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# Extraction phytochemical screening and development of phytosomes of hydroalcoholic extract of *Terminalia chebula* Retz for hepetoprotection

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## ABSTRACT

Evidences from ethnopharmacological practices have shown that *Terminalia chebula* Retz are traditionally used to treat symptoms of the liver disorder. In the current days, most of the prevailing diseases and nutritional disorders are treated with natural medicines. The effectiveness of any herbal medication is dependent on the delivery of effective level of the therapeutically active compound. But a severe limitation exists in their bioavailability when administered orally or by topical applications. Phytosomes are recently introduced herbal formulations that are better absorbed and as a result produced better bioavailability and actions than the conventional phyto molecules or botanical extracts. The aim of the present study was to evaluate qualitative and quantitative phytochemical analysis, HPLC, optical microscopic study and in vitro antioxidant activities of leaf of *Terminalia chebula* Retz collected from Bhopal region of Madhya Pradesh. The Hydroalcoholic extract of phytosome was prepared in phospholipids:cholesterol. Characterization of phytosome was done by FTIR, Entrapment efficiency, Particle size and size distribution, optical microscopic study, HPLC and stability studies, In vitro dissolution studies, and in vitro antioxidant activity by DPPH model. Combination of phospholipids and *Terminalia chebula* Retz can result in synergistic effect, Synergistic effect measure with free radical scavenging activity use DPPH model.

**Keywords:** *Terminalia chebula* Retz, Phytosome, Phospholipids, DPPH model, free radical scavenging activity

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## INTRODUCTION

Liver is the largest organ which can be damaged by numerous causes including pathogen infections, harmful chemicals and alcohol or drug abuse [1]. Liver is well-known to have a high potential of regeneration and recovery from injury[2]. Rarely, severe and acute case of liver injury can lead to life-threatening clinical syndromes including jaundice, severe coagulopathy, and high rates of mortality. There are still medical tasks for elucidating its pathophysiological mechanisms and development of efficient therapy for especially severe hepatic injury[3]. Terminalia chebula (T. chebula) Retz. (Combretaceae) which exhibited a number of medicinal activities due to the presence of a large number of different types of phytoconstituents. The fruit of the tree possesses diverse health benefits and has been used as traditional medicine for household remedy against various human ailments since antiquity[4-6]. T. chebula has been extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine. The observed health benefits may be credited to the presence of the various phytochemicals like polyphenols, terpenes, anthocyanins, flavonoids, alkaloids and glycosides. The phytosome (technology was developed by Indena s.p.a of Italy), are used to enhance the bioavailability of phytomedicines by incorporating phospholipids into standardized plant extract[7]. It is novel drug delivery system in which hydrophilic choline moiety (head) binds to phytoconstituents (polar) and lipophilic phosphatidyl moiety surrounds choline bound phytoconstituents or form outer layer, hence water soluble phytoconstituents become lipid soluble[8]. Phytosomes contains naturally occurring phospholipid, phosphatidylcholine (PC) like soyl ecithin. It is also a cellular component which is biodegradable and has reported hepatoprotective activity. Phytosomes have improved pharmacokinetic and pharmacological parameter[9-11].

## MATERIALS & METHODS

### Materials

The leaves of T. chebula were collected from local area of Bhopal (M.P.).

### Chemical and reagents

All the chemicals used in this study were obtained from HiMedia Laboratories Pvt. Ltd. (Mumbai, India), SigmaAldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-

Chem Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

**Extraction procedure**

Following procedure was adopted for the preparation of Hydroalcoholic extracts from the shade dried and powdered herbs of T. chebula.

**Defatting of plant material**

Leaves of T. chebula were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether. The extraction was continued till the defatting of the material has been taken place.

**Extraction by maceration process**

Dried powdered leaves of T. chebula has been extracted with hydro alcoholic solvent (30:70) using maceration process for 48 hrs. The extracts were evaporated above their boiling points and stored in an air tight container free from any contamination until it was used. Finally the percentage yields were calculated of the dried extracts.

**Qualitative phytochemical analysis of plant extract**

The T. chebula leaves extract obtained was subjected to the preliminary phytochemical analysis following standard methods by Khandelwal and Kokate[12,13] . The extract was screened to identify the presence or absence of various active principles like phenolic compounds, carbohydrates, flavonoids, glycosides, saponins, alkaloids, fats or fixed oils, protein and amino acid and tannins.

**Total flavonoids determination**

The total flavonoid content was determined using the method of Olufunmiso et al[14]. 1 ml of 2% AlCl3 methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; the absorbance of the reaction mixture was measured at 420 nm using UV/visible spectrophotometer The content of flavonoids was calculated using standard graph of quercetin and the results were expressed as quercetin equivalent (mg/g).

**DPPH free radical scavenging assay**

DPPH scavenging activity was measured by modified method[14]. DPPH scavenging activity was measured by the spectrophotometer. Stock solution (6 mg in 100ml methanol) was prepared such that 1.5 ml of it in 1.5 ml of methanol gave an initial absorbance. Decrease in the absorbance in presence of sample extract at different concentration (10-100 µg/ml) was noted after 15 minutes. 1.5 ml of DPPH solution was taken and volume made till 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 1.5 ml of DPPH and 1.5 ml of the test sample of different concentration were put in a series of volumetric flasks and final volume was adjusted to 3 ml with methanol. Three test samples were taken and each processed similarly. Finally the mean was taken. Absorbance at zero time was taken for each concentration. Final decrease in absorbance was noted of DPPH with the sample at different concentration after 15 minutes at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation: % inhibition = [(absorbance of control - absorbance of sample)/absorbance of control] × 100%. Though the activity is expressed as 50% inhibitory concentration (IC50), IC50 was calculated based on the percentage of DPPH radicals scavenged. The lower the IC50 value, the higher is the antioxidant activity.

**Formulation development of phytosomes**

**Preparation of phytosomes**

The complex was prepared with phospholipids: Cholesterol and T. chebula in the ratio of 0.5:0.4:1, 1:0.8:1, 1.5:1.2:1, 2:1.6:1 respectively. Weight amount of extract and phospholipids and cholesterol were placed in a 100ml round-bottom flask and 50ml of methanol was added as reaction medium. The mixture was refluxed and the reaction temperature of the complex was controlled to 50°C for 3 h. The resultant clear mixture was evaporated and 20 ml of n-hexane was added to it with stirring. The precipitated was filtered and dried under vacuum to remove the traces amount of solvents. The dried residues were gathered and placed in desiccators overnight and stored at room temperature in an amber colored glass bottle Table 1.

**Process variables used for optimization**

The developed formulation was optimized by selecting following process variables.

- Effect of ethanol concentration
- Effect of lecithin concentration
- Drug concentration

**Table 1 Different formulation of phytosomes**

**Optimization of Phospholipid and cholesterol**

| Formulation | Ratio of Phospholipid and Cholesterol | Drug Concentration (%) | Alcohol Concentration | % Entrapment Efficiency |
|---|---|---|---|---|
| F1 | 0.5:0.4 | 1 | 50 | 51.25±0.52 |
| F2 | 1.0:0.8 | 1 | 50 | 63.23±0.65 |
| F3 | 1.5:1.2 | 1 | 50 | 76.56±0.32 |
| F4 | 2.0:1.6 | 1 | 50 | 65.32±0.45 |

*Average of three determination

**Optimization of Drug Concentration**

| Formulation | Ratio of Phospholipid and Cholesterol | Drug Concentration (%) | Alcohol Concentration | % Entrapment Efficiency |
|---|---|---|---|---|
| F5 | 1.5:1.2 | 0.5 | 50 | 65.65±0.14 |
| F6 | 1.5:1.2 | 1.0 | 50 | 65.98±0.65 |
| F7 | 1.5:1.2 | 1.5 | 50 | 55.69±0.47 |
| F8 | 1.5:1.2 | 2.0 | 50 | 63.12±0.58 |

*Average of three determination

**Optimization of Alcohol Concentration**

| Formulation | Ratio of Phospholipid and Cholesterol | Drug Concentration (%) | Alcohol Concentration | % Entrapment Efficiency* |
|---|---|---|---|---|
| F9 | 1.5:1.2 | 1.0 | 25 | 65.45±0.54 |
| F10 | 1.5:1.2 | 1.0 | 50 | 77.56±0.62 |
| F11 | 1.5:1.2 | 1.0 | 75 | 63.32±0.58 |
| F12 | 1.5:1.2 | 1.0 | 100 | 58.98±0.85 |

*Average of three determination

## Characterization

### Determination of interaction between T. chebula and phospholipids

Fourier transform infrared spectrophotometer (FT-IR Spectrometer, Bruker alpha) was used to study the interaction between T. chebula and phospholipids. The IR spectra of T. chebula extract, phospholipids, their complex and physical mixture were obtained by the KBr method.

### Entrapment efficiency

Phytosome preparation was taken and subjected to centrifugation using cooling centrifuge (Remi) at 12000 rpm for 30min. The clear supernatant was siphoned off carefully to separate the non-entrapped quercetin and the absorbance of supernatant for non-entrapped T. chebula was recorded at $\lambda_{max}$ 420.0 nm using UV/visible spectrophotometer (Labindia 3000+). Sediment was treated with 1ml of 0.1 % Triton x 100 to lyse the vesicles and diluted to 100 ml with phosphate buffer saline (7.4) and absorbance taken at 420.0 nm. Amount of quercetin in supernatant and sediment gave a total amount of T. chebula in 1 ml dispersion. The percent entrapment was calculated by following formula.

$$\text{Percent Entrapment} = \frac{\text{Amount of drug in sediment}}{\text{Total amount of drug added}} \times 100$$

### Particle size and size distribution

The particle size, size distribution and zeta potential of optimized phytosomes formulation were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetamaster ZEM 5002, Malvern, UK). The electric potential of the phytosomes, including its Stern layer (zeta potential) was determined by injecting the diluted system into a zeta potential measurement cell.

### Quantitative HPLC Study

### Instrumentation

A thermospectronic model of Labindia 3000 + UV/VIS Spectrophotometer with 1cm. matched quartz cells was used for determination of $\lambda_{max}$. The HPLC system (Waters) consisted of a pump, a U.V. Visible detector, a Thermo C18 (250 X 4.6 mm, 5µm) column, a Data Ace software.

### Chromatographic conditions

The chromatographic analysis was performed at ambient temperature on a RP-C18 analytical column with a mobile phase composed of acetonitrile: methanol (50:50 v/v) and was isocratically eluted at a flow rate of 1 mL min-1. A small sample volume of 20 µL was used for each sample run, being injected into the HPLC system. The chromatogram was monitored with UV detection at a wavelength of 256 nm.

### Sample Preparation

10 mg phytosomes was taken in 10 ml volumetric flask and dilute upto the mark with Methanol; resultant solution was filtered through Whatmann filter paper and finally volume made up to mark with same solvent to obtain concentration of 1000 µg/ml. The resulting solution was again filtered using Whatmann filter paper no. 41 and then sonicated for 10 min.

### *In vitro* drug release study

*In vitro* drug release of the sample was carried out using USP- type II dissolution apparatus (Paddle type). The dissolution medium, 900 ml 0.1N HCl was placed into the dissolution flask maintaining the temperature of 37±0.50c and rpm of 50. Equivalent to 100 mg of phytosomes was placed in each bowl of dissolution apparatus. The apparatus was allowed to run for 10 hours. Sample measuring 5 ml were withdrawn after every 1 hour up to 10 hours using 10ml pipette. The fresh dissolution medium ($37^0$C) was replaced every time with the same quantity of the sample. From this take 0.5 ml and dilute up to 10ml and take the absorbance at 420.0 nm using spectroscopy.

### Preparations of hydroalcoholic extract of T. chebula phytosomes

In this study, we prepared the T. chebula -phospholipids complex to improve the lipophilic properties of T. chebula. We prepared the complex with different quantity ratios of phospholipids and T. chebula such as 0.5, 1, 1.5, and 2. The results showed that when the ratio was lower than 1, the stability of the T. chebula –phospholipids complex was worse. To get the best complex and use the smallest quantity of phospholipid, we finally prepared a T. chebula -phospholipids complex with a 1:1.5:1.2 ratios of ingredients.

### Process variables used for optimization

Three process variables as concentration of lipid, concentration of drug and concentration of alcohol were used to optimize the best formulation of Hydroalcoholic extract of T. chebula. T. chebula loaded phytosomes and all the formulations were preliminary evaluated for drug entrapment efficiency and particle size.

### Optical microscopic study

Phytosome was observed under Microscopy, Cippon (Japan). One drop of diluted extract-loaded phytosome suspension was deposited on a glass slide and it was. Excess of solution was drained off with a filter paper and then slide was allowed to dry. The sample was then examined by optical Microscopy.

### Stability studies

Results of stability studies clearly indicates that optimized batches of phytosomes were stable over the chosen temperature and humidity conditions up to 3 months as were found no significant variation in physical appearance and % drug content.

## RESULTS AND DISCUSSION

Organoleptic evaluation represents those properties of materials that can be done by using the sense organs. It thereby defines some specific characteristics of the material which can be considered as a first step towards establishing the identity and degree of purity of the material. The results of qualitative phytochemical analysis of the crude powder of leaves of T. chebula are shown in Table 2.

The total flavonoids content of the extracts was expressed as percentage of quercetin equivalent per 100 mg dry weight of sample. The total flavonoids estimation of hydroalcoholic extracts of leaves of T. chebula showed the content values of 0.891 Table 3.

**Table 2 Phytochemical screening of hydroalcoholic extract of T. chebula**

| S. N. | Constituents | *Terminalia chebula* |
|---|---|---|
| 1. | **Alkaloids** [Hager's test] | -ve |
| 2. | **Flavonoids**<br>Lead acetate<br>Alkaline test | <br>-ve<br>+ve |
| 3. | **Phenolics**<br>$Fecl_3$ | <br>-ve |
| 4. | **Proteins and Amino acids**<br>Xanthoproteic test | <br>+ve |
| 5. | **Carbohydrates**<br>Fehling's test | <br>+ve |
| 6. | **Saponins**<br>Foam test | <br>+ve |
| 7. | **Diterpins**<br>Copper acetate test | <br>+ve |

**Table 3 Total flavanoid content of hydroalcoholic extract of T. chebula**

| S. No. | Extracts | Total flavanoid (QE) (mg/100mg) |
|---|---|---|
| 1. | Hydroalcoholic extract of *Terminalia chebula* | 0.891 |

There is increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in herbs and medicinal plants. Antioxidant activity of Hydroalcoholic extract of T. chebula is measured by free radical scavenging activity and reducing power assay. The tested plant extracts showed strong antioxidant activity Table 4 and Fig 1.

**Table 4 % Inhibition of ascorbic acid and hydroalcoholic extract of T. chebula using DPPH method**

| S. No. | Concentration | % Inhibition | |
|---|---|---|---|
| | | Ascorbic acid | Hydroalcoholic extract of *Terminalia chebula* |
| 1 | 10 | 36.65 | 37.66 |
| 2 | 20 | 63.56 | 48.65 |
| 3 | 40 | 76.00 | 59.08 |
| 4 | 60 | 89.01 | 71.30 |
| 5 | 80 | 92.713 | 87.44 |
| 6 | 100 | 96.41 | 89.01 |
| $IC_{50}$ | | 8.19 | 25.28 |

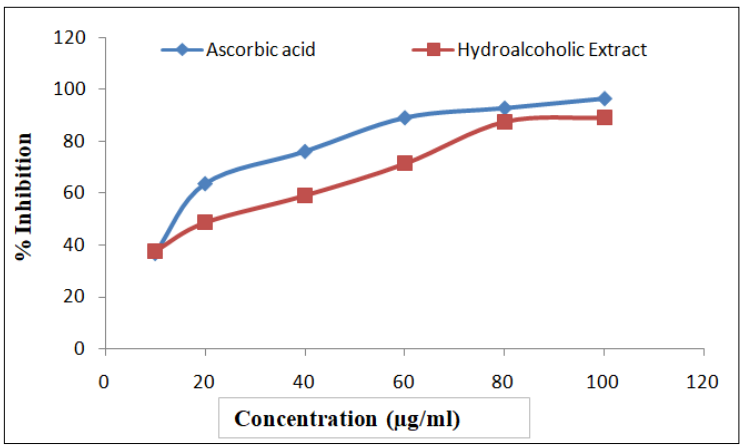


**Figure 1 % Inhibition of ascorbic acid and hydroalcoholic extract of T. chebula using DPPH method**

From the FTIR data of the extract and phytosome formulation it is clear that functionalities of drug have remained unchanged including intensities of the peak. This suggests that during the process extract and Phospholipid-Cholesterol has not reacted with the drug to give rise to reactant products. So there is no interaction between them which is in favor to proceed for formulation of phytosomes drug delivery Fig 2 & 3.

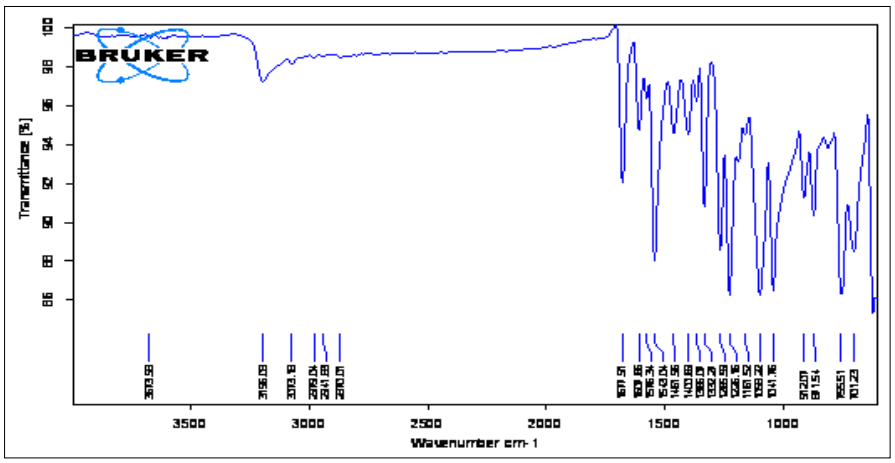


**Figure 2 FT-IR spectrum of hydroalcoholic extract of T. chebula**

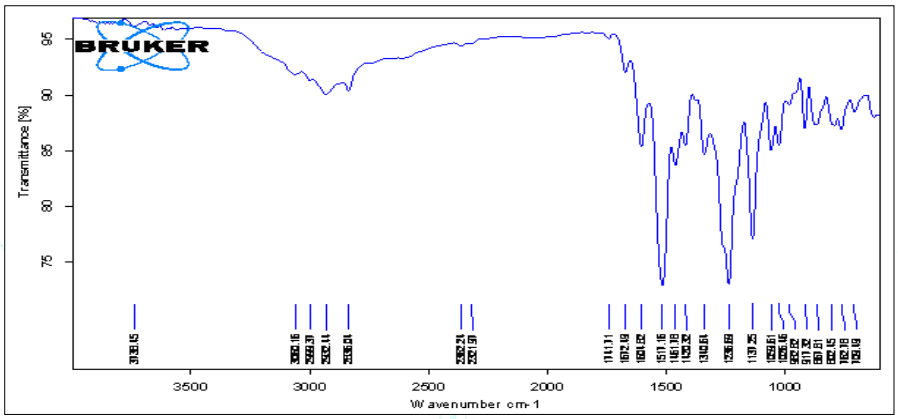


**Figure 3 FT-IR spectrum of hydroalcoholic extract of T. chebula loaded phytosomes**

Entrapment efficiency is an important parameter for characterizing phytosomes. In order to attain optimal encapsulation efficiency, several factors were varied, including the concentration of the lipid, concentration of drug and concentration of alcohol. The entrapment efficiency of all the prepared formulations is shown in Table. The entrapment efficiency of the phytosomes was found in the range of 51.25±0.52 to 77.56±0.62%. Particle size of all formulations found within range 226.32±0.45 to 338.89±0.85nm. Concentration of lipid has shows significant impact on size of phytosomes. Formulation F10 was found best one which is further evaluated for drug release study, solubility studies and UV spectroscopy. The best formulation of *T chebula* - phospholipid complex (1.5:1.2) formulation F10 was subjected to structural analysis by drug release study Table 5 & 6 and Fig 4-8.

**Table 5 Particle size and entrapment efficiency of drug loaded phytosomes**

| Formulation Code | Particle size | Entrapment Efficiency |
|---|---|---|
| **F1** | 245.56±0.45 | 51.25±0.52 |
| **F2** | 239.45±0.32 | 63.23±0.65 |
| **F3** | 280.23±0.41 | 76.56±0.32 |
| **F4** | 305.45±0.35 | 65.32±0.45 |
| **F5** | 338.89±0.85 | 65.65±0.14 |
| **F6** | 285.65±0.65 | 65.98±0.65 |
| **F7** | 296.45±0.41 | 55.69±0.47 |
| **F8** | 278.98±0.32 | 63.12±0.58 |
| **F9** | 269.56±0.25 | 65.45±0.54 |
| **F10** | 226.32±0.45 | 77.56±0.62 |
| **F11** | 292.56±0.23 | 63.32±0.58 |
| **F12** | 291.45±0.14 | 58.98±0.85 |

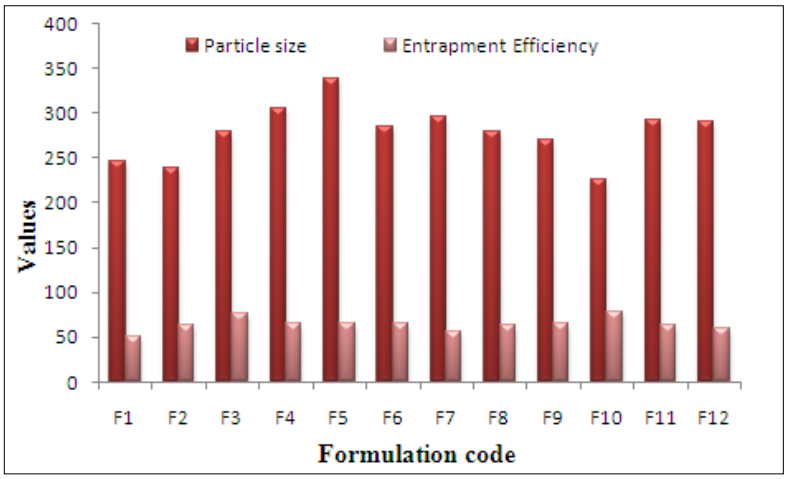


**Figure 4 Particle size and entrapment efficiency of drug loaded phytosomes**

**Entrapment efficiency**

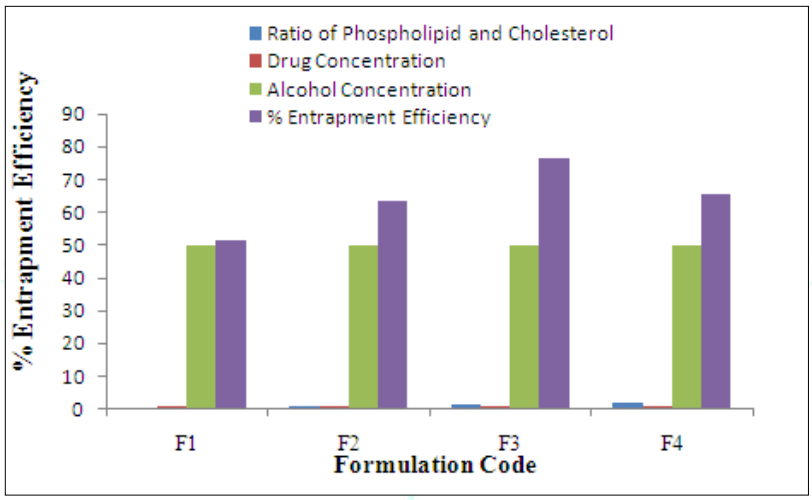


**Figure 5 Optimization of phospholipid and cholesterol**

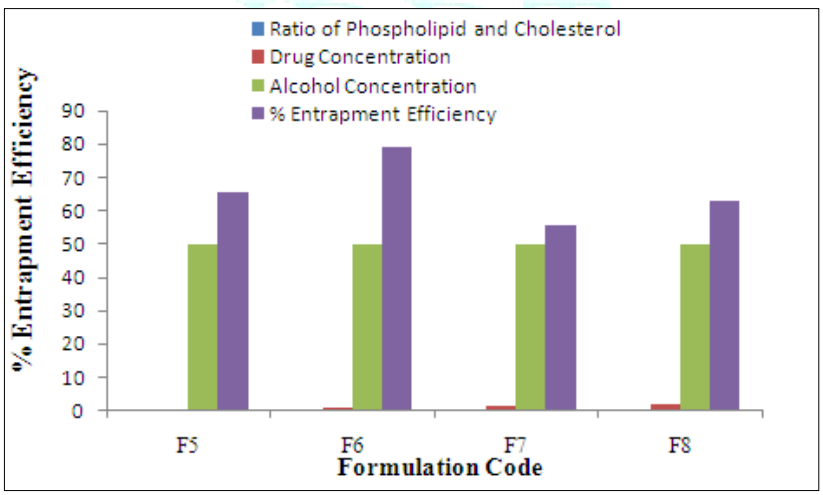


**Figure 6 Optimization of drug concentration**

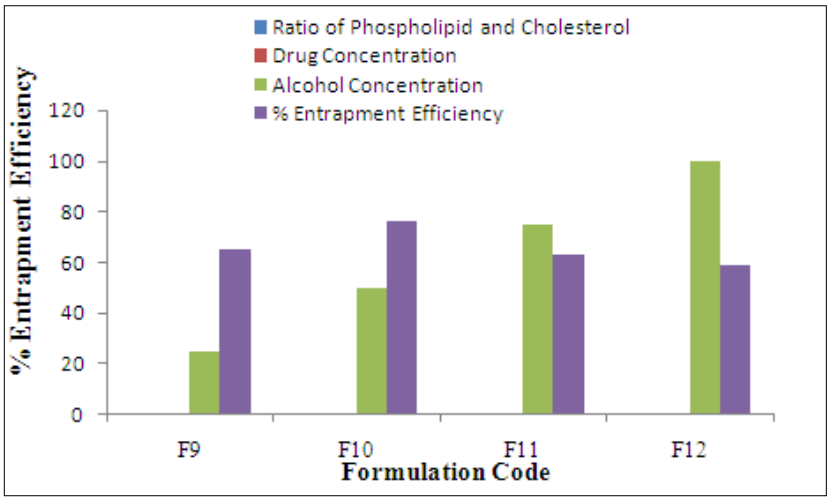


**Figure 7 Optimization of alcohol concentration**

**Table 6 Composition of best formulation**

| Formulation Code | Lecithin Concentration (%) | Drug Concentration (%) | Alcohol Concentration |
|---|---|---|---|
| F10 | 1.5:1.2 | 1.0 | 50 |

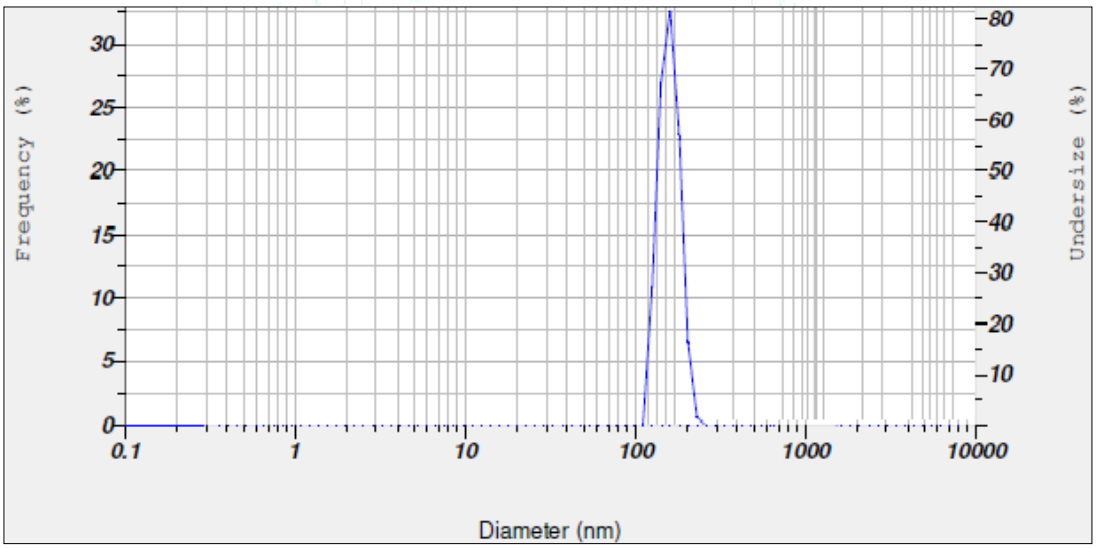


**Figure 8 Particle size of optimized batch F10**

A reverse phase C-18 column equilibrated with mobile phase methanol: acetonitrile (50:50 v/v) was used. Mobile phase was filtered through Whatmann filter paper and degassed. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 256 nm. The sample was injected using a 20 µl fixed loop, and the total run time was 10 min. The sample solution was chromatographed and a concentration of quercetin in Extract sample was found out using regression equation Table 7 & 8 and Fig 9 & 10.

**Preparation of the calibration curve of quercetin**

Each of the standard drug solutions were injected 3 times and the mean peak area of drug was calculated and plotted against the concentration of the drug. The regression equation was found out by using this curve.

**Table 7 Characteristics of the analytical method derived from the standard calibration curve**

| Compound | Linearity range µg/ml | Correlation co-efficient | Slope | Intercept |
|---|---|---|---|---|
| Quercetin | 5-25 | 0.999 | 51.18 | -10.11 |

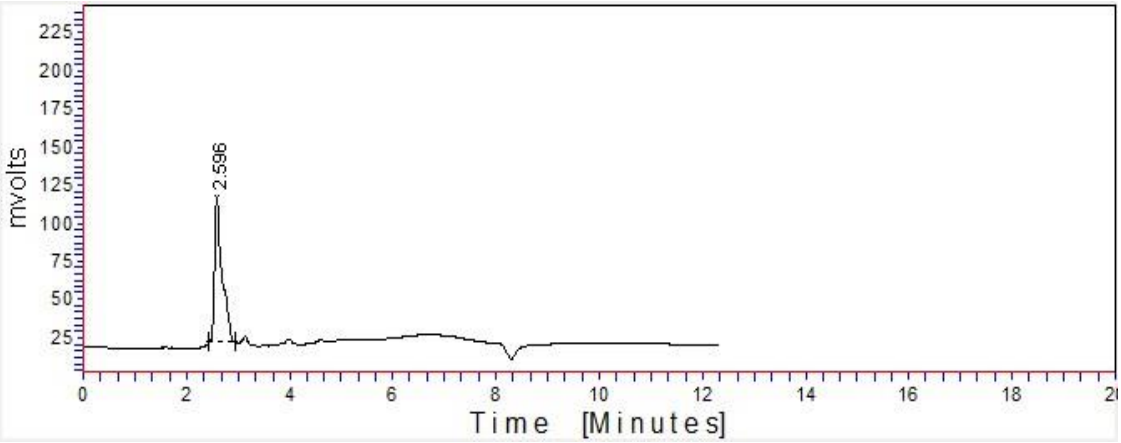


**Figure 9 Chromatogram of standard quercetin**

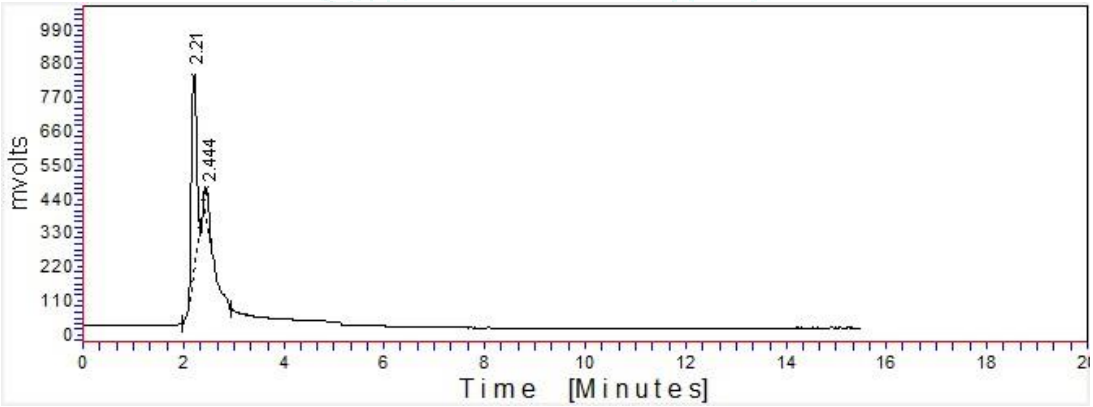


**Figure 10 Chromatogram of extract loaded phytosomes**

**Table 8Quantitative estimation of quercetin in phytosomes**

| S. No. | Formulation | RT | Area | % Assay |
|---|---|---|---|---|
| 1. | **Phytosomes** | 2.444 | 215.156 | 0.401 |

In vitro dissolution study of F10 indicated that the phytosomes had extended release dissolution patterrn. The phytosomes show of 6 hr. 69.98 % release table 9 & Fig. 11.The kinetic release profile of phytosome shows that formulation F10 was best formulation and it follows pappas release profile. phytosome released drug in controlled release manner in 6hr Table 9& 10 Fig 11-14 .

**Table 9 *In vitro* drug release data for F10**

| S. No. | Time (Hrs) | Square Root of Time | Log Time | Cumulative* Percentage Drug Release±SD | Log Cumulative Percentage Drug Release | Cumulative Percent Drug Remaining | Log cumulative Percent Drug Remaining |
|---|---|---|---|---|---|---|---|
| **1** | 1 | 1 | 0 | 12.25 | 1.088 | 87.75 | 1.943 |
| **2** | 2 | 1.414 | 0.301 | 25.45 | 1.406 | 74.55 | 1.872 |
| **3** | 3 | 1.732 | 0.477 | 33.12 | 1.520 | 66.88 | 1.825 |
| **4** | 4 | 2 | 0.602 | 43.12 | 1.635 | 56.88 | 1.755 |
| **5** | 5 | 2.236 | 0.699 | 60.25 | 1.780 | 39.75 | 1.599 |
| **6** | 6 | 2.449 | 0.778 | 69.98 | 1.845 | 30.02 | 1.477 |

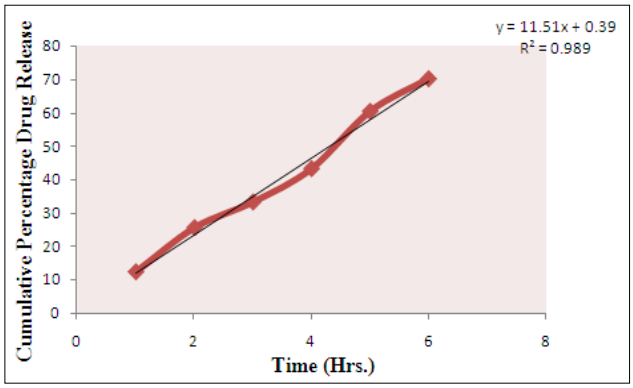


**Figure 11 Cumulative percent drug released Vs time (Zero Order Plots) of formulation F10**

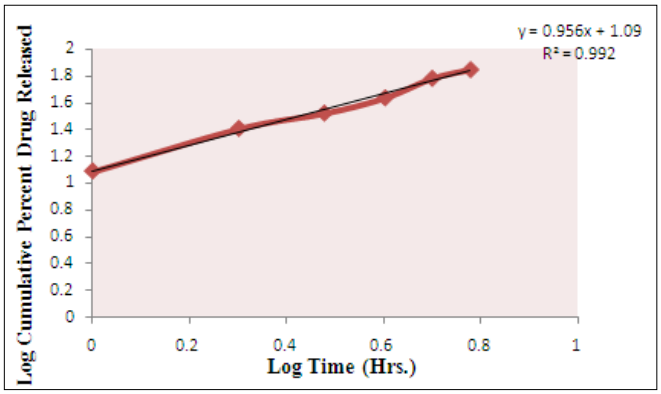


**Figure 13 Log Cumulative Percent Drug Released Vs Log Time (Peppas Plots) of Formulation F10**

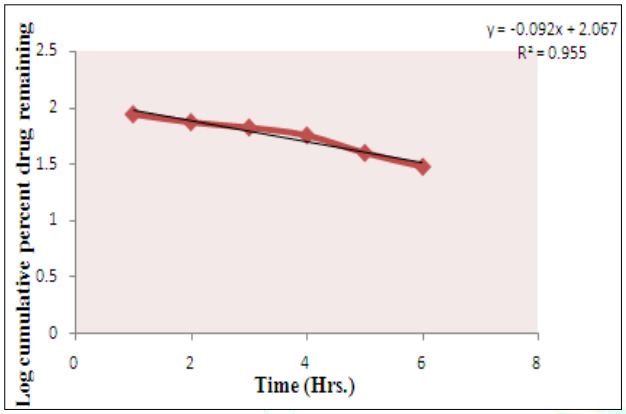


**Figure 12 Log cumulative percent drug remaining Vs time (First Order Plots) of formulation F10**

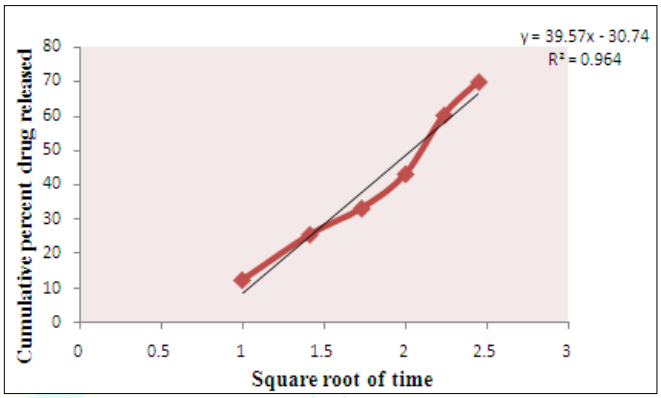


**Figure 14 Cumulative percent drug released Vs Square root of time**

**Table 10 Regression analysis data of phytosome formulation**

| Formulation | Zero order | First order | Pappas plot | Higuchi plot |
|---|---|---|---|---|
| F10 | y = 11.51x+0.39<br>$R^2$ = 0.989 | y = -0.092x + 2.067<br>$R^2$ = 0.955 | y = 0.956x + 1.09<br>$R^2$ = 0.992 | y = 39.57x-30.74<br>$R^2$ = 0.964 |

The sample was then examined by optical Microscopy Fig 15.

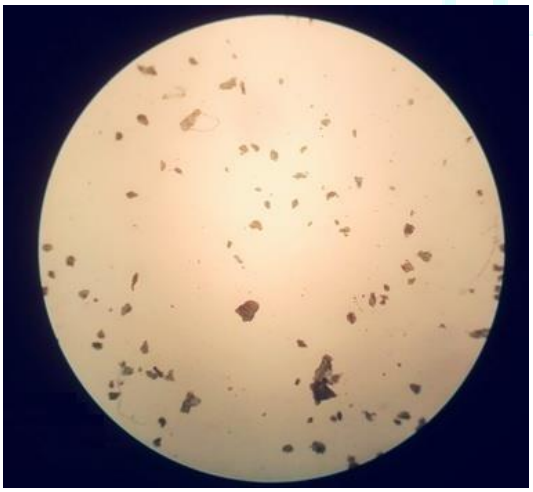

**Figure 15 Optical Microscopic study of optimized formulation**

Results of stability studies clearly indicates that optimized batches of phytosomes were stable over the chosen temperature and humidity conditions up to 3 months as were found no significant variation in physical appearance and % drug content.

## CONCLUSION

It can be concluded that from present investigation the HPLC and preliminary phytochemical investigation study of T. chebula leaves yielded a set of standards that can serve as an essential basis of evidence to determine the identity and to determine the quality and purity of the plant material as per its future perspectives. The phytochemical investigation gave valuable information about the different phytoconstituents present in the plant, which helps the future investigators concerning the selection of the particular extract for further investigation of isolating the active principle and also gave idea about different phytochemical have been found to possess a wide range of activities. The total flavonoid content in hydroalcoholic leaves extract was found to be higher which is further proved by in vitro antioxidant studies. Potential antioxidant activity has good correlations with the therapeutic use in the treatment of liver disorders. Further research to isolate individual compounds, their in-vivo antioxidant activities with different mechanism is needed.

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