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ORIGINAL ARTICLE

Development and Characterization of *Costus Speciosus* Rhizome Extract Based Antimicrobial Liposomal Gel

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ABSTRACT

Objective: *Liposome based formulations are quite popular nowadays for effectively treating different dermal disorders. Different synthetic and plant based drugs are successfully used for liposome preparation for better potential effect. Use of herbal extract into liposome results reduction in side effects as in case of synthetic drugs. Costus speciosus rhizomes possess good antibacterial potential, assuring its greatness as potent plant active of this plant. Alcoholic extract of Costus speciosus rhizomes were found to be more active towards the bacterial species than the aqueous extract. Therefore, this rhizomes extract was incorporated into liposomes for enhanced activity, upon topical application. The main objective of the present research work is to develop this potent rhizome extract into a nano formulations i.e. liposomes and to fabricate its novel topical liposomal gel for anti-microbial activity. Methanolic Rhizome Extract (MeRE) was incorporated into liposomes by thin film hydration method. The batch having lipid ratio i.e. Soya lecithin: Cholesterol (3:1); MeRE concentration 70 mg with entrapment efficiency 71.5 ± 0.9% was finalized. The vesicle size was found to be 3.3µm ± 0.4. In vitro drug diffusion and skin retention from liposomal gel was found to be 63.3% ± 1.2 and 24.02% ± 0.28 respectively. Stability studies indicated that formulation was stable over a period of 3 months when stored at 2-8°C.*

Conclusions: *The fabricated gel formulation showed a promising drug delivery vehicle for topical delivery of Costus speciosus rhizome extract and could be successfully used for the treatment of dermal microbial infections.*

Keywords: *Costus speciosus, Methanolic Rhizome Extract (MeRE), Antibacterial, In vitro drug diffusion.*

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INTRODUCTION

The fruitful management of pharmacokinetics as well as the tissue distribution of any drug is the main goal during the development of new drug delivery system. To achieve the above target, variety of delivery systems such as microspheres, nanoparticles, lipoproteins, micellular systems and liposomes are used in the past for several years. Among these, most useful delivery system has been liposomal drug delivery system. Liposome has the ability of to carry a wide variety of substances. Based on their structural properties and harmless nature of their components, liposomes have been very popular to treat variety of therapeutic conditions [1]. Liposomes are the promising carriers as they are having potential to incorporate with variety of small drug molecules, proteins, nucleotides and plasmids as well. Liposomes can be easily formulated and refined to different sizes, compositions, charges and lamellarity [2].

On topical application of liposomal formulation, the liposomes are easily absorbed and merged with the cellular membranes of the skin. During this process, the drug loaded liposomes release the active materials into the cells. Due to the interaction of liposomal formulation with the corneocytes and of the intercellular lipids, it results in the softening and smoothening of skin [3]. Liposomes are capable of reaching the deeper layers of skin with high dose of drugs as well as it reduces the percutaneous absorption and unwanted side effects [4].

A wide variety of synthetic and herbal drugs are successfully incorporated into liposome for enhanced efficacy [5]. Liposomes are most suitable for plant extract delivery vehicles. Examples like turmeric, carrot extract, papaya extract, aloe-vera, green tea extract are reported for successful delivery through

liposomes [6,7]. As compared with the side effects related to synthetic drugs, herbal extract loading into liposome is more beneficial [8].

Costus speciosus belongs to family *Costaceae (Zingiberaceae)* is a medicinally as well as ornamental plant which is largely cultivated in India [9]. The common name of this plant is Kewkand and sometimes it is also known as spiral, crepe or wild ginger. This herbaceous and rhizomatous plant is having different names in many Indian languages like in Hindi - Keukand; in Tamil - Kostum; in Telgu - Cengalva Kostu; in Sanskrit - Kuslita, Kashmira, Shura, Pushkarmula, Katar Katar etc. It is widely distributed in India. Tuberous rhizome of this plant is used as vegetable, medicine and in pickle, *Badi* and *chatni* preparation by the local community of Chhattisgarh [10]. The plant has been found to possess many pharmacological activities such as antibacterial, antifungal, anticholineesterase, anthelmintic, antioxidant, antihyperglycemic, anti-inflammatory, analgesic, anti-pyretic, anti-diuretic, larvicidal, anti-stress and estrogenic activity [9]. The tribal communities are utilized this plant extensively for head-ache, fever, cough, cut and wounds, scabies, snake bite antidote, jaundice and arthritis. The potent antibacterial activity against Gram positive (*Staphylococcus aureus, Staphylococcus epidermidis)* and Gram negative bacteria (*E. coli, Pseudomonas aeruginosa, Salmonella typhimurium*) was analyzed previously by using this plants rhizome extract [11].

The antibacterial and antifungal activity of this plant was strongly reported in a study, in which different extracts of this plant were made by using hexane, chloroform, ethyl acetate and methanol. This study concluded that among all of the extracts, the hexane extract exhibited potent activity against Gram positive bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus subtilis* and fungi like *Trichophyton mentagrophytes, Epidermophyton floccosum, Trichophyton rubrum* and *Magnaporthe grisea*. Costunolide and eremanthin were isolated from the extract and showed good inhibition against pathogenic fungi at a very low concentration. This result proves the potent antifungal and antibacterial activity of this plant [12].

Emeritus scientists were also analyzed the alcoholic extract of *Costus speciosus* against *Aspergillus species* isolated from pulmonary infections. They also supported to the antifungal activity of the extract [13].

The objective of the present research work is to develop a nano formulation of the rhizome extract of this traditionally marvelous plant i.e. liposomal gel.

MATERIAL AND METHODS

Cholesterol and soya lecithin were obtained from Hi Media Laboratories Ltd., Mumbai, Maharashtra. All the other materials along with solvents like acetone, chloroform and methanol were used of analytical grade. The solvents were collected from S.D. fine-chem limited, Mumbai, Maharashtra.

The fresh rhizomes of mature plants were collected from places in and around forest hill regions of Bilaspur district, Chhattisgarh, India. The marker compound Quercetin was collected from Ultra purity, Mumbai, Maharashtra.

Preparation Of Extract

The total Methanolic Rhizome Extract (MeRE) was made by using maceration process. During extract preparation, the rhizomes of *Costus speciosus* mature plants were carefully washed with water to remove the soil from it. Then these rhizomes were allowed to shed drying at 21-24˚C for three to four weeks for eliminating its resident moisture. Then these dried rhizomes (500 gm) were ground finely in a mortar and pestle and then extracted with 1.5 liter methanol and then subjected to rotary vacuum evaporator for evaporation at 45˚C. Finally the total methanolic extract was preserved at 4°C until being analyzed [14, 15].

Standardization Of Methanolic Extract Of The Rhizomes Of Costus Speciosus By Using Marker Compound (HPLC Technique)

HPLC condition:

The chromatographic analyses were performed by using the stationary phase [C18 column Phenomenex (250×4.60 mm)] and the mobile phase [acetonitrile: water with 0.1ml of ortho-phospharic acid (40:60)]. The flow rate (1.0 ml/min) and injection volume (20 µl.) were adjusted. By comparing the retention time, the peaks of the analytes were confirmed. The Spinchrom software was utilized. Shimadzu isocratic HPLC system (with Rhyodyne manual injector) was used for the analysis. The detection was carried out by UV detector at 369nm. All of the chromatographic processes were operated within the ambient temperature. Three separate extractions of each sample were carried out and by extract injection in triplicate, the analysis was performed.

Preparation Of Sample Solution:

To prepare stock solution of sample, 1 gm of accurately weighed Methanolic Rhizome Extract (MeRE) were taken in a 100 ml volumetric flask and dissolved in the mobile phase and the volume was made up.

With the use of above solution, sample solution was prepared. A 0.45 µm membrane was use to filter the supernatant, then 20 µl of the filtrate was injected to HPLC.

Procedure: After setting-up the instrument an aliquot of 20µl of standard solution and sample solutions was injected respectively and chromatograms were recorded [16, 17, 18].

Formulation Of Liposomes

A lipid phase was prepared by dissolving accurately weighed quantities of Methanolic Rhizome Extract (MeRE), Soya lecithin and Cholesterol in the 2:1 v/v mixture of chloroform- methanol placed in a round bottom flask (250 ml) containing the beads. From the above solution mixture the solvents were removed by rotary evaporator (Superfit) at 45- 50°C, under reduced pressure, to obtain a thin film of lipids on the wall of the flask and the surface of beads. Then the resultant dry lipid film was made hydrous with the addition of phosphate buffer pH (6.4) at 60± 2°C temperature. Then this dispersion was kept aside and remain uninterrupted for 2-3 hours at room temperature to achieve complete swelling of the lipid film and hence to obtain vesicular suspension [19].

A. Optimization Of Formulation

Different process variables are involved in the preparation of Methanolic Rhizome Extract (MeRE) liposome such as ratio of Soya Lecithin: Cholesterol, various concentration of Methanolic Rhizome Extract (MeRE), and effect of sonication time.

B. Entrapment Efficiency

UV-spectrophotometer Systronics 2203 fitted with deuterium and tungsten lamp (Systronics) was used to analyze the entrapment efficiency of prepared formulation. In order to quantify the content of Methanolic Rhizome Extract (MeRE) in the supernatant and pellets in samples, series of standard solutions were prepared. To measure the encapsulation efficiency of Methanolic Rhizome Extract (MeRE) liposomes, Quercetin was chosen as standard. The stock solution of quercetin (10µg/ml) was prepared by dissolving different amounts of quercetin in methanol and dilute them. Standard solutions were then prepared from stock solution. The absorbance was measured at 369 nm based on the spectral analysis. A calibration curve of quercetin was developed by plotting absorbance versus concentration of standard solutions. The prepared Liposomal dispersion was then subjected to centrifugation at 5000 rpm for 20 min. The supernatant and pellets were each dissolved in methanol. The analysis was performed in triplicate. Encapsulation efficiency was measured by applying the following formula:

$$\text{Entrapment efficiency (EE\%)} = A/(A+B) \times 100$$

Where, A is the amount of quercetin in the pellet and B is amount of quercetin in the supernatant. Based on the entrapment efficiency, final Methanolic Rhizome Extract (MeRE) concentration was finalized [18].

C. Particle Size Reduction

Liposomal dispersion was subjected to ultrasonic irradiation for 30 min with continuous sonication bath. The sample was left to cool down and placed in the fridge at 4°C for 1 day prior to further test. Then this sample was analyzed by microscopy after suitable dilution to determine the particle size. Optical microscopy was used with oil immersion lens. By measuring the diameters of 50 liposomes, the mean geometric diameter along with standard deviation was calculated.

D. Transmission Electron Microscopy

Transmission electron microscopy (TEM) analysis of the prepared liposomes was carried out from Indian Institute of Technology, Mumbai, using TEM-PHILIPS, CM200. For this analysis, all the samples are previously negatively stained with phosphotungustic acid (PTA) aqueous solution (1% w/v).

E. Preparation Of Liposomal Gel

Topical Methanolic Rhizome Extract (MeRE) liposomal gel formulations were prepared by incorporation of liposome dispersions into the structured vehicle of 1% carbopol 940 and 0.5% HPMC with 5 minutes of gentle mechanical mixing at 25 rpm. Triethanolamine was added for neutralization. The same procedure was followed to prepare blank liposomal gel which does not contain Methanolic Rhizome Extract (MeRE) [7].

F. Evaluation Of Liposomal Gel

Physicochemical Evaluation

Physical examination: The Methanolic Rhizome Extract (MeRE) Liposomal gel was prepared by the procedure mentioned and evaluated for colour, odor and transparency.

pH: pH meter which was previously calibrated using buffers of pH 4 and pH 7, was used for the measurement of pH values of 1% aqueous solutions of the prepared gels.

Drug Content uniformity: 100 mg of gel sample was taken for this analysis and drug content (MeRE i.e. Quercetin as standard), was analyzed by UV spectrophotometer at 369 nm. Likewise, to determine the content uniformity, drug concentration was analyzed in gel withdrawn from 3 to 4 different points of the

container. For liposomal gel, methanol in sufficient quantity was used to shake the liposomal gel in order to extract the drug and then it was subjected to UV spectrophotometer for analysis at 369 nm.

Spreadability: Spreadability of liposomal gel was examined through wooden block and glass slide equipment. Approximately 20g weights are put on to the pan and the time (in seconds) to detach the upper movable slide completely from the lower fixed slides was recorded. Then the gel spreadability was calculated by the following equation:

$$S = M.L / T$$

Where,

S = Gel spreadability

M = Weight (g) tied to the upper glass slide

L = Length (cm) moved on the glass slide

T = Time (seconds) taken to separate the slide completely from each other

1) *Homogenicity:* The prepared liposomal gel is placed in a container and then it was analyzed for homogenicity by visual inspection. This was tested for their appearance and presence of any aggregates.
2) *Viscosity Studies:* The rheological property of formulated gel is determined by using Brookfield Viscometer. By revolving the spindle (64) at 100 rpm, apparent viscosity was determined at room temperature.

In-Vitro Drug Diffusion Study:

Preparation of Skin

This study was performed on the abdominal skin of rat, from which subcutaneous fatty tissues are removed by a scalpel and surgical scissors. Then the surface of the skin surface was thoroughly rinsed by saline solution for cleanup. The skin was stored in saline solution at 4°C, and then used within one day.

Experiment

Franz diffusion cell was employed to carry out this experiment. Rat abdominal skin (completely shaved) was mounted between the donor and the receptor compartments in such a manner that the side of stratum corneum is facing to the donor compartment. The divisional area of the cell is about 0.785cm^2 and 10 ml is the receptor compartment. The donor medium consisted of 1 gm liposomal gel. A ratio of 3:1 of phosphate buffer (pH 6.4): Ethanol was placed in receptor compartment as medium for retaining the sink condition. Stirring rate and temperature were kept 400 rpm and 37°C respectively. Regularly at fixed time intervals i.e.,1, 2, 4, 8, 12 and 24 hrs, the samples from receptor compartment were removed and replaced with fresh receptor medium. Then the above removed samples were analyzed for drug (MeRE, as per quercetin) content by spectrophotometer at 369 nm. The cumulative drug release was measured as a function of time and the rate of drug release was then calculated.

Skin Retention

The skin mounted on the Franz diffusion cells was cautiously detached after the permeation study was carried out. Then the above skin was cleaned by spatula remaining formulation adhering to the skin was scraped with a spatula followed by cleaning with cotton then it was soaked in phosphate buffer (pH 6.4). After this process the skin was firmly dried out by pressing between two tissue papers. Then this cleaned skin was mashed and on to this mashed portion, methanol (50 ml) was added, then it was subjected to a water shaker bath for mechanical shaking (1 hr.) at 37 ºC for the exhaustive extraction of the drug material. The filtrate was then separated out and the drug (MeRE as per quercetin) content in filtrate was examined by UV spectrophotometer at 369 nm. The measurement was done in triplicate.

Stability Studies of Optimized Formulation

The optimized liposomal gel was studied for stability. Optimized Methanolic Rhizome Extract (MeRE) liposomal gel formulation was sealed in amber colored bottles with cap covered by aluminum foil and these packed formulations was stored in different temperature viz i) room temperature (R.T) ii) 2°-8°C and according to ICH guidelines, maintained at 40^0 C ± 2^0 C at for 3 month. The formulation was evaluated before and after periodic interval for change in appearance, pH, viscosity, drug content, and *in vitro* drug diffusion.

RESULTS AND DISCUSSIONS

The MeRE obtained by maceration technique is observed for color, odor and appearance. Results of description of MeRE were found to be similar as mentioned in literature. The extract is thick semisolid, dark-green in color and possessing the aromatic odor.

Determination Of Solubility

The MeRE was dissolved in various solvents to evaluate its solubility characteristics. The result shows its solubility in methanol, Phosphate buffer pH (6.4): Ethanol in the ratio 3:1, ethanol and in di-methyl sulfoxide.

Standardization Of Methanolic Extract Of The Rhizomes Of Costus Speciosus By Using Marker Compound (HPLC Technique)

After injecting the sample and the standard solutions, the HPLC chromatograms of MeRE and quercetin were obtained respectively. The MeRE chromatogram shows a sharp peak with retention time of 6.127 min. and that of quercetin was at 6.147 min. The HPLC data of MeRE, was compared with that of standard quarcetin. It was found that the peak of qurcetin was eluted in the chromatogram of MeRE at 6.127 min. Hence, the methanolic rhizomes extract of *Costus speciosus* was standardized and concluded that it contains the potent marker quercetin.

Optimization Of Formulation

Soya Lecithin (SL) and cholesterol (CH) concentrations are very important in the formulation and stabilization of liposomes. Cholesterol plays a critical role in stability of formulation by diminishing the permeability. Non rigidity and irregular shape of formulated liposome is observed in absence of cholesterol. That's why the various molar ratios of Soya Lecithin and cholesterol were used during the liposomes preparation. To establish the appropriate lipid ratio, placebo liposomes were formulated instead of adding the drug (Table 1). The uniform multilamellar vesicles are formed with the batch of placebo liposomes having lipid ratio 3:1 i.e. 135 mg SL: 45 mg CH and hence this batch was selected.

TABLE 1: OPTIMIZATION OF LIPID CONCENTRATION

S.No.	Formulations	Molar Ratio	Remarks
1.	SL : CH	1 : 1	Non rigid Vesicles formed
2.	SL : CH	2 : 1	Aggregates formed
3.	SL : CH	3 : 1	Uniform, regular and rigid vesicles were formed
4.	SL : CH	4 : 1	Uniform vesicles were formed but aggregates are also seen
5.	SL : CH	6 : 1	Irregular and non rigid vesicles were formed

Then the numbers of liposomal formulations are prepared by using different concentrations of MeRE employed with SL: CH (3:1). According to the results of entrapment efficiency, concentration of drug was finalized. Findings are tabulated in (Table 2). Finally, by results of entrapment efficiency the appropriate concentration of MeRE was selected e.g. 70 mg.

TABLE 2: OPTIMIZATION OF METHANOLIC RHIZOME EXTRACT (MERE) CONCENTRATION

S. No.	Soya Lecithin: Cholesterol Ratio	Extract Concentration (Mg)	Entrapment Efficiency (% ±SD)
1.	3 : 1	10	33.8 ± 1.3%
2.	3 : 1	15	36.3 ± 1.1%
3.	3 : 1	20	41.3 ± 0.8%
4.	3 : 1	25	48.5 ± 0.7%
5.	3 : 1	35	58.4 ± 1.0%
6.	3 : 1	50	63.3 ± 1.4%
7.	**3 : 1**	**70**	**71.5 ± 0.9%**
8.	3 : 1	100	62.3 ± 1.1%

The size or size distribution and entrapment efficiency of liposome was quite affected by the sonication process. In order to determine the optimal conditions for sonication, it was necessary to perform a number of trials and evaluate the impact of duration of sonication on the properties of liposome. Sonication parameters were analyzed with reference to vesicle size by using SL: CH (3:1) liposomal compositions. A sonicator bath with ice was used to sonicate the liposomes for a number of time duration. Results showed the reduction in vesicle size with increasing the time duration. Results are tabulated in (Table 3).

TABLE 3: EFFECT OF SONICATION ON SIZE OF LIPOSOMES

S.No.	Time in minutes	Particle size in (µm) ± SD
1.	5	10.9 ± 0.7
2.	10	8.3 ± 0.4
3.	15	6.4 ± 1.8
4.	20	4.3 ± 0.6
5.	30	3.3 ± 0.4

Evaluation of Liposomes

Vesicle size by microscopy

Vesicle size was evaluated by optical microscopy. For this analysis, 100X oil immersion lens was used to determine the spherical lamellar vesicles containing drug. The average particle size of MeRE liposomes was found to be 3.3µm ± 0.4 (Figure 1).

Transmission Electron Microscopy

TEM analysis was carried out for observing spherical lamellar vesicles and this was done by analyzing the liposomal suspension at 50 nm scale. TEM images of prepared liposomes are shown in Figure 2.

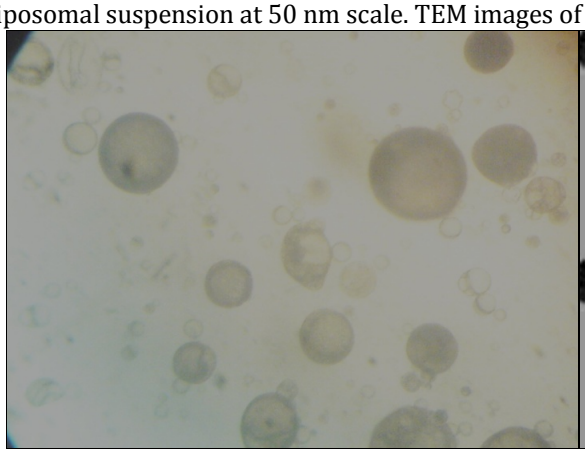

FIGURE 1: IMAGE OF LIPOSOME UNDER 100X OIL IMMERSION LENS

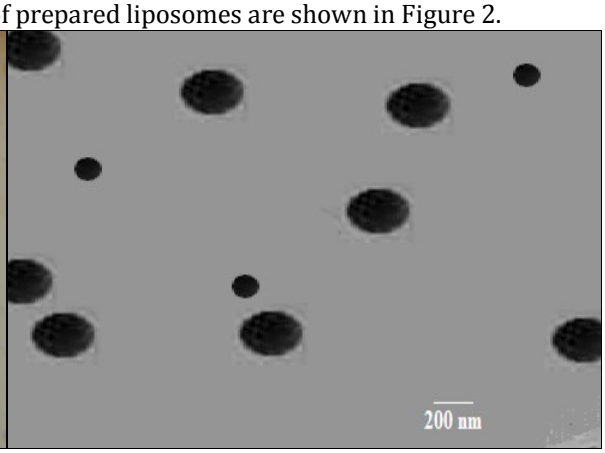

FIGURE 2: TEM IMAGE OF LIPOSOME

Evaluation Of Liposomal Gel

Physicochemical Evaluation

The prepared liposomal gel was subjected to various evaluation studies like, colour, clarity, transparency, pH, drug content, viscosity, spreadability, homogenicity, *in vitro* diffusion and skin retention. The particle size was observed to be 3.3µm ± 0.4, while the pH was 6.4. The spreadability value of 12.9 ± 0.3 g.cm/sec concludes that the gel is simply spreadable when a little shear is applied.

In-vitro drug diffusion study:

The *in-vitro* drug diffusion of optimized liposomal formulation batch was observed to be 63.3% ± 1.2 up to 24 hours (Table 4). Percent (%) drug release form liposomal gel formulation batch was shown in figure 3.

TABLE 4: *IN-VITRO* DRUG DIFFUSION STUDY OF LIPOSOMAL GEL

S.No.	Time in Hrs	% Drug Release ± SD
1.	0	0.0
2.	1	10.9 ± 0.5
3.	2	17.4 ± 0.7
4.	3	21.6 ± 0.4
5.	5	30.7 ± 0.4
6.	7	39.9 ± 0.8
7.	9	48.4 ± 1.1
8.	12	52.8 ± 0.3
9.	18	57.1 ± 0.3
10.	24	63.3 ± 1.2

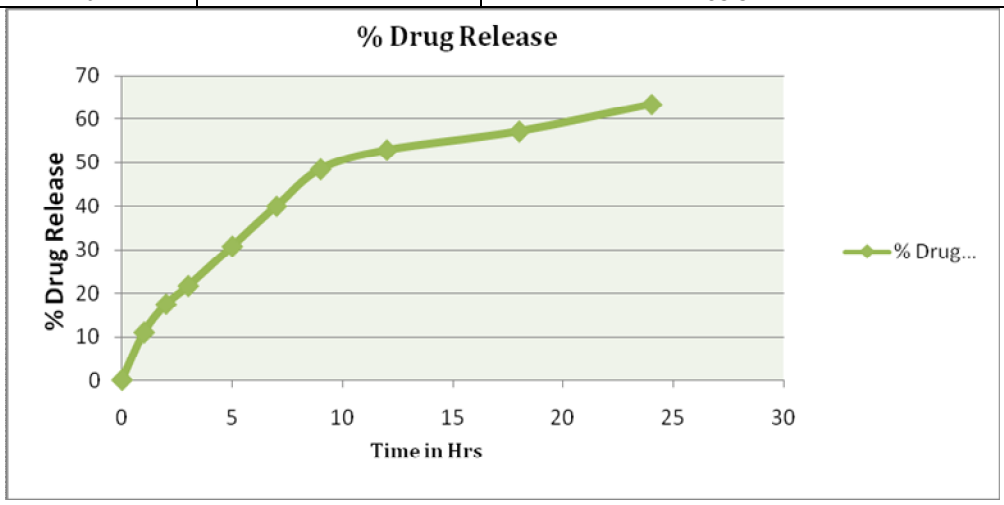

FIGURE 3: *IN-VITRO* DRUG DIFFUSION OF LIPOSOMAL GEL

Skin retention:

The skin retention is calculated from the calibration curve of Quercetin in methanol. The skin retention of optimized MeRE after 24 hrs was found to be 24.02% ± 0.28. The skin retention effect of drug may be due to deposition of other components of liposomes with drug into the skin and it increases drug retention capacity of the skin.

Stability Studies of optimized formulation:

It was observed that at room temperature and in 40˚C temperature, the formulated liposomal gel shows instability.

When the formulated liposomal gel was subjected to stability studies, it concludes that the characters of liposomal gel like physical appearance, rheological properties and drug release remain unchanged upon storage at 2-8°C for 3 months (Table 5).

TABLE 5: STABILITY OF LIPOSOMAL GEL AT 2-8˚C

S. No.	Evaluation parameter	After one month observation	After two month observation	After three month observations
1.	Colour	Green	Green	Green
2.	Transparency	Transparent	Transparent	Transparent
3.	pH	6.4	6.4	6.4
4.	Drug content (%)±SD	82.9 ± 0.5	81.8 ± 0.6	81.2 ± 0.2
5.	Viscosity ±SD	4448 ± 0.6	4417 ± 0.2	4401 ± 1.2
6.	Spreadability ±SD (g.cm/sec)	12.4 ± 0.5	12.3 ± 0.5	12.9 ± 0.3
7.	Homogenicity	homogenous	homogenous	homogenous

CONCLUSION

The Methanolic Rhizome Extract (MeRE) of *Costus speciosus* was incorporated into liposomal drug delivery system to increase the rate of permeation into the skin and exhibiting a better topical antimicrobial activity. The SL: CH ratio (3:1) is optimized for the preparation of liposomes. The batch having lipid ratio i.e. SL: CH (3:1); MeRE concentration 70 mg with entrapment efficiency 71.5 ± 0.9% was finalized. This was evaluated for vesicle size by Optical microscopy and by Transmission electronic microscopy. The vesicle size was found 3.3µm ± 0.4. This was incorporated into gel. Carbopol 940 (1%), HPMC (0.5 %), was used for gel preparation. The formulated gel was evaluated for various parameters of physical appearance and rheological properties (colour, clarity, transparency, pH, drug content, viscosity, spredability, and homogenicity). *In vitro* drug diffusion and skin retention from liposomal gel was found to be 63.3% ± 1.2 and 24.02% ± 0.28 respectively. Stability studies confirm the stability of prepared liposomal formulation, when it is stored at 2-8˚C for a period of 3 months. The results showed that the physical appearance, rheological properties and drug release pattern of the prepared liposomal gel formulation, remain unchanged upon storage at 2-8°C for 3 months.

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