FORMULATION AND EVALUATION OF EPOXIDES DERIVED FROM OXYGEN HETEROCYCLIC COMPOUNDS AND INDANE -1, 3-DIONES AS POSSIBLE SPECIFIC TARGETING AGENTS FOR CYTOTOXIC ACTIVITY

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BY

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INDIA

2008

DECLARATION

I here by declare that the Ph.D thesis "**Formulation and Evaluation of Epoxides derived from Oxygen Heterocyclic compounds and Indane -1, 3-dione derivatives as possible Specific site Drug targeting agents for Cytotoxic Activity**" submitted to **The Tamilnadu Dr. M.G.R. Medical University, Chennai** is a record of independent work carried out by me in K.M. College of Pharmacy, Department of Pharmaceutics, Madurai - 625107 under the supervision and guidance of **Prof. M. Nagarajan, HOD of Pharmaceutics, K. M. College of Pharmacy, Madurai** and the thesis has not formed previously the basis of any Degree, Diploma, Associateship, Fellowship or other Similar Title.

Date :

Place :

(S.VENKATARAMAN, B.Sc., M.Pharm,)

CERTIFICATE

This is to certify that this thesis "**Formulation and Evaluation of Epoxides derived from Oxygen Heterocyclic compounds and Indane -1, 3-dione derivatives as possible Specific site Drug targeting agents for Cytotoxic Activity**" is a bonafide record work of independent research carried out by **Mr. S. VENKATARAMAN**, **M. Pharm** under my supervision and guidance in the institute of **K. M. College of Pharmacy, Madurai - 625107** and this thesis has not formed previously the basis of any Degree, Diploma, Associateship, Fellowship or other Similar Title.

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CHAPTER – I

PART – I

1. INTRODUCTION [1,2,3,4]

1.1 CANCER AND CANCER CHEMOTHERAPEUTIC AGENTS

The chemotherapy of neoplastic disease has become increasingly important in recent years. Most cancer patients now receive some form of chemotherapy even though it is merely palliative in many cases. The relatively high toxicity of most anticancer drugs has fostered the development of supplementary drugs that may alleviate these toxic effects or stimulate the regrowth of depleted normal cell.

The term cancer and neoplastic disease actually encompass more than 100 different tumors each with its own unique characteristics. At present, at least 10 different neoplasms can be cured by chemotherapy in most patients. Cure is defined here is an expectation of normal longevity. These neoplasms are acute leukemia in children, Burkitt's lymphoma, choriocarcinoma in women, Ewing's sarcoma, Hodgkin's disease, lymphosarcoma, mycosis fungoides, rhabdomyosarcoma, retinoblastoma in children, and testicular carcinoma. Only these relatively rare neoplasms are readily curable.

There are cogent reasons why cancer is more difficult to cure than bacterial infections. There are qualitative differences between human and bacterial cells. Cancer cells overexpress certain antigens, and antibodies produced by recombinant

DNA technology exert a selective cytotoxic effect on them. Anti-neoplastic agents kill cells by first order kinetics.

1.2 CELL DIVISION AND CYTOTOXIC DRUGS

The cells of the body vary enormously in appearance and function but have some characteristics which are common to all types of cell. All cells consist of nucleus surrounded by cytoplasm. The most important component of the nucleus is deoxyribonucleic acid (DNA), which consists of two chains of molecules arranged rather like a spiral staircase. DNA is very important because it contains the code which determines the type of protein that is made by the cell and thus ultimately how the cell functions.

One of the important components of the cell cytoplasm is ribonucleic acid (RNA). This is the substance which receives instructions from the DNA in the cell nucleus and is actually responsible for the manufacture of protein.

Most cytotoxic drugs interfere with DNA or RNA and thus they have a profound effect on cells and their functions. Unfortunately these actions are not confined to the malignant cells but affect normal cells as well.

Some cells in the body divide frequently to replace the cells which have become worn out, particularly the cells of the bone marrow, the lymphatic system and the lining of the intestinal tract and are particularly sensitive to the action of cytotoxic drugs.

During its life a cells passes through a series of changes, and cell division itself is a complicated process. The newly formed cell enters the G stage, which is a period of protein synthesis. This may last for a variable time, from a few hours to many years. Many cells remain in this phase through out life of the organism, but

some undergo division and enter the S phase. This phase is short and is concerned with DNA and RNA synthesis, so that the DNA strands may split when cell division occurs. It is a period of great metabolic activity. The G2 phase which follows is a short period of consolidation before cell division occurs. In the mitotic phase, the DNA spiral splits longitudinally so that each daughter cell has its full complement of DNA which is exactly the same as that in the parent cell. Some cytotoxic drugs will affect cells at any phase in their life cycle; others will only act at a single phase of the cell cycle, usually when the cell is dividing, and are called “phase specific”. It follows therefore that when using phase specific drugs, repeated dosage is necessary, if maximum effect is to be achieved.

The cell life cycle

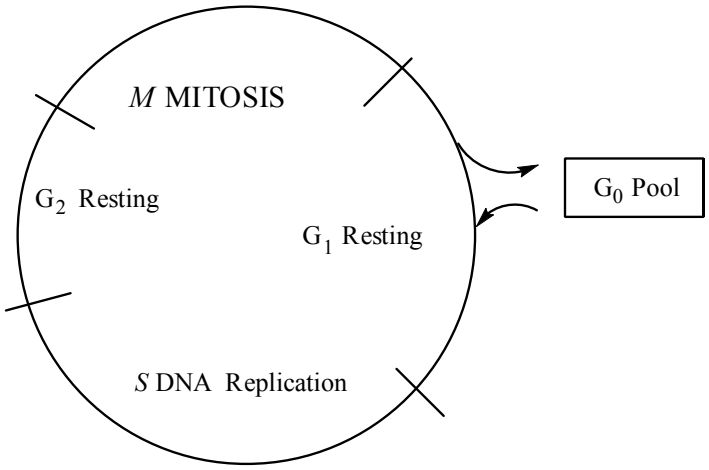


Fig. (1)

1.3 ETIOLOGY

Certain categories of agent or factors have been implicated in the carcinogenic process. Theses include viruses' physical agent, chemical agent, genetic or familial factors, dietary factors and hormonal agent.

1.3.1 Viruses

Viruses as a cause of human cancer are hard to ascertain because viruses are difficult to isolate. Infectious causes are considered or suspected when specific cancers appear in clusters. Viruses are thought to incorporate themselves in the genetic structure of cells, thus altering future generation of that cell population perhaps leading to a cancer. For example, the Epstein-Barr virus is highly suspected as a causative agent in Burkitt's lymphoma and nasopharyngeal cancers. Herpes simplex type II virus, cytomegalovirus, and human papillomavirus (HPV), type 16 and 18, all have been associated with dysplasia and malignancy of the uterine cervix; the hepatitis B virus has been implicated in hepatitis virus (HTLV-I) has been associated with some lymphocytic leukemia and lymphomas, especially among individual in southern Japan; and the HIV virus is associated with Kaposi's sarcoma.

1.3.2 Physical Agent

Physical factors associated with carcinogenesis include exposure to sunlight or to radiation, chronic irritation or inflammation, and tobacco use.

Excessive exposure to the ultraviolet radiation of the sun, especially in people who are fair-skinned and have blue or green eyes, increases the risk of skin cancer. Factors such as clothing styles (sleeveless dresses or shorts), use of sunscreens,

occupation, recreational habits, as well as environmental variables such as humidity, altitude, and latitude all play a role in the exposure risk of ultraviolet light.

Exposure to ionizing radiation can occur with repeated diagnostic radiographic procedures or when radiation therapy is used to treat disease. Improved radiographic equipment present in minimal risk of extensive radiation exposure when used appropriately. Radiation therapy used in disease treatment or exposure to radioactive material at nuclear weapon sites or nuclear power plants is associated with higher incidences of leukemia, multiple myeloma, and cancer of the lung, bone, breast, thyroid, and other tissues. Natural background radiation (radon) occurring from the natural decay of the earth has also been associated with increased lung cancer. Home displaying high level of trapped radon should be ventilated to allow the radon gas to disperse into the atmosphere.

Exposure to electromagnetic field (EMF) from power lines, microwaves, and cellular phones, may also increase cancer risk. Currently, findings of epidemiologic studies related to EMF that suggest an increase in leukemia, central nervous system tumors, skin malignancies and testicular cancers remain controversial.

Chronic irritation or inflammation is thought to damage cell, leading to abnormal cell differentiation. Cell mutations secondary to chronic irritation or inflammation are associated with lip cancer among pipe smokers. Oral cancer is associated with prolonged tobacco use or ill-fitting dentures. Melanomas are associated with chronically irritated moles: colorectal cancers with ulcerative colitis; and liver cancers with cirrhosis.

1.3.3 Chemical Agent

Eightly–five percent of all cancers are thought to be related to the environment. Tobacco smoke is a potent chemical carcinogen that accounts for at least 35% of cancer deaths. Smoking is strongly associated with cancers of the lung,head and neck, esophagus, pancreases, cervix, and bladder. Tobacco may also act synergistically with other substances such as alcohol, asbestos, uranium, and viruses to promote cancer development. Chewing tobacco is associated with cancers of the oral activity and primarily occurs in men under 40 years of age.

Many chemical substances found in the workplace have proved to be carcinogens or co-carcinogens in the cancer process. The extensive list of suspected chemical substances continues to grow. Chemical carcinogens include aromatic amines and aniline dyes, arsenic, soot and tars, asbestos, benzene, betel nut and lime; cadmium; chromium compounds; nickel and zinc ores, wood dust, beryllium compounds, and polyvinyl chloride.

Most hazardous chemicals produce their toxic effects by altering DNA structure in body site distant from chemical exposure. The liver, lungs, and kidneys are the organ systems most often affected, presumably because of their roles in detoxifying chemical.

1.3.4. Genetic and Familial Factors

Genetic factors also play a role in cancer cell development. If DNA damage occurs in cells where chromosomal patterns are abnormal, mutant cell may develop. Abnormal chromosomal patterns and cancer have been associated with extra chromosomes, too few chromosomes, or translocated chromosomes. Specific cancers

with underlying genetic abnormalities include Burkitt's lymphoma, chronic myelogenous leukemia, meningiomas, acute leukemias, retinoblastomas, Wilms' tumor, and skin cancer.

Some cancer of adulthood and childhood display familial predisposition. These cancers tend to occur at an early age and at multiple sites in one organ or pair of organs. In cancer with a hereditary predisposition, commonly two or more first-degree relatives share the same cancer type. Cancers associated with familial inheritance include retinoblastomas, nephroblastomas, pheochromocytomas, malignant neurofibromatosis, leukemias, and breast, endometrial, colorectal, stomach, prostate, and lung cancers.

1.3.5. Dietary Factors

Dietary factors are thought to be related to 40% to 60% of all environmental cancers. Dietary substance can be proactive (protective), carcinogenic, or co-carcinogenic. The risk of cancer increases with long-term ingestion of carcinogens or co-carcinogens or chronic absence of proactive substances in the diet.

Dietary substances associated with an increased cancer risk include fats, alcohol, salt-cured or smoked meats, and food containing nitrates and nitrites, and a high caloric dietary in take. Food substances that appear to reduce cancer risk include high-fiber food, cruciferous vegetables (cabbage, broccoli, cauliflower, brussel sprouts, kohlrabi), carotenoids (carrots, tomatoes, spinach, apricots, peaches, dark green and deep vegetables), and possibly vitamins E and C and selenium.

1.3.6. Hormonal Agents

Tumor growth may be promoted by disturbances in hormonal balance by either the body's own (endogenous) hormonal production or administration of exogenous hormones. Cancers of the breast, prostate, and uterus are considered to be dependent on endogenous hormonal levels for growth. Oral contraceptives, diethylstilbestrol (DES), and prolonged estrogen replacement therapy have been associated with hepatocellular carcinomas, vaginal carcinomas, and breast cancer, respectively.

1.4. PATHOPHYSIOLOGY OF THE MALIGNANT PROCESS

Cancer is a disease process that begins when an abnormal cell is transformed by the genetic mutation of the cellular DNA. This abnormal cell forms a clone and begins to proliferate abnormally, ignoring growth-regulating signals in the environment surrounding the cell.

A stage is then reached in which the cells acquire invasive characteristics, and changes occur in surrounding tissues. The cells infiltrate these tissues and gain access to lymph and blood vessels, by which they can be carried to other areas of the body to form metastases (cancer spread) in other parts of the body.

Although the disease can be described in the general terms just used, cancer is not a single disease with a single cause; rather it is a group of distinct diseases with different causes, manifestations, treatments, and prognoses.

1.4.1. Benign Versus Malignant Proliferative Patterns

During a person's life span, various body tissues normally experience periods of rapid or proliferative growth that must be distinguished from malignant growth activity. Several patterns of cell growth exist and are designated by the terms hyperplasia, metaplasia, dysplasia, anaplasia, and neoplasia.

1.4.1.1 **Hyperplasia:** Hyperplasia, an increase in the number of cells of tissue, is a common proliferative process during periods of rapid body growth and during regeneration of skin and bone marrow. It is a normal cellular response when a physiologic demand exists; it becomes an abnormal response when growth exceeds the physiologic demand such as may chronic irritation.

1.4.1.2 **Metaplsia**: Metaplsia occurs when one type of mature cell is converted to another type by means of a stimulus that affects the parent stem cell. Chronic irritation or inflammation, vitamin deficiency, and chemical exposure may be factors leading to metaplasia. Metaplastic changes may be reversible or may progress to dysplasia.

1.4.1.3 **Dysplasea**: Dysplasia is bizarre cell growth resulting in cells that differ in size, shape, or arrangement from other cells of the same type of tissue. Dysplasia can occur from chemicals, radiation, or chronic inflammation or irritation. It can be reversible or can precede irreversible neoplastic change.

1.4.1.4 **Anaplasea:** Anaplasia is lower degree of differentiation of dysplastic cells. Anaplastic cells are poorly differentiated and irregularly shaped or disorganized

with respect to growth and arrangement. Anaplastic cells lack normal cellular characteristics and are nearly always malignant.

1.4.1.5 **Neoplasia:** Neoplasia, described as uncontrolled cell growth that follows no physiologic demand, can be either benign or malignant. Benign and malignant neoplastic growths are classified and named by tissue of origin.

Benign and malignant cells differ in many cellular growth characteristics including method and rate of growth, ability to metastasize or spread, general effects, destruction of tissue, and ability to cause death. The degree of anaplasia (Lack of differentiation of cells) ultimately determines the malignant potential.

1.5. CHARACTERISTICS OF MALIGNANT CELLS.

Despite their individual differences, all cancer cells share some common cellular characteristics in relation to the cell membrane, the presence of special proteins, the nuclei, chromosomal abnormalities, and the rate of mitosis and growth. The cell membranes are altered in cancer cells, which affect fluid movement in and out of the cell. The cell membrane of malignant cells also contains proteins called tumor-specific antigens and prostate specific antigen (PSA), which develop as they become less differentiated (mature) over time. These proteins distinguish the malignant cell from a benign cell of the same tissue type and may be useful in measuring the extent of disease in a person and in tracking the course of illness during treatment or relapse. Malignant cellular membranes also contain less fibronectin, “cellular cement”; thus they are less cohesive to adjacent cells and do not adhere as readily.

Nuclei of cancer cells are often large and irregularly shaped (pleomorphism). Nucleoli, structures within the nucleus that house ribonucleic acid (RNA), are larger and more numerous in malignant cells, perhaps because of increased RNA synthesis. Chromosomal abnormalities (translocations, deletions, addition) and fragility of chromosomal abnormalities are commonly found on analysis of cancer cells.

Mitosis occurs more frequently in malignant cells than in normal cells, thus increasing the growth fraction of the tumor cell population. Additionally, cancer cells have altered amounts of cyclic adenosine monophosphate (AMC) and cyclic guanosinemonophosphate (GMP). These substrates, which are the building blocks of nucleic acid, facilitate the use of nutrients and the synthesis of RNA. As a result, cell growth and division are promoted, requiring high levels of glucose and oxygen. If these glucose and oxygen stores are unavailable, malignant cells will use anaerobic metabolic channels to produce energy. Anaerobic means of energy production make the cell less dependent on the availability of a constant oxygen supply.

1.6. CANCER CHEMOTHERAPEUTIC AGENTS

1.6.1. Alkylating Agents

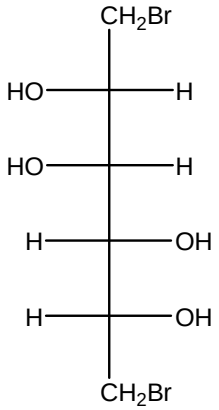
Alkylation is defined as the replacement of hydrogen on an atom by an alkyl group. The alkylation of nucleic acids or proteins involves a substitution reaction in which a nucleophilic atom of the biopolymers displaces a leaving group from the alkylating agent.

The reaction rate depends on the nucleophilicity of the atom (S, N, O), which is greatly enhanced if the nucleophile is ionized.

Reaction orders on the structure of the alkylating agent. Methane sulfonates; epoxides and aziridines give second-order reactions that depend on concentrations of the alkylating agent and nucleophile.

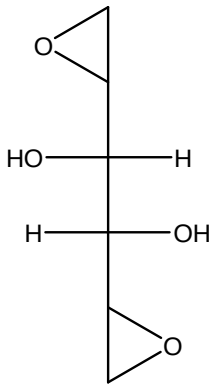
Ethylene imines and epoxides are strained ring system, their reactions are second order and enhanced by the presence of acid (eg triethylenemelamine and thiotepa).

The use of epoxides as cross-liking agents in textile chemistry suggested that they be tried in cancer chemotherapy. Simple diepoxides such as 1,2,3, 4-diepoxybutane showed clinical activity against Hodgkin's disease Dibromomannitol (mitobronitol) **(Fig 2)** gives the corresponding diepoxides. This diepoxide (1,2,5, 6-dianhydro-D-mannitol) **(Fig 3)** shows potent alkylating activity against experimental tumors, thus suggesting that dibromomannitol and related compound such as dibromoduclcitol act by way of the diepoxides.



Mitobromitol

(Fig. 2)



Dianhydro-D-mannitol

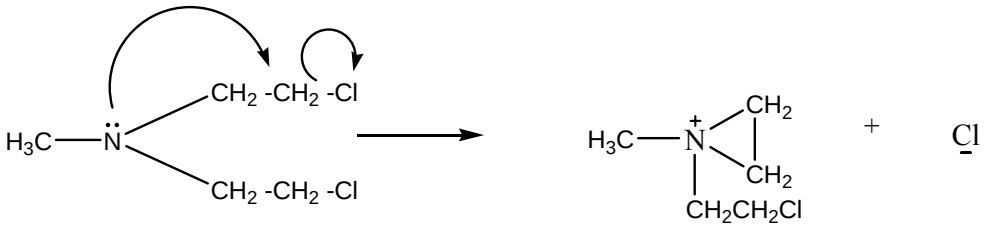
(Fig. 3)

1.6.2. Nitrogen Mustard:-

Mechlorethamine hydrochloride (Fig. 4)

Mechanism of action

The primary site of attack is atom 7 of guanine, then, in decreasing order of sensitivity, atom 3 of adenine and atoms 1 of adenine and cytosine. Alkylation with subsequent cross linking not only inhibits DNA synthesis but also causes deletion of guanine with concomitant formation of an easily hydrolysable apurinic link in the ribose-phosphate backbone of DNA.

(i) 

(Fig. 4)

(ii) Nucleophilic attack

-SH of protein

-N of protein DNA base

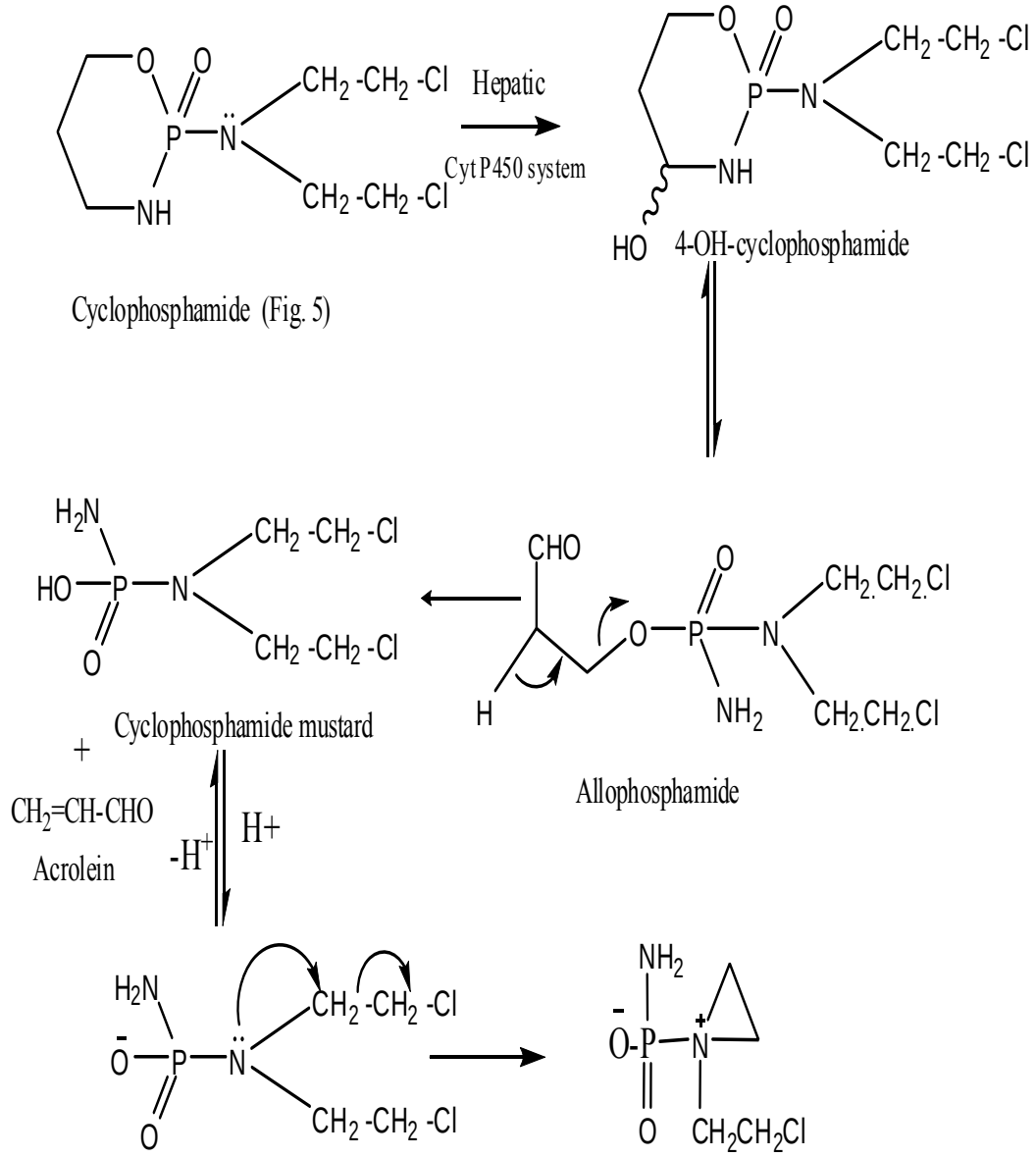
=O of DNA base

Cyclophosphamide (Fig. 5)

Mechanism of action

In the body, cyclophosphamide is converted to the active compound mainly in the liver rather than in tumor. It is converted by hepatic cytochrome P-450 into the corresponding 4-hydroxyderivative. The 4-hydroxyintermediate is a carbinolamine in equilibrium with the open-chain aminoaldehyde form. Nonenzymatic decomposition of the latter form generates phosphotamide mustard and acrolein. Recent studies based on NMR have shown that the conjugate base of phosphoramide mustard cyclizes to the aziridine ion which is the principal cross-linking alkylator at maximal rate at pH 7.4.

It was suggested that selective toxicity certain neoplastic cells might be based on their abnormally low pH. This would afford a slower formation of aziridinun ion and they would persist longer because of decreased inactivation by OH^-.The phosphorus atom is asymmetric, and the levorotatory form has twice the therapeutic index of the dextro rotatory form.

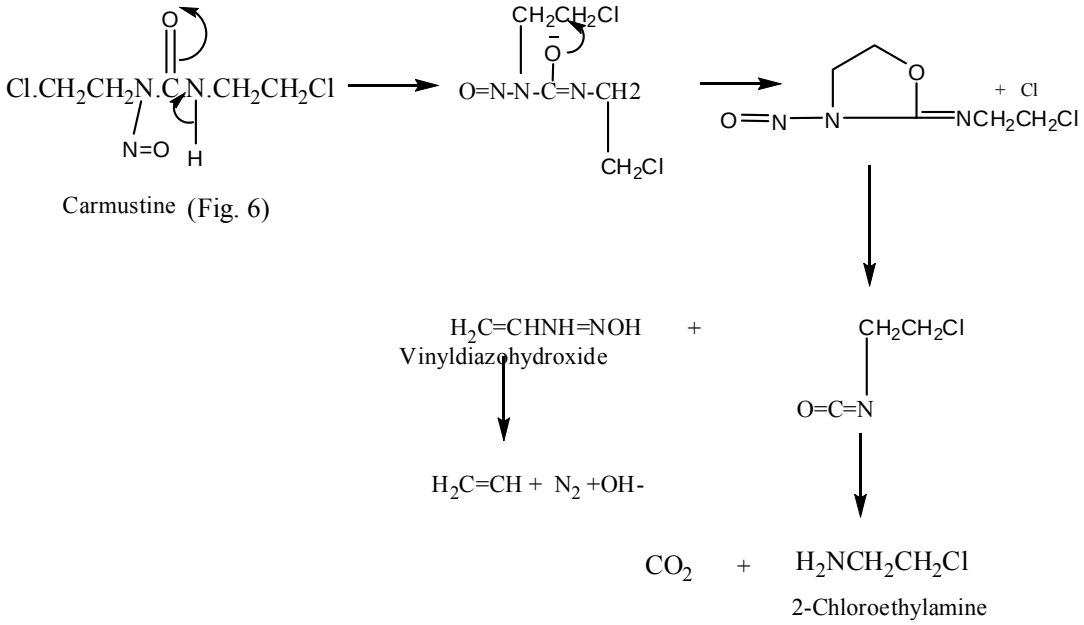

Hepatic
Cyt P450 system
4-OH-cyclophosphamide
Cyclophosphamide (Fig. 5)
Cyclophosphamide mustard
Allophosphamide
Acrolein
H+

1.6.3 Nitrrosoureas

Carmustine (Fig. 6)

Mechanism of action

Carmustine undergoes an abnormal decomposition in which the urea oxygen displaces a chlorine intermediate, which decomposes to vinylcarbonim ion, and 2-chloroethlisocyanate



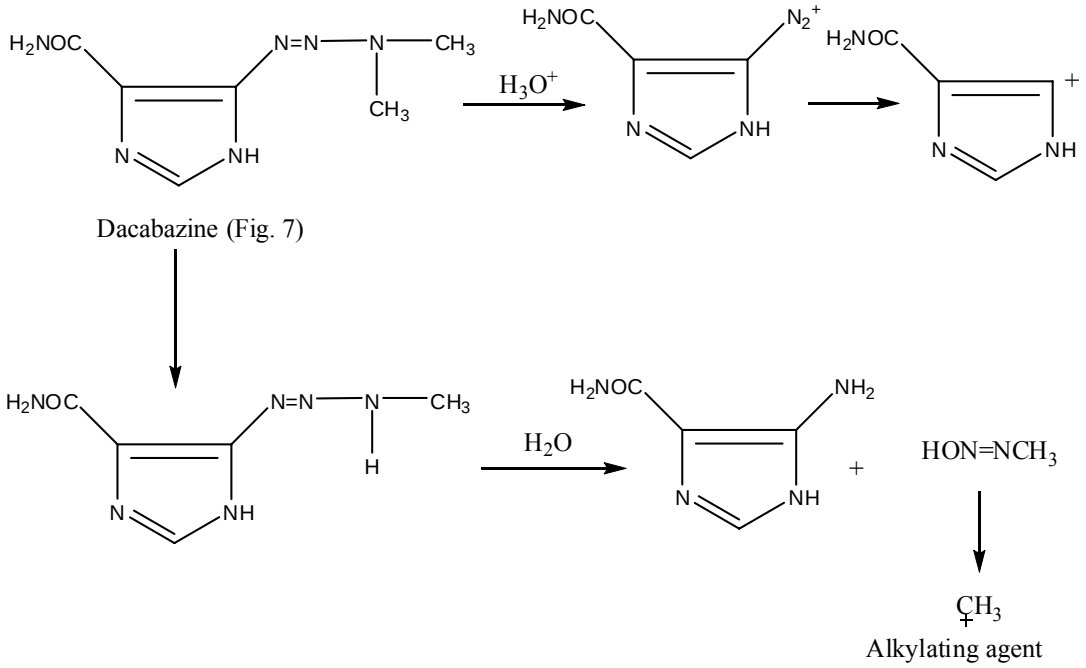
Carmustine (Fig. 6)

1.6.4. Uracil Mustard:-

Dacarbazine (Fig. 7)

Mechanism of action

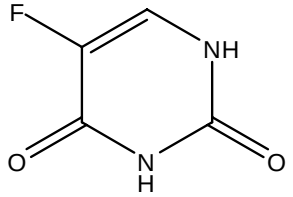
Dacarbazine, earlier thought to be an antimetabolite, now appears to be an alkaylating agent. Dacarbazine undergoes acid-catalyzed hydrolysis to a diazonium ion, which can react in this form or decompose to the corresponding carbonium ion. Support for the mechanism was afforded by a correlation between the hydrolysis rates of phenyl-substituted dimethyltriazines and antitumor activities.



1.7. ANTIMETABOLITES

Antimetabolites are compounds that prevent the biosynthesis or use of normal cellular metabolites. Nearly all of the clinical agents are related to metabolites and cofactors in the biosynthesis of nucleic acids. They usually are closely related in structure to the metabolites that is antagonized. Many antimetabolites are enzyme inhibitors. They may combine with the active site as if they were the substrate or cofactor. Alternatively, they may bind to an allosteric regulatory site, especially when they resemble the end product of a biosynthetic pathway under feedback control. Sometimes, the antimetabolite must be transformed biosynthetically into the active inhibitor.

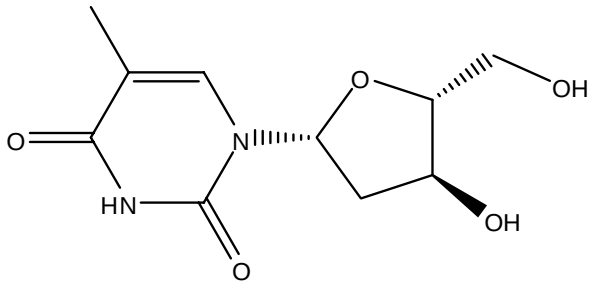
5-Fluorouracil (Fig. 8)



5-fluorouracil (Fig. 8)

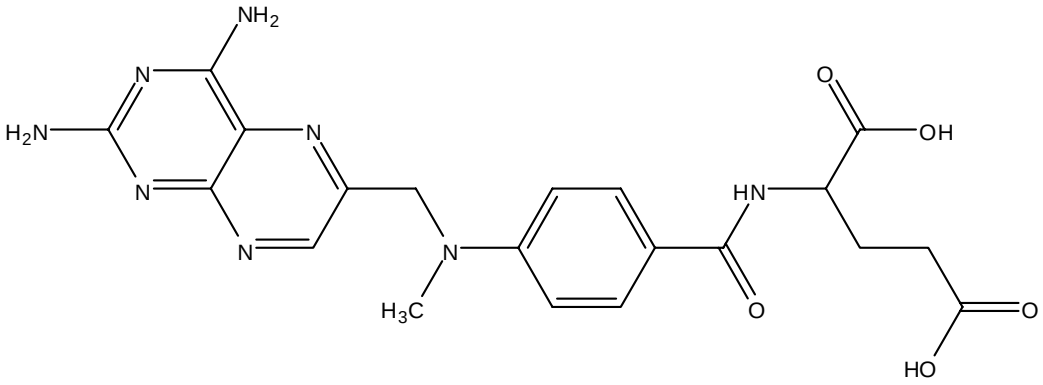
Mechanism of action

5-Fluorouracil **(Fig. 8)** is activated by anabolism to 5-fluoro-2′-deoxyuridylic acid. This is a powerful competitive inhibitor of thymidylate synthetase, the enzyme that converts 2′-deoxyuridylic acid to thymidylic acid. This blockade is probably the main effect of 5-florouracil and its metabolites. In this inhibiting reaction, the sulphydryl group of a cysteine residue in the enzyme adds to the 4-position of the fluorouracil moiety. The 5-position then binds to the methylene group of 5, 10-methylene tetrahydrofolate. This bond is extremely stable and precludes addition of a methyl group in the 5-position, preventing the formation of thymidine. (Fig. 9)



thymidine (Fig. 9)

Methotrexate (Fig. 10)



(Fig. 10)

Mechanism of action

Methotrexate acts as an antifolate by binding almost irreversibly to the enzyme dihydrofolate reductase, and preventing the formation of the coenzyme tetrahydrofolic acid, essential for DNA synthesis and for replication of animal cells. The basis of this binding strength is in the diaminopyrimidine ring, which is protonated at physiological pH. At pH 6, methotrexate binds stoichiometrically with dihydrofolate reductase ($K^1 \approx 10^{-10}$M).

1.8. ANTIBIOTICS

Antibiotics play a significant role in the current treatment of neoplastic diseases, and it is reasonable to expect antibiotics are to assume more importance in the future. However, a discussion of antitumor antibiotics is complicated as they form a heterogeneous group with different structures and mechanisms of action.

Nine different antibiotic and their semisynthetic analogues are established clinical anticancer agents, and other antibiotics are undergoing clinical development. Some of these agents have been approved recently; however, others have been known for a long time. For example, dactinomycin (actinomycin D) was first isolated 1940 by Waksman and Woodruff, although its activity against neoplasams was not described until 1958. Furthermore, plicamycin, originally discovered as aureolic acid in 1953, had to be rediscovered twice before its antitumor activity was established in 1962.These compounds were originally rejected as antibacterial agent because of their cytotoxicity. Only later was it found that this toxicity could be an advantage in the chemotherapy of cancer. Many laboratories routinely screen extracts of microorganism cultures for antitumor activity in cell cultures.

The mode of action of actinomycins has been studied extensively, and it now is generally accepted that they intercalate into double- helical DNA.

Actinomycins, anthracyclines, and certain other intercalating agent, including mitoxantrone and amsacrine, inhibit the enzyme topoisomerase II. Topoisomerase regulate the topological state of DNA by unwinding and unlinking coiled double-strand DNA molecules. They are thought to be critical for DNA replication and

transcription, and they act by cleaving and rejoining one or both strand of the phosphodiester backbone of DNA.

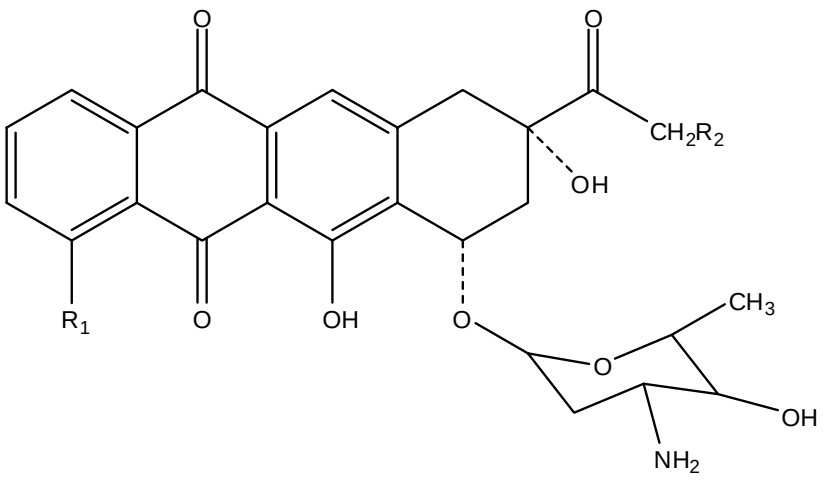
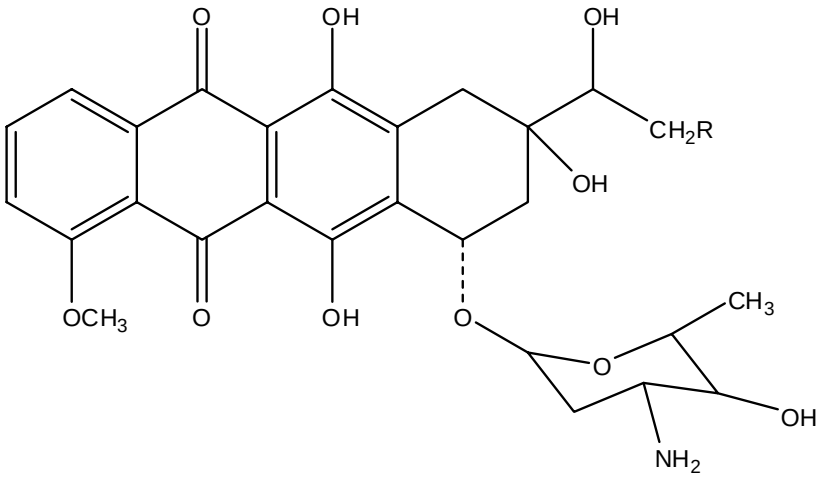


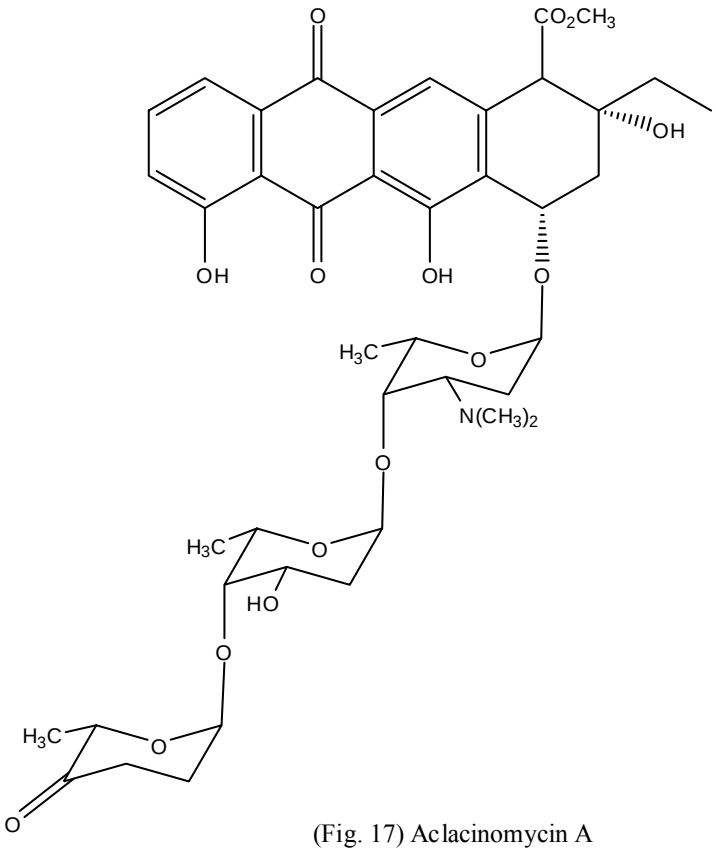
fig 11	Daunorubicin	$R_1=OCH_3, R_2=H$
fig 12	Doxorubicin	$R_1=OCH_3, R_2=OH$
fig 13	Idarubicin	$R_1=H, R_2=OH$
fig 14	Carminomycin	$R_1=OH, R_2=H$

Daunorubicin **(Fig. 11)** proved to be active against acute leukemias, and it became an established clinical agent. It was pushed into the background, however, by the discovery of doxorubicin (Adriamycin) in *S.peucetius* var. *caesius* in 1969. Doxorubicin is active against a broad spectrum of tumors, including both solid and hematological types. It became one of the most widely used antineoplastic agents.



(Fig. 15) Daunomycinol, R=H

(Fig. 16) Adriamycinol,R=OH

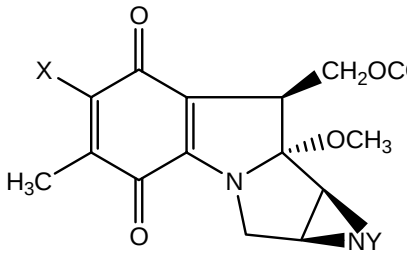


(Fig. 17) Aclacinomycin A

Daunorubicin and doxorubicin **(Fig.11)** exhibit biological effects similar to those of actinomycin, and they are thought to intercalate into double-helical DNA and inhibit topoisomerase II. Reduction of doxorubicin followed by intercalation cause

DNA strand scission. This scission is thought to result from the attack of hydroxyl radicals generated from redox cycles involving doxorubicin. In contrast to daunorubicin, aclacinomycin **(Fig. 17)** and related compounds do not induce lysogenic phage in bacteria. They are believed to interfere more with RNA syntheses than with DNA synthesis. Aclacinomycin lacks the cardiotoxicity shown by daunorubicin and doxorubicin.

The mitomycins were discovered in Japan in the late 1950s, and one of them, mitomycin C, was rapidly developed as an anticancer drug. Unfortunately, the initial clinical experience with this compound in the United States was disappointing. It was not approved until 1974, following extensive studies and the establishment of satisfactory dosage schedules. Porfiromucin **(Fig. 20)**, the N-methyl homologue of mitomycin C **(Fig.19),** was discovered at the Upjohn Company. It has received clinical study, but it is not yet an approved agent

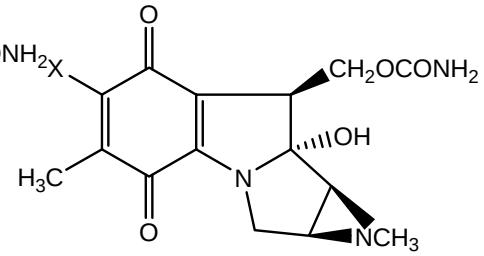


(Fig. 18) Mitomycin A,X = CH_3O, Y = H

(Fig. 19)Mitomycin C,X = NH_2,Y = H

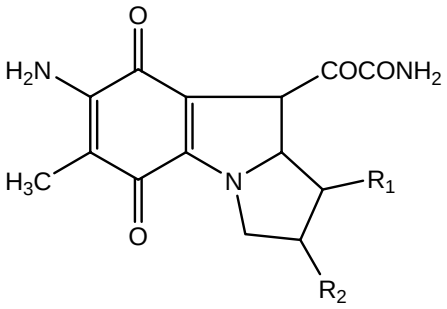
(Fig.20) Porfiromycin,X = NH_2,Y = CH_3

(Fig. 21) BMY-25067,X = $NO_2C_6H_4SS(CH_2)_2NH$



(Fig. 22) Mitomycin B,X = CH_3O

(Fig. 23)Mitomicin D,X = NH_2



(Fig. 24) 2,7-Diamino-1-hydroxymitosene, R_1= OH,R_2 = NH_2

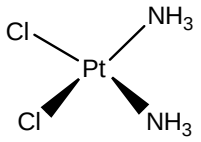
(Fig. 25) 1,2-Aziridino-7-aminomitosene, R1,R2 = 

Structures of the mitomycin were elucidated at Lederle Laboratories. These compounds have an unusual combination of three different carcinostatic function; quinine, carbamate, and aziridine. They are arranged in such a way that the molecule is relatively unreactive in its natural state. Chemical or enzymatic reduction to the corresponding hydroquinone is, however, followed by the loss of methanol (water from mitomycin B **(Fig. 22),** and the resulting indolhydroquinone becomes a bifunctional alkylating agent capable of cross-linking double-helical DNA. Mitomycines bound to DNA may undergo successive redox cycles, each of which results in the generation of hydrogen peroxide. This potent oxidizing agent can cause single- strand cleavage of the DNA.

Mitomycins are unstable in both acids and bases. Mild acid hydrolysis results in opening of the aziridine ring and loss of methanol or water to give motosenes such as 2,7-diamino-1-hydroxymitosene **(Fig.24).** Catalytic hydrogenation followed by reoxidation gives aziridinomitosenes **(Fig. 25),** which retain a significant amount of antitumor activity in animals. Many mitomycin analogues have been prepared by partial synthesis, and two of them have received clinical trails. Unexpected toxicity has led to their withdrawal, however. The present clinical candidates, BMY-25067 and KT6149 **(Fig.21),** contain disulfide substituents on the 7-amino group. Control of the quinone reduction potential is especially stressed in analogue studies, because reduction is the key step in bioactivation of these molecules.

1.9 INORGANIC COMPOUNDS

Cisplatin (Fig. 26)



Cisplatin (Fig. 26)

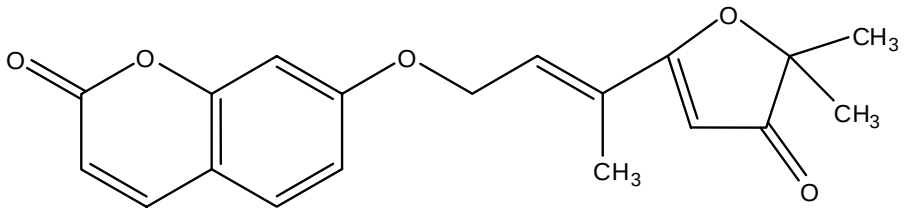
The platinum co-ordination complexes represent a new class of cytotoxic agents whose antitumor potential was initially identified by Rosenberg. One of these compounds, cisplatin has been actively studied in clinical trials sponsored by the National Cancer Institute. Although its full therapeutic potential was slow in being recognized, knowledge of its spectrum of activity means of overcoming its nephrotoxicity and exploration of high-dose and combination regimens are recent developments that have led to widespread interest in the role of cisplatin and other platinum compounds in cancer chemotherapy.

Mechanism of antitumor action

The principal target of the platinum compounds appears to be DNA, as suggested by the selective inhibition of DNA synthesis that can be demonstrated in mammalian cells. The differential cytotoxic activity of the cis and trans cisplatin may be related to a differential ability to cross-link DNA strands thus bearing a similarity to bifunctional alkylating agents. Chemically, the chloride ligands in cisplatin act as leaving groups, permitting alkylation's of nucleophylic moieties in DNA and protein.

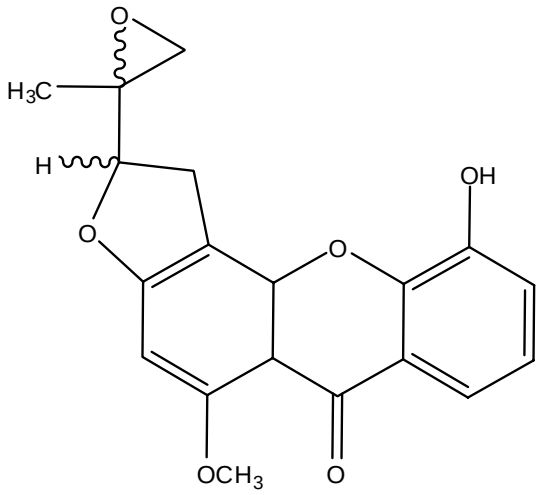
1.10 PLANT PRODUCTS

Early studies led to the isolation of the cytotoxic agents hyrcanoside and deglucohyrcanoside from *Coronilla varia*. Geiparvarin is a cytotoxic coumarin.



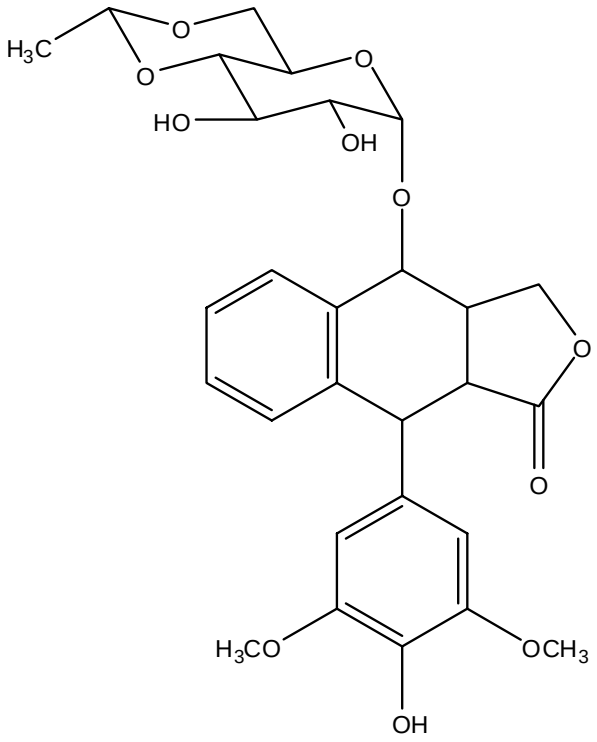
GEIPARVARIN (Fig. 27)

An examination of antitumor activities of simple substituted xanthones reveals that among 30- 40 tested at NCI (National Cancer Institute) all are active.

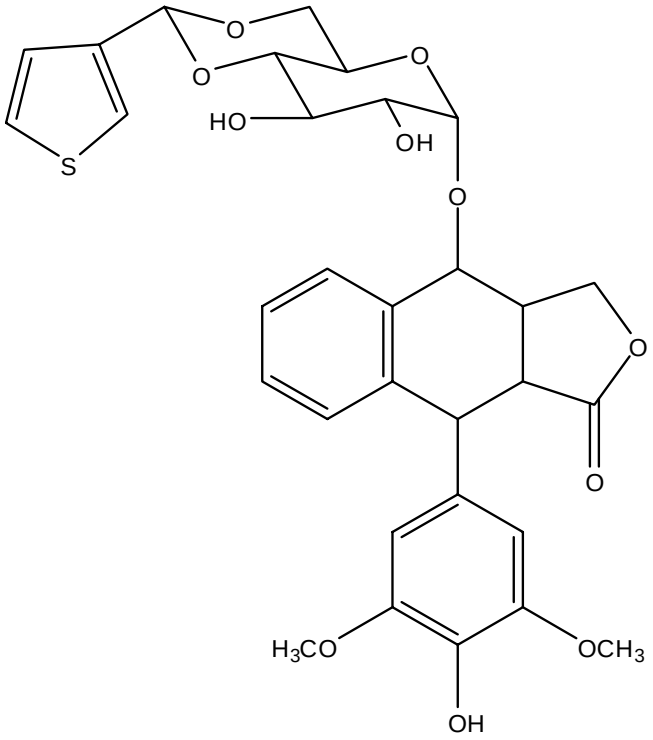


PSOROSPERMIN (Fig. 28)

The use of higher plants in treating neoplastic disease dates to antiquity. Dioscorides described the use of colchicines for this purpose in the first century. In more recent years, scientists have attempted to select and screen systamatically plants reputed to have antitumor activity. If activity is established for one member of a plant family, other members of this family are selected and tested. A major impetus to this research was given by Hartwell at the NCI, who established an extensive system of plant collection, screening, and isolation. More than 100,000 plants have been screened under this program.

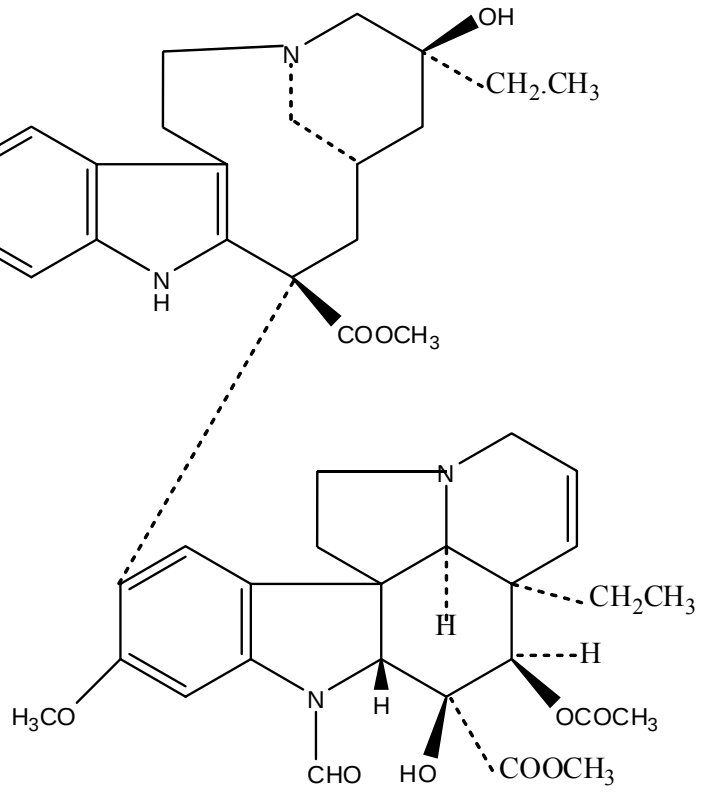


Etoposide **(Fig. 29)**

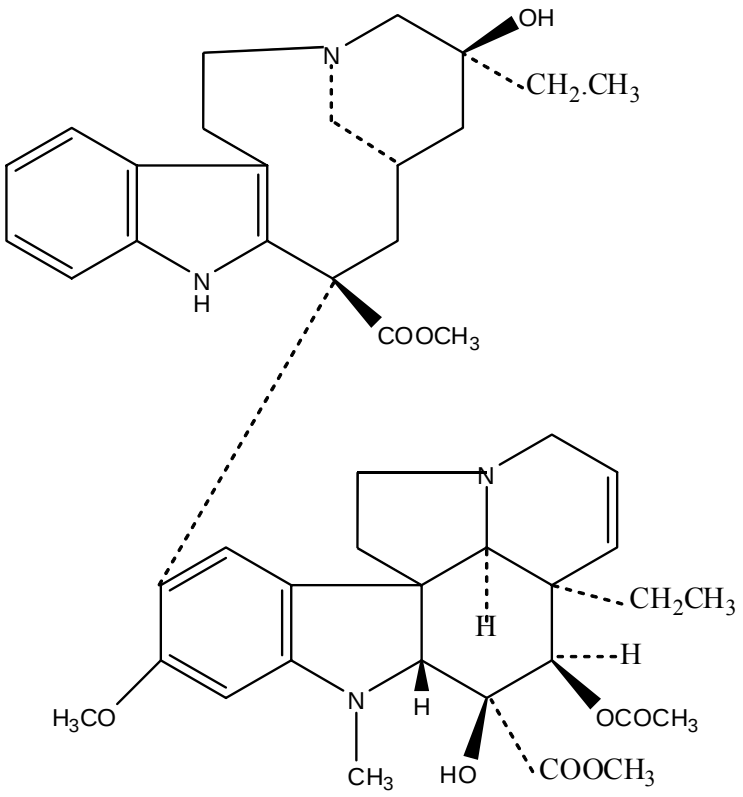


Teniposide **(Fig. 30)**

Resin of the may apple, *Podopyllum peltatum*, has long been used as remedy for warts. One of its constituents, podophyllotoxin, has antineoplastic activity, but it is highly toxic. This lignin inhibits mitosis by destroying the structural organization of the mitotic apparatus. Early derivatives of podophyllotoxin showed poor clinical activity, but newer analogues, such as the epipodiphyllotoxin derivatives etoposide **(Fig. 29)** and teniposide **(Fig. 30),** are much better. Both of these analogues differ from podophyllotoxin in inhibiting topoisomerase II rather than microtubule assembly.

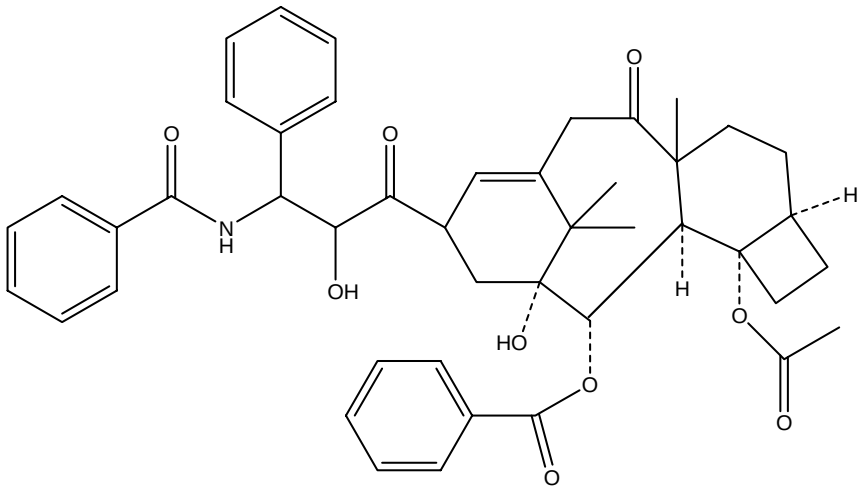


Vinblastine **(Fig. 31)**



Vincristine **(Fig. 32)**

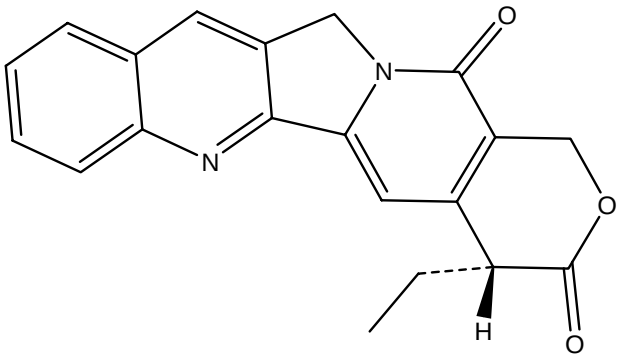
The vinca alkaloids are a family of important antitumor agents from plants. These compounds were isolated from the periwinkle *Catharanthus rosea* at Eli Lilly Company. They have complex structures composed of an indole-containing moiety, catharanthine, and an indoline-containing moiety, vindoline. Four closely related compounds have antitumor activity; vincristine **(Fig.32),** vinblastine **(Fig.31),** vinrosidine, and vinleurosine. Among this group, vincristine and vinblastine are proved clinical agents.



Paclitaxel (Fig. 33)(Taxol)

A plant product of high current interest in cancer chemotherapy is paclitaxel **(Fig.33)** (Taxol). This compound was isolated from the bark of the Pacific yew tree *Taxus brevifolia* by Wani et al. in 1971. At that time, it was found to have antitumor activity; however, there was little enthusiasm for its further development until recently, when its potential for human clinical activity was suggested by screening against human tumors in immunodeficient mice. It is now the world's leading antineoplastic agent in terms of sales. Paclitaxel is active against refractory ovarian cancer, metastatic breast cancer, metastatic melanoma, and non-small cell lung cancer.

Paclitaxel inhibits mitosis by acting as a spindle poison; however, it acts by a unique mechanism in promoting the assembly of microtubules and a stabilizing them against depolymerization. This mechanism is in contrast to that of compounds like the vinca alkaloids, which prevent the assembly of microtubules.



Camptothecan (Fig. 34)

Irinotecan has been approved recently for first-line therapy, in combination with 5-FU and leucovorin, for patients with metastatic colon or rectal carcinomas. It is semi synthetic analogue of camptothecan. Camptothecin **(Fig.34)** was isolated from camptotheca acuminate, an ornamental tree found in China. It is very insoluble in water, but its sodium salt, prepared by alkaline hydrolysis of the lactone ring, showed promising antitumor activity. Clinical trials were eventually discontinued because of unpredictable toxic effects. Irinotecan has a basic tertiary amine group, which can be protonated to solublize the drug. The lactone ring remains intact and increases activity above that of the ring opened sodium salt. Camptothecin and irinotecan inhibit topoisomerase I. cytotoxicity is caused by double-strand DNA damage, which occurs during DNA synthesis when replication enzymes interact with the ternary complex formed from DNA, topoisomerase I, and the drug.

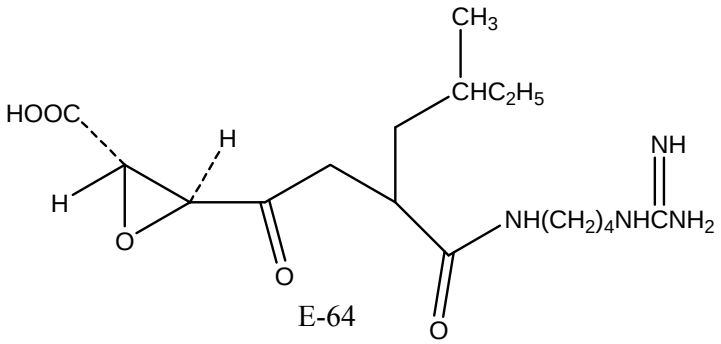
1.11 FUTURE ANTINEOPLASTIC AGENTS

New approaches to cancers chemotherapy of special interest: inhibition of proteases involved in metastasis, angiogenesis inhibitors, antisense technology, and telomerase inhibitors.

1.11.1 Proteases and Metastasis

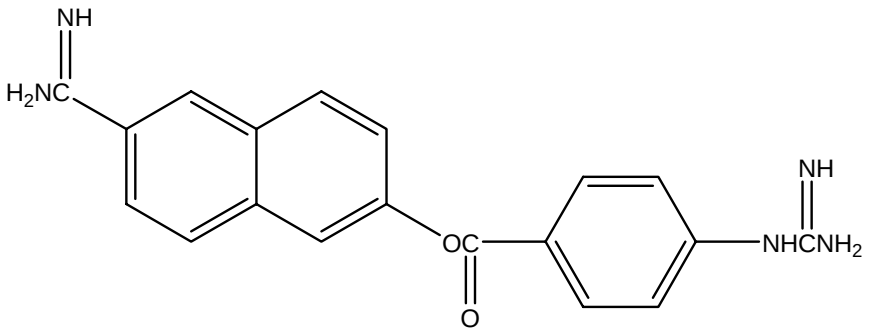
The ability of cells from primary tumors to colonize secondary sites (metastasis) is the major cause of cancer mortality. Metastasis involves tumor cells entering and leaving the circulation and invading adjacent tissue. It requires degradation of the exracellular matrix by the concerted action of proteases. This process occurs normally, but it is controlled by the elaboration of protease inhibitors.

Among the synthetic compounds currently under investigation are E-64 **(Fig. 35)** an inhibitor of the cysteine proteinase cathepsin B and nafamostat **(Fig. 36)** a seine protease inhibitor. Sumarin blocks melanoma and mammary tumor invasiveness, possibly by inhibiting heparinase, cathepsin D secretion, and urinary plasminogen activator receptor **(Fig. 35)**



E-64

(Fig. 35)

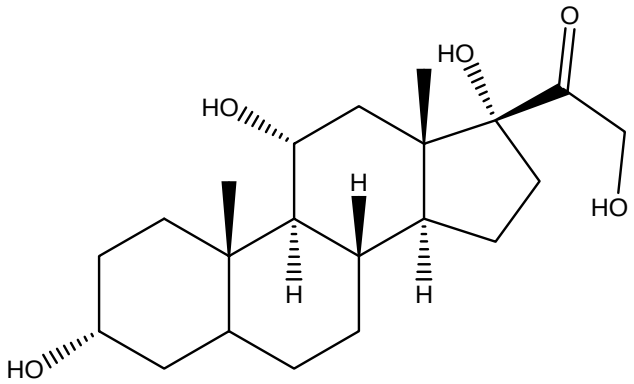


Nafamostat

(Fig. 36)

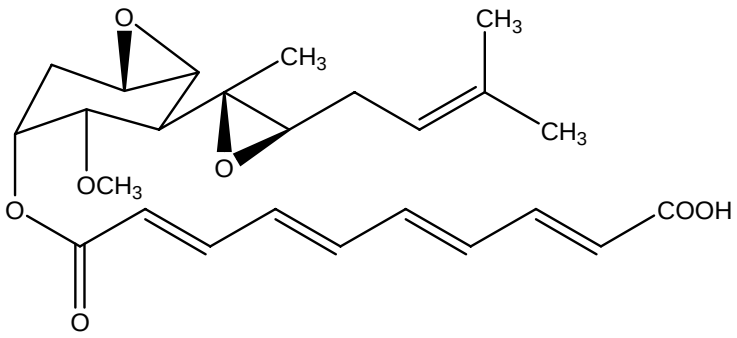
1.11.2 Angiogenesis inhibitors

Angiogeneis is the formation of new blood vessels. It is a necessary but carefully regulated component of normal growth and wound healing. Uncontrolled angiogenesis is a driving factor in solid tumor growth. The process of angiogenesis is complex and requires the coordinated interaction of multiple cell types. Multiple sites for drugs intervention are expected example cartilage derived inhibitor.



tetrahydrocortisone

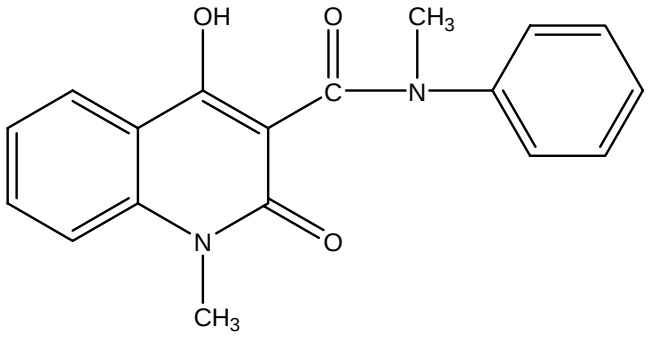
(Fig. 37)



(Fig. 38)

Agents used are steroids (eg) Tetra hydrocortisone **(Fig. 37)** antibiotic (eg) Fumagillin **(Fig. 38)** inhibits angiogenesis in tumor and a more potent analogue, AGM-1470 reduces the growth rate of lung cancer and melanoma in mice.

TNF-α is a powerful stimulant to angiogenesis. The secretion of this factor by macrophages in prostatic cancers is decreased significantly by linomide **(Fig. 39)** a quinoline-3-carboxamide derivatives.



Linomide

(Fig. 39)

1.11.3 Antisense technology

Single-stranded messengers RNA (m-RNA) can undergo sequence-specific; high-affinity binding to a complementary oligonucleotide sequence by Watson-Crick hydrogen bonding. Such a complementary agent is called an antisense oligomer. By binding with m RNA, the antisense oligomer can interfere with its translation into protein by ribosomal blockade. Complexes between mRNA and DNA-like antisense oligonucleotides also can activate RNase H, an enzyme that specifically cleaves the RNA strand of a RNA/DNA duplex. Eg. for Antisense agents are phosphorothiote and peptide Nucleic acid.

1.11.4 Telomerase Inhibitors

Telomeres are nucleoprotein structure, located at the ends of eukaryotic chromosomes. They protect chromosome ends from fusion and degradation and ensure complete replication of chromosomal DNA. In human somatic cells, telomeres have 1,000 to 3,000 repeats. They gradually shorten with every cell division. This shortening is though to limit their proliferative capacity. Cancer cells, in contrast, can maintain their telomere length and they become immortalized. They do this by reactivating telomerase; a specific reverse transcriptase with an endogenous RNA template.

1.11.5 Cytoprotective agents

Highly cytotoxic anti neoplastic agents produce a variety of serious side effects in patients. This problem has stimulated the search for compounds that protect patients from certain specific toxicities. Three widely different cytoprotective agents have been approved for clinical use in the United States.

$HSCH_2$, CH2.SO3Na (Mesna) **(Fig. 40)**

Mesna **(Fig. 40)** is the sodium salt of mercaptoethane sulfonic acid. Although it is oxidized to the corresponding disulfide in blood, it is reduced back to the free thiol in the kidney. This property led to its use in preventing hemorrhagic cystitis in patients.

Dexrazoxane is the S-(+) isomer of razoxane. It is a potent intracellular chelating agent that is used for cardioprotection in patients receiving doxorubicin.

Amifostine **(Fig. 41)** is an organic hypophosphate used to toxify the reactive metabolites of cisplatin especially in the kidney. It is dephosphorylated by alkaline phosphatase to the active free thiol. This transformation occurs selectively in normal tissues because they have higher alkaline phosphatase activity, higher pH, and better vascularity.

$H_2N\ (CH_2)_3NH\ (CH_2)\ SPO_3H_2$ **(Fig. 41)**

CHAPTER –II

LITERATURE REVIEW

2.1 OXYGENATED HETEROCYCLIC COMPOUNDS

Adam et al[5] postulated intramolecular electron-transfer mechanism was postulated for the formation of the spiroepoxide, which subsequently dimerizes. Only the benzofuran epoxide**,** besides the benzofuran dioxeranes**,** was mutagenic in the ***Salmonella typhimurium*** strain TA100. Therefore, they implicate the epoxide **as** the ultimate mutagen responsible for the high mutagenic activity observed with dioxerane in the Ames test. They have postulated that in the oxidative metabolism of polycyclic arenes and heteroarenes the corresponding epoxides are generated from the intermediary dioxeranes by deoxygenation with sulfides.

William et al[6] reported the preparation of (flavonoxy) propanolamine esters (42 and 43)as antiarrhythmic agents. The compounds **(Fig. 42)** [I; R^1 = $R^2R^3NCH_2CH$ (OR_4) CH_2O; R^2 = alkyl; R^3 = H, $PhCH_2$; R^4 = H, alkanoyl, naphthoyl, (Un) substituted Bz, etc.] were prepared. Flavonoxy propanolamine esters compound **(Fig. 43)** reversed ouabain-induced arrhythmiacs in anesthetized dogs at 1 and 3 mg/kg i.v.

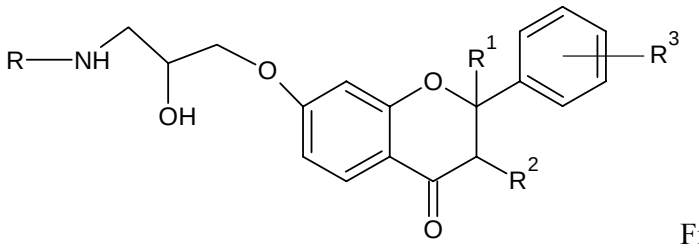


Fig. 42

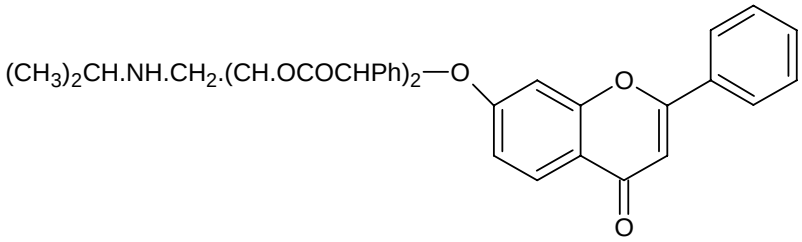


Fig. 43

Kamelia et al[7] synthesised antiallergic coumarinyloxyamines (44 and 45)

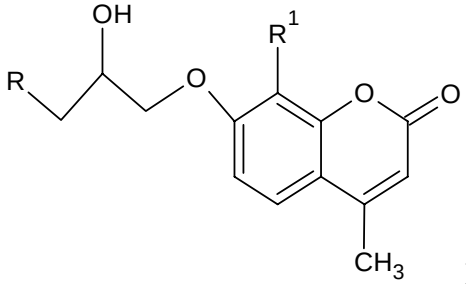


Fig. 44

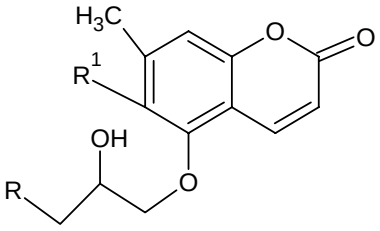


Fig. 45

Rudi et al[9] prepared coumarin derivatives [R = H, Bu, $Et_2NCH_2CH_2$, allyl or 2-(1-pyrazinyl)ethyl, R^1 = Me, Pr or Ph, X = $(CH_2)_2$, $(CH_2)_3$, CH_2CHOH or 2-(3,4,5-trimethoxybenzoyloxy)-1,3-propylene, Y = $(CH_2)_2$, $(CH_2)_3$, $(CH_2)_4$, or CH_2CHMe] which were found to be useful as coronary dilators.

Laura et al[10] synthesized and studied α_1-adrenoceptor binding properties of new arylpiperazine derivatives bearing a flavone nucleus as the terminal heterocyclic molecular portion. In his research project aimed at obtaining new compounds with high affinity and selectivity toward $\boldsymbol{\alpha}_1$-adrenoceptors (AR), a new class of piperazine derivatives was designed, synthesized and biologically tested. The new compounds characterized by a flavone system linked, through an ethoxy or propoxy spacer, to a phenyl- or pyridazinone-piperazine moiety. Biological data showed an interesting profile for the phenylpiperazine subclass found to have a nanomolar affinity toward α_1-AR, and less pronounced affinity for α_2-AR and the 5-HT1A serotoninergic receptor. A discussion on the structure activity relationship (SAR) of such compounds was also reported, on the basis of the flavone substitution pattern, length and functionalization of the spacer, and disruption of the phenylpiperazine system.

Peter et al[11] prepared non-nucleosidic coumarin derivatives as polynucleotide-crosslinking agents Novel coumarin derivatives e.g. **(Fig. 46)** comprising of a coumarin moiety linked to a non-nucleosidic backbone moiety were prepared and incorporated into DNA. The resulting molecules were typically used as photo-activate cross linking groups when incorporated into polynucleotides as replacements for one or more of the complementary nucleoside bases present in probes used in procedures involving nucleic acid hybridization reactions.

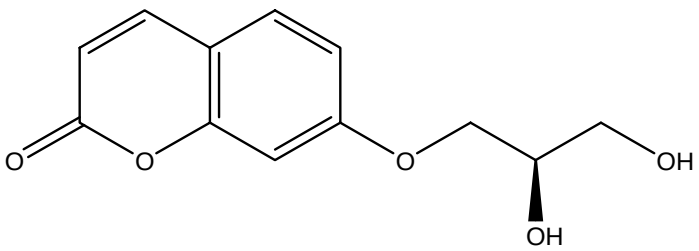


Fig. 46

Gyogyszeres et al[12] prepared 7-substituted benzopyrans and studied their use in medicine. (aminohydroxyalkoxy)benzopyrans **(Fig. 47)**, (R = H, alkyl, Ph, and R^1 = H; R^2 = H, Ph, or R^1R^2 form a direct bond; R^3 = R^4 = H, or R^3R^4 = O; n = 1, 2; R^5 and R^6 are H, alkyl, hydroxyalkyl, alkenyl, cycloalkyl, phenylalkyl, dimethoxyphenylalkyl, or NR^5R^6 form a heterocycle) and they showed antihypertensive activity.

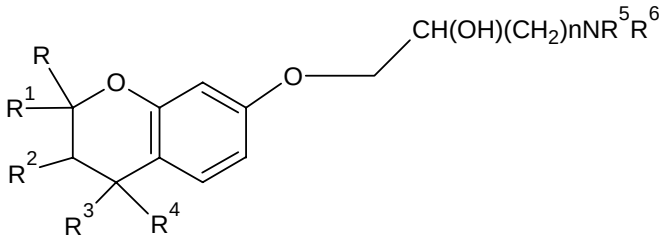
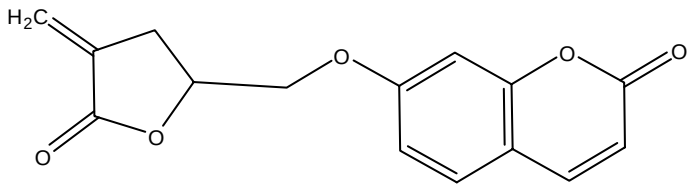
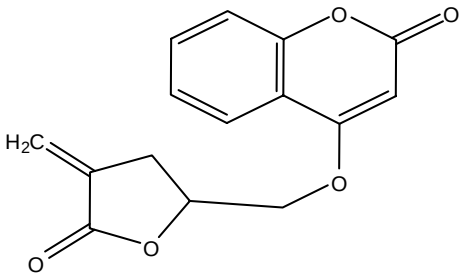


Fig. 47

Long et al[13] certain coumarin α-methylene-γ-butyrolactones were synthesized and evaluated for antiproliferative and vasorelaxing activities. These compounds were synthesized via alkylation of six hydroxycoumarins followed by oxidation and the Reformatsky-type condensation. Compd. **(Fig. 48)**, with an IC_{50} value of 9.4 μM against pig coronary arterial contraction induced by KCl, was the most potent vasorelaxant. Compd. **(Fig. 49)** exhibited the most potent antiproliferative activity in tests with MCF7, NCI-H460, and SF-268 cell cultures with IC_{50} values of 6.97, 14.68 and 8.36 μM respectively.

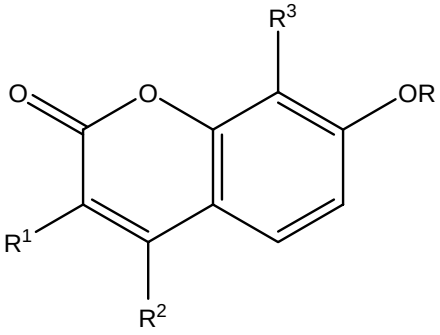


(Fig. 48)



(Fig. 49)

Desai et al[14] synthesised [3-(substituted amino)propoxy]-2'-hydroxycoumarin **(Fig.50)** derivatives as possible β -blockers.



(Fig. 50)

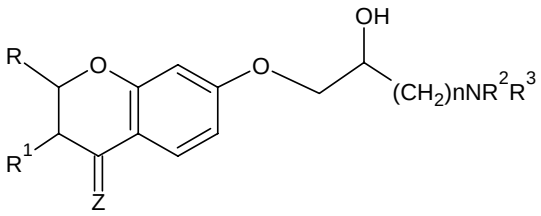
Buckle et al[15] prepared number of N-benzylpiperazino derivatives of 3-nitro-4-hydroxycoumarin and they have been shown to combine potent H_1-antihistamine activity with that of mast cell stabilization as demonstrated by their activity as antagonists of histamine on guinea pig ileum.

Re et al[16] In search for new β-adrenergic blocking drugs with enhanced therapeutic properties is still very active and there has recently been an increasing interest in heterocyclic (e.g., benzodioxane, indole, 6-dibenzofuran, quinoline etc.) derivatives. They have reported the preparation of some new and α-adrenergic blocking derivatives of the chromone and flavone group, with which the versatility of the benzo-γ-pyrone molecule as a carrier moiety in medicinal research. They have also prepared two analogous xanthone derivatives on the basis of the results obtained among the CNS stimulants of the same the basic chain was located, according to the synthetic possibilities.

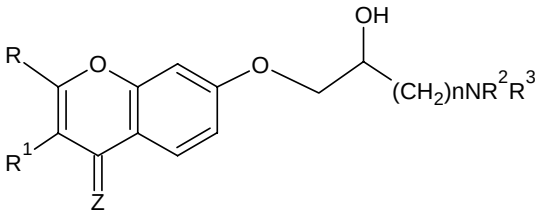
Shtacher et al[17] synthesized several conformationally defined aryloxypropanolamines of the type $ArOCH_2CH$ (OH) CH(R) NHR^1have been synthesized and tested *in vivo* for β-adrenoceptor blockade. Key intermediates in the synthesis were the appropriate *cis*- and *trans*-disubstituted olefins. Epoxidation of the olefins, followed by amination of the resulting *cis*- and *trans*-epoxides, yielded the desired diastereomeric model compounds with a defined threo and erythro stereochemistry respectively. All active compounds in this series exhibit a simple, bimolecular, competitive antagonism at β-adrenoceptors.

Xiaoshu et al[18] synthesised flavone and flavanone-7-O-isopropanolamine derivatives for their antiarrhythmic activity. Compounds were prepared starting from 7-hydroxyflavones or 7-hydroxyflavanones and were tested on rats for the ventricular arrhythmia induced by aconitine.

Dezso et al[19] prepared 7-(Aminohydroxyalkoxy)benzopyrans **(Fig.51)** and **(Fig.52)** (R = H, alkyl, Ph; R^1 = H, Ph; Z = H_2, O; n = 1, 2; R^2, R^3 = H, alkyl, hydroxyl, alkyl, alkenyl, cycloalkyl, phenylalkyl, dialkoxy phenylalkyl; NR^2R^3=heterocycle) and which exhibited antihypertensive activity.



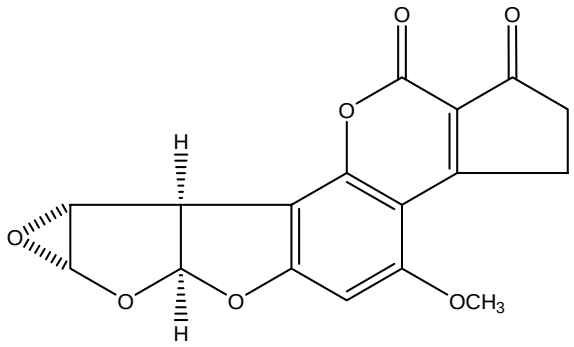
(Fig.51)



(Fig.52)

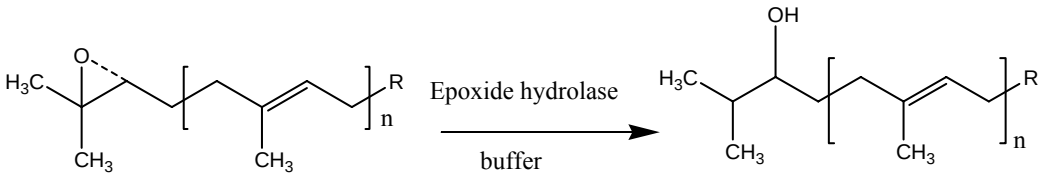
Hildebrandt et al[20] studied the *in vitro* effects of two coumarin anticoagulants, warfarin and difenacoum, on rat liver microsomal vitamin K dependent carboxylase, vitamin K epoxidase, vitamin K epoxidereductase, and cytosolic vitamin K reductase (DT-diaphorase) from the livers of normal and a warfarin-resistant strain of rats have been determined.

Gopalakrishnan et al[21] prepared aflatoxin B_1 8,9-epoxide **(Fig.53)**. Two intercalation sites would be available with d (ATGCAT). Intercalation provides excellent positioning for nucleophilic attack by guanine N7 on aflatoxin B, 8, 9-epoxide, which probably accounts for the observed efficiency of adduct formation despite the relatively low DNA binding affinity observed for aflatoxin B.



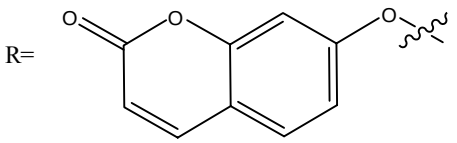
(Fig.53) Aflatoxin B_1-8,9-epoxide

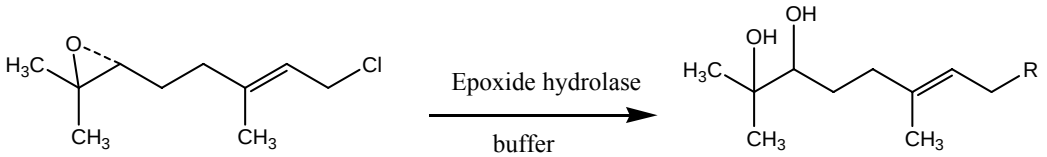
Edegger et al[22] accomplished asymmetric biohydrolysis of trisubstituted terpenoid oxiranes **(Fig.54)** (rac-1a–rac-3a) by employing the epoxidehydrolase activity *Rhodococcus* and *Streptomyces* spp. Depending on the biocatalyst, the biohydrolysis proceeded in an enantio-convergent fashion and gave the corresponding *vic*-diols in up to 97% yield at conversions beyond the 50%-threshold. In order to avoid a depletion of the yield of product by further oxidative metabolism, bioconversions had to be conducted in an inert atmosphere with exclusion of molecular oxygen. The synthetic applicability of this method was demonstrated by the asymmetric total synthesis of the monoterpenoid coumarin (R)-(+)-Marmin in 95% yield.



rac 1 a = n =2
rac 2 a = n = 1

Karatvicinol 1 b
Marimin 2 b





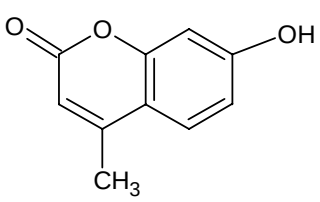
i = 2,2-dimethyl propane/H^+Cat.
ii = $NaOH/H_2O$
iii = H^+/H_2O

3b = R= Cl
3c = R = OH

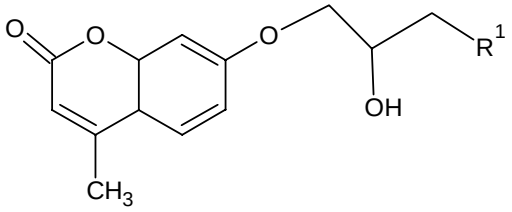
(Fig.54)

Karal et al[23] synthesised a new series of 4-(3-coumarinyl)-3-cyclohexyl-4-thiazoline-2-one benzylidene hydrazones were synthesized and evaluated for antituberculosis activity against *Mycobacterium tuberculosis* H37Rv.

Mangel et al[25] synthesised and evaluated the pharmacological activities of 7-(3-substituted amino or aryloxy-2-hydroxypropoxy)-4-methyl-2H-1-benzopyran-2-ones and related compounds. Benzopyranol **(Fig.55)** was converted to ethers (R^1 = substituted amino) via etherification with epichlorohydrin. (R^1 = morpholino) exhibited anti-inflammatory activity, while antihypertensive activity was shown by **(Fig.56)** (R^1 = N+HPrMe I-).



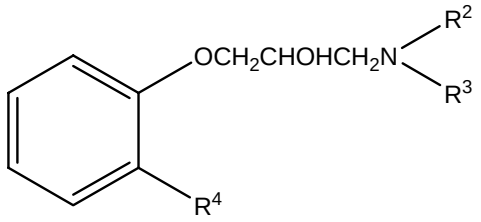
(Fig.55)



(Fig.56)

Fasco et al[26] reported that warfarin is a potent inhibitor of vitamin K and 2,3-epoxide reduction to vitamin K *in vitro* and *in vivo*. Dithiothreitol, an *in vitro* reductant for the vitamin K 2, 3-epoxidereductase, antagonizes inhibition of the reductase by warfarin via mechanisms that have not been determined.

Nakanishi et al[27] synthesised of several l-amino-3-(substitutedphenoxy-2-propanols **(Fig.57)** examined for adrenergic *β*-receptor blocking activity.



(Fig.57)

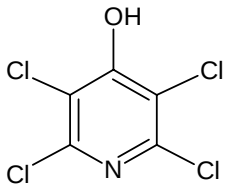
Cox et al[28] the synthesised of a series of stilbene, biaryl, tolane, diaryl ether, sulfide, sulfoxide, and sulfone oxypropanolamines as potential antiobesity agents.

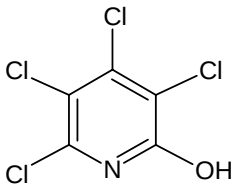
Ghate et al[29] synthesised 4-(Bromomethyl)coumarins and have been screened for their anti-inflammatory activity. Out of these the 5,6-benzo-4-2-benzo[b]furanyl) coumarin and the aryloxymethylcoumarin with p-formyl group were found to be most active.

Rastogi et al[30] synthesised and pharmacological screening of 1-[*p*-(alkanoy1, aralkanoyl and aroy1)phenoxy and thiophenoxy]-3-(N^4-arylpiperaziny1)propanes and propan-2-ols, l-[*p*-(alkylsulfinyl, alkylsulfonyl, alkenyl, cyclopropylcarbonyl, cinnamoyl, ethoxycarbonyl, acetonyl, and a hydroxyalky1)phenoxyl-3-(N^4-arylpiperazinyI)propan-2-ols and 1-(p-acylanilino and 4-propionyl-1-naphthyloxy)-3-(N^4-arylpiperaziny1)-propan-2-01s and some related compounds have been carried out. 1-(*p*-propionylphenoxy)-3-(N^4-phenylpiperazinyl)propan-2-ol was found to possess marked antidepressant activity.

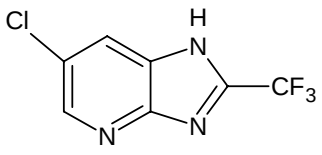
Rastogi et al[31] synthesised and screned for pharmacological action for (±)-1-(alkanoylphenoxy)-3-(N^4-arylpiperazinyl)propan-2-ols and some related compounds. It has been found that *o*-alkanoyl compounds possess marked hypotensive and local anesthetic activity, *m*-alkanoyl compounds showed tranquillizing activity, while *p*-alkanoyl compounds have antidepressant activity; this dissociation in biological action is particularly marked in N^4-phenylpiperazinyl compounds. the marked local anaesthetic activity of (±)-*o*-acetylphenoxy)-3-[N^4(3,4-dimethylphenyl)piperazinyl]propan-ol (centxylazine), in surface, infiltration, and spinal anesthesia, and its higher safety margin than known local anesthetics make it a promising candidate for clinical evaluation.

Friedman et al[32] The compounds 2,3,5,6-tetrachloro-4-pyridinol (TCP) **(Fig.58)** and the structurally related imidazopyridines (IP) cause hemorrhage and lower the plasma prothrombin level in animals. *In vitro*, TCP and the IP are more potent inhibitors of both the vitamin K dependent carboxylase which catalyzes the posttranslational γ-carboxylation of specific glutamyl residues in proteins and the related vitamin K epoxidase activity than they are either of vitamin K epoxide reductase or of NAD-(P)H-K oxidoreductase. TCP and IP, as is the case with the coumarin and indane dione anticoagulants, are competitive inhibitors of NAD (P) H-K oxidoreductase with respect to NADH. The epoxide reductase from coumarin-resistant rats is quite resistant to inhibition not only by warfarin but also by the IP, and to a lesser extent by TCP. When interpretedin light of published *in vivo* experiments, the data suggest that the principal site of anticoagulant action of the IP, but not TCP, is the epoxide reductase. The anticoagulant effect of TCP may be inhibition of the carboxylase itself. TCP is a significantly more potent inhibitor of the carboxylase and epoxidase than the IP; it inhibits both the enzymatic activities to the same degree with 50% inhibition observed at about 10^{-5} M.

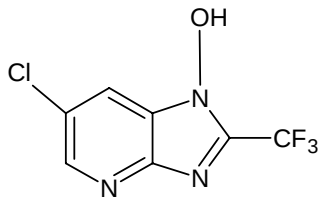




2,3,5,6-tetrachloropyridin-4-ol **(Fig.58)** 3,4,5,6-tetrachloropyridin-2-ol



6-chloro-2-(trifluoromethyl)-1*H*-imidazo [4, 5-*b*] pyridine



6-chloro-2-(trifluoromethyl)-1*H*-imidazo [4, 5-*b*] pyridin-1-ol

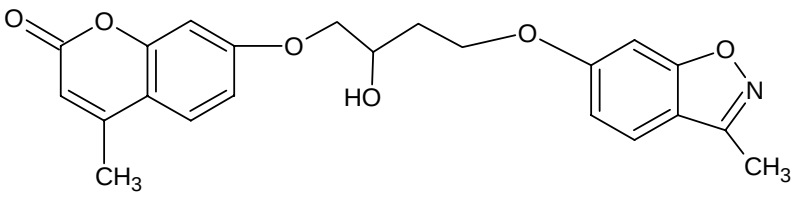
Ganesan et al[33] reported that azapeptide epoxides are a novel class of irreversible inhibitors that are highly specific for clan CD cysteineproteases. The five crystal structures of caspase-3-aza-peptide epoxide inhibitor complexes.

Sadowski et al[34] reported that postmitochondrial supernatants from vitamin K deficient rat liver catalyze both the vitamin K dependent conversion of microsomal precursor proteins to prothrombin, and the conversion of vitamin K to its 2,3-epoxide. Requirements for the latter reaction have been studied, and the possible relationship of the two reactions have been investigated. The epoxidase activity is located in the microsomes and, if NAD (P) H is provided, no cytosolic component is required.

Sadowski et al[35] reported that the 2, 3-epoxide of phylloquinone is a normal metabolite of the vitamin, and it has been demonstrated that coumarin anticoagulants inhibit an enzyme system that converts this metabolite to the vitamin. It has been postulated that this epoxide is a competitive inhibitor of vitamin K at the metabolic site where it is involved in prothrombin synthesis, and that coumarins act as anticoagulants because they increase the tissue levels of this epoxide.

Hans et al[36] prepared of 7-[3-(propylamino)-2-hydroxypropoxy] flavone useful as antihypertensive, by reacting 7-(2, 3-epoxypropoxy) flavone with $PrNH_2$, the corresponding **β**-chalcone by product obtained was reacted with maleic acid.

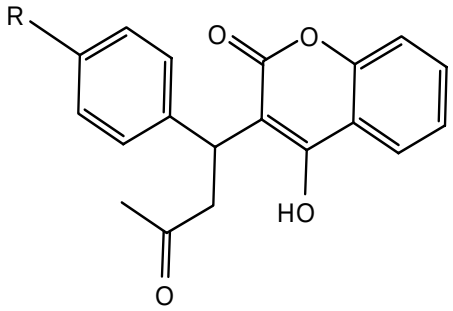
Shinde et al[37] synthesised 1-(Substituted coumarinyloxy)-3-(substituted coumarinyloxy/ benzisoxazolyloxy)-2-hydroxypropane **(Fig.59)** by treating substituted hydroxycoumarins and 6-hydroxy-3-methylbenzisoxazole with epichlorohydrin and their PCA activities determined.



(Fig.59)

Yasunobu et al[38] synthesized about 60 (2-hydroxy-3-aminopropoxy) coumarin derivatives from 5-7-and 8-hydroxycoumarin derivatives and the **β**-adrenergic blocking activities were examined. 5-Methyl-8-(2-hydroxy-3-tert-butylaminopropoxy) coumarin was most favorable as a **β**-adrenergic blocking agent.

Almeda et al[39] reported purification of NAD (P) H dehydrogenase in four steps from a homogenate of rat liver. The final step was affinity chromatography on Sepharose coupled to 3, 3′-(m-hydroxybenzylidene)bis(4-hydroxycoumarin) **(Fig.60)** The purified enzyme was inhibited competitively with respect to NADH by 3-(cy-acetonyl-p-nitrobenzyl)-4-hydroxycoumarin **(Fig. 61)**.

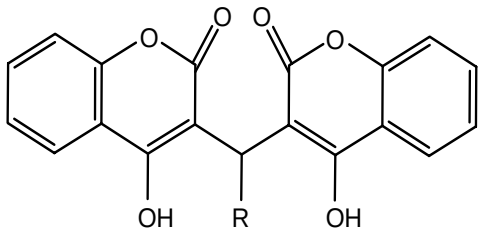


R = H (Warfarin)

$R = NO_2$ (acenocoumarin)

$R = N_3$ (azidowarfarin)

(Fig.61)



R = H dicoumarol

$R = 3\text{-}OH\text{-}C_6H_4$

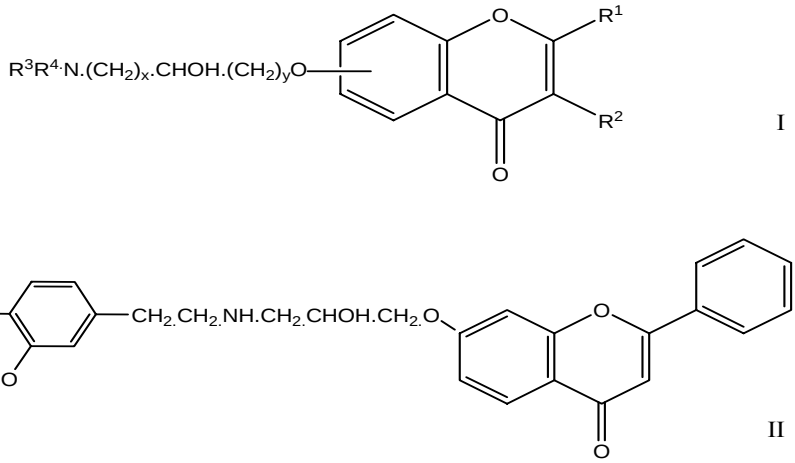
[3,3'-(m-hydroxy benzylidine)-bis(4-hydroxy coumarin)]

(Fig. 60)

Burke et al[40] reported the synthetic routes using of the isomeric 2-pyranyl monoether derivatives of 4-hydroxysalicylaldehyde and 2,5-dihydroxyacetophenone. The corresponding *o*-allyl ethers were converted to substituted phenols by Baeyer-Villiger oxidation and the propanolamine side chain elaborated using epichlorohydrin, followed by oxirane ring opening with isopropylamine. Each of the hydroxylated metabolites is about ten times less potent than oxprenolol as an antagonist to the isoproterenol-induced relaxation of guinea pig tracheal strips.

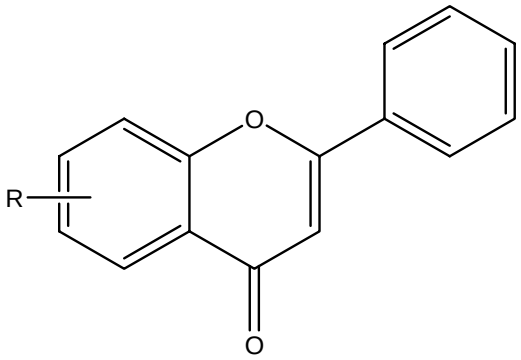
Jen et al[41] Two distinct chemical classes, namely, phenylethanolamines resembling the natural biogenic catecholamines and l-alkylamino-3-aryloxy-2-propanol (aryloxypropanolamines), exert a distinct action as agonists and/or antagonists of adrenergic receptors.

Chou et al[42] preparation of [amino (hydroxyalkoxy)] flavonoids as inhibitors of leukotrienes and 5-lipoxygenase the compounds. **(Fig.62)** (I; R^1, R^2 = H, Ph; R^3 = H, C1-5 alkyl; R^4 = Ph, $PhCH_2$, substituted alkyl, heterocyclyl; NR^3R^4 = heterocyclyl; x, y = 1-5) flavone **(Fig.63)** R = Me) were prepared.



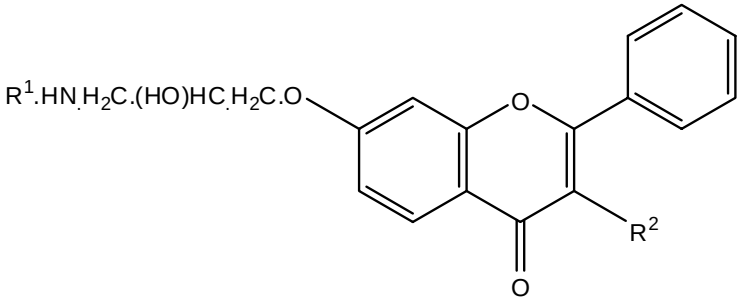
(Fig.62, 63)

Chou et al[43] flavones Synthesised and structure activity relationship of flavodilol and its analogs. A novel class of antihypertensive agents with catecholamine depleting properties. A series of simple flavonyloxypropanolamines was prepared to further explore the structural requirements for the antihypertensive effect of these compounds. Of these analogs tested, the most effective one was flavodilol **(Fig.64)** [R = $PrNHCH_2CH$ (OH) CH_2O]. Only **(Fig.64)** [R = 8-$Me_2CHNHCH_2CH$ (OH) CH_2O] was a **β**-antagonist. Flavodilol was chosen for in-depth pharmacology, toxicology, and clinical evaluation.



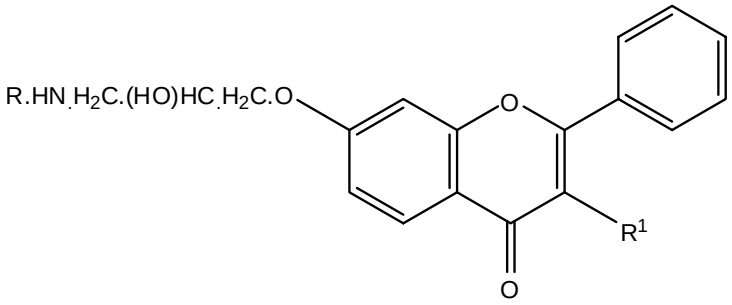
(Fig. 64)

Chou et al[44] chromones (R^1 = Pr, cyclopropyl, Me_2CH; R^2 = Ph, H) and their salts, useful as antihypertensives, were prepared. Thus, etherification of 7-hydroxy-2,3-diphenylchromone with epichlorohydrin gave 7-(2,3-epoxypropoxy)-2,3-diphenylchromone, amination of which with Me_2CHNH_2 gave **(Fig.65)** R^1 = Me_2CH, R^2 = Ph).



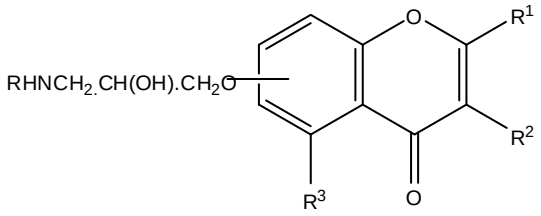
(Fig.65)

Chou et al[45] reported the synthesis of Aminohydroxypropoxy flavones **(Fig.66)** (R = $CHMe_2$, Pr, cyclopropyl; R^1 = Ph, H), which showed antihypertensive activity. 7-Hydroxy-2, 3-diphenylchromone was etherified by epichlorohydrin and NaOH, and the glycidyl ether obtained was treated with Me_2CHNH_2 to give **(Fig. 67)** (R = $CHMe_2$, R^1 = Ph).

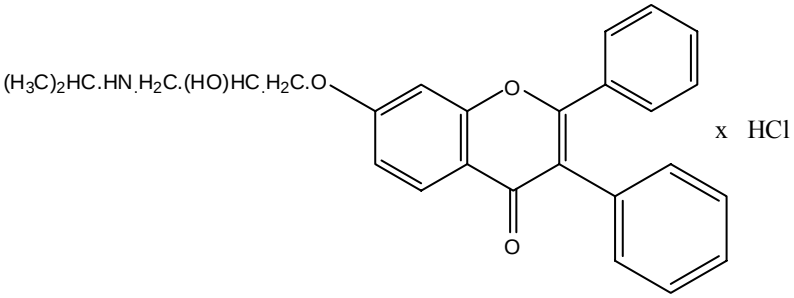


(Fig.66, 67)

Chou et al[46] reported the synthesis of Antihypertensive chromones **(Fig.68)** (R = H, alkyl, cycloalkyl; R^1 = H, alkyl, F_3C, Ph, *o*-ClC_6H_4, *p*-ClC_6H_4; R^2 = H, Ph; R^3 = H, HO). Thus, 7-hydroxy-2, 3-diphenylchromone was treated with epichlorohydrin to give 7-(2, 3-epoxypropoxy)-2, 3-diphenylchromone, which was treated with Me_2CHNH_2 to give the chromone **(Fig.69)** had antihypertensive activity at 8 mg/kg.



I



x HCl

II

(Fig.68, 69)

Whitlon et al[47] reported that the liver microsomal preparations carried out carboxylation converted the vitamin to its 2, 3-epoxide (epoxidase activity) and reduced the epoxide to the vitamin (epoxide reductase activity). The effect of the coumarin anticoagulant Warfarin on these reactions were studied.

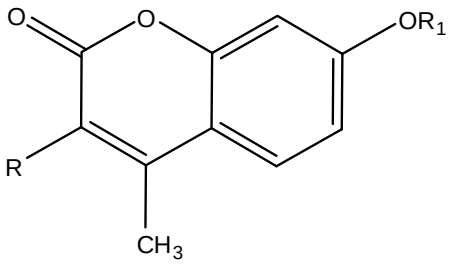
Xu et al[48] reported the discovery of two novel epoxide hydrolases in mung bean (*Phaseolus radiatus* L.) for the first time, either of which can catalyze enantioconvergent hydrolysis of styrene epoxides. Their regioselectivity coefficients are more than 90% for the *p*-nitrostyrene oxide. Further more, the crude mung bean powder was also shown to be a cheap and practical biocatalyst, allowing a one-step asymmetric synthesis of chiral(R)-diols from racemic epoxides, in up to >99% yield and 68.7% overall yield (after recrystallization).

Wendel et al[49] synthesised of the isomeric ring methoxyoxprenolols accomplished from the isomeric methoxy salicylaldehydes by *o*-allylation, followed by Baeyer-Villiger oxidation. The propanolamine side chain was elaborated by 0-alkylation of the Bayer-Villiger product with epichlorohydrin and subsequent oxirane opening with isopropylamine.

Chen et al[50] certain coumarin α-methylene-γ-butyrolactones were synthesized and evaluated for antiproliferative and vasorelaxing activities.

Tyagi et al[51] reported the synthesis of the six novel 4-methylcoumarins bearing different functionalities such as amino, hydroxy, *N*-acetyl, acetoxy and nitro and they were examined for their effect on NADPH dependent liver microsomal lipid peroxidation *in vitro*, and the results were compared with other model 4-methylcoumarin derivatives to establish the structure activity relationship.

Zawadzka et al[52] reported the synthesis of Coumarin derivatives. **(Fig.70)** (R = H, CH_2CH_2OH; $R^1 = R^2NHCH_2CH$ (OH) CH_2; $R^2 = Me_2CH$, Me_3C] as potential drugs of the circulatory system.



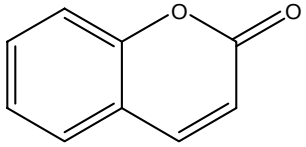
(Fig.70)

Zaagsma et al[53] reported the synthesis of a series of bis (2-hydroxy-3-isopropylaminopropyl) ethers of dihydroxyarenes and investigated *in vitro* for β-adrenergic blocking activity, antagonism of ouabain-induced arrhythmias, inotropic and chronotropic effects, and local anesthetic activity.

2.2 IMPORTANCE OF NATURALLY OCCURRING OXYGEN HETEROCYCLES AND INDANE- 1, 3-DIONES AS THERAPEUTIC AGENTS

Coumarins [61,62,63]

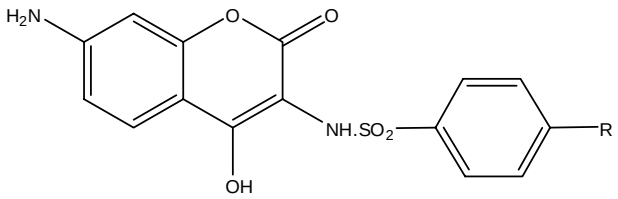
Among the naturally occurring oxygen heterocycles, coumarins and flavones occupy prominent position as therapeutic agents. Coumarins **(Fig 71)** (2H-benzo[b] pyran-2-ones) form an important class of oxygen containing heterocycles and are widely distributed in nature.



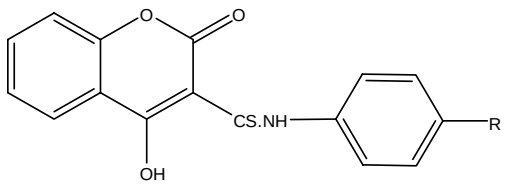
(Fig. 71)

Coumarin and its derivatives have attracted considerable interest because of their various physiological and biochemical properties. They act as growth regulators in number of plants. Plant extracts and synthetic products containing coumarin nucleus are used as anticoagulants.

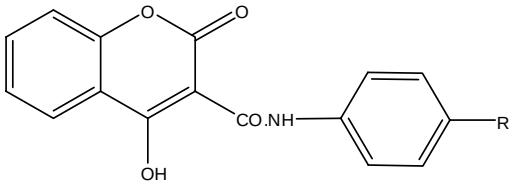
Some coumarins exhibit useful antibiotic properties. Compound **(Fig 72)** inhibits *Mycobacterium tuberculosis*; **(Fig 73)** is a fungicidal agent and **(Fig 74)** is an insecticidal agent.



(Fig. 72)



(Fig. 73)



(Fig. 74)

6-Substituted coumarins act as potential anticancer agents. Coumarin (1) itself has been reported to be a moderately potent inhibitor of chemical carcinogen induced neoplasia.

Flavones [64]

Flavonoids exhibit broad spectrum of physiological activity. The anti spasmolytic, antianginal, antiulcer, antihepatotoxic, anti-inflammatory, antiallergic, antimicrobial and antiviral effects of flavonoids are note worthy. In recent years, a number of flavonoids like baicalin, taxifolin, gossypin, proanthocyanidine, nepetin, diosmin, fisetin, (+)-catechin, (-)-epicatechin and 5,7-dimethyoxyflavone have been reported to have anti-inflammatory effects. Antiviral activity of a few flavonoids were investigated by Pusztai etal., and observed that hydroxylation in 3-position appears to be prerequisite for this activity. Antirhinovirus and antipicronovirus activity of certain flavonoids have been reported. Some flavonoids such as flavones, isoflavones, flavanones and flavonols exhibited useful anti-tumour activity.

Recently, 7,8-di-O-substitued flavans, biflavans, and flavones showed cytotoxic activity and it has been established that the activity is due to methoxy and/or hydroxyl group in the structure.

Inhibition of Human Immunodeficiency (HIV) reverse transcriptase is currently considered a useful approach in the prophylaxis and intervention of Acquired Immunodeficiency Syndrome (AIDS) and natural products have been extensively explored as inhibitors of this enzyme to discover drugs active against AIDS. Recently, 150 pure natural products have been examined and polyphenolic compounds were found to be responsible for this activity; among flavonoids tested quercitin exhibited moderate activity.

Indane-1,3-diones [65,66,67,68]

Indane 1,3-dione constitutes a unique group of compounds. Indane-1,3-dione and its derivatives exhibit a wide range of biological activity ranging from anticoagulant, neurotropic, antiphlogistic radioprotective effects, zoocidal, insecticidal, fungicidal, bacteriocidal, hypoprothrombinemic and cytotoxic properties.

CHAPTER - III

3.0 AIM OF THE PRESENT WORK

SYNTHESIS AND CYTOTOXIC EVALUATION OF EPOXIDES DERIVED FROM NATURAL OXYGEN HETEROCYCLES AND INDANE-1,3-DIONES.[55,56,57,58]

Among naturally occurring oxygen heterocyclic compounds, derivatives of coumarins (2*H*-chromen-2-one) and flavones (2-aryl-4*H*-chromen-4-one) exhibit useful cytotoxicity. Likewise, certain derivatives of indane-1,3-dione showed, in addition to their anticoagulant activity, marked cytotoxic activity.

It has been well established that the mutagenic activity of many naturally occurring and synthetic compounds is attributed to the presence of epoxide (oxirane) moiety. The marked mutagenic activity of epoxide pharmacophore is due to its high reactivity toward nucleophilic group (NH_2, OH) of purine and pyrimidine derivatives of DNA. Epoxides being strained ethers function as reactive electophiles and alkylate the nucleophilic moieties of DNA especially N-7 of guanosine. In other words, epoxide moiety serves as a reactive mutagenic pharmacophore.

In our attempts to find cytotoxic compounds with reduced toxicity, we have anticipated that oxygen heterocyclic compounds of natural origin such as coumarins and flavones and synthetic indane-1,3-dione derivatives incorporating epoxide pharmacophore may possess useful cytotoxic activity. With this objectives, we have synthesized epoxides of derivatives of coumarins and flavones and indane-1,3-diones as depicted in Schemes I, II and III with a view to evaluate them for cytotoxic activity

by MTT assay; special attention has been paid to those compounds that exhibit favorable ED_{50} (<40 mg/Kg body weight of animal).

3.1 PLAN OF WORK

PART - I

The scheme of the proposed work is as follows

- Synthesis of epoxides of Coumarin, Indane -1, 3-dione and Flavone
- Evaluation for Anti cancer activity of Coumarin epoxide, Indane-1, 3-dione epoxide and Flavone epoxide

PART - II

- Process optimization by changing speed of rotation in flash evaporator, hydration time, volume of hydration fluid
- Formulation of empty vesicles
- Formulation development of Methotrexate liposomes, coumarin epoxide liposomes and Indane-1,3-dione epoxides liposomes respectively
- Separation of unentrapped drug from liposomal preparation
- Evaluation of liposomes
- HPLC analysis of Methotrexate, Coumarin epoxide and Indane-1,3- dione epoxide
- Stability studies
- Statistical evaluation of release kinetics

MATERIALS USED

3.2 List of the materials used

Chemicals	Brand Name
2-(Chloromethyl)oxirane	Loba chemicals
Potassium carbonate	Merck India
Piperidine	S.d fine chemicals
Alcohol	S.d fine chemicals
Sodium hydroxide	Merck India
Hydrogen peroxide	Merck India
Benzaldehyde	Merck India
Anisaldehyde	Merck India
2-Chloro benzaldehyde	Merck India
4-Nitro benzaldehyde	Merck India
4-Chloro benzaldehyde	Merck India
Furfuraldehyde	Merck India
Pyridin-3-aldehyde	Merck India
3-Nitro benzaldehde	Merck India
Hydrochloric acid	S.d fine chemicals
Acetic acid	S.d fine chemicals
Acetone	S.d fine chemicals
Benzoyl chloride	S.d fine chemicals
2-Chloro benzoyl chloride	S.d fine chemicals
4-Chloro benzoyl chloride	S.d fine chemicals
Dimethyl formamide	Merck India

4-Methoxy benzoic acid	Merck India
3,4-Dimethoxy benzoic acid	Merck India
3,4,5-Trimethoxy benzoic acid	Merck India
3-Chloro benzoic acid	Merck India
3-Nitro benzoic acid	Merck India
4-Nitro benzoic acid	Merck India

3.3 Name of the Instruments Used

IR:	Jasco 410 FT-IR Spectrometer
NMR:	300 MHz Bruker (Avance) NMR Spectrometer
Elemental Analysis:	Perkin Elmer 2400 series II, Elemental CHNS analyser

RESEARCH ENVISAGED

4.0 Synthesis of Epoxide derived from oxygen heterocyclic and Spiro Epoxide from Indane - 1,3-dione

The experimental section of this present dissertation includes two major parts:

Part I:

a) Synthesis of Coumarin epoxide, Indane -1, 3-dione epoxide and Flavone epoxides.

b) Determination of melting point, solubility, elemental analysis, IR, ^{1}H-NMR, ^{13}C-NMR, DEPT-135, MASS.

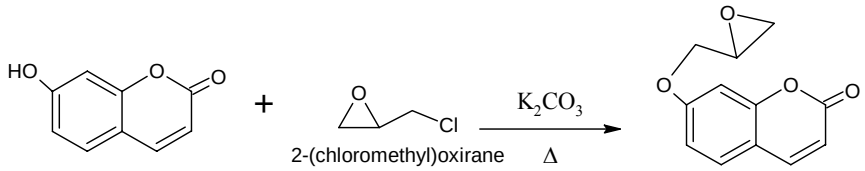
c) Biological screening of the synthesized compounds for cytotoxic activity by MTT assay.

Part II:

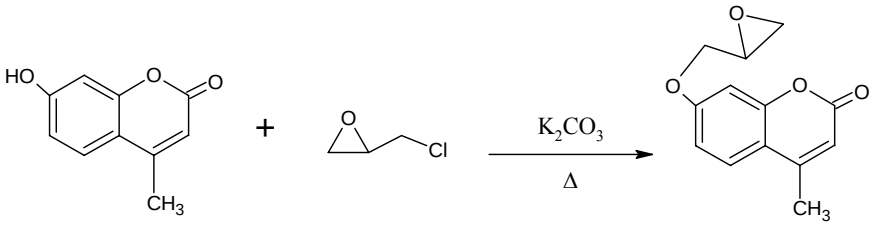
a) Formulation of the synthesized compounds and Methotrexate

b) Evaluation of the optimized formulation.

SCHEME I



7-hydroxy-2*H*-chromen-2-one 2-(chloromethyl)oxirane 7-(2'3'-Epoxy propxy)-4- coumarin **(Cu 1)**



7-hydroxy-4-methyl-2*H*-chromen-2-one 2-(chloromethyl)oxirane 7-(2'3'-Epoxy propxy)-4-methyl coumarin **(Cu 2)**

SCHEME – I

7-(2′3′-Epoxy propxy)-4- coumarin (Cu 1)

7-Hydroxy coumarin (1.6 g, 0.01) was refluxed with excess of 1-chloro 2,3-epoxy propane 10 ml and anhydrous potassium carbonate (3 g) for 3h.The reaction was monitored by TLC. After completion of the reaction, the residue was washed with hot acetone, filtered and excess solvent was removed *in vacuo*. The residue was treated with cold water and the separated solid was filtered, washed with water and dried. It was crystallized from ethyl acetate-pet ether as colourless solid.

Yield: 75 % m.p:101^{0}C

IR: γmax (KBr): 2910, 1720(CO), 1612,1398,1122,833, 763cm^{-1}

PMR (300MHz, $CDCl_3$, δ):2.79 (dd,1H,C_3^1,Hd) 2.79 (dd,1H,C_3^1Hc), 3.79 (m,1H,OCH), 3.94 (dd,1H,C_2^1-Hb), 4.32 (dd,1H,C_2^1-Hb), 6.83-7.63 (m,5H,Ar-H)

^{13}C-NMR (75 MHz, $CDCl_3$, δ): 44.93 (C_3^1), 50.17 (C_2^1), 69.63(C_1^1), 102.00(C_8), 113.30(C_6), 113.82(C_{10}), 114.35(C_3), 129.28(C_5), 143.77(C_4), 156.13(C_9), 161.52(C_7), 161.94(C_2)

7-(2′3′-Epoxy propxy)-4-methyl coumarin (Cu 2)

7-Hydroxy -4-methylcoumarin (1.75 gm 0.01mol) was refluxed with excess of 1-chloro 2,3-epoxy propane(10ml)and anhydrous potassium carbonate (3 g) for 3h. The reaction was monitored by TLC. After the completion of the reaction the residue was washed with hot acetone, filtered and excess solvent was removed *in vacuo*. The residue was treated with cold water and the separated solid was filtered, washed with water and dried. It was crystallized from ethyl acetate-pet ether as colourless solid.

Yield: 77 % m.p:120^{0}C

IR: γmax (KBr): 2923, 1722(CO), 1619,1392,1265,1072,912, 848cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 2.40 (s,3H,4-CH_3), 2.79 (dd,1H,C_3^1Hd), 2.87 (dd,1HC_3^1-Hc) 3.37 (m,1H,OCH), 3.84 (dd,1H, C_2^1-H_9), 4.34 (dd,1H, C_2^1-Hb), 6.15 (s,1HC_3-H) 6.82-7.53 (m,3H,Ar-H) **^{13}C-NMR (75 MHz, CDCl$_3$, δ):** 19.08 (C_4-CH_3), 44.93 (C_3^1), 50.20(C_1^1), 69.57(C_2^1), 101.98(C_8), 112.56(C_6), 112.96(C_{10}), 114.36(C_3), 126.08(C_5), 152.99(C_9), 155.48(C_4),161.64(C_7),161.74(C_2)

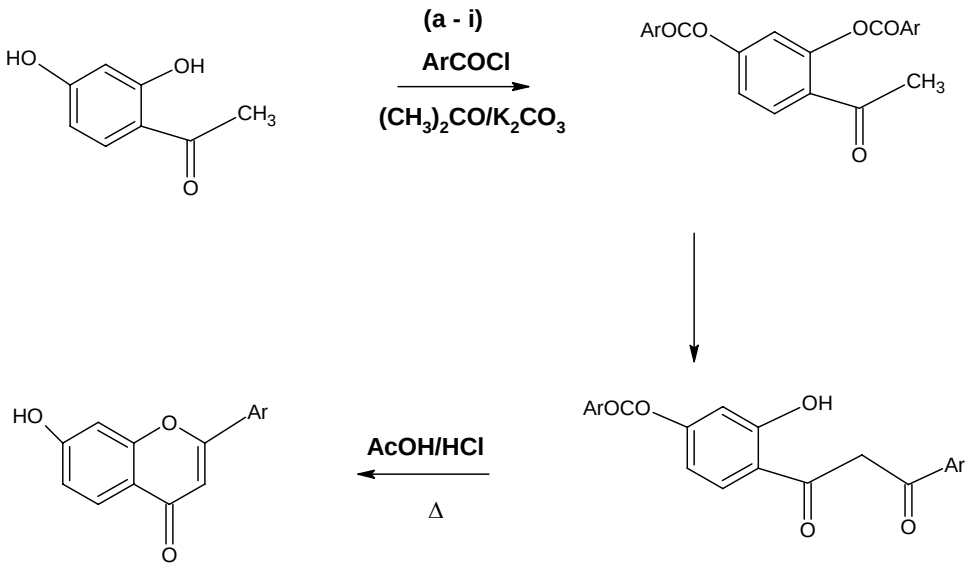
CH-COSY and HMBC data for (Cu – 2)

POSITON	**CH-COSY**	**HMBC**
C_3-H (8.615)	114.36	19.08(C_4-CH_3),112.96(C_{10}), 61.74(C_2)
C_4-CH_3 (2.40)	19.08	112.96(C_{10}), 114.36(C_3)
C_5-H (7.50)	126.08	152.99(C_9),155.48(C4), 161.64(C_7)
C_6-H (6.82)	112.56	101.98(C_8), 112.96(C_{10})
C_8-H (6.89)	101.98	112.56(C6), 112.96(C_{10})
C_1^1-H_9 (3.84) & C_1^1-Hb (4.34)	69.57	44.93(C_3^1), 161.64(C_7)
C_2^1-H (3.37)	50.20	44.93(C_3^1)
C_3^1-Hc (2.87) & C_3^1-Hd	44.93	50.20(C_2^1), 69.57(C_1^1)

Synthesis of Substituted flavone epoxide

SCHEME - II

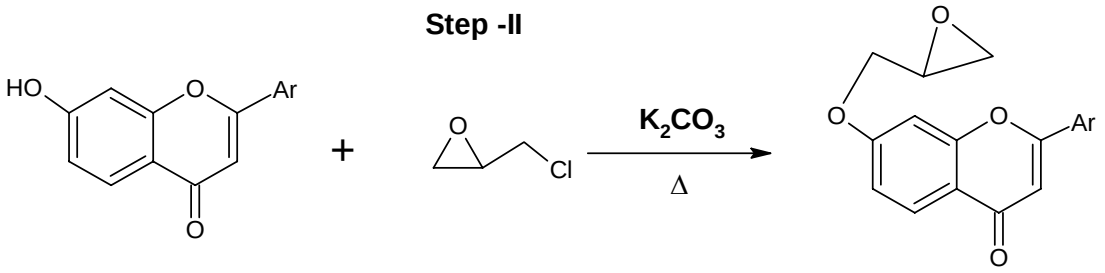
Step - I Synthesis of 7- HYDROXY FLAVONE



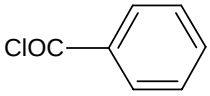
Fa - i

Synthesis of 7-(oxiranyl 2'-methoxyflavones)

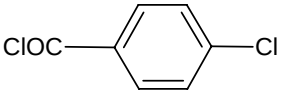
Step -II



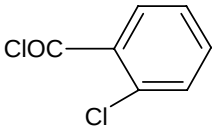
Ar **FEa -i**

a = 

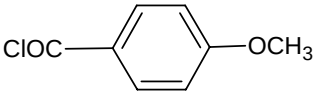
Benzoylchloride

b = 

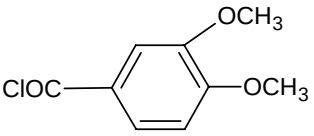
p -chlorobenzoyl chloride

c = 

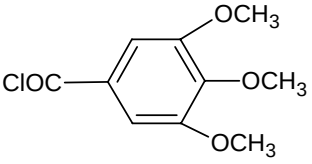
o -chlorobenzoyl chloride

d = 

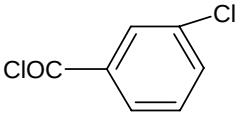
p -methoxybenzoyl chloride

e = 

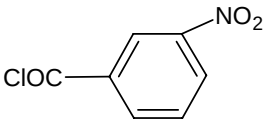
1,2-dimethoxybenzoyl chloride

f = 

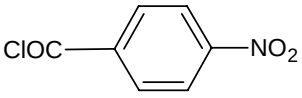
1,2,3- trimethoxybenzoyl chloride

g = 

m -chlorobenzoyl chloride

h = 

m -nitrobenzoyl chloride

i = 

p -nitrobenzoyl chloride

SCHEME - II

SYNTHESIS OF SUBSTITUTED FLAVONE EPOXIDE

Step I:

2, 4-DIHYDROXY ACETOPHENONE

In a 100 ml beaker, freshly fused and powdered zinc chloride (10 g) was dissolved in glacial acetic acid (16ml). After adding fused and powdered resorcinol (10g) the mixture was heated to about 140^0 and kept the reactant for about 10 – 15 minutes. It was then cooled and poured into ice cold hydrochloric acid. The solid separating out was collected on a Buckner funnel under suction, washed with water and dried. It was crystallized from hot water saturated with sulphurdioxide.

m.p. 145° C (Lit.[59] m.p. 147° C)

General procedure for the preparation of acid chlorides (d – i)[24]

To a suspension of aromatic acids (0.1 mol) in 2 drops of DMF, thionylchloride (0.2 mol) was added and refluxed for 1h. The excess of thionyl chloride was removed *in vacuo*. The separated acid chloride was used as such for further reactions.

Step II:

General procedure for the synthesis of Substituted Diketones (Ka – Ki)

To a solution of reacetophenone (0.1mol) in acetone (300 ml) taken in a 500ml round bottom flask, appropriate substituted benzoyl chloride (0.21 mol) and anhydrous potassium carbonate (25 g) were added and the mixture refluxed on a water bath for 20h. The mixture was filtered hot and the potassium salts washed with

warm acetone. The filtrate was distilled to remove the solvent and the residue was treated with water. The diketone separating out was filtered, washed with water, dried and recrystallized from ethanol.

3-Hydroxy-4-(3-oxo-3phenylpropanoyl) phenyl benzoate (K-a)

Yield: 67% m.p:217^{0}C (aq.acetone)

Elemental analysis: the molecular formula $C_{22}H_{16}O_5$ requires C, 73.33: H, 4.48; and found to have C, 73.29; H, 4.46%

4-(3-(4-Chlorophenyl)-3-oxopropanoyl)-3-hydroxyphenyl-4-chlorobenzoate(K-b)

Yield: 65% m.p:236^{0}C (aq.acetone)

Elemental analysis: the molecular formula $C_{22}H_{14}Cl_2O_5$ requires C, 61.56: H, 3.29; and found to have C, 61.52; H, 3.26%.

4-(3-(2-Chlorophenyl)-3-oxopropanoyl)-3-hydroxyphenyl-2-chlorobenzoate (K-c)

Yield: 62% m.p:217 ^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{22}H_{14}Cl_2O_5$ requires C, 61.56: H, 3.29; and found to have C, 61.53; H, 3.31%

3-Hydroxy-4-(3-(4-methoxyphenyl)-3-oxopropanoyl)phenyl4-ethoxybenzoate (K-d)

Yield: 64% m.p:202 ^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{24}H_{20}O_7$ requires C, 68.55: H, 4.80; and found to have C, 68.53; H, 4.82%

4-(3-(3,4-Dimethoxyphenyl)-3-oxopropanoyl)-3-hydroxypheny3,4-dimethoxybenzoate (K-e)

Yield:66 % m.p:235^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{26}H_{24}O_9$ requires C, 65.00: H, 5.03; and found to have C, 65.3; H, 5.07%.

3-Hydroxy-4-(3-oxo-3-(3,4,5-trimethoxyphenyl)propanoyl)phenyl3,4,5-trimethoxybenzoate (K-f)

Yield:61 % m.p:207^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{28}H_{28}O_{11}$ requires C, 62.22: H, 5.22; and found to have C, 62.19; H, 5.24%.

4-(3-(3-Chlorophenyl)-3-oxopropanoyl)-3-hydroxyphenyl-3-chlorobrnzoate (K-g)

Yield: 67% m.p:196^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{22}H_{14}Cl_2O_5$ requires C, 61.50: H, 3.29; and found to have C, 61.53; H, 3.25%

3-Hydroxy-4-(3-(3-nitrophenyl)-3-oxopropanoyl) phenyl-3-nitrobenzoate (K-h)

Yield: 63% m.p:172^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{22}H_{14}N_2O_9$ requires C,58.67: H,3.13;N,6.22; and found to have C,58.62; H,3.10;N,6.23%.

3-Hydroxy-4-(3-(3-nitrophenyl)-3-oxopropanoyl) phenyl-4-nitrobenzoate (K-i)

Yield:65% m.p:183^{0}C (aq.alcohol)

Elemental analysis: the molecular formula $C_{22}H_{14}N_2O_9$ requires C, 58.67: H, 3.13; N, 6.22; and found to have C, 58.64; H, 3.09; N, 6.18%.

Step III:

General procedure for the synthesis of Substituted 7- hydroxyl flavone (Fa –Fi)

In a 200ml round bottom flask fitted with water cooled condenser, the appropriate substituted diketone (0.1mol), acetic acid (50ml) and concentrated hydrochloric acid (50ml) were taken and the mixture refluxed for 2h. After cooling the mixture was poured into ice and the solid separating out was filtered, washed first with 5% sodium bicarbonate solution and then with water. After drying it was recrystallized from ethanol.

7-Hydroxy-2-phenyl-4H-chromen-4-one (F-a)

Yield: 85% m.p:240 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.32 (s,1H,C_3-H), 6.41-7.68(m,84,Ar-H),10.06 (s,1H,-OH)

2-(4-Chlorophenyl)-7-hydroxy-4H-chromen-4-one (F-b)

Yield: 72% m.p:232 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.29 (s,1H,C_3-H), 6.38-7.31 (m,7H,Ar-H), 10.12 (s,1H,-OH)

2-(2-Chlorophenyl)-7-hydroxy -4H-chromen-4-one (F-c)

Yield: 79% m.p:217 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.31 (s,1H,C_3-H), 6.42-7.87 (m,7H,Ar-H), 9.97(s,1H,-OH)

7-Hydroxy -2-(4-methoxyphenyl)-4H-chromen-4-one (F-d)

Yield: 78% m.p:238 ^{0}C (aq.alcohol)

PMR(300MHz,$CDCl_3$,δ): 3.69(s,3H,-OCH_3), 6.37(s,1H,C_3-H), 6.48-7.93 (m,7H,Ar-H), 10.32 (s,1H,-OH)

2-(3,4-Dimethoxyphenyl) -7-hydroxy -4H-chromen-4-one (F-e)

Yield: 73% m.p:213 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 3.59 (s,3H,-OCH_3), 3.61 (s,3H,-OCH_3), 6.33 (s,1H,C_3-H), 6.41-7.86 (m,6H,Ar-H), 10.11 (s,1H,-OH)

7-Hydroxy -2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (F-f)

Yield: 81% m.p:225 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 3.58 (s,3H,-OCH_3), 3.59(s,3H,-OCH_3), 3.61(s,3H,-OCH_3), 6.19(s,1H,C_3-H), 6.32-7.99 (m,6H,Ar-H), 10.13 (s,1H,-OH)

2-(3-Chlorophenyl)-7-hydroxy-4H-chromen-4-one (F-g)

Yield: 73% m.p:207 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.33 (s,1H,-C_3-H), 6.72-7.78 (m,7H,Ar-H), 9.98 (s,1H,-OH)

7-Hydroxy -2-(3-nitrophenyl)-4H-chromen-4-one (F-h)

Yield:75% m.p:217 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.29 (s,1H,-C_3-H), 6.66-7.91 (m,7H,Ar-H), 9.93 (s,1H,-OH)

7-Hydroxy -2-(4-nitrophenyl)-4H-chromen-4-one (F-i)

Yield: 72% m.p:227 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 6.11 (s,1H,-C_3-H), 6.43-7.77 (m,7H,Ar-H), 10.08 (s,1H,-OH)

Step IV:

General procedure for the synthesis of substituted flavone epoxide (FEa to FEi)

To the appropriate substituted 7-hydroxyl flavone (0.1mol) taken 500 ml round bottom flask, epichlorhydrin (15 ml) and anhydrous potassium carbonate (3 g) were added mixture refluxed on a water bath for 2h. the reaction mixture was cooled, epoxide separated as mass. The product was collected with suction and washed with hot acetone evaporate the solvent *in vacuo*, the residue obtained was treated with water, filter the product, dried and crystallized in ethanol.

7-(Oxiran-2-ylmethoxy)-2-phenyl-4H-chromen-4-one (FEa)

Yield:72 % m.p:134 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 2.67 (m, 2 H, C_3'- CH_2), 3.21 (m, 1 H, C_2'- CH), 4.11 (m, 2 H, -OCH_2), 6.70 (s, 1H, C_3 – H), 6.89- 8.21 (m, 8H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(4-chlorophenyl)- 4H-chromen-4-one (FEb)

Yield:70 % m.p:103 ^{0}C (aq.alcohol)

PMR (300MHz,CDCl$_3$,δ): 2.31 (m, 2 H, $C_3^{'}$- CH_2), 3.28 (m, 1 H, $C_2^{'}$- CH), 4.20 (m, 2 H, -OCH_2), 6.68 (s, 1H, C_3 – H), 6.72-7.43 (m, 7H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(2-chlorophenyl)- 4H-chromen-4-one (FEc)

Yield: 75 % m.p:117 ^{0}C (aq.alcohol)

PMR (300MHz,CDCl$_3$,δ): 2.37 (m, 2 H, $C_3^{'}$- CH_2), 3.21 (m, 1 H, $C_2^{'}$- CH), 4.22 (m, 2 H, -OCH_2), 6.57 (s, 1H, C_3 – H), 6.77- 7.43 (m, 7H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(4-methoxyphenyl)- 4H-chromen-4-one (FEd)

Yield:68 % m.p:128 ^{0}C (aq.alcohol)

PMR (300MHz,CDCl$_3$,δ): 2.33 (m, 2 H, $C_3^{'}$- CH_2), 3.23 (m, 1 H, $C_2^{'}$- CH), 3.83 (s, 3H, -OCH_3), 4.12 (m, 2 H, -OCH_2), 6.69 (s, 1H, C_3 – H), 6.71- 7.69 (m, 7H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(3,4-dimethoxyphenyl)-4H-chromen-4-one (FEe)

Yield: 77 % m.p:135 ^{0}C (aq.alcohol)

PMR (300MHz,CDCl$_3$,δ): 2.31 (m, 2 H, $C_3^{'}$- CH_2), 3.01 (m, 1 H, $C_2^{'}$- CH), 3.81 (s, 6H, 2-OCH_3), 4.12 (m, 2 H, -OCH_2), 6.70 (s, 1H, C_3 – H), 6.72- 7.32 (m, 6H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one (FEf)

Yield: 69 % m.p:119 ^{0}C (aq.alcohol)

PMR (300MHz,CDCl$_3$,δ): 2.39 (m, 2 H, $C_3^{'}$- CH_2), 3.03 (m, 1 H, $C_2^{'}$- CH), 3.82 (s, 9H, -OCH_3), 3.98 (m, 2 H, -OCH_2), 6.69 (s, 1H, C_3 – H), 6.71- 7.93 (m, 5H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(3-chlorophenyl)- 4H-chromen-4-one (FEg)

Yield: 73 % m.p:143 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 2.32 (m, 2 H, C_3'- CH_2), 3.31 (m, 1 H, C_2'- CH), 4.22 (m, 2 H, -OCH_2), 6.67 (s, 1H, C_3 – H), 6.69- 7.72 (m, 7H, Ar-H).

7-(Oxiran-2-ylmethoxy)-2-(3-nitrophenyl)- 4H-chromen-4-one (FEh)

Yield: 75 % m.p:132 ^{0}C (aq.alcohol)

PMR (300MHz,$CDCl_3$,δ): 2.36 (m, 2 H, C_3'- CH_2), 3.12 (m, 1 H, C_2'- CH), 4.18 (m, 2 H, -OCH_2), 6.73 (s, 1H, C_3 – H), 6.75- 8.20 (m, 7H, Ar-H).

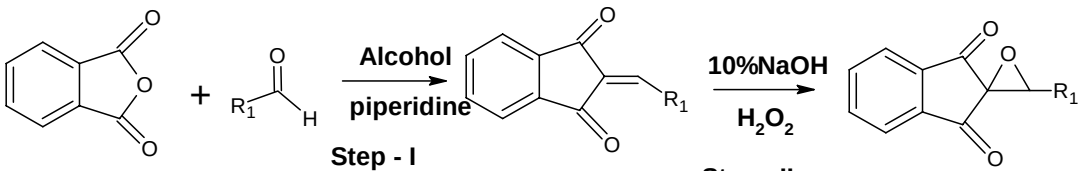
7-(Oxiran-2-ylmethoxy)-2-(4-nitrophenyl)- 4H-chromen-4-one (FEi)

Yield: 77 % m.p:127 ^{0}C (aq.alcohol)

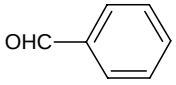
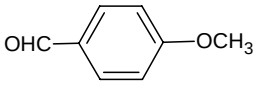
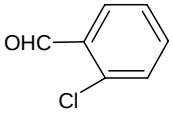
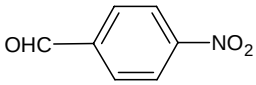
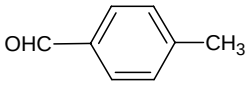
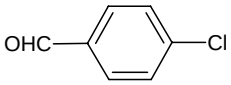
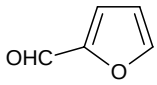
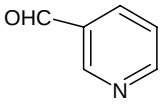
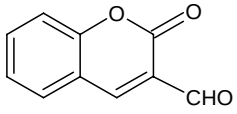
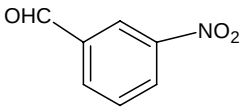
PMR (300MHz,$CDCl_3$,δ): 2.33 (m, 2 H, C_3'- CH_2), 3.14 (m, 1 H, C_2'- CH), 4.19 (m, 2 H, -OCH_2), 6.76 (s, 1H, C_3 – H), 6.78- 8.12 (m, 7H, Ar-H).

Synthesis of spiro epoxides of indane-1,3-dione

SCHEME III



R_1 = a to j

a	=		Benzaldehyde
b	=		Anisaldehyde
c	=		*o* -chlorobenzaldehyde
d	=		*p* -nitrobenzaldehyde
e	=		*p* -methylbenzaldehyde
f	=		p -chlorobenzaldehyde
g	=		Furfuraldehyde
h	=		Pyridin-3-aldehyde
i	=		Coumarin-3-aldehyde
j	=		*m* -nitrobenzaldehyde

SCHEME - III

Synthesis of Indane-1, 3-dione; 1, 2-substituted Indane-1, 3-dione and Indane- 1, 3-dione epoxide:[59]

A solution of diethyl phthalate (106.5 mL, 0.563 mol) and sodium wire (25 g, 1.09 mol) were placed in a dry two necked flask fitted with a reflux condenser and dropping funnel; each were protected with a calcium chloride tube. The flask was heated on a steam bath. A mixture of dry ethyl acetate (136 ml, 1.39mol) and absolute ethanol (2.5 ml) were added over a period of 90 minutes. The heating was continued for 6 hours. Then it was cooled and ether 50 ml was added. The sodium salt was filtered on a sintered glass funnel, and washed by stirring with ethyl acetate. After the filtration the sodium salt was dissolved in hot water (1400 ml). The solution was cooled to 70^0C and stirred vigorously. Then the solution was acidified with dilute sulphuric acid (100 ml, 3 parts of concentrated acid to 1 part of water). Finally the mixture was cooled to 15^0C in an ice bath and the indane-1, 3-dione was collected by vacuum filtration. Indane-1, 3-dione was rexcrystallized from a dioxane-benzene (1:1) mixture by the addition of light petroleum to give the pure compound.

Yield: 85% M.p: 122^0c

Step II

General procedure for the synthesis of 2-(substituted phenylmethylene) indane - 1, 3-dione (In -a to In -j)

To an alcoholic solution of Indane-1,3-dione (0.1mol), freshly distilled apprppriate aromatic (0.1 mol) was added followed by a drop of piperidine. The reaction mixture was refluxed for 3h. After 3h the excess of alcohol was removed *in vacuo*. The reaction mixture was cooled in ice water and diluted by adding small

amount of distilled water. The solid that separated was filtered using vacuum pump and washed repeatedly with ice cold 4N ethanol.

2-Benzylidene-1H-indene-1,3(2H)-dione (In-a)

Yield: 85% m.p:154^{0}C (aq.alcohol)

IR:γmax (KBr): 3066, 2927, 2838, 1705(CO), 1687(CO), 1592cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 7.26-8.01 (m,9-H,Ar-H), 8.48 (s,1H=CH-Ar)

Elemental analysis: the molecular formula $C_{16}H_{10}O_2$ requires C, 82.04: H, 4.30; and found to have C, 82.13; H, 4.22%.

2-(4-MethoxyBenzylidene)-1H-indene-1,3(2H)-dione (In-b)

Yield: 87% m.p:164^{0}C (aq.alcohol)

IR:γmax (KBr): 3072, 2919, 2838, 1690(CO), 1683(CO), 1587, 1508cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 3.60(s,3H,-OCH_3), 6.87-7.83(m,84,Ar-H),

8.63 (s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{17}H_{12}O_3$ requires C, 77.26: H, 4.58; and found to have C, 77.30; H, 4.47%

2-(2-Chlorobenzylidene)-1H-indene-1,3(2H)-dione (In-c)

Yield:91% m.p:173^{0}C (aq.alcohol)

IR:γmax (KBr): 3079, 2919, 1963, 1754(CO), 1695(CO), 1590, 1473, 1428cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 7.26-7.83 , (m,8H,Ar-H), 8.32 (s,1H=CH-Ar)

Elemental analysis: the molecular formula $C1_6H_9ClO_2$ requires C, 71.52: H, 3.38; and found to have C, 71.49; H, 3.33%

2-(4-Nitrobenzylidene)-1H-indene-1,3(2H)-dione (In-d)

Yield: 83% m.p:230^{0}C (aq.alcohol)

IR:γmax (KBr): 3116, 2917, 1733 (CO), 1695(CO), 1348cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 7.63-8.23 (m,8H,Ar-H), 8.27(s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{16}H_9NO_4$ requires C, 68.82: H, 3.25; N, 5.02; and found to have C, 68.85; H, 3.27; N, 5.05%

2- (4-Methylbenzylidene)-1H-indene-1,3(2H)-dione (In-e)

Yield: 88 % m.p:183^{0}C (aq.alcohol)

IR:γmax (KBr): 3012, 2982, 1727(CO), 1689(CO), 1576, 1465cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 2.07 (s,3H,-CH_3), 7.43-7.83 (m,8H,Ar-H),

7.96 (s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{17}H_{12}O_2$ requires C, 82.24: H, 4.87; and found to have C, 82.28; H, 4.83%

2-(4-Chlorobenzylidene)-1H-indene-1,3(2H)-dione (In-f)

Yield: 73% m.p:192^{0}C (aq.alcohol)

IR:γmax (KBr): 3076, 2972, 1736(CO), 1687(CO), 1432, 1327cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 7.41-7.83 (m,4H,Ar-H), 7.87 (s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{16}H_{19}ClO_2$ requires C, 71.52: H, 3.38; and found to have C, 71.56; H, 3.35%

2-(Furan-2-yl-methylene)-1H-indene-1,3(2H)-dione (In-g)

Yield: 72% m.p:163^{0}C (aq.alcohol)

IR:γmax (KBr): 3010, 2985, 1739(CO), 1692(CO), 1568, 1422cm^{-1}

PMR (300MHz, CDCl3, δ): 6.82-8.31(m,8H, Ar-H and =CH-Ar merged together)

Elemental analysis: the molecular formula $C_{14}H_8O_3$ requires C, 75.00: H, 3.60; and found to have C, 75.03; H, 3.62%

2-(Pyridine-3-ylmethylene)-1H-indene-1,3(2H)-dione (In-h)

Yield: 73% m.p:186^{0}C (aq.alcohol)

IR:γmax (KBr): 3061, 2871, 1726(CO), 1685(CO), 1428, 1371cm^{-1}

PMR (300MHz, CDCl3, δ): 7.32-8.73(m,8H, Ar-H and =CH-Ar merged together)

Elemental analysis: the molecular formula $C_{15}H_9NO_2$ requires C, 76.59: H, 3.86; and found to have C, 76.54; H, 3.81%

2-((2-Oxo2-H-chromen-3-yl)-1H-indene-1,3(2H)-dione (In-i)

Yield: 73% m.p:179^{0}C (aq.alcohol)

IR:γmax (KBr): 1714(CO str.of coumarin), 1702(CO), 1691(CO) 1525, 1431cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 7.21-7.87 (m,9H,Ar-H), 8.91 (s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{19}H_{10}O_4$ requires C, 75.49; H, 3.33; and found to have C, 75.52; H, 3.38%

2-(3-Nitrobenzylidene-1H-indene-1,3(2H)-dione (In-j)

Yield: 69% m.p:172^{0}C (aq.alcohol)

IR:γmax (KBr): 3025, 2823, 1725(CO), 1687(CO), 1535, 1426cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 7.58-8.29 (m,8H,Ar-H), 8.37 (s,1H,=CH-Ar)

Elemental analysis: the molecular formula $C_{16}H_9NO_4$ requires C, 68.82: H, 3.25;N,5.02; and found to have C,68.87; H,3.29;N,5.05%

EPOXIDE PREPARATION:

Step III:

General procedure for the synthesis of (1,3-diketo indane)-2-spiro-2′-(3′-aryl oxirane) (IE a to IE -j)

To a stirred solution of appropriate 2-(substituted phenyl methylene)-indane-1,3-dione (0.1 mol) in acetone (25ml),50% hydrogen peroxide (1.0 mL) was added followed by sodium hydroxide (10%, 2 mL) solution. The reaction mixture was cooled during the addition after a short while the epoxide separated as a mass of pale yellow crystals. The product was collected with suction and washed with small amount of and acetone dried and crystallized in an ethanol.

3′-Phenylspiro [indene-2,2′-oxirane] -1,3-dione (IE-a)

Yield: 68% m.p:165^{0}C (aq.acetone)

IR:γmax (KBr): 2956, 2809, 1709(CO), 1656(CO), 1596, 1457cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 5.12 (1H,s,-CH-oxirane) 6.23-7.34 (m,9H,Ar-H),

Elemental analysis: the molecular formula $C_{16}H_{10}O_3$ requires C, 76.79: H, 4.03; and found to have C, 76.75; H, 4.08%

3′-(4-Methoxyphenyl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-b)

Yield: 72% m.p:236^{0}C (aq.acetone)

IR:γmax (KBr): 2911, 1704(CO), 1648(CO), 1596, 1517cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 5.15 (1H,s,-CH-oxirane) 6.20-7.51 (m,8H,Ar-H),

Elemental analysis: the molecular formula $C_{17}H_{12}O_4$ requires C, 72.85: H, 4.32; and found to have C, 72.81; H, 4.29%

3′-(4-Methoxyphenyl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-c)

Yield: 67% m.p:198^{0}C (aq.acetone)

IR:γmax (KBr): 3079, 2919, 1734(CO), 1698(CO), 1473cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 4.75 (1H,s,-CH-oxirane) 7.10-8.12 (m,8H,Ar-H), Elemental analysis: the molecular formula $C_{16}H_9ClO_3$ requires C,65.50: H,3.19; and found to have C,65.47; H,3.16%

3′-(4-Nitroyphenyl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-d)

Yield: 76 % m.p:212^{0}C (aq.acetone)

IR:γmax (KBr): 2846, 2013, 1729(CO), 1698(CO), 1517, 1444cm^{-1}

PMR (300MHz,$CDCl_3$,δ): 4.81 (1H,s,-CH-oxirane) 7.56-8.09 (m,8H,Ar-H), Elemental analysis: the molecular formula $C_{16}H_9NO_5$ requires C,65.09: H,3.07;N,4.74; and found to have C, 65.07; H, 3.12; N, 4.77%

3′-p-Tolylspiro [indene-2,2′-oxirane] -1,3-dione (IE-e)

Yield: 71% m.p:207^{0}C (aq.acetone)

IR:γmax (KBr): 3021, 2816, 1701(CO), 1652(CO), 1585, 1497cm^{-1}

PMR (300MHz, $CDCl_3$, δ): 2.32 (s, 3H, CH_3),4.31 (1H, s,-CH-oxirane) 7.13-8.07 (m, 8H, Ar-H),

Elemental analysis: the molecular formula $C_{51}H_{36}O_9$ requires C, 77.26: H, 4.58; and found to have C, 77.31; H, 4.59%

3′-(4-Chloroyphenyl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-f)

Yield: 65% m.p:>300^{0}C(aq.acetone)

IR:γmax (KBr): 2986, 2872, 1715(CO), 1639(CO), 1527cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 4.32 (1H,s,-CH-oxirane) 7.28-8.01 (m,8H,Ar-H), Elemental analysis: the molecular formula $C_{16}H_9ClO_3$ requires C,67.50: H,3.19; and found to have C,67.54; H,3.22%

3′-(Furan-2-yl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-g)

Yield: 72% m.p:295^{0}C (aq.acetone)

IR:γmax (KBr): 3012, 2816, 1721(CO), 1691(CO), 1528cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 5.07 (1H,s,-CH-oxirane) 6.32-7.91 (m,7H,Ar-H), Elemental analysis: the molecular formula $C_{14}H_8O_4$ requires C,70.00: H,3.36; and found to have C,69.97; H,3.38%

3′-(Pyridin-3-yl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-h)

Yield: 74% m.p:>300^{0}C(aq.acetone)

IR:γmax (KBr): 3025, 2972, 1718(CO), 1682(CO), 1535, 1491cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 4.72(s,1H,-H,-CH-oxirane) 7.31-8.32(m,7H,Ar-H)

Elemental analysis: the molecular formula $C_{15}H_9NO_3$ requires C,71.71: H,3.61; N,5.58; and found to have C,71.69; H,3.58; N,5.56%

3′-(2-Oxo-2H-chromen-3-yl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-i)

Yield: 68% m.p:>300^{0}C (aq.acetone)

IR:γmax (KBr): 3022, 2896, 1716(CO str.of coumarin), 1702(CO), 1692(CO), 1532, 1427cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 4.33(s,1H,-CH-oxirane) 7.31-8.09(m,9H,Ar-H)

Elemental analysis: the molecular formula $C_{19}H_{10}O_5$ requires C, 71.70: H, 3.17; and found to have C, 71.68; H, 3.14%

3′-(3-Nitrophenyl)spiro[indene-2,2′-oxirane] -1,3-dione (IE-j)

Yield: 67% m.p:>300^{0}C(aq.acetone)

IR:γmax (KBr): 3015, 2871, 1709(CO), 1687(CO), 1638, 1597cm^{-1}

PMR (300MHz,CDCl$_3$,δ): 4.71(s,1H,-CH-oxirane) 7.69-8.23(m,8H,Ar-H)

Elemental analysis: the molecular formula $C_{16}H_9NO_5$ requires C,65.09: H,3.07;N,4.74; and found to have C,65.11; H,3.03; N,4.72%

RESULTS

The following coumarins epoxides were synthesized as depicted in the Scheme I

7-(2′3′-Epoxy propxy)-4- coumarin **(Cu -1)**

7-(2′3′-Epoxy propxy)-4-methyl coumarin **(Cu 2)**

As depicted in the Scheme II the following epoxides were synthesized

a) 7-(Oxiran-2-ylmethoxy)-2-phenyl-4H-chromen-4-one **(FEa)**

b) 7-(Oxiran-2-ylmethoxy)-2-(4-chlorophenyl)- 4H-chromen-4-one **(FEb)**

c) 7-(Oxiran-2-ylmethoxy)-2-(2-chlorophenyl)- 4H-chromen-4-one **(FEc)**

d) 7-(Oxiran-2-ylmethoxy)-2-(4-methoxyphenyl)- 4H-chromen-4-one **(FEd)**

e) 7-(Oxiran-2-ylmethoxy)-2-(3,4-dimethoxyphenyl)-4H-chromen-4-one **(FEe)**

f) 7-(Oxiran-2-ylmethoxy)-2-(3,4,5-trimethoxyphenyl)-4H-chromen-4-one **(FEf)**

g) 7-(Oxiran-2-ylmethoxy)-2-(3-chlorophenyl)- 4H-chromen-4-one **(FEg)**

h) 7-(Oxiran-2-ylmethoxy)-2-(3-nitrophenyl)- 4H-chromen-4-one **(FEh)**

i) 7-(Oxiran-2-ylmethoxy)-2-(4-nitrophenyl)- 4H-chromen-4-one **(FEi)**

As depicted in scheme III the following epoxides were synthesized

(a). 3′-Phenylspiro [indene-2,2′-oxirane] -1,3-dione **(IE-a)**

(b). 3′-(4-Methoxyphenyl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-b)**

(c). 3′-(4-Methoxyphenyl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-c)**

(d). 3′-(4-Nitroyphenyl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-d)**

(e). 3′-(4-Methylphenyl)spiro [indene-2,2′-oxirane] -1,3-dione **(IE-e)**

(f). 3′-(4-Chloroyphenyl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-f)**

(g).3′-(Furan-2-yl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-g)**

(h).3′-(Pyridin-3-yl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-h)**

(i). 3′-(2-Oxo-2H-chromen-3-yl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-i)**

(j). 3′-(3-Nitrophenyl) spiro [indene-2, 2′-oxirane] -1, 3-dione **(IE-j)**

The synthesized epoxides were purified and characterized by spectral data (IR, ^{1}HNMR and ^{13}C NMR) as given in the text.

5. MTT ASSAY OR TESTING THE ANTIPROLIFERATIVE PROPERTIES OF TEST COMPOUNDS [8, 54]

Principle

The Microculture Tetrazolium assay is based on the metabolic reduction of 3-(4-5 dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide to water insoluble blue formazan crystal with mitochondrial dehydrogenase enzyme. This gives direct correlation of viable cells (Alley et al., 1988; Scudiero et al., 1988). The assay is suitable for measuring the cell proliferation, cell viability or cytotoxicity. Procedure involves culturing the cells in a 96 well micro titreplate (MTP) and then incubating them with MTT solution for approximately 2 hours. During incubation period, viable cells convert MTT to a water insoluble formazan dye. It is solublized and quantified with a ELISA plate reader. The absorbance directly correlates with cell number. This is applicable for adherent cells cultured in MTP.

Material:

Cell culture: Ehrlich Ascites carcinoma

Cell number for subculture: one million cells per flask of 30 ml capacity

Cell loading into plate: 5000 cells per well (96 well plate)

a) Preparation of RPMI-1640 Medium

1. RPMI-1640 media (Himedia labs, Mumbai) of 10.39 gm was weighed in a sterile conical flask.

2. The contents were dissolved in 900 ml of triple distilled water by stirring and the antibiotic solutions were added (100 units/mL of penicillin-G sodium, 50 μg /mL of Streptomycin and 2 μg/mL of Amphotericin-B).
3. Once all constituents of the medium were completely added, the pH was adjusted to 7.2-7.5 with 0.1N HCl.
4. The volume was made upto one litre with triple distilled water.
5. Contents were transferred to the Millipore filtration Kettle (Schott Duran, Germany) supported by 0.22 micron membrane filter, kept in laminar flow hood that was connected to the outlet by negative pressure pump and filtered.
6. Pressure was adjusted to a flow rate of 100 mL/min.
7. Medium of 5 mL is taken in t_{25} tissue flask; kept for 24h in CO_2 incubator for sterility check.
8. Fetal Bovine serum of 100 mL sterile was added to 900 mL medium.

b) Selection of flasks for plating

1. Removed the flasks from incubator and checked under microscope for contamination and cell morphology.
2. The flask used for plating should be between 70-80% confluent in a state of healthy log phase growth. The colour of the medium in the flask should not be too yellow or too red.

c) Preparation of cells for plating

1. The media was aspirated from each flask containing the cells, make sure to change pipettes between cell lines to prevent cross contamination.

2. Monolayer was rinsed with 5-10 mL Phosphate Buffer Saline (PBS) to remove the traces of serum and aspirated with the rinsing solution.
3. Approximately 1 mL of 0.25% trypsin-EDTA (Himedia Labs, Mumbai) was added to each flask and spread evenly over cell monolayer. Depending on cell type, the flask was either placed in hood or in incubator for 2 to 5 minutes.
4. The flask was gently tapped for dislodging the cells.
5. Resuspended the cells in 8 mL medium containing serum to stop the action of trypsin. Gentle pippetting was carried out up and down for breaking up of the clumps.
6. Cell suspension was transferred to a properly labeled 15 mL centrifuge tube.
7. The tubes were centrifuged at 1000 rpm for 5 minutes.
8. The pellet was resuspended in 5 – 10 mL of medium depending upon the size of the pellet or cell number.

d) Procedure

1. The adherent cells were trypsinized according to protocol and were resuspended in fresh medium after centrifugation. Cell suspension was mixed thoroughly by pippetting several times to get a uniform single cell suspension.
2. Dilutions of drug solutions were made in media with final DMSO concentration in the well to be less than 1%.
3. Cell suspension of 100 µL was transferred aseptically to each well of a 96 well plate and to it 100 µl of 1% media/drug solution in media was added.
4. The plate was incubated at 37^0C for 72 h in CO_2 incubator.

5. After 72 h of incubation, 20 µL of MTT (4mg/mL stock) was added to each well and the plate was wrapped in an aluminium foil to prevent the oxidation of dye. The plate was again incubated for 2 h.
6. Lysis Buffer of 80 µL (15% sodium dodecyl sulfate in 1:1 mixture of DMF and water) was added to each well and the plate was placed on a shaker for overnight.
7. The absorbances were recorded in ELISA reader at 562 nm.
8. The absorbances of the test were compared with that of DMSO control to get % inhibition.

$$\% \text{ inhibition} = [1 - \frac{test}{control}] \text{ X } 100.$$

Table 1

MTT Assay-Growth Inhibition Using Ehrlich Ascites Carcinoma Cell Lines

COUMPOUND CODE	CONCENTRAION (µg/mL)	% INHIBITION
MTX	30	64.3
Cu-1	30	14.6
IE-ii	30	8.5
FE-a	30	14.6
IE-i	30	32.9
Cu -2	30	39.0

Standard drug- Methotrexate (MTX)

Solvent-DMSO (Dimethyl sulphoxide)

Results and discussion

MTT assay of various test compounds against Ehrlich ascites carcinoma cell line (Table No 1) proved that compound Cu-2 and IE-i, were significantly effective. Hence, these two compounds were selected for formulation into liposomal drug delivery system further.

It was found that 7-(2′,3′-epoxypropoxy)-4-methylcoumarin **(Cu-2)** exhibited favorable ED_{50} and hence they were subjected to cytotoxic evaluation by MTT assay using methotrexate as a standard anticancer drug.

All of them registered significant cytotoxic activity. However, **Cu-2** was found to be significantly more active and toxicity study[55,56,57,58] has been well established. Therefore, **Cu-2** was selected for formulation studies using liposomes as drug carriers. In order to compare the efficiency of the formulation of **Cu-2**, standard anti-cancer drug methotrexate was also selected for formulation in a liposomal form.

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CHAPTER IV

PART II

1.1 LIPOSOMES AS DRUG RELEASE CARRIERS [1,2,3]

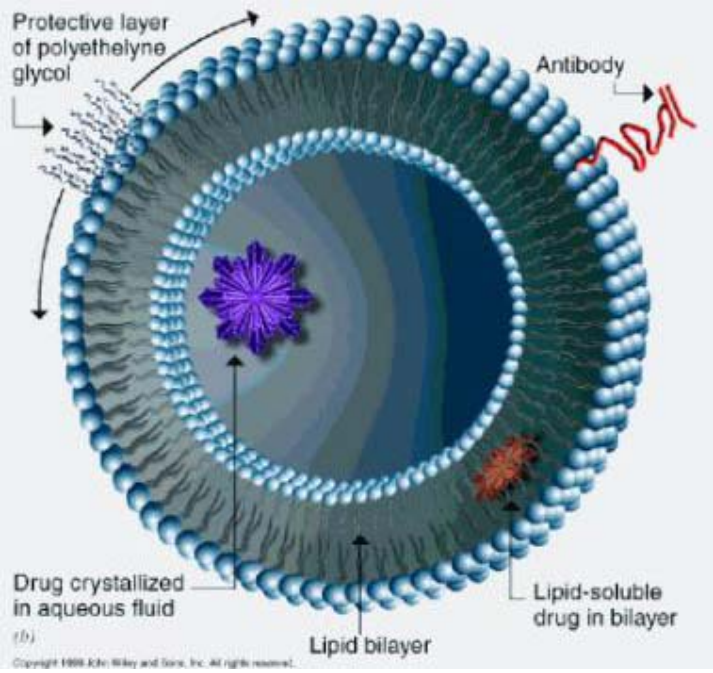
An ideal drug delivery system delivers the drug at a rate dictated by the need of the body over the period of treatment and it channels the active entity solely to the site of action. At present no available drug delivery system can achieve all these goals.

Liposomes were discovered in the early 1960's by Bangham and colleagues and subsequently become the most extensively expected drug delivery system. Liposomes have covered predominantly medical, albeit some non–medical areas like bioreactors, catalysts, cosmetics and ecology. However, their predominance in drug delivery and targeting has enabled them to be used as therapeutic tool in fields like tumor targeting, gene and anticancer therapy, genetic vaccination, immuno modulation, lung therapeutics, fungal infections, and skin care and topical cosmetic products.

Liposomes are concentric bilayered vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids.

Drugs incorporated in liposome are not inactivated under physiological conditions and do not cause unfavorable side effects as well.

Liposomes are microscopic fluid–filled pouch whose walls are made of layers of phospholipids identical to the phospholipids that make up cell membranes. It was found that phospholipids combined with water immediately formed a sphere because one end of each molecule is water soluble, while the opposite end is water insoluble. Water-soluble medications added to the water were trapped inside the aggregation of the hydrophobic ends; fat-soluble medications were incorporated into the phospholipid layer.



Liposomes attach to cellular membranes, releasing their contents into the cell. Sometimes they are taken up by the cell and their phospholipids are incorporated into the cell membrane while the drug trapped inside is released. In case of phagocytic cells the liposomes are taken up, the phospholipids walls are acted upon by organelles called lysosomes, and the medication is released.

The encapsulation of drugs with liposomes alters drug pharmacokinetics, and this may be exploited to achieve targeted therapies. Alteration of the liposome surface is necessary in order to optimize liposomal drug targeting.

Liposomes can be administered intravenously to carry lipid – soluble drug to improve drug stability to target organs with fenestrated capillaries, to achieve an intravascular slow drug release system and to reduce drug levels in certain organs sensitive to toxicity.

Liposomes are inherently targeted to liver and spleen When the drug encapsulated liposomes are introduced into the bloodstream, the body's defense systems consider them as foreign particles and an antigenic response is produced. As a result the antigenic particles, the opsonins, adhere to the liposomes. Once the opsonins attach to the liposomes, the circulating monocytes (macrophages) engulf the liposomes and carry them to the liver, spleen for detoxification. This is called as natural or passive targeting of liposomes to the organs like liver and spleen. This property of liposome is exploited for treating infections of liver because of the selective distribution of liposomes to those sites. When a drug encapsulated liposomes engulfed by macrophages undergo detoxification, the encapsulated drug is released slowly.

Liposomes stay within circulation and can only leave at certain sites where the capillary is fenestrate (e.g: - liver, thus enabling targeting to the liver parenchyma cell) or alternatively where the endothelium is disturbed by necrosis or inflammation. Liposomes were actively taken up by phagocytes of Reticulo endothelial system

(RES) including tissue macrophages (mainly located in liver and spleen) and circulating blood monocytes.

1.2 CLASSIFICATION OF LIPOSOMES [3]

Liposomes are classified based on its pharmaceutical and therapeutical aspects, which include structural parameters, method of preparation, and components and its applications. **Table-1**

Based on structural parameters	
TYPE	SPECIFICATIONS
MLV	Multilamellar large vesicles >0.5μm
OLV	Oligolamellar vesicles 0.1-1μm
UV	Unilamellar vesicles (all size range)
SUV	Small unilamellar vesicles 20-100 nm
MUV	Medium sized unilamellar vesicles
LUV	Large unilamellar vesicles >100nm
GUV	Giant unilamellar vesicles >1μm
MV	Multivesicular vesicles >1μm
Based on method of liposome preparation	
REV	Single or oligo lamellar vesicles made by reverse-phase evaporation method.
MLV-REV	Multilamellar vesicles made by reverse phase evaporation method
SPLV	Stable plurilamellar vesicles
FAT MLV	Frozen and thawed MLV
VET	Vesicles prepared by extrusion technique
DRV	Dehydration – rehydration method

1.3 CHARACTERIZATION OF LIPOSOMES [3]

Liposomal formulations after their formulation are characterized to ensure their predictable *in vitro* and *in vivo* performances. The characterization parameters could be classified into three broad categories which include physical, chemical and biological parameters. The following table shows the liposome characterization with their quality control assays.

Table-2

Physical characterization

Characterization parameters	**Analytical methods / instrumentation**
Vesicle shape and surface morphology	Transmission electron microscopy, freeze-fracture electron microscopy.
Vesicle size and size distribution	
Submicron range	Dynamic light scattering, .transmission electron microscopy, zetasizer.
Micron range	Transmission electron microscopy, freeze- fracture electron microscopy, photon correlation spectroscopy, laser light scattering, gel permeation and gel exclusion
Surface charge	Free- flow electrophoresis
Electrical surface potential and surface pH	Zetapotential measurements and pH sensitive probes.
	Small angle X- ray scattering, freeze-fracture electron microscopy, p- NMR
Lamellarity	Freeze–fracture electron microscopy, differential scanning calorimetry.
Phase behaviour	Minicolumn centrifugation, gel exclusion,
Percent capture/ percent of free drug	lon-exthange chromatography, protamine aggregation , radiolabelling,
Drug release	Diffusion cell / dialysis.

Chemical characterization	
Characterization parameters	**Analytical methods / instrumentation**
Phospholipid concentration	Lipid phosphorus content using Barlett assay / stewart assay, HPLC.
Cholesterol concentration	Cholesterol oxidase assay and HPLC.
Drug concentration	Appropriate methods given in the monograph for individual drug(s)
Phospholipid Hydrolysis	HPLC and TLC and fatty acid concentration.
Cholesterol auto-oxidation	HPLC & TLC.
Anti-oxidant degradation	HPLC & TLC.
pH	pH meter.
Osmolarity	Osmometer.
Biological characterization	
Sterility	Aerobic or anaerobic cultures
Pyrogenicity	Rabbit fever response test or limulus Amebocyte Lysate (LAL) test.
Animal toxicity	Monitoring survival rates, histology and pathology.

1.4 METHODS OF LIPOSOME PREPARATION [3,4]

I. Preparation of MLVs

A. Thin film hydration using hand shaking (MLVs) and non-shaking methods (LUVs)

The lipids are casted as stacks of film from their organic solution using flash rotary evaporator under reduced pressure (or by hand shaking). Upon hydration using buffer or drug solution the lipids swell and peel off from the round bottom flask and forming MLVs. The large unilamellar vesicles (LUVs) are produced by non-shaken methods with encapsulation efficiency as high as 30%.

B. Pro-liposomes

The lipid mixture is dried over a finely divided particulate support, such as powdered sodium chloride, or Sorbitol or other polysaccharides. These dried lipid coated particulates are pro-liposomes. Pro–liposomes form dispersion of MLVs on adding water into them, where support is rapidly dissolved and lipid film hydrates to form MLVs.

C. MLVs formed by freeze drying SUV dispersions

A simple method for preparing MLVs with high entrapment efficiency was developed by ohsawa et al (*1984*) and kirbygregoriadis (*1984*). The aqueous phase containing the molecules to be encapsulated are mixed with preformed suspension of SUVs and the mixture freeze dried by conventional means. Large MLVs are formed when the dry lipid is rehydrated usually with small volume of distilled water.

II. Preparation of Suvs

A. Sonicated Vesicles

The exposure of MLVs to ultrasonic irradiation and still remains the method most widely used for producing small vesicles.The MLV preparation is subsequently sonicated using either probe or bath ultrasonic disintegrators. Probe tip sonicators causing lipid degradation and also tend to release titanium particles into the liposome dispersion.

Sonication of an MLV dispersion is accomplished by placing a test tube containing the dispersion in a bath sonicator (or placing the tip of the sonicator in a probe sonicator) and sonicating for 5-10 min (1, 00, 000 g)

B. Micro emulsification liposomes (MEL)

"Micro fluidizer" is used to prepare small MLVs from concentrated lipid dispersion .The lipids can be introduced into the fluidizer, either as a dispersion of large MLVs, or as slurry of unhydrated lipid in an organic medium. The fluid collected can be recycled through the pump and interaction chamber until vesicles of the spherical dimension are obtained. After a single pass the size of vesicles is reduced to a size 0.1 & 0.2 μm in diameter.

III. Preparation of uni- or oligo – lamellar vesicles

One of the first & still very useful method developed is extrusion of preformed large liposomes in a French press under very high pressure [9] .This technique yields rather uni- or oligo – lamellar liposomes of intermediate size (30-80 nm in diameter depending on the applied pressure). These liposomes are more stable as compared to sonicated vesicles.

IV Solvent dispersion methods

A. Ethanol injection

This method has been developed for the preparation of SUVs without sonication. An ethanol solution of lipids is injected rapidly through a fine needle into an excess of saline or other aqueous medium. This procedure yields a high proportion of SUVs (~ 25nm)

Advantages

1. This method is extremely simple and has low risk of degradation of sensitive lipids.
2. The vesicles of 100nm size may obtained by little modification in this method.

Disadvantages:

1. Limitation of the solubility of lipids in ethanol and volume of ethanol that can be introduced into the medium (7.5% v/v max) limits quantity of lipids dispersed. So that resulting liposomal dispersion gets diluted.
2. Difficulty to remove residual ethanol from phospholipid membranes.

Ether injection

A method introduced by Deamer and Bangham in 1976 provides a means of making SUVs by slowly introducing a solution of lipids dissolved in diethyl ether or ethanol / methanol mixtures into warm water. Typically the lipid mixture is injected into an aqueous solution of the material to be encapsulated using a syringe type of pump at 55-65ºc or under reduced pressure. Subsequent removal of residual ether under vacuum leads to the formation of single layered vesicles. Depending upon the condition used, the resulting diameter of vesicles range from 50-200nm.

V. Preparation of large unilamellar vesicles

A. Detergent depletion methods of passive loading

A different approach to produce liposome is dependent on the removal of the detergent molecule from dispersions of phospholipids/ detergent mixed micelles. As the detergent is removed, the micelles progressively richer in phospholipids and finally coalesces to form closed single bilayer vesicles. Three methods of detergent removal are appropriate for this purpose.

1. Dialysis
2. Column chromatography.
3. Biobeads.

1. Dialysis

Kagawa and Racker (*1971*) were the first to introduce dialysis method for lipid vesicle preparation. Although these authors were interested primarily in reconstituting biological membrane solubilized with detergents, their method is applicable to the liposomes as well.

Detergents commonly used for this purpose exhibit a reasonable high CMC or the order of 10-20 nm. In order to facilitate their removal the bile salts like sodium cholate, sodium deoxycholate & synthetic detergents such as octylglucoside can be included. The treatment of egg PC with a 2:1 molar ratio of sodium cholate followed by dialysis results in the formation of vesicles in the 100 nm diameter range within a few hours.

2. Column chromatography

Phospholipids in the form of either sonicated vesicles or as a dry film, at a molar ratio of 2:1 with deoxycholate form unilamellar vesicles of 100nm on removal of deoxycholate by column chromatography. This could be achieved by passing the dispersion over a sephadex G-25 column pre–saturated with constitutive lipids and pre-equilibrated with hydrating buffer.

3. Detergent adsorption using Bio-beads

Levy et al., 1990 developed this method. Detergent (non–ionic) /Phospholipid mixtures can form large LUVs upon removal of non-ionic detergent like Triton X -100 using appropriate adsorbents for the detergents. The ability of Bio–beads SM -2, to adsorb Triton X-100 selectively and rapidly, makes them a suitable candidate for LUV preparation by detergent solubilization method. On hydrating the casted lipid film with 0.5-1.0% Triton X-100, washed bio- beads are added to the dispersion and locked for about 2h at 4±1ºC.

B. Reverse phase evaporation technique (REV)

LUVs can also be prepared by forming water-in-oil emulsions of phospholipids and buffer in excess of organic phase followed by removal of the organic phase under reduced pressure. The two phases are emulsified by bath sonication. Removal of the organic solvent in a rotary evaporator causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent results in the collapse of the gel into a smooth suspension of LUVs. The vesicles formed are unilamellar and have an average diameter of 0.5 μm. The encapsulation percentage is found to be nearly 50%.

Advantages

1. This method is used to encapsulate both small and large molecules.
2. Biologically active macromolecule such as RNA and various enzymes can be encapsulated without loss of activity.

Limitations

In this method, exposure of the material to be encapsulated to organic solvents and mechanical agitation can lead to the denaturation of some proteins or breakage of DNA strands.

a. Advantages and Disadvantages of Liposomes [3,5]

Advantages of Liposomes

2. Liposomes are chiefly composed of natural or synthetic phospholipids especially lecithins hence can be metabolized in vivo and generally non-toxic and non-antigenic.

3. Entrapment of drug leads to different pharmacokinetics. Hence can result in better therapeutic index and enhanced cellular uptake.

4. Liposomes are biodegradable and biocompatible drug package.

5. Due to protection of drug from first pass metabolism associated with various routes, dose of drug can be reduced.

6. The liposomes can be designed to interact with specific tissue, improving the drug selectivity and decreasing toxicity.

7. Liposomes function as superior immunological adjuvant by enhancing the immune response to encapsulated antigens.

8. Liposomes cross biomembranes and facilitate the transport of drugs through normally impermeable barriers. In particular, liposome facilitates the intracellular

penetration of encapsulated compounds Liposome increases the efficacy and therapeutic index .

9. Liposomes produce site avoidance effect. The most promising examples are liposomal Doxorubicin (reduced cardio toxicity) and liposomal Amphotericin B (reduced nephrotoxicity).

10. Liposomes improving the pharmacokinetic effects by reducing the rate of elimination and increasing the circulation life times.

11. Reduction in toxicity of the encapsulated agent.

Disadvantages of Liposomes

1. Formulation stability is less.
2. The materials used in the formulation of liposomes are expensive.
3. Variable purity of natural phospholipids.

1.6 Applications of Liposomes

1. Liposomes can be used as carrier for both water-soluble and lipid-soluble drugs. Substances entrapped in liposomes include enzymes, glycolipids, immunoglobulins, and monoclonal antibodies. Drugs entrapped most notably are anti tumor, antiarrithmic and antiparasitic, antigens, biological response modifiers, antifungal agents, chelating agents, markers, antibiotics, ions, vaccines, and chromosomes.
2. Water soluble drugs with low molecular weight given intravenously in liposomes maintain in the blood cells for a longer period of time than obtained using the free drugs as the rate of excretion of entrapped drug is decreased.
3. Another use is to form intra muscular depots of water-soluble compounds.

4. Liposomes containing coagulation factor VII and heparin have been used orally for the treatment of hemophilia. Some oral applications of liposomes can pass through the doubt whether liposomes can pass through the gut mucosa into the blood stream still persists.
5. Apart from use as drug carries, perhaps the most promising immunological property of liposomes is their action as adjuvants.
6. Liposomes markers have been used in radiology to improve the image quality.
7. In cell physiology liposomes find its application in liposomes reconstitution various techniques to intercalate proteins to liposomes have been reviewed by Racker.
8. Liposomes based drug delivery patch consisting of progesterone involved agarose matrix was observed to impose zero order kinetics.
9. Liposomes were used in gene delivery in the field of gene and antisense therapy and genetic (DNA) vaccination.
10. Liposomes are most widely used in tumour therapy. They act as a carrier of small cytotoxic molecule and as a vehicle for macromolecules like cytokines or genes.
11. Liposomes are also used for antimicrobial, antifungal, (lung therapeutics) and antiviral (anti-HIV) therapy.
12. Liposomes are potential nasal drug delivery system [2]

CHAPTER - V

2. LITERATURE REVIEW

2.1 LIPOSOME AS A CARRIER IN CANCER CHEMOTHERAPY

Nallamothu et al[6] developed an efficient Tumor Vasculature Targeted Liposome Delivery System for Combretastatin A4. Liposomes were prepared by the lipid film hydration and extrusion process and characterized in terms of size, lamellarity, ligand density, drug loading and leakage properties. Electron microscopic observations revealed the presence of small unilamellar liposomes of ~ 120 nm in diameter. HPLC determination indicated that more than 99% of RGD peptides were reacted with maleimide groups on the liposome surface of 3 mg/ml. In the *invitro* cell culture studies, targeted liposomes showed significantly higher binding to their target cells than nontargetted liposomes.

Distefano et al[7] prepared Maleic – and Fumaric- diamides of (0, 0- diacetyl) –L-Dopa- methylester as anti-Parkinson prodrugs in liposomal formulation. The new compounds were characterized by evaluating solubility, chemical stability, apparent partition coefficient and comparing neostriatum dopamine (DA) levels in freely moving rats after I.P administration of prodrugs [(+)-4, (+)-5] with prodrugs in liposomal formulation [(+)-4Lip, (+)-5lip]. The results suggest that cis dimeric prodrug (+)-4 and (+)-4lip can improve the release of DA in rat brain.

Cereda et al[8] developed Liposomal formulations of Prilocaine, Lidocaine and Mepivacaine for prolonged analgesic duration. Large unilamellar vesicles were prepared by extrusion (400 nm), at pH 7.4. The encapsulated formulations induced total anesthetic effect (35.3%, 26.1%, and 57.1%) the liposomes provided effective drug – delivery systems for intermediate – duration local anesthetics.

Siripon et al[9] investigated antitumor activity of Liposomal Naphthoquinone esters isolated from Thai medicinal plant *Rhinacanthus nastus* Kurz. Liposomal formulation of rhinacanthus-C,-N & -Q showed antiproliferative activity.

Linfyun et al[10] studied the effects of Cholesterol component on molecular interactions between Paclitaxel and Phospholipid within the lipid monolayer at the air–water interface. Langmuir film technique, atomic force microscopy & FTIR are employed for a systematic investigation. The existence of cholesterol in DPPC monolayer can restrict the drug penetration into the monolayer.

Johnston et al[11] studied therapeutically optimized rates of drug release by varying the drug-to-lipid ratio in Liposomal Vincristin formulations. In this work, the effects of very high D/L ratios on Vincristin release rates were investigated, and the antitumor efficacy was characterized in human xenograft tumor model. It was found that the anticancer activity of the Liposomal Vincristin formulations increased as D/L ratio increased from 0.025 to 0.1 (w/w). In Liposomal Vincristine formulation, the antitumor activity increased as D/L ratio increased from 0.025 to 0.1 (w/w) (T1/2 =6.1-15.6 h) but decreased at higher D/L ratios (D/L=0.6) (T1/2=117h)

Shah et al[12] developed the Proliposomes containing Cyclosporine A (CSA). The proliposomes were prepared by spraying a solution of CSA, Egg lecithin and Cremophor EL in methanol – choloroform mixture onto directly compressible lactose into a rotary evaporator. The liposomes exhibited good entrapment of about 99%. The results of bioavailability study indicated that the difference in mean drug concentration of free drug and liposomes was found to be statistically significant ($P<0.05$, P value is 0.032). The absorption constant was much greater (10.26 $h^{-1}$and Vd was found to be less [7629.88 ml / kg]

Afouna et al[13] developed Demeclocycline (DEM) Liposomal formulations and assessment of their intraocular pressure lowering effects. Different Liposomal formulations of DEM were prepared and characterized for their drug entrapment.

Fang et al[14] studied the effect of liposome encapsulation of Tea catechins on their accumulation in Basal Cell Carcinomas (BCCs). Liposomes containing Egg Phosphatidyl choline, Cholesterol, or anionic surfactant in presence of 15% ethanol were prepared. Liposomes containing (-) – Epigallocatechin gallate (EGCG) were injected into BCCs, malenomas & colon tumors. EGCG encapsulated in liposomes with Deoxycholic Acid (DA) and ethanol increased drug deposition by 20-fold as compared to free form.

Cintia et al[15] reported the development and *in vivo* evaluation of a Liposomal Prilocaine with Egg Phosphatidyl choline , Cholesterol and α– Tocopherol (4:3:0.07 molar ratio). The size of the Liposomes was measured by laser light scattering analysis, showed vesicle population with ca 400 nm. The ESR results which characterized the Prilocaine – Liposome interaction, showed a decrease in the

orientation of the phospholipids molecules into the Liposomes. A prolongation of anesthetic effect was produced by Liposomal Prilocaine in comparison to plain Prilocaine so that the encapsulation of Prilocaine in Liposomes facilitates the controlled release of Prilocaine.

Mari et al[16] determined the incorporation capacity of Camptothecin in Liposomes. CPT-saturated Liposomes were prepared by dispersing freeze-dried blends of lipids and drug in phosphate buffer, and subsequently probe sonicated. In the liposomes made of neutral and anionic phospholipids, the solubility of CPT in the buffer was improved by a factor of 10 (from ~ 2.7 to 15-50 μg/ml) as compared with buffer, cationic liposomes containing 1,2 – dioleoyl-3-trimethyl-ammonium –propane (DOTAP) , a maximum CPT-solubilization of ~ 100 – fold. Increasing DOTAP fractions within Egg-Phosphatidylcholine (EPC)/ DOTAP liposomes reached a CPT-incorporation plateau at ~ 20 mol% DOTAP.

Bhamra et al[17] studied activity, pharmacokinetics and tissue distribution of TLC ELL -12. It is a liposomal formulation of the novel antineoplastic compound 1-0-octadecyl -2-0-methyl-Sn-glycero-3-phosphocholine (L-ET-18-OCH (3)). Growth – inhibitory activity of L-ET-18-OCH (3) and TLC ELL-12 against methylnitrosourea (MNU)-induced tumors grown *in vitro* was evaluated. Concentration –time profiles for selected tissues indicated rapid distribution of L-ET-18 – OCH (3) from the circulation into tissues with highest concentrations in spleen, liver, lungs, kidneys and GIT. When i.v. administered, L-ET-18 –OCH (3) from ELL-12 is well distributed and slowly eliminated by metabolism in tissues.

Vyas et al[18] designed liposomal aerosols for improved delivery of Rifampicin to alveolar macrophages. Neutral Liposomes were prepared by cast film method with PC: CHOL of 7:3 molar ratios. Pressurized packed systems based on preformed Liposomal formulation in chlorofluorocarbon aerosol propellants were prepared. *In vitro* airway penetration efficiency of the Lisposomal aerosols was recorded 1.5-1.8 times higher as compared to plain drug solution-based aerosol and higher lung drug concentration was recorded in ligand anchored Liposomal aerosols. Percent viability of *Mycobacterium smegmatis* inside macrophages was 7-11% for ligand-anchored Liposomal aerosol, while it was 45.7 & 31.6% for plain drug and plain neutral Liposomal aerosol.

Jain et al[19] formulated the liposome formulation of NSC-639829 using Halothane as a solvent. Liposomes were prepared by thin-film hydration method using various solvents like chloroform, ether or halothane. The desired particle-size (200-300 nm) was obtained by probe sonication. The drug concentration was determined by HPLC assay. Liposomes with similar morphology, particle size, incorporation efficiency, and stability were obtained with halothane, chloroform, and ether. Halothane provides additional ease in formulation because of its higher volatility and safety.

Dodov et al[20] prepared 5-Fluorouracil in topical liposome gels for anticancer treatment and drug release properties *in vitro* have been evaluated. Liposomes were prepared by the film hydration method with lipid: cholesterol ratio of 9:1, 12:1, 15:1. Liposome formulation prepared with 15:1 lipid: cholesterol ratio showed the highest encapsulation efficiency. Liposome gels were prepared by incorporation of liposomes

into a structured vehicle (1% mm, chitosan gel base) and also hydrogels were prepared.

Zhang et al[21] Developed and characterized a novel liposome based formulation of SN-38. The formulation contains liposomes of uniform size distribution (<200nm). Drug entrapment efficiency is >95%. Long term stability studies indicate that the lyophilized LE-SN-38 is physically and chemically stable for at least 6 months at 2-8°C

Shulman et al[22] characterized sterically stabilized Cisplatin liposomes by nuclear magnetic resonance. The liposomal Cisplatin (STEALTH cisplatin, formerly known as SPI-77) shows excellent stability in plasma and has a longer circulation time, greater efficacy and lower toxicity than much free Cisplatin. Since NMR detects only the soluble Cisplatin in the Liposomes and not the insoluble drug, containing NMR and atomic absorption data enables one to determine how much of the encapsulated drug is soluble in the intraliposomal aqueous phase

.

Hirabayashi et al[23] developed a complex of polyinosinic- polycytidylic acid and cationic liposome (LIC) inhibited the growth of many tumor cells lines. The IC_{50} values of LIC against the tumor cells ranged from 0.1 to 1000 ng/ml. The mechanism of the antiproliferative effect of LIC against malignant cells was the induction of apoptosis. LIC induced the fragmentation of nuclear DNA and the degradation of rRNA in tumor cells. LIC reduced the incidence and the size of metastatic liver-cancer tumors. Both the fragmentation and the inhibition of cancer cell growth were suppressed by a nuclease inhibitor. In contrast, laspase inhibitors did not affect the antiproliferative activity of LIC. Histochemical analysis revealed that the km12-HX

cells in the tumor nodules were undergoing apoptosis which induced the apoptosis of tumor cells *in vivo.*

Maswadeh et a[24] developed encapsulation of Vinblastine into new liposome formulations prepared from Triticum lipids and its activity against human leukemic cell lines. Two Liposomal formulations (I&II), with encapsulated Vinblastine, and formulation III (empty liposomes) have been prepared by thin film hydration method. For the cytotoxic /cytostatic activity, the parameters GI (50), TGI, LC (50) were estimated against nine human leukemic cell lines. The results show that formulation III, exhibited a growth inhibiting activity. Formulation II showed mean of LC (50) at 124.6 nm, mean of TGI at 71.6 nm & mean of GJ (50) at 30.8nm.

Katrgadda et al[25] examined the encapsulation, stability, and *in vitro* release characteristics of Liposomal of Stavudine (D4T). Liposomes were prepared by reverse phase evaporation method and encapsulation was studied by adding different amounts of cholesterol. Stability studies were performed by storing at 4, 25, and 37°C for 12 weeks. Encapsulation was found to be maximum (48%) in DSPC liposomes containing equimolar amounts of cholesterol. *Invitro* release was found to be biphasic. The release was inhibited in the presence of charge inducer (30%). Particle size distribution ranged from 0.6μm to 1.4μm and was polydisperse and liposomes were stable for a period of 4 weeks.

Lacy et al[26] developed studies on coumarin coumarin related compounds to determine their therapuetic role in the treatment of cancer. A recent study has shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. Esculetin inhibits growth and cell cycle progression by

inducing arrest of the G1 phase in HL-60 leukaemia cells, resulting from the inhibition of retinoblastoma protein phosphorylation. Genistein is a well-known isoflavone and is a tyrosine kinase inhibitor. These studies suggest that both genistein and esculetin exerted the most potent inhibitory effect on cell growth in comparison to the other two compounds.

Stephanie et al[27] *In Vitro* Kinetics of Coumarin 3, 4-Epoxidation: application to species differences in toxicity and carcinogenicity. In human liver microsomes (n = 12), the kinetics of *o*-HPA formation were best described by a single enzyme model, with the K_m for *o*-HPA formation ranging from 1320–7420 μM. In the most active human sample, the intrinsic clearance of coumarin via the 3, 4-epoxidation pathway was 1/9 and 1/38 that of the rat and mouse, respectively. The *in vitro* kinetics of *o*-HPA formation, and in particular, the large quantities of coumarin required for *o*-HPA production in human liver microsomes, strongly suggest that humans are unlikely to produce toxicologically relevant concentrations of this metabolite following low level coumarin exposures.

Vyas et al [28] The present study was aimed at preparation, characterization, and performance evaluation of rifampicin-loaded aerosolized liposomes for their selective presentation to alveolar macrophages. These results suggest that the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high-drug concentration in lung with high population of alveolar macrophages but also maintain the same over prolonged period of time.

Moreira et al [29] Characterized the interaction of antagonist G (H-Arg-D-Trp-Leu-Met-NH_2). The cellular association of antagonist G-targeted (radio labeled)

liposomes was 20-30-fold higher than that of non-targeted liposomes. There data suggest that a maximum of 12,000 antagonist G-targeted liposomes were internalized/cell during 1-h incubation at 37°C. confocal microscopy, experiments using pyranine-containing liposomes further confirmed that receptor-mediated endocytosis occurred, specifically in the case of targeted liposomes. The improved cellular association of antagonist G-targeted liposomes, relative to non- targeted liposomes, resulted in an enhanced nuclear delivery (evaluated by fluorimetry) and cytotoxicity of encapsulated doxorubincin for incubation periods as short as 2 h.

Batavia et al[31] may be used to estimate the maximum incorporation (<5 mole% drug) of beclomethasone dipropionate (BDP) in dipalmitoylphosphatidycholine (DPPC) multilamellar liposomes. Differential interference contrast (DIC) microscopy and cross-polarisation microscopy have been used to measure the concentration at which BDP crystals become apparent in BDP containing liposome preparations. An alternative method is described whereby the unentrapped solid drug is separated from the liposomes via suspension in D_2O folled by centrifugation and HPLC analysis.

Joshi et a[32] liposomes composed of saturated egg phospatidyl choline (EPC) and cholesterol (CHOL) were prepare by lipid film hydration and sonicated to have the desired size (<5 μml). Charaterization of liposomal dispersion was done for size, lamellarity, entrapped volume and oxidation index. Process optimization revealed that a vacuum, 20 in presumption hydration, 60 min; post sonication hydration, 2 h and purification by dialysis gave maximum encapsulation efficiency. It may be concluded that an optimal bulk and mass ratio of sucrose, relative to the dose of liposomes is

necessary for effective cryoprotection. In this investigation, DPI of liposomal KF was successfully prepared and delivered to the required site in the lungs.

Kirabayashi et a[33] A complex of polyinosonic-polycyctidylic acid [poly(I)-poly©] and cationic liposome (LIC) inhibited the growth tumor cell lines at low concentration *in vitro*, but poly(I)-poly(C) alone had no such antiproliferative effect. The IC_{50} values of LIC against the tumor cells ranged from 0.1 to 1000 ng/ml. LIC had strong cytotoxic effects on malignant cells of epithelial and fibroblastic origin from various tissues and was also effective against adriamycin-resistant tumor cells. LIC did not significantly affect the growth of lymphoma cells, leukemia cells, normal diploid fibroblasts, or primary liver cells at concentration up to 10 µg/ml.

Berger et al[34] liposomes were prepared by stepwise extrusion through 5, 1, 0.4, 0.2, 0.1 and 0.05 µm pore sizes using two different filter-extruders, the continuous high pressure device Dispex Maximator *CE) or alteranatively the discontinuous Avestin LiposoFast (DE. The liposome dispersions obtained were compared in terms of particle, size, lamellarity and encapsulation efficiency of calcein.

Dass[35] Cationic liposomes are traditionally used for delivering macromolecules such as nucleic acids to mammalian and plant cells. The study also evaluated the utility of a colorimetric method for quantification of cationic liposomes.

Goyal et al[36] liposomes have been widely investigated since 1970 as drug carriers for improving the delivery of therapeutic agents to specific sites in the body.

Kozubek et al[37] Commercial liposome-based drugs have already been discovered, registered and introduced with great success on the pharmaceutical market. However, further studies, focusing on the elaboration of more efficient and stable amphiphile-based vesicular (or non-viral) drug carriers are still under investigation.

Park et al[38] Drug delivery system can in principle provide enhanced efficacy and reduced toxicity for anticancer agents. Long circulating macromolecule carriers such as liposomes can exploit the enhanced permeability and retention effect for preferential extravasation from tumor vessels.

Patil et al[39] investigated the use of novel lipoplexes composed of physiological components for plasmid DNA delivery into mammalian cells *in vitro*.

Williams et al[40] determined whether liposomal preparations containing a phospholipids conjugate of methotrexate and dimyristoylphosphatidylethanolamine (MTX-γ-DMPE) incorporated within their lipid membranes are effective in suppressing established joint inflammation in a monoarticular model of arthritis in the rat.

Gaber et al[41] Folate receptor as Trojan horse to deliver folate-targeted liposomes bearing diverse cargo represents a novel therapeutic strategy to target folate receptor-expressing cells.

Huwyler et al[42] Immunoliposomes (antibody-directed liposomes) were used to delivery of the antineoplastic agent daunomycin to the rat brain.

Mayer et al[43] In there study Murine fibrosarcoma tumors arising from subcutaneous inoculation of FSa-N cells exhibit 4-fold higher tumor-associated macrophage(TAM) levels than those from the FSa-R line. These solid tunors were used to assess the role of TAMs in the accumulation of liposomal anticancer drugs. Two liposomal formulations of doxorubicin were investigated; a conventional formulation composed of distearoylphosphatidylcholine (DSPC) and cholesterol and a sterically stabilized lioposomal formulation composed of DSPC/cholesterol/poly (ethylene glycol) – modified distearoylphosphatidy thanolamine (PEG-PE). Circulating concentrations of PEG-PE containing liposomes 24 h after i.v. administration were 3-fold greater than hose observed after administration of conventional liposomes.

Drummond et al[44] The effective delivery of a drug in its active form to solid tumors. Most small-molecule chemotherapeutic agents have a large volume of distribution on i.v. administration (Speth et al., 1988; Chabner and longo, 1996). The result of this is often a narrow therapeutic index due to a high level of toxicity in healthy tissues. Through encapsulation of drugs in a macromolecular carrier, such as a liposome, the volume of distribution is significantly reduced and the concentration of drug in the tumor is increased.

Lijeoma et al[45] The toxic side effects associated with the administration of anticancer drugs makes these drugs ideal candidates for drug targeting. Anticancer agents have been encapsulated within liposomes in an effort to target such agents to tumours. The use of liposomes as anticancer drug delivery systems was originally hampered by the realization that liposomes are rapidly cleared from the circulation and largely taken up by the liver macrophages. Liposomes used as Vaccine adjuvants,

anti-infective agents. Active targeting of liposomes – Immunoliposomes, Ligand bearing liposomes.

Zeisberger et al[46] Tumour-Associated Macrophages, TAMs, play a pivotal role in tumour growth and metastasis by promoting tumour angiogenesis. Treatment with clodronate encapsulated in liposomes (clodrolip) efficiently depleted these phagocytic cells in the murine F9 teratocarcinoma and human A673 rhabdomyosarcoma mouse tumour models resulting in significant inhibition of tumour growth ranging from 75 to > 92%, depending on therapy and schedule.

Buchholz et al[47] optimized and simplify photodynamic therapy using a new liposomal formulation of the photosensitizer meta-(tertrahydroxyphenyl)chlorine [m-THPC(Fosan); liposomal m-THPC(Fospeg)] and to reduce systematic reactions to the photosensitizer.

Liu et al[48] Topotecan (TPT), a topoisomerase I inhibitor, is presently undergoing clinical evaluation worldwide. Previous entrapping TPT within multi-lamellar vesicle liposome can stabilize the lactone moiety, which is structurally in however, low drug: lipid ratios due to the amphipathic character and small entrapment volume in the unilamellar development of pharmaceutically acceptable liposomal formulation.

Zerouga et al[49] Synthesized and characterization of a lipophilic phosphatidylcholine containg the omega-3 fatty acid and the cytotoxic drug methotrexate (MTX). This is novel phospholipids combines the fatty acid's and the drug's anticancer amenable to a liposome bilayer for sale, simultaneous delivery.

Desjardins et al[50] Prolonged tumor exposure to topoisomerase I inhibitors has been correlated to enhance the efficacy of water-soluble camptothecin analog were formulated as a liposomal drug.

Schwonzen et al[51] The combination of paclitaxel and doxorubicin or epirubicin is highly active against metastatic breast cancer congesive heart failure. liposome-encapsulated doxorubicin is a new formulation of doxorubixin with no toxicity.

Kanter et al[52] A preclinical toxicology study of liposome encapsulated vincristine, free vincristine and empty liposomes and dogs by single and multiple (daily for 5 days) intravenous injection. Single and multiple dose intravenous mice showed the encapsulated form of vincristine to be less toxic than free vincristine.

CHAPTER - VI

3.0 AIM OF WORK

Coumarin epoxide (Cu-2) is a synthetic compound which may be useful in the treatment of certain neoplastic disorders. Cu-2 is highly toxic drug. Since LD_{50} value of 40 mg produced toxicity within one week in mice. For drugs with high toxicity, a properly designed targeted release may be advantageous.

Liposomal formulation is prepared to provide selective passive targeting to tumor tissues, to increase the stability via encapsulation, to reduce the toxicity of the encapsulated agent and to improve the pharmacokinetic effects[3]. There are certain drugs with high toxicity available in the liposomal formulation (E.g. Doxorubicin, Doxil, Evacet, Kozubek A.). Hence formulation of Coumarin epoxide in liposome form will be useful.

The aim of the present study was to develop liposomal Coumarin epoxide and compare its effect with liposomal methotrexate.

4.0 MATERIALS AND METHODS

4.1 List of the materials used

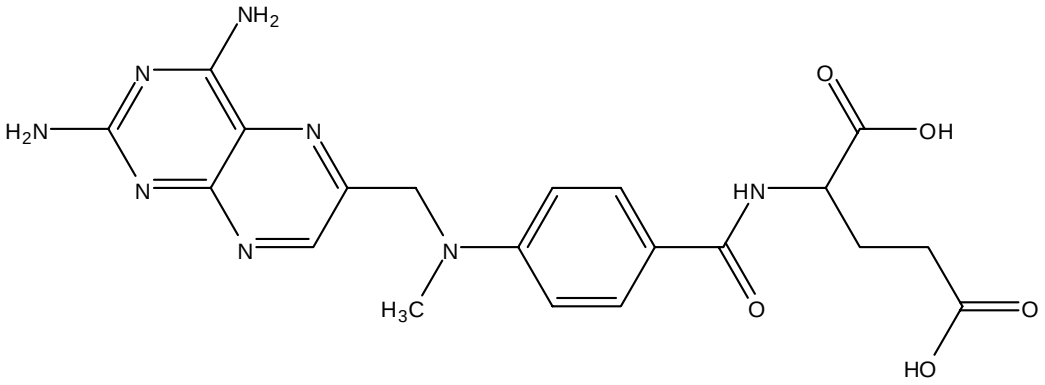
Materials	Name of the Supplier
Methotrexate	United Technologies Limited – UJETI-Halol.
Coumarin Epoxide(Cu-2)	Synthesized compounds
Soy phosphatidyl choline (Lipoid S 100)	Sigma Chemicals
Cholesterol	Loba Chemicals
Chloroform AR	Qualigen fine chem.
Methanol AR	SD fine Chem.
Stearylamine	Sigma Aldrich
Disodium hydrogen phosphate	SD fine chem.
Potassium dihydrogen phosphate	SD fine chem.
Sodium chloride	SD fine chem.
Sodium hydroxide	SD fine chem.
Dialysis bag	Himedia, Mumbai.
Triton X-100	Himedia, Mumbai.
Acetronitrile	Merck, Mumbai.
Ehrlich carcinoma cell lines	NCCS, Pune.

4.2 Name of the Instruments used:

Rotary Flash Evaporator	Equitron
Probe Sonicator	Bandelin, Germany
Single Beam UV –Visible Spectrophotometer	1201, Shimadzu Corporation, Japan.
Double Beam UV- Visible Spectrophotometer	Perkin Elmer
HPLC With binary Pump and Spectrophotometric detector	Perkin Elmer
Stability Chamber of 120 liters capacity	Osworld, Mumbai.
Single pan Electronic balance	Shimadzu Corporation.
Magnetic stirrer	Remi
pH meter	Elico LI 810

4.3 DRUG PROFILE

4.3.1 METHOTREXATE



Chemical name:

2-(4-(((2,4-Diaminopteridin-6-yl)methyl)(methyl)amino)benzamido)pentanedioic acid

Molecular Formula: $C_{20}H_{22}N_8O_5$

Molecular Weight: 454.44

Methotrexate is a mixture of 4-amino-10-methylfolic acid and closely related compounds. It contains not less than 98 % and not more than 102 % of $C_{20}H_{22}N_8O_5$, calculated on the anhydrous basis.

Caution – Great care should be taken to prevent inhaling particles of Methotrexate and exposing the skin to it.

Category: cytotoxic

Correct Dose: For adults 2.5 to 10mg

For children 2.5 to 5mg

Pharmacokinetic Data

Availability (oral) %	:	70 ± 27
Urinary excretion %	:	48 ± 18
Bound in plasma %	:	34 ± 8
Clearance (ml .min^{-1}.kg^{-1})	:	2.1 ± 0.8
Volume of distribution (lr/kg)	:	0.55 ± 0.19
Half – life	:	7.2 ±2.1[c]

c-A faster half-life (2hrs) is seen initially

Description: yellow to orange – brown crystalline powder.

Solubility:

Practically insoluble in water, in 1, 2-dichloroethane, in ethanol (95%) and in ether. It dissolves in dilute solutions of mineral acids and in dilute solution of alkali hydroxides and carbonates.

Storage:

Store in tightly – closed, light-resistant containers.

Mechanism of action:

Methotrexate inhibits dihydrofolate reductase (DHFRase) – blocking the conversion of dihydrofolic acid (DHFA) to tetrahydrofolic acid (THFA) which is an essential coenzyme required for one carbon transfer reactions in denovo purine synthesis and amino acid interconversions.

Methotrexate has cell cycle specific action, kills cells in S phase; primarily inhibits DNA synthesis, but also affects RNA and protein synthesis.

Adverse Drug Reaction:

CNS:

Arachnoiditis,(within hrs of intrathecal use), subacute neurotoxicity, necrotizing demyelinating leukoencephalopathy (may occur a few yrs later), malaise, fatigue, dizziness, headache, drowsiness, seizures.

EENT

Pharyngitis, gingivitis, blurred vision.

GI

Stomatitis, diarrhoea, abdominal distress, anorexia, GI ulceration and bleeding, enteritis, nausea, vomiting.

GU

Nephropathy, tubular necrosis, renal failure, hyperuricemia, hematuria, menstrual dysfunction, defective spermatogenesis, cystitis

Hematologic:

WBC and Platelet count nadirs occuring on day 7; anemia, leukopenia, thrombocytopenia.

Hepatic:

Acute toxicity (elevated transaminase level),

Chronic toxicity (cirrhosis, hepatic fibrosis).

Metabolic: Diabetes

Musculoskeletal: Arthralgia, myalgia, osteoporosis.

Respiratory:

Pulmonary fibrosis, pulmonary interstitial infiltrates, pneumonitis; dry, nonproductive cough

Skin:

Alopecia, urticaria, pruritus, hyperpigmentation, erythematous rash, ecchymoses, psoriatic lesions, rash, photo sensitivity.

Other:

Fever, chills, reduced resistance to infection, soft tissue necrosis, osteonecrosis, septicemia, sudden death.

Condraindications and Precautions

Condraindicated in patients hypersensitive to drug and during pregnancy or breast - feeding. Also contraindicated in patients with psoriasis or rheumatoid arthritis who also have alcoholism, alcoholic liver, Chronic liver disease, immunodeficiency syndroms, or preexisting blood dyscrasiasis.

Use cautiously in very young or geriatric or debiliated patients and in those with impaired renal or hepatic function, bone marrow suppression, alplasia, leukopenia, thrombocytopenia, anemia, folate deficiency, infection, peptic ulcer, or ulcerative colitis. Drug exits slowly from third space compartments resulting in a prolonged terminal plasma half-life & risk of toxicity.

Overdose and Treatment:

Signs and symptoms of overdose include myelosuppression, anemia, nausea, vomiting, dermatitis, alopecia and melena.

The antidote for hematopoietic toxicity of methotrexate is calcium leucovorin, started as soon as possible and with in 1 hr after the administration of methotrexate. The dosage of leucovorin should produce plasma levels higher than those of methotrexate.

Absorption, Fate, and Excretion:

Methorexate is readily absorbed from the gastrointestinal tract at doses of less than 25mg/m^2, but larger doses are absorbed incompletely and are routinely administered intravenously. Peak concentration in the plasma of 1 to 10 mμ are obtained after 25-100mg/m^2 doses, and 0.1-1mM are achieved after high-dose infusions of 1.5g/m^2 or more. Distribution of methotrexate into body spaces, such as pleural or peritoneal cavities occurs slowly.

Of the drug absorbed from 40 to 50% of a small dose (2.5-15 μg/kg) to about 90% of a larger (150 μg/kg) is excreted unchanged in the urine within 48 hrs mostly within first 8 hrs. A small amount of methotrexate is also excreted in the stool.

Metabolism of methotrexate in man is usually minimal. After high doses, metabolites do accumulate; these include 7-hydroxymethotrexate, which is potentially nephrotoxic.

It is important to emphasize that concentration of methotrexate in cerebrospinal fluid are only 3% of those in the systemic circulation at steady state; so when high doses of methotrexate are given (>1.5g/m^2) , followed by leucovorin 'rescue', cytotoxic concentrations of methotrexate may be attained in the CNS.

Preparations, Dosage, and Routes of Administration:

In acute lymphocytic leukemia the daily oral dosage of methotrexate has been 2.5 to 5 mg for children and 2.5 to 10 mg for adults.

Methotrexate is of great value in the maintenance of remissions, particularly when administered intermittently at doses of 30mg/m^2 intramuscularly, twice a week, or by intensive 2 day 'pulses' of 175-525 mg/m^2 at monthly intervals.

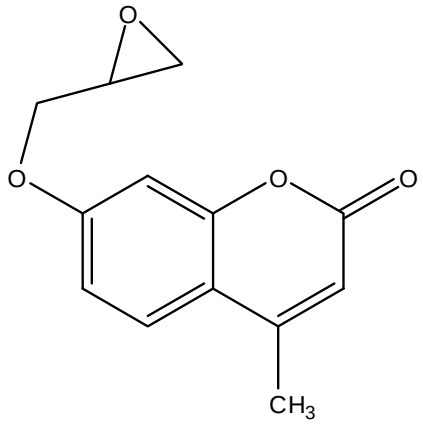
The intrathecal administration of Methotrexate has been employed for treatment of prophylaxis of meningeal leukemia or lymphoma or for treatment of meningeal carcinomatosis. The recommended intrathecal dose is 12 mglm^2 upto a maximum total dose of 12 mg for every 4 days.

In the treatment of choriocarcinoma with methotrexate 1mg/ kg is administered intramuscularly every other day for four doses, alternating with leucovorin (0.1 mg / kg every other day).

Methotrexate has been used in the treatment of psoriasis in doses of 2.5 mg orally for 5 days, followed by a rest period of at least 2 days, or 10-25 mg intravenously weekly.

4.3.2 Coumarin Epoxide: (Cu-2)

Structure:



CHEMICAL NAME: 4-Methyl-7-(Oxiran-2-yl-methoxy)-2-*H*-chromen-2-one

Nature: Powder

Molecular formula: $C_{13}H_{12}O_4$

Molecular weight: 232.23

Melting point: 181°C

Solubility: Insoluble in water, freely soluble in Acetonitrile, chloroform

Coumarin toxicity:

Coumarin is often found in tobacco products and artificial vanilla substitutes, though it has been banned as a food additive in numerous countries since the mid-20th century because it is moderately toxic to the liver and kidneys, with an LD_{50} of 40 mg /kg, low compared to related compounds. Although only moderately dangerous to humans, coumarin is a potent rodenticide. Rats and other rodents largely metabolize it to 3,4-coumarin epoxide, a toxic compound that can cause internal hemorrhage and death. Humans largely metabolize it to 7-hydroxycoumarin, a compound of lower toxicity.

Coumarin was banned as a food additive in the United States in 1940. OSHA considers this compound to be only a lung-specific carcinogen, and "not classifiable as to its carcinogenicity to humans". Coumarin was banned as an adulterant in cigarettes by tobacco companies in 1997, but due to the lack of reporting requirements to the US Department of Health and Human Services it was still being used as a flavoring additive in pipe tobacco. Coumarin is currently listed by the United States Food and Drug Administration (FDA) among "Substances generally prohibited from Direct Addition or Use as Human Food", according to 21 CFR 189.130 but some natural additives containing coumarin (such as sweet woodruff) are allowed "in alcoholic beverages only" (21 CFR 172.510). In Europe, such beverages are very popular, for example *Maiwein* (white wine with woodruff) and *Żubrówka* (vodka flavored with bison grass).

European health agencies have warned against consuming high amounts of cassia bark, one of the four species of cinnamon, because of its coumarin content.

Physilogical Properties:

Coumarin moiety is not effective anti-coagulant, but its derivatives containing Coumarin nucleus like warfarin are used as anticoagulant, for example warfarin. Anticoagulant activity is through the inhibition of vitamin K-epoxide reductase and vitamin K quinine reductase.

Biological Evaluation:

Coumarin derivatives possessed other biological activities like anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial.

4.4. REAGENTS USED

Preparation of release media

pH 7.4 Phosphate buffered saline (*I.P. 1996*)

In 1 liter of water, 2.39 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8 g of sodium chloride were added and dissolved. The pH is adjusted if necessary.

0.1M sodium hydroxide

In 1 liter of water, 4 g of sodium hydroxide was added and dissolved.

4.5 Construction of standard curve for Methotrexate in pH 7.4 phosphate buffered saline and analysis by UV (*I.P. 1996):*

Methotrexate of 100 mg was dissolved in small amount of 0.1M sodium hydroxide and made up to 100 ml with phosphate buffered saline (PBS) of pH 7.4 to get 1 mg/ml solution. From this 10 ml was pipetted out and diluted to 100 ml with phosphate buffered saline of pH 7.4. Aliquots were prepared ranging from 4 to 10 µg/ml and the absorbances were measured at 303 using phosphate buffered saline as blank.(Table 3,Fig 1)

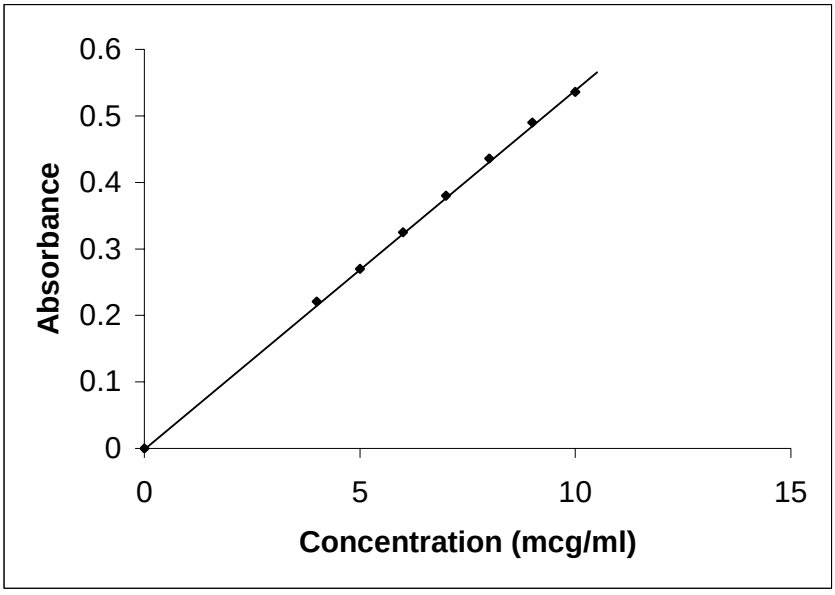
Table: 3

Standard curve data for Methotrexate by UV spectroscopy

Concentration µg/ml	Absorbance at 303 nm
4	0.221
5	0.270
6	0.325
7	0.380
8	0.436
9	0.490
10	0.536

Figure: 1

Standard curve of Methotrexate by UV



r^2 = regression = 0.999396

4.6 Construction of standard curve for Methotrexate and analysis by HPLC

The mobile phase was prepared by mixing the acetonitrile and HPLC grade water [Lichrosolv] in the ratio of 1:1.

A primary stock of Methotrexate 1 mg/ml was prepared by dissolving in minimum quantity of 0.1M NaoH solution and then made up with HPLC grade water. Then secondary stock of 100µg/ml was prepared using mobile phase.

Standard solutions were prepared using the secondary stock with the mobile phase so that the final concentrations are 2 to 6 µg/ml.

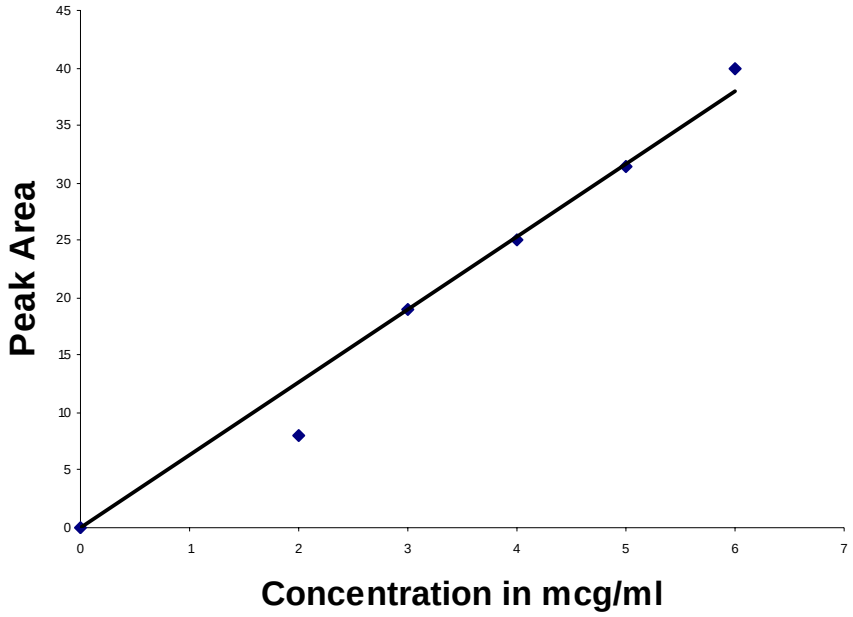
The chromatographic system consisted of the following Perkin Elmer components like Series 200 binary pump, degasser and series 200 spectrophotometric detector variable wavelength. Chromatographic analysis was performed at ambient temperature, using a Perkin Elmer G-ODS precolumn (21 mm x 4 mm internal diameter 5 µm particle size) and a chromosil LC-18 column (250 mm x 4.6mm internal diameter, 5µm particle size). The mobile phase consisted of acetonitrile – HPLC grade water [50: 50 v/v] and was pumped at a flow rate of 1.0 ml\min. The analytes were detected at 303 nm. The injection volume was 20 µl. The retention time for Methotrexate was found to be 1.6 min.(Table 4, Fig 2)

Table: 4
Standard Curve Data of Methotrexate analysed by HPLC

Concentration (µg/ml)	Peak area[uV*sec]
2	84913.06
3	193903.19
4	255354.95
5	306544.54
6	403843.19

Figure: 2

Standard curve of Methotrexate by HPLC



Regression (r^2) = 0.982533

CHAPTER VII

RESEARCH ENVISAGED

FORMULATION AND EVALUVATION OF LIPOSOMES OF METHOTREXATE AND COUMARIN EPOXIDE (Cu-2)

5.0 Optimization process for liposomal preparation

Empty vesicles were prepared by thin film hydration technique. A liquid phase was prepared by dissolving accurately weighed quantities of phosphatidylcholine and cholesterol in the chloroform – methanol mixture (1:1v/v) in 250 ml round – bottom flask. The solvent mixture was removed from the liquid phase by rotary evaporation at 45-60°C by varying speed of rotation like 75 rpm, 100 rpm, 125 rpm, and 150 rpm to obtain a thin film of lipids on the wall of the flask. Simultaneously the flask was kept under vacuum to ensure the complete removal of residual solvent. The film was hydrated with phosphate buffered saline of pH 7.4 by varying hydration volume like 5 ml, 10 ml and hydration time as 30 min, 45 min, and 60 min at a temperature of 60°C ±2°C. The vesicle size and appearance of empty vesicles were noted.

5.1 Results obtained for process optimization by forming empty vesicles

Various product influencing variables viz. vacuums, speed of rotation, hydration media, hydration volume and hydration time were studied in order to

produce drug loaded liposomes with desired attributes. For this, empty vesicles were formed and the vesicles were observed under the optical microscopy. Vesicle size and appearance were noted (Table: 5). From the obtained results, the speed of rotation, vacuum required, hydration time and hydration volume for the formulation were finalized.

The vacuum used for drying of film was 355 mm Hg. Below 355 mm Hg it was found to be insufficient for complete removal of the solvents and resulted in aggregation of the Liposomes on hydration. The vacuum above 355 mm Hg resulted in rapid evaporation of solvents, leading to entrapment of air bubbles on lipid film surface. Hydration of these films resulted in Liposomes with poor drug entrapment. At an optimal vaccum of 355 mm Hg, the lipid films were translucent and on hydration gave a better drug entrapment in Liposome formulation.

The hydration time of the lipid film was increased from 30 to 60 min. At hydration time of 60 min, the formed Liposomes were spherical and smaller vesicle size ranging from 3 µm to 8 µm was obtaining. Hence 60 min was selected for the prepared formulations with the 5 ml of hydration volume.

Rotational speed of the flask demonstrated marked influence on the thickness and uniformity of the lipid film. The rotational speed was increased from 75 rpm to 150 rpm. The optimum speed was noted to be 150 rpm as the same yielded a uniform thin film on the flask and subsequently homogenous population of Liposomes.

In the rotary evaporator, the water bath temperature was maintained at 45-60°C for the removal of solvent mixture to obtain a thin film of lipids. The dry lipid film was hydrated at a temperature of 60 ± 2°C. With regard to the influence of

formulation variables on the vesicle size, different compositions with varying ratios of phosphatidyl choline and cholesterol were studied.

Table: 5

Vesicle size obtained by varying Speed of Rotation, Hydration time

CHOL: PC Mass Ratio	Speed of Rotation (rpm)	Hydration Time (Min)	Vesicle Size-in µm (Mean ± S.D)
1: 5	75	30	15.5 ± 0.12
	100	45	13.5 ± 0.23
	125	60	10.5 ± 0.32
	150	60	09.5 ± 0.11
1:10	75	30	16.5 ± 0.33
	100	45	14.25 ± 0.36
	125	60	11.05 ± 0.19
	150	60	09.25 ± 0.27
1:15	75	30	12.50 ± 0.12
	100	45	09.50 ± 0.32
	125	60	07.50 ± 0.42
	150	60	05.45 ±0.23

6.0 Methotrexate liposomes

6.1 Development of Methotrexate Liposomes

Liposomes were prepared by thin film hydration technique. A lipid phase was prepared by dissolving accurately weighed quantities of the phosphatidylcholine and cholesterol in the chloroform–methanol mixture [1:1 v/v] in 250 ml round bottom flask. The solvent mixture was removed from the lipid phase by rotary evaporation at 45-60°C to obtain a thin film of lipids on the flask. Simultaneously the flask was kept under vacuum to ensure the complete removal of residual solvent. An appropriate amount of Methotrexate was dissolved in small amount of 0.1 M sodium hydroxide and then added 5 ml of phosphate buffered saline (PBS) of pH 7.4. The dry lipid film was hydrated with drug solution at a temperature of 60°C ±2°C for a period of 1 hour until the formation of liposomes. Liposomes containing charge inducing agent like Stearylamine was also prepared (Table: 6).

Preparation of SUVs

This was first achieved by exposure of MLVs to ultrasonic irradiations. MLVs were sonicated using probe sonicator (Bandelin,Germany) for 5 min with a cooling interval of 2 min (Table: 6).

Table: 6

Composition of Methotrexate Liposomes

Ingredients	**Category**	**Batch Number**							
		M_1	M_1S	M_2	M_2S	M_3	M_4	M_5	M_6S
Methotrexate	Active ingredient (mg)	50	50	50	50	50	50	50	50
Soy phosphatidyl Choline	Lipid (mg)	250	250	500	500	750	750	750	750
Cholesterol	Lipid (mg)	50	50	50	50	50	50	-	50
Chloroform	Solvent (ml)	5	5	5	5	5	5	5	5
Methanol	Solvent (ml)	5	5	5	5	5	5	5	5
Stearylamine	Charge inducing agent (mg)	-	-	-	-	-	25	-	-

6.2 Evaluation of Methotrexate Liposomes

6.2.1 Removal of Unentrapped Drug from Liposomes

The unentrapped drug from liposome was removed by dialysis method. The prepared liposome dispersion was placed in 3 cm x 8 cm long dialysis bag with a molecular weight cut off 12,000 (Himedia Labs,Mumbai). The dialysis bag was then placed in 250 ml beaker containing phosphate buffered saline of pH 7.4 with constant stirring by means of a magnetic stirrer. Dialysis was carried out for 24 hours replacing the buffer with fresh buffer for every 3 hours. Dialysis membrane would allow only free drug and unencapsulated lipid molecules of molecular weight less than 12,000.

6.2.2 Size Evaluation

Preliminary evaluation was done by optical microscopy. If the vesicles are unmeasurable by optical microscopy, it indicates that they are smaller in size. Such formulations were analysed by Atomic Force Microscopy (AFM).

6.2.3 Percentage Drug Encapsulation Efficiency

The encapsulation efficiency describes the percent of the aqueous phase and hence the percent of water soluble drug that becomes ultimately entrapped during preparation of lipsomes and is usually expressed as percentage entrapment per mg lipid. 10 % Triton X-100 of 0.5 ml solution was added to 0.5ml of liposomal preparation and made up to 10 ml with phosphate buffered saline of pH 7.4. Then it was incubated at 37°C for 1.5 hours to completely break the liposome membrane and release the enclosed material. Then the sample was filtered through a millipore membrane filter (0.45 μm) under vacuum. Filtrate of 1 ml was diluted to 10 ml with PBS and the absorbance was measured at 303 nm (as explained in section 3.5)

$$\text{Percentage Drug Loading (PDL)} = \frac{Entrapped(mg)}{\text{Total drug added}(mg)} x100$$

6.2.4 *In vitro* drug release and analysis by UV

The Liposome preparation was placed in the dialysis bag and the bag was placed in a 250 ml beaker containing phosphate buffered saline of pH 7.4. The temperature of the receptor medium was maintained at 37°C ±2 °C and the medium was agitated at a moderate speed using magnetic stirrer. The samples were collected at 6 hrs interval with replacement of equal volume of PBS. The samples were analyzed spectrophotometrically at 303 nm for the drug content against the respective buffer blank. The mean percentage of Methotrexate at various time intervals was calculated from standard graph and plotted against time. (Ref. Section 3.5)

6.2.5 *In vitro* release study for the optimized formulation and analysis by HPLC

The Liposome preparation was placed in the dialysis bag and the bag was placed in a 50 ml beaker containing phosphate buffered saline of pH 7.4. The temperature of the receptor medium was maintained at 37°C ±2 °C and the medium was agitated at a moderate speed using magnetic stirrer. The samples were collected at 6 hrs interval with replacement of whole volume of PBS. The withdrawn samples were filtered using sartorious membrane filter and analyzed.

Analysis of samples:

The filtered samples were analyzed by HPLC for drug concentration at 303 nm using LC-18 column having particles of 5µm pore size at ambient temperature and the flow rate was maintained at 1ml/min. The mobile phase was HPLC grade water: Acetonitrile (1:1). Membrane filtered sample of 20µl was spiked and noted the peak area of Methotrexate at the retention time of 1.6 minutes. Percentage of the drug released was calculated from the standard graph [Ref. Section 3.6].

6.2.6 Stability Studies

The optimized liposomal formulation was sealed in 10 ml vials and stored at different temperatures 4°C, 25±2°C, 40°C/75%RH for a period of one month. The samples from the batch at each temperature were withdrawn at definite time intervals. The residual amount of the drug in vesicles (i.e. entrapment) and *in vitro* release were determined.

7.0 COUMARIN EPOXIDE LIPOSOMES

7.1 Construction of Standard curve of Coumarin epoxide(Cu-2) by UV Spectrophotometer

An accurately weighed 100 mg of Coumarin epoxide was dissolved in small amount of acetonitrile and made up to 100 ml with phosphate buffered saline pH 7.4 to get 1 mg/ml. From this 10 ml was pipetted out and made up to 100 ml with phosphate buffered saline of pH 7.4. From this 2 ml, 4 ml, 6 ml, 8 ml were pipetted out and made up to 10 ml with PBS. The absorbances were measured at 320 nm using phosphate buffered saline of pH 7.4 as blank. (Table 7, Fig 3)

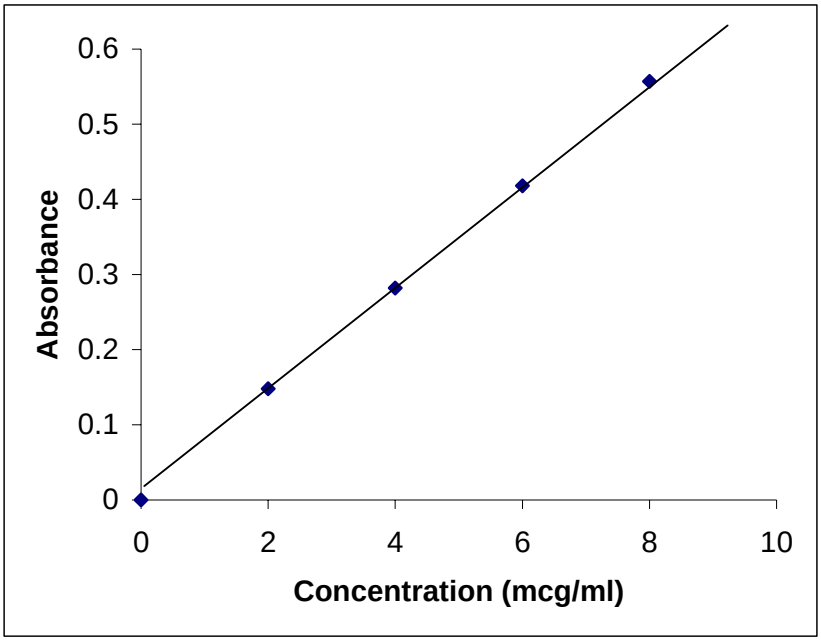
Table: 7

Standard Curve Data of Coumarin epoxides(Cu-2) by UV

Concentration (µg/ml)	Absorbance At 320 nm
2	0.148
4	0.282
6	0.417
8	0.557

Figure: 3

Standard curve of Coumarin epoxides (Cu-2) by UV



Correlation coefficient = r^2 = 0.999718

7.2 HPLC determination of Coumarin epoxides (Cu -2)

The mobile phase was prepared by mixing the acetonitrile and HPLC grade water [Lichrosolv] in the ratio of 1:1.

A primary stock of Coumarin epoxide 1 mg/ml and a secondary stock of 100 µg/ml were prepared using Acetonitrile (HPLC Grade).

Standard solutions were prepared using the secondary stock with the mobile phase so that the final concentrations are 2.5 to 10µg/ml.

The chromatographic system consisted of the following Perkin Elmer components like series 200 binary pump, degasser and series 200 spectrophotometric detector variable wavelength. Chromatographic analysis was performed at ambient temperature, using a Perkin Elmer G-ODS precolumn (21 mm x 4 mm internal diameter 5 µm particle size) and a chromosil LC-18 column (250mm x 4.6mm internal diameter, 5µm particle size). The mobile phase consisted of acetonitrile –HPLC grade water [50: 50 v/v] and was pumped at a flow rate of 1.5 ml\min .The analytes were detected at 320 nm. The injection volume was 20 µl .The retention time for Coumarin epoxide was found to be 3.6 min. (Table 8, Fig 4).

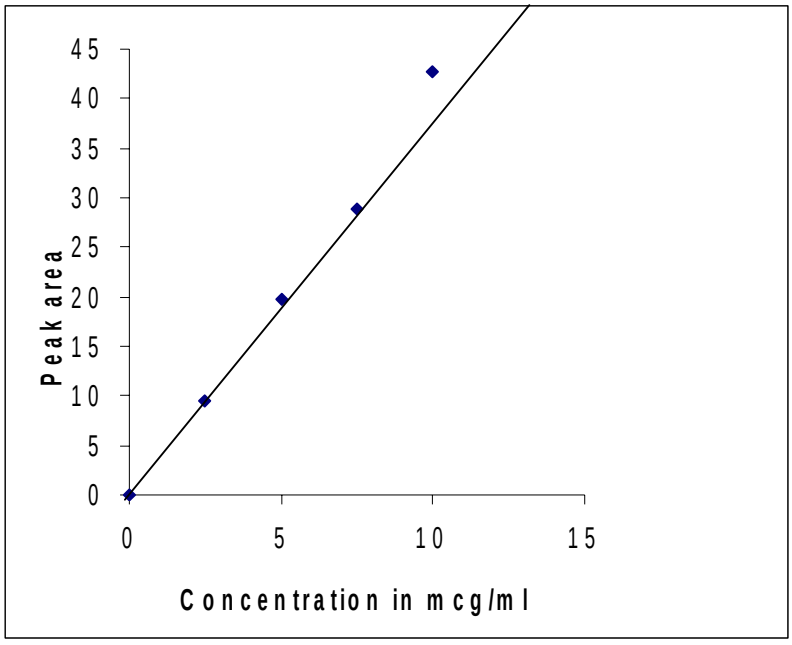
Table: 8

Standard curve data of Coumarin epoxides (Cu -2) by HPLC

Concentration in µg/ml	Peak area [uV*sec]
2.5	85177.91
5	197135.47
7.5	288833.83
10	427025.56

Figure: 4

Standard curve of Coumarin epoxide(Cu -2) by HPLC



r^2 = 0.9993347

7.3 Development of Coumarin Epoxide(Cu-2) liposomes

Coumarin epoxide liposomes were developed as mentioned in the section 2.1.

Table: 9

Composition of Coumarin epoxide(Cu-2) Liposomes

S.No	Ingredients	Category	Batch Number			
			C_1	C_2	C_3	C_2S
1	Coumarin Epoxide (Cu-2)	Active Ingredient (mg)	50	50	50	50
2	Soy phosphatidyl Choline	Lipid (mg)	750	750	750	750
3	Cholesterol	Lipid (mg)	-	50	50	50
4	Chloroform	Solvent(ml)	5	5	5	5
5	Methanol	Solvent(ml)	5	5	5	5
6	Stearylamine	Charge inducingAgent (mg)	-	-	25	-

S =Sonicated

7.4 Evaluation of Coumarin epoxide Liposomes

7.4.1 Removal of Unentrapped drug from liposome

The unentrapped drugs from liposomes were removed as mentioned in the section3.2.1.

7.4.2 Size Evaluation

Size of the vesicles was determined as mentioned in the section 6.2.2.

7.4.3 Percentage Encapsulation Efficiency

Amount of drug encapsulated was determined as mentioned in the section 6.2.3.

7.4.4 *In vitro* Release Study and estimation by UV

Method was similar as explained in the section 6.2.4. The coumarin was analyzed by UV spectrophotometer as explained in section 3.1

7.4.5 *In vitro* release studies for the optimized formulation C_2 in phosphate buffered saline of pH 7.4 and estimation by HPLC

The liposome preparation was placed in the dialysis bag and the bag was placed in a 50 ml beaker containing phosphate buffered saline of pH 7.4. The temperature of the receptor medium was maintained at 37°C ±2 °C and the medium was agitated at a moderate speed using magnetic stirrer. The samples were collected at 6 hrs interval with replacement of whole volume of PBS. The withdrawn samples were filtered using sartorious membrane filter and analyzed.

Analysis of samples:

The filtered samples were analyzed by HPLC for drug concentration at 320 nm using LC-18 column having particles of 5µm pore size at ambient temperature and the flow rate was maintained at 1.5 ml/min. The mobile phase was HPLC grade water: Acetonitrile (1:1). Membrane filtered sample of 20µl was spiked and noted the peak area of Coumarin epoxide at the retention time of 3.6 minutes. Percentage of the drug released was calculated from the standard graph. (Ref. Section 6.2).

7.4.6 In vitro release studies for the optimized formulation C_2 in water by HPLC method

Instead of phosphate buffer saline of pH 7.4 as the release medium, triple distilled water was used as release medium. The coumarin ring got opened in presence of alkali present in PBS. Hence, water was used as release medium. (Ref. Section 6.2).

7.5.7 Stability Studies

The optimized liposomal formulation was sealed in 10 ml vials and stored at different temperatures 4°C, room temp, 40°C/75RH for a period of one month. The samples from batch at each temperature were withdrawn at definite time intervals. The residual amount of the drug in vesicles (i.e. entrapment) and *in vitro* release were determined.

RESULTS AND DISCUSSIONS

8.0 Methotrexate Liposomes

8.1 Development of Methotrexate Liposomes

An effective liposomal targeted delivery system should incorporate high drug loading with stable encapsulation and should possess good physical and chemical stability during self life. Drug should not leak during this *in vivo* transit.

The targeted liposome delivery system is composed of Egg phosphatidyl choline, drug, and cholesterol. Cholesterol is included in the formulation to give further rigidity to the bilayer. Presence of cholesterol in liposome formulation was also known to enhance retention of entrapped solutes and reduce serum – induced instability caused by binding of serum proteins to liposome membrane. Through these effects cholesterol improves both *in vitro* and *in vivo* stability of the liposomes *(Nallamothu R., 2006*). With regard to the influence of formulation variables on the percent drug loading (PDL), different compositions with varying ratios of drug, PC and cholesterol were studied. The effect of charge on PDL was also investigated. At a lower level of PC, the entrapment efficiency was found to be less and on increasing the same while keeping the other constituent, CHOL, at a constant level the entrapment efficiency could be enhanced. On this basis various formulations with increasing amount of PC (Table: 6) were prepared and evaluated. In our study, positive charge imparting agent stearylamine did not affect the vesicular entrapment but inhibited the *in vitro* release. This was further evident and consistent from the work of (*Katragadda A. K., 1999*).

8.2 Evaluation of Methotrexate Liposomes

8.2.1 Removal of unentrapped drug from liposomes

The unentrapped drug from liposomes was removed by dialysis technique for 24 hrs. The results are presented in the table 10. Maximum encapsulation of drug was seen in formulation M_3. This was evident as the percentage of drug dialyzed was less.

Table: 10

Physiochemical parameter of Methotrexate liposomes

Formulation code	Drug: PC:CHOL	Amount of drug dialyzed in mg	Percentage of drug dialysed	Vesicle size in μm	Percentage Entrapment efficiency
M_1	1:5:1	17.70 ± 0.35	35.40 ± 0.70	8.52±0.19	66.10 ± 0.26
M_1S	1:5:1	25.25 ± 0.25	50.50 ± 0.50	<0.5	49.50 ± 0.24
M_2	1:10:1	12.75 ± 0.40	25.50 ± 0.80	7.25±0.26	75.90 ± 0.48
M_2S	1:10:1	19.75 ± 0.35	39.50 ± 0.70	<0.5	60.50 ± 0.30
M_3	1:15:1	06.98 ± 0.12	13.96 ± 0.24	100nm	92.10 ± 0.62
M_4	1:15:1(stearylamine)	08.50 ± 0.15	17.00 ± 0.30	3.2±0.36	80.10 ± 0.32
M_5	1:15:0	12.48 ± 0.32	25.36 ± 0.64	4.5±0.15	83.00 ± 0.63
M_6S	1:15:1	13.52 ± 0.16	27.04 ± 0.32	<0.5	79.06 ± 0.52

8.2.1 Vesicle size determination

Morphology

The prepared Liposomal vesicles were mostly spherical in shape and some vesicles are slightly elongated. The vesicles are slightly yellow in color. There is a thickening around the inner compartment in the liposome prepared with choline and cholesterol. But in the case of vesicles without cholesterol, there is a very thin film around the inner compartment. In some vesicles the film was not found. Sometimes it might be absent.

The maximum number of globules of M_1 (5:1 PC: CHOL), M_2 (10:1 PC: CHOL ratio), M_4 (15:1 PC: CHOL and stearylamine) and M_5 (liposomes with no CHOL) formulations were observable under optical microscopy with size range of 1-9 μm. Though the maximum no of particles of M_3, M_1S, M_2S, M_6S formulations were not observable under optical microscopy, the *in vitro* release of M_3 formulation was higher than M_6 and encapsulation efficiency was also higher than M_6. Hence liposome formulation M_3 was selected for AFM study. The atomic force microscopy(AFM) images of selected M_3 liposome formulation which contains 15:1 PC: CHOL ratio were presented in fig: 5. The liposome vesicles shown under AFM are discrete globules with sharp boundaries that range in size from 50 – 100 nm with 90% of liposomal population equal or below 100 nm. The particle sizes of the various Methotrexate liposomal formulations are presented in the table: 10.

8.2.3 Percentage Drug Entrapment efficiency

Relatively hydrophilic Methotrexate was incorporated into the liposome bilayer by dissolving it into the hydration medium. Phosphate buffered saline of pH 7.4 was used as the hydrating medium that ensured better drug stability. The studies of various liposomes consisting of different ratios of drug, PC and cholesterol describes the effect of these variables on the degree of entrapment (Table: 10). The inclusion of charger into the lipidic layers could avoid the aggregation and fusion of vesicles to maintain their integrity and uniformity. At a lower level of PC, the entrapment efficiency was found to be less as 66.1%, 49.5%, 60.5% and 75.9% for M_1, M_1S, M_2 and M_2S respectively. On increasing the PC concentration while keeping the other constituent, CHOL, at a constant level, the entrapment efficiency could be enhanced (M_3 to M_6) (*Agarwal R. et al., 2001*). This may be accounted to the saturation of lipid domains with reference to drug where low PC content provides limited entrapment capacity *(Patel & Mishra., 1999*). The increase in the entrapment efficiency is attributed to the ability of CHOL to cement the leaking space in the bilayer membranes, which in turn allow enhanced drug level in liposomes (*Plessis et al., 1966*).

As the cholesterol content of the formulation increased, drug leakage decreased (*Nallamothu R., 2006*). Hence it is important to select the appropriate proportions of the two (PC & CHOL) to maximize the entrapment. In case of CHOL free soy PC liposomes (M_5) maximum drug loading of 80.5% could be achieved using 1:15 w/w drug: lipid ratio. A further improvement of 10% in the entrapment was noted with the addition of CHOL in M_3 formulation. Formulation containing stearylamine has not shown any significant changes in percentage encapsulation efficiency. In sonicated (probe sonication) vesicles (M_1S, M_2S, M_6S formulation) the entrapment efficiency was decreased which may be due

to the breakage of lipid layer. Hence 15:1 PC: CHOL mass ratio in the formulation M_3 was found to be optimum for maximum entrapment efficiency.

8.2.4 *In vitro* release study

The study was done for 7 days (168 hours). *In vitro* release was found to be biphasic as the release was controlled by the dialysis membrane and the lipid bilayer. Incorporation of cholesterol affected the release rate of the encapsulated drug.

M_1 formulation with 5:1 PC: CHOL ratio has shown only 66.22% drug release in a 168 hour period. As the cholesterol content of the formulation increased, drug release decreased (*Nallamothu. R et al., 2006*). (Table.11, Fig.6)

In M_2 formulation with 10:1 PC: CHOL ratio, the concentration of PC was increased and it has shown 73.09% drug release in 168 hour. (Table.13, Fig.8)

Formulation M_1S and M_2S which are probe sonicated has shown release of 58.95%, 67.2% respectively. (Table.12, Fig.7) (Table.14, Fig.9)

In M_3 formulation, slightly increased the amount of Phosphatidyl choline and the release was found to be satisfactory.

By increasing the amount of cholesterol in the lipid phase (PC/CHOL mass ratio from 15:1, to 5:1), the release rate of Methotrexate decreased, which could be related to increased rigidity of the phospholipids bilayer , followed by its decreased permeability for the encapsulated drug [*Marija glaves-DODOV et al., 2003]*.(Table.15,Fig 10)

In M_4 formulation containing positive charge inducer stearylamine the release was inhibited by 10% *(Katragadda A. K., 1999).* The release was extended upto 168 hours.(Table 16, Fig 11)

M_5 formulation with no cholesterol in them released their content rapidly (*Nallamothu .R, 2006)*. Compared to M_3, the M_5 formulation showed release of 83.72% for 102 hour. (Table 17, Fig 12)

In M_6 formulation which is probe sonicated for 5 min, showed the satisfactory release. Compared to M_3 it showed release of 85.91% for 120 hours. Sonicated vesicles show lower encapsulation efficiency and faster release than the unsonicated liposomes. (Table 18, Fig 13)

Hence, M_3 formulation is the optimized one. In vitro release from M_3 formulation was again carried out and the collected samples were analysed by HPLC. The cumulative % of release was 85 %.(Table 19, Fig 14)

Table: 11

The i*n vitro* release data of M_1 formulation

[1:5:1 Drug: Pc: Chol]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	1	3.02	3.02
2	12	1.75	5.29	5.3
3	24	2.51	7.56	7.58
4	30	4.26	12.85	12.88
5	36	6.01	18.15	18.2
6	48	8.24	24.96	25.03
7	54	9.76	29.50	29.60
8	60	11.02	33.28	33.40
9	72	12.50	37.8	37.95
10	78	13.75	41.6	41.75
11	84	14.51	43.8	44.03
12	96	16.75	50.68	50.85
13	102	17.5	52.95	53.15
14	108	18.24	55.21	55.43
15	120	19.49	59.0	59.22
16	126	20.25	61.27	61.50
17	132	21.0	63.5	63.78
18	144	21.26	64.32	64.58
19	150	21.44	64.87	65.12
20	156	21.64	65.47	65.73
21	168	21.80	65.96	66.22

Figure: 6

The i*n vitro* release profile of M_1 formulation

[1:5:1 Drug: PC: Chol]

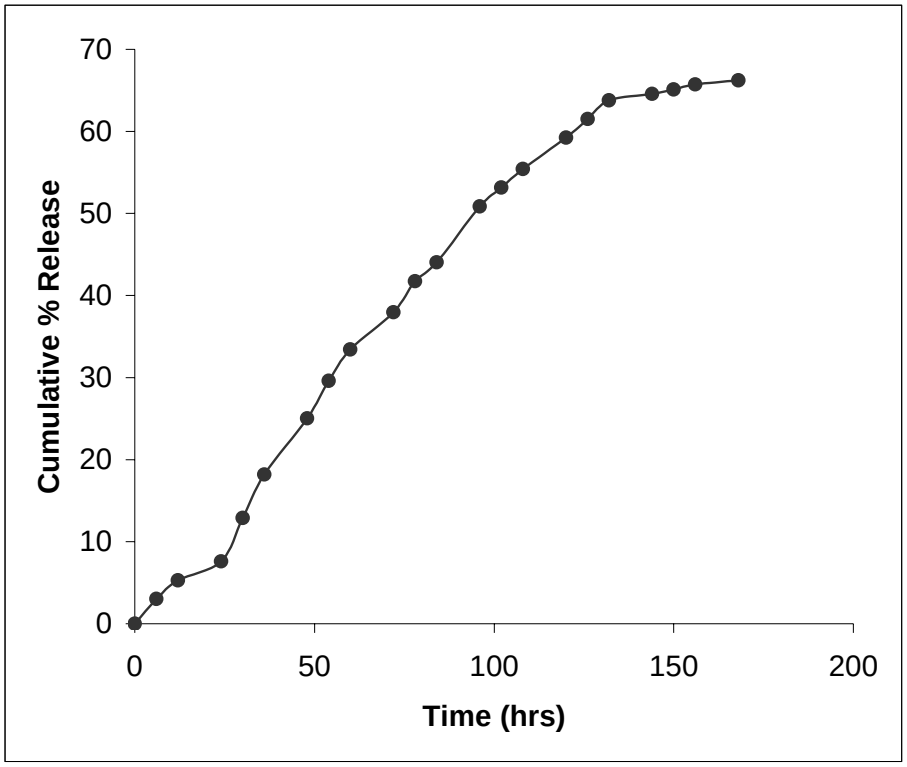


Table: 12

The *in vitro* release data of M_1S formulation

[1:5:1 Drug: PC: Chol, sonicated]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	0.71	2.86	2.86
2	12	1.60	6.46	6.47
3	24	3.28	13.25	13.27
4	30	4.52	18.26	18.28
5	36	5.71	23.07	23.10
6	48	6.58	26.58	26.64
7	54	7.15	28.88	28.96
8	60	7.68	31.03	31.14
9	72	8.12	32.80	32.92
10	78	8.85	35.75	35.88
11	84	9.50	38.38	38.52
12	96	10.95	44.24	44.39
13	102	11.55	46.66	46.82
14	108	12.14	49.05	49.22
15	120	12.86	51.95	52.15
16	126	13.25	53.53	53.73
17	132	14.15	57.17	57.38
18	144	14.45	58.38	58.70

Figure: 7

The *in vitro* release profile of M_1S formulation

[1:5:1 Drug: PC: Chol, sonicated]

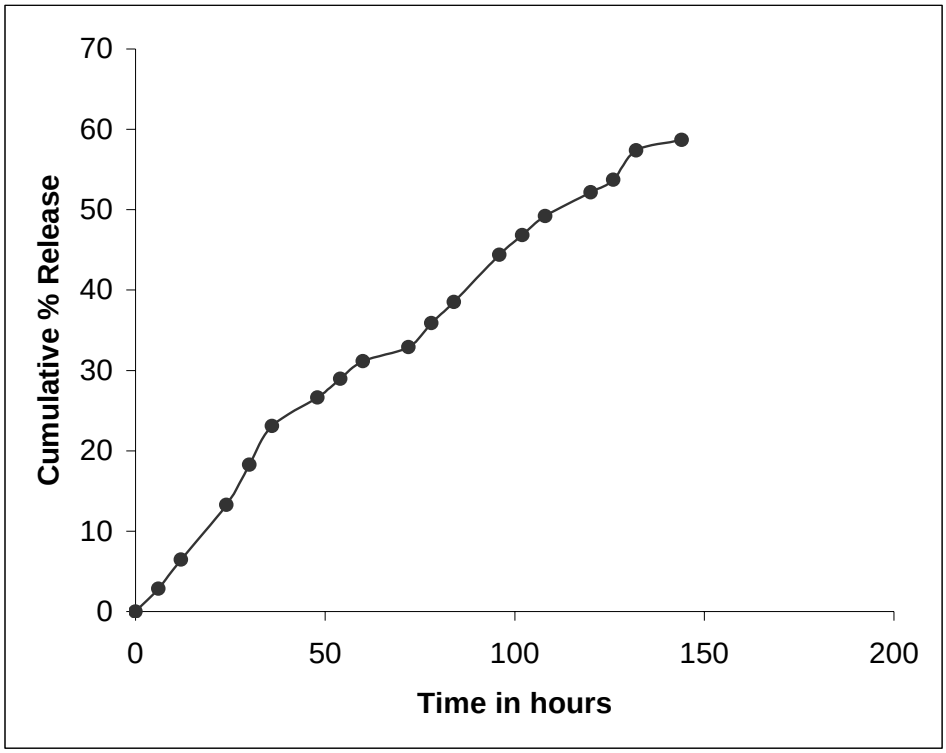


Table: 13

The i*n vitro* release data of M_2 formulation

[1:10:1 Drug: PC: Chol]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	1.25	3.29	3.29
2	12	2.01	5.27	5.28
3	24	3.25	8.56	8.58
4	30	5.51	14.49	14.52
5	36	7.25	19.1	19.16
6	48	9.75	25.69	25.76
7	54	11.26	29.64	29.74
8	60	12.50	32.93	33.05
9	72	15.51	40.84	40.97
10	78	16.74	44.13	44.30
11	84	18.25	48.08	48.26
12	96	20.5	56.65	56.86
13	102	22.75	59.94	60.17
14	108	25.34	66.77	67.01
15	120	25.61	67.48	67.75
16	126	26.14	68.88	69.15
17	132	26.81	70.64	70.92
18	144	27.0	71.14	71.41
19	150	27.28	71.88	72.16
20	156	27.32	71.98	72.27
21	168	27.63	72.80	73.09

Figure: 8

The i*n vitro* release profile of M_2 formulation

[1:10:1 Drug: PC: Chol]

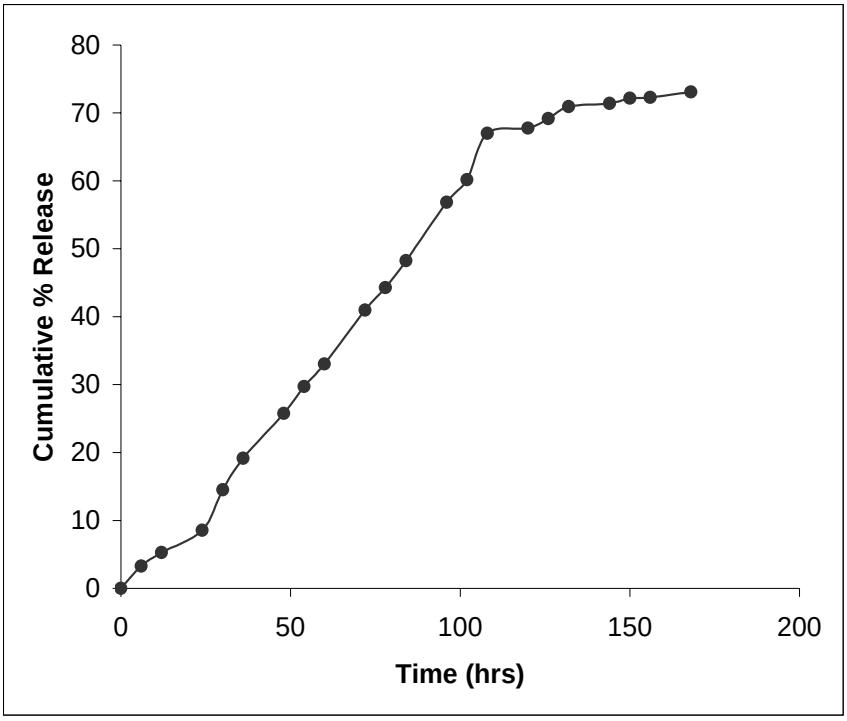


Table: 14

The i*n vitro* release data of M_2 S formulation

[1:10:1 Drug: PC: Chol, sonicated]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	0.75	2.47	2.47
2	12	1.50	4.95	4.97
3	24	2.75	9.09	9.11
4	30	3.25	10.74	10.78
5	36	4,50	14.87	14.92
6	48	6.25	20.66	20.74
7	54	7.50	24.79	24.87
8	60	8.75	28.92	29.03
9	72	10.86	35.90	36.04
10	78	11.92	39.40	39.55
11	84	13.17	43.53	43.69
12	96	15.28	50.51	50.68
13	102	16.68	55.14	55.32
14	108	17.05	56.19	56.38
15	120	19.64	64.92	65.13
16	126	20.25	66.94	67.20

Figure: 9

The i*n vitro* release profile of M_2S formulation

[1:10:1 Drug: PC: Chol, sonicated]

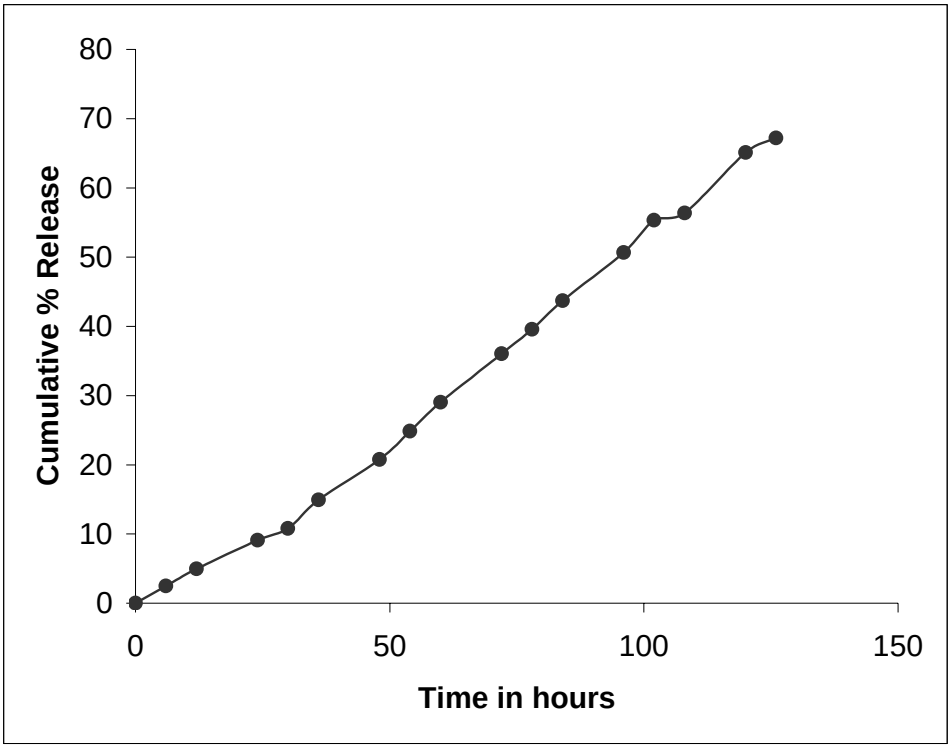


Table: 15

The i*n vitro* release data of M_3 formulation

[1:15:1 Drug: PC: Chol]

S.No	Time in hrs	Drug Concentration in mg	Percentage drug Release	Cumulative % Release
1	6	2.50	5.42	5.42
2	12	4.25	9.22	9.24
3	24	6.93	15.04	15.07
4	30	9.90	21.48	22.08
5	36	12.20	26.47	26.56
6	48	16.84	36.54	36.65
7	54	19.56	42.45	42.59
8	60	22.24	48.26	48.43
9	72	26.85	58.27	58.46
10	78	29.57	64.17	64.41
11	84	32.00	69.45	69.70
12	96	35.97	78.06	78.34
13	102	37.02	80.34	80.65
14	108	37.89	82.23	82.55
15	120	39.44	85.59	85.99
16	126	39.78	86.33	86.67
17	132	40.11	87.05	87.39
18	144	41.40	89.85	90.20
19	150	41.77	90.65	91.01
20	156	42.08	91.32	91.69
21	168	42.08	91.32	91.69

Figure: 10

The i*n vitro* release profile of M_3 formulation

[1:15:1 Drug: PC: Chol]

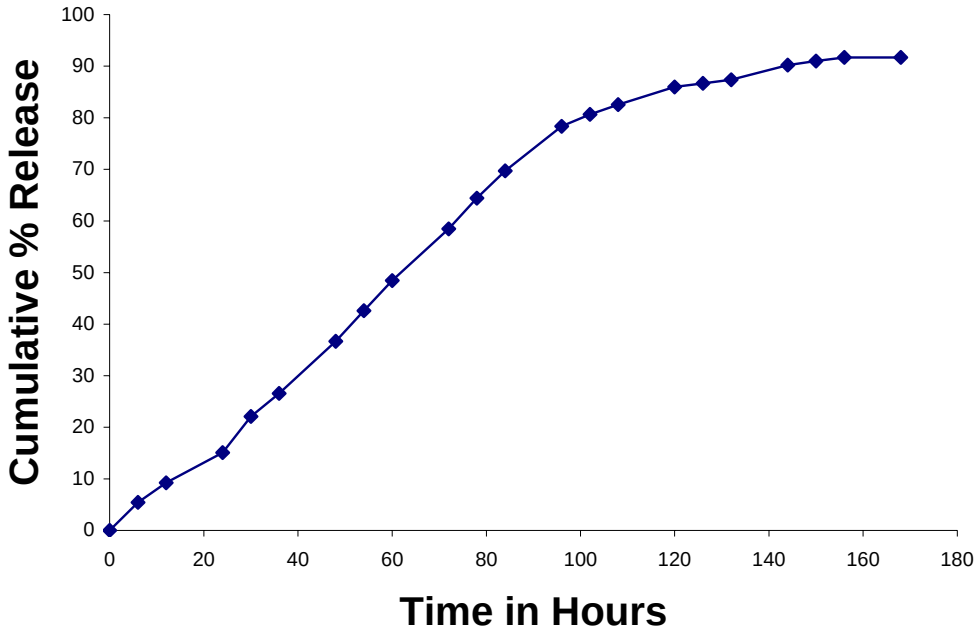


Table: 16

The i*n vitro* release data of M_4 formulation

[1:15:1 Drug: PC: Chol + Stearylamine]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	1.75	4.36	4.36
2	12	3.00	7.49	7.50
3	24	4.90	12.23	12.26
4	30	7.50	18.72	18.77
5	36	10.09	25.19	25.26
6	48	13.71	34.23	34.33
7	54	14.94	37.30	37.44
8	60	16.30	40.69	40.84
9	72	18.27	45.61	45.78
10	78	19.77	49.36	49.54
11	84	21.52	53.73	53.93
12	96	24.17	60.34	60.56
13	102	24.53	61.24	61.48
14	108	26.18	65.36	65.61
15	120	28.33	70.73	70.99
16	126	29.08	72.60	72.89
17	132	29.60	73.90	74.19
18	144	31.08	77.60	77.89
19	150	31.43	78.47	78.78
20	156	32.04	80.00	80.31
21	168	32.38	80.84	81.16

Figure: 11

The i*n vitro* release profile of M_4 formulation

[1:15:1 Drug: PC: Chol + Stearylamine]

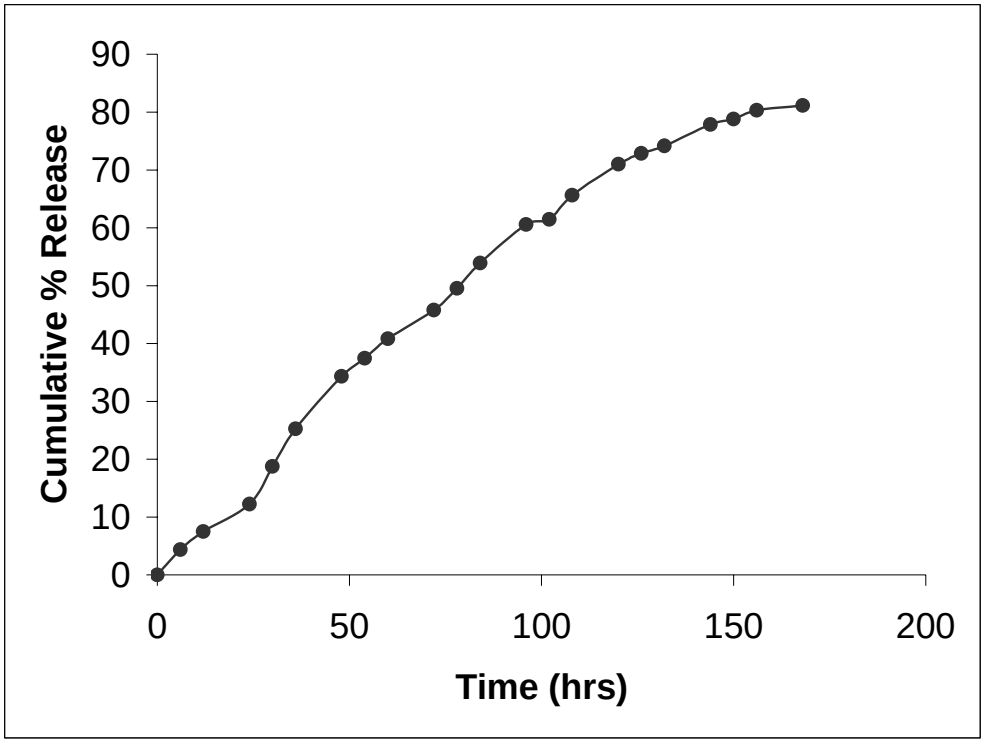


Table: 17

The i*n vitro* release data of M_5 formulation

[1:15:0 Drugs: PC: Chol]

S. No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	1.81	4.36	4.36
2	12	3.37	8.12	8.13
3	24	5.62	13.54	13.56
4	30	7.92	19.08	19.21
5	36	12.37	29.80	29.85
6	48	19.87	47.87	47.96
7	54	23.83	57.42	57.59
8	60	26.30	63.37	63.58
9	72	30.39	73.22	73.55
10	78	31.40	75.66	75.92
11	84	32.63	78.62	78.88
12	96	34.25	82.53	82.81
13	102	34.62	83.42	83.72

Figure: 12

The i*n vitro* release profile of M_5 formulation

[1:15:0 Drug: PC: Chol]

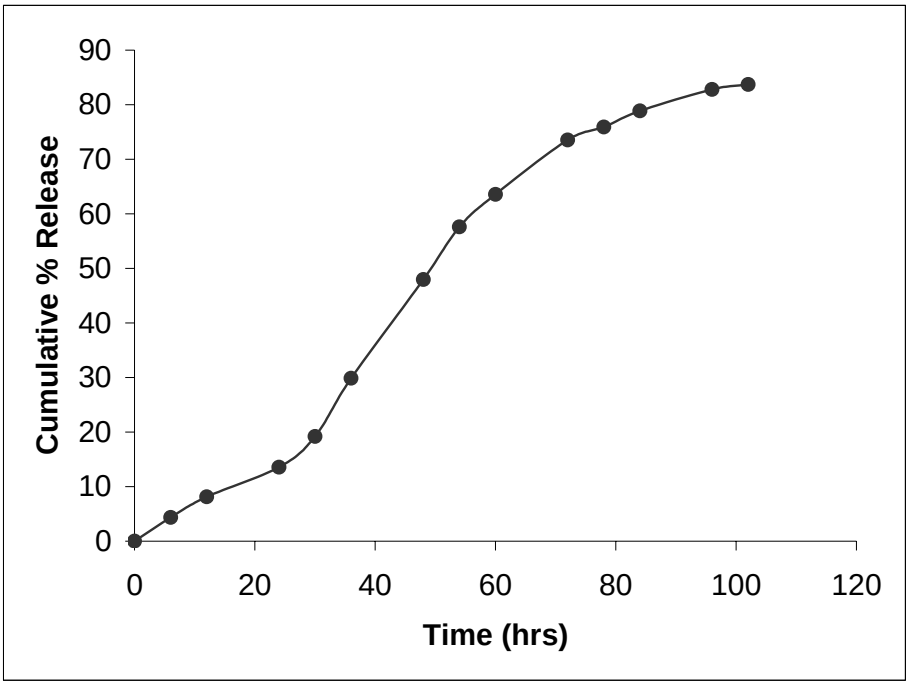


Table: 18

The *in vitro* release data of M_6 formulation

[1:15:1 Drug: PC: Chol, sonicated]

S.No	Time in hrs	Drug Concentration (mg)	Percentage drug Release	Cumulative % Release
1	6	2.25	5.65	5.65
2	12	4.5	11.3	11.32
3	24	9.0	22.6	22.65
4	30	11.5	28.89	28.98
5	36	13.30	33.43	33.53
6	48	19.0	47.7	47.87
7	54	22.25	55.9	56.09
8	60	24.95	62.7	62.91
9	72	29.01	72.9	73.14
10	78	31.0	77.88	78.18
11	84	31.84	80.0	80.31
12	96	32.35	81.3	81.60
13	102	32.99	82.91	83.21
14	108	33.38	83.88	84.20
15	120	34.06	85.60	85.91

Figure: 13

The *in vitro* release profile of M_6S formulation

[1:15:1 Drug: PC: Chol, sonicated]

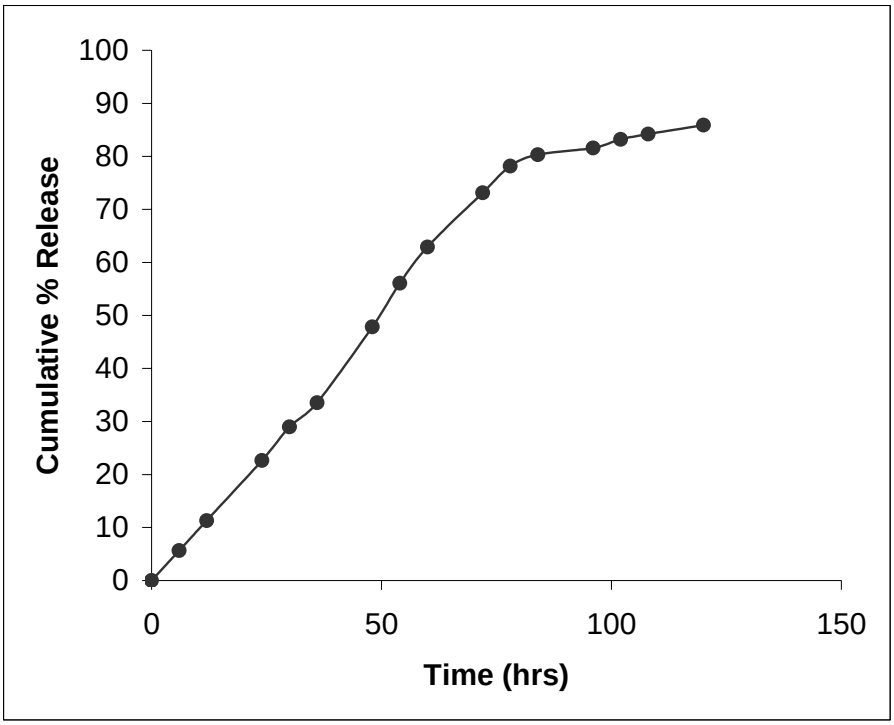


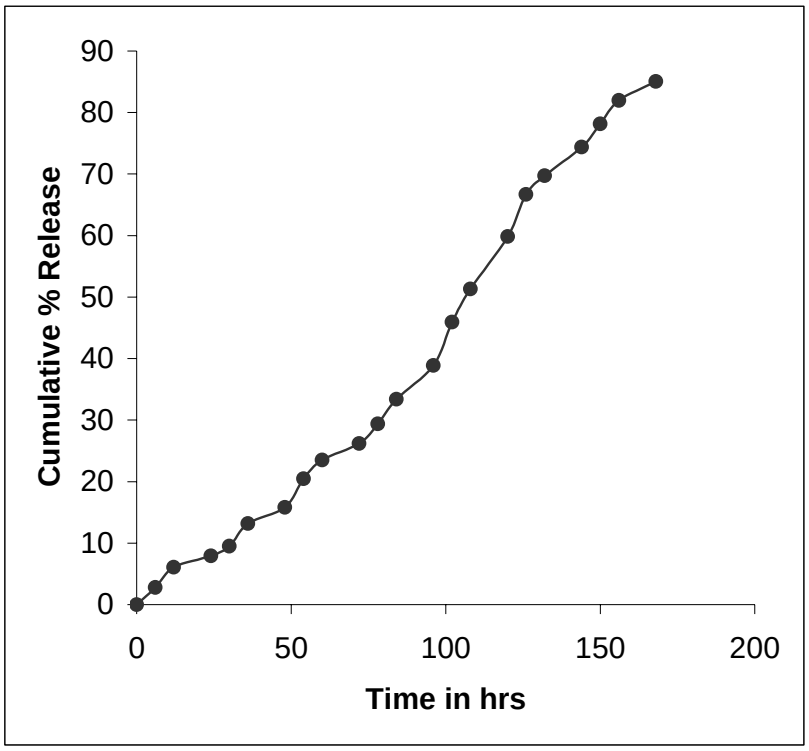
Table: 19

In vitro release data of optimized formulation M_3 and estimation by HPLC

S.No	Time in min	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	1.15	2.80	2.80
2	12	1.35	3.29	6.09
3	24	0.75	1.83	7.92
4	30	0.65	1.586	9.5
5	36	1.50	3.66	13.16
6	48	1.1	2.68	15.84
7	54	1.9	4.63	20.47
8	60	1.25	3.05	23.52
9	72	1.1	2.68	26.20
10	78	1.3	3.17	29.37
11	84	1.65	4.02	33.39
12	96	2.25	5.49	38.88
13	102	2.9	7.07	45.95
14	108	2.2	5.36	51.31
15	120	3.5	8.54	59.85
16	126	2.8	6.832	66.68
17	132	1.25	3.05	69.73
18	144	1.9	4.63	74.37
19	150	1.55	3.78	78.15
20	156	1.57	3.83	81.98
21	168	1.25	3.05	85.03

Figure: 14

In vitro release data of optimized formulation M_3 and estimation by HPLC



Stability studies

The bar diagram of percent drug remaining in liposomes after 15 and 30 days of exposure to 4, 27 and 40°C has been represented in Fig. 15. Liposomes have shown a fairly high retention of the drug in vesicles at a refrigerated temperature upto a period of one month (~90%), while storage at high temperature (40°C/75RH) lead to a substantial loss (~40%) of drug from liposomes at the end of one month period. The drug leakage at elevated temperatures may be related to the degradation of lipids in the bilayers resulting in defects in membrane packing making them leaky. The stability of liposomes may be explained on the basis of the greater affinity of phospholipid bilayers towards the drug molecule and also due to their better structural integrity. (Table: 20)

The *in vitro* release of the batch exposed to stability study at 4^0C did not show any marked decrease even after exposure of one month (Table: 21). Whereas the batch exposed to 40^0C/75%RH showed very less release exposed to that of unexposed batch (Table: 21). Hence, it is recommended to store the formulation at 4^0C.

Table: 20

Stability data of M_3 formulation

Temperature	Percentage of drug remaining	
	After 15 days exposure at	After 30 days exposure at
4°C	91.50	90.75
Room temperature	85.25	81.50
40°C / 75% RH	75.65	62.70

Figure: 15

Percent drug remaining of M_3 formulation at 4°C, Room temp, 40°C/75%RH

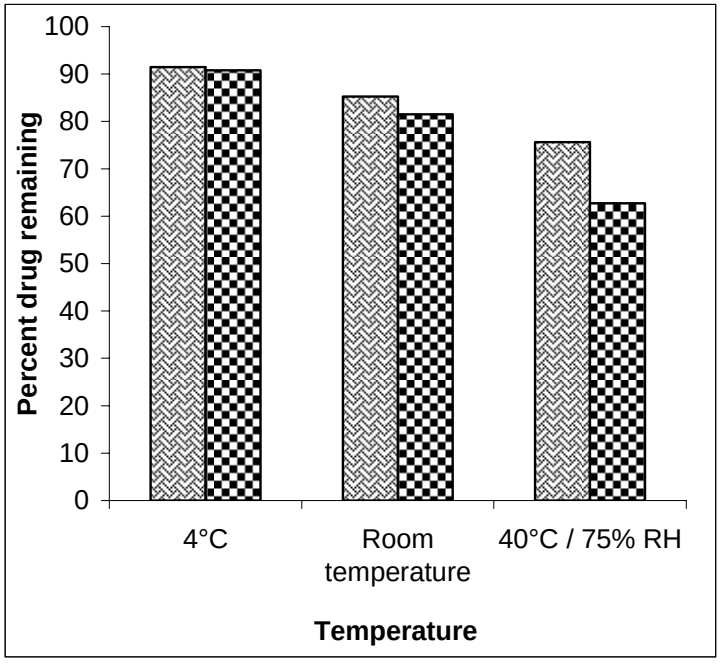


Table: 21

Invitro release data for optimized formulation M_3 subjected to stability study

Time in hrs	Cumulative % release for the sample stored at					
	4^0C		RT		40^0C/RH	
	After 15 days of exposure	After 30days of exposure	After 15 days of exposure	After 30days of exposure	After 15 days of exposure	After 30 days of exposure
6	5.97	4.88	4.23	3.80	2.71	2.32
12	9.79	9.05	8.37	7.09	7.61	4.89
24	14.69	14.21	12.97	12.51	10.34	7.28
30	20.68	20.51	19.24	16.33	14.15	12.50
36	26.68	26.09	25.59	20.15	20.14	16.32
48	36.47	35.17	33.76	29.39	27.76	23.39
54	42.49	41.57	39.76	35.40	33.22	27.86
60	48.37	47.39	45.21	40.85	38.13	30.40
72	56.91	56.56	53.38	49.02	44.12	33.75
78	62.65	62.87	57.21	52.85	49.03	37.56
84	69.17	68.11	64.83	59.92	52.85	41.20
96	75.19	75.73	70.42	67.01	60.47	44.10
102	80.10	79.02	75.19	70.84	61.58	47.37
108	83.38	80.70	76.84	73.57	63.43	49.55
120	85.34	83.92	77.93	74.66	64.85	53.44
126	87.20	85.56	78.92	75.75	65.92	55.00
132	87.75	87.09	80.19	77.39	67.03	56.09
144	88.84	87.53	81.20	78.48	68.67	56.75
150	89.93	87.64	82.29	79.03	69.22	57.73
156	89.93	88.77	83.38	80.11	69.98	59.36
168	-	-	83.93	80.12	70.31	60.45

Table: 22

Comparison of optimized Methotrexate formulation M_3 with the batch exposed to stability study

S.No	Parameters	M_3	After 15 days exposure at			After 30 days exposure at		
			4ºC	Room temp	40ºC/75 %RH	4ºC	Room temp	40ºC/75 %RH
1	Percentage entrapment efficiency	92.10	91.50	85.25	75.65	90.75	81.50	62.75
2	Cumulative % release	91.69	89.93	84.75	70.31	88.77	80.12	60.45

Table: 23

Physio chemical parameter of Liposomal formulations of Methotrexate

Form. Code	Composition Drug:PC:Chol	Vesicle Size (µm)	Drug % entrapped	Max. Release till(h)	Cumulative % Release
M_1	1:5:1	8.52±0.19	66.10 ± 0.26	168	66.22
M_1S	1:5:1 sonicated	<0.5	49.50 ± 0.24	144	58.70
M_2	1:10:1	7.25±0.26	75.90 ± 0.48	168	73.09
M_2S	1:10:1 sonicated	<0.5	60.50 ± 0.30	126	67.20
M_3	1:15:1	**100nm**	92.10 ± 0.62	168	**91.69**
M_4	1:15:1 Stearylamine	3.2±0.36	83.00 ± 0.63	168	81.16
M_5	1:15:0	4.5±0.15	80.10 ± 0.32	102	83.72
M_6S	1:15:1 sonicated	<0.5	79.06 ± 0.52	120	85.91

S - Sonicated

9. Coumarin epoxide Liposomes

9.1 Development of Coumarin epoxide liposomes

Based on results obtained from Methotrexate liposomes, Coumarin epoxide loaded liposomes with high entrapment efficiency and desired drug release were formulated. MLVs and SUVs of Coumarin epoxide were prepared by thin film hydration method, characterized and evaluated for their *in vitro* drug release and stability on different storage conditions.

9.2 Evaluation of Coumarin epoxide Liposomes

9.2.1 Removal of unentrapped drug from liposomes

The unentrapped drug and unencapsulated lipid molecules were removed from liposomes by dialysis technique. Maximum encapsulation was seen in C_2 since the percentage of drug dialysed was less.

Table: 24

Physiochemical parameter of Coumarin Epoxide (Cu-2) liposomes

Formulation code	Drug: PC:CHOL	Amount of drug dialysed in mg	Percentage of drug unentrapped	Vesicle size in (mean ±SD)µm	Percentage Entrapped
C_1	1 : 15 : 0	09.50 ± 0.5	19.00 ± 0.26	4.5 ± 0.12	83.2 ± 0.48
C_2	1 : 15 : 1	07.65 ± 0.24	15.30 ± 0.48	100 nm	94.4 ± 0.26
C_3	1 : 15 : 1 (with stearylamine as charge inducer)	10.80 ± 0.17	21.60 ± 0.34	3.75 ± 0.15	81.2 ± 0.44
C_2S	1 : 15 : 1 sonicated	14.35 ± 0.33	28.70 ± 0.66	< 100 nm	77.2 ± 0.22

9.2.2 Vesicle size determination

The atomic force microscopy images of selected C_2 Liposome formulation which contains 15:1 PC: CHOL ratio is presented in fig: 16. The size ranges of all other liposomal formulations were found to be 0.5 μm-5 μm. The maximum number of particles of C_1, C_3 is observable under ordinary optical microscope but the maximum numbers of particles of C_2 formulation are not observable under ordinary microscope. Inclusion of charge inducing substance stearylamine does not influence the particle size. Sonicated vesicles have shown the vesicles size in the range below 100 nm. The vesicle size of the various Coumarin epoxide liposomes are shown in the table: 24.

9.2.3 Percentage Drug Entrapment Efficiency

The percentages of drug encapsulated in liposomes are given in Table: 24. Percentage of drug encapsulation varies when the lecithin, cholesterol proportion were varied. A good percentage of drug encapsulation was achieved from C_1, C_2, C_3 formulations and the least percentage of drug encapsulation are observed in C_2S formulation. Formulation containing stearylamine(C_3) has not shown any significant changes in percentage encapsulation efficiency.

9.2.4 *In vitro* drug release of Coumarin epoxide (Cu-2)

In vitro drug release was studied for all the batches of Coumarin epoxide Liposomes. The cumulative percentage release of Coumarin epoxide from various formulations was found to be 83.47%, 90.41%, 79.74% and 90.04% for C_1, C_2, C_3 and C_2 S respectively.

An almost constant drug release was observed in all formulations indicating zero order release pattern.

The results indicated that C_1 formulation with no cholesterol in them released their content rapidly. Compared to C_2, the C_1 formulation has shown release of 83.47% for 102 hours. (Table: 25, Fig 17)

The inclusion of charge inducing substance Stearylamine slows the release rate as well as overall release in formulation C_3. (Table: 27, Fig 19)

A sonicated vesicle (C_2S formulation) has shown satisfactory release but faster release rate. (Table: 28, Fig 20)

C_2 formulation shows the higher encapsulation efficiency and desired sustained release of drug up to 168 hours. Hence, C_2 formulation is the optimized one. (Table: 26, Fig 18)

HPLC datas of *in vitro* samples (pH 7.4 buffer as the release medium) was found to be low (Table: 31). This might be due to the opening of epoxide ring in alkaline environment. Hence, it was decided to run the *in vitro* study using water as the release medium. The UV (Table: 29) and HPLC (Table: 30) data were found to be well correlated when water was used as release medium.

Table: 25

The *in vitro* release data of C_1 formulation

[Formulation without cholesterol]

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	1.75	4.21	4.21
2	12	3.25	7.83	7.85
3	24	6.50	15.66	15.69
4	30	10.25	24.69	24.76
5	36	14.75	35.54	35.64
6	48	20.75	50.00	50.14
7	54	23.50	56.62	56
8	60	26.50	63.85	64.08
9	72	32.25	77.71	77.96
10	78	33.25	80.12	80.43
11	84	34.00	81.92	82.24
12	96	34.25	82.53	82.85
13	102	34.50	83.13	83.47

Figure: 17

The *in vitro* release profile of C_1 formulation

[Formulation without cholesterol]

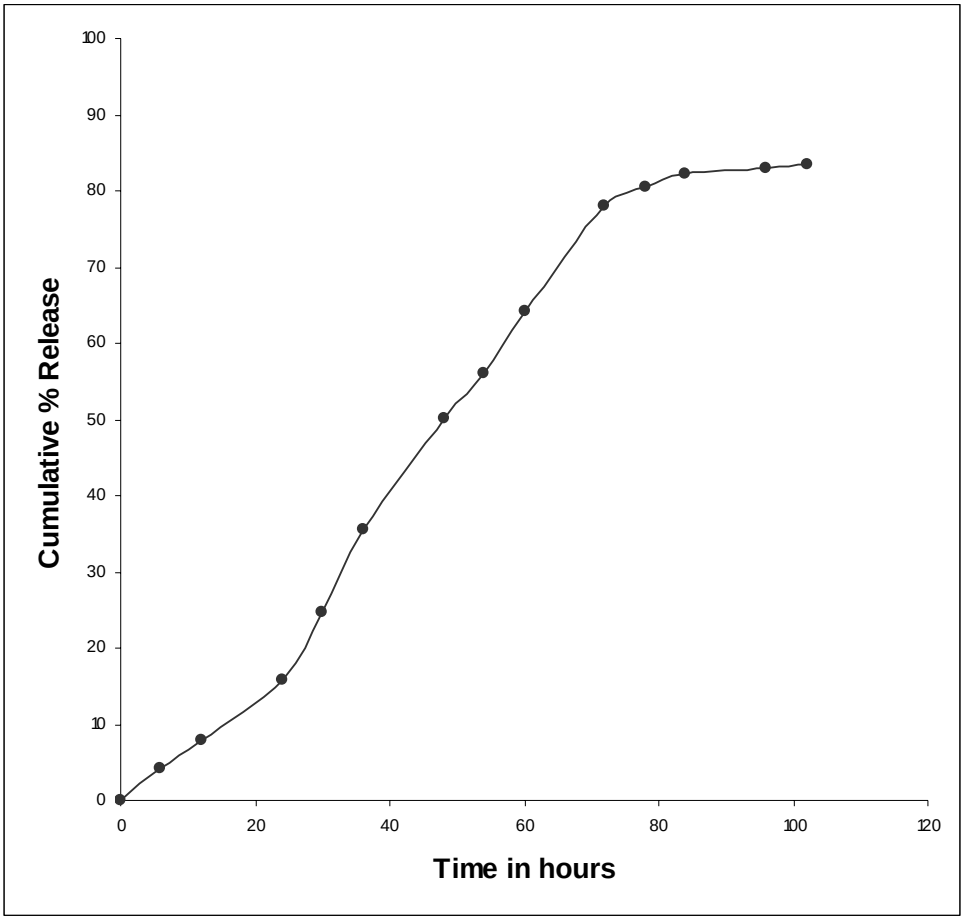


Table: 26

The i*n vitro* release data of C_2 formulation

[1:15:1 Drug: PC: Chol]

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	1.25	2.76	2.76
2	12	2.50	5.52	5.54
3	24	4.75	10.49	10.51
4	30	8.50	18.78	18.82
5	36	10.25	22.65	22.72
6	48	15.50	34.25	34.34
7	54	18.75	41.43	41.57
8	60	21.25	46.96	47.12
9	72	24.75	54.69	54.88
10	78	26.50	58.56	58.78
11	84	29.25	64.64	64.87
12	96	33.50	74.03	74.29
13	102	34.75	76.79	77.09
14	108	35.50	78.45	78.76
15	120	36.75	81.53	81.53
16	126	37.25	82.64	82.64
17	132	38.50	85.08	85.40
18	144	39.25	86.74	87.08
19	150	39.75	87.84	88.19
20	156	40.25	88.95	89.30
21	168	40.75	90.15	90.41

Figure: 18

The i*n vitro* release profile of C_2 formulation

[1:15:1 Drug: PC: Chol]

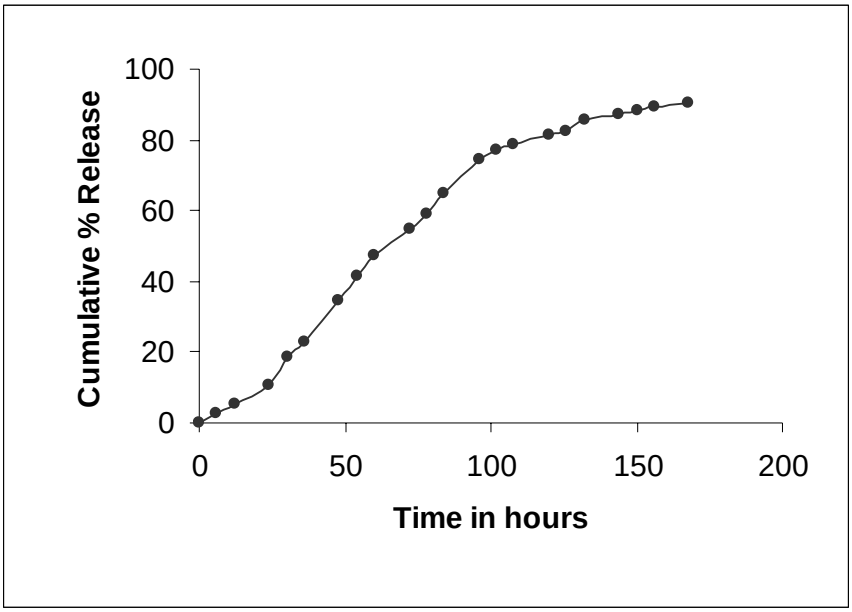


Table: 27

The i*n vitro* release data of C_3 formulation

[Formulation containing Stearylamine]

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	01.25	3.07	3.07
2	12	02.50	6.15	6.16
3	24	04.75	11.68	11.70
4	30	06.25	15.37	15.42
5	36	08.50	20.91	20.97
6	48	11.75	28.90	28.98
7	54	13.25	32.59	32.71
8	60	15.50	38.13	38.26
9	72	18.75	46.12	46.32
10	78	20.25	49.81	49.99
11	84	21.50	52.89	53.08
12	96	24.75	60.88	61.07
13	102	25.50	62.73	62.92
14	108	26.25	64.57	64.85
15	120	28.15	68.88	69.05
16	126	28.75	70.72	70.99
17	132	29.50	72.57	72.87
18	144	30.25	74.41	74.62
19	150	31.50	77.49	77.72
20	156	32.00	78.72	79.02
21	168	32.25	79.43	79.74

Figure: 19

The i*n vitro* release profile of C_3 formulation

[Formulation containing Stearylamine]

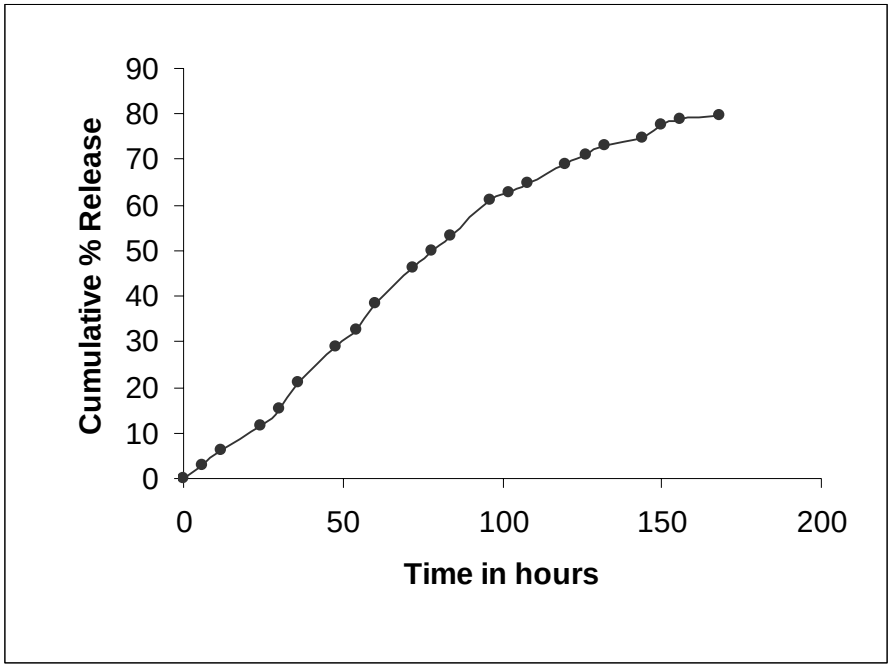


Table: 28

The *in vitro* release data of C_2S formulation

[1:15:1 Drug: PC: Chol, sonicated]

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	3.00	7.77	7.77
2	12	4.50	11.65	11.68
3	24	8.04	20.83	20.87
4	30	9.24	23.96	24.02
5	36	12.00	31.00	31.18
6	48	15.11	39.15	39.26
7	54	17.75	45.98	46.14
8	60	20.00	51.80	51.99
9	72	25.27	65.23	65.67
10	78	27.00	69.94	70.21
11	84	29.50	76.42	76.70
12	96	32.58	84.42	84.70
13	102	33.00	85.49	85.82
14	108	33.74	87.43	87.75
15	120	34.50	89.37	89.72
16	126	34.62	89.71	90.04

Figure: 20

The *in vitro* release profile of C_2S formulation

[1:15:1 Drug: PC: Chol, sonicated]

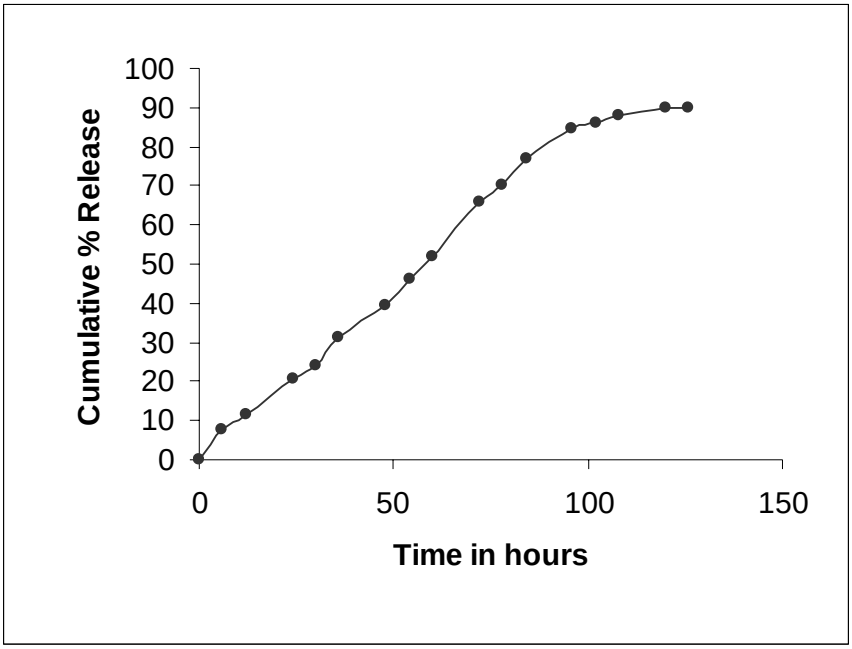


Table: 29

***In vitro* release data of optimized formulation C_2**

[1:15:1 Drug: PC: Chol] in water and estimation by UV

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	0.9	2.06	2.06
2	12	1.7	3.90	5.96
3	24	2.2	5.05	11.01
4	30	2.45	5.63	16.65
5	36	4.25	9.77	26.41
6	48	3.75	8.62	35.03
7	54	3.25	7.47	42.5
8	60	4.5	10.34	52.84
9	72	2.65	6.09	58.93
10	78	2.75	6.32	65.25
11	84	2.35	5.40	70.65
12	96	1.75	4.02	74.67
13	102	1.5	3.44	78.11
14	108	1.75	4.02	82.13
15	120	0.75	1.72	83.85
16	126	1.00	2.29	86.14
17	132	1.50	1.15	87.29
18	144	0.75	1.72	89.01
19	150	0.50	1.15	90.16
20	156	0.50	1.15	91.31
21	168	0.50	1.15	92.46

Figure: 21

***In vitro* release profile of optimized formulation C_2**

[1:15:1 Drug: PC: CHOL] in water and analysis by UV

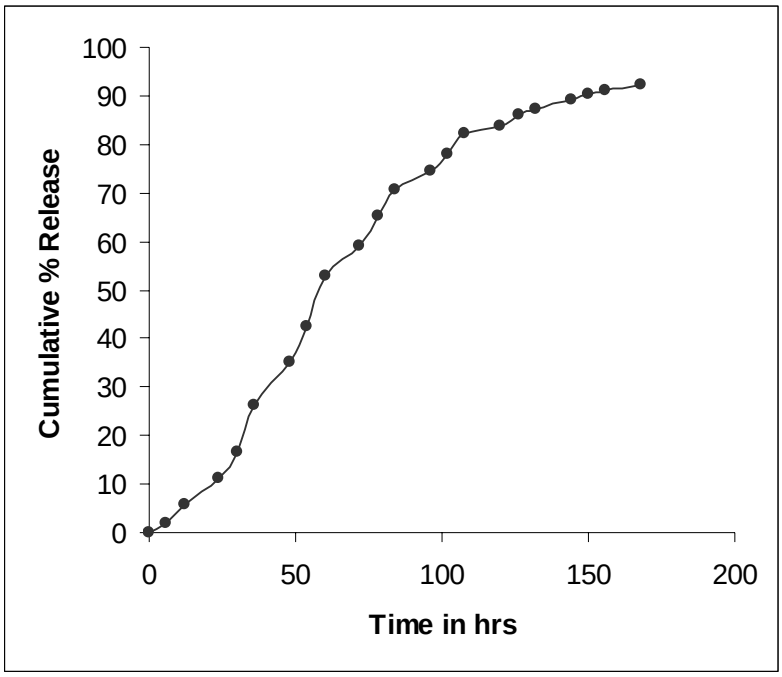


Table: 30

***In vitro* release data of optimized formulation C_2**

[1:15:1 Drug: PC: Chol] in water and analysis by HPLC

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	0.375	0.86	0.86
2	12	0.750	1.72	2.58
3	24	2.250	5.17	7.75
4	30	2.270	6.32	14.07
5	36	4.375	10.05	24.12
6	48	4.125	9.48	33.6
7	54	2.750	6.32	39.92
8	60	4.500	10.34	50.26
9	72	4.000	9.19	59.45
10	78	2.625	6.03	65.48
11	84	1.625	3.73	69.21
12	96	1.250	2.87	72.08
13	102	1.500	3.44	75.52
14	108	1.250	2.87	78.39
15	120	0.875	2.01	80.40
16	126	0.625	1.43	81.83
17	132	0.500	1.15	82.98
18	144	0.625	1.43	84.41
19	150	1.000	2.30	86.71
20	156	0.500	1.15	87.86
21	168	0.500	1.15	89.01

Figure: 22

***In vitro* release profile of optimized formulation C_2**

[1:15:1 Drug: PC: Chol] in water and analysis by HPLC

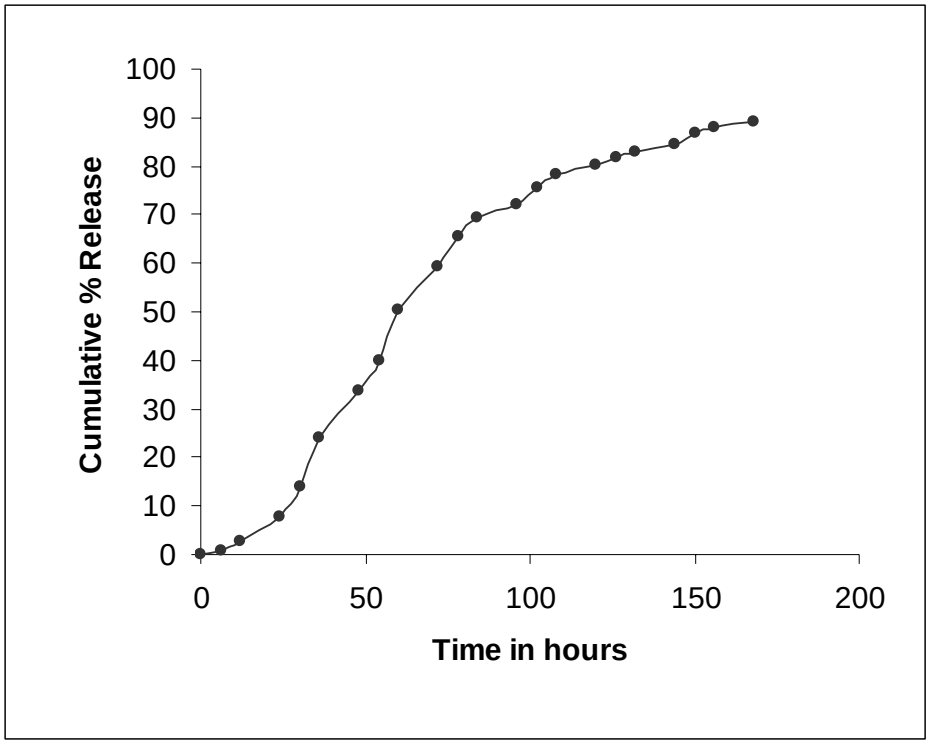


Table: 31

***In vitro* release data of optimized formulation C_2 [1:15:1 Drug: PC: Chol]**

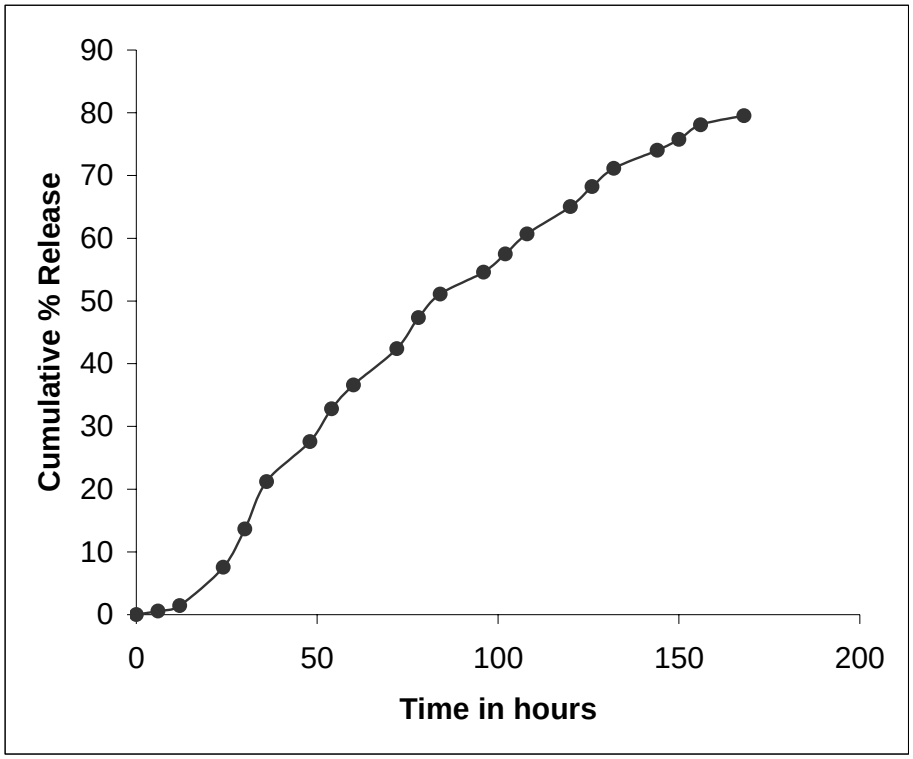
In phosphate buffer saline pH 7.4 and estimation by HPLC

S.No	Time in hrs	Drug concentration in mg	Percentage drug release	Cumulative % release
1	6	0.25	0.58	0.58
2	12	0.375	0.872	1.45
3	24	2.625	6.10	7.55
4	30	2.625	6.10	13.65
5	36	3.25	7.55	21.20
6	48	2.75	6.39	27.59
7	54	2.25	5.23	32.82
8	60	1.625	3.77	36.59
9	72	2.5	5.81	42.40
10	78	2.125	4.94	47.34
11	84	1.625	3.77	51.11
12	96	1.5	3.48	54.59
13	102	1.25	2.90	57.49
14	108	1.375	3.19	60.68
15	120	1.875	4.36	65.04
16	126	1.375	3.19	68.23
17	132	1.25	2.9	71.13
18	144	1.25	2.9	74.03
19	150	0.75	1.74	75.77
20	156	1.00	2.32	78.09
21	168	0.625	1.45	79.54

Figure: 23

***In vitro* release profile of optimized formulation C2 [1:15:1 Drug: PC: Chol]**

in phosphate buffer saline pH 7.4 and analysis by HPLC



Stability Studies

The bar diagram (Figure: 24) depicts the percent drug remaining from liposomes over the 30 days period, at different storage temperatures. Substantial loss of drug, i.e., approximately12% to 25% was evident from the samples stored at elevated temperatures, i.e., room temperature and 40ºC, respectively. On the other hand, at lower temperature conditions, i.e., 4ºC, the liposomes could retain 95% of the incorporated drug.

Table: 32

Percentage drug remaining of C_2 formulation after stability studies at 4°C, Room temp, 40°C/75%RH

Temperature	Percentage drug remaining	
	After 15 days	After 30 days
4°C	89.75	88.50
Room temp	84.50	80.50
40°C /75%RH	73.75	64.25

The *in vitro* release of the batch exposed to 4^0C did not show much change in its release pattern when compound to unexposed batch. Where as the batch exposed to higher temperature shows much decreases in its release (Table: 33)

Figure: 24

Bar diagram of Percentage drug remaining of C_2 formulation after stability studies at 4°C, Room temp, 40°C/75%RH

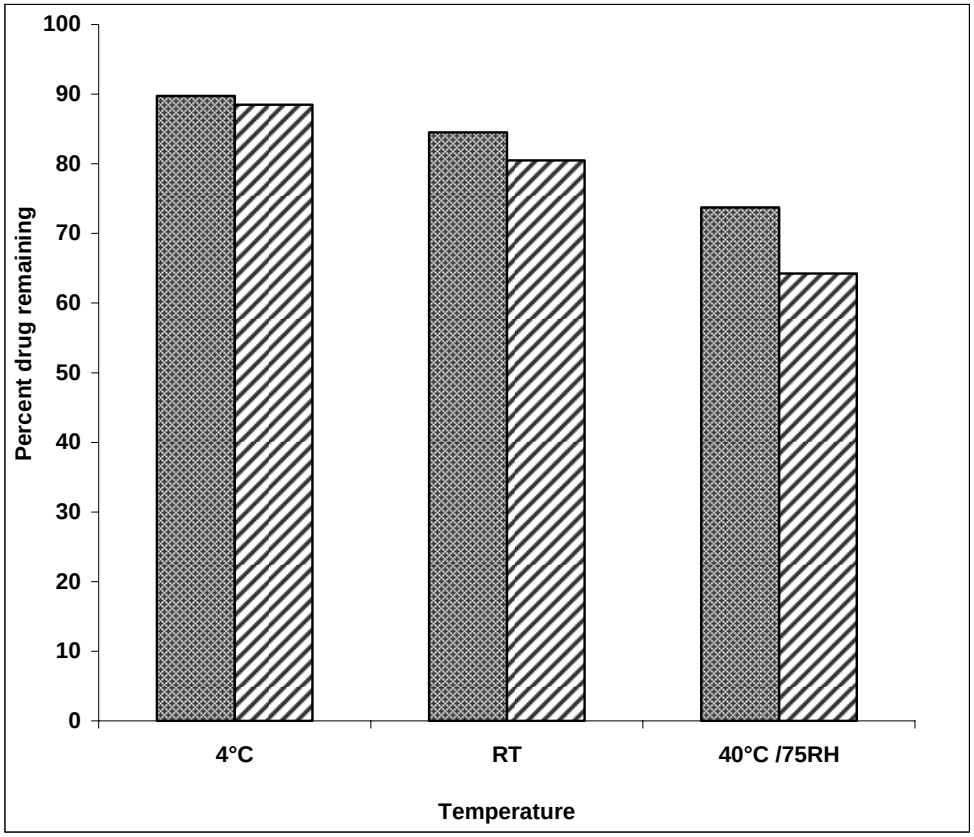


Table: 33

Invitro release data for optimized formulation C_2 subjected to stability study

Time in hrs	Cumulative % release for the sample stored at					
	4^0C		RT		40^0C/RH	
	After 15 days of exposure	After 30days of exposure	After 15 days of exposure	After 30days of exposure	After 15 days of exposure	After 30 days of exposure
6	3.32	2.91	2.47	1.98	1.32	1.10
12	5.58	4.87	4.54	3.83	3.81	3.32
24	9.45	9.20	7.40	7.13	8.52	8.15
30	17.20	16.75	11.65	10.12	11.53	11.55
36	21.11	20.45	15.86	13.49	16.35	16.45
48	33.87	29.61	22.81	18.17	22.39	23.39
54	40.53	34.52	28.70	22.46	28.13	26.89
60	46.05	41.82	32.41	26.14	33.11	30.98
72	53.80	48.27	38.60	30.28	38.55	33.78
78	57.15	52.29	44.01	34.91	43.64	41.83
84	60.99	59.39	48.72	38.28	48.64	44.62
96	70.39	66.82	55.09	44.80	53.71	47.61
102	74.45	71.58	58.11	47.26	56.98	50.32
108	77.01	75.81	61.89	55.13	59.46	52.21
120	78.75	78.28	66.31	61.93	64.82	53.52
126	81.51	81.46	69.12	64.71	66.83	56.70
132	83.18	83.81	73.71	70.54	68.24	58.08
144	84.85	84.75	76.73	74.60	69.28	59.74
150	85.42	86.47	79.19	75.99	72.13	60.55
156	88.18	86.99	81.71	77.92	73.41	62.26
168	89.08	87.45	84.12	80.09	75.12	63.54

Table: 34

Comparison of Coumarin epoxide in optimized formulation C_2 with the batch exposed to stability study

S.No	Parameters	C_2	After 15 days of exposure at			After 30 days of exposure at		
			4ºC	Room temp	40ºC/75 %RH	4ºC	Room temp	40ºC/75 %RH
1	Percentage entrapped	91.20	89.75	84.5	73.75	88.5	80.50	64.25
2	*cumulative* release	90.41	89.08	84.12	75.12	87.45	80.09	63.54

Table: 35

Comparison of Vesicle size, Entrapment efficiency, Cumulative release and % drug remaining of Coumarin epoxide liposomes

Form. Code	Compo-Sition(Drug:PC:Chol	Charge induced	Vesicle Size (μm)	Drug % entrapped	Max. Release till(h)	Cumulative % Release
C_1	1:15:0	No	4.5±0.12	83.2±0.48	102	83.47
C_2	1:15:1	No	100 nm	90.5±0.26	168	90.41
C_3	1:15:1	Yes	3.75±0.15	81.2±0.44	168	79.74
C_2S	1:15:1	Yes	< 200nm	77.2±0.22	126	90.04

S - Sonicated

10.0 KINETICS OF DRUG RELEASE

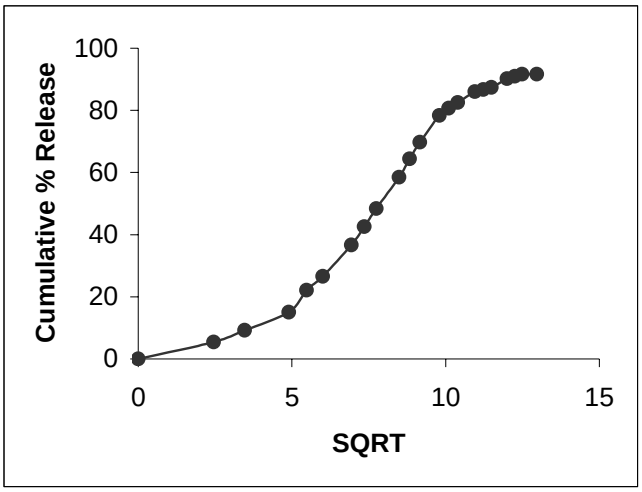
The optimized formulation M_3 (Methotrexate) and C_2 (Coumarin epoxide) were subjected to graphical treatment to assess the kinetics of drug release.

A plot of cumulative % release versus time showed linearity in both optimized formulations of methotrexate and coumarin epoxide. Hence it follows zero order kinetics. (Ref. Fig. 25, 28)

Higuchi's plot confirms that the release is diffusion mediated in both M_3 and C_2 formulation.

Figure: 25

Higuchi's plot for optimized formulation M_3



Regression = 0.9865

Figure: 26

Zero order plot for optimized formulation M_3

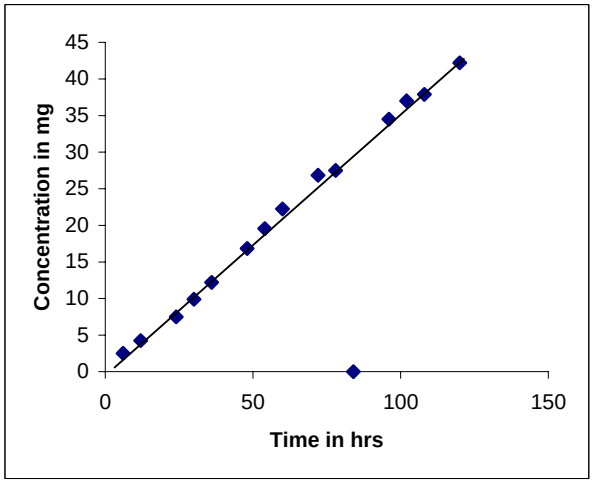
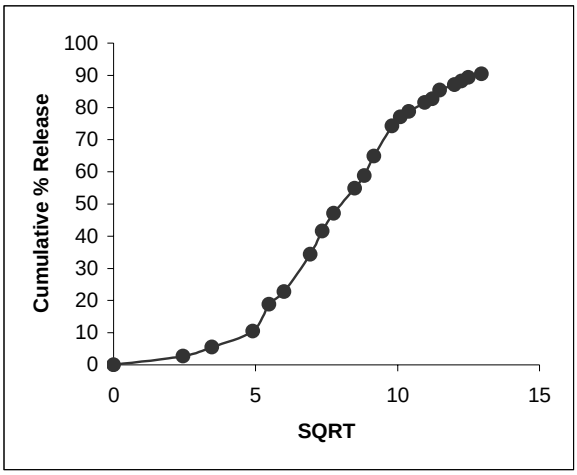


Figure: 27

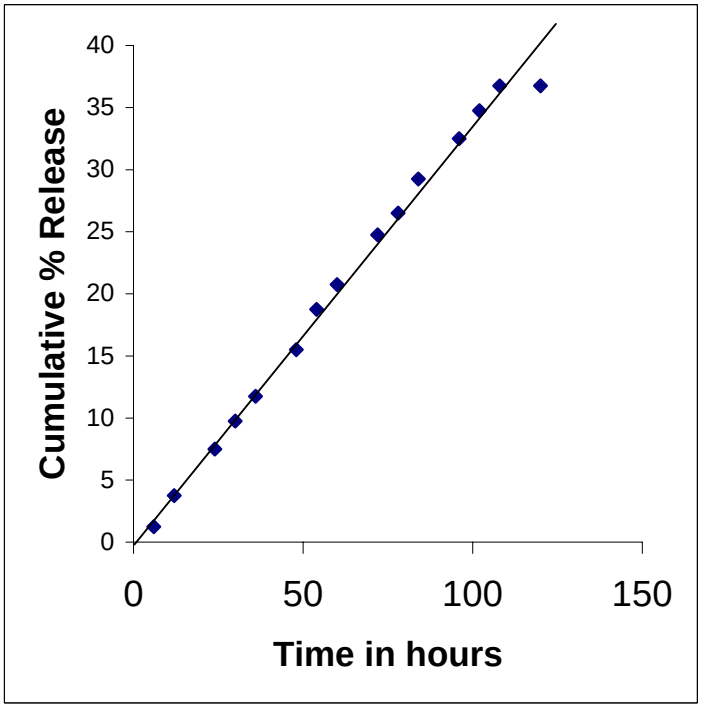
Higuchi's plot for optimized formulation C_2



Regression = 0.9832

Figure: 28

Zero order plot for optimized formulation C_2



10.1 CONCLUSION

An effort was made to formulate the liposomes by thin film hydration technique. Process variables influencing the formulation were vacuum pressure maintained for evaporation of solvent, speed of flask rotation, hydration volume, hydration time and hydration temp.

At an optimal vacuum of 355mm Hg, the lipid films were translucent and on hydration gave a better drug entrapment. The speed of flask rotation in flask evaporator was optimized at 150 rpm with the hydration time of 60 min. Hydration volume of 5 ml and hydration temperature of 60 ± 2^0C produced the liposomes of required vesicle size. The optimized ratio of Cholesterol: Phosphatidyl Choline was 1:15.

Based on the above optimized variables, an effort was made to formulate methotrexate and coumarin epoxide liposomal delivery system.

The hydration medium used for methotrexate liposomal formulation was pH 7.4 buffer. While using the buffer as hydration and in vitro release medium, the coumarin epoxide liposome formulation showed a decrease in cumulative percentage release. This fact was evident when the invitro samples were analysed by HPLC than that of by UV method. The presence of alkali in pH 7.4 buffer might have opened the ring of coumarin epoxide. This would have been the reason for decreased release. Hence, it was decided to use only double distilled water as hydration medium and also as in vitro release medium.

Compared to sonicated type, the unsonicated liposomes have shown the release for an extended duration. Similarly, the addition of charge inducer Stearylamine was not beneficial in extending the release of encapsulated drug.

Among the methotrexate formulations tried, the batch coded as M_3 containing 1:15:1 ratio of Drug Phosphatidyl Choline:Cholesterol showed an extended cumulative percentage release of 85.03% (in vitro release samples estimated by HPLC) for 168h; Atomic florescence microscopy (AFM) revealed the average vesicle size as 100 nm. The amount of drug originally got entrapped in the vesicles was 92.10%.

Among the coumarin epoxide liposomes tried, the batch coded as C_2 containing 1:15:1 ratio of drug:phosphatidylcholine:cholesterol showed an extended cumulative percentage release of 89.01% for 168h (in vitro release samples estimated by HPLC). AFM showed the average particle size as 100 nm. The percentage of drug entrapment originally was found to be 90.5%

From the above discussion, we can conclude that formulation M_3 of methotrexate and C_2 of coumarin epoxide were the optimized batches. They were found to follow zero order release pattern as revealed by the linearity shown from the plot of time versus concentration. Higuchi plot proved that the release from formulation M_3 and C_2 was diffusion mediated.

Optimized formulations of M_3 and C_2 were stable with respect to the amount of drug retained for a period of 4 weeks at 4^0C and affirm that the drug leakage increased at a higher temperature.

Thus, liposomal coumarin epoxide of formulation C_2 (drug:phosphatidylcholine:cholesterol::1:15:1) has achieved the objectives of better drug entrapment, minimum leakage and smaller vesicle size showing an extended release equivalent as that of standard, methotrexate.

As an extension of this work, the formulation C_2 may be linked to a suitable monoclonal antibody to produce conjugated immuno liposomes.

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