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Proniosomal formulation of curcumin having anti-inflammatory and anti-arthritic activity in different experimental animal models

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Curcumin, the active ingredient of the spice turmeric, has a long history as an herbal remedy for a variety of diseases. Transdermal drug delivery has been recognized as an alternative route to oral delivery. Proniosomes offer a versatile vesicle delivery concept with the potential for drug delivery via the transdermal route. In this study, different proniosomal gel bases were prepared by the ether injection method, using Span 60 and Span 80, Tween 20, cholesterol, and formulation PA2. They were characterized by scanning electron microscopy, revealing vesicular structures, and assessed for stability and effect on *in vitro* skin permeation using rat skin. Anti-inflammatory and anti-arthritic effects of formulation PA2 and PB1 were compared with a standard market product containing indomethacin. The effect of formulation PA2 and PB1 was evaluated for acute inflammation in carrageenan induced rat paw edema and for chronic inflammation in complete Freud's adjuvant (CFA) induced arthritis in rats. Further histopathological and radiographic evaluation was performed. The investigated curcumin loaded proniosomal formula proved to be non-irritant, non-toxic, but had lower anti-inflammatory and anti-arthritic effects than the marketed indomethacin products.

1. Introduction

Curcumin [(1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the active ingredient of the spice turmeric, used in cooking in India and other regions of Asia. Its origin is the plant *Curcuma longa* L., which belongs to the Zingiberaceae family and can be found in India (Wahlstrom et al. 1978). Curcumin is poorly absorbed in the lower GIT and has a short elimination half-life of ~0.39 h. The poor bioavailability (< 1%) of the molecule owing to the insolubility at gastric pH and degradation at alkaline pH of intestine in the human body has severely limited its clinical application. High oral doses (8 g/day) in humans result in C_{max} of $< 2\,\mu M$ (Apisariyakul et al. 1995). However, curcumin has substantial anti-inflammatory activity. It inhibits several enzymes involved in the onset of inflammation, including cyclooxygenase-2, or COX-2. By suppressing this enzyme, curcumin reduces the production of prostaglandins (Kumar et al. 1998).

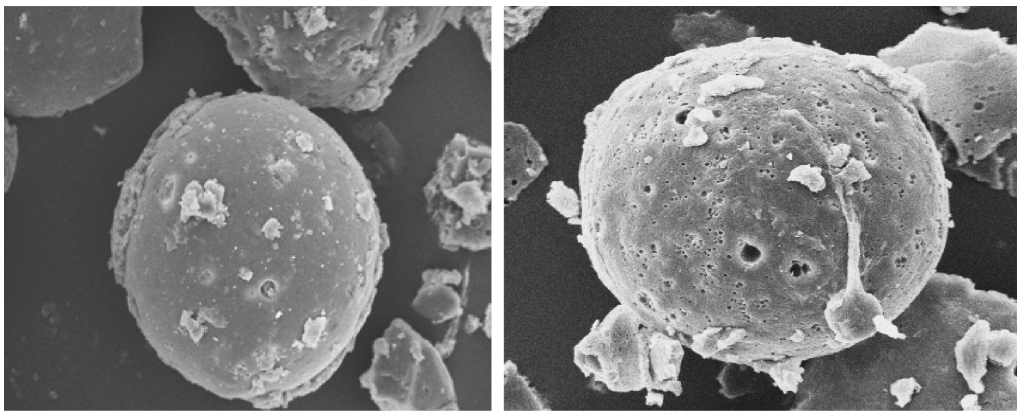
Proniosomes are liquid crystalline-compact niosomal hybrids which could be converted into niosomes upon hydration with water offering a versatile vesicle delivery concept with potential for transdermal drug delivery. Upon skin application proniosomes get hydrated with water from skin under occlusion (Manconi et al. 2006). Proniosomes should be hydrated to form niosomal vesicles before the drug is released and permeates across the skin. Both phospholipids and non-ionic surfactants in proniosomes can act as penetration enhancers, since it was found that some phospholipids are able to fluidize the stratum corneum lipid bilayers and diffuse through them (Balakrishnan 2009). Proniosomes provide additional convenience of transportation, distribution, storage and dosing. They are known to avoid many of the problems associated with either the aqueous niosome dispersion, as problems of physical stability (aggregation, fusion, leaking), or liposomes, as degradation by hydrolysis or oxidation as well as sedimentation, aggregation or fusion during storage (Vora et al. 1998). Proniosomes should be hydrated to form niosomal vesicles before the drug is released and permeates across the skin. The niosomes can be prepared from the proniosomes by adding the aqueous phase with the drug to the proniosomes with brief agitation at a temperature greater than the mean transition phase temperature of the surfactant (Almira et al. 2001).

Thereby, the present study aims at designing a new transdermal formulation for curcumin characterized by safety and high therapeutic efficacy, through designing an optimum proniosome gel formulation so as to reduce the daily administered dose of curcumin with a subsequent improvement in patient compliance and drug safety.

2. Investigations, results and discussion

Prepared niosomes reveal that they are discrete and spherical shape, and some vesicles are slightly elongated (Fig. 1).

In vitro skin permeation studies show a maximum release for formulation PA1 i.e. 89.9% in 24 h and formulation PB2 shows a minimum release i.e. 58.614% and formulation PA2 and PB2 shows 84.919% and 58.614% respectively (Fig. 2). The amount of drug retained within the vesicles under defined conditions ultimately governs the shelf life of the drug. The results showed



(a) (b)

Fig. 1: SEM of (a) formulation PA2 and (b) PB1

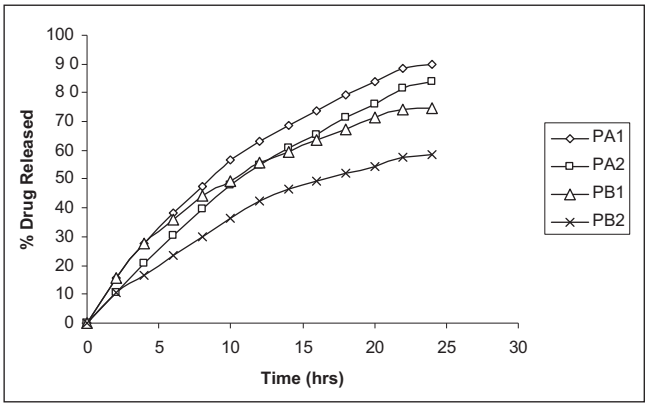


Fig. 2: Cumulative amount of drug permeated through rat abdominal skin from proniosomal formulations

that proniosomal gel formulation was quite stable at refrigeration and room temperatures as not much leakage of drug was found at these temperatures (Fig. 3).

Percent drug retained at 45 °C might have decreased due to the melting of the surfactant and lipid present in the formulation. Therefore, the proniosomal gel formulations can be stored at either refrigeration or room temperature. Formulation PA2 shows 99.5% drug content at refrigeration condition and 99% and 91% at room temperature and oven temperature respectively. Formulation PB1 shows 99.49% drug content at refrigeration condition and 99.22% and 93% at room temperature and oven temperature respectively in a study of four weeks.

The selected proniosome formulation PA2 and PB1 showed an irritation potential of 0.33, and 0.28 thus proving to be non-irritant as it was mentioned by Van-Abbe et al. (1975), that a value between 0 and 9 in an irritancy test indicates that the applied formulation is generally non-irritant to human skin. No obvious erythema, oedema or inflammation was observed on rabbits' skin after one week of application of the selected formulation. Local injection of carrageenan into rat hind paw induces acute inflammatory responses such as edema (Sidhapuriwala et al. 2007). The development of the edema induced by carrageenan has been described as a biphasic event. A rapid early phase (up to 2 h) is triggered by the concerted release of histamine, bradykinin, 5-hydroxytryptamine or cyclooxygenase products. And a more sustained late phase (2 to 5 h) is regulated by neutrophil infiltration and sustained production of arachidonic metabolites (prostanoids) (primarily by cyclooxygenase) or nitric oxide from inducible nitric oxide synthase (Maleki et al. 2001). Standard indomethacin shows 34.861% mean paw volume percentage inhibition, while PA2 and PB1 shows 8.5976% and 20.172% respectively (Table 1).

Mean percentage inhibition of paw thickness and volume by standard and formulation indomethacin and treatment is shown in Fig. 4.

Adjuvant-induced arthritis in rats is a well established experimental model that has features similar to the human rheumatoid arthritis. In addition, it is a good chronic inflammatory model for development of potential analgesic and/or anti-inflammatory drugs useful for arthritis treatment. Adjuvant arthritis is characterized by chronic proliferative and inflammatory reactions in synovial membranes, producing pain, disability and eventu-

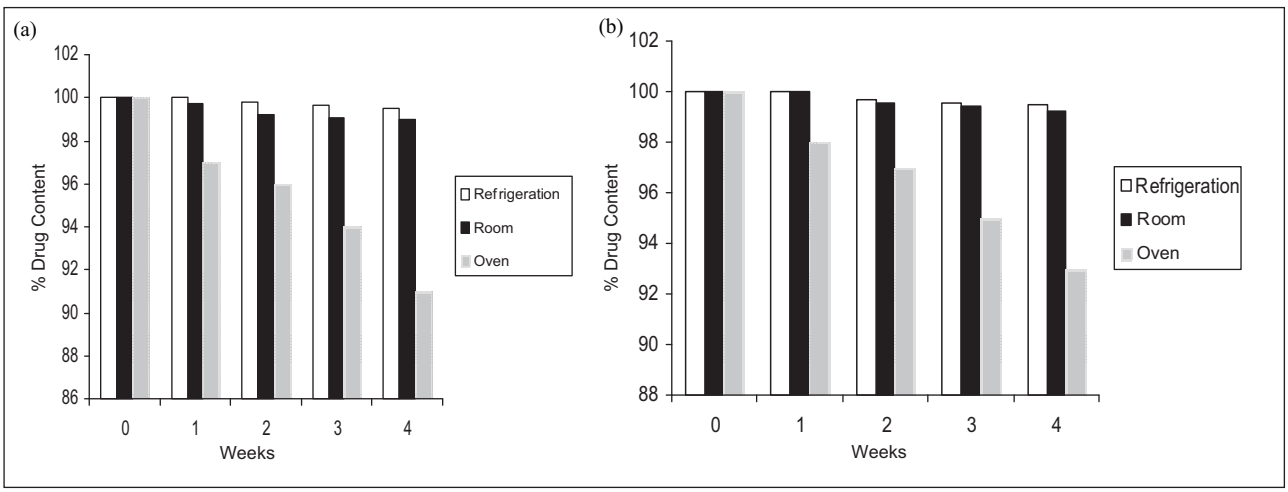


Fig. 3: Stability study of proniosomal formulation (a) PA2 and (b) PB1 at different conditions

Table 1: Percentage inhibition on CFA induced paw edema volume by different treatment

S. N.	Treatment	Percent inhibition of paw edema				Mean of % inhibition
		Day 5	Day 10	Day 15	Day 20	
1.	Standard (10 mg/kg, I.P.) (indomethacin)	30	64	80	98	68
2.	Formulation PA2, 2 g	9	20	45	64	34.5
3.	Formulation PB1, 2 g	15	45	65	88	53.25

N = 6, $p < 0.05$

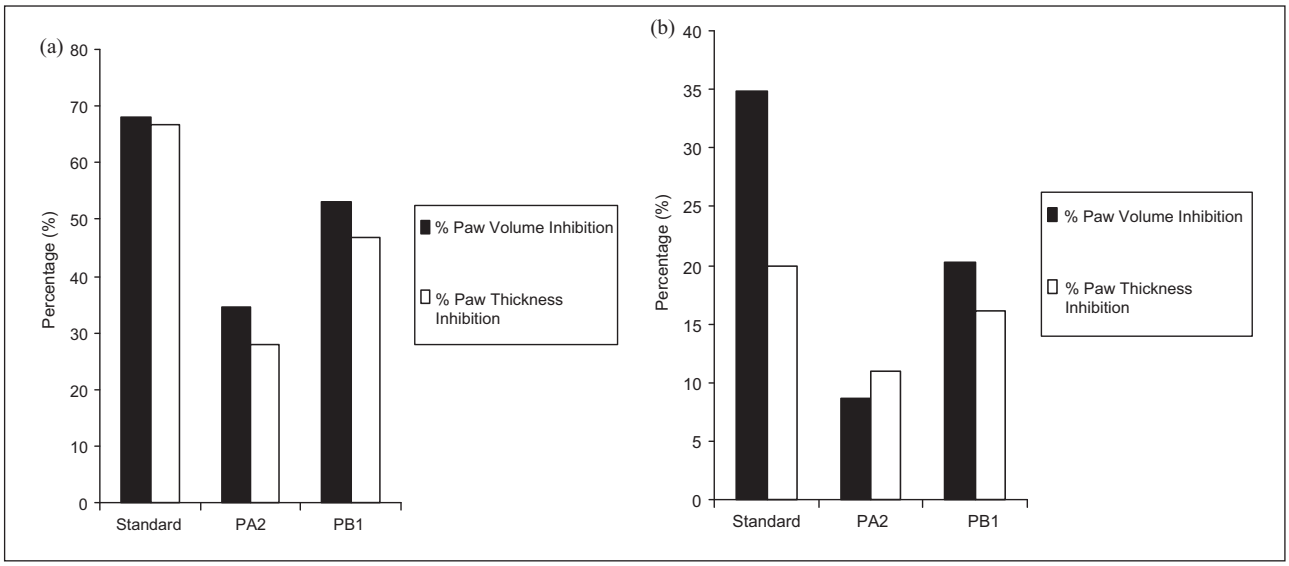


Fig. 4: Mean % edema inhibition by different treatment induced by (a) carrageenan and (b) CFA

ally destruction of joints (Holoshitz et al. 1983). Proniosomal formulation PA2 and PB1 shows significant prevention of paw edema, as compared to standard indomethacin 10 mg/kg. Radiographic examination of curcumin and CFA injected hind paws of the arthritic control rats on 20 day revealed the severe soft tissue swelling, narrowing of the joint spaces and the subsequent destruction of the bones and cartilages in the knee joint as compared to the normal control (Fig. 5).

The treatment with proniosomal formulation PA2, PB1 and indomethacin 10 mg/kg showed marked inhibition of the soft tissue swelling and the destruction of the knee joints as compared to the normal control. The tissue sections of joints of carrageenan and CFA injected in hind paws rat revealed the pathological changes, which can be correlated with arthritis, as compared to the normal control (Fig. 6). In particular massive influx of inflammatory cells, synovial hyperplasia, and accumulation of abundant mono- and poly-morphonuclear cells in the joint and congestion of vessels was evident. The treatment with proniosomal formulation PA2, PB1 and indomethacin 10 mg/kg (Fig. 6) was shown marked reduction of the histological injury of joint tissue sections and most of the histological changes were minimized and found negligible as compared to the arthritis control. In particular, it was shown the regeneration of synocytes and disappearance of inflammatory exudates, mild focal infiltration of cells in synovial region and few cuboidal cells lining the synovial membrane. In arthritic condition, there is a mild to moderate rise in WBC count due to the release of IL-IB inflammatory response, IL-IB increases the production of both granulocyte and macrophages colony stimulating factors. In the present study, the migration of leucocytes into the inflamed area is significantly suppressed by formulation PA2 and PB1 when compared to standard drug indomethacin, as seen from the significant reduction in the total WBC count. Erythrocyte Sedimentation Rate (ESR) is an estimate of the suspension stability of RBC in plasma. As the incidence and severity of arthritis increased, the changes in the body weights of the rats also occurred during the course of the experimental period.

Hematological parameters of the investigated animals were recorded and are given in Table 2.

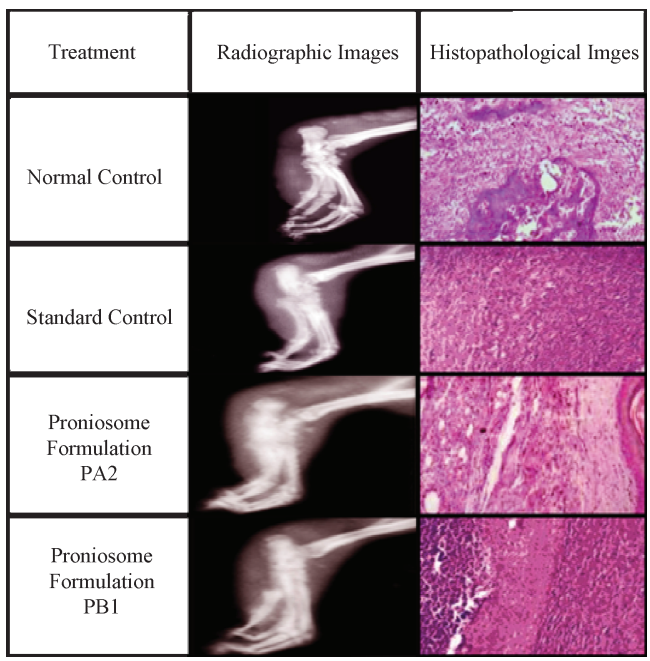


Fig. 5: Effects of different treatment on radiographic evaluation and histopathological changes induced by CFA in arthritic rat after 20 days

Table 2: Effects on hematological parameters and body weight in rats after 20 days

Treatment	Total WBC count (cells/cu. mm)	RBC count (million/cu. mm)	Hb (g %)	ESR (mm/h)	Mean body weight (g)	
					Before Induction (g)	After Induction (g)
Control	7.45 ± 0.05	5.3 ± 0.06	13.9 ± 0.04	4.06 ± 0.07	158 ± 0.21	163 ± 0.41
Standard (indomethacin)	7.12 ± 0.04	5.26 ± 0.02	14.05 ± 0.07	3.28 ± 0.09	156 ± 0.55	190 ± 0.31
Formulation PA2	7.08 ± 0.03	5.45 ± 0.08	14.20 ± 0.04	3.24 ± 0.04	154 ± 0.49	176 ± 0.28
Formulation PB1	7.06 ± 0.09	5.52 ± 0.09	14.45 ± 0.03	3.20 ± 0.03	153 ± 0.63	170 ± 0.82

N = 6, $p < 0.05$

Table 3: Composition of formulations

S.N.	Formulation Code	Diethyl ether (ml)	Drug (mg)	Surfactant:Cholesterol ratio	Span 60 (mg)	Span 80	Cholesterol (mg)
1.	PA1	2	100	1:4	50	–	200
2.	PA 2	2	100	1:3	50	–	150
3.	PB1	2	100	1:2	–	50	100
4.	PB 2	2	100	1:1	–	50	50

In conclusion, both developed formulation shows stability at room temperature, under refrigeration conditions and at oven temperature and significant controlled *in-vitro* skin permeation release. Proniosomal formulations PA2, PB1 used in this study showed significant reduction of paw edema thickness and volume at 8 h or more after carrageenan injection, demonstrated that the proniosomal gel possess fairly good anti-inflammatory activity. Proniosomal formulations PA2, PB1 shows sufficient anti-arthritic activity in CFA induced model for chronic level studies of 20 days. Anti-inflammatory and anti-arthritic activity of both formulations was similar to indomethacin but not as good as a commercial product. All formulation i.e. standard market product indomethacin and proniosomal formulations PA2, PB1 showed potent anti-inflammatory and anti-arthritic activity and the potency of the treatment follows the order

Standard > PA2 > PB1

However, future studies with inclusion of penetration enhancer in proniosomal gel formulation may provide a curcumin loaded proniosomal gel and herbal formulation comparable to topical NSAIDs can be produced.

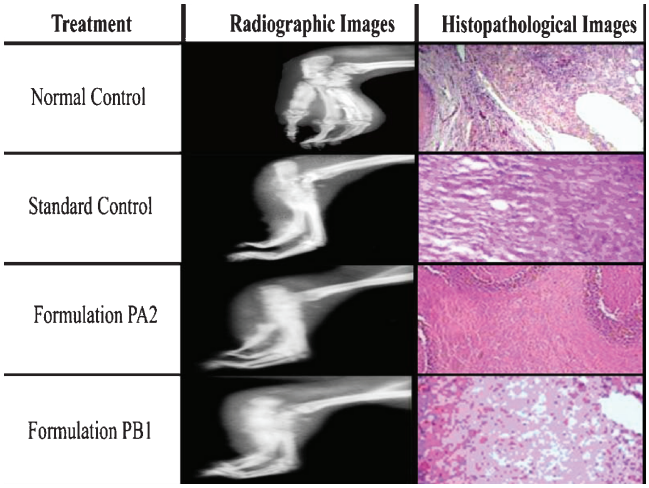


Fig. 6: Effects of different treatment on radiographic evaluation and histopathological changes during inflammation induced by in rat in a study of 8 h

3. Experimental

3.1. Materials

Curcumin was procured as the gift sample from Krish Enteprizes, Mumbai, India. Span 80 was purchased from CDH, Delhi. All other chemicals used were of analytical grade.

Different proniosomal formulations were prepared by ether injection method (Liberman et al. 1989). Proniosomes containing curcumin of 1:1 ratio was prepared by taking cholesterol (50 mg), Span-80 (50 mg equivalent) in a 50 ml beaker. The mixture was dissolved in diethyl ether and the solution was slowly injected into a beaker containing curcumin in phosphate buffer saline (pH 7.4). The temperature maintained during the injection was 40–60 °C. The differences in temperature between phases cause rapid vaporization of ether resulting in spontaneous vesiculation. Similarly other three ratios 1:2, 1:3 and 1:4 were prepared.

3.2. Scanning Electron Microscopy (SEM) studies

Selected formulation PA2 was sputtered coated using pelco gold palladium coaters. The surface morphology of the layered sample was examined using SEM. The sample were placed in an evacuated chamber and scanned in a controlled pattern by an electron beam. Interaction of the electron beam with the specimen produces a variety of physical phenomenon that detected, are used to form images and provide information about the specimens (Mahmoud et al. 2009).

3.3. Stability studies

The ability of vesicles to retain the drug (drug retention behaviour) was assessed by keeping the proniosomal gel at three different temperature conditions, i.e., refrigeration temperature (4–8 °C), room temperature (25 ± 2 °C) and oven (45 ± 2 °C) .Throughout the study, proniosomal formulations were stored in aluminium foil-sealed glass vials. The samples were withdrawn at different time intervals over a period of one month and drug leakage from the formulations was analyzed for drug content spectrophotometrically (Solanki et al. 2008).

3.4. In-vitro skin permeation study

The *in vitro* rat skin permeation study was carried out as per the guidelines compiled by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animal, Ministry of Culture, Government of India) and all the study protocols were approved by the local institutional Animal Ethics Committee (PSIT, Kanpur, India). Also an international protocol for conducting experiments on animals was followed (Uchegbu et al.1998). The abdominal hair of albino rats (wistar strain), weighing 200 ± 20 g, was shaved using hand razor. Care was taken not to damage the skin surface. Rats were sacrificed by administration of excess chloroform inhalation and the abdominal skin of the rat was separated. The skin was stored at −20 °C and used within three days for the permeation study. Before the permeation study, the skin was hydrated in phosphate buffer pH 7.4 (containing 0.02% sodium azide as a preservative) at 4 °C over night and the adipose tissue layer of the skin was removed by rubbing with a cotton swab. The permeation of drug from proniosomal gel formulations was determined in a Franz diffusion cell. The excised rat skin was mounted on the receptor compartment

with the stratum corneum side facing up-wards into the donor compartment. The donor compartment was filled with the proniosomal gel formulation. A 15 ml of pH 7.4-phosphate buffer containing 10% PEG was used as receptor medium to maintain sink conditions. The available diffusion area of cell was 3.14 cm^2. The receptor compartment was maintained at $37 \pm 1\,^{\circ}C$, with magnetic stirring at 600 rpm. The samples from the receptor compartment were withdrawn at predetermined time intervals and immediately replaced by an equal volume of fresh buffer solution. Initial experiments confirmed the maintenance of sink conditions by this procedure. The samples withdrawn from the receptor compartment were then analyzed by an UV spectrophotometers.

Curcumin formulations that are administered into the animals for *in vivo* evaluation in rats were selected based on *in vitro* release studies and stability studies. Pharmacokinetics was evaluated using all the compositions containing curcumin selected in this study.

3.5. Skin irritancy test

Irritancy test was carried out to determine possible localized reaction of the selected formula on the skin since skin safety is a major concern in transdermal drug delivery. A single dose of 200 mg of the selected medicated formulations (PA2 and PB1) was applied to the left side of the shaved back of male albino rabbits (1.5 ± 0.5 kg) and the right side was considered as control. The control area was further divided into two sub areas, one receiving the selected formulation unloaded with the drug (positive control) and the other receiving no treatment (negative control). The development of erythema was monitored daily for 6 days. Extents of development of erythema were indicated on the basis of the following 0: no erythema development; 2: barely visible few blood vessels and light erythema development; 4: main blood vessels visible and slight erythema development; 6: main blood vessels more obvious and slight erythema development. Irritation potential was calculated using the following equation (Van et al. 1975).

$$\text{Resulttant index} = \frac{\text{A.B}}{\text{No. of days of observation}} \quad (1)$$

where *A* and *B* represent erythema value and corresponding day, respectively.

3.6. Anti-inflammatory studies

Healthy albino rats of either sex (Wistar strain) weighing 160–190 g were used for the present study. The animals were kept in plastic cages with soft bedding (6 per cage). The animals had free access to food and water and were maintained under controlled temperature (27 ± 2 C) and a 12 h:12 h light and dark cycle. They were allowed to acclimatize for one week before the experiments. Initial body weight of each animal was recorded. Before the experiment, food was withdrawn overnight but adequate water was given to the rats.

This study was performed according to Alol et al. (1993). Due to painful condition imposed on animals the numbers of subjects used were restricted to the minimum six per group that allowed reliable statistical analysis of the results. The animals were divided into four groups of 6 animals each. The control group received normal saline, the standard group was treated with indomethacin given i.p., the test group received 2 g proniosomal gel of formulation PA2 and PB1 applied over 9 cm^2 as transdermal patch on the dorsal skin after removing the hair with a clipper. The area of application was occluded with bandages and it was left in place for 2 h. The dressing was then removed and the gel remaining on the surface was wiped off with cotton. The animals were then injected with 0.1 ml of 1% carrageenan solution in saline in the plantar region of left hind paw and the paw volume was measured after 1, 2, 4, 6, 8 h with a water plethysmometer. The right hind paw served as a reference non inflamed paw for comparison.

3.6.1. Paw volume

The initial rat paw volume was measured using a plethysmometer (Model 7150, UGO Basile, Italy). The paw was marked with ink at the level of lateral malleolus and immersed in mercury up to the mark in the plethysmometer. Mean changes in paw volume were calculated and % inhibition of paw edema was calculated using formula:

$$\%\,\text{Inhibition} = \frac{\text{Vc} - \text{Vt}}{\text{Vc}} \times 100 \quad (2)$$

where, Vc is the mean changes in paw volume of control group and Vt is mean changes in paw volume treated group.

3.6.2. Paw thickness

Paw thickness was measured by compressing the joint by rotating the screw of micrometer screw gauge till the pain elicited as indicated by squeaking or leg withdrawal. The distance moved by the screw gauge was recorded and % inhibition of paw thickness was calculated using formula:

$$\%\,\text{Inhibition} = \frac{\text{Tc} - \text{Tt}}{\text{Tc}} \times 100 \quad (3)$$

where, Tc is mean change in paw thickness of arthritis control group and Tt is mean changes in paw thickness of treated group.

3.7. Anti-arthritic activity

Freud's adjuvant induced arthritis model was used to assess the anti-arthritic activity in albino rats (Newbould 1963). Animals were divided into three groups of six animals each. Group I served as control, which received 5% gum acacia suspension, Group II served as reference standard, which received 10 mg/kg body weight IP of indomethacin, and Group III and IV served as test, which received 2 g proniosomal gel of formulation PA2 and PB1 applied over 9 cm^2 as transdermal patch on the dorsal skin after removing the hair with a clipper. Arthritis was induced by injecting 0.05 ml of suspension of killed *Mycobacterium tuberculosis* bacteria (0.5% w/w) homogenized in liquid paraffin into the left hind paw. Drug treatment was started from the initial day i.e. from the day of adjuvant injection (0 day), 30 min before adjuvant injection and continued till 20 day. Paw volume and thickness were measured on 5th, 10th, 15th and 20th day with the help of a plethysmometer and micrometer screw gauge respectively. The mean changes in injected paw edema with respect to initial paw volume and thickness, were calculated on respective days and percentage inhibition of paw edema with respect to untreated group (control) was calculated.

3.8. Radiographic evaluation

The rats were anaesthetized using ketamine (100 mg/kg, i.p.) and radio graph was recorded on a digital system and seamen's X-ray machine after 20 days (Van Ede et al. 1998).

3.9. Histopathological studies

The hind paw was amputed above the knee joint and fixed in 10 % formalin solution. The sections were stained with haemtoxylin and eosin and were examined microscopically for histopathological changes

3.10. Hematological parameters and body weight

The changes in body weight were recorded daily. After 20 day, blood was withdrawn through the retro-orbital vein puncture of all groups after anaesthetizing the animals with diethyl ether. The biochemical parameters such as hemoglobin content, total WBC count, ESR and RBC were analyzed.

3.11. Statistical analysis

Data was expressed as means $\pm$ SEM and analyzed for statistical significance using Student's t test, one-way analysis of variance (ANOVA) followed by Dunnett's test or two-way ANOVA followed by Bonferroni test. $P < 0.05$, $p < 0.01$ and $p < 0.001$ was considered to be significant

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