WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.453
Volume 13, Issue 9, 1399-1419. Research Article ISSN 2277–7105

DEVELOPMENT, CHARACTERIZATION AND EVALUATION OF POLYPHENOLIC PHYTO-PHOSPHOLIPID COMPLEXES (PHYTOSOMES) FOR IMPROVED SOLUBILITY AND SCAVENGING ACTIVITY

Pushpa Patiram Harijan[1], Ran Vijay Singh[2] and Dr. Sanjay Kumar Kushwaha*[3]

Director, Bhavdiya Institute of Pharmaceutical Sciences and Research, Ayodhya, UP.

Article Received on
15 March 2024,

Revised on 04 April 2024,
Accepted on 25 April 2024

DOI: 10.20959/wjpr20249-32216

***Corresponding Author**
Dr. Sanjay Kumar Kushwaha
Director, Bhavdiya Institute of Pharmaceutical Sciences and Research, Ayodhya, UP.

ABSTRACT

The aim of present study was to develop and characterise phytosomes of some herbal extracts to improve the solubility of its phytoconstituents. The poor solubility and dissolution rate of the phytoconstituents present in herbal extracts limit its oral absorption and bioavailability. The Standardised extracts of *Citrus Limon* (SCE), were used for development of phytosomes by solvent evaporation technique. The optimized phytosomes were characterized by various parameters like solubility studies, particle size determination, infrared absorption (FTIR), Differential scanning calorimetry (DSC), X-ray diffraction (XRD), Scanning electron microscopy (SEM), entrapment efficiency and *in vitro* antioxidant activity. SEM and XRD reveal the reduction in crystallinity of extract in the phytosome. FTIR and DSC confirm the formation of phyto-phospholipid complex. The apparent solubility and *in vitro* dissolution studies indicated a significant improvement in the solubility and the drug release of SCE, from phytosomes. It has been observed that thecomplex formed an effective scavenger of DPPH radicals. The result of the study revealed that the phytosomes may be considered as a promising drug delivery system that improves the solubility and absorption of plant constituents. As far as the potential of phytosome technology is concerned, it has a great future for use in formulation technology. Th e developed phytosomes in suitable dosage form may prove to be very effective in clinical use.

KEYWORDS: *Citrus Limon*; phytosomes, DPPH, Antioxidant, *SEM*, *In-vitro* study Phospholipid, Phytosome, Polyphenolic, Herbal, Citrus lemon.

INTRODUCTION

The current remedy uses botanical constituents using plant extracts that were formerly employed by conventional home treatments. Due to their lengthy side chains and strong polarity, several plant constituents were unable to pass though lipidic membranes across diffusion that is passive. According to phytochemical investigation, several plant-based items have medicinal properties, differing structures, and the ability to improve overall wellness [Kennedy et al., 2011]. The accessibility for numerous medicinal herbs and plant-based constituents because they're poorly soluble in lipids as well as less metabolised, has to be improved. Due to its beneficial impacts on medical treatment, extracts of herbs are utilised to treat a variety of disorders that are both microbiological and non-microbial biological origin. Due to their popularity and acceptance by all faiths for use in therapeutic treatments, herbs and botanical extracts are trustworthy, inexpensive, less poisonous, and easily accessible. Vegetables are in fact the first substance utilised in alternative medical treatments for a variety of ailments. According to Vaou et al. (2020), a number of plants have medicinal and pharmacological effects that function as antibacterial, anticancer, anti-infectious, and antitumor substances. In many nations, botanical extracts constitute a staple of the main medical system. A potential source of recently found antibacterial compounds could be plants used for medicine. 80% of mankind reside in poor nations [Yadav et al., 2015].

Recent approach on traditional system of medicine

Range of requirements including supplies of nutrition, energy, building products, fibers, bulk substances personal care products and medications. The maintenance of global health relies heavily on the use of medicinal plants, which are widely accessible and plentiful. These plants provide instant access to safe and efficient compounds that can be used to treat a variety of ailments. There are four main ways that plant-based remedies are useful in contemporary medical practice.

- Topical medicinal products are derived from herbs.
- Chemical compositions obtained from plant-based substances can be utilised as models for developing novel somewhat synthetic molecules. Crops are used as the foundation for the fabrication of complicated partially synthetic molecules.
- The application of plant species as taxonomical identifiers to discover novel chemicals.

Recently, it was discovered that there are more than 20,000 different kinds of medicinal plants within India. Of these, 5000 indigenous peoples are known to use 800 different plant species to treat a variety of illnesses. Due to its lack of side effects, plant-based medicines are now used as the primary form medical treatment approximately 80% of the population worldwide. Medicinal plants are a crucial source. A while ago, at least one component manufactured from plants is found in 25% of American medications for pharmaceuticals. According to Verma et al. (2013), 121 medicinal items were created using conventional wisdom. The herbs that are utilised by rasayana are included in herbal medicines in the standard Indian healthcare system as a whole and the majority of professionals create and administer their own formulations. Worldwide, there are 21,000 plants used for medicinal uses, according to the Global Health Organisation (WHO). Out of the 2500 Indian different species, 150 varieties are widely used extensively. Indian is renowned as the world's botanical park and is the country that has created the most healing plants.

Phyto-phospolipid complex vesicles: Phytosome

A number of the most effective ways to improve the accessibility of phyto-pharmaceuticals is by the use of an innovative phyto-phospholipid interaction technology known for its "phytosome."

According to Lu et al. (2019), several plant-based constituents possess poor solubility therefore cannot pass membranes in biological systems. Many plant actives, despite having powerful in vitro medicinal properties, have not produced the same in vivo reaction. These establish actions could be made greater effectively successful by combining them with common food products lipids like the fatty acid to create new, amphipathic in structures within cells. To add fatty acids to homogenised extract in order to enhance its absorption, its bioavailability and utilisation, phytosome is a top element in neutraceuticals.

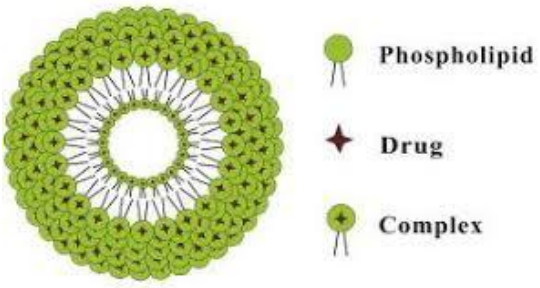

Figure 1: Structure of Phytosome.

Advantages of Phytosome

- The prolonged length of effectiveness for medicinal products is guaranteed by phytosomes.
- They increase the rate of bioavailability and oral, topical in nature, and additional routes of absorbing polymeric phytoconstituents.
- They develop a tiny cell that shields the curative qualities of botanical extracts against microbes in the gut or secretions from the gastrointestinal tract.
- Phytosomes encourage effective delivery of medication to the appropriate organs.
- Through using phytosomes to deliver the medicinal medication, nutritional security is not required to be compromised.
- Phytosomes can be easily carried from inside the cell membranes and taken up by the living cells.
- The oral absorption of the medication noticeably improves.
- Because the primary ingredients are absorbed to their fullest extent, the dose requirement has decreased.
- They decrease the dosage needed and enhance physiologically active constituent absorption.
- The phosphodiesterase molecule plus plant nutrients form bonds of chemicals, which contribute to the phytosomes' excellent stability.
- Because of their better penetration through the skin and elevated lipid content, phytosomes as agents are frequently utilised in cosmetology to improve cutaneous absorption of plant-based constituents
- Its phytoconstituent within phytosomes can be more absorbed and can pass through the tissue of the intestine walls with ease.
- The whole thing is sustainable, and phytosomes do not have issues concerning binding to drugs.

MATERIAL AND METHOD

Citrus Limonia

Within primary genus within the Rutaceae category is Citrus. Tanaka, the author a Japanese expert in citrus taxonomy, concluded that the origins of citrus species are in the northeast of India, northwestern Burma, Yunnan County and the adjacent regions of China. Many businesses, including the alimentary and cosmetics sectors, use diverse plants from these

species. Pure sources of sugars, dietary fibre, vitamins that are water-based, and antioxidants like flavonoids, or limonoids, and carotene include various portions of the citrus species. A few of their most well-known conventional applications were briefly covered [Rao et al., 2020]. In this article, the consequences of Citrus species discovered in multiple studies were evaluated. Clinical research have focused mostly on anxiety, renal inequalities, and lipid counts.

Figure 2: ***Citrus Limonia.***

Method of Preparation

1. Rotary evaporation methods

In 30 millilitres of tetrahydrofuran, a precise quantity of botanical extract and fatty acids were submerged in a rotating RBF. Three hours were spent stirring the flask at an internal temperature of no more than 40 °C. The material was reduced to a thin film before a solution of was introduced and whirled repeatedly with a magnetic stirrer. The resulting precipitate has been gathered, processed, then kept at ambient humidity in amber-coloured glass containers.

2 Solvent evaporation Methods

Every 100ml RBF was filled with precisely one tablespoon of material from plants and phospholipid, or and then it was heated to between fifty- and sixty-degrees Celsius while being stirred with 20 ml of ethanol for twenty minutes. In order to acquire the residue, which has been separated and collected, the combination was diluted to 5–10 ml. In an amber-coloured glassware bottle, a dried precipitation phytosome complexes was put before being kept at ambient humidity.

3 Ether-Injection Technique

With this method, an organic solvent was used to dissolve simultaneously the resulting extract or the bilayer. Vesicles are were created by slowly injecting this combination into an aqueous agent that had been heated. Amphiphiles' condition is dependent on dosage. Amphiphiles provide a single-molecule state while their amount is lower, yet as increases, a range of constructions, including those of the circular, cylinder-shaped, disc, and cubic, and hexagonal types, may emerge.

Characterization and evaluation of Phytosomes

Actual size, permeability of membranes, proportion of imprisoned solvents, and chemical makeup, along with the amount and quality of the founding ingredients, all affect how phytosomes behave in both psychological and biological contexts Phytosomes are analysed usingfollowing physical parameters.

Physical properties

The subsequent methodologies were utilized to describe the physical characteristics of phytosomes.

a) Visualization

Transmission electron microscopy, also known as TEM, can be used to observe phytosomes as agents and may offer information concerning the way they are made as well as a variety of their properties, including their shape, crystallisation, capacity for stress, as well as domains of magnetic attraction. SEM emphasises on the phytosome membrane and reveals physiological details by analysing the chemical makeup.

b) Particle size and zeta potential

Employing a computerised inspection system of a technique called photon correlation spectroscopy (PCS), dynamic light dispersion (DLS) can be used for measuring size of particles as well as zeta potential.

c) Effectiveness of the trap

The process called ultra-method can be used to assess the drug's trapping effectiveness and capacity in phytosomes. It provides information on the percentage of medication that is efficiently retained inside phytosomes.

d) Frequency of transition

The method of differential scanning calorimetry is a method used to find the vesicle phospholipid systems' switching temperature.

e) Measuring surface tension activity

The ring-tensile methods in a Du Nouy ring tensiometer can be used to assess the drug's tension at the surface function in an a solution of water.

f) Cellular stability

Vesicle persistence can be assessed by tracking changes in their shape and measurement throughout the years. DLS evaluates the median size, and TEM keeps track of structural alterations.

g) Drug content (g)

A customised HPLC or an appropriate spectroscopic technique can be used for measuring the quantity of medication.

Spectroscopic Analysis

Spectroscopic analyses are frequently employed to not only validate the development of complexes across the extract of herbs and fatty acids or a compound called moiety but also to learn about the equivalent connection between groups.

PRE-FORMULATION STUDY

Identification of drug

Characterization of SCE, SPE and SBE were done by obtaining FTIR spectra using FTIR spectrophotometer. Applying a DSC, the medicinal product's temperature reactivity was assessed, and the chemical enthalpy as well as melting pointspectrum were identified.

Infrared spectroscopy

The potassium bromide disk method was used for the preparation of sample. IR spectrum of Drug dissolution in magnesium bromide was used for determining it in its solid state. The straps were simply given (cm-1).

DSC study

Applying a DSC, the isolates' temperature performance was examined and the boiling point range, temperature, and temperature were calculated. The medication was warmed at an average speed of 10 C/min throughout ambient temperature categories of 50-300 ^{0}C while sealed securely in ventilated metal pans.

Melting point

Applying a melting point equipment, the melted points of all extracts have been determined.

Solubility study

While extracting 10 ml of different fluids and adding incremental amounts of the drug to create a fully saturated medication the solution, the soluble content of isolates was measured.

Violet spectrophotometry Scan

SCE, SPE, plus SBE were produced as a 10 g/ml solution in alcohol and a buffered phosphate solution (pH 7.4). Employing a dual beam UV-VIS spectrophotometer, the produced solution was checked for ultraviolet radiation in the 200–400 nm frequency spectrum. The maximum absorption of SCE, SPE and SBE were shown in table no. 5.3.

Table Maximum absorbance wavelength (λ max) of SCE, SPE and SBE.

Sample	Maximum wavelength (nm)	
	Phosphate buffer pH 7.4	Methanol
SCE	268	260
SPE	273	270
SBE	330	360

Preparation of Phytosomes

The approach of resolving and evaporating a solvent was used to create the phytosomes. Several phosphodiesterase extracting ratios, such as 0.5:1, 0.75:1, 1:1, 2.5:1, and 3:1, were used to create phytosomes. This compound and ethanol were utilised to disperse phosphodiesterase and extracts, and correspondingly. Both mixtures were combined and poured into a 200 ml flask witha circular bottom. The combination was refluxed for a variety of times, ranging from 1 to 4 hours, and at temperatures between 45 and 65 °C. The accumulated transparent substance was vaporised, dried beneath vacuum, and kept in desiccants for later use.

PHYSICOCHEMICAL EVALUATION

Apparent Solubility

After pouring additional extract with phytosome for 5ml of either water or n-octanol in covered glass bottles at the ambient temperature (25–30oC), it became possible to evaluate the apparent soluble capacity of the extracted and phytosomes. After being stirred for a full 24 hours, the resulting solution was spun down for 20 minutes at 1,000 rpm to eliminate excessive extract. In order to analyse the specimens spectrophotometrically at 268 nm employing an ultraviolet (UV) spectrophotometer, the resulting solution had been passed over

a Membrane Filter (0.45 m), followed by 1 ml of the filtration was blended with 9 ml of boiled water or n- alcohol [Sikarwa 2008].

Entrapment capacity

With the aid of a visible-UV spectrophotometer, entrapment efficiency (EC) was assessed. 50 mlcontaining ethanol were then added to estimated amounts of phytosomes has equal to 10 mg contained isolates in a beaker that contained 100 millilitres of methanol. The mixture was agitated for four hours using a magnetic stirrer, and then let to stand for an additional hour. The transparent fluid was removed from the bottle and whirled for fifteen minutes at a rate of 5000 rpm. Before centrifugal force the resulting solution passed through 0.45-inch Whatman filter cloth, when the drug concentrations was determined by measuring transmittance at 268 wavelengths in the Ultraviolet after a sufficient diluting. A triple of every measurement was made.

Particle size Analysis

The newly created materials' particle sizes were analysed employing spectroscopy for photon correlation and dynamic light scattering methods. The phytosomes that are were mixed with the isopropyl alcohol mixture for a ten-minute period using a stirrer with a magnet. A size analyzer was used to assess the distribution.

X-Ray diffraction (XRD) Analysis

X-ray diffractometer was used to assess the polymorphism configurations of the material. Voltage 45 kV, supply 0.8 mA, and scanned frequency 1/min constituted the operational parameters. Employing a Cu-Anode X-ray source with oscillation detection device, each of the specimens were photographed with a diffraction angle that varied throughout an interval of 5- 60°.

DSC

DSC was used to perform thermal studies on the samples. The testing was completed using a high-purity environment for calibrating the analyzer's heat flow and heating the capacity under a purge procedure of dryness gaseous nitrogen. The five milligramme samples were kept in lidded metal pans. Every one of the samples underwent an individual warming cycle from 0 to 300 degrees Celsius at a rate of 10 degrees Celsius per minute [Durowoju et al., 2017].

FTIR Analysis

To determine the composition plus chemical property of the generated phytosomes as well as to analyse the connection involving extracts and PC, a FTIR was implemented. Utilising the KBr technique, the IR spectroscopy of the materials were acquired. By carefully combining a little amount of material (1 mg) using 100 mg of KBr, the pellets of KBr have been generated. A little amount of specimen was placed close towards the probe, it was then tightly fastened to the surface and interrogated in the 4000-500 cm-wavenumber range. The collected infrared (IR) spectra have been examined for categories of function at each wave position (cm-1) using the results.

SEM

Samples were provided with a gold coating. A tiny amount of the specimen with coating was analysed by putting it in the SEM, where its exterior appearance was seen and recorded to examine the nanoparticle structure.

In-vitro diffusion analysis

Throughout the purpose of drug diffusion examinations, a Franz cell diffusion system with a 25- milliliter capacity for cells was applied. Across the tissue paper membrane's appear, 1 g of the solution was equally applied over a specific region. Phospho buffer mixture pH 7.4 was just prepared and poured into a receptor chamber. A stirring magnet with a star-shaped blade swirled within the receptor chamber. During the appropriate time intervals, 1.0 ml a portion of the material were collected and regenerated with new buffer solution. After the proper dilutions, materials were examined for drug content using an UV-visible spectrophotometer with a wavelength at 270 wavelength. According to time, the entire quantity of medication transported through the barrier was calculated [Salamanca et al., 2018].

5.7 Phytosome Stability evaluation

In order to assess any chemical or physical modifications that occur on preservation, tests for stability on the optimised phytosomes as agents were conducted. According to ICH criteria, an evaluation of stability was conducted. The goal of stability investigation is to demonstrate how a drug substance's or the item's performance varies over time underneath the influence of several environmental conditions like light, transpiration, and warmth. For six months, stability tests for the optimised composition were conducted at 25 2 °C and 60 5% RH and 40 2 °C and 75 5%RH, respectively. The samples' colour, physical characteristics, and pH

were evaluated after 0, 3,and six months. [Ali et al., 2013].

5.8 Statistical Analysis

The data is displayed as mean exceptional deviations. One-way evaluation of variation (ANOVA) and the Student's t-test were used for the statistical examination. We considered P values < 0.05 to be scientifically substantial.

RESULT AND DISCUSSION

Infrared spectroscopy

The FT-IR spectrum of SCE along with interpretation has been showed in figure number 6.1 aswell as table 6.1 accordingly.

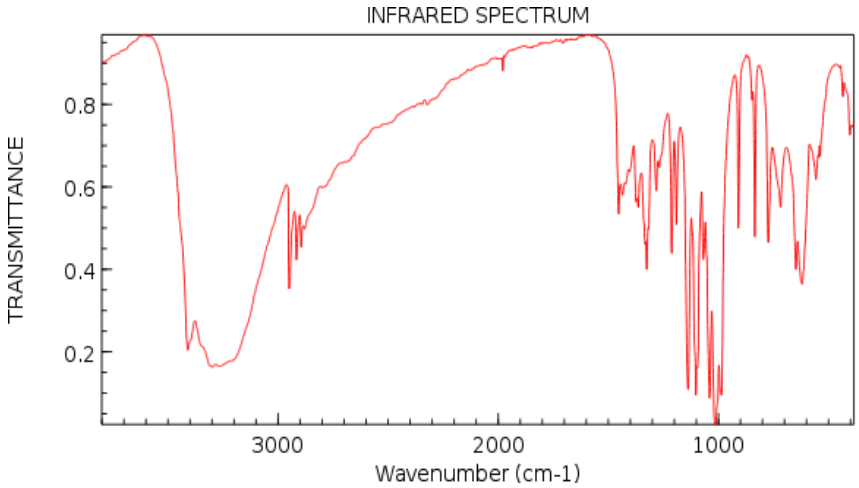

Figure 3: FTIR spectra of SCE.

Table 1: Interpretation of IR spectra for SCE.

SN	Stretching	Peak (cm^{-1})
1	O-H (stretching)	3556
2	Aro C-H (-do-)	3100
3	Ali C-H (-do-)	2900
4	C=O (-do-)	1660
5	C=C (-do-)	1559
6	C-O-C (-do-)	1300

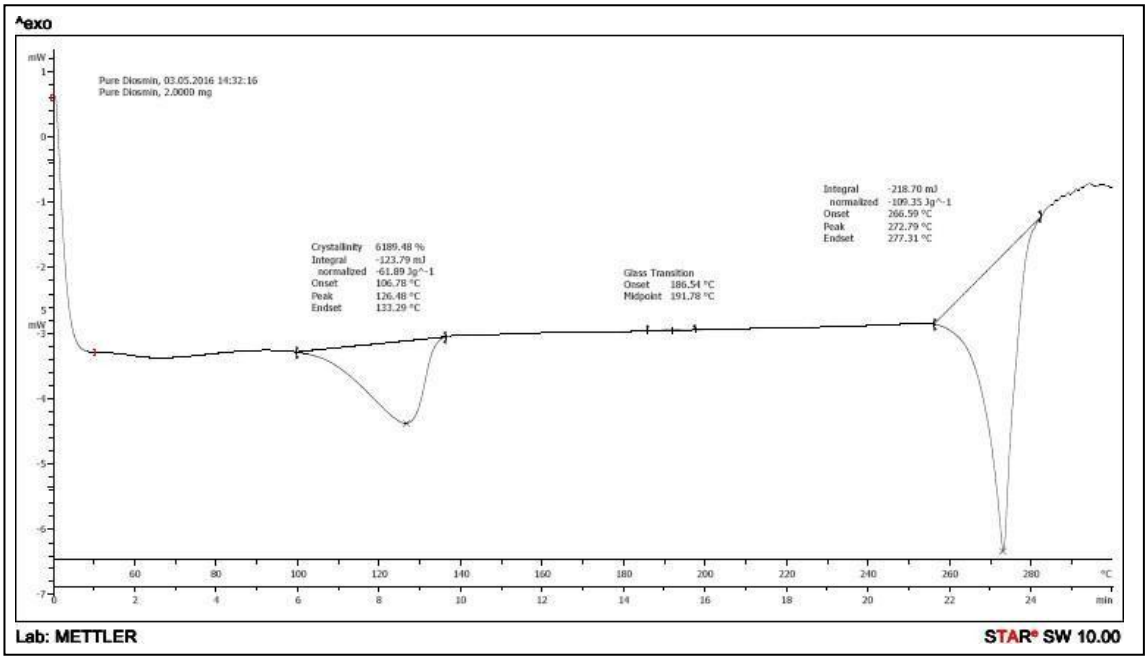

Figure 4: DSC of (A) SCE and (B) CP.

Solubility study

The dissolution and friendliness of the medication in the lecithin in each solvent are the major factors in choosing the best vehicle for the creation of phytosome filled combination. Numerous chemicals, including organic ones such as acetone, which aqueous dimethyl sulfoxide, or (DMSO), water from distillation, alcohol, and a pH 7.4 phosphate buffer solution (PBS), were used to test the soluble form of SCE. Table 6.2 displays the investigation of SCE's solubility in several solvents. Every other solvent, excluding acetone, acetone, DMSO, and distilled water, had demonstrated a favourable dispersion characteristic for SCE.

Table 2: Solubility analysis of SCE.

SN	Solvent	Concentration (µg/ml)
01	Acetone	1.3 ± 0.12
02	Acetonitrile	5.32 ± 0.54
03	DMSO	8.2± 1.03
04	Distilled Water	2.35± 0.31
05	Ethanol	10.42± 0.84
06	Methanol	12.56± 1.04
07	Phosphate buffer pH 7.4	14.21± 0.23

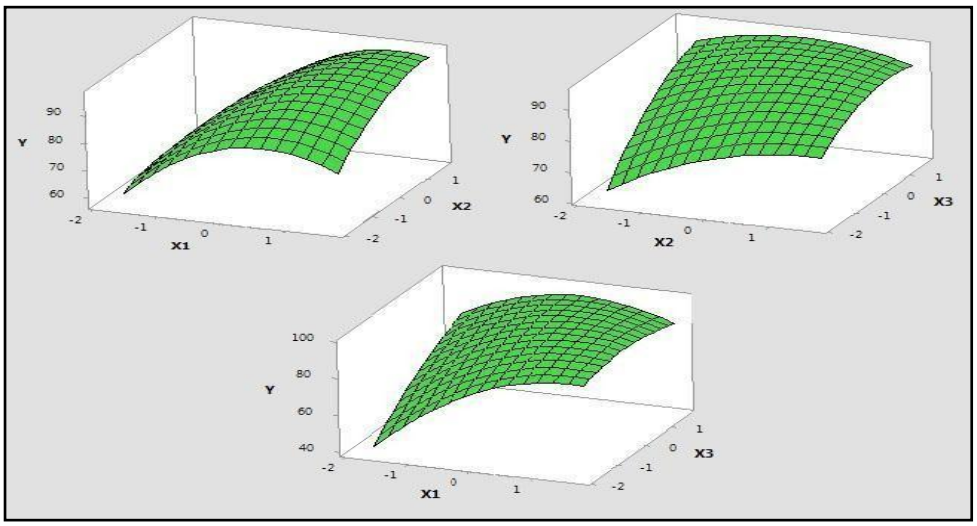

Figure 5: Responsive curve map demonstrating the effects of the process temp (X2, °C), the reaction duration (X3, h), plus the phospholipids-drug concentration (X1, w/w) affect the effectiveness of SCE encapsulation.

% Drug release

In illustration 6.19 as well as column 6.14, the outcomes of in vitro release of drugs investigations are demonstrated. The simplest form of SCE demonstrated the least rapid rate of breakup, with only around 44% compared to w of SCE breaking down at the conclusion of the 12-hour dissolving interval in a phosphate buffer (pH 6.8). When compared with pure SCE, the biological combination dissipated at a pace that didn't seem significantly different (49% weight to weight in 12 hours). The planned CP showed a noticeably quicker breakdown of SCE at the conclusion of the disintegration phase. Over 89 percent w/w SCE was seen to be discharged out of CP after 12 hours. The fluidity and crystallised structure of the materials have a significant impact on the breakdown rate, and the enhanced dissolution and heterogeneous shape of the produced combination may contribute to the enhanced disintegration speed of SCE compared to CP. The improved water solubility and slightly more aggregate state found in the phytosome could help the medication's continuous distribution.

Table 3: Characterization of the in-vitro disintegration of SCE, PM, as well as CP.

Time	% Drug Release		
	SCE	PM	CP
1	22.0811	26.7838	35.1784
2	29.4324	30.7027	40.3212
3	34.3243	36.1351	46.3242
4	36.4054	37.8108	57.2367

5	38.6487	40.1892	63.1324
6	39.1892	43.1351	67.4532
7	41.3478	44.1345	70.1276
8	42.4653	46.5632	73.3498
9	42.7236	47.2378	75.6532
10	43.3489	47.9876	80.5609
11	44.1256	48.3465	85.5432
12	44.7896	49.5097	89.7865

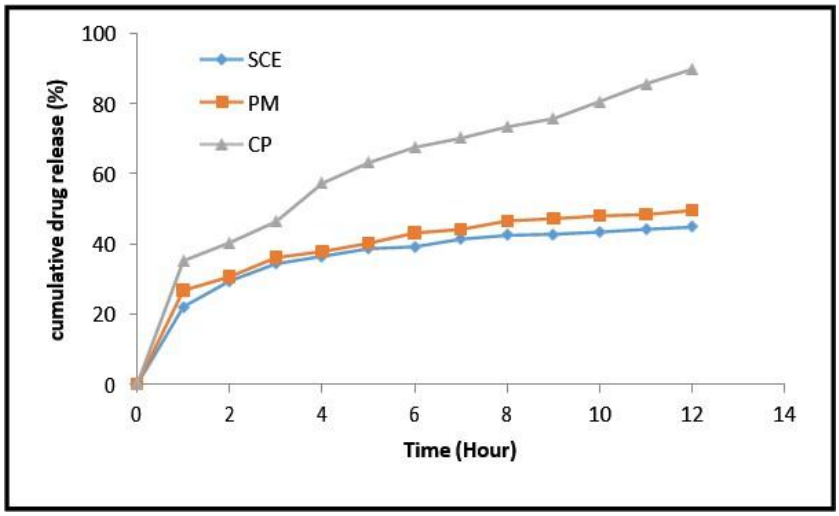

Figure 6: Characterization of the in-vitro disintegration of SCE, PM, as well as CP.

6.7 Antioxidant potential by DPPH analysis

The ability of the extract to scavenge radicals created by DPPH has been shown as shown in Figure 6.20 as well as Table 6.15. The DPPH assay used DPPH absorbance to calculate an antioxidant's antiradical potency. For a dosage of 1000 g/mL, the percentage scavenger effects of DPPH as a radical increased when the amount of CP, SCE, as well as vitamin C increased in the sequence vitamins C > CP >'SCE, with values of 95.56%, 92.74%, and 74.43%, correspondingly.

Table 4: DPPH radical scavenging activity SCE, CP and Vit C.

SN	Concentration(µg/ml)	DPPH (% Inhibition)[a]		
		SCE	CP	Vit C
1	100	17.21±0.16	19.70±0.23	23.92±0.13
2	200	31.23±0.31	48.09±0.43	54.68±0.14
3	400	51.57±0.17	63.18±0.21	68.04±0.17
4	600	62.32±0.02	72.18±0.03	78.08±0.15
5	800	70.08±0.22	82.62±0.32	85.34±0.46
6	1000	74.43±0.14	92.74±0.42	95.56±0.26

[a]All values are mean ± SD (n=3)

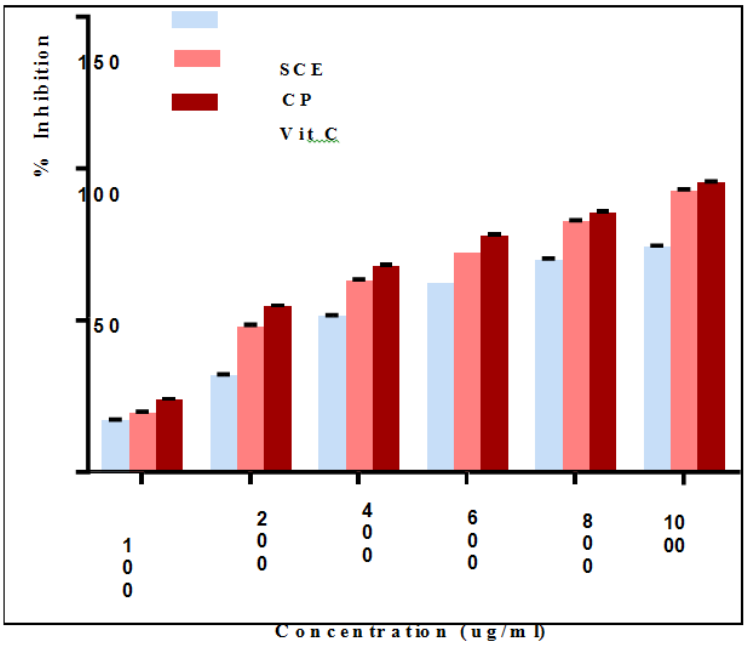

Figure 7: DPPH radical scavenging activity of SCE and CP.

DISCUSSION

Although herbal remedies are effective in treating a variety of disorders, it is crucial to discover effective ways to deliver medications from plants and animals. The herbal extracts are very useful for health maintenance and wellbeing. Certain limitation such as poor miscibility with oil and lipids causes poor solubility and bioavailability. The effectiveness of extracts is depending on the applications of drug. The pharmacological study of phytomedicine shows the therapeutic applications of active constituents. Also, The full spectrum of nutrients, metabolites, which and medicinal products are naturally compatible using fatty acids. Therefore, by using the plant extracts and phospholipids enhance the bioavailability of plant constituent.

The goal of the current investigation was to investigate the preparation and characterization of phytosomes of some herbal extracts such as Standardized Citrus Limon (SCE), The primary objective of the current work would to determine whether it had been possible to increase the permeability of standardised preparations by creating a vascular combination out of them using phosphotidylcholine. Orange Phytosome (CP) is the name given to this complex. They were created by evaporating solvents. Applying an Improvement by Designs (QbD) methodology, the formulation as well as the procedure factors for the production of the the phytosomes were optimised.

The produced the phytosomes have been studied chemically for stability and dissolving rate research, fluidity and crystallisation (FT-IR), thermal investigation (DSC), surface structure (SEM), and chemical interactions (FT-IR). All the phytosomes were also evaluated for antioxidant and hepatoprotective activity. Analysis of SEM of extracts revealed that it exists in crystalline form. On the other hand, in the photomicrograph of phytosome formulation, the corresponding features of extracts signifying the formation of complex. This observation suggests the existence of physical interaction of extract with phosphatidylcholine leading to decreased drug crystallinity. The phytosomes was found to have amorphous nature.

The botanical extracts had an influenced by concentration ability to scavenge radicals that were unstable and suppress DPPH. DPPH levels can be frequently used to assess how well different antioxidant substances scavenge reactive oxygen species. Antioxidants in the botanical extract can convert the stable radicals DPPH used in the DPPH test into the yellow-colored diphenyl picryl hydrazine. Antioxidants' capacity to scavenge radicals that are free is measured using a reagent known as DPPH. In order to transform from a free radical that is stable into a stable electromagnetic molecule, DPPH needs a single electron or proton radical. Reduction capacity is enhanced by antioxidant action and could serve as a useful indicator of this process.

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