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Design and Development of Myricetin-Phospholipid Nanocarrier for Enhanced Intestinal Permeation.

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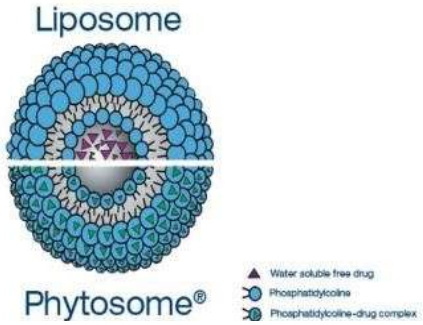
ABSTRACT: INTRODUCTION: For the treatment of intracellular infections Conventional chemotherapy is ineffective, due to limited permeation of drugs into cells. This can be overcome by use of vesicular drug delivery systems. Phytosome is a patented technology which incorporates standardized plant extracts or water soluble phytoconsituents into phospholipids to produce lipid compatible molecular complexes, so as to improve their absorption and bioavailability.
MATERIALS AND METHODS: In this study Myricetin-Phospholipid Nanocarriers were formulated by solvent evaporation method. The optimized complex was evaluated for various physicochemical parameters.The anti-proliferative effect of the complex and free drug was determined by MTT assay.
RESULTS: The process was optimised and the complex was evaluated for various physicochemical parameters. Myricetin-phospholipid complex showed better permeation than the Myricitin. The cytotoxic effect was more for complex than free drug. This can be due to increased uptake of complex by the cells.
KEY WORDS: Phytosomes, Myricetin, Encapsulation.

I. INTRODUCTION:

Most of the bioactive constituents from plants are water soluble molecules.However, low systemic bioavailability of such polar water soluble constituents likeflavonoids, terpenoid and tannins are mainly due to either multiple rings, large size, highlypolar and water soluble, or strong lipophilic nature.

Phytosome is a patented technology which incorporatesstandardized plant extracts or water soluble phytoconsituentsinto phospholipids to produce lipid compatible molecularcomplexes, called as phytosomes and so as to improve theirabsorption and bioavailability.[12]

The term "phyto" means plant while "some" means cell-like, thereforephytosomes are little cell like structures that protects the valuable components of the herbal extract from destruction by digestive secretions and gut bacteria.[13]The drug phospholipid complex was first developed in the year 1989 in Italy by chemically reacting polyphenolic extracts with phospholipids containing phosphatidyl choline. The mixture markedly increased the bioavailability of polyphenolics when compared with the pure extract.[14]



MYRICETIN

Myricetin is a member of the flavonoid class of polyphenolic compound, with antioxidant and anticancer properties. It is commonly derived from vegetables, fruits, nuts, berries and also found in red wine. Dihydromyricetin is frequently sold as a supplement and has controversial function as a partial GABAA receptor potentiator and treatment in Alcohol Use Disorder (AUD).[26]

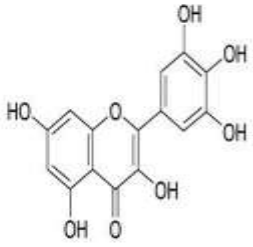


Fig 1.3: structure of Myricetin

Myricetin is able to scavenge for ROS and can chelate intracellular transition metal ions that

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ultimately produce ROS. It can induce the enzyme glutathione S-transferase (GST). GST has been suggested to protect cells against oxidative stress by protecting cells against free-radicals. In vitro studies have shown that myricetin significantly increased GST activity. Research does not indicate any side-effects on the intake of Myricetin

Reported average intake of myricetin per day varies depending diet but has been shown to be 23 mg/day. There is no specific recommended dosage of Myricetin

II. MATERIALS AND METHOD

Table 2.1: Chemicals and suppliers

Chemicals	Manufacturer/Supplier
Myricetin	Nutra Green Biotechnology
Phosphatidyl choline	Yarrow chem, Mumbai
Dichloromethane	Finar chemicals , Ahamadabad
n- hexane	Central drug house Pvt Ltd , Delhi
Sodium hydroxide	Central drug house Pvt Ltd , Delhi
Potassium dihydrogen orthophosphate	Nice chemicals, Kochi

All other chemicals and reagents used were of analytical grade.

Reverse osmosis water was prepared by Hi Media reverse osmosis water purifier used for all studies.

2.1 PREPARATION OF MYRICETIN – PHOSPHOLIPID COMPLEX

Myricetin- phospholipid complex was prepared by solvent evaporation method. 3.18 g of Myricetin and 8g phospholipid were taken in a 100 ml round bottom flask and refluxed with 30 ml of dichloromethane at 40^0C for 3 hrs. The mixture was concentrated to 5- 10 ml by placing in temperature controlled water bath over stirring for 4 hours. 10 ml of n-hexane added carefully with continuous stirring to get the precipitate which was

The morphology of the Myricetin phospholipid complex was examined usingJeol/JEM 2100at 70 kV After dilution with the original dispersion medium of thenanoemulsion, the samples were negatively stained with 1% (w/v) phosphotungsticacid for observation.

filtered and collected, stored in vacuum desiccator overnight. The dried precipitate crushed via mortar and sieved through 100 meshes. Powdered complex was stored in amber colored bottle and stored at room temperature.[66]

2.2DETERMINATION OF ENCAPSULATION EFFICIENCY[6]

10 mg of complex was transferred into centrifugation tube and dispersed in 50 ml distilled water. The dispersion was centrifuged for 5 min at 5000 rpm in REMI R-8C centrifugation apparatus. Aftercentrifugation 48 ml supernatant was collected and residue was filtered through Whatman filter paper of pore size 45 µm. It was then suitably diluted and the amount of free Myricetin was determined spectrophotometrically(ʎmax= 253 nm). The encapsulation efficiency has been determined according to the following equation:

$$EE\% = \frac{WA - WB}{WA} \times 100$$

Where, WA= Total amount of drug added before centrifugationandWB= amount of free drug measured in the lower chamber of the centrifugation tube after centrifugation.

1.2 PROCESS OPTIMISATION

Process optimisation of Myricetin phospholipid complex was done usingDesign expert software 9.0.5.1 by response surface method. A circumscribed central composite statistical design with 1 factor, 5 levels, and 13 runs was selected for the study using Design-Expert software9.0.0.6 (State-Ease Inc, Minneapolis USA).

2.3 CHARACTERIZATION OF OPTIMISED DRUG-LIPID COMPLEX

2.3.1 SEM[70]

The morphology of Myricetin phospholipid complex was examined by JEOLModel JSM – 6390LV. The samples were stained with 2% (w/v) phosphotungstic acidfor 30 s and placed on copper grids with films for viewing.

2.3.2 TEM[71]

2.3.3 ZETA POTENTIAL[72]

Zeta potential ofMyricetin phospholipid complex was determined using Malvern Zeta sizer version 6.34 at 25^0C after suitable dilution with water.

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2.4IN VITRO O INTESTINAL PERMEATION STUDIES[76]

The in vitro permeability study of optimized drug phospholipid complex and pure Myricetin was conducted using Franz diffusion cell.The donor compartment of diffusion cell was filled with 5ml of pure drug (10 mg/ml) and 5 ml of complex (equivalent to 10 mg/ml). The fluid in receptor compartment was filled with pH7.4 phosphate buffer which was maintained at 37^0c and stirred continuously at a very low speed, using thermostatically controlled magnetic stirrer with 40eflon coated bead. The external jacket of Franz diffusion cell was connected to a water bath so as to maintain temperature in cell. The excised goat intestinal mucosal membrane was mounted in between two5 ml of samples were withdrawn periodically from the receptor compartment, diluted accordingly and drug content was determined by uv spectrophotometer at 253 nm.

The volume of withdrawn sample was replaced by the same amount of receptor fluid.

Volume of drug taken inside the donor compartment = 5ml

Volume of phosphate buffer taken inside the receptor compartment = 25 ml

Cumulative amount of drug diffused (µg/cm) = C*D*V/ A

2.5 invitro Antipro liferative Effect Determination By Mtt Assay[77]

HeLa (cervical cancer) cellswere initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified Eagles medium (Gibco, Invitrogen).

The viability of cells was evaluated by direct observation of cells by Invertedphase contrast microscope and followed by MTT assay method.

Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 3 0µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubatorfor 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm (Laura B. Talarico et al., 2004).

III. RESULTS AND DISCUSSION

3.1 DEVELOPMENT OF MYRICETIN-PHOSPHOLIPID COMPLEX

Myricetin- phospholipid complex was prepared by solvent evaporation method. The dried residue was dispersed in water and stored in desiccator. Formation of complex was confirmed with the help of IR spectra.20 different formulations were prepared varying drug phospholipid ratio, reaction time and temperature and encapsulation efficiency was determined. Among 20 formulations the optimized the formulation was selected using design expert software 10.0.1.0 ®. The intestinal permeation of the prepared complex in 7.4pH buffer was determined by Franz diffusion method and compared with permeability of free drug.

3.2 DETERMINATION OFENCAPSULATION EFFICIENCY

Entrapment efficiency of Myricetin-phospholipid complex was determined by ultracentrifugation method. The results obtained is given in table 3.1.

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Table3.1:Entrapment efficiency

Absorbance (nm)	Concentration (µg)	Encapsulation Efficiency (%)
0.1223	2446	75.54

Weight of added drug = 10 mg
Free drug measured = 2446 µg

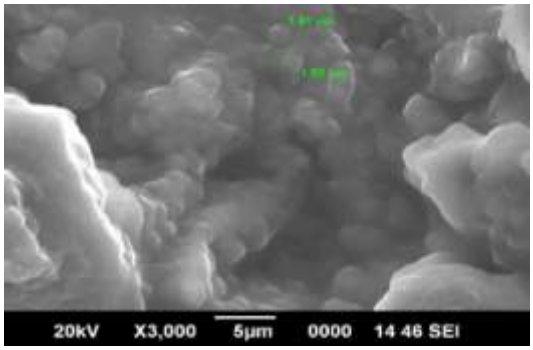
Table 3.2: PREDICTED & EXPERIMENTAL VALUES OBTAINED BASED ON OPTIMISED FORMULA

Predicted values based on optimized formula			
Factors			Responses
Phospholipid drug ratio	Time (hr)	Temperature (^{0}c)	Entrapment efficiency(%)
1.0029	2.198	69.8743	70.5665
Experimental values based on optimized formula			
Factors			Responses
Phospholipid drug ratio	Time (hr)	Temperature (^{0}c)	Entrapment efficiency(%)
1.0029	2.198	69.8743	75.54
Percentage prediction error			2.63

3.3CHARACTERIZATION OF OPTIMISED DRUG PHOSPHOLIPID COMPLEX

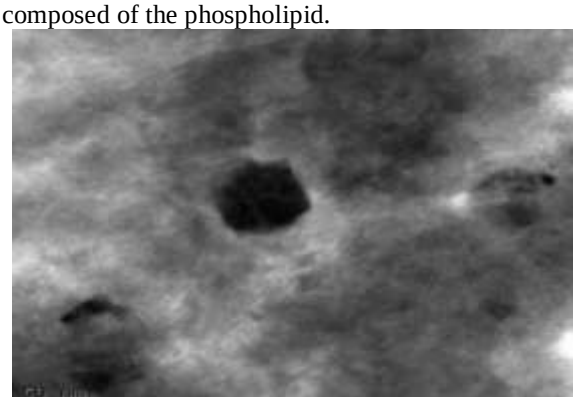
3.3.1 SEM

The surface morphology of Myricetin phospholipid complex was shown in SEM in figure. The complex was found to be of disc shaped with rough surface morphology. The surface was found to be sticky in nature



3.3.2 TEM

TEM images of drug complex shows small dark structures with a lighter envelope probably composed of the phospholipid.



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3.3.3 ZETA POTENTIAL

Zeta potential of drug phospholipid complex was found to be -4.35mV and conductivity of 0.227 mS/cm.

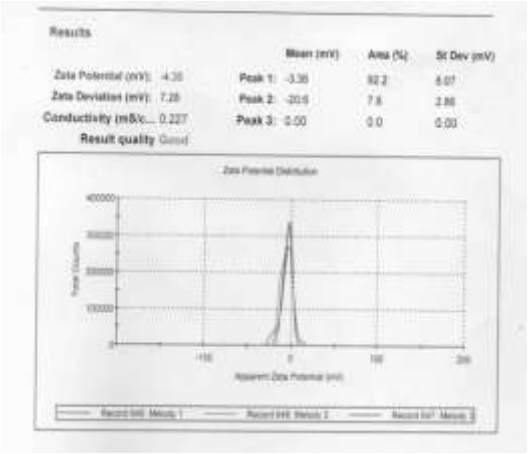

Fig: Zeta potential of complex

3.4 INVITRO DRUG PERMEATION

Invitro permeation of drug phospholipid complex and plain Myricetin was studied in Franz diffusion cell using excised goat intestine.

Table 3.3 Invitro permeation of drug phospholipid complex and free Myricetin

Drug phospholipid complex				Free drug			
Time (min)	Absorbance nm	Concentration of drug in receptor medium (µg)	Cumulative amount of drug diffused (µg)	Time (min)	Absorbance nm	Concentration of drug in receptor medium (µg)	Cumulative amount of drug diffused (µg)
15	0.0348	385.63	393.5	15	0.0171	189.49	193.35
30	0.0432	478.72	488.48	30	0.0195	216.09	220.50
45	0.0512	567.37	578.95	45	0.0238	263.74	269.12
60	0.0641	673.75	687.50	60	0.0239	287.01	292.85
90	0.0703	789.21	724.84	90	0.0392	434.39	443.25
120	0.0839	929.74	948.71	120	0.0457	508.42	516.75

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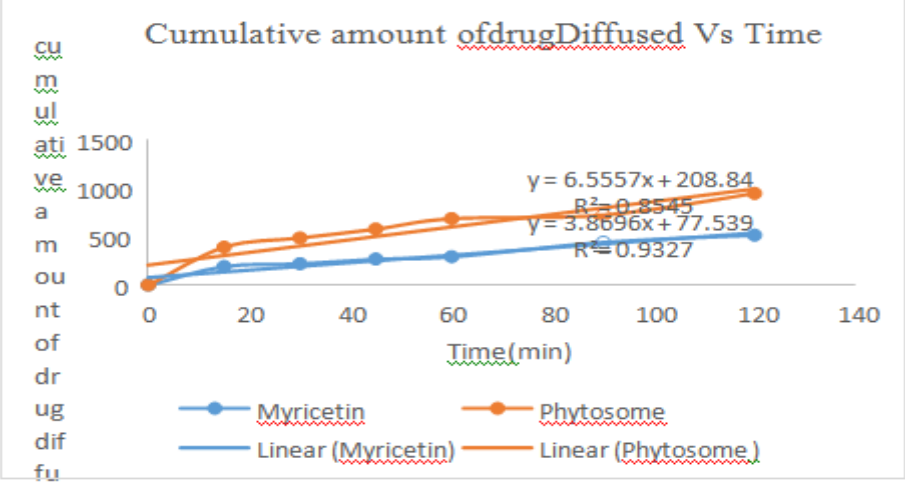

Fig 3.1:Cumulative amount of drug diffused vs time

At the end of 2hr 948.71 µg of Myricetin phospholipid complex was diffused by permeation via intestinal membrane in pH 7.4 buffer. Only 516.75 µg amount of free drug was diffused and it indicates the less drug permeation. Myricetin-phospholipid complex showed better permeation than the Myricetin.

3.5 INVITRO ANTI-PROLIFERATIVE EFFECT DETERMINATION BY MTT ASSAY

The results of anti-proliferative effect of Myricetin – phospholipid complex and pure Myricetin are as follows.

Table 3.4: Percentage Viability of drug phospholipid complex and free Myricetin

Sample Concentration (µg/ml)	**Average OD at 540nm**	**Percentage Viability**
Control	0.8291	
Sample-1 -std		
6.25	0.6035	72.78977
12.5	0.5791	69.84682
25	0.5327	64.25039
50	0.3993	50.85032
100	0.3383	46.653
Sample-2 -mp		
6.25	0.5236	63.15282
12.5	0.4734	57.09806
25	0.4455	53.73296

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50	0.4216	48.16066
100	0.3868	40.80328

The Myricetin - phospholipid complex and plain Myricetin was studied for antiproliferative effect. MTT assay was performed to study the anti-proliferative effect. The results obtained indicated that percentage viability of complex decreased with increasing concentration. The cytotoxic effect was more for complex than free drug. This can be due to increased uptake of complex by the cells. This further strengthens the fact that phospholipid complex are the herbal products wherein the individual components of herbal extracts binds to phosphatidylcholine which provides better pharmacological activity and bioavailability as compared to its pure molecular adducts.

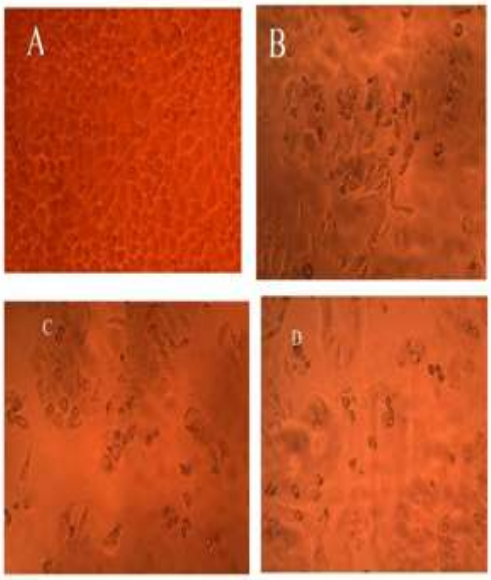

Fig. 3.2: Photos of MTT assay of Myricetin on HeLa cell lines. A. Control B.6.25µg/ml C.50 µg D.100 µg/mCONCLUSION:

Phospholipid complexation is one of the better advancements in the field of solubility enhancement of both phytopharmaceuticals and other drugs. In the present study a novel Myricetin phospholipid nanocarrier was prepared by simple and reproducible method. Encapsulation efficiency of the complex was determined by ultracentrifugation method and was found to be 70%. The process was optimised and the complex was evaluated for various physicochemical parameters. In vitro permeation of Myricetin phospholipid nanocarrier was also increased as compared to free drug. The anti-proliferative effect of the complex and free drug was determined by MTT assay. The complex shows greater decrease in viability than free drug.

The results of the present study show that the prepared phospholipid complex is a promising delivery system for enhancing the cellular uptake of Myricetin. However, more studies are essential to establish the in vivo bioavailability and efficacy.

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