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Synthesis and Characterization of Transfersomes and Evaluation of Entrapment and Unentrapment Efficiency of the Phenol Extracted from *Camellia sinensis* with the Physical and Chemical Stability of Transdermal Patch

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ABSTRACT

*Camellia sinens*is (green tea) is widely known for its medicinal quality in treating ailments and acts as an excellent antioxidant and stimulator for decades. It is rich in phyto constituents and has high amount of active compounds like phenols, polyphenols and caffeine. Polyphenols found in *Camellia sinensis* (green tea) exhibits many beneficial biochemical effects *in vitro* and *in vivo*. Nano biopharmaceutics is the recent technology, which is used for drug delivery system. Transdermal is an application process by which, skin permeation enhancers promotes skin fluidity and 'Nano-porosity' which increase the capability of low molecular weight lipid- based vesicles to cross the skin. Using different lipid mediated carriers as nanovesicle loaded with desired effective drugs to the targeted sites in treating diseases has been an effective method in the recent past. The present study focuses on extraction and quantification of phenolic content present in the green tea leaves extract by means of physical and chemical stability, λ max determination, Entrapment efficiency and SEM analysis. Optimizing the effective Nanovesicle tranfersomes is done by various process variables like Soy lecithin with Tween 80 (1:1 and 1:2 ratios) and Cholesterol with span 60. The entrapment efficiency is found to be high at Soy lecithin with Tween 80 (1:1 ratio) 6000 rpm at room temperature. Thus, the present finding infers that the transferosomes entrapped with Green tea leaf extract would be a promising approach in preparing transdermal patches that would treat many diseases and be an efficacious targeted drug delivery system.

***Key words*:** *Camellia sinesis*, Nanobiopharmaceutics, Transdermal patch, Nanovesicles, Transfersomes

Nanoscale science in supplementary words nanoscience, is the study of a materials properties, response and features at the molecular or atomic level. The relationship of nanobiotechnology to nanomedicine serves numerous applications in the pharmaceutical industry [1]. It incorporates diagnostics and therapeutics as a part of clinical application, by which extremely accurate systems have been developed. Nanomaterial micelles structures like liposomes, transfersomes, capsosomes, ethosomes and niosomes have been used as vesicular carriers to effectively transport drug to the site of requirement [2-3]. Carrier mediated transdermal drug delivery has been in modern focus as an emerging and most powerful technology in drug administration. Colloidal drug carrier systems have been in trend due to its high encapsulation efficiency and biocompatibility. The main objective of such formulations is optimized drug loading and release capability, long shelf-life, high bioavailability and zero toxicity [4]. One of the greatest prominent morphological features of the normal skin is its stratification. Using skin permeation enhancer which promotes skin fluidity, 'Nano-porosity' increases the capability of low molecular weight lipid- based vesicles to cross the skin [5]. Transdermal patches have a cutting-edge advantage over other drug delivery systems as they have a rate limiting membrane which controls the out flux of drug into the blood stream. The drug permeation characteristics can be controlled by managing the porosity of the membrane used and also its nature [6]. When a patch is placed over the

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skin, the drug compartment containing the active drug is at a higher concentration, while, the blood is at a lower concentration. Green tea has been used as an antioxidants and stimulator for decades [7]. The challenge of nanotechnology is to develop nanoparticles for medical and biotechnology applications to deliver the pharmaceutical advances in the right place at the right time [8]. Keeping this in mind, the present investigation was carried out to synthesis the phenolic compound present in green tea, along with characterization of nanovesicle transfersomes and to evaluate its application in transdermal drug delivery as an effective nanovesicle in treating diseases.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the study were purchased from Merck & Co., India.

Collection of samples

The Green tea leaf samples were collected form Coonoor, India and authenticated by Professor M. Kumar, Department of Botany, MCC, Chennai, India.

Extraction of phenol from green tea

The collected green tea leaves were surface sterilized with running tap water thoroughly and used for subsequent experiments. Screened active material is subjected to extraction using methanol at room temperature. For this purpose, 10g of fresh green tea leaves was crushed and suspended in 200 ml of methanol. Mixture was kept for stirring for the period of 30 min. The total crude extract was centrifuged and the supernatant was separated out for isolation and purification of phenol [9].

Purification of phenol

The supernatant was subjected to purification using column chromatography. A thin column was packed with silica gel of 230-400 mesh. Hexane was used as an eluent. Four ml of hexane is first used to activate the powder to mesh form. Different column fractions were collected. The extracted purified phenol was confirmed with authentic by using commercially available pre-coated TLC plate [10-11].

Quantification of phenolic content

The absorbance of phenol content of different fractions of green tea was analyzed at 750 nm using UV-Spectrophotometer. Total phenolic content of green tea was determined using Folin-Ciocalteu assay. The standard solution was prepared by weighing 1 g of gallic acid and dissolve in 1000 ml of double distilled water. From the stock solution the working standard was prepared by drawing 1 ml of stock and made upto 100 ml using double distilled water. From this 2 ml to 10 ml of working standard solution is taken in a series of test tubes in the concentration ranges from 0.00, 0.25, 0.50, 0.75 and 1 mM. Briefly, 200 mL of green tea leaf extract (10 mg/mL) and 2 ml of solution A (10 ml of 2% sodium carbonate with 0.1 ml of copper sulphate and 0.1 ml of sodium and potassium tartrate) were mixed, after 4 min, 0.4 ml of 0.5 M sodium hydroxide was added. After 10 min, 0.2 ml of Folin-Ciocalteu reagent (1:1 v/v with water) was added. The solution was allowed to stand for 30 min and its absorbance was measured at750 nm in UV–Vis. Spectrophotometer [12].

Preparation of transfersomes

Transfersomes were prepared using thin film hydrated method where mixture of surfactant (Tween 80-Polysorbate 80) and phospholipid (Soy lecithin) as source dissolved in a volatile organic solvent (diethyl ether, chloroform and ethanol) in a round bottom flask. Various trials for surfactants and lipids at different concentrations were carried out. The organic solvent is removed by vigorous hand shaking over a hot water bath maintained at 80°C. A thin film is obtained at the bottom of the round flask which is then rehydrated with phosphate buffer.

Phosphate buffer preparation for nanovesicle formation

Phosphate buffer is prepared by mixing 80.2 ml of 1M Dipotassium hydrogen phosphate and 19.2 ml of 1M Mono potassium hydrogen phosphate. Constant stirring is required until both salts dissolve in distilled water. The pH of this mixture is then checked, using a pH meter, and maintained at 7.4 so that the integrity of transfersomes is not disrupted. Buffer is added drop by drop to the transfersomes in round bottom flask with constant handshaking over the water bath at 80°C. Once the hydration is done the vesicles move into the solution and appear as a colloidal dispersion.

Encapsulation of phenol

Vesicular structures prepared previously were stored at 10°C. They were brought to room temperature before encapsulation. Transfersomal suspension and purified phenol was added in a ratio of 1:1 into a round bottom flask. This was kept in the orbital shaker for a period of 30 min by that the encapsulation is said to be complete.

Entrapment efficiency of phenol loaded transfersomes

Previously prepared drug encapsulated vesicle sample was taken and divided into two portions. One was centrifuged at 3000 rpm for 30 min to separate the unentrapped drug from the entrapped drug. Another portion was centrifuged at 6000 rpm to analyze the effect of centrifugation on entrapment efficiency. The supernatant of both the portion was discarded and 50 % of 5 ml of iso-propanol was added to the pellet. Dissolved pellet was again centrifuged at 4000 rpm to separate the drug from lysed vesicles. Folin Ciocalteu assay was performed on the pellet and the absorbance was measured at 750 nm under UV-Vis spectrometer.

Entrapment efficiency = (R/O) * 100

R-Concentration of drug released from vesicle

O- Original concentration of drug taken

Encapsulation of phenol into transfersomes

Out of the various trials performed a best composition was finalized by judging the entrapment efficiency (% EE) of each sample. Since phenol is highly hydrophobic in nature, it occupies the space between lipid bilayers, so that it cannot be encapsulated after the transfersomes have been formed. Hence phenol stock was added to the solution containing phospholipid and surfactant during the hand shaking process to produce loaded transfersomes [13].

Preparation of hydrogel

To facilitate easy application and to enhance the drug permeation ability through the skin, a hydrogel of the drug loaded transfersomes was prepared. Carbopol 940 is a cross linked poly-acrylate polymer which is initially in white powder form. On addition of water and heated to about

60°C, it polymerizes to form a gel base. Definite amount of polymer was sprinkled to drug and vortexed for a period of 25-30 min. About 6 ml of known concentration of transfersomal suspension was added during polymerization to form the hydrogel.

Drug release through patch

A film of 4 cm was cut and placed between the donor and receiver compartments of the Franz diffusion cell. Subsequently, drug loaded hydrogel was applied on it. The drug was left to elute in the phosphate buffer solution with constant stirring at 100 rpm to which fresh PBS was replaced at every 30 minutes interval on removal of 3 ml of sample. Each sample was stored carefully in different vials and subject to UV spectroscopic studies at 230 nm.

Preparation of transdermal patch

A complete transdermal patch was obtained by loading the films, previously synthesized with a hydrogel containing the drug. The hydrogel acts as a drug reservoir to allow movement of the drug containing nanovesicle to seep through the patch. Commercially available bio adhesive gel Gantez™ was used to seal all four side of the film. The final result was a transdermal patch easy to apply on the skin with a lining and backing layer similar to that of band aid [14].

Characterization study: SEM analysis of transdermal patch

The phenol encapsulated transdermal patch was subjected to electron microscopic study to determine the morphology and size of the synthesized transfersomes. SEM data helped to shape out the morphology and used to confirm the encapsulation of phenol in the patch.

RESULTS AND DISCUSSION

Estimation of total phenol content

The amount of standard phenol content and unknown phenol content, i.e the phenol extracted from green tea leaves extract was determined by Folins' assay and subjected to UV Spectroscopic measurement of absorption at 750 nm. The obtained O.D is directly proportional to the amount of total phenol concentration present in the sample. The results taken as a triplicate value were analyzed using SPSS, and the data was calculated (Table 2).

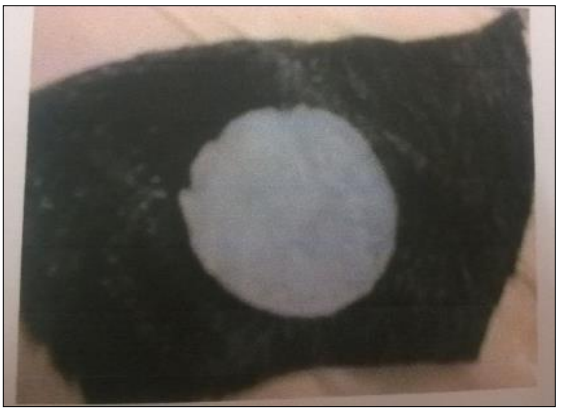

Fig 1 Preparation of nanovesicle [Soy lecithin + Tween 80 (1:1)]

Table 1 Estimation of standard and unknown phenol

Sample (ml)	Concentration (µg/ml)	O.D @ 750 nm for standard solution	O.D @ 750 nm for Unknown sample
Blank	0	0.00	0.00
2	0.00	0.03	0.05
4	0.25	0.06	0.10
6	0.50	0.09	0.15
8	0.75	0.12	0.20
10	1.00	0.15	0.25

Trails conducted

Various phospholipid and surfactants sources were taken at different ratios and the vesicles were prepared to obtain the best composition. To obtain the best composition a batch preparation was done.

Trial 1 consist of preparation of samples (T_1 - T_4) using the following ratios by slow hand shaking at 80°C using boiling water bath until film formation, expressed in (Table 1).

Trial 2 consist of preparation of samples using the same ratios and placing them in the orbital shaker at 70 rpm at room temperature until the film was formed. From this table and image shows that the sample T2 shows best result compared to other samples. So, it taken for further assays.

Table 2 Trials conducted on different formulations

Sample	Phospholipid source	Surfactant	Solvent
T_1	Soy lecithin	Tween 80 (1:2)	5 ml:5 ml
T_2	Soy lecithin	Tween 80 (1:1)	5 ml:5 ml
T_3	Cholesterol	Span 60 (1:1)	5 ml:5 ml
T_4	Cholesterol	Span 60 (1:2)	5 ml:5 ml

Folin's test on evaluating unentrapped drug

The amount of un-entrapped drug was determined by Folin Ciocalteau assay and subjected to spectroscopic measurement of absorption at 750 nm. Data depicted in (Table 3) represents the different centrifugation rate of the unentrapped samples shows different OD at 750 nm. From this result it is evident that, the OD is increased with increase in the concentration of the sample.

Entrapment Efficiency of phenol in transfersomes

The entrapment efficiency of phenol into transfersomes can be expressed as percentage of entrapped to total drug concentration. Among the four different concentrations of the samples, increased percentage of entrapment efficiency was observed in 1ml of sample with 99.4% EE at 3000 rpm.

Table 3 Evaluation of unentrapped drug samples centrifuged at 3000 & 6000 rpm

Sample (ml)	Concentration (µg/ml)	O.D @ 750 nm with 3000 rpm	O.D @ 750 nm with 6000 rpm
Blank	0	0.00	0.00
0.5	2.5	0.015	0.035
1.0	5	0.030	0.070
1.5	7.5	0.045	0.108
2.0	10	0.060	0.140
0.5	2.5	0.015	0.035

Table 4 Evaluation of entrapped drug Samples centrifuged at 3000 & 6000 rpm

Sample (ml)	Optical density @ 725 nm for 3000 rpm	Percent entrapped efficiency	Optical density at 725 nm for 6000	Percent entrapped efficiency
0.5	0.158	97.82	0.153	97.6
1.0	0.030	99.4	0.029	99.3
1.5	0.063	99.0	0.070	98.7
2.0	0.098	98.6	0.100	97.0

The proportion of unentrapped and entrapped was determined by centrifuging 0.5–2.0 mL of the transfersome formulation at 3000 and 6000 rpm for 10 min at 37°C and measured at 725 nm. The above table shows that 1.0 ml sample exhibits maximum entrapment efficiency.

Drug release through patch

Sodium alginate, a natural polymer used in the form of buccal and transdermal patches now a day. On observing the sustainability of the patch no tearing was found over a period of 3 hours. The slope of the graph within the first 60 minutes was steep around 0.14 implying that the patch had observed no leakage.

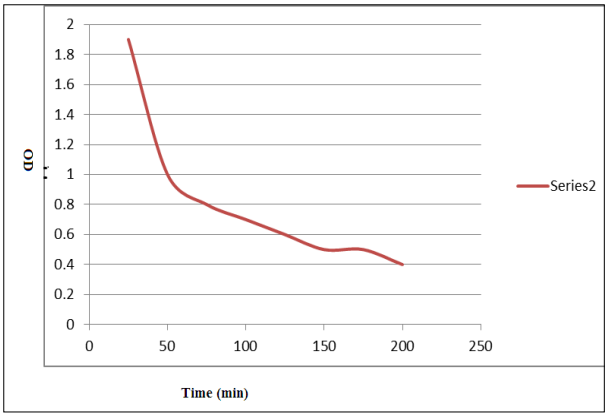

Fig 2 Release of drug through the patch

The drug release from the patch was analyzed from the figure, it can be interpreted that sustainability is strong enough to hold the entrapped materials. The graph revealed that the patch had observed no leakage.

Physical stability and chemical stability

The Stability tests were performed to see the effect of green tea extract on the drug delivery of patches by means of Drug release, Physical stability and Chemical stability was analyzed by means of agglomeration.

Scanning electron microscope (SEM)

The morphology of Transfersome was evaluated using scanning electron microscopy (SEM). The microspheres were suspended in absolute alcohol and sonicated for 5 s to break up the aggregates. One drop of suspension was spread onto an aluminum stub covered with double-sided adhesive tabs. After the alcohol evaporated completely, the microspheres were vacuum-coated with a gold-palladium film and analyzed with SEM.

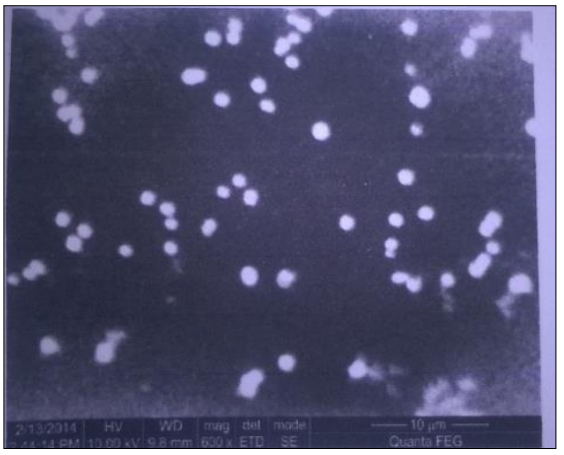

Fig 3 SEM image of transfersomes

According to Rita Muzzalupo *et al.* 2011 the concept of novel drug delivery systems used for therapy has been wide area of research times. Optimizing the dosage and other therapeutic requirements for patients is the need of the hour. The role of nanotechnology in the field of diagnostics and therapeutics, theranostics has been a great contribution to mankind. Nano biotechnology, which combines the positives of small size of nanotechnology and biological aspects of biotechnology, has been of great interest of researchers worldwide. Scaling down materials to nano size has made detection of any disease accurate to the genetic level [15-18]. Uses of molecular markers and nano fluorescing materials have helped to analyze plausible defects of patient even at the chromosomal level [19].

The synthesis and development of vesicles which mimic the structure and function of a cell has been designed by nanotechnologists globally to carry drugs ranging from low to high molecular weight and also peptides from the site of administration to the target. Incorporating these vesicles into a transdermal patch is a painless mode of drug administration through the layers of the skin [20]. Transfersomes are one of the best among many carriers. The self-assembly of non-ionic surfactants into vesicles is first reported in the 70's by researchers in the cosmetic industry.

The non-ionic surfactants form a closed bilayer vesicle in aqueous media based on its amphiphilic nature using some energy like heat and physical agitation to form this structure.

The entrapment efficiency is found to be high at soy lecithin with Tween 80 (1:1 ratio) which is prepared by two trials. In trial one it is conducted at 80°C using boiling water bath. In second trail a film and nanovesicle formation was carried out using orbital shaker at 7000 rpm at room temperature (Table 2). It is observed that green tea extract has maximum phenolic content when comparing the standard at the same concentration (Table 1). There is an increase in phenolic content observed in case of unentrapped drug which is evident from increasing concentration. Based on the study by Effionora Anwar *et al.* 2018 [21]. It can be concluded that the green tea leaf extracts have strong antioxidant activity.

The entrapment of phenol in terms of quantity to load in transfersome is calculated by means of entrapment efficiency. From (Table 4), it is clear that 1 ml has maximum of 99.4% EE. Whereas the microspheres formed from the tea extract had an entrapment efficiency of 53.45% only [21]. To check its entrapped and unentrapped drug in Nanovesicle and using Transfersomes as a lipid vehicle for the encapsulation of the phenol (drug) and to evaluate its physical and chemical stability. The preparation and characterization of transdermal patches is the aim of this small study which has to be studied further which has many applications especially in the field of nano-biopharmaceutics.

Stability is an important factor, which determines the integrity of the transfersomes present in the suspension. No agglomeration or flocculation is found in the prepared suspension, stored over a period of 40 days at 10°C. This shows that the transfersomes have had a constant closed 3D structure within its phospholipid and surfactant. The physical stability is analyzed by means of agglomeration or flocculation. The slope of the graph within the first 60 minutes is steep around 0.14 implying that the patch had observed no leakage. No changes in λ max value of drug-loaded transfersomes were observed on UV analysis conducted every 10 days. This showed that the synthesized transfersomes did not show any kind of drug leakage or instability in its structure over the given time period which in turn proves its chemical stability.

The Scanning Electron Microscope (SEM) focuses on the sample where the beam of high energy electrons generates a variety of signals at the surface of specimens. The signals derived from electron- sample interactions reveal the information about external morphology (texture). Based on the SEM results, of the transdermal patch with sample, this has the most spherical shape compared previous literatures with different samples for different transdermal treatments. The data collected over a selected area on the sample surface generates a 2-dimensional image which displays the spatial variations in its properties.

CONCLUSION

It is evident from the above parameters that the green tea leaves contain phenolic compound and by using transdermal patches the drug can entrapped efficiently and can be examined in different disease conditions. Among the diseases breast cancer being one of the 5th largest causes of deaths in the world has been the focus of this nanovesicle. The recent studies have shown transdermal patch containing a formulation of chemotherapeutic drug is synthesized successfully. The suspension has also been checked for its physical and chemical stability. No agglomeration of flocculation is observed over time. The penetration of the drug-based hydrogel is validated under *in vitro* studies. Thus, the use of transdermal patch as a plausible replacement to painful chemotherapeutic injections could be an option. Depending upon the need of the patient and the dosage, the patch can be designed as a sustained and controlled release one. Hence the plausible further study of this paper is to establish the Nanovesicle transfersomes entrapped efficiently with *Camellia sinensis* (phenols) which helps siRNA slip inside the cells can treat especially breast cancer in women which can be also cost effective and therapeutic in treating many more diseases.

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