RESEARCH ARTICLE

Enhanced Antifungal Efficacy of Clove Oil using Nanosponges: A Novel Topical Delivery System

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ABSTRACT

Clove oil (CO), derived from *Syzygium aromaticum*, is recognized for its broad-spectrum antimicrobial, antifungal, and anti-inflammatory properties. Still, its pharmaceutical uses are narrow due to poor aqueous solubility, chemical instability and potential skin irritation. This study aimed to enhance CO's therapeutic efficacy using nanosponges as a novel delivery system. A 3^2 complete factorial design was used to optimise the synthesis of ethyl cellulose nanosponges, which were achieved utilising the emulsion solvent diffusion method. optimized formulation (F4) exhibited high entrapment efficiency (94.38%) and practical yield (93.23%). The nanosponge-loaded gel exhibited excellent physicochemical properties, including a pH of 5.4, viscosity of 13,092 cps, and sustained drug release (92.3% over 12 hours) following zero-order kinetics. *In vivo* studies in an immunosuppressed rat model of *Candida albicans* infection exhibited significantly improved antifungal efficacy with the nanosponges-loaded CO gel compared to pure CO gel. Histopathological analysis confirmed enhanced skin recovery, while the Draize patch test indicated no skin irritation. Stability studies further validated the formulation's long-term integrity, with consistent drug release over 90 days. The results suggest that nanosponges-based CO delivery enhances antifungal activity, improves bioavailability, and minimizes skin irritation, making it a promising approach for topical antifungal therapy.

Keywords: Clove oil, Nanosponges, Antifungal therapy, Topical drug delivery, *Candida albicans*, Sustained release

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INTRODUCTION

Clove (*Syzygium aromaticum*) is a extensively cultivated evergreen tree, and its dried flower buds are the cradle of clove oil (CO), a valuable essential oil used extensively in food, cosmetics, and pharmaceuticals.[1] In the pharmaceutical sector, CO is recognized for its broad-spectrum pharmacologic activities, including antimicrobial, antifungal, antiviral, and anti-inflammatory properties, making it a versatile candidate for both topical and oral formulations.[2] The primary bioactive component of CO is eugenol (70–85%), which exhibits potent antimicrobial and analgesic effects.[3-5]But when exposed to heat, light, and oxygen, eugenol degrades rapidly, resulting in oxidation and a loss of therapeutic effectiveness.[6-8] Moreover, CO's poor aqueous solubility and potential to cause skin irritation limit its effectiveness in pharmaceutical formulations.[9]

To address these challenges, nanosponges have emerged as an innovative drug delivery system. These nanosized, porous carriers can encapsulate both lipophilic and hydrophilic compounds, thereby enhancing drug solubility, stability, and bioavailability while enabling sustained release.[10] Incorporating nanosponges into topical hydrogel formulations improves skin retention, minimizes irritation, and enhances therapeutic performance.[11,12] Researchers in the pharmaceutical industry are interested in nanosponges for regulated medication delivery because they can circumvent the drawbacks of traditional formulations.[13]

Because of its biocompatibility, hydrophobicity, and capacity to regulate the release of encapsulated active substances, EC finds extensive application in the production of nanosponge.[14,15] In order to create a topical hydrogel, study targets to create & characterize Ethyl cellulose nanosponges loaded with clove oil. Influence of formulation factors on % practical yield and % entrapment efficiency was explored by a 3^2 complete factorial design. The next step was to test the improved formulation in a rat model for antifungal activity, skin irritation potential, & *in vitro* drug diffusion.[16]

MATERIALS AND METHODS

Clove oil was purchased from Veda oils. Ethyl cellulose (EC) and polyvinyl alcohol (PVA) were purchased from S.D. Labchem Mumbai. Dichloromethane was purchased from Research Lab Fine Chem Industries, Mumbai. Carbopol 940 was purchased from S.D. Labchem Mumbai. High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were procured from Merck (Darmstadt, Germany).The remaining chemicals & reagents were all of analytical grade.

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Table 1: 3^2 full factorial experimental scheme

Factors	Levels		
	-1	0	+1
Ethyl cellulose (mg)	250	375	500
PVA (% w/v)	0.2	0.3	0.4
Dependent variables	% practical yield & % Entrapment efficiency		

Determination of Minimum Inhibitory Concentration(MIC) and Minimum Fungicidal Concentration (MFC) of Clove Oil

By employing the broth macro dilution method in accordance with Clinical & Laboratory Standards Institute (CLSI) M27-A3 criteria for yeasts, with minor adjustments, the MIC and MFC of clove oil were ascertained. A solution of 10% aqueous dimethyl sulfoxide (DMSO) with 0.5% Tween 80 was created by serially diluting clove oil twice, resulting in final concentrations ranging from 0.02 to 20 µl/ml. Candida albicans ATCC 100231 suspensions were added to Sabouraud dextrose agar tubes at a concentration of 2.5 × 10^3 CFU/ml. Aerothermal incubation at 35°C for 48 hours followed the addition of clove oil dilutions to the tubes. MIC of clove oil suggesting fungistatic action. In order to identify the MFC, 10 µl of the substance from every tube that did not exhibit apparent growth and from the first tube that did show growth was colonised onto Sabouraud dextrose agar plates. The plates were left to incubate at 35°C for an extra 48 hours. As a measure of fungicidal action, the minimum concentration of clove oil (MFC) at which no fungal growth was seen was determined. For the sake of reproducibility, every experiment was carried out in triplicate and then repeated three times.[17]

Design and Preparation of CO-loaded Nanosponges[4,17]

The emulsion solvent diffusion method was referred to manufacture clove oil nanosponges. The technique involved by ethyl cellulose as the polymer and aqueous phase consisting of PVA in distilled water. The necessary amount of solvent was used to dissolve the clove oil and

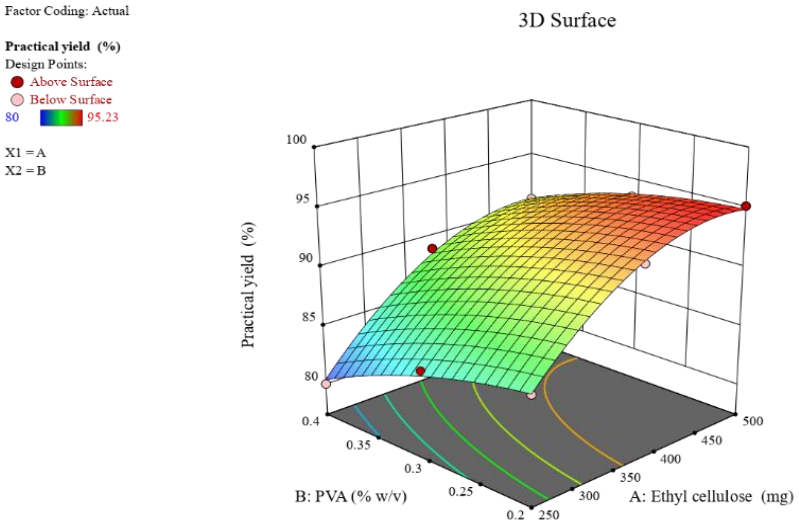

Figure 1: Response surface plot of effect of X1 & X2 on % practical yield of prepared nanosponges (Y1)

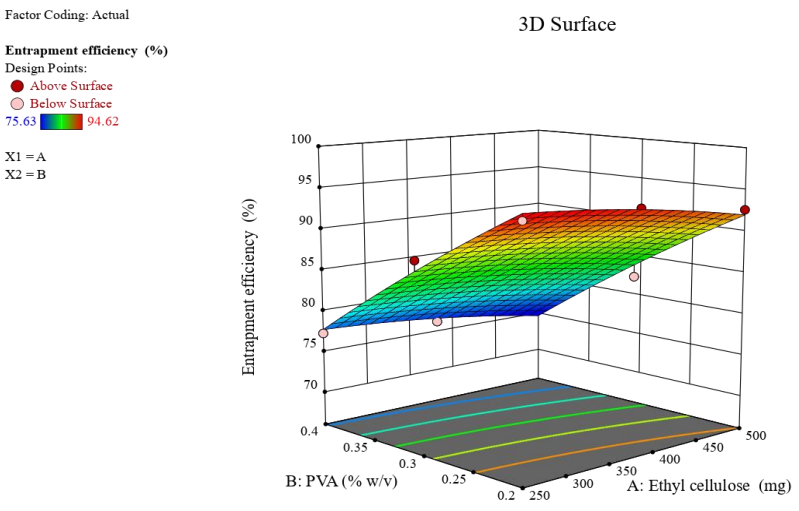

Figure 2: Response surface plot of effect X1 & (X2) on % entrapment efficiency of prepared nanosponges (Y2)

Table 2: Formulations of CO loaded nanosponges

Code	Clove oil (µl)	Ethyl cellulose (mg)	PVA(%w/v)	Dichloromethane (ml)	Distilled water(ml)
F1	100	250	0.2	20	100
F2	100	250	0.3	20	100
F3	100	250	0.4	20	100
F4	100	375	0.2	20	100
F5	100	375	0.3	20	100
F6	100	375	0.4	20	100
F7	100	500	0.2	20	100
F8	100	500	0.3	20	100
F9	100	500	0.4	20	100

Table 3: Percent practical yield and entrapment efficiency (%EE)

Code	Ethyl cellulose (mg)	PVA (%w/v)	Percent practical yield (%)	Entrapment efficiency (%)
F1	250	0.2	86.23± 0.3	94.62± 0.4
F2	250	0.3	84.61± 0.8	88.36± 0.3
F3	250	0.4	80± 0.2	77.3± 0.4
F4	375	0.2	93.23± 0.1	94.38± 0.8
F5	375	0.3	91.61± 0.4	86.32± 0.7
F6	375	0.4	89± 0.5	76.32± 0.6
F7	500	0.2	95.23± 0.9	92.65± 0.2
F8	500	0.3	93.61± 0.5	82.36± 0.4
F9	500	0.4	91± 0.7	75.63± 0.3

polymer. This solution was slowly added to a definite amount of PVA in the aqueous continuous phase.

For two hours, mixture was agitated by a magnetic stirrer set at 1000 rpm. The formed clove oil nanosponges were collected by filtration and dried in an oven at 40°C for 24 hours. To make solvent removal easier, dried nanosponges were placed in a vacuum desiccator.

Effect of the independent variables, EC (X1) and polyvinyl alcohol (X2), on the in vitro properties of the produced formulations was investigated using a 3^2 complete factorial design.The following concentrations of EC and PVA 0.2, 0.3 and 0.4 (in % w/v) were studied: 250, 375, and 500 mg, respectively (Table 1). Nine batches were prepared by merging the three levels of everyvariable; Table 2 details these formulations. The nanosponges' percent practical yield (Y1) and their percent entrapment efficiency (Y2) were dependent variables that were evaluated. The trials were carried out with Stat-Ease Inc.'s (Minneapolis, MN) Design Expert application (Version 13).

Preparation of CO Loaded Nanosponges Gel

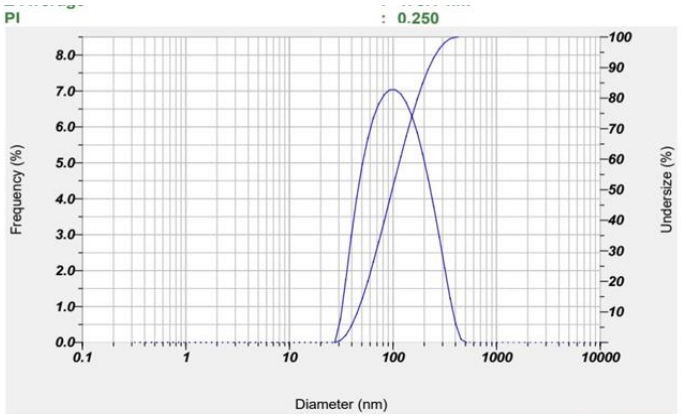

Figure 3: Particle size of optimized batch

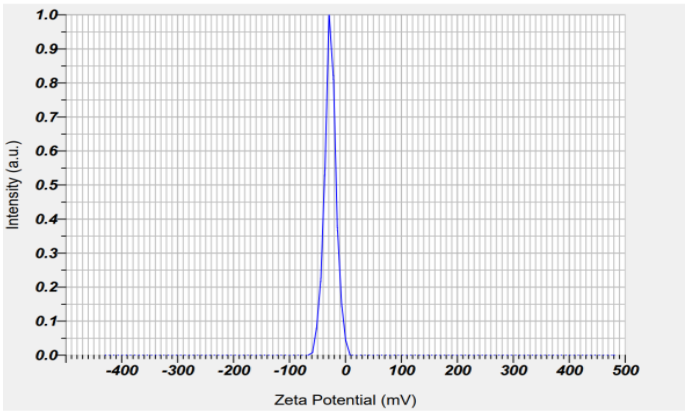

Figure 4: Zeta potential of optimized batch

Carbopol gel was prepared by dispersing 1% of Carbopol 934 in 100 ml of distilled water & left over night meant for swelling, and then pH of gel was adjusted between 6 to 7 using triethanolamine (TEA). The optimized formulation (F4) of CO loaded nanosponges was integrated in gel base.

Measurement of Percent Practical Yield

Here is the equation to calculate % practical yield of nanosponges:

$$\text{Percent practical yield} = \frac{\text{Weight of nanosponges}}{\text{Weight of solids (drug + polymer)}} \times 100$$

In order to determine the yield, precise measurements were taken of the starting weight of the medication and polymer, as well as the ending weight of the synthesised nanosponges.[21]

Measurement of Drug Entrapment Efficiency (%EE)

% EE of eugenol in CO-loaded nanosponges was determined by analyzing drug content in supernatant subsequently ultracentrifugation. Dispersion was subjected to ultracentrifugation at 16,900 × g and 4°C for 30 minutes using an REMI Equipment Pvt. Ltd, Mumbai. Supernatant was filtered & evaluated by RP-HPLC at 280 nm to quantify unencapsulated eugenol.

Eugenol Content Determination in CO Loaded Nanosponges

A system controller (CBM-20A) from Shimadzu was used in conjunction with a photodiode array detector (SPD-M20A) and an isocratic pump (LC-10 AD) from Shimadzu to measure the concentration of eugenol in CO-loaded nanosponges. A reversed-phase column (RP18, Nova-Pak, Waters Associates, USA) measuring 250 mm in length, 4 mm in internal diameter & 5 µm in particle size was used for study. The mobile phase included acetonitrile, water, and methanol (50:35:15, v/v), with a flow rate of 1.0ml/min. Analysis was performed at 280 nm, and eugenol concentrations in the samples were determined based on a calibration curve of total peak areas in contradiction of nominal eugenol concentrations. In terms of accuracy, precision, linearity, and % recovery, the approach was validated.

% of eugenol incorporated into CO-loaded nanosponges was determined using an HPLC assay. A 250 mg sample of the CO-loaded nanosponge formulation was dispersed in 10 ml of methanol, followed by sonication (Enertech Electronics Pvt. Ltd.) for 1 hour. After that, the mixture was spun in a centrifuge (REMI Equipment Pvt. Ltd, Mumbai) at 4,500 rpm and 4°C for half an hour. An HPLC analysis was performed on the collected supernatant after it had been passed through a Millipore 0.22 µm membrane filter.[15,22]

The percentage of eugenol incorporated in the nanosponges was measured by:

$$\% \text{ Eugenol} = \frac{C_E}{C_{Th}} \times 100$$

Where, C_E= Experimental concentration, C_{Th} = Theoretical concentration

Particle Size and Zeta Potential

Utilising DLS in conjunction with a Horiba SZ-100 Zeta Sizer (Horiba Scientific Nano Partica SZ-100, Kyoto, Japan), the average particle size of the optimized nanosponge formulation (F4) was measured. In order to get

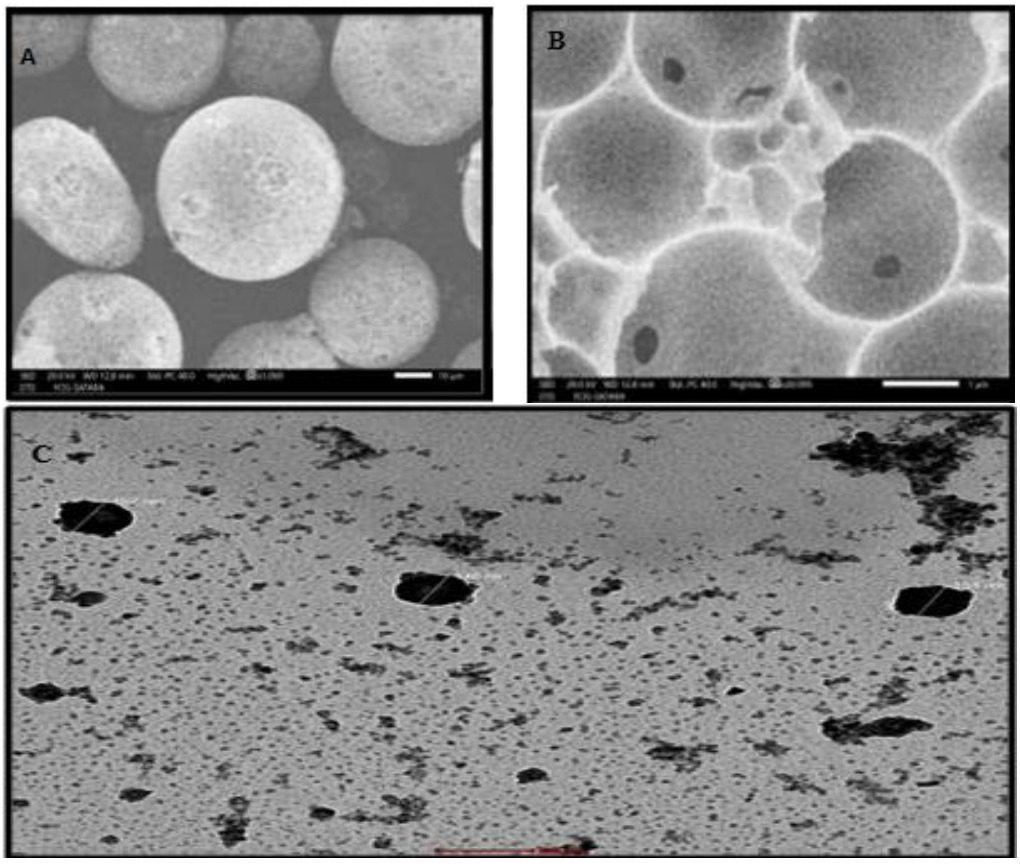

Figure 5: Morphological results of optimized formulation F4. (A) SEM of dried nanosponges (×3,000)'(B) SEM of dried nanosponges (×30,000), (C) TEM of clove oil loaded nanosponges (×2,500).

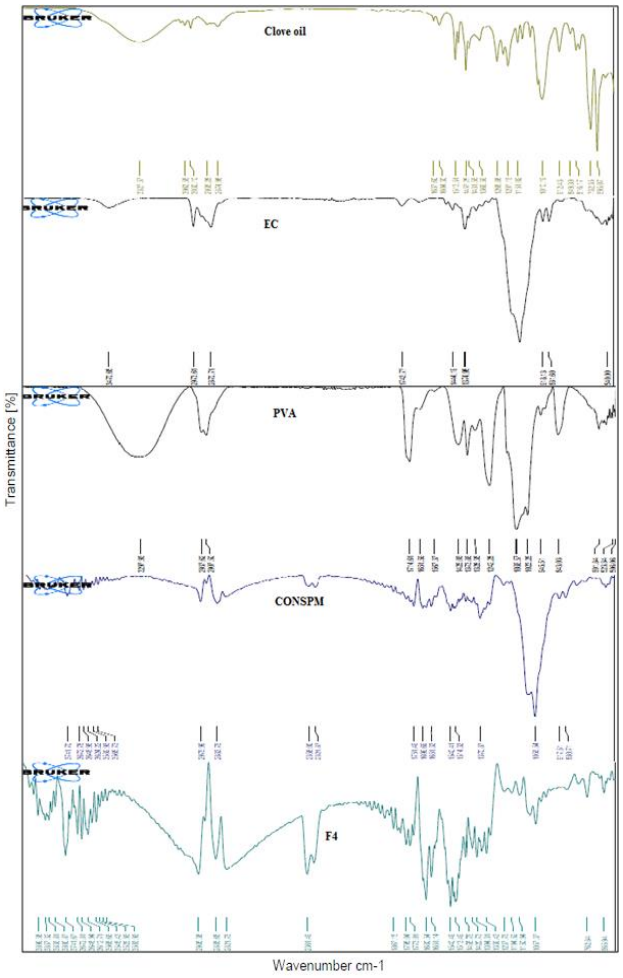

Figure 6: FTIR Spectra of Clove Oil, EC, PVA, and Formulation (F4)

the best possible scattering conditions, sample was diluted 20-fold with double-distilled water for evaluation.

Surface Morphological Studies

SEM (JEOL Benchtop SEM-EDS) was used to analyse surface morphology of optimized nanosponge formulation (F4). A circular aluminium stub was used to mount the sample using double-adhesive carbon tape. Then, a HUS-5GB vacuum evaporator was used to coat the sample with gold, which increased its conductivity. The surface structure and porosity of the nanosponge were examined in detail by doing the SEM analysis at an acceleration voltage of 20 kV and magnifications of 3000x and 30,000x.

We used TEM (TALOS L120C, Thermo Fisher Scientific, USA) to further examine morphology of the optimized nanosponge formulation (F4). Sample of nanosponge suspension was put onto a film-coated 200-mesh gold support grid, followed by drying under ambient conditions. TEM imaging provided high-resolution structural insights, allowing for the detailed examination of the nanosponges' shape, porosity, and particle uniformity.[23]

Compatibility Study of Optimized F4 Formulation

The compatibility study was carried out for optimized formulation (F4) using FT-IR (Bruker INDIA) Limited) at wavelength range of 4000-400 cm^{-1}spectrum.[24]

Evaluation CO Loaded Nanosponges Gel

pH Measurement

pH of CO-loaded nanosponge gel formulation was measured by a digital pH meter (Systronics pH System 362, India). Prior to measurement, the instrument was calibrated by adjusting the pH of distilled water to 7.0. The electrode was then immersed directly into the gel formulation, and the pH reading was recorded at room temperature.

Rheological Characterization

Viscosity of CO-loaded nanosponge gel was assessed using a Brookfield Viscometer (LVDV-II+, Brookfield Engineering Laboratories, INC., Middleboro, USA). The formulation was placed in a beaker and maintained at room temperature. Viscosity measurements were performed using a T-shaped spindle (spindle no. 42) at a speed of 50 rpm with 50% torque applied. The viscosity value was determined by averaging three independent readings for accuracy.[25]

Spreadability Test

An instrument comprised of a pulley system on one end of a wooden block was used to measure spreadability of gel containing CO-loaded nanosponges. Sandwiching the formulation was a 2 g sample of the gel placed on a ground glass slide with another glass slide of the same dimensions on top. To ensure uniform distribution and removal of air, a 1 kg weight was placed over the slides for a specific duration. Excess formulation was carefully removed from edges.

Next, a hook-and-string operation was used to apply a pulling force of 80 g to the upper slide. Upper slide had to move a predetermined distance of 7.5 cm, & time it took to do so was measured in seconds. The gel formulation's improved spreadability was shown by a shorter time interval.[26]

In vitro Drug Diffusion Study

CO-loaded nanosponge gel was evaluated by a Franz diffusion cell (Sri Sai Precision Instrument & Research Centre Pvt Ltd.) with a cellulose acetate dialysis membrane (HiMedia). A 25 ml phosphate buffer (pH 6.8) was mixed to receptor compartment, &a specific quantity of CO-loaded nanosponge gel was distributed uniformly across dialysis membrane in donor partition. In order to mimic physiological conditions, the donor and receptor compartments were tightly clamped together and retained at a persistent temperature of 37 ± 0.5°C.

Table 4: Evaluation CO loaded nanosponges gel

Physical appearance	White gel
Consistency	Good
pH	5.4± 0.4
Viscosity	13092±25.90 cps
Spreadability	30.05± 0.8 (g.cm/s)
In-vitro drug diffusion at 12 hrs	92.3± 0.5 %

Table 5: Release kinetics of gel

Formulation	R^2				Flux	Kp cm/min
	Zero order	First order	Higuchi	Korsmeyer Peppas	$\mu g/cm^2/min$	
Clove oil nanosponges gel	0.992	0.889	0.915	0.887	0.312	0.0322

Table 6: Erythemal score

Formulation	Erythemal score (n=6)		
	24 h	48 h	72 h
Group I- Control	0	0	0
Group II- DS	0	1	1
Group III- Placebo gel	0	0	0
Group IV- Marketed Ketocip cream 2%	0	0	0
Group IV- CO loaded nanosponges gel	0	0	0

Table 7: WBC counts in normal, infected& treated groups (mean ± standard deviation, n = 3)

Subject	White Blood Cells/mcl
Group I (normal)	9782 ± 125
Group II (infected + untreated)	4294 ± 230
Group III (infected + treated with pure CO gel)	6806 ± 298
Group IV (infected + treated with marketed Ketocip cream 2%)	9697 ± 190
Group V (infected + treated with CO loaded nanosponges gel)	9587 ± 160

*(mean ± standard deviation)

Receptor media was constantly mixed by means of externally powered magnetic bars to guarantee even mixing. Compartment was refilled with an identical volume of new phosphate buffer (pH 6.8) kept at the same temperature at specified intervals after aliquots were removed using a pipette. Utilising a previously established HPLC assay method, the eugenol concentration in the gathered samples was ascertained. To guarantee repeatability, every experiment was carried out three times.[27]

Diffusion Kinetics Study

By fitting data to different mathematical kinetic models, like Zero-order, First-order, Higuchi, & Korsmeyer-Peppas equations, principle of drug release from optimized CO-loaded nanosponge gel formulation could be understood. Drug's diffusion mechanism and release kinetics were determined using these models.[27]

Calculation of Flux[28]

The In vitro percutaneous flux (Jss) (mg/cm²·h⁻¹) of clove oil (CO) from the formulation was determined by plotting time versus the cumulative amount of the active compound permeated through the skin.

Skin Irritation Test

The gel containing CO-loaded nanosponges was tested for its ability to cause skin irritation using the Draize patch test, in contrast to a placebo gel. Approval number (1197/PO/Re/S/08/CCSEA) was granted to this study methodology by the institutional animal ethical committee, which consists of the chairperson and member secretaries of the Institutional Animal Ethical Committee (247/PO/Re/S/08/CCSEA). Each of the five groups of thirty Wistar albino rats had six animals. As a control, Group I got nothing, whereas Group II (DS) got a medication solution that didn't have a gel composition. Group III (placebo gel) received a placebo gel devoid of active drug, and Group IV and Group V was treated with the marketed cream (Ketocip cream 2%) and CO-loaded nanosponges gel respectively. The dorsal skin of each rat was shaved 24 hours before the test to ensure uniform application. A measured quantity of each formulation was evenly applied over a 4 cm² hair-free skin area. The treated sites were scrutinized at 24, 48, & 72 hours post-application for signs of irritation. Skin reactions, including erythema (redness) and edema (swelling), were evaluated using a standardized scoring system ranging from 0 (no erythema/edema) to 4 (severe erythema/edema). The mean erythema scores for each formulation were documented and compared to determine the irritation potential.[29]

In vivo Antifungal Activity

The purpose of the study was to determine whether certain gels containing CO-loaded nanosponges were beneficial in treating more severe cases of fungal infections. Approval number (1197/PO/Re/S/08/CCSEA) was granted to this study methodology by the institutional animal ethical committee, which consists of the chairperson and member secretaries of the Institutional Animal Ethical Committee.

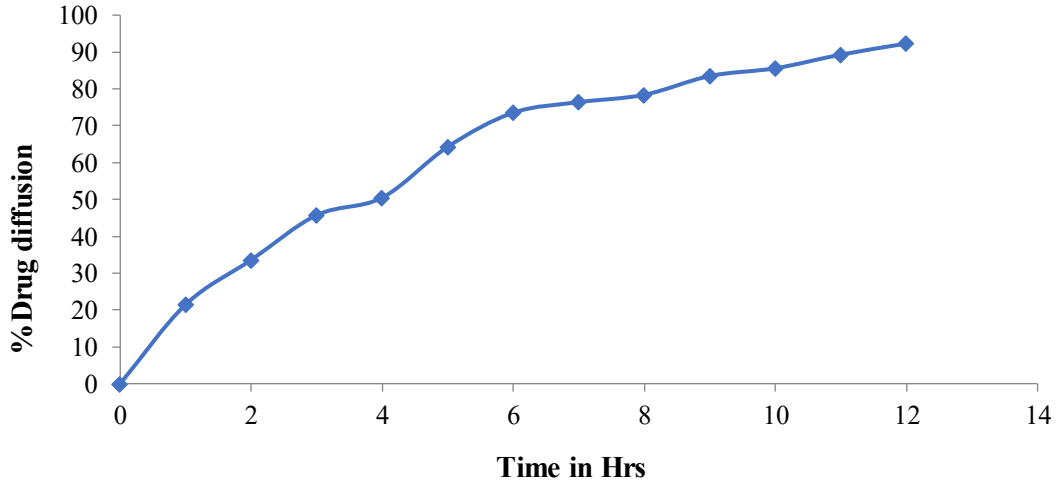

Figure 7: Cumulative drug diffusion profile vs time

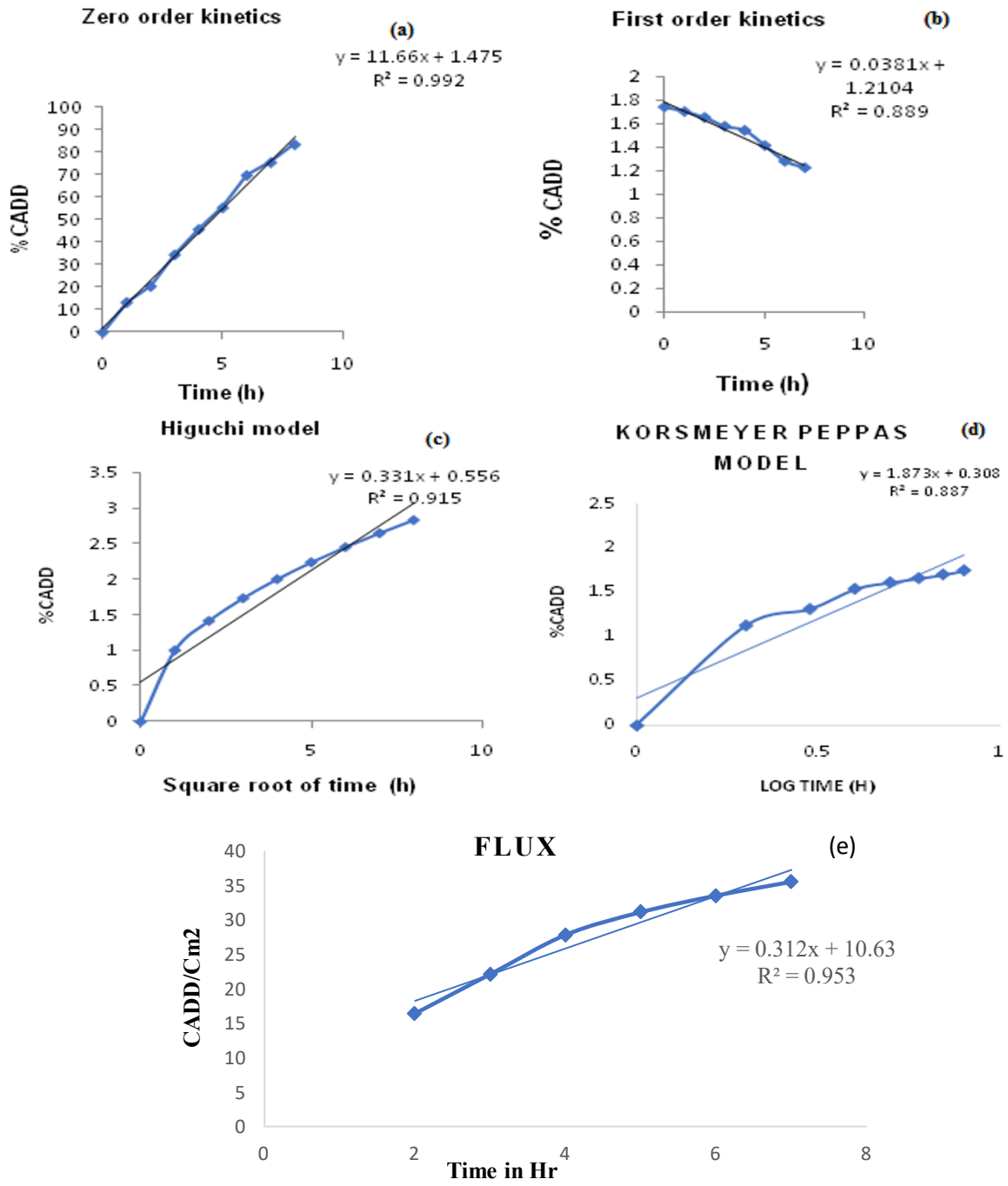

Figure 8: Kinetic Models and Flux Analysis of CO loaded nanosponges gel (a) Zero order kinetics (b) First order kinetics (c) Higuchi (d) Korsmeyer peppas (e) Flux

The immunosuppression of rats was done to mimic a severe cutaneous illness.[30] Each of the five groups consisted of three rats (n=6).

The following was the method of random grouping:

- Group I(Control group).
- Fungus infection without treatment (untreated) was found in Group II, the positive control group.
- Group III, which received a 10-day treatment of pure CO gel.
- Group IV consisted of infected rats that were treated for 10 days with marketed cream (Ketocip cream 2%).
- Group V consisted of infected rats that were treated for 10 days with gel containing CO loaded nanosponges.

To create an immunosuppressive state, all rats except those in Group I were given 5 mg/kg of intravenous Prednisolone for three days. The rats' backs were shaved one centimetres per square centimetre four hours before to the test, but the skin remained unharmed. The concentration of the fungus strain, Candida albicans, was adjusted to 10^6 CFU/ml. Injecting 0.3 ml of Candida albicans suspension intradermally into centre of exposed skin of each rat (except Group I) caused minor oedema at injection site, which was eliminated by aggressively massaging the area. The injection site showed symptoms of a fungal infection after 72 hours. Separate cages were used to keep the rats from licking each other's skin.

Table 8: Differential WBC counts in normal, infected & treated groups (mean ± standard deviation, n = 3)

Subject	Lymphocytes (%)	Eosinophils (%)
Group I (normal)	41±2.5	1±0.5
Group II (infected + untreated)	17±3	5±1
Group III (infected + treated with pure CO gel)	28±2	2±0.5
Group IV (infected + treated with marketed Ketocip cream 2%)	41±2	2±0.5
Group V (infected + treated with CO loaded nanosponges gel)	40±2	1±0.5

Table 9: Stability Study

Parameter	Initial	30 Days	60 Days	90 Days
pH	5.4 ± 0.4	5.2 ± 0.4	5.0 ± 0.3	5.0± 0.3
In-vitro drug diffusion at 12 hrs	92.3± 0.5 %	91.2 ± 0.9 %	89.8 ± 1.3 %	89.0 ± 1.5 %

Group III rats received 1 gramme of pure CO gel, while Group IV rats received 1 gramme of gel containing CO loaded nanosponges. The rats' ability to recover from skin injury was then evaluated. The results of the blood tests were also recorded.

Histopathological Analysis

Rats were sedated before being sacrificed at the end of the study. A 1:10 mixture of ketamine (0.2 ml/100 mg) and xylazine (50 mg/kg) was used to induce anesthesia. Skin from the affected area was collected and fixed with 10% formalin, then embedded in paraffin. Slides were prepared from the paraffin blocks using a rotary microtome & cut into 5 µm-thick sections. Haematoxylin and eosin dyes were used to stain these sections. We tested every single group of animals and compared their outcomes to those of control groups. In order to detect alterations in dermis and epidermis, as well as inflammation, skin samples were examined under a compound microscope.

Assessment of Blood Parameters

On 10^{th}day after animals were sacrificed, blood was collected in a glass vial that contained EDTA in order to examine the haematological parameters. The white blood cell (WBC), lymphocyte (L cell), and eosinophil (Eos) counts in the normal, untreated & treated rat groups were determined using a conventional method.

Stability Study

The CO-loaded nanosponges gel (F4), which was optimised according to ICH requirements, was kept for 90 days in a container that was hermetically sealed at 40 ± 2°C and 75 ± 5% relative humidity. Researchers tested the pH of nanosponge gel and conducted *in vitro* drug diffusion experiments.[31]

RESULTS AND DISCUSSION

MIC and MFC of Clove Oil

In a test against Candida albicans strain ATCC 100231, clove oil shown strong antifungal activity. Significantly greater than the MIC was the MFC. In a nutshell, MIC was 1 µl/ml & MFC was 2 µl/ml. Both the MIC and MFC values that were calculated were greater than the ones that had been previously documented for clove oil. Using different sources of oil could be the reason for the variance. Due to its high concentration of the potent antibacterial eugenol, clove oil has fungistatic and fungicidal effects.[32]

Percent Practical Yield

The percent practical yield of batches F1 to F9 ranged from 80 ± 0.2% to 95.23 ± 0.9% (Table 3). A statistical analysis was conducted to evaluate the influence of ethyl cellulose (X1) and polyvinyl alcohol (PVA, X2) on the % practical yield using a quadratic regression model (Figure 1). A full factorial design was employed, and the statistical analysis was performed using Design-Expert software**.**

ANOVA results confirmed the statistical significance of model **(**F-value = 150.09, p = 0.0009**),** indicating that the observed variations were unlikely due to random error. Both ethyl cellulose (p=0.0002) and PVA (p=0.0013) were identified as significant factors influencing practical yield. The quadratic effect of ethyl cellulose (p = 0.0043) was also significant**,** demonstrating a nonlinear relationship**,** whereas the interaction term (X1X2) and the quadratic effect of PVA ($X2^2$) were not statistically significant (p > 0.10)**.**

Model fit statistics further support edits strong predictive capability, with R^2 = 0.9960, Adjusted R^2 = 0.9894, and Predicted R^2 = 0.9542**,** confirming the model's reliability. The regression equation indicated that ethyl cellulose exhibited a positive impact on practical yield up to an optimal concentration, beyond which a decline was observed, whereas PVA negatively influenced yield**.**

Practical Yield = 91.83 + 4.83X1 - 2.45X2 + 0.5000X1X2 - 2.83$X1^2$ - 0.8283$X2^2$

Entrapment Efficiency (%EE)

The entrapment efficiency (%EE) of batches F1 to F9 ranged from 75.63 ± 0.3% to 94.62 ± 0.4%. A quadratic regression model based on a full factorial design (Figure 2) was used to statistically assess effects of ethyl cellulose (X1) & polyvinyl alcohol (PVA, X2) on %EE.

ANOVA results confirmed arithmetical significance of model (F-value = 45.30, p = 0.0050), indicating that the observed variations were unlikely to be attributed to random error. Among the independent variables, PVA exhibited a highly significant negative impact on entrapment efficiency (p = 0.0007), while ethyl cellulose was not statistically significant (p = 0.0726). Moreover, interaction term (X1X2) and the quadratic terms ($X1^2$, $X2^2$) were not significant (p > 0.10), suggesting minimal combined or nonlinear effects.

The model demonstrated strong predictive power, as evidenced by R^2 = 0.9869, Adjusted R^2 = 0.9651, and Predicted R^2 = 0.8425, confirming its reliability and robustness. Regression analysis further indicated that PVA significantly reduced entrapment efficiency, whereas ethyl cellulose had only a minor effect. The final regression equations provided valuable predictive insights, with PVA exerting a considerably stronger negative influence compared to ethyl cellulose.

%

Entrapment Efficiency=86.03−1.61X1−8.73X2+0.0750X1 X2−0.5200X1^2−0.5300X2^2

Optimized Batch Selection

Choice of optimized nanosponges formulation was decided on maximizing both percent practical yield and entrapment efficiency while ensuring formulation stability. Among the evaluated batches, Batch F7 exhibited the highest practical yield (95.23 ± 0.9%), whereas Batch F1 achieved the highest entrapment efficiency (94.62 ± 0.4%). However, statistical analysis indicated that ethyl cellulose positively influenced practical yield up to an optimal concentration, while PVA had a significant negative effect on entrapment efficiency, highlighting the necessity of maintaining a lower PVA concentration. Batch F4 (375 mg Ethyl Cellulose, 0.2% PVA) exhibited an optimal balance, achieving a high practical yield (93.23 ± 0.1%) along side excellent entrapment efficiency (94.38 ± 0.8%). This formulation effectively minimized the adverse effects associated with excessive polymer usage while maintaining structural integrity. Consequently, Batch F4 was identified as the optimized formulation, offering the most favorable trade-off between yield and entrapment efficiency, making it the most suitable candidate for additional pharmaceutical expansion.

Particle Size and Zeta Potential Measurements

Particle size of optimized nanosponges formulation (F4) was measured at 190.4 ± 12.03 nm, confirming its nanoscale nature with minimal size variation. PDI was recorded at 0.250 ± 0.0221, indicating a moderately uniform size distribution, which is essential for consistent drug release. Furthermore, zeta potential was measured at -27.1 ± 1.63 mV, suggesting good colloidal stability. The high negative charge prevents particle aggregation through electrostatic repulsion, thereby enhancing formulation stability and ensuring efficient drug encapsulation (Figure 3 and 4).[15]

Surface Morphological Studies

The provided images (Figure 5 - A, B and C) depicted the SEM & TEM analyses of optimized nanosponge formulation (F4), highlighting its surface morphology, size, and structural properties.

The SEM images (A and B) displayed the surface morphology of the nanosponges at different magnifications. The nanosponges appeared spherical with a porous structure, confirming their suitability for drug encapsulation. The uniform shape and smooth surface suggested efficient polymerization and the formation of well-defined nanosponges.

The TEM image (C) revealed the internal structure and particle distribution of the nanosponges. The particles appeared well-dispersed with a nanoscale size, confirming the data obtained from particle size analysis. The presence of small pores and internal cavities supported great drug loading.

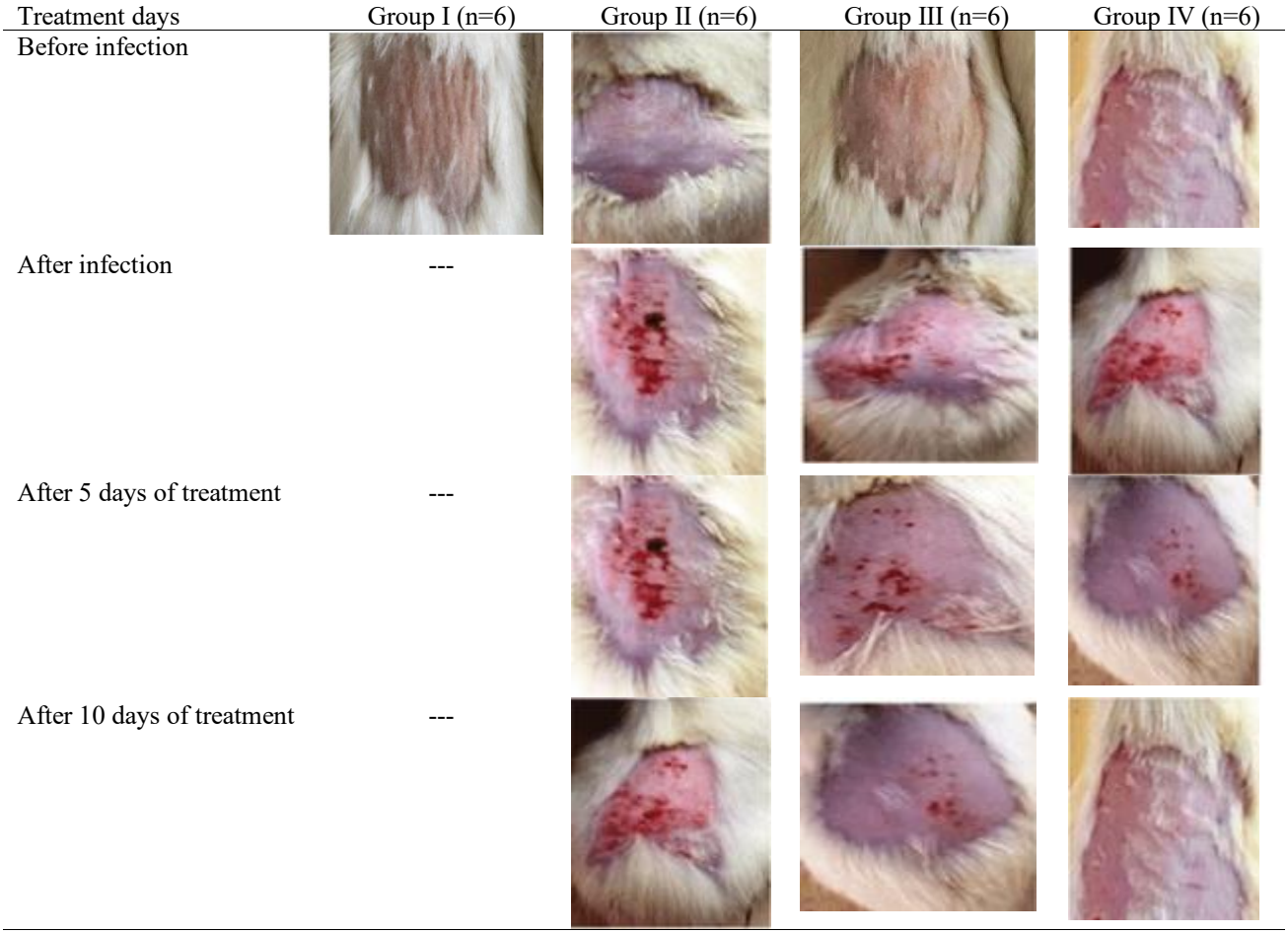

Figure 9: *In vivo* antifungal activity

Overall, these images confirmed that the optimized F4 formulation consisted of uniform, spherical, and porous nanosponges, making it a suitable candidate for drug delivery applications.[33]

Compatibility Study of Optimized F4 Formulation

The FTIR spectra of the individual components and the final formulation (F4) were analyzed to assess compatibility (Figure 6). The spectrum of clove oil exhibited characteristic absorption bands corresponding to its major active compound, eugenol, with O-H stretching (phenolic group) at approximately 3400 cm^{-1}, C-H stretching (aromatic) at around 3000 cm^{-1}, C=C stretching (aromatic ring) near 1600 cm^{-1}, and C-O stretching (ether bond) at approximately 1250 cm^{-1}. These functional groups confirmed the presence of eugenol. FTIR spectrum of ethyl cellulose (EC) displayed characteristic peaks, including C-O-C stretching (ether group) at ~1100 cm^{-1}, C-H stretching (alkane group) at ~2900 cm^{-1}, and CH_2 bending vibration at ~1375 cm^{-1}. These peaks indicated the structural integrity of EC, a hydrophobic polymer commonly used in controlled drug release and film-coating applications. Similarly, the spectrum of polyvinyl alcohol (PVA) revealed significant peaks such as O-H stretching (hydroxyl group, strong hydrogen bonding) at ~3300 cm^{-1}, C-H stretching (alkane group) at ~2950 cm^{-1}, and C=O stretching (carbonyl group from acetate impurities) at ~1720 cm^{-1}, confirming its role as a biocompatible, water-soluble polymer used for film formation and drug delivery. The CONSPM representing the physical mixture of clove oil loaded nanosponges displayed combined peaks of clove oil, EC, PVA without major peak shifts. The O-H stretching (~3400 cm^{-1}) from clove oil and PVA remained unchanged, suggesting no interaction affecting hydrogen bonding. The C-O-C ether peaks (~1100 cm^{-1}) from EC and clove oil were preserved, indicating no structural modifications. Additionally, the aromatic C=C stretching (~1600 cm^{-1}) from clove oil was still present, confirming that eugenol had not undergone degradation. No significant peak shifts, disappearances, or new peak formations were observed in the F4 spectrum compared to the individual components, demonstrating the absence of major chemical interactions or incompatibilities. The lack of spectral changes indicated that clove oil, EC, and PVA remained pharmaceutically compatible, ensuring the physical and chemical stability of the formulation.

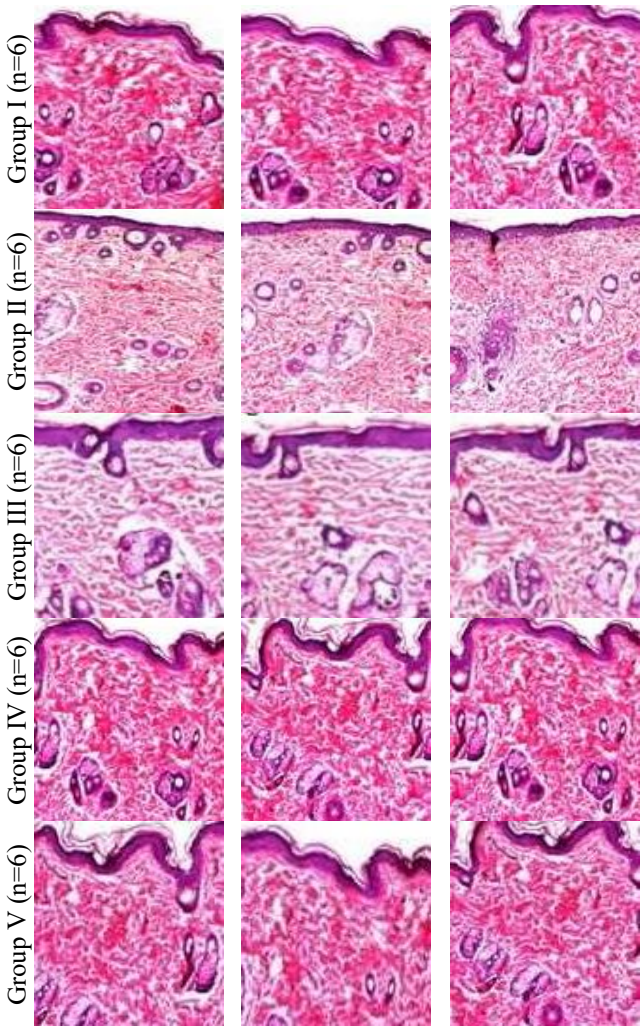

Figure 10: Histopathological Analysis

Evaluation CO Loaded Nanosponges Gel

The evaluation of the nanosponge-based gel formulation (Table 4) demonstrated its desirable physicochemical properties for topical drug delivery. The gel appeared as a uniform white formulation with good consistency, ensuring proper texture and homogeneity. The pH was recorded as 5.4 ± 0.4, indicating its compatibility with skin's natural pH & minimizing threat of irritation. The viscosity was measured at 13,092 ± 25.90 cps, suggesting a suitable texture that balanced spreadability and retention on the skin. The gel exhibited excellent spreadability (30.05 ± 0.8 g·cm/s), which ensured even distribution upon application. Additionally, *in-vitro* drug diffusion studies revealed a 92.3 ± 0.5% drug release over 12 hours (Figure 7), demonstrating its potential for sustained drug delivery. Generally, findings indicated that nanosponge-based gel was well-suited for effective topical application, ensuring stability, ease of use, and controlled drug release.

Diffusion Kinetics Study and Calculation of Flux

Zero-order kinetics model (Figure 8-a) was most well-fitting mathematical model among all the models employed to analyse drug release kinetics of clove oil nanosponges gel. Drug was released at a constant rate regardless of its beginning concentration because the greatest R^2 value was 0.992, indicating a strong linear association.

Zero-order kinetics was highly desirable in pharmaceutical formulations, particularly for sustained and controlled drug release, as it helped to preserve consistent therapeutic drug levels over prolonged period. This ensured better results compared to models where release rate fluctuated with drug concentration. The first-order model; Figure 8- b (R^2 = 0.889) was less fitting, indicating that drug release was not significantly reliant on concentration.

While the Higuchi model; Figure 8- c (R^2 = 0.915) & Korsmeyer-Peppas model; Figure 8- d (R^2 = 0.887) also showed a good fit, they primarily described diffusion-controlled and mechanism-based drug release, respectively. Thus, based on the R^2 values and theoretical considerations, zero-order model was determined to be best fit, confirming that clove oil nanosponges gel provided a steady and

prolonged release profile, building it appropriate for topical therapeutic applications.

Moreover, flux study demonstrated a drug permeation rate of 0.312 mg/cm²/min and a permeability coefficient (K) of 0.0322 cm/min, confirming a steady and consistent drug permeation profile. The comprehensive analysis, as summarized in the table 5, confirmed that the clove oil nanosponges gel primarily followed zero-order kinetics, ensuring sustained and controlled drug release. These findings suggested that the formulation was well-suited for prolonged therapeutic efficacy and enhanced topical application.[34]

Skin Irritation Test

Outcomesst indicated that CO-loaded nanosponges gel (Group IV) did not cause any significant skin irritation, as mean erythema & edema scores remained at 0 throughout the observation period. Similarly, the Control group (Group I), Placebo gel (Group III), and Marketed Ketocip cream 2% (Group IV) also showed no signs of erythema, maintaining a score of 0 at all time points (Table 6). In contrast, the Drug Solution (Group II) exhibited mild to moderate erythema, with scores increasing to 2 by 72 hours.. These findings suggested that incorporating CO into a nanosponges gel formulation effectively reduced the skin irritation potential associated with the drug solution.

In vivo Antifungal Activity

The efficacy of antifungal treatments has been commonly assessed using *Candida albicans* as a model pathogen. Figure 9 illustrated the progression of infection and subsequent skin recovery following treatment with marketed Ketocip cream 2%,CO-loaded nanosponges gel and pure CO gel over a 10 days period.

Prior to fungal induction, all animals exhibited a normal skin structure with no visible signs of infection, like redness, swelling, edema, or scaling. However, after inoculation with *Candida albicans*, infected skin developed distinct symptoms of inflammation, including red patches, edema, scaling, and cracking. Symptoms were monitored daily, with initial redness observed within 24 hours post-inoculation, which progressed to pronounced scaling and erythema by day 3.

By day 5 of treatment, infected sites exhibited shedding of infectious scales, depicting underlying light pink skin. After 10 days of treatment, the pure CO gel demonstrated partial symptom resolution, with a reduction in inflammation and edema; however, residual scarring remained visible. In contrast, the marketed Ketocip cream 2% and CO-loaded nanosponges gel facilitated complete restoration of normal skin structure, indicating superior antifungal efficacy.

Among the tested formulations, the CO-loaded nanosponges gel exhibited the most significant reduction in fungal infection, underscoring its potential as an effective therapeutic option. The enhanced antifungal activity was attributed to the nanosponges' ability to provide controlled and sustained drug release, which enabled deeper penetration into infected skin layers and prolonged drug retention at the infection site. Furthermore, the nanocarrier system's synergistic effect with permeation enhancers improved drug bioavailability, maximizing antifungal efficacy. Conversely, the pure CO gel, lacking a flexible delivery system, remained confined to the surface layers of stratum corneum, limiting both skin penetration & therapeutic effectiveness.

Histopathological Analysis

To judge effects of treatment & identify any changes in tissue, a histological analysis was performed on the skin of both healthy albino rats (serving as a negative control) and infected rats. Group I (negative control) & was not infected; Group II was a positive control and was infected without treatment; Group III was infected and treated with pure CO gel Group IV was infected and treated with marketed Ketocip cream 2% and Group V was infected and treated with CO loaded nanosponges gel. Figure 10 displays the histological pictures of these five experimental groups. Over the course of the ten days of monitoring, the expected negative control group (Group I) showed no abnormalities in histological architecture. There were no pathological changes to the structural integrity of the dermis and epidermis. It seemed like the size and shape of the skin's components had been conserved. In contrast, the infected but untreated group (Group II) displayed significant pathological changes over the 10-day period without any signs of recovery. Notable histopathological findings included focal acanthosis accompanied by a compact hyperkeratotic layer. Chronic inflammation was evident in the dermal layer, characterized by extensive infiltration of inflammatory cells and necrotic tissue. Additionally, fungal hyphae were observed within the overlying epidermis, and focal interface dermatitis became prominent. Following treatment with pure CO gel (Group III), only minimal improvement was observed. While there was a gradual restoration towards normal skin morphology, inflammatory cell infiltration persisted, and the skin structure remained incompletely recovered. In contrast, rats treated with marketed Ketocip cream 2% and CO-loaded nanosponges gel (Group IV and V) exhibited significant improvement, demonstrating complete tissue recovery. The treated skin showed a nearly normal histological appearance with well-defined dermal and epidermal layers. This enhanced therapeutic effect may be attributed to the nanosponge formulation, which facilitates deeper skin penetration due to its nanoscale size, thereby accelerating the resolution of fungal infection.[35]

Assessment of Blood Parameters

A haematological research was carried out to determine the test animals' immune state. The white blood cell (WBC) counts, both total and differential, were assessed in rats that were either healthy, sick, or given a treatment. Table 7 shows that equated to untreated group, treated group had a substantially increased mean WBC count in the current study. It appears that the CO-loaded nanosponges gel offered protection against Candida albicans infection, as the raised WBC count showed the lack of infection and a restored immunological response.

A greater lymphocyte count was found in the treated group equated to untreated group, according to differential WBC analysis. The normal and treated groups also had significantly greater lymphocyte counts than the infected and untreated ones. Both the untreated and infected groups showed a significant decrease in lymphocyte counts after

receiving the immunosuppressant prednisolone (5 mg/kg body weight). Eosinophil numbers, on other hand, showed the reverse pattern, as seen in Table 8.

Traditional semisolid formulations, including lotions and creams, have a reputation for having slower skin penetration. But the gel laden with nanosponges penetrated the skin quickly, allowing the active chemicals to be delivered more deeply and efficiently. The infection was successfully cured and skin conditions were normalised with help of this improved delivery mechanism.

Stability Study

A 90-days stability analysis was conducted to evaluate physicochemical properties of optimized nanosponges gel formulation (table 9), with evaluations performed at predetermined intervals. The pH of the formulation was initially recorded as 5.4 ± 0.4. A slight decrease was observed over time, with pH values of 5.2 ± 0.4 at 30 days and 5.0 ± 0.3 at 60 days, where it remained stable until the 90-days mark.

The *in-vitro* drug diffusion profile initially exhibited a drug release of 92.3 ± 0.5% at 12 hours. Over time, a gradual decline in drug release was observed, with values of 91.2 ± 0.9% at 30 days, 89.8 ± 1.3% at 60 days, and 89.0 ± 1.5% at 90 days. These results suggested that while minor variations in pH and drug diffusion occurred, the formulation maintained acceptable stability throughout the study period, with no significant impact on drug release or efficacy.

CONCLUSION

This study successfully formulated and evaluated a clove oil loaded nanosponges based gel designed to enhance topical antifungal therapy. Utilizing a 3^2 full factorial design, the optimized formulation demonstrated high practical yield and entrapment efficiency, along with desirable physicochemical attributes such as skin-compatible pH, suitable viscosity, good spreadability, and nanoscale particle size with stable zeta potential. In-vitro diffusion studies confirmed a sustained drug release over 12 hours, following zero-order kinetics, while permeation and flux analysis indicated efficient transdermal delivery. In vivo evaluation in an immunosuppressed rat model of Candida albicans infection revealed significantly improved antifungal efficacy of the nanosponges gel compared to both the pure clove oil gel and a marketed antifungal cream. Histopathological examination confirmed complete restoration of skin architecture in treated groups, and haematological analysis demonstrated enhanced immune response. The Draize skin irritation test further validated the formulation's dermal safety. Additionally, stability studies over 90 days affirmed the formulation's robustness, with minimal changes in pH and drug diffusion profile. Overall, the clove oil loaded nanosponges gel presents a promising, safe, and efficacious topical delivery system, offering controlled drug release, improved skin penetration, and superior therapeutic outcomes over conventional formulations in the treatment of fungal infections.

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