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Phytosomes: A Potential Carrier for Herbal Drugs as Novel Drug Delivery System

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ABSTRACT

Liquorice is a well-known traditionally used root for various ailments. Constituents of the Liquorice such as Glycyrrhetinic acid, Glycyrrhizin, Licochalcone, Isoliquiritigenin and Glabridin possess good antitumor activity. Glycyrrhizin was believed to be the marker component in Liquorice for its anticancer properties. Liquorice thus shows good potential against breast cancer, MCF-7 Cell Lines by inhibiting the cancer cell growth by the induction of apoptosis which could be mediated through an evident disruption of the mitochondrial membrane potential, release of Cytochrome C and activation of caspase-9 cycle. In Guggul, Guggulsterone is responsible for its anticancer activity. Literature reveals various mechanisms which are responsible for the incidence and development of breast carcinogenesis. Both these traditional drugs which are easily available and have reported good anticancer activity may be used alone or in combination in cancer therapeutics. The lower the IC50 value obtained from the MTT assay and data obtained from diffusion studies indicates better therapeutic activity. Evaluation of other characterization such as UV- vis spectroscopy, FTIR, HPLC, Particle size and zeta potential are the indicators of stability and presence of constituents in the formulation.

KEYWORDS: *Liquorice, Guggul, Glycyrrhizin, Guggulsterone, anticancer*

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The main objectives of the research are

1. Phytochemical evaluation of the Liquorice and guggul extract and Characterization of both the hydroalcoholic extract by TLC and UV-Vis spectrophotometric analysis.
2. Formulation and characterization of phytosomes of *Glycyrrhizia glabra* and *Commiphora weighti.*
3. Qualitative analysis of the phytosomes by HPLC and *In vitro* evaluation of the anticancer activity of the phytosomes on human breast cancer (MCF-cell lines)
4. Formulation and evaluation of both the phytosomal tablets.

INTRODUCTION:

Medicinal plants primarily based ancient systems of medicines are enjoying vital role in providing health care to massive section of population, particularly in developing countries. Herbal medicines are now being considered as a potential source of anti-cancer agents and are widely used due to availability of the material, affordability and their therapeutic efficacy. Phytochemicals obtained from medicinal plants play a crucial role in the treatment of cancer mostly plant secondary metabolites and their derivatives have been applied to combat cancer. Toxicity and absorption problem limit the use of these constituents. Apart from those herbal extracts are destroyed by the digestive secretions and gut bacteria. But Novel herbal formulation techniques have assured the researchers to delivers the plant based secondary metabolites to their systemic targets. Phytosome Represents advanced herbal technology that offers defined bioavailability of plant drug over herbal extract. The research project was aimed to "enhance the absorption of lipid insoluble polar phytoconstituents through oral route showing improved bioavailability", therefore extensively better therapeutic profit.

Experimental:

Phytochemistry of Liquorice:

Liquorice contains more than 30 species which are widely distributed all over the world. Glycyrrhiza root

of ethanolic extract contains alkaloids, glycosides, carbohydrates, saponins, lipids, tannins, sterols, and steroids. Phytoconstituents such as triterpenoids saponins which include glycyrrhizin, glycyrrhetinic acid. Other phytoconstituents are is flavonoids, chalcones, triterpenoids, steroids, amines, Gums, lignans, and volatile oil. Therapeutic effects of liquorice can be affected due to variation in their phytoconstituents because of different geographical conditions, harvesting, and processing.

Phytochemistry of Guggul:

Guggul contains just about six photochemical, which mostly contains gallic acid, quercetin, and guggulsterone E and Z. It shows the presence of steroids, sterols, terpenoids, flavonoids, tannins, and lignans. It shows gum (32%), oleo-gum resin (38%), and essential oil (1%) Gallic acid. Most of the plants based biologically active constituents are polar or water-soluble. However, water-soluble phytoconstituents like flavonoids, tannins, glucosidal aglycones, etc. are poorly absorbed either due to their greater molecular size, which cannot be absorbed by passive diffusion, or due to which resulting in their poor bioavailability. But due to the high lipophilicity of phytosomes they improved bioavailability and therapeutic. Phytosomes are a new form of herbal extract that has improved pharmacokinetic and pharmacological parameters.

Collection

Drug was procured from Dr. Paleps research lab.

Extraction

Root was dried at 40°C in an oven and then ground to a fine powder and passed through a sieve with mesh No 20.

Soxhlet extraction

Soxhlet extraction was performed in a Soxhlet apparatus. Exhaustive extraction with different solvent (ethanol 80 %, methanol 80 % or water) was performed using 25 g Glycyhhriza glabra powder, wrapped in filter paper and impregnated with solvent. The extract was then filtered through Whatman No 1 filter paper and the extraction yield was calculated.

Preparation of extracts:-

Hot extraction:-

Plant materials were extracted sequentially with Hexane (69°C), ethyl acetate (77°C), acetone (56°C), methanol (64°C) in order of increasing polarity. Fifteen grams (15g) of the plant resin was soaked in 100 ml of the extraction solvent and extraction was carried out in Soxhlet apparatus until they became colourless. The extract obtained from the method were kept in the Petri plate for 48hrs at room temperature (30°c±2) to evaporate the solvent. All the extracts were kept in fridge (7-8°C) until use.

Phytochemical evaluation

Both the extract was subjected to phytochemical evaluation to determine the presence of various phytoconstituents viz. flavonoids, terpenoids, alkaloids, glycosides, saponins, tannins, phenols, carbohydrates and steroids.

Result-The preliminary phytochemical screening of hydroalcoholic extract of Liquorice and Guggul shows the presence of Phyto constituents namely Alkaloids, Tannins, Carbohydrate, Phenol, Saponins, and Flavonoids.

1. TLC ANALYSIS OF METHANOLIC EXTRACT

A. Liquorice:

Thin layer chromatography was carried out on a precoated silica gel 60 F254 plate. The mobile phase was optimised using different solvent system. Final mobile phase was n butanol: Acetic acid: water (5.7:2.85:1.45). The plates were examined under ultraviolet light examined at 254 nm and 366 nm.

B. Guggul:

Thin layer chromatography was carried out on a precoated silica gel 60 F254 plate. The mobile phase was optimised using different solvent system. Final mobile phase was chloroform: Ethyl acetate (7:3). The plates were examined under ultraviolet light examined at 254 nm and 366 nm.

2. Determination of wavelength (λ_{max}) of maximum absorption by UV-VIS spectrophotometer

A. For Liquorice:

10 mg of *Glycyrrhiza glabra* extract was dissolved in 10 of hydro alcoholic solution to get a concentration of 1000ug/ml. From this 1000ug/ml stock solution 1ml was taken and diluted to 100 ml with hydro alcoholic solution. The prepared solution was scanned in the range of 200-800 nm on a thermos scientific 300 UV-Vis spectrophotometers. An absorbance maximum was found to be 248nm.

B. For Guggul:

5 mg of *Commiphora wightii* extract was dissolved in 10 ml of hydro alcoholic solution to get a concentration of 500ug/ml. From this 500ug/ml stock solution 1ml was taken and diluted to ml 10 with hydro alcoholic solution. The prepared solution was scanned in the range of 200-800 nm on a thermo scientific 300 UV-Vis spectrophotometer. An absorbance maximum was found to be 253nm.

Preparation of Standard Calibration curve

Prepare the calibration curve of Hydroalcoholic extract of Licorice and Guggul

Formulation

SELECTION OF LIPID AND METHOD FOR PREPARATION OF PHYTOSOME:
Lipid, cholesterol was used for the preparation of Phytosome on the ideas of most typically used, cost, availability, compatibility with the hydro alcoholic extract result on formation of phytosomes. Various phytosome trial batches were prepared by varied lipids and with entrapment efficiency, Lipoid P-100 as lipid shows good entrapment efficiency and no sedimentation was observed.

Procedure-
The Licorice extract was dissolved in 3ml of 1:1 hydroalcoholic solvent. And Guggul extract was dissolved in 5ml of 1:1 hydro alcoholic solvent. Cholesterol and the lipid (Soy lecithin) were dissolved in ethanol and the solution was sonicated.

The drug extract solution was treated as an aqueous phase and was made up to volume with the remaining Phosphate buffer pH 7.4. The lipid phase was added dropwise to the aqueous phase using the magnetic stirrer at 1300rpm for 2 hrs.

Tween-80 was added as a surfactant to reduce the interfacial tension between the aqueous and lipid phase and stirring was continued.

Optimization of phytosome
Phytosomes of each batch were optimized.

EVALUATION AND CHARACTERIZATION
Entrapment efficiency-Entrapment efficiency of phytosomal batches was determined using the centrifugation method. The prepared phytosomes were placed in an Eppendorf tube and centrifuged at 12000 rpm for 60 minutes. The supernatant (1ml) was withdrawn and diluted with phosphate buffer (pH 7.0) up to 10 ml and buffer is used as blank. Accordingly, calculations were done to find out the amount of unentrapped drug.

$$\% \text{ Entrapment efficiency} = \frac{\text{Total Drug-Amount of unentrapped drug}}{\text{Total drug}} \times 100$$

Result- Entrapment efficiency of optimized phytosomal batch of liquorice and guggul were found to be 76 and 80%, respectively.

Particle size and zeta potential measurement
Determination of particle size and zeta potential was carried out which is a critical parameter for the stabilization and should comply within the set ranges.

Particle size measurement of the optimized phytosome formulation was carried out using Malvern Zeta sizer.

Particle size of the optimized batched of liquorice and guggul phytosome were found to be 221nm and 131nm respectively.

Zeta potential analysis was carried out using HORIBA Scientific. Zeta potential of liquorice and guggul were found to be 1.26 and 0.0361 respectively.

DRUG EXCIPIENT COMPATIBILITY STUDY
The drug compatibility studies were carried by FTIR. The FTIR spectra of the mixture of liquorice and Guggul extract and the excipient used in formulation of phytosomes were obtained and observed with the FTIR –spectra of the phytosomal formulation to determine the interaction between the extract of liquorice and guggul and excipients used in the formulation of the phytosomes.

In-vitro release studies
The release study was carried in 250 ml beaker containing 200ml of diffusion medium phosphate buffer solution pH 7.4. Dialysis membrane previously soaked nightlong within the diffusion medium was used for the study. 10 cm of Dialysis membrane was taken and one end of membrane was tied with the help of thread and 5ml was filled from another end into the dialysis membrane and tie with thread. The dialysis membrane containing the phytosomes was suspended into the medium. Beaker assembled on a magnetic stirrer and equilibrated at 37 ^{0}C. Content of beaker were kept to stirred at 100rpm and aliquots were withdrawn at 0min, 5min, 15min, 30min, 45min, 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 24hrs and replaced by 3ml volume of phosphate buffer pH 7.4 to maintain the sink condition.[53]

HPLC Analysis
Qualitative automated HPLC gradient method was performed.

Preparation of sample: 5 mg of the extract was solubilised in 2 ml ACN and water mixture and sonicated. The sonicated solution was then filtered and filled in vials for injection.

Chromatographic system conditions:
System: Water 2695 HPLC system

Stationary phase: Haemachrome c 18 column, 5u, 15cm length.

Mobile phase: Acetonitrile (solvent A) and 0.005% TFA (solvent B) set to gradient elution.

Run time: 75 minutes

Injection volume: 10ul

Flow rate: 1ml/min

Detector: PDA 2996

Detection wavelength: 254

Stability studies:

The stability of phytosomes was carried out as per ICH guideline [52]. The optimized formulation were stored at different temperature ranges 4^0C +_2^0C, 25^0C+_2^0C for a period of 3 months and studied for drug entrapment. Phytosomal batches of both the extract in the duration of 3 months was studied for entrapment efficiency and particle size

FREEZE DRYING OF FORMULATION

The final phytosomal formulation was freeze dried using 10%W/V D- (+)- Trehalose dihydrate as a cryoprotectant. Phytosome were freeze using a deep freezer (Thermo Scientific, India)- 80^0C for 16-17 hrs in order to remove the water content and to obtain the phytosome in dry solid form with an objective to enhance the stability and shelf-life of the phytosome.

The phytosomal batch was lyophilized and a free-flowing solid was obtained at the end of 17 hrs.

FORMULATION OF TABLET

Preparation of powder blend for tablet:

A. Preparation of powder blends for tablet:

The solid product obtained after lyophilisation of the optimized batch F5 is blended and passed through 40# mesh sieve to ensure uniformity of the particles. Talc was added as a lubricant and glidant.

B. Pre compression parameter of the blended mixture:

The extract of Liquorice and Guggul blended with the excipients is evaluated for, parameters like, bulk density, tapped density, Carr s index, Hausner s ratio and angle of repose. To determine the flow properties and compressibility of the prepared blend.

a. Angle of repose:
b. Bulk density:
c. Tapped density:
d. Carr s (Compressibility) index:
e. Hausner s ratio:

Formulation of tablet:

Hydro alcoholic Liquorice and Guggul phytosomal tablet was prepared by direct compression technique. All the ingredients were thoroughly mixed then the powder was passed through sieve mesh 40# to get uniform size of particles. Talc was finally added as lubricant and glidant. Powdered were compressed on 10 mm, diameter, flat faced punches using tablet compression machine each tablet contains 100 mg of hydro alcoholic liquorice and guggul.

EVALUATION OF LICORICE AND GUGGUL PHYTOSOME TABLET

a. Weight variation
b. Drug content uniformity test
c. Thickness and diameter
d. Hardness test
e. Friability test
f. Disintegration time

In vitro dissolution study:

This study was carried out using type 2 apparatus at 50 rpm. The dissolution media used were 900 ml phosphate buffer pH 7.4 was used as dissolution media for two hrs and maintained at 37^0 C -^{0} +0.5 C. 5 ml aliquots were withdrawn at the specified time intervals. An equal volume of fresh media was replenished after each sampling to maintain the constant volume of the medium. The samples were analysed at 248nm for liquorice and 253nm for Guggul using UV-visible spectrophotometer.

***IN VITRO* CYTOTOXICITY EVALUATION OF LICORICE AND GUGGUL EXTRACT AND THEIR OPTIMIZED PHYTOSOMAL BATCHES:**

The *in vitro* cytotoxicity evaluation of the phytosmes of licorice and guggul was carried out on MCF (Human myeloid leukemia) by MTT assay.

Maintenance of cell lines:

The MCF7 (Human Breast adenocarcinoma cell line) is purchased from NCCS, Pune, India. The cells were maintained in DMEM high glucose media supplemented with 10 % FBS along with the 1% antibiotic-antimycotic solution in the atmosphere of 5% CO_2, 18-20% O_2at 37^0C temperature in the CO_2incubator and subculture for every 2days.

Background of the study:

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on reduction of the yellow colour water soluble tetrazolium dye MTT to formazan crystals. Mitochondrial lactate dehydrogenase produced by live cells reduces MTT to insoluble formazan crystals, which upon dissolution into an appropriate solvent exhibits purple colour, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570nm.

RESULT:

Formulation code	Amount of Extract	Amount of Lipid-100	Amount of cholesterol	Volume of Phosphate buffer	Volume of Ethanol
F1	10mg	10mg	3mg	15ml	5ml
F2	10mg	20mg	5mg	15ml	5ml
F3	30mg	20mg	5mg	15ml	5ml
F4	14mg	7mg	5mg	15ml	5ml
F5	20mg	30mg	5mg	15ml	5ml
F6	30mg	70mg	8mg	15ml	5ml
F7	15mg	10mg	5mg	15ml	5ml

Optimization of phytosome of *glycyrrhizia glabra*

Formulation code	Amount of Extract	Amount of Lipid-100	Amount of cholesterol	Volume of Phosphate buffer	Volume of Ethanol
F1	10mg	10mg	3mg	15ml	5ml
F2	20mg	40mg	5mg	15ml	5ml
F3	25mg	15mg	5mg	15ml	5ml
F4	30mg	60mg	10mg	15ml	5ml
F5	20mg	30mg	5mg	15ml	5ml
F6	40mg	60mg	5mg	15ml	5ml
F7	20mg	80mg	5mg	15ml	5ml

Optimization of phytosome of *commiphora wightii*

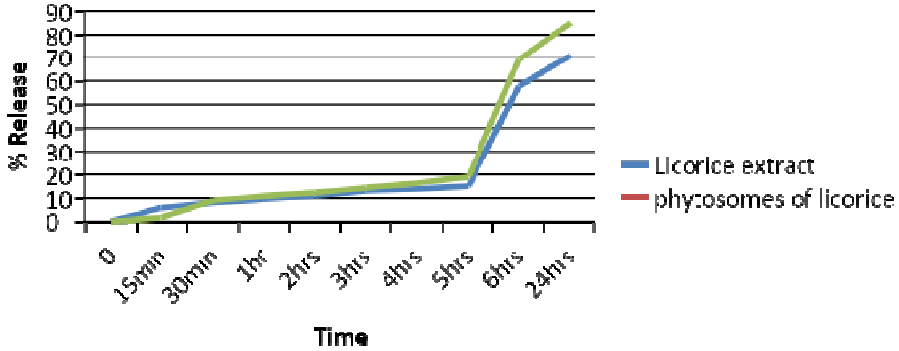
Formulation code	Entrapment efficiency
F1	55.2%
F2	56.38%
F3	57.39%
F4	24.25%
F5	76.45%
F7	70.79%

Entrapment efficiency of phytosomal batch of Licorice

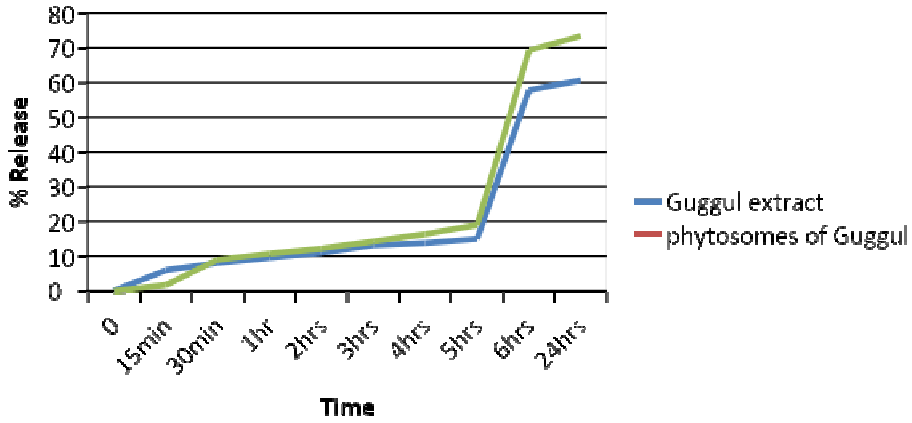
Formulation code	Entrapment efficiency
F1	45.95%
F2	55.86%
F3	57.39%
F4	59.45%
F5	80.40%
F6	60.8%
F7	71.25%

Entrapment efficiency of phytosomal batch of Guggul

- Zeta potential analysis was carried out using HORIBA Scientific. Zeta potential of licorice and guggul were found to be 1.26 and 0.0361 respectively.
- **In vitro release studies-** The optimized Phytosomal batch of both the extract Liquorice and Guggul showed maximum release at 85% and 71 % respectively at 24.



***In vitro* diffusion studies of the Phytosomes of licorice**



***In vitro* diffusion studies of the Phytosomes of licorice**

Stability testing:

Sr. No.	Temperature	Time	Particle size (nm)	Entrapment efficiency (%)
1	$4^0C \pm 2^0C$	Initial	221	76
	$4^0C \pm 2^0C$	1 months	225	75
	$4^0C \pm 2^0C$	2 months	270	72
	$4^0C \pm 2^0C$	3 months	285	70
2	$25^0C \pm 2^0C$	Initial	221	76
	$25^0C \pm 2^0C$	1 months	237	72
	$25^0C \pm 2^0C$	2 months	275	68
	$25^0C \pm 2^0C$	3 months	288	55

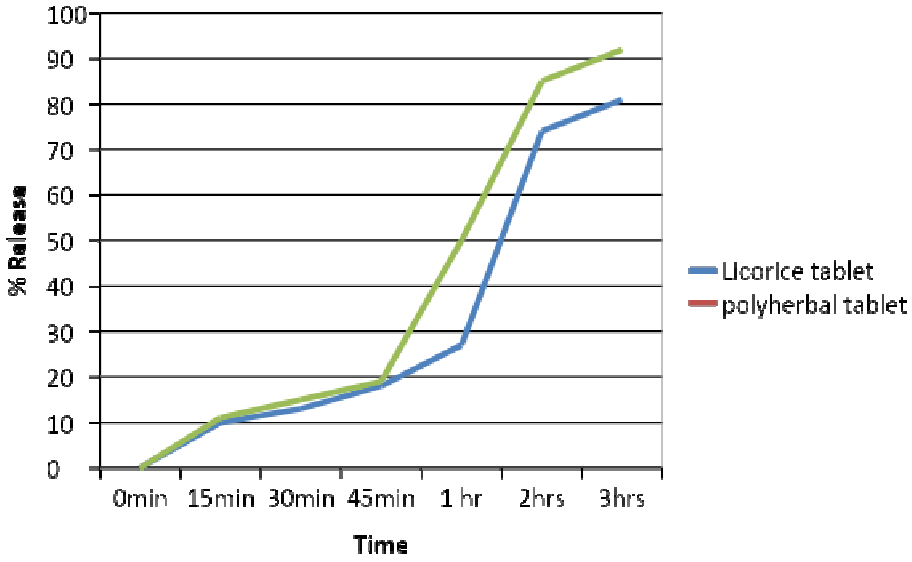
Stability studies of the optimized Phytosomal batch of Licorice (F5)

Sr. No.	Temperature	Time	Particle size (nm)	Entrapment efficiency (%)
1	$4^0C \pm 2^0C$	Initial	131	80
	$4^0C \pm 2^0C$	1 months	135	78
	$4^0C \pm 2^0C$	2 months	180	76
	$4^0C \pm 2^0C$	3 months	185	73
2	$25^0C \pm 2^0C$	Initial	131	80
	$25^0C \pm 2^0C$	1 months	138	72
	$25^0C \pm 2^0C$	2 months	170	65
	$25^0C \pm 2^0C$	3 months	185	60

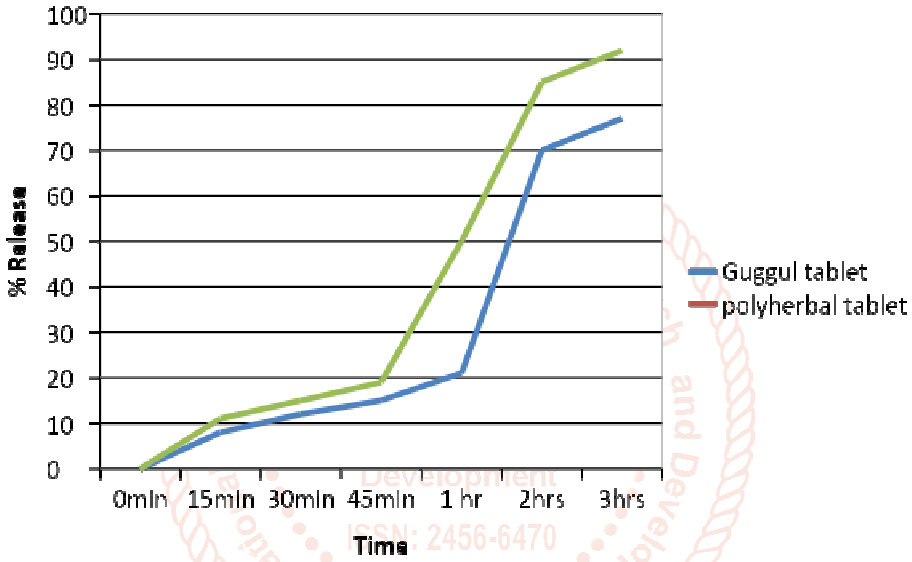
Stability studies of the optimized Phytosomal batch of Guggul (F5)

EVALUATION OF LICORICE AND GUGGUL TABLET

Evaluation parameter	Result (Licorice)	Result (Guggul)
Weight variation (mg)	250	230
Diameter (mm)	11	12
Thickness	4±0.5	3±0.5
Hardness (kg/cm^2)	5	4
Friability	0.5	0. 7
Disintegration (min)	5	3



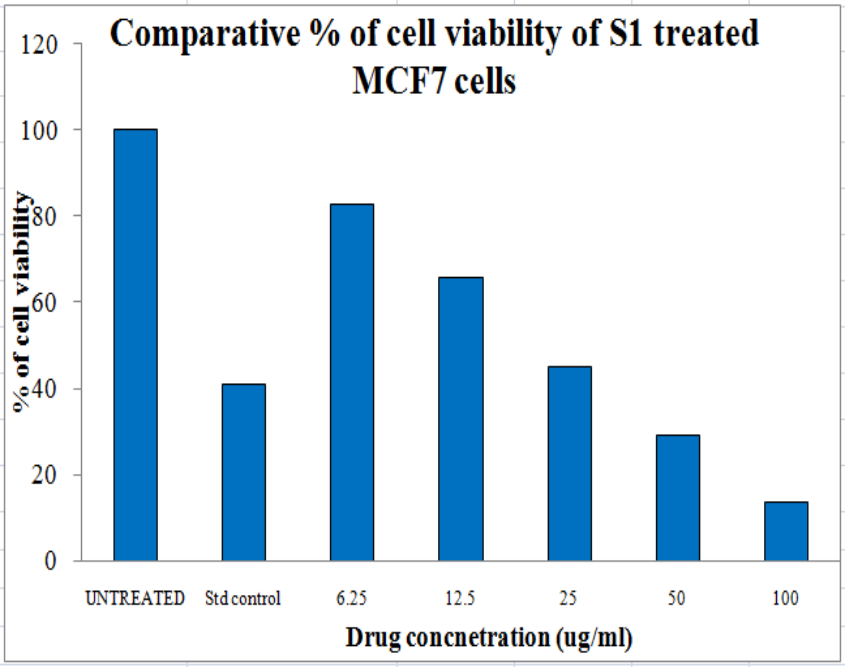
Dissolution study of polyherbal phytosomes tablet at 229nm and Licorice phytosomes tablet



Dissolution study of polyherbal phytosomes tablet at 229nm and Guggul phytosomes tablet

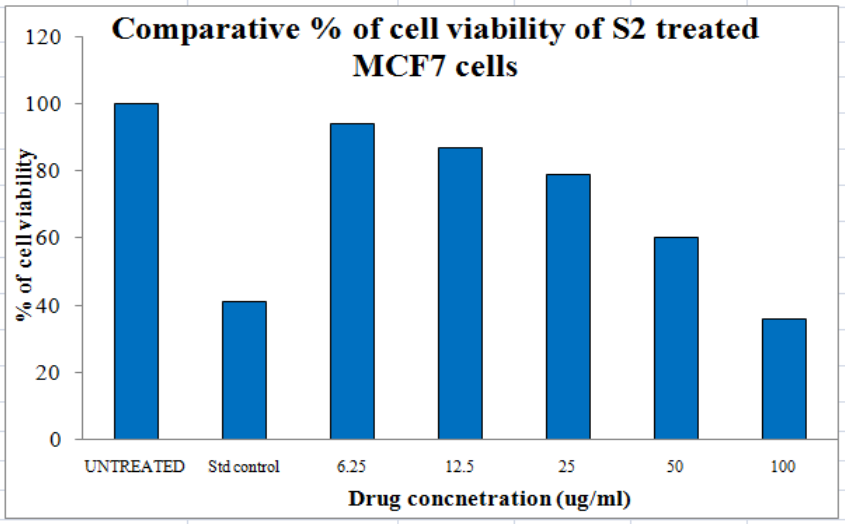
***IN-VITRO* CYTOTOXICITY EVALUATION OF LICORICE AND GUGGUL EXTRACT AND OPTIMIZED PHYTOSOMAL BATCHES**

Effect of licorice extract on MCF7 cell lines



Comparative % cell viability of Licorice phytosome on MCF-7 cell lines

Effects of phytosomes of Guggul on MCF-7 cell lines

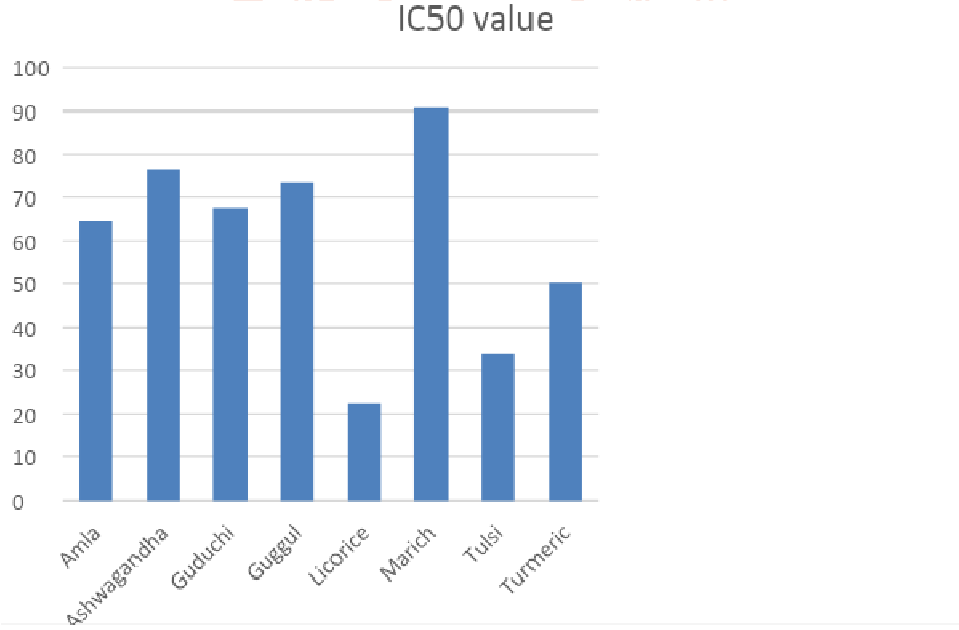


Comparative % cell viability of Guggul phytosome on MCF-7 cell lines

MTT TEST RESULTS

S. NO	Sample	TEST PARAMETER-MTT IC_{50} (ug/ml)
1	S1	22.53
2	S2	73.43

IC50 value of all the herbs



Graph of IC50 value of all the herbs

CONCLUSION

1. Herbal medicines are now being considered as a potential source of anti-cancer agents and are widely used due to availability of the material, affordability and their therapeutic efficacy Several traditionally used herbal medicines, including astragalus, Turmeric, Licorice, Ginseng, are commonly used by cancer patients to either "treat" cancer or "reduce the toxicity" induced by chemotherapy or radiotherapy.
2. The phytoconstituents presents in the extract of Licorice and Guggul have large molecular weight and poor lipid solubility which in turns leads to decreased bioavailability of these extracts. It was considerably thought worldwide to convert this traditional formulation into a novel drug delivery system as a phytosomes.
3. The Phytosome containing the Phospholipids closely mimic the bioavailability and efficacy. The Licorice and Guggul extracts were formulated in to phytosomes using solvent injection technique.
4. The preliminary phytochemical screening of Licorice and Guggul extracts revealed the presence of phytoconstituents alkaloids, tannins, saponins, glycosides, phenols, flavonoids, and

carbohydrates. Drug excipient compatibility studies were carried out by FTIR spectra.

5. FTIR spectra of cholesterol, Phospholipids, P_100, extracts of licorice and Guggul was obtained. no significant compatibility resulting due to interaction of extract and excipient Qualitative HPLC analysis confirmed that the major peaks obtained in the extract and phytosomal formulation are of the same phytoconstituents.
6. Different phytosomal batches prepared by solvent injection technique. Optimized batch of Licorice and Guggul showed particle size of 221nm and 131nm respectively. Zeta potential of licorice and guggul was observed 1.26 and 0.0361 respectively.
7. Entrapment efficiency of the optimized Phytosomal batch of Licorice and Guggul were found to be 76% and 80% respectively. Phytosomes of licorice and Guggul showed release of 85% and 71% respectively.
8. The formed phytosomal batches were subjected to stability studies for a period of 3 months and were found to be stable.
9. The phytosomes of Licorice and Guggul were lyophilized to obtain a flow able solid which was blended with excipients like Avicel, anhydrous lactose, aerosil, talc to obtain a tablet.
10. The powder blend was evaluated and tested for precompressiom parameters like Hausner's ratio, bulk density, tap density, and carr's index and powder blend showed good flow properties.
11. The prepared tablet of licorice and Guggul showed the release of 90% and 91% at 2 hrs respectively. In vitro cytotoxic studies performed on MCF-7 cell lines showed that extract of licorice and Guggul showed greater cytotoxic activity.
12. The phytosomes of polyherbal extract showed IC50 value 61.78ug/ml and licorice and Guggul showed IC50 value 22.53ug/ml and 73.43 respectively.

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