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PHYTOCHEMICAL SCREENING AND DEVELOPMENT OF PHYTOSOMES OF HYDROALCOHOLIC EXTRACT OF WRIGHTIA TINCTORIA (ROXB.) R. BR FOR EFFECTIVE TREATMENT OF HEPATIC DISEASE

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Abstract:

Wrightia tinctoria (Roxb.) R.Br. (W. tinctoria) has been reported to exhibit number of therapeutic uses such as astringent, stomachic, febrifuge, skin diseases and tonic in India. 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, formulation and evaluation of phytosomes of hydroalcoholic leaf extract of W. tinctoria. The phytosome was prepared by the hydroalcoholic extract of plant and phospholipids: cholesterol by simple method. Characterization of phytosome was done by FTIR, entrapment efficiency, particle size and size distribution, optical microscopic study, stability studies and In vitro dissolution studies. Phytochemical analysis revealed the presence of alkaloids, glycosides, flavonoids, proteins, carbohydrates and saponins. The total flavonoids and alkaloid content of hydroalcoholic leaves extract of W. tinctoria was found to be 0.752 and 0.489mg/100mg respectively. Particle size and entrapment efficiency of optimized batch F3 was founds to be196.65±0.36nm and73.32±0.14. Combination of phospholipids and W. tinctoria can result in synergistic effect, For the future purpose it can used as a targeting drug delivery system as a liver targeting, brain targeting, cardio protective etc. Novel approach for herbal drug delivery is more prominent than conventional which improves bioavailability of polar extract and also patient compliance.

Keywords: *Wrightia tinctoria, Phytosome, Phospholipids, Phytochemical analysis, Characterization*

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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]. *W. tinctoria* (Apocynaceae), commonly known as pala indigo plant or sweet indrajao in english and dudhi in hindi. It is a small deciduous tree with a light gray, scaly smooth bark and native to India and Burma [4]. It is very popular as medicinal agent in ethnic medicine; the plant was mentioned in ancient Ayurvedic text like Rajanighantu and Sva. It is used in Ayurveda, Unani and Siddha medicines. Traditional system of medicine claim usefulness of *W. tinctoria* for the treatment of stomachic, febrifuge, skin diseases, abdominal pain and used as tonic [5]. The leaves are useful in psoriasis, non-specific dermatitis, anthelmentic, aphrodisiac, haemorrhoids dipsia and dropsy. The bark contains β-sitosterol, β-amyrin and its acetate and lupeol benzoate and seeds yield up to 40% fixed oil [4]. Previous studies have reported that *W. tinctoria* possesses the antibacteria, anti-fungal, antinociceptive and wound healing properties**.** Phytochemical screening of the plant has shown the presence of indole, flavonoid, sterols, fixed oil and triterpenoid compounds [6]**.** Phytosomes are multifaceted of natural products and natural phospholipids like soy phospholipids. This multifaceted is produced by the stoichiometric reaction of substrate and phospholipids in a suitable solvent. Phytosomes are superior exploit, absorbed and create better consequences than conservative herbal extracts [7]. The improved bioavailability of phytosomes above the non-complexed botanical imitative has been established by pharmacokinetics studies and by pharmacodynamic examinations in investigational animals and in human subjects [8]. When indulgenced with water, phytosomes obtain a micellar shape forming liposome-like structures, but while in liposomes the active part is dissolved in the interior core or is floating in the membrane layer, in phytosomes the active part is bound to the polar head of the phospholipids and works as an integral part of the membrane [7]. The ethanolic extract phytosomes were forecasted to show better hepatoprotective, antioxidant and renal protective results contrasted to extract due to their enhanced bioavailability. In addition, phosphatidylcholine molecules themselves are absorption enhancers [9]. In this study, phytosomes of hydroalcoholic leaf extract of *W. tinctoria* was prepared and evaluated. Characterization of phytosome was done by FTIR, entrapment efficiency, particle size and size distribution, optical microscopic study, stability studies and *In vitro* dissolution studies.

MATERIALS & METHODS:

Materials:

The leaves of *W. tinctoria* were collected from Bhimbetka Bhojpur, Raisen (Madhya Pradesh) in the month of February, 2021.

Chemical and reagents:

All the chemicals used in this study were obtained from Hi Media Laboratories Pvt. Ltd. (Mumbai, India), Sigma Aldrich Chemical Co. (Milwaukee, WI, USA), SD Fine-Chem. Ltd. (Mumbai, India) and SRL Pvt. Ltd. (Mumbai, India).All the chemicals used in this study were of analytical grade.

Extraction by Soxhlet apparatus:

Defatted dried powdered leaves of *W. tinctoria* has been extracted with hydroalcoholic solvent (ethanol: water: 75:25) using soxhletion process for 48 hrs, filtered and dried using vacuum evaporator at 40°C 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.

Phytochemical screening:

Phytochemical screening to detect the presence of bioactive agents was performed by standard procedures [10, 11]. After the addition of specific reagents to the solution, the tests were detected by visual observation of color change or by precipitate formation.

Total flavonoids content:

The total flavonoids content was estimated using the procedure described by Olufunmiso et al [12]. A total of 1 ml of plant extracts were diluted with 200 µl of distilled water separately followed by the addition of 150 µl of sodium nitrite (5%) solution. This mixture was incubated for 5 min and then 150 µl of aluminium chloride (10%) solution was added and allowed to stand for 6 min. Then 2 ml of sodium hydroxide (4%) solution was added and made up to 5 ml with distilled water. The mixture was shaken well and left it for 15 min at room temperature. The absorbance was measured at 510 nm. Appearance of

pink colour showed the presence of flavonoids content. The total flavonoids content was expressed as rutin equivalent mg RE/g extract on a dry weight basis using the standard curve.

Determination of alkaloids:
A total of 200 ml of 20% acetic acid was added to 5 g of root powders taken in a separate 250 ml beaker and covered to stand for 4 h. This mixture containing solution was filtered and the volume was reduced to one quarter using water bath. To this sample, concentrated ammonium hydroxide was added drop-wise until the precipitate was complete. The whole solution was allowed to settle and the precipitate was collected by filtration and weighed. The percentage of total alkaloid content was calculated as:

Percentage of total alkaloids (%)=Weight of residue×100/Weight of sample taken

Preparation of phytosomes:
The complex was prepared with phospholipids: cholesterol and extract of leaves of *W. tinctoria* in the ratio of 1:0.5:1, 1:1:1, 2:0.5:1, 2:1:1 respectively. Weight amount of extract and phospholipids and cholesterol were placed in a 100ml round-bottom flask and 25ml of dichloromethane 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 [13]. The dried residues were gathered and placed in desiccators overnight and stored at room temperature in an amber colored glass bottle Table 1.

Table 1 Different formulation of phytosomes

Formulation	Ratio of Phospholipids and Cholesterol (%)	Extract Concentration (%)	Dichloromethane Concentration (ml)
F1	1:0.5	1	25
F2	1:1	1	25
F3	2:0.5	1	25
F4	2:1	1	25

Characterization of Phytosomes:
Microscopic observation of prepared Phytosomes:
An optical microscope (cippon, Japan) with a camera attachment (Minolta) was used to observe the shape of the optimized Phytosome formulation.

Drug Excipients compatibility study by FT-IR:
IR spectra of physical mixture of extract and excipients were recorded by ATR (Attenuated total reflection) techniques using Fourier transform infrared spectrophotometer. A base line correction was made and the sample was directly mounted in IR compartment and scanned at wavelengths 4000 cm^{-1} to 400 cm^{-1}.

Entrapment efficiency:
Phytosome preparation was taken and subjected to centrifugation using cooling centrifuge (Remi) at 12000 rpm for an hour at 4. The clear supernatant was siphoned off carefully to separate the non entrapped flavonoids and the absorbance of supernatant for non entrapped *W. tinctoria* was recorded at λ_{max} 420.0 nm using UV/visible spectrophotometer (Labindia 3000+). Amount of flavonoids in supernatant and sediment gave a total amount of *Delonix regia* in 1 ml dispersion. The percent entrapment was calculated by following formula.

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

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.

***In vitro* dissolution rate studies:**
In vitro drug release of the sample was carried out using USP- type I dissolution apparatus (Basket type). The dissolution medium, 900 ml 0.1N HCl was

placed into the dissolution flask maintaining the temperature of 37±0.5^{0}C and 75 rpm. 10 mg of prepared phytosomes was placed in each basket of dissolution apparatus. The apparatus was allowed to run for 8 hours. Sample measuring 3 ml were withdrawn after every interval (30 min, 1 hrs, 2 hrs, 4 hrs, 6 hrs, 8 hrs, and 12 hrs.) up to 12 hours using 10 ml pipette. The fresh dissolution medium (37^{0}C) was replaced every time with the same quantity of the sample and takes the absorbance at 316.0 nm using spectroscopy [14, 15].

RESULTS AND DISCUSSION:

The crude extracts so obtained after soxhlet extraction process was concentrated on water bath by evaporation the solvents completely to obtain the actual yield of extract. The yield of extracts obtained from the leaves of the plants using hydroalcoholic (ethanol: water: 75:25) as solvents are depicted in the Table 2. The results of qualitative phytochemical analysis of the crude powder of leaves of *W. tinctoria* are shown in Table 3. Total flavonoids content was calculated as quercetin equivalent (mg/100mg) using the equation based on the calibration curve: y = 0.032x + 0.004, R^2=0.999, where X is the quercetin equivalent (QE) and Y is the absorbance. Total alkaloid content was calculated as atropine equivalent mg/100mg using the equation based on the calibration curve: y = 0.007x + 0.007, R^2=0.999, where X is the Atropine equivalent (AE) and Y is the absorbance. The total flavonoids estimation of hydroalcoholic extracts of leaves of *W. tinctoria* showed the content values of 0.752. The total alkaloids estimation of hydroalcoholic extracts of leaves of *W. tinctoria* showed the content values of 0.489mg/100mg Table 4 & Fig. 1& 2. 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. 3 & 4. 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 5. The entrapment efficiency of the phytosomes was found in the range of 63.32±0.15 to 73.32±0.14%.Particle size of all formulations found within range 196.65±0.36 to 285.32±0.45nm Table 5 & Fig. 5. Concentration of lipid has shows significant impact on size of phytosomes. Zeta potential of optimized formulation F3 was found to be -34.50mV Fig. 6.

Formulation F3 was found best one which is further evaluated for drug release study, transmission electron microscopy (TEM), and stability studies. In vitro dissolution study of F3 indicated that the phytosomes had extended release dissolution pattern. The phytosomes show of 12hr. 98.85 % release Table 6. When the regression coefficient values of were compared, it was observed that 'r^2' values of Korsmeyer Peppas was maximum i.e. 0.976 hence indicating drug release from formulations was found to follow Korsmeyer Peppas kinetics Table 7, Fig. 7-10. The sample was then examined by optical Microscopy Fig. 11.

Table 2 % Yield of leaves extracts of *W. tinctoria*

Extracts	% Yield (w/w)
Pet ether	2.37%
Hydroalcoholic	5.85%

Table 3 Phytochemical screening of leaves extracts of *W. tinctoria*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Dragendroff's test	+ve
	Hager's test	+ve
2.	**Glycosides**	
	Legal's test	+ve
3.	**Flavonoids**	
	Lead acetate	+ve
	Alkaline test	+ve
4.	**Phenol**	
	Ferric chloride test	-ve

5.	**Proteins** Xanthoproteic test	+ve
6.	**Carbohydrates** Fehling's test	+ve
7.	**Saponins** Foam test	+ve
8.	**Diterpenes** Copper acetate test	-ve

Table 4 Estimation of total flavonoids and alkaloid content of *W. tinctoria*

S. No.	Total flavonoids content (mg/ 100 mg of dried extract)	Total alkaloid content (mg/ 100 mg of dried extract)
1.	0.752	0.489

Table 5 Particle size and entrapment efficiency of drug loaded phytosomes

Formulation Code	Particle size (nm)	Entrapment Efficiency (%)
F1	285.32±0.45	63.32±0.15
F2	265.56±0.32	68.85±0.23
F3	196.65±0.36	73.32±0.14
F4	220.32±0.25	65.45±0.36

Average of three determinations (n=3)

Table 6 *In-vitro* drug release data for optimized formulation F3

Time (h)	Square Root of Time(h)$^{1/2}$	Log Time	Cumulative*% Drug Release	Log Cumulative % Drug Release	Cumulative % Drug Remaining	Log Cumulative % Drug Remaining
0.5	0.707	-0.301	22.25	1.347	77.75	1.891
1	1	0	39.98	1.602	60.02	1.778
2	1.414	0.301	46.65	1.669	53.35	1.727
4	2	0.602	59.95	1.778	40.05	1.603
6	2.449	0.778	65.58	1.817	34.42	1.537
8	2.828	0.903	82.23	1.915	17.77	1.250
12	3.464	1.079	98.85	1.995	1.15	0.061

Table 7 Regression analysis data of optimized formulation F3

Batch	Zero Order	First Order	Higuchi	Korsmeyer Peppas
	R^2	R^2	R^2	R^2
F3	0.945	0.856	0.976	0.963

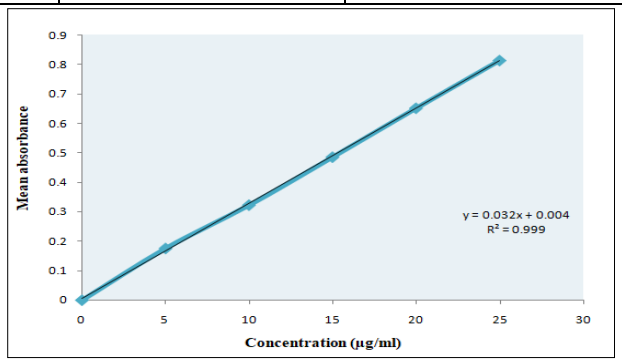


Fig 1Graph of estimation of total flavonoid content

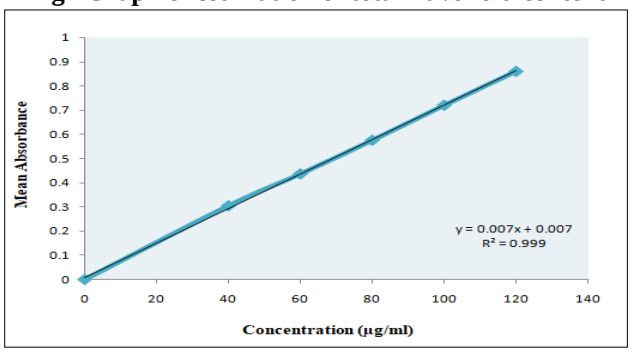


Fig 2Graph of estimation of alkaloid content

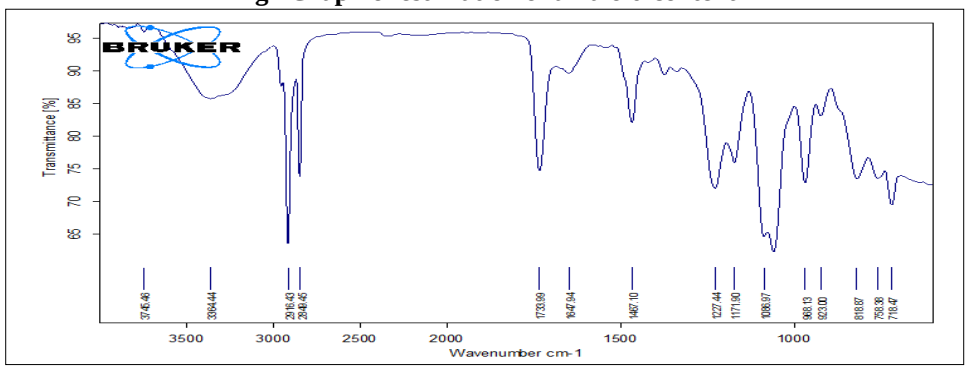


Figure 3 FT-IR spectrum of hydroalcoholic extract of *W. tinctoria*

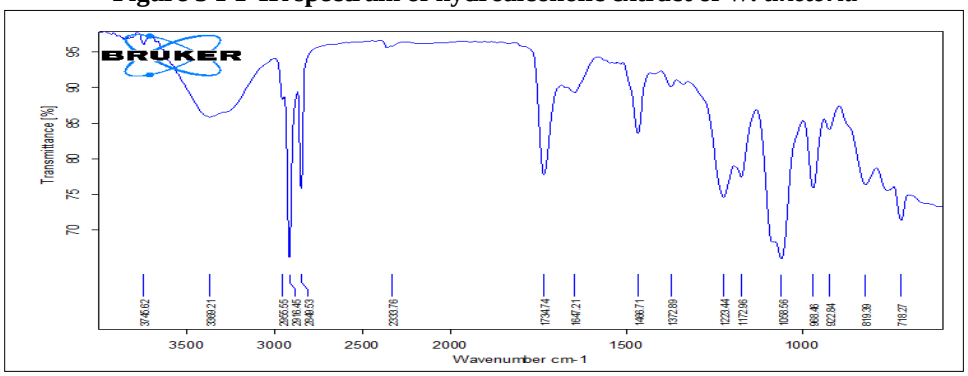


Figure 4 FT-IR spectra of prepared phytosomes optimized formulation F3 formulation

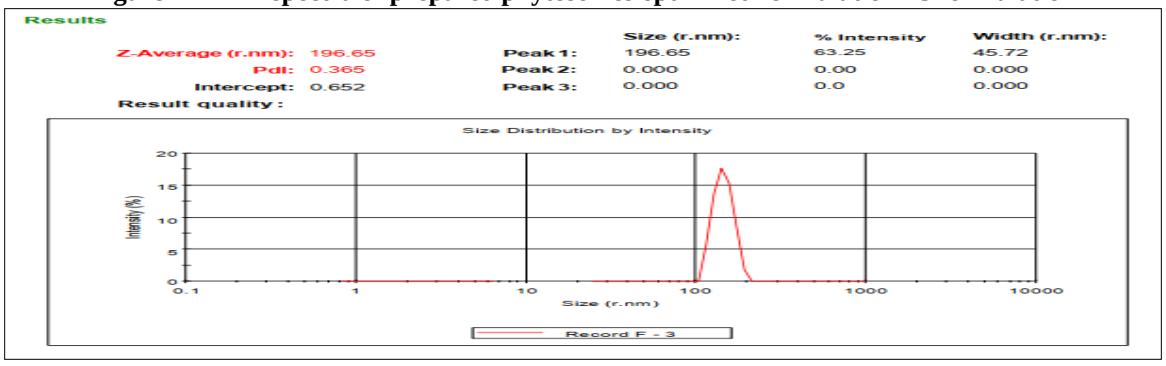


Figure 5 Particle size of optimized batch F3

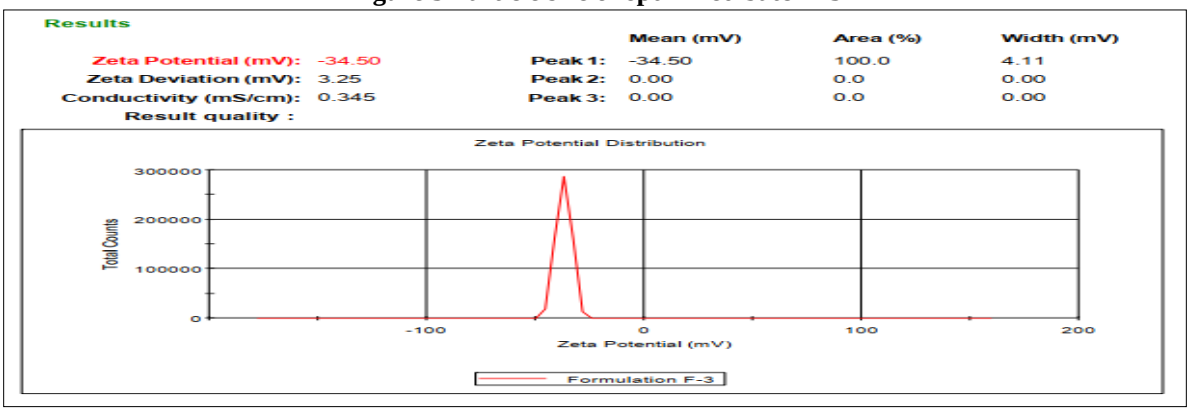


Figure 6 Zeta potential of optimized batch F3

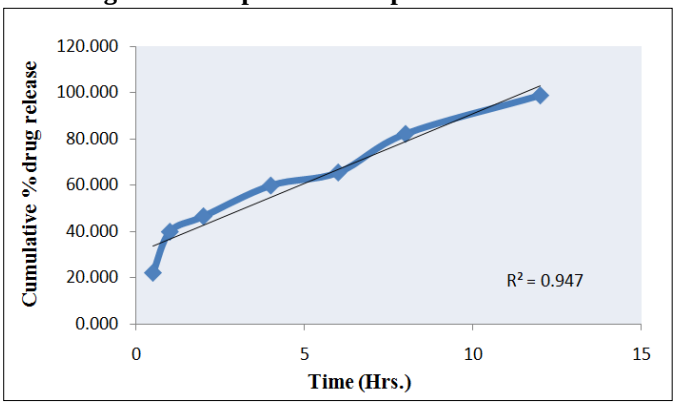


Figure 7 Cumulative % drug released Vs Time

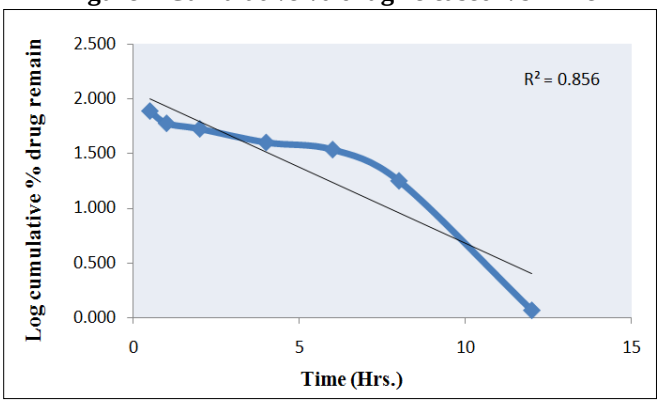


Figure 8 Log Cumulative % drug remain Vs Time

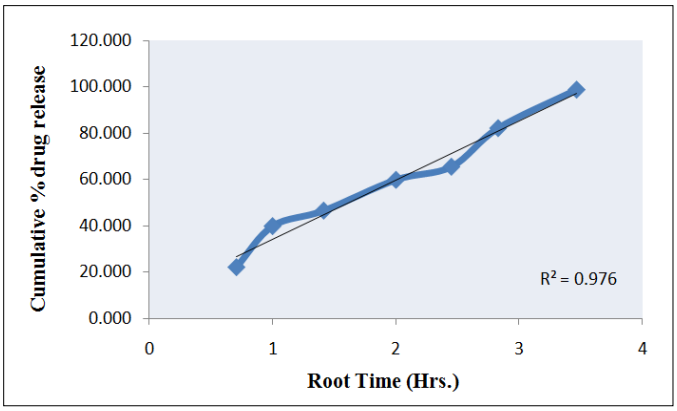


Figure 9 Cumulative % drug release Vs Root time

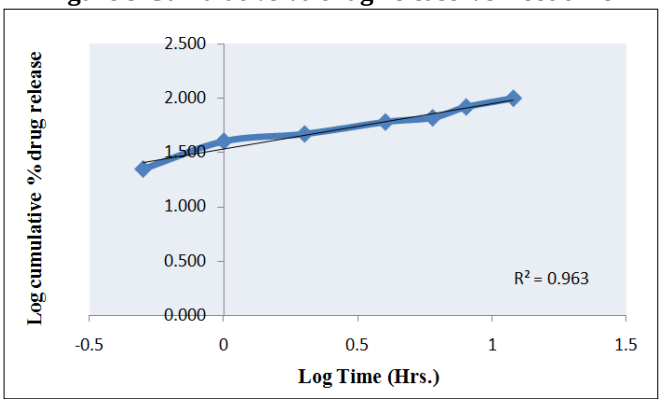
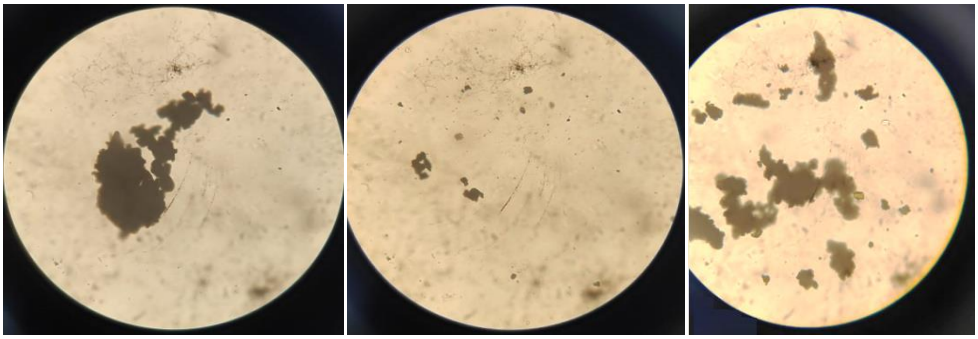
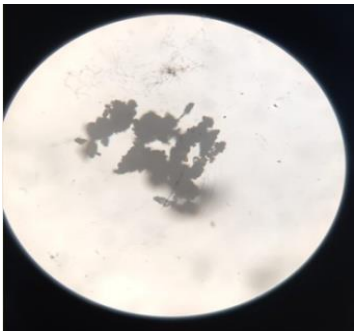


Figure 10 Log Cumulative % drug release Vs Log time



F1 **F2** **F3**



F4

Figure 11 Microscopic observation of formulation F1-F4

CONCLUSION:

From above studies we are concluded that phytosomes has better physical characteristics than that of extract. 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. Further research to isolate individual compounds, their *in-vivo* activities with different mechanism is needed.

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