Journal of Current Pharma Research (JCPR)

Volume 20, Issue 9, September 2024 **jcpr.humanjournals.com** ISSN: 2230-7842, 2230-7834

Enhanced Rheumatoid Arthritis Therapy: Formulation, Optimization, and *In Vitro* and *Ex Vivo* Characterization of Polyherbal-Loaded Invasomal Gel

Chetan T. Bhat [1], Panchaxari M. Dandagi [1*], Kishori P. Sutar[1], Pradnya G. Patted[1], Shivaprasad S. Bevinakoppamath[1]

Department of Pharmaceutics, KLE College of Pharmacy, Constituent Unit of KLE Academy of Higher Education and Research, Belagavi, Karnataka, INDIA.

Received: 2024-09-08 Revised: 2024-09-19 Accepted: 2024-09-23

ABSTRACT

Background: This study aims to develop, optimize, and characterize a polyherbal-loaded invasomal gel as an alternative to traditional medicines for enhanced rheumatoid arthritis (RA) therapy. **Methods:** Ginger and Boswellia serrata extracts were used for their anti-inflammatory and anti-arthritic properties. Invasomes were prepared using the ethanol injection method and optimized using a Box-Behnken design. The optimized invasomes were then incorporated into a gel formulation and evaluated for various parameters including vesicle size, zeta potential, entrapment efficiency, and surface morphology. The gel's pH, viscosity, spreadability, and drug content were also assessed. *In vitro*, drug diffusion and anti-arthritic studies, ex vivo permeation using goat skin, and stability studies under different storage conditions were conducted. **Results:** The optimized invasomal gel exhibited a vesicle size of 95.3 nm, a zeta potential of -28.7 mV, and an entrapment efficiency of 85.4%. Transmission electron microscopy confirmed spherical vesicles. The gel formulation showed a pH of 6.4, a viscosity of 42,000 cps, and excellent spreadability. In vitro drug diffusion studies demonstrated sustained release over 24 hours. In vitro, anti-arthritic activity revealed significant inhibition of protein denaturation. Ex vivo permeation studies indicated enhanced skin penetration and drug deposition. Stability studies confirmed the formulation's stability, particularly under refrigerated conditions. **Conclusion:** The polyherbal-loaded invasomal gel showed promising results in enhancing the bioavailability and therapeutic efficacy of the herbal extracts for rheumatoid arthritis treatment. This novel formulation offers a potential alternative to conventional therapies with improved stability and reduced side effects.

Keywords: Polyherbal-loaded invasomal gel, Rheumatoid arthritis therapy, Invasomes, Bioavailability, Anti-inflammatory properties, Box behnken Design.

1 INTRODUCTION

Rheumatoid arthritis (RA) is a serious and progressive autoimmune condition defined by systemic inflammation that promotes joint destruction and dysfunction. Approximately 0.5-1% of the world's population is impacted by this condition, with a higher likelihood of women being affected than men[1]. Early diagnosis and prompt initiation of therapy are crucial to mitigating its adverse effects and preventing irreversible joint damage. Despite advancements in treatment, the etiology of RA remains incompletely understood, with dysregulated citrullination and the production of anti-citrullinated protein antibodies (ACPAs) implicated in its pathogenesis [2].

RA is primarily treated with nonsteroidal anti-inflammatory drugs, corticosteroids, disease-modifying anti-rheumatic drugs (DMARDs), and biological response modifiers [3] Synthetic drugs used in this have high treatment costs and many side effects along with drug resistance which can be overcome by the use of traditional medicine. Natural products and supplements can reduce the pain and morbidity associated with RA.

Ginger (*Zingiberaceae*), Bears antiarthritic[4], antiplatelet[5], antitumor[6], antioxidant[7], anti-inflammatory[8], antiviral, anti-diabetic[9], and anti-emetic properties[10]. Modern research has emphasized the ability of gingerols, that inhibit cyclooxygenase (COX) and potentially treat inflammatory conditions like rheumatoid arthritis [11] ginger is a traditional medicinal plant, that provides polyphenolic compounds, such as 6-gingerol, 8-gingerol, 10-ginger gallic acid, chlorogenic acid, and caffeic acid [12]. The compound 6-gingerol (5-hydroxy-1(4-hydroxy-3-methoxyphenyl l)-decan-3-one) is the most abundant constituent, which has been reported to possess various pharmacological and physiological effects including anti-inflammatory activity [13].

Boswellia serrata (*Burseraceae*) is widely used in traditional medicine due to its anti-oxidative, anti-inflammatory, and anti-arthritic properties. Boswellic acids (BAs) are triterpenes present in the oleo gum resins of Boswellia species. Among them, β-boswellic acid and 3-acetyl (11-keto-beta-boswellic acid) have received significant pharmacological interest in inhibiting pro-inflammatory cytokines [14,15].

In addition, the presence of cineole in invasomal formulation (*Eucalyptol*) in invasomes preparation and camphor oil **(***Cinnamomum camphora***)** and menthol in final gel formulation will also be effective in reducing inflammation and joint pain and improve the overall bioavailability of these herbal ingredients. These natural remedies may help RA patients live better overall, rely less on allopathy drugs, and experience fewer adverse effects [15].

To improve the permeation and bioavailability of these herbal ingredients, invasomes were introduced as vesicular systems containing phospholipids, ethanol, and terpenes. Compared to liposomes, invasomes offer additional advantages due to the presence of terpenes, which disrupt the tight bilayers and lipid packing in the stratum corneum (SC), promoting drug permeation through intercellular lipids. Ethanol disturbs the ordered multilamellar lipid domain, enhances the SC's fluidity, and provides the vesicles with enough flexibility to deeply permeate skin layers, thereby increasing the permeation of active ingredients. The capability to entrap multiple drugs combined with high skin permeability makes these vesicles an excellent option for developing effective skin formulations [16,17].

To target the diseased area, The transdermal administration will be more effective, Transdermal delivery systems offer distinctive benefits, including targeted drug delivery, non-invasiveness, steady and prolonged drug release, low toxicity, and an ability to surmount hepatic metabolism, all because this route uses the skin as a portal for medicine absorption [18,19].

The study involves the preparation of invasomes using the ethanol injection method and optimization through a 3^3 Box-Behnken design to analyze the impact of independent variables on the formulation. The optimized invasomes will be incorporated into gels and evaluated for characteristic parameters. Additionally, the polyherbal-loaded invasomal gel will be assessed through *in vitro* diffusion and release kinetics studies, *in vitro* anti-arthritic studies, and stability studies under different storage conditions.

MATERIALS & METHODS

Materials

Boswellia serrata was received as a gift sample from Biomed Pharmaceuticals Pvt. Ltd, (Goa, India), Phosphatidylcholine 90G was obtained as a gift sample from Lipidome Lifesciences (Ahmedabad, India), The ginger rhizome was purchased from the local market of Belagavi. Polyethylene glycol 400, Ethanol, Chloroform, and Xanthan gum were acquired from SD Fine Chemicals (Mumbai, India), Cineole and camphor oil were purchased from Naturals (Malur, Karnataka). Triethanolamine and chlorocresol were procured from Fischer Scientific (Mumbai, India).

Extraction of 6-gingerol

The ginger rhizomes were cleaned and dried under the shade. Dry ginger was crushed to a coarse powder and passed through sieve No. 60. From the fine ginger powder, 6 gingerol was extracted with optimum methanol concentration by a simple maceration process for 3 continuous days. The prepared extract solution was kept water bath for 30 min and filtered using Whatman filter paper. The solvent was evaporated using a rotary evaporator to obtain a thick pasty mass. These samples were dried and used as crude extracts of 6-gingerol for further formulation [20,21].

Preparation of invasomes

Polyherbal loaded invasomal nanoparticles were formulated using an ethanol injection method with slight modifications [22]. Briefly, Phospholipon 90G (Lipid) Gingerol extract (200mg), Boswellia serrata (50mg), and Cineole (Terpene) were dissolved in ethanol to produce the organic phase and bath sonicated for 10 minutes to ensure proper solubilization of all ingredients. The organic phase was then added to the aqueous phase in a stream flow (2 ml/min) with continuous stirring at 1200 rpm for 30 minutes using a magnetic stirrer. The invasomes were formed spontaneously, turning the resulting hydroalcoholic solution slightly turbid. These were then subjected to 5 minutes of ultrasonication for size reduction. The final invasomal dispersion was stored in a refrigerated condition till further evaluation.

Optimization of invasomes using box-behnken design (BBD)

Primary screening studies were done using different terpenes and different ratios of lipid to identify the Critical Quality Attributes (CQAs). After the determination of desirable parameters, a three-factor Box-Behnken design (BBD) was employed using design

expert software (version 13.0). BBD is applied to ascertain the impact of lipid concentration (A_1), cineole concentration (A_2), and ethanol concentration (A_3) on response variables vesicle size (Y_1) and Zeta potential (Y_2). Table 1 summarizes the design matrix containing the selected factors at low (-1), medium (0), and high (+1) levels accounting total of 15 experimental runs with triplicate runs of center points. Various formulations were formulated based on the suggested concentrations of independent variables, as indicated in Table 1.

Table 1: Variables and their levels for optimization

Independent factor	**Low level (-1)**	**Medium level (0)**	**Highest level (+1)**
A_1 = PL 90 G (%w/w)	0.5	1	1.5
A_2 = Cineole (%w/v)	0.5	1	1.5
A_3 = Ethanol (%v/v)	20	30	40
Dependent factor	**Desired outcome**		
A_1 = Vesicle size (nm)	Minimum		
A_2 = Zeta Potential (mV)	In range (-25 mV to -35 mV)		

Characterization Invasomes

Vesicle size, polydispersity index (PDI), and zeta potential.

The Malvern zeta sizer (ZSU3100) was utilized to ascertain the particle size, polydispersity index, and zeta potential of the invasomes. Samples were diluted with Mill-Q water type I (1 ml in 10 ml). The measurements were conducted at a constant angle of 90° and a temperature of 25 ± 2°C [23].

Entrapment efficiency

The Indirect method using ultracentrifugation was employed to assess the entrapment efficiency of invasomes [24,25]. The samples were stored overnight, and then 2 ml of each formulation was taken into Eppendorf tubes and subjected to ultracentrifugation at 4°C at 15,000 rpm for 10 minutes. The supernatant containing free herbal drugs was separated, diluted with a solvent (2:10 methanol : water), and analyzed using a UV spectrophotometer (Shimadzu-1900, Japan) at two different wavelengths 244nm and 279nm. The % entrapment efficiency was calculated using the following formula,

$$\% \text{ Entrapment efficiency} = \frac{\text{Total drug content} - \text{Unentrapped drug}}{\text{Total drug content}} \times 100$$

Optimized invasomal surface morphology analysis

Transmission electron microscopy (Tecnai G2 Spirit Biotwin) was used to analyze the morphology of the prepared invasomes. A 0.5 µL sample was taken and diluted with 4 ml distilled water. Pipette out 0.2 µL diluted sample and coated on cu-corban grid (200 mesh). Then, it was negatively stained with a 2% PTA (Phosphotungustic Acid) solution and dried at room temperature. These samples were observed at 100 kV [26].

Formulation of polyherbal loaded invasomal gel:

To achieve prolonged precorneal residence for extended drug release, the optimized polyherbal invasomal dispersion (contains 1%w/w gingerol 6 and 0.25%w/w boswellia serrata) was incorporated into a gel by simple cold technique along with the addition of camphor oil and menthol to enhance permeation and to increase the retention time of formulation on the skin for a longer period. Both menthol (a non-opioid pain reliever) and camphor oil have anti-inflammatory and analgesic properties that assist in lessening inflammation and joint pains. The gel was prepared by adding a defined amount of different gelling polymers (Table 2) in distilled water and left overnight to swell. Later, polyethylene glycol 400 (plasticizer) and chlorocresol (preservative) were added, then optimized polyherbal invasomes were added dropwise to attain a homogenous gel mixture. The gel was then neutralized by the dropwise addition of triethanolamine with continuous mixing, to get the final homogeneous transparent gel base [27,28].

Table 2: Formulation composition of polyherbal invasomal gel

Ingredients (%)	ING 1	ING 2	ING 3	ING 4
Carbopol 934 (% w/w)	1.5%	-	-	-
Xanthan gum (% w/w)	-	2%	-	-
HPMC K4M (% w/w)	-	-	3%	4%
Camphor oil	2% v/v	2% v/v	2% v/v	2% v/v
Menthol	0.5% w/w	0.5% w/w	0.5% w/w	0.5% w/w
Triethanolamine (%v/w)	q.s.	q.s.	q.s.	q.s.
PEG 400 (%w/w)	5%	5%	5%	5%
Chlorocresol (%v/w)	0.1%	0.1%	0.1%	0.1%
Distilled Water	Up to 100 %	Up to 100 %	Up to 100 %	Up to 100 %

Evaluation of polyherbal invasomal Gel

pH measurement

The pH of the gel formulations was measured by taking 0.5 g of gel in 20 mL distilled water and for proper mixing, the formulation was stirred on a magnetic stirrer for 30 minutes at room temperature. The pH sensor probe electrode was then immersed into the dispersed gel, and the pH of the formulation was noted as displayed on a digital monitor [29].

Determination of viscosity:

The viscosity of the topical gel is significant because it impacts the ease of administration and the gel's capacity to stay on the skin, ensuring proper delivery of active chemicals. The viscosity of the optimized invasomal gel formulation was estimated using a Brookfield viscometer. After keeping the 1 g of the gel sample on the viscometer base and allowed to settle for 5 minutes. After that, spindle number 1 revolved at 100 revolutions per minute at a temperature of 25 ± 2°C to analyze viscosity [30].

Estimation of spreadability

The spreadability of the topical gel was evaluated using the petri dish method. Initially, 1 g of the gel was placed at the center of a petri dish, and its diameter was recorded. A second petri dish was carefully placed on top, followed by the application of a standardized weight (100g). The assembly was left undisturbed for 5 minutes, after which the weight and the upper petri dish were removed. The final diameter of the spread gel was measured. The spreadability was calculated using standard formula [31].

Determination of drug Content

The drug concentration of the gel formulation was measured by diluting 1 g of gel to 2 mL methanol and volume was made up to 100 mL with distilled water. The solution was stirred for 2 h using a magnetic stirrer and examined by UV spectrophotometer at 244 nm and 279nm. Drug content is calculated using the following formula, [32]

$$\text{Drug Content} = \frac{\text{Actual drug content in vesicles}}{\text{Theoretical drug content in vesicles}} \times 100$$

Screening and optimization of polyherbal invasomal gel

Among the formulations, the gel with excellent homogeneity, acceptable viscosity, ideal spreadability, compatible pH, and complete drug content is considered as an optimized gel base and utilized for further study.

***In vitro* drug diffusion study**

The *in vitro* drug diffusion comparative study of a polyherbal loaded optimized gel and a plain gel was examined using the Franz diffusion cell. A dialysis membrane, preactivated by soaking the membrane in 0.1M phosphate buffer pH 6.8 for 12 hours was inserted between the two chambers of the Franz diffusion cell. The receptor chamber was filled with 12ml of the 0.1M phosphate buffer pH 6.8 as the diffusion medium. Accurately weighed gel formulations (1 g), each equivalent to 10mg gingerol and 2.5mg boswellia serrata, were placed in the donor compartment. The receptor media was maintained at 37 ± 2 °C with continuous stirring at 100 rpm. Samples of 0.5 ml were withdrawn at certain intervals (0.5, 1, 2, 3, 4, 6, 8, and 24 hours) and replaced with fresh buffer medium. Drug release quantification was carried out using a UV spectrophotometer at two different absorbances 279 nm

and 244nm. The % drug release was calculated and a graph of % drug release against time was plotted, release studies were performed in triplicate for each formulation. [33]

***In vitro* anti-arthritic activity**

The protein denaturation method was employed to assess the *in vitro* antiarthritic activity of polyherbal invasomal optimized gel, plain gel, and diclofenac sodium gel. The mechanism involves alteration in disulfide bonding and electrostatic hydrogen during denaturation. The 0.5 ml of reaction mixture was prepared by taking a 5% aqueous solution of Bovine serum albumin (4.5 ml) and formulation (0.5 g) and then pH was adjusted to 6.3 by adding 0.1 N HCl. Further, samples were incubated at 37 ºC for 20 min and then heated for 30 min at 60 ºC. Then after cooling the sample, PBS of pH 6.8 (2.5 ml) was added to each sample. Similarly, a control solution was prepared, and using a UV spectrophotometer, turbidity was measured at 600 nm. The percentage inhibition was determined using the formula: [34]

$$\text{Percentage inhibition} = \frac{100 - (\text{Absorbance of test} - \text{Absorbance of control})}{\text{Absorbance of control}} \times 100$$

***Ex vivo* permeation study using goat skin**

Preparation of a goatskin

Fresh goatskin was obtained from a local slaughter house. After thorough washing, the hair on the skin was removed using an electronic trimmer, and the skin was separated from the underlying cartilage and subcutaneous fat using a scalpel and cut into appropriate sizes. The skin was washed with isopropyl alcohol and stored in a buffer solution at 4 °C. [35].

***Ex vivo* permeation study**

The full-thickness goatskin was used for the *ex-vivo* permeation experiment using a Franz diffusion cell. The skin samples were inserted into the Franz diffusion cell with dermal skin tissue facing toward the receiver compartment with an effective penetration area of 1.76 cm^2. The receiver chamber is filled with 12 mL of Phosphate buffer pH 6.8. Then 1 g of the polyherbal gel formulation corresponding to 10mg gingerol and 2.5mg boswellia serrata was placed in the donor compartment. The receptor media was maintained at 37 ± 2 °C with continuous stirring at 100 rpm. Samples of 0.5 ml were withdrawn at certain intervals (0.5, 1, 2, 3, 4, 6, 8, and 24 hours) and replaced with fresh buffer medium. The cumulative drug permeated was determined by using a UV spectrophotometer, analyzed at two different absorbances 279 nm and 244nm. The cumulate drug diffused against the time graph was plotted [36].

Drug Deposition Study

The flux study is essential for determining the amount of drug deposited in the skin layers, providing critical insights into the efficacy and penetration of the topical formulation. To carry out the drug deposition study, goat skin mounted on the Franz diffusion cell was carefully removed after 24 hours and washed with phosphate buffer (pH 6.8) the chopped skin pieces were bath-sonicated for 10 minutes in methanol to prepare the tissue suspension for 10 minutes then centrifuged at 10,000 rpm and 4°C, they. After being filtered through a 0.45 µm syringe filter, the supernatant was further diluted (1:10) and subjected to UV spectrophotometer analysis at 279 nm and 244nm [37].

Stability studies

Stability studies were conducted for polyherbal-loaded invasomes and invasomal gel at two distinct temperatures 4±2°C and 25±2°C with relative humidity of 60±5%, as per ICH Q recommendations. Polyherbal loaded optimized invasomal vehicle's particle size and zeta potential were analyzed and the optimized invasomal gel's pH and viscosity were evaluated [38].

RESULTS

Preformulation study

Preformulation studies were carried out on active ingredients 6-gingerol and boswellia serrate. Both drugs showed maximum solubility in methanol and the melting point was found to be 26.95 ºC and 288.32 ºC respectively. UV estimation of 6-gingerol and Boswellia serrata was carried out by taking methanol: water (2:10) combination of solvents as a mobile phase, 6-gingerol showed the maximum absorbance at 279nm with a linearity range of 10-100 µg/ml. Boswellia serrata showed maximum absorbance at 244nm with a linearity range of 5-60 µg/ml.

Formulation of Poly-herbal loaded invasomes

The invasomes were formulated using the ethanol injection method with Box-Behnken optimization. A total of 15 formulations with 3 center point formulations were prepared with varying concentrations of independent variables as suggested by the Design Expert software. The experimental designs for the preparation of poly-herbal loaded invasomes are given in Table 3.

Table 3: Results of the responses from experimental Box-Behnken optimization of polyherbal loaded invasomes

Formulation	Independent variable			Dependent variable	
	A_1	A_2	A_3	Y_1	Y_2
1	1	1.5	40	120.2 ± 0.8	- 63.5 ± 0.07
2	1.5	1	40	255.9 ± 0.6	- 53.5 ± 0.18
3	1.5	0.5	30	259.8 ± 3.2	- 42.94 ± 0.5
4	1	0.5	20	170.7 ± 2.6	- 48.56 ± 0.09
5	0.5	1	20	210.3 ± 1.4	- 36.29 ± 0.6
6	0.5	0.5	30	195.5 ± 0.8	- 19.04 ± 0.04
7	1.5	1	20	271.2 ± 0.9	- 50.57 ± 0.21
8	1	0.5	40	180.4 ± 0.7	- 29.73 ± 0.65
9	1	1	30	203.8 ± 3.8	- 34.82 ± 0.68
10	1	1.5	20	175.6 ± 1.2	- 52.84 ± 0.21
11	0.5	1.5	30	146.9 ± 4.3	- 35.27 ± 0.87
12	1.5	1.5	30	236.9 ± 2.8	- 55.77 ± 1.24
13	0.5	1	40	186.5 ± 2.6	- 23.77 ± 1.8
14	1	1	30	191.9 ± 3.4	- 33.37 ± 0.04
15	1	1	30	184.7 ± 2.8	- 30.21 ± 1.02

Quadratic model	R^2	Adjusted R^2	Predicted R^2	S.D.	% CV
Response (Y_1)	0.9880	0.9665	0.9119	7.70	3.86
Response (Y_2)	0.9908	0.9744	0.9186	2.07	5.10

Values are expressed as mean ± standard deviation.

Optimization using Box-Behnken design

Optimization of invasomes was done using Design Expert Software (Version 13). The BBD was used to optimize the invasomal formulation. The responses were subsequently subjected to statistical analysis through response surface analysis employing ANOVA. By examining the 3D surface plot and polynomial equation, the impact of independent variables on dependent responses was explored. The quadratic model proved to be the most suitable fit for analyzing vesicle size and zeta potential. The fit statistics results are given in Table 3 showing satisfactory R^2, adjusted R^2, predicted R^2, S.D., and % C.V.

Effect of independent variables on response Y_1 vesicle size

Vesicle size is a significant characteristic, influencing the efficacy and stability of the formulation. This investigation focused on examining the impact of various independent factors on vesicle size, including PL90G, Terpene, and ethanol. The analysis revealed that all these factors significantly influenced vesicle size, with a *p-value* of 0.003.

The vesicle sizes of all 15 formulations ranged from 120.2 nm to 271.2 nm. The polynomial equation of the quadratic model describes the vesicle size as:

Vesicle size = 193.47 + 35.75 *A -15.85 *B -10.60 *C + 6.63 *AB +2.13 *AC -16.28 *BC

The quadratic model was selected based on the highest predicted and adjusted R^2 compared to the other models. The model F-value of 45.89 and P-value of less than 0.05 indicate that the model is statistically significant. The difference between adjusted R^2 and predicted R^2 from the fit statistics data is less than 0.2. The P-value for lack of fit is 0.731, which is insignificant. An insignificant lack of fit is beneficial to the model.

As depicted in the graphs (Figure 1(A_1&A_2)). By increasing the lipid concentration, vesicle size increased. It was discovered that as the concentration of soya phosphatidylcholine 90 was increased from 0.5 to 1.5%, the vesicle size was also increased from 210.3 ± 0.42 nm (F5) to 271.2 ± 0.52 nm (F7). Similar results were seen with F11 (146.9 ± 0.25 nm) and F12 (236.9 ± 0.91 nm). This could be because of more bilayer formation ultimately leading to the formation of multilamellar vesicles occurs [36].

By increasing the concentration of terpene particle size was decreased. When the concentration of cineole was increased from 0.5 to 1.5 %, the vesicle size of invasomes was reduced from 259.8 ± 0.76 nm (F3) to 146.9 (F11). This behavior is likely due to the adsorption of surfactants on the vesicle surface, which prevents the growth of invasomal vesicles and reduces surface-free energy, thereby minimizing agglomeration [39].

Ethanol also has a negative effect on vesicular size; as its concentration increases, vesicle size decreases. The vesicle size gets reduced from 175.6 ± 0.52 nm to 120.2 ± 1.31nm, 259.8 ± 0.42 nm to 146.9 ± 2.09 nm as observed in formulations 10 & 1, 3 & 11 respectively. The ethanol could be due to the disruption of the bilayer structure of the cellular membrane beyond their certain concentration Ethanol acts as a cosurfactant, reducing interfacial tension and thus decreasing particle size. It also causes the formation of smaller vesicles by modifying the net charge of the system, providing some degree of steric stabilization, which ultimately leads to a reduction in vesicle size.

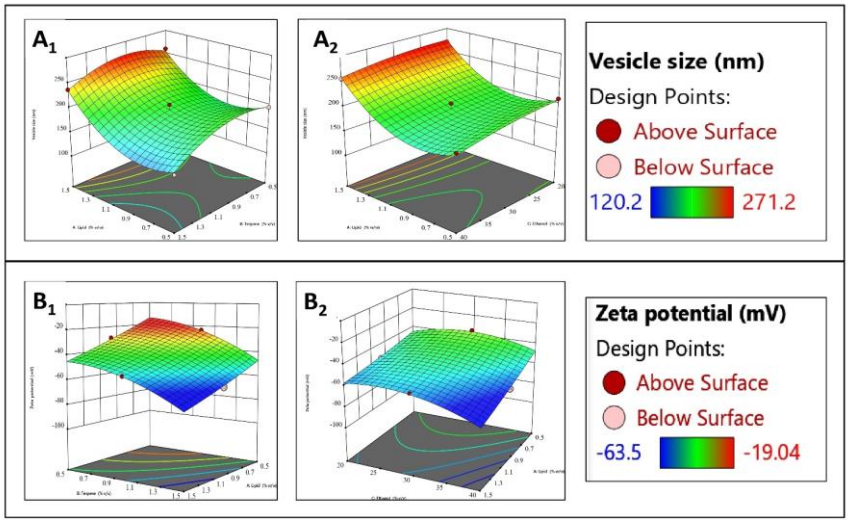

Figure 1: 3D surface plot exhibiting the influence of lipid, terpene, and ethanol concentrations on A_1&A_2) Vesicle size B_1&B_2) Zeta potential

Effect of independent variables on response Y_2 Zeta Potential

Determining the zeta potential (ZP) of polyherbal-loaded invasomes is crucial for evaluating their stability. As the repulsive charges within the formulation increase, its stability improves. All independent variables with a *p-value* <0.0001 significantly impacted the ZP of invasomes. A considerable change in zeta potential was associated with higher concentrations of cineole, ethanol, and lipid, indicating that all three independent factors mixed influence on ZP (Figure 1(B_1&B_2)). The results of the zeta potential analysis revealed that all the prepared invasomes had negatively charged zeta potential, ranging from -19.04 to -63.5 mV. The polynomial equation for zeta potential is as follows;

Zeta Potential = -32.80 - 11.05 * A - 8.39 * B +2.22 * C +0.85 * AB -3.86 * AC -7.37 * BC

Lipid concentration showed a positive impact on zeta potential as the concentration of lipid increases in formulations F6 and F12 the zeta potential increased from -19.04 to -55.77 mV. The terpene concentrations showed a positive impact on the zeta potential of invasomes because of their negative charges. This would reduce the aggregation of invasomal vesicles and hence enhance the stability of these nanocarriers. Ethanol concentration showed a minor impact on the overall charge as it is a nonionic surfactant.

The findings showed that all formulated invasomes exhibited a negative zeta potential. Negatively charged vesicles demonstrate superior skin permeation compared to positively charged vesicles.

Percentage drug entrapment efficiency evaluation

The entrapment efficiency of prepared formulations was found to be 42 to 89 % for 6-gingerol and 40-78 % for boswellia serrata. As a vesicle size increases the entrapment efficiency also increases. As compared to the boswellia serrata, the 6-gingerol showed a higher encapsulation rate because of its solubilization property. The % entrapment efficiency is illustrated in Figure 2.

Journal of Current Pharma Research (JCPR)
Volume 20, Issue 9, September 2024 **jcpr.humanjournals.com** ISSN: 2230-7842, 2230-7834

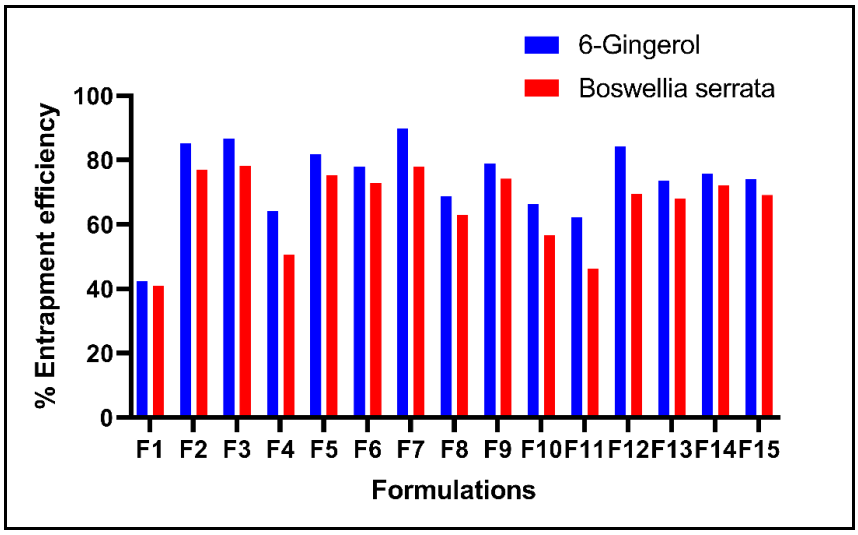

Figure 2: Entrapment efficiency (%) of 6-gingerol and boswellia serrata in invasomal formulation

Optimized polyherbal loaded Invasomes preparation.

In this study, the objective was to minimize particle size and obtain zeta potential within a standard range. Out of 70 given solutions, the best desirability showed solution was chosen as an optimized formulation. Figure 3 displays the design space (overlay plot), with the shaded yellow region indicating the range where the specified requirements are met. Based on the above experimental results, an optimized formulation was prepared with 0.601 %w/w of phospholipid 90G, 1.20 %w/w of cineole, and 38.72 % v/v of ethanol as per the formula generated by the point prediction method. The polyherbal loaded invasomes exhibited a vesicle size of 167.2 nm (Figure 4(A)) with PDI 0.4766, entrapment efficiency of 6-gingerol is 81.23% and boswellia serrata is 74%, and zeta potential of -35.27 mV (Figure 4(B)) which were close to the predicted values generated by the Box-Behnken design.

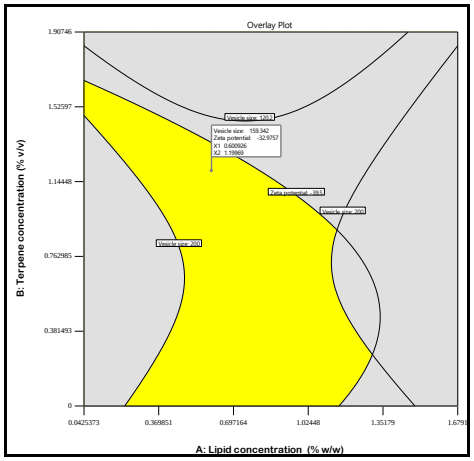

Figure 3: Overlay plot of optimized polyherbal loaded invasomal formulation

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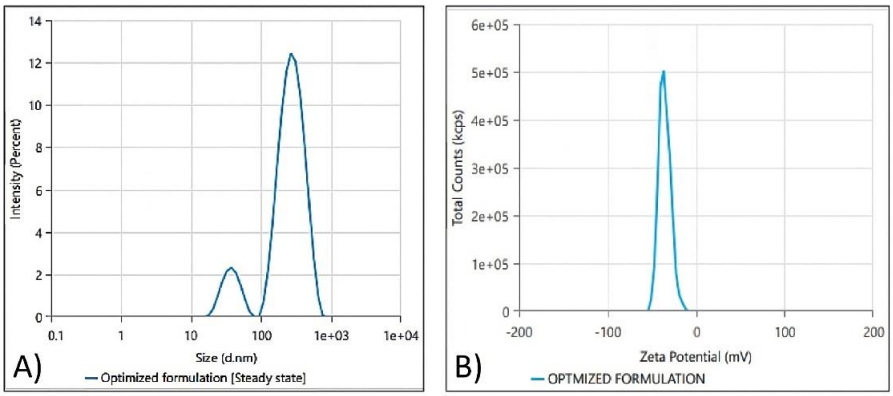


Figure 4: Optimized invasomes characterization A) Vesicle size B) Zeta potential

Surface morphological study

The invasomes were observed under a magnification of 200nm. The transmission electron microscopy (TEM) examination of the polyherbal loaded optimized invasomes revealed that the prepared vesicles were spherical with a well-defined sealed structure and uniform size distribution (Figure 5).

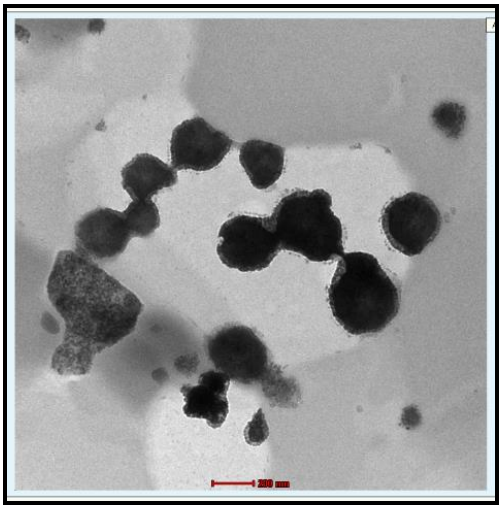


Figure 5: Transmission electron microscopic images of optimized invasomal formulation

Characterization of invasomal gels

The gel was prepared using varying concentrations of Carbopol 934, HPMC K4M, and Xanthan gum. These formulations are characterized by their pH, viscosity, spreadability, and drug content. The results are detailed in Table 4.

The pH of the gel formulations ranged from 6.01 to 7.12, ensuring compatibility with skin pH. The formulation pH similar to skin pH will minimize the possible skin irritation. The viscosity measurements varied between 744 cP to 1128 cP, indicating a consistent and desirable thickness for topical application. Spreadability values were found to be between 9.83 and 15.13 $g \cdot cm^2/s$, demonstrating ease of application. Additionally, the drug content ranged from 92.35% to 98.71%, confirming efficient drug loading and uniform distribution within the gel matrix. Among the various gels tested, the gel formulated with natural xanthan gum (ING 2) was identified as the optimal formulation. Consequently, further comparative evaluation studies were conducted using this optimized gel.

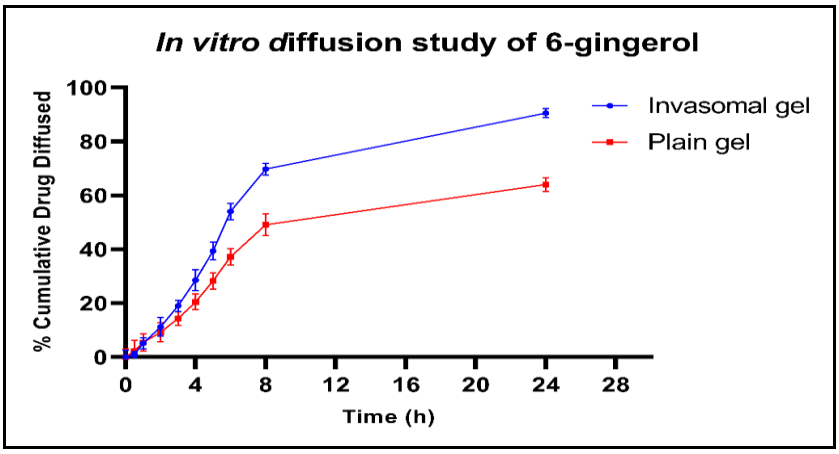
Table 4: Characterization results of invasomal gels

EVALUATION PARAMETERS	ING 1 (Carbopol 1.5 %)	ING 2 (Xanthan 2%)	ING 3 (HPMC 3%)	ING 4 (HPMC 4%)
Colour	Pale white	white	white	white
pH	7.12 ± 0.02	6.77 ± 0.02	6.51 ± 0.03	6.01 ± 0.01
Viscosity (cP)	744 ± 19	971 ± 26	956 ± 12	1128 ± 16
Spreadability ($g.cm^2/s$)	10.38 ± 0.35	15.13 ± 0.47	9.83 ± 0.56	11.51 ± 1.05
Drug Content (%)	95.42 ± 0.29	98.71 ± 0.09	92.35 ± 0.58	95.78 0.5

***In vitro* drug diffusion study**

A comparative *in vitro* drug diffusion study between polyherbal loaded optimized invasomal gel and plain gel was carried out using the Franz diffusion cell. At each time point, a significant drug release was achieved. The invasomal gel demonstrated sustained release with a higher diffusion rate of approximately 90.55% of gingerol and 84.57% of boswellia serrata, surpassing that of the plain- gel which showed only 64.01 % of gingerol and 44.26 % of boswellia serrata for 24 hours. Figure 6(A) shows *in vitro* diffusion of 6-gingerol and Figure 6(B) shows *in vitro* diffusion of boswellia serrata. This release profile of the polyherbal invasomal gel demonstrated a controlled and sustained release, making it more suitable for transdermal drug delivery.

A)



B)

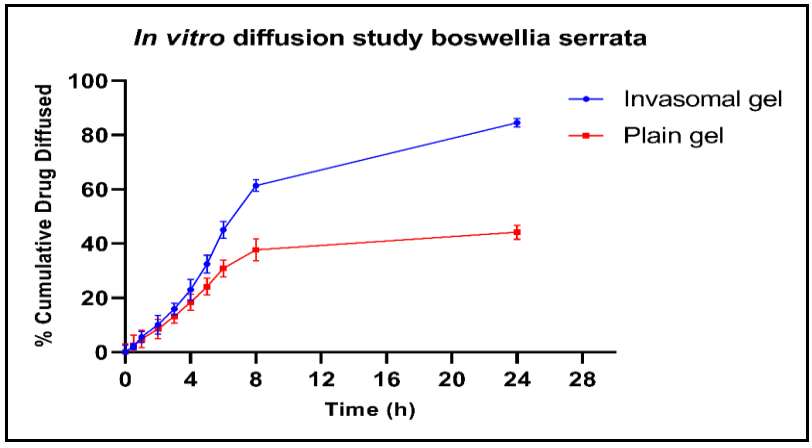
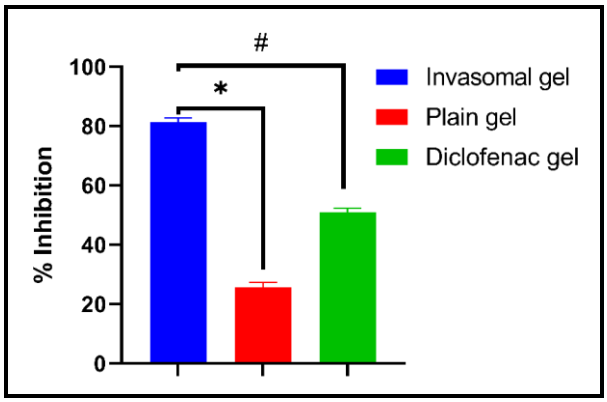


Figure 6. *In vitro* drug diffusion comparative study of A) 6-Gingerol B) Boswellia serrata

***In vitro* antiarthritic activity**

Protein denaturation is a critical factor in the progression of RA. as it leads to the production of autoantigens that the immune system mistakenly targets, resulting in chronic inflammation and joint damage. Rheumatoid arthritis can result in protein denaturation which results in autoantigens production. *In vitro* antiarthritic activity of polyherbal invasomal gel was compared with plain gel and diclofenac sodium gel as a standard gel. The *In vitro* antiarthritic activity of polyherbal invasomal gel had a

percent inhibition of inflammation of 81.29 ± 1.38%, significantly higher than the ± 1.29% observed with the standard diclofenac sodium gel and the 25.6 ± 1.75% with the plain gel (Figure 7). Polyherbal invasomal gels superior inhibition of protein denaturation (81.29 %) makes it a more effective option for the treatment of rheumatoid arthritis compared to both the standard diclofenac sodium gel and the plain gel.



*&# indicates significance among compared groups with *p-value* <0.005

Figure 7: Protein inhibition (%) of Invasomal gel comparison with plain gel and standard diclofenac gel.

***Ex vivo* permeation study**

An *ex vivo* study of the polyherbal loaded invasomal gel reveals the permeation of drugs through the skin layer Figure 8 illustrates the percentage of drug permeation through goat skin. The study revealed the permeation of 83.46% of gingerol and 76.45% of Boswellia serrata at 24 hours. The superior permeation of these polyherbs from invasomal gel formulation can be attributed to the presence of terpene and permeation enhancers in formulation, which imparts flexibility to the vesicles, allowing them to deform and pass through the narrow obstructions in subcutaneous tissue and thereby increases bioavailability.

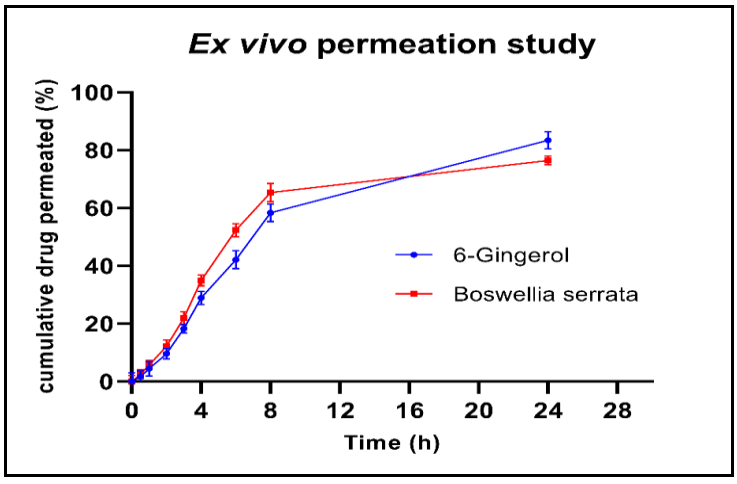
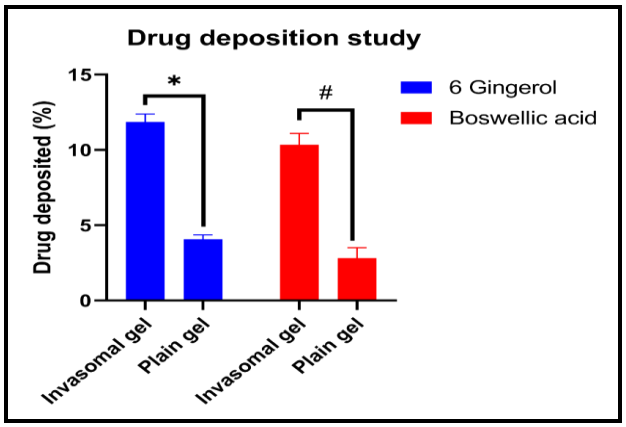


Figure 8: *Ex vivo* drug permeation study of 6-Gingerol and Boswellia serrata.

Drug deposition study

After 24 hours, the goat skin was removed from the Franz diffusion cell, and the drug deposited in skin layers was separated using the centrifugation technique and analyzed using UV spectroscopy. The % Skin deposition is illustrated in Figure 9. The deposition of 6-gingerol was 11.86% which is 2.91 fold higher than the normal gel which was found to be 4.07%. Boswellia serrata also showed similar findings as gingerol, the % skin deposition of boswellia serrata was found to be 10.35% which was 3.58 folds greater than the deposition from the plain gel formulation (2.82%) Therefore, it can be inferred that the developed invasomal gel exhibits enhanced skin deposition due to the presence of ethanol, terpene, and lipids, which collectively facilitate drug absorption into deeper skin layers and deposition on the skin.

Journal of Current Pharma Research (JCPR)
Volume 20, Issue 9, September 2024 **jcpr.humanjournals.com** ISSN: 2230-7842, 2230-7834



*&# indicates significance among compared groups with *p-value* <0.005

Figure 9: Drug deposition comparison between invasomal gel and plain gel formulations

Stability study

The formulation was stored in glass vials in a humidity control oven at 25±2°C/60±5% RH and refrigerated at 4±2°C respectively. The stability study results are tabulated in Table 5. Poly-herbal loaded invasomes showed better stability at refrigerated conditions for 30 days, but in case of room temperature, these nanoparticles showed an increase in their vesicle size, and a drastic decrease in zeta potential indicates its instability.

The stability parameters of the polyherbal loaded invasomal gel were measured at the beginning (Day 0) and at 30 and 90 days. At room temperature, the pH showed a slight decrease over 90 days, while under refrigerated conditions, the pH remained more consistent, indicating better pH stability when refrigerated. The viscosity demonstrated stability over time, with a slight decrease at room temperature and insignificant changes under refrigeration, suggesting good viscosity retention under both conditions with slightly better stability when refrigerated.

Table 5: Stability studies under different storage conditions

EVALUATION PARAMETERS	**BEFORE**	**ROOM TEMPERATURE (25°C ± 2°C)**		**REFRIGERATED CONDITION (4°C ± 2°C)**	
	Day 0	**30 days**	**90 days**	**30 days**	**90 days**
Invasomes					
Particle size (nm)	167 ± 3.4	183 ± 2.6	231 ± 4.2	165 ± 1.8	158 ± 1.9
Zeta potential (mV)	-35.2 ± 0.6	-30.6 ± 0.9	-21 ± 1.8	-33.5 ± 0.3	-32.2 ± 0.8
Invasomal gel					
Ph	6.77 ± 0.02	6.68 ± 0.04	6.45 ± 1.56	6.74 ± 0.11	6.65 ± 0.08
Viscosity (cP)	971 ± 26	956 ± 14	930 ± 17	966 ± 8	951 ± 16

DISCUSSIONS:

This research focused on developing an optimized invasomal gel formulation by incorporating ginger and boswellia serrata extracts for enhanced rheumatoid arthritis (RA) therapy. The study employed a Box-Behnken design to optimize the invasomes, which demonstrated significant improvements in vesicle size, zeta potential, and entrapment efficiency.

The results of this study demonstrate that the polyherbal-loaded invasomal gel significantly enhances the bioavailability and therapeutic efficacy of ginger and Boswellia serrata extracts for rheumatoid arthritis therapy. These findings align with previous research that has shown the potential of herbal formulations in managing inflammatory conditions.

In comparison to traditional delivery systems, the invasomal gel formulation in our study offered superior skin permeation and drug deposition, likely due to the presence of ethanol and terpenes, which enhance the skin's permeability. The optimized invasomal gel showed a vesicle size of 167.2 nm with PDI 0.4766, and a zeta potential of -35.27 mV indicating stable and well-dispersed nanoparticles. The high entrapment efficiency of 81.23% of gingerol and 74% of boswellia serrata, suggests the

effective incorporation of the active herbal ingredients into nanovesicles. These findings are consistent with those of prior studies, such as the work by Wang et al. (2018), which highlighted the role of terpenes in increasing transdermal drug delivery through enhanced disruption of the stratum corneum [1]. Transmission electron microscopy confirmed the spherical morphology of the invasomes, supporting their potential for improved skin permeation.

Physicochemical evaluation of the gel formulation revealed a suitable pH of 6.77, high viscosity of 971 cP, and excellent spreadability, all of which are crucial for topical application. The in vitro drug diffusion studies demonstrated a sustained release of the active ingredients over 24 hours, which is beneficial for prolonged therapeutic effects. Furthermore, in vitro anti-arthritic studies showed significant inhibition of protein denaturation, underscoring the formulation's potential in alleviating RA symptoms.

Furthermore, the *ex vivo* results indicate that the invasomal gel formulation provides a controlled and sustained release of the active ingredients. The sustained release observed in our formulation is advantageous for maintaining consistent therapeutic levels of the active compounds over extended periods, as suggested by previous research in the field. Stability studies confirmed that the formulation remains stable under various storage conditions, particularly when refrigerated, ensuring a longer shelf life.

Despite the promising outcomes of the polyherbal-loaded invasomal gel for enhanced rheumatoid arthritis therapy, this study has certain limitations. The research may not fully replicate the complexities of real-world clinical scenarios. Additionally, the sample size used for ex vivo studies was relatively small, which could limit the generalizability of the findings. The long-term stability and efficacy of the formulation under varying environmental conditions were not assessed, and further studies are required to evaluate these aspects. Moreover, while the ex vivo permeation study using goat skin provided valuable insights into skin absorption, it may not fully mimic human skin's response. Future studies should include in vivo assessments in animal models and clinical trials to validate the therapeutic potential and safety of the formulation in humans. Additionally, exploring the pharmacokinetics and bioavailability in a living system would provide a more comprehensive understanding of the formulation's performance.

Overall, the polyherbal-loaded invasomal gel presents a promising alternative to conventional RA therapies, offering improved bioavailability and therapeutic efficacy with reduced side effects. This novel formulation leverages the benefits of natural anti-inflammatory agents and advanced drug delivery systems, potentially enhancing patient outcomes in RA management.

CONCLUSION:

The study successfully formulated polyherbal-loaded invasomes by ethanol injection method, optimized using Box Behnken design, and characterized a polyherbal-loaded invasomal gel for different parameters for enhanced rheumatoid arthritis therapy. The optimized invasomal gel demonstrated significant improvements in both *in vitro* and *ex vivo* drug permeation and skin deposition, which is because of the incorporation of terpenes and permeation enhancers. Stability studies revealed that the formulation maintained its integrity better under refrigerated conditions compared to room temperature. Overall, the invasomal gel showed promising potential for effective delivery of polyherbal extracts, offering a viable alternative for rheumatoid arthritis treatment with enhanced bioavailability and stability.

LIST OF ABBREVIATIONS:

INV: Invasomes, RA: Rheumatoid arthritis, SC: Stratum corneum, ING: Invasomal gel, PBS: Phosphate buffer solution, EE: entrapment efficiency.

ACKNOWLEDGEMENTS

The authors would like to express their sincere gratitude to the Department of Pharmaceutics, KLE College of Pharmacy (KLE Academy of Higher Education and Research), Belagavi for providing the facilities necessary to conduct this research. We extend our appreciation to Dr. Prabhakar Kore Basic Science Research Center (BSRC) Belagavi for their instrumental role in facilitating the research.

DECLARATIONS:

Ethics approval and consent to participate: Not applicable. No studies involving human participants, or no human data were done in this research.

Consent to publication: All authors consent to the publication of this research material.

Availability of data and material: Authors declare to produce the data and material on demand/request.

Competing interests: Authors do not have any conflict of interest.

Funding: This research work was funded by KAHER's Research and Development cell, Belagavi. Ref. No. KAHER/RD/23-24?D.29092316

Authors' contributions: All the authors equally contributed to experimental works and manuscript preparation. All authors have read and approved the manuscript.

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How to cite this article:
Panchaxari M. Dandagi et al. Jcpr.Human, 2024; Vol. 20 (9): 1-15

Conflict of Interest Statement: All authors have nothing else to disclose.