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Pharmacognostical Study, Phytochemical Screening And Formulation Development From Achras Sapota Bark Extract For Anti-Inflammatory Anti-Arthritis And Skin Irritation Study

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Abstract
This study examines the pharmacognostic, phytochemical, and pharmacological characteristics of the bark extract of Achras sapota (Sapodilla) to facilitate the development of a phytopharmaceutical formulation. Extraction utilized solvents of increasing polarity petroleum ether, chloroform, and methanol resulting in yields of 2.5%, 1.9%, and 10.0% (w/w), respectively. Initial phytochemical analysis indicated the presence of alkaloids, steroids, and Triterpenoids in the petroleum ether extract; glycosides, alkaloids, and carbohydrates in the chloroform extract; and tannins and flavonoids in the methanol extract. The chromatographic analysis via TLC and HPTLC revealed unique phytoconstituents with RF values between 0.79 and 0.98. UV spectral analysis indicated a peak absorption at 229.8 nm, implying the existence of conjugated systems. IR spectroscopy of the isolated product identified typical functional groups, including hydroxyl, carbonyl, and aromatic moieties, signifying the presence of phenolic and carbonyl-containing compounds. ^1H NMR spectroscopy validated the identification of Gallic acid, a Polyphenolic molecule, as a principal ingredient. The antioxidant capacity was evaluated with the DPPH radical scavenging test. The bark extract demonstrated significant free radical scavenging activity, with percent inhibition between 39% and 52.89%, in comparison to normal ascorbic acid (40.5%–65.04%), suggesting considerable antioxidant capacity. In conclusion, the results indicate that Achras sapota bark is a substantial source of bioactive phytochemicals, including gallic acid, which may possess antioxidant properties. These findings warrant additional investigation and application in the manufacture of herbal remedies for therapeutic purposes.
Keywords: Achras sapota, *pharmacognosy, phytochemicals, herbal formulation, bark extract, medicinal plants, skin irritation test, anti-inflammatory activity, anti-arthritis activity.*

INTRODUCTION

Plants have been the main source of healthcare for most of the last two thousand years of human civilization. [1] Approximately 6,000 of the numerous species of plants that have been found are recognized to offer some kind of medical use. This is true even if a lot of different types of higher plants have been found. There has been a significant rise in the need for studies on these plants because of the huge potential they have to improve medical care throughout the world.[2,3] The World Health Organization (WHO) says that almost 80% of people on Earth depend on traditional medicine to take care of their fundamental health needs[4] Traditional medicine often uses plant-based extracts or the active parts of plants.[5,6] In the last several decades, a lot of work has gone into finding out how these more common methods work.[7,8] The commercial value of these plants has risen significantly in recent decades as a direct result of the meteoric expansion of the acceptance of alternative medical practices. In the Himalayan region specifically, the use of herbal remedies plays a very vital role. [9, 10] These regions are home to a plethora of plant species, many of which have the ability to treat a range of diseases. [11] The increasing demand for plant-based therapeutics has revitalized interest in the pharmacognostical and phytochemical study of traditionally used medicinal plants. Among these, Achras sapota (L.), also known as Manilkara zapota, is a member of the Sapotaceae family and is widely grown in tropical and subtropical regions. Though primarily cultivated for its sweet and fleshy fruit commonly referred to as sapodilla or chikoo various other parts of the plant, including the bark, leaves, and seeds, have been employed in folk medicine across Asia and Central America for their potential therapeutic benefits [12, 13].The bark of A. sapota has been traditionally used to treat ailments such as diarrhea, inflammation, and fever [14]. Once

the pharmacognostical and chemical properties of A. sapota bark are known, extracts from it might be used in topical or oral medicines. The Achras sapota tree is an evergreen tree that grows in tropical areas. It is grown a lot in tropical areas for its edible fruit, which is also called chikoo. People eat and study the fruit a lot, but folk medicine has utilized different parts of the plant, such the bark, to cure diarrhea, fever, wounds, and inflammation for a long time [15].

MATERIALS

Standardization of plant material

Collection-plant material was collected from ....

Authentication-Pharmacognostic study of the plant material was authenticated by Vineet Rawat deputy director Botanica Survey of India, Koregaon road, Pune

METHODS

Extraction methodology [14, 15, 16, 17]

I. Petroleum Ether (60-80°C) Extract
II. Chloroform Extract
III. Methanol Extract

Preliminary phytochemical screening for various extracts of plant Achras sapota

Test for Carbohydrates, reducing sugars, monosaccharides, Proteins, amino acids Steroids Cardiac Glycosides Anthraquinones Glycosides saponin Glycoside Tannins and Phenolic compounds and Flavonoids.

Phytochemical investigations

a. TLC of methanolic & EAS extract of plant Achras Sapota
b. Development of HPTLC Technique of methanolic extract of plant of Achras Sapota.
c. Column Chromatography
d. Spectral analysis of isolated fraction of extract of Achras Sapota bark

CHROMATOGRAPHIC SEPARATION:

A. Thin Layer Chromatography:

Methanolic extract and Ethyl acetate soluble fraction of methanolic extract was evaluated by TLC to identify the presence of number of phytoconstituents present in an extract using specific solvent system which was found to give proper separation.

Steps involved in performing TLC of extracts:

Preparation of TLC plate: Prepared the slurry of adsorbent media (silica gel-G) in distilled water and poured the slurry on the TLC glass plates to obtain a thin layer.

Activation of TLC plate: Heating in oven for 30 min. at 105°C activated TLC plate.

Sample application: Dipping the capillary into the solution to be examined and applied the sample by capillary touched to the thin layer plate at a point about 2 cm from the bottom. Air-dried the spot.

Chromatogram development: After the saturation of chamber and spotting of samples on plate, it was kept in chamber. The solvent level in the bottom of the chamber must not be above the spot that was applied to the plate, as the spotted material will dissolve in the pool of solvent instead of undergoing chromatography. Allowed the solvent to run around 10-15 cm on the silica plate

Chamber saturation: The glass chamber for TLC should be saturated with mobile phase. Mobile phase was poured into the chamber and capped with lid. Allowed saturating about 30 min.

Visualization: Plates were removed and were examined visually, under UV and suitable visualizing agent (Alcoholic ferric chloride solution) after that R_F was calculated by following formula.

$$R_F = \frac{\text{Distance traveled by solute from origin line}}{\text{Distance traveled by solvent from origin line}}$$

B. High performance thin layer chromatography (HPTLC):

Reagents and other materials.

STD Gallic acid and, toluene, ethyl acetate, and methanol [all reagents of analytical grade, E-Merck] and silica gel 60F254 precoated TLC aluminium plates [E-Merck].

Apparatus

Spotting device: Linomat V Automatic Sample Spotter; CAMAG (Muttenz, Switzerland)

Syringe: 100µL Hamilton (Bonadzu, Switzerland)

Thin layer chromatography (TLC) Chamber: Glass with trough chamber (20×10 ×4 cm) (CAMAG)
Densitometer: TLC scanner 3 linked to WinCats Software (CAMAG)
HPTLC plates: 10×10 cm, 0.2 mm thickness precoated with silica gel 60 F254 (E. Merck, Mumbai, India)
Experimental conditions: Temperature 25 ± 2 °C, relative humidity 40 %
Mobile phase: - Toluene: Ethyl acetate: methanol (5:4:1)
Preparation of standard and sample solutions
Standard solution of Gallic acid
A stock solution of Gallic acid was prepared by dissolving 10 mg of accurately weighed gallic acid in methanol and making up the volume to 10 ml with methanol to get the final concentration of 1mg/ml.
Preparation of sample solutions.
Sample solution was prepared by dissolving 50 mg of the ethyl acetate fraction of methanolic extract in methanol and making up the volume to 5 ml to get the concentration of 10 mg/ml.

METHODOLOGY:
Exactly 10 µL of each of the standard solution of Gallic acid, methanol sample solution were applied in four spots of each on 10×10 cm TLC plates. The plates were developed in a solvent system of toluene: ethyl acetate: methanol (5:4:1) at 25 ± 20C temperature and 40% relative humidity and allowed to travel up to a distance of 8 cm. After development the plates were dried in air and scanned densitometrically at 254 nm for Gallic acid, & sample solution. The colour of the spots, peak areas and peak heights were recorded.
Specificity:
The specificity of the method was ascertained by analyzing the standard drug and extract. The spot for Gallic acid in the sample was confirmed by comparing the R_F values and spectra of the spot with that of the standard. The peak purity of the Gallic acid was assessed by comparing the spectra at three different levels, viz. peak start, and peak apex and peak end positions of the spot.
C. Column chromatography
Preparation of column:
150 Gms of silica gel for column chromatography was activated in hot air oven at 110°C for 1 hr. The adsorbent bed was developed in mobile phase which was initially packed with glass wool. The glass wool is fixed at the bottom of the column. The slurry of activated silica gel was made in Toluene : Ethyl acetate : methanol (5 : 4: 1) & charged into column in small portions by keeping knob open with gentle taping after each addition in order to ensure uniform packing. The small quantity of solvent was allowed to remain on the top of the column. The air bubbles present in the column were removed by gentle tapping to get uniform bed of adsorbent. The adsorbent bed was allowed to develop overnight taking care to prevent the drying of column by plugging open end with cotton and aluminium foil. The column was run fast for some times with mobile phase in order to remove any impurities. Prepared sample was then charged in to the column & was allowed to settle. A small cotton pad was placed above the sample to prevent the mixing of dust particles with the sample. Then it was eluted with mobile phase to collect fractions. Fractions collected were further concentrated. Each fraction was evaluated by TLC to detect the number of phytoconstituents present in it.
TLC of Isolated fraction after column chromatography:
Stationary phase : Silica gel G
Mobile phase : Toluene: Ethyl acetate: methanol
Proportion : 5: 4: 1
Visualizing agents: Alcoholic Ferric chloride reagents.
All fraction showed two spots on TLC plates.
Recolumn:
Preparation of sample for Recolumn: All fraction are collected in a beaker & concentrate on water bath to about 5ml fraction are left in a beaker. These fraction was mixed well with 2gm silica gel & dried in vacuum at 45°C. The adsorbed material obtained was transferred to the column. Then it was eluted with mobile phase to collect fractions. Fractions collected were further concentrated. Each fraction was evaluated by TLC to detect the number of phytoconstituents present in it.
TLC of Isolated fraction after Recolumn:
Stationary phase : Silica gel G
Mobile phase : Toluene: Ethyl acetate: methanol
Proportion : 5: 4: 1

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Visualizing agents: U.V 254nm

Fraction showing same number of compounds with same R_F values were combined, concentrated & evaporated to dryness.

Spectral analysis of isolated fraction-A:-

1. U.V. Spectrum:
2. Fourier transform infrared spectroscopy (FTIR):
3. H_1 Nuclear magnetic resonance. (NMR):

FORMULATION AND EVALUATION OF GEL

Preparation of Topical Gel

Different combinations of Achras Sapota Bark Extract (2.5%, 5%) were tried with different types of polymers (Sodium CMC, Carbopol 934) using various formulae. The following few combination with Carbopol 934 resulted in the best gel formulation, which was smooth and stable. Control sample also was prepared for testing of animal to check the activity of control ingredients.

Method for Preparation of Gel Containing Extract

1. 1 g of Carbopol 934 was dispersed in 50 ml of distilled water with continuous stirring.
2. 5 ml of distilled water was taken and required quantity of methyl paraben and propyl paraben were dissolved by heating on water bath.
3. Cool the solution, then to that added Propylene glycol 400.
4. Further required quantity of Achras Sapota Bark Extract was mixed to the above mixture and volume made up to 100 ml by adding remaining distilled water.
5. Finally full mixed ingredients were mixed properly to the Carbopol 934 gel with continuous stirring and triethanolamine was added drop wise to the formulation for adjustment of required skin pH (6.8-7) and to obtain the gel at required consistency.

Experimental design

The response surface methodology (RSM) was employed to perform Quality by Design approach for constructing and investigating the polynomial models, using fewer experimental runs. Central composite Design comprising of 2-factors and 3- levels was employed to examine the quadratic response surfaces by assessing the effect of pre-defined independent variables on different response dependent variables pH, Viscosity and Spreadability was coded as Y1 and Y2 and Y3. Two independent variables namely concentration of Bark Extract (%) and Concentration of carbopol (mg) were chosen. Each of the variables was varied at three different levels, known as high, medium and low levels. All the finalized independent variables and the response variables are described in Table 1.

Table No.1: dependent and independent variables

Coded level	**-1 (low)**	**+1 (high)**
Independent variables		
X1-Concentration of bark extract (%)	2.5	7.5
X2- Concentration of Carbopol 934 (mg)	500	1000

Dependent variables	
pH	Optimum
Viscosity	Optimum
Spreadability	Optimum

Table No.2 Formulation run of designed batches

Formulation Code	Concentration Bark extract (%)	Concentration of carbopol 934
F1	2.5	500

F2	2.5	1000
F3	8.53553	750
F4	7.5	1000
F5	1.46447	750
F6	5	1103.55
F7	7.5	500
F8	5	396.447
F9	5	750

EVALUATION OF TOPICAL GEL FORMULATION

A. Physical Evaluation

Physical parameters such as color and appearance were checked.

B. Measurement of pH

pH of the gel was measured by using pH meter.

C. Spreadability

Spreadability was determined by the apparatus which consists of a wooden block, which was provided by a pulley at one end5. By this method spreadability was measured on the basis of slip and drag characteristics of gels. An excess of gel (about 2g) under study was placed on this ground slide. The gel was then sandwiched between this slide and another glass slide having the dimension of fixed ground slide and provided with the hook. A. 1 kg weighted was placed on the top of the two slides for 5 minutes to expel air and to provide a uniform film of the gel between the slides. Excess of the gel was scrapped off from the edges. The top plate was then subjected to pull of 80 Gms. With the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5 cm be noted. A shorter interval indicates better spreadability. Spreadability was calculated using the following formula:

$$S = M \times L / T$$

Where,
S = Spreadability,
M = Weight in the pan (tied to the upper slide),
L = Length moved by the glass slide and
T = Time (in sec.) taken to separate the slide completely each other.

D. Viscosity

Viscosity of gel was measured by using Brookfield viscometer with spindle.

E. Stability Study

The stability study was performed as per ICH guidelines 6. The formulated gel was filled in the collapsible tubes and stored at different temperatures and humidity conditions, viz. 250 C ± 20C/ 60% ± 5% RH, 300 C ± 20C/ 65% ± 5% RH, 400 C ± 20C/ 75% ± 5% RH for a period of three months and studied for appearance, pH, viscosity and spreadability.

F. Acute Dermal Irritation Study

Acute Dermal Irritation Study of [F8 (optimized formulation of gels)] in Wistar Rats as per OECD Guideline No. 404

To evaluate the potential of the test item to produce dermal irritation or corrosion when applied to the skin of Wistar rats.

Test Guidelines

The study was conducted following the OECD Guideline No. 404 – "Acute Dermal Irritation/Corrosion".

Test System

Parameter	Details
Species	Wistar albino rats
Sex	Male
Weight Range	180–220 g
Number of Animals	3
Housing	Polycarbonate cages, standard laboratory conditions
Acclimatization	Minimum 5 days before dosing
Ethics Approval	[Institutional Animal Ethics Committee (IAEC) approval number]

Test Item

Parameter	Description
Name	F8 (OPTIMIZED FORMULATION OF GELS)
Appearance	[SEMISOLID Liquid]
Solubility	[Soluble in water AND other solvents]
Dose	0.5 g per animal (for semi-occlusive patch)
Application Area	6 cm² shaved dorsal skin

METHODOLOGY

- The fur was removed from the dorsal trunk area of each rat at least 24 hours before application.
- Dose applied: 0.5 g of test item on a 6 cm² area.
- The area was covered with a gauze patch and held in place with a non-irritating adhesive tape under a semi-occlusive dressing.
- Exposure duration: 4 hours.
- After removal of the dressing, residual test substance was gently wiped off with moistened cotton.
- The skin site was observed for erythema and edema at:
- 1 hour
- 24 hours
- 48 hours
- 72 hours' post-application

Scoring System

Score	Erythema & Eschar Formation	Edema Formation
0	No erythema	No edema
1	Very slight erythema	Very slight edema
2	Well-defined erythema	Slight edema
3	Moderate to severe erythema	Moderate edema
4	Severe erythema (beet redness) or eschar	Severe edema (raised > 1 mm, beyond exposure area)

G. ANTI-INFLAMMATORY ACTIVITY

Animal Model: carrageenan induced paw edema
Test Item: - F8 (optimized formulation of gels)
Dose: -
Inducer: - 1% freshly prepared suspension of carrageenan to normal distilled water (0.1 ml per animal)

- F8 (OPTIMIZED FORMULATION OF GELS)

Experimental design (Grouping)

Group	Description
Group I (Positive control Group)	Rats were subjected to normal distilled water.
Group II (Negative control Group)	Rats were subjected to after carrageenan injection of the hind paw and did not undergo any treatment.
Group III (Standard Group)	Rats were subjected to after carrageenan induced inflammation paw and treated with Diclofenac (10mg/kg) for topically administrate.

Group IV (Treatment group)	Rats were subjected to after carrageenan induced inflammation paw and treated with F8 for topically administrate.

- **Procedure:**

1. Wistar rats were divided into 5 groups, with 6 rats in each. The second group was inflamed by carrageenan injection and did not undergo any treatment.
2. The inflammation of the second group, used as reference, and that of the third to fifth group was treated by a topically administrate with test item (F8) and Diclofenac (5mg/kg), respectively, 1/2 hour before the carrageenan injection. The doses Test item (F8) and Diclofenac chosen during treatments were proportional in the size of the edema and covered the whole swelling. Test item and Diclofenac topically administrate.
3. In all treated groups, the size of the edema was measured before and after the inflammatory injection using a digital caliper. Edema was expressed as the relative increase in paw volume induced by the inflammation injection (i.e., the edema was proportional to the volume difference between 0 hours and the other times, 30 min, 60, 120, and 180 min, after carrageenan injection).
4. Percentile edema inhibition was calculated according to following

Formula: - Percentile inhibition = [1-(VT/V0)] X 100

VT represents the edema volume in the drug treated group.

V0 represents the edema volume in the Carr group.

H. Anti-arthritis Activity

Animal Model: formaldehyde induced paw edema

Test Item: - F8 (optimized formulation of gels)

Dose: -

Inducer: - 2% V/V freshly prepared to formaldehyde in normal distilled water (0.1 ml per animal)

Chronic phase of inflammation was induced in rats by subcutaneous injection of 0.1 ml (2 % v/v in normal saline) formaldehyde solution into the sub plantar region of right hind paw of Wistar albino rats on first and third day of the experiment.

The groups were as follows:

Group 1: Received distilled water (3 ml/kg p.o.)

Group 2: Received 2% formaldehyde solution (0.1ml) sub plantar injection.

Group 3: Received indomethacin (3 mg/kg p. o.)

Group 4: Received F8 (OPTIMIZED FORMULATION OF GELS)

Baseline paw edema of the right hind paw was measured on day 0 as the average of two measurements using a digital caliper, 3 hours prior to arthritis induction. [125,126] Paw edema measurements of the right hind paw of each rat were repeated at 2-day intervals up to day 10 after arthritic induction.

The change in paw edema, indicative of edema, was calculated as the difference between subsequent measurements.

- **Parameters:**

% inhibition of paw edema concerning untreated groups was calculated using the same formula:

I. RESULT AND DISCUSSION

A. Physical Evaluation

Table: Physical Evaluation

Batch Code	Color	Appearance / Texture
F1	Pale brown	Runny, slightly fluid gel
F2	Brown	Thick, opaque, uniform
F3	Light brown	Smooth, semi-translucent
F4	Dark brown	Thick, slightly opaque
F5	Light tan	Smooth, glossy gel
F6	Deep brown	Very thick, slightly lumpy

F7	Medium brown	Smooth, spreadable
F8	Light beige	Smooth, translucent gel
F9	Brown	Smooth, homogeneous gel

DISCUSSION:

The color of the formulations ranged from pale brown to deep brown, which is primarily attributed to the concentration of Achras sapota bark extract. Extracts rich in phenolic compounds, flavonoids, and tannins typically impart a brownish hue due to their inherent chemical composition. Formulations with higher extract concentrations such as F4 and F6 exhibited darker shades (dark brown and deep brown respectively), whereas lower extract concentrations (e.g., F1, F5, and F8) resulted in lighter colors like pale brown, light tan, and light beige. This progressive color deepening is an expected outcome and can serve as a preliminary indicator of phytochemical load. Formulations such as F3, F5, F7, and F9 displayed smooth, semi-translucent to homogeneous textures, which are ideal characteristics for topical application. These batches balanced extract concentration and polymer content effectively to provide a stable and aesthetically pleasing formulation. Specifically, F5 and F9 were noted for their glossy and homogeneous appearance, respectively, which may indicate proper gelling and compatibility between components without phase separation or precipitation.

B. Measurement of pH

Table: Measurement of pH

Batch Code	pH
F1	6.9
F2	6.7
F3	6.8
F4	6.6
F5	7.1
F6	6.5
F7	6.9
F8	7.0
F9	6.8

Discussion: All batches exhibited pH values within the acceptable range for topical application (6.5–7.1).Batch F5 (7.1) was slightly above the upper limit of the ideal pH range, which may be attributed to lower extract concentration and possibly higher neutralization with triethanolamine. However, it is still generally safe for short-term skin application. Batch F6 (6.5) showed the lowest pH, possibly due to a higher extract concentration and lower amount of triethanolamine added for neutralization. The other formulations (F1, F2, F3, F4, F7, F8, and F9) were well-balanced, with pH values between 6.6 and 6.9, which is considered optimal for dermal compatibility. There was no significant deviation in pH values, indicating a consistent formulation process and effective buffering capacity of the gel base.

C. Spreadability

Table: Spreadability

Batch Code	Spreadability (g·cm/s)
F1	13.5
F2	10.2
F3	12.4
F4	9.5
F5	14.2
F6	9.0
F7	13.0
F8	15.0
F9	12.8

Discussion: F8 (15.0 g·cm/s) showed the highest spreadability, indicating an excellent balance of gel consistency and polymer concentration, allowing for smooth and easy application. This may be due to the lower concentration of Carbopol 934 (396.447 mg) used, which resulted in a softer gel matrix.F5 (14.2), F1 (13.5), and F7 (13.0) also exhibited good spreadability, suggesting that moderate levels of Carbopol and extract resulted in gels with desirable texture and spreadability. F6 (9.0) showed the lowest spreadability, likely due to the highest concentration of Carbopol 934 (1103.55 mg), making the gel very viscous and resistant to spreading.F2 (10.2) and F4 (9.5) also had relatively lower spreadability, correlating with their higher polymer concentration (1000 mg), which increased viscosity and hindered smooth application. F3 (12.4) and F9 (12.8) presented intermediate values, indicating a satisfactory gel structure that balances consistency with ease of application.

D. Viscosity

Table: Viscosity

Batch Code	Viscosity (cps)
F1	3200
F2	4200
F3	3700
F4	4700
F5	3000
F6	4900
F7	3400
F8	3800
F9	3600

Discussion: Highest viscosity was observed in Batch F6 (4900 cps), followed by F4 (4700 cps) and F2 (4200 cps). These formulations had high concentrations of Carbopol 934 (1000 mg and above), which is directly responsible for increasing the gel matrix density and resistance to flow. Lowest viscosity was found in Batch F5 (3000 cps) and F1 (3200 cps), which corresponds with lower polymer concentrations. These lower values may lead to runny gels with reduced physical stability, although they often show better spreadability. Intermediate viscosities were recorded for Batches F3 (3700 cps), F8 (3800 cps), and F9 (3600 cps). These batches demonstrate a good balance of viscosity and spreadability, Batch F8 (3600 cps) also falls within an acceptable range, with a viscosity that allows for ease of application while maintaining adequate gel structure.

E. Stability study

Table: Stability study

Batch Code	Stability (3 Months)
F1	Stable
F2	Stable
F3	Stable
F4	Slight phase separation
F5	Stable
F6	Unstable (viscosity drop)
F7	Slight separation
F8	Stable
F9	Stable

Discussion: Formulations F1, F2, F3, F5, F8, and F9 exhibited no significant changes in their physical characteristics over the test period and were categorized as stable. This indicates that the formulation components in these batches were compatible and maintained their integrity under storage conditions. Formulation F4 showed slight phase separation, suggesting a potential incompatibility between the emulsifying agents or inadequate homogenization. This could compromise product consistency and necessitates reformulation or adjustment of stabilizers. Formulation F6 was found to be unstable due to a noticeable drop in viscosity. A decrease in viscosity may indicate polymer degradation, improper gelling agent concentration, or breakdown of emulsion structure, which may affect spreadability and application performance. Formulation F7 exhibited slight separation, possibly due to borderline emulsion instability. Although minor, this may progress over time and should be monitored further or improved via optimization of surfactant levels or processing conditions.

F. Acute Dermal Irritation Study

Results:

The skin irritation potential of the test item was evaluated in Wistar rats following OECD Guideline 404. The test item (0.5 g or mL) was applied dermally to the shaved dorsal skin of animals for a 4-hour exposure period under semi-occlusive conditions. Observations for erythema and edema were made at 1, 24, 48, and 72 hours after patch removal.

The scoring of skin reactions was done according to the Draize scoring system.

Animal No.	Time (hr)	Erythema Score	Edema Score
1	24	1	1
	48	0	0
	72	0	0
2	24	1	1
	48	0	0
	72	0	0
3	24	1	1
	48	0	0
	72	0	0

Mean Scores (24–72 hrs.):

- **Erythema:** (1 + 0 + 0)/3 = **0.33**
- **Edema:** (1 + 0 + 0)/3 = **0.33**

Interpretation (as per OECD 404)

- If the mean score (24 - 72 hrs.) for erythema or edema is:
- **< 2:** Non-irritant
- **2 to < 4:** Mild irritant
- **4 to < 6:** Moderate irritant
- **≥ 6:** Severe irritant

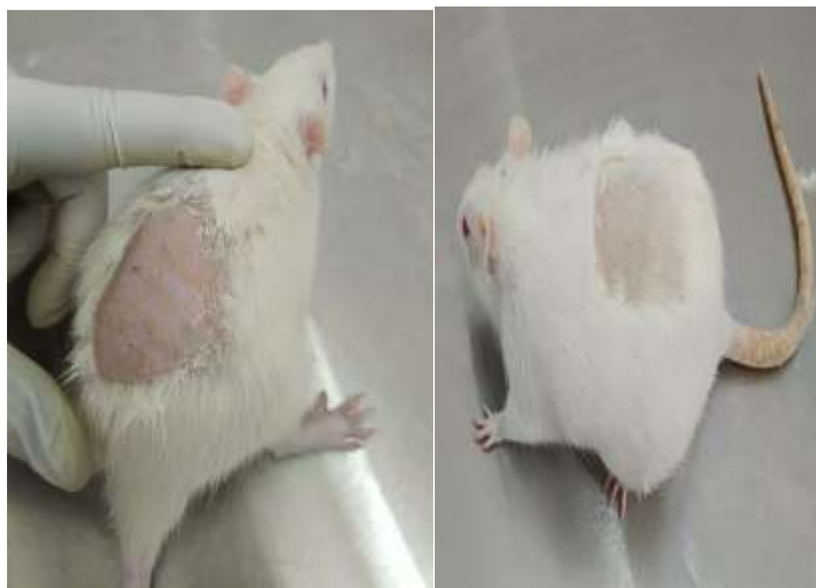

Fig. no. Acute Dermal Irritation Study

Discussion:

The application of the test substance caused very slight erythema and edema in all test animals at 24 hours, which completely resolved by 48 hours. No other adverse skin reactions such as necrosis, bleeding, or eschar formation were noted. The mean erythema and edema scores over the 24, 48, and 72-hour observation periods were both 0.33, indicating that the test item caused only minimal and reversible irritation.

According to OECD Guideline 404 and GHS (Globally Harmonized System) classification:

- Substances with mean scores less than 2.3 for erythema or edema (in at least 2 of 3 animals) are considered non-irritant.
- Since the effects were mild, transient, and fully reversible within 48 hours, the test item does not require classification as a skin irritant.

Conclusion:
Based on the observed effects and mean irritation scores:
- The test item exhibited no significant dermal irritation under the test conditions.
- The test item can be classified as "Non-Irritant" to skin according to OECD 404 and GHS guidelines.

Classification (CLP / GHS)

Classification	Criteria
Category 2 (Irritant)	Mean score ≥ 2.3 for erythema or edema in ≥ 2 of 3 animals
Category 1 (Corrosive)	Severe lesions or necrosis
No classification	Mean score < 2.3 and reversible within 72 hrs.

G. Anti-inflammatory activity result

Sr.no	Treatments Group	Mean Paw volume in different time intervals					% edema inhibition in different time intervals			
		0 min	30 min	60 min	120 min	180 min	30 min	60 min	120min	180 min
1	Group I	3.46 ± 0.17	3.57 ± 0.63	3.63 ± 0.76	3.61 ± 0.086	3.37 ± 0.16				
2	Group II	4.16 ± 0.025	8.09 ± 0.087	7.89± 0.032	7.76 ± 0.048	7.66 ± 0.085	-	-	-	-
3	Group III	5.01 ± 0.089	7.91 ± 0.087	7.44 ± 0.154	6.31 ± 0.056	5.55 ± 0.086	2.224	5.70%	18.68%	27.54%
4	Group IV	5.02 ± 0.125	8.34 ± 0.087	8.00 ± 0.185	6.40 ± 0.023	5.45 ± 0.079	-3.09%	1.39%	17.52%	28.85%

Fig. no. Anti-inflammatory activity Paw volume

H. Anti-arthritis Activity

Group	Day 0 (mm)	Day 2 (mm)	Day 4 (mm)	Day 6 (mm)	Day 8 (mm)	Day 10 (mm)	% Inhibition (Day 10)
Group I	3.93 ± 0.05	3.95 ± 0.23	3.96 ± 0.56	3.99 ± 0.42	3.98 ± 0.15	3.77 ± 0.19	N/A
Group II	4.65 ± 0.08	8.92 ± 0.17	11.62 ± 0.34	11.54 ± 0.19	11.57 ± 0.34	11.62 ± 0.39	0% (reference)
Group III	3.79 ± 0.18	7.31 ± 0.26	9.72 ± 0.34	7.5 ± 0.35	4.91 ± 0.42	4.83 ± 0.26	58.43%
Group IV	4.69 ± 0.24	8.84 ± 0.54	10.33 ± 0.23	9.42 ± 0.24	6.19 ± 0.47	5.85 ± 0.15	49.65%

Results are in mean ± SEM (n=6), one-way ANOVA followed by Dunnet t test. No significant, p≥0.05. *P<0.05, **P<0.01, ***P<0.001 compared with arthritic control.

Fig. no. Anti-arthritis Activity

Body weight determination in formaldehyde induced arthritis rats

Groups	Treatment and Dose	Body weight in gm					
		Day-0	Day-2	Day-4	Day-6	Day-8	Day-10
1	Group I	$297±17^{ns}$	$303±14^{ns}$	$317±26^{ns}$	$309±18^{ns}$	$327±29^{ns}$	$328±31^{ns}$
2	Group II	$283±26^{ns}$	$269±39^{*}$	$254±43^{*}$	$269±37^{*}$	$242±13^{*}$	$258±28^{*}$
3	Group III	$306±27^{ns}$	$287±32^{**}$	$264±23^{**}$	$292±22^{**}$	$319±37^{**}$	$325±43^{**}$
4	Group IV	$278±31^{ns}$	$271±47^{***}$	$283±36^{***}$	$267±27^{***}$	$296±32^{***}$	$306±38^{***}$

Results are in mean ± SEM (n=6), one-way ANOVA followed by Dunnet t test. No significant, p≥0.05. *P<0.05, **P<0.01, ***P<0.001 compared with arthritic control.

Body weight determination

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