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Original Article

LIPID AND SURFACTANT-BASED PHYTOFORMULATION FOR AMELIORATION OF INFLAMMATION

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ABSTRACT

Objective: Observations from traditional medicine and findings of modern science recommend use of curcuminoids and piperine in inflammatory ailments such as rheumatoid arthritis. Therapeutic potential of these phytoconstituents cannot be exploited to the maximum extent because of poor solubility and low bioavailability. The objective of this study was to overcome these challenges and harness the potential of these phytoconstituents by developing lipid and surfactant-based formulations.

Methods: A microemulsion was prepared by selecting lipids, surfactants and cosurfactants on the basis of the solubility and stability of phytoconstituents. It was further converted into a transparent gel for topical application. The phytoformulation was characterized by physicochemical tests. Its hemocompatibility and irritation potential was determined. Further phytoformulation was studied in RAW 264.7 cells for cell internalization and antiarthritic potential was investigated in Complete Freund's Adjuvant (CFA) induced arthritic rats. The disease progression was recorded. At the end of the study hematological, biochemical and oxidative stress parameters were measured.

Results: A stable phytoformulation containing 0.75% w/w curcuminoids and 0.25% w/w piperine was developed. At the end of 24 hours, the amount of curcuminoids and piperine permeated through the skin from phytoformulation was 4.38 and 1.38 times that of the oil. It had good hemocompatibility and poor irritation potential. Internalization of phytoformulation in RAW 264.7 cells was concentration dependent. There were significant changes in rats due to disease induction by CFA and results indicated regression of the disease progress due to phytoformulation.

Conclusion: Lipid and surfactant-based formulation improved solubility and permeability of phytoconstituents. The developed phytoformulation could recover inflammatory changes in rats and it can be further studied in human beings.

Keywords: Curcuminoids, Piperine, Microemulsion, Polyherbal, RAW 264.7, CFA

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INTRODUCTION

Inflammation is a complex physiological and pathological process that is frequently a protective reaction brought on by harmful stimuli and situations to maintain the homeostasis of the body [1]. There are two types of inflammation, acute inflammation and chronic inflammation. Acute inflammation normally benefits the host and lasts just a short period, while chronic inflammation, which lasts for an extended period, can cause several chronic conditions, including arthritis, metabolic and neurodegenerative diseases, and certain types of cancer [2]. To treat inflammatory conditions like arthritis, NSAIDs are frequently used, but their efficacy is modest. The unavoidable adverse effects of gastrointestinal bleeding, gastrointestinal toxicity, and nephrotoxicity as well as therapeutic gaps also put a cap on the long-term use of these drugs [3]. Researchers are thus becoming more and more interested in the active components of natural herbal plants due to their wide range of sources, strong therapeutic impact, and fewer side effects [4].

"Curcuminoids" is a mixture of curcumin, demethoxycurcumin and bisdemethoxycurcumin, wherein curcumin is the major component and demethoxycurcumin and bisdemethoxycurcumin are minor components. These are phenolic compounds obtained from the rhizome of the turmeric plant i. e., *Curcuma longa* (L). Various allergic and inflammatory respiratory diseases, liver diseases, and wound healing have all been traditionally treated with curcuminoids [5]. In modern medicine, its antioxidative, antiproliferative, and anti-inflammatory characteristics have been extensively studied for a broad range of medical disorders, including inflammatory bowel syndrome (IBS), psoriasis, rheumatoid arthritis, and different cancers [6]. Another popular spice is black pepper i. e., *Piper nigrum* (L). It acts as a stimulant to skin when applied externally [7]. Piperine, the alkaloid found in it has shown many promising effects in the treatment of arthritis [8]. Piperine inherits anti-oxidant, anti-pyretic and anti-tumor properties which have a chief role in the management of inflammatory conditions [9]. Kumar *et al.* demonstrated the efficiency of piperine as an anti-inflammatory agent, during the study piperine hindered the expression of TNF-α induced cell adhesion on the endothelial cells, the phenomenon was observed due to the inhibition of nuclear factor-κB (NF-κB) activation in the endothelial cells [10].

Curcuminoids physicochemical characteristics, particularly its poor water solubility, bioavailability, and low stability, including photodegradation provide significant difficulties which restrict the therapeutic potential of conventional formulations containing curcuminoids [11]. These observations and drawbacks open the potential for the development of innovative curcuminoid delivery systems. The solubility, permeability, and stability of antioxidant compounds like curcumin can be enhanced by colloidal systems since they are designed to function in all regions of drug delivery [12]. Until now, topical and transdermal delivery of curcumin has been developed using metal complexes, polypeptides, solid lipid nanoparticles, biodegradable nanoparticles, elastic vesicles, nanoemulgels etc [13]. Scientists have developed novel drug delivery systems like solid lipid nanoparticles [14], solid self-emulsifying drug delivery system [15], chitosan-coated liposomes [16] etc. for piperine.

Effect of the combination treatment of curcuminoids and piperine has been studied clinically and preclinically. An oral formulation of 500 mg of curcuminoids and 5 mg of piperine was administered orally twice a day to 44 osteoarthritic patients in Mumbai. Amelioration of pain and stiffness was observed [17]. In separate studies carried out in rodents, combination treatments have shown promising hypocholesterolemic [18], antigenotoxic [19] and cardioprotective [20] effects.

In the present work, we have used lipids and surfactants to make a successfully co-loaded formulation. The objective is to incorporate phytoconstituents at high concentrations by overcoming challenges of poor solubility and to have a formulation with good permeability which can be used to treat inflammation. Further objective is to develop the formulation which can be scaled up easily.

MATERIALS AND METHODS

Materials

Curcuminoids were extracted in–house and were found to contain total curcuminoids of 98.30% and curcumin 81.80%. Potency was determined using curcumin (total curcuminoids 97% and curcumin 84%) purchased from Sigma-Aldrich Chemie GmbH, India. The following chemicals were received as gift samples viz. Capmul MCM from Abitec Corporation, USA, Carbopol 974 P from Lubrizol, India, Kolliphor RH 40 and Kollisolve PEG 400 were gifts from BASF, India. Following chemicals were purchased - Piperine of Sigma Aldrich, India, Polysorbate 80 of SD Fine Chem, India; Isopropyl alcohol of E. Merck, India, Eucalyptus oil of Yucca Enterprise, India; Sesamol of Sigma Aldrich, India. Other chemicals used were of laboratory grade or HPLC grade.

Preformulation studies

Solubility studies

In separate glass vials, 5 g of each of the excipients was taken. To it, curcuminoids were added in increments of 10 mg under continuous stirring, using a magnetic stirrer 1MLH, Remi Electrotechnik Ltd., Mumbai, India. The procedure was continued till the undissolved phytoconstituents were visible. The mixture was further stirred at room temperature for 24 h. Undissolved solids were separated from the rest of the liquid by centrifugation using Eltek RC 4100 D (Eltek India Pvt. Ltd., Mumbai, India.) The supernatant was filtered through a 0.45 µm membrane syringe filter and diluted with methanol for quantification by UV-Visible spectrophotometer (Evolution 300™, Thermo Fisher Scientific, Hemel Hempstead, UK). The same procedure was followed to determine the solubility of piperine.

Drug excipient compatibility studies

Individual phytoconstituents and selected excipients were mixed in 1:4 ratio. The mixture was analysed for the phytoconstituent content by UV-Visible spectrophotometer. Then the mixture was placed in a glass vial and sealed with a rubber plug and aluminum cap. The vials were stored at 60 °C for 3 days in an oven. The phytoconstituent content was reanalysed after exposure by the same procedure.

Selection of surfactant-mix

Polysorbate 80 and Kolliphor RH 40 were mixed in various ratios viz, 1:1, 2:1, 3:1 and 4:1. 3g of Capmul MCM was emulsified with 1g of surfactant mix and purified water using the magnetic stirrer at 900 rpm for 30 min. Globule size of the emulsion was measured using an optical microscope and calibrated eyepiece micrometer.

Development of microemulsion system

In the present study, Capmul MCM was spiked with eucalyptus oil and sesamol to form the oil phase of the formulation. Polysorbate 80 and Kolliphor RH 40 (4:1) formed the surfactant component; Kollisolv PEG 400 and isopropyl alcohol (IPA) (3:1) were taken as the cosurfactant component. Surfactants and cosurfactants together formed Smix. 1 gram of different combinations of oil and Smix in the ratios 1:9, 2:8, 3:7, 4:6. 5:5. 6:4, 7:3, 8:2 and 9:1 was taken in a stoppered test tube. Mixture was warmed to 40 °C for 15 minutes and mixed thoroughly. As per the water titration method, water was added in small increments and mixed thoroughly. Mixture was allowed to stand for an hour before the next addition of water. The mixtures were characterized visually for clarity. This procedure was continued till the solution turned hazy.

Construction of experimental design space

Microemulsions are formed when oil, surfactant and water are mixed in the correct concentrations. These combinations of concentrations are represented graphically by triangular diagrams known as ternary diagrams. Three sides of triangles represent 3 components viz. oil, surfactant and water. Since, surfactants alone cannot form good microemulsions, cosurfactants are often added in the system. This fourth component is taken in a fixed ratio and one side of the triangle is represented by the total concentration of surfactant and cosurfactant, often termed as Smix. Because of this fourth component, ternary diagrams are referred to as pseudo-ternary diagrams. For each ratio of surfactant and cosurfactant a separate diagram is plotted. The clear region obtained after water titration was marked in the pseudo-ternary diagrams. No further discretion was done based on microstructure in the pseudo-ternary diagrams. Three phase diagrams, each for surfactant mix: cosurfactant mix ratio (Smix) of 1:1, 2:1 and 4:1, were constructed using Microsoft Excel-based application.

Preparation of phytoconstituents loaded microemulsions

Oil phase was saturated with curcuminoids. Mixture of surfactants and cosurfactants (Smix) was added to it and stirred at room temperature for 24 h. Piperine was dissolved in it. The required quantity of water was added in small aliquots.

Selection of optimum microemulsions

Following batches of microemulsions as indicated in table 1 with varying ratios of oil: Smix and surfactant mix: cosurfactant mix were prepared.

Table 1: Composition of various drug-loaded formulations

Formulation code	Oil: Smix	Surfactants: cosurfactants
F1	1:4	1:1
F2	1:6	1:1
F3	1:8	1:1
F4	1:4	2:1
F5	1:6	2:1
F6	1:8	2:1
F7	1:4	4:1
F8	1:6	4:1
F9	1:8	4:1

All the formulations contained 50 % w/w water

Stress studies

Following stress studies were carried out to evaluate physical stability of the preparation [21]. The formulations were centrifuged at 15000 rpm for 30 min and observed visually. Freeze-thaw study consisted of exposing the formulations to -21 °C for 24 h and exposing the same to +25 °C for 24 h in one cycle. Three such cycles were carried out. In the heating-cooling cycle the formulations were studied for three cycles, where formulations were kept at refrigerated temperature of 4 °C for 24 h and exposing the same for 24 h to 40 °C in one cycle.

Interaction with metal ion

The stability of the microemulsion against metal ion solution was studied. To 4 ml of the microemulsion, 1 ml of 1.5% copper sulphate pentahydrate solution was added and vortexed for 5 minutes. The solution was incubated for 2 hours. The resultant mixture was centrifuged at 15000 rpm for 30 min and observed visually. Samples collected after freeze thaw study and heating cooling cycle were used in this study.

In vitro release of phytoconstituents

A regenerated seamless cellulose dialysis membrane (LA 387-5MT) manufactured by HiMedia Laboratories Pvt Ltd, India was used in the study. This membrane was partially permeable, with a molecular weight cut off between 12,000 to 14,000. The membrane was hydrated by soaking in the diffusion media for 12 hours prior to use. One end of the tubing was tied with the cotton thread. Approximately 200 mg of the sample was introduced in it and then the other end was closed. The

sac was placed in a glass beaker containing 60 ml of medium [22]. The release medium was composed of phosphate buffer pH 5.5 and 90% ethanol (1:1). The medium was maintained at 37 ± 1°C and stirred with the magnetic stirrer (1 MLH, Remi, India) at 400 rpm. 5 ml aliquot was taken and analysed for content of curcuminoids by HPLC, Agilent 1260 Infinity II (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) equipped with a UV/Vis Detector. Acetonitrile: 0.1 % orthophosphoric acid in HPLC water (35:65 % v/v) was used as a mobile phase for the analysis.

Characterization of selected microemulsion

Particle size and Zeta potential measurement

The microemulsion was diluted 10 times with water. The mean droplet size of the optimized formulation was determined by nanoparticle tracking analysis (Nanosight NS 500, Malvern, UK). All measurements were obtained at 25 °C.

The microemulsion was filled in the disposable cuvettes of Nanopartica SZ100 (Nanoparticle Analyzer Horiba Scientific, Kyoto, Japan) and zeta potential was recorded.

Transmission electron microscopy (TEM)

The phytoconstituent loaded microemulsion was diluted with ethanol-water at 25 °C to get a very slightly turbid solution. The solution was ultrasonicated and was dropped on carbon-coated grids of 200 mesh and then dried for a day under reduced pressure. The TEM images were obtained on JM 2100 (JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV, camera length = 499.606 mm and resolution = 0.24 nm.

Development and characterization of gel phytoformulation

Carbopol 974P was dispersed in water under continuous stirring using an overhead stirrer (RQ 121/D, Remi Electrotechnik Ltd, India). It was left undisturbed for 24 hours to complete the hydration. The pH of the gel was adjusted to 5.5 using triethanolamine. The gel was homogenized using homogenizer (RQ 127A/D, Remi Electrotechnik Ltd, India). Phytoconstituents loaded microemulsion was prepared as described above using only part of the water. The required quantity of the concentrated microemulsion was taken into the glass pestle and mortar. Carbopol 974P gel prepared as above was added to it under slow trituration. Weight of the hydrogel was adjusted with water. The gel was homogenized using the homogenizer.

pH

The pH of the gel was measured using a PHAN pH meter (Lab India, India) at 25 ± 1 °C.

***In vitro* hemocompatibility study**

Fresh human blood was collected in tubes lined with EDTA (anticoagulant). The red blood cells (RBCs) were collected by centrifugation at 3000 rpm for 20 min in a centrifuge (Eltek RC 4100 D, Mumbai, India) and washed thrice with saline solution and suspended in saline. Gel was diluted with saline. To 2.5 ml of erythrocyte suspension, 1 ml of gel solution was added and volume was made up to 5 ml. It was incubated for 4 h at 37 °C. After the incubation period, the reaction mixture was centrifuged again. The supernatant was collected and its optical density was measured at 574 nm [23] similarly, for negative and positive control blood was incubated with saline and deionized water, respectively. To study the effect of phytoconstituents, methanolic solution containing 15 µg and 5 µg of curcuminoids and piperine respectively was taken into the tube. Phytoconstituents were precipitated by evaporation of solvent and then resuspended in 1 ml of saline. Hemolytic activity was calculated as follows

$$\% \text{ Hemolytic Activity} = (As - An)/(Ap - An) \times 100 \text{ .... Equation 1}$$

Where, As, An and Ap are the absorbance of sample, negative and positive control, respectively.

Irritation potential

Collagen swelling as an indicator for irritation potential has been used for medical devices and formulations [24, 25] Collagen sheets (Colldrez; Synerheal Pharmaceuticals) were removed from the media and pat dried. It was cut into small pieces of 2.5 X 2.5 cm. Weights of the pieces were recorded. Phytoformulation was diluted with saline (1:4) and taken in the Petri dish. Collagen pieces were placed in it. After 42 h the pieces were taken out of the incubation medium and collagen swelling was determined gravimetrically [26]. The weight of the saline absorbed per gram of collagen was calculated.

***Ex vivo* skin permeation studies**

The *ex vivo* skin permeation studies were performed on pig ear skin [27] obtained from a local slaughterhouse. The whole ear skin was excised from domestic pigs and stored at −20 °C and were utilized within a month. Before the conduct of the study, the skin was thawed and mounted on a vertical Franz diffusion cell with stratum corneum facing the donor compartment. The receptor compartment was filled with phosphate buffer pH 5.5: 90% ethanol (1:1) of 10 ml. The receptor phase was kept under constant stirring at 37 ±1 °C. The formulations were applied to the pig ear skin. The aliquot (3 ml) was withdrawn at predetermined time intervals and an equal volume of fresh medium was added to the receptor compartment to maintain the sink condition. The samples were analyzed using a HPLC. The detection was carried out at a wavelength of 420 nm and 342 nm for the curcuminoids and piperine, respectively. The amount of phytoconstituents permeated through per unit area of the skin surface was estimated. Graph of the amount of phytoconstituent permeated against time was plotted. The flux was calculated as the slope of the linear part of the relation. Permeability coefficient was calculated as follows

$$Kp = Jss/Cd \text{.... Equation 2}$$

Where Kp = permeability coefficient, Jss= Flux and Cd= concentration of phytoconstituent in the donor compartment

Skin retention

At the end of the experiment, the skin was removed from the Franz diffusion cell, washed with 30 ml saline. The permeation area of skin was then excised, soaked in 10 ml of ethanol and homogenised. The resultant solution was filtered using nylon filter 0.22µm and the amount of phytoconstituents retained in the skin was measured by HPLC [28].

MTT test assay in RAW 264.7 cells

The study was carried out on Raw 264.7 (Murine macrophage cell line). In a 96-well plate, 20,000 cells were seeded per well and allowed to grow for about 24 hours. The cells were treated with various concentrations of phytoformulation or phytoconstituents at various concentrations viz. curcuminoids+ piperine: 3.125 + 1.041, 6.25 +2.083, 12.5 +4.16, 25 +8.33 and 50+16.66 µg/ml. The plate was incubated for 24h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, plates were taken out from the incubator, and the media containing test formulations were removed. MTT reagent was added to final concentration of 0.5 mg/ml of total volume. The plates were covered with aluminum foil and kept in the incubator for 3 h. The internalization of the reagent by the cells was terminated by aspiration of the media and the cells were lysed with 100 µl dimethyl sulfoxide kept at 37 °C for 30 min. The mixture was gently shaken. The absorbance was recorded on an ELISA reader at 570 nm wavelength. % Cell viability was calculated using below formula:

$$\% \text{ Cell viability} = (\text{Abs of treated cells})/(\text{Abs of untreated cell}) \times 100 \text{ .... Equation 3}$$

The half-maximal inhibitory concentration (IC_{50}) values were determined.

Evaluation of anti-inflammatory activity

The experimental protocol (KMKCP/IAEC/13/2017) was approved by the Institutional Animal Ethics Committee. Wistar rats of either sex (150–200 g;), procured from Bharat Serum Ltd, India, were kept in solid bottom cages and were fed with standard rat chow with water available ad libitum. The animals were divided into 4 groups of 6 rats each. For induction of RA, rats were injected with a single 0.1 ml (0.05% w/v) of CFA into the sub-planter region of the left

hind paw [29]. This day was assigned as day "0". Dosing with the gel phytoformulation and with the standard formulation, were started two days prior and continued for 28 days. The placebo, phytoformulation and standard formulation were gently rubbed with index finger on the plantar surface of hind paw of rats in respective groups. The paw thickness was measured for each animal using a vernier caliper. Further rats were observed for change in body weight. The visual arthritis scoring system was used to evaluate the severity of arthritis. Inflammation in paw was graded for all rats using a 5-point scale of 0-4. The scale for assigning the score was as follows 0: no evidence of edema or swelling; 1: slight swelling and erythema limited to foot or ankle joint; 2: slight edema and erythema from the ankle to the midfoot; 3: moderate swelling and erythema extending from the ankle to the metatarsal joints; 4: severe swelling and erythema encompassing the ankle, foot and digits. The mean arthritic index was used for the comparison. The readings were taken on 0, 7th, 14th, 21st, and 28th day. The data was statistically evaluated. Hematological, biochemical and oxidative parameters were evaluated at the end of the study. The rats were sacrificed by cervical dislocation. Just prior to that the blood was collected from the rats by retro-orbital puncture for the estimation of various parameters. The blood was centrifuged at 3000 rpm for 10 min within 1 h after collection and frozen at -20 °C until the analysis of biochemical and oxidative parameters.

RESULTS AND DISCUSSION

Preformulation studies

From the solubility studies as described above, it was observed that curcuminoids showed poor solubility in sunflower oil, soybean oil, sesame oil and isopropyl myristate. In ethyl oleate, glycerin, polyglycerol oleate, span 80 it showed intermediate solubility. High solubility was found in Capmul MCM, Kollisolv PEG 400, polysorbate 80, Kolliphor RH 40 and Transcutol P. Though the amount of piperine dissolved was more than curcuminoids, the solubility pattern was similar. This could be due to lower molecular weight and lower partition coefficient of piperine than curcuminoids.

The therapeutic moieties were lipophilic and the desired dosage form was with oil as the internal phase. Since Capmul MCM had maximum solubilising capacity for phytoconstituents, it was selected as an oil in further studies.

In drug excipient compatibility studies, less than 5% reduction in curcuminoids content was observed in polysorbate 80, Kollisolv PEG 400, Kolliphor RH 40, IPA and Capmul MCM. Slightly higher reduction in curcuminoids content was observed in Transcutol P. Piperine was found to be stable in all high solubility solvents.

While selecting surfactant-mix, it was observed that emulsions prepared with polysorbate 80 and Kolliphor RH 40 at ratios 1:1, 2:1, 3:1, 4:1; exhibited globule size of 5.76 ±0.44, 5.49 ±0.57, 5.11 ±0.36, 4.43 ±0.71 (µm) respectively. Batches prepared with 4:1 had the smallest globule size. This ratio was selected for further studies.

Development of microemulsion system

Capmul MCM was found to be a good choice as an oil phase as it had high solubilisation capacity and being predominantly a mixture of mono- and diacylglycerol could easily form microemulsion. Polysorbate 80 and Kolliphor RH 40 had good solubilisation capacity for both oil and phytoconstituents. This was essential, as the chosen surfactant needs to efficiently disperse selected oil and it should have good drug solubilisation capacity. Non-ionic nature, low cost and good safety profile were the added advantages [30]. Kollisolv PEG 400 and isopropyl alcohol were used as cosurfactants. Cosurfactants decrease interfacial tension. They influence fluidity of the interfacial film resulting in modification of curvature of the interface thereby affecting the size and shape of the internal phase. They also influence the polarity of internal and external phases. Phytoconstituents had high solubility in Kollisolv PEG 400. IPA was used in the formulation to make Smix more fluid. It is a good penetration enhancer and gives a drying effect on application. Microemulsions, being thermodynamically stable, could be prepared without the incorporation of energy. This ensured ease of formulation and easy scale up ability.

Construction of experimental design space

Microemulsion regions obtained using different ratios of surfactant and cosurfactant mix viz 1:1, 2:1 and 4:1 are depicted in fig. 1(a), (b) and (c) respectively. As the concentration of surfactant in comparison to cosurfactants increased, microemulsion area also increased. This could be linked to higher HLB of polysorbate 80 and Kolliphor RH 40 over Kollisolv PEG 400 and IPA.

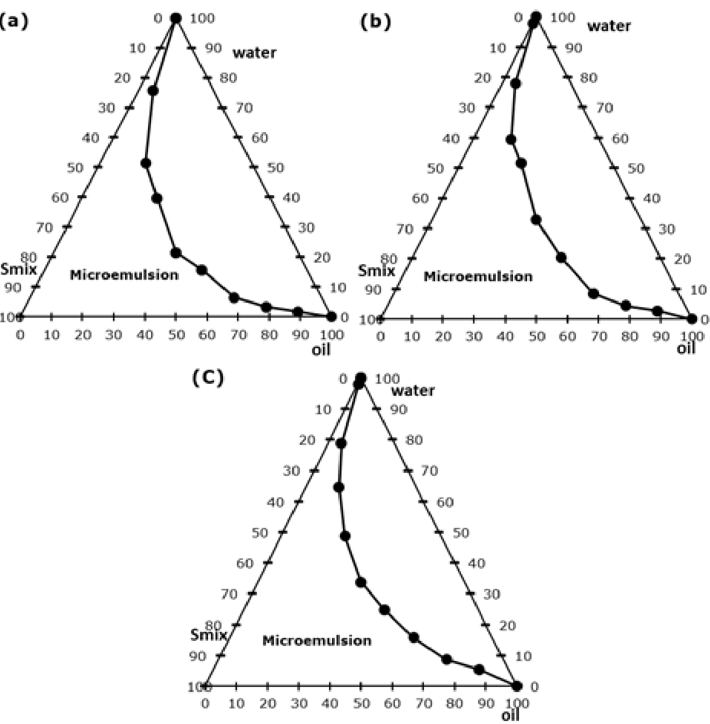

Fig. 1: Microemulsion region in pseudoternary phase diagrams at different Smix ratios (a) 1:1, (b) 2:1 and (c) 4:1

Preparation and selection of phytoconstituents loaded microemulsion

Oil- Smix phase was saturated with phytoconstituents and then water was added to form the microemulsion. Addition of water would lead to reduction in oil and phytoconstituent holding capacity of Smix, with the possibility of phase separation and precipitation of phytoconstituents. Hence, physical stability of the emulsion was evaluated using various stress parameters like centrifugation and effect of temperature. Identification of batches with separation of insoluble curcuminoids was further confirmed by interaction with metal ions. Cuprous ions bind to at least two curcumin molecules [31] This reaction is possible only with free curcumin and not with encapsulated curcumin [32] Phase separation was not observed in any batch. All batches passed the centrifugation test. F1 to F3 batches showed separation of free curcuminoids in temperature effect studies, indicating physical instability hence, were not included in further studies.

***In vitro* release of phytoconstituents**

Objective of the formulation was to improve solubility and to increase the availability of phytoconstituents at the inflamed synovial joints. Higher availability of phytoconstituents will help in amelioration of inflammation. Surfactants at higher concentration will cause over solubilization of drug and poor release from the formulation [33]. To confirm release of curcuminoids from the phytoformulation *in vitro* release studies were carried out. Observations of *in vitro* release studies are presented in fig. 2. At the end of 24 h, curcuminoids release was found to be in the range of 58.89% to 88.60%. Lowest curcuminoids release were for batch F9 and highest release were for F7. Hence, the batch F7 with highest release of curcuminoids at the end of 24 h was selected.

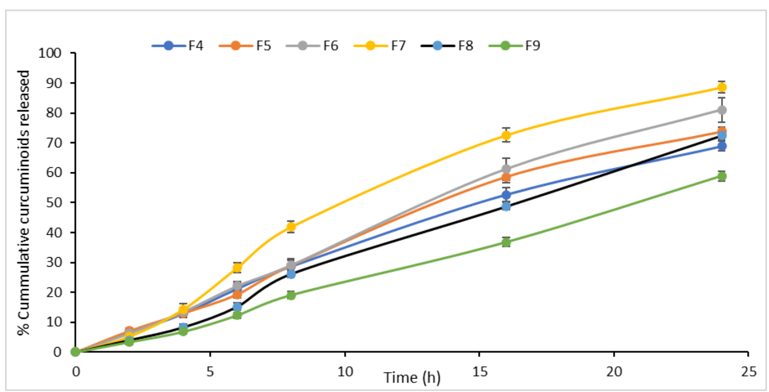

Fig. 2: *In vitro* release of curcuminoids from different phytoformulations. Results are expressed as mean ± SD (n = 3)

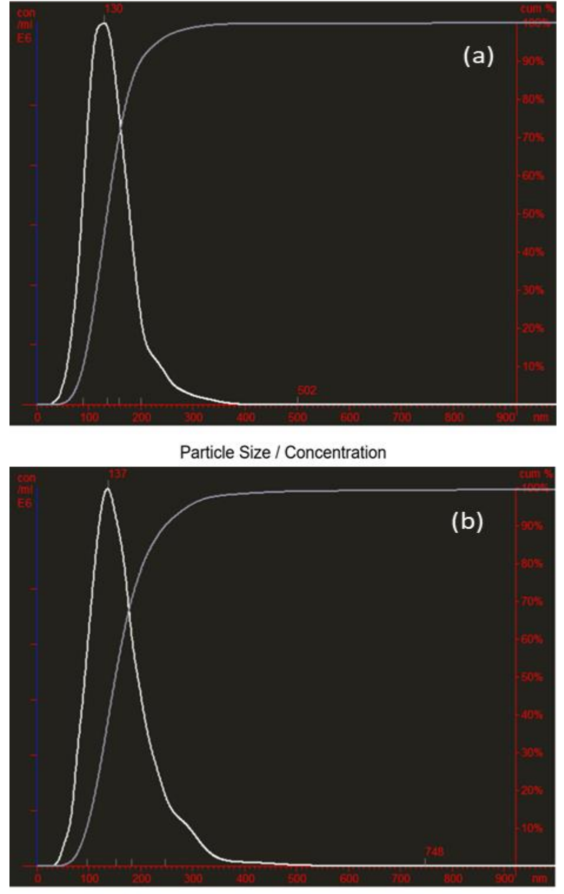

Fig. 3: Globule size of (a) placebo PF7 and (b) phytoconstituent loaded microemulsion F7

Characterization of phytoconstituent loaded microemulsion

Particle size and zeta potential

The mean droplet size of the placebo PF7 was 143 nm with a standard deviation of 46 nm (fig. 3a) as determined by NTA (Nanosight NS 500, Malvern, UK). Slight enlargement of globules was observed on incorporation of phytoconstituents and the batch F7 showed mean discontinuous phase droplet size of 169 nm with standard deviation of 94 nm (fig. 3b). The globule size for both the formulations was well below 200 nm indicating good stability and good penetrability.

Zeta potential measurement

Placebo PF7 had a zeta potential of -13.9 mV (fig. 4a). The low negative zeta potential can be linked to use of non-ionic surfactants. The batch F7 had zeta potential of -21.1 mV (fig. 4b). This could be due to entrapped phytoconstituents in the formulation. When all of the particles have strong zeta potentials, either positive or negative, they repel one another and the system is said to be stable. Another mechanism by which the biphasic system is stable, in spite of low zeta potential, is by steric stabilization [34]. Systems containing polysorbate 80 often show steric stabilization [35].

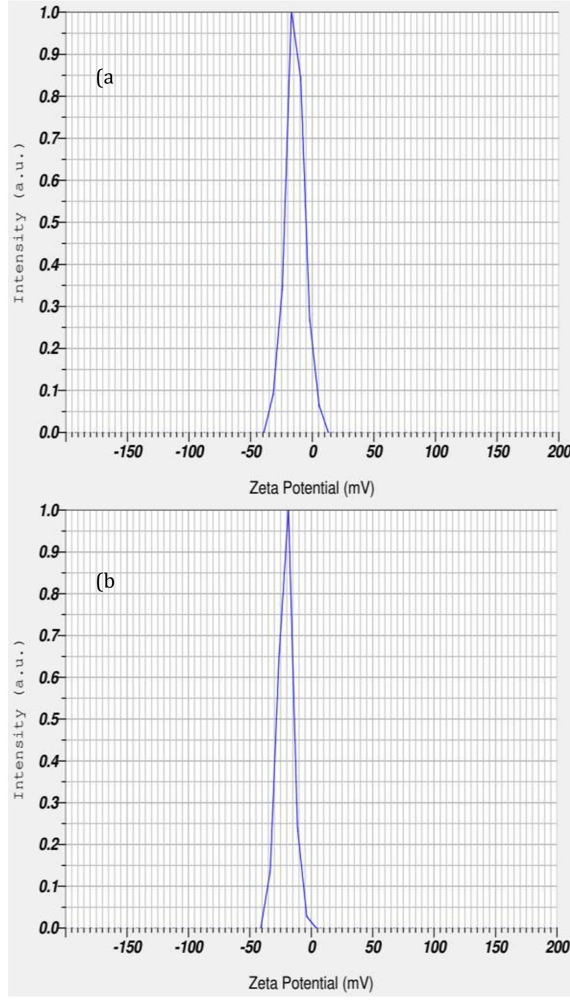

Fig. 4: Zeta potential of (a) placebo PF7 and (b) phytoconstituent loaded microemulsion F7

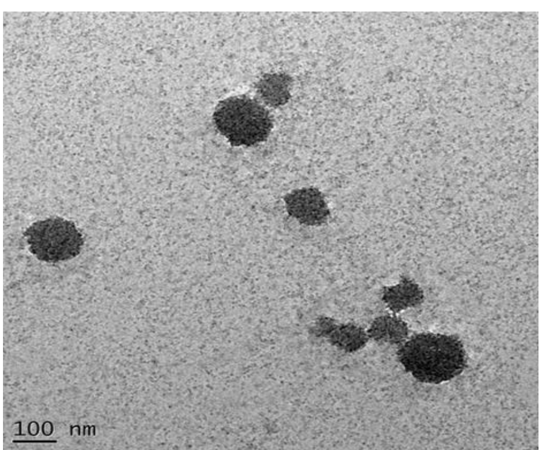

Fig. 5: Transmission electron microscopy (TEM) image of F7 microemulsion

Transmission electron microscopy (TEM)

The TEM image of the microemulsion is as shown in fig. 5. TEM uses a beam of electrons instead of light for imaging. This produces images at high resolution and captures microstructural transitions. The observed particles were spherical in nature and average globule sizes obtained from differential light scattering technology (NTA) were not significantly different from those obtained using the TEM.

Development and characterization of Gel phytoformulation

The microemulsion was converted in the gel using carbomer. Topical gel form has many advantages. It avoids first pass metabolism and helps in providing primarily localized effects. Smooth transparent gel provides better patient compliance. As compared to other gelling agents like HPMC, gelling was possible at very low concentration of carbomer. It was an orange-red transparent semisolid preparation.

pH

The pH of the gel was 5.5 ± 0.2. The curcuminoids are stable in acidic pH. Also, skin pH is 5.5. Thus, this pH range ensured stable, skin compatible formulation.

***In vitro* hemocompatibility study**

Phytoconstituents (Curcuminoids + Piperine) and the gel phytoformulation showed 2.87±0.24 and 3.01±0.16 % hemolysis. All formulations showed less than 5% hemolysis indicating excellent blood compatibility [36, 37].

Irritation potential

Weight of the saline absorbed per gram of collagen on exposure to normal saline, gel phytoformulation and 2% SLS solution was 2.91, 2.32 and 6.81 respectively. In comparison to SLS, gel phytoformulation caused significantly less swelling. This is a predictive indicator test [38]. Researchers have found a good correlation between collagen test and *in vivo* studies [39].

***Ex vivo* skin permeation studies**

Ex vivo skin permeation studies were performed to determine the permeability of the phytoconstituents from formulations using pig ear skin as a biological membrane. For the initial assessment of skin permeation in humans, porcine skin is a suitable substitute. In terms of hair coat, hair follicle distribution, epidermis thickness, epidermal turnover kinetics, lipid composition, carbohydrate biochemistry, lipid biophysical characteristics, and the positioning of dermal collagen and elastic fibres, porcine skin is the most comparable to human skin [40, 41].

The cumulative amount of curcuminoids permeated through skin form microemulsion gel formulations were much higher than that of oil formulations over the period of 24 h (fig. 6). From the oils containing curcuminoids only and curcuminoids with piperine, 36.20±3.16 and 50.96±4.77 μg/cm² of curcuminoids permeated through the skin respectively whereas for gel phytoformulation these values were 175.98±4.20 and 223.47±6.94 μg/cm² respectively. Piperine acted as a penetration enhancer for both the types of formulation. The amount of piperine permeated through microemulsion gel containing piperine with curcuminoids in comparison to oil formulation was 1.38 times (fig. 7). Summary of various permeation parameters is given in table 2. The nano size of droplets had provided the larger surface area for phytoconstituents release and permeation through the skin. Besides smaller particle size, piperine played an important role in modification of the skin permeability.

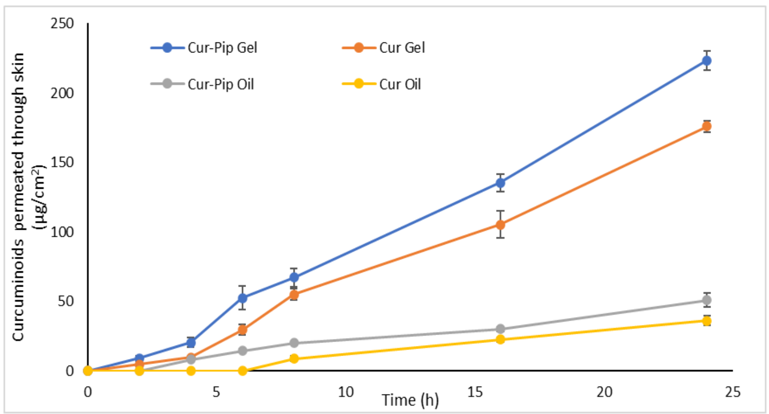

Fig. 6: *Ex vivo* skin permeation profiles of curcuminoids from different formulations through pig ear skin for 24 h. Results are expressed as mean ± SD (n = 3)

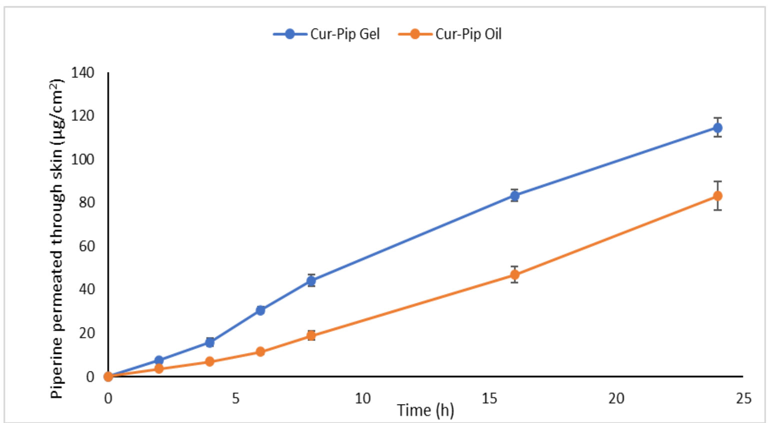

Fig. 7: *Ex vivo* skin permeation profiles of piperine from different formulations. Results are expressed as mean ± SD (n = 3)

Table 2: Summary of phytoconstituent's penetration fluxes and permeability coefficients

Phytoconstituent	Formulation	Flux (Jss) (μg/cm²/min) 10^{-3}	Permeability coefficient (Kp) (cm/min) x 10^{-5}
Curcuminoids	Cur-pip gel	157.70	10.51
	Cur gel	130.30	8.69
	Cur-pip oil	32.10	2.14
	Cur oil	28.50	1.90
Piperine	Cur-pip gel	48.40	9.68
	Cur-pip oil	41.2	8.24

Skin retention

Microemulsion based gel phytoformulation showed higher skin retention of phytoconstituents as indicated in Fig. 8 and 9. Phytoconstituents retained in the skin will provide prolonged release.

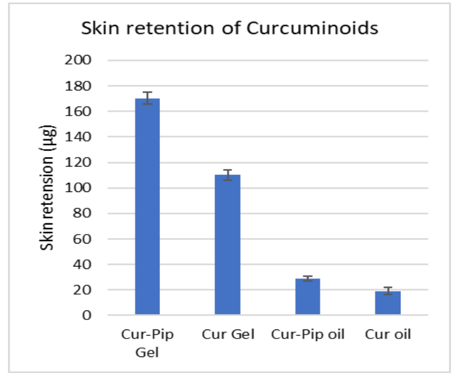

Fig. 8: *Ex vivo* skin retention profiles of curcuminoids from different formulations. Results are expressed as mean±SD (n = 3)

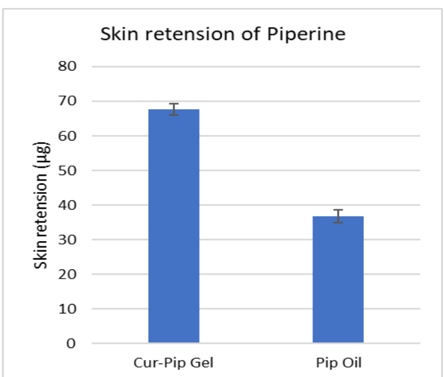

Fig. 9: Ex vivo skin retention profiles of piperine from different formulations. Results are expressed as mean±SD (n = 3)

MTT test assay in RAW 264.7 cells

As shown in fig. 10, at concentration 4.16 μg/ ml pure phytoconstituents as well as the gel phytoformulation showed high cell viability. As the concentration increased cell viability decreased for the pure phytoconstituents as well as for the gel phytoformulation. Cell viability was more in case of phytoconstituents as compared to gel phytoformulation at higher concentrations. IC_{50} value of polyherbal formulation was found to be 30.34 μg/ml, whereas IC_{50} value of the phytoconstituents (3:1) was 38.77 μg/ml. This is due to better internalization of phytoconstituents by cells from the lipid- surfactant environment.

The MTT assay is based on a reaction between the mitochondrial dehydrogenase enzymes from viable cells with the tetrazolium rings of MTT (a yellow reagent), which produces dark blue formazan crystals. These crystals are impermeable to cell membranes, resulting in their accumulation in healthy cells. Solubilization of the cells causes the release of the crystals. The number of surviving cells correlates to the level of the formazan product generated. The color was then quantified using a colorimetric assay. Higher the internalization of the compound, lower is the cell viability. We studied cell internalization in the RAW 264.7 cell line, which is a macrophage cell line that was established from a tumor in a male mouse induced with the Abelson murine leukemia virus. Cell viability was high at concentration below 5 μg/ml. This is in line with the earlier reported observations [42]. We observed that internalization of therapeutic agents was concentration dependent and was more in novel polyherbal formulation. Macrophages are the targets for the treatment of inflammation [43, 44]. Many novel anti-inflammatory formulations have reported higher internalization as compared to pure drugs [45, 46].

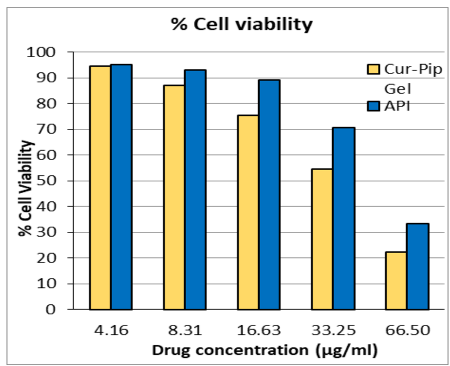

Fig. 10: Viability of RAW 264.7 cells in presence of phytoconstituents and the gel loaded with it

Evaluation of anti-inflammatory activity

Pearson developed Complete Freund's adjuvant induced arthritis model. The CFA is based on injection of heat-killed mycobacterium tuberculosis to generate inflammatory response. Pathophysiology of joints in CFA induced arthritic rats is similar to the changes observed in human arthritic patients, hence it has become a popular tool in preclinical research [47].

As shown in fig. 11, CFA could induce arthritis in rats. The progressive swelling of CFA injected hind paws increased in arthritic rats over time until the 14th day and then had plateau effect till experimental endpoint at day 28. Treatment groups receiving gel phytoformulation and standard formulation (diclofenac gel) separately showed significantly reduced paw oedema volume when compared with the placebo group. Weight gain in the placebo group was significantly impaired compared with non-arthritic rats. Groups receiving treatment showed weight gain (fig. 12). The similar pattern was observed for arthritis score (fig. 13).

Table 3 summarizes the effect of gel phytoformulation on various parameters in experimental rats. Arthritis caused reduction in hemoglobin, RBC and packed cell volume. It increased WBC and platelets. AST, ALT and ALP values increased. LPO increased and SOD, GPx, CAT decreased. Polyherbal treatment could significantly reverse these changes and the effect was comparable to that of the standard formulation.

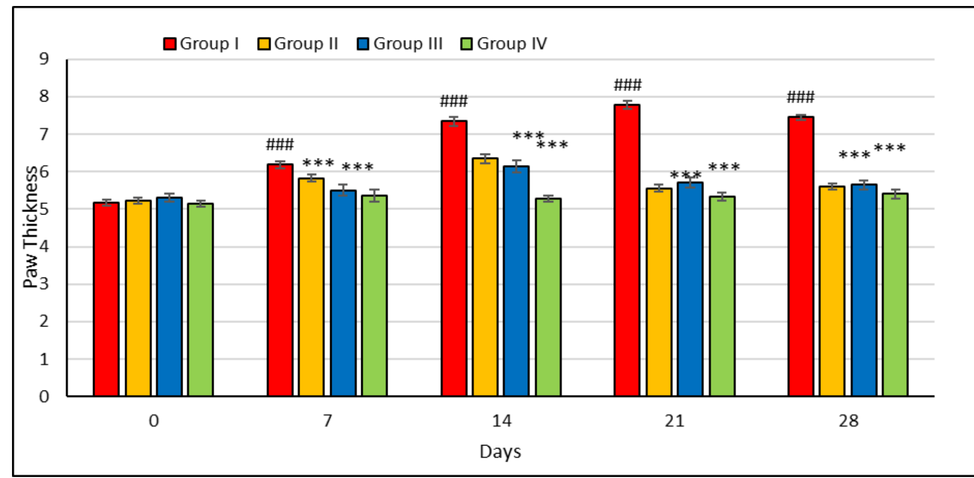

Fig. 11: Effect of gel phytoformulation on paw thickness in CFA induced arthritic rats (Results expressed as mean±SEM (n = 6) and analysed by one- way ANOVA followed by Tukey's post hoc test. Group I (arthritic control) compared with Group IV (normal control) and Group II and Group III (treatment groups) compared with Group I (arthritic control). #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001versus Group IV. **P* < 0.05, ***P* < 0.01, ****P* < 0.001versus Group I.)

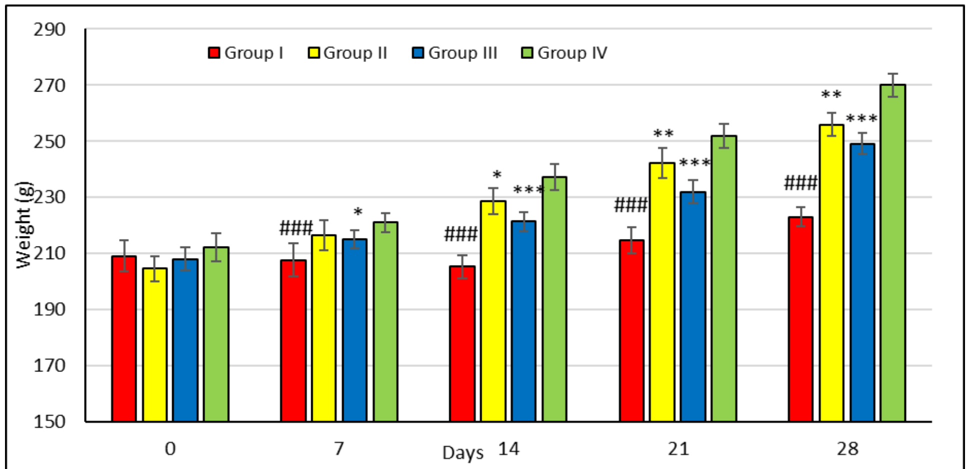

Fig. 12: Effect of gel phytoformulation on weight gain in CFA induced arthritic rat. (Results expressed as mean ± SEM (n = 6) and analysed by one- way ANOVA followed by Tukey's post hoc test. Group I (arthritic control) compared with Group IV (normal control) and Group II and Group III (treatment groups) compared with Group I (arthritic control). #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 versus Group IV. **P* < 0.05, ***P* < 0.01, ****P* < 0.001versus Group I.)

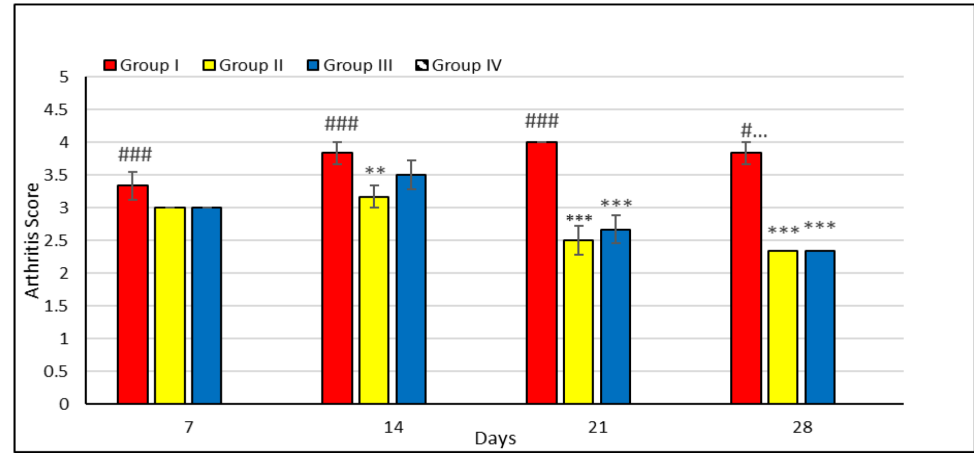

Fig. 13: Effect of gel phytoformulation on arthritis score in CFA induced arthritic rats. (Results expressed as mean±SEM (n = 6) and analysed by one- way ANOVA followed by Tukey's post hoc test. Group I (arthritic control) compared with group IV (normal control) and Group II and Group III (treatment groups) compared with Group I (arthritic control). #*P* < 0.05, ##*P* < 0.01, ###*P*< 0.001 versus Group IV. **P*<0.05, ***P*<0.01, ****P*<0.001versus Group I.)

Table 3: Effect of gel phytoformulation on blood parameters, biochemical markers and oxidative stress parameters in CFA induced arthritic rats

Parameters	Group I	Group II	Group III	Group IV
Toxicant	CFA	CFA	CFA	-
Treatment	Placebo	Gel phytoformulation	Diclofenac gel	-
Hb (gm %)	12.73 ± 0.28$^{###}$	14.35 ± 0.25***	14.05±0.2**	15.45±0.2
RBC (x 10^6/cmm)	7.48 ± 0.19$^{##}$	8.41 ± 0.16*	8.13±0.15**	9.16±0.24
WBC (x 10^3/cmm)	9.2 ± 0.23$^{##}$	8.03 ± 0.16**	8.17±0.16*	7.32±0.18
PLT (x 10^3/cmm)	380.67 ± 5.28$^{###}$	281.33 ± 5.25***	289.67±7.64***	272±5.69
PCV (%)	38.38 ± 0.47$^{###}$	43.55 ± 0.51***	43.88±0.85***	43.63±0.44
AST (IU/l)	238.83 ± 3.28$^{###}$	115.83 ± 3.49***	122±3.65***	108.17±3.54
ALT (IU/l)	187 ± 1.84$^{###}$	114.5 ± 3.37***	117.83±2.41***	99.5±2.29
ALP (IU/l)	259.33 ± 1.91$^{###}$	127.83 ± 1.4***	139.67±1.74***	108.5±1.5
SOD (U /mg protein)	11.94 ± 0.25$^{###}$	19.07 ± 0.36***	18.37±0.37***	22.55±0.8
GPx (mcg/mg protein)	2.73 ± 0.04$^{###}$	3.8 ± 0.05***	3.67±0.06***	4.35±0.13
CAT (U /mg protein)	118.33 ± 3.5$^{###}$	185.5 ± 3.73***	172.33±2.62***	207.17±4.02
LPO (Nmole / g Hb)	128.23 ± 2.79$^{###}$	104.25 ± 3.43***	108.06±1.73***	99.86±2.43

The data were expressed as mean ± SEM of 6 rats and analysed by one- way ANOVA followed by Tukey's post hoc test. Group I (arthritic control) was compared with Group IV (normal control) and Group II and Group III (treatment groups) were compared with Group I (arthritic control). #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus Group IV. *P<0.05, **P<0.01, ***P<0.001versus Group I.

Mowat studied hematologic abnormalities in rheumatoid arthritis [48]. Pathophysiological changes due to arthritis included abnormal storage of iron in the body and the failure of bone marrow to respond to anemia. He concluded that "Most of these abnormalities are related to the activity of the disease and return to normal when this is controlled." We observed similar effects in experimental animals for hemoglobin content. CFA stimulated the immune system and caused an increase in WBC count. The treatment had an immunomodulatory effect and a decrease in WBC count was observed.

AST and ALT are released by hepatocytes and elevated levels indicate liver injury. ALP is the indicator of bone mineralization [49]. Higher AST, ALT and ALP levels are observed in arthritic patients [50]. Reduction in levels of AST, ALT and ALP implies that the gel phytoformulation can relieve the liver toxicity induced by CFA and prevent the bone changes.

SOD is an essential enzyme found in all living cells. It eliminates superoxide radicals by conversion to hydrogen peroxide and oxygen. The activated neutrophils must be safely removed to control the inflammation. SOD induces neutrophil apoptosis [51]. Decreased levels of SOD are observed in arthritic patients [52]. The upregulation of SOD, GPx and CAT in treatment groups indicated the restoration of the antioxidant defense mechanism. The gel phytoformulation helped to restore free radical scavenging ability. Antioxidants have been found to be useful in the treatment of arthritis [53]. Curcuminoids and piperine, being antioxidants in nature, were found to be effective in the treatment. Oxygen-free radicals produced in the hypoxic environment of synovium react with unsaturated fatty acids to form lipid peroxidases. Thus, Group I arthritic untreated rat showed elevated LPO. This LPO can disrupt cell membranes and proteins, leading to further aggravation of disease. Group II and Group III (the arthritic rats which received treatment) showed LPO levels under control.

CONCLUSION

Curcuminoids and piperine are abundantly available phytoconstituents with known antiarthritic activity. They have poor solubility and permeability. Carefully selected combination of lipid, surfactant and cosurfactant could form a stable and patient compliant dosage form. The selected microemulsion gel showed promising results in physico-chemical studies. It had good cellular compatibility. The results revealed that repeated application of gel phytoformulation could significantly inhibit the complications associated with arthritis in CFA induced arthritic rats. It is an easy to scale up formulation and can be further studied in human volunteers.

ABBREVIATIONS

Complete Freund's adjuvant (CFA), Hemoglobin (Hb), Red Blood Cells (RBC), White Blood Cells (WBC), platelets (PLT), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidase (LPO).

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AUTHORS CONTRIBUTIONS

Data acquisition and drafting of the manuscript was done by Roopam Raut. Design of study and data analysis was done by Roopam Raut and Jessy Shaji. Final approval was given by Jessy Shaji.

CONFLICT OF INTERESTS

Author declares no conflict of interest.

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