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Fabrication of Hydrogel Infused with Geranium Oil Loaded Nanosponges: Pharmaceutical and In-Vivo Evaluation

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KEYWORDS:
Nanosponges,
Geraniol,
HPLC method,
Validation,
In- vitro evaluation

ABSTRACT:

Introduction: Geranium oil is a volatile oil that is derived from the leaves of *Pelargonium graveolens* and has a variety of pharmacologic effects. The main constituents of the essential oil were citronellol (37.5%), geraniol (6.0%), caryophyllene oxide (3.7%), menthone (3.1%), linalool (3.0%), β-bourbonene (2.7%), iso-menthone (2.1%) and geranyl formate (2.0%).

Objectives:

The formulation was developed to improve safety and efficacy of active component. The rate of drug delivery at targeted site can be achieved through this dosage form. Furthermore, the volatile nature of its main active component may cause volatilization and interaction with other formulation elements. To address these issues, our study intends to develop geranium-loaded nanosponges with improved antifungal properties.

Methods: Solvent Evaporation Technique

Results: Particle size, zeta potential, entrapment efficiency, FESEM, and in vitro release were all tested on the formulated formulations. The nanosponge dispersions were all nanosized, and the nanosponges had a spherical uniform shape with a spongy structure, according to FESEM data.

Conclusions:

The nanosponges made using PVA: EC (1:1) were determined to be superior in the in-vitro release testing, as they displayed sustained drug release from nanosponges and were subsequently selected for antifungal potential investigation. It was evaluated for antifungal efficacy against *C. albican*, with findings confirming the nanosponges' excellent antifungal activity.

1. Introduction

Essential oils are aromatic and volatile liquids extracted from plant material such as flowers, roots, bark, leaves, seeds, peel, fruits, timber, and whole plants. These oils have piqued people's interest throughout history, and while many of their applications have faded with time, it is widely assumed that humans have been extracting them from fragrant plants since the dawn of time. Essential oils are used for a variety of functions, including not only enhancing the taste and health benefits of food in the kitchen, but also in the production of fragrances and cosmetics. Essential oils were employed by the ancient Egyptians in medicine, perfumery, and the art of embalming and mummification. Essential oils and perfumes have been employed by many cultures throughout history for a variety of purposes, including religious rites, perfume manufacture, and treatment of infectious illnesses.[1]

Steam distillation is the most applied method to extract these complicated chemical mixtures from plants. Essential oils, as stated above, are complex mixes made up of a variety of ingredients that are usually liquid but may also be solid. When newly distilled, these mixes range in colour from colourless to slightly yellowish in colour at room temperature, have a fragrant scent, are extremely clean to the touch, and are easily absorbed by the skin. They are soluble in most common organic solvents, including ethanol and diethyl ether, and blend well with vegetable oils, fats, and waxes, despite their limited solubility in water. There are about 3000 distinct essential oils that have been identified. Around 300 of these are utilized commercially in the flavouring and fragrance industry

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[2]. Secondary metabolites are the most common source of essential oils. They are frequently kept in non-differentiated cells (Lauraceae), secreted organs (Lamiaceae and Asteraceae), secretory ducts (schizogenous in Myrtaceae and schizolysigenous in Rutaceae), or cavities (Conifers). Sometimes, as in the instance of valeriana or garlic, the essential oil is not created by the plant itself, but rather by the hydrolysis of some of the plant's components [3-4.] Numerous views exist concerning their potential involvement as antioxidants in that they contribute hydrogen in oxidative processes, particularly when light is present. Antifungal and antibacterial properties are also suggested to protect the plant against pathogenic assault [3]. *Pelargonium graveolens* (Family-Geraniaceae), often known as rose scented Geranium, is a highly regarded fragrant plant. It is primarily farmed for its essential oil, Geranium oil, which has a strong rose scent. Geraniol and Citronellol, the oil's main components, are responsible for the oil's rose aroma. Geranium oil is one of the world's top 20 essential oils, with a wide range of uses in the perfumery, cosmetics, and flavour industries. It's found in a lot of high-end fragrances [5]. It also has insect repellent [6], antifungal [7], and antibacterial [8] characteristics, making it beneficial in the medical and agricultural fields. It has a wide range of applications in aromatherapy and the food sector. Geraniol ($C_{10}H_{18}O$) is an acyclic monoterpene alcohol with the chemical formula 3, 7-dimethylocta-trans-2, 6-dien-1-ol. Geraniol has a rose-like odour and is described as sweet flowery rose-like, citrus with fruity, waxy undertones (at 10 ppm) [9]. This monoterpene alcohol is a common scent ingredient. Geraniol also has a variety of metabolic and pharmacological characteristics. Geraniol has been demonstrated to be an effective plant-based insect repellent by researchers [10], and its antibacterial potential has been emphasised in multiple studies[11]. Geraniol inhibits the growth of murine leukemia, hepatoma, and melanoma cells *in vitro* and in-vivo [12-14]. The administration of drug at higher dose produced toxicity. The components are found to be instable and difficult in applying in smaller amount. Thus the present study aimed to developed nanosponges.

2. Methods

The entire chemicals were procured from the S. G. Fine given as below.

Table 1: List of chemicals with their uses

Ingredients	Uses
Polyvinyl alcohol	Hydrophilic polymer
Hydroxypropyl methylcellulose (HPMC) K15 M	Hydrophilic polymer
Agar	Hydrophilic polymer
Ethyl cellulose	Hydrophobic polymer
Carbopol 940	Gelling agent
Dichloromethane	Cross linking agent and solvent
Ethanol	Cross linking agent and solvent
Triethanolamine	Used to neutralize pH
Water	For dispersion

Method:

Selection of raw material:

The different polymers were tried during the development of formulation including Hydroxypropyl methyl cellulose, agar, ethyl cellulose, poly vinyl alcohol, Eudragit S100. The solubility of these polymers was studied in various solvents (including water, methanol, ethyl cellulose, dichloromethane) single or in combined form.

Fabrication of hydrogel infused with Geranium Oil Nanosponges: Selection of combination of a hydrophilic and a hydrophobic polymer and cross linking agent done by performing a solubility study **(Table 2).**

Table 2: Solubility study of raw material:

Solubility of HPMC K15M	Solubility of Ethyl cellulose (EC)	Solubility of Eudragit S100	Solubility of polyvinyl alcohol (PVA)	Solubility of Agar
HPMC+ ethyl acetate = soluble	EC+ ethyl acetate = soluble	Eudragit+ ethyl acetate = soluble	PVA+ ethyl acetate = insoluble	Agar+ ethyl acetate = insoluble
HPMC+ water = soluble	EC+ water = insoluble	Eudragit+ water = insoluble	PVA+ water = slightly soluble in cold water (soluble in hot water)	Agar+ water = insoluble in cold water(swells in boiling water)
HPMC+ ethanol = insoluble	EC+ ethanol = soluble	Eudragit+ ethanol = soluble	PVA+ ethanol = insoluble	Agar+ ethanol = insoluble
HPMC+ dichloromethane = soluble	EC+ dichloromethane = soluble	Eudragit+ dichlomethane = insoluble	PVA+ dichloromethane = insoluble	Agar+ dichloromethane = insoluble
HPMC + methanol = insoluble	EC+ methanol = soluble	Eudragit+ methanol = soluble	PVA+ methanol = insoluble	Agar+ methanol = insoluble
HPMC+ ethanol: water 1:1=	EC+ ethanol: water (1:1)= insoluble	Eudragit+ ethanol: water = insoluble	PVA+ ethanol: water 1: 1 = insoluble	Agar+ ethanol: water = insoluble

Based on the solubility study of polymers, the different combinations of polymers like HPMC and EC, HPMC and Eudragit S100, PVA and Eudragit S100, agar and ethyl cellulose (EC), PVA and EC were tried to formulate nanosponges.

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General procedure for preparation of nanosponges:
The one form polymer from given polymer dissolved in 20 ml of water kept on vortex. Another selected polymer was dissolved in 10 ml of ethanol. The dispersed phase was then added to aqueous phase kept on magnetic stirrer at 1000 rpm for 2 $^{1/2}$ hr. in drop wise manner. The formed nanosponges were filtered and air dried.

Preparation of 1% hydrogel using carbopol:
The 1 % of hydrogel was prepared by dissolving 0.1 gm of carbopol 940 in 10 ml of distilled water kept on vortex on magnetic stirrer to form the gel. Dried nanosponges containing oil equivalent to 100 mg were weighed and added to the gel kept on vortex. Stirred for 20 minutes so that the nanosponges were uniformly dispersed and then the gel was neutralized using TEA.

Evaluation of Geraniol Content in Oil and Formulation by Developed and Validated HPLC Method:

HPLC Method Development:
The determination was done using Agilent Tech. (1100) system. The separation of chromatogram was carried out on column Fortis C18 (100 x 4.6 mm id with 2.5µm particle size) using various solvent systems such as acetonitrile, methanol, water were tried for development of HPLC method for analysis of geraniol. The effect of flow rate was determined by setting flow rates at 0.5 ml/min, 0.7 ml/min, 1.0 ml/min, 1.1 ml/min, 1.2 ml/min and 1.5 ml/min. The solution was scanned between the wavelength range 400-200 nm using the UV spectrophotometer. Quantification of drug was estimated by calculating peak are using CHEMSTATION 10.1 software.

Preparation of mobile phase:
Mobile phase was prepared by mixing HPLC grade acetonitrile and water in the ratio of 98:2 v/v. The content was sonicated for 15 min and filtered through 0.45 µm membrane filter. Mixed solvents were degassed and used as mobile phase.

Preparation of reference standard and sample solution:
Geraniol is the main active content present in geranium oil. Therefore geraniol is used as reference standard. 1000 mg of formulation equivalent to 10 mg of geranium oil used to prepared standard and sample solution.

Method validation parameters:
The validation of the developed HPLC method was carried out in accordance with ICH guidelines. The linearity was analysed for concentration ranging from 2- 10 µg/ml by using Least-square regression analysis where, peak areas were plotted against the corresponding concentrations. The intra- day and inter-day precision and repeatability was evaluated by triplicates of three different concentrations of each quercetin was spotted and analysed on same day for intra-day study and two different days for inter-day study with respective chromatographic conditions.
Recovery study method was employed to evaluate accuracy. The samples were spiked with 80, 100 and 120 % of median concentrations of standards.

Accuracy= (spiked concentration - mean concentration)/ (spiked concentration) ×100

Robustness was carried out by making deliberate changes in the wavelength, flow rate and mobile phase and evaluated their effect on the retention factor. The estimation of LOD and LOQ were done by standard deviation method.
Detection limit =3.3σ /S and quantitation limit=10 σ /S (σ is residual standard deviation of a regression line and S is the slope of the calibration curve).

Percent Content of Geraniol in Geranium oil and formulation:
The determination of percent content of geraniol was carried out using previously developed and validated HPLC method. The gel equivalent to 100 mg of oil was weighed and dissolved in 100 ml of HPLC grade methanol to get 1mg/ml of solution was further diluted in 10 ml of acetonitrile.

Evaluation of Nanosponges:

Particle size:
The measurements of particle size were done on Zetasizer instrument at 25°C. This technique produces the mean particle diameter and particle size distribution. The analysis was done by the software provided by Malvern Instruments. Before analysis samples were placed in refrigerator maintained at 4°C.

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Polydispersity Index:

Polydispersity index is indicative of uniformity in the particle size and hence it should be as low as possible. The measurement of PDI was obtained during the analysis of particle size.

Zeta potential:

The formulation of nanocochleate was tested for zeta potential using Malvern Zetasizer instrument. Zeta potential was determined by zeta potentiometer. The sample was filled into the cell; an electrode inserted was placed under the microscope and connect them to the zeta meter. The analysis was carried out at 25°C.

Determination of Entrapment efficiency:

The nanosponge formulation (10 mcg/ml) was centrifuged at 4000 rpm for 18 min at 4°C temperature by using Remi cooling centrifuge to separate the oil. A supernatant contains the nanosponges in suspending stage and oil on the wall of centrifuge tube. The supernatant was again centrifuged at 15000 rpm for 30 min at 4°C temperature. As a result, a transparent solution of supernatant and nanosponge was attained which was redispersed in mobile phase (100 ml). The resulting solution sonicated, the nanosponges were disrupted to discharge the oil. The discharged oil was determined for the drug entrapment. The amount of etoposide was estimated by using HPLC system.

Percentage Entrapment Efficiency= Wc/Wt×100

Where amount of drug content (entrapped) in the liposomes is denoted as Wc and total amount of drug in the dispersion is denoted as Wt.

Surface morphology:

The particle size and morphology of nanoparticles was examined by transmission electron microscope (TEM) (Tenai G2 20 Twin, FEI Company, Netherland).

***In-vitro* drug release study:**

In-vitro release study of geraniol from the formulation was carried out in phosphate buffer pH 5.5 acetate buffer by the dialysis membrane method.

The amount of formulations (1000 mg) equivalent to 10 mg of oil and oil dispersion was taken in dialysis bags for pH 5.5 medium (by cut-off of 12,000 Da, Sigma). The drug dispersion and formulation containing dialysis bags suspended in a beaker with 100 ml of acetate buffer maintained at pH 5.5 was kept on a magnetic stirrer which is rotated at 100 rpm, with temperature adjusted to 37±0.5°C for a selected time intervals. 5 ml sample was withdrawn for analysis and replaced with the same quantity of a fresh media. The samples were then filtered through 0.45µm filter. The samples were analysed for drug release by determining absorbance using UV spectrophotometer, the rate of etoposide release obtained using the standard curve.

Animal Safety Profile Study:

The acute, sub-acute and chronic toxicity study for geranium oil (10% of geranium oil in coconut oil as carrier oil) at 2000 mg/kg dose was performed. The drug was administered in the form of suspension to each rat by a single oral gavage. The animals were dosed using a stainless-steel intubation needle fitted onto a suitably graduated syringe. The dosage volume administered to individual rat was adjusted according to its most recently recorded body weight. Acute oral toxicity was studied in the experimental rats by administration of suspension. For seven days, mortality was checked.

Sub-acute oral toxicity was studied in the experimental rats by administration of suspension. After 14 days, rats were sacrifice and evaluated for various parameter such as body weight, histopathology and blood analysis (LFT, Kidney functioning test, lipid profile, haematological studies with mild anaesthesia by isoflurane) for toxicity.

Chronic oral toxicity was studied in the experimental rats by administration of suspension. After 90 days, rats were sacrifice and evaluated for various parameter such as body weight, histopathology and blood analysis (LFT, Kidney functioning test, lipid profile, haematological studies with mild anaesthesia by isoflurane) for toxicity.

In-vivo Antifungal Study:

The in- vitro study has been carried of MIC was found to be 0.21 µg/ml by Clevenger method. Based on the above study further in vivo study was performed to estimate antifungal potential of formulation.

The experimental study was carried out in both sex Wister albino rats. The rats with weighted between 150- 200 gm. were selected trough approval of the committee. The experimental protocol was approved by Institutional Animal Ethics Committee (lAEC) of Rajaram and Tarabai Bandekar college of Pharmacy, Ponda, as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines (Protocol No.

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PESRTBCOP/IAEC, 2022R-96). Animals were housed in a well-ventilated animal unit with normal day light (12 hr. light/dark cycles). Group II to IV animals were changed with intravenous methylprednisolone (5mg/kg) for 3 days for induction and maintenance of cell-mediated immunosuppression (Organisms from stock isolates were stored in nutrient agar at 27°C, streaked onto nutrient broth, and incubated at 37°C for 24 hr and included culture was used for further experiment). An area of 3 cm^2 was marked on each animal shaved back except normal control group and gently rubbed with *candida* suspension using a sterile swab to cause infection. An occlusive dressing was applied to the infected area, fixed in place by a sterile adhesive bandage and kept covered for 48 hrs. to allow for the infection. The colony growth was confirmed by counting colony-forming-unit. The animals which as CFU value of more than 3 CFU/ml ware included in the study. The animals were treated for week period and visually observed its physical changes. The swab culture was collected on initial day, 4th and 7th day of the experiment for microscopical evaluation. End of the experiment the animals were sacrificed and infected skin was excised from all the experimental animals and washed. The samples were placed into saboured dextrose agar culture media and incubate for 48 hrs. at 37°C ± 1°C. The CFU values were then recorded. The rats were divided in the five groups given as below.

Group I- Normal control

Group II- *Candida albican* control

Group III- Standard Treatment Group

Group IV- Nanosponges enriched gel treatment group (I)

Group V- Nanosponges enriched gel treatment group (II)

3. Results & Discussion

Evaluation of prepared nanosponges:

Table 3: Evaluation of Nanosponges prepared with different combinations of polymers

Sr. No.	Combinations of polymers	Results		
		0.5:1	1:1	1:2
1	HPMC : EC	Less yield, white powder	Less yield, white powder	Good yield, fluffy white powder
2	HPMC : Eudragit	No product formed	No product formed	No product formed
3	PVA : Eudragit	Gel like product formed	Gel like product formed	Gel like product formed
4	Agar : EC	Film type flaky product (less yield)	Flaky product	Flaky product, high yield

*HPMC-Hydroxypropyl methyl cellulose, EC- Ethyl cellulose, PVA- Polyvinyl alcohol

Table 4: Evaluation of nanosponges containing PVA and EC polymers

Sr. No.	Combinations of polymers	Results				
		1:1	1:2	2:1	3:2	2:3
1	PVA:EC	Fluffy powder Product	Product formed	Product formed	Product formed	Product formed

*PVA- Polyvinyl alcohol, EC- Ethyl cellulose

The various nanosponges were prepared using different combinations of bases with various ratios. The formulation were prepared using polymer HPMC: EC, HPMC: Eudragit, PVA: Eudragit, Agar: EC in the ratio of 0.5:1, 1:1 and 1:2. The nanosponges prepared by using HPMC and Eudragit polymers do not formed any product. Whereas product with HPMC and EC produced white powder, PVA and Eudragit produced gel like product and Agar and EC produced flaky product. The PVA and EC formulated in different ratios (1:1, 1:2, 2:1, 3:2 and 2:3) produced fluffy powdered product. The primary evaluation concluded that nanosponges prepared using HPMC and EC, Agar and EC and PVA and EC produced the products which were used for further evaluations.

Development and validation of HPLC method:

Optimization of RP- HPLC method:

The optimization of the RP-HPLC chromatographic parameters were carried out by using different compositions of mobile phase and flow rates. The separation was carried out on Agilent Tech. (1100) system using acetonitrile and water in the ratio of 98:2 v/v with the flow rate 0.7 ml/min as it gave well resolved peak. Based on peak area quantification was carried out at 252 nm. The Rt for geraniol was found to be 6.26 min given in **figure 1, 2, 3.**

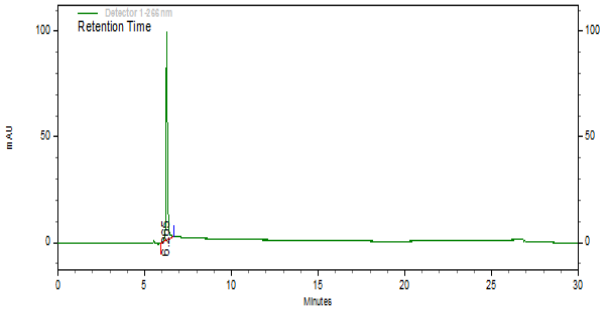

Figure 1: Chromatogram of standard geraniol

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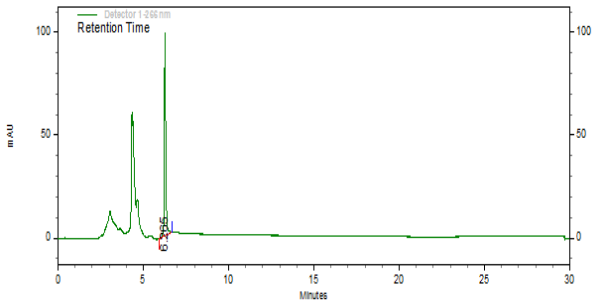

Figure 2: Chromatogram of geraniol in geranium oil

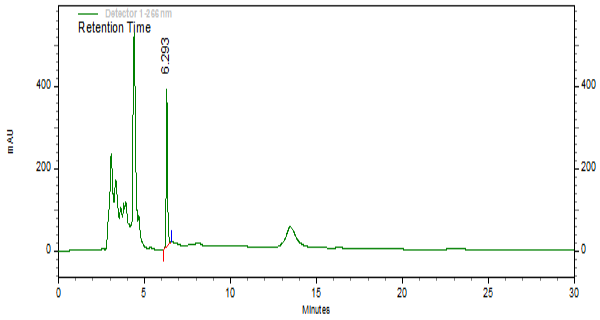

Figure 3: Chromatogram of geraniol in formulation

Method validation parameters

Linearity:

The linearity of the method was determined by diluting the standard stock solution to produce the concentration ranges from 2 to 10 µg/ml. The results show excellent correlation existed between peak area and concentration of analyte. By plotting the AUC versus the concentration of analyte, the calibration curve was prepared and analysed through linear regression **(Figure 4).**

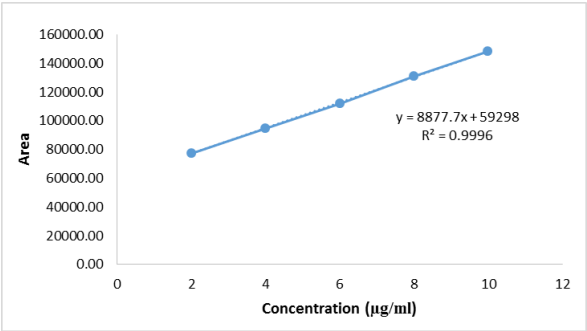

Figure 4: Linearity curve of geraniol

Accuracy:

Good recovery study of the drug was carried out at three different concentrations levels indicating the method was accurate. A known amount of standard drug (80, 100, and 120%) was added into pre-analysed sample and subjected them to the proposed HPLC method. The recoveries were found to be in the range of 99.50- 101.54%. The % recovery was found to be within the limits.

Precision:

Data on repeatability and instrumental variation were obtained in triplicate. Method precision was evaluated by repeatedly introducing 6 µg/ml concentration of geraniol. The developed method was found to be precise as % RSD was found to be 1.85. Intraday and interday precision was done in triplicate at 3 distinct concentration levels. The % RSD was found to be 0.15, 1.39 and 1.03 for interday precision and 0.42, 1.00, and 0.34 for intraday precision. RSD < 2%, proved that the method was highly precise.

Robustness:

Robustness was done by small changes in the chromatographic conditions like mobile phase, flow rate and wavelength. It was observed that there were no marked changes in the chromatograms. The developed method was found to be robust as the % RSD values were< 2.0 %.

Limit of Detection (LOD) and Limit of Quantification (LOQ):

This data showed that the sensitivity of method to determine the geranium oil. The LOD and LOQ were found to be 0.10 µg/ml and 0.30µg/ml respectively.

Evaluation of Percent Content of Geraniol from Geranium oil and formulation:

The developed and validated method used for determination of percent content of geraniol in geranium oil. The developed method provided a well resolved chromatogram, with no alterations in peaks of geraniol **(Table 5).**

Table 5: Percent content of Geraniol

Sr. No.	Nanosponge formulations	% Content of Geraniol
1	HPMC and EC	13.80
2	Agar and EC	13.09
3	PVA and EC	14.80

Evaluation of formulation:

The nanosponges prepared by using Agar: EC (1:2), PVA: EC (1:1), HPMC: EC (1:2) were further studied for different parameters such as particle size, poly dispersibility, zeta potential, % drug loading and entrapment efficiency given in table 6.

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Table 6: Evaluation of Nanosponges

Sr. No.	Formulation	Particle size (nm)	PDI	Zeta potential (mV)	% Drug loading	% Entrapment efficiency
1.	Nanosponges using Agar: EC (1:2)	187.9	1.041	-21.90	67.31	34.18
2.	Nanosponges using PVA: EC (1:1)	100.2	0.566	-21.97	73.11	64.28
3.	Nanosponges using HPMC: EC (1:2)	5035.0	2.039	-16.63	68.34	48.86

Nanosponges prepared by using PVA and EC combination showed good results as the particle size and PDI was less. The zeta potential was also found in acceptable range. The particle size of nanosponges increases during the analysis as nanosponges were formed a colloidal solution with water. As the formulation comes in contact with water it swells. After swelling PVA: EC combination has shown lower range.

FESEM:

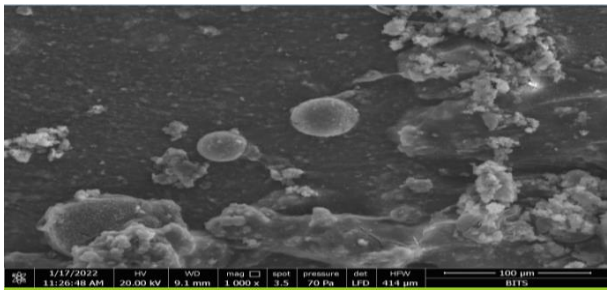

Fig. 5A

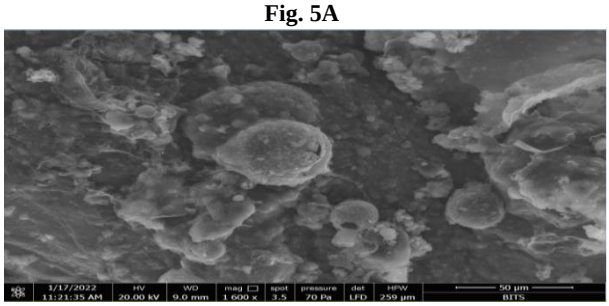

Fig. 5B

Figure 5A and 5B: FESEM evaluation of nanosponges using Agar: EC 1:2

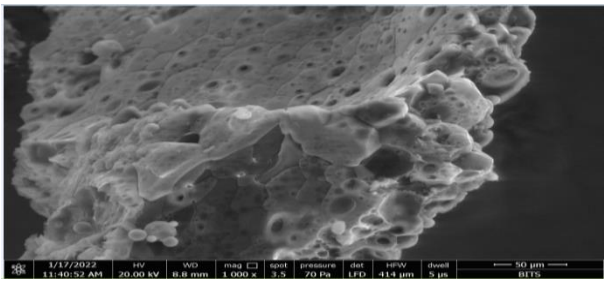

Fig. 6A

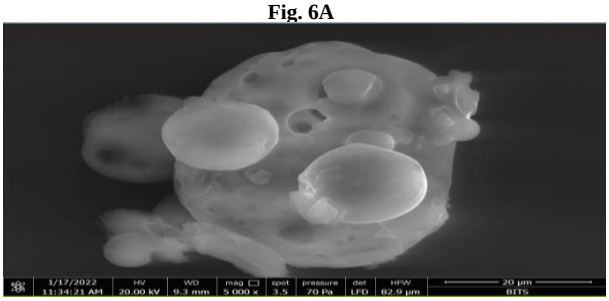

Fig. 6B

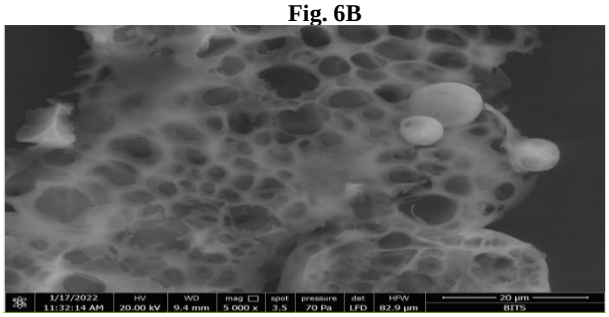

Fig. 6C

Figure 6A- 6C: FESEM evaluation of nanosponges using PVA:EC 1:1

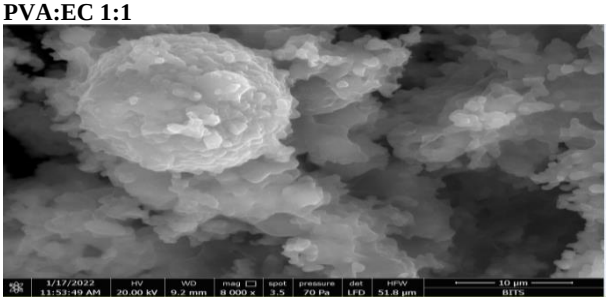

Fig. 7A

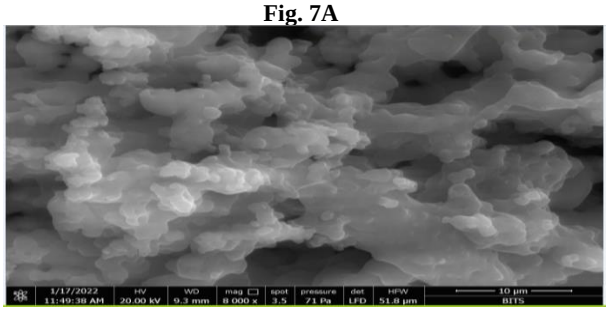

Fig. 7B

Figure 7A-7B: FESEM evaluation of nanosponges using HPMC: EC 1:2

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Table 7: FESEM data of all formulations

Sr. No.	Formulation	Size (Micron)	Structural arrangements
1.	Nanosponges using PVA:EC	10	spongy network, smooth, uniform, spherical sphere
2.	Nanosponges using Agar:EC	50	No proper network and rough surface
3.	Nanosponges using HPMC:EC	10	Formed dense network and having smooth surface

The nanosponges prepared by using PVA: EC combination showed distinct spongy network along with smooth spherical uniform sphere at 10 micron, whereas nanosponges prepared using agar: EC combination were rough at the surface with no proper network even at 50 micron. Nanosponges prepared using HPMC showed smooth surface but these nanosponges were very much agglomerated with a dense network at 10 micron table 7.

***In- vitro* % drug release study**

The % drug release from nanosponges prepared by using Agar: EC (1:2), PVA: EC (1:1) and HPMC: EC (1:2) in first half an hour is 8.59%, 0.03% and 1.86% respectively. At the end of 24 hrs around 95.82%, 84.45% and 91.04% drug was released from nanosponges prepared using Agar: EC (1:2), PVA: EC (1:1) and HPMC: EC (1:2) respectively given in table 8 and figure 8. The nanosponges prepared using PVA: EC (1:1) were found to be better as they showed sustained release of drug from nanosponges.

Table 8: *In-vitro* study of nanosponges containing Agar: EC (1:2), PVA: EC (1:1), HPMC: EC (1:2) at pH 5.5

Time (hrs.)	% Drug Release		
	Agar: EC	PVA: EC	HPMC: EC
0	0	0	0
0.5	8.59± 1.18	0.03± 0.01	1.86± 0.29
1	13.61± 1.03	0.33± 0.12	3.90± 0.77
2	23.80± 0.32	0.19± 0.15	8.12± 0.63
4	33.69± 1.97	7.09± 0.25	13.89± 1.79
6	56.77± 1.74	17.54± 1.56	19.52± 0.68
8	70.04± 0.33	22.23± 1.32	42.82± 0.63
10	86.35± 0.87	32.98± 0.19	60.34± 1.90
12	94.53± 0.44	41.83± 1.40	89.64± 1.47
24	95.82± 0.62	84.45± 0.77	91.04± 1.16

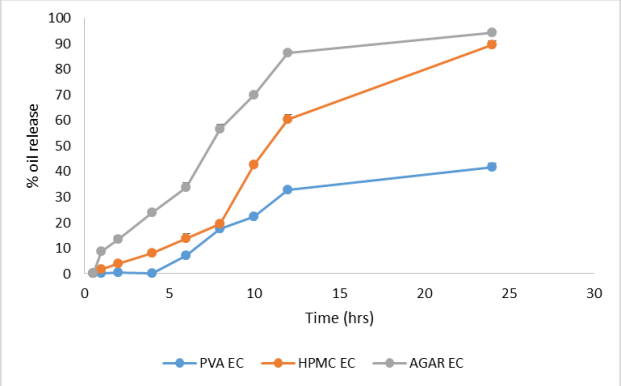

Figure 8: *In-vitro* study of nanosponges containing Agar: EC (1:2), PVA: EC (1:1), HPMC: EC (1:2) at pH 5.5

Safety Profile Study:

Acute Toxicity

For acute toxicity evaluation, careful general clinical observations were made every day. There was a gradual increase in the body weight of both the treated and control rats (Table 9).

The acute toxicity study did not show any toxicity sign and symptom at 2000mg/kg. No morbidity or mortality was observed in the treated groups at 2000mg/kg dose during acute toxicity study. As a result, the LD50 of the drug sample could be greater than 2000mg/kg body weight. As, 2000mg/kg dose was totally safe, and it was carried forward for sub-acute and chronic toxicity studies.

Table 9: Evaluation data on acute toxicity study

Mean difference in body weight after 7 days of treatment		
Group	0 day	7 day
Normal	156.33 ± 1.63	166.00 ± 1.67
Test	157.00 ± 1.67	163.00 ± 1.90

Sub-Acute oral Toxicity:

Throughout the study period no sign of toxicity and mortality was observed on treated rats, which received 2000 mg/kg. During the subacute experimental period all groups of rats showed gradual and normal increase in their body weight. The hematological parameters (WBC, RBC, PLT, HGB, HCT, MCV, MCH, and MCHC) given in table 10 and biochemical parameters in table 11 of the treated group were within the reference range for rats and were not significantly.

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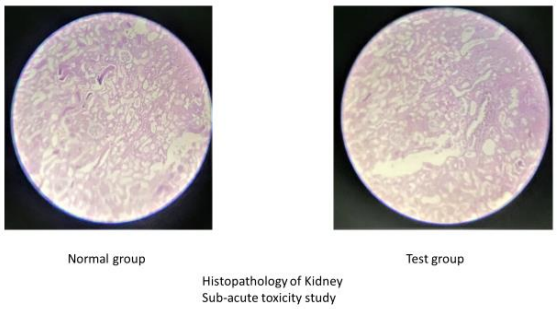
Table 10: Evaluation of hematological parameters based on sub- acute toxicity study

Hematological Parameters	Group	Mean	P value
WBC x103/µL	Normal	8.38 ±0.05	0.13
	100mg/kg	8.55 ±0.32	
RBC x 106/µL	Normal	7.38 ± 0.14	0.05
	100mg/kg	7.61 ± 0.21	
HGB (g/dL)	Normal	14.51 ± 0.34	0.19
	100mg/kg	14.80 ± 0.50	
HCT (%)	Normal	48.93 ± 0.60	0.14
	100mg/kg	49.23 ± 0.61	
MCV (fL)	Normal	67.17 ± 1.26	0.03
	100mg/kg	68.38 ± 0.53	
MCH (pg)	Normal	21.47 ± 0.60	0.05
	100mg/kg	20.80 ± 1.24	
MCHC (g/dL)	Normal	31.38 ± 0.50	0.20
	100mg/kg	31.72 ± 0.61	
PLT x 103/µL	Normal	699.46 ± 0.68	0.40
	100mg/kg	699.34 ± 0.72	

Table 11: Evaluation of biochemical parameters based on sub -acute toxicity study

Biochemical parameters	Doses	Mean	P Value
AST (IU/L)	Normal	288.08 ± 0.90	0.00000
	100mg/kg	302.07 ± 1.42	
ALT (IU/L)	Normal	199.33 ± 0.85	0.00001
	100mg/kg	209.73 ± 1.65	
Urea (mg/dl)	Normal	51.21 ± 0.72	0.00018
	100mg/kg	56.49 ± 1.13	
Creatinine (mg/dl)	Normal	0.73 ± 0.01	0.11949
	100mg/kg	0.75 ± 0.05	
Total Protein (mg/dl)	Normal	6.46 ± 0.08	0.00086
	100mg/kg	6.78 ± 0.17	
Glucose (mg/dl)	Normal	81.56 ± 0.73	0.08378
	100mg/kg	80.96 ± 0.29	



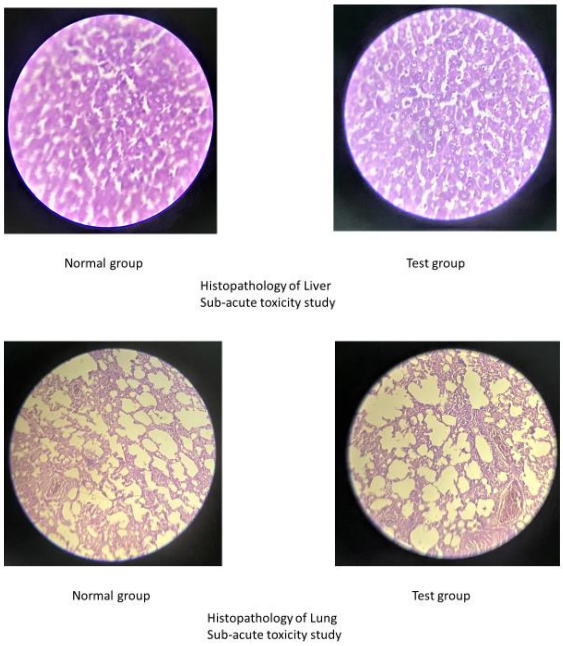


Figure 9: Histopathology of Kidney, Liver and Lung

Chronic Oral Toxicity:

General clinical observations were made at least once a day throughout the study period of 90 days considering the period of anticipated effects after dosing. Throughout the study period no sign of toxicity and mortality was observed on treated rats, which received 2000 mg/kg. In the sub-chronic acute toxicity study, the hematological and biochemical parameters of the treated group were within the reference range for rats and were not significantly different from the control group. Histopathological studies of the vital organs; heart, lung, liver, kidney, and spleen sections of rats treated with doses of 2000 mg/kg showed no significant microscopic changes compared with the controls.

Pharmacological Action:

C. albican was used to test the antifungal efficacy of the prepared geranium oil loaded nanosponge formulation (Figure 9). Before the administration of the formulation, all of the rats showed infection ranges from 3.49–3.50 log10 CFU/ml at day 0. After four days of therapy, both groups of animals showed decreased in CFU/ml values (2.49 log10 CFU/ml in group treated with formulation) as compared to day 0 ($P<0.05$). The ability of the formulation to diminish *C. albican* load in the skin might be regarded proof for its antifungal efficacy in the oil content. This is most likely due to

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the possibility of oil-loaded nanoparticles penetrating and accumulating in the skin layers. As a result, the oil antifungal activity of the oil may last longer and be more effective.

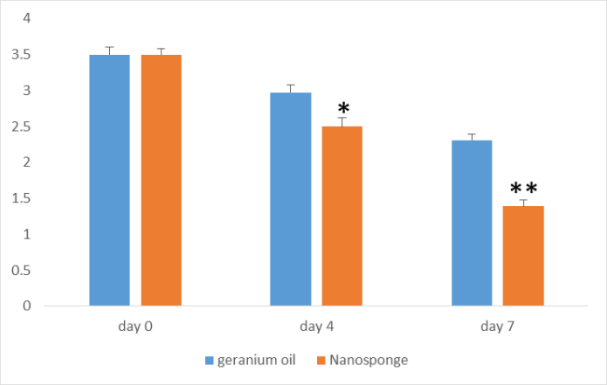

Figure 9: Antifungal activity of prepared formulation of geranium oil loaded nanosponges

Conclusion:

The geranium oil obtained by Clevenger method used to produce nanosponges using different combinations of bases such as HPMC and EC, HPMC and Eudragit S100, PVA and Eudragit S100, agar and ethyl cellulose, PVA and EC. The formulation were prepared using polymer HPMC: EC, HPMC: Eudragit, PVA: Eudragit, Agar: EC in the ratio of 0.5:1, 1:1 and 1:2. The nanosponges prepared by using HPMC and Eudragit polymers do not formed any product. Whereas product with HPMC and EC produced white powder, PVA and Eudragit produced gel like product and Agar and EC produced flaky product. The PVA and EC formulated in different ratios (1:1, 1:2, 2:1, 3:2 and 2:3) produced fluffy powdered product. The primary evaluation concluded that nanosponges prepared using HPMC and EC, Agar and EC and PVA and EC produced the products which were used for further evaluations. The nanosponges prepared by using Agar: EC (1:2), PVA: EC (1:1), HPMC: EC (1:2) were further studied for different parameters such as particle size, poly dispersibility, zeta potential, % drug loading and entrapment efficiency. The formulations were further studied for % drug released and FESEM. The nanosponges prepared using PVA: EC (1:1) were found to be better as they showed sustained release of drug from nanosponges. Based on the *in-vitro* fungal study the in-vivo study was carried out to evaluate the antifungal potential of formulation in *C. albican* rats. The ability of the formulation to reduced fungal infection in the skin might be regarded proof for its antifungal efficacy in the oil content. As a result, the oil antifungal activity of the oil may last longer and be more effective. The results concluded that nanosponges prepared by using PVA and EC based were good in reducing fungal infection.

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