"DEVELOPMENT AND OPTIMIZATION OF APIGENIN NANOPARTICLE FOR TARGETING BREAST CANCER"

Thesis submitted to

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH (BELAGAVI)

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For the award of the degree of

Doctor of Philosophy
In the Faculty of Pharmacy

By

Priya P.Shetti M.Pharm.

(Registration No: KLEU/Ph.D./18-19/DO1218013)

Under the Guidance of

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February - 2022

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Date: **Mrs. Priya P Shetti**

Place: Belagavi

Table of content

List of abbreviations

ACN	:	Acetonitrile
FA	:	Formic acid
ALP	:	Alkaline phosphatase
ALT	:	Alanine transaminase
AST	:	Aspartate transaminase
ANOVA	:	Analysis of variance
API	:	Apigenin
APISL	:	Apigenin loaded stealth liposome
BC	:	Breast Cancer
Chol	:	Cholesterol
cm	:	Centimeter
CO_2	:	Carbon dioxide
DDS	:	Drug delivery system
DMEM	:	Dulbecco's Modified Eagle's medium
DMSO	:	Dimethyl sulphoxide
DSC	:	Differential Scanning Calorimetry
DMBA	:	7, 12-Dimethylbenz[a]anthracene
EI	:	Ethanol injection
EE	:	Entrapment efficiency
EPR	:	enhanced permeation and retention
FBS	:	Fetal bovine serum
FT-IR	:	Fourier Transform infrared spectroscopy.
g	:	Gram
IC50	:	Half maximal inhibitory concentration

ICH	:	International Council for Harmonization
KBr	:	Potassium bromide
LOD	:	Limit of detection
LOQ	:	Limit of quantification
mg	:	Milligram
ml	:	Milliliter
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	:	Number of theoretical plates
ng	:	Nanogram
nm	:	Nanometer
ns	:	No significant
P	:	Probability
PBS	:	Phosphate buffer saline
PDI	:	polydispersity index
PEG	:	Polyethylene glycol
pH	:	power of hydrogen
PS	:	Particle size
ROS	:	Reactive oxygen species
RP-HPLC	:	Reversed-phase high performance liquid chromatography
RSD	:	Relative standard deviation
S	:	Slope
SD	:	Standard deviation
T	:	Tumor stage
T	:	Tailing factor
$t_{1/2}$	:	Half life
UV spectroscopy	:	Ultra violet spectroscopy

VS	:	Vesicle size
ZP	:	Zeta potential
°C: %	:	Percentage
µg	:	Microgram
µl	:	Microliter
µm	:	Micrometer
v/v	:	Volume by volume
w/w	:	Weight by weight
<	:	Less than
>	:	Greater than

List of tables

Abstract

Background

Breast cancer (BC) the most frequent type benign tumor in women, and it's amongst the most common type of tumor deaths worldwide, comprising 26-288 of their cancer cases and roughly 19% of breast cancer-related fatalities in underdeveloped nations, and it is the second largest cause of death (45%) next to cervical cancer. BC expanded by 10% in the female population during the last two decades. Clinicians find it challenging to cure the disease because of its destructive character. As a result, novel approaches for BC are urgently needed, as the usefulness of existing cytotoxic medications in controlling BC-related mortality has approached a level. Antioxidant molecules derived from plants that have various cancer treatment and chemoprevention techniques have been found that have anti-tumor characteristics. Apigenin (API) is a component of natural flavonoid medication with cancer-fighting effects. However, due to its poor pharmacokinetics properties, their clinical applicability is limited.

Objectives

The present research project aims to investigate the effect of Apigenin loaded stealth liposomes in BC. Hence the objectives of the study are:

1. To formulate, evaluate and optimize Apigenin loaded stealth liposomes.
2. To study anticancer activity of optimized Stealth liposomes against Breast Cancer (BC) by *in vitro* and *in vivo* methods.

Methodology

Apigenin loaded stealth liposomes (APISL) were prepared by ethanol injection method followed by probe sonication. The APISL was optimized using Design-Expert®

software and a 3^2 factorial design. Entrapment efficiency, particle size, shape, and Polydispersibility index were all evaluated in the developed formulations. In vitro release tests and *in-vitro* cell culture experiments (cytotoxicity) were performed on the optimized APISL8 formulation. 7, 12-Dimethylbenz[a]anthracene (DMBA carcinogen) caused (mammary gland tumors) BC to Female Sprague-Dawley (SD) rats were used to conduct in-vivo pharmacokinetic and pharmacodynamic (prophylactic and therapeutic treatment) tests with API and improved APISL.

Results

The improved stealth liposomes formulation (APISL) has a spherical particle size (PS) of 369nm, a Polydispersibility index (PDI) of 0.92, and a 73 percent encapsulation efficiency (EE). In-vitro drug release of API from APISL8 according to reports remained faster at pH 7.4 and drug release pattern was found up to 48hrs. In vitro cytotoxicity experiments in MCF-7 cells revealed that APISL8 has better antitumor effect than free API. When tried to compare to the free API group, pharmacokinetic analysis of APISL8 revealed greater API localization. When comparing the tumours of APISL8-treated rats to those of free API, pharmacodynamic analysis demonstrated a substantial reduction in tumours. Furthermore, when BC-bearing (mammary tumor) Sprague-Dawley (SD) rats were given APISL8. In comparison to the disease-free group, the liver bio marker enzymes (alanine transaminase, alkaline phosphatase, and aspartate transaminase) reduced dramatically. Histopathological investigation confirmed these findings, indicating that the DMBA-induced harmful effect on rat tumor formation could be reduced by APISL8 formulation therapy.

Conclusion

The treatment of API-loaded stealth liposomes greatly lowered the severity of the disease, according to these data. (Rat mammary tumor) They're a satisfactory nanoparticle drug delivery technology in treatment of BC, making it an attractive nanoparticle drug delivery technology for cancer therapy.

Keywords: Apigenin; stealth liposomes; breast carcinoma; cytotoxicity; pharmacokinetic; pharmacodynamic.

1. INTRODUCTION

1.1Background

Cancer cells have a number of distinct traits that set them apart from normal cells. To begin with, tumour cells struggle to regulate cell division and survival. There's also a lot of harm to the genome. Normal cells, on the other hand, often have genetic mutations; tumor cells' ability to spread is the second distinct property. Breast Cancer (BC) is the furthermost recurrent malignancy in females. Tumor grown at the site of terminal collecting duct of the breast. Prevalence of breast malignancy pattern changes across all over world and increased by different risk factors. [1]

In 2018, Breast cancer has been diagnosed in around 2.1 million women for the first time. The global occurrence of BC has been increased with yearly rises of 3.1%, commencement with 641,000 cases in 1980 and rise in breast cancer patient by >1.6 million trend is till continued. Indeed, the worldwide breast cancer severity and burden in women is increasing in countries irrespective of revenue level, change in daily routine life, and rise in population and on getting old population. [2]

1.2 Cause of BC & Pathogenesis of Breast cancer:

Breast cancer (BC) has a few biologically molecular causes, including that of the beginning of the receptor for human epidermal growth factor, the beginning of hormone receptors, the Oestrogen receptor, the progesterone receptor, and genetic abnormalities. The different risk factors are connected with breast cancer like patient age, generative factors, family history of patients, disease history of patient, genetic pre-disposition and ecological, economically issues are have been linked with a chance in risk for the growth of breast tumor [3].We predicted normal

adenocarcinoma to be the forerunner phase to invasive breast tumour growth. This type of cancer also progresses further to a metastatic level of disease, which are located at axillary duct nodes of the breast and are the most common at sites, but distant metastases can also be observed in bones and region of bone marrow and the most major bone sites. Tissue will be examined, and a biopsy or surgery would be performed. The breast can determine whether a tumour is benign or malignant by observing the tumour cells. [4, 5]

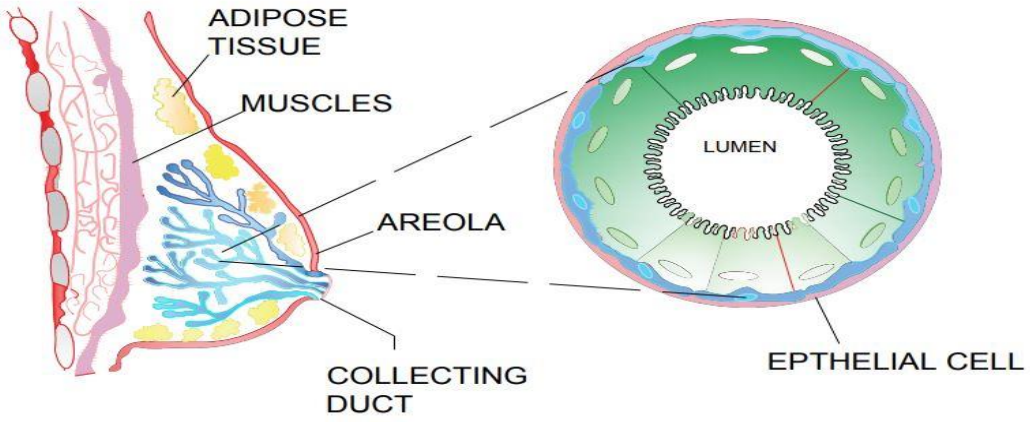


Fig no: 1 representing normal breast tissue

Classification of the breast cancer:

Breast cancer is divided into two categories. There may be significant differences in the cell types that give way to the malignant tumour within a specific tissue. Pericanalicular fibroadenoma, Intracanalicular fibmadenoma-cyto sarcomaphyllodes, and fibrosing adenomatosis are the two major histological groups of breast carcinomas. Breast cancers are divided into two kinds based on their invasiveness. [6, 7, 8]

- **Benign Type:** In this sort of cancer tumors not spread or occupy its surrounds.
    a) Epithelial - papilloma, adenorna
    b) Mixed (epithelial and mesodermal) - intra canalicular fibroadenoma, cystosarcoma phyllodes (Soft variant).
    c) Mesodermal - Lipoma, angioma, fibroma, myoma.
- **Malignant Type:** In this type of cancer tumor that spread its surrounds.

I. Mammary ducts

a) Non-Filtrating carcinoma - Papillary comcdocarcinoma

b) Infiltrating carcinoma (adcnoma sarcoma) - Papillary and comedocarcinoma - Carcinoma simplex - Colloid carcinoma - Pagct's disease.

II. Mammary Lobules

a) Non-infiltrating carcinoma in situ

b) Infiltration - lobular adenosarcoma

III. Epithelial or mesodermal origin from the skin and supporting tissue

a) From the skin of the breast cancer and its appendages - Dermoid cyst, sweat gland tumors, squarnous cell carcinoma of the skin

b) From the supporting tissues of the breast-sarcoma, liposarcoma etc

Because the choice of treatment between surgery, radiotherapy, and chemotherapy is dependent upon whether tumor is benign or malignant, and if the specific histological subgroup is responsive to radiotherapy or chemotherapy, such differentiation is critical for clinical diagnosis. The sensitivity of different breast

cancers to various cytotoxic medicines varies significantly. As a result, only when the tumour tissue has been precisely diagnosed can suitable therapy be provided.

Facts about Breast Cancer:

Sign & Symptoms of BC

Basically, symptoms observed skin irritation, lump or mass formation, Pain, and has uneven distribution of edges is more likely to be cancer. Breast cancer symptoms vary from person to person. Some individuals show no indications or symptoms at all. Breast cancer diagnostic indications are

• A new breast or underarm lump.

• Inflammation or thickening of a portion of the breast

• Surface roughness or irritation of the breast skin.

• Streaky tissue or redness in the nipple area or breast.

• Nipple pinching in or soreness in the nipple area.

• Red nipple discharge in combination to breast milk.

• Any alteration in the size or shape of the breasts.

• Breast discomfort in any region.

Risk Factors for Breast Cancer:

Most of the studies found that it's caused mostly in the women especially at a younger age group 30-40yrs. The main risk factors that may cause Breast malignancy are shown below chart.[9]

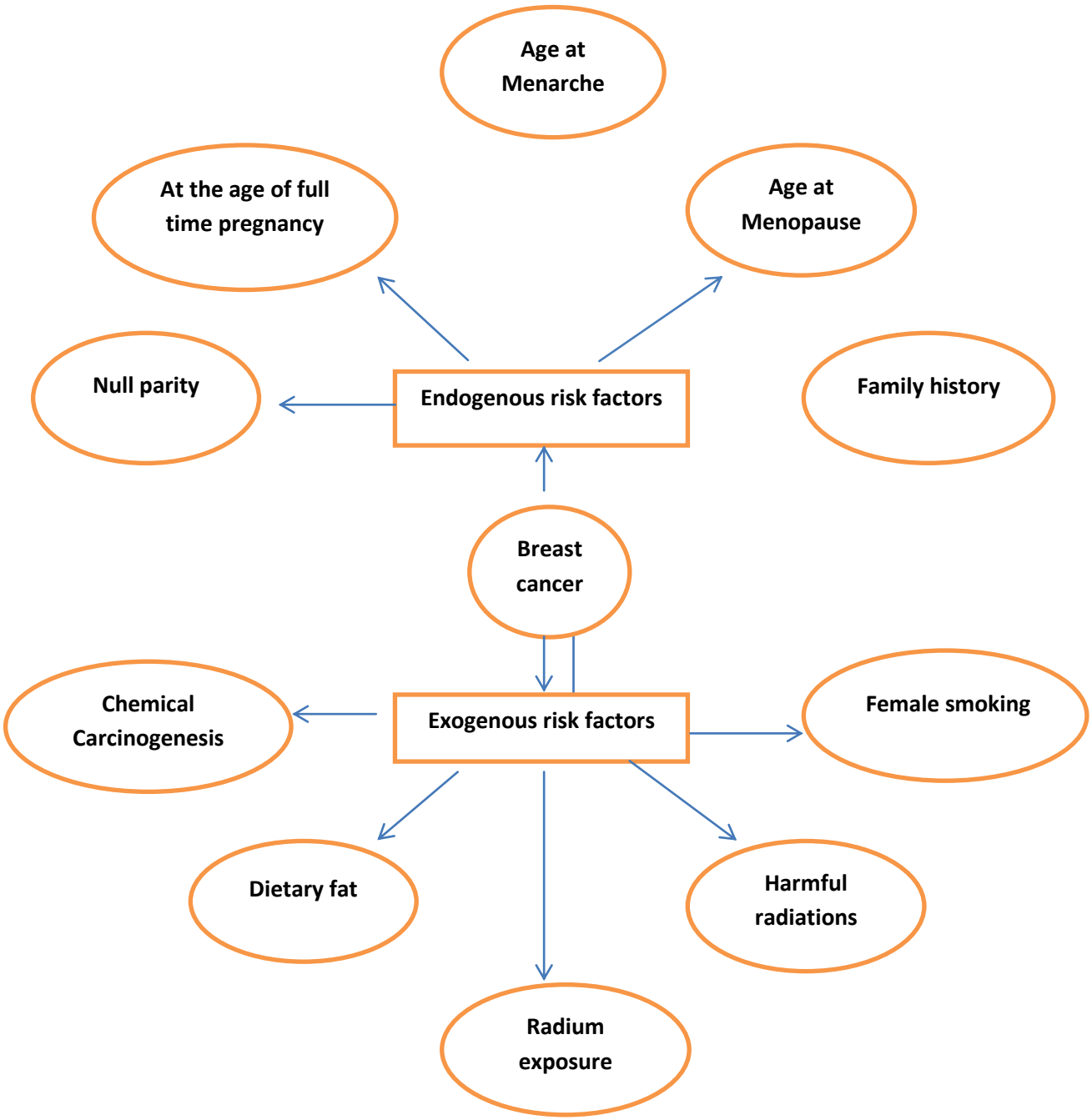


Fig no: 2 representing the risk factors of BC

Treatments of Breast Cancer:

The BC treatment option varies and is dependent on the phase of cancer. There are mainly two pathways to arrest the breast cancer growth. [10] These are of two main types:

1. **Local Treatment:** This type of treatment is limited to certain organ specific localized area i.e it includes only the tumor only and excludes the whole body.
   - **Surgery:** This treatment strategy is dependent on the features of the tumour. The tumour is removed along with the tissue in this type of malignancy.
   - **Radiation Therapy:** In this type of treatment such as radiation rays are used abolish cancer cells.
2. **General Treatment:** The normal chemotherapeutic agents used to inhibit the breast cancer growth are considered for the general treatment.

Chemotherapy: To minimize the risk of the cancer reoccurrence chemotherapy given prior to surgery to minimize the risk of extensive surgery.

- **Hormonal therapy:** The sex hormones like estrogen and progesterone therapy are used to treat the cancer.
- **Targeted therapy:** Targeted therapies, such antibodies, vaccinations, and gene therapy, are used to treat tumour growth over the coming years in a few steps.

Breast Cancer and a Natural Phytoconsituent:

Herbs and plants have been used for both medical and culinary purposes. Thus researchers have focused on various plant species that have immune-stimulating and anti-tumor capabilities. Different varieties of herbs include a wide range of phytochemicals that are active, sulphides, lignans, and plant sterols, as well as carotenoids, flavonoids, ligands, polyphenolic, and terpenoids, sulphides, lignans, and terpenoids. These flavonoids have a variety of mechanisms of action. They either inhibit cells from multiplying or activate a protective enzyme like glutathione transferase. Allium sativum, Echinacea, Curcuma longa, Arctium lappa, Camellia sinensis, Panax ginseng and Flax seed have biochemical characteristics. Studies have shown that the Extracts and juices of Withania somnifera, Amoora rohituka, Dysoxylum binectariferum, and Vaccinium macrocarpon were employed as anti-breast cancer agents, these herbs natural volatile oils, and extracts inhibit gene transcription, which slows cancer growth and cholesterol production. [11, 12, 13]

Apigenin, an active natural isolated compound:

The use of alternative medicine, herbs to prevent, treats, and control cancer-related issues has gotten a lot of press lately. Chamomile plant belongs to family Asteraceae and its among most widely used medicinal herb in world .Its scientific name is Matricaria chamomilla or Matricaria recutita. Chamomile has a wide range of biotic and pharmacologic actions, which includes including anti-inflammatory, antibacterial, anticancer, antispasmodic, and sedative. Despite numerous researches examining the possible effects of chamomile on metabolic markers in diabetics, no systematic review has been published that summarizes the findings. [14]

Fig no: 3 representing isolated compound Apigenin from Chamomile flower.

Apigenin and luteolin are now the most often utilized flavones for breast cancer treatment since they are low in toxicity and non-mutagenic. Apigenin, In MCF-7 and MDA-MB468 cells, this suppresses Rb phosphorylation and the MAPK pathway causes G2/M arrest in breast cancer cells, resulting in antiproliferative and apoptotic effects. In our research we have selected Apigenin. Apigenin is a common flavonoid; it is originated from a variety of fruits, vegetables. It includes onion plant, orange tree, Tea, chamomile, wheat at different seasoning.[15]. It arrests the cell proliferation in a different cancer cell and cell lines, which includes different types of the organ e.g Breast, Colon, Skin, Thyroid, Leukaemia, Prostate cancer [16]. Apigenin which is potentially used in cancer therapy. Apigenin is not mutagenic, unlike other structurally similar flavonoids. Although it has historically suppressed the proliferation of a number of different cancer cells, there are few findings demonstrating that it also inhibits the arrest the growth of human bladder cancer.

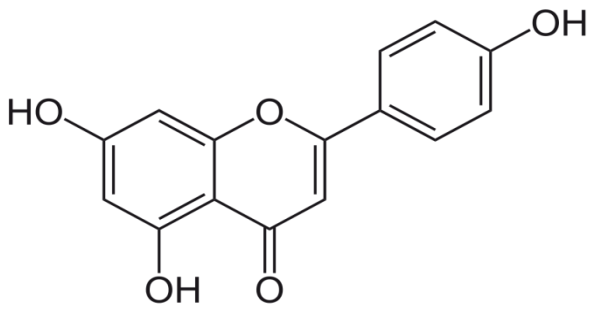


Fig no: 4 Chemical structural representation of Apigenin

Obtained from the different sources of plants, seeds, fruits. The isolated compound IUPAC name was with molecular formula $C_{15}H_{10}O_5$, which is also known as 5,7 -dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran 4-on and mass: 270.24 g mol-1. The chemical abstract number (CAS): 520- 36-5. It is isolated active from commercially as a light-yellow in colour and crystalline product. [17] Chemically it is flavonoid which is mostly abundant in fruits and vegetables. Apigenin is a possible active isolated flavonoid against different sort of ailments, used as actual potent antioxidant, especially in different types of cancer, and has anti-inflammatory activity. [18,19]. Apigenin is usually accessible in different dosage forms. Basically, the solubility of isolated active apigenin in different solvents which involves aqueous, Methanol, Ethanol. At different temperature T = 288.2 K to 328.2 K and at normal P = 0.1 MPa. [20, 21].

Apigenin as therapeutic agent for treatment of various diseases: [22]

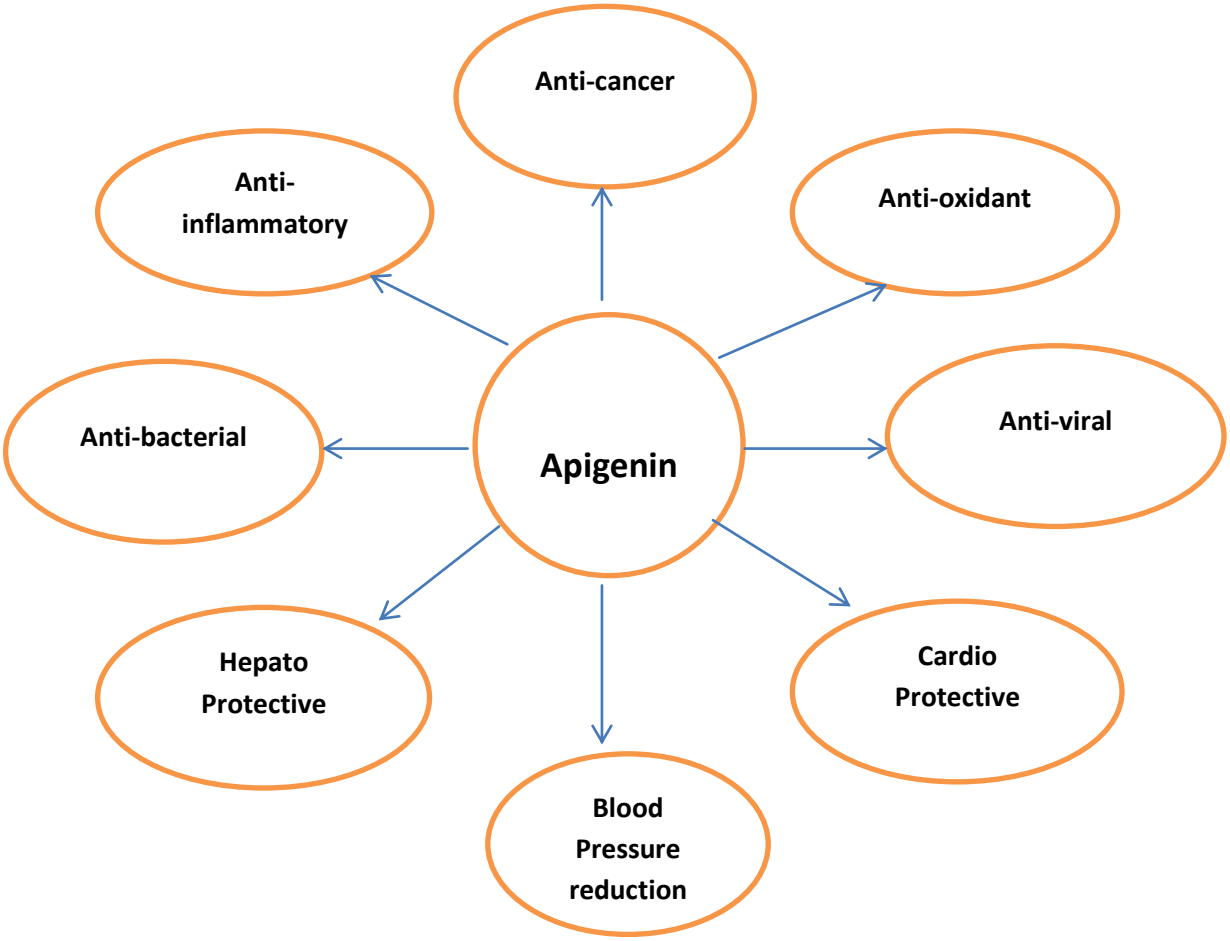


Fig no: 5 Apigenin used in different treatment

Drug Delivery system:

DDS is an innovative field in pharmaceutical research that relies on the process of releasing medications and payloads at a precise time and rate. Heterogeneous capabilities of drug delivery carriers have been revealed, resulting in better, improved therapeutic efficacy and reduced drug toxicity. Because of their ability to improve commercial therapeutic markets, the pharmaceutical industry is paying attention to nanoparticle delivery technologies. [23, 24]. The pharmaceutical ingredient is encased in a Tran's drug delivery system (TDDS).

The advantages like.

1. Improved patience compliance.
2. Improved product shelf life.
3. Reduced costing
4. Reduce in toxicity.
5. Achieve better therapeutic effect.

The current pharmaceutical drug delivery research, break through the approaches focused on TDDS are on the rise. The role of nanotechnology to drug delivery is well documented for its ability to deliver diverse therapeutic agents to damaged tissue with extreme accuracy, as well as it is diagnostic and therapeutic potential. Therefore, developments of techniques which are focused on TDDS are on rise in the present pharmaceutical drug delivery research. [25]

Role of Nanocarrier in drug delivery system:

Nanocarriers are solid, colloidal, particles with size in the range of varying 100-1000 nanometer sizes. These are well considered for their application in various sectors and healthcare system which is increasing and replacing helpful system. [26, 27] Different carriers involved in drug delivery system represented below are

1. Dendrimers
2. Liposomes
3. Micelles
4. Solid lipid particles
5. Self-nanoemulsifying drug delivery system.

Merits of nanocarrier drug delivery system.

1. Improves the water solubility of drug.
2. Inhibits the photo (UV- light) degradation of drug.
3. Facilitates sustained release of drug.
4. Enhance the drug's bioavailability.
5. It helps in reduction of side effects of drug.
6. Provide adequate possibilities for various drug administration routes.
7. Help in the choice of route of drug administration.
8. Allowed rapid formulation and developments of drugs.

Liposomal drug delivery system:

A liposome seems to be a spherical vesicle with a phospholipid bilayer membrane that can be utilized to transport medications or genetic material into cells. Pure components or organic phospholipids with mixed lipid chains (such as egg phosphatidyl choline) can be used to make liposomes (such as Dioleoyl phosphatidyl ethanol amine). [28]

By precisely managing the quantity and position of a medicine in the body, adverse effects are decreased, smaller dosages are frequently required, and novel treatments are conceivable. Liposomes have attracted a lot of attention as a vehicle for improved medication delivery. Bangham was investigating phospholipids and blood clotting at the time, created the first liposomes in England in 1961. Thus every molecule has one end that is water soluble and the other end that is water insoluble, it was observed that when phospholipids and water were combined, they rapidly formed a spherical shape. Fat-soluble drugs were integrated into the phospholipids layer, whereas water-soluble pharmaceuticals were confined inside the aggregation of the

hydrophobic ends.[29] Liposome technologies have developed as "first-generation liposome" toward "second-generation liposome," Long-circulating liposomes is created through changing the lipid content, size, and charge of the vesicle. PEG on surface of liposomal carrier, it's observed to improve blood circulation although lowering absorption by the mononuclear phagocyte system (stealth liposome). As a result of importance of this strategy wide range of liposome formulations encapsulating active chemicals in the past produced, which are all highly efficient and active at their target [30]. Due to a major chemical modification of the final PEG molecule, stealth liposomes could eventually be systematically targeted using monoclonal antibodies as well as legends. Preclinical besides clinical data on the most popular liposome formulations are presented in this research, this emphasizes on stealth technology, as well as talking about new developments in this intriguing technology [31, 32].

Classification:

1. Multilamellar liposome is the Onion structure

2. Conventional liposome is stabilized natural Synthetic identical- Chain phospholipids Glycolipid containing liposome

3. Specialized liposome specially the Antibody directed Lipoprotein coated

4. Stealth liposome is the Polyhydroxy ethyl L-asparagine coated PEG Coating H-PG-PEG Coated and Dope Coated.

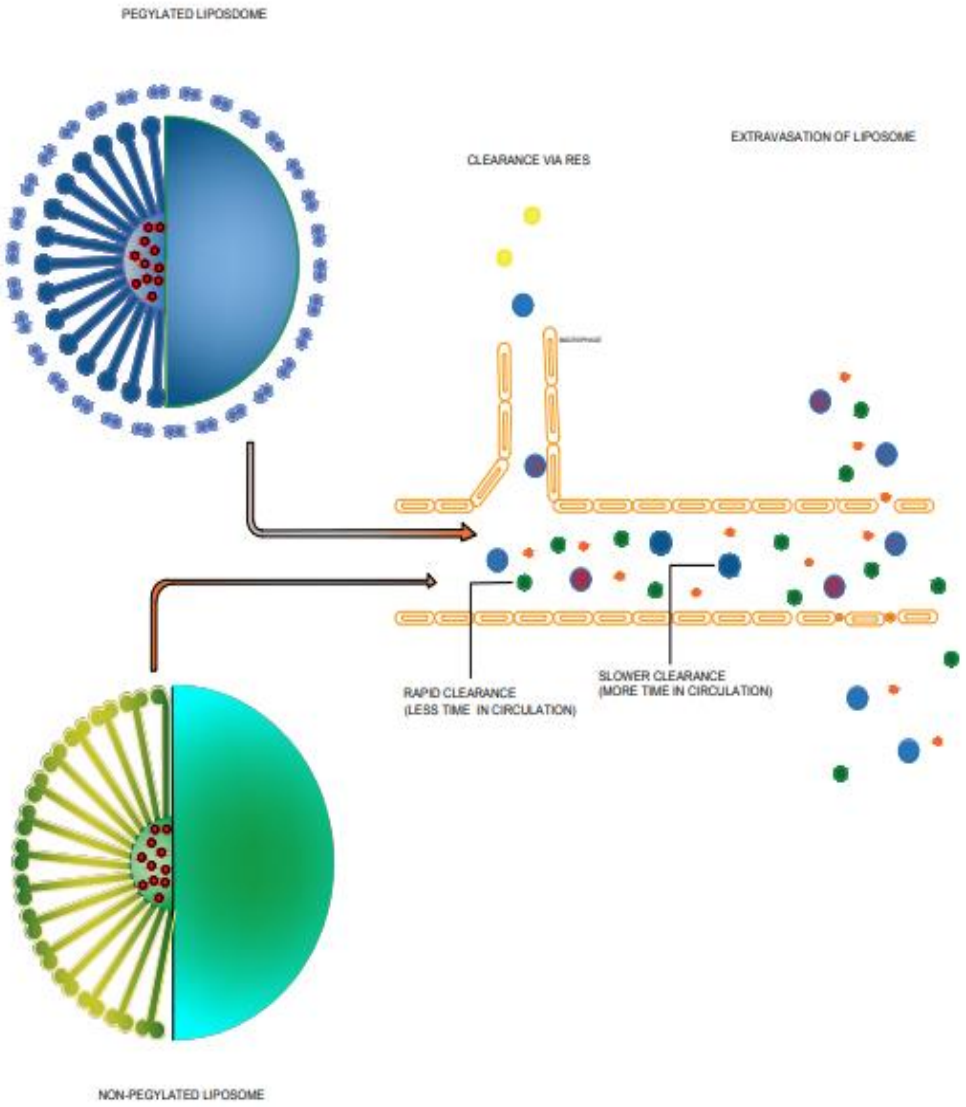


Fig no: 6 representing the formation of stealth liposome nanoparticle]

Stealth liposomal drug delivery:

In the formation of liposomes that circulate for a long time. A crucial step was the addition of synthetic polymer poly-ethylene glycol (PEG) to the liposome mixture. The inclusion of PEG on small molecule carrier's surface is shown to increase blood circulation time while decreasing phagocyte system uptake. [33] (Stealth liposome)

Liposomes that have been in circulation for a long time are being phased out too quickly. The second significant finding is that successfully leveraging the leaky endothelium effect took a while. The 'stealth effect' of encapsulating liposomes with PEG slows macrophage intake, resulting in a notice of liposomes in the circulation or enough time for them to flee through a leaky endothelium [34]. Stealth liposomes were also spherical vesicles with such a phospholipid bilayer membrane for transferring genetic material into cells. Liposomes are made up of naturally occurring phospholipids containing heterogeneous lipid chains coated or stabilized by PEG polymers. "Innovative medication delivery and controlled release technology are on the rise, thanks to stealth liposomes." [35] Liposomes that circulate for a long time, may serve as a reservoir for therapeutic agents to be released over time.

"Stealth liposomes were introduced, which were proven to have blood circulation duration of many days (2days compared to minutes rather than hours for ordinary liposomes and may essentially elude detection by the immune system."[36]

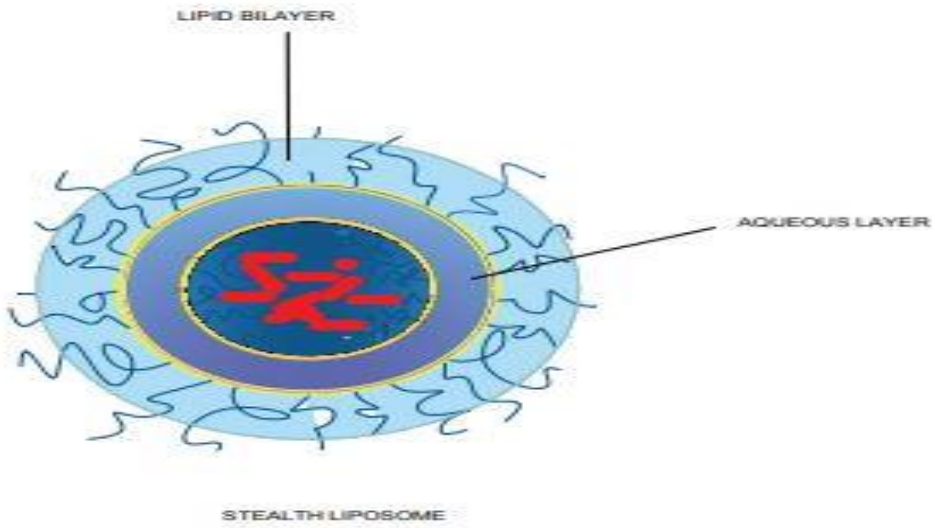


Fig no: 7 representation of stealth liposome

Characteristics of Stealth liposome:

1. These are of double layered composed of cholesterol and phospholipids, the host cell's makeup and structure remain unchanged.

2. The surfaces of stealth liposomes have always had an aqua phobic tail shape and a hydrophilic head structure.

3. Double lipid layers of stealth liposomes form smaller vesicles same toward the physique cells along with their organelles.

4. These are spherical in shape which establish minor deposits that can be hold an antigen, antibiotic or different drugs.

5. Their size ranges from 50 to 5000nm, hence are stable in nature.

6. The size, shape of stealth liposome can be altered depending on drug and material used.

7. The nature of the stealth lipid nanoparticles is colloidal and consistent.

8. They are incorporated drug are delivered at different tissue and cells and site-specific drug delivery.

9. The substance is protected throughout different tissues and cells of the body until it is released from the liposome, at which point it becomes toxic.

10. Depending on the medicine and the product's potential purpose, the size, charge, other characteristics can be altered.

11. The reticuloendothelial system (RES) is unable to absorb them, that result in poor drug release.

12. In stealth liposomes phospholipids, spingolipids, glycolipids and sterols are commonly used lipids.

MECHANISM:

1. Liposomes communicate with cells in a number of ways. Endocytosis of phagocytic cells such as macrophages and neutrophils by the reticuloendothelial system

2. Nonspecific weak hydrophobic or specific hydrophobic adsorption to the cell surface

3. Interactions between cellular components produce electrostatic forces.

4. Penetration of the liposome's lipid bilayer further into plasma membrane, following by release of the liposomal payload into the cytoplasm, results in fusion only with plasma cell membrane.

5. Liposomal lipids are transported from liposomes to cellular and otherwise intracellular membranes without the contents of the liposome being linked. [37,38, 39]

STORAGE CONDITIONS

It can be challenging to retain the physical characteristics of lipid particles once they have been produced. The size distribution of the components might alter during storage owing to component deterioration. The membrane's permeability might cause encapsulated substances to seep out. Lipid compounds have a general problem with stability owing to hydrolytic breakdown. Since the presence of high water in lipid preparations induces rapid hydrolytic breakdown, aqueous formulations of medicinal products are less stable. Lipid suspensions should be kept at a pH of around

7 when the sizing procedure is completed. Acid and base hydrolysis can affect lipids with ester-linked hydrocarbon chains. Because temperature has a significant impact on the rate of hydrolysis, Lipid suspensions should always be kept cool at all times throughout storage. Lipid suspension must not be frozen since freezing destroys the lipids. Process may cause the vesicles to fracture or burst, resulting in a shift in size distribution and loss of interior contents. Cryoprotectant like dextrose, sucrose, and trehalose can help enhance hydrolysis stability. Additionally, samples may oxidize during storage. Small doses of antioxidants added during preparation may aid in suspension stabilization and product oxidation reduction. The lipid is quickly hydrolyzed, yielding monoacyl derivatives (Lyso lipids), which act as detergents then degrade membrane, causing it to permeabilize. Internal contents will begin to leak after 5-7 days at 4-8°C, suggesting hydrolytic breakdown of the lipid. Vesicles can be preserved for 1-2 months with limited (10%) hydrolytic breakdown if membrane structure is not a significant feature. Temperature, pH, medium, and other variables all influence storage time. Liposomes were maintained in a buffer over 5-7 days at pH 7.4 at 4°C hence does not show membrane structural changes, the stability of trapped fluorescent marker is proof. This marker continues to leak out of the liposome after that. It's possible that a membrane-destabilizing component exists. [40]

APPLICATIONS:

- Cancer treatment: Because of their lengthy circulation period and focused medication delivery, stealth liposomes are employed in cancer treatment. In both the United States and Europe, PEGylated liposomal doxorubicin was the first and remains just one stealth liposome formulation to be intended for treatment of Kaposi's sarcoma and recurrent ovarian cancer. Another stealth liposome

formulation contains cisplatin in the aqueous core of sterically stabilized liposomes.

- Vaccines: Antigens from bacteria, viruses, and tumours have been encoded in genetic vaccines these showed promise in preserving humoral and cellular immunity. Clinical experiments have shown that liposome-based vaccinations are effective.[41]
- Gene transfection: Most suitable transfecting vectors in cationic form are stealth liposomes. The therapeutic protein gene is enclosed in liposomal vesicles that allow the DNA plasmid to compress into a well-ordered shape while preserving DNA throughout storage and circulation. Furthermore, the structural architecture of the gene-delivery mechanism must prevent DNA destruction in the lysosomal compartment by bypassing the cell membrane and facilitating endosome escape.
- Targeted medication delivery: Individual drugs are targeted using polymers or antibodies providing a number of benefits over targeted stealth liposomes. The substantial increase in medication quantity that can be given to the targeted site is one of the most intriguing advancements, Because drug molecules will diffuse into neighboring tumour cells, It is possible to improve the number of ligand molecules displayed on the liposome surface, enhancing ligand avidity and uptake.
- In diagnostic imaging: Stealth vesicles are utilized in a membrane or hydrophilic chamber, to transport multiple different molecules making these good for gamma scintillation, magnetic resonance imaging (MRI), computed tomography imaging (CTG), and sonography, among other contrast techniques as their ability to deliver the chemical to the target location specifically to combine numerous contrast

moieties, improve the contrasting signal in stealth liposomes to diagnosis the substance

- To treat inflammation: Lipids having long and saturated hydrocarbon chains, as well as cholesterol, make up stealth liposomes, which have been shown to concentrate near inflammation sites. Liposomes of this kind were utilized for diagnostic reasons. Liposomes carrying corticosteroids, which work as a sustained release method, are administered intravenously into inflammation sites, notably in arthritic joints. A range of release kinetics can be generated by altering the lipid composition.[42]
- Pharmaceutical: As an oxygen therapy, liposome-encapsulated hemoglobin has also been developed. The spatial separation of hemoglobin by a lipid bilayer may help to reduce other modified types of hemoglobin have been linked to cardiovascular hemodynamic effects. These liposomes, on other hand, when delivered intragastrically, various water insoluble medications and vitamins have a higher systemic bioavailability. They're being studied in various compositions with low pH, extracellular enzymes, and bile salts also made up of several bilayers with varying chemical stabilities and programmed degradation kinetics, it can also be found in ecofriendly gels or shells, polymer-coated liposomes, other related products. These are also available as a cream on top, lotion, or tinctures. Hence formulations can be used topically in addition to subcutaneous or intramuscular pharmaceutical depots.[43]

Methods of Preparation of APISL:

Stealth liposomes are created using the same method as normal liposomes. PEGylation, on the other hand, is a crucial step in these procedures. This process of permitting a polymer enclosed with liposome is known as PEGylation. [44, 45]

1. Hand shaking method

2. Reverse phase evaporation

3. Freeze-dried Rehydration

4. Detergent replication

5. Sonication method:

a) Bath Sonicator.

b) Probe Sonicator

6. Solvent Injection:

a). **Ethanol Injection method**: - A considerable amount of buffer is rapidly injected with an ethanol lipid solution. Liposomes are developed instantly, the above liposomes are extremely thin, As a result, it's tough to get rid of all of the ethanol since it forms a zeotrope with water, as well as the fact that a variety of physiologically active macromolecules, in the influence of even minimal levels of ethanol, it can be inactivated.[46]

b**). Ether injection method**: - At 55-65°C or under decreased pressure, a lipids-in-ether/methanol solution combination is gently injected inside an aqueous medium of the encapsulated material. Production of liposomes is caused by the subsequent

elimination of ether under vacuum. The exposure of encapsulated chemicals to organic solvents or high temperatures.[47]

Preparation methods of stealth Liposomes:

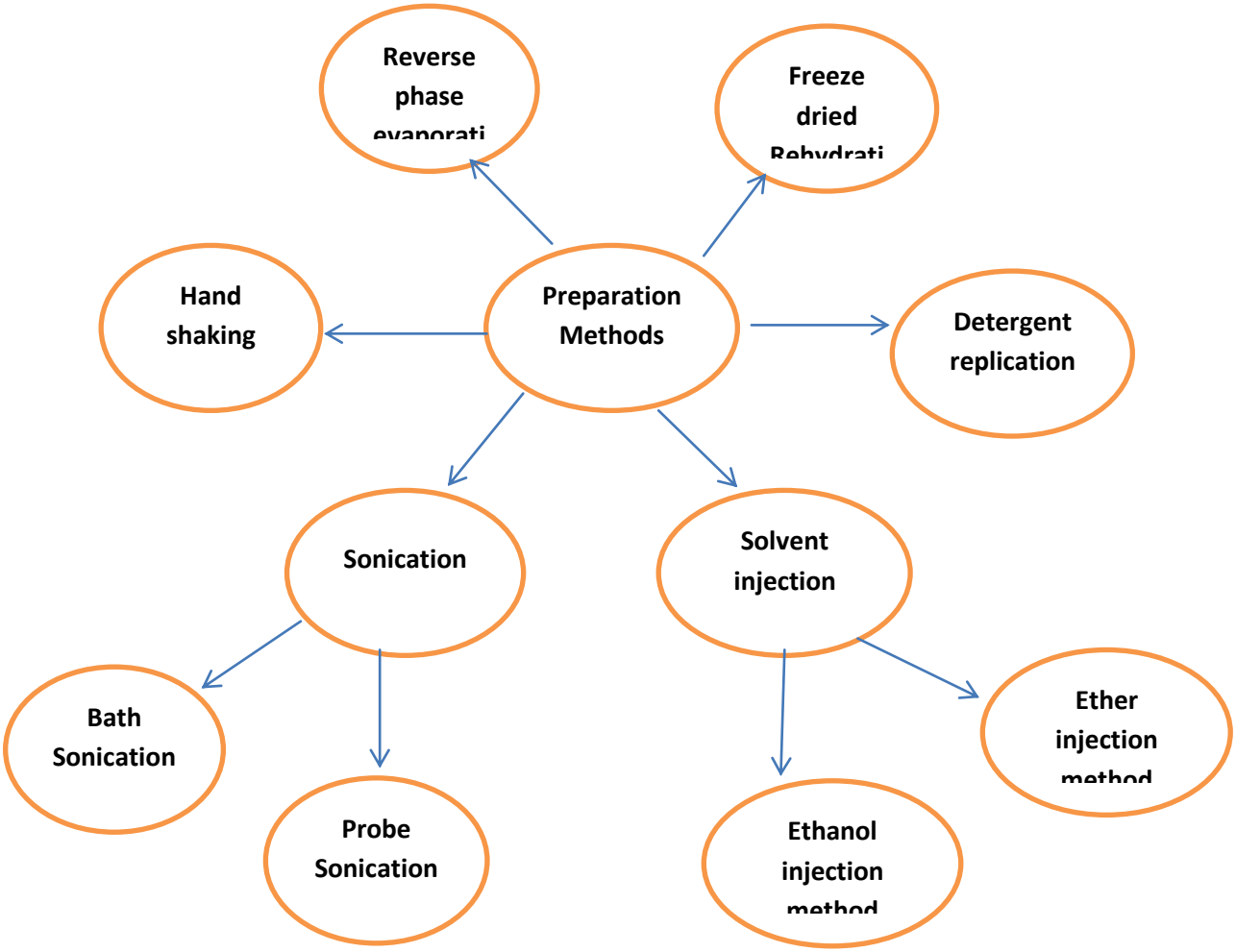


Fig no: 8 various methods used in preparation of stealth liposomes

Review of literature:

Kataria Sahil et al reviewed that BC is the biggest cancer - related deaths among women; it has long been seen as a societal burden due to its high rate of mortality and disability, with an estimated 29 percent of cases and 15 percent of deaths related to breast cancer. ***Javed iqbal et al*** found that the anti-cancer activities of phytochemicals have been investigated. Carotenoids, alkaloids, biamines, terpenes, polyenes, cynogenics, and phenolic chemicals are obtained from natural products and lessen the risk of breast cancer. Flavonoids aid people with cancer by inhibiting breast cancer cell proliferation, inducing apoptosis and cell cycle arrest. Avoid metastasis through modulating many signaling pathways. Which results, apigenin is one of the most often employed flavones for breast cancer treatment because of its low toxicity and lack of mutagenesis effects. [1,2]

Sreemanti Das et al reviewed that flavonoids have an important role in cancer prevention; hydroxylated flavonoids such as apigenin, luteolin, quercetin, and kaempferol have been shown to suppress cellular growth and metabolism in human breast cancer cells. Apigenin inhibits the spread of cancer by regulating a number of processes, including (ROS) and DNA damage and repair.[48]

Gao, y, Qian, S et al found Apigenin-loaded micelles with a prolonged release feature were made using a thin-film dispersion approach. As apigenin is a poorly water soluble medication, polymeric micelle formulations could be used as nanocarriers. [49]

Bokyung Sung et al studied on stimulation of autophagy in various human cancer cell lines is apigenin's role in chemoprevention. As a result, we go through apigenin,

apoptosis, autophagy, and the significance of apigenin in cancer prevention and treatment via apoptosis and autophagy activation.[50]

Hye‐sook seo et al found Apigenin's ability to combat drug resistance was studied in the current investigation. Using adriamycin-resistant breast cancer cells (MCF7/ADR), determine whether apigenin may reverse drug resistance. Apigenin inhibited cell proliferation and colony formation in both MCF7/ADR as well as parental MCF7 cells in our studies.[51]

Weiwei Wu et al concluded that compared to raw apigenin, nanoparticles of apigenin inhibited HepG2 cells more effectively with a lower IC50. Apigenin nanoparticles were studied in attempt to improve their solubility and bioavailability, liquid anti-solvent precipitation (LAP) approach was chosen.[52]

Gopal Venkatesh Shavi et al confirmed that the PEGylated liposomes offer promising results in nanodrug, Because of their lengthy circulation and long-term delivery properties, formulation will be useful in treatment of BC. As a result, anastrozole (ANS)-containing conventional and use of PEGylated (longer circulated) particles in the breast cancer treatment was found to be beneficial.[53]

Loaye Al Shaal et al has worked on the preparation and characterization of nanocrystal formulation of apigenin which have been used in the novel UV skin protective formulations and in order to reduce its poor solubility, suspension of apigenin loaded formulation by using combination technology like milling & homogenization. By bead milling method there was as distinct reduction in particle size and polydispersity index. [54]

Yingjie Zhai et al reviewed to increase its solubility, polymeric micelles have been produced coating dispersion approach was used to make these micelles. And these formulations were optimized by CCD. Further the obtained micelles showed the sustained release property. [55]

Haihua Bai et al found that the apigenin shows effective inhibition of the growth of tumor cells MCF-7. Very minute concentration which cause little damage to the normal cells and shows best cell inhibition tumor cells. Hence apigenin is used to treat all kinds of cancer cells.[56]

Rajendra Jangde et al prepared optimized formulation using the factorial design approach and have chosen as best for optimized drug (quercetin) loaded nanoformulation for wound healing, These formulations provided long-term medication release in wound regions. Hence quercetin loaded nanoformulation were prepared for effective wound treatment.[57]

Lucia Ruxandra Tefas et al attempted to find out how formulations affect physico-chemical properties of quercetin loaded liposomes and its optimization. During preparation used an experimental design approach with three factors and three levels this showed increase in lipid ratio causes a decrease in size and more or less high PDI. This D-optimal Experimental Design was used to obtain successfully QU-loaded liposome with optimized characteristic.[58]

P. Arun, T. Amol et al developed UV– method of Apigenin (APG) in Apigenin - HSPC Complex, here Apigenin showed good linearity in concentration range between 2-12µg/ml. This developed method was economic; As a result, it is employed as a quality control method for APG analysis. [59]

Boyong LI et al has assessed the properties of apigenin and its analytical method development. The study of apigenin, a plant flavonoid with promise chemo preventive efficacy against skin cancer, requires preliminary based on analytical tools.[60]

Noorfaiza A et al reviewed that apigenin increased Cisplastin inhibitory effects on cancer cells and also has emerged as a promising strategy to combat cancer cells. This study showed the additive effect of apigenin highly dependent and is different across the cell lines [61]

Simon Silvan, Shanmugam Manoharan et al throughout DMBA-induced oral carcinogenesis, Apigenin therapies stopped the growth of oral tumor completely, while somehow restoring lipid peroxidation, antioxidants, phase I and phase II detoxifying enzymes to relatively close values. Apigenin might well have decreased oral carcinogenesis during DMBA-induced rat buccal pouch tumorigenesis by enhancing antioxidative defense mechanisms and altering the processes of phase I and phase II detoxification pathways into increased outflow of the active form of DMBA.Therefore, apigenin has chemopreventive potential in an animal model of 7,12-dimethylbenz(a)anthracene-induced oral carcinogenesis.[62]

Kalyani Chowdary Karnama , Maheswara Ellutla discovered that intragastric administration of 7, 12-dimethylbenz[a]anthracene (DMBA) at a concentration of 80 mg/kg of body composition caused breast cancer in Sprague Dawley (SD) rats. Berberine has been given to breast tumour producing rats at a dose of 50 mg/kg of body weight, and it was shown to be efficacious against DMBA-induced mammary cancer. Histopathological tests confirmed that berberine is effective against DMBA-induced carcinogenis. It also proved that berberine has a protective role against DMBA-induced breast cancer in SD rats. [63]

Justification:

BC is one of type of cancer which is also responsible for the leading cause of cancer related mortality, as well as its prevalence is on the rise in developed world. It is the most challenging to treat and due to high resistance of radiation and conventional chemotherapy.

According to GLOBOCAN REPORT an estimate of 11.6% of newly patient diagnosed with the 6.6% deaths or mortality rates. BC is associated with factors like age, Hormone exposure, past and family history, lifestyle conditions, also exposure to suspected carcinogens such as obesity, UV radiation, diet, and other substances. Particularly mutations in genetic risk factor, BC is caused by the BRCA1 and BRCA2 genes. Hence it has been reported that such as flavonoids reduce the risk of BC. Apigenin a flavonoid exhibits promising antitumor potential against Breast cancer. Apigenin is a natural phytoconstituents and is almost found in vegetables and fruits like orange, tomatoes, parsley, and chamomile flower. Hence Apigenin is taken from chamomile flower extract.

It possesses a strong cytotoxity and is used to reduce inflammation and in smooth muscle cramps and it's also used as effective treatment in skin condition its essential oil is shown to kill up to 93% of breast cancer cells. Use of apigenin is limited due to the poor solubility and the absorption. As a result, these limitations could be avoided by using Apigenin-loaded stealth liposomes as a component of nanocarriers or nanoformulation. Different ADRs and toxicity to healthy cells have been seen in recent BC treatment.

Antioxidant molecules derived from plants which have anti-tumor properties have been explored as alternative malignancy prevention and therapy strategies.

Various phytochemicals, as well as minerals and dietary factors, have been discovered to be beneficial against a variety of malignancies. Due to their ability to target cancer cells despite reducing toxicity to healthy cells, nanoparticle formulation, a novel method coated with natural medicines, is used as a drug delivery in BC treatment. Nanoformulation coated with the polymer membrane show promising decrease or reduction in the toxicity. Hence these nanoformulation coated with polymer membrane are known as stealth liposomes and these were introduced because They have been demonstrated to have blood circulation time over many days and may largely elude detection by the immune system, these stealth liposomes are stable in nature, colloidal and uniform and also improves the stability problem. As a result, it functions as a dispersing agent, inhibiting multidrug resistance and increasing anticancer medication absorption. As a result, Apigenin-loaded Stealth liposomes will be developed to improve solubility, absorption, and retention, along with anticancer effects. Hence in the proposed project the Apigenin loaded Stealth nanoparticle will be prepared. An emerging technique has been used by unique possible nanocarrier stealth liposomes, to enhance the stability, safety, and efficacy of apigenin for targeting breast cancer. These stealth liposomes possess bio adhesion, high thermal stability, high loading capacity, penetration enhancement property, and the ability to encapsulate hydrophilic, lipophilic, and amphiphilic medicines, allowing them to outperform other nanocarriers in BC. Apigenin has been shown by researchers to be a possible phytoconstituent for the treatment of BC. However, no evaluation of the efficacy of apigenin-loaded stealth liposome nanoparticle as a novel drug delivery strategy for malignancy has been done yet. As a result, with this background the current study aims to construct Apigenin-loaded nanoparticles and test their efficacy in the treatment of breast cancer.

1.4 AIM AND OBJECTIVES OF RESEARCH STUDY:

Aim:

To investigate the effect of Apigenin loaded stealth liposome in Breast Cancer.

Objective:

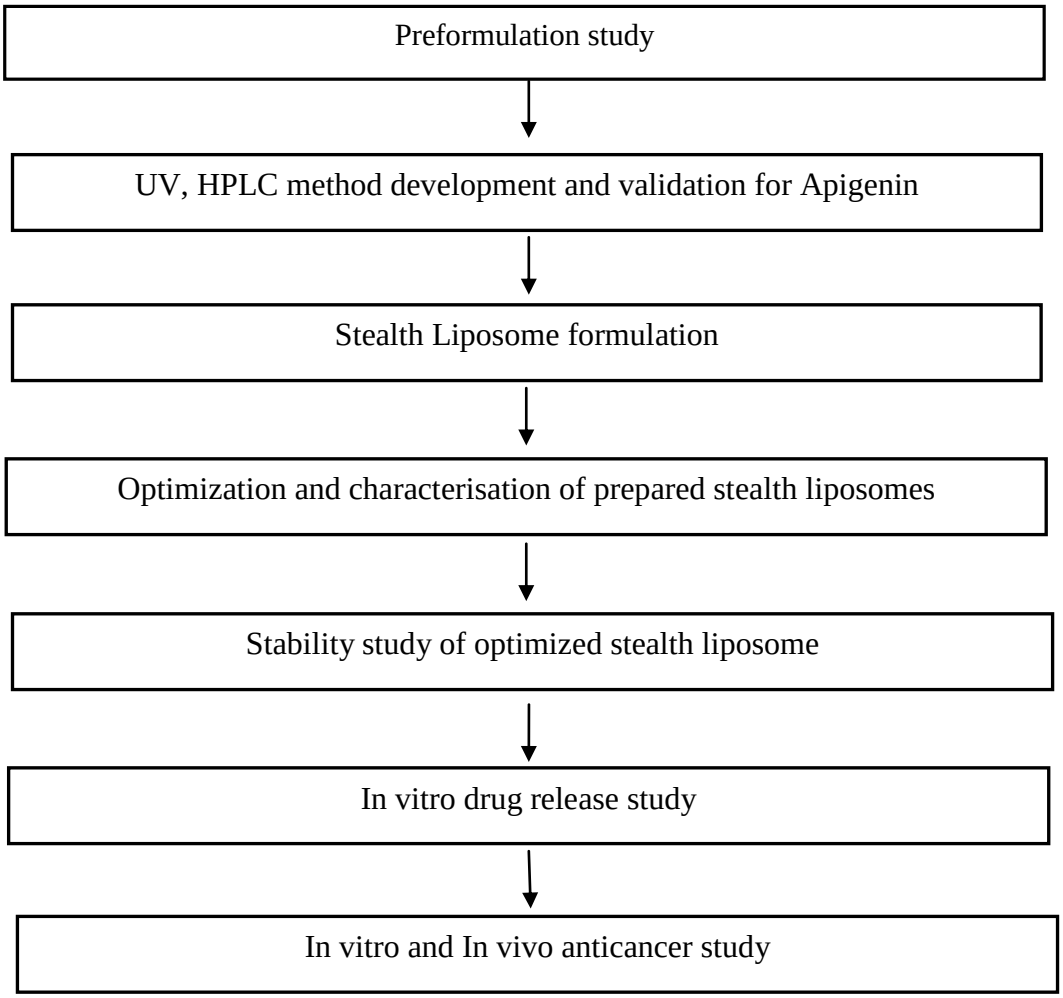
1. Analytical UV method development and validation.

2. Analytical RP-HPLC method development and validation.

3. To formulate and optimize Apigenin loaded stealth nanoformulation.

4. To study in vitro and in vivo anticancer activity of optimized stealth nanoformulation against Breast Cancer.

STUDY PLAN


Preformulation study
UV, HPLC method development and validation for Apigenin
Stealth Liposome formulation
Optimization and characterisation of prepared stealth liposomes
Stability study of optimized stealth liposome
In vitro drug release study
In vitro and In vivo anticancer study

2. MATERIALS AND METHOD

Table No. 1. List of instruments

S. No	Instruments name	Model	Make
1	Electronic balance	AUW220D	Shimadzu
2	Magnetic stirrer	RCT basic	IKA
3	FTIR	IRA affinity-1S	Shimadzu
4	Differential –scanning Calorimetry	DSC-60	Shimadzu
5	High performance liquid chromatography	LC-20AD Prominence	Shimadzu
6	PH meter	Cyber scan PH 510	Eutech instrument
7	Probe sonicator	Rivotek	Riviria Glass Private limited
8	Bath Sonicator	CPX1800 H-E	Branson
9	Inverted Microscope	TCM – 400	Labomed
10	Scanning electron microscope	H-7500	Hitachi
11	Homogeniser	RQT-127A	Remi laboratory system
12	Freez dryer	Alpha1-2 LD Plus	Christ
13	CO2 incubator	Galaxy 170R	New Brunswick

Table No.2 List of chemicals

1	Apigenin	Aktin chemical China
2	DSPE-PEG	Lipoid Gmbh Germany
3	Ethanol absolute	SD Fine chem Ltd, India
4	Cholesterol	Loba Chemie
5	Sodium hydroxide	Merck India
6	Mannitol	Himedia labs
7	MTT	Sigma Aldrich
8	AST, ALP, ALT kit	ERBA diagnostics
9	DMEM	Himedia labs
10	MCF7 Cell line	NCCS, Pune
11	DMBA	Sigma Aldrich

A. Preformulation study:

2.1 Fourier transforms infrared spectroscopy (FTIR):

FTIR spectrum of Apigenin, two lipids, and cholesterol studies, as well as graphics, have all been completed. Fourier Transformer Infrared (FTIR) Spectroscopy of Apigenin was performed using an FT-IR Spectrophotmeter (JASCO FT/IR-410). The spectra were analysed at a resolution of 4cm-1cm throughout a wavelength range of 400 – 4000 cm-1.During the operation, samples were dispersed in KBr and compressed onto discs over a 5-minute period using an HP of 5 tons. Placing the pellets in the light path yielded the spectrum.

2.2. UV spectroscopy:

A UV spectrophotometer was used to evaluate apigenin spectra in a pre-formulation analysis (Schimadzu UV-1800) UV spectrum of methanolic drug solutions (Apigenin 10mg/10ml) were obtained in the 200–400 nm range. Solubility of apigenin was tested using several solvents such as ethanol and methanol. Several investigations were conducted in order to determine the solvent system, and methanol was eventually selected as the carrier. Apigenin was generated as a primary and secondary stock solution by diluting it in methanol. Operating sample solution containing 10 g/mL apigenin was scanned across the wavelength ranges of 400 - 200 nm against methanol as a blank from these solutions. The UV spectra and absorption curve showed distinctive absorption maxima at 267 nm, which was chosen for apigenin analysis. [64]

2.3. Analytical HPLC Method development for the estimation Apigenin:

HPLC method development was carried out utilizing a Shimadzu (LC20-AD,Japan) High-performance liquid chromatography system, which included an LC (20 AD) pump and (SIL-20AC HT) Auto sampler, as well as a (SPD-M20A),PDA detector, Luna C18 (2)5 100A phenoxmenex C18 analytical columns with a size of 250mm X 4.6.Mobile phase was changed to ACN:0.1 percent FA (formic acid) (55:45v/v) A continuous flow rate of 1ml/min is provided. All of the samples had a 10 ml sample volume. Different parameters must be established; the mobile phase and its composition, as well as the flow rate and temperature conditions are factors to be considered. The validation procedure was then carried out. [65]

2.3.1 Preparation of calibration standards:

The primary stock solutions (1000g/ml) were made by combining 10 mg of medication with methanol (10 ml). Apigenin working solution was made by diluting the stock solution IS (10 μg/mL) in mobile 0.5-16 g/ml is a good starting point. Before usage, the standard solution was kept at 40°C.

2.3.2 Validation studies:

Specificity, linearity, and consistency LOD during validation of LOQ, the following accuracy, stability, ruggedness, and robustness of the approach were noted.

2.4. Experimental design:

Apigenin loaded stealth liposome was prepared by using a two-factor, three-level (3^2) factorial design, with X1 indicating lipid content (DSPE-PEG) and X2 representing cholesterol as independent variables, Y1 representing particle size, Y2 Polydispersity Index, and Y3 representing % EE as dependent variables, as indicated

in the table no 3 ,[66,67] Design –Expert Software was used to optimize formulation design.

Table: 3. FD (Factorial design) for APISLNF.

Coded level	Low (-1)	Medium (0)	High (+1)
Independent variables			
Lipid (X1)	3	6	9
Cholesterol (X2)	1(0.5)	2 (1)	3 (1.5)
Dependent variables			
Particle size (Y1)	Minimum		
Polydispersity Index (Y2)	Maximum		
% Entrapment Efficiency (Y3)	Maximum		

2.4.1Preparation of stealth liposome:

Ethanol injection method was used to generate apigenin-loaded stealth liposomes, which is a terrific way to make single unilamellar vesicles (SUVs) without using sonication or a high-pressure homogenizer,. The weighed quantity of DSPE-PEG2000, stearic acid, and medication were gradually warmed on a hot plate at a temperature of not more than 50^0C in a beaker containing 5ml of ethanol. 10 mL phosphate buffer pH7.4 was poured into the monophasic ethanolic drug lipid mixture, which was held on a magnetic stirrer for uniform mixing at a temperature of not more than 60°C at 500 rpm for 90 minutes, the resulting blend was sonicated. The suspension was stored at 4 ^{0}C, it was used for future characterization study.[68]

B. Characterization of the stealth liposomal formulation:

2.5.1 Determination of PS, PDI, and ZP.

At room temperature, the PS, PDI, and ZP of the stealth liposome formulation was influenced by PS analyzer (Zetasizer, Malvern Instruments, and Malvern, UK). To evaluate the limited nature of the particle size distribution, the PDI.

2.5.2Determination of the entrapment efficiency (EE)

The ultracentrifugation technique determines the quantity of APISL trapped inside the liposomal system. The dispersal was centrifuged at 4°C for 10 minutes at 2000 rpm (using rotor type AG1212) using only a Kubota 6500 (Japan). A tube containing the stealth liposome preparation was put in (eppendrof) tubes and centrifuged (Kubota 6500, Japan) at 3000rpm (using rotor model AG 1212) for 3 hours at 4 ºC. Free API drug present in the supernatant was separated and diluted. The amount of Hydroquinone present was determined by using the HPLC analysis. To check the total amount of APISL present in the designed formulation, the particle dispersion was sonicated after being lysed with mobile phase.[69] The solution was filtered via 0.45mm filters, diluted, and HPLC was used to analyze it. The proportion of EE was calculated as follows:

EE percent = (T* S)/T x 100,

Where, S is the amount of APISL present in supernatant only, and

T*S is the amount of API present inside the formulation.

2.5.3Drug *release study (In - vitro) of prepared Nanoformulation:*

By using dynamic dialysis technique, the best batch of plain AP was employed to release APSL in vitro (USP II Method). An Electrolab EDT-08LX tablet dissolving testing equipment equipped with an LVC kit (EDT08L/08L 150 ml, Figure 7) created specifically for nanoformulation was used to conduct the drug release study. In a dialysis bag, 5 mL APISL was inserted and submerged in 100 mL PBS (pH 7.4). The dissolving media was heated to 37°C and at 500 rpm. To alter the sink condition, aliquots of the drug (1ml) release media were obtained in regular breaks; the appropriate capacity of fresh buffer was used to replace the deleted aliquots. As previously stated, the amounts of APISL unconfined in the medium were measured using the HPLC technique. The mechanism of drug release from Stealth liposomal solution is identified using numerous drug release kinetic models.[70,71]

2.6 Freeze drying: lyophilization

Stealth Liposomes were lyophilized using Mannitol 5 percent w/v as a cryoprotectant in order to perform DSC and FTIR tests on improved APISL. Samples were lyophilized using such a freeze dryer after being frozen at - 20°C for 24 hours. (Christ, Germany). Primary drying was carried out under typical conditions of -55°C condenser temperature, vacuum pressure was 0.023 mbar, while major drying carried out under -60°C condenser temperature and 0.0054 mbar vacuum pressure. For further research, A desiccator was being used to dry the powder.

2.6.1 DSC Studies:

The samples were enclosed in aluminium pans cells and heated at 1000 degrees Celsius per minute i.e 300 to 5000 degrees Celsius in a nitrogen environment.

The peak transition maximum temperature was compared using a thermal analyzer (TA- DSC-6060WS, Shimadzu, Japan).

2.6.2Stability studies of liposomes:

The stability of liposome dispersion and lyophilized compositions, and also different characteristics such as VS, PDI, ZP, and % EE, were evaluated for 3 months at storage conditions of 2-8°C, according to ICH guidelines.[71,72]

2.7 In vitro assay of APISL8 by MCF 7 cell lines.

The NCCS in Pune, India provided breast cancer (MCF7) cell lines for this study. Cells cultured in a CO2 incubator (ENBG170G) at 37°C at regulated humidity in a mixture of Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum FBS, with 1% Pen step antibiotics.

2.7.1 In vitro cytotoxicity study:

The cytotoxic activity were influenced using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) test for both API and APISL8 on the MCF-7 cell line (48). Both cultures were planted in 96 well plates (Nest Biotechnology) at a density of 5 x 10^4 cells per well then raised at 37°C for 24 hours at 5% CO2 incubator. Time period of 24, 48, and 72 hours, the cells treated with varying doses (2.5 - 80.0 g/ml) of pure API and optimized APISL8 dose. As a control, untreated cells in culture media were employed .[73,74]At the end of therapy, the culture media from each well was scraped clean, and also the cells were rinsed twice with PBS solution. Each one received 20 µl of MTT solution (5 mg/ml in PBS). 4 hours, the cells subsequently incubated overnight at 37 °C. After that, the supernatant was discarded, and each well plate was filled with 100 µL of DMSO.

(Lisa Plus, Rapid Diagnostic Pvt. Ltd. India) and the plates were shaken to disperse the insoluble formazan crystals.[75] This micro plate reader had been used to measure the absorbance at 490 nm. The percent cell viability estimated by multiplying the ratio of the treatment group's absorbance by the absorbance of the control group.

2.8 In vivo studies:

Female SD rats weighing 200 - 250 grams were obtained from KLE College of Pharmacy (KAHER) in Belagavi, India's animal facility. The Institutional Animal Ethics Committee (KLECOP/Reg.No.221/Po/Re/S/2000/CPCSEA/17/03/2021) in KAHER, India, authorized all the animals experiment techniques. Animals(SD Rats) were housed in groups of six at 25–30 °C with a 12-hour light-dark cycle for one week prior to the trials which were given free access to pellet diet (VRK Nutritional Solutions, Sangali, Maharashtra, India) and water.

2.8.1Organ toxicity tests in vivo:

Hepatic and renal biochemical markers such as alkaline phosphate (ALP), alanine transaminase (ALT), and aspartate transaminase (AST). Furthermore, histopathological evaluation of liver, kidney was performed after staining with hematoxylin and eosin.

2.8.2 Prophylactic and Therapeutic anti-cancer activity:

Female SD rats weighing 200-250 g were given DMBA at a rate of 45mg/kg body weight for 3 consecutive weeks and fed with pellet diet up to 10 weeks to produce breast cancer.[76,77] Six groupings of animal experiments were represented in (Table 4) with each group consisting of six animals. [78]Two extra rats were added to Group 2 (therapeutic and preventive groups) at the conclusion of the 16th week of

the experiment to confirm the induction of Breast cancer. And during 16th week, each group animals were sacrificed. Plasma was collected first from blood after dissection using centrifugation at 3000 rpm for 5 minutes at 4°C for biochemical examination of Alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total RBC, & WBC levels. The tumour was isolated, the tumour size was measured, and the samples were kept in buffered formalin for histological evaluation.[79,80,81]

Table No.4: Animal grouping was done to study the effect of prophylactic and therapeutic treatment in BC induced in female SD rats.

Groups	Treatment
I. (Normal)	Normal saline and standard diet
II (Disease control)	DMBA (carcinogen) 45mg/kg body weight oral route administration.
Prophylactic Treatment	
III. (API)	API dose 20 mg/kg body weight, two times in weeks. (intraperitoneal route)
IV (APISL8)	Apigenin loaded stealth liposomes 20 mg/kg body weight, two times in weeks.(intraperitoneal route)
Therapeutic treatment after 10th Week.	
V (API)	Dose 20 mg/kg body weight intraperitoneal two times in weeks.
IV (APISL8)	Dose 20 mg/kg body weight two times in weeks.

2.8.3In vivo BC study

All rats throughout the study were placed into six groups, that each have six animals. (Table 4). The drug treatment of formulation was continued up to the 16th week. The tumor volume and body weight were measured on each week using a Vernier caliper and digital weighing balance respectively [82,83]. Tumor volume was used as a parameter for tumor inhibition in anti-tumor activity [84] and must be calculated by using the equation given.

$$\textit{Tumor volume} = \frac{\textit{(length X (width)}^2}{2}$$

2.8.4 *Histopathological analysis:*

At the end of the sixteenth week, female SD rats from each group were beaten, the tumour tissues were then treated with just a 10% formalin solution. Hematoxylin and eosin dye were used to stain sections of tumour tissues, and the images were viewed under a light microscope (Olympus, Delhi).

3. Statistical analysis

The APISL formulations were optimized using Design-Expert® software version 7.0.0. Data analysis of in-vitro testing was performed using Prism software version, and all of the values were written as mean SEM for 6 groups (n=6). One-way ANOVA was used to compare groups, followed by Tukey's data were analyzed. There were comparisons to be made between the Normal Control and the Disease Control groups. In comparison, Disease Control vs. API-Prophylactic, APINP-Prophylactic, API-Therapeutic, and APINP-Therapeutic.

4. RESULTS

Results:

4.1Identification of DRUG API:

4.1.1FTIR: FT-IR Spectrophotmeter (JASCO FT/IR-410) was used to perform Apigenin Fourier Transformer Infrared (FTIR) Spectroscopy. The spectra were scanned at a resolution of 4cm throughout a wavelength range of 4000 to 400cm-1. The infrared spectrogram of pure APG (Figure No: 9), the functional group peaks of the API loaded stealth formulation did not interfere with FTIR analyses. Indicating they were chemically compatible. The stretch at 3092.82 cm^{-1}confirms the presence of the O-H group and the stretch at 1656.85 cm^{-1} confirms the presence of C=O, the stretch 1537.42 cm^{-1} confirms C=C and 2980.33 at the aromatic benzene ring C-H stretch (Figure No: 11). The FTIR spectrum of the physical mixture showed a change in the peak configuration; hence the compatibility is confirmed between the drug and the excipients.

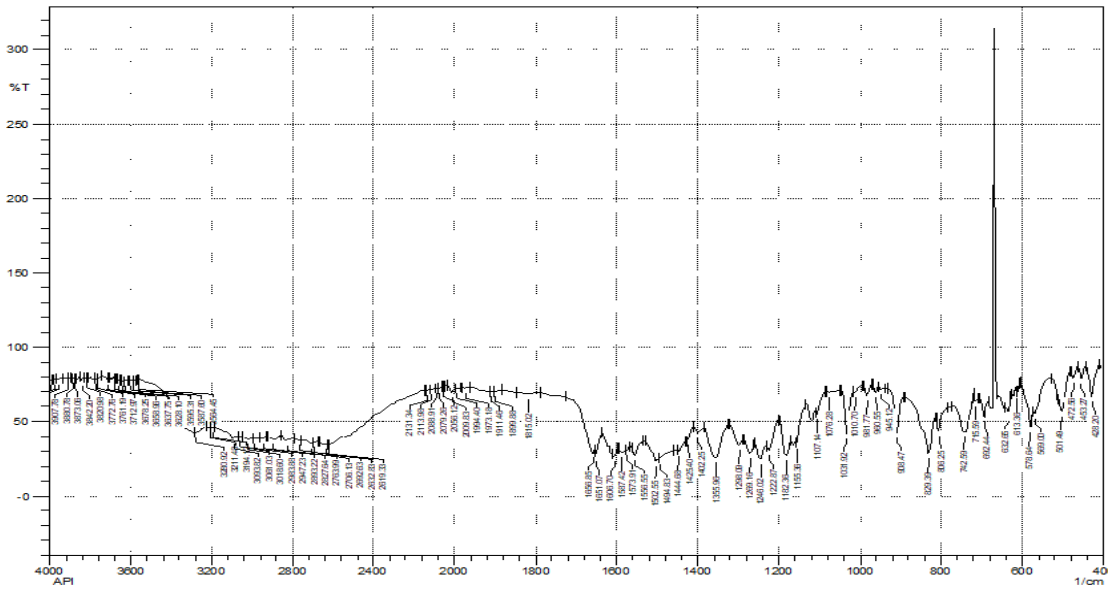


Fig no: 9 (A) Apigenin:

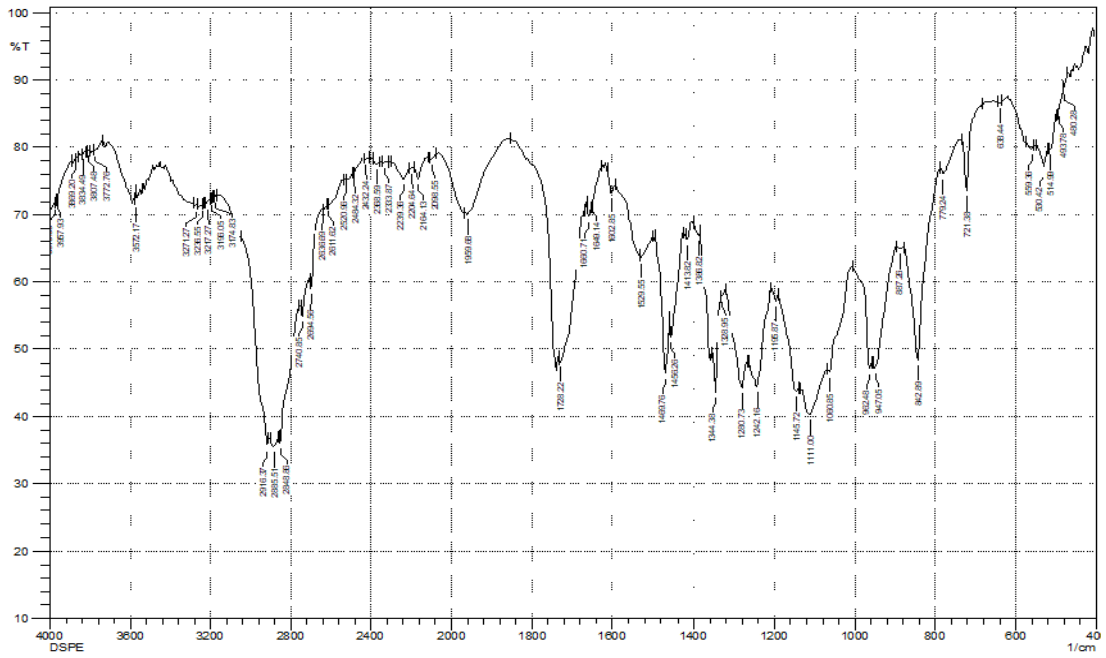


Fig no : 10 (B) DSPE-PEG2000

4.1.2 DSC Study: API AND APISL8

DSC thermo gram of Apigenin and optimized Apigenin loaded stealth liposome8 is shown in figure no: 11 below. The pure drug Apigenin released a sharp endothermic peak at 365.22 ^{0}C,

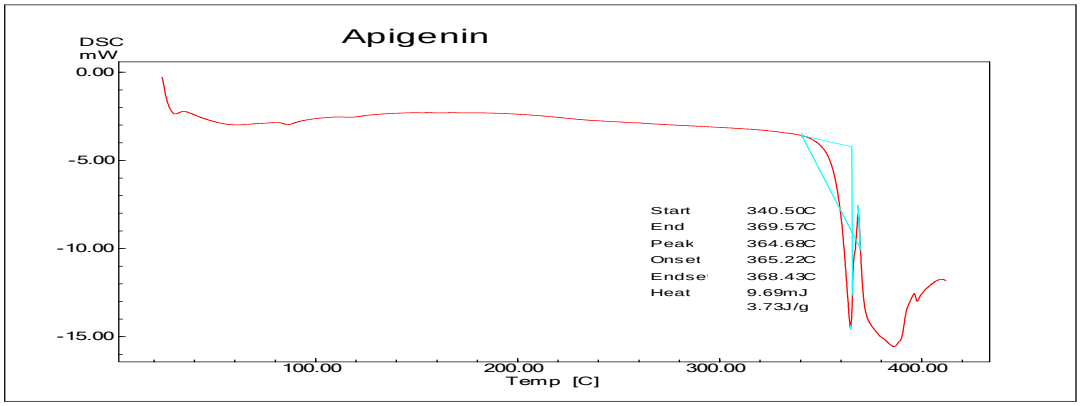


Fig no: 11 DSC thermogram of Apigenin

4.2Method Development and validation of Apigenin UV Spectrophotometric :

UV spectrophotometric procedure began with the selection of a solvent system and the determination of the absorption maximum wavelength. Apigenin solubility was tested in various solvents, including ethanol and methanol, both experimentally and through a literature review. In identifying the optimal solvent solution, a variety of analyses carried out and methanol was ultimately chosen. To reach a concentration of 10µg/ml, this working stock solution was diluted with methanol (1 ml to 10 ml).

This sample was scanned against methanol as a blank here between wavelength ranges of 400-200 nm. The UV spectra were displayed in Figure no 12, and the absorption curve revealed two distinct absorption maxima at 269 nm and 335

nm. As a result, both (max) were chosen for API examination. Dilution of the working sample solution with methanol yielded a sequence of concentration-range calculated values of 2–10 g/ml at 269 nm. The UV spectra of apigenin were obtained by scanning between 400 and 200 nm. The wavelength of maximum absorbance for apigenin was 269 nm.

Apigenin UV spectrum was investigated using a UV spectrophotometer (Schimadzu UV-1800). Against methanol as a blank, the sample was scanned between the different wavelengths of 200-400nm. The absorption curve of the UV spectra (Fig.12) revealed two distinct peaks at 335 and 269nm. Because the absorption curve at 269nm had a prominent peak, it was chosen for Apigenin investigation.

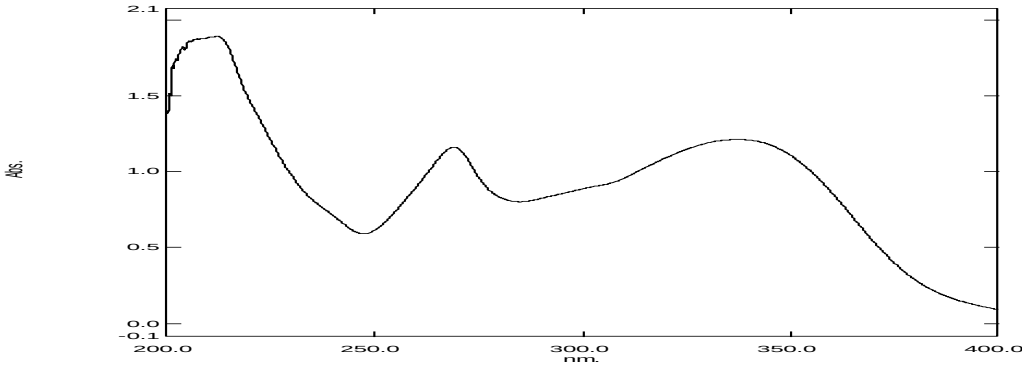


Fig no : 12 of UV spectra of Apigenin

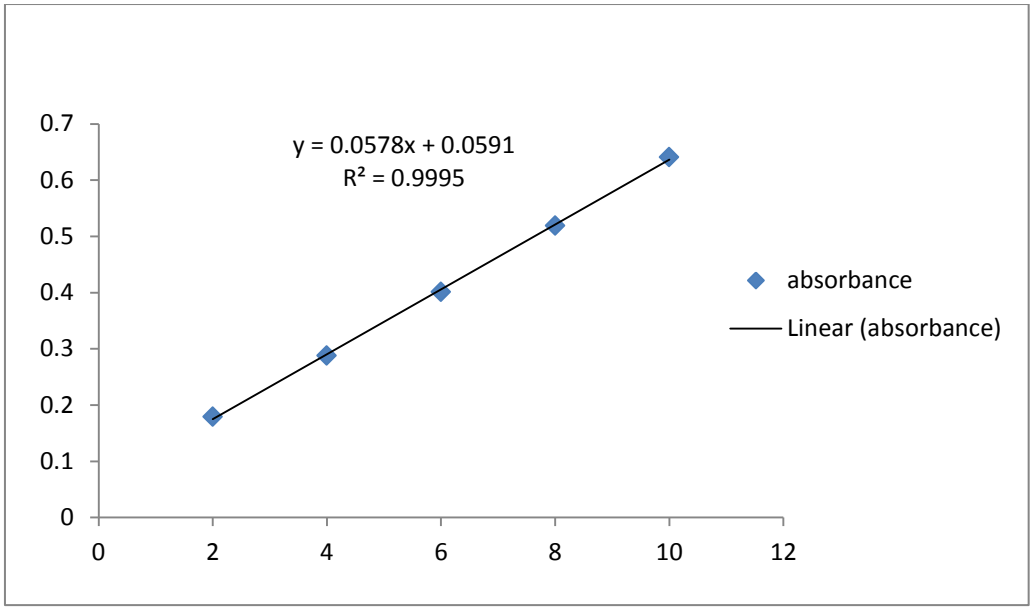


Fig no: 13 linearity of apigenin UV method

4.3 Analytical HPLC method for the estimation of API:

To develop a method for quantification of apigenin, an HPLC system comprised of a shimadzu HPLC (LC-2010) (Kyoto, Japan), an LC-20AD pump system with solvent cabinet, a DGU-20A5 degasser, a CTO-10ASVP oven, an auto-injector SIL-20ACHT, SPD-M20A detector, and a computer software (Lab solution, version 1.25) equipped with for the elution of apigenin, A C-18 analytical column was used (250 4.6 mm, 5m), flow rate of 1 mL/min as well as a column temperature of 35 °C, the mobile phase was made up of acetonitrile with 0.1 percent v/v Formic acid in a 45:55 v/v ratio. The injection volume was 10µl. The calibration curve for the described HPLC technique was created using apigenin standard solutions produced in methanol Basic sense, The (LOD) and (LOQ) of the HPLC technique were determined relying on the signal-to-noise ratio (S/N) methodology (LOQ).

In general, S/N of 3.3:1 is adequate for evaluating LOD, while a S/N of 10:1 is utilized to measure LOQ. The following equation was used to calculate S/N: S/N=H/h

2, the height of said apigenin-responsive peak here on chromatogram is H, while the background noise just on chromatogram is h.

An S/N of 3.3:1 is often appropriate for calculating LOD and an S/N of 10:1 is used to evaluate LOQ. S/N was determined using the subsequent equation: S /N =H/h 2 (1). While H is the height of the chromatogram's apigenin-responsive peak, and h is the height of the chromatogram's background noise.

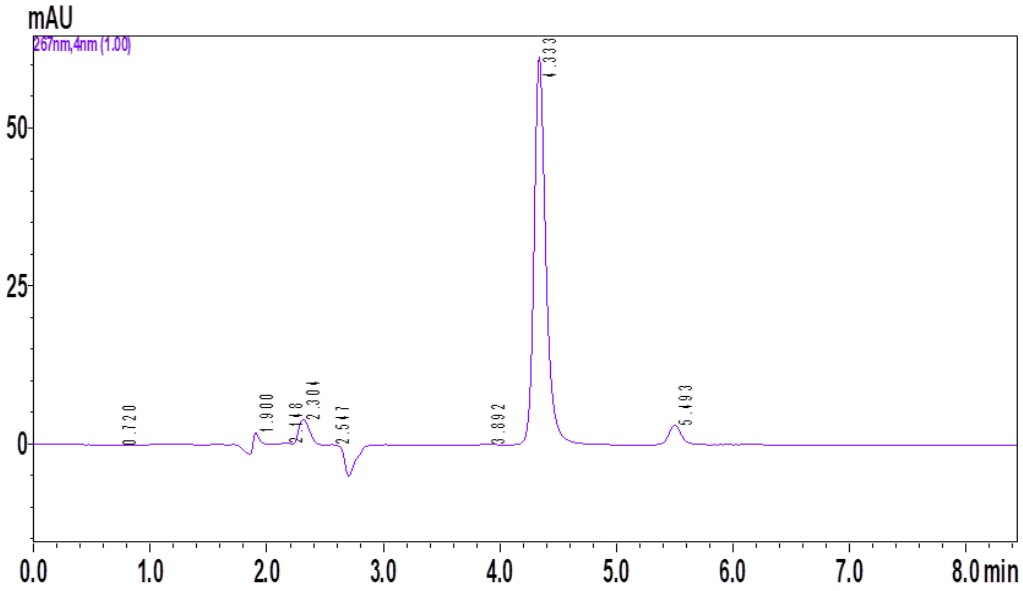


Fig no: 14 HPLC chromatogram of apigenin with Linearity chromatogram of apigenin.

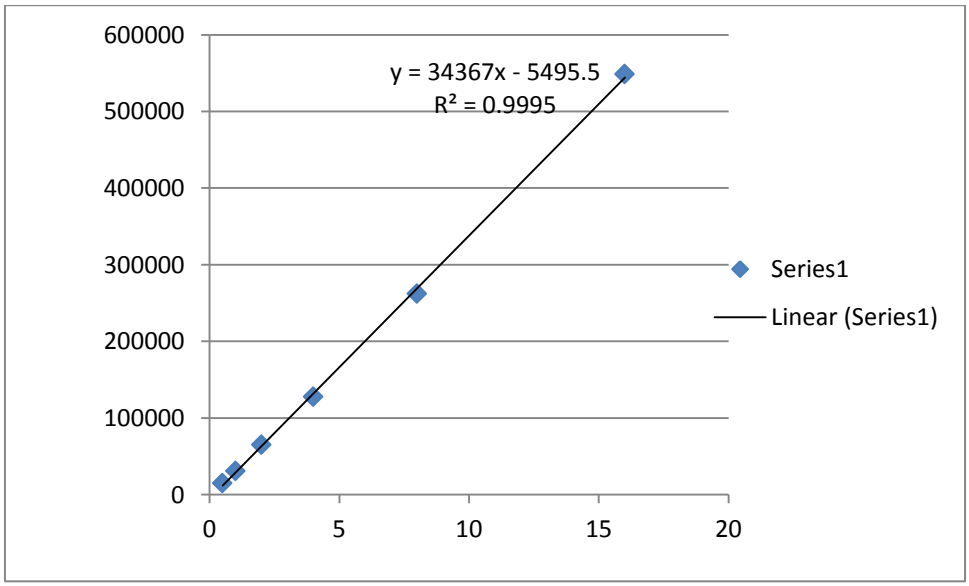


Fig no: 15 linearity of chromatogram of Apigenin

4.4 Experimental design of the study

A 3^2 factorial designs was used to determine the optimized batch. As a result, lipid concentrations of 3, 6, and 9% w/w were chosen as independent variables, whereas particle size, entrapment, % drug release was chosen as dependent variables.

Table No.5 Selected batches of Apigenin Stealth formulation preparation

Coded level	Low (-1)	Medium (0)	High (+1)
Lipid	3	6	9
Cholesterol	1(0.5)	2(1)	3(1.5)

Table No: 6 formulation 9 batches apigenin loaded stealth liposomes

Formulation batches	Lipid	Cholesterol
Apigenin 1	30	10
Apigenin 2	30	20
Apigenin 3	30	30
Apigenin 4	60	10
Apigenin 5	60	20
Apigenin 6	60	30
Apigenin 7	90	10
Apigenin 8	90	20
Apigenin 9	90	30

Preparation and optimization of Apigenin:

Ethanol Injection Method was followed.

- 20 mg of drug Apigenin,20mg of stearic acid was dissolved in 2ml of ethanol, later weighed amt of lipid and cholesterol was added in the same beaker pre heated at temp 40ºC which is above the melting range of selected lipids. This drug lipid phase was mixed under continuous stirring at about 400 -500 RPM using a magnetic bead for about 45mins. (Drug lipid phase)
- 500ml of PBS was prepared using Millipore water ph. 7.4 (Aqueous phase)
- With the help of injection syringe drop by drop solution of PBS was placed to the drug lipid phase.
- When the aqueous phase was dropped to drug lipid phase. The drug lipid phase immediately turned milky indicating the vesicle formation.
- The volume was made up to 10 ml and the system was kept under stirring at 500 RPM using a magnetic bead for up to 3-4 hrs.
- To achieve further size uniformity the formulation was sonicated for 30mins with the help of probes sonicator and stored at 4ºC for further use.

Table No:7 The independent variables, observed responses according to 3^2 factorial designs of ASL PS, ZP and %EE.

Batch Code	Lipid	Cholesterol	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment Efficiency (%)
Apigenin 1	30	10	898	0.45	-10.7	81.7
Apigenin 2	30	20	925	0.53	-11.2	75.2
Apigenin3	30	30	700	0.30	-14.2	29.5
Apigenin 4	60	10	670	0.47	-3.76	63.3
Apigenin 5	60	20	562	0.56	-7.18	80.7
Apigenin 6	60	30	820	0.51	-8.45	48.2
Apigenin 7	90	10	578	0.65	-4.28	51.1
Apigenin 8	90	20	369	0.92	-2.44	73
Apigenin 9	90	30	565	0.76	-6.25	91.5

Coded level: (-1 Low), (0 Medium), (+1 High), Independent variables: X1- amount of lipid, X2- amt of Cholesterol, Dependent variable: Y1- Particle size, Y2- % EE, Y3- PDI.

4.4.2Particle size analysis:

The particle size of APISL ranged between 369-925nm (above table no 7). The equation was taken based on polynomial equation which was generated from the Design –Expert Software showing the impact of independent variables on APISL particle size.

Where PS is particle size which was taken as X1 lipid (DSPE-PEG2000), Cholesterol concentration (percent w/w) is represented by X2. The effect of (X1) DSPE-PEG2000 and (X2) Cholesterol on PS is depicted in the below. Fig no 18. The negative sign for both coefficients (independent variables) shows their antagonistic

effect on particle size. As the percentage of DSPE-PEG 2000 and cholesterol increases the particle size decreases. The suggested model for PS is linear model where the r^2=0.6744

PS = + 678.56-6.83 XA -171.83XB ---------- (1)

4.4.3 PDI analysis:

The Poly dispersibility index of the developed APISL ranged within 0.30-0.92, the equation was selected based on the suggested model for PDI is Quadratic model where the r^2=0.9691, which was with acceptable range 1.

PDI= +0.6111+0.0000XA+0.1750XB+0.0650XAB- 0.146XA^2 + 0.0883XB^2 ……(2)

4.4.4Entrapment Efficiency analysis:

1. The positive sign of coefficient for X_1 (DSPE-PEG 2000) indicates its synergistic effect on entrapment efficiency. As the concentration of DSPE-PEG 2000 increases, the EE increases. As the coefficient of X_2 is negative the concentration of Cholesterol has antagonistic effect on EE. As the percentage of cholesterol increases, the EE decreases. As the coefficient values of both are comparable, both the factors are having nearly equal contribution in EE. The suggested model for EE is 2FI model where the r^2=0.7748.

EE= 66.24- 4.15XA + 5.20XB+23.65XAB ---------- (3)

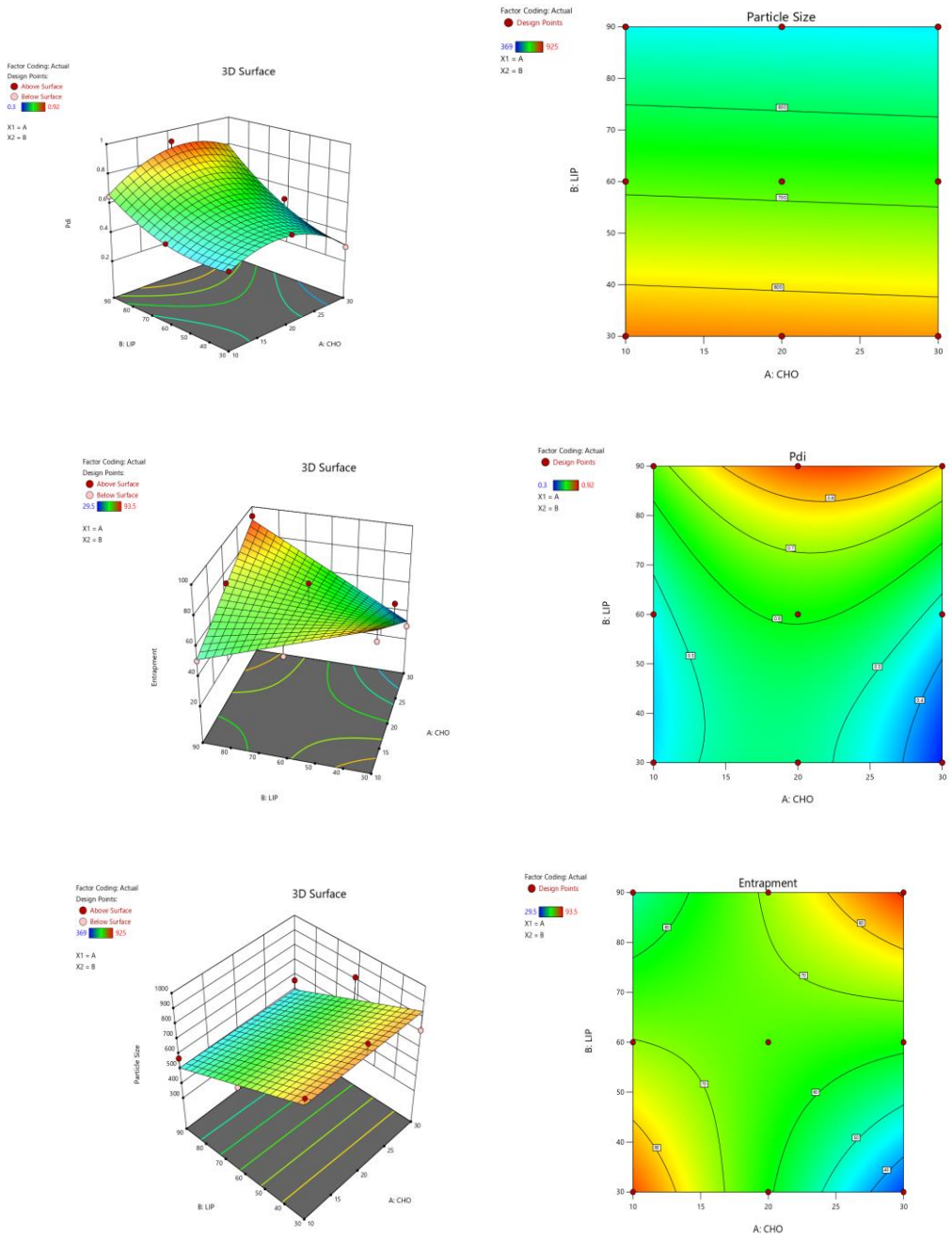


Figure no: 16 : DES: (ABC) 3D Response Surface plots. (DEF) Counter plots displaying the effect of lipid (X1) and Chol (X2) on PS, PDI and EE of ApiSL Nanoformulation

4.4.5 Selection of optimized stealth nanoformulation:

Based on the requirements of highest entrapment efficiency as well as smallest particle size, the optimum APISL batch was chosen among nine stealth liposome formulations. APISL8 batch containing ratios of DSPE-PEG(90 w/w) with CHOL (20 w/w) was chosen for further outcome measures results acquired for dependent variables, since it has the highest EE (73.4 percent for API correspondingly) with the smallest particle size (369 nm). Further assessment trials were conducted using the optimized stealth liposome batch (APISL8).

4.5 Characterization of stealth liposomes

4.5.1 PS ,PDI and ZP:

APISL8 (DSPE-PEG:Chol ratio 90:20 mg) was shown to meet the requirements of an ideal formulation after a full assessment. The optimized Apigenin loaded stealth liposomes (APISL8) had EE, PS, ZP, with PDI of 73.4 percent, 369 nm, -2.44mV, and 0.92, respectively. Figure 16 shows overall particle size distribution including ZP of optimized Stealth liposomes (APISL8).

Particle size range of prepared stealth liposome formulation was with the range ie 100-1000nm, as shown in below fig no: 17, was checked on the instrument nano track. Optimized formulation selected for both in vitro and in vivo study was 369nm. This showed lowest particle size with higher PDI AT 0.92.

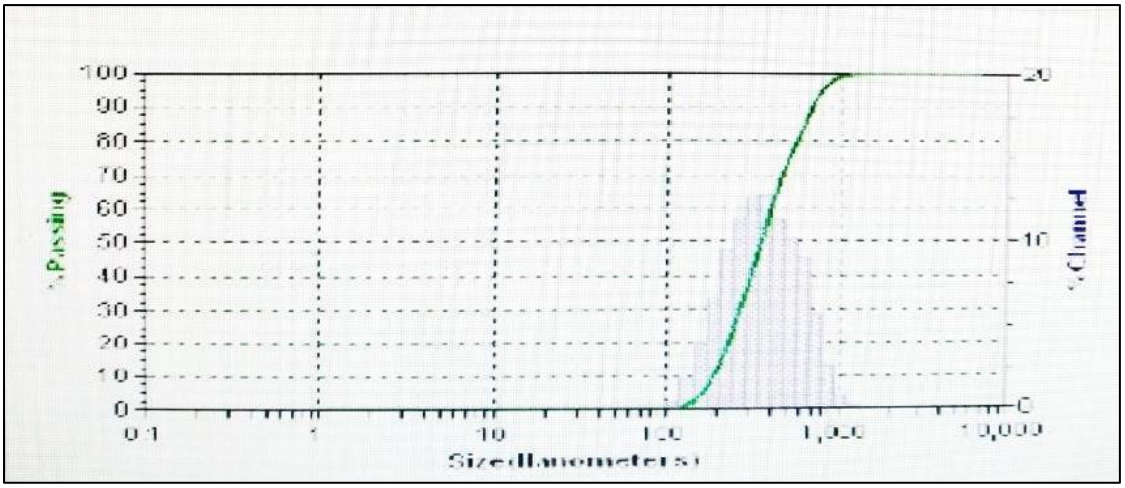


Fig no: 17 PARTICLE SIZE DISTRIBUTION OF APISL8

4.5.2 FTIR of optimized APISL8:

The FTIR spectrum of the physical mixture showed a change in the peak configuration; hence the compatibility is confirmed between the drug and the excipients. Ethanol softens the vesicle membrane and causes hydrocarbon chain (ethanol) interpenetration in the vesicular lipid double layer, resulting in a substantial reduction in vesicle membrane thickness. (Figure No 18).

"Ethanol gives the system a net negative charge and some steric stabilization, which may eventually lead to a decrease in vesicular size".

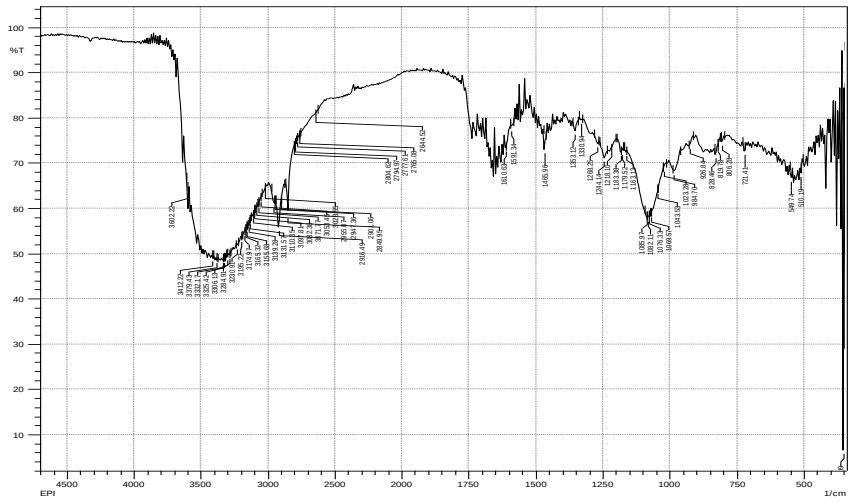


Fig no:18 FTIR OF OPTIMIZED BATCH APISL8

4.5.3 DSC of optimizedAPISL8 :

The DSC peak of the physical mixture showed a change in the peak configuration; where the peak of drug Apigenin was at hence the compatibility is confirmed between the drug and the excipients. The pure drug Apigenin released a sharp endothermic peak at 365.22 ^{0}C, where in lipid peak was at 115.06^{0}C, cholesterol at 62.60^{0}C respectively**.**

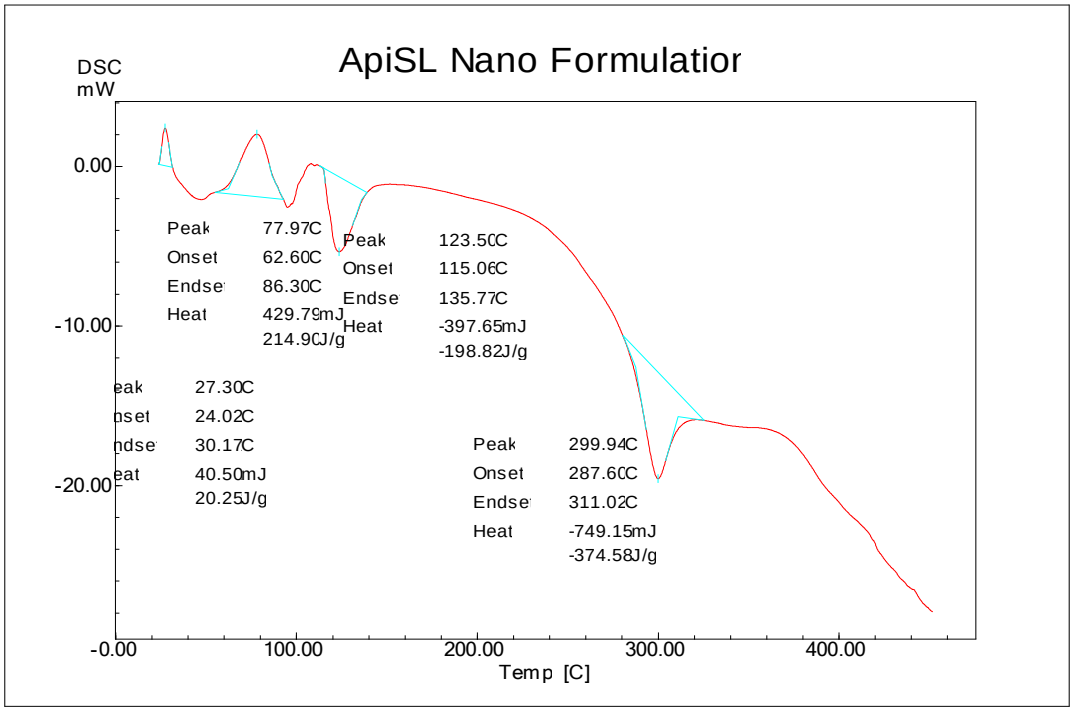


Fig no:19 DSC thermogram of Apigenin loaded stealth liposome

4.5.4 SEM

The vesicle morphology and shape were determined by Scanning electron microscopy (SEM). Images of vesicles were observed to be in nano-sized and unilamellar. The photographs showed that the vesicles are almost spherical in shape and uniform in size. SEM image is presented in figure no 20.

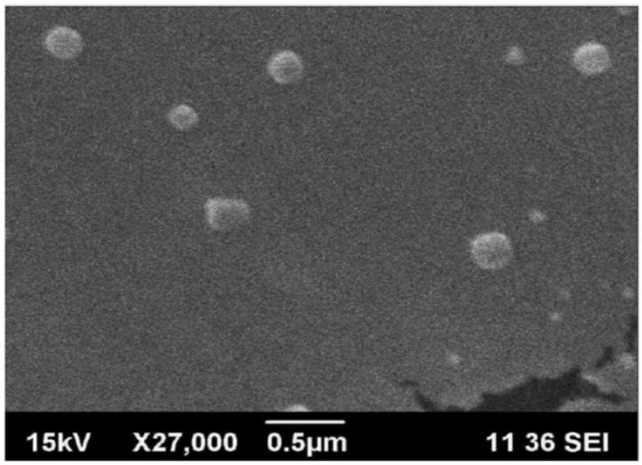


Fig no: 20 representing the spherical morphology (shape)

4.5.5Drug release study of optimized batch:

The optimized liposomes dispersion ApiSl8 showed 15.28% drug release in 48 hours when compared to plain API is 13.24. The drug release data shows the pattern which the drug release follows. The release pattern was determined by various kinetic models such as zero order kinetics, first order kinetics, Higuchi model and Korsmeyer- peppas model. The release of formulation Apigenin loaded stealth liposome and pure Apigenin followed Higuchi model kinetic pattern with highest R^2= 0.6752, which states that release pattern was through diffusion.

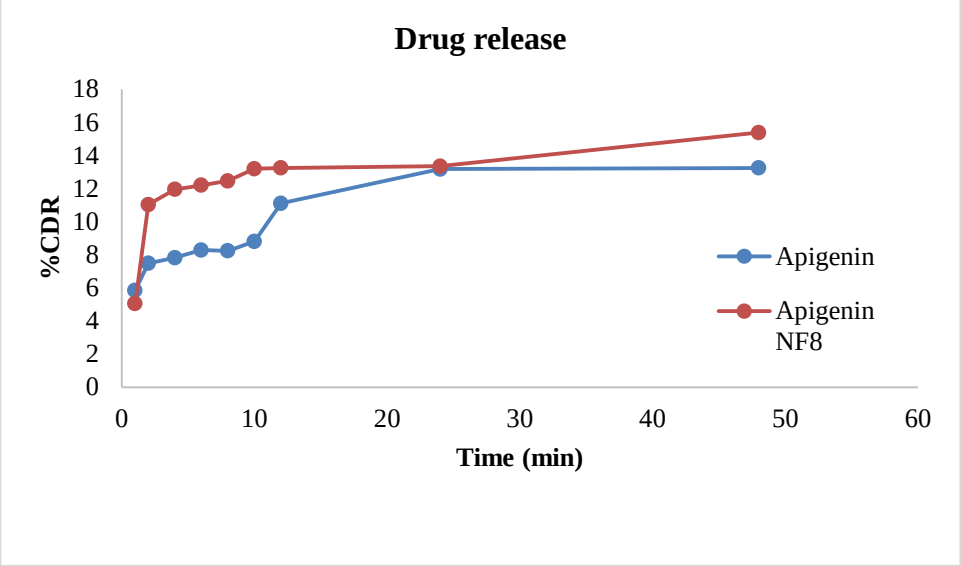


Fig no: 21 Dissolution profile of apigenin released from API, APISL8.

Sr.no	Time (hrs)	APISL8	API
1	1	5.057682403	5.842918
2	2	11.02729614	7.483433
3	4	11.94386266	7.819571
4	6	12.20085837	8.283691
5	8	12.45660944	8.238798
6	10	13.19158798	8.808412
7	12	13.24257511	11.1018
8	24	13.35124464	13.16979
9	48	15.38180258	13.24567

TABLE NO: 8 DRUG RELEASE BY DISSOLUTION

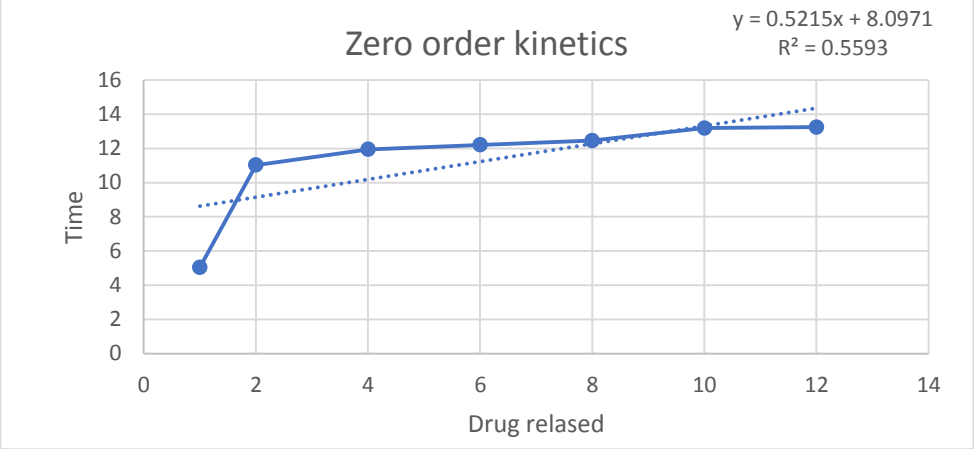


Figure No. 21 .a zero order kinetics

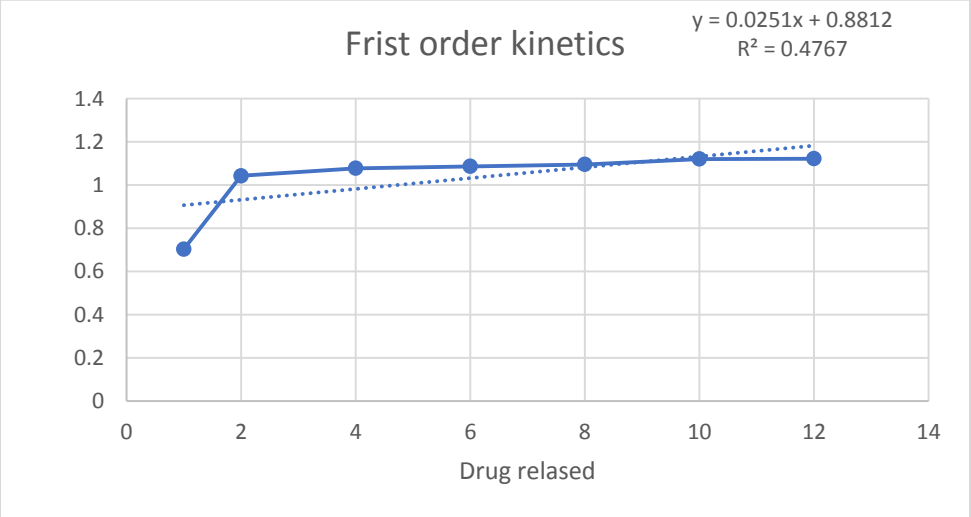


Figure No. 21 b. first order kinetics.

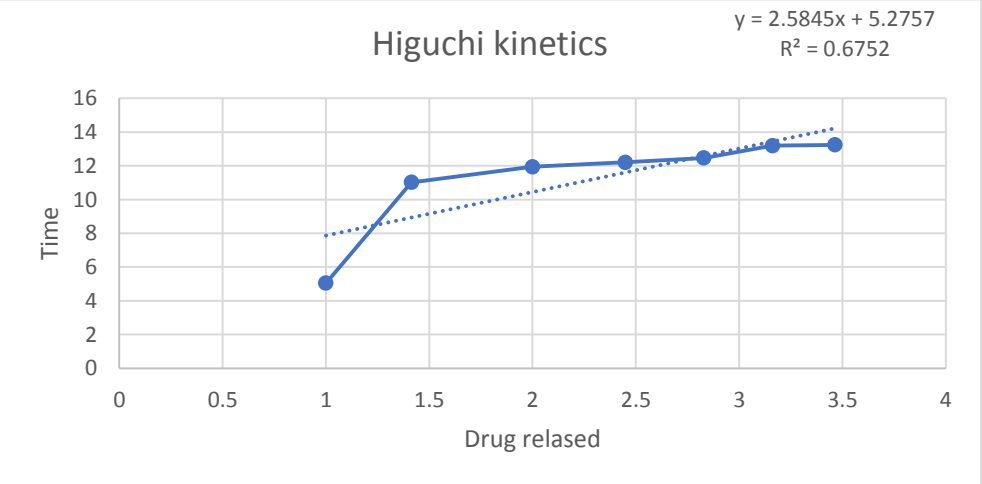


Figure No. 21 .c. Higuchi model.

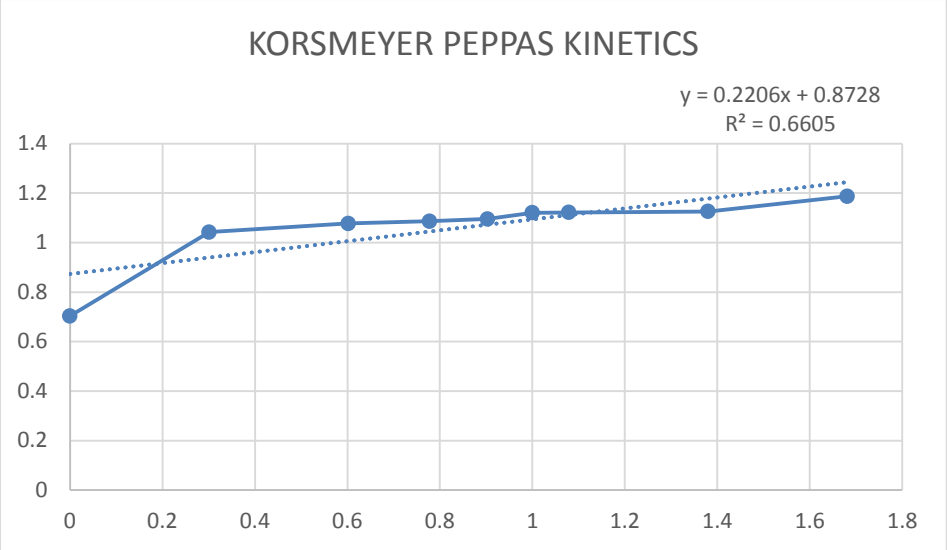


Figure No. 21 d. korsmeyer peppas kinetics

4.5.6 Stability study:

The optimized apigenin loaded stealth liposomes dispersion APISL8 were studied for the stability study; this prepared formulation was stored, the PS, PDI, ZP, EE, and %DR of the improved stealth liposome dispersion were characterized after being stored at 4 ºC at a period of 4 months at room temperature. The table no 9 summarizes the findings, results showed neglible changes as compared to previous results.

Table no: 9 Stability *study for optimized batch APISL8*

Optimized batch after 3 months	Particle size (nm)	PDI	Zeta (Mv)	% EE	%DR
APISL 8 (Initial)	369.2	0.92	-2.44	73.09	73.7
APISL 8 (After 4 months)	378.2	0.97	-2.75	71.00	77.2

In vitro anti-cancer activity for optimized apigenin loaded stealth liposome:

a) The *In-vitro* cytotoxicity study:

An MTT assay was used to test the cytotoxicity of apigenin (API) and optimized Apigenin loaded stealth liposome batch (APISL8) against the MCF7 cell line for 24, 48, and 72 hours. The viability of MCF-7 cells was tested at concentrations ranging from (2.5 to 80 µg/ml). The vitality of the cells was significantly reduced after treatment with the plain medication (API) and the improved formulation (APISL8) The results suggest that the cytotoxicity of API and APISL8 liposomes was dosage and time dependent, while APISL8 demonstrating considerably higher cytotoxicity against MCF-7 than API. Pure apigenin (API) had IC_{50} values of 70.78, 64.95, and 52.9µg/ml for 24, 48, and 72 hours, respectively, whereas optimized stealth liposome (APISL8) had IC50 values of 63.37, 52.65, and 43.02µg/ml, respectively.

Table no : 10 representing the IC_{50} value for 24, 48,72hrs

IC_{50} (µM)	**Apigenin**	**Apigenin loaded stealth liposome(APISL8)**
24 Hours	70.78	63.37
48 Hours	64.95	52.65
72 Hours	52.95	43.02

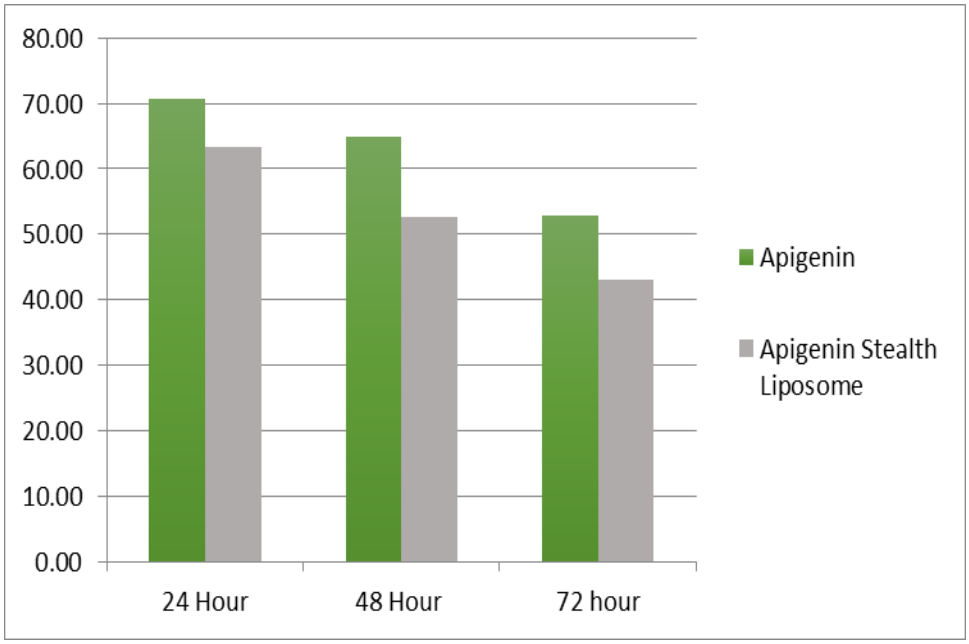


Fig no: 22 Representing API & APISL8 cytotoxicity studied for 24,48 &72hrs

4.6 In vivo study:

4.7.1 Estimation of liver marker enzymes:

Different markers were used to profile the toxicity of API and APISL8. ALP, ALT and AST levels have been measured intended for nephrotoxicity. Untreated animals were served as a control in the study. Animals given plain apigenin; apigenin loaded stealth liposomes did not develop liver or kidney damage, as illustrated in the image. Furthermore, histological investigations verified the biochemical findings, indicating that free API and APISL8 are harmless. Liver and kidney histological sections (Figure 24), there was no indication of inflammation or necrosis, indicating normal parenchymal cell physiology.

In-vivo toxicity studs:

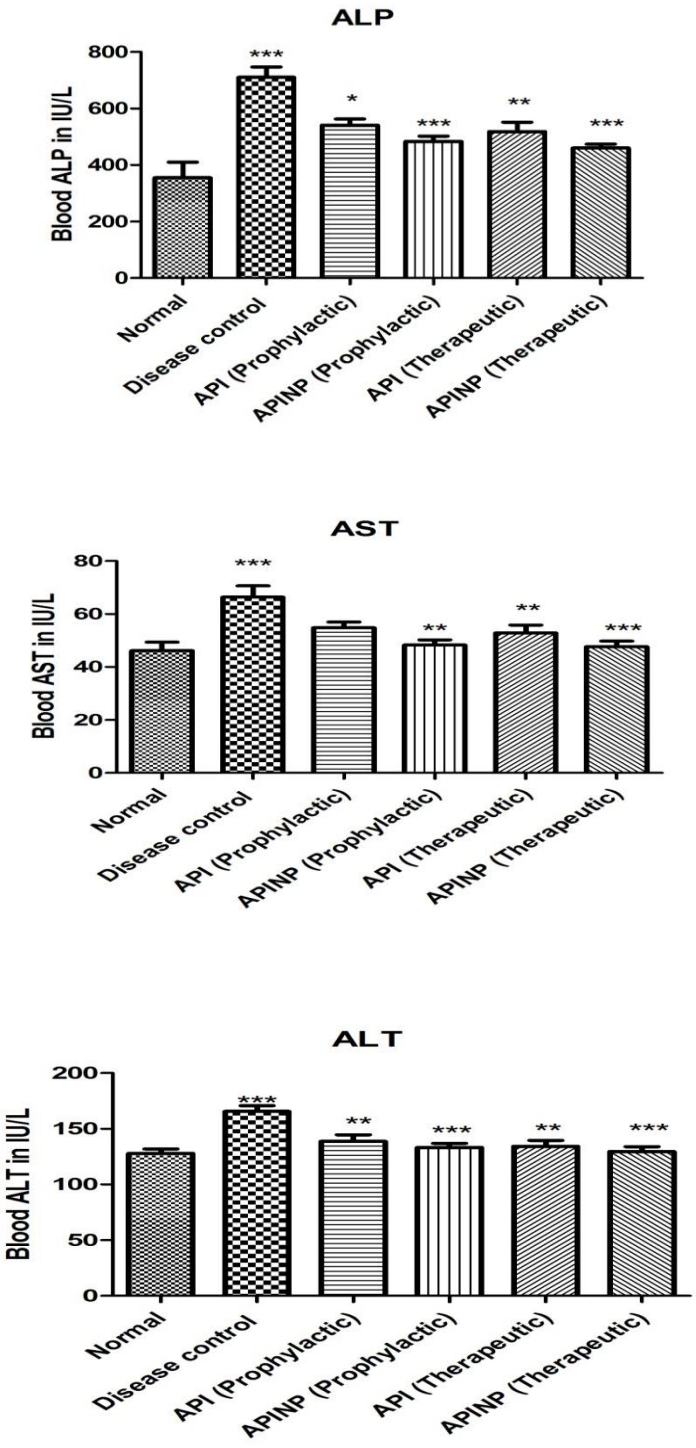
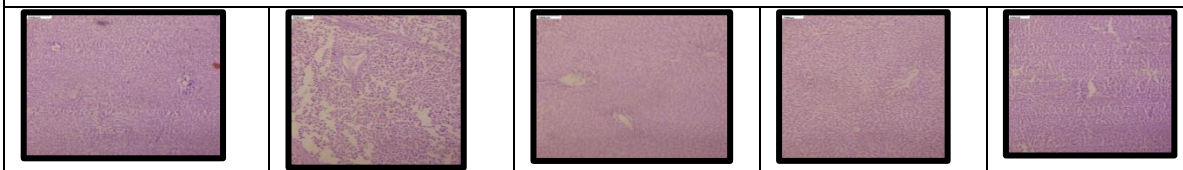


FIG 23: Results of biochemical liver marker enzymes ALP, AST and ALT (A, B, C)

In the liver marker enzymes ALP, AST and ALT .All values were expressed as mean ± SEM for 6 groups (n=6) Values are statistically significant at*p < 0.05, ** p < 0.001 & *** p < 0.0001. An intergroup comparison was done by one way ANOVA followed by Tukey's – multiple comparison test. Comparisons were made between Normal Control vs Disease control . Whereas Disease Control vs API-Prophylactic, APINP-Prophylactic , API-Therapeutic & APINP-Therapeutic

Histopathology examinations of liver and kidney

In isolated liver:



In isolated kidney:

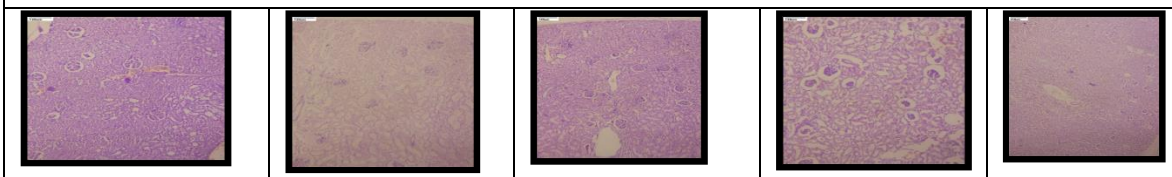
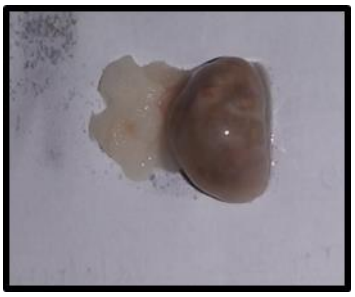
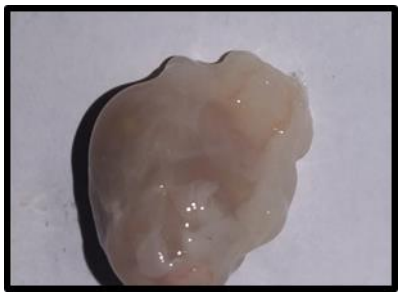


Fig no: 24 represents that the liver and kidney histopathological examination we observed higher nontoxic behaviour of Api and Apisl8

4.*8 In vivo* anticancer study of API and APISL8 in DMBA induced animal model

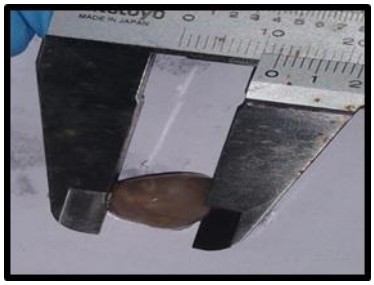
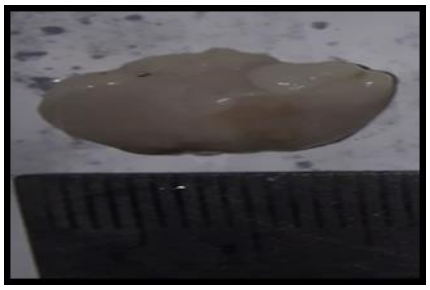
APISL8's prophylactic and therapeutic anticancer activities were tested in female SD rats using DMBA-induced BC. The DMBA control group had a 100% tumour incidence, but the prophylactic and therapeutic (preventative and treatment) groups had a lower tumour incidence. To compare the efficacy of APISL8 with free API, the study looked at tumour morphology, histology, relative tumour volume , tumour nodules and liver biochemical test (ALT), (ALP), and (AST). Mammary tissues from the disease control group (Figure 23 ABC) a significant change in size, colour, consistency, and surface texture were observed. The material in necrotic tissue was pale in colour and expanded in size which shows nodules in the prophylactic and therapeutic API treated groups. In rats, the prophylactic and therapeutic APISL8 treated group (Figure 25 BC) demonstrated a significant reduction in tumour size with necrotic tissue damage produced by DMBA Administration of API and APISL8 (groups III–VI) revealed very few differences when compared to DMBA alone treated SD rats (group II) . As rats were injected with APISL8 in both prophylactic and therapeutic conditions (groups IV and VI), the formation of tumours was significantly slowed, with substantial changes in size and morphology, when compared to DMBA-treated rats. (Figure 25 A) shows a sharp rise in AST, ALT, and ALP total in DMBA-treated rats, which is indicated of mammary injury. When compared to DMBA-induced BC, APISL8 therapy resulted in a considerable reduction in AST, ALT, and ALP levels in BC-induced mice (group II). In comparison to (Disease control) group II BC carrying rats, preventive and curative APISL8 therapy led to a significant reduction in cancer development without necrosis.

❖ **Disease control group:**

Observed large tumor with necrotic tissue and reduced

❖ **Prophylactic group:**

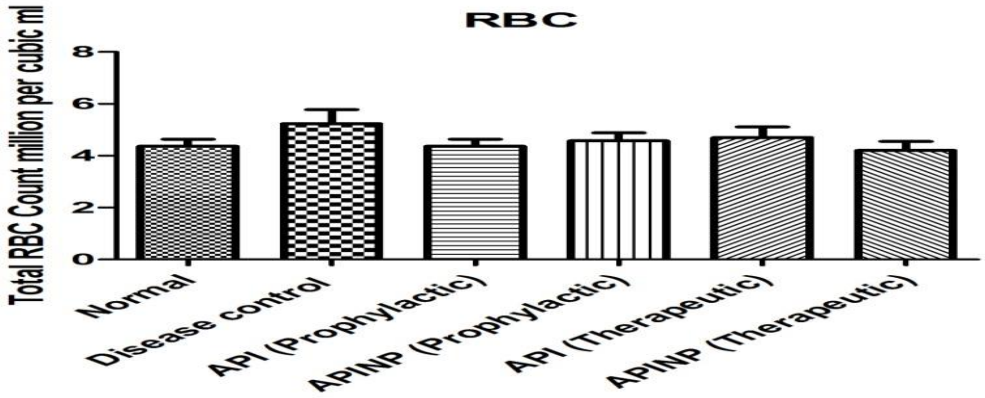
Observed tumor with necrotic tissue and reduced tumor size in female SD rats.

❖ **Therapeutic group :**

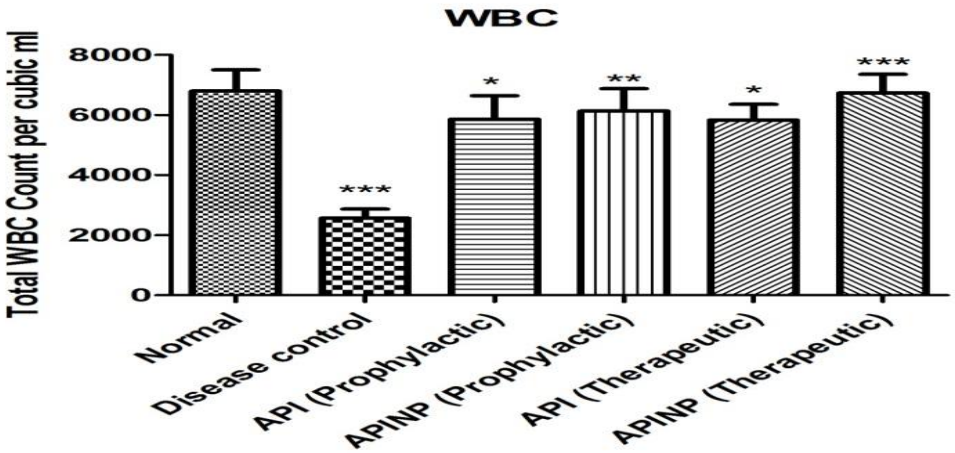

Comparatively observed reduced in tumor size without necrotic tissues in female SD rats.

Fig no :25 respresenting the tumor difference obsevered when compared to disease control group(A), prophylatic group(B) and therapeutic group(C)

Estimation of blood parameters:



All values were expressed as mean ± SEM for 6 groups (n=6). Intergroup comparisons were done by one way ANOVA followed by Tukey's – multiple comparison test. Comparisons were made between Normal Control vs Disease control (5.23 ± 0.53). Whereas Disease Control vs API-Prophylactic (4.36 ± 0.26), APINP-Prophylactic (4.57 ± 0.30), API-Therapeutic (4.70 ± 0.40) & APINP-Therapeutic (4.20 ± 0.34)



All values were expressed as mean ± SEM for 6 groups (n=6) Values are statistically significant at*p < 0.05, ** p < 0.001 & *** p < 0.0001. An intergroup comparison was done by One way ANOVA followed by Tukey's – multiple comparison test. Comparisons were made between Normal Control vs Disease control (2567 ± 300.7). Whereas Disease Control vs API-Prophylactic (5867 ± 776.6), APINP-Prophylactic (6133 ± 743.3), API-Therapeutic (5833 ± 525.1) & APINP-Therapeutic (6733 ± 622.2)

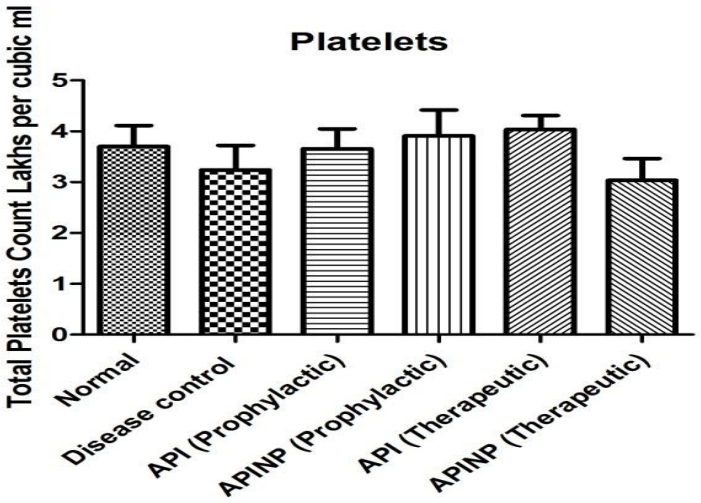


Fig 26 (A,B,C) differences observed from blood parameters RBC,WBC and PLATELETS

All values were expressed as mean ± SEM for 6 groups (n=6). Intergroup comparisons were done by one way ANOVA followed by Tukey's – multiple comparison test. Comparisons were made between Normal Control vs Disease control (3.23 ± 0.48). Whereas Disease Control vs API-Prophylactic (3.65 ± 0.39), APINP-Prophylactic (3.91 ± 0.50), API-Therapeutic (4.03 ± 0.27) & APINP-Therapeutic (3.03 ± 0.43)

In vivo BC study on tumor volume:

All rats throughout the study were placed into six groups, that each group had six animals. (Table 4). The drug treatment of formulation was continued up to the 16th week. The tumor was removed after dissection on the last day of experiment ie 16^{th} week using a Vernier caliper respectively and tumor size was measured , Tumor volume was used as a parameter for tumor inhibition in anti-tumor activity which was statistically represented in fig no 27. And at the end of the sixteenth week, female SD rats from each group were beaten, the tumour tissues were then treated with just a 10% formalin solution. Hematoxylin and eosin dye were used to stain sections of tumour tissues, and the images were viewed under a light microscope (Olympus, Delhi).

Tumor volume:

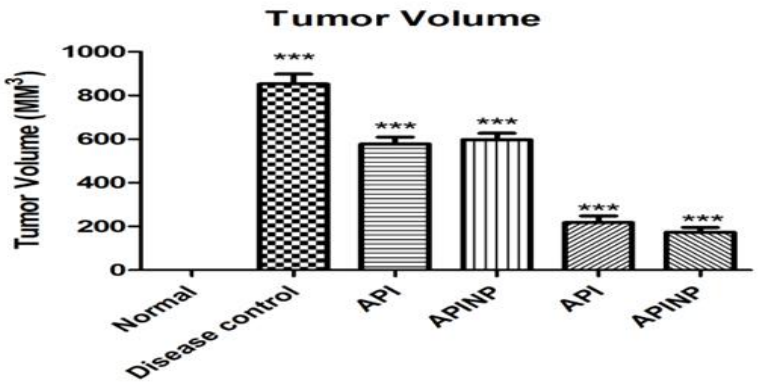


Fig no : 27 Tumor volume

All Comparisons were made between Normal Control vs Disease control & Disease Control vs API & APINP. In case of tumor volume statistically significant results were observed as shown in the graph. There was significant reduction in tumor volume in therapeutic group treated ApiSL formulation when compared to disease control group and prophylactic group.

Histopathology of mammary tissue:

Difference observed in the histopathology of mammary tissue when compared to disease control, prophylactic group and therapeutic group from fig 28 shows that there was dedifferentiated cells and mucin in mammary tissue for disease control group, there was only adenosis and necrosis with fibrosis were present in prophylactic group and finally there was no mucin and foreign material without necrosis in therapeutic group , which proves that the optimized apigenin loaded stealth liposome was effective the treatment of mammary tissue when compared to plain apigenin.

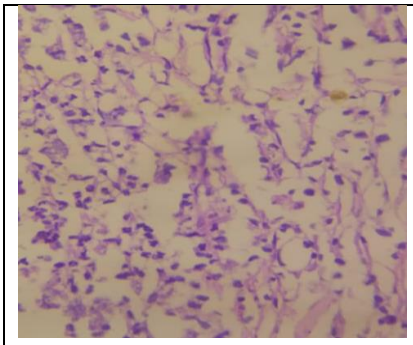
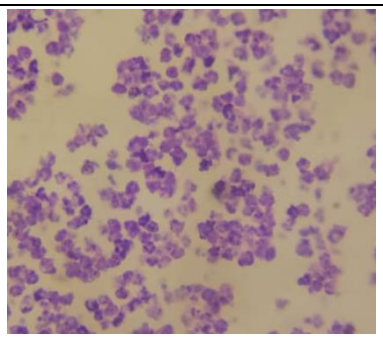
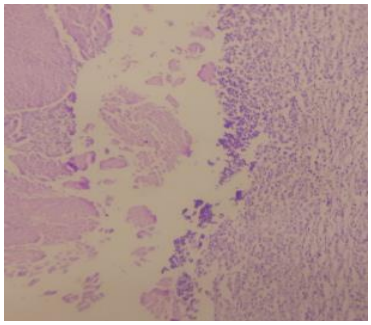
		
Mammary tissue segment of a disease control (DMBA treated group) SD rat.	**Prophylactically administered group's.**	**Therapeutic group shows Mammary tissues having no necrosis.**

Fig no: 28 (A,B,C) representing the histopathology of mammary tissue observed from the disease control group, prophylactic group and therapeutic group.

5. DISCUSSION:

The identification of pure drug was made by FTIR and DSC to test the identity of the API obtained from Aktin Chemical China. The FTIR spectra API displayed all the feature peaks in the number of different waves corresponding to the various active groups present in the API. A sharp endotherm peak of API displayed at 365.45 °C confirmed its glass transition temperature/melting point. FTIR spectrum of API was in conformity with the literature reports. Thus the results confirmed that the obtained drug was API. A simple and precise RP-HPLC method validated as per ICH guidelines was used for the estimation of API. All the parameters were found to be within the acceptable limits. The essential criteria to consider throughout all phases of formulation, particularly those recommended for parenteral administration, when establishing a new drug delivery system, are physicochemical qualities and stability. This ensures acceptable bioavailability of administered drugs and biocompatibility. The APISL acquired through factorial design (3^2) fixed that independent factors like (X1) DSPE-PEG, (X2) Chol, independent variables (X1) and (X2) concentration both had significant on the current study, effects on dependent variables such as PS, PDI, and EE were investigated. The total amount of medication successfully entrapped inside the stealth liposomes is expressed as percent entrapment efficiency (percent EE). One of the most important parameters to examine is the percent EE, which indicates a system's medication retaining capacity and decisive delivery capability. Although both hydrophilic and hydrophobic medications may be encased in this stealth liposome in this stealth liposome vesicle, it has been reported in the literature that the percent EE for hydrophobic pharmaceuticals is significantly larger than for lyophobic drugs. The EE of the liposomal formulation was determined by the molar concentrations of DSPE-PEG and cholesterol involved in their manufacture, as well

as the amount used. Increasing in the DSPE-PEG: Chol molar ratio increased in the drug EE. The lipophilic interaction of the hydrophobic drug with the vesicle membrane, which enhances the percent EE, is attributable to the beneficial influence of phospholipid content on percent EE. (68). Furthermore, increasing the concentration of phospholipid for drug molecules improved the accessibility of the lipid phase, leading in lipid bilayer accommodation. In the Stealth liposomes, a nonlinear increase in APISL encapsulation efficiency was found. The liposome size distribution is a crucial characteristic, especially when the stealth liposomes are intended for medicinal usage via inhalation or parenteral administration.(69) the reticulo - endothelial system (RES) filters vesicles from circulation, phagocytosis by Kupffer cells, and distribution and accumulation in certain organs are all influenced by PS. PS of optimized stealth vesicles in this study was in the range of 369 nm, indicating that these vesicles have a lower absorption into the RES and a slower clearance rate from plasma. Furthermore, due to the EPR effect, nanoparticles of sufficient size (upto 4,000 kDa/500nm) can penetrate through the tumour vascular walls and move into the neoplastic lesion. The amount of DSPE-PEG in APISL formulations has a direct positive effect on particle size. During the formulation development process, all formulations were tested for ZP. This is a valuable method for determining the surface charge of vesicles, which is used to predict colloidal dispersion stability. The balance of surface positive and negative charges is known as ZP, and it is proportional to the potential size. (66). Basis of the findings, the observed ZP of the optimized stealth liposomal formulation in this research was 38.03. Particle size distribution and homogeneity of nanoparticles are evaluated using parameters such as PDI. It's also used to assess the preparation technique's consistency and efficiency. A homogeneous population is defined by low PDI values, whereas a

diverse population is defined by high PDI values. The PDI of all liposomal formulations under this study was about 0.92, representing restricted size uniformity with a high grade of homogeneity. SEM is used to examine morphology of liposomes, and the images clearly demonstrated a homogeneous size distribution and a spherical shape. The hydrophobic nature of lipids may be responsible for the creation of sphere-shaped morphology. When lipids were exposed to an aqueous environment, they spontaneously form sealed bilayers, releasing the stealth liposomes' exterior surface hence becomes polar hydrophilic. (24), the liposome's structure and stealth liposomes' surface shape was smooth, with no vesicular aggregation, indicating that they were stable. DSC analysis was used to confirm the physical state of the medication put into the nanoparticles. DSC is the most widely used thermal analytical technique in the pharmaceutical industry, as well as in analytical, quality assurance, and research and innovation laboratories, to investigate a diverse range of physicochemical phenomena such as purity, polymorphism, crystallization, glass transition, drug–excipient compatibility, degradation, and stability determinations. The removal of the API endothermic melting peak in APISL8 thermograms indicated that the crystalline substance was converted to an amorphous state when encapsulated into the liposomal system. APISL8, but at the other hand, had a clear Mannitol peak at 166.0°C, It was used in the lyophilization process as a cryoprotectant. FTIR experiments were carried out to establish the API's entrapment in liposomal systems. The FTIR spectra of APISL8 showed all of the peaks associated to Apigenin, DSPE-PEG, Cholesterol, and Mannitol, indicating that these excipients haven't yet changed. Furthermore, the absence of typical API peaks in the APISL8 FTIR spectra may imply efficient API entrapment in nanocarrier systems. The API molecules were trapped in the inner core of the spherical nanovesicles and physical instability Nano dispersions may appear

throughout the formulation process, the agglomeration or fusion of unstable vesicles, or after long-term storage, resulting in a particle size increase. Stealth Liposomes grow in diameter, resulting in a brief half-life due to fast approval by RES method, followed by elimination (26,28). As a result, the most important part of medicinal product development is preparing stealth liposomes with a tiny and homogenous size distribution. After 3months storage period, there was no discernible difference in PS, PDI, or EE at 4^0C of the improved stealth liposome formulation in dispersion form, indicating that the formulation had high long-term stability. The in-vitro drug release studies were completed in order to get useful insight into the in-vivo performance of drug-loaded formulations (Stealth liposomes). API from APISL8 drug release profiles in vitro at pH 7.4 which is blood Ph demonstrated sustained and full drug release in 48 hours. Over the course of the investigation, no significant differences in API release from APISL8 were discovered. Our findings support those of Karve et al., who investigated the properties and DPPC (16 carbon containing lipid; 1,2-dipalmitoyl-sn-glycero-3-phosphocholine, sodium salt) lipids, DPPC and DPPA lipids, and DSPC and DSPA lipids) in various ph. The drug release mechanism is the method through which the payload is released from the nanocarrier. The characteristics of the polymer/lipid used in the formulation, as well as the release conditions, which can include dissolution, diffusion, swelling, erosion, and degradation, determine it to investigate the APISL8 and API release mechanism; four kinetic models were used to suit the release data. The Higuchi model had the highest R2 value for APISL8 (R2 value 0.6752), according to the research. As a result, API from APISL8 is released using the Higuchu model, which involves surface dissolution and is reliant on the surface area and diameter of the vesicle. It is critical to assess the in-vitro cytotoxicity of the formulated formulation for probable anticancer treatment

before initiating the in-vivo test. The MTT assay was used to assess the cytotoxic activity of API and APISL8 against MCF7. API and APISL8 cytotoxicity against MCF7 was dosage and time dependent, with APISL8 exhibiting considerably higher cytotoxicity than API. APISL8 may have better efficacy than API due of the increased absorption of Stealth liposomes and API solubility is improved in the formulation of nanoliposomes. The physico-chemical features of nanocarriers such as particle size, shape, and surface charge have a significant impact on their cellular absorption effectiveness. One of the crucial parameters is the size of the nanoparticle (50-5000 nm), which has the ability to enter the cell without restriction. (46,47). In this study, we discovered that the optimized APISL8 formulation had higher API absorption in MCF7 cells than the native API solution. In comparison to normal cell surfaces, In addition to PS, tumour cell surfaces have strong negatively charged components (72,73). Because of a fundamental electrostatic interaction, cationic liposomes have a high proclivity for endocytosis in tumor cells. (81). Toxicity testing for new chemicals is crucial in the drug development process. The experimental product's species, organ, and dose-specific adverse effects are revealed through preclinical toxicity research on a variety of biological systems. Different toxicity markers have been used to profile the toxicity of the API and APISL8 formulations. The levels of ALT, AST, and ALP in the blood were measured. API and its stealth liposomal version (APISL8) did not cause liver or kidney damage after one week of treatment. Histopathological studies validated the biochemical findings, indicating that free API and APISL8 Apigenin loaded Stealth liposomal formulations are harmless. The liver and kidney results of the histological examination demonstrated normal parenchymal cell physiology, with no evidence of inflammation or necrosis. Liposomes drug delivery systems (DDS) are great choices for overcoming medication characteristics that aren't perfect. In

comparison to the animal group that received higher concentrations of API from APISL8 were found in the plasma and liver. The opsonization and phagocytosis of the liposomes could explain the increase in API plasma concentrations in animals treated with APISL8. Furthermore, because of the electrostatic interaction with the negative surface of the peritoneal mesothelium, Positive liposomes are poorly absorbed by peritoneal macrophages because their positive charge limits absorption. The PS distribution for the improved stealth liposomes is mostly responsible for tissue distribution and clearance of the API after intraperitoneal injection of APISL8.As a result, the mechanism stated which indicates usefulness of stealth liposomes as a promising breast cancer affecting method. DMBA-induced BC in SD rats was used to evaluate the prophylactic and therapeutic anticancer abilities of API and APISL8. 7,12-Dimethyl-Benz-anthracene (DMBA) is a well-known carcinogen that has been used to produce breast cancer in animal models. Reactive oxygen species have been implicated in a variety of cancer in experimental, clinical, and epidemiological research. Due to the increased generation of free radicals, DMBA has been linked to oxidative stress and cellular damage. (83,84).

In ability to predict the capability of stealth liposomes, scholars have found that APISL8 supplied prophylactically or therapeutically significantly ($P<0.001$) reduced the number of malignant tumors without producing any changes in body weight in SD rats when compared to DMBA administered rats. Although the animals in the free API group had fewer mammary tissue, the results were not as significant as those in the stealth liposomes group. API dramatically reduced the incidence and quantity of mammary tissue in a chemically generated BC animal model, thus according previous information . Apigenin (API) was shown to restrict. The lowered accessibility of cells to malignancies as a result of API's enzyme regulation could be

related to the inhibition of carcinogen activity. The administration of DMBA to SD rats caused the AST, ALT, and ALP values in the blood were significantly increased, indicating mammary injury. The cohort of (ROS) during DMBA metabolism in the mammary gland causes oxidative stress, which leads to cellular damage in Prophylactic and therapeutic treatment of BC-bearing rats with APISL8 resulted in considerable decrease when there was comparison to that same disease control group, there had been a rise in the level of ALT, AST, and ALP. When compared to group II Breast Cancer bearing rats, the results demonstrated that preventive and curative treatment with APISL8 considerably decreased ($P<0.001$) the AFP levels. Histopathological tests of liver tissues provided additional strong estimony supporting APISL's beneficial consequences in battling DMBA-induced BC, which improved the reliability and repeatability of the diagnostic pathology. Administration of DMBA caused substantial histopathological abnormalities in the mammary tissue, which could be due to the formation of multinucleate normal and cancer cells with genetic anomalies and improper divisions associated with malignant variants. API was used in some other study to target many cellular targets such as invasion, metastasis, angiogenesis, cellular proliferation and growth - apoptosis, all of which help to prevent or halt the progression of inflammatory diseases such as cancer. APISL8 showed enhanced drug accumulation, promising anticancer activity and appropriate tolerability in DMBA-induced BC in SD rats in all in-vitro and in-vivo experiments.

6. SUMMARY

Apigenin is a polyphenolic molecule with anti-aging, anti-diabetic, cardio protective, anti-obesity, antioxidant, and anti-tumor properties. API has anti-inflammatory, antibacterial, anti-cancer, anti-spasm, and sedative properties, according to evidence. Multiple studies looking into Apigenin used as a potential marker in various cancer studies, however no systematic review of the findings has been published. API is an excellent choice for the treatment of BC due to its strong therapeutic potential and multi-targeting capacity. Its formulation development was hampered by its low water solubility and susceptibility to oxidative breakdown. Apigenin-loaded Stealth liposomes (APISL) nanoparticles were developed and tested in this study to see if they could be used to treat BC. The next paragraphs provide a summary of the current study.

1. FTIR and DSC tests were used to make a preliminary API identification and compatibility study. For the quantification of API, a UV Spectrophometric and a reversed phase-HPLC method verified in accordance with ICH criteria were utilized. Preliminary screening investigations were carried out to find the levels of formulation variables that potentially influence liposome properties. Using 3^2 factorial design (two-factor, three-level) in Design-Expert® software, the APISL was successfully created and optimized. Each of the two independent variables/factors (DSPE-PEG: Chol molar ratio) were evaluated at 3 levels differently i.e low, medium and high. To have a better understanding of how the dependent and independent variables interact, contour graphs and response surface plots were plotted. Nine formulations were created using a factorial design, and the best batch was chosen based on PS, PDI and %EE. SEM analysis was used to examine the morphology of liposomes, which

revealed a spherical shape. For more research the loading of API in Stealth liposomes was validated by DSC thermograms and FTIR spectroscopic investigations. Using ready-to-use dialysis tubes, in-vitro drug release of API and optimized APISL8 at pH 7.4 was done and sample was then subjected to a variety of release kinetic models. The results showed that drug release was sustained and complete in 48 hours, and the Higuchi model was shown to be the best fit model for APISL8 release from Stealth liposomes, with the highest R_2 value 0.6752. After 3 months of storage at 4^0C, the optimized APISL8 formulation was confirmed to be stable in both dispersion and lyophilized form.

2. API and APISL8 demonstrated dose and time dependent alterations in cytotoxicity assays, with APISL8 confirming considerably higher cytotoxicity against MCF7 cells than free API.

3. Animals treated with API and APISL8 did not develop liver or kidney toxicity in *in-vivo* toxicity testing. The biochemical assessment was backed up by histological studies, indicating that free API and APISL8 are harmless.

4. When compared to free API, APISL8 treated SD rats had significantly less necrotic and liver marker enzymes, according to our pharmacodynamic study. Histopathological investigation confirmed the findings, demonstrating that the DMBA-induced adverse effect with APISL8 formulation therapy, the rat mammary tissue was successfully reversed.

7. CONCLUSION

API's application is limited by physiological characteristics such as poor bioavailability, short half-life, and water solubility, despite its well-known utility in cancer therapies. Stealth liposomes are one of the FDA-approved prospective formulation technologies for cancer therapy. The use of cationic liposomes as a BC carrier, we aimed to increase the bioavailability and kinetic characteristics of API in our study. The 3^2 factorial designs were used to successfully develop and optimize API. The optimized stealth liposomal formulation (APISL8) demonstrated a spherical morphology with uniform size, suitable vesicle size, zeta potential, and improved entrapment efficiency. The API was successfully entrapped in the Stealth liposomal vesicular formulation, according to the DSC and FT-IR measurements. *In-vitro* cytotoxicity tests revealed the APISL8 had a higher killing capability against cancer cells than the free drug. In the *in-vivo* study APISL8-treated animals had higher drug concentrations in breast cancer tissue than untreated animals. API had a chemo preventive and therapeutic impact on DMBA-induced BC, according to *in-vivo* pharmacodynamic studies. According to our findings, the APISL delivery system has a great potential for treating BC, but it will require a large-scale preclinical efficacy research to verify the concept.

8. References

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Animal Ethical Committee Approval letter

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BELAGAVI

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
Reg.No.221/Po/Re/S/2000/CPCSEA

CERTIFICATE

Date: 17/03/2021

This is to certify that the project proposal no ...12..... entitled," Development and optimization of Apigenin loaded nanoparticles for targeting Breast Cancer " submitted by Dr./ Mr. / Ms., Priya P Shetti under the guidance of Prof. (Dr.) Sunil S. Jalalpure has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 13/03/2021, resolution No. 30 has been sanctioned .....36..... Rats/ Mice/ Rabbits/Guinea pig (animals) sex female under this proposal for a duration of next.....months.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

Authorized by	Name	Signature	Date
Member Secretary:	Dr. N.A. Khatib	MEMBER SECRETARY Institutional Animal Ethical Committee. KLES's College of Pharmacy, BELGAUM - 590010	17/03/2021
Main Nominee of CPCSEA:	Dr. Vinod Kumar cs	CPCSEA Nominee Institutional Animal Ethics Committee KLES's College of Pharmacy, BELGAUM.	17-3-2021

Original Article

Optimization of a Validated UV-Spectrophotometric Methodology for Assessment of Apigenin in Bulk Powder

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ABSTRACT

Introduction: Apigenin (4,5,7 Trihydroxyflavone) is a natural compound of flavonoids class, which is found in various plant constituents. Since primitive years, Apigenin has been used as a traditional medicine, because it possesses biological functions. Common ayurvedic formulations comprising apigenin are in market. Hence quality control of preparation covering apigenin is desirable. **Objectives:** In present study, pointed to enhance and validate UV Spectrophotometric method to evaluation of apigenin in bulk powder. **Methods:** Using methanol as solvent, UV Spectrophotometric method was developed. Apigenin showed supreme absorbance wavelength at 267 nm. New technique enhanced and validated in terms parameters such Specificity, Discrimination, Linearity, Correctness, Ruggedness, Solution stability as per each ICH guidelines. **Results:** The detector response for apigenin was linear in the sure concentration range of 2μg/mL-10μg/mL with correlation coefficient of 0.9995. Newly developed method was found to be specific, Selective linear, Precise, Rugged, reproducible for estimation of apigenin with %RSD values less than 2%. **Conclusion:** In this study Development and Validation of UV-Spectrophotometric method can be employed for the apigenin assessment in bulk powder.

Key words: Apigenin, Absorbance, ICH guidelines, Ruggedness, Spectrophotometric, Ayurvedic Formulations.

INTRODUCTION

Apigenin is a very significant natural compound existing in various fruits and vegetables such as parsley, celeriac, celery, chamomile tea, orange, apple, tomatoes.[1] Apigenin in Figure 1 is a flavonoid that is used as a traditional medicine for decades, as it establish to show DPPH, antioxidant, *in vivo* animal anti-inflammatory, cytotoxic study, anti-bacterial, anti-viral and numerous biological activities. One of the very significant activities of apigenin is anticancer activity.[2-4] Numerous ayurvedic formulations comprising apigenin were marketed for health care delivery systems.[5] The quality control of formulations containing apigenin is important in the ayurvedic industries. Literature survey revealed that analytical methods such as spectrophotometric, HPLC and HPTLC methods[6-9] were reported for the estimation of apigenin in various plant extracts. The stated methods were having their own confines such as extra time consuming, use of costly and hazardous solvents. As per the literature review, there was no established UV spectrophotometric method for valuation of apigenin in the form of pure form. Henceforth, it is needed to develop and validate UV spectrophotometric method estimation of apigenin by using newer solvent system. In the present research an effort has been made to develop

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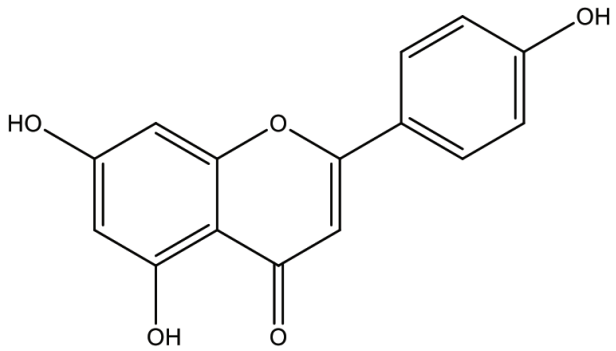

5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one

Figure 1: 2D structure of Apigenin.

and validate new UV-spectrophotometric method for estimation of apigenin in bulk powder.

MATERIALS AND METHODS

Chemicals

Apigenin was achieved as gift sample from Aktin chemicals, China. Beneficial chemicals and reagents used for the analysis pure, high analytical grade gotten from KAHER'S Dr. Prabhakar Kore Basic Science Research Center, Belagavi. UV-Spectrophotometer of Shimadzu make and model UV-1900 having UV probe software were used for analysis. Calibrated weighing balance was used for weighing drug sample in the study.

Method Optimization

Development also optimization of new UV Spectrophotometric method was accepted by selecting the proper solvent system the wavelength were recognized from review literature. Solubility of apigenin was screened by taking various solvents ethanol, methanol. In order to select the solvent system few trials were carried out, finally methanol was chosen as a solvent. Primary and secondary stock solution of apigenin was prepared by dissolving it in methanol. From these solutions working standard solution containing 10 µg/mL of apigenin was scanned between the wavelength regions of 400 - 200 nm against methanol as blank. The UV spectra were shown in and absorption curve showed characteristic absorption maxima at 267 nm and it was selected for analysis of apigenin.[10,11]

Method Validation

In order to validate newly developed method parameters as per the ICH guidelines (ICH guidance Q2A; Q2B) was followed.[12-15]

Specificity and selectivity

Employed standard solution containing 2 µg/mL Apigenin was observed between the range of 400-200nm. Spectrum of solvent as blank was obtained, analyzed for any interference of solvent at maximum wavelength of absorbance.

Linearity and range

While execution the study for linearity, 10mg of apigenin weighed and transferred into 10ml of volumetric flask and volume was attuned to the mark using methanol to obtained 1000µg/mL solution of apigenin. From this standard stock the working stock was prepared which consist of 1ml of standard stock was taken placed in another 10 mL volumetric flask covering methanol solvent and the volume was made up to the mark to 10mL, later further serial dilutions were made to prepare 2, 4, 6, 8 and 10 µg/mL solutions of apigenin. Solutions were ready in triplicates and absorbance's was measured at 267 nm.

Limit of Detection and Limit of Quantification

LOD and LOQ was calculated by using statistical calculations using formulas:

LOD = 3.3 × SD of y-intercept/ Slope of the calibration curve and LOQ= 10 x SD of y- intercept/ Slope of the calibration curve.

LOD = 3.3 × standard deviation of regression/ Slope

LOQ = 10 × standard deviation of regression/ Slope

Precision

Precision was measured in six replicates of solution containing apigenin that was prepared and the absorbance was recorded at 267nm on same day at different time intervals. On altered days the obtained system precision, intraday precision and interday precision data and absorbance were measured and % RSD was calculated.

Ruggedness and Reproducibility

In determine the ruggedness normally six replicates of solutions containing apigenin were prepared and absorbance of each replicate was measured by different analyst also by using different instruments and %RSD as calculated for absorbance.

Solution and standard stock solution stability

In order to check solution stability and obtained stability, fresh stock was prepared and dilutions were made using fresh solvent, absorbance's of each dilutions containing

apigenin was compared with that of old stock dilutions and % RSD for absorbance's was calculated.

RESULTS AND DISCUSSION

Optimization and validation

Solvent development step involves the use of methanol in which apigenin showed spectrum with maximum absorbance at 267 nm. Developed method parameters were presented below.

Specificity and selectivity

By obtaining solvent spectrum there was no interference of absorbance at 267nm hitch highlighted the specificity and method selectivity. The UV spectrum of solvent and apigenin is represented in Figure 2 and Figure 3 respectively.

Linearity and Range

Linearity and range was resolute by plotting standard calibration curve using concentration UV absorbance's obtained by linear dilution of apigenin. The absorbance range was between the concentration of 2, 4, 6, 8 and 10µg/mL. The regression equation of apigenin was 0.999. Linearity data as signified in Table 1. Overlay spectrum of linearity of apigenin is shown in Figure 4 with standard calibration curve was presented in Figure 5.

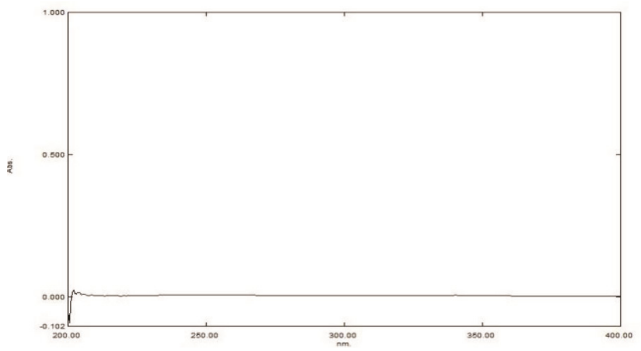

Figure 2: UV-Spectrum of Methanol.

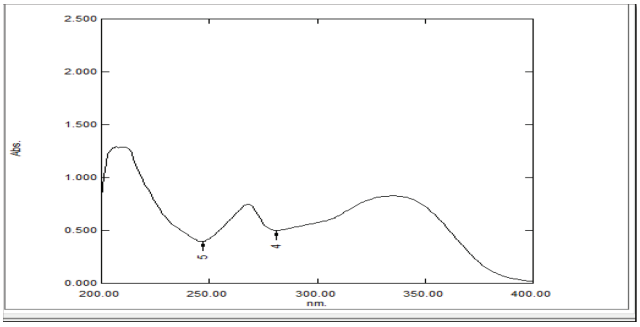

Figure 3: UV-Spectrum of Apigenin.

Table 1: Linearity and range data of apigenin.

Sr. No	Concentration	Absorbance at 267nm
1	2 µg/mL	0.179
2	4 µg/mL	0.288
3	6 µg/mL	0.401
4	8 µg/mL	0.519
5	10µg/mL	0.641
r^2		0.999
Slope		0.05775
LOD		0.27 µg/mL
LOQ		0.83 µg/Ml

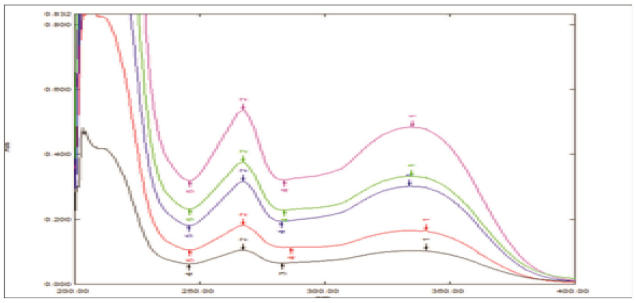

Figure 4: Overlay spectrum of Apigenin.

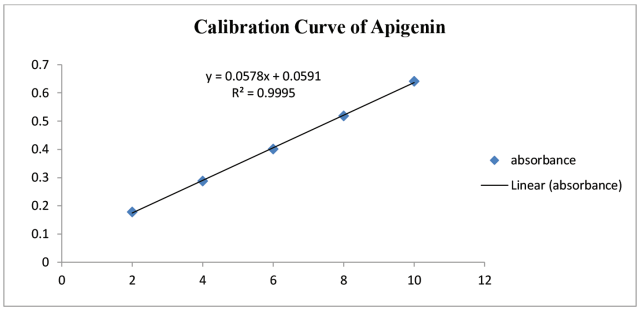

Figure 5: Standard calibration plot of Apigenin.

Limit of Detection and Limit of Quantification

By statistical calculation LOD and LOQ was found to be 0.27µg/mL and 0.83 µg/ml respectively.

Precision

Method was found to be precise as the %RSD calculated for six replicates solution of apigenin at each precision level was found to be less than 2% (Table 2).

Ruggedness and Reproducibility

The % RSD values calculated for apigenin was found to be less than 2% which indicates that the method was robust with slight change in the % composition of solvent system and reproducible as %RSD obtained for

Table 2: System, intraday and interday precision data of apigenin.						
Precision	**System precision**	**Intraday 1st hr**	**Intraday 5thhr**	**Interday -1 Precision**	**Interday -2 Precision**	**Interday -3 Precision**
2 µg/mL	0.164	0.171	0.168	0.171	0.155	0.161
2 µg/mL	0.167	0.174	0.172	0.174	0.159	0.165
2 µg/mL	0.168	0.172	0.172	0.172	0.154	0.169
2 µg/mL	0.166	0.167	0.174	0.167	0.157	0.167
2 µg/mL	0.167	0.164	0.176	0.164	0.159	0.164
2 µg/mL	0.166	0.166	0.177	0.166	0.161	0.166
%RSD	**0.82%**	**0.90%**	**1.87%**	**0.90%**	**1.69%**	**1.65%**
6 µg/mL	0.396	0.357	0.407	0.357	0.382	0.382
6 µg/mL	0.4	0.363	0.407	0.363	0.382	0.383
6 µg/mL	0.4	0.365	0.408	0.365	0.383	0.382
6 µg/mL	0.407	0.365	0.409	0.362	0.39	0.389
6 µg/mL	0.408	0.362	0.41	0.369	0.392	0.39
6 µg/mL	0.409	0.363	0.412	0.365	0.389	0.39
%RSD	**1.32%**	**0.81%**	**0.47%**	**0.81%**	**1.16%**	**1.04%**
10 µg/mL	0.633	0.591	0.639	0.633	0.631	0.619
10 µg/mL	0.634	0.597	0.641	0.634	0.636	0.622
10µg/mL	0.639	0.594	0.64	0.639	0.634	0.62
10 µg/mL	0.64	0.589	0.641	0.64	0.637	0.622
10 µg/mL	0.641	0.591	0.642	0.641	0.64	0.623
10 µg/mL	0.641	0.595	0.643	0.641	0.641	0.624
%RSD	**0.56%**	**0.50%**	**0.22%**	**0.56%**	**0.58%**	**0.29%**

Table 3: Ruggedness data of apigenin.					
Conc	**Absorbance**	**Conc**	**Absorbance**	**Conc**	**Absorbance**
2 µg/mL	0.168	6 µg/mL	0.368	10 µg/mL	0.633
2 µg/mL	0.172	6 µg/mL	0.369	10 µg/mL	0.639
2 µg/mL	0.172	6 µg/mL	0.369	10 µg/mL	0.64
2 µg/mL	0.174	6 µg/mL	0.362	10 µg/mL	0.641
2 µg/mL	0.177	6 µg/mL	0.363	10 µg/mL	0.641
2 µg/mL	0.176	6 µg/mL	0.365	10 µg/mL	0.642
%RSD	1.87%	%RSD	0.84%	%RSD	0.51%

absorbance's of each replicate of solutions was within the acceptance by change in the analyst and instrument (Table 3).

Solution stability

The solution stability for apigenin was determined by the %RSD or absorbance was calculated from prepared fresh solution and old solution containing apigenin. Results analyzed were found to be within the acceptance and data obtained revealed the standard stock solution.

Table 4: Solution stability data of apigenin.			
Solution stability		**Fresh stock dilutions**	**Old stock dilutions**
Replicates	**Conc.**	**Apigenin**	**Apigenin**
1	2 µg/mL	0.168	0.179
2	2 µg/mL	0.172	0.167
3	2 µg/mL	0.172	0.172
4	2 µg/mL	0.174	0.172
5	2 µg/mL	0.177	0.171
6	2 µg/mL	0.176	0.169
% RSD		1.87%	1.17%

Solution stability study was done for 4days and data have been displayed in (Table 4).

CONCLUSION

This paper presents in detail the development of a portable low-cost UV spectrophotometer which, by using an isolated compound, allows detection of developed, validated parameters, providing effective accurate results. Hence, the developed method is accurate, precise, reproducible and used as a quality control tool for analysis of apigenin in bulk. In future the methods

desirable to apply for estimation of apigenin in its various extract and other marketed dosage forms.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

µg: Microgram; **mL:** Milliliter; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **UV:** Ultra violet spectrophotometer; **RSD:** Relative Standard Deviation; **ICH:** International Council of Harmonization.

Author Contribution

All the authors have equally contributed for the development and validation of apigenin. Ms. Priya Shetti contributed to develop and validate and method for apigenin in its pure form. Dr. Sunil S. Jalalpure guided for the present research work also in the reviving literature and writing the paper work.

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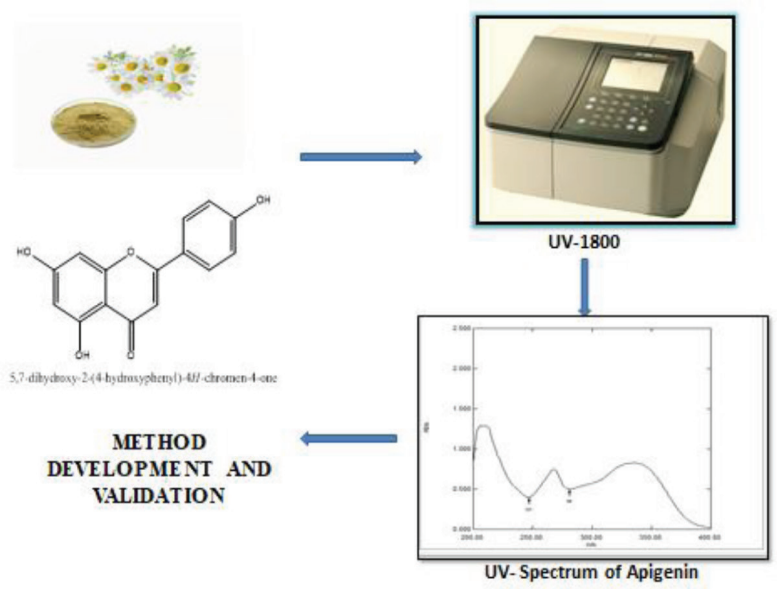
SUMMARY

The present study delivers a reference line data and analytical methods to contribution in the evaluation of apigenin, Plant flavonoid having capable chemo preventive activity counter to various cancers. Apigenin has low solubility which soluble in DMSO. Many studies on HPLC, HPTLC analysis for apigenin have been reported but till date UV Spectrophotometric method for apigenin isolated compound has not been reported. Here in this study the UV Spectra of apigenin was recorded by taking methanol as solvent, which sturdily absorbed gave two absorption spectra at a wavelength of 267 and 336nm herein we selected 267 nm as it produced a sharp peak compared to 336nm. Upright linearity was found with in concentration range of 2-10µg/mL with correlation coefficient (r^2) at 0.999. The data of precision study (interday and intraday) represent a good reproducibility with the RSD lower than 2.0% which shows that the method is precise. The use of costly analytical methods like HPLC, HPTLC limit the realizations of *in situ* studies because of their high cost, their limited portability, and even extend the time duration of the study. Henceforth the simple UV method was developed for the estimation of Apigenin in various extracts and in the marketed formulation and this developed method is validated according to the ICH guidelines.

PICTORIAL ABSTRACT



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RESEARCH Open Access

A single robust stability-indicating RP-HPLC analytical tool for apigenin quantification in bulk powder and in nanoliposomes: a novel approach

Priya Shetti[1] and Sunil S. Jalalpure[2*]

Abstract

Background: Apigenin (4′, 5, 7-trihydroxyflavone), a flavonoid, is present usually in fruits and vegetables possessing numerous biological properties like antioxidant, anti-viral, antibacterial, anti-inflammatory, and chemoprevention activity. So present study was aimed to prepare and characterize nanoliposomes of apigenin and estimate its encapsulation efficiency by stability-assisted reverse-phase (RP)-HPLC method.

Results: The stability indication of the RP-HPLC method developed for apigenin-loaded nanoliposomes was successfully demonstrated and parameters were mainly the retention time which was 4.21 min, limit of detection (LOD) 0.49 μg/mL, limit of quantification (LOQ) 1.48 μg/mL, and %relative standard deviation (RSD) less than 2%. Therefore, the stability indication of the developed reverse-phase HPLC method for apigenin-loaded nanoliposomes was demonstrated successfully and parameters like accuracy, linearity, LOD, LOQ, precision, and %RSD were within the limit range and found to be satisfactory.

Conclusion: The developed RP-HPLC method was found to be suitable for the quantification or estimation of apigenin with its stability in apigenin-loaded nanoliposomes, and this method will be a powerful tool in the future for the estimation of apigenin present in any pharmaceutical preparations.

Keywords: Apigenin, Nanoliposomes, Stability-indicating method, RP-HPLC, Validation

Background

Apigenin (4,5,7-trihydroxyflavone) (Fig. 1) is one of the most novel flavonoids in plants and formally belongs to the flavone sub-class. Apigenin possess moderate antioxidant property, anti-hyperglycemic [1], anti-inflammatory [2], myocardial ischemia, and anti-apoptotic effects [3]. A review by Zhou et al. summarized various biological effects of apigenin being effective against cytostatic and cytotoxic activities of a number of cancer cells and anti-atherogenic effects and also protective effects on hypertension, cardiac hypertrophy, and autoimmune myocarditis [4]. Novel approach has led to the development of apigenin in the pharmaceutical era, and quantification and estimation of apigenin in plant extracts has need raised. Nanoliposomes are similar to nano-metric kind of liposomes which tend to be the further most applied in encapsulation and controlled release systems [5]. These deliver more surface area, compared to liposomes, hence enhancing the bioavailability and controlled release, facilitating accurate target of the encapsulated material to a larger amount [6]. Apigenin having potential to increase the performance of the bioactive agent helps in enhancing solubility, bioavailability, and stability reported in vitro and in vivo. Due to prevention in unnecessary interactions with other molecules with this support, these nanoliposomes have diverse applications; current study was

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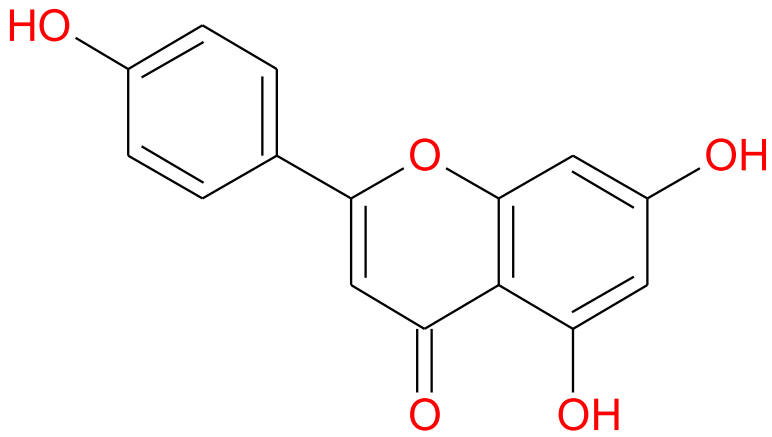

Fig. 1 Chemical structure of apigenin

commenced for the preparation, characterization, and confirmation of apigenin-loaded nanoliposomes for its encapsulation stability by RP-HPLC method.

Methods

Chemicals and reagents

Apigenin was a gift sample from Aktin Chemicals Ltd., China. Methanol, acetonitrile, and formic acid (HPLC grade) were purchased from Merck Limited (Mumbai, India). Other common laboratory reagents were of analytical grade.

Instrumentation

Shimadzu HPLC (LC- 2010) (Kyoto, Japan), pump system (LC-20 AD), degasser (DGU-20A5), a column oven (CTO-10ASVP), an auto-injector (SIL-20ACHT), PDA Detector (SPD-M20A), and a computer software (lab solutions, version 1.25) were used.

Chromatographic conditions

The chromatographic separation was carried out by injecting (10 μL) a sample to the HPLC system connected to a C-18 analytical column (Phenomenex Luna 5 μm, 250 mm × 4.6 mm) (set at 35 °C) operating at 1 ml/min flow rate, and detection was done at 269 nm. Acetonitrile and 0.1% formic acid, 55:45 (v/v) at pH = 7.4, was used as a mobile phase.

Table 1 Results of system suitability studies of quality control samples of apigenin

Parameter	Mean ± SD	% RSD
Retention time (min)	4.21 ± 0.00[a]	0.13
Peak area	127512 ± 273[a]	0.21
Theoretical plates	7010 ± 38.23[a]	0.54
Tailing factor	1.35 ± 0.00[a]	0.22

RSD relative standard deviation
[a]*n* = 6

Standard solution preparation

Stock solution was prepared (100 μg/mL) in the mobile phase. Further dilutions were carried out to get the calibration curve ranging from 0.5 to 16 μg/mL.

Preparation of stealth apigenin-nanoliposomes

Formulating the stealth apigenin-nanoliposomes, an ethanol injection technique was used in the preparation of nanoliposomes as described by Sudhakar et al. [7]. Briefly, apigenin (10 mg) and stearic acid (10 mg) were dissolved in 2 ml of absolute ethanol with gentle heating (< 50 °C) on a hot plate. Then, lipid di-stearoyl-sn-glycero-phosphoethanolamine–N (polyethyleneglycol) (DSPE-PEG) (0.003%) and cholesterol (0.0015%) were added into the same mixture with stirring. Phosphate-buffered saline (PBS) (pH 7.4) (10 ml) was added to mixture dropwise with constant stirring for 90–120 min to remove ethanol. The nanoliposomes were optimized for various concentrations of lipid. The optimized batch was homogenized at 10,000×*g* for 10 min followed by sonication for 15 min, which were further filtered through 0.45-μm and 0.2-μm sterile syringe filters and were allowed to equilibrate for 1 day and then stored at 4 °C. In the time, apigenin-loaded nanoliposomes were also assessed for their average particle size, poly dispersity index (PDI) according to Sharma et al. [8]. Moreover, encapsulation efficiency of nanoliposome was also assessed by centrifugation at 10,000×*g* for 30 min, and supernatant solution obtained after centrifugation was mixed with mobile phase and analyzed by using HPLC [9, 10].

Method development

The chromatographic conditions were optimized and steady baseline was obtained. Chromatogram was assessed by injecting standard apigenin solution, and analyses were repeated in a similar way for six times.

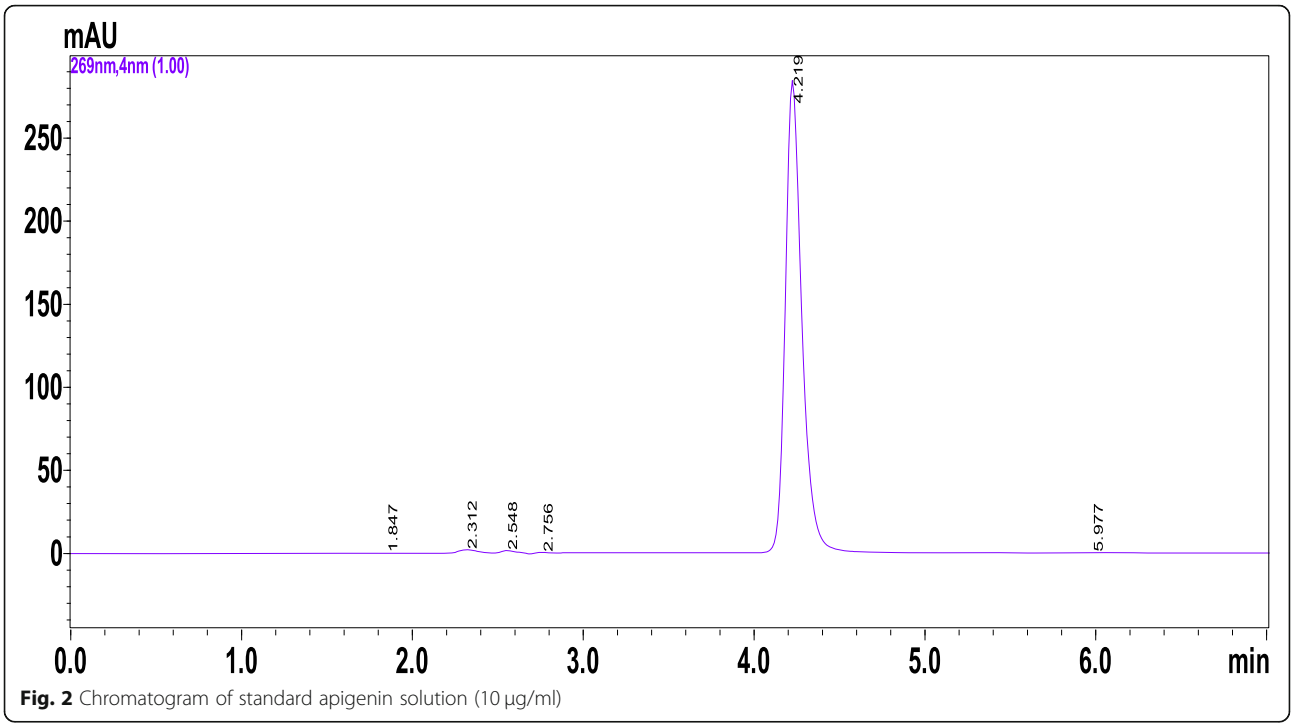

Fig. 2 Chromatogram of standard apigenin solution (10 μg/ml)

Method validation

Linearity

Linearity studies reveal the standard calibration curve was plotted from 0.5 to 16 μg/mL concentrations. Standard calibration curves were determined for concentrations versus peak area. Sample run in triplicate of all the sample solutions was assessed, and a chromatogram was recorded.

Precision and accuracy

The apigenin samples were subjected to three levels of quality control; namely low, medium, and high respective concentrations of 0.5, 4, and 16 μg/mL were prepared and used in determining the precision and accuracy. For intra-day precision and accuracy, triplicates of standard solutions were injected on the same day and also for interday; triplicates of standard solutions (50%, 100%, and 150%) were injected over three consecutive days. %relative standard deviation (RSD) and % RE were calculated respectively.

Table 2 Results of linearity and regression analysis of apigenin

Analyte	Apigenin at 269 nm
Concentration range (μg/ml)	0.5–16
Slope	34,367
Intercept	5495
R^2	0.999
LOD(μg/ml)	0.49
LOQ (μg/ml)	1.48

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD was determined by a series of dilutions of apigenin stock solutions to find a signal to noise (S/N) ratio of at least 3.3:1 for LOD and 10:1 for LOQ. Both parameters obliged the quantity of analyte that can be quantified.

System suitability

To validate the chromatographic system, suitability test is carried out, and various factors such as tailing factor, theoretical plates, and peak area and resolution data were also considered.

Robustness

The robustness of the method was evaluated by deliberately making a slight change in the optimized value. The evaluated parameters were as follows: detector wavelength (± 1 nm), flow rate (± 0.2 ml/min), and oven temperature (± 2 °C). Apigenin peak areas and the %RSD of robustness testing were statistically measured.

Specificity

The specificity study chance of interferences from PBS and apigenin-loaded nanoliposomes at the retention time of apigenin was analyzed by comparing the chromatograms obtained from the standard apigenin solution and PBS.

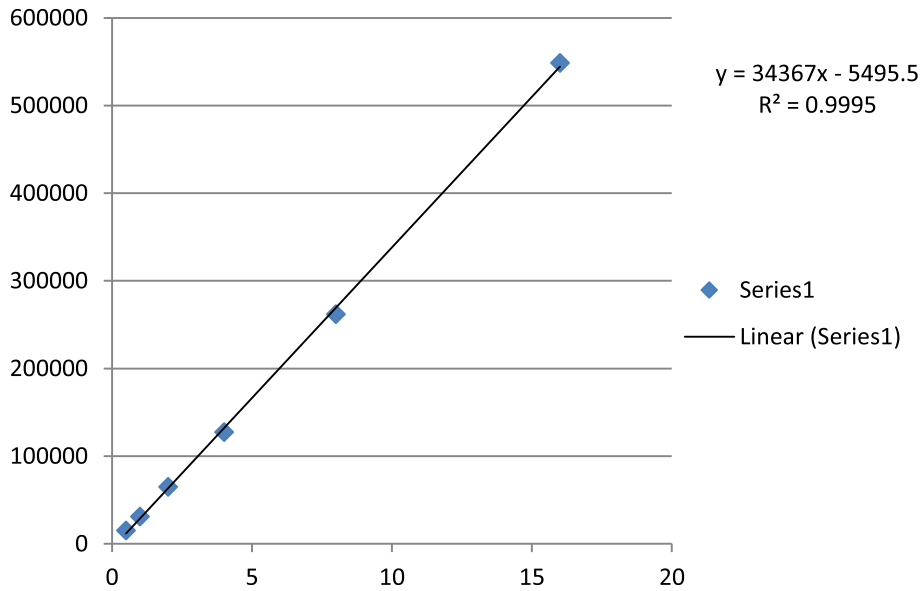

Fig. 3 Calibration curve for apigenin 269 nm

Force degradation studies

The stability-indicating property of the HPLC method which was developed was carried out as per the International Conference on Harmonization (ICH) guidelines [11]. Forced degradation studies of apigenin were conducted by exposing the working sample to oxidation, photolytic, acidic, and alkaline and heat conditions.

Results

Optimization of sample preparation and chromatographic conditions

Various trials were performed by changing the composition of the mobile phase. Finally, composition of ACN, 0.1 % FA (55:45 v/v, pH = 7.4), gave the better separation. During these studies, the 10 µl injected volume and the flow rate (1 ml/min) found to be constant.

Analytical method validation suitability of system

The system suitability results are tabulated in Table 1. The peak retention time was found to be 4.21 min (Fig. 2), the average theoretical plate was > 7000, and the tailing factor was < 2. Presence of higher theoretical plates with a lower tailing number indicates the efficiency of the system. Additionally, the % RSD values for all the parameters including retention time, peak area, and theoretical plates and tailing number were less than 1% justifying the better suitability of the system.

Table 3 Results of accuracy study of apigenin

Sl. No.	Conc. (µg/ml)	Level in (%)	Added qty (µg/ml)	Found qty (µg/ml)	Recovery (%)	RSD (%)
1	2	50	1	1.02	99.96	0.76
2	2	100	2	2.00	100.20	0.28
3	2	150	3	3.15	101.40	0.12

RSD relative standard deviation

Linearity and range

The linearity and regression are tabulated in Table 2 and Fig. 3. None of the constituents from the nanoliposomes or the derivative products of stress treatment interfered with apigenin peaks. A calibration curve was generated by plotting concentration of apigenin (x) against the peak area. A linearity (R^2 = 0.9995; equation y = 34367x–5495.5) was achieved for the concentration range of 0.5–16 µg/mL. Apigenin was detected employing C_{18} column with methanol to 0.2% phosphoric acid mobile phase and had an accuracy and recovery of 93.82 and 88.35% respectively.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of apigenin were found to be 0.49 and 1.48 µg/ml, respectively. A higher LOD (1.94 µg/ml) and LOQ (6.26 µg/ml) of apigenin was reported.

Accuracy and precision

The obtained results are expressed in Tables 3, 4, and 5. The intra-day precision for apigenin was between 0.11 and 1.38%, while the accuracy ranged between 0.12 and 0.76%. The interday precision was 0.27% and 1.69%, respectively. Three separate samples injected showed a best recovery of 99.96–101.4% indicating a higher accuracy of the method.

Table 4 Results of intra-day precision of apigenin solution

Experiment time for day 1	Concentration (μg/ml)	Peak area	Retention time (min)	(%)RSD of peak area
9:00 am	0.5	19,404.17	4.28	1.38
	4	129,483	4.29	0.39
	16	673,361.8	4.30	0.78
1:00 pm	0.5	19,404.17	4.31	1.38
	4	129,707.2	4.29	0.27
	16	673,361.8	4.28	1.05
6:00 pm	0.5	19,497.17	4.27	0.34
	4	129,882.5	4.27	0.32
	16	682,828	4.28	0.11

RSD relative standard deviation

Robustness and ruggedness

The results of the robustness study are presented in Table 6. Parameters including mobile phase, flow rate, detection wavelength, column oven temperature, and the type of column did not hinder the resolution in terms of similar retention time and lower RSD values.

Stability-indicating study

The results of the stress degradation studies (acid, alkali, oxidation, heat, and light) for the standard apigenin solution are shown in Table 7. The chromatograms of the stressed standard apigenin solution after 2 h under various stress conditions are shown in Figs. 4 and 5. In the current study, apigenin showed a degree of degradation ranging from as low as 5.87% for alkaline hydrolysis to 20% by photolytic damage. The degradation of apigenin was perfectly estimated by the developed method. HPLC analysis of aqueous and ethanolic chamomile extracts exposed to different temperature and pH showed that apigenin-7-O-glucosidedegraded by 6% at pH 8, 9% at pH 9, and 11–12% at pH 10–12; 48% at pH 13.0, with no loss below pH 5.0. Long-term storage of extracts at room temperature revealed conversion of glycoside to aglycone as compared to storage at − 20 °C.

Table 5 Results of interday precision data of apigenin samples

Day / time	Concentration (μg/ml)	Peak area	Retention time (min)	(%)RSD of peak area
Day 1 10:00 am	0.5	18,728.5	4.28	0.93
	4	129,084.2	4.29	0.28
	16	647,448.3	4.30	1.16
Day 2 10:00 am	0.5	19,404.17	4.31	1.38
	4	129,707.2	4.29	0.27
	16	673,361.8	4.28	1.05
Day 3 10:00 am	0.5	20,680	4.30	1.69
	4	130,179	4.29	0.27
	16	721,931.5	4.28	1.02

RSD relative standard deviation

Table 6 Analysis of robustness and ruggedness using apigenin solution

Parameters	Variations	Time (min)	%RSD
Mobile phase(± 2 v/v)	55:45	4.30	0.08
	53:47	4.51	0.10
	57:43	4.00	0.03
Flow rate(± 0.1 ml)	1 ml/min	4.33	0.08
	0.9 ml/min	4.71	0.10
	1.1 ml/min	3.86	0.09
Detection wavelength (± 2 nm)	267	4.24	0.13
	269	4.23	0.11
Column oven temp(±1 °C)	35 °C	4.21	0.13
	34 °C	4.22	0.15
	36 °C	4.20	0.17
Column	Luna 5u(250X4.6)	4.33	0.08
	Luna 5u(150X4.6)	3.84	0.09

RSD relative standard deviation

Preparation and characterization of stealth liposomes

The apigenin-loaded nanoliposomes were successfully prepared by the ethanol injection technique; the nanoliposomes was 124 nm of the blank liposome and apigenin-loaded liposome was 151 nm. The average polydispersity index for blank and drug-loaded liposome was 0.23 and 0.16, respectively (Table 8). These results summarized that the PDI value is < 0.3 and indicated narrow homogeneous presence of particle size distribution. Meanwhile, these apigenin-loaded liposomes were also subjected to the encapsulation efficiency (EE) of apigenin in the formulation. The chromatogram showed an intense characteristic apigenin peak in nanoliposomes, and t 80.18% of encapsulation efficiency was achieved by this method (Fig. 6, Table 8).

The average particle size of apigenin-loaded lecithin liposomes was in the range of 158.9 ± 6.1 to 260.3 ± 11.1 nm. The PDI ranged from 0.205 ± 0.02 to 0.331 ± 0.063 with a remarkable encapsulation efficiency of more than 92% and possessing high potential for nutraceutical formulations. In another study, apigenin-loaded distearoylphophatidylcholine (DSPC) liposomes showed a particle

Table 7 Results of the stress degradation studies of apigenin solution at 2 h

Stress degradation studies	Conc. used (μg/ml)	Conc. left after degradation (μg/ml)	% Degradation
Acid hydrolysis	2.0	1.62	18.88
Alkaline hydrolysis	2.0	1.88	5.87
Oxidation	2.0	1.70	14.61
Thermal	2.0	1.66	16.55
Photolytic	2.0	1.60	20.00

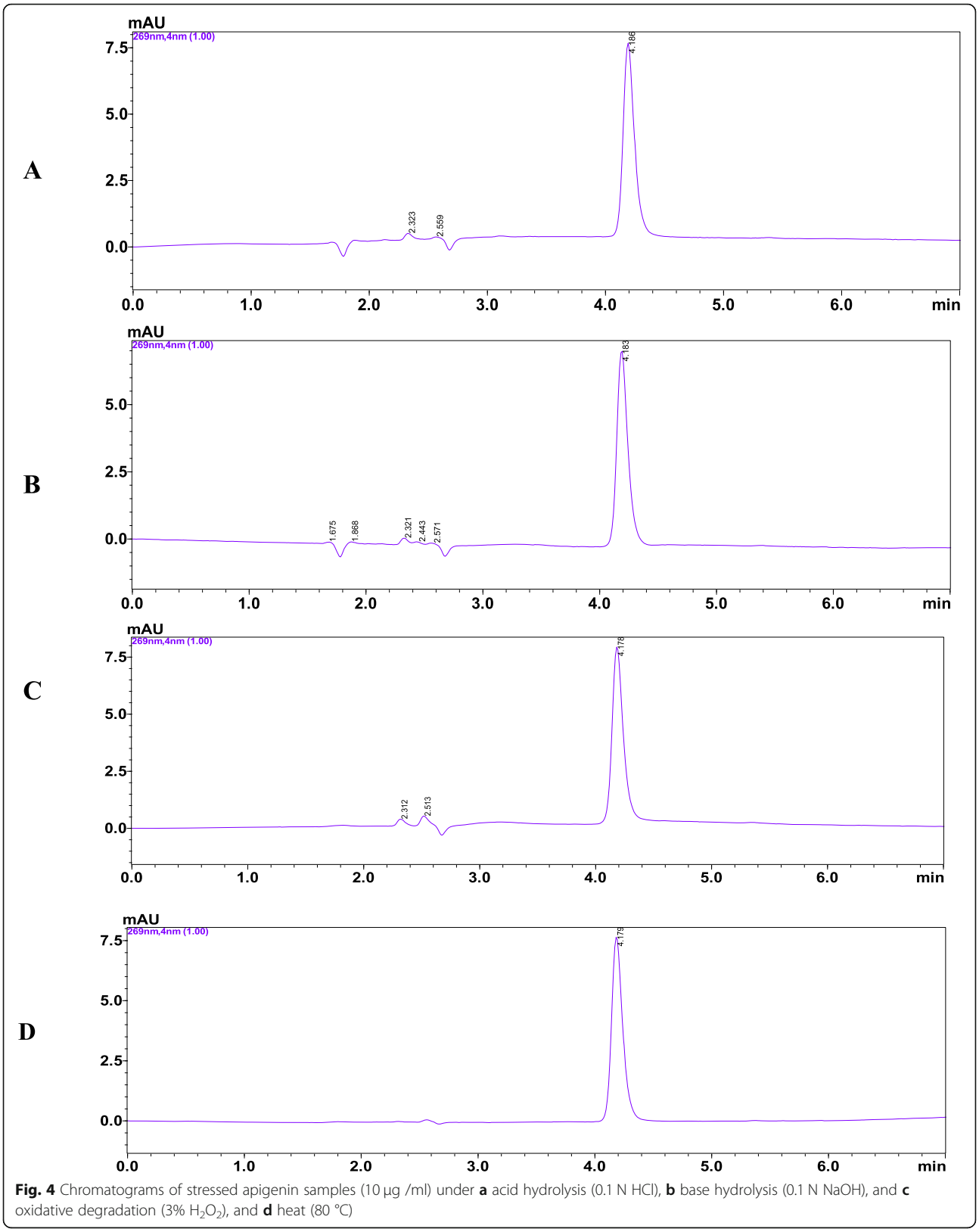

Fig. 4 Chromatograms of stressed apigenin samples (10 µg /ml) under **a** acid hydrolysis (0.1 N HCl), **b** base hydrolysis (0.1 N NaOH), and **c** oxidative degradation (3% H_2O_2), and **d** heat (80 °C)

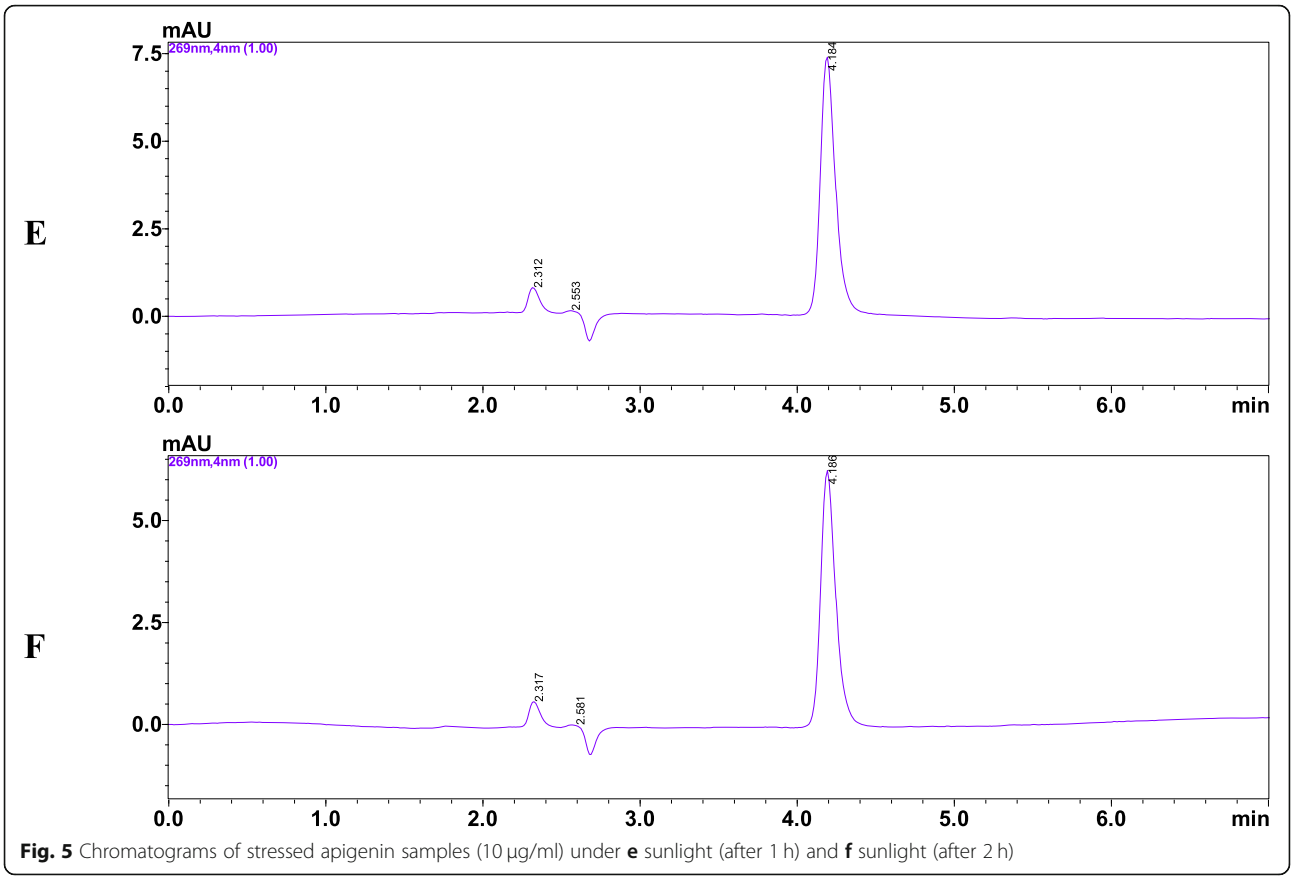

Fig. 5 Chromatograms of stressed apigenin samples (10 μg/ml) under **e** sunlight (after 1 h) and **f** sunlight (after 2 h)

size of 104.3 ± 1.8 nm, PDI of 0.204, and a high EE of 89.9%. The apigenin-loaded DSPC liposomes were effective in delivering the drug within bacterial cells and thus were antibacterial in nature.

Discussion

Presence of higher theoretical plates with a lower tailing number indicates the efficiency of the system. Additionally, the % RSD values for all the parameters including retention time, peak area, and theoretical plates and tailing number were less than 1% justifying the better suitability of the system [12].

None of the constituents from the nanoliposomes or the derivative products of stress treatment interfered with apigenin peaks. A calibration curve was generated by plotting concentration of apigenin (x) against the peak area. A linearity of r = 0.9995 with equation y = 34367x−5495.5 was achieved for the concentration range of 0.5–16 μg/ml. Apigenin was detected utilizing C_{18} column with methanol to 0.2% phosphoric acid mobile [13]. A higher LOD (1.94 μg/ml) and LOQ (6.26 μg/ml) of apigenin was reported, which could be due to variance in the mobile phase [13] and depending on the type of analysis and analytical conditions used [12]. Three separate samples injected showed a best recovery of 99.96–101.4% indicating a higher accuracy of the method. The RSD values of intra- and interday precision of the method was less than 2% [14–16].

Parameters including mobile phase, flow rate, detection wavelength, column oven temperature, and the type of column did not hinder the resolution in terms of similar retention time and lower RSD values. Triplicate analysis of the samples showed the better robustness of the developed method which was similar to those reported [17].

In the current study, apigenin showed a degree of degradation ranging from as low as 5.87% for alkaline

Table 8 Results of characterization of apigenin-entrapped liposomes

Liposome formulation	Particle size diameter (nm)	PDI	% Entrapment efficiency
Blank liposome	124	0.23	NA
Apigenin-loaded liposomes	151	0.16	80.18%

PDI polydispersity index

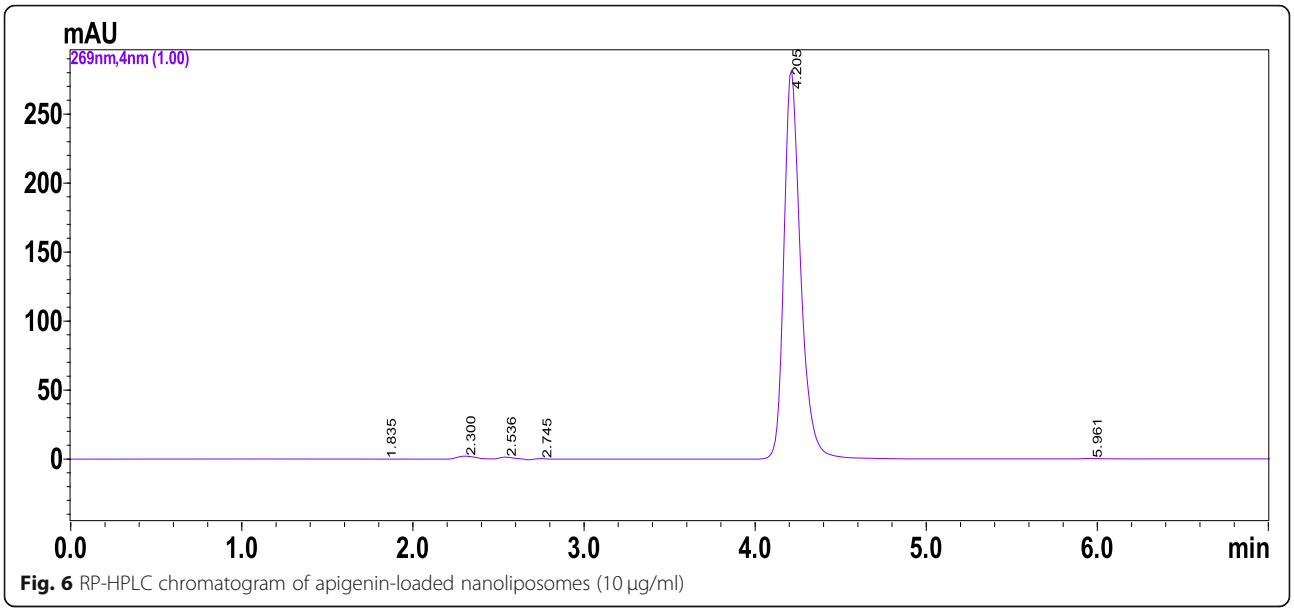

Fig. 6 RP-HPLC chromatogram of apigenin-loaded nanoliposomes (10 μg/ml)

hydrolysis to 20% by photolytic damage. The degradation of apigenin was perfectly estimated by the developed method [18]. Apigenin was also found to be structurally unstable when subjected to temperatures of 37 °C [19, 20].

A multiple emulsion solvent evaporation technique was employed to encapsulate apigenin in poly(lactide-co-glycoside), PLGA, which showed a drug loading efficacy of 19.14% [21]. The EE of apigenin-loaded liposomes was better than that reported and similar to encapsulation of apigenin in lecithin liposomes [22]. The average particle size of apigenin-loaded lecithin liposomes was in the range of 158.9 to 260.3 nm. The PDI ranged from 0.205 to 0.331 with a remarkable encapsulation efficiency of more than 92%. In another study, apigenin-loaded distearoylphophatidylcholine (DSPC) liposomes showed a particle size of 104.3 ± 1.8 nm, PDI of 0.204, and a high EE of 89.9%. Apigenin-loaded nanoliposome from the current study would thus function as an efficient drug delivery system [23–25].

Conclusion

A sensitive, simple, specific, and stability-indicating RP-HPLC tool for the quantification of apigenin bulk and apigenin-loaded nanoliposomes were developed effectively. Interferences of peaks with apigenin by the encapsulation substances were not detected. The method could be used in herbal drug industries for quantification of apigenin.

Abbreviations

RP-HPLC: Reverse-phase high-performance liquid chromatography; PDI: Polydispersity index; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation; PBS: Phosphate-buffered saline; EE: Encapsulation efficiency; DSPC: Distearoylphophatidylcholine; ICH: International Conference on Harmonization; DSPE-PEG: Di-stearoyl-sn-glycero-phosphoethanolamine (polyethyleneglycol)

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Authors' contributions

PS contributed for experimental work and manuscript preparation. SSJ contributed in hypothesis and finalization of manuscript. All authors have read and approved the manuscript.

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Availability of data and materials

Authors declare to produce the data and material on demand/request.

Declarations

Ethics approval and consent to participant

Not applicable.

Consent for publication

Not applicable.

Competing interests

Authors do not have any conflict of interest.

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