
**“FORMULATION, OPTIMIZATION AND
CHARACTERIZATION OF Fisetin LOADED CUBOSOMES
FOR THE TREATMENT OF LUNG CANCER:
AN EXPERIMENTAL STUDY”**

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**Accredited ‘A⁺’ Grade by NAAC (3rd Cycle)
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**For the award of the degree of
Doctor of Philosophy
In the Faculty of Pharmacy**

By

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AO	Acridine orange
A549	Human lung cancer cell line
Cm	Centimeter
CO ₂	Carbon dioxide
P	Centipoises
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
DAPI	4', 6-diamidino-2-phenylindole
DLS	Dynamic Light Scattering
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
EE	Entrapment efficiency
EB	Ethidium bromide
FBS	Fetal bovine serum
FIS	Fisetin
FISCUB	Optimized fisetin cubosomal nanoformulation
FT-IR	Fourier Transform infrared spectroscopy
GMO	Glyceryl monooleate
HPLC	High Performance Liquid Chromatography
HR-TEM	High Resolution Transmission Electron Microscopy

IAEC	Institutional Animal Ethics Committee
IC50	Half maximal inhibitory concentration
ICH	International Council for Harmonization
kg	kilogram
LOD	Limit of detection LOQ: Limit of quantification
min	minute
mm	millimetermg: Milligram ml: Milliliter
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	Nanometer
P	Probability
PBS	Phosphate buffer saline
PDI	polydispersity index
PEG	Polyethylene glycol
pH	power of hydrogen/ pouvoir hydrogen
PDA	Prominence diode array
PI	Propidium iodide
PS	Particle size
r^2	Correlation coefficient
ROS	Reactive oxygen species
rpm	Revolution per minute
RSD	Relative standard deviation
SAED	Selected area electron diffraction
SD	Standard deviation

TEM	Transmission electron microscopy
USFDA	United States Food and Drug Administration
USP	United States Pharmacopeia
v/v	Volume by volume w/v: Weight by volume w/w: Weight by weight
XRD	X-Ray Diffraction
ZP	Zeta potential 2θ: Diffraction angle
2FI	Two factor interaction μg: Microgram
μm	Micrometer
°C	Degree centigrade
%	Percentage
<	Less than
>	Greater than

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ABSTRACT

Background

Lung cancer is reported very commonly in all the diagnosed human cancer types with the highest death rate. It is treated with surgical resection, radiation, and chemotherapy but it has many adverse effects, toxicity, drug resistance and disease recurrence. Many effective anticancer drugs are used in the treatment but there is a need to improve the therapeutic potential of existing and development of new anticancer drugs. Phytoconstituents are effective and have fewer side effects than synthetic chemotherapeutic drugs, many are present in clinical trials and report improved therapeutic efficacy in their nanoformulations.

Flavonoid is a class of phytoconstituents that have reported prominent anticancer property but possess solubility problem. A flavonoid fisetin was selected for lung anticancer study as it reports promising activity and prepared its cubosomal nanoformulation for improving solubility. Cubosomal nanoformulation is a stabilized liquid dispersion of crystalline cubic nanoparticles of an amphiphilic lipid glyceryl monooleate. Cubosomes have high drug loading capacity, site-specific drug release, are highly stable, and are more suitable for fisetin to improve therapeutic efficacy.

Objectives

The undertaken study aims to investigate the effect of cubosome-loaded fisetin in lung cancer with the following objectives

- 1) To formulate and characterize fisetin-loaded cubosome nanoparticles.
- 2) To evaluate anticancer activity in lung cancer *in vitro* and *in vivo*.

Methodology

A top-down cubosome formulation method was used, optimized using a 3²-full factorial design in Design Expert[®] software by formulating and evaluating 16 pilot and 9 test batches. The melted GMO was combined with 40 mg of fisetin and this mass was added in Pluronic F-127 at 65 °C and mixed. Then dropwise added in to 40 milliliters of water at the same temperature, cooled and homogenized at 14,500 rpm for 20 min, sonicated for 10 minutes and stored. The characterization of the formulation for particle size, entrapment efficiency, viscosity, pH, polydispersity index, FTIR, XRD, DSC and HRTEM examination were done. The release study performed using cellulose dialysis membrane and stability for three months. Performed in vitro study using human lung cancer A549 cells for MTT, clonogenic, flow cytometry, quantitative cell uptake and fluorescent staining assay. The animal study performed using known lung carcinogen B(a)P as inducing agent in male Swiss albino mice.

Results

The optimized FISCUB (batch B2) contains 2% lipid (GMO) and 1% stabilizer (Pluronic F-127) reported less particle size (93.07 ± 5.41 nm) and maximum fisetin entrapment (80.10 ± 0.36 %), and stable during its 3-month stability study. The statistical analysis reveals that 2FI is the best-fit model for particle size with GMO and Pluronic F-127, then 2D contour, 3D response surface plots, and statistical equation of the particle size indicate that the increase in GMO resulted in a rise of FISCUB particle size.

The FTIR spectra for FIS, GMO, Pluronic F-127, and FISCUB indicate the absence of chemical interaction between fisetin and formulation ingredients. The DSC thermograms also indicate the absence of drug-excipient interactions and fisetin

was completely entrapped in cubosomes formed from GMO and Pluronic F-127. The HRTEM and XRD analysis results confirm the cubic structure of the particles, scattered, well separated from each other, present in the nano range and fisetin entrapment in FISCUB. The in vitro fisetin release study shows 50% more release from FISCUB indicating an increase in fisetin aqueous solubility.

The change in A549 cellular absorption, cytotoxicity, and anticancer potential for Fisetin and FISCUB was analyzed through an in vitro study. The observation of a decrease in IC_{50} value, mean colony number, and increase in cytotoxicity, apoptosis, and 40 % fisetin absorption by FISCUB when compared with native fisetin confirms the increased anticancer effect and applicability of formulation in fisetin delivery to human lung cancer A549 cells. In the in vivo study, changes in the animal body and lung weight, concentration of serum proteins, SOD and CAT antioxidant enzymes, and histological lung tissue observations indicate that the protective and therapeutic effect of fisetin against lung-specific carcinogen B(a)P-induced toxicity was found to be increased for FISCUB.

Conclusion

In the proposed study, formulated, characterized, and optimized a stable, novel fisetin cubosomal nanoformulation and investigated its effect on lung cancer. Study findings for the FISCUB report, there was an increase in the anticancer and anti-inflammatory potential of fisetin in human lung cancer A549 cells and mice lungs because of the improved solubility and cellular absorption resulting from fisetin entrapment in the nanosized cubic crystalline particles of the prepared formulation.

Keywords: Fisetin; A549 cells; cubosome; lung cancer; cell uptake; drug release

1. INTRODUCTION

1.1 Background

Lung cancer

The most frequent type of cancer in humans is lung cancer and has the highest number of cancer associated deaths in males and after breast cancer in females. Worldwide, there were 2.1 million detected fresh cases and 1.8 million deaths in 2018; incidence differs in different areas. Tobacco exposure, asbestos, and other factors are responsible for lung cancer ^(1,2).

Types and subtypes of lung cancer depend on the types of involved lung cells and the location of the tumor in the lung

a) Non-small cell lung cancer – It contains 85% of total lung cancer cases, less malignant, primarily treated with surgical resection and chemotherapy.

I. Adinocarcinoma II. Squamous cell carcinoma III. Large cell carcinoma.

Staging is important for selection of treatment and predicting five-year survival rate of the patient for Stage I to IV are 47, 26, 8.4 and 1.6 in percent respectively.

b) Small-cell lung cancer – It contains 15% of the total lung cancer reports, highly malignant and more rapid growth, primarily treated with chemotherapy and radiation⁽³⁾. It also called oat cell carcinoma because of flat cell shape and is primarily treated with chemotherapy and radiation.

I. Limited Stage – It is limited to one lung or lymph node.

II. Extensive Stage – It spreads beyond the lungs.

Symptoms – Coughing, coughing up phlegm or blood, chest pain, weight loss, shortness of breath, loss of appetite, weakness and fatigue.

Diagnosis – Chest imaging, sputum cytology, bronchoscopy etc.

Treatment - Surgery, radiation, chemotherapy (Cisplatin, Etoposide, Carboplatin and for recurrence Doxorubicin, Cyclophosphamide, Vincristine and Paclitaxel), targeted treatment and immunology (Nivolumab), each of this has its side effects and limitations.

Herbal phytoconstituents for lung cancer

Various phytoconstituents and their derivatives reported potential anticancer activity from alkaloids, carotenoids, terpenes, flavonoids which shows less toxicity and side effects in clinical practice. In current research literatures, many phytoconstituents are reported with anticancer activity by inhibiting tumor cell growth and metastasis ⁽⁴⁾.

Flavonoid is a class of bioactive polyphenolic phytoconstituents reported for various pharmacological properties including anticancer activity and therefore extensively studied for its drug development ^(5,6). Problem in drug development of this class of phytoconstituent is its low aqueous solubility and more lipid solubility. Delivery of such flavonoids with help of nanoformulation technique is reported to be effective for solving solubility and biopharmaceutical issues which increase efficacy of such phytoconstituent^(7–11). Interests in flavonols as an anti-cancer drug development is growing because of its remarkable pharmacological properties, less toxic and non-mutagenic effects.

Flavonol fisetin (FIS) is selected for study because it reports *in vitro* and *in vivo* anticancer properties^(12–14) but possesses low aqueous solubility limiting its anticancer properties^(15,16). FIS is found in fruits, vegetables, nuts and teas. It is crystalline powder, MP 330 °C, low aqueous solubility (10.45 µg/ml) and high solubility in DMSO (30 mg/ml) therefore Class II drug as per BCS and have limited bioavailability⁽¹⁷⁾.

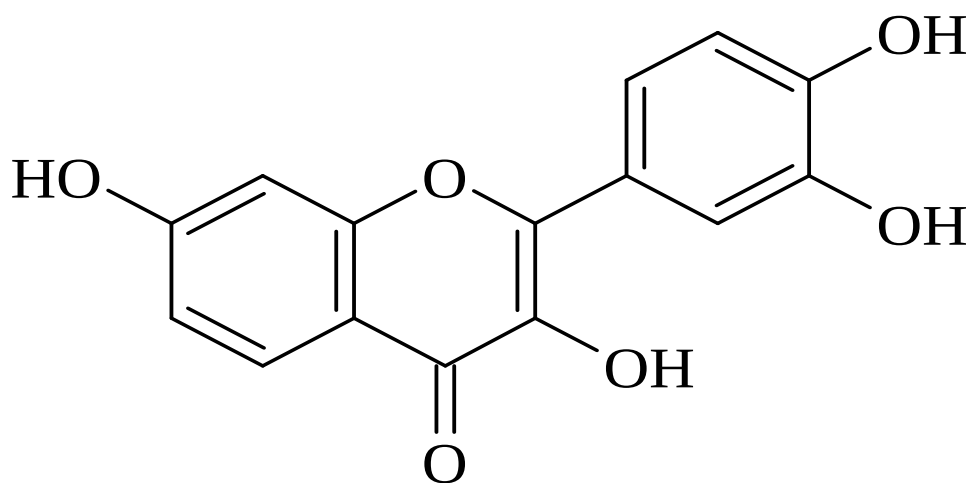


Figure 1. Fisetin

The use of such phytoconstituents with the help of novel drug delivery system with active or passive tumor cell targeting principle is reported to be more effective than pure phytoconstituents against various forms of cancer^(18–22).

FIS is a plant-derived dietary flavonoid chemically a flavanol (3, 3', 4', 7-tetrahydroxyflavone) and available in the market as a nutraceutical. It possesses many biological and pharmacological effects (Figure 2), including anticancer activity in various types of cancer^(23–26).

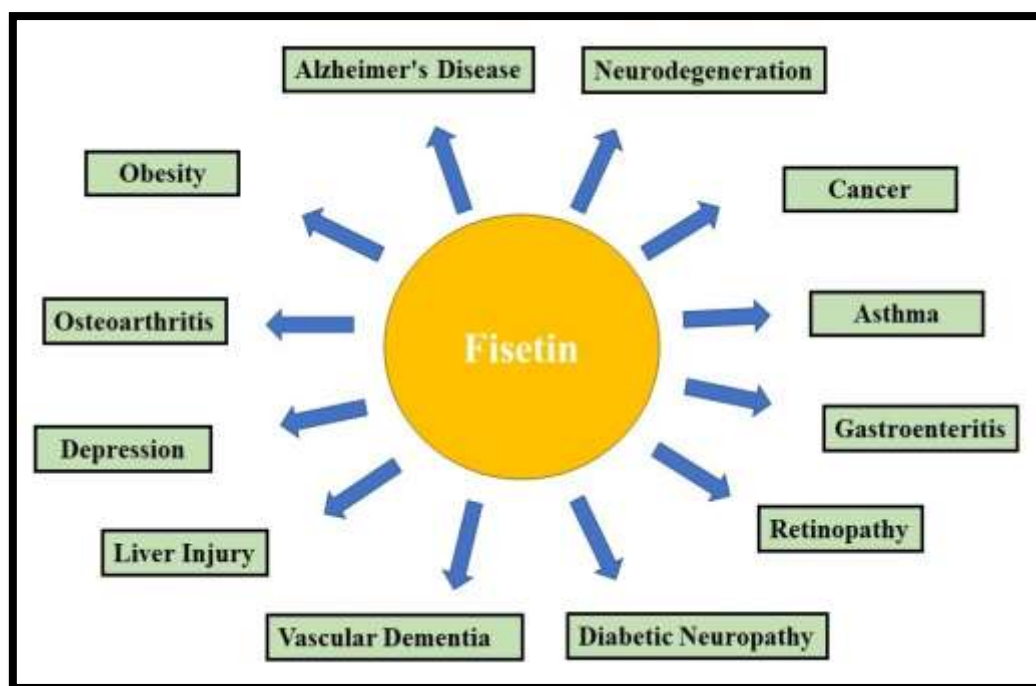


Figure 2. Biological and pharmacological activities of Fisetin

It acts on cancer cells via cell cycle arrest, apoptosis, metastasis reduction, angiogenesis and cellular oxidation process ⁽²⁷⁾. It has also shown a synergistic effect with other anticancer drugs, with previous researchers reporting the suppression of various types of cancer cell proliferation. Although wide promising pharmacologic activities of FIS have been reported, because it is a BCS class II drug, FIS possesses the problem of low aqueous solubility and bioavailability. This restricts the application of FIS as a promising lead molecule along with its maximum therapeutic benefits against its anticancer and other uses ⁽¹⁵⁾.

Cubosomes

Nanostructured particles of bicontinuous cubic liquid crystalline phase of submicron size of amphiphilic lipids like Gluceryl monooleate, Phytanyl gegerate and Glyceryl monolinolate. Cubosomes offer several benefits over liposomes, including as being simpler to prepare, having more stability, and having unique liquid crystalline characteristics ^(28–30).

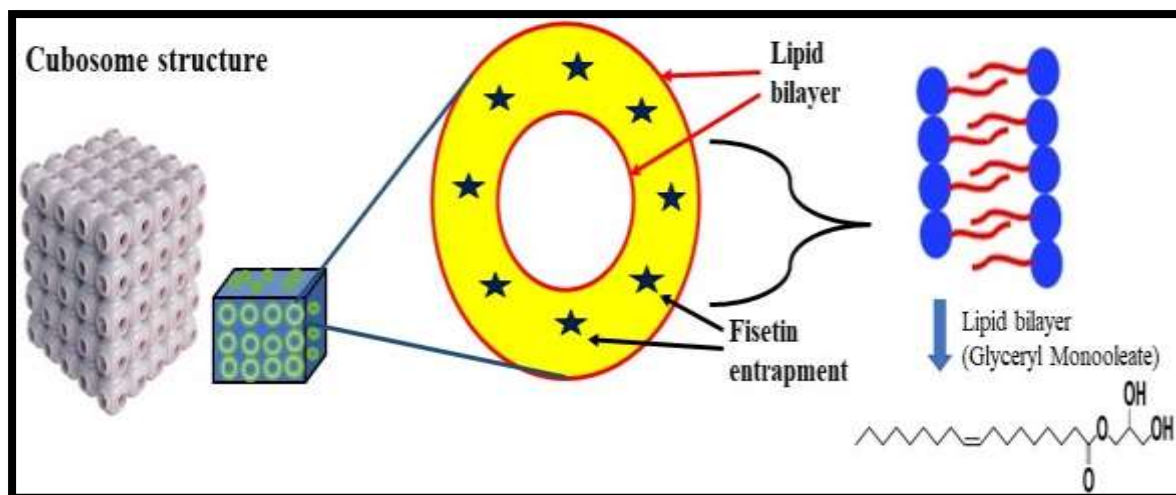


Figure 3. Cubosome membrane structure and fisetin entrapment

Advantages of cubosome over other nanoparticles ^(31–33)

1. Controlled and target release profile
2. Lipids used are biocompatible, non-hazardous, no allergic reports, nonirritating
3. Provide an increased rate of encapsulation efficiency.
4. Thermally stable, accommodate both hydrophilic and lipophilic drugs
5. Easy method of preparation.
6. Bio-adhesion property to the biological membrane.

Disadvantages

1. On long storage, they lead to the growth of particles and therefore create problems for parenteral delivery.
2. Because of high viscosity large production is difficult.
3. As more water is entrapped in cubosomes, low entrapment of lipophilic drugs.

Cubosome preparation

Two methods are commonly employed for the preparation of cubic liquid crystalline particles from suitable amphiphilic lipids and other ingredients.

Top-down approach: Lipid (GMO) with Stabilizer (Poloxamer) added into the water with stirring followed by sonication or vortex.

Bottom-up approach: Cubosomes are formed by crystallizing from precursors. In this method lipid, stabilizer, and a hydrotrope are added in sufficient water along with small shear^(33–37).

Potential routes of delivery for Cubosome

The cubosomes and their formulations are administered using various routes depending on the drug and formulation, disease condition and other pharmacokinetic and pharmacodynamic factors such as oral, transdermal, ophthalmic, and parenteral^(38–45). Out of all these routes for cubosome administration, the oral route is more suitable for poorly water-soluble drugs because

1. Poorly water-soluble drugs solubilize and encapsulate in the lipid bilayer part which avoids drug precipitation in GIT⁽⁴⁶⁾.
2. Close contact of cubosome with intestinal cell membrane increases bio-adhesion⁽²⁸⁾.
3. As lipid-based nano-particles, they have a significant role in drug absorption^(47,48).

To increase the aqueous solubility and anticancer activity of FIS, we developed a novel optimized FIS cubosomal formulation using a top-down method. As compared with other lipid-based carriers, the cubosomes were reported to be good solubilizers, biocompatible and biodegradable; to require only a simple preparation procedure; ability to entrap a greater number of water-insoluble drugs (Figure 3), and to increase cellular uptake and bioavailability.

Therefore, we prepared this cubosomal nanoformulation to study its effect on FIS solubility and cellular absorption. The developed formulation method is simple, yields thermodynamically stable cubosomes, and requires a smaller number of formulation ingredients than the previously reported FIS nanoformulations. To evaluate its effect, we tested the optimized cubosomal formulation for its stability, particle characteristics, encapsulation efficiency, *in vitro* FIS release, and anticancer activity against A549 lung cancer cells.

1.2 REVIEW OF LITERATURE

Freddie Bray et al., (2018) explain global cancer statics, worldwide new cancer cases 18.1 million and deaths occurred 9.6 million in 2018. The primary reason of cancer incidence and death is still lung cancer, with 2.1 million (11.6% of the total cases) new lung cancer reports, 1.8 million (18.4% of the total reported mortality) deaths predicted in 2018. There are twenty-fold variations in lung cancer rates in different regions and depends on tobacco exposure intensity and duration with other factors ^(1,2).

Latimer K et al., (2015) explains diagnosis and treatment for lung cancer. They explained symptomatic diagnosis through hemoptysis, dyspnea, lack of appetite, weight loss, chest or rib discomfort, and a rise in the frequency of visits to assess a persistent cough along with chest radiography, tissue diagnosis, staging, and functional evaluation, methods for handling nonsmall and small cell lung cancer with their types and stages ⁽³⁾.

Seca A M et al., (2018) explains importance of plant secondary metabolites as active molecule against various types of cancer. About 40% of FDA drugs are being natural substances or compounds inspired by them and 74% of those molecules are utilized in anticancer therapy. Natural remedies are thought to be less harmful to normal cells and more physiologically friendly and there for more studied for newer drug molecules. Successful cases of anticancer drugs from plant origin are explained such as paclitaxel, homoharringtonine and vincristine and many are in various stages of clinical trials like ingenol mebutate, curcumin and betulinic acid ⁽⁴⁹⁾.

Eskandar Qaed et al., (2023) uncovers different mechanism and targets of the fisetin acting as a anti-cancer molecule. Fisetin Induces apoptosis mechanism by caspases

mediated cell death. Various signaling molecules activation like P53, Bax, Bak and BCL-2 leads to activation of caspases 3, 7, 8, 9, and 10 which results into cell apoptosis. Autophagy pathway also induced due to fisetin and self-degradation of broken cell components in the lysosome. It is observed via Beclin 1 protein and ATG-ATG5:ATG7 complex activation. Fisetin reported a decrease in animal cells migration and invasion. It also reporting reductive role over the reactive oxygen species and synergistic effect with other anti-cancer drugs. Antitumor effect of fisetin have been reported on animal experiments. It is reported effective against A549 lung cancer cell line and other forms of cancer via different mechanisms⁽¹⁴⁾.

Kirtana Supramaniam et al., (2024) explored updated information on the role of various phytochemicals in the form of nanoformulation for the management of lung diseases. Many types of nanoformulations like nanoemulsion, solid-lipid nanoformulation, nanostuctured lipid carrier, liposomes, polymeric nanoformulations and cyclodextrin complexes are prepared and successfully used for phytochemicals such as curcumin effectively against lung cancer cells. Prepared nanoformulations were reported to be effective to minimize the solubility and bioavailability of used phytochemicals in order to increase its effectiveness. This type of study using herbal medicinal compounds in combination with nanoformulations has been frequently reported for the management of lung cancer cell growth⁽⁵⁰⁾.

Chenhui Zhou et al., (2023) summarized the therapeutic effect and molecular mechanisms of fisetin as anticancer molecule for lung and other types of cancer. It found to be affecting various molecular mechanisms which leads into decrease in cancer cell propagation, movement and assault, ROS and inflammation through vascular endothelial growth factor, caspase 3 whereas promotes apoptosis and autophagy through AKT/mTOR mechanism. Based on various molecular

mechanisms, fisetin have been reported as promising therapeutic molecule for cancer therapy in combination with existing anticancer drugs as supplementary or in its pure form ⁽¹²⁾.

Agarwal A. D., (2011) explained pharmacologically active flavonoids which are classified as flavones, flavanols, flavanones, catechins and anthocyanins, many of which exhibits anticancer activity like apigenin, fisetin and luteolin ⁽⁵¹⁾.

Ravichandran N. et al., (2011) reports the attenuation of lung carcinogenic potential of fisetin *in-vivo* and confirm remarkable free radical carrying as well as anti-oxidant actions. Lung cancer developed into the mice using B(a)P and administered fisetin and cyclophosphamide in combination and alone and assessed ⁽⁵²⁾.

Piyush Mehta et al., (2018) reported bioactive flavonol fisetin anticancer activity and various novel drug delivery strategies being used to overcome dissolution, bioavailable issue for effective administration like Polymeric nanocarriers, Polymeric micelles, Polymeric nanoparticles, Nanoemulsion, Solid lipid nanoparticles, Liposomes, Cochleates, Cocrystals and Cyclodextrins complexes ⁽¹⁶⁾.

Wang H. et al., (2012) reviewed natural phytoconstituent and its druggability in the prevention and treatment of cancer. He explained the effect of fisetin as lung anticancer by reviewing inhibition of PI3K/Akt and mTOR signaling, Wnt signaling, G1-phase arrest and disrupted Wnt/ β catenin signaling ⁽⁵³⁾.

Azarmi S et al., (2008) explains targeted delivery of nanoparticles to treat pulmonary conditions in which he explained lung targeting delivery by various routes like intravenous, Oral and nasal in details ⁽⁵⁴⁾.

Sethuraman V et al., (2019) reports site specific drug release using cubosomal nanoformulation. Passive tumor targeting because of solubility of calcium phosphate nanoparticles in lower pH of cancer cell than normal cell ⁽⁵⁵⁾.

Naga M. L. et al., (2014) explains the characteristics of cubosome that makes them targeted drug delivery systems especially in case of cancer cells. Because the lipids employed in cubosome nanoformulation are stable, the nanoformulation will remain stable throughout its shelf life. Cubosomal formulation methods include top-down, bottom-up, and automated cubosome preparation using high pressure homogenization, and sonication techniques are highlighted ⁽³¹⁾.

Karami Z et al., (2016) explained the structural components of cubosomes, the depiction of drug dissolution, applications, and route of delivery in detail. Cubosomes comprise curved bi-continuous lipid bi-layers, arranged in two internal aqueous channels that are separated into three-dimensional honeycomb patterns for a variety of bioactive substances ⁽³³⁾.

Yara E. Elakkad et al., (2019) studied different approaches for targeting cancer cells by cubosomes for improving drugs cellular uptake and thereby anticancer effect. Cubosomal formulations now play an important function in many different drug delivery issues ⁽⁵⁶⁾.

Ahirrao, M., & Shrotriya, S. (2017) Prepared cubosomes of resveratrol using top down method from GMO and Lutrol F127, used a 3²-factorial design was employed for the optimization of the cubosome. Report assessment of cubosome in situ nasal gel comprising resveratrol for neuronal delivery ⁽³⁹⁾.

Kumar R. et al., (2019) prepared Self-emulsifying nano drug delivery system of fisetin. Details of its preparation, optimization, characterization and cytotoxic evaluation are explained in this literature. He reported that solubility problem and enhancement in drug release is increased in his formulation ⁽⁵⁷⁾.

Rajan Kumar et al., (2020) Prepared Liquisolid powder formulation of fisetin. In liquisolid technology a non-volatile liquid (PEG), adsorbs on solid ingredients, called as carriers. The carriers like microcrystalline cellulose and coating material such as amorphous silicon dioxide were used. He reported the preparation of liquisolid formulation of fisetin in this article ⁽⁵⁸⁾.

Kurangi, B. et al., (2020) reported the development and assessment of a cubosome nano formulation loaded with resveratrol. Top-down method with homogenization technique and optimization using factorial design was reported. Characterizations depending on particle size, charge, and entrapment efficacy were done and further evaluated for *in vitro* dissolution and stability study ⁽³⁴⁾.

Skiba M. et al., (2021) reported increased aqueous solubility and release of fisetin by sphere-shaped amorphous solid mixture in the polymeric form of cyclodextrin. To increase the fisetin solubility, the spray-drying solid dispersion technique with cyclodextrin polymer is used, to improve less water-soluble compounds like fisetin ⁽⁵⁹⁾.

Mohsen A. M. et. al., (2021) reported Cubosomes as a prospective oral medication delivery method for coenzyme Q10. Prepared and used cubosomes for oral administration of medicaments with large molecular size and poorly soluble drugs. Cubosomes are able to facilitate with excellent loading capacity and protect drugs from degradation ⁽⁶⁰⁾.

Chettupalli, A. K. et. al., (2021) An antiretroviral drug Atazanavir in cubosomal gel formulation, *in-vitro* and *in-vivo* studies discovered the transdermal administration of drug cubosome gel considerably increases the absorption and the bioavailability of the drug of drug compared to that of oral drug solution ⁽⁴⁰⁾.

Liu, W. Y., et. al., (2021) Fisetin-loaded nanoparticles using polyvinyl alcohol (PVA) and polylactic-co-glycolic acid were prepared method. Their *in vitro* study results shows that the increase in absorption and the bioavailability of Fisetin due to nanoformulation for oral use ⁽⁶¹⁾.

Jiang, K.,et. al., (2021) fisetin action on oxidative stress and the inflammation in a model of endometritis generated by lipopolysaccharide and a bovine epithelial cells and reported that it is strongly associated with induction of the Nrf2/HO-1 pathways and suppression of TLR4-mediated ROS/NF- κ B ⁽¹²⁾.

Lorenzo G. D. et al., (2022) proved the supplementary application of Fisetin in the managment of prostate cancer individuals taking androgen-deprivation treatment. Men on such long treatment are protected against some of the most clinically significant side effects. Fisetin may potentially increase the anticancer effects of ADT or other concurrent medications ⁽²⁷⁾.

Afroze N. et al., (2022) reported that fisetin reduces cell proliferation, induces apoptosis, and increases oxidative tension and irritation in HeLa cell. It downregulates the appearance of AKT and MAPK paths changes propagation and improves apoptosis of cancer cells. By controlling the JAK-STAT/NF-kB channels, fisetin therapy also reduces irritation and enhances oxidative stress ⁽⁶⁾.

Eguchi, H., et al., (2022) report the Fisetin increases the toxicity of chemotherapeutic drugs in lung adenocarcinomas decreased expression of claudin-2 in A549 spheroid cells ⁽⁶²⁾.

Mitra S. et al., (2022) reported that In a mouse study, oral fisetin administration decreases the symptoms of acute allergic asthma. Oral use in an aqueous form, a small dosage of fisetin (50 nM) may provide a cost-effective and safe treatment option for asthma ⁽²⁵⁾.

Summary for review of literature

From the literature review, global cancer statistics states that the lung cancer has highest incidence among all the cancer types, which is now being treated with chemotherapy and radiation therapy which have noticeable side effects. FIS reported activity against lung cancer and reduction in lung cancer induced using B(a)P in Swiss albino mice. It reduces AKT, MAPK pathways, cell proliferation, induces apoptosis, increases oxidative pressure and irritation in HeLa cell, and anti-cancer molecule activate P53, Bax, Bak and BCL-2 signaling molecules, ROS and inflammation. The changes in anticancer molecule associated toxicity by FIS in human lung cancer A549 cells.

The current developments in FIS nanoformulations to overcome the solubility and bioavailability problem of FIS were reported in many literature^(63–65). Previously SLNP, microemulsion, transferosome like many types of nano formulations were reported to improve therapeutic benefits of FIS. Recently self-emulsifying nano drug delivery, liquisolid powder preparation and dispersion of cyclodextrin polymer have been prepared and evaluated ^(57–59,61,66–72).

Reports on preparation and evaluation of cubosomal nanoformulation of various phytoconstituents are also shows increased therapeutic benefits of studied phytoconstituents. Cubosomal formulations were reported for oral use to increase solubility and bioavailability of various drugs which has solubility and bioavailability issue. Cubosomes are more stable and effective nanoformulations for delivery of less water-soluble phytochemicals like flavanols and also reports site specific drug delivery in various types of cancer cells and other body tissues.

1.3 JUSTIFICATION

Reports on the cancer study states that the lung cancer is major reason for death within all other types of cancers. Chemotherapy and radiation therapy used in lung cancer have many adverse effects, drug resistance and disease recurrence. Many successful anticancer drugs are present in market like cisplatin, paclitaxel etc. and many new phytoconstituents are in clinical trial study. Phytoconstituents are effective and have less side effects than synthetic chemotherapeutic drugs.

Flavonoids is a class of phytoconstituents which have reported for prominent anticancer effect but possess solubility problem. Many reports are available on use of such flavonoids as anticancer agents by improving its solubility through various nanoformulation techniques. FIS, a flavonoid is selected for lung anticancer study depending on literature reports and cubosomal nanoformulation for improving its solubility to achieve its maximum therapeutic effect.

Properties of cubosome like high drug loading capacity, site specific drug release and highly stable at different physiological conditions makes it more suitable type of nanoformulation for FIS than earlier reported nanoformulation. In proposed study, cubosomes have been used to overcome the limitations like poor aqueous solubility, poor cellular absorption, and poor pharmacokinetic of FIS to get its maximum anticancer effect.

1.4 AIM, OBJECTIVES AND STUDY PLAN

Aim: To investigate the effect of cubosome loaded fisetin in lung cancer.

Objectives

- To formulate and characterize fisetin loaded cubosome nanoparticles.
- To evaluate anticancer activity in lung cancer *in-vitro* and *in-vivo*.

Study Plan

Preformulation Study

The compatibility study of Fisetin with the other excipients. Fourier transform Infrared (FTIR) spectroscopy, Differential Scanning Calorimetry (DSC) methods will be used for their compatibility study



Analytical method development and validation by HPLC and UV-Spectroscopy technique

HPLC method development for fisetin will be done using suitable mobile phase, other parameter will be fixed like flow rate and oven temperature and validation should be done using ICH Q2 (R1) guidelines. UV-spectroscopy method will be developed using suitable solvent and its validation.



Fisetin Cubosome formulation

Top-down approach will be selected. Polymer and lipid in different ratio will be used. Glyceryl monooleate (GMO) and poloxamer 407 are selected and other lipids will be studied depending their requirement in study. Independent variables like lipid and polymer concentrations and dependent variables like percent encapsulation efficiency and particle size will be selected for optimization.



Optimization and characterisation of prepared cubosomes

Pilot study and then application of Design Expert software, Cubosome particle characteristics like Particle size, Polydispersibility index, encapsulation efficiency, HR-TEM, DSC and drug release study



Stability study of optimized Cubosomes

Optimized batch will be selected and tested for 3 months' time duration for particle size, encapsulation efficiency and drug release.



***In vitro* fisetin release study**

Effect of Fisetin cubosomal formulation on its solubility and dissolution will be studied in different dissolution medium. Drug release of fisetin will be compared between unprocessed fisetin, Fisetin cubosomal formulation and available marketed formulation.



***In vitro* lung anticancer study**

Purchase of authentic sample of A549 cells. MTT, Clonogenic and Flow-cytometry assay, Flurocent staining study, Fisetin cell uptake study will be used to study effect of Fisetin cubosomal formulation in comparison with free Fisetin



***In vivo* lung anticancer study**

It will be performed using male Swiss albino mice, anticancer effect will be studied for Fisetin cubosomal formulation, marketed formulation and pure Fisetin in lung cancer bearing mice. Lung toxicity in Swiss albino mice will be induced using lung carcinogen B(a)P orally and studied various parameters like body weight, lung weight, level of total proteins and enzymatic antioxidants (SOD and CAT) and histopathology.



Statistical methods for result interpretation

Effect of Fisetin cubosomal formulation on *in vitro* fisetin release and anticancer study, *in vivo* animal study in comparison with unprocessed Fisetin was interpreted using appropriate statistical method like use of Design Expert and Graph Pad Prism software. Study for improvement in the fisetin solubility, cellular absorption and therapeutic effect will be carried out.

2. MATERIALS AND METHODOLOGY ADAPTED

Table1. Instruments used in the study

Sr. No.	Instrument name	Company	Model
1	High-performance liquid chromatography	Shimadzu, (Japan)	Prominence, LC-20AD,
2	Dissolution apparatus	Electrolab	EDT-08LX
3	FTIR	Shimadzu, (Japan)	IR Affinity-1S
4	Differential scanning calorimetry	Shimadzu, Kyoto, (Japan)	DSC-60
5	Electronic balance	Shimadzu, (Japan)	AUW 220D
6	pH meter	Eutech Instruments	Prominence
7	Homogenizer	IKA (Germany)	T25 digital Ultra Turax
8	Probe sonicator	Rivo TEK, (Mumbai, India)	T25 digital Ultra
9	Bath sonicator	Branson	Rivotek™ Ultrasonic
10	Inverted microscope	Labomed	Sonic
11	High resolution-TEM	Jeol/JEM 2100	200 kV LaB6 Electron Gun, CPX 1800 H-E
12	Zetasizer	Malvern Instruments, (UK)	TCM 400
13	High speed refrigerated Centrifuge	Eppendorf, Hamburg, Germany	EI 6500
14	Micro centrifuge	Tarson	Lisa Plus

15	Homogenizer	Remi Laboratory	Floor model 7000
16	Microscope (fluorescence)	Olympus	V6B-02
17	Ultra-low temperature freezer	New Brunswick	RQT-127A
18	Lyophilizer	CHRIST, (Japan)	ALPHA1–2 LD plus, BX41
19	X-ray diffractometer	Philips (Holland)	Automated, SmartLab SE, U410-86
20	CO ₂ incubator	New Brunswick	Alpha 1-2 LD plus
21	Microtiter plate reader	Rapid Diagnostic	PW-3710
22	Viscometer	Brookfield, CAP 2000+(USA)	Galaxy 170R
23	Cellulose dialysis membrane	Himedia, (India)	150, LA401-5MT

Table 2. Materials and chemicals used

Sr. No.	Chemicals	Company details
1	Fisetin	Shaanxi Yi An Biological, (China)
2	Glyceryl monooleate	Mohini Organics, (India)
3	Pluronic [®] F-127	Sigma Aldrich, (MO, USA)
4	Mannitol	Himedia Laboratories, (India)
5	Potassium dihydrogen phosphate	Merck, (India)
6	Sodium hydroxide	Merck, (India)
7	A549 cells (Human lung cancer cells)	NCCS, (Pune, India)
8	Phosphate-buffered saline	Himedia Labs, (India)
9	3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT)	Sigma Aldrich, (USA)
10	Ethidium bromide	Himedia Laboratories, (India)
11	Acridine orange	Himedia Laboratories, (India)
12	4',6-Diamidino-2-phenylindole	Sigma Aldrich, (USA)
13	Dimethyl sulfoxide	Fisher Scientific, (Mumbai, India)
14	Acetonitrile	Fisher Scientific
15	Methanol	Fisher Scientific
16	Chloroform	Fisher Scientific
17	Ortho phosphoric acid	Fisher Scientific, (Mumbai, India)
18	Formic Acid	Qualigens, (India)
19	Fetal bovine serum	Himedia Labs, (India)
20	Dulbecco's modified Eagle's medium	Himedia Labs, (India)
21	Benzo(a)pyrene	Merck, (India)

Preformulation study

To test the compatibility of FIS with the other excipients, we used Fourier-transform infrared spectroscopy and differential scanning calorimetry. The preformulation study of FIS was performed by using FTIR, DSC and UV spectroscopy method. For FTIR and DSC, samples of pure drug FIS, Pluronic F-127, GMO, and FISCUB were tested. FTIR is a suitable technique for characterizing various molecular species, which reveals drug-excipient compatibility by providing information on the interaction of various molecules and functional groups when mixed in the formulation. DSC provides the thermal behavior and interactions of FIS with cubosomal formulation excipients. We performed FTIR analysis at SAIF IIT Bombay, Mumbai (Vertex 80 with Spectral resolution of FTIR 0.2 cm^{-1} , focal plane array: 128×128 , range: $4000\text{-}900\text{ cm}^{-1}$), and we used DSC (Shimadzu DSC-60, Kyoto, Japan; temperature range of $0\text{-}400\text{ }^{\circ}\text{C}$) to obtain thermograms of FIS, GMO, Pluronic F-127 and cubosomal formulation. Using the UV spectroscopy method, we scanned the appropriate concentration to find out maximum absorption wavelength and compared with the reported value for identification and characterization of received FIS sample.

2.1 Development of analytical method for estimation of FIS

The FIS quantification from the unprocessed form and prepared cubosomes is very important for the cubosome characterization parameters like drug entrapment efficiency of cubosome formulation. There is a need of simple, accurate, and reliable FIS estimation method that can be used for all types of FIS formulations and FIS estimation from various natural and synthetic sources also. We developed and

validated two analytical methods, one is very sensitive and reliable chromatographic RP-HPLC and other is a simple and time saving UV-Spectroscopic method ^(73–75).

2.1.1 RP- HPLC

To evaluate cubosomal formulations, it is important to accurately estimate the amount of FIS from different types of FIS samples. The FIS quantification analytical method is also required to carry out its cell line and animal study. The new RP-HPLC methodology has been developed and employed to estimate FIS from prepared cubosomal nanoformulation as well as testing several FIS sources ^(76–80).

Chromatographic instrument and requirements

A liquid chromatographic system (RP-HPLC) Shimadzu prominence liquid chromatography (LC-20AD, Japan) containing pump - LC-20AD, autosampler - SIL-20AC HT, detector - SPD-M20A PDA, degasser - DGU-20A5, oven – CTO -10AS VP column and 20 µl loop rheodyne injection. The software used was version 1.25 of Shimadzu LC solution for data analysis and interpretation. An analytical column Phenomenex (Canada, USA) Luna 5u C18 (2) 100A0 (inner diameter – 150 x 4.6 mm, particle diameter - 5µm) with a 35 °C column temperature was used for separation and analysis of FIS. The 25:75 v/v mixture of optimized mobile phase acetonitrile (ACN): formic acid (0.1%) was used and chromatograms were obtained at 360 nm PDA wavelength by using a fixed flow rate of 1ml/min.

FIS stock and sample preparation

The accurately weighed 10 mg of FIS was dissolve in 10 ml of methanol to prepare 1 mg/ml solution A and further diluted using mobile phase to get 50 µg/ml concentration of solution B.

For preparation of the calibration curve, concentrations between 0.1 to 16 µg/ml were prepared, stored in dark at 4°C temperature and tested using RP-HPLC. The sample C was prepared from cubosome nanoformulation by diluting 1 ml formulation up to 10 ml using methanol (100 µg/ml).

Method development

The analytical method was developed by testing several solvents as mobile phase and chromatographic conditions. The RP-HPLC method development for FIS was performed with mobile phase as methanol, and acetonitrile at different pH conditions.

Method validation

The analytical technique was developed and validated in accordance with ICH Q2(R1) requirements ⁽⁸¹⁾. The various parameters like linearity, precision, accuracy, system suitability, ruggedness, LOQ, LOD, and robustness were tested.

Calibration curve development

The solution B was used to prepare concentrations of 0.10, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, and 16.0 µg/ml to develop standard calibration graph.

Accuracy

The three concentrations of solution B as 0.50, 1.0, and 1.5 ml were mixed with 0.50 ml of sample C and diluted to 10 ml using mobile phase. We get the 7.5, 10 and 12.5 µg/ml final concentrations for testing and calculation of % drug concentration recovered was done using following equation

Actual % recovery = (Actual concentration recovered/Theoretical concentration)
X100 ...(1)

Precision

The percent RSD of intra-day and inter-day drug estimation using developed method were calculated. The three concentrations 5, 10, and 15 µg/ml of the FIS were tested with same laboratory conditions. To find the precision, readings were taken in triplicate for every concentration and percent relative standard deviation were calculated as % RSD = (Standard deviation of peak area/ Average peak area) X 100..... (2)

Ruggedness and robustness

The ruggedness and robustness states about the effect of flexibility in developed method conditions over performance of the method and tested by making small intentional modifications in method conditions. Triplicate readings of the 10 µg/ml concentration were taken and chromatogram characteristics along with percentage recovery were evaluated.

Methods Suitability

The method suitability was assessed from recorded values for theoretical plate, peak purity index, tailing factor and HETP.

Sensitivity

The estimated concentrations of the LOD and LOQ represents the sensitivity of the HPLC method. The response's standard deviation (σ) and the calibration curve (S) slope were used to determine LOD and LOQ.

$$\text{LOD} = 3.3 \times \sigma / S \dots\dots\dots (3)$$

$$\text{LOQ} = 10 \times \sigma / S \dots\dots\dots (4)$$

Application of the method for fisetin estimation in cubosomal nanoformulation and other fisetin samples

To estimate FIS from cubosomal formulation, 1 ml of sample C diluted up to 10 ml and analyzed. The linearity curve equation was used to estimate percentage of FIS loading as per following equation

$$\% \text{ FIS Loading} = (\text{Actual amount of FIS present} / \text{Total amount of FIS added}) \times 100 \dots\dots\dots (5)$$

The 20 FIS capsules were used for assay calculation and plant extracts were diluted in mobile phase and evaluated for FIS quantification using developed RP-HPLC method.

FIS stress degradation study

This study was conducted as per test conditions give in ICH guidelines for acidic, alkaline, thermal, oxidative, and photolytic stress conditions. Study was performed in light resistant amber volumetric flasks due to photosensitivity of FIS ⁽⁸²⁾.

2.1.2 UV-Spectroscopic method

UV-visible spectroscopy is a very simple, fast, and sensitive technique for the estimation of FIS. Previously developed and used UV-visible spectroscopic methods were not validated or not used for estimation of FIS from cubosomal formulation. Therefore, we designed and validated a UV-visible spectroscopy method that allows for quick and easy FIS measurement from a variety of FIS samples. The found

method is economical and can be applied to the detection and estimation of FIS from produced cubosome formulation with the highest level of accuracy, and precision using methanol as solvent. A Japan made Shimadzu UV-1800 double beam UV spectrophotometer was used for the method development and validation ^(83–86).

Preparation of stock and standard solution

The 10 mg of FIS was completely dissolve in the 10 ml of methanol with sonication and 1 ml of this solution was further diluted up to 10 ml to prepare 100 µg/ml concentration. Serial dilutions of 2 to 10 µg/ml were prepared and absorbance was measured to prepare calibration curve.

Method development and validation

The solubility study of FIS in different solvents was performed and among methanol, ethanol, acetone, and dimethyl sulfoxide, methanol was selected for method development by considering its better solubility in methanol. FIS was scanned between 200 and 800 nm using methanol as a solvent. The maximum absorbance was noted at 362 nm wavelength in methanol as solvent. Similar to RP-HPLC method, ICH guidelines were followed for validation of UV-Spectroscopic method and tested for its accuracy, precision, system suitability, linearity, LOQ, LOD, ruggedness, and robustness. The linearity curve was developed using 0, 2, 4, 6, 8, and 10 µg/ml dilutions and used. For measurement of FIS entrapment in cubosomal formulation, high speed centrifugation was performed and supernatant was taken for absorption measurement.

$$\% \text{ FIS Entrapment (EE)} = \frac{(\text{Total FIS added in formulation} - \text{FIS in supernatant})}{\text{Total FIS added in formulation}} \times 100 \dots\dots\dots (6)$$

2.2 Experimental design of the study for cubosomal formulation

We prepared and tested 16 pilot batches before the optimization study to select a suitable range for the lipid and stabilizer (GMO and Pluronic F-127) concentrations. A 3^2 -full factorial design from the Design Expert[®] software (version 7.0.0) was applied to optimize cubosomal nanoformulation. Particle size and entrapment efficiency were chosen as two dependent factors, while the quantities of the stabilizer and lipid were selected as two independent factors. Three level of concentrations were assigned to each independent variable: low, medium, and high. The dependent variables particle size and entrapment efficiency were set to minimize and maximize respectively^(34,39,40). The Design Expert[®] software suggested 9 batches for development of FIS cubosomal nanoformulation optimized batch ⁽⁸⁷⁾.

Table 3. Coded levels in 3^2 factorial design of independent variables for FIS Cubosomal nanoformulation

Selected Levels	Independent Variables (Lipid and Stabilizer concentrations)	
	GMO (%w/w)	Pluronic® F-127 (%w/w)
Low (-1)	2	0.5
Medium (0)	4	1
High (+1)	6	1.5
FIS cubosomal formulation batches suggested by software		09

2.3 Preparation of FIS Cubosome (FISCUB)

A milky white aqueous dispersion of the FIS cubosomal nanoformulation was prepared using the top-down method from GMO and Pluronic F-127. In the top-down method, nano-sized cubic liquid crystalline particles were separated from their honeycomb-like molecular assembly using homogenization and sonication techniques. We used various reported methods using different concentrations of lipid GMO, and Pluronic F-127 polymer to prepare the stable cubosome nanoparticle structures loaded with FIS. The method was modified and established for FIS cubosomal nanoformulation based on the physical observations and obtained particle size ^(28,45,46).

The melted GMO was combined with 40 mg of FIS over a thermostatic water bath and this mass was added in Pluronic F-127 at 65 °C and mixed. This molten mass was gradually added in to 40 milliliters of warm water at the same temperature. This results in the formation of a white liquid dispersion, cooled and homogenized (IKA T25, Germany) in cool conditions at 14,500 rpm for 20 min further sonicated (Rivo TEK, India) for 10 minutes and stored overnight to equilibrate properly ^(34–37).

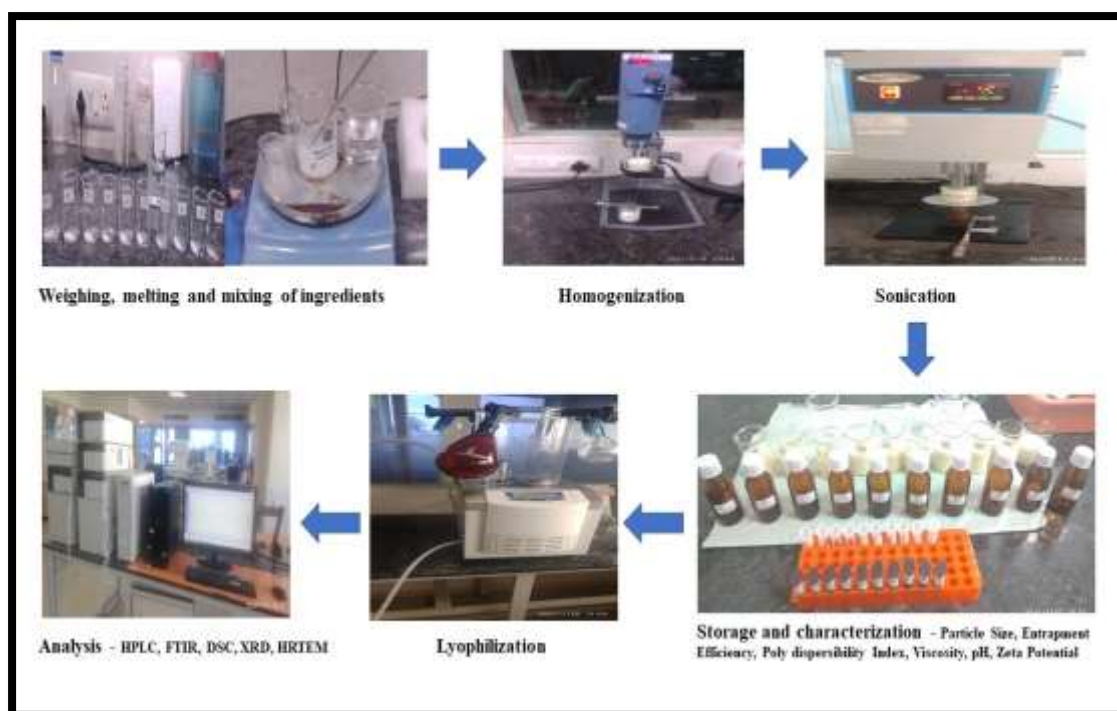


Figure 4. Cubosome nanoformulation preparation

The particle size, PDI and ZP values for cubosomal nanoformulation were investigated using a Zetasizer (Malvern Instruments, UK) ⁽³⁸⁾.

The FIS present in the formulation supernatant was measured using RP-HPLC method. Formulation was centrifuged 30 minutes at high speed 14,500 rpm (Kubota 6500, Osaka, Japan) and supernatant separated and mixed with selected mobile phase of the HPLC FIS estimation method. The amount of FIS entrapped inside the cubosomes in nanoformulation was estimated using standard curve equation developed for HPLC method.

2.4 Lyophilization

Thee FISCUB and its blank nanoformulation were stored at -80°C overnight and then lyophilized (CHRiST, ALPHA 1–2 LD plus) to get a dense sample for HRTEM and XRD study. The selection of cryoprotectant and its concentration is

crucial in lyophilization of the liquid cubosomal nanoformulation. Although there have been reports for application of a variety of cryoprotectants for the general lyophilization procedure, mannitol was utilized for the same in numerous lipid-based nanoformulations. In the lyophilization procedure for FIS cubosomal nanoformulation, 3% mannitol was used as a cryoprotectant to minimize damage to cubosome particles during freeze-drying. The lyophilized form of the nanoformulation was tested for HRTEM and XRD analysis and examined the cubosomal appearances^(88,89).

2.5 Characterization of FISCUB

2.5.1 Physicochemical Characterization of FISCUB

Various analytical and instrumental tools were used for the physicochemical characteristic evaluation of the prepared FIS cubosomal formulation. It includes structural and chemical characteristics of cubosome liquid crystalline particles like size, shape, size distribution, charge developed over the surface, pH, viscosity, interaction between the chemical components used. The samples of pure drug FIS, Pluronic F-127, GMO, and FISCUB were tested for ingredient compatibility and structural study of the cubosomal formulation by using fourier-transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), XRD, and HRTEM^(41,42).

Particle size, PDI, ZP

Zetasizer instrument with its standard protocol was used to accomplish the physicochemical characterization of FISCUB for the average particle size, PDI, and ZP values which works on the dynamic optical scattering principle.

FTIR

FTIR is a suitable technique for characterizing various molecular species, which reveals drug-excipient compatibility by providing information on the interaction of various molecules and functional groups when mixed in the formulation. The FTIR examination conducted at SAIF IIT Bombay, Mumbai (Vertex 80 with Spectral resolution of FTIR 0.2 cm⁻¹, focal plane array: 128×128, range: 4000–900 cm⁻¹)

XRD

The XRD examination demonstrates the crystallinity of the produced cubosome particles present in the FIS nanoformulation. The XRD spectrum for FIS, FIS cubosomal nanoformulation, and blank cubosomes was taken from SAIF KUD, Dharwad (Automated XRD SmartLab SE, Philips Holland with x-ray tube-CuAngular). For XRD analysis, the instrument's 2θ range was kept in-between 2° and 150° throughout sample testing⁽⁶¹⁾.

pH and viscosity

The pH and viscosity tests were performed on the prepared FIS cubosomal nanoformulation for examination of pH measurement and rheological performance (Brookfield viscometer, CAP 2000+; MA, USA)^(40,44,90).

DSC

The cubosomal nanoformulation excipients and FIS chemical interactions and their thermal behavior are studied using DSC. The DSC instrument (Shimadzu DSC-60, Kyoto, Japan; temperature range of 0–400 °C), the thermograms for FIS, and formulation excipients were obtained and analyzed.

2.5.2 FIS loading/ Entrapment efficiency (EE) testing

The high-speed centrifuge machine (Eppendorf, Hamburg, Germany) was used to separate the FIS-entrapped cubosomes from the prepared nanoformulation at 14,500 rpm for 30 minutes. We used the established HPLC method to measure the drug content in the liquid supernatant. Equation 6 was used to calculate the proportion of entrapped FIS in the cubosomal nanoformulation.

2.5.3 HRTEM

The HRTEM instrument (Jeol/JEM 2100 with 200 kV LaB6 Electron gun) was employed for revealing the physical structural characteristics of the cubosomes with arbitrarily chosen locations over the slide for electron diffraction. A copper grid covered with carbon was utilized inside the 2% w/v phosphotungstic acid ^(88,91).

2.5.4 *In vitro* FIS & FISCUB release

Drug dissolution, release and permeability study of the FIS from prepared cubosomal formulation was performed using a cellulose dialysis membrane (HIMEDIA - 150) and different dissolution media were used. The dissolution media used were water, HCL buffer (pH 1.2), and phosphate buffer (pH 6.8), and a dissolution instrument (Electrolab EDT-08LX) with modification to conduct a dissolution study for low-volume nanoformulations (100 ml basket, 100 rpm speed rotating paddle) ^(34,92).

The dialysis film was dipped in particular dissolution medium overnight and 5 ml cubosomal nanoformulation was loaded. The drug release study carried out at 37 °C and 1 ml sample for RP-HPLC analysis was collected from baskets with replacement of equal amount.

2.6 Stability study

The optimal cubosomal formulation was kept in a stability chamber for 3 months between 4 and 8 °C in properly sealed, amber-colored glass vials covered with aluminum foil. Three-month stability samples were evaluated for their particle characterization ⁽⁴³⁾.

2.7 *In vitro* study

The change in the therapeutic efficacy of FIS in its unprocessed and cubosomal form was tested using an animal cell culture study. An appropriate cell culture model was selected to study the changes in proliferation effect in lung cancer cells ^(10,11,93).

Purchase of A549 cell and maintenance

For *in vitro* lung anticancer study, the human lung cancer A549 cell line model is commonly used and therefore selected for testing the effect of cubosome formulation on the anticancer effect of FIS. An authentic sample of the A549 cell line was purchased from NCCS Pune, subcultured in DMEM, and studied for MTT, clonogenic, flow cytometry assay, and staining using fluorescent stains like acridine orange-ethidium bromide, and DAPI.

2.7.1 MTT Assay

The A549 cells were grown in DMEM media, supplemented with a 1% antibiotic combination (potassium penicillin and streptomycin sulphate) and 10% FBS in a CO₂ incubator at 37 °C. A confluent grown monolayer of A549 cells was removed from the flask and the cells number were maintained at 10⁵ cells/ml, 100 µl and added in each well of a 96-well tissue culture plate incubated at 37 °C for one

day. The concentrations 0, 12.5, 25, 50, 100, 150, and 200 µg/ml of serial samples in cells culture media were created and 100 µl of each concentration was added triplicate and cultured at 37 °C using 5% CO₂ incubator for two days. The culture medium was replaced by 20 µl MTT (5 mg/ml in sterile PBS) and stored in a CO₂ incubator at 37 °C for 5 hours. The upper MTT solution from wells was removed and added 100 µl DMSO to take absorption readings at 570 nm. The IC₅₀ concentrations for FIS, FISCUB, blank formulation, and cisplatin were calculated employing GraphPad Prism 5.01 software ^(93,94).

2.7.2 Clonogenic Assay

The formation of a 32-cell colony from the growth of a single animal cell represents the proliferative and reproductive capability of the animal cell and is reduced in the presence of chemical insults. The retention of the replication capability of A549 cells after treatment with FIS and FISCUB will result in their comparative antiproliferative effect. The 6-well animal cell culture plates were seeded with 1 ml A549 cell suspension of 300 cells/ml in triplicate and cultured for 12 hours. The 1 ml of IC₅₀ concentrations of FIS, FISCUB, and cisplatin were placed in wells and incubated up to 24 hours. Then solutions were replaced with freshly prepared DMEM and grow the cells for 12 days to develop individual cells into colonies. The developed colonies were washed using PBS and treated with 1% crystal violet for visual counting ⁽⁹⁵⁾.

2.7.3 Flow Cytometry assay

The 1 ml of 10⁵ cells/ml cell suspension of A549 cells added in 24 well tissue culture plates and cultured in CO₂ incubator overnight at 37 °C. In the partial monolayer-grown cells, the calculated IC₅₀ concentrations of FIS, FISCUB and

cisplatin were added and kept for 12 hours. Cells were cleaned with PBS, centrifuged and again suspended in 1X cold binding buffer at 4 °C to form 10^5 cells/ml. Then put 1 µl of Annexin V-FITC and 5 µl PI and stored on ice for 15 minutes away from light. Finally, added 400 µl of cold 1X binding buffer and flow cytometric analysis was performed ^(26,70).

2.7.4 Acridine orange & ethidium bromide (EB/AO) staining

Based on the integrity of the cell wall, this differential fluorescence staining technique assesses apoptosis and survival of cells. In each 6-well tissue culture plate well, 1 ml of 10^5 cells/ml A549 cells were added over a glass coverslip, and drug treatment was given with IC_{50} concentrations for 48 hours. Acridine orange (0.5%) and then subsequently ethidium bromide (0.1%) was used for staining the drug-treated cells and cleaned with PBS. The acridine orange gives green fluorescence upon binding DNA of live cells and ethidium bromide deep orange under a blue filter of the fluorescence microscope (Nikon OPTIPHOT-2)

2.7.5 DAPI staining

The fluorochrome DAPI (4, 6-diamidino-2-phenylindole) 1 µg/ml was used in the staining of treated and untreated A549 cells over the glass coverslip. DAPI stains nuclear DNA by binding the adenine-thymine-rich area in the dsDNA strands. The cells were treated with the drug using IC_{50} values for 48 hours and observed for the morphology of A549 cell nuclei and its apoptotic changes ⁽²⁶⁾.

2.7.6 Procedure for quantitative Cell uptake study

Cells were trypsinized, prepared 1×10^5 cells/ml in DMEM and 1.5 ml of this cell suspension was added in each well of 6 well plates. Then 6 well plates were

incubated for 24 hours in a CO₂ incubator at 37 °C. Removed the medium and added 1 ml FIS and FISCUB (equivalent to 10 mcg/ml) prepared in sterile DMEM. The time given for drug uptake inside the A549 cells was 5 hours. Then the medium was separated, gently washed three times with sterile PBS to separate the free drug. For cell lysis, 1 ml of 0.1% Triton X-100 was supplemented and mixed properly. This cell lysate content was transferred in a 2 ml plastic tube and added 1 ml ACN for total FIS extraction. This content was centrifuged at 10000 rpm for 15 min to separate cell lysate and the supernatant was tested for FIS quantification using previously developed and reported RP-HPLC method ^(94,96).

2.8 In vivo animal study

The animal study protocol was approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA) and the Institutional Animal Ethics Committee (IAEC) (Letter: KLECOP 221/Po/Re/S/2000/CPCSEA/14-17/03/2021). The Swiss albino male mice were used for toxicity and anticancer studies. The mice were maintained in the cages at an ambient temperature for one day prior to the light-dark cycle.

Animals: Swiss albino mice, Total animals taken: 60 animals, Age: 6 -8 weeks, Sex: Male

For the development of cancer in disease control, 50mg/kg body weight. Benzo(a)pyrene (inducing agent) was given twice a week for four weeks orally with corn oil to develop lung cancer at the end of 16 weeks. For drug treatment, 25 mg/kg body weight pure drug or its nanoformulation was given orally ^(52,97-99).

2.8.1 Animals and grouping (Experimental design)

To study the change in therapeutic efficacy of FIS due to its cubosomal formulation on induced lung toxicity, both preventive and therapeutic effect study was designed using known cigarette smoke carcinogen benzo(a)pyrene model protocol. For evaluation of B (a) P-induced lung toxicity between native FIS and its cubosomal nanoformulation, the experimental design contains five animal groups containing 6 animals in every individual group as given in the following Table 4.

Table 4. Animal Grouping and *in vivo* study design

Sr. No.	Treatment Groups (N=6)	Number of animals	
		Preventive Study	Therapeutic Study
I	Normal	6	6
II	Control (Disease induced)	6	6
III	Fisetin	6	6
IV	Fisetin Cubosomal Formulation	6	6
V	Standard drug (Cyclophosphamide)	6	6

Chemicals used and dose given

- 1) Benzo(a)pyrene 98 % pure (50 mg/kg body weight)
- 2) FIS and FIS cubosomal nanoformulation (25 mg/kg of the body weight)
- 3) Cyclophosphamide (30 mg/kg body weight)
- 4) Corn Oil

The details of the dosing for each group are given in following Table 5.

Table 5. Animal group dosing details

Animal group	Treatment given
A) Preventive study	
Group I	Normal Control -Given normal feed and water
Group II	Diseased control - Given Benzo(a)pyrene 50mg/kg body wt. dissolved in corn oil, orally given twice a week for 4 weeks, Lung cancer will develop by 16 weeks.
Group III	Pure drug -25mg/kg, Started one week before first dose of B(a)P
Group IV	Cubosomal nanoformulation – Orally 25mg/kg, Started one week before first dose of B(a)P and continued up to 4th week.
Group V	Standard - 30mg/kg cyclophosphamide injected subcutaneously, Started one week before first dose of B(a)P and continued up to 4th week.
A) Therapeutic Study	
Group I	Normal Control -Normal feed and water
Group II	Diseased control - Given Benzo(a)pyrene 50mg/kg body wt. dissolved in corn oil, orally given twice a week for 4 weeks, Lung cancer will develop by 16 weeks.
Group III	Pure drug -25mg/kg, Start on 8 th week of first dose of B(a)P
Group IV	Cubosomal nanoformulation – Orally 25mg/kg, Start on 8th week of first dose of B(a)P and continue up to 16th week.
Group V	Standard - 30mg/kg cyclophosphamide injected subcutaneously, Start on 8th week of first dose of B(a)P and give up to 16th week.

2.8.2 Physiological parameter –

Body and lung weight, level of total proteins, and enzymatic antioxidants superoxide dismutase and catalase (SOD and CAT) parameters concentrations were measured for the interpretation of the effect of the FIS cubosomal nanoformulation against benzo(a) pyrene-induced lung toxicity. The body weight of all the animals

was measured every week using a digital weighing machine. The total protein estimation were performed using Lowry method (100), SOD by Misra et al ^(101,102). and CAT by Takahara et al. ^(103,104) method.

2.8.3 Histopathological analysis

At finishing of the 16th week, blood were collected by retro-orbital puncture method from all the mice, anesthetized, sacrificed, and dissected for isolation of lungs, and stored in 10% formalin. The hematoxylin and eosin (H&E stain) dye was utilized for staining the sections of the lung tissues. The images of lung tissue sections from all the groups were observed using the light microscope ⁽¹⁰⁵⁾.

In vivo study planned to explain the comparative protective role of FIS with its cubosomal formulation on B(a)P-induced lung toxicity by evaluating body weight, lung weight, serum total proteins, levels of enzymic anti-oxidants such as superoxide dismutase (SOD), catalase (CAT) and histopathological study.

3. STATISTICAL ANALYSIS

Design-Expert® software (Stat-Ease Inc., MN, USA) was used for the optimization of the FIS cubosomal nanoformulation. The one-way analysis of variance (ANOVA) was utilized for establish the significance of the model and associate the *in vivo* effect in animal study for various parameters. To find the relevant statistically significant parameters of the *in vitro* and *in vivo* studies, GraphPad Prism software (GraphPad Software Inc., CA, USA) was used.

4. RESULTS

4.1 RP-HPLC method development and validation

Method development

For the RP-HPLC method development, different mobile phase mixtures of organic and aqueous solvents like methanol, ACN, HCl acidified water, orthophosphoric acid, formic acid and glacial acetic acid were analyzed. The combinations of methanol and acidified water with different concentrations of HCl, orthophosphoric acid, and formic acid were analyzed. Then the combinations of ACN with acidified aqueous phase with different concentrations of glacial acetic acid, orthophosphoric acid, and formic acid were analyzed.

These mobile phase combinations were analyzed over RP-HPLC by using a FIS stock concentration of 10 μ g/ml and 10 μ l injection volume. The developed chromatographic peaks for FIS were analyzed. Better resolved peaks were observed for methanol with water at 45:55 v/v and for acetonitrile and acidified water at 30:70 v/v ratio. The ACN and formic acid 0.1% (30:70 v/v) combination was shown highest peak area (507435), theoretical plates (5236.056), and tailing factor (1.257) and therefore further investigated to get better separation and quantification results using their modified proportions. It was found that ACN: formic acid 0.1 % in a mixture of 25:75 v/v at a flow rate of 1 ml/min gives the highest values for peak area (901804.33) and theoretical plates (6184.87) and was chosen for further validation study.

Method Validation

Calibration curve development and linearity range

The FIS stock solution B was further diluted with the mobile phase solution to get different concentrations in the range of 0.1 to 16 $\mu\text{g/ml}$ and found a straight line in the linearity graph when plotted concentration versus area as given in Figure 5 ($R^2 = 0.9999$).

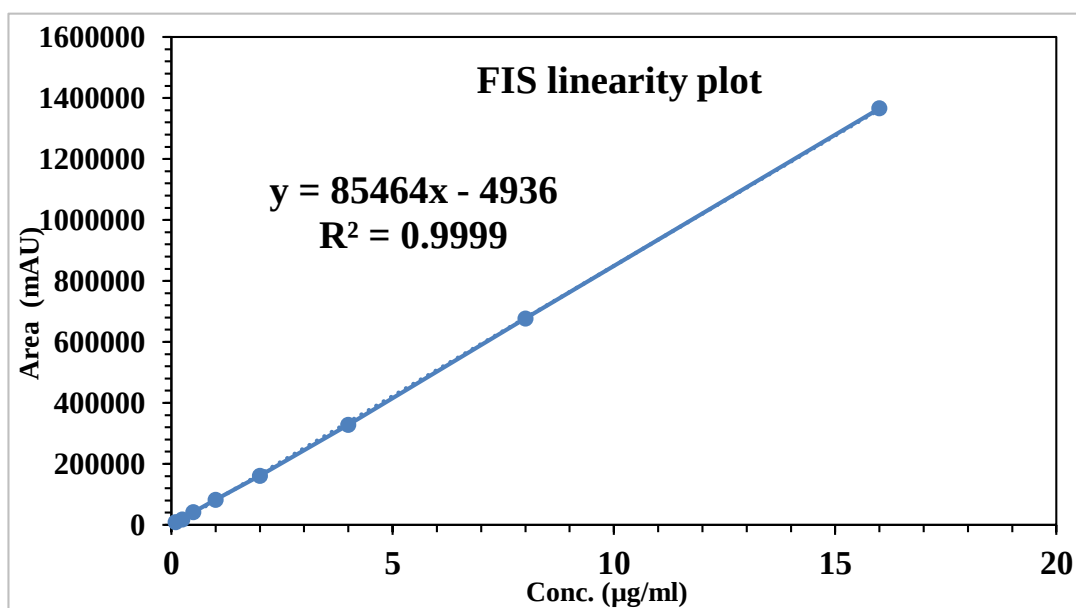


Figure 5. Linearity graph of FIS using RP-HPLC

Accuracy

Using the above-developed linearity equation, the spiked FIS concentration in cubosome nanoformulation produces three concentration levels 7.5, 10 and 12.5 $\mu\text{g/ml}$, and the analyzed mean % recovery was between 98.24 to 100.65 % (prescribed limit of 95 to 105%). This indicates good accuracy for the FIS estimation using the developed RP-HPLC method.

Table 6. Accuracy study results of FIS (n=3)

FIS content in formulation ($\mu\text{g/ml}$)	Level (%)	Spiked qty. ($\mu\text{g/ml}$)	Total qty. ($\mu\text{g/ml}$)	Recovered qty ($\mu\text{g/ml}$)	% Drug Recovered	Mean % of Drug Recovered
5	50	2.5	7.5	7.54 $\pm$ 0.04	100.65	99.67
5	100	5	10	10.01 $\pm$ 0.03	100.12	
5	150	7.5	12.5	12.27 $\pm$ 0.02	98.24	

Precision

The intraday and inter-day precision were analyzed using three-level concentrations as given in Table 7. It was observed that the developed procedure was quite precise as % RSD estimation was found to be less than 2 for intra and inter-day analysis at defined chromatographic conditions.

Table 7. Precision study results of FIS solution (n=3)

FIS conc. (µg/ml)	Intra-day		Inter-day					
			Day 1		Day 2		Day 3	
	Found Conc. ± SD	% RSD	Found Conc. ± SD	% RSD	Found Conc. ± SD	% RSD	Found Conc. ±SD	% RSD
5	4.83 ± 0.01	0.09	5.34 ± 0.01	0.18	5.42 ± 0.01	0.03	5.55 ± 0.01	0.08
10	10.05± 0.01	0.09	10.63 ± 0.03	0.31	10.79 ± 0.13	1.20	11.07 ± 0.01	0.13
15	15.65 ± 0.03	0.20	15.79 ± 0.19	1.19	15.46 ± 0.12	0.81	15.26 ± 0.01	0.07

SD= Standard deviation, RSD= Relative standard deviation

Ruggedness and Robustness

The less than 2% changes measured in % RSD values of peak areas estimated in the FIS chromatogram after making small internal changes in chromatographic conditions for flow rate, mobile phase ratio, oven temperature, formic acid concentration, and absorption wavelength, indicates ruggedness and robustness of the developed RP-HPLC method.

Table 8. Ruggedness and robustness study results (n=3)

Variable	Value	Peak Area		Retention Time	
		Mean $\pm$ SD	% RSD	Mean $\pm$ SD	% RSD
Flow rate (ml/min)	0.8	1051067.00 $\pm$ 2079.96	0.20	8.55 $\pm$ 0.00	0.05
	1	844452.33 $\pm$ 801.85	0.09	6.89 $\pm$ 0.00	0.00
	1.2	705836.67 $\pm$ 822.63	0.12	5.72 $\pm$ 0.03	0.49
Mobile Phase Ratio	23:77	845998.33 $\pm$ 5787.99	0.68	8.65 $\pm$ 0.00	0.03
	25:75	846071.00 $\pm$ 1234.45	0.15	6.86 $\pm$ 0.00	0.04
	27:73	848818.67 $\pm$ 1260.44	0.15	5.64 $\pm$ 0.00	0.02
Oven Temperature ($^{\circ}$ C)	30	842032.67 $\pm$ 2290.91	0.09	7.21 $\pm$ 0.02	0.04
	35	843733.00 $\pm$ 4021.38	0.48	6.89 $\pm$ 0.01	0.08
	40	843383.67 $\pm$ 5602.74	0.66	6.68 $\pm$ 0.00	0.07
Formic Acid conc (%)	0.01	841030.33 $\pm$ 3069.88	0.37	6.92 $\pm$ 0.00	0.01
	0.1	840095.33 $\pm$ 3511.48	0.42	6.93 $\pm$ 0.01	0.12
	0.3	839874.33 $\pm$ 3821.63	0.46	6.85 $\pm$ 0.00	0.04
Absorption wavelength (nm)	358	842414.33 $\pm$ 2681.05	0.32	6.88 $\pm$ 0.00	0.05
	360	843685.00 $\pm$ 2822.21	0.33	6.88 $\pm$ 0.01	0.09
	362	840489.33 $\pm$ 2761.03	0.33	6.88 $\pm$ 0.01	0.09

System suitability

The established RP-HPLC method was observed suitable in the FIS estimation as we found peak purity index value 1, theoretical plate value 6184.87 (>2000), and tailing factor 1.19 (near to 1). The obtained chromatograph for cubosomal nanoformulation shows a distinctive FIS peak and no extra peak near to FIS peak (Figure 6).

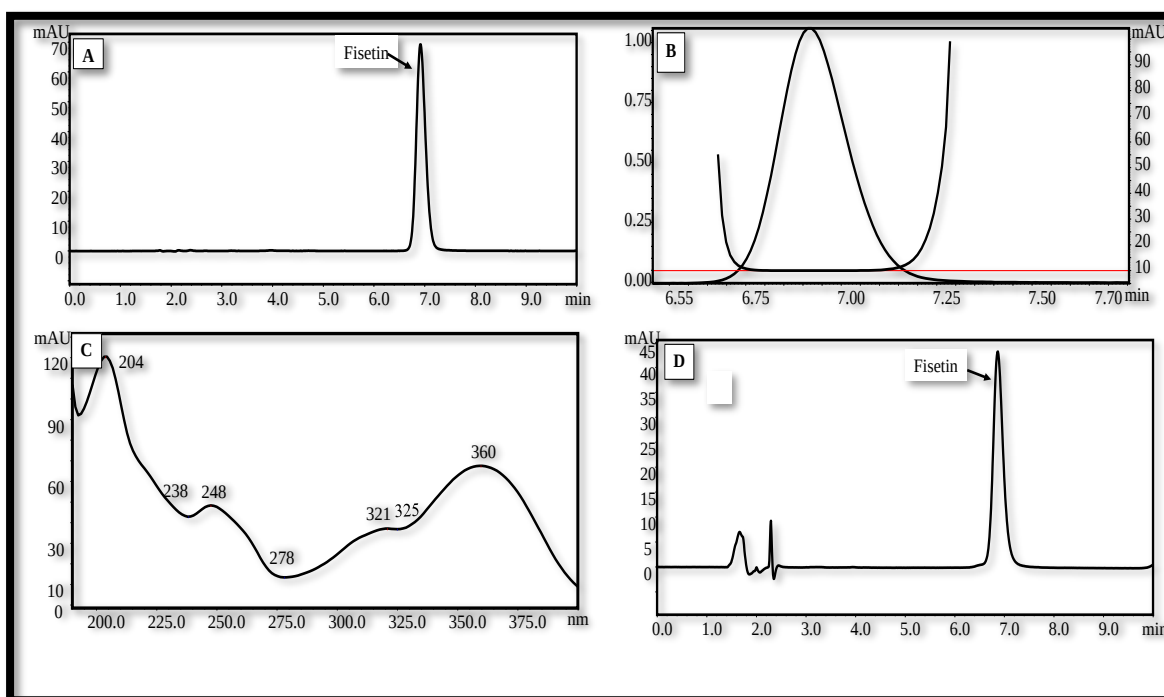


Figure 6. HPLC chromatograms at 360 nm for FIS (A), Peak purity curve (B), FIS UV-Visible absorption spectrum (C), FIS loaded cubosome (D)

Sensitivity

The developed method was very sensitive for FIS estimation from cubosomal formulation as well as other sources also as LOD and LOQ values were 17.26 and 52.31 ng/ml correspondingly.

Fisetin estimation in cubosomal nanoformulation, marketed formulation, and plant extract

In the developed FIS cubosome formulation, the drug loading and entrapment efficiency were found to be 99.60 and 80.10 % respectively. The marketed FIS capsules (Doctor's Best ® Fisetin) contain 98.94 ± 0.08 mg/Capsule fisetin (Label quantity– 100 mg/capsule). The estimated fisetin content in strawberry fruits was found to be 2.39 ± 0.15 µg/gm using the developed RP-HPLC method.

Stress degradation study

The stress degradation study demonstrates how stable FIS is under different stress circumstances and assists in designing formulations and other studies on FIS. The RP-HPLC chromatograph of FIS in the standard experimental condition is given in Figure 7A. The FIS chromatograph developed after acidic treatment (1N HCl at 80 °C for 1 hour) reports only 6.14 % degradation and, therefore reports stability in acidic conditions (Figure 7B). For the alkali treatment study using 1 N NaOH at 80°C for 1 hour, all the FIS was degraded. An additional three peaks were observed for its metabolites (Figure 7C) and found highly unstable for basic conditions.

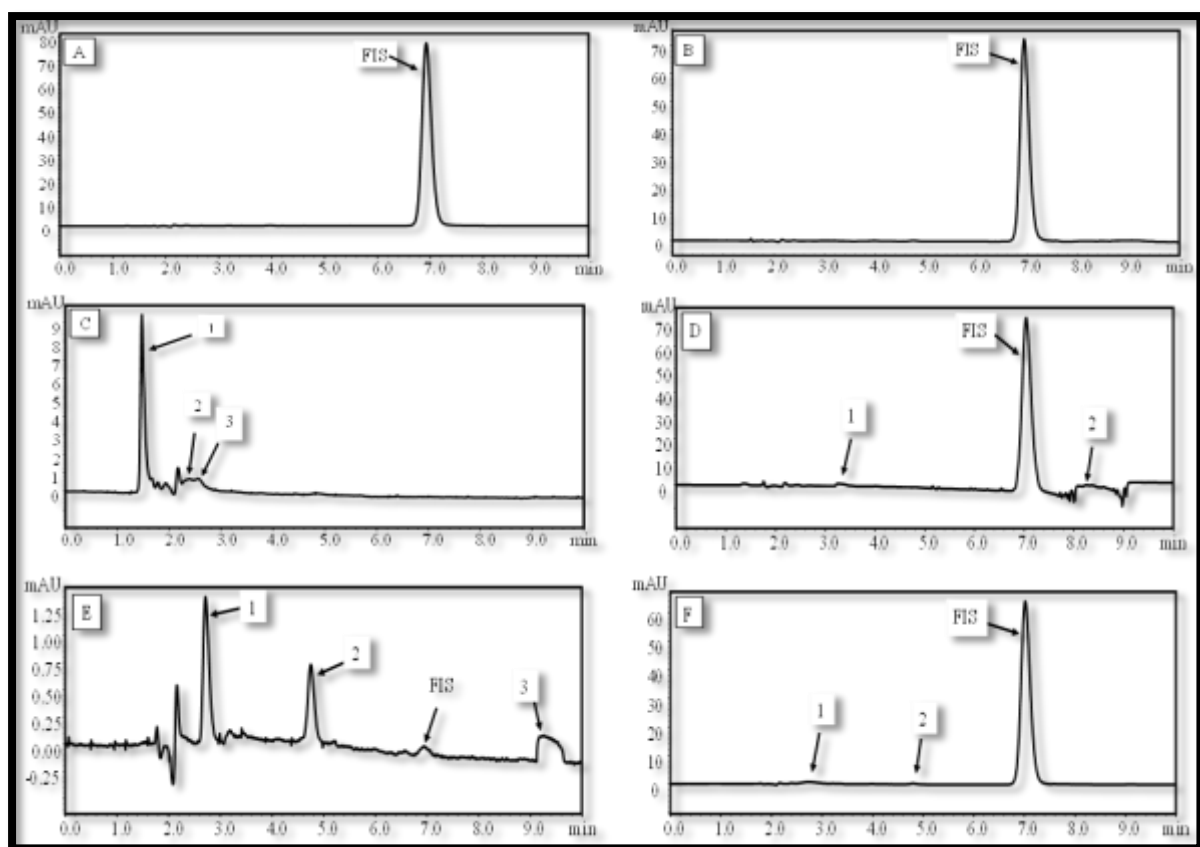


Figure 7. HPLC chromatogram of FIS in forced stress degradation study under normal (A), acid (B), alkaline (C), thermal (D), oxidative (E), and photolytic (F) stress conditions

It reports 11.7 % FIS degradation at 80°C for 1 h with two degradation peaks and found stable for thermal treatment. The treatment of FIS with 30% H₂O₂ for 1 hour found more FIS degradation and showed three metabolite peaks reporting instability for oxidation (Figure 7E). When the FIS sample is taken in a transparent volumetric flask and exposed to sunlight for 1 hour then 15.28 % drug was degraded and reports sensitivity to sunlight exposure.

4.2 UV-Spectroscopic method development

Method development and validation

The FIS 10 µg/ml concentration was used for measuring the absorbance between wavelength 200 to 800 nm. using solvents methanol, ethanol, and DMSO. The reported λ max for the used solvents was 362 nm (Table 9)

Table 9. UV-Spectroscopic method development

Parameter	Details
Solute drug/Analyte	Fisetin
Solvents used	Ethanol, Methanol, Dimethyl Sulfoxide
Fisetin Absorption max (λ max) for methanol	362 nm
Instrument Used	Double Beam Scanning, Shimadzu UV-1800

Since FIS using methanol as a solvent system exhibits a distinct and well-defined peak for FIS absorption at 362 nm, it was chosen as the solvent. The method was validated as per the ICH Q2 (R1) standards like selectivity and specificity, accuracy, precision, ruggedness and robustness, and sensitivity.

Selectivity and specificity

The absorption spectra obtained for FIS and solvent methanol exhibited a sharp and clear peak at 362 nm with no interference confirming the selectivity and sensitivity of the method.

The following figure shows the FIS absorption spectrum using solvent methanol.

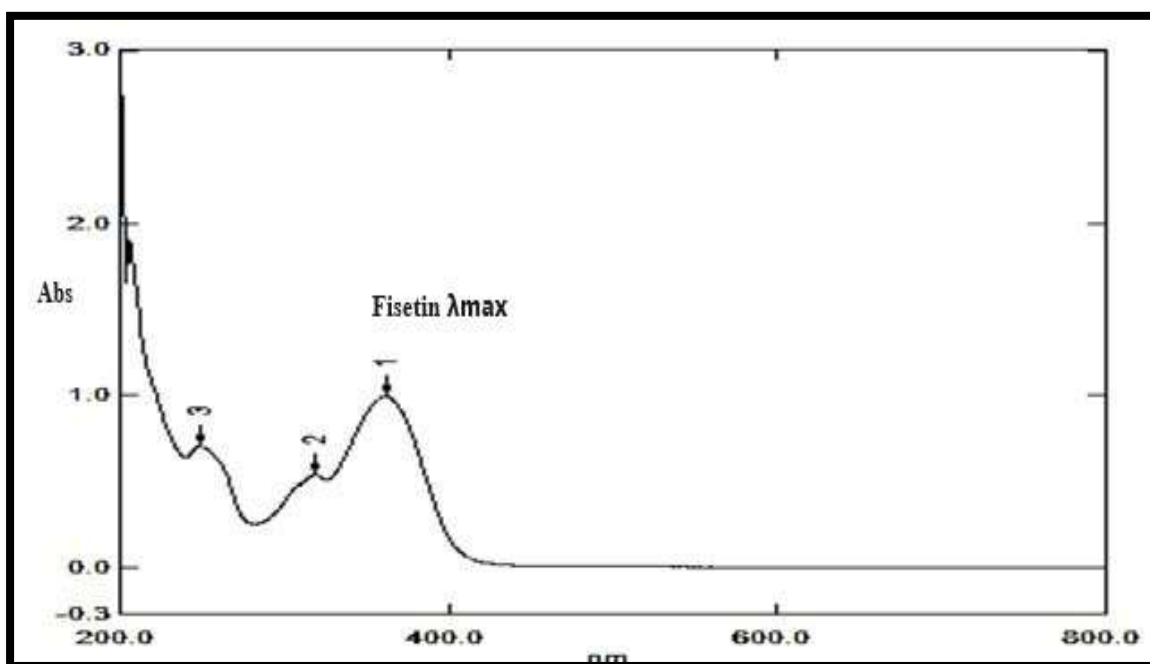


Figure 8. UV-Spectrum of Fisetin showing absorption maximum wavelength at 362 nm

Linearity curve development

The amount of 2 to 10 µg/ml FIS in methanol was used for the linearity curve development and triplicate absorbance was taken at 362 nm (Table 10).

Table 10. Linearity curve development for fisetin using UV-Spectroscopy

Sr. No.	Fisetin Conc. (µg/ml)	Fisetin Abs. at 362 nm
1	2	0.197
2	4	0.388
3	6	0.578
4	8	0.756
5	10	0.953
R^2		0.9998
Slope		0.0947
LOD (µg/ml)		0.119
LOQ (µg/ml)		0.250

The linearity curve was taken to establish the linearity equation, the correlation coefficient (R^2) was found > 0.9998 , and the slope value was measured 0.0947 (Figure 9).

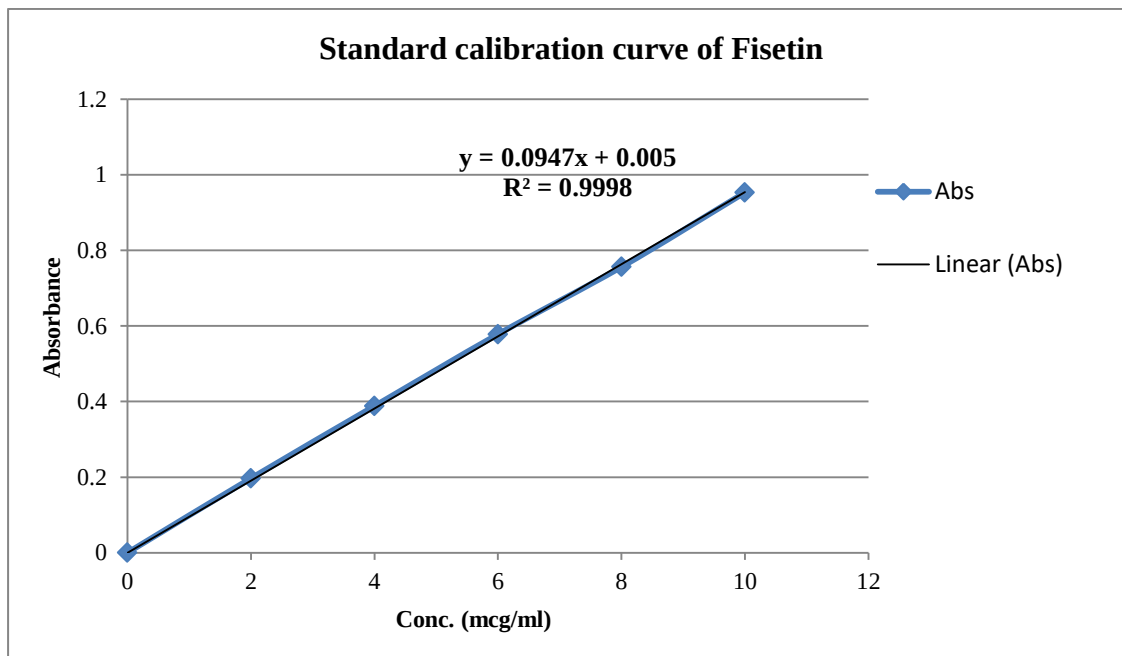


Figure 9. Linearity curve of fisetin using UV-Spectroscopic method

Accuracy

The three level concentrations of the median (5 μ g/ml) were taken for the measurement of absorbance and % of FIS recovered was measured. The % of FIS recovered using this method was more than 95% therefore reported accurately for FIS estimation (Table 11).

Table 11. Accuracy data for Fisetin by UV-Spectroscopic method

Level %	Fisetin Conc. (μ g/ml)	Absorbance	Amount recovered (μ g/ml)	%Mean recovery $\pm$ SD
50	2.5	0.239	2.471	99.683 $\pm$ 0.422
		0.24	2.482	
		0.241	2.492	
100	5	0.477	4.984	101.373 $\pm$ 0.921
		0.478	4.995	
		0.485	5.069	
150	7.5	0.713	7.476	98.557 $\pm$ 0.695
		0.714	7.487	
		0.705	7.392	

Precision

The inter and intraday precision was measured for FIS estimation using three levels as low, medium, and high concentrations. The % RSD values were calculated for noted absorbance values and found <2 indicating the preciseness of the developed method (Table 12).

Table 12. Precision data for Fisetin using UV-Spectroscopic method

Conc. (µg/ml)	Interday Abs. reading			Intraday Abs. reading	
	Day 1	Day 2	Day 3	Morning	Evening
2.5	0.238	0.237	0.237	0.235	0.237
	0.240	0.235	0.231	0.239	0.231
	0.237	0.235	0.239	0.234	0.234
%RSD	0.641	0.490	1.767	1.121	1.282
5	0.482	0.479	0.475	0.475	0.477
	0.480	0.477	0.481	0.478	0.476
	0.476	0.470	0.475	0.474	0.479
%RSD	0.637	0.994	0.726	0.438	0.320
7.5	0.711	0.712	0.703	0.714	0.719
	0.701	0.703	0.713	0.712	0.716
	0.712	0.716	0.706	0.709	0.718
%RSD	0.859	0.937	0.725	0.354	0.213

Ruggedness and Robustness

The ruggedness was assessed by changing analyst and instrument for measurement of absorbance and finding change in the % RSD values of three-level concentrations. The robustness of the method was assessed from the calculation of changes in % RSD values of absorbance after making a small change in λ max. The change in % RSD values after changing analyst, instrument, and λ max was less than 2 therefore method was found to be robust for the estimation of FIS.

Sensitivity

The calculated LOD and LOQ concentration values for FIS estimation using the developed UV-spectroscopic method were found 0.119 and 0.250 $\mu\text{g/ml}$ respectively. This values of LOD and LOQ indicate that the method was very sensitive.

Application of the method for fisetin estimation in cubosomal nanoformulation, marketed formulation

By using the developed UV-spectroscopic method, the FIS estimation from cubosomal nanoformulation and marketed FIS capsules was performed. The total drug content (amount of FIS loading) of FIS was quantified as 99.48 ± 0.23 percent and to the market available (Doctor's Best ® Fisetin) 98.82 ± 0.18 mg/capsule formulation.

4.3 Preparation, optimization, and characterization of FISCUB

4.3.1 Preparation of FISCUB

For the development of FIS cubosomes, we employed the top-down approach, utilizing homogenization and sonication stages to develop an aqueous liquid dispersion. The various amounts of lipid and polymer were reported for various cubosomal drug entrapment methods. The changes in the concentration of independent variables GMO and Pluronic F-127 directly affect the values of dependent variables like particle size and FIS entrapment. The 16 pilot batches were prepared using 3 to 9% GMO and 0.5 to 2% Pluronic F-127 and studied the effect of concentration changes in independent variables over the dependent variables. The evaluation of the effect of GMO and Pluronic F-127 concentration effect over the dependent variables cubosomal particle size and FIS entrapment efficiency was performed for the selection of the appropriate concentration range of GMO and Pluronic F-127 to be employed for optimization study using Design Expert[®] software.

Table 13. Pilot study results of the dependent variables

Batch No	Batch Details	Particle size (nm)	PDI	Entrapment Efficiency (%)
1	GMO 3%, Pluronic F-127 0.5%	128.3±4.12	0.26±0.01	88.96±0.59
2	GMO 3%, Pluronic F-127 1%	77.76±2.58	0.65±0.07	82.91±0.42
3	GMO 3%, Pluronic F-127 1.5%	50.83±7.31	1.40±0.01	80.45±0.86
4	GMO 3%, Pluronic F-127 2%	38.36±5.44	1.87±0.03	72.81±0.49
5	GMO 5%, Pluronic F-127 0.5%	175.6±7.62	0.18±0.06	90.48±0.55
6	GMO 5%, Pluronic F-127 1%	122.53±3.46	0.36±0.03	80.96±0.41
7	GMO 5%, Pluronic F-127 1.5%	693.66±5.81	1.36±0.09	77.04±0.74
8	GMO 5%, Pluronic F-127 2%	611±3.91	1.27±0.07	69.27±0.63

9	GMO 7%, Pluronic F-127 0.5%	526±8.13	1.50±0.05	88.35±0.77
10	GMO 7%, Pluronic F-127 1%	311.9±4.62	0.59±0.04	78.42±0.35
11	GMO 7%, Pluronic F-127 1.5%	269.4±7.23	0.89±0.09	73.28±0.59
12	GMO 7%, Pluronic F-127 2%	609.33±8.72	1.00±0.05	60.38±0.72
13	GMO 9%, Pluronic F-127 0.5%	421±3.96	0.37±0.06	84.54±0.58
14	GMO 9%, Pluronic F-127 1%	821±5.14	0.72±0.01	75.13±0.67
15	GMO 9%, Pluronic F-127 1.5%	1017±2.94	1.03±0.07	66.63±0.52
16	GMO 9%, Pluronic F-127 2%	748±5.34	0.85±0.03	75.43±0.69

Values represents $\pm$ SD (n=3)

4.3.2 Optimization & characterization of formulation FISCUB parameters

To optimize FIS cubosomes, we applied 3^2 full factorial designs of Design Expert[®] software was employed the top-down production approach. The three-level concentrations low, medium, and high for GMO and Pluronic F-127 were decided for the selected 3^2 full factorial design study as shown in the table. The dependent variables, particle size, and entrapment efficiency were set for minimum and maximum outcomes in the Design Expert[®] software trials. The software suggested all nine batches were prepared and evaluated for the cubosomal particle characteristics like particle size, Poly dispersibility index, drug entrapment efficiency, and zeta potential (Table 14).

Table 14. Optimization study using Design Expert[®] software

Batch No.	Batch Details	Particle size (nm)	PDI	Entrapment Efficiency (%)	Zeta Potential
B1	GMO 2%, Pluronic F-127 0.5%	116.9±3.23	0.13±0.01	72.50±0.72	-53.20±0.61
B2	GMO 2%, Pluronic F-127 1%	93.07±5.41	0.20±0.01	80.10±0.36	-31.60±0.57
B3	GMO 2%, Pluronic F-127 1.5%	117.2±6.57	0.50±0.11	78.80±0.57	-37.40±1.18
B4	GMO 4%, Pluronic F-127 0.5%	127.2±3.72	0.15±0.07	77.67±0.74	-43.30±0.84
B5	GMO 4%, Pluronic F-127 1%	118±5.18	0.16±0.08	75.80±0.91	-41.90±0.41
B6	GMO 4%, Pluronic F-127 1.5%	148.7±2.64	0.42±0.08	69.10±1.21	-5.87±0.72
B7	GMO 6%, Pluronic F-127 0.5%	214.6±4.63	0.27±0.03	75.47±0.85	-30.30±0.85

B8	GMO 6%, Pluronic F-127 1%	129.5±6.27	0.16±0.05	77.79±0.77	-32.10±0.45
B9	GMO 6%, Pluronic F-127 1.5%	123.2±1.91	0.23±0.02	70.52±0.46	-18.1±0.76
B10 (Blank)	GMO 2%, Pluronic F-127 1%	148.7±7.23	0.42±0.08	0	-26.0±0.47
B2 (Stability)	GMO 2%, Pluronic F-127 1%	90.76±3.67	0.221±0.4	81.36±0.71	-17.1±0.34

Values represents $\pm$ SD (n=3)

4.3.2.1 Particle size, PDI and ZP

The result for particle size and FIS entrapment efficiency of the resultant 9 batches were put in to the Design Expert[®] software to find statistical results for particle characteristics. The statistical findings and developed equations revile the relations between undertaken particle characteristics. For the entrapment efficiency and particle size, statistical regression study proposed the quadratic and 2FI best-fit numerical models with calculated precisions 3.99 and 5.50 separately (Table 15).

Table 15. Model fit summary statistics

Source	Particle size					Entrapment efficiency				
	Std. Dev.	R ²	Adjusted R ²	Predicted R ²	PRESS	Std. Dev.	R ²	Adjusted R ²	Predicted R ²	PRESS
Linear	29.68	0.43	0.24	-0.50	14116.53	4.04	0.15	-0.12	-1.12	247.56
2FI	25.24	0.66	0.45	-0.58	14807.02	3.65	0.42	0.08	-1.10	244.97
Quadratic	23.37	0.82	0.53	-1.10	19691.47	3.20	0.73	0.29	-2.12	363.93
Cubic	7.83	0.99	0.94	-0.19	11164.04	1.47	0.98	0.85	-2.38	394.72
Adequate precision	For 2FI model = 5.50					For Quadratic model = 3.99				

Due to the unique liquid crystalline structural properties of prepared cubosome particles, the FIS solubility and membrane absorption was considerably increased confirms the applicability of cubosomal nanoformulation preparation for FIS use. The particle size results of the tested batches show 93.07 to 214.6 nm range in formulation optimization study.

The relation between measurement of cubosome particle size and its influencing independent parameter GMO and Pluronic F-127 concentrations obtained from statistical interpretation and depicted in following equation.

$$PS = 132.04 + 23.35A - 11.60B - 22.92AB \dots\dots\dots (7)$$

4.3.2.2 Entrapment efficiency

The ability of the cubosomal unites to enclose drug FIS inside its cubic structure is very important for delivering the FIS to desired tissue location and in the results of the formulation optimization study reports between 69.10 and 80.10% (Table 14)

The obtained statistical results gives an equation for predicting the entrapment efficiency from the measured values of lipid (GMO) and stabilizer (Pluronic F-127) for the developed FIS cubosomal nanoformulation and correlation shown in above figure .

$$EE = 76.78 - 1.27A - 1.20B - 2.81AB + 1.67A^2 - 3.89B^2 \dots\dots\dots (8)$$

From the Figure 10A & 10B, it was suggesting that there is increase in particle size and reduction in entrapment efficiency when there is increase in the independent parameter lipid (GMO) concentration. Figure 10C & 10D suggests that entrapment efficiency reduced when there was an increase in (A) lipid (GMO) and (B) stabilizer (Pluronic F-127).

4.3.2.3 Selection of optimized batch

The Design Expert[®] software generated 2D contour and 3D surface graphs for the FIS cubosomal particle size and entrapment efficiency and used for the interpretation of changes in the amount of Lipid GMO and Stabilizer Pluronic F-127 on them.

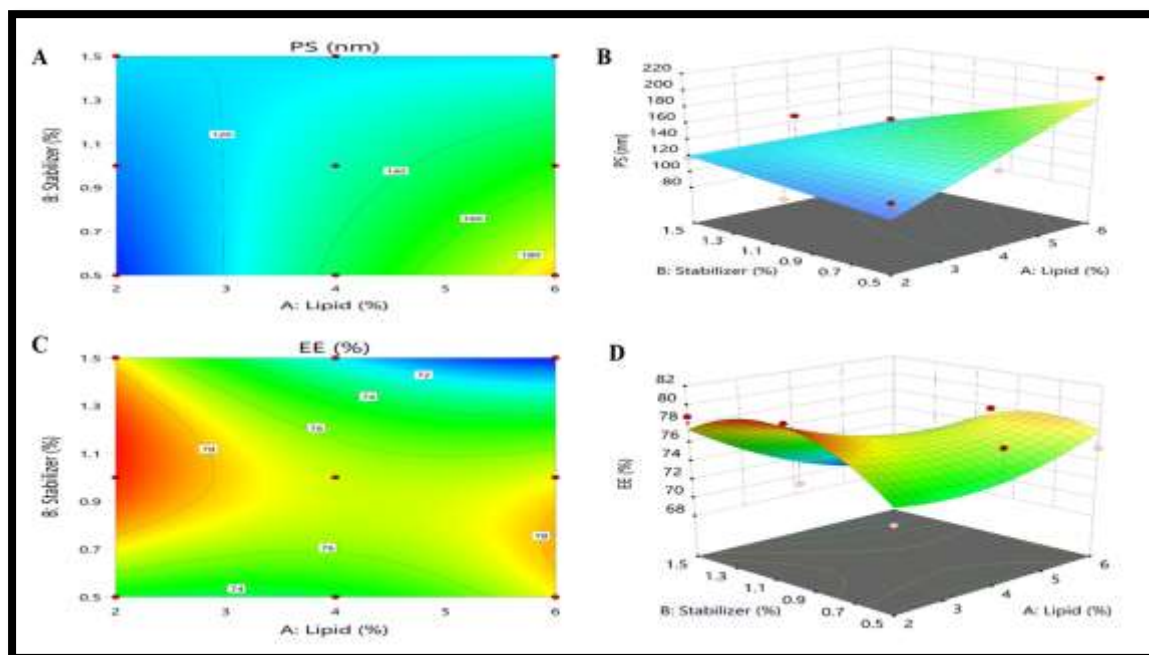


Figure 10. 2D contour and 3D response surface for PS and EE with the concentration of lipid and stabilizer

The interpretation of the relation between the independent and dependent FIS cubosomal formulation variables using plotted 2D contour and 3D response surface concludes that particle size is directly proportional to lipid concentration and inverse with stabilizer concentration. The drug loading was found to be reduced when there is increase in lipid and increasing with stabilizer concentration.

4.3.2.4 FTIR

The FTIR Spectrum and DSC thermograms of FIS, GMO, Pluronic F-127, and FISCUB, the compatibility of the substances utilized for FIS cubosomal formulation was investigated. After the development of FTIR spectra for each component, a study of their compatibility was conducted. The following FTIR spectra (Figure 11) show the characteristics of absorption bands of various functional groups present in the chemical components used in the cubosomal formulation preparation.

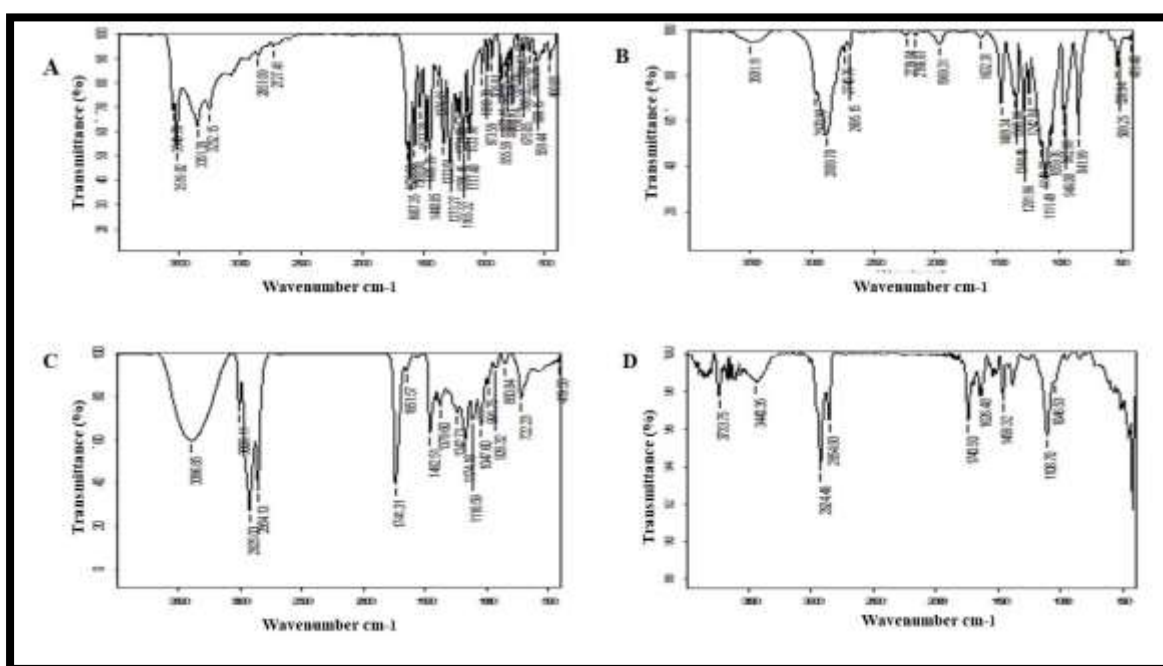


Figure 11. FTIR Spectrum – A. FIS, B. Pluronic F-127, C. Glyceryl monooleate (GMO), D. FISCUB

4.3.2.5 DSC

The DSC technique commonly used in material science, we used for checking the physical and chemical interactions between FIS and formulation ingredients. The characteristic thermogram peaks were obtained for FIS and other ingredients of the nanoformulation at specified conditions and shown in the following Figure 12.

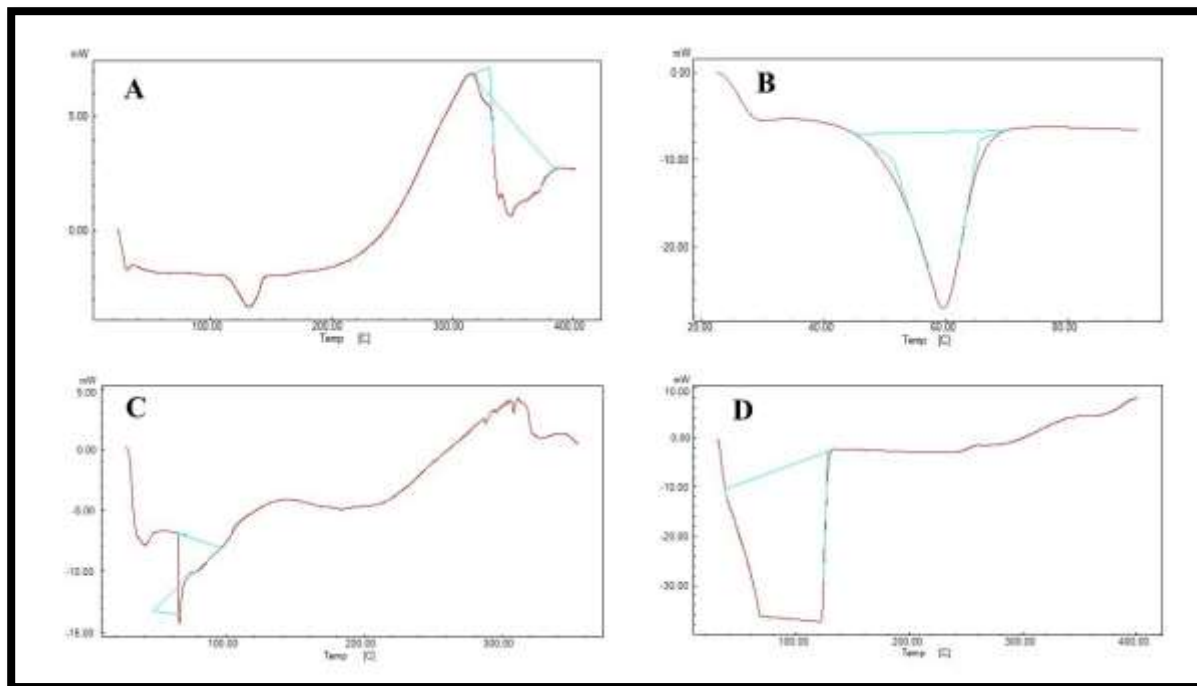


Figure 12. DSC Thermograms – DSC thermograms of A. FIS, B. Pluronic F-127, C. GMO, D. FISCUB

4.3.2.6 XRD

To study the nano-sized particle crystal characteristics of the prepared cubosomes, we recorded the XRD spectra of pure drug and prepared nanoformulation. At specified instrumental conditions, XRD spectra were obtained and given below figure for FIS, FISCUB and blank cubosomes (Figure 13).

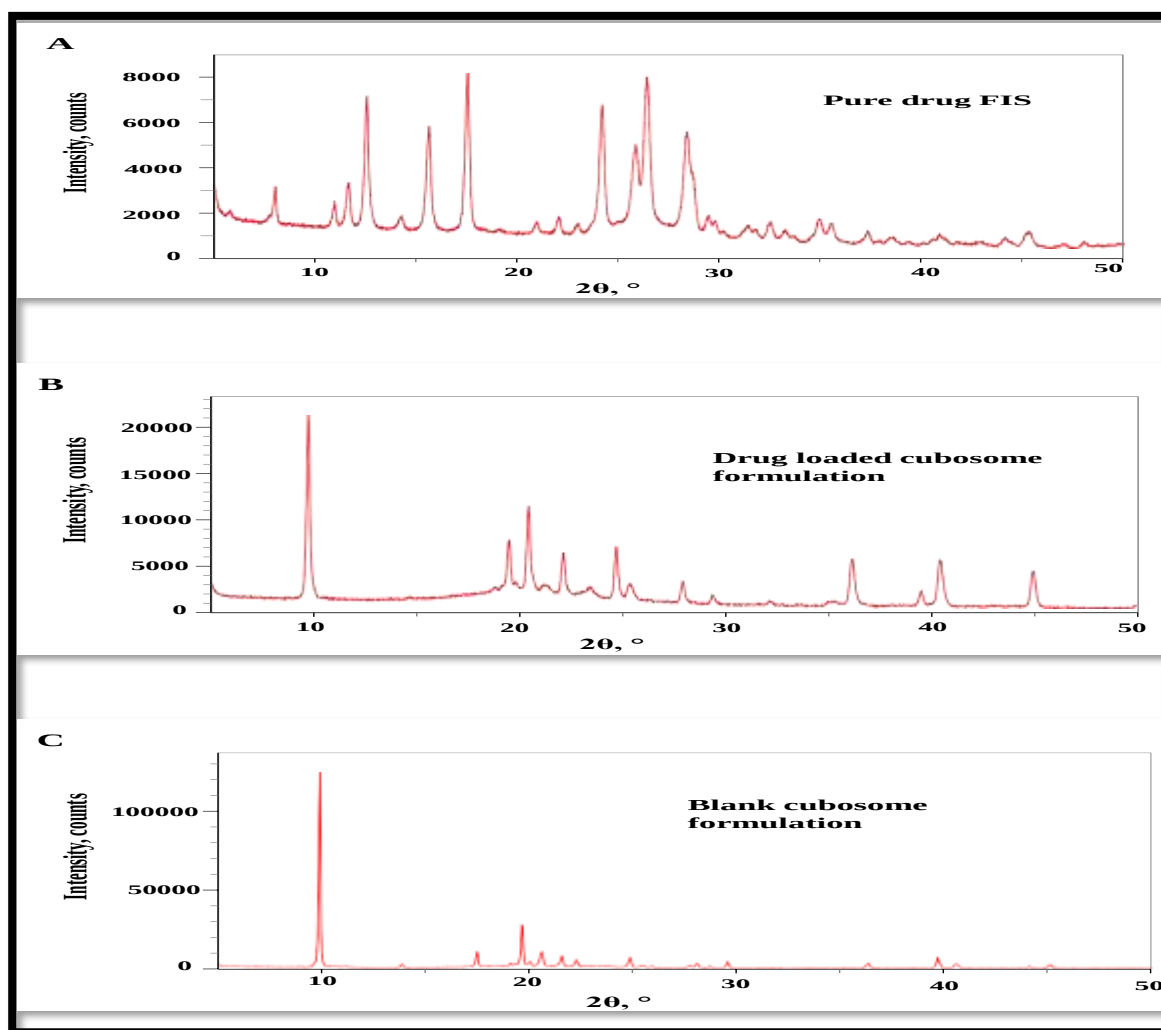


Figure 13. XRD spectra of A. FIS, B. FISCUB, C. Blank nanoformulation

4.3.2.7 HR-TEM

Using the specified instrumental specifications, the lyophilized sample of FISCUB formulation was observed using high-resolution transmission electron microscope for 100, 50 and 20 nm resolution to elucidate structural details of the liquid crystalline particles of GMO (Figure 14). The observations of the HR-TEM study revile the size and shape characteristics of the prepared FIS cubosome nanoparticles.

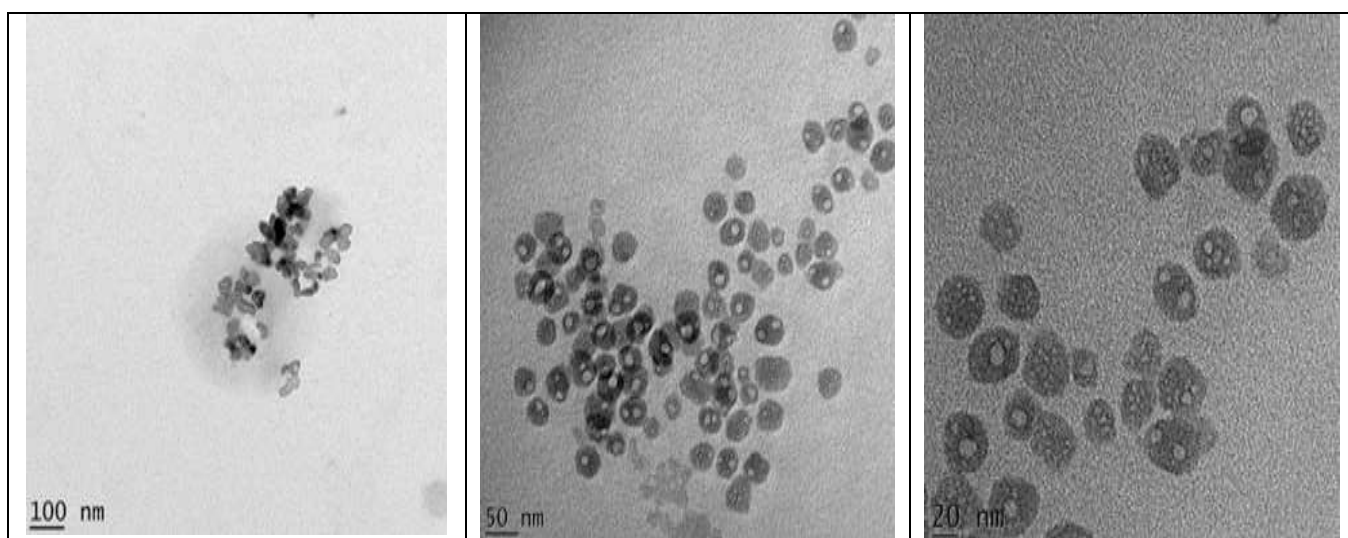


Figure 14. HRTEM images for FISCUB at various magnifications

4.3.2.8 pH and viscosity

The pH and viscosity of the formulation are important for the stability and acceptability of the FIS cubosomal formulation. It was measured for freshly prepared optimized FISCUB formulation and formulation without FIS initially and after three-month storage also for stability testing purpose (Table 16).

Table 16. Viscosity and pH of the formulations

Batch No	FIS Cubosome Batch Details	Initial Readings		Three-month stability reading	
		Viscosity (cP)	pH	Viscosity (cP)	pH
B2	GMO 2%, Pluronic F-127 1%	5.76± 0.03	6.38±0.07	5.60±0.02	6.41±0.03
B10 (Blank)	GMO 2%, Pluronic F-127 1%	5.96±0.02	6.36±0.05	5.89±0.06	6.46±0.08

Values represents ± SD (n=3)

4.4 *In vitro* drug release study

An *in vitro* FIS release study was performed using different dissolution mediums using a dissolution apparatus modified for a nanoformulation release study.

The % release of FIS from the native unprocessed FIS and FISCUB were quantified up to 8 hours and plotted against time to interpret the result. The results of this *in vitro* FIS release study suggests that there was a substantial rise in FIS release from FISCUB when compared with its native form.

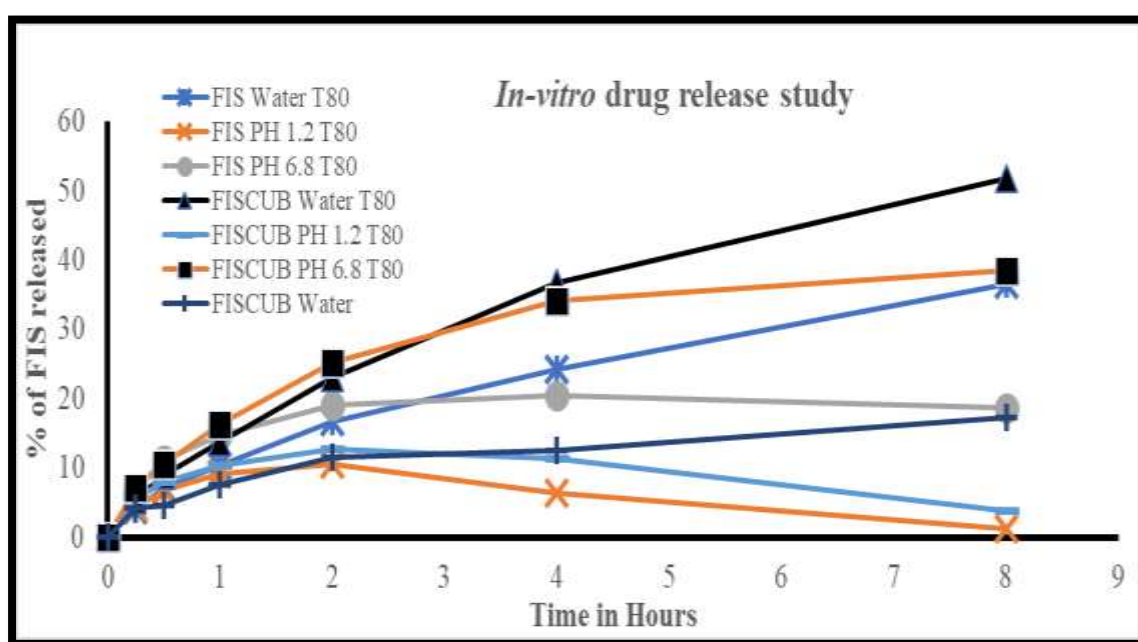


Figure 15. In-vitro drug release study of pure drug FIS and FISCUB in different dissolution medium

4.5 Stability study

The optimized FISCUB formulation particle characteristics were evaluated after storage in a stability chamber for three months and the obtained results were interpreted. The observations indicate there were no major alterations in particle size, PDI, entrapment efficiency and ZP measurement (Table 14). There was not much change in the viscosity and pH indicating stability after 3 months of storage (Table 16).

4.6 *In vitro* activity study

The results of the various *in vitro* assays in order to test the change in the activity of FIS due to the formation of its cubosomal formulation were noted using an authentic sample of A549 cells.

4.6.1 MTT Assay

The living animal cells metabolize yellow color MTT and change into purple color metabolite by mitochondrial enzymes and it's a measure of viability and metabolic activity. The purple color intensity depends on the number of A549 living cells present in the tissue culture plate and more the viable A549 cells more the formation of a purple color in the assay. The MTT assay used to study cytotoxicity of FIS, FISCUB and cisplatin treated A549 cells. IC₅₀ values of the above drugs were calculated from observed mean absorbance of the seeded drug concentrations and further used to carry *in vitro* study over A549 cells. FIS and its cubosomal nanoformulation were used to measure the mean absorbance value of the purple color intensity. The GraphPad Prism 5.01 software was used to calculate the IC₅₀ concentrations and found 16.59, 12.18, and 2.131 µg/ml to the FIS, nanoformulation, and cisplatin correspondingly. The IC₅₀ values of a certain chemical molecule show efficiency to suppress cell proliferation in cancer cells when used as a therapeutic

agent. The IC₅₀ concentration of the FISCUB corresponds to the cytotoxicity potential. of FISCUB nanoformulation and compared with values of native FIS.

Table 17. MTT assay absorbance readings

Concentration of FIS and FISCUB used (µg/ml)	Mean Absorbance at 570 nm		Concentration of Cisplatin (µg/ml)	Mean Absorbance at 570 nm
	FIS	FISCUB		
			0.00	0.46±0.01
0	0.45±0.02	0.46±0.01	0.01	0.48±0.04
12.5	0.34±0.01	0.28±0.01	0.10	0.48±0.01
25	0.23±0.01	0.14±0.01	0.25	0.46±0.01
50	0.14±0.01	0.15±0.01	0.50	0.46±0.03
100	0.17±0.01	0.18±0.02	1.00	0.35±0.02
150	0.18±0.01	0.14±0.01	5.00	0.28±0.01
200	0.22±0.01	0.23±0.01	10.00	0.15±0.01
IC₅₀	16.59	12.18	2.13	-

Values represents ± SD (n=3)

4.6.2 Clonogenic assay

For this experiment, we employed FIS IC_{50} values and triplicate samples of the optimized formulation. When comparing the colony number of blank cubosomes to untreated A549 cells, we did not see any considerable variation, suggesting that the formulation's constituents did not affect the A549 cell ability to proliferate. Colony numbers were 8.33 ± 0.58 for FISCUB and 11.67 ± 1.15 for FIS, suggesting a greater extent of drug absorption by the A549 cells from the prepared nanoformulation, responsible for the greater colony formation inhibition effect. The lowest colony count was reported with cisplatin (6.33 ± 0.58) as the standard drug. The developed colonies were counted after A549 cells treatment with native FIS, Optimized FIS formulation and blank cubosome formulation.

Table 18. Clonogenic assay

Treatment for A549 cells	Number of colonies observed			Average number of colonies $\pm$ SD
STD (Untreated cells in DMEM)	56.00	58.00	57.00	57.00 $\pm$ 1.00
Blank Cubosome	52.00	53.00	53.00	52.67 $\pm$ 0.58
Fisetin	13.00	11.00	11.00	11.67 $\pm$ 1.15
Optimized Fisetin formulation	8.00	9.00	8.00	8.33 $\pm$ 0.58
Cisplatin	7.00	6.00	6.00	6.33 $\pm$ 0.58

Values represents $\pm$ SD (n=3)

Image of 6 well plates showing colonies for different test samples in clonogenic assay and clonogenic assay graph plotted for mean colony number against different samples is shown in following Figure 16.

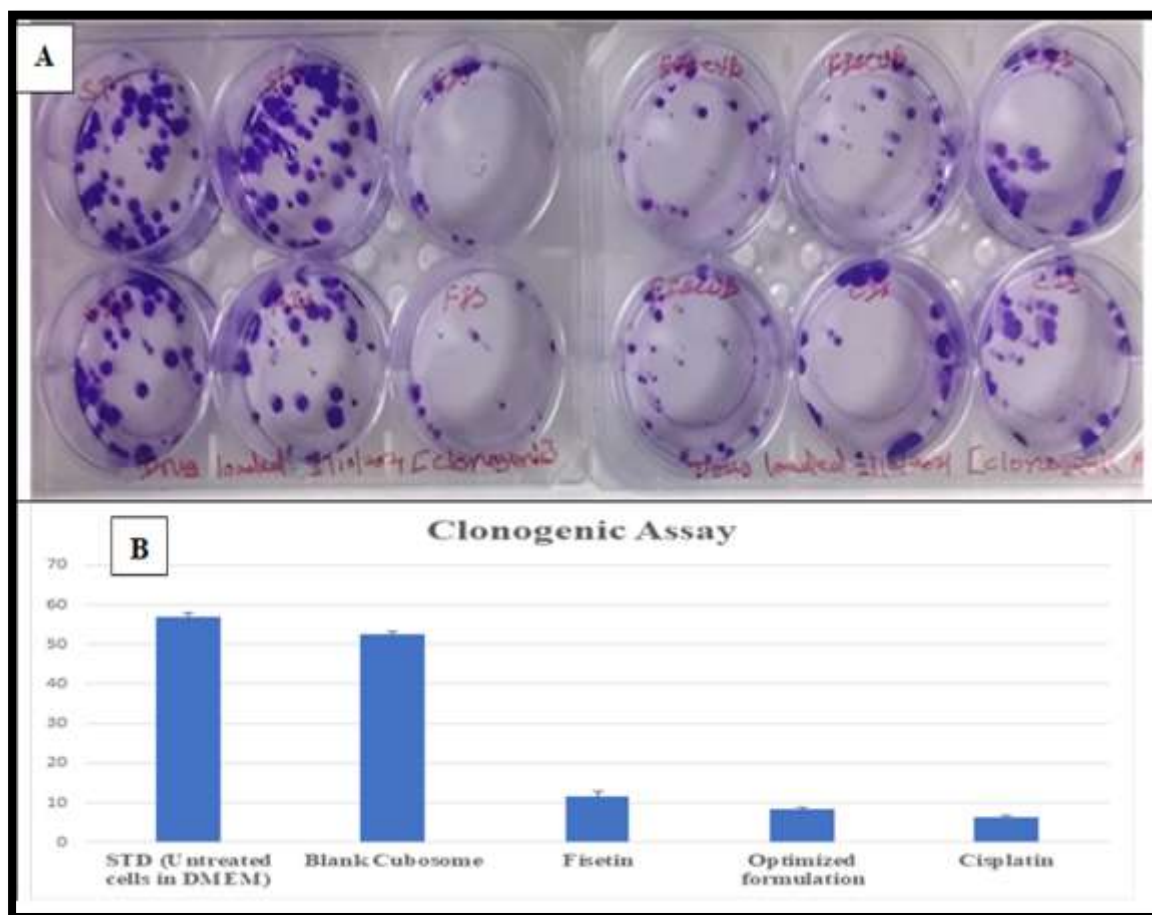


Figure 16. Clonogenic assay plate image and colony number graph

The optimized Cubosomal nanoformulation (FISCUB) was reported a smaller number of colonies than the native FIS drug.

4.6.3 Flow cytometry study

Flow cytometric analysis is an important and credible cytological evaluation method for examining the histological characteristics of animal cells. It is based on detecting the fluorescence and scattering of light from the RNA and specific tagged surface markers. In this study, we conducted a flow cytometry assay for the cell cycle study of A549 cells with and without FIS treatment to understand the impact of FIS, FISCUB and cisplatin as a reference drug for comparison. Untreated cells were found to be healthy and unaffected, as the percentage of live cells was 97.1 and cisplatin was most cytotoxic and apoptotic, as only 35.5% live cells were present.

Table 19. Flow cytometry study results

Sample Type	Live Cells (%)	A549 Cell Apoptosis (%)		Dead (%)
		Early	Late	
Control	97.1±0.72	1.96±0.58	0.74±0.66	0.2±0.40
Native Sample FIS	86.9±0.75	3.19±0.86	3.64±0.80	6.27±0.58
Formulation	58.6±0.93	13.6±0.71	19.8±0.94	7.91±0.78
Cisplatin	35.5±0.65	5.81±0.94	32.1±0.83	26.6±0.81

The results of the flow cytometric fluorescence scatter plot analysis are shown in following Figure 17 for A Untreated cells A549, B FIS, C FISCUB, D Cisplatin, and E Graph showing percentage of apoptotic cells in all four samples.

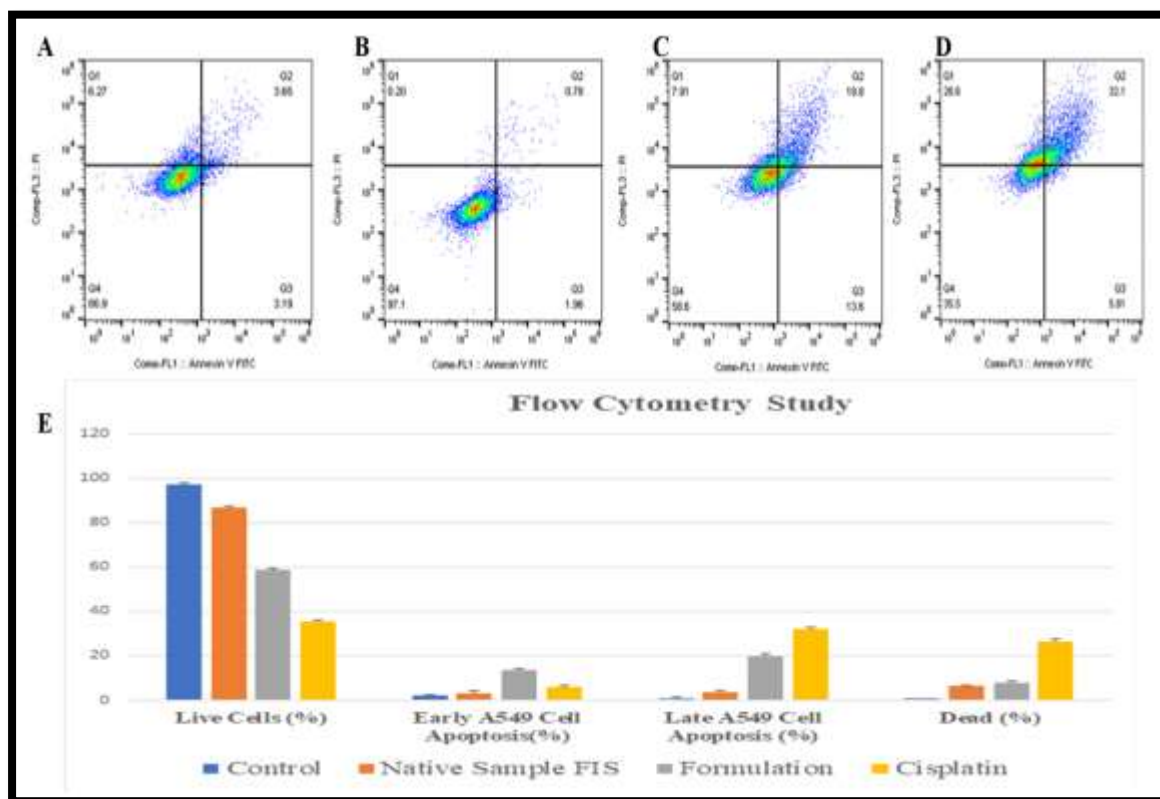


Figure 17. Flow cytometry results for assessment of apoptosis after A549 cells staining using Annexin V-FITC and PI

It was observed that, the FISCUB reported increase in both early as well as late apoptosis than the native FIS drug.

4.6.4 Acridine orange-ethidium bromide (EB/ AO) study

The fluorescent stain AO colors living cells and is observed with a green nucleus, whereas ethidium bromide colors the nucleus of dead cells and observed with orange fluorescence. Normal untreated A549 cells and FIS, FISCUB and cisplatin-treated A549 cells were observed after EB-AO staining under a fluorescent microscope for changes in the cell membrane, cell morphology, and nuclear changes under 10x and 45x magnification.

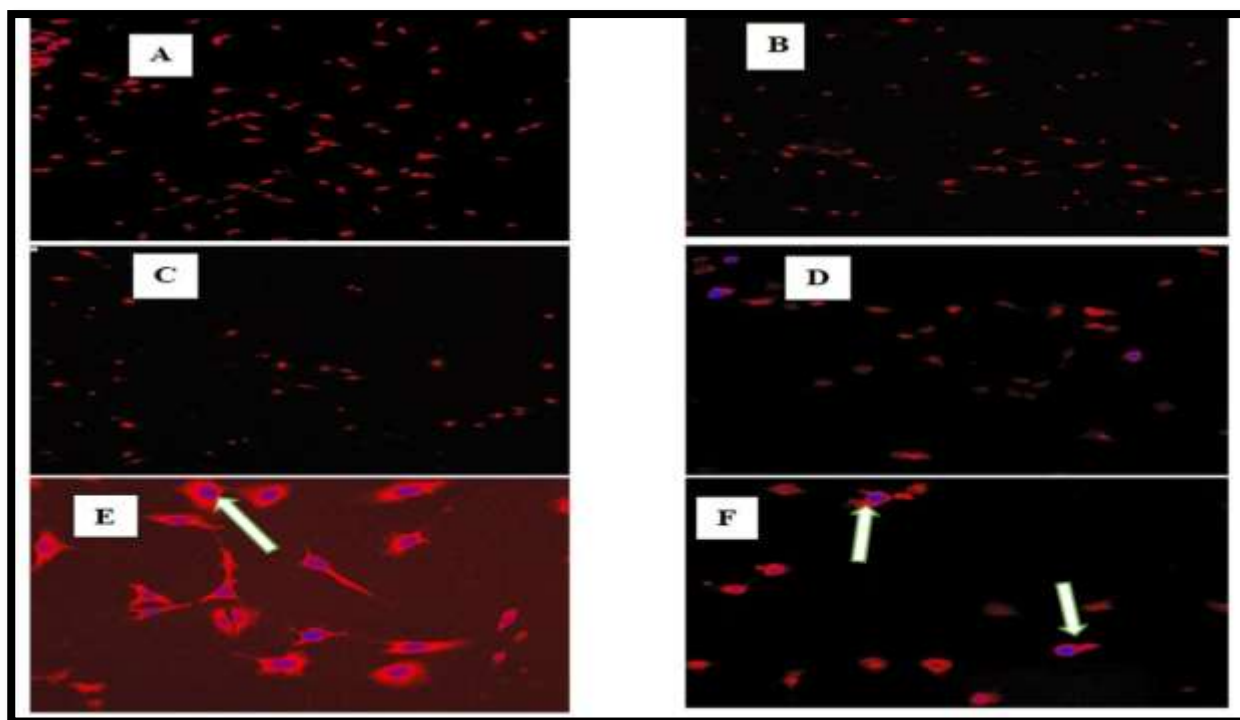


Figure 18. Staining with acridine orange and ethidium bromide

The observations after drug treatment and AO/EB staining as shown in Figure 18. A. Normal cells (10X), B. FIS treated (10X), C. FISCUB treated (10X), and D. Cisplatin treated (10X). The E and F show normal and FISCUB treated cells observation under 40X magnification, found healthy cells with no apoptotic bodies and abundant apoptotic bodies respectively.

4.6.5 DAPI staining study

DAPI is a popular blue fluorescent DNA stain for distinctly seeing the nucleus in both living and dead cells. We observed blue nuclei of the A549 cells for untreated and treated samples with FIS, FISCUB, and cisplatin.

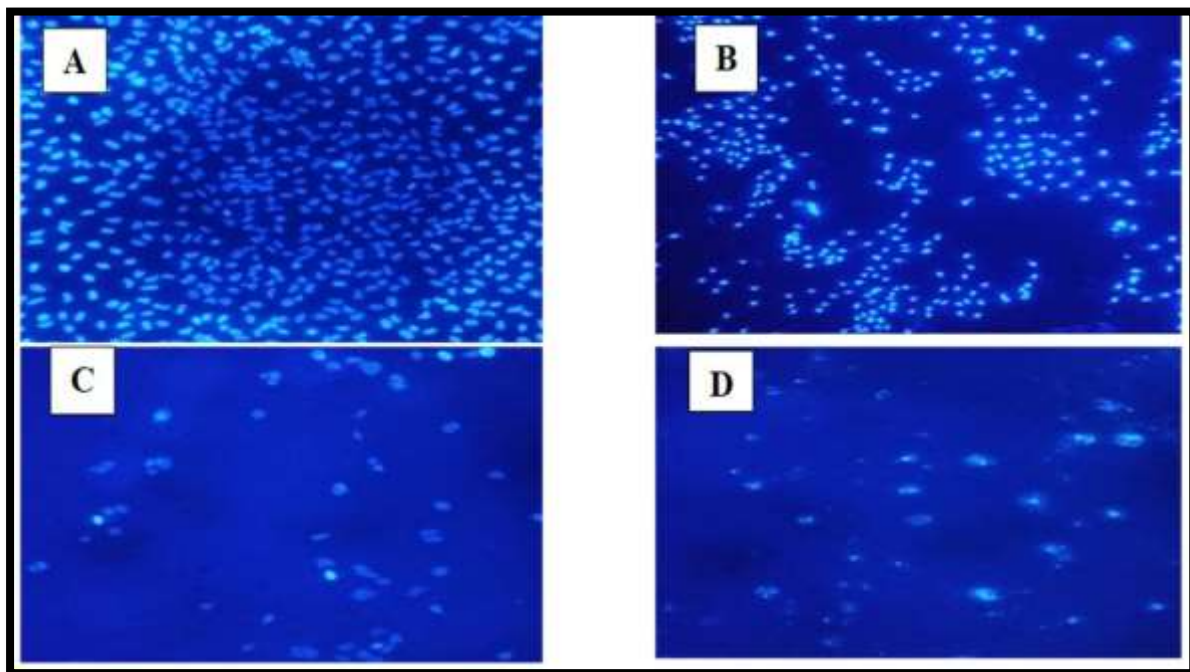


Figure 19. A549 cells observed after DAPI staining

The A549 cells observed under 10X after DAPI staining and shown in figure as A. Normal cells and cells treated with B. FIS, C. FISCUB and D. Cisplatin.

4.6.6 Cell uptake study

The A549 cells were treated with native FIS and FISCUB formulation, the drug entered in cells were extracted in acetonitrile and quantified using previously reported RP-HPLC method (Table 20). The concentration of FIS from both the samples were measured in µg/ml and compared with initial concentration used (10 µg/ml) to find the comparative percentage of FIS uptake in both the samples.

Table 20. Fisetin uptake in Human lung cancer A549 cells

Sr. No.	Native Fisetin		FISCUB Formulation	
	Area	Conc. (µg/ml)	Area	Conc. (µg/ml)
1	125050	1.521	166416	2.005
2	121842	1.483	166409	2.005
3	122606	1.492	171852	2.069
4	121557	1.480	171716	2.067
5	122682	1.493	172400	2.075
6	121080	1.474	171750	2.067
Mean	122469.5	1.474	170090.5	2.067
X	14.745		20.674	
Y	100		140.217	

X – % FIS uptake of A549 cells from FISCUB treated sample

Y - % FIS uptake to native FIS

4.7 In Vivo study

Benzo(a)pyrene is a polycyclic aromatic hydrocarbon (PAH) found in cigarette smoke, forest fires, fossil fuels, and volcanic eruptions that is a key molecule in cancer development by producing an alteration in lung pathologies. The cytochrome P4501A1 and dihydrodiol dehydrogenases enzyme metabolize B(a)P to form carcinogen mutagenic adduct and more quantity of free radicle reactive oxygen species (ROS) which produce chronic inflammation, alveolar epithelial damage and results in lung cancer development. Primarily oxidative stress developed by B(a)P is responsible for lung cancer development.

B(a)P exposure is linked to lung cancer development through various mechanisms, inducing oxidative stress, chronic inflammation in lung tissues, tissue damage and can lead to alterations in protein metabolism. The body and lung weight changes due to B(a)P exposure provide insights into lung toxicity, inflammation, and cancer development in mice models. Usually reports state that there is a decrease in body weight due to metabolic changes, loss of appetite, and an increase in lung weight due to inflammation in lung cancer studies induction using B(a)P as a carcinogen. Many reports are available mentioning changes in total serum protein levels, and enzymatic antioxidants (SOD and CAT) after the exposure of B(a)P in mice and rats, and this relationship is multifactorial. The use of phytochemicals like FIS has been reported as effective during lung cancer studies.

4.7.1 Physiological parameters

Ravichandran et al. reported the loss of body weight, SOD, and CAT concentration where as an increase in lung weight and total serum protein concentration in mice when induced lung cancer using B(a)P as a carcinogen, and this effect was reduced after use of FIS. In the conducted study, the results of the body and lung weight after the treatment of native FIS and its cubosomal nanoformulation are given in the following table (Table 21).

Table 21. Effect of FIS cubosomal nanoformulation on the body and lung weight

Sr. No.	Animal group (n=6)	Weight in Preventive Study		Weight in Therapeutic Study	
		Body (g)	Lung (mg)	Body (g)	Lung (mg)
I	Normal control	38.42±2.56	290±22.76	38.10±2.14	292±12.88
II	Negative (Treated with B(a)P inducing agent)	30.36±3.83	327±35.31	30.89±2.34	325±31.55
III	Treatment (FIS)	32.40±2.78	305±31.45	32.98±3.36	302±25.47
IV	Treatment (FISCUB)	33.71±2.23	314±28.59	32.57±2.42	311±23.27
V	Treatment (Cyclophosphamide)	36.21±2.69	298±30.73	37.02±3.11	297±34.71

Values are expressed as mean ±S.D., Statistical significance $p < 0.05$.

For both preventive and therapeutic studies, the negative control (Group II) was compared with the normal control (Group I), FIS treatment group III was compared with the negative control group II, FIS cubosomal formulation treatment group IV was compared with both negative control (Group II) and FIS treatment group III, and the cyclophosphamide treated group V was compared with negative control group II. The FIS cubosome nanoformulation treated group IV of both the preventive and the therapeutic studies were compared with each other.

The above table shows the comparative changes in body weight and lung weight between the animal groups and found that body weight was reduced whereas lung weight was increased for all treated groups in comparison to normal in both preventive and therapeutic (pre- and post-treated animals) study groups.

The study results for the concentrations of serum total proteins, SOD, and CAT for all the animal groups were given in the following table (Table 22) and compared to find out the effect of cubosomal nanoformulation preparation on lung toxicity and anticancer effect in the mice using B(a)P lung cancer-inducing agent.

Table 22. Effect of FIS cubosomal nanoformulation on level of total proteins and enzymatic antioxidants (SOD and CAT) induced using B(a)P in Swiss albino mice lungs

Sr. No.	Animal group (n=6)	Preventive Study			Therapeutic Study		
		Serum proteins	SOD	CAT	Serum proteins	SOD	CAT
I	Normal control	5.39±0.49	6.51±0.55	249±15.69	5.48±0.30	6.18±0.72	258±13.92
II	Negative (Treated with B(a) P inducing agent)	8.31±0.52	3.42±0.29	134±19.40	8.11±0.61	3.37±0.22	155±11.60
III	Treatment (FIS)	6.93±0.27	4.86±0.71	207±31.37	6.20±0.22	4.37±0.57	211±74.82
IV	Treatment(FISCUB)	5.59±0.41	5.09±0.34	221±11.92	5.17±0.39	4.93±0.41	226±18.77
V	Treatment (Cyclophosphamide)	5.86±0.09	5.87±0.64	234±16.55	5.35±0.42	5.62±0.81	247±27.41

Values in the table are represented as $\pm$ S.D. for every group of 6 animals. Serum total proteins – g/dl., SOD- units/min/mg protein, CAT - μ moles of H_2O_2 consumed/min/mg protein., Statistical significance $p < 0.05$

The above table shows the concentrations of serum total proteins and cellular enzymatic antioxidants such SOD, and CAT measured in serum samples of all animal groups. The significant increase in serum protein concentration and reduction in concentration of enzymatic antioxidants (SOD and CAT) were observed in comparison with the normal control group in both preventive and therapeutic (pre- and post-treated animals) study groups.

4.7.2 Histopathological study

The histopathological samples for all the animals were prepared and observed under 100x for changes in the alveolar epithelial cell morphology, architecture of cells, and presence of macrophages and lymphocytes. We observed different extents of chronic inflammation development in lung alveolar epithelial cells of all the lung tissue samples but no inflammation was observed in the normal group (Figure 20). The cancer development was not seen in the treated as well as untreated lung histopathological slides but chronic inflammation was seen which was associated with administration of B(a)P lungs carcinogen in treated animals.

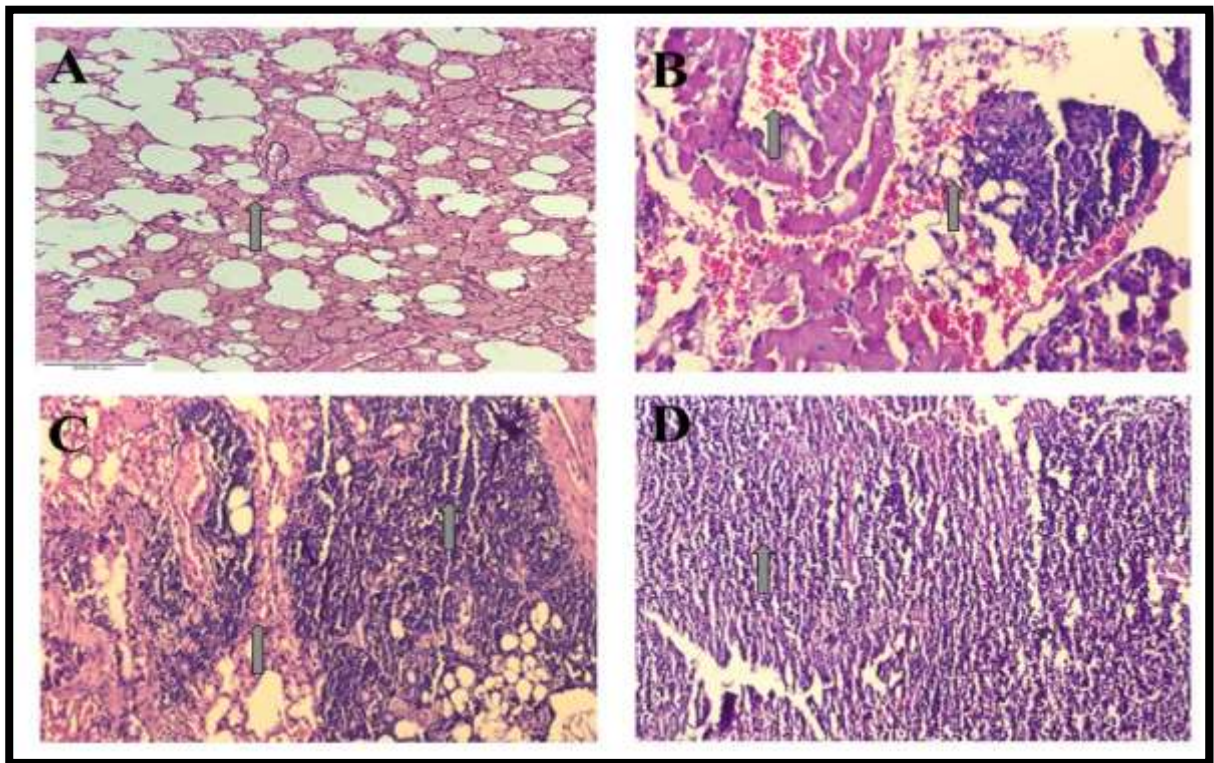


Figure 20. Histopathological examination for lung tissue observations

The mice lung alveolar epithelial cells were observed using the light microscope under 100 x magnification after staining with hematoxylin-eosin (H&E) staining. The extent of inflammatory responses in histopathological examination of all the treated and untreated animals from this study was graded in four levels normal,

scant, moderate, and dense for histopathological evaluation of the result and representative images are given in above figure.

The normal lung cells of the untreated mouse lung were observed for the histological examination (normal control Group I) mice were observed normal architecture of cells with an abundance of alveolar structure. All cellular structures were normal and any type of inflammation or cancer development was not observed (Figure 20A). The scant level of inflammation was characterized by very little damage to alveoli and cell architecture. A moderate level of chronic inflammation was characterized by a small extent of alveolar, cell architecture damage and accumulation of macrophages and lymphocytes (Figure 20B). The moderate level of chronic inflammation was characterized by a considerable extent of alveolar, cell architecture damage and more accumulation of macrophages and lymphocytes (Figure 20C). The dense level of chronic inflammation was characterized by a high extent of alveolar and cell architecture damage along with the high accumulation of macrophages and lymphocytes (Figure 20D). The observed levels of inflammation were noted for various study groups and reported in the following table (Table 23).

Table 23. Histopathological inflammation grading levels

Sr. No.	Animal group (n=6)	Level of developed chronic inflammation	
		Preventive Study	Therapeutic Study
I	Normal control	Normal control cells without inflammation	Normal control cells without inflammation
II	Negative (Treated with B(a)P inducing agent)	Dense	Dense
III	Treatment (FIS)	Moderate	Moderate
IV	Treatment (FISCUB)	Scant to moderate	Scant to moderate
V	Treatment (Cyclophosphamide)	Scant	Scant

The results of the histopathology study of FIS cubosome nanoformulation show a reduced level of chronic inflammation developed due to the administration of lung carcinogen B(a)P in lung epithelial cells than native FIS. These results indicate that cubosomal nanoformulation of FIS decreases the level of mice lung cell inflammation, which was associated with administering lung cancer-inducing agent B(a)P to Swiss albino mice animals.

5. DISCUSSION

5.1 RP-HPLC method

The linearity range was observed between 0.1 to 16 µg/ml for FIS ($R^2 > 0.999$). The accuracy for the FIS estimation of the developed method was very high (recovery 98.24 to 100.65 %), precise, robust, and high level of sensitivity as very small concentrations can be detected and identified. It was proved to be widely applicable for FIS estimation from its formulation, marketed formulation (Doctor's Best[®] Fisetin), and plant source. From the FIS forced degradation study results, it was unstable under oxidative and alkaline stress scenarios and stable for acidic, light and heat conditions. We developed and validated a new, robust, cost-effective, and highly sensitive RP-HPLC technique for FIS measurement that is broadly applicable.

5.2 UV- Spectroscopic method

A simple, fast UV-visible spectroscopic technique with solvent methanol was developed and validated for FIS estimation. This novel method was economical, more sensitive and can be used for FIS identification and quantitation. The linearity graph was prepared for the 2 to 10 µg/ml concentrations and observed maximum absorbance wavelength at 362 nm which was characteristic for FIS. The FIS recovered was more than 95%, percent RSD was less than 2 for the validation parameters. It was used for FIS quantification from various sources.

A straightforward, economical, quick, precise, sensitive UV-spectroscopic technique applicable for routine quality checking of FIS samples and its pharmaceutical formulations.

5.3 Preparation and evaluation of FISCUB

The milky white cubosomal nanoformulation for FIS was prepared by using a simple top-down method with of homogenization and sonication techniques. During

formulation, GMO and 40 mg of FIS were melted together and mixed into Pluronic F-127 at 65°C. Then mixture was slowly added to 40 mL of warm water, cooled and homogenizing at 14,500 rpm, sonicated, then stored overnight for proper equilibration. The concentrations of the cubosomal nanoformulation ingredients for optimization study were selected from the initial 16 pilot batches. The pilot study results of the cubosomal particle size and FIS entrapment exhibits relatively a smaller amount particle size and increased entrapment efficiency at 3 to 5% GMO and 1% Pluronic F-127 concentrations. Therefore, FIS cubosomal nanoformulation was optimized using 2–6% lipid and 0.5–1.5% polymer concentrations. From the nine optimization study batches based on the less particle size (93.07 nm) and maximum FIS entrapment, batch B2 was chosen the optimized formulation and tested in the succeeding study.

5.3.1 Particle size, PDI, and ZP

The results of the optimization study were analyzed for particle size and FIS entrapment using Design Expert[®] software for regression analysis. The statistical models and equation for stating the relation between GMO and Pluronic F-127 concentration with particle size and entrapped FIS were developed. The statistical analysis reveals 2FI is best fit model for GMO and Pluronic F-127 with particle size. The observations of 2D contour, 3D response surface plots and statistical equation of the particle size indicates that the increase in GMO results in to rise in FIS cubosomal particle size (Table 14, Figure 10) and 1% concentration of Pluronic F-127 gives the least particle size. The particle size distribution (PDI) value was not affected by used lipid GMO concentration and increasing with the increase in Pluronic F-127 concentration. The prepared optimization batches were reported sufficient zeta potential values (More than -20) for stability of cubosome particles.

5.3.2 Entrapment efficiency (EE)

The observation results show maximum entrapment in batch B2 formulation and found decreasing with increase in GMO concentration. From the regression analysis for results of the nine batches gives an equation for entrapment efficiency and 2D contour and 3D surface graphs (Figure 10) stating relation between used GMO and Pluronic F-127 concentration with FIS entrapment in cubosomal particles. The best fit statistical model for entrapment efficiency was Quadratic model (Adequate precision = 3.99) based on relationship between GMO and Pluronic F-127 with FIS entrapment efficiency in prepared cubosomal formulation.

5.3.3 Optimization

After the result characterization of all the nine batches suggested by 3^2 full factorial optimization study design, formulation B2 prepared by using 2% lipid (GMO) and 1% stabilizer (Pluronic F-127) was selected as the optimized formulation as reported least value of particle size 93.07 nm and highest drug entrapment of 80.10%.

5.3.4 FTIR

The FTIR spectra scanned between 4000 to 450 cm^{-1} for FIS, GMO, Pluronic F-127, and FISCUB are shown in the figure. In the spectrum of FIS, wavenumbers were observed for -C-OH stretching vibrations 1129, 1112, C=O band 1619 to 1507 and 1592.57, 1463 cm^{-1} because of aromatic-ring vibration. The significant FIS peak of O-H stretching observed at 3519.82 cm^{-1} was shifted to 3733.75 cm^{-1} and intensity is very much reduced in the FISCUB spectrum, indicating complete loading of FIS in the prepared cubosomes. Characteristic peaks observed in Pluronic F-127 and GMO at 3501.11, 2925.03, and 2854.13 cm^{-1} are also observed in FISCUB spectra with the same intensity and no or very little shift at 3440.35, 2924.46 and

2854.93 cm^{-1} which indicates that there is no chemical interaction between FIS and these formulation ingredients (Figure 11).

5.3.5 DSC

DSC thermograms were analyzed to understand the interaction between native FIS and cubosome formulation excipients. In the thermograms of FIS, Pluronic F-127, and GMO, sharp endothermic peaks are present at 314.9, 59.9, and 96.2°C respectively, and absent of these sharp endothermic peaks in the FISCUB thermogram. A prominent characteristic endothermic peak is observed for FISCUB between 62 to 121°C due to the liquid crystallinity of prepared cubosomes loaded with FIS (Figure 12). This confirms that FIS is completely entrapped in cubosomes formed from GMO and Pluronic F-127.

5.3.6 XRD

In the obtained x-ray diffractograms of 2θ versus intensity plots, high-intensity sharp crystal peaks at a diffraction angle (2θ) of pure native FIS are observed at 8.05, 12.57, 15.65, 17.55, 24.18, 26.40 and 28.37, suggesting its crystal characteristics (Figure 13). However, these prominent peaks are absent in FISCUB and blank cubosome formulation, suggesting that the FIS is entrapped in the prepared FISCUB. Sharp peaks of both FISCUB and blank cubosome overlap with each other, suggesting no change in FISCUB structure after FIS entrapment. The obtained peaks are characteristics of the liquid crystalline structure of prepared cubosomes.

5.3.7 HR-TEM

In aqueous environment, self-assembling of certain amphiphilic lipids forms stable nanostructured liquid cubic crystalline particles capable of carrying lipophilic drug molecules inside their crystalline structures. The confirmation of cubosome formation using amphiphilic lipides like GMO usually confirmed using TEM

analysis. We confirmed the formation of cubosomes from the HRTEM image observations. We observed HRTEM images at 100-, 50- and 20-nm scales, and the structural observations confirmed that the cubic structures of particles were scattered, well separated from each other, and present in the nanorange with respect to their size (Figure 14). HRTEM cubosome image observations confirm the cubic structures of nanosized, well-separated particles.

5.3.8 pH and viscosity

The pH and viscosity of the optimized FISCUB formulation were found to be 6.38 and 5.76 cp respectively, which was in normal range of acceptability of the formulation for its administration. The both parameters were noted for blank formulation also and found very close readings as that of optimized FISCUB formulation indicating least effect of FIS entrapment on these parameters.

5.4 *In vitro* release study

Initially, it was carried out without a permeation enhancer but the FIS release rate was very low; therefore, 1.5% Tween 80 was used as a permeation enhancer. In the results, we found that a very low FIS release rate was observed for both FIS and FISCUB in dissolution medium HCl buffer pH 1.2. When phosphate buffer pH 6.8 was used then release rate was greatly increased and found maximum for water. For all the above cases, it was found that FISCUB has a greatly increased rate of drug release than native FIS (Figure 15). This study clearly indicates that the aqueous solubility was increased more than 50% in the FISCUB than native FIS.

5.5 Stability Study

In the three-month stability study, optimized FIS cubosomal nanoformulation were found to be stable and no significant change in the particle size, PDI, entrapment

efficiency, and zeta potential values were observed. The stability study confirms the stability of formulation as well as FIS along with formulation ingredients.

5.6 *In vitro* study using A549 cells

The *in vitro* study findings using A549 cells indicates there was increase in anticancer activity of the FIS when used in cubosomal nanoformulation rather than use in its native unprocessed form. The outcomes of the different *in vitro* assays using optimized FISCUB formulation and pure FIS were analyzed for anticancer study.

5.6.1 MTT assay

The prepared cubosomal nanoformulation, MTT assay reports less IC₅₀ value of FIS indicating a more cytotoxic effect in A549 cells than the free drug (Table 17). This suggests that more absorption of FIS was present from the nanoformulation than from pure FIS and confirms the applicability of prepared nanoformulation in anticancer studies.

5.6.2 Clonogenic assay

There was no remarkable change in the colony number of blank cubosome as compared to untreated A549 cells which indicates formulation ingredients are not affecting on proliferative characteristic of cells. FISCUB and FIS shows 8.33 ± 0.58 and 11.67 ± 1.15 colony number, suggesting more extent of drug absorption by the A549 cells from prepared nanoformulation and responsible for more colony formation inhibition effect (Table 18). The lowest colony count was reported with cisplatin (6.33 ± 0.58) as the standard drug.

5.6.3 Flow cytometry assay

The outcomes of the flow cytometry study report that the FISCUB observed an increased percentage of early and late apoptosis compared with the native FIS sample. Graph plotted using the percentage of cells against live, apoptotic, and dead cells

shows that a greater extent of anticancer effect over A549 cells was observed by FISCUB than the native FIS sample, which suggests that the nanoformulation increases the FIS activity (Figure 17). This suggests that the more FIS absorption and accumulation in the A549 cells when applied in cubosomal nanoformulation compared with native unprocessed FIS. As per the reports of the flow cytometry study, the anticancer activity of FIS was increased for its prepared cubosomal nanoformulation.

5.6.4 EB/AO staining study

In the observations of EB/AO staining, normal untreated cells were found to be healthy, more in number than all other samples, with an intact nucleus and cell membrane attached to the coverslip. FIS and FISCUB-treated cells were not properly attached to a glass coverslip, and cell shrinkage, and nucleus divisions were observed, which indicates the presence of apoptosis in the A549 cells. A reduced number of cells in FISCUB compared with native FIS was observed, which indicates more effect of formulation than native FIS. The cisplatin-treated sample contains a minimum number of A549 cells, and a greater number of the cells were rounded, detached, fragmented nuclei of apoptotic bodies (Figure 18).

5.6.5 DAPI staining study

In DAPI-stained cells, untreated A549 cells were more in number, intact, rounded, normal nuclei and no apoptotic bodies were present. Whereas treated A549 cells were detected as round, constricted with membrane blebbing, many as apoptotic bodies, signified in the figure using arrows. The anticancer drug cisplatin-treated sample observed a greater quantity apoptotic bodies and least quantity of total cells as compared with other samples. The FISCUB-treated sample contains a larger quantity

of apoptotic cells than native FIS, indicating that the prepared FIS formulation shows a higher anticancer effect than native FIS (Figure 19).

5.6.6 Cell uptake study

The change in amount of FIS absorption by human lung cancer A549 cells after treatment with a specific concentration 10 µg/ml for 5 hours of native FIS and optimized FISCUB formulation were studied to understand the effect of cubosomal formulation preparation on amount and extent of absorption within cancer cells. Results of cell uptake study show that there was 14.74 and 20.67 percent FIS absorption from native FIS and optimized FISCUB formulation respectively and confirms the applicability of prepared formulation to overcome the FIS solubility and cellular absorption with the help of prepared formulation. When the increase in absorption compared with the native FIS absorption, it's found that there is 40 percent more absorption from optimized cubosomal formulation (Table 20). This is because of nano size FIS cubosome particles and other characteristics of cubosomes resulted for the increase in cellular absorption and overcome the aqueous solubility and bioavailability problem of FIS.

5.7 In Vivo study

The increase in the protective and therapeutic effect of the FIS due to its cubosomal nanoformulation on toxic effect of lung carcinogen B(a)P on mouse lungs were studied using changes in body and lung weight, total serum proteins, anti-oxidant enzymes and lung tissue histopathology.

5.7.1 Physiological parameters

The observations of the effect of B(a)P, FIS, FISCUB and cyclophosphamide administration on body and lung weight in mice were noted in Table 21 and used to compare control and experimental groups. The administration of B(a)P resulted in a

reduction in body weight of negative control group II animals when compared with control group I, whereas lung weight observed a significant increase in both preventive and therapeutic types of study ($p < 0.05$). This effect of decreased body weight and increased lung weight was due to the toxic and carcinogenic properties of B(a)P in mouse and documented in the literature. The FIS-treated group III reported an increased body weight and a decreased lung weight in comparison with negative control group II indicating a reduction in lung toxicity due to administration of B(a)P in mice ($p < 0.05$). The FISCUB formulation-treated group IV was compared with both negative control group II and FIS-treated group III to assess the effect of FIS cubosomal formulation on B(a)P induced lung toxicity. The results of group IV reported an increased body weight and a decreased lung weight when compared with group II and further this effect over body and lung weight was found to be increased when compared with group III indicating a further reduction in toxic effect of B(a)P ($p < 0.05$). The effect of cyclophosphamide administered group V on body and lung weight was compared with group I and results showed no significant changes indicating its protective role against the toxic and carcinogenic effect of B(a)P in mice ($p < 0.05$).

The free radicals have an important impact on the complicated process of inflammation and multi-step carcinogenesis. The first line of defense against harmful oxygen radicals is antioxidant enzymes, which scavenge metabolites of oxygen reduction. To prevent the inflammation and carcinogenesis process induced due to various carcinogens, used antioxidants can either directly absorb free radicals or interfere with their formation. The components of the antioxidant defense system SODs and CAT, which convert superoxide radicals into hydrogen peroxide (H_2O_2) and finally into water. The effects of oxidant molecules on animal cells are also

restricted by the anti-oxidant enzymes SOD and CAT, which are activated against cell damage (Ramakrishnan et al., 2006, Lee et al., 2010, Gaetani et al., 1989). The lipid peroxidation is induced by the potent inflammatory and carcinogenic agent B(a)P by its interaction with membrane lipids.

The estimated concentrations for total serum proteins and antioxidant enzymes (SOD and CAT) for both preventive and therapeutic studies have been given in Table 22, depicting the effect of B(a)P, FIS, FISCUB formulation, and cyclophosphamide on mice. The normal control group I represents the serum concentrations of total serum proteins and antioxidant enzymes in healthy mice without any treatment and is used to assess experimental effects on other animal groups. There was a significant increase in total serum protein concentrations and a decrease in both antioxidant enzymes (SOD and CAT) of negative control group II when compared with normal control group I ($p < 0.05$), which indicated an inflammatory and toxic effect of B(a)P in mice groups. The FIS-treated group III shows a reduction in serum protein concentration and increased values for SOD and CAT in comparison with the negative control group II, indicating the protective effect of FIS against B(a)P induced toxicity in mice. The FISCUB formulation treated group IV shows a significant reduction in serum protein level and increased for SOD and CAT in comparison with the negative control group II. Further, this effect for serum protein and antioxidant enzymes was found to be increased when compared with group III indicating more reduction in the toxic effect of B(a)P than treatment of native FIS ($p < 0.05$). The results of the cyclophosphamide-treated group V compared with group I found no significant changes indicating its protective role against the toxic and carcinogenic effect of B(a)P in mice ($p < 0.05$).

From the above study, FIS cubosomal formulation increases mice lung weight, total serum proteins and decreases body weight, antioxidant enzymes (SOD and CAT) concentration than native FIS administration. The above study findings confirm that the prepared FIS cubosomal formulation (FISCUB) increases the protective effect of FIS for B(a)P induced lung toxicity in mice. The study results indicate that the protective effect of FIS and FISCUB against B(a) P-induced toxicity were significant in preventive treatment than the therapeutic treatment groups in mice.

5.7.2 Histopathological study

The microscopic sections of lung samples of all group animals were observed for histopathology study and reported. The prepared animal group samples show various levels of chronic lung inflammation in mice (Table 23) as a normal untreated control group with a normal level of alveolar epithelial tissue inflammation, lung carcinogen B(a)P (50 mg/kg) treated animal groups show dense level chronic inflammation and extensive lung alveolar architecture damage with alveolar epithelium necrosis, native unprocessed FIS treated (25 mg/kg body weight) lung samples show protection to the B(a)P-induced lungs damage and inflammation level was moderate, FIS cubosomal nanoformulation (equivalent to 25 mg/kg body weight formulation) treated group showed scant to moderate level inflammation lung morphology (observed reduced inflammation than native FIS treated group), cyclophosphamide (30 mg/kg body weight) treated lung cells observed very little inflammation and rated scant level of inflammation due to highest protective effect of it.

The histological study reports show that the animal group provided with the FISCUB formulation reported a stronger protective effect in lung samples for B(a)P induced lung toxicity than the usage of native FIS. Thus, this histological report

supports the above biochemical results of total serum protein and antioxidant enzymes (SOD and CAT) concentration. The histological study results also indicate that the protective effect of FIS and FISCUB on the B(a)P induced toxicity was significant in preventive treatment than the therapeutic treatment groups in mice.

6. SUMMARY

Lung cancer is the fetal and more abundantly diagnosed human cancer and treated with chemotherapy and radiation which has side effects, harmful and toxic to normal body cells. Various herbal phytoconstituents and their derivatives reported potential anticancer activity from alkaloids, flavonoids, terpenes, and carotenoids which show less toxicity and side effects in clinical practice.

Fisetin is a plant metabolite from the group of flavonoids that shows promising therapeutic potential including both *in vitro* and *in vivo* anticancer activity. The low aqueous solubility and bioavailability are the problems in drug development of bioactive flavonoids like fisetin which restricts its utilization of maximum therapeutic benefits against cancer and other uses. These issues can be resolved by designing nanoformulations and other phytoformulations therefore we designed a cubosomal nanoformulation of FIS and tested it for lung anticancer activity.

Before the cubosomal nanoformulation preparation, a novel and very sensitive RP-HPLC analytical method was developed and validated using ICH Q2 (R1) guidelines because it was required for FIS estimation from free form and its cubosomal formulation. An alternative fast and simple UV-visible spectroscopic method was also developed and used for FIS estimation.

Cubosomal nanoformulation is a stabilized liquid dispersion of crystalline cubic nanoparticles of an amphiphilic lipid glyceryl monooleate. A top-down cubosome formulation method was used to prepare a novel FIS cubosomal formulation and optimize it. The 3^2 -full factorial design using Design Expert[®] software was used for formulation optimization. The particle size, entrapment efficiency, viscosity, pH, and polydispersity index were tested.

The FIS cubosome formulation was optimized using 2 to 6 % GMO and 0.5 to 1.5 % Pluronic F-127 concentrations. The batch B2 prepared using 2% lipid (GMO) and 1% Pluronic F-127 was selected as an optimized batch because of less particle size (93.07 nm) and maximum FIS entrapment (80.10 %), found to be stable during its 3-month stability study.

The statistical models and equation for stating the relation between GMO and Pluronic F-127 concentration with particle size and entrapped FIS were developed. The statistical analysis reveals that 2FI (Adequate precision = 5.50) is the best fit model for particle size with GMO and Pluronic F-127. The observations of 2D contour, 3D response surface plots, and statistical equation of the particle size indicate that the increase in GMO results rise in FIS cubosomal particle size. The best fit statistical model for entrapment efficiency was the Quadratic model (Adequate precision = 3.99) based on the relationship between GMO and Pluronic F-127 with FIS entrapment efficiency in prepared cubosomal formulation.

The FTIR spectra scanned between 4000 to 450 cm⁻¹ for FIS, GMO, Pluronic F-127, and FISCUB indicate that there is no chemical interaction between FIS and formulation ingredients and complete loading of FIS in the prepared cubosomes. The observations of DSC thermograms noted the absence of the fisetin's sharp endothermic peaks in the FISCUB thermogram, confirming the absence of drug-excipients interactions and FIS was completely entrapped in cubosomes formed from GMO and Pluronic F-127.

High-resolution transmission electron microscopy observed nanoscale cubosome images at 100-, 50- and 20-nm, confirming the cubic structure of the particles, scattered, well separated from each other, and present in the nano range concerning their size. The x-ray diffraction study was performed for the cubosome

particles and prominent peaks due to FIS being absent in FISCUB and blank cubosome formulation, suggesting that the FIS is entrapped in the prepared FISCUB and peaks are characteristic to the cubic crystalline structure of particles. The *in vitro* release study confirms that the FIS release was about 50% more in the prepared cubosomal formulation compared to native FIS and the aqueous solubility was increased.

The *in vitro* study of the optimized cubosomal formulation was carried out to study the effect of cubosomal formulation on cellular absorption and cytotoxicity studies. In the *in vitro* study, MTT, clonogenic, flow cytometry assay, and acridine orange-ethidium bromide staining were performed. The MTT assay reports less IC₅₀ value for FISCUB indicating a more cytotoxic effect and solubility in A549 cells than the free drug. In the clonogenic assay, more extent of drug absorption by the A549 cells from prepared nanoformulation and responsible for more colony formation inhibition effect. As per the reports of the flow cytometry study, the graph plotted using the percentage of cells against live, apoptotic, and dead cells shows that a greater extent of anticancer effect over A549 cells was observed by FISCUB than the native FIS sample. The increased anticancer activity for prepared cubosomal nanoformulation than native FIS was reported in a flow cytometry study. In the observations of EB/AO and DAPI staining, a reduced number of A549 cells with more shrinkage, and nucleus divisions indicates the presence of apoptosis in FISCUB compared with native FIS, indicating an increased anticancer effect of formulation than native FIS. The optimized cubosomal formulation reported more cytotoxicity results than the native FIS. In the A549 FIS uptake study, the FISCUB reports a 40 percent increase in absorption compared with the native FIS because of nano-size FIS

cubosome particles which resulted in the increase in cellular absorption and overcame the aqueous solubility issue of FIS.

In the *in vivo* study, an increase in the protective and therapeutic effect of FIS due to its cubosomal nanoformulation on the toxic effect of lung carcinogen B(a)P on mouse lungs. The treated animal group reported an increase in body weight and a decrease in lung weight when compared with FIS treated group reduction in the toxic effect of B(a)P. The administered B(a)P in mice increase total serum protein concentrations and a decrease in both antioxidant enzymes (SOD and CAT). This effect was suppressed more in the treated group than FIS alone, indicating more effectiveness of the prepared formulation. The histological study reports show that the animal group administered with the FISCUB formulation reported a stronger protective effect by reducing B(a) P-induced chronic inflammation and toxicity in mice lungs than the usage of native FIS.

In the FIS cubosomal nanoformulation study, we have prepared, characterized, and optimized FIS cubosomal nanoformulation with a simple top-down procedure and a smaller number of ingredients for better delivery of FIS. Further, the FIS delivery effect of the prepared cubosomal nanoformulation was investigated over human lung cancer using *in vitro* and *in vivo* studies. In both studies, the anticancer and cytotoxic effect of FIS was found more in its cubosomal nanoformulation than native form. This increase in the therapeutic potential of FIS is because of the enhancement of solubility and cellular absorption due to nanosized cubic particles of the prepared formulation.

7. CONCLUSION

In the present study, cubosomal nanoformulation of fisetin was formulated, characterized, and investigated for an increase in the lung anticancer efficacy of FIS due to the preparation of its novel cubosomal nanoformulation. The formulated cubosomal nanoformulation of the bioactive flavonoid FIS was stable, needed a smaller number of formulation ingredients, and was optimized based on cubosomal size and FIS entrapment efficiency

The FIS cubosomes were formulated using top-down method, found optimized batch B2 using a 3²-full factorial design applied using Design Expert[®] software, consist of 2% lipid (GMO) and 1% stabilizer (Pluronic F-127) reported particle size (93.07 nm) and maximum drug entrapment (80.10 %). The *in vitro* FIS release from FISCUB confirms 50% more release than native FIS suggesting increase in aqueous solubility. For quantitative FIS estimation, a novel, sensitive RP-HPLC and a simple, fast UV-Spectroscopic analytical methods were developed and validated.

The FISCUB was more effective than FIS in anticancer activity using the A549 cancer cells when tested for MTT, clonogenic, flow cytometry assay, and acridine orange-ethidium bromide staining study. The lung cancer A549 cells flow cytometry study confirm the applicability of cubosomes for FIS delivery as FISCUB reported reduction in live cell content and enhanced apoptotic effect than native FIS. The FISCUB reported more than 40 % increase in A549 cellular absorption in drug uptake study compared with the native FIS due to nano-size FIS cubosome particles which resulted in the increase in cellular absorption and overcame the aqueous solubility issue of FIS.

In line with the *in vitro* study, FISCUB exhibited an increased protective effect against the toxicity of lung carcinogen B(a)P during *in vivo* study in Swiss

albino mice lungs. When FISCUB treated animal groups compared with FIS treated, reported an increase in body weight and antioxidant enzyme concentration, a decrease in lung weight and total serum proteins indicates reduction in the toxic effect of B(a)P. The histopathological observations found improved protective effect by reducing B(a)P induced chronic inflammation and toxicity in mice lungs for FISCUB treated groups than the native FIS. The *in vivo* study results for FISCUB reported increased anti-inflammatory and anticancer potential than native FIS in Swiss albino mice lung cells.

In conclusion, study reports the formulation and optimization of FIS cubosomal nanoformulation with a simple top-down method and a smaller number of ingredients for better delivery of FIS in lung cancer cells. The anticancer and cytotoxic effect of FIS was found more in FIS cubosomal nanoformulation than its native form. This increased therapeutic efficacy of FIS was due to the enhanced solubility and cellular absorption resulting from FIS entrapment in the lipid bilayer of nanosized cubic crystalline particles of the prepared formulation.

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8. BIBLIOGRAPHY

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* 2018;68(6):394–424.
2. Ferlay J, Colombet M, Soerjomataram I, Mathers C, Parkin DM, Piñeros M, et al. Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods. *Int J Cancer.* 2019;144(8):1941–53.
3. Latimer KM, Mott TF. Lung cancer: Diagnosis, treatment principles, and screening. *Am Fam Physician.* 2015;91(4):250–6.
4. Heneczkowski M, Kopacz M, Nowak D, Kuźniar A. Infrared spectrum analysis of some flavonoids. Vol. 58, *Acta Poloniae Pharmaceutica - Drug Research.* 2001. p. 415–20.
5. Kashyap D, Garg VK, Tuli HS, Yerer MB, Sak K, Sharma AK, et al. Fisetin and quercetin: Promising flavonoids with chemopreventive potential. *Biomolecules.* 2019;9(5):1–22.
6. Afroze N, Pramodh S, Shafarin J, Bajbouj K, Hamad M, Sundaram MK, et al. Fisetin Deters Cell Proliferation, Induces Apoptosis, Alleviates Oxidative Stress and Inflammation in Human Cancer Cells, HeLa. *Int J Mol Sci.* 2022;23(3) :1707.
7. Jeong HM, Kang HN, Lee YR, Kim EA, Lee EH, Shim JH. Improved low water solubility of fisetin by enzymatic encapsulation reaction using cycloamylose produced by cyclodextrin glucanotransferase. *Process Biochem.* 2023;130(January):138–46.

8. Talaat SM, Elnaggar YSR, Gowayed MA, El-Ganainy SO, Allam M, Abdallah OY. Novel PEGylated cholephytosomes for targeting fisetin to breast cancer: in vitro appraisal and in vivo antitumoral studies. *Drug Deliv Transl Res.* 2023;433–54.
9. Awadeen RH, Boughdady MF, Zaghloul RA, Elsaed WM, Abu Hashim II, Meshali MM. Formulation of lipid polymer hybrid nanoparticles of the phytochemical Fisetin and its in vivo assessment against severe acute pancreatitis. *Sci Rep.* 2023;13(1):1–20.
10. Khorsandi L, Mansouri E, Rashno M, Karami MA, Ashtari A. Myricetin Loaded Solid Lipid Nanoparticles Upregulate MLKL and RIPK3 in Human Lung Adenocarcinoma. *Int J Pept Res Ther.* 2020;26(2):899–910.
11. Mehdi SH, Zafaryab M, Nafees S, Khan A, Ahmad I, Hafeez Z Bin, et al. Chrysin Sensitizes Human Lung Cancer Cells to Tumour Necrosis Factor Related Apoptosis-Inducing Ligand (TRAIL) Mediated Apoptosis. *Asian Pacific J Cancer Biol.* 2019;4(2):27–33.
12. Zhou C, Huang Y, Nie S, Zhou S, Gao X, Chen G. Biological effects and mechanisms of fisetin in cancer: a promising anti-cancer agent. *Eur J Med Res.* 2023;28(1):297.
13. Kumar RM, Kumar H, Bhatt T, Jain R, Panchal K, Chaurasiya A, et al. Fisetin in Cancer: Attributes, Developmental Aspects, and Nanotherapeutics. *Pharmaceuticals.* 2023;16(2):196.
14. Qaed E, Al-Hamyari B, Al-Maamari A, Qaid A, Alademy H, Almoiliqy M, et al. Fisetin's Promising Antitumor Effects: Uncovering Mechanisms and

- Targeting for Future Therapies. *Glob Med Genet.* 2023;10(03):205–20.
15. Szymczak J, Cielecka-Piontek J. Fisetin—In Search of Better Bioavailability—From Macro to Nano Modifications: A Review. *Int J Mol Sci.* 2023;24(18):14158.
 16. Mehta P, Pawar A, Mahadik K, Bothiraja C. Emerging novel drug delivery strategies for bioactive flavonol fisetin in biomedicine. *Biomed Pharmacother.* 2018;106:1282–91.
 17. Kashyap D, Sharma A, Sak K, Tuli HS, Buttar HS, Bishayee A. Fisetin: A bioactive phytochemical with potential for cancer prevention and pharmacotherapy. *Life Sci.* 2018;194:75–87.
 18. Sundarraj K, Raghunath A, Perumal E. A review on the chemotherapeutic potential of fisetin: In vitro evidences. Vol. 97, *Biomedicine and Pharmacotherapy.* 2018. p. 928–40.
 19. Rengarajan T, Yaacob NS. The flavonoid fisetin as an anticancer agent targeting the growth signaling pathways. *Eur J Pharmacol.* 2016;789:8–16.
 20. Im WK, Park HJ, Lee KS, Lee JH, Kim YD, Kim K-H, et al. Fisetin-rich extracts of *Rhus verniciflua* Stokes improve blood flow rates in mice fed both normal and high-fat diets. *J Med Food.* 2015;00(0):1–7.
 21. Kadari A, Gudem S, Kulhari H, Bhandi MM, Borkar RM, Kolapalli VRM, et al. Enhanced oral bioavailability and anticancer efficacy of fisetin by encapsulating as inclusion complex with HP β CD in polymeric nanoparticles. *Drug Deliv.* 2017;24(1):224–32.

22. Mohtar N, Taylor KMG, Sheikh K, Somavarapu S. Design and development of dry powder sulfobutylether- β -cyclodextrin complex for pulmonary delivery of fisetin. Vol. 113, *European Journal of Pharmaceutics and Biopharmaceutics*. 2017. p. 1–10.
23. Wang Y, Wu X, Ren W, Liu Y, Dai X, Wang S, et al. Protective effects of fisetin in an A β 1-42-induced rat model of Alzheimer's disease. *Folia Neuropathol*. 2023;61(2):196–208.
24. Huard CA, Gao X, Dey Hazra ME, Dey Hazra RO, Lebsock K, Easley JT, et al. Effects of Fisetin Treatment on Cellular Senescence of Various Tissues and Organs of Old Sheep. *Antioxidants*. 2023;12(8).
25. Mitra S, Ghosh N, Paul P, Banerjee ER. Orally Administered Fisetin Reduces the Symptoms of Acute Allergic Asthma in a Preclinical Mouse Model. *Biomed Res Ther*. 2022;9(3):4953–70.
26. Chen Y, Wu Q, Song L, He T, Li Y, Li L, et al. Polymeric micelles encapsulating fisetin improve the therapeutic effect in colon cancer. *ACS Appl Mater Interfaces*. 2015;7(1):534–42.
27. Lorenzo G Di, Scafuri L, Costabile F, Pepe L, Scognamiglio A, Crocetto F, et al. Fisetin as an adjuvant treatment in prostate cancer patients receiving androgen-deprivation therapy. *Futur Sci OA*. 2022;8(3):11–3.
28. Cytryniak A, Nazaruk E, Bilewicz R, Górzyńska E, Żelechowska-Matysiak K, Walczak R, et al. Lipidic cubic-phase nanoparticles (Cubosomes) loaded with doxorubicin and labeled with ^{177}Lu as a potential tool for combined chemo and internal radiotherapy for cancers. *Nanomaterials*. 2020;10(11):1–14.

29. Sivadasan D, Sultan MH, Alqahtani SS, Javed S. Cubosomes in Drug Delivery—A Comprehensive Review on Its Structural Components, Preparation Techniques and Therapeutic Applications. *Biomedicines*. 2023;11(4).
30. Pushpa Ragini S, White J, Kirby N, Banerjee R, Reddy Bathula S, Drummond CJ, et al. Novel bioactive cationic cubosomes enhance the cytotoxic effect of paclitaxel against a paclitaxel resistant prostate cancer cell-line. *J Colloid Interface Sci*. 2023;649:966–76.
31. Lakshmi N, Yalavarthi P, Vadlamudi H, Thanniru J, Yaga G, K H. Cubosomes as Targeted Drug Delivery Systems - A Biopharmaceutical Approach. *Curr Drug Discov Technol*. 2014;11(3):181–8.
32. Pan X, Han K, Peng X, Yang Z, Qin L, Zhu C, et al. Nanostructured Cubosomes as Advanced Drug Delivery System. *Curr Pharm Des*. 2013;19(35):6290–7.
33. Karami Z, Hamidi M. Cubosomes: Remarkable drug delivery potential. *Drug Discov Today*. 2016;21(5):789–801.
34. Kurangi B, Jalalpure S, Jagwani S. Formulation and evaluation of resveratrol loaded cubosomal nanoformulation for topical delivery. *Curr Drug Deliv*. 2021;18:1–13.
35. Bei D, Marszalek J, Youan BBC. Formulation of dacarbazine-loaded cubosomes-part I: Influence of formulation variables. *AAPS PharmSciTech*. 2009;10(3):1032–9.
36. Bei D, Marszalek J, Youan BBC. Formulation of dacarbazine-loaded cubosomes-part II: Influence of process parameters. *AAPS PharmSciTech*. 2009;10(3):1040–7.

37. Bei D, Zhang T, Murowchick JB, Youan BBC. Formulation of dacarbazine-loaded cubosomes. Part III. physicochemical characterization. *AAPS PharmSciTech*. 2010;11(3):1243–9.
38. Chishti N, Jagwani S, Dhamecha D, Jalalpure S, Dehghan MH. Preparation, optimization, and in vivo evaluation of nanoparticle-based formulation for pulmonary delivery of anticancer drug. *Med*. 2019;55(6).
39. Ahirrao M, Shrotriya S. In vitro and in vivo evaluation of cubosomal in situ nasal gel containing resveratrol for brain targeting. *Drug Dev Ind Pharm*. 2017;43(10):1686–93.
40. Chettupalli AK, Ananthula M, Amarachinta PR, Bakshi V, Yata VK. Design, formulation, in-vitro and ex-vivo evaluation of atazanavir loaded cubosomal gel. *Biointerface Res Appl Chem*. 2021;11(4):12037–54.
41. Nasr M, Younes H, Abdel-Rashid RS. Formulation and evaluation of cubosomes containing colchicine for transdermal delivery. *Drug Deliv Transl Res*. 2020;10(5):1302–13.
42. Hundekar YR, Saboji JK, Patil SM, Nanjwade BK. Preparation and evaluation of Diclofenac sodium Cubosomes for percutaneous administration. *World Jpurnal Pharm Pharm Sci*. 2014;3(5):523–39.
43. Nasr M, Ghorab MK, Abdelazem A. In vitro and in vivo evaluation of cubosomes containing 5-fluorouracil for liver targeting. *Acta Pharm Sin B*. 2015;5(1):79–88.
44. Teba HE, Khalil IA, El Sorogy HM. Novel cubosome based system for ocular delivery of acetazolamide. *Drug Deliv*. 2021;28(1):2177–86.

45. Alshawwa SZ, El-Masry TA, Nasr M, Kira AY, Alotaibi HF, Sallam AS, et al. Celecoxib-Loaded Cubosomal Nanoparticles as a Therapeutic Approach for Staphylococcus aureus In Vivo Infection. *Microorganisms*. 2023;11(9):1–17.
46. Sultan AA, Nashar NFE, Ashmawy SM, Maghraby GME. Cubosomes for Enhancing Intestinal Absorption of Fexofenadine Hydrochloride: In situ and in vivo Investigation. *Int J Nanomedicine*. 2022;17(August):3543–60.
47. Flak DK, Adamski V, Nowaczyk G, Szutkowski K, Synowitz M, Jurga S, et al. At101-loaded cubosomes as an alternative for improved glioblastoma therapy. *Int J Nanomedicine*. 2020;15:7415–31.
48. Shi X, Peng T, Huang Y, Mei L, Gu Y, Huang J, et al. Comparative studies on glycerol monooleate- and phytantriol-based cubosomes containing oridonin in vitro and in vivo. *Pharm Dev Technol*. 2017;22(3):322–9.
49. Seca AML, Pinto DCGA. Plant secondary metabolites as anticancer agents: Successes in clinical trials and therapeutic application. *Int J Mol Sci*. 2018;19(1).
50. Supramaniam K, Gazzali AM, Noor NM, Abdul-aziz A. REVIEW ARTICLE A Review on Supporting Roles of Phytochemicals and Advances in Their Nanoformulations for Lung Diseases. 2024;20:198–211.
51. Agrawal AD. Pharmacological Activities of Flavonoids : A Review. *Int J Pharm Sci Nanotechnol*. 2011;1394–8.
52. Ravichandran N, Suresh G, Ramesh B, Siva GV. Fisetin , a novel flavonol attenuates benzo (a) pyrene-induced lung carcinogenesis in Swiss albino mice. *Food Chem Toxicol*. 2011;49(5):1141–7.

53. Hu W, Khor T, Shu L, Su Z. Plants Against Cancer: A Review on Natural Phytochemicals in Preventing and Treating Cancers and Their Druggability. *Anticancer Agents Med Chem.* 2012;12(10):1281–305.
54. Azarmi S, Roa WH, Löbenberg R. Targeted delivery of nanoparticles for the treatment of lung diseases ☆. 2008;60:863–75.
55. Sethuraman V, Janakiraman K, Krishnaswami V, Natesan S, Kandasamy R. pH responsive delivery of lumefantrine with calcium phosphate nanoparticles loaded lipidic cubosomes for the site specific treatment of lung cancer. *Chem Phys Lipids.* 2019;224.
56. Kamal M, Younis M, Elakkad YE, Younis MK. Challenge Of Cubosomes In Advanced And Targeting Drug Delivery Systems. *Int J Sci Eng Res.* 2019;10(7):1740–9.
57. Kumar R, Khursheed R, Kumar R, Awasthi A, Sharma N, Khurana S, et al. Self-nanoemulsifying drug delivery system of fisetin: Formulation, optimization, characterization and cytotoxicity assessment. *J Drug Deliv Sci Technol.* 2019;54(May):101252.
58. Kumar R, Khurana N, Sharma N, Khurana S, Kumar R, Pandey NK, et al. Formulation of liquisolid powder of Fisetin. *Eur J Mol Clin Med.* 2020;07(07):2020.
59. Skiba M, Gasmi H, Milon N, Bounoure F, Lahiani-Skiba M. Water Solubility and Dissolution Enhancement of Fisetin by Spherical Amorphous Solid Dispersion in Polymer of Cyclodextrin. *Austin J Biotechnol Bioeng.* 2021;8(1):1106.

60. Mohsen AM, Younis MM, Salama A, Darwish AB. Cubosomes as a Potential Oral Drug Delivery System for Enhancing the Hepatoprotective Effect of Coenzyme Q10. *J Pharm Sci.* 2021;110(7):2677–86.
61. Liu W, Lin C, Hsieh Y. Nanoformulation Development to Improve the Biopharmaceutical Properties of Fisetin Using Design of Experiment Approach. *Molecules.* 2021;26(10):3031.
62. Eguchi H, Kimura R, Matsunaga H, Matsunaga T, Yoshino Y, Endo S. Increase in Anticancer Drug-Induced Toxicity by Fisetin in Lung Adenocarcinoma A549 Spheroid Cells Mediated by the Reduction of Claudin-2 Expression _ Enhanced Reader.pdf. *Int J Mol Sci.* 2022;23:7536.
63. Ragelle H, Crauste-Manciet S, Seguin J, Brossard D, Scherman D, Arnaud P, et al. Nanoemulsion formulation of fisetin improves bioavailability and antitumour activity in mice. *Int J Pharm.* 2012;427(2):452–9.
64. Aboushanab AR, El-Moslemany RM, El-Kamel AH, Mehanna RA, Bakr BA, Ashour AA. Targeted Fisetin-Encapsulated β -Cyclodextrin Nanosponges for Breast Cancer. *Pharmaceutics.* 2023;15(5):1–26.
65. Ghosh P, Singha Roy A, Chaudhury S, Jana SK, Chaudhury K, Dasgupta S. Preparation of albumin based nanoparticles for delivery of fisetin and evaluation of its cytotoxic activity. *Int J Biol Macromol.* 2016;86:408–17.
66. Lorthongpanich N, Mahalapbutr P, Rungrotmongkol T, Charoenwongpaiboon T, Prousoontorn MH. Fisetin glycosides synthesized by cyclodextrin glycosyltransferase from *Paenibacillus* sp. RB01: characterization, molecular docking, and antioxidant activity. *PeerJ.* 2022;10:1–19.

67. Seguin J, Brullé L, Boyer R, Lu YM, Ramos Romano M, Touil YS, et al. Liposomal encapsulation of the natural flavonoid fisetin improves bioavailability and antitumor efficacy. *Int J Pharm.* 2013;444(1–2):146–54.
68. Mignet N, Seguin J, Ramos Romano M, Brullé L, Touil YS, Scherman D, et al. Development of a liposomal formulation of the natural flavonoid fisetin. *Int J Pharm.* 2012 Feb;423(1):69–76.
69. Moolakkadath T, Aqil M, Ahad A, Imam SS, Sultana Y, Mujeeb M, et al. Development of transethosomes formulation for dermal fisetin delivery : Box – Behnken design , optimization , in vitro skin penetration , vesicles – skin interaction and dermatokinetic studies. *Artif Cells, Nanomedicine, Biotechnol.* 2018;46(S2):S755–65.
70. Xiao X, Zou J, Fang Y, Meng Y, Xiao C, Fu J, et al. Fisetin and polymeric micelles encapsulating fisetin exhibit potent cytotoxic effects towards ovarian cancer cells. *BMC Complement Altern Med.* 2018;18(1):1–12.
71. Moolakkadath T, Aqil M, Ahad A, Imam SS, Praveen A, Sultana Y, et al. Fisetin loaded binary ethosomes for management of skin cancer by dermal application on UV exposed mice. *Int J Pharm.* 2019;560:78–91.
72. Moolakkadath T, Aqil M, Ahad A, Imam SS, Praveen A, Sultana Y, et al. Preparation and optimization of fisetin loaded glycerol based soft nanovesicles by Box-Behnken design. *Int J Pharm.* 2020;578(October 2019):119-125.
73. Kedar T, Jalalpure S, Kurangi B, Kazi T. Development and Validation of Stability-Indicating RP-HPLC Method for the Estimation of Fisetin in Novel Cubosomal Nanoformulation: Application to the Marketed Formulation and

- Selected Plant Extracts. *Curr Pharm Anal.* 2022;18(10):983–92.
74. Bouzid K, Toumi Benali F, Chadli R, Bouzouina M, Bouzid A, Benchohra A, et al. Extraction, identification and quantitative HPLC analysis of flavonoids from fruit extracts of *Arbutus unedo* L from tiaret area (Western Algeria). *Eur J Mol Biotechnol.* 2014;6(4):160–9.
75. Kurangi B, Jalalpure S, Jagwani S. A validated stability-indicating HPLC method for simultaneous estimation of resveratrol and piperine in cubosome and human plasma. *J Chromatogr B.* 2019;1122–1123(February):39–48.
76. Kumar R, Kumar R, Khursheed R, Awasthi A, Ramanunni AK, Kaur J, et al. Validated reverse phase-high-performance liquid chromatography method for estimation of Fisetin in self-nanoemulsifying drug delivery system. *Assay Drug Dev Technol.* 2020;18(6):274–81.
77. Abdulrahman SK, Rasheed AS, Qassim AW. Identification and determination of Fisetin in black and green teas by ZIC-HILIC column coupled with UV detection. *Syst Rev Pharm.* 2021;12(2):289–94.
78. Moolakkadath T, Aqil M, Ahad A, Imam SS, Sultana Y, Mujeeb M, et al. Analytical quality by design (AQbD) approach based HPTLC method for quantification of fisetin with superior recovery in formulations. *Curr Anal Chem.* 2020;16(2).
79. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: Extraction Methods, Antioxidative Action, Bioavailability and Anticarcinogenic Effects. *Molecules.* 2016;21(7).
80. Boukhary R, Aboul-ElA M, Al-Hanbali O, El-Lakany A. Phenolic Compounds
-

- from *Centaurea horrida* L Growing in Lebanon. *Int J Pharmacogn Phytochem Res.* 2017;9(1):3–6.
81. ICH EMA. Validation of analytical procedure: Methodology International Conference on Harmonization. “ICH Harmonised tripartite guideline - Validation of analytical procedures: Text and methodology Q2(R1). 1995;
82. Kurangi B, Jalalpure S. A validated stability-indicating rp-hplc method for piperine estimation in black pepper, marketed formulation and nanoparticles. *Indian J Pharm Educ Res.* 2020;54(3):s677–86.
83. Kedar T, Jalalpure S, Kurangi B. A Validated UV-Spectrophotometric Method For Fisetin Estimation From Cubosomal Nanoformulation, Marketed Formulation And Strawberry Fruit Extract. *African J Biol Sci (South Africa).* 2024;6(Si 2):3242–51.
84. Kumar R, Kumar R, Khursheed R, Kapoor B, Sharma N, Khurana S, et al. Development and validation of UV spectroscopic method for estimation of fisetin in self nanoemulsifying drug delivery system. *Res J Pharm Technol.* 2020;13(3):1179–82.
85. Chadha R, Bhalla Y, Chadha K, Karan M. Crystal engineering of fisetin: a step towards improved biopharmaceutical parameters. *SDRP J Food Sci Technol.* 2019;4(2):597–613.
86. Chimagave SS, Jalalpure SS, Patil AK, Kurangi BK. Development and Validation of Stability Indicating UV-Spectrophotometric Method for the Estimation of Hesperidin in Bulk Drugs, Plant Extract, Ayurveda Formulation and Nanoformulation. *Indian J Pharm Educ Res.* 2022;56(3):865–72.

87. Kedar T, Jalalpure S, Kurangi B. Cubosomal nanoformulation increase in vitro dissolution and anticancer activity of Fisetin in A549 lung cancer cells. *Ther Deliv.* 2024;15(5):355–69.
88. Hakeem EA, El-Mahrouk GM, Abdelbary G, Teaima MH. Freeze-Dried Clopidogrel Loaded Lyotropic Liquid Crystal: Box-Behnken Optimization, In-Vitro and In-Vivo Evaluation. *Curr Drug Deliv.* 2020;17(3):207–17.
89. Kumar KN, Mallik S, Sarkar K. Role of freeze-drying in the presence of mannitol on the echogenicity of echogenic liposomes. *J Acoust Soc Am.* 2017;142(6):3670–6.
90. Ahmed LM, Hassanein KMA, Mohamed FA, Elfaham TH. Formulation and evaluation of simvastatin cubosomal nanoparticles for assessing its wound healing effect. *Sci Rep.* 2023;13(1):1–14.
91. Pramanik A, Xu Z, Shamsuddin SH, Khaled YS, Ingram N, Maisey T, et al. Affimer Tagged Cubosomes: Targeting of Carcinoembryonic Antigen Expressing Colorectal Cancer Cells Using In Vitro and In Vivo Models. *ACS Appl Mater Interfaces.* 2022;14(9):11078–91.
92. Zhang X, Xiao Y, Huang Z, Chen J, Cui Y, Niu B, et al. Smart phase transformation system based on lyotropic liquid crystalline@hard capsules for sustained release of hydrophilic and hydrophobic drugs. *Drug Deliv.* 2020;27(1):449–59.
93. Choi IS, Kim BS, Cho KS, Park JC, Jang MH, Shin MC, et al. Amiodarone induces apoptosis in L-132 human lung epithelial cell line. *Toxicol Lett.* 2002;132(1):47–55.

94. Jagwani S, Jalalpure S, Dhamecha D, Jadhav K, Bohara R. Pharmacokinetic and Pharmacodynamic Evaluation of Resveratrol Loaded Cationic Liposomes for Targeting Hepatocellular Carcinoma. *ACS Biomater Sci Eng.* 2020;6(9): 4969–84.
95. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Clonogenic assay of cells in vitro. *Nat Protoc.* 2006;1(5):2315–9.
96. Jagwani S, Jalalpure S, Dhamecha D, Hua GS, Jadhav K. A Stability Indicating Reversed Phase HPLC Method for Estimation of trans-Resveratrol in Oral Capsules and Nanoliposomes. *Anal Chem Lett.* 2019;711–26.
97. Selvendiran K, Prince Vijeya Singh J, Sakthisekaran D. In vivo effect of piperine on serum and tissue glycoprotein levels in benzo(a)pyrene induced lung carcinogenesis in Swiss albino mice. *Pulm Pharmacol Ther.* 2006;19(2):107–11.
98. Touil YS, Seguin J, Scherman D, Chabot GG. Improved antiangiogenic and antitumour activity of the combination of the natural flavonoid fisetin and cyclophosphamide in Lewis lung carcinoma-bearing mice. Vol. 68, *Cancer Chemotherapy and Pharmacology.* 2011. p. 445–55.
99. Selvendiran K, Singh JPV, Krishnan KB, Sakthisekaran D. Cytoprotective effect of piperine against benzo w. 2003;74:109–15.
100. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193(1):265–75.
101. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*

- [Internet]. 1972;247(10):3170–5. Available from:
[http://dx.doi.org/10.1016/S0021-9258\(19\)45228-9](http://dx.doi.org/10.1016/S0021-9258(19)45228-9)
102. Marklund S, Marklund G. Involvement of the Superoxide Anion Radical in the Autoxidation of Pyrogallol and a Convenient Assay for Superoxide Dismutase. *Eur J Biochem.* 1974;47(3):469–74.
103. Sinha AK. Colorimetric assay of catalase. *Anal Biochem.* 1972;47(2):389–94.
104. Takahara S, Hamilton HB, Neel J V., Kobara TY, Ogura Y, Nishimura ET. Hypocatalasemia: a new genetic carrier state. *J Clin Invest.* 1960;39(4):610–9.
105. Wang Y, Rouggly L, You M, Lubet R. Animal models of lung cancer: Characterization and use for chemoprevention research. 1st ed. Vol. 105, *Progress in Molecular Biology and Translational Science.* Elsevier Inc.; 2012. 211–226 p.

ANNEXURE A. ANIMAL ETHICAL CLEARANCE LETTER

	KLE College of Pharmacy A Constituent Unit of KLE Academy of Higher Education and Research (Deemed to be University) DEPARTMENT OF PHARMACOLOGY <i>JNMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India</i> <u>INSTITUTIONAL ANIMAL ETHICS COMMITTEE</u> <i>Reg.No.221/Po/Re/S/2000/CPCSEA</i>	
CERTIFICATE		
This is to certify that the project proposal no <u>14</u> entitled, "Formulation, optimization and characterization of fisetin loaded cubosomes for the treatment of lung cancer: An experimental study" submitted by Dr./ Mr. / Ms., Tukaram Kisan Kedar 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 <u>60</u> ~~Rats~~ <u>Mice</u> ~~Rabbits~~ ~~Guinea pig~~ (animals) sex <u>male</u> 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:	<u>Dr. N. A. Khatib</u>	<u>13/03/2021</u>
MEMBER SECRETARY Institutional Animal Ethical Committee, KLE's College of Pharmacy, BELGAUM - 590010		
Main Nominee of	<u>Dr. Vinodkumar CS</u>	<u>17-3-2021</u>
CPCSEA:	CPCSEA Nominee Institutional Animal Ethics Committee KLE's College of Pharmacy, BELGAUM.	

ANNEXURE B. PUBLICATIONS

Sr No.	Title	Journal Name	Publisher	Indexing
1	Cubosomal nanoformulation increase in-vitro dissolution and anticancer activity of Fisetin in A549 lung cancer cells	Therapeutic Delivery	Taylor and Francis	Scopus Q2 IF 4.2
2	Development and Validation of Stability-Indicating RP-HPLC Method for the Estimation of Fisetin in Novel Cubosomal Nanoformulation: Application to the Marketed Formulation and Selected Plant Extracts	Current Pharmaceutical Analysis	Bentham Science	Scopus Q3 IF 0.6
3	A Validated UV-Spectrophotometric Method For Fisetin Estimation From Cubosomal Nanoformulation, Marketed Formulation And Strawberry Fruit Extract	African Journal of Biological Sciences (South Africa)	Institute for Advanced Studies	Scopus Q4 IF 0.12
4	Cubosomal nanoformulation augments fisetin uptake in A549 human lung cancer cells and reduces inflammation and toxicity in the Swiss albino mice lungs	In process	-	-

Poster Presentation/ Conference/ Workshop

Sr. No.	Presentation	Details
1	Poster Presentation	Presentation at 25th National Convention of Society of Pharmacognosy at Graduate School of Pharmacy, Gujarat Technological University, 27&28 Nov 2021
2	Poster Presentation	Presentation in national conference on recent advancements and challenges in analytical chemistry at JSS College of Pharmacy, Mysuru held on 20th May 2022
3	Workshop	Anchome HPTLC training at BSRC, Belagavi
4	Conference/ Webinar	“Regulatory compliances : Laboratory practice” conducted by Dr. Prabhakar Kore Basic Science Research center, KLE University on 20th April 2021
5	Workshop	Participated in Training Programme on ‘Laboratory Procedures’ organized by KAHER’S Dr. Prabhakar Kore BSRC Belagavi during 17th to 21st December 2020
6	Workshop	Workshop on Study Designs and Statistical Methods using SPSS/EXCEL held from 16th September to 20th September 2019 organized by Department of Epidemiology and Biostatistics in collaboration at KAHER, Belagavi
7	Online SWAYAM	ICMR-NPTEL course exam on “Health Research Fundamentals” Jan-March 2020 (8 Weeks)



Cubosomal nanoformulation increase *in vitro* dissolution and anticancer activity of Fisetin in A549 lung cancer cells

Tukaram Kedar, Sunil Jalalpure & Bhaskar Kurangi

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Cubosomal nanoformulation increase *in vitro* dissolution and anticancer activity of Fisetin in A549 lung cancer cells

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Aim: To prepare fisetin (FIS) cubosomal nanoformulation to increase aqueous solubility and anticancer activity. **Methods:** Top-down method using glyceryl monooleate (GMO) and Pluronic F-127. **Results:** Optimized using 2% GMO and 1% Pluronic F-127, reported 93.07 nm particle size, 80.10% drug entrapment, and reports more than 50% enhanced *in vitro* drug release than native FIS. MTT assay reports IC₅₀ Values of FIS 16.59 µg/ml and optimized cubosomal FIS nanoformulation (FISCUB) 12.18 µg/ml. The colony numbers observed in clonogenic assay for FISCUB were 8.33 ± 0.58 and FIS 11.67 ± 1.15. In flow cytometry study, apoptotic cells in FISCUB and FIS-treated A549 cells were found to be 33.4 and 6.83% respectively. **Conclusion:** A stable cubosomal nanoformulation of FIS showed enhanced aqueous solubility and anticancer activity.

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Keywords: A549 cells • characterization • fisetin • fisetin cubosome nanoformulation • *in vitro* anticancer study

Fisetin (FIS) is a plant-derived dietary flavonoid that is chemically a flavanol (3,3',4',7-tetrahydroxyflavone) and available in the market as a nutraceutical. It possesses many pharmacologic effects, including anticancer activity in various types of cancer [1–3]. It acts on cancer cells via cell cycle arrest, apoptosis, metastasis reduction, angiogenesis and cellular oxidation process [4,5]. It has also shown a synergistic effect with other anticancer drugs, with previous researchers reporting the suppression of various types of cancer cell proliferation [6]. Although wide promising pharmacologic activities of FIS have been reported, because it is a BCS class II drug, FIS possesses the problem of low aqueous solubility and bioavailability [7]. This restricts the application of FIS as a promising lead molecule along with its maximum therapeutic benefits against its anticancer and other uses [8–10].

Researchers have reported many novel nanoparticulate drug-delivery systems and other phytoformulation options for overcoming solubility and bioavailability [11–13]. These formulations are prepared for *in vivo* application of FIS as nanoemulsions used in mice induced with Lewis lung carcinoma [6,14,15], liposomes with 73% encapsulation used for intravenous and the intraperitoneal administration in mice [16,17], albumin based nanoparticles with 84% encapsulation used for *in vitro* study on MCF-7 cells [18], transthesomes with 68.31% encapsulation used for skin cancer [19], polymeric micelles with 98.53% encapsulation used for ovarian cancer cells [20], binary ethosomes with 99.89% encapsulation used for skin cancer in mice [21,22], self-nano emulsifying drug delivery tested for *in vitro* cytotoxicity, formulated liquisolid powder evaluated for *in vitro* release [23,24], cyclodextrin solid dispersion for improving aqueous solubility [25], nanoparticles using polyvinyl alcohol and poly (lactic-co-glycolic acid) [26], and synthesis of FIS glycosides using microbial enzyme [27,28].

To increase the aqueous solubility and anticancer activity of FIS, we developed a novel optimized FIS cubosomal formulation using a top-down method. As compared with other lipid-based carriers, the cubosomes were reported to be good solubilizers, biocompatible and biodegradable; to require only a simple preparation procedure; ability to entrap a greater amount of water-insoluble drugs; and to increase cellular uptake and bioavailability. Therefore,



we prepared this cubosomal nanoformulation to study its effect on FIS solubility and cellular absorption [29–32]. The developed formulation method is simple, yields thermodynamically stable cubosomes, and requires a smaller number of formulation ingredients than the previously reported FIS nanoformulations. To evaluate its effect, we tested the optimized cubosomal formulation for its stability, particle characteristics, encapsulation efficiency, *in vitro* FIS release, and anticancer activity against A549 lung cancer cells.

Materials & methods

Materials used

Shaanxi Yi An Biological Technology Co., Ltd (Xi'an, China), provided 98% high-performance liquid chromatography (HPLC)-grade pure FIS, and Mohini Organics Pvt. Ltd (Mumbai, India) provided GMO as gift samples. Pluronic® F-127 (oxirane, methyl-, polymer with oxirane) was procured from Sigma-Aldrich, (MO, USA). HPLC-grade acetonitrile, methanol, acetic acid, orthophosphoric acid and formic acid were purchased from Fisher Scientific (Mumbai, India). The used A549 cells (human lung cancer cell line) were purchased from NCCS (Pune, India). DMEM, fetal bovine serum, MTT, 4',6-diamidino-2-phenylindole (DAPI), ethidium bromide, phosphate-buffered saline (PBS), and other chemicals required for cell line study were purchased from Hi-media.

Selection of chromatographic parameters for the FIS estimation

A reversed-phase (RP-HPLC) was performed to estimate FIS in pure form and the cubosomal nanoformulation using Shimadzu prominence liquid chromatography (LC-20AD, Japan) and currently reported [33].

Preparation of cubosomal nanoformulation

We used the top-down method [28,31,34,35] with the homogenization technique for separating cubosomes from their honeycomb-like bulk molecular structure and to disperse them in water [36,37]. We used various reported methods using different concentrations of lipid GMO, and Pluronic F-127 polymer to prepare the stable cubosome nanoparticle structures loaded with FIS [38,39]. The method was established for FIS cubosome formulation using these previously reported formulations by evaluating the physical observation of the prepared formulations, particle size and polydispersity index (PDI) [40,41].

A thermostatic water bath (Water Tech) was used to completely melt GMO and Pluronic F-127 separately. In the molten mass of GMO, FIS 40 mg was added and mixed properly along with heating. We added this mixture of FIS and GMO to Pluronic F-127 at 65°C along with proper mixing continuously at 65°C to obtain a homogeneous mass of the above three ingredients. This molten mass was added dropwise to 40 ml of preheated water at the same temperature with continuous stirring. A milky liquid dispersion was obtained, allowed to cool, and processed for homogenization (IKA T25 digital Ultra Turax, Germany) at 14,500 rpm for 20 min. This solution was sonicated using a probe sonicator (Rivo TEK, Mumbai, India) for 10 min under cool conditions. Before this formulation was investigated further, it was stored at room temperature for 24 h to allow it to equilibrate properly.

The prepared cubosomes were then analyzed using a Zetasizer (Malvern Instruments, Malvern, UK) for particle size, zeta potential and PDI values [42]. We checked the FIS entrapment efficiency of the cubosomal nanoformulation using the high-speed centrifugation method at 14,500 rpm (Kubota 6500, Osaka, Japan) for up to 30 min. After centrifugation, the supernatant was separated and mixed with the mobile phase, and the FIS concentration was estimated using the developed HPLC method to analyze the quantity of FIS entrapped in prepared cubosomes.

Optimization & characterization of the formulation parameters

We selected a wide concentration range of GMO and Pluronic F-127 and prepared 16 pilot batches to determine a useful concentration range. The formulation was optimized using Design expert® software (version 7.0.0, Stat-Ease Inc., MN, USA), using a 3² factorial design in which lipid and stabilizer concentrations were chosen as two independent parameters, and particle size and entrapment efficiency were measured as two dependent parameters. We tested nine formulation batches for particle characteristics [43,44].

Lyophilized samples of prepared cubosomes for HRTEM & XRD study

Optimized and blank formulations were cooled at -80°C overnight for lyophilization (CHRIST, ALPHA 1–2 LD plus) to prepare solid samples for high-resolution transmission electron microscopy (HRTEM) and x-ray diffraction (XRD) study. The selection of cryoprotectant and its concentration is crucial in lyophilization of the liquid cubosomal nanoformulation. The use of various cryoprotectants were reported for lyophilization process, however

the mannitol was used for many lipid based nanoformulations. Therefore, the prepared cubosome formulations were lyophilized using 3% mannitol as a cryoprotectant [45,46]. We sent the freeze-dried samples for HRTEM and XRD analysis to understand cubosome particle characteristics.

Compatibility study of the formulation ingredients

To test the compatibility of FIS with the other excipients, we used Fourier-transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), XRD, and HRTEM. For FTIR and DSC, samples of pure drug FIS, Pluronic F-127, GMO, and FISCUB were tested [47–49]. FTIR is a suitable technique for characterizing various molecular species, which reveals drug-excipient compatibility by providing information on the interaction of various molecules and functional groups when mixed in the formulation. DSC provides the thermal behavior and interactions of FIS with cubosomal formulation excipients. We performed FTIR analysis at SAIF IIT Bombay, Mumbai (Vertex 80 with Spectral resolution of FTIR 0.2 cm⁻¹, focal plane array: 128 × 128, range: 4000–900 cm⁻¹), and we used DSC (Shimadzu DSC-60, Kyoto, Japan; temperature range of 0–400°C) to obtain thermograms of FIS, GMO, Pluronic F-127 and cubosomal formulation.

XRD analysis revealed the crystallinity of the prepared cubosomes. We obtained XRD spectra for FIS, FISCUB, and blank cubosomes from SAIF KUD, Dharwad (Automated XRD SmartLab SE, Philips Holland with x-ray tube-CuAngular), and its 2θ range was maintained between 2° and 150° during analysis.

The HRTEM using randomly selected area electron diffraction (Jeol/JEM 2100 with 200 kV LaB6 Electron gun) was used to understand the structural morphology of cubosomes. A copper grid coated with carbon was used in 2% w/v phosphotungstic acid.

Viscosity & pH

The viscosity for rheological behavior (Brookfield viscometer, CAP 2000+; MA, USA) and pH of the prepared formulations were tested [44,50,51].

Drug loading/entrapment efficiency of the prepared cubosomal formulation

To separate cubosomes from liquid dispersion, a high-speed centrifugation method (Eppendorf, Hamburg, Germany) at 14,500 rpm for 30 min was used. We measured the drug content in the supernatant liquid using the developed HPLC method. The percentage of entrapped FIS in the cubosomal formulation was estimated using the following formula (Equation 1):

$$\% EE = \left(\frac{\text{total FIS added in formulation} - \text{FIS in supernatant}}{\text{total FIS added in formulation}} \right) \times 100 \quad (\text{Equation 1})$$

Stability study

The optimized cubosomal nanoformulation B2 was stored in amber-colored, tightly closed glass vials wrapped in aluminum foil at a temperature of 4 to 8°C. FISCUB batch B2 was kept for three months in a stability chamber that controlled the temperature. Then the sample was tested for its particle characteristics [49].

In vitro release study of FIS & FISCUB

The water, HCl buffer (pH 1.2), and phosphate buffer (pH 6.8) were used as dissolution medium with and without Tween-80 (1.5%). For the drug release study, partially permeable cellulose dialysis membrane (HIMEDIA, 150, LA401-5MT; diameter 25.4 mm with capacity 5.07 ml/cm) were used. The release was studied using a tablet dissolution apparatus (Electrolab EDT-08LX) with a low-volume conversion kit (100 ml capacity, 100 rpm speed rotating paddle) specially designed for nanoformulation dissolution and release studies [37,49,51]. The dialysis membrane was soaked overnight in the respective dissolution medium, and 5 ml FISCUB was loaded in dialysis bags for release study at 37°C. After specific time intervals, 1 ml sample was withdrawn from each basket, and this volume was replaced by using the respective dissolution medium to maintain the constant volume of the dissolution medium [37,52]. The developed HPLC method was used to quantify the released drug from all selected media.

In vitro study of the optimized cubosomal formulation

For the *in vitro* study, human lung carcinoma cells A549 were selected and received from NCCS. Various types of cell culture studies like MTT assay, clonogenic assay, flow cytometry study, dead and live cell assay with acridine orange-ethidium bromide, and DAPI staining were performed [33–35].

MTT assay

An authentic sample of A549 cells was purchased from NCCS, observed for no contamination and subcultured in DMEM medium containing 10% fetal bovine serum, and 1% antibiotic–antimycotic solution (potassium penicillin 10,000 U/ml, streptomycin sulphate 10,000 µg/ml). Cells were cultured for 10 days in a T25 cell culture flask in a CO₂ incubator at 37°C. A monolayer culture of A549 cells was trypsinized and the cell count was adjusted to 10⁵ cells/ml. A cell suspension of 100 µl was added in each well of a 96-well plate and incubated at 37°C for 24 h to form a partial monolayer. The serial dilutions 0, 12.5, 25, 50, 100, 150 and 200 µg/ml of FIS, FISCUB, cisplatin and blank formulation were prepared in triplicate in DMEM, and 100 µl of these concentrations were added, and incubated at 37°C in 5% CO₂ incubator for 48 h. The medium was flicked off, and 20 µl of freshly prepared MTT solution (5 mg/ml in sterile PBS) was added and incubated in a CO₂ incubator at 37°C [56]. We removed excess MTT and added 100 µl of DMSO and readings were obtained at 570 nm. Assay was performed in triplicates to assess the cytotoxicity effect of pure drug, optimized formulation, formulation without drug, and cisplatin as a standard drug [54,57]. We calculated cytotoxic concentration (IC₅₀) values from the absorbance readings using GraphPad Prism 5.01 software.

Clonogenic assay or colony formation assay

The *in vitro* colony formation ability of an animal cell was tested using this assay. Retaining proliferative and reproductive ability of an animal cell after treatment of FIS and FISCUB will give the comparative antiproliferative effect of free drug and drug in prepared novel cubosomal formulation. Tissue culture six-well plates were taken, and 1 ml of 300 cells/ml A549 cells were added and incubated for 12 to 24 h. The medium was removed, and cells were treated with a concentration of IC₅₀ value of FIS, FISCUB and cisplatin for 24 h. The drug was removed, and fresh medium was added, and incubated for up to 12 days to produce 32 cell colonies from individual cells in six-well plates. To count the cell colonies, they were washed with PBS and stained with 1% crystal violet in methanol [58].

Flow cytometry study

The A549 cells were added to a flat-bottom 24-well microplate with coverslips and incubated at 37°C in a CO₂ incubator overnight. These cells were treated for 12 h with concentrations of IC₅₀ values of FIS, FISCUB and cisplatin; cleaned with PBS, and centrifuged for 5 min at 500 × g at 4°C. The 1X cold binding buffer was used to resuspend the pellets to get 1 × 10⁵ cells per ml. Add 1 µl of annexin V-FITC solution and 5 µl propidium iodide, incubated for 15 min in the dark on ice. Added 400 µl of ice-cold 1X binding buffer and mixed gently and analyzed cells in the 30 min using flow cytometry [20,59].

Staining with acridine orange & ethidium bromide

This differential fluorescent staining method evaluates apoptosis and cell viability depending on cell membrane integrity. We added a glass coverslip to the tissue culture six-well plates. We seeded 1 ml of A549 cell suspension of 10⁵ cells/ml, which were then treated with IC₅₀ concentrations of FIS, and FISCUB for 48 h. Cells on glass coverslip were first stained with acridine orange and then with ethidium bromide and washed with PBS. In viable cells, the acridine orange (0.5% in PBS) binds with the DNA and appears green, whereas in nonviable cells, ethidium bromide (0.1% in PBS) binds with DNA and appears bright orange under fluoresce microscope (Nikon OPTIPHOT-2) with a blue 480-nm filter.

DAPI staining study

We used the blue fluorescent stain DAPI (4',6-diamidino-2-phenylindole) for staining the cell nuclei in fluorescence microscopy because it binds with the adenine-thymine rich region in the dsDNA sequence and therefore preferentially stains cell nuclei. DAPI 1 µg/ml solution was used for staining of A549 cells chromosome and to observe the integrity of cell nuclei in normal, FIS and FISCUB treated cells with respective IC₅₀ concentrations for 48 h. Drug-treated cells were washed with PBS and observed under a fluorescent microscope for qualitative assessment of apoptosis induction and nuclear changes [59].

Table 1. Optimization study using design expert software.

Batch no.	Batch details	Particle size (nm)	PDI	Entrapment efficiency (%)	Zeta potential
B1	GMO 2%, Pluronic F-127 0.5%	116.9 ± 3.23	0.13 ± 0.01	72.50 ± 0.72	-53.20 ± 0.61
B2	GMO 2%, Pluronic F-127 1%	93.07 ± 5.41	0.20 ± 0.01	80.10 ± 0.36	-31.60 ± 0.57
B3	GMO 2%, Pluronic F-127 1.5%	117.2 ± 6.57	0.50 ± 0.11	78.80 ± 0.57	-37.40 ± 1.18
B4	GMO 4%, Pluronic F-127 0.5%	127.2 ± 3.72	0.15 ± 0.07	77.67 ± 0.74	-43.30 ± 0.84
B5	GMO 4%, Pluronic F-127 1%	118 ± 5.18	0.16 ± 0.08	75.80 ± 0.91	-41.90 ± 0.41
B6	GMO 4%, Pluronic F-127 1.5%	148.7 ± 2.64	0.42 ± 0.08	69.10 ± 1.21	-5.87 ± 0.72
B7	GMO 6%, Pluronic F-127 0.5%	214.6 ± 4.63	0.27 ± 0.03	75.47 ± 0.85	-30.30 ± 0.85
B8	GMO 6%, Pluronic F-127 1%	129.5 ± 6.27	0.16 ± 0.05	77.79 ± 0.77	-32.10 ± 0.45
B9	GMO 6%, Pluronic F-127 1.5%	123.2 ± 1.91	0.23 ± 0.02	70.52 ± 0.46	-18.1 ± 0.76
B10 (Blank)	GMO 2%, Pluronic F-127 1%	148.7 ± 7.23	0.42 ± 0.08	0	-26.0 ± 0.47
B2 (Stability)	GMO 2%, Pluronic F-127 1%	90.76 ± 3.67	0.221 ± 0.4	81.36 ± 0.71	-17.1 ± 0.34

A total of nine batches were suggested (3^2 factorial design) by Design Expert software, prepared and evaluated. The cubosome characteristic parameters were noted, such as particle size, poly dispersibility index, drug entrapment efficiency and zeta potential values. Values represents $\pm$ SD ($n = 3$).

Results

Development of the analytical method for FIS estimation

We developed and validated an efficient RP-HPLC analytical method using the ICH Q2R1 guidelines for estimating FIS. The previously reported method was found to be linear in the range of 0.1 to 16 $\mu\text{g/ml}$ ($y = 8546X - 4936$, $R^2 = 0.999$) [33].

Preparation of cubosomal nanoformulation

We used the top-down method for FIS cubosome preparation with homogenization and sonication steps to obtain liquid dispersion in water. In previously reported studies, different concentrations of lipid and polymer were used for various drug entrapment methods. We selected two independent parameter concentrations of GMO 3% to 9% and Pluronic F-127 0.5–2% of formulation volume and prepared 16 pilot batches to understand the effect of lipid and polymer concentration on dependent parameters like particle size and entrapment of FIS (Supplementary Table 1).

Optimization & characterization of formulation parameters

To optimize of FIS cubosomes, we applied 3^2 full factorial design of Design Expert software to the top-down method. Lipid and stabilizer concentrations were coded for low, medium, and high concentrations whereas particle size was set to minimize and entrapment efficiency to maximize the result [47]. Table 1 reports the outcomes of the nine batches suggested by the software.

Supplementary Table 2 shows the statistical results obtained for particle size and entrapment efficiency using Design Expert software. The results of the regression analysis suggest that the 2FI and quadratic were the best-fit statistical models, and the adequate precision values were 5.50 and 3.99 for particle size and entrapment efficiency, respectively.

The usefulness of the prepared formulation for increasing FIS solubility and cellular absorption was greatly influenced by nanosized cubic crystalline particles. The prepared batches for FIS cubosomal nanoformulation optimization had a mean particle size [30] in the range of 93.07 to 214.6 nm (Table 1). The following equation (Equation 2) shows the effect of %w/w concentration of independent variables (A) lipid (GMO) and (B) stabilizer (Pluronic F-127) on the particle size.

$$PS = 132.04 + 23.35A - 11.60B - 22.92AB \quad (\text{Equation 2})$$

Delivery of FIS to the target site or cells is governed by the drug-loading ability of the prepared cubosome particles. In the conducted formulation study, it was found to be between 69.10 and 80.10% (Table 1). The correlation of independent parameters (A) lipid (GMO) and (B) stabilizer (Pluronic F-127) with entrapment efficiency was shown in Figure 1C & D and the following equation (Equation 3).

$$EE = 76.78 - 1.27A - 1.20B - 2.81AB + 1.67A^2 - 3.89B^2 \quad (\text{Equation 3})$$

RESEARCH ARTICLE



Development and Validation of Stability-Indicating RP-HPLC Method for the Estimation of Fisetin in Novel Cubosomal Nanoformulation: Application to the Marketed Formulation and Selected Plant Extracts



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Abstract: Background: Fisetin (FIS) is a bioactive flavonoid found in various plants, reported for many pharmacological activities, and presently marketed as a nutraceutical. To overcome less water solubility and bioavailability issues, FIS cubosomal nanoformulation has been prepared and characterized.

Objective: To estimate FIS in prepared novel cubosomes, an RP-HPLC analytical method development with the most sensitivity, economical, robust, and wide applicability in marketed FIS formulations and plant extracts also.

Methods: An RP-HPLC method was developed and validated as per ICH Q2R1 guidelines by using C-18 Phenomenex Luna 5μ, 100Å column, LC-20 AD pump, and Shimadzu LC solution 1.25 software. The combination of acetonitrile and formic acid (0.1%v/v) in the ratio of 25:75 v/v was used as a mobile phase for chromatographic separation using a PDA detector at 360 nm and a flow rate of 1 ml/min.

Results: The developed method was remarkably linear in the range of 0.1 to 16 μg/ml ($R^2 > 0.999$). This method was found to be accurate (recovery 98.24 to 100.65 %), precise, robust (% RSD < 2), and more sensitive than the earlier reported method with LOD and LOQ values of 17.26 and 52.31 ng/ml, respectively. The FIS estimation was also performed using the developed method in the marketed FIS formulation Doctor's Best® Fisetin, and different plant extracts such as strawberry, grapes, black tea, and green tea. The forced degradation study suggests that FIS was unstable in alkaline and oxidative stress conditions.

Conclusion: For FIS estimation in cubosomal nanoformulation, a widely applicable, novel, robust, most sensitive, and economical RP-HPLC method was developed and validated and also applied to marketed formulations and plant extracts.

Keywords: Fisetin, cubosome, RP-HPLC, stability study, doctor's best® fisetin, plant extract.

1. INTRODUCTION

Bioactive flavonoid Fisetin (FIS) is a naturally synthesized yellow-colored secondary metabolite present in various plants such as green tea and strawberry. It is a 15 carbon-containing polyphenolic flavonol 3,3',4',7-Tetrahydroxyflavone (Fig. 1.) usually extracted from the wood of smoke tree *Cotinus coggygria Scop* and other plants at a

commercial scale [1-3]. Recently, more studies have been carried out on FIS because of its broad pharmacological interest [4]. Even though FIS has good pharmacological activities, it possesses therapeutic challenges due to low water solubility and low bioavailability. Researchers working to address these issues by preparing different nanoformulations to utilize the maximum therapeutic benefit of FIS [3, 5, 6]. To overcome different therapeutic challenges, various types of FIS formulations are reported [7-14]. As per the literature review, HPLC methods are used in the reported FIS formulations [7, 8, 10] and in FIS extraction from some plants [15, 16] without validation. Some HPLC

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methods are reported with validation data, but the FIS stress degradation study is not mentioned [5, 17, 18]. Information on FIS stress degradation is reported using another chromatographic technique (HPTLC) [19]. Bioanalytical HPLC methods were reported for FIS estimation in biological samples [8, 20-22]. Other analytical techniques like LC-MS/MS [6] and UV-Visible [23] are reported for FIS estimation but are less commonly used. The previously used HPLC methods are reported without validation data, some are with incomplete validation study, without stress degradation study, and not applied for marketed formulation.

In the present work, we developed a novel FIS loaded cubosomal nanoformulation for more therapeutic benefit. No analytical method is available for the determination of entrapped FIS in cubosomal nanoformulation. Hence there is a need to develop a robust and sensitive HPLC method for the estimation of FIS in developed cubosomal nanoformulation.

Thus, the present study aims to develop and validate a single-widely applicable, economical, more sensitive, reproducible, and superior RP-HPLC method with complete validation parameters to estimate FIS in the prepared FIS cubosome nanoformulation. The developed method is also applied to estimate FIS in the marketed formulation and some plant extracts.



Fig. (1). Chemical structure of Fisetin.

2. MATERIALS AND METHODS

2.1. Chemicals Used

FIS (98% HPLC grade) was received as a gift sample from Shaanxi Yi An Biological Technology Co., Ltd. China. Poloxamer 407 and Glyceryl monooleate were received as gift samples from BASF, Mumbai, and Mohini Organics Pvt. Ltd, Mumbai, India. The HPLC grade acetonitrile, methanol, acetic acid, orthophosphoric acid, and formic acid were obtained from Fisher Scientific, Mumbai, India. Marketed formulation of FIS, Doctor's Best® Fisetin purchased from the market.

2.2. Chromatographic Instrument and Requirements

A liquid chromatographic system (RP-HPLC) Shimadzu prominence liquid chromatography (LC-20AD, Japan) containing pump - LC-20AD, autosampler - SIL-20AC HT, detector - SPD-M20A PDA, degasser - DGU-20A5, oven - CTO-10AS VP column and 20 µl loop rheodyne injection. The software used was version 1.25 of Shimadzu LC solu-

tion for data analysis and interpretation. An analytical column Phenomenex (Canada, USA) Luna 5u C18(2) 100A⁰ (inner diameter - 150 x 4.6 mm, particle diameter - 5 µm) with a 35°C column temperature was used for separation and analysis of FIS. The 25:75 v/v mixture of optimized mobile phase acetonitrile (ACN): formic acid (0.1%) was used and chromatograms were obtained at 360 nm PDA wavelength by using a fixed flow rate of 1 ml/min.

2.3. Preparation of Cubosomal Nanoformulation

For the preparation of FIS cubosomes using the top-down method, glycerol mono-oleate (0.8 gm) and poloxamer 407 (0.6 gm) were taken in a separate beaker and heated at 65°C on a thermostatic water bath until it melts completely. In the molten mass of glycerol mono-oleate, FIS 40 mg was added and mixed properly along with heating. This mixture of FIS and glycerol mono-oleate was added in poloxamer at 65°C along with proper mixing. Mix continuously at 65°C to make the homogeneous mass of the above three ingredients. This molten mass was added dropwise in preheated 40 ml water at the same temperature with continuous stirring. Milky liquid dispersion was obtained, allowed to cool, processed for homogenization (IKA T25 digital Ultra Turax, Germany) at speed of 14500 rpm for 20 min. and sonicated for 10 min.

These prepared cubosomes were then analyzed using the dynamic light scattering method for their mean particle size, zeta potential, and polydispersity index values. The FIS entrapment efficiency of prepared cubosomal nanoformulation was performed using the centrifugation method at high speed of 15000 rpm (Kubota 6500, Japan) for up to 30 min. After centrifugation, the supernatant was separated and mixed with the mobile phase and FIS concentration was estimated by the developed RP-HPLC method to analyze the quantity of FIS entrapped in prepared cubosomes [24].

2.4. Plant Sample Collection and Preparation

Black tea, green tea, and fresh fruit samples of strawberries and grapes were purchased from the local market. All plant samples were dried until constant weight and then crushed. FIS extraction was done in the dark by using the maceration technique (solvent methanol: water 80:20 v/v for 72 hours) followed by filtration. The filtrate was taken in glass separation assembly and mixed for 20 min with 30 ml chloroform. The chloroform layer was separated, filtered through filter paper, and evaporated at room temperature. The final extract obtained was filtered through 0.45 µm syringe filters and processed for FIS estimation by RP-HPLC [15, 18].

2.5. FIS stock and Sample Preparation

A primary FIS stock solution was prepared by solubilizing 10 mg of FIS in 10 ml of methanol (Solution A, 1 mg/ml). Further drug dilutions were prepared using the selected mobile phase as a dilution medium. The working stock was produced by diluting 2.5 ml of solution A up to 50 ml (Solution B, 50 µg/ml). The standard solutions of FIS were prepared in the range of 0.1-16 µg/ml by the dilution of this solution B using the mobile phase used for liquid chromatography. Primary stock and all standard solutions

were kept in an amber-colored, light-resistant, properly closed volumetric flask at 4°C temperature, before HPLC analysis. The FIS cubosomal nanoformulation 1 ml was diluted up to 10 ml using methanol in an amber-colored volumetric flask (Sample solution C, 100 µg/ml).

2.6. Method Development

In previously reported chromatographic methods, FIS identification and quantification were performed by using methanol, and acetonitrile as mobile phase along with acidified water or buffer [15-17, 20, 25, 26]. The development of the proposed analytical method was done by using various mobile phase concentration ratios, pH, and flow rate.

2.7. Method Validation

The developed method was validated as per the ICH Q2(R1) guidelines for various validation parameters such as accuracy, precision, system suitability, linearity, the limit of quantification (LOQ), and limit of detection (LOD), ruggedness, and robustness. The performance of the system was further assessed by taking note of parameters such as the theoretical plate, the height equivalent to the theoretical plate, and the tailing factor [27].

2.7.1. Calibration Curve Development

Suitable aliquots of solution B were diluted up to 10 ml to get final concentrations of 0.1, 0.25, 0.5, 1, 2, 4, 8, and 16 µg/ml, respectively, and the calibration curve was developed.

2.7.2. Accuracy

The developed chromatographic method's accuracy was evaluated by spiking FIS solution into cubosomal nanoformulation solution (5 µg/ml of FIS) at 50 %, 100%, and 150% of the labeled claim of formulation *i.e.*, 5 µg/ml. To prepare these three-level concentrations, aliquots of 0.5, 1, and 1.5 ml of solution B were added in 10 ml amber-colored volumetric flasks separately with 0.5 ml of solution C. The final volume, up to 10 ml was adjusted with the mobile phase. The resultant three solutions (7.5, 10 and 12.5 µg/ml of FIS) were injected thrice and % drug concentration was calculated. The following equation was used to find the absolute percentage recovery of FIS.

$$\text{Actual \% recovery} = (\text{Actual concentration recovered} / \text{Theoretical concentration}) \times 100 \quad (1)$$

2.7.3. Precision

The precision of the developed method was decided by finding intra-day and inter-day values of percentage relative standard deviation. Intra-day precision was determined in a single day, after triplicate injection of the low, medium, and high concentrations (5, 10, and 15 µg/ml) under a similar laboratory setup. The estimation of three-level samples in triplicate for three consecutive days was done for inter-day analysis. Recorded mean data was used for the finding of percentage relative standard deviation (% RSD) by the following equation

$$\% \text{ RSD} = (\text{Standard deviation of peak area} / \text{Average peak area}) \times 100 \quad (2)$$

2.7.4. Ruggedness and Robustness

It was measured by making intentionally small changes in the analytical method parameters. Triplicates of the stock concentration 10 µg/ml were injected to obtain respective chromatograms and mean values of peak area, retention time, and percentage recovery was used to interpret the results.

2.7.5. Methods Suitability

Obtained chromatogram characteristics like theoretical plate, peak purity index, tailing factor and HETP were recorded for checking the system suitability for validation of the new method.

2.7.6. Sensitivity

To check the sensitivity of the developed method, the calibration curve was used to find out the values of LOD and LOQ. The response's standard deviation (σ) and the calibration curve (S) slope were used to determine LOD and LOQ. The standard deviation of the Y intercepts of the regression line was the value of σ in the following equation.

$$\text{LOD} = 3.3 \times \sigma / S \quad (3)$$

$$\text{LOQ} = 10 \times \sigma / S \quad (4)$$

2.8. Application of the Method for FIS Estimation in Cubosomal Nanoformulation, Marketed Formulation, and Plant Extracts

For estimation of FIS in cubosome nanoformulation, solution C (1 ml) was mixed with 10ml of the mobile phase. Then the sample was analyzed on RP-HPLC. The calibration curve equation was used to process the observed area to calculate the drug loading.

$$\% \text{ drug loading} = \text{Actual amount of drug present} / \text{Total amount of drug added} \times 100 \quad (5)$$

For quantification of FIS in marketed formulation, the average weight of 20 capsules of blend powder was taken and processed for FIS assay calculation. Dried chloroform extracts of plants were diluted with mobile phase and analyzed using RP-HPLC.

2.9. FIS Stress Degradation Study

The stress degradation study was performed for FIS using the developed RP-HPLC method with acidic, alkaline, thermal, oxidative, and photolytic stress conditions suggested in ICH guidelines. As FIS was reported as a light-sensitive drug, the amber-colored volumetric flasks were used with parafilm sealing, except for the photodegradation study, and kept for 1 hour degradation time. For degradation with acid and base, FIS 1 mL stock solution (100µg/ml) was treated separately using solutions of 1 mL of 1 N HCl and NaOH for 1 h at 80°C. Neutralize the above samples and run on HPLC for analysis. Heat degradation study was done using 1 ml drug stock solution with 2 ml methanol, heated at 80°C for 1 h, and then analyzed. For oxidative stress degradation, 1 ml of stock solution (100µg/ml) was mixed with 1 ml, 30% H₂O₂ solution for 1 h and analyzed. In the photochemical degradation study, 1 ml of drug stock solution (100µg/ml) was diluted with mobile phase up to 10 ml in a

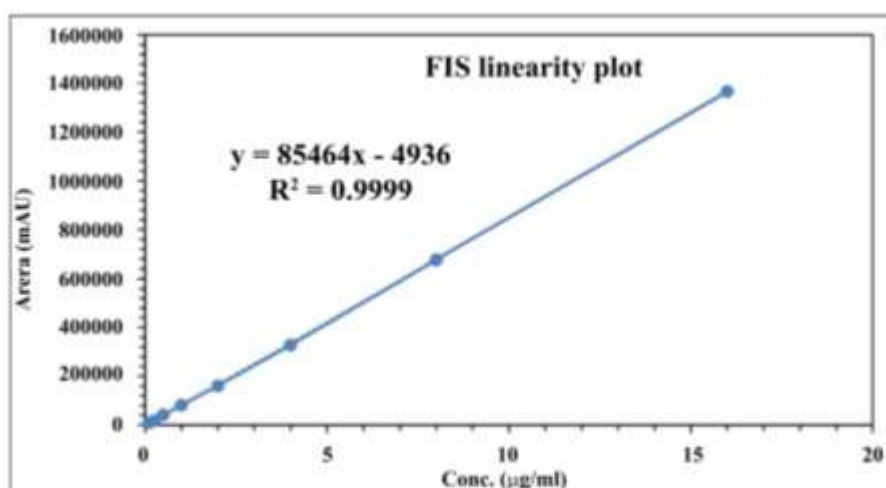


Fig. (2). Linearity graph of FIS. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

10 ml transparent volumetric flask and directly exposed to sunlight for 1 h. HPLC analysis in the above tests as performed by adjusting the final volume up to 10 ml anfiltering through 0.2 µm syringe filters [28-30].

3. RESULTS AND DISCUSSION

3.1. Development and Validation of RP-HPLC Method

An efficient RP-HPLC method was developed by exploring various reported and new combinations of mobile phases (one organic and one aqueous phase with different pH). The methanol and ACN were combined with acidified aqueous phase using HCL, orthophosphoric acid, formic acid and glacial acetic acid with their increasing concentrations. For use of methanol with aqueous phase combination, aqueous phase contains concentration of HCL (0, 0.1, 0.2 % v/v), orthophosphoric acid (0.01, 0.1, 0.2% v/v), and formic acid (0.1, 0.2, 2 %). For use of ACN with aqueous phase combination, concentration of orthophosphoric acid (0.01, 0.1, 0.2 % v/v), formic acid (0.1, 0.2 % v/v) glacial acetic acid (0.1, 0.2 % v/v) were tested. Above mobile phase, combinations were explored with increasing concentrations of aqueous phase (30:70, 40:60, 60:40, 80:20 v/v) with 10 µl injection volume of standard stock solution (10 µg/ml) and FIS peaks were detected and analyzed using a PDA detector at 360 nm. When using methanol with acidified water, a better resolved FIS peak was observed in the ratio of 45:55 v/v and for acetonitrile and acidified water, it was in a 30:70 v/v ratio.

Peak sharpness, retention time, and theoretical plates were compared for the above mobile phase. It was observed that a single sharp peak was present for the combination of ACN: formic acid 0.1% (30:70 v/v) mobile phase and gives the highest value for peak area (507435), theoretical plates (5236.056), and tailing factor (1.257) in an acceptable range. For the final selection of the mobile phase, further this ACN: formic acid 0.1 % combinations were studied over and below its 30:70 v/v range. For this, ACN: formic acid 0.1 % in combinations of 15:85, 20:80, 25:75, 30:70, 35:65 and 40:60 v/v were tested using RP-HPLC method.

In the above-tested ratio of ACN and formic acid 0.1%, the ratio of 25:75 v/v at a flow rate of 1 ml/min shows the highest values for peak area and theoretical plates. For this combination ratio, at a flow rate of 1 ml/min and 20 µl injection, it shows a peak area of 901804.33, the retention time of 6.91 min., theoretical plates 6184.87, and tailing factor 1.195 was finalized for the further validation study.

3.2. Calibration Curve Development and Linearity Range

The calibration curve was developed by injecting serial dilutions of solution B using mobile phase solution and found to be linear in the concentration range 0.1-16 µg/ml as shown in Fig. (2) (Coefficient of regression, $R^2 = 0.9999$).

3.3. Accuracy

After analyzing the known quantity of FIS spiked triplicate in the cubosomal formulation sample to get three-level concentrations (7.5, 10 and 12.5 µg/ml of FIS), the mean percent recovery of FIS was found to be in the range of 98.24 to 100.65 %. It shows that the mean percent of FIS recovered in all the selected three levels was within the prescribed limit of 95 to 105% (Table 1), which indicates good accuracy of the developed RP-HPLC method for FIS quantification in prepared cubosomal formulation.

3.4. Precision

Low, medium, and high concentration solutions at intra-day and inter-day levels were used. The % RSD values for both intra-day and inter-day analysis were observed < 2, which proves that the developed RP-HPLC method was found to be highly precise under given specified experimental conditions (Table 2).

3.5. Ruggedness and Robustness

After doing intentional minor changes in various parameters like flow rate, mobile phase ratio, oven temperature, change in formic acid concentration in percent, and absorption wavelength show < 2% changes in % RSD of peak area readings. This shows the ruggedness and robustness of the



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A Validated UV-Spectrophotometric Method For Fisetin Estimation From Cubosomal Nanoformulation, Marketed Formulation And Strawberry Fruit Extract

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Abstract

Fisetin (3,3', 4'7-tetrahydroxyflavone) is an active flavanol phytoconstituent, available in the market as a nutraceutical but poor aqueous solubility limits its therapeutic use. Even though sensitive and modern technique based fiestin estimation was reported earlier, there is a need of simple, fast and cost-effective method based on UV-Spectrophotometric technique for routine application. Developed and validated a UV-spectrophotometric method and used for estimation of fisetin from cubosomal nano-formulation, marketed formulation, and strawberry fruits. A standard calibration graph was developed for fisetin using methanol in the 2-10 µg/ml concentration range using λ_{max} 362 nm. The linearity equation for fisetin absorbance was $y=0.0947x + 0.005$ with a correlation coefficient of 0.9998. The method was accurate as fisetin recovery > 95%, precise and robust as % RSD < 2 for absorbance after a change in analyst, instrument, and wavelength. The fisetin loading and cubosomal entrapment percentage was found to be 99.48 ± 0.23 and 79.83 ± 0.65 respectively. Fisetin estimated in 'Doctor's Best * Fisetin' was 98.82 ± 0.18 mg per capsule (Label claim 100mg/capsule) and in strawberry 2.59 ± 0.21 µg/gm of its dry powder. We report a new, simple, cost-effective, fast, accurate, and sensitive UV-spectroscopic method for routine quality control of fisetin formulations and other samples.

Keywords: Fisetin, UV-spectroscopy, Fisetin cubosome nanoformulation, Doctor's Best * Fisetin, Strawberry

1. INTRODUCTION

A plant polyphenolic secondary metabolite Fisetin, is extracted from various plants like strawberry, green tea, apple, kiwi, grapes, chamomile flowers, and lotus root. It is a yellow powder extracted commercially from various plants like *Rhus succedanea* and *Cotinus coggygia* Scop and reported in varying amounts. Fisetin attracts more study attention because of the wide pharmacotherapeutic potential in the in vitro and in vivo study reports (1). Four hydroxy groups in the fisetin structure are responsible for considerable antioxidant potential, inhibit many types of angiogenesis by binding

with many anticancer receptor sites, and have anti-inflammatory effect by inhibiting inflammatory cytokines (Figure 1). However, its limited cellular absorption and bioavailability restrict its therapeutic development (2,3). Various pharmaceutical drug delivery strategies have been reported to overcome this problem. Liposomes, Polymeric micelles, Trans and Binary ethosomes, Self-nano emulsions, Polyvinyl alcohol and poly (lactic-co-glycolic acid) nanoparticles have been reported (4-10). Cubosome nanoformulation of fisetin was prepared because of its simple procedure, use of very few formulation ingredients, increased water solubility and bioavailability, and targeted drug delivery effect (11-14).

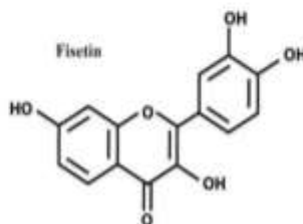


Figure 1. Fisetin Chemical Structure

Several sensitive analytical methods were used for the estimation of fisetin from plant and biological samples like HPLC (15), HP-TLC, and LC-MS/MS but are expensive, need skill and not used frequently (16).

Earlier we reported a very sensitive and reproducible RP-HPLC method using formic acid and acetonitrile mixture as mobile phase for estimation of fisetin from cubosomal formulation, various plants extract and marketed formulation along with stress degradation study of fisetin (17). UV-visible spectroscopy is a very simple, fast, and sensitive technique for the estimation of fisetin and very few reports on developed and validated methods are available. In many reports on fisetin identification and estimation from plants, the UV-visible spectroscopic method was used without method development and validation therefore its accuracy and reproducibility are questionable.

A single developed and validated UV-visible spectroscopic method was reported using solvent methanol for quantification of FIS in Self-Nanoemulsifying Drug Delivery System but not applied for plant samples and marketed formulation and was used only for their in-house formulation (18). In earlier literatures, validated UV-visible spectroscopic methods were not used for the estimation of fisetin from plant samples and formulations (19,20). Therefore, we developed and validated a UV-visible spectroscopic method for fast and simple estimation of fisetin from various types of samples. The present study aims to develop a simple, better, economical, more sensitive, and reproducible UV-visible spectroscopic method to evaluate the prepared Fisetin cubosome nanoformulation and some plant extracts for its quantification, drug entrapment efficiency, evaluation of marketed FIS formulation. The developed method is cost-effective and used for both the identification and quantification of fisetin with maximum accuracy, precision, and reproducibility from plant samples, marketed formulation, and prepared cubosomal nanoformulation. We report a validated, novel UV-visible spectroscopic method using methanol as solvent which can be used for the estimation of fisetin from different categories of samples.

2. MATERIALS AND METHODS

2.1 Materials

A gift sample of Fisetin was provided by Shaanxi Yi an Biologicals, Technology Co., Ltd. China. The Poloxamer 407 received from BASF, Mumbai, and Glyceryl monooleate from Mohini Organics Pvt. Ltd, Mumbai, were received. The analytical grade methanol, ethanol, and dimethyl sulfoxide were





Errata submission:

Observation	Clarification	Correction undertaken
1. Page no. 30 (As per Index Page 8) Freddie Bray et al., (Globocan 2018)	Corrected the referencing style	1. Page no. 30 Freddie Bray et al., (2018)
2. Page no. 34 (As per Index Page 12) Kumar R., Khursheed et al., (2019)	Corrected	2. Page no. 34 Kumar R., et al., (2019)
Skiba M, Gasmi H et al., (2021)	Corrected	Skiba M et al., (2021)
3. References		
Reference no. 6: Afroze N, Pramodh S, Shafarin J, Bajbouj K, Hamad M, Sundaram MK, et al. Fisetin Deters Cell Proliferation, Induces Apoptosis, Alleviates Oxidative Stress and Inflammation in Human Cancer Cells, HeLa. Int J Mol Sci. 2022;23(3).	Missing page number added in the reference	Afroze N, Pramodh S, Shafarin J, Bajbouj K, Hamad M, Sundaram MK, et al. Fisetin Deters Cell Proliferation, Induces Apoptosis, Alleviates Oxidative Stress and Inflammation in Human Cancer Cells, HeLa. Int J Mol Sci. 2022;23(3):1707.
Reference no. 13: Kumar RM, Kumar H, Bhatt T, Jain R, Panchal K, Chaurasiya A, et al. Fisetin in Cancer: Attributes, Developmental Aspects, and Nanotherapeutics. Pharmaceuticals. 2023;16(2).	Missing page number added in the reference	Kumar RM, Kumar H, Bhatt T, Jain R, Panchal K, Chaurasiya A, et al. Fisetin in Cancer: Attributes, Developmental Aspects, and Nanotherapeutics. Pharmaceuticals. 2023;16(2):196.
Reference no. 72: Moolakkadath T, Aqil M, Ahad A, Imam SS, Praveen A, Sultana Y, et al. Preparation and optimization of fisetin loaded glycerol based soft nanovesicles by Box-Behnken design. Int J Pharm. 2020;578(October 2019):119-125.	Corrected the typing error	Moolakkadath T, Aqil M, Ahad A, Imam SS, Praveen A, Sultana Y, et al. Preparation and optimization of fisetin loaded glycerol based soft nanovesicles by Box-Behnken design. Int J Pharm. 2020;578(October 2019):119-125.

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