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Dr. Pankaj Sharma

Preliminary investigation of the effects of topical gel of *Cardiospermum halicacabum* and *Ricinus communis L.* leaves extract for the treatment of arthritis in animal model

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Abstract

The aim of this research is to develop and assess the anti-arthritic properties of a topically herbal gel including leaf extracts from *Cardiospermum halicacabum* and *Ricinus communis L.* in rats. Utilizing gelling agents carbopol 940 (2.5, 5, 7.5 g), nine herbal gel compositions were created. They were then assessed for physical appearance, spreadability, viscosity, net content, pH, extrudability, in vitro diffusion profile, and main skin irritant tests. According to ICH recommendations, the stability research for the topical herbal gel composition was completed, and Freund's Complete Adjuvant (FCA) induced arthritis technique was used to assess the anti-arthritic efficacy. Additional procedures included measuring the body weight, paw volume, biochemical and haematological variables, histological analysis, and in vitro serum biomarker detection. The prepared gels followed the instructions and were uniform and stable. F5 performed better than the other compositions in terms of release kinetics (97.20%). The gel proved safe and non-toxic since no erythema nor edema was seen during the skin irritation test. Comparing the herbal gel F5 comprising carbopol 940 to rats with arthritis, topical treatment showed considerable ($p<0.001$) anti-arthritic effect. The anti-arthritic action of the gel formulations was confirmed by decreased paw volume, absence of agglutination in reacting protein and rheumatic factor, a decline in TNFα level, restoration to baseline biochemical and haematological characteristics, decrease in thymus and spleen weight, and histopathological study.

Keywords: *Cardiospermum halicacabum, Ricinus communis*, Topical gel**,** Drug release, Paw volume**,** Haematological

Introduction

An auto immune condition called arthritis affects 0.51–0.6% of people globally. Steroid, non-steroidal anti-inflammatory, disease-modifying, anti-rheumatic, and immunosuppressive medications are frequently used for rheumatoid arthritis. These medications have the potential to cause gastrointestinal issues, immunodeficiency, and humoral changes, among other adverse effects. An alternative method of treating arthritis is becoming more widely acknowledged: the Siddha and Ayurvedic systems of medicine. The two kinds of plants that are most frequently used in conventional medicine to treat arthritis are *Ricinus communis L.* and *Cardiospermum halicacabum.*

Chinese medicine has historically utilized *Cardiospermum halicacabum* (CH) (Sapindaceae) to treat rheumatism, inflammation, and other conditions [1]. In RAW264.7 cells, the anti-inflammatory properties of CH's ethanol extract have been shown to suppress LPS-induced production of COX-2, TNF-α, and iNOS [2]. The results from laboratory pharmacological investigations have demonstrated the following: anti-pyretic activity toward yeast-induced pyrexia in rats [3], anti-oxidant activity [4], anti-malarial activity [5], anti-ulcer activity against ethanol-induced gastric ulcer in rats [6] and suppression of TNF-α and the generation of nitric oxide in mononuclear cells of human peripheral blood [7]. Several chemicals have been determined to be present in CH, including luteolin-7-O-glucuronide, apigenin, chrysoeriol-7-O-glucuronide, and arachidic acid [8].

Ricinus communis L. (RC) (Euphorbiaceae) is used for treating edema, gout, and skin issues [9]. Polyphenols and flavonoids, the plant's primary components, have anti-inflammatory and antioxidant qualities [9, 10].

CH and RC were used to make an external gel in an effort to look into the scientific backing for the usage of these types of plants for the treatment of arthritis. The anti-arthritic efficacy of the external gel was tested because there is a lack of information on CH and RC's preclinical study on this topic. Applying gel directly to the affected area, ensuring it causes no discomfort or irritation during use, having no first pass effect, not deteriorating the gastrointestinal tract, and being easy to use make it a practical option to supply CH and RC. For the greatest part, traditional medicine treats arthritis with the leaves of the plant, despite the fact that CH and RC are utilized medicinally in all sections of the plant [11]. To make a topical gel composition, extracts from the leaves of both of the plants were used.

Material and Methods

Polymer carbopol 940 (gelling agent) was obtained from Loba Chemie Pvt. Ltd. Mumbai. diclofenac sodium, triethanolamine (stabilizer), propylene glycol (moisturizing agent) and disodium edetate (for maintain consistency such as pH, odour, texture etc.) were purchased from Himedia Laboratories Pvt. Ltd., Delhi, India.

Preparation of the extracts

Dry leaves of *Cardiospermum halicacabum* were collected from the village of Ganj Rampur in Morena, Madhya Pradesh, India. A plant taxonomist at the Agra Agriculture College herbarium in Agra, Uttar Pradesh, India, recognized and verified the leaves. Using a mechanical grinder (Sujata, 900, India), the leaves that had dried were roughly pulverized. The powder was kept for later use in a container that was airtight after being run using sieve No. 40. After being macerated in a mixture of methanol and water (80:20) and shaking for a whole day, the leaves that had been powdered were passed through using Whatman filter paper No. 1. Three times the maceration procedure was carried out. The screened extraction (herbal extract ratio: 19%) had been concentrated in a rotating evaporator (Singla Scientific Glass Industries, Vadodara, Gujrat, India) at 40 °C. Water was then removed using a freeze dryer (Bioline Technologies, Thane, India). Until it was utilized, the dry extract was kept in a cold area.

Fresh leaves of *Ricinus communis* were obtained from hamlet Ganj Rampur, Morena, Madhya Pradesh, India were recognized and validated and deposited comparable to *Cardiospermum halicacabum*. The leaves were then ground into powder using a machine grinder after being dried in the shade. As with CH (the herbal extract ratio was 22%), the leaves that were powdered and had been macerated in water and shaken for 24 hours. We administered the herbal extracts at identical quantities as stated in the sources [12–14], yet we weren't comparing the effects of adding other extract dosages.

Animals

In the animal house of ShriRam College of Pharmacy, Banmore, Morena, India, Wistar strain rats (12-week old, healthy) weighing 150-200 g of either sex were chosen for the anti-arthritic assessment. Acute toxicity studies were conducted on female albino mice weighing 20–30 g, while primary skin irritation tests were conducted on albino rabbits with an average weight of 2.2 kg. The temperature (23±2), humidity (50±5), and light and dark cycles (10–14 hours) were carefully regulated in their housing. With hygienic rice husk bedding and unlimited access to food and water, each animal was kept in a separate polypropylene cage.

Designing and carrying out the tests in compliance with ethical guidelines authorized by the Institutional Animal Ethical Committee (SRCP/M.Pharma/IAEC/87/22-23) and the Committee for the Purpose of Control and Supervision on tests on Animals (CPSCEA).

Preparation of gel base

To prevent agglomeration, carbopol 940 was dissolved gradually and stirred in 60 mL of distilled water for one hour. After that, 10 mL of distilled water was used for dissolving triethanolamine and disodium edetate, and the mixture was agitated for ten minutes then stirred for ten minutes while adding 5 g of propylene glycol to 11 mL of water that had been distilled. After adding triethanolamine solution and sodium edetate to the carbopol solution, the mixture was stirred for ten minutes to bring the pH down to 7.4. After stirring for ten minutes, the propylene glycol solution was added, and the mixture became transparent and uniform with a gel basis [15].

Optimization of primaquine loaded transdermal patch by 3^2 factorial designs

The purpose of the 3^2 factorial design (3^2- 2 variables at three levels) was to select the stages of different independent variables (Table 1) of H_{mix} ratio (R_1) and Carbopol 940 concentration (R_2) with drug release (Y_1), viscosity (Y_2), and spreadability (Y_3) [16].

Table 1: Actual unit with coded labels (3^2 factorial designs)

Factors	Level used		
	Level -1 (Low)	**Level 0 (Medium)**	**Level 1 (High)**
Independent variables			
$R_1 = H_{mix}$ (CH and RC ratio)	1:1	1:2	2:1
R_2 = Carbopol 940 (g)	2.5	5	7.5
Dependent variables			
Y_1 = *In-vitro* drug release			
Y_2 = Viscosity			
Y_3 = Spreadability			

Preparation of gel formulation

Nine topical gel formulations were prepared using different H_{mix} (methanolic leaf extract of *Cardiospermum halicacabum and Ricinus communis L.)* ratios: 1:1 (CH 3g and RC 3g), 1:2 (CH 2g and RC 4g), and 2:1 (CH 4g and RC 2g). All nine formulations F1-F9 were prepared using the gelling agent carbopol 940 at different levels or quantities (2.5, 5, 7.5 g) [15]. All

other excipients were used as per manual described in Table 2. Details of formulation compositions are recorded in Table 2.

Table 2: Composition of a topical gel according to factorial design

Formulation code	Components (g)					
	H_{mix} (R_1) (6 g)	Carbopol 940 (R_2)	Triethanol-amine	Disodium EDTA	Propylene Glycol	Distilled water (100 g)
F1	1:1 (-1)	2.5 (-1)	2	0.01	5	q.s
F2	1:1 (-1)	5 (0)	2	0.01	5	q.s
F3	1:1 (-1)	7.5 (1)	2	0.01	5	q.s
F4	1:2 (0)	2.5 (-1)	2	0.01	5	q.s
F5	1:2 (0)	5 (0)	2	0.01	5	q.s
F6	1:2 (0)	7.5 (1)	2	0.01	5	q.s
F7	2:1 (1)	2.5 (-1)	2	0.01	5	q.s
F8	2:1 (1)	5 (0)	2	0.01	5	q.s
F9	2:1 (1)	7.5 (1)	2	0.01	5	q.s

Characterization of CH and RC loaded topical gel

Estimating the net quantity of active ingredients in gel formulation

The active ingredients in the methanol were dissolved by thoroughly shaking every formulation (2 g) in a 100 mL volumetric flask filled to the brim with methanol. Following the solution's filtering using Whatman filter paper, 0.2 mL of the filtrate was taken out and adjusted with methanol to total 20 mL. The UV spectro photometer was used to determine the concentration of active components by plotting the λ_{max} of the extracts at 268 nm on a standard curve [17].

pH evaluation

By fully submerging the electrode made of glass into the gel solution and completely covering it, a digital pH tester was used to test the gel's pH. The evaluation was made three times, and the mean of the triplicates measurements was noted [18].

Extrudability

A crimping device was used to stop any rollback when a closed, collapsible tube with around 25 g of gel was forcibly squeezed at the crimped end. The gel was extruded once the cap was taken off. Weighing was done on the quantity of extruded gel that was recovered. It was estimated what proportion of the extruded gel was [19].

Viscosity

A viscometer (Brookfield Ametek, DVE, USA) was used to test the viscosities of gel specimens. In a viscometer, a two-number spindle was employed, dipped in gel specimen, and revolved at 5, 10, and 20 rpm at 25˚C [12]. The viscometer recorded measurements at each speed. Mean values were computed when the specimens were carried out three times [15].

Spreadability

There were taken two sets of standard-sized glass slides. One of the slides was covered by the herbal gel mixture. A space of 10 centimeters was taken up along the slides by the gel between the opposite slide and the gel itself, which was positioned on atop of the gel. On the upper slides, a hundred grams of gel mass was applied, and the gel was evenly compressed to create a thin layer between the two slides. After the load was taken off, the extra gel that was sticking to the slides was scraped off. The two slides in place were securely fastened to a stand so that only the upper slides could come loose from the weight that was fastened to them. Carefully, a 25 g weight was fastened to the upper slide. It was recorded how long it took the top slide, bearing the weight of the lower slide, to move 10 cm and detach from it. Three iterations of the study were conducted, and the average time was calculated [15].

The following formula was used to determine spreadability:

$$S = \frac{m \bullet l}{t}$$

where, S= spreadability, m-mass tied to upper slides (25 g), l- length of the glass slide (10 cm), t- time taken in sec.

In-vitro drug release studies

Franz diffusion cells were used to conduct in vitro drug release tests for each composition. The apparatus for the diffusion cell was made locally and consisted of an open-ended, cylindrical tube of 3.8 cm2 in size and 100 mm in elevation that had a diffusion surface of 4 cm2. As receptor medium, phosphate buffer (pH 7.4) was employed. Dialysis membranes were made from the abdomen skin of rats. The stratum corneum adjacent to the skin was in close proximity to the formulation's releasing area on the donor cell due to the skin's attachment to the diffusion cell. A donating chamber was filled with 100 mL of isotonic phosphate buffer solution (pH 7.4) before it was put on the diffusion cell. A weighed amount of composition, equal to two grams of gel, was applied to the rat's skin and submerged just a little in 100 milliliters of receptor media while being constantly agitated. The system's overall

temperature was kept at 37±1 ºC. At predetermined intervals for up to 10 hours, an aliquot of 5 mL was extracted, and its spectrophotometric value was determined at 268 nm. The diffusion media was changed out with a new one of the same amount following subsequent withdrawal. Every time period (in hours) was computed to determine the cumulative percent release [20].

Release kinetics

The collected data was mapped to many mathematical models in order to determine the release behavior of the active ingredient from herbal gel. First order kinetics, which involves drug release by either diffusion or swelling and subsidence, relies on quantity, whereas zero order kinetics is concentration independent. To determine the response, data were verified using Higuchi, Korsmeyer-Peppas, and Hixon-Crowell's model. Then the values of the correlation coefficient were calculated from the linearity of curves [21].

Stability studies of optimized formulation

The primary aim of the stability analysis is to furnish proof about the temporal variations in the medication product's quality due to moisture and temperature fluctuations. Using a stability chamber for six months, the topical herbal gel formulation's stability research was conducted in accordance with ICH criteria. The optimized topical herbal gel preparations was added into a floor-standing model, three units in one, in a particular moisture and temperature system, 15–60°C, Patel Scientific Instruments Private Limited, India) at three different temperatures: 25°C ± 2°C/60% RH ± 5% RH, 32°C ± 2°C/60% RH ± 5% RH, and 40°C ± 2°C/75% RH ± 5% RH. Specimens were taken at the start of the first, second, third, and sixth months, and measurements were made of their odor, color, consistency, viscosity, pH, net content, microbiological load, and sterility test findings [22, 23].

Anti-arthritic activity

FCA (Freund's complete adjuvant) induced arthritis model [24] in rats was used to examine the effectiveness of the topical herbal gel composition. A total of four groups, each with six rats, were created from the rats. Using gel base as a standard control, Group 1 was administered topically. Using a 0.1 mL (0.1% w/v) solution of dead Mycobacterium TB bacteria (Jiwaji University, Gwalior) homogenized in liquid paraffin, arthritis was produced in rats in groups 2 through 4. In terms of arthritis control, group 2 was regarded. For a period of 21 days, groups 2 through 4 that received FCA were permitted to experience arthritis. Utilizing an electronic Vernier caliper (Labworld Vernier Caliper, India), the body mass and

rat paw volume of both treatment and control groups were taken on the fourth, eighth, fourteenth, and twenty-first day of the study.

Following the confirmation of the onset of arthritis, Group 3 (the standard) and Group 4 used topically for 21 to 42 days the diclofenac sodium gel (Voveran gel, bought from a local medical store) and the herbal gel composition F4.

A digital Vernier caliper (Labworld Vernier Caliper, India) was used to measure the rat volume of paws of both the treatment and control groups, as well as the total body mass of the rats on days 21, 25, 29, 35, and 42 of the course of treatment. The animals' visual scores on the pain test were documented at the conclusion of the 42nd day [25].

Biochemical estimation

A part of the blood specimens were centrifuged for 10 minutes at a speed of 10,000 rpm in order to separate the serum, and the resulting serum was tested by autoanalyser and an authorized kit (the Sigma-Aldrich assay kit),for several biochemical markers, including total protein, globulin, albumin, creatinine, uric acid, urea, triglycerides, VLDL levels, SGPT, SGOT, and ALP [26].

Serum and haematological parameters estimation

Ketamine (20 mg/kg, i.p.) was used to anesthetize the animals that had fasted for the entire night. Blood specimens were then drawn via the retro-orbital sinus and centrifuged for 10 min at 10,000 rpm to determine hematological characteristics, such as the amount of red blood cells, white blood cells, erythrocyte sedimentation rate (ESR), hemoglobin (Hb), and using standard laboratory procedures [27]. A combination of pre-made kits (Omega diagnostics Limited, Scotland, UK), ELISA reagents (ELISA Kit, Gen-Probe, France), and serum biomarkers (CRP, RF, TNFα, IL-1ß, and IL6) were used to measure the levels of uric acid, urea, and serum biomarkers in the divided serum.

Histopathological investigations

The organs of the animals—the thymus, the spleen, and the ankle joint's bone joints—were separated from the surface fat before the animals were slain by cervical dislocation. Using Cal-Ex^{TM} Decalcifying solutions CSS10-1D (Fischer Scientific, India), the isolated ankle joint was submerged for nine days. Subsequently, the ankle joint was fixed in paraffin, divided utilizing a microtome in a serial fashion (6 μ), placed on slides for the microscope, and colored with Harris hemotoxylin and eosin [24, 28]. Digital pictures were obtained and histopathological alterations in the rats' joints were studied underneath a microscope.

Statistical analysis

Data gathered from assessed formulations were statistically analyzed using one-way analysis of variance (ANOVA) [29].

Skin sensitivity test

A healthy male New Zealand White rabbit weighing between two and three kilograms was subjected to a skin irritancy test. As per guideline 404 of the OECD (Organisation for Economic Co-operation and Development), the dwelling standards were upheld prior to the test. A skin sensitivity testing was conducted in accordance with OECD guideline 404. Before the experiment, the rabbit's left and right dorsal skin hairs were cut off without harming the skin, around 24 hours prior. Rabbits without braided skin were used for the skin sensitivity test. The left dorsal surface of the rabbits received conventional gel (diclofenac gel), while the right dorsal surface of the rabbits received produced gel administered to an area of around 4 cm^2. Skin response was assessed once daily at 1, 24, 48, 72 hours, 7 and 10 days (post-test observation period) for any erythema and edema at the location of the rabbit's left and right dorsal surfaces [30, 31].

Results and discussion

Among the various applied topically semisolid compositions, gel preparations is usually chosen due to its long skin residence time, substantial viscosity, hydrating effects on flaky skin because of its occlusive characteristics, greater bio adhesiveness, fewer irritations, simple use, and superior release characteristics [32]. Multiple investigations have demonstrated the anti-inflammatory and anti-arthritic properties of flavonoids found in plants, including luteolin and apigenin. Furthermore, luteolin and apigenin, two polyphenolic flavonoids, have been shown to enter human skin [33]. As a result, an external herbal gel composition combining these flavonoids was created to cure arthritis.

Formulation and evaluation of topical herbal gel

The gelling agent carbopol 940 was used at different levels or quantities (2.5, 5, 7.5 g) to create nine different gel formulations (F1 to F9) that were made using varying concentrations Hmix ratios: 1:1 (CH 3g and RC 3g), 1:2 (CH 2g and RC 4g), and 2:1 (CH 4g and RC 2g) because they are biodegradable, bioadhesive, biocompatible, irritation-free, and not absorbed into the body). The creation of the gel involved the utilization of every other component (Table 2). The manufactured gel formulations were evaluated for drug release, pH, spreadability, and other characteristics. They were also homogenous, well-looking, and consistent.

Estimating the net quantity of active ingredients in gel formulation

At doses ranging from 10–60 µg/ml, the drug's linear response was confirmed. After the absorbance versus concentration data was shown, a linear regression analysis was used to create the calibration curve. The calibration curve for the acquired active ingredient was determined to be linear in the amounts described above, with a correlation coefficient (R^2) of determination of 0.9928. The equation of the calibration curve was y = 0.0263x + 0.3012. This suggests that a rise in concentration value results in a rise in R^2.

Prepared gel characterization

Physical appearance, spreadability, pH, viscosity, extrudability, net content, and in vitro diffusion profile were assessed for gel formulations F1 to F9 made with carbopol polymers. The study's findings were within acceptable bounds of the ICH guidelines, and the specifics are listed in Table 3.

Table 3: Data for various evaluated parameters of prepared gel

Formulation	pH	Spreadability g.cm/sec	Viscosity (mPa·S)	Net content % w/w	Extrud-ability	Physical appearance
F1	5.6±0.12	4.79 ± 0.10	14764.0±0.44	99.7±0.21	Good	Transparent, uniform, smooth, and dark green
F2	5.6±0.11	4.87 ± 0.11	14677.3±0.40	101.1±0.30	Good	Transparent, uniform, smooth, and dark green
F3	5.5±0.13	4.35 ± 0.12	14800.1±0.50	100.7±0.32	Good	Transparent, uniform, smooth, and dark green
F4	5.5±0.11	4.98 ± 0.10	14633.5±0.44	99.9±0.22	Good	Transparent, uniform, smooth, and dark green
F5	5.5±0.10	5.10 ± 0.11	14671.9±0.43	100.1±0.20	Good	Transparent, uniform, smooth, and dark green
F6	5.5±0.13	4.35 ± 0.12	14821.0±0.42	101.9±0.33	Good	Transparent, uniform, smooth, and dark green
F7	5.6±0.11	5.00 ± 0.10	14555.8±0.40	99.4±0.25	Good	Transparent, uniform, smooth, and dark green
F8	5.5±0.10	4.99 ± 0.11	14500.6±0.43	99.1±0.23	Good	Transparent, uniform, smooth, and dark green
F9	5.4±0.13	4.60 ± 0.11	14815.3±0.40	99.6±0.27	Good	Transparent, uniform, smooth, and dark green

The already made gels were discovered to be uniform, well-appearing, and consistent. The skin irritation investigation supports the pH values of all compositions being within the narrow range of neutral pH (5.4-5.6), meaning that they did not cause any skin irritation. In order to achieve maintaining a medication level within the therapeutically useful spectrum, polymers have been incorporated into the topical formulations that were devised. Differences in viscosities were noted across all gel compositions when the polymer content was adjusted

to 2.5, 5, and 7.5 g. Additionally, Sharma et al. (2020) [15] revealed that the optimal viscosity value for topical gel formulations made with carbopol polymers was found to be between 14500.6 and 14800 mPa.s.

Scores of the spreadability suggested that the gel compositions are readily spreadable. Over ninety percent (good) of the contents of gel compositions F1 to F9 could be extruded, suggesting that they had good extrudability.

In vitro diffusion profile and release kinetics

Figure 1 shows the in vitro diffusion profile of the F1 to F9 compositions. Phosphate buffer saline pH 7.4 was utilized for the in vitro release experiments of the gel formulations since the pH of the membrane employed was within the range of 5 to 7.8. Within six hours, about 97% of the nine formulations created with carbopol 940 showed release characteristics in vitro.

The developed topical herbal gel formulations showed promising in vitro release properties that matched commercially available gel. Out of all the formulations, F5 exhibited superior release characteristics (97.20%) in comparison to the remaining preparations (Figure 1).

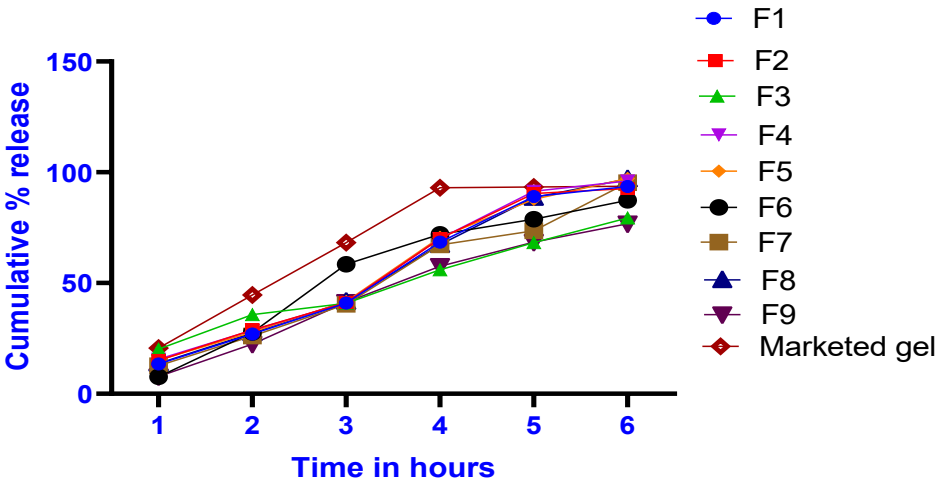


Figure 1: Topical herbal gels (F1-F9) versus commercial gels' in-vitro diffusion profiles

We found that the F1, F2, F4, F5, F7, F8, and F9 formulations adhered to zero order and F3 and F6 showed first order and Higuchi's kinetics based on our kinetic release investigation (Table 4). For in vivo experiments, gel formulation incorporating Hmix (F5) was used since zero order kinetics is desired for prolonged release. The F5 composition, which contains 1:2 ratio of CH to RC, released nearly 97% of its active ingredients for up to 6 hours, compared to the commercial diclofenac sodium gel formulation, which released almost 93.10% of its content in 4 hours (Figure 1). This formulation is ideal for sustained release and increased compliance among patients. Gel formulation F5 therefore demonstrated zero order release

kinetics based on the release data acquired using various mathematical models (Table 4). For in-vivo research, the gel formulation F5 containing was chosen as zero order kinetics followed regulated release.

Table 4: *In vitro* release kinetic study of topical herbal gel formulated with Carbopol 940

Formulation	Zero order	First order	Higuchi's model	Hixon Crowell's model	Korsmeyer Peppas's model	Best fitted model
F1	0.9812	0.9186	0.9329	0.9278	0.9481	**Zero order**
F2	0.9640	0.9231	0.9292	0.9184	0.9010	**Zero order**
F3	0.9563	0.9600	0.9476	0.9345	0.9289	**First order**
F4	0.9972	0.9813	0.9312	0.9576	0.9323	**Zero order**
F5	0.9981	0.9734	0.9489	0.9421	0.9400	**Zero order**
F6	0.9697	0.9354	0.9740	0.9500	0.9198	**Higuchi's model**
F7	0.9801	0.9700	0.9500	0.9167	0.9360	**Zero order**
F8	0.943	0.9744	0.9371	0.9051	0.9467	**Zero order**
F9	0.9678	0.9615	0.9390	0.9584	0.9612	**Zero order**

Fitting of data to the model

Using response surface methods and Design-Expert software, all nine formulations of the generated gel's responses were observed and fitted to different models. The response surface technique revealed that the best-fitted model (Figures- 2a, and 2b) exhibited linearity and p-value was <0.0001 (significant).

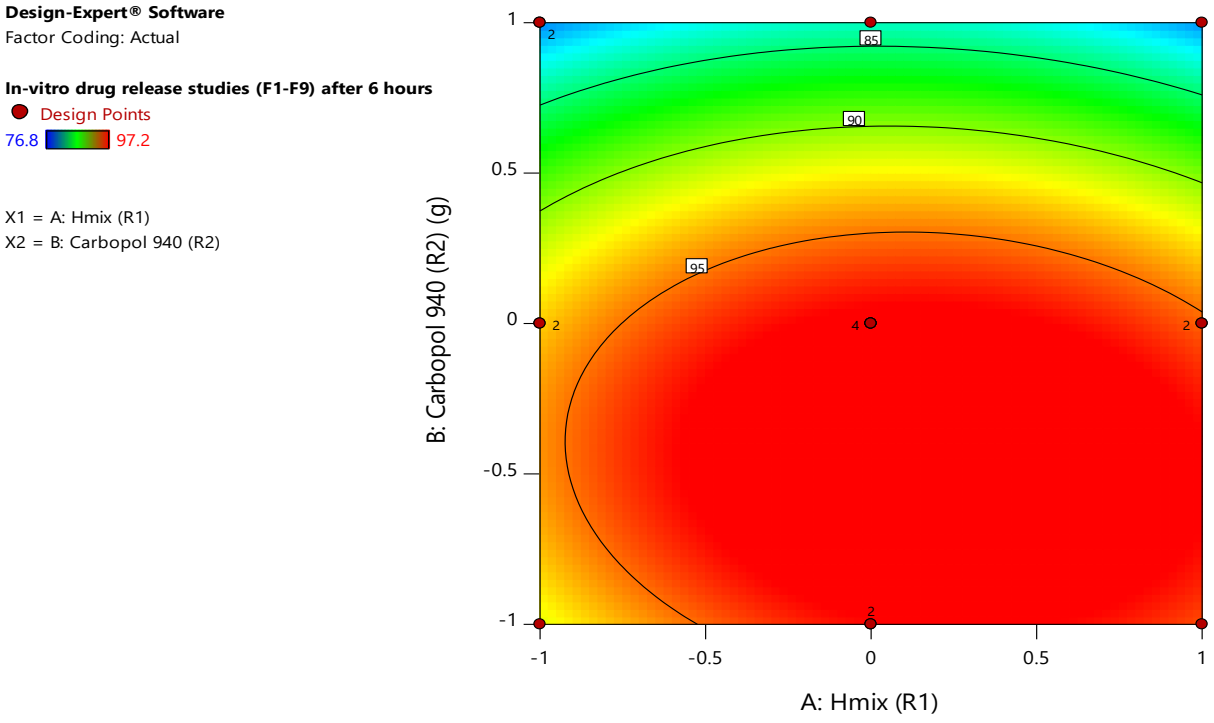


Figure 2a: Contour plot of % drug release

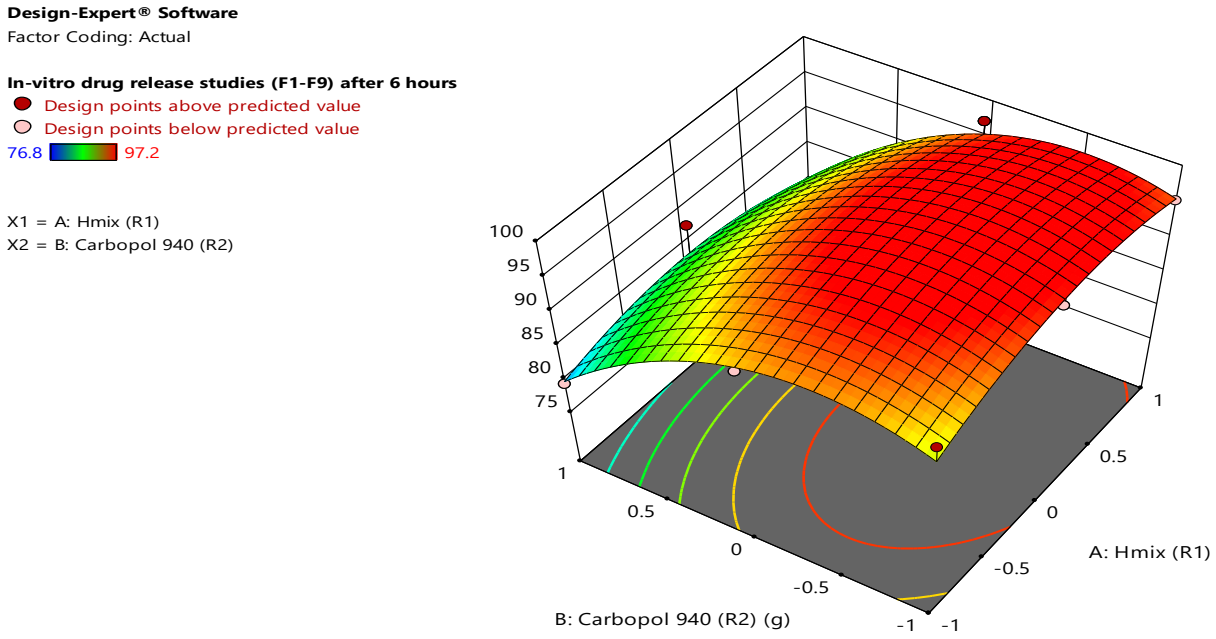


Figure 2b: Response surface plot of % drug release

Skin irritation test

Table 5: Scoring for skin irritation test

Time of film detachment	Edema	Erythema
Acute singled skin irritation test		
1 hour after application	0	0
24 hours after application	0	0
48 hours after application	0	0
72 hour after application	0	0
7 days after application	0	0
10 days after application	0	0
Acute repeated skin irritation test		
1 hour after application	0	0
24 hours after application	0	0
48 hours after application	0	0
72 hour after application	0	0
7 days after application	0	0
10 days after application	0	0

After a 10-day testing period, no erythema or edema was seen in any of the composition (Table 5), suggesting that the created herbal gel formulation (F5) was deemed safe based on its evaluation for skin irritating impact.

Stability testing

In order to ensure the quality, safety and efficacy throughout the shelf life, stability study was performed as per ICH guidelines for F5 formulation (prepared using No change in color, odour, homogeneity, pH, viscosity and net content of the topical herbal gel formulation was observed for this formulation after 0,1,2,3 and 6 months (short term) of stability testing. Results of the study clearly revealed that the formulated topical gel F5 is found to be stable (Table 6).

Acute oral toxicity study

Our extensive investigation into the acute and sub-chronic toxicity of these plants, which we have previously published, showed that the CH and RC extracts were safe at doses up to 2000 mg/kg [34, 35].

Anti-arthritic activity

Body weight

After causing arthritis in the rats, a mean increase or decrease in body mass was noted for each group (Table 7). Comparing the arthritic control group to the normal group of rats, the marketed gel and topical herbal gel composition F5 treated groups showed gains in body weight, while the arthritic control group showed a reduction in body weight.

Paw volume

On the 21st, 25th, 29th, 35th, and 42nd days following topical administration of the commercial gel and the herbal gel composition (F5), alterations in rat paw volume were noted (Table 8). The paw volume increased in the arthritic control groups, indicating the onset of arthritis. On day 21, following FCA induction, a significant ($p<0.01$) decrease in rat paw volume was noted in the groups treated with commercialized gel and the topical herbal gel composition F5 treated groups.

According to Laird et al. (2001) [25] visually arthritic grading systems were used to determine the extent of arthritis. The pain related to FCA-induced arthritis was greatly reduced in the groups treated with market-driven gel and topical herbal gel composition F5, according to Table 9's assignment of arthritic test scores. When comparing the treated group of rats to the arthritic control group, notable changes were seen in the flexion pain test rating, movement score, and stance rating. The topical herbal gel composition F5's anti-arthritic properties are supported by the change in arthritic test scores.

Table 6: Optimized topical herbal gel formulation (F5)

Parameters	Storage conditions														
	25°C±2°C / 60%RH ± 5% RH					32°C±2°C / 60% RH ± 5% RH					40°C±2°C / 75%RH ± 5% RH				
	Time					Time					Time				
	0	1	2	3	6	0	1	2	3	6	0	1	2	3	6
Odour	No change in odour					No change in odour					No change in odour				
Color	No change in colour					No change in colour					No change in colour				
Consistency	Smooth					Smooth					Smooth				
pH	5.54	5.55	5.55	5.54	5.54	5.54	5.54	5.55	5.55	5.54	5.54	5.56	5.54	5.55	5.54
Viscosity (mPas.)	14671.9	14677.0	14676.3	14671.6	14671.8	14671.9	14672.8	14671.8	14670.7	14672.1	14671.9	14671.7	14671.9	14672.0	14670.8
Net content (%)	100.1	99.99	100	100.1	100.1	100.1	100.0	100.1	99.97	100.1	100.1	99.99	99.89	100.0	99.99
Microbial load (Fungi & Bacteria)	At 24, 48, and 72 hours, no microbial growth was seen					At 24, 48, and 72 hours, no microbial growth was seen					At 24, 48, and 72 hours, no microbial growth was seen				
Sterility test	At 24, 48, and 72 hours, no microbial growth was seen					At 24, 48, and 72 hours, no microbial growth was seen					At 24, 48, and 72 hours, no microbial growth was seen				

Table 7: Marketed gel and produced gel (F5): impact on body weight variations in rats with induced arthritis by FCA

Groups	Initial body weight (g)	Body wt after 21 days of FCA induction	Body wt after treatment 25th day	Body wt after treatment 29th day	Body wt after treatment 35th day	Body wt after treatment 42nd day	Weight gain (g)
Normal Control	140.6±0.56	159.3±1.59	161.1±1.78	164.60±1.50	170.00±1.00	175.89±1.15	~ 21
Arthritic control	139.5±0.78	137.20±0.59	136.30±0.86	135.34 ±1.00	133.78 ± 1.11	132.34±1.26	~ - 4
Marketed topical gel	140.0± .99	141.35±1.05	143.50±1.31	144.10±1.25	145.60±1.53	148.70±1.54	~ 4
Prepared gel (F5)	140.5±1.00	141.00±1.22	141.70±1.35	142.05±1.44	143.90±1.45	145.85±1.62	~ 2.5

*Data represented as mean ± SEM (n=6) in table

Table 8: Rats with FCA-induced arthritis were used to test the herbal gel formulation F5's anti-arthritic efficacy (Paw volume)

Groups	Rat paw volume (mm)					
	Initial	**21st days**	**25th days**	**29th days**	**35th days**	**42nd days**
Normal Control	4.70±0.15	5.10±0.22	5.05±0.19	5.25±0.10	5.55±0.15	5.72±0.20
Arthritic control	4.65±0.14	10.52±0.10[a]	10.25±0.20[a]	10.58±0.15[a]	10.70±0.20[a]	10.70±0.10[a]
Marketed topical gel	5.05±0.12[a]	10.10±0.13[a]	10.15±0.20[ns]	9.75±0.15[b]	8.88±0.21[c]	8.10±0.08[c]
Prepared gel (F5)	4.99±0.10[a]	10.35±0.12[a]	10.25±0.11[ns]	9.71±0.15[c]	9.10±0.12[c]	8.70±0.15[c]

*Data represented as mean ± SEM (n=6) in table [a]p<0.001 Normal control Vs Arthritic control; [b]p<0.05 Arthritic control Vs Treated groups; [c]p<0.001 Arthritic control Vs Treated groups (ns = not significant).

Table 9: Variations in different pain test ratings in rats with arthritis induced by FCA

Groups	Testing for pain		Score for mobility	Score for stance
	Extension	Flexion		
Arthritic control	8.5±0.35	8.25±0.30	1.35±0.20	1.45±0.20
Marketed topical gel	5.15±0.15[d]	4.65±0.19[d]	2.65±0.20[d]	2.35±0.20[d]
Prepared gel (F5)	4.64±0.20[d]	3.65±0.30[d]	3.17±0.22[d]	2.85±0.16[d]

*Data represented as mean ± SEM (n=6) in table and [d]p<0.001 Arthritic control Vs Treated groups

Table 10: Impact of formulation F5 on biochemical markers in rats with arthritic conditions caused by FCA

Groups	Urea (mg/dL)		Uric acid (mg /dL)	
	21st day	**42nd day**	**21st day**	**42nd day**
Normal control	15.00±0.50	16.50±0.60	2.80±0.10	2.95±0.06
Arthritic control	44.45±0.90[a]	46.45±0.95[a]	7.10±0.18[a]	7.50±0.15[a]
Marketed topical gel	45.25±0.8[a]	27.95±0.95[b]	6.75±0.55[a]	5.20±0.20[b]
Prepared gel (F5)	42.17±0.50[a]	34.80±0.55[b]	7.10±0.15[a]	5.30±0.15+

*Data represented as mean ± SEM (n=6) in table and [a]p<0.001 Normal control Vs Arthritic control; [b]p<0.001 Arthritic control Vs Treated groups

Biochemical parameters

In comparison to the arthritic control group, a significant ($p<0.01$) reduction in uric acid and urea concentration was noted in the marketed gel and herbal gel composition F5 treatment groups (Table 10).

Serum and haematological parameters estimation

Comparing rats treated with commercial gel and gel composition (F5) to arthritic control groups revealed a rise in Hb and RBC count, a reduction in WBC count, and an elevation in ESR (Table 11). Topical treatment of both commercial and herbal gels resulted in characteristic hematological abnormalities, including decreased WBC count and increased Hb. It has been suggested that lower erythropoietin levels, a weakened effect of erythropoietin in the bone marrow, and early red blood cell death because the fall in Hb count associated with arthritis. According to the current study, topical herbal gel and gel containing diclofenac sodium have a tendency to bring the WBC count back to normal.

Agglutination was seen in the serum of the examined animal groups prior to medication, however it was not shown in the serum of the groups receiving topical herbal gel F5 composition and commercial topical gel.

Since agglutination offers better sensitivity and straightforwardness, auto antibodies, also known as "Rheumatoid Factor," are the most important prognostic indicator for the diagnosis of rheumatoid arthritis. When examined animal groups were given topical herbal gel F5 composition and commercial topical gel, there was no sign of agglutination in their serum.

The TNFα level in rats given an arthritic stimulus was 42.00 pg/mL. It was discovered that the TNFα level was lower in the group treated with commercialized topical gel and topically herbal gel composition F5 than in the rats with arthritic conditions. According to Table 12, this study showed that the TNFα levels were lowered in the topical herbal gel composition F5 and marketed topical gel treatment groups.

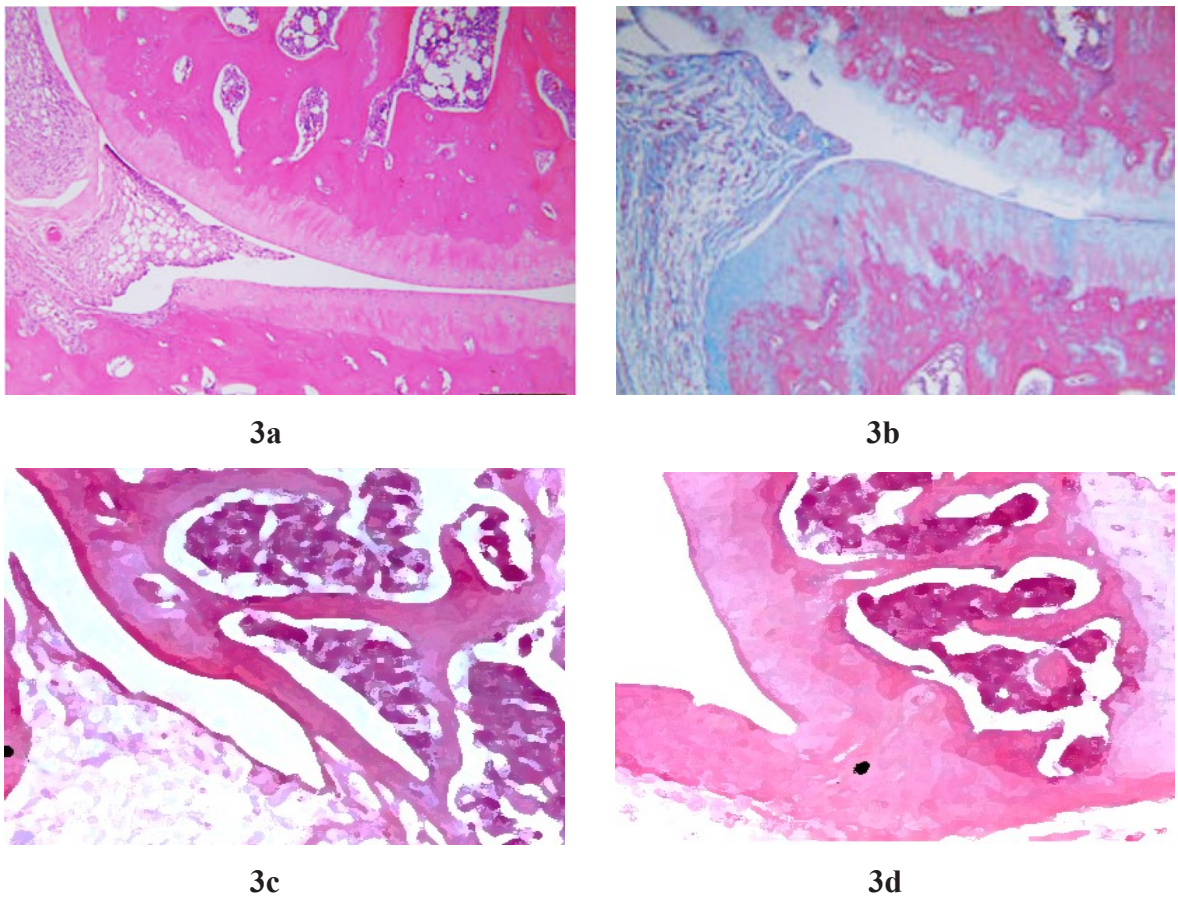
Table 12 shows that the cytokine levels in the arthritic control group were considerably ($p<0.01$) lower than those in the herbal gel composition (F5) and marketd gel treatment groups.

Histopathological investigations

After the 42nd day of rat sacrifice, the relative weights of the spleen and thymus were noted. Comparing the arthritic control group to the groups that received herbal gel composition F5 and marketing gel, a significant ($p<0.01$) decrease in spleen and thymus weight was noted (Table 12).

Because elevated blood levels of RF, CRP, IL1β,TNF-α, and IL6 are hallmarks of RA, in vitro measurements of these biomarkers were made for both the arthritic control group and each of the treatment groups. The research reveals that external herbal gel composition may have the ability to modulate pro-inflammatory cytokines, as evidenced by the substantial suppression of TNF-α, IL-1ß, and IL-6 production [36, 37].

Normal joint space, neighboring synovium, soft tissues, and cartilage were all evident upon histological inspection of a normal joint material (Figure 3a). An extensive inflammatory response was seen in the soft tissue surrounding the joint in the arthritis control samples. Diagrams 3b depict normal cortex, cartilage, and marrow in the joint specimens of the marketed topical gel administered groups. Histopathological analysis of arthritic control rats and animals given marketed gel and topical herbal gel composition F5 revealed decreased soft tissue inflammation around the joints (Figure 3c and 3d).



3a **3b**

3c **3d**

Figure 3a, 3b, 3c, and 3d: 3a-Ankle joint histological slice of the rat control group and demonstrates a normal joint surface. **3b-** Ankle joint histological section of the rat arthritis control group demonstrating inflammation. **3c-** Ankle joint histological slice of rats treated with marketed gel demonstrating intact cortex, cartilage, and marrow. **3d-** Ankle joint histopathology in the prepared gel (F5)-treated group demonstrating mild inflammation.

Table 11: Rats given FCA-induced arthritis: Impact of composition F5 on haematological parameters

Groups	Hb (mg%)		WBC (x103/mm3)		RBC (x106/mm3)		ESR (mm/h)	
	21st day	42nd day	21st day	42nd day	21st day	42nd day	21st day	42nd day
Normal control	15.75±0.15	16.05±0.12	6.50±0.10	6.70±0.10	9.60±0.05	10.15±0.19	5.45±0.40	5.10±0.35
Arthritic control	11.00±0.26[a]	10.00±0.15[a]	12.00±0.15[a]	10.78±0.12[a]	7.95±0.21[a]	7.28±0.15[a]	34.58±1.50[a]	40.75±1.25[a]
Marketed topical gel	11.25±0.20[a]	15.00±0.22[b]	11.30±0.18[a]	8.15±0.10[b]	7.29±0.22[a]	8.96±0.20[b]	39.15±0.80[a]	26.15±1.95[b]
Prepared gel (F5)	12.25± 0.15[a]	13.50±0.13[b]	11.95±0.30[a]	9.55 ±0.50[b]	7.15 ±0.20[a]	8.18±0.11[b]	41.00± 0.50[a]	31.35±1.76[b]

Data represented as mean ± SEM (n=6) in table and [a]p<0.001 Normal control Vs Arthritic control; [b]p<0.001 Arthritic control Vs Treated groups

Table 12: Rats given FCA-induced arthritis: Impact of composition F5 on serum TNFα, Interleukin levels, and changes in thymus and spleen

Groups	TNFα (pg/mL)	Serum interleukin (pg/mL)		Thymus weight (g)	Spleen weight (g)
		IL-1β	IL-6		
Normal control	1.269±0.19	1.41±0.22	20.00±1.00	0.250±0.050	0.525±0.050
Arthritic control	41.50±2.00[a]	68.10±1.15[a]	730.44±29.55[a]	0.750±0.060[a]	1.41±0.071[a]
Marketed topical gel	6.13±0.81[b]	21.25±0.60[b]	315.15±10.00[b]	0.395±0.050[b]	0.760±0.040[b]
Prepared gel (F5)	18.05±0.81[b]	37.25±1.15[b]	429.25±10.17[b]	0.420±0.050[b]	0.925±0.050[b]

Data represented as mean ± SEM (n=6) in table; [a]p<0.001 Normal control Vs Arthritic control; [b]p<0.0001 Arthritic control Vs Treated groups.

Conclusion

The anti-arthritic properties of the prepared topical herbal gel formulation could be attributed to the luteolin and apigenin noticed in the methanol leaf extracts of CH and RC. The produced formulation F5, which contains 5g of carbopol 940 and a 1:2 ratio of CH to RC (2g and 4g), has been shown to be an intriguing topical herbal gel to be used in the management of arthritis. Additional clinical research can support the use of this version for arthritic patients.

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Conflict of interest

The authors say they have no competing interests.

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