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PREPARATION, VALIDATION, EFFICACY AND SAFETY ASSESSMENT OF TOPICAL ANTI-INFLAMMATORY FORMULATION

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ABSTRACT

Inflammation is a biological defence mechanism, but chronic inflammation can contribute to conditions such as arthritis and musculoskeletal disorders. This study focuses on the formulation, validation, and evaluation of an anti-inflammatory formulation (ointment) containing glucosamine sulphate, methyl salicylate, magnesium sulphate, clove oil and sodium chloride as active pharmaceutical ingredients. The ointment was developed using a modified fusion method and assessed for physicochemical and pharmacological efficacy. Among the three formulations; F1, F2, and F3, F2 exhibited optimal pH, spreadability, viscosity and compatibility and demonstrated significant analgesic and anti-inflammatory activity making it the most suitable for topical application. Stability studies confirmed that F2 maintained its efficacy over twelve months without significant degradation. UV-visible spectroscopy analysis validated the presence of glucosamine sulfate and methyl salicylate at 210 nm and 270 nm, respectively, while FTIR analysis confirmed the structural integrity and compatibility of the formulation components. Skin sensitivity tests revealed no redness, irritation or adverse reactions. These findings indicate that F2 is a promising, stable, and effective anti-inflammatory ointment formulation.

Keywords: Anti-inflammatory, Ointment, Glucosamine Sulfate, Methyl salicylate, Magnesium sulphate, Clove oil, Validation

INTRODUCTION

Inflammation is the body's natural defence mechanism against injury, infections, and harmful stimuli. While acute inflammation is essential for healing, chronic inflammation can contribute to various musculoskeletal disorders such as arthritis

and osteoarthritis **[1]**. Long-term inflammation leads to excessive production of inflammatory mediators like cytokines and prostaglandins, which cause pain, swelling, and tissue damage **[2]**. Common treatments for inflammatory conditions include nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. However, prolonged use of these medications is linked to adverse effects, including gastrointestinal disturbances and cardiovascular risks **[3]**. To minimize these side effects, topical formulations are being explored as an alternative, offering localized treatment with reduced systemic absorption **[4]**. Various bioactive compounds have demonstrated anti-inflammatory and analgesic properties, making them effective for topical applications. Glucosamine sulfate is recognized for its joint-protective and anti-inflammatory benefits. It provides resilience of cartilage, helps to slow down the breakdown of cartilage whereas methyl salicylate acts as a counterirritant and pain reliever. Magnesium sulfate is known to aid in muscle relaxation and inflammation reduction, while clove oil, rich in eugenol, exhibits strong antioxidant and anti-inflammatory effects. Additionally, sodium chloride plays a role in maintaining formulation stability and enhancing drug absorption **[5-8]**.

This study aims to develop and evaluate a topical anti-inflammatory ointment containing glucosamine sulfate, methyl salicylate, magnesium sulfate, clove oil, and sodium chloride. The prepared formulations were assessed for their physical and chemical properties, pharmacological activity, and safety profile. The results contribute to the advancement of stable and effective topical anti-inflammatory therapies for potential clinical use.

MATERIALS AND METHODS

Materials: Hard Paraffin, White soft paraffin, Magnesium Sulphate, Wool Fat, Cetostearyl Alcohol, Sodium Chloride, and Methyl salicylate were purchased from Research Lab Fine Chem Ind. Mumbai. Glucosamine Sulphate was procured from Ontop Pharm. Pvt. Ltd. Bengaluru. Clove oil was purchased from local Pharmacy, Nashik.

Formulation Development: The ointment was formulated using a fusion technique **[9]**. Three different formulations—F1, F2, and F3—were developed, each containing varying proportions of the key active ingredients. These variations were designed to enhance the formulation's overall efficacy, stability, and user acceptability, ensuring an optimal balance between therapeutic benefits and ease of application **(Table 1)**.

Table:1: Ingredients and their Composition used in the Ointment Formulation

Sr. No.	Ingredients	F1	F2	F3	Properties
1	Glucosamine Sulphate	1.0g	1.0g	1.0g	Maintains the strength, elasticity, and resilience of cartilage, helps to slow down the breakdown of cartilage
2	Methyl Salicylate	1.0ml	1.0ml	1.0ml	Relieves pain in muscle, joint and tendons. It also reduces inflammation
3	Magnesium Sulphate	0.8	0.7g	0.6g	Muscle relaxant and anti-inflaammatory
4	Sodium Chloride	0.2g	0.1g	0.2g	To draw water out of swollen area, anti-inflammaotry.and muscle relaxant
4	Clove Oil	0.8 ml	0.9ml	0.7ml	Anti-oxidant, anti-inflammatory and anti-microbial
5	Hard Paraffin	1.0g	0.8g	1.0g	Emollient
6	White Soft Paraffin	4.0g	5.0g	4.0g	Ointment Base
7	Wool fat	0.5g	0.2g	0.8g	Emollient
8	Cetostearyl Alcohol	0.5g	0.3g	0.7g	Emulsifying Agent

Preparation of Ointment: Each ingredient was accurately weighed before formulation. Cetostearyl alcohol and hard paraffin were melted in an evaporating dish using a water bath. Once melted, white soft paraffin and wool fat were incorporated into the mixture and stirred continuously until a homogeneous blend was formed. When the temperature of the mixture dropped to approximately 50°C, glucosamine sulfate, sodium chloride, magnesium sulphate (finely powdered) methyl salicylate and clove oil were added. The formulation was then thoroughly mixed to ensure uniform distribution of all components. After cooling to room temperature, the prepared ointment was transferred into an appropriate container, labelled accordingly, and subjected to quality control assessments and anti-inflammatory screening.

Validation: Formulations (F1, F2 and F3) were evaluated through various of quality control tests as per ICH guidelines. The following parameters were assessed:

(a) **Appearance:** Each formulation was subjected to visual assessment to evaluate its color, texture, phase separation, and consistency **[9] (Table 2)**.

(b) **Homogeneity**: A small quantity of the formulation was applied between the thumb and index finger to assess its homogeneity and texture. The texture and uniformity of the formulations were evaluated based on immediate sensory characteristics, including grittiness, stiffness, stickiness, greasiness, and the presence of any coarse particles **[10] (Table 2)**.

(c) **pH:** Determined to be compatible with the pH of the skin using a calibrated pH meter. (Skin pH ranges from 4.5 to 5.7.) 50 ml of water was added to a dry beaker containing around 2.5 g of each formulation and were heated to 60–70°C in a water bath. A pH meter was used to determine the ointments' pH. The three readings were averaged after the

measurements were made in triplicate **[10] (Table 3)**.

(d) **Spreadability:** Spreadability was evaluated using the parallel-plate method to assess the ease of application and uniform distribution of the formulation on the skin. Formulation weighing 100 g weight was sandwiched between the two glass slides, each positioned on a pulley, for a duration of five minutes to ensure uniform thickness. After this, a 250 g weight was placed on the pan. The spreadability was measured by recording the time (in seconds) taken for the two slides to separate **[10] (Table 3)**. Three reading were taken to check the reproducibility.

Spreadability was measured using the formula S = m x l/t

Where, S is Spreadability, m – weight tied on upper slide, l – length of glass slide and t – time in seconds

(e) **Viscosity:** The rheological properties of the formulations were assessed using a Brookfield viscometer. A 50 g sample was allowed to equilibrate in a beaker for five minutes before measurements were taken using a T-D spindle (No. 7). The dial readings were recorded at rotational speeds of 10, 40, 50, and 100 rpm. The readings were noted at each speed, and the spindle speed was then gradually reduced, with the dial values recorded again. All measurements were conducted at room temperature and repeated three times. The viscosity in centipoise (cP) was calculated by multiplying the dial readings by the appropriate calibration factors from the Brookfield Viscometer catalog **[10] (Table 3)**.

(f) **Stability Studies**: Stability testing of the formulated ointments was conducted in accordance with the guidelines established by the International Council for Harmonisation (ICH). The ointments were stored in suitable containers for six months under three distinct conditions: 25°C±2°C with 60%±5% relative humidity, 30°C±2°C with 65%±5% relative humidity, and 40°C±2°C with 75%±5% relative humidity. During this period, the formulations were periodically evaluated for changes in pH, appearance, spreadability, and viscosity **[11] (Table 3)**.

(g) **Anti-inflammatory Activity**:

Institutional animal ethical committee approval was taken to conduct the study. Standard guidelines were adopted for the screening. The anti-inflammatory activity of the ointment was evaluated in Wistar rats using the carrageenan-induced paw edema model. Each animal received a subcutaneous injection of 0.1 mL of carrageenan solution into the left hind paw. The swollen paw was then topically treated with approximately 100

mg of the ointment. Paw volume measurements were taken at 0, 1, 3, and 5 hours after ointment application. The ointment was re-applied every two hours. The percentage of paw edema inhibition was calculated based on the reduction in paw volume compared to the untreated control group.

% Inhibition = To-Tt/ To x100, Where, To=Paw thickness of rats of control group & Tt= Thickness of paw of rats given ointment at corresponding time **[12] (Table 4)**.

(h) **Skin irritation test**: For the skin irritation test, albino rats weighing between 150 and 200 g were divided into four groups, each consisting of five animals. The dorsal region of each rat was shaved 24 hours prior to the experiment. The rats were housed individually and had unrestricted access to distilled water. A 1 cm^2 area of the shaved skin was treated with 100 mg of each formulation. A 0.5% formalin solution was used as the standard irritant. Skin irritation and sensitization were monitored daily for up to seven days, with observations for signs of erythema, edema, redness, and inflammation **[10] (Table 4)**.

(i) **Validation, Analytical Profile**:

UV- visible spectroscopy: To identify and quantify compounds, evaluate purity, and monitor reactions, providing critical information regarding the chemical composition and stability of the final product

Sample Preparation: 10 mg of the ointment formulation was weighed and dissolved in 10 mL of a methanol-water mixture (1:1 v/v). The solution was sonicated for 15–20 minutes to ensure proper dissolution of the active ingredients. It was then filtered through What man filter paper No. 1 or a 0.45 µm membrane filter. The filtrate was used for UV-Vis analysis (LABINDIA UV3200). Pure sample were used for comparison. Methanol-water (1:1) as the blank/reference **(Figure 1)**.

(j) **FTIR:** The FTIR spectrum of the ointment formulation was obtained and analyzed to verify the chemical identity and compatibility of the ingredients. Prior to analysis, the ATR crystal was cleaned by wiping it with a lint-free cloth moistened with a cleaning solvent to remove any residual contaminants, and then allowed to dry completely. A small amount of the ointment was carefully applied to the ATR crystal surface using a spatula, ensuring a thin, uniform layer. The ointment was gently pressed to establish good contact with the ATR crystal. A background scan was conducted with a clean ATR crystal across the relevant spectral range (4000–400 cm^{-1}), which was later subtracted

from the sample spectrum. The ointment-covered ATR crystal was then placed in the spectrometer, the scan parameters were set, and the FTIR spectrum was acquired. The background spectrum was automatically subtracted by the software during the process **(Figure 2)**.

RESULTS AND DISCUSSION:

Three distinct ointment formulations (F-A, F-B, and F-C) were developed for the treatment of joint and muscular pain, with variations in their component compositions. The formulations were thoroughly evaluated for their stability, therapeutic efficacy, and physicochemical properties, in accordance with ICH guidelines. Assessments included appearance, texture, homogeneity, pH, spreadability, viscosity, and stability. Biological evaluations were also conducted to assess skin irritation and anti-inflammatory effects using in vivo models. The objective was to identify the formulation that offered optimal therapeutic efficacy, stability, and patient acceptability. Among the three, F2 demonstrated superior performance across various parameters, making it the most suitable candidate for topical application. The following discussion outlines the results of each quality control test and their implications.

Statistical Analysis: All physical parameters and stability data were analyzed statistically to assess significant differences over time between the formulations (F1, F2, and F3) and within each formulation. One-way ANOVA, followed by Tukey's post hoc test, was employed to analyze the data. The results are presented as mean ± SD for each of the three independent measurements. A p-value of less than 0.05 was considered statistically significant.

All formulations exhibited a pale yellow, opaque appearance, indicating consistent mixing and stability, which is important for consumer acceptance. F1 had a hard texture, potentially affecting user comfort and ease of application, while F3's softness suggested lower viscosity, which may reduce stability and skin adhesion. F2* displayed a smooth texture with an ideal balance of hardness and softness, enhancing spreadability and user satisfaction. All formulations were homogeneous with no signs of phase separation, indicating uniform distribution and stability. Even though F1 and F2 provided a pleasant feel, free from grittiness, stickiness, or greasiness, improving patient compliance. F2 was found to be the most user-friendly, offering the best skin sensation.

One-way ANOVA indicated significant differences between F2 and both F1 and F3 for pH, spreadability, and viscosity. F2's pH (5.42 ± 1.01) is optimal for skin application, aligning with the physiological pH (4.5–6.0), promoting skin barrier function and reducing irritation risk. F1's lower pH is less

suitable for delicate skin, while F3's higher pH may increase irritation potential. F2 also showed better spreadability (105.02 ± 0.01 g.cm/s) compared to F1, and although F3 had slightly higher spreadability, it may affect skin adhesion. F2's viscosity (30,000 ± 0.34 CPS) provided optimal application ease and stability, unlike F1's higher viscosity, which may hinder spreadability, and F3's lower viscosity, which could impact stability. Over 12 months, F2 maintained consistent pH, spreadability, and viscosity, suggesting superior skin compatibility and stability. Based on these findings, F2 was selected for further anti-inflammatory and skin sensitivity evaluation.

Anti-Inflammatory Activity

The carrageenan-induced paw edema model demonstrated that F2 significantly reduced paw edema at all time points compared to the control group ($p < 0.05$). After 2 hours, F2 exhibited a 60.54% inhibition of edema, showing potent anti-inflammatory effects similar to the reference, which showed 63.26% inhibition. In the skin sensitivity test, F2 showed no signs of erythema, edema, or other adverse reactions over seven days, with a severity score of zero throughout the observation period. This indicates that F2 is safe for topical application and does not cause irritation or sensitization, even with prolonged use.

UV- visible spectroscopy

In UV-visible spectroscopy indicated the absorbance maxima of glucosamine sulphate 0.6563 at 210nm and methyl salicylate 0.9843 at 270nm **(Table 5)**. This absorbance is similar compared with each API absorbance values and results shows in ointment the presence of Glucosamine sulphate and Methyl salicylate confirmed. Correlation coefficient (R^2) is 0.949.

Concentration and absorbance of ointment by UV-visible spectroscopy

FTIR

Broad peak around 3200–3600 cm^{-1} corresponds to O-H (hydroxyl) stretching from cetostearyl alcohol, wool fat, and methyl salicylate; N-H stretching (glucosamine sulfate) also contribute here. Strong peaks near 2900 cm^{-1} represents C-H stretching from paraffins (hard and soft), cetostearyl alcohol, and wool fat. Peak around 1700–1750 cm^{-1} likely corresponds to the C=O stretching (ester and fatty compounds) from methyl salicylate and wool fat. Peaks between 1500–1600 cm^{-1} associated with C=C stretching from methyl salicylate's aromatic ring. Strong peak at 1050–1200 cm^{-1} indicates S=O stretching from glucosamine sulfate. Peak in the range of 1000–1100 cm^{-1} C-O stretching from cetostearyl alcohol and methyl salicylate. Fingerprint Region (Below 1000 cm^{-1}) Multiple peaks indicate complex bending and skeletal vibrations from various components like wool fat, paraffins, and

glucosamine sulfate and eugenol. 1645 cm -1 indicates the C-H stretching vibration of benzene (eugenol) and 1379 cm -1 shows C-H deformation vibration of eugenol methyl group indicating the clove oil **(Table 6)**.

Table 2: Preliminary Physical Evaluation of ointment formulation

Formulation	Colour/Appearance	Texture	Homogeneity	Skin feel test
F1	Pale yellow, opaque	Little Hard	Homogeneous	Absence of stickiness grittiness and greasiness
F2*	Pale yellow, opaque	Smooth	Homogeneous	Absence of stickiness grittiness and greasiness
F3	Pale yellow, opaque	Soft	Homogeneous	Absence of stickiness grittiness and greasiness

Table 3: Evaluation of Physical properties of ointment formulations and Stability Studies (after twelve months)

Formulation	F1	F2*	F3	Standard (Moov)
pH	4.04±0.11*	5.42±0.01**	5.73±0.04*	5.43±0.11**
Spreadability (g.cm/s)	104.00±1.34*	105.02±0.01**	105.24±1.02*	105.08±1.01**
Viscosity (CPS)	32000±1.34*	30000±0.34**	29920±2.04*	30000±0.02**
	Stability Studies: After 12 months			
pH	4.98 ± 0.20	5.45 ± 0.95	5.70 ± 1.98	5.43±0.11**
Spreadability (g.cm/s)	33,200 ± 1.30	29,200 ± 1.10	29,000 ± 1.20	105.08±1.01**
Viscosity (CPS)	101.80 ± 1.60	104.20 ± 1.70	104.60 ± 1.40	30000±0.02**

Values are presented as Mean ± S.E.M. (n=3); One-Way ANOVA and the Tukey post hoc test were used for analysis; *P<0.05 is regarded as significant when compared to the standard. **Statistically significant (p< 0.05)

Table 4: Anti-Inflammatory Activity of Formulation F2 in Carrageenan-Induced Paw Edema Model and Skin sensitivity test

Treatment	Paw Volume in mL(Mean ± SEM)			
	0 h	30 min	60 min	120 min
Control (Ointment base-50mg)	1.47 ± 0.12	2.01 ± 0.04	2.16 ± 0.02	2.92 ± 0.02
F2 (100mg)	1.47± 0.82	0.99 ± 0.06*	0.68± 0.04*	0.58 ± 0.02*
Standard (100mg-Moov)	1.47 ± 0.62	0.97 ± 0.02*	0.66 ± 0.02*	0.54 ± 0.01*

Skin sensitivity test [Severity Score (0-5)]						
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 6
Score	0	0	0	0	0	0

The data are expressed as Mean ± S.E.M. (n=5). One-Way ANOVA followed by the Tukey post hoc test was performed for statistical analysis, with *P<0.05 considered statistically significant when compared to the control. [0- indicates no erythema or edema)

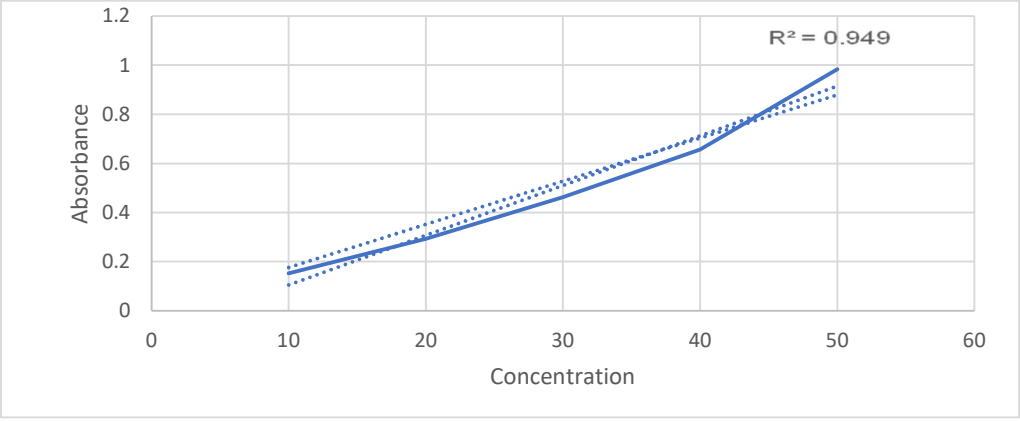

Figure 1: UV spectrum of Anti-inflammatory ointment

Table 5: Wave length and absorbance

Compound	Range	Absorbance
Glucosamine Sulphate	210nm	0.6563
Methyl Salicylate	270nm	0.9843

Table 6: Peak interpretation (Carried out only to check the stability status of methyl salicylate and glucosamine sulfate)

Wave number Range (cm^{-1})	Functional Group & Vibration	Source Components
3200–3600	O-H stretching, N-H stretching	Cetostearyl alcohol, wool fat, methyl salicylate, glucosamine sulphate
~2900	C-H stretching	Paraffins (hard and soft), cetostearyl alcohol, wool fat
1700–1750	C=O stretching (ester, fatty compounds)	Methyl salicylate, wool fat
1500–1600	C=C stretching (aromatic ring)	Methyl salicylate
1050–1200	S=O stretching	Glucosamine sulfate
1000–1100	C-O stretching	Cetostearyl alcohol, methyl salicylate
Below 1000	Fingerprint region (bending & skeletal vibrations)	Wool fat, paraffins, glucosamine sulfate
1645 cm -1	C-H stretching vibration of benzene	Clove oil
1379 cm -1	C-H deformation vibration of eugenol methyl	Eugenol-Clove oil

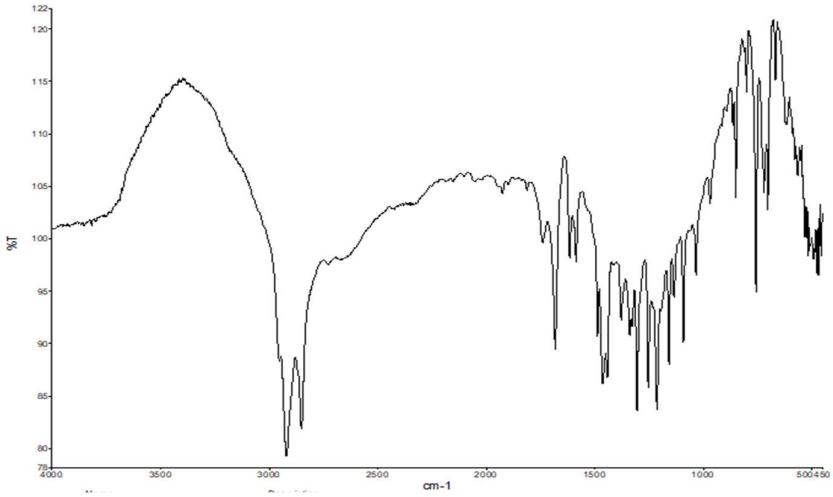

Figure 2: FTIR spectrum of Anti-inflammatory ointment

CONCLUSION

The optimized anti-inflammatory ointment F2 exhibited superior physicochemical stability, optimal rheological properties, and significant ($p < 0.05$) anti-inflammatory activity compared to control. Stability and biocompatibility studies confirmed its long-term shelf life and safety, while spectroscopic analyses (FTIR/HPLC) verified drug-excipient compatibility. These findings position F2 as a viable topical therapy for inflammatory musculoskeletal conditions, though clinical validation remains essential.

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