC_{50} value of Cisplatin is 11.67µg/ml.



CONCLUSION

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