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FORMULATION AND CHARACTERIZATION OF PHOSPHOLIPID COMPLEX OF *APIUM GRAVEOLENS* LINN FOR EFFECTIVE DIABETES TREATMENT

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Abstract:
The present investigation aim to prepare phyto-phospholipid complexes of Apium graveolens has recently received increased attention for treatment of diabetes. The Hydroalcoholic extract of leaves of Apium graveolens had revealed the presence of flavonoids, phenols, protein, carbohydrates, tannins and saponins. Alkaloids, glycosides and diterpenes were found to be absent. 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 50.23±0.36 to 73.32±0.25%. When the regression coefficient values of were compared, it was observed that 'r^2' values of KorsmeyerPeppas was maximum i.e.0.998 hence indicating drug release from formulations was found to follow KorsmeyerPeppas kinetics. 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 % drugcontent.

Key words: Apium graveolens, Phospholipid complex, Diabetes treatment

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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[1-3].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[1]. 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[1].

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)[4], 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].

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[5-6].

Novel vesicular drug delivery systems aim to deliver the drug at a rate directed by need of body during the period of treatment, and channel the active entity to the site of action. A number of novel vesicular drug delivery systems have been emerged encompassing various routes of administration, to achieve targeted and controlled drug delivery. Targeted drug delivery is a mode of delivering the therapeutic agent to the tissues of interest while reducing the relative concentration of therapeutic agent in remaining tissues which improves the therapeutic efficacy and reduces the side effects. Drug targeting means the delivery of drugs to receptor, organs or any other specific part of body to which one wishes to deliver the entire drug[7-8].

A variety of solutions have been proposed to counter the problem of poor absorption, such as the preparation of emulsions, liposomes, and nanoparticles, as well as the modification of chemical structures and delivery as prodrugs. Among the potential strategies, phyto-phospholipid complexes (known as phytosomes) haveemerged as a promising strategy to enhance the bioavailability of active constituents.Phyto-phospholipid complexes are prepared by complexing active constituents atdefined molar ratios with phospholipids under certain conditions.

Celery (*Apium graveolens* L) is a plant from the apiaceae family, and is one of the annual or perennial plants that grow throughout Europe and the tropical and subtropical regions of Africa and Asia. Presence of compounds such as limonene, selinene, frocoumarin glycosides, flavonoids, and vitamins A and C are the reason that celery is the most widely used plant in traditional medicine. Therefore, the present investigation aim to prepare phyto-phospholipid complexes of *Apium graveolens* has recently received increased attention for treatment of diabetes.

MATERIAL AND METHODS:

Extraction procedure

Following procedure was adopted for the preparation of extracts from the shade dried and powdered herbs [9-10]:

Defatting of plant material

53.6 gm of dried powdered leaves of *Apium graveolens* 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 *Apium graveolens* has been extracted with hydroalcoholic solvent (ethanol: water: 70:30) using maceration method for 48 hrs, filtered and dried using vacuum evaporator at 40^0C.

Phytochemical screening

Phytochemical screening: Phytochemical examinations were carried out for the extract as per the standard methods[11].

1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Wagner's Test: Filtrates were treated with Wagner's reagent (Iodine in Potassium Iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Hager's Test: Filtrates were treated with Hager's reagent (saturated picric acid solution). Presence of alkaloids confirmed by the formation of yellow coloured precipitate.

2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Fehling's Test: Filtrates were hydrolysed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Legal's Test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

4. Detection of saponins

Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 gm of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

5. Detection of phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

6.Detection of tannins

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

7. Detection of flavonoids

Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

8. Detection of proteins

Xanthoproteic Test: The extracts were treated with few drops of conc. Nitric acid. Formation of yellow colour indicates the presence of proteins.

9. Detection of diterpenes

Copper acetate Test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

Formulation development ofphytosomes[12]

Preparation of phytosomes

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

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[13]

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*Apium graveolens* was recorded at λ_{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 0.1 N HCl and absorbance taken at 420.0 nm. Amount of quercetin in supernatant and sediment gave a total amount of *Apium graveolens* 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[14]

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*anti diabetic activity of hydroalcoholic extract of *Apium graveolens*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.

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

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 (37^0C) was replaced every time with the same quantity of the sample and takes the absorbance at 256.0 nm using spectroscopy[15].

RESULTS AND DISCUSSION:

The results of phytochemical screening of leaves of *Apium graveolens* 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. The Hydroalcoholic extract of leaves of *Apium graveolens* had revealed the presence of flavonoids, phenols, protein, carbohydrates, tannins and saponins. Alkaloids, glycosides and diterpenes were found to be absent.

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 50.23±0.36 to 73.32±0.25%.

Particle size of all formulations found within range 313.25-465.32nm. 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. When the regression coefficient values of were compared, it was observed that 'r^2' values of KorsmeyerPeppas was maximum i.e.0.998 hence indicating drug release from formulations was found to follow KorsmeyerPeppas kinetics. 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 % drugcontent.

Table 2: % Yield of leaves extractof *Apium graveolens*

S. No.	Extracts	% Yield (w/w)
1	Pet ether	3.56%
2.	Hydroalcoholic	5.22%

Table 3: Phytochemical screening ofextractof *Apium graveolens*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids** Wagner's Test Hager's test	 -ve -ve
2.	**Glycosides** Legal's test	 -ve
3.	**Flavonoids** Lead acetate Alkaline test	 +ve +ve
4.	**Phenol** Ferric Chloride Test	 +ve
5.	**Proteins** Xanthoproteic test	 +ve
6.	**Carbohydrates** Fehling's test	 +ve
7.	**Saponins** Froth Test Foam test	 +ve +ve
8.	**Diterpins** Copper acetate test	 -ve
9.	**Tannins** Gelatin Test	 +ve

Table 4: Particle size and entrapment efficiency of drug loaded phytosomes

Formulation Code	Particle size (nm)	Entrapment Efficiency (%)
F1	365.45	52.32
F2	350.26	57.74
F3	347.85	59.96
F4	362.12	63.32
F5	347.74	65.74
F6	356.65	67.74
F7	347.85	62.25
F8	326.65	64.78
F9	325.65	61.25
F10	**290.45**	**72.25**
F11	314.56	65.45
F12	345.65	68.87

Average of three determinations (n=3)

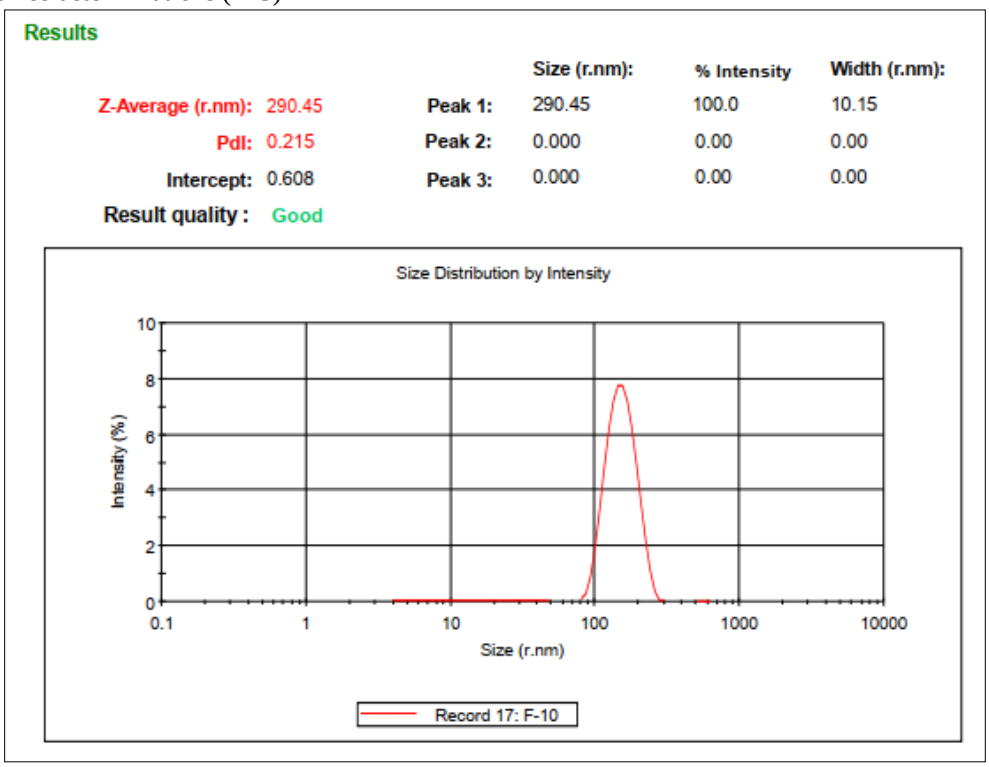

Figure 1: Particle size of optimized batch F10

Table 5: % Inhibition of hydroalcoholic extract of *Apium graveolens and* prepared phytosomes

S. No.	Concentration (µg/ml)	Acarbose	*Apium graveolens* extract	Phytosomes formulation
		% Inhibition		
1.	100	49.58	43.32	42.32
2.	200	68.85	51.14	56.65
3.	300	73.12	69.98	71.25
4.	400	82.25	75.65	76.69
5.	500	89.95	83.32	85.45
IC 50 value		**58.51**	**160.38**	**145.47**

Table 6: Regression analysis data of optimized formulation F10

Batch	Zero Order	First Order	Higuchi	KorsmeyerPeppas
	R^2	R^2	R^2	R^2
F10	0.886	0.928	0.974	0.998

CONCLUSION:

In conclusion, in this study, the combined hydroalcoholic extract of *Apium graveolens* 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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