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DEVELOPMENT AND EVALUATION OF PHYTOSOMES OF EXTRACT OF *HELICTERES ISORA* FOR EFFECTIVE TREATMENT OF DIABETES

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Abstract:
Helicteres isora L. is a large arborescent shrub of the family Sterculiaceae. It is used as an anti gastro spasmodic, anthelmintic, antispasmodic, antipyretic, anti diarrheal, anti dysenteric and as a tonic after childbirth. Stems of this plant are used as anthelmintic, colic, and aphtha, while fruits are used as colic, anticonvulsant, and abdominalgia. The aim of present work to prepare and characterize Helicteresisora extracts loaded phytosomes for effective treatment diabetes. 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 7.6. The entrapment efficiency of the phytosomes was found in the range of 55.85 to 70.25%. Particle size of all formulations found within range 310.32-385.45nm. 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, transmission electron microscopy (TEM), and stability studies. In this study, the combined hydroalcoholic extract of Helicteres isora in ratio of 2:1:1 found to exhibit significant results. Phytosomes has better physical characteristics than that of extract. In-vitro studies revealed that phytosomes showed control release of phytoconstituents. Hence, phytosomal formulation of this herbal drug combination can be used for clinical application to enhance the therapeutic effect.

Key Words: *Helicteresisora, Phytosomes, Formulation, Evaluation.*

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INTRODUCTION:

Diabetes mellitus is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycaemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both [1]. Such complications arise due to derangements in the regulatory systems for storage and mobilization of metabolic fuels, including the catabolism and anabolism of carbohydrates, lipids and proteins emanating from defective insulin secretion, insulin action, or both [2-3].

Classification of diabetes mellitus is based on its aetiology and clinical presentation. As such, there are four types or classes of diabetes mellitus viz; type 1 diabetes, type 2 diabetes, gestational diabetes, and other specific types. Type 1 diabetes is said to account for only a minority of the total burden of diabetes in a population although it is the major type of the diabetes in younger age groups at majority of well-to-do countries. The incidence of type 1 diabetes is increasing in both rich and poor countries. Furthermore, a shift towards type 1 diabetes occurring in children at earlier ages is imminent.

85 to 95% of all diabetes in high-income countries is of type 2 accounting for an even higher dominance in developing countries. It is intimately associated with improper utilization of insulin by target cells and tissues. It is currently a common and serious health concern globally. According to WHO, (1994), this problem has been aggravated by rapid cultural and social dynamics, ageing populations, increasing urbanization, dietary changes, reduced physical activity and other unhealthy lifestyle and behavioural patterns. Diabetes mellitus and lesser forms of glucose intolerance, particularly impaired glucose tolerance, can now be found in almost every population in the world and epidemiological evidence suggests that, without effective prevention and control programmes, diabetes will likely continue to increase globally [4].

In 2010, about 285 million people in the age group 20-79 were envisaged to have diabetes worldwide, about 70% of whom live in developing nations. This estimate is expected to increase to about 438 million, by 2030. Further, by 2030, the number of people with IGT is projected to increase to 472 million, or 8.4% of the adult population.

The debilitating effects of diabetes mellitus include various organ failures, progressive metabolic complications such as retinopathy, nephropathy, and/or neuropathy [5]. Diabetics are accompanied by risk of cardiovascular, peripheral vascular and cerebrovascular diseases. Several pathogenetic processes are involved in the development of diabetes, including destruction of pancreatic β-cells that lead to lowered sensitivity of insulin action [6].

Phytochemicals are bioactive polyphenolic compounds naturally found in plants that have been studied extensively due to their potential medicinal and nutritional benefits to humans. They not only play a protective role for the plant but are responsible for its color, aroma, and flavor. These compounds have attracted the attention of scientists worldwide, owing to their potent bioactivity against different diseases, their low cytotoxicity and their ability to be utilized in the production of cosmetics and dietary supplements [7-9].

Phytochemicals are categorized into three major categories based on their structural elements: terpenoids, alkaloids and polyphenolic substances. Numerous flavor and aromatic molecules are trepeniods, including menthol, linalool, geraniol, and caryophyllene, while catechols, lignins, tannins, stilbenes, and flavonoids are phenolic compounds. Alkaloids are further divided into Pyrrolidine, Pyrrolidine-pyridine, Pyridine-piperidine, and Isoquino-line alkaloids based on their heterocyclic ring systems [10]. There are a variety of ways in which phytochemicals can exhibit their influence, including acting as substrates, cofactors, or inhibitors of enzymatic reactions [11-13]. By demonstrating their chelating properties they can remove undesirable constituents from the gastrointestinal tract. Additionally, phytochemicals can be utilized to enhance the uptake and stability of a variety of essential nutrients [14]. The antioxidant activity of phytochemicals is one of their potential properties that has an important function in scavenging free radicals in human tissue [15].

An example of the utility of phytochemicals is salicin, which is a compound found in willow tree bark. It has anti-inflammatory and analgesic activity, and is also used as a precursor to numerous conventional non-steroidal anti-inflammatory drugs (NSAIDs). Due to its widespread use and high demand, greater quantities of salicin were prepared using chemical synthesis [16]. Certain phytochemicals also have nutritional and energetic properties, which is why they are referred to as nutraceuticals [17]. Efforts have been made to identify bioactivities in different phytochemicals compounds extracted from a variety of plant species that can be implemented in the treatment of different diseases. Despite the potential bioactivity of phytochemicals, there remain concerns about their quality, safety and efficacy [18].

A number of chief constituents of herbal medicine are easily soluble in water (glycoside, flavonoid); however, these constituents are bounded in their potency because they may be partially soluble or hydrophobic in nature, so when applied topically shows less therapeutic efficacy. Numerous efforts have been put forward to enhance the bioavailability of such drug by formulating them to target drug delivery system such as phytosomes and liposomes are good options. The use of these techniques in formulation development process may lead to good bioavailability of herbal drugs as compare to conventional herbal extracts [19].

Phytosomes means herbal drug loaded in vesicles, which is available in the Nano form. The phytosome provide an envelope, like coating around the active constituent of drug and due to this the chief constituent of herbal extract remains safe from degradation by digestive secretion and bacteria. Phytosome is effectively able to absorb from a water loving environment into lipid loving environment of the cell membrane and finally reaching to blood circulation [20].

The term "Phyto" means plant and "some" means cell like. It is also mentioned as herbosomes. This is a new patented technology, where standardized plant extracts or water soluble phytoconstituents are complexed with phospholipids to produce lipid compatible molecular complexes, there by greatly increasing absorption and bioavailability. Phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol are the phospholipids used, but phosphatidylcholine are widely used because of their certain therapeutic value in case of liver diseases, alcoholic steatosis, drug induced liver damage and hepatitis. Phospholipids are as natural digestive aids and as carriers for both fat miscible and water miscible nutrients. Phytosomes can easily traverse the lipophilic path of the enterohepatic cell membranes and also stratum corneum layer of the skin [21].

Helicteresisora L. is a large arborescent shrub of the family Sterculiaceae. It is used as an antigastrospasmodic, anthelmintic, antispasmodic, antipyretic, antidiarrheal, antidysenteric and as a tonic after childbirth. Stems of this plant are used as anthelmintic, colic, and aphtha, while fruits are used as colic, anticonvulsant, and abdominalgia. The aim of present work to prepare and characterize *Helicteresisora* extracts loaded phytosomes for effective treatment diabetes.

MATERIAL AND METHODS:

Extraction procedure

Following procedure was adopted for the preparation of extracts from the shade dried and powdered herbs[22-23].

Defatting of plant material

40.5 gm of dried powdered leaves of *Helicteresisora* were shade dried at room temperature. The shade dried plant material was coarsely powdered and subjected to extraction with petroleum ether by maceration. The extraction was continued till the defatting of the material had taken place.

Extraction by maceration process

Defatted dried powdered leaves of *Helicteresisora* has been extracted with hydroalcoholic solvent (ethanol: water: 80:20) using maceration method for 48 hrs, filtered and dried using vacuum evaporator at 40°C.

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

Phytochemical screening

Phytochemical screening: Phytochemical examinations were carried out for the extract as per the standard methods[24].

Quantitative estimation of bioactive compounds

Estimation of Total protein content

The amount of protein was estimated by Lowry's method [25].

Reagents A. 2% Na_2CO_3 in 0.1 N NaOH

B. 1% NaK Tartrate in H_2O

C. 0.5% CuSO4.5 H_2O in H_2O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 partFolin-Phenol [2 N]: 1 part water.

1 ml of each BSA (Bovine serum albumin) working standard 50-250 µg/ml or test in test tubes. The test tube with 1 ml distilled water was serving as blank. Added 4.5 ml of reagent I and incubated for 10 minutes. After incubation added 0.5 ml of reagent II and incubated for 30 minutes. Measure the absorbance at 660 nm and plot the standard graph.

Total flavonoids content estimation

Determination of total flavonoids content was based on aluminium chloride method[26].

Preparation of standard: 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.

Preparation of extract: 10mg of dried extracts of were dissolved in 10 ml methanol and filter. Three ml (1mg/ml) of this solution was used for the estimation of flavonoid.

Procedure: 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

Preparation of phytosomes

The complex was prepared with phospholipids: Cholesterol and *Helicteresisora* in the ratio of 1:1:1, 1:2:1, 2:1:1, 2:3:1 respectively [27]. 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. The dried residues were gathered and placed in desiccators overnight and stored at room temperature in an amber colored glass bottle.

Formulation development of phytosomes

Table 1: Different formulations of phytosomes

Formulation	Ratio of Phospholipids and Cholesterol	Extract Concentration (%)	Dichloromethane Concentration
Optimization of Phospholipids and Cholesterol			
F1	1:1	1	20
F2	1:2	1	20
F3	2:1	1	20
F4	2:3	1	20
Optimization of Drug Concentration			
F5	2:1	0.5	20
F6	2:1	1.0	20
F7	2:1	1.5	20
F8	2:1	2.0	20
Optimization of solvent concentration			
F9	2:1	1.0	5
F10	2:1	1.0	10
F11	2:1	1.0	15
F12	2:1	1.0	20

Characterization of prepared phytosome

Microscopic observation of prepared phytosome

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

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-entrappedflavonoids and the absorbance of supernatant for non-entrapped*Helicteresisora* was recorded at λ_{max} 420.0 nm using UV/visible spectrophotometer (Labindia 3000+) [28]. Sediment was treated with 1ml of 0.1 % Triton x 100 to lyse the vesicles and diluted to 100 ml with 0.1 N HCl and absorbance taken at 420.0 nm. Amount of quercetin in supernatant and sediment gave a total amount of *Helicteresisora* 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 of optimized phytosomes formulation were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetamaster ZEM 5002, Malvern, UK) [29]. 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.

Transmission Electron Microscopy
Surface morphology was determined by TEM, for TEM a drop of the sample was placed on a carbon-coated copper grid and after 15 min it was negatively stained with 1% aqueous solution of phosphotungustic acid. The grid was allowed to air dry thoroughly and samples were viewed on a transmission electron microscopy (TEM Hitachi, H-7500 Tokyo, Japan).

***In vitro*anti diabetic activity of hydroalcoholic extract of *Helicteresisora*and Phytosome**
Inhibition of alpha amylase enzyme:
Preparation of standard: 10 mg acarbose was dissolved in 10 ml methanol, and various aliquots of 100- 1000µg/ml were prepared in methanol.

Preparation of sample: 100 mg of dried extract was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. 500 µl of this extract was for the estimation of enzyme inhibition.

Method: A total of 500 µl of test samples and standard drug (100-500µg/ml) were added to 500 µl of 0.20 mM phosphate buffer (pH 6.9) containing α-amylase (0.5mg/ml) solution and were incubated at 25°C for 10 min. After these, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added to each tube. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1.0 ml of 3, 5 dinitrosalicylic acid colour reagent. The test tubes were then incubated in a boiling water bath for 5 min, cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm. Control represent 100% enzyme activity and were conducted in similar way by replacing extract with vehicle.

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} X\ 100$$

Where A_0 is the absorbance of the control and A_t is the absorbance of the sample.

***In vitro* dissolution rate studies**
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±0.5^{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 256.0 nm using spectroscopy.

RESULTS AND DISCUSSION:

The results of phytochemical screening of leaves of *Helicteresisora*using Hydroalcoholic as solvents were depicted in table. However, the physiological and biosynthetic reactions taking place inside the plant, responsible for absence of some phytochemicals in one parts and its presence in the other part of plant sample. Hydroalcoholic extract of leaves of *Helicteresisora*had revealed the presence of flavonoids, protein and tannins. Alkaloids, saponins, glycosides, diterpenes, phenols and carbohydrates were found to be absent.

Total protein content was calculated as Bovine serum albumin equivalent mg/100mg using the equation based on the calibration curve: Y=0.001X+ 0.001, R^2=0.999, where X is the BSA equivalent (BE) and Y is the absorbance.

The content of total flavonoid compounds (TFC) content was expressed as mg/100mg of quercetin equivalent of dry extract sample using the equation obtained from the calibration curve: y = 0.036x + 0.018, R^2= 0.998, where X is the quercetin equivalent (QE) and Y is the absorbance.

Table revealed the results of total protein and flavonoid contents in the hydroalcoholic extract of leaves of *Helicteresisora*. The total protein and flavonoid contents of leaves of *Helicteresisora* showed the content value 0.319mg BE/100mg extract and 0.587mg QAE/100mg extract respectively. The *Helicteresisora*showed the highest concentration of flavonoid followed by protein indicate plant may hold better therapeutic application.

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 7.6. The entrapment efficiency of the phytosomes was found in the range of 55.85 to 70.25%.

Particle size of all formulations found within range 310.32-385.45nm. 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, transmission electron microscopy (TEM), and stability studies.

TEMs are capable of imaging at a significantly higher resolution than light microscopes, owing to the small de Broglie wavelength of electrons. This enables the instrument's user to examine fine detail even as small as a single column of atoms, which is thousands of times smaller than the smallest resolvable object in a light microscope. TEM forms a major analysis method in a range of scientific fields, in both physical and biological sciences. TEM characterization revealed that the Phytosomes are spherical in shape (Figure 1). However, some variation in size distribution was observed in the TEM image, which might be attributed to an uncontrolled charge neutralization process involved between oppositely charged chains occurring during the formation of phytosomes.

Table 2: % Yield of leaves extractof *Helicteresisora*

S. No.	Extracts	% Yield (w/w)
1.	Pet ether	0.53%
2.	Hydroalcoholic	6.87%

Table 3: Phytochemical screening ofextract *of Helicteresisora*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Wagner'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**	
	Froth Test	-ve
	Foam test	-ve
8.	**Diterpins**	
	Copper acetate test	-ve
9.	**Tannins**	
	Gelatin Test	+ve

Table 4: Total protein and total flavonoid content of *Helicteresisora*extract

S. No.	Total protein (mg/100mg)	Total flavonoid (mg/100mg)
1.	0.319	0.587

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

Formulation Code	Particle size (nm)	Entrapment Efficiency (%)
F1	385.45	55.85
F2	362.25	62.23
F3	347.85	62.23
F4	358.85	63.32
F5	347.74	65.74
F6	321.12	70.25
F7	345.78	67.85
F8	326.65	64.78
F9	325.65	61.25
F10	310.32	74.65
F11	314.56	65.45
F12	345.65	68.87

Average of three determinations (n=3)

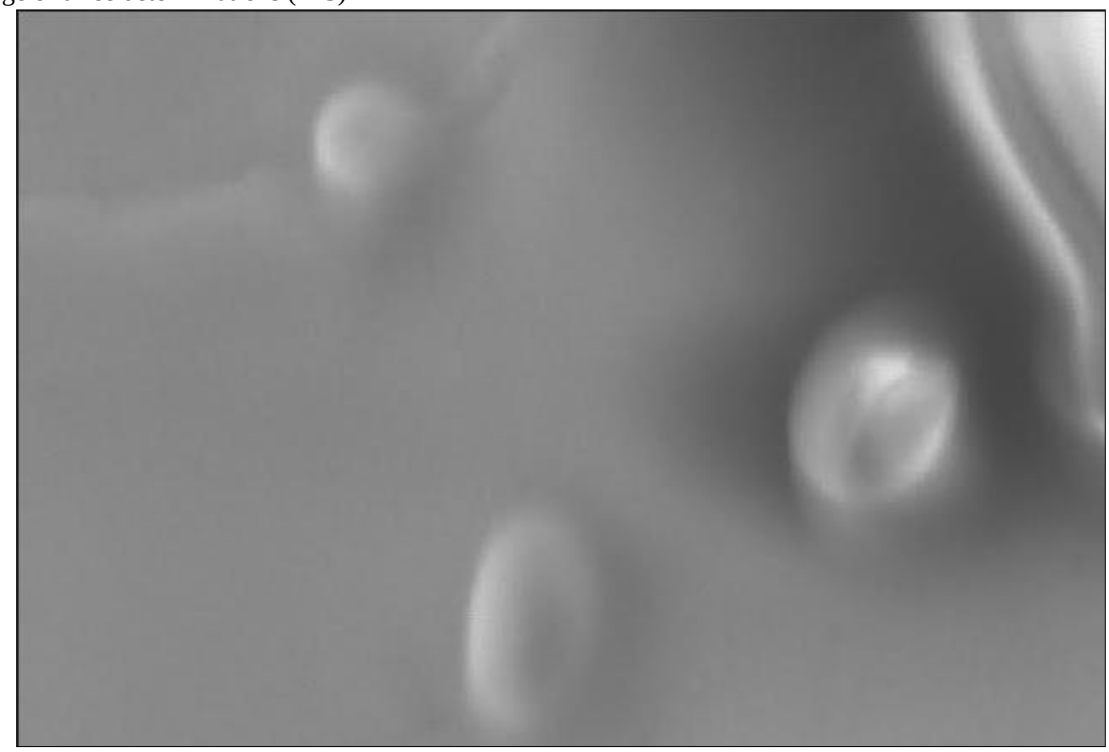

Figure 1: TEM image of phytosomes

Table 6: % Inhibition of hydroalcoholic extract of *Helicteresisora and* prepared phytosomes

S. No.	Concentration (µg/ml)	Acarbose	*Helicteresisora*extract	Phytosomes formulation
		% Inhibition		
1.	100	48.85	42.32	41.15
2.	200	63.32	52.26	55.65
3.	300	70.14	65.58	70.23
4.	400	81.74	73.32	75.65
5.	500	88.74	82.21	83.32
IC 50 value		**90.91**	**328.8**	**326.73**

Table 7: *In-vitro* drug release data for optimized formulation F10

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	26.65	1.426	73.35	1.865
1	1	0	35.58	1.551	64.42	1.809
2	1.414	0.301	48.65	1.687	51.35	1.711
4	2	0.602	65.98	1.819	34.02	1.532
6	2.449	0.778	76.65	1.885	23.35	1.368
8	2.828	0.903	89.98	1.954	10.02	1.001
12	3.464	1.079	99.12	1.996	0.88	-0.056

Table 8: Regression analysis data of optimized formulation F10

Batch	Zero Order	First Order	Higuchi	KorsmeyerPeppas
	R^2	R^2	R^2	R^2
F10	0.924	0.931	0.990	0.997

CONCLUSION:

When the regression coefficient values of were compared, it was observed that 'r^2' values of KorsmeyerPeppas was maximum i.e. 0.997 hence indicating drug release from formulations was found to follow KorsmeyerPeppas kinetics.

In conclusion, in this study, the combined hydroalcoholic extract of *Helicteresisora* in ratio of 2:1:1 found to exhibit significant results.Phytosomes has better physical characteristics than that of extract. *In-vitro* studies revealed that phytosomes showed control release of phytoconstituents. Hence, phytosomal formulation of this herbal drug combination can be used for clinical application to enhance the therapeutic effect.

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