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# Design and development of a Quercetin Phospholipid Complex for enhanced Permeability and Therapeutic efficacy

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## ABSTRACT

Flavonoids are one of the most important phytochemicals in terms of its significant pharmacological activity. Quercetin is one of the most abundant dietary flavonoids with poor aqueous solubility and poor oral absorption. It is rapidly cleared from the body following oral administration. Development of lipid-drug complex is a potential approach for improving the therapeutic efficacy of Quercetin. The present study aimed to formulate Quercetin Pharmacosomes and Biotin-Chitosan conjugated Quercetin Pharmacosomes for tumour targeted delivery. The Characterisation and *In vitro* drug release studies indicate that the prepared Phospholipid complexes are promising candidates for further studies in the delivery of Quercetin and other herbal drugs.

## INTRODUCTION

From ancient times, herbal medicines are used to cure illnesses due to their high potential and low side effects. Herbal medicinal products, also known as phytopharmaceuticals, are pharmaceutical products of plant origin having certain therapeutic activities. They are dosage forms consisting of one or more plant parts, processed plant parts, crude or purified extracts, isolated compounds, or combinations thereof, which provide specific or other benefits in the diagnosis, treatment and prevention of diseases. The mode of drug delivery can become the key element in a drug's therapeutic success and failure, as the choice of a drug is often influenced by the way the medicine is administered. Herbal Drug Delivery Research during the past decades had been focused on the development of better drug delivery systems like mouth dissolving tablets, sustained and extended-release formulations, mucoadhesive systems, transdermal dosage forms, microparticles, microcapsules, nanoparticles, implants etc. However, these dosage forms were not able to successfully deliver the required quantity of the active entity to the appropriate site of action in a desired rate and desired duration as needed for the therapy[1].

Novel drug delivery is a novel approach that aims to address the limitations of traditional drug delivery systems. They utilise methods by which an optimum amount of the concerned drug is administered to the patient in such a way that it reaches the appropriate site of action in the required therapeutic concentration. In phytoformulation research, developing nano dosage forms like polymeric nanoparticles and nanocapsules, liposomes, solid lipid nanoparticles, phytosomes, nanoemulsion etc. have a number of advantages, including enhancement of solubility, bioavailability, pharmacological activity, stability, etc., sustained delivery, protection from toxicity, physical and chemical degradation, etc. The nanocarrier based novel drug delivery systems of herbal drugs have a potential future for enhancing the activity and overcoming the problems associated with plant medicines.Flavonoids are phytochemical compounds most commonly found in fruits, herbs, stems, cereals, nuts, vegetables, flowers and seeds with potential applications in

medicinal chemistry. The reason for the use of many herbal products in traditional herbal medicines may be attributed to the presence of flavonoids in them. They are secondary metabolites, which mainly consists of a benzopyrone ring bearing a phenolic or poly-phenolic group at different positions. Flavonoids are found to have anticancer, antioxidant, anti-inflammatory, and antiviral properties. But when they are administered orally or topically, a poor absorption profile results, due to the hydrophilic nature of the molecules, gastric and hepatic degradation or formation of non-absorbable complexes with other substances present in the gastrointestinal tract. Flavonoids' ability to cross the biological membrane and be absorbed systemically after oral administration is limited by their water solubility and gastrointestinal stability. Flavonoids are poorly absorbed in their native form due to complex mechanisms of gastrointestinal absorption. Even when in the form of glycosides, flavonoids have low water solubility, poor bioavailability, and are rapidly altered by environmental conditions such as temperature, pH, and light. Flavonoids are thought to be extensively digested by intestinal microbes and/or enzymes, resulting in a variety of metabolites. Even if these metabolites are absorbed, they are exposed to the hepatic enzymatic system, where new metabolites of altered/decreased bioactivity can be produced. Therefore, the development of novel delivery systems for this class of compounds is highly desirable in order to achieve improved bioavailability. Development of amphiphilic drug-lipid complexes is a potential approach for improving therapeutic efficacy of the drugs by increasing solubility, release profile and oral bioavailability. Cancer is the second worldwide cause of death, exceeded only by cardiovascular diseases. It is characterised by uncontrolled cell proliferation and an absence of cell death. Cancer is caused by damage or mutations in the genetic material of the cells due to environmental or inherited factors. Targeted Nanocarriers are colloidal nano-scale systems capable of transporting anticancer agents, so that, the normal tissues are avoided and gets accumulated in tumors, achieving a cytotoxic concentration several-fold higher with a reduced toxicity for the rest of the body. In addition, these nanocarriers protect the drug from degradation and, reduce the renal clearance and increase its half-life in the bloodstream, augment the payload of cytotoxic drugs, and allow the control of the release kinetics of the anticancer drugs. Quercetin (3, 3', 4', 5, 7-pentahydroxyflavone), a polyphenolic flavonoid, is one of the most abundant dietary flavonoids found in many fruits, vegetables, leaves, seeds, and grains. It shows several biological effects like anti-inflammatory, anti-cancer, antiproliferative, antimutagenic and apoptosis induction. Quercetin is a BCS Class IV drug. Hence, it exhibits poor aqueous solubility and poor oral absorption even when ingested in large amounts. The bioavailability of quercetin in humans is low and highly variable (050%), and it is rapidly cleared with an elimination half-life of 12 hours after ingesting quercetin foods or supplements.

Biotin is water soluble vitamin (vitamin H) having essential roles in cell growth, signal transduction and many other cellular functions. Biotin receptors are over expressed in the majority of cancer cells like breast cancer, prostate cancer, pancreatic cancer and ovarian cancer. As cancer cells are under rapid proliferation, the transporters are highly expressed which desires more Biotin for rapid cell divisions. Biotin has been used as a targeting moiety with drug-loaded carriers like liposomes to increase cellular uptake in tumour cells. Biotin can ensure the selectivity, deliverability and stability of the formulation. Chitosan is a natural biodegradable polymer which increases the biocompatibility of a matrix for controlled release. It has a tendency to degrade under lysosomal enzymes at acidic pH of tumor microenvironment. Chitosan can be covalently conjugated with the acid group of Biotin and the conjugate has strong electrostatic interaction between biomolecules and nanocarriers. The Biotin-Chitosan conjugates have been explicitly proven using various studies to be an efficient targeting moiety for the delivery of nanocarrier based anticancer drug delivery systems.

In the present study, a phospholipid complex of Quercetin has been developed which can improve the solubility, permeability and stability and result in potentially improved bioavailability and increased retention in the tumour tissues. The prepared Quercetin phospholipid complex was conjugated with Biotin-Chitosan conjugates to facilitate tumor targeting.

## MATERIALS AND METHODS

Quercetin Hydrate, Biotin, N,N- Dicyclohexylcarbodiimide & Lecithin from soybean were purchased from Tokyo Chemical Industry, Japan. Dichloromethane, Dimethyl sulfoxide, Acetone & Glacial acetic acid were purchased from Merck Life Science Pvt. Ltd, Mumbai. Chitosan was purchased from Kanton Laboratories, Kerala. N- Hydroxy Succinimide was purchased from Sigma Aldrich, Sodium Hydroxide was purchased from Central Drug House, India & Potassium Dihydrogen Phosphate was purchased from LOBA Chemie Pvt Ltd, Mumbai. All other chemicals and reagents used in the study were of analytical grade. Millipore Ultra purified water had been used throughout the study.

## METHODS

PREPARATION OF QUERCETIN PHARMACOSOMES [2]

Quercetin loaded pharmacosomes were prepared by solvent evaporation method. 150 mg of Quercetin and Phosphatidylcholine was taken in a 100 ml round bottom flask and dissolved in 25 ml of Dichloromethane. The solution was refluxed and the final traces of solvent was evaporated under vacuum in a rotary vacuum evaporator. A thin lipid film was formed at the bottom of the round bottom flask. Then the film was hydrated with 30 ml of pH 7.4 phosphate buffer and vesicular suspension of pharmacosomes was formulated.

PREPARATION OF BIOTIN-CHITOSAN CONJUGATED QUERCETIN PHARMACOSOMES [3]

**Preparation of Biotin Chitosan Conjugates :**

Biotin 50 mg in 2 ml DMSO was treated with 67.3 mg Dicyclohexyl carbodiimide and 31.3 mg N- Hydroxy Succinimide for 30 min at 4°C in order to activate Biotin. The Biotin -activated solution was added dropwise to 25 ml solution of 1% w/v Chitosan in 0.1 M Acetic acid under slowly stirring at 500 rpm in a REMI 2MLH magnetic stirrer at room temperature for 24 hours. The Biotin - Chitosan conjugates was precipitated by adding 15 ml acetone and stored at 4°C.

**Preparation of Biotin-Chitosan Conjugated Quercetin Pharmacosomes:**

10 mg Biotin Chitosan conjugates were dissolved in 2 ml DMSO and sonicated for 10 minutes. Then added this solution to the above suspension of Quercetin Pharmacosomes, under stirring at 500 rpm in REMI 2MLH magnetic stirrer for 6 hours and lyophilized.

OPTIMIZATION OF BIOTIN-CHITOSAN CONJUGATED QUERCETIN PHARMACOSOMES

Optimization of Biotin-chitosan conjugated Quercetin pharmacosomes were done by varying the lipid-drug concentration, time and temperature. For this a 5 level three factor central composite design with 20 runs were selected. Biotin-Chitosan Conjugated Quercetin pharmacosomes was prepared as per the formula proposed by the Design Expert software 7 and characterized for their particle size and entrapment efficiency.

EVALUATION OF THE PREPARED PHARMACOSOMES

PARTICLE SIZE

The prepared Quercetin and Biotin-Chitosan Conjugated Quercetin Pharmacosomes were evaluated for particle size evaluated using Photon Dispersibility Spectroscopy (PSC) on a Malvern Zetasizer 3000 Ver.7.02, at a fixed angle of 90º at 25º C.

ZETA POTENTIAL

The Zeta potential is an indication of the stability of the colloidal systems and indicates the charge present on the particles of the colloidal systems. Zeta potential of the prepared Pharmacosomes were determined using Malvern Zeta sizer Ver.7.02 at 25º C after suitable dilution with distilled water. Samples were placed in clear disposable zeta cells, and results were recorded.

ENCAPSULATION EFFICIENCY

The encapsulation efficiency of the prepared pharmacosomes were determined by calculating the amount of entrapped Quercetin in the pharmacosomes. 10 ml of the pharmacosomes vesicular suspension placed in a centrifuge tube and centrifuged at 4000 rpm for 1 hour in a REMI-C24 cooling centrifuge. After centrifugation the supernatant was collected and filtered through a Whatman filter paper of pore size 45µm. It was then suitably diluted and the amount of free Quercetin was determined spectrophotometrically at 256 nm. The amount of Quercetin was determined by calibration curve method and the calibration plot was generated from a series of Quercetin solution with different concentrations. The encapsulation efficiency has been determined according to the following equation

EE% = (Total Drug  Free Drug) / Total Drug x 100

DRUG LOADING EFFICIENCY

Evaluation of drug loading efficiency of the prepared pharmacosomes were done by taking the UV-Visible absorbance for initial Quercetin content in the solution and the residual Quercetin content in the collected supernatent at 256 nm.

DLE% = (Total Drug  Free Drug) / Total amount of Nanoparticles x 100

SCANNING ELECTRON MICROSCOPY: The surface morphology of the prepared pharmacosomes were determined using a scanning electron microscope by JOEL Model JSM-6390 LV.

*IN VITRO* DRUG RELEASE STUDY[4,5]

Dissolution test was carried out using USP dissolution type II (paddle) apparatus using 150 mg Quercetin and the prepared nonconjugated and conjugated Quercetin Pharmacosomes equivalent to 150 mg pure Quercetin filled in size 1 hard Gelatin Capsules. The stirring speed was maintained at 50 rpm at 36°C±0.5°C for 24 hours in the dissolution medium of pH 7.4 phosphate buffer and pH 5 acetate buffer solutions (900 ml). At predetermined time intervals, samples were withdrawn and was replaced with fresh buffer solution. The absorbance of the sample was measured at 256 and 254 nm respectively. The cumulative release of the sample was calculated by using suitable equations with the help of the standard curve.  Drug release kinetics was studied by the kinetic equations such as zero order, first order, Higuchi model, KorsmeyerPeppas model etc.

INTESTINAL  PERMEATION STUDY [6]

Chicken ileum was kept in phosphate buffer for 20 min to equilibrate. The The Franz Diffusion cell receptor compartment was comprised of 20 ml of pH 7.4 phosphate buffer. The temperature of the receptor compartment was maintained at 36°C±0.5°C  under magnetic stirrer at 100 rpm. After 20 minutes, 150 mg Quercetin was converted to an aqueous paste and placed in the donor chamber. At predetermined time points, 1 ml samples were withdrawn from the receptor compartment, replacing the sampled volume with fresh phosphate buffer after each sampling, for a period of 24 hours. The samples were filtered and analysed for drug content by UV visible spectrophotometer at 256 nm. The cumulative amount of drug release across the mucosal membrane was determined. The same procedure was repeated using the prepared Quercetin and conjugated Quercetin pharmacosomes in the quantity equivalent to Quercetin 150 mg.

NATURAL SKIN PERMEATION STUDY[6]

Fresh Goat's skin was kept in phosphate buffer for 20 min to equilibrate. The The Franz Diffusion cell receptor compartment was comprised of 20 ml of pH 7.4 phosphate buffer. The temperature of the receptor compartment was maintained at 36°C±0.5°C  under magnetic stirrer at 100 rpm. After 20 minutes, 150 mg Quercetin was converted to an aqueous paste and placed in the donor chamber. At predetermined time points, 1 ml samples were withdrawn from the receptor compartment, replacing the sampled volume with fresh phosphate buffer after each sampling, for a period of 24 hours. The samples were filtered and analysed for drug content by UV visible spectrophotometer at 256 nm. The cumulative amount of drug release across the mucosal membrane was determined. The same procedure was repeated using the

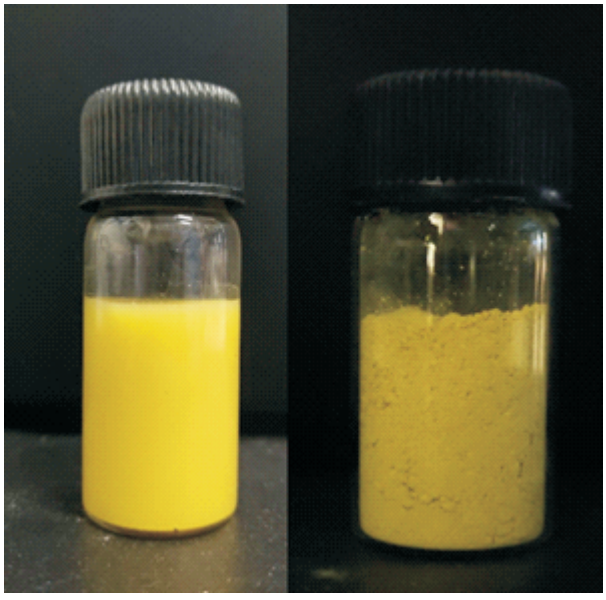

**Fig 1 :** Pharmacosomal Suspension and Pharmacosomes

prepared Quercetin and conjugated Quercetin pharmacosomes in the quantity equivalent to Quercetin 150 mg.

STABILITY STUDIES

Stability of the drug in the prepared Quercetin and conjugated Quercetin pharmacosomes were studied. Stability studies were conducted by storing the pharmacosomes at 30°C± 2°C and 70% RH ± 5% for 45 days. The samples were withdrawn at initial, 30th & 45th day and analyzed for the Encapsulation efficiency and Particle size.

## RESULTS

OPTIMISATION OF PARAMETERS AND PREPARATION OF PHARMACOSOMES

The optimised formula having Quercetin 150 mg Soy lecithin 450 mg Dichloromethane 25 ml with reflux for 3 hours at 60°C was used for preparing the pharmacosomes. (Table No. 1; Figure No. 1)

CHARACTERISATION STUDIES

The average Particle Size, Poly Dispersity Index, Zeta

**Table 1 :** Predicted and Experimental values obtained based on optimized formula for Pharmacosome preparation

| Predicted values based on optimized formula | | | | |
|---|---|---|---|---|
| Factors | | | Responses | |
| Lipid drug concentration | Time | Temperature | Entrapment efficiency | Particle size |
| 3.00 | 3.00 | 60.00 | 84.32 | 51.47 |
| Experimental values based on optimized formula | | | | |
| Factors | | | Responses | |
| Lipid drug concentration | Time | Temperature | Entrapment efficiency | Particle size |
| 3.00 | 3.00 | 60.00 | 85.95 | 52.25 |
| Percentage prediction error | | | 1.89 | 1.49 |

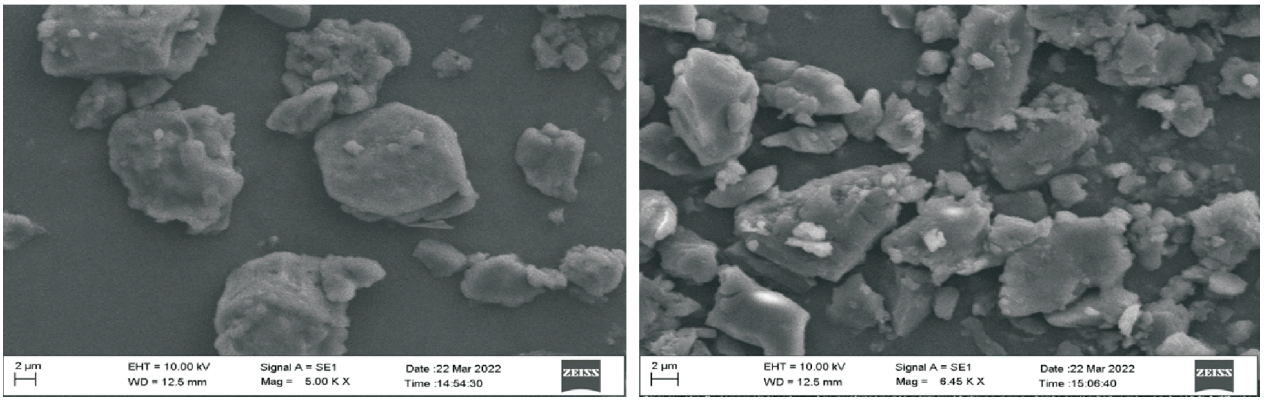


**Fig 2 :** Particle Size and Zeta Potential of Non-Conjugated and Conjugated Pharmacosomes

**Table 2 :** Physical characterisation of the Optimised Quercetin Pharmacosomes

| | Quercetin loaded Pharmacosomes | Conjugated Quercetin Pharmacosomes |
|---|---|---|
| Particle Size | 68.84 nm | 52.25 nm |
| Poly Dispersity Index | 0.435 | 0.258 |
| Zeta Potential | -46.9 mV | -28.4 mV |
| Entrapment Efficiency | 77.82% | 85.95% |
| Drug Loading Efficiency | 19.45% | 21. 48%. |

Potential, Drug Entrapment and Drug Loading Efficiency are shown in Table No. 2. The Scanning Electron Microscopic images are shown in Figure No. 2.

*IN VITRO* DRUG RELEASE

*In Vitro* drug release from Biotin-Chitosan conjugated Pharmacosomes are shown in Table No. 3 and Figure No. 3.

SKIN AND INTESTINAL PERMEATION STUDY

The skin and intestinal permeation study are shown in Table No. 4, Figure No. 4 and 5.

STABILITY STUDIES

The results of the stability studies are shown in Table No. 5.

**Table 3 :** *In vitro* drug release characterisation of the Quercetin Pharmacosomes

| % Drug Release | Ph 5 | Ph 7.4 | Kinetics |
|---|---|---|---|
| Quercetin | 20.32 | 24.73 | First Order |
| Non Conjugated Pharmacosome | 62.14 | 41.39 | Higuchi |
| Conjugated Pharmacosome | 72.72 | 39.19 | Korsemeyer Peppas |

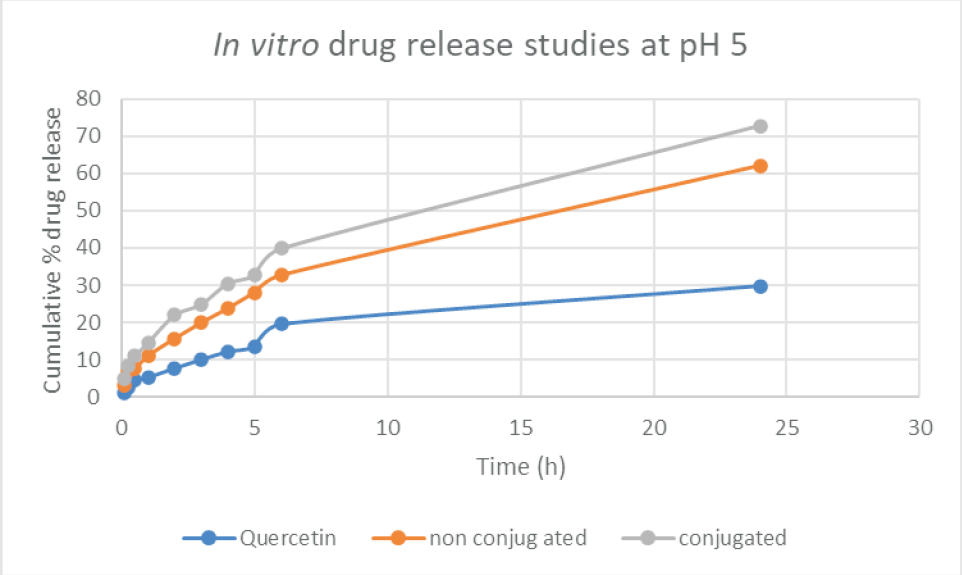


**Fig 3 :** *In vitro* Drug Release Studies

**Table 4 :** Skin and Intestinal Drug release characterisation of the Quercetin Pharmacosomes

| % Drug Release | Skin | Kinetics | Intestine | Kinetics |
|---|---|---|---|---|
| Quercetin | 24.84 | Higuchi | 16.92 | Korsemeyer Peppas |
| Non Conjugated Pharmacosome | 35.61 | Korsemeyer Peppas | 35.58 | Korsemeyer Peppas |
| Conjugated Pharmacosome | 45.33 | Korsemeyer Peppas | 36.41 | Korsemeyer Peppas |

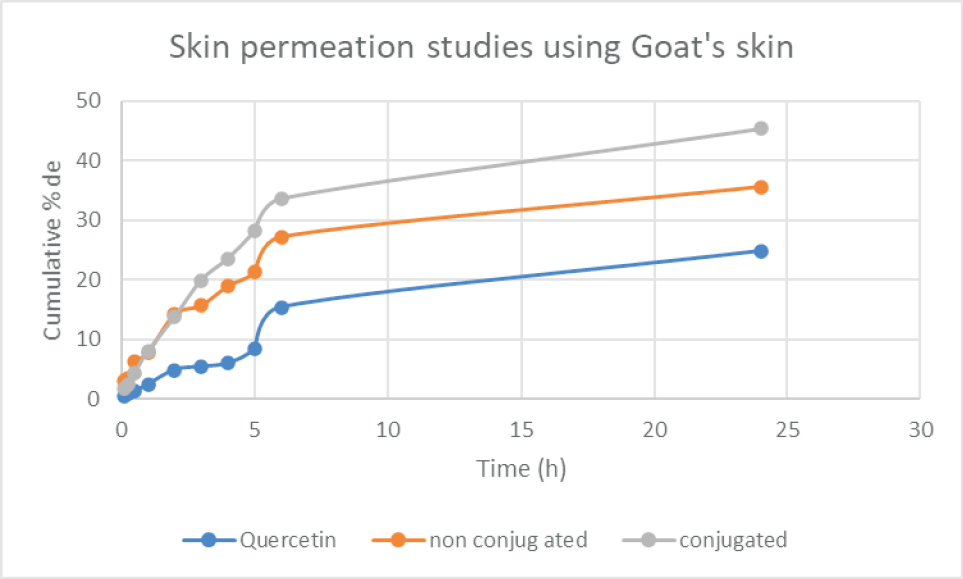


**Fig 4 :** Skin Permeation Studies

**Table 5 :** Stability studies of the prepared Pharmacosomes

| Day | Encapsulation efficiency | | | Particle size | | |
|---|---|---|---|---|---|---|
| | 0 | 30 | 45 | 0 | 30 | 45 |
| Conjugated pharmacosomes | 85.32±1.33 | 85.11±0.43 | 84.32±1.45 | 52.76±1.33 | 52.11±0.15 | 53.14±1.33 |
| Non-conjugated pharmacosomes | 78.12±1.13 | 78.23±0.11 | 77.45±1.32 | 68.13±1.56 | 67.12±0.58 | 68.32±0.11 |

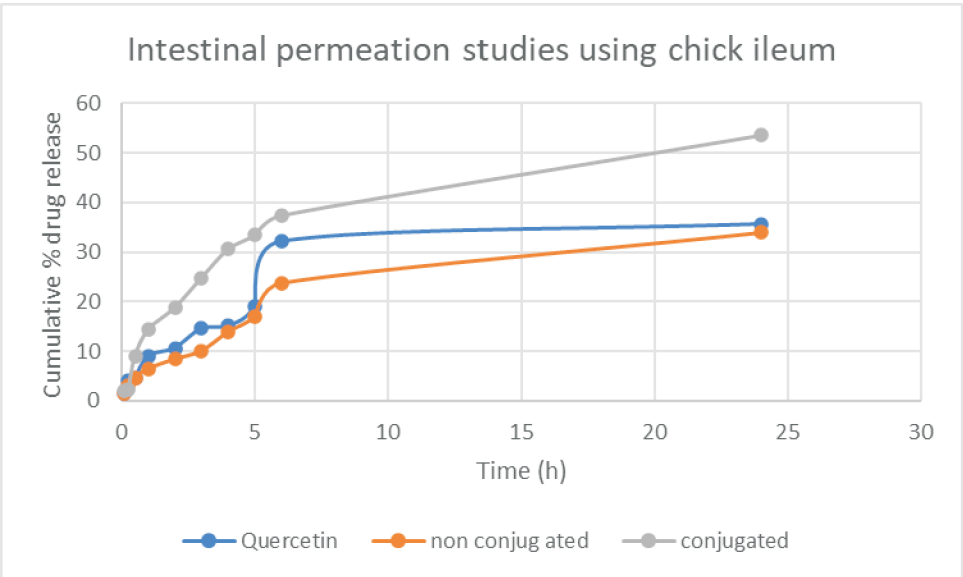


**Fig 5 :** Intestinal Permeation Studies

## DISCUSSION

The optimized formula was determined after studying the effect of independent variables on response, i.e. encapsulation efficiency and particle size. The goals were set as maximum encapsulation efficiency and minimum particle size. It was observed that the percentage of prediction error (%) was low which indicates the accuracy of the prediction by the software and utility of the experimental design for process optimization.

The average Particle Size, Poly Dispersity Index, Zeta Potential, Drug Entrapment and Drug Loading Efficiency of the prepared Pharmacosomes were found to be in satisfactory range proving that the product is thermodynamically stable and having adequate drug loading efficiency.

The Scanning Electron Microscopic images revealed that the crystalline nature of Quercetin has been lost completely and roughly spherical vesicular structures have formed. The particle size and surface characteristics of the conjugated pharmacosomes have been found to be different from that of the unconjugated pharmacosomes, probably due to the surface functionalisation.

The *In Vitro* drug release from Biotin-Chitosan conjugated Pharmacosomes were carried out at pH 7.4 and pH 5. The pH 5 has been chosen to imitate the conditions prevalent in the tumour site, and to assess the drug release parameters in tumour tissue environment. The comparison of drug release from conjugated and non-conjugated pharmacosomes has shown that the conjugated pharmacosomes in pH 5 showed the greatest drug release. At pH 5 it was found that the in vitro drug release of Quercetin from conjugated pharmacosome was best explained by Korsmeyer-Peppas Model, and the plot showed the highest linearity with a regression coefficient of 0.9964. Here the obtained 'n' value was between 0.43 and 1.0, which shows that the mechanism of drug release from conjugated pharmacosomes followed non-Fickian diffusion. At pH 7.4 it was found that the in vitro drug release of Quercetin from conjugated pharmacosomes was best explained by Higuchi model, and the plot showed the highest linearity with a regression coefficient of 0.9824. From the skin and intestinal release studies, the conjugated pharmacosomes showed higher release compared to non-conjugated pharmacosomes. It was found that the Quercetin release from the pharmacosomes was best explained by Korsmeyer-Peppas Model, which shows that the mechanism of drug release followed a non-Fickian diffusion pattern.

Not much published data was found to be available regarding the *in vitro* and *ex vivo* drug release properties of pharmacosomal delivery systems of Quercetin. In a recent study by Ammu Shaji [7], the *in vitro* drug release of Quercetin proliposomes at pH 5 and 7.4 were reported to be 60.27% and 19.41% respectively, which was lesser than what has been found in the present study. *Ex vivo* skin and intestinal release studies were not conducted. The drug release pattern was found to be Non Fickian.

The stability studies have shown that no change has occurred in the Encapsulation and Particle Size of the prepared Pharmacosomes, which indicates that the prepared product is stable under the given conditions.

## CONCLUSION

Quercetin pharmacosomes conjugated with Biotin-Chitosan has been found to be a suitable and effective drug delivery system for cancer. Chitosan conjugation with the ligand allows for pH-sensitive medication delivery at the tumour location. The overall results from the Characterisation and *In vitro* drug release studies indicate that the prepared Phospholipid complexes are promising candidates for further studies in the delivery of Quercetin and other herbal and non-herbal drugs. However, more *in-vivo* tests will be conducted to establish the formulation's safety and efficacy.

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