**Research Article**

**Formulation preparation and evaluation of Apigenin ointment for anti-inflammatory and wound healing activities**

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**Abstract**

**Objective:** Objective of research work was to prepare the formulation and evaluation of Apigenin (Ap)-loaded ointment (Ont) for anti-inflammatory and wound healing activities. **Material and Methods:** Ap-loaded ointment was prepared by fusion method and evaluated using different parameters. The optimized ointment formulation was further used for the anti-inflammatory and wound healing activities studies. The anti-inflammatory and wound healing activities was performed on albino wistar rats by using carageneen paw edema method and wound healing activity was performed using Incision wound model. **Results:** Optimized F6 formulation was found to be good physical properties such as colour, odour, pH, spreadability, extrudability and diffusion method used. The diffusion of F6 was indicating a 72% drug release; and was inhibited 59.97% paw edoema when compared to the control group. Result indicated that apigenin is a bioactive anti-inflammatory molecule. It was key components for producing anti-inflammatory property. According to the results of measurement of tensile strength, hydroxyproline estimation, hexosamine and protein estimation were showed F6 topical formulation have potential wound healing activity. It may be due to its antioxidants and possible inhibitors of cyclo-oxygenase, lipoxygenase, and nitric oxide synthase action. **Conclusion:** Apigenin (Ap)-loaded ointment (Ont) for anti-inflammatory and wound healing activities can be used as successful drug-delivery system for wound healing. **Keywords:** Apigenin, ointment, wound treatment, topical formulation

**Introduction**

Numerous researchers have developed poly-herbal topical formulations for the treatment of inflammatory skin diseases like acne, wounds, itching, fungal infections, psoriasis, and wrinkles. More studies with corroborating scientific evidence are still required, though. The literature review summarises the works of a few scientists who have developed and been tested poly-herbal topical preparations. Topical preparation increases the drug’s bioavailability and inhibits the drug’s liver metabolism. Topical medications take effect immediately after application (Kaur et al., 2013).

The complicated biological response to injury or damaging stimuli, such as pathogens, damaged cells, or irritation, is thought to include inflammation. Due to numerous related changes like vasodilation, increased vascular permeability, and plasma extravasation, this response causes numerous physical symptoms like fever, discomfort, and edoema. In typical circumstances, the body's response to inflammation is self-limiting via the upregulation of anti-inflammatory protein expression, the downregulation of pro-inflammatory protein expression, and a reversal of the vascular alterations that aided the initial process of immune cell recruitment. Injury that breaks the skin or other body tissues is called a wound. They consist of skin punctures,
scrapes, scratches, and cuts (Linlin et al., 2018). However, surgery, sutures, and stitches can also result in wounds. Wounds frequently occur as a result of accidents. Hemostasis, inflammation, proliferation, and remodelling are all important biological processes that take place during wound healing. Many different types of cells, including as neutrophils, macrophages, lymphocytes, keratinocytes, fibroblasts, and endothelial cells, are involved in this process (Guo et al., 2010). The phytochemicals in oregano species are mostly abundant and have been extensively investigated in the past. There have been reports of potential antioxidant, anti-inflammatory, and anti-cancer health effects from the flavonoids and phenolic chemicals found in oregano species (Erick et al., 2018). In order to generate topical dosage forms containing herbal antibacterial/antiseptic agents for the treatment of topical infectious diseases, it is necessary to construct an effective method of ointment formation. It can be highly beneficial for the treatment and prevention of skin conditions. The aim of present research work is to develop ointment containing Apigenin formulation for the treatment of skin disease, using of simple fusion method and will the pharmacological screening of anti-inflammatory and wound healing activity. The objective of research work will to develop novel ointment semisolid dosage form for the treatment of wound healing and inflammation diseases in economic way. On the basis of review and literature survey indicated that no any topical formulations of Apigenin still not available and also not any systemic scientific data related topical formulation. Various literatures proofed that Apigenin bioactive molecule having very potential antimicrobial compounds. It can be used for the formulation of topical formulation for topical diseases. Therefore I have chosen this bioactive molecule for research work.

**Material and methods**

**Collection and procurement of chemicals of analytical grade**

The Apigenin was procured from sigma Aldrich Pvt. Ltd. The only additional compounds utilised were of an analytical grade. All other chemicals used are analytical grade.

**Preparation of topical ointment formulation**

The ingredients were all taken in the measured amounts as report and an ointment was made using the fusion process (Datar et al., 2013; Lodhi et al., 2010). In the fusion method, all of these components were placed in a melting pan and heated to 70°C. After melting, the mixture was gently mixed while being kept at a temperature of 70°C for about 5 minutes, and it was then cooled to 40°C while being agitated constantly. The ointments were then swirled to achieve a smooth consistency, stored at room temperature (25°C), and used for additional analysis. The process for creating an ointment with 5% Apigenin added to the formulation and triturating the mixture using a spatula to create 100 g. Ointment was made using the specified constituent ratio. Based on the evaluation criteria, the best ointment formulation was chosen.

**Characterization of prepared ointment formulation**

All of the prepared ointments underwent characterization tests for factors like spreadability, hardness, water number,

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**Table 1. Composition of simple ointment base**

<table>
<thead>
<tr>
<th>Ingredients (%)</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
<th>F5</th>
<th>F6</th>
</tr>
</thead>
<tbody>
<tr>
<td>White petrolatum</td>
<td>94</td>
<td>95</td>
<td>96</td>
<td>97</td>
<td>98</td>
<td>99</td>
</tr>
<tr>
<td>White bees wax</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Wool fat</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Hard paraffin</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Cetostearyl alcohol</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 2. Drug Content determination of different ointment formulations**

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>% Drug Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>80.31</td>
</tr>
<tr>
<td>F2</td>
<td>83.52</td>
</tr>
<tr>
<td>F3</td>
<td>91.84</td>
</tr>
<tr>
<td>F4</td>
<td>86.31</td>
</tr>
<tr>
<td>F5</td>
<td>88.52</td>
</tr>
<tr>
<td>F6</td>
<td>97.84</td>
</tr>
</tbody>
</table>
homogeneity, odour, colour, and homogeneity (Viswanad et al., 2019; Singh et al., 2013).

**Organoleptic characteristics**

All "blank formulations" (i.e., formulations devoid of active component) and formulations containing medication underwent physical appearance, colour, texture, phase separation, and homogeneity testing. These characteristics were evaluated visually. The homogeneity and texture of the produced cream and gels were assessed by rubbing a small amount between the thumb and index finger. The consistency of the formulations and the presence of coarse particles were used to evaluate the texture and homogeneity of the formulations. The instantaneous sensation of the skin was also evaluated (including stiffness, grit, and greasiness).

**pH**

50 ml of water and 2.5 g of each formulation were added to a dry beaker. In a beaker over a water bath, ointments were heated to 60 to 70 °C. Determine the pH of the ointment using a pH metre (pH Tutor, Eutech Instruments). The computations were performed three times, and the three readings' averages were recorded.

**Spreadability**

The spreadability of the formulation was assessed using a modified multimer device. It comprises of a pulley fixed to one end of a glass slide mounted on a wooden block. Three grammes of additional ointment were put on a ground plate. The ointment was sandwiched between these two glass plates using a third plate that had the dimensions of a permanent ground plate and a hook. A 1 kg weight was placed on top of the two plates for five minutes in order to press out the air and produce a uniform film of ointment between them. Extra ointment has been scraped off the edges. Next, 240 g was used to pull the top plate. It was noted how long it took the top plate, with the help of a spring attached to the hook, to move 10 cm. A shorter interval is an indication of a more efficient distribution. The spreadability was calculated using the formula below:

\[ S = M \times L / T \]

Where

- **S** = Spread ability
- **M** = Weight tide to the upper slide
- **L** = Length of glass slide
- **T** = Time taken to separate the slides

**Viscosity**

A Brookfield Synchro-Lectric Viscometer (Model RVT) with a Helipath Stand was used to conduct the rheological testing. The sample (50 g) was placed in a beaker and allowed to equilibrate for 5 min before being used to measure the dial reading with a T-D spindle at 10, 20, 30, 50, 60, and 100 rpm. At each speed, the corresponding dial reading on the viscometer was noted. The matching dial reading was noted each time the spindle speed was reduced. Three duplicates of each measurement were made at room temperature. The viscosity in centipoises was calculated by directly multiplying the dial values by the coefficients specified in the Brookfield Viscometer catalogue (CPS). Three times of each measurement were made at room temperature.

**Skin irritation study**

Models for skin sensitization and irritancy were developed using healthy albino rats weighing 25–30 g. Under standard conditions (12 h light/dark cycles, 22 °C, and 35–60% humidity), the animals were housed in polypropylene cages. There was free access to tap water and regular palletized food. The study was approved by the Indian Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), which is a member of the RKDF University in Bhopal's institutional animal ethical committee (registration No.). For this experiment, albino rats weighing 150–200 g were employed. There are five different animals in each of the four groupings of creatures. The rats were housed in separate cages and had their dorsal hairs removed a day before the trial began in order to avoid contact with the other rats. The optimised formulation F6, which contains sunflower wax and USP (T1) and IP (T2) simple ointments, was used to compare rat skin irritation studies. There were two groups of each for the standard irritation and the control. Tests were administered to the other two groups. A square centimetre of both healthy and injured animal skin received 50 mg of each formulation. 0.8% formalin aqueous solution was employed as a common irritant. At the end of the experiment, the impact of skin irritancy and sensitization on animal skin was evaluated. We observed the animals for seven days, looking for any signs of erythema and edoema. The potential for the images to aggravate skin was evaluated.

**Diffusion study**

A modified Franz diffusion cell was used to conduct in vitro drug release testing on substances. The donor and receptor compartments were separated by a dialysis membrane that had been pre-soaked in phosphate buffer at pH 7.4. 10 mg of the formulation were administered to the donor compartment. The diffusion medium volume in the receptor compartment was held at 25 ml, the temperature was kept at
34 0.5°C, and the rpm was kept at 25 using a hot plate magnetic stirrer. At intervals of 15, 30, 45, 1 hour, up to 6 hours, aliquots were removed and replaced with equal quantities of diffusion media. Aliquots were appropriately diluted with pH 7.4 before being subjected to UV Spectrophotometer analysis at 220 nm.

**Drug Content**

The ointment’s 10mg dosage was taken and dissolved in distilled water. Then, using a UV-Visible spectrophotometer, absorbance at 220 nm was determined.

**Assessment of anti-inflammatory activity of ointment formulation** (Viswanad et al., 2019)

The present investigation used Albino Wistar rats (180-200 g BW). Animals were maintained in a regular setting and fed a conventional pellet diet. The animals were given a period of seven days prior to the experiment to get used to the lab environment. Before the 18-hour experiment, they were denied meals. They were taken for an experiment after that. Animals were cared for properly in accordance with CPCSEA, New Delhi requirements after the experimental protocol had been examined and approved by RKDF University's Institutional Animal Ethical Committee (IAEC).

Three groups of Albino Wistar Rats (each with six animals; n = 6) were created: the positive control group, the standard treatment group, and the test group. The standard group, consisting of Apigenin ointment and Diclofenac Sodium gel 1.0%, was compared to the positive control group (Test group). Carrageenan (0.1 ml, 1% w/v in normal saline) was injected into the sub-plantar tissue of the right hind paw of each animal in each group to cause edema. Digital screw gauze was used to measure the linear paw circumference. The measurements of the paw circumference were taken both before and four hours after the introduction of edema. Control group did not get any therapy at all. The animal’s right hind paw’s sub-plantar tissue was treated with both the standard and test formulations by gently rubbing it 50 times with the index finger. Using the following formula, the percentage value of edema inhibition was determined:

\[
\text{\% inhibition} = 1 - \frac{y - x}{b - a} \times 100
\]

Where x is the initial paw thickness of the test group animal, y is the paw thickness after treatment, is the initial paw thickness of the control group animal, and b is the paw thickness after treatment.

**Wound healing activity**

**Animals**

All of the current in vivo trials used healthy wistar rats of either sex that weighed 150–200 g and had never received any pharmacological treatment. The animals were fed a commercial pellet diet from Hindustan Lever in Bangalore, India, along with unlimited amounts of water. Before beginning the experiment, the animals spent 10 days adjusting to the hygienic conditions of the facility. With proper authorization from the institutional animal ethics council, an animal study was carried out in the department of pharmaceutical sciences at RKDF University in Bhopal (MP).

In each of the three models-incision, excision, and dead space wound—54 animals of either sex weighing between 150 and 200 g were separated into three groups, each consisting of six animals: Simple ointment base was utilised for group I, 5% Apigenin ointment for group II, and 0.005% Fluticasone propionate (Apigenin) ointment for group III.

**Excision wound model**

The animals had their backs shaved and sanitised with 70% ethanol before a surgical blade was used to cut a 7 x 7 mm excision wound from a preset shaved area on each animal’s back (Nagoba et al., 2019, Lodhi et al., 2013, Lodhi et al., 2006). All animals in each group were anaesthetized using the open mask approach with anaesthetic ether prior to wound formation. The wound was left exposed to the open environment without the administration of any local or systemic antibacterial treatments. Cutting away 500 mm² of skin's complete thickness from a specific location caused an excision wound, which was then left exposed to the outside environment. To the standard group, control group, and treated group, respectively, the standard medicine ointment (Povidone iodine), simple ointment base BP., and Apigenin ointment (0.5%, w/w) were applied topically until the wound had fully healed. This model tracked the rate of wound contraction and wound closure. The amount of wound contraction was calculated as a percentage over the first two days following wound creation. Each rat had a sample of tissue removed from the healed wound for histological analysis. The following formula was used to determine the proportion of wound contraction:

\[
\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total wound area}} \times 100
\]

Every two days, the wound margin was traced on a clear piece of paper to determine the wound area, which was then subtracted from to determine the healed area (Nagoba et al., 2019, Lodhi et al., 2013, Lodhi et al., 2006).

Group 1: served as test group treated with Apigenin ointment for 24 days.

Group 2: served as reference standard treated with wound healing ointment (Povidone iodine).
Group 3: served as negative control treated with ointment base. On day 24, complete epithelization and healing were visible in control rats.

**Incision wound model**

All animals were sedated prior to the production of wounds, and on each side of the rat's depilated back, two paravertebral long incisions were created into the skin at a distance of roughly 1.5 cm from midline. The entire trial was conducted without the use of any topical or systemic antimicrobials. All groups received the same care as in the excision model, with the edges held together and sewn with black silk surgical thread (number 000) and a curved needle (number 11). The continuous threads on both wound borders were getting tighter to ensure a complete healing of the wound. Following stitching, the wound was left uncovered while basic ointment base, Apigenin-ointment, and standard ointment were used daily for up to nine days. Once the wounds had completely healed, the sutures were removed.

**Measurement of tensile strength**

This approach estimated the wound-breaking strength as the weight of water per area of the specimen at the time of the wound-breaking.

**Hydroxyproline estimation**

In a hot air oven set to 60–70 C, tissues were dried to a uniform weight before being hydrolyzed in 6N HCl at 130 C for four hours in sealed tubes. After being neutralised to pH 7.0, the hydrolysate underwent 20 minutes of Chloramine-T oxidation. The Ehrlich reagent was used at 60 °C to produce colour, and a spectrophotometer was used to detect the colour at 557 nm to terminate the reaction.

**Histopathological studies**

After the incision wound had fully healed, wound tissue samples from the control, test, and standard groups were collected. Following the usual processing, 6-mm thick sections were cut and stained with haematoxylin and eosin. Under a light microscope, sections were evaluated qualitatively for fibroblast proliferation, collagen production, angiogenesis, and epithelialization.

**Statistical analysis**

For n = 6, the values are shown as the mean standard error of the mean. One-way ANOVA was used to statistically assess the outcome, and Tukey’s multiple comparison tests were then performed. Statistically significant as compared to the control was the p-value of 0.05.

**Results and discussion**

**Formulation preparation and characterization**

All of the manufactured ointments were characterised using parameters such measures for viscosity, hardmess, water number, homogeneity, pH, spreadability, and appearance, odour, and colour. The produced formulations’ organoleptic characteristics were white colour, acceptable homogeneity, and good consistency.

All of the manufactured ointments were characterised using parameters such measures for viscosity, hardness, water number, homogeneity, pH, spread ability, and appearance, odour, and colour. The synthesised formulations were found to have a white colour, good homogeneity, and good consistency according to organoleptic observations. The results of the three independent measurements revealed a pH range of 5.5–6.5. A good spread ability of around 5 seconds was discovered. At room temperature, the

<table>
<thead>
<tr>
<th>Group/treatment</th>
<th>8th days</th>
<th>16th days</th>
<th>24th days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apigenin</td>
<td>12.88±0.56</td>
<td>15.88±0.56</td>
<td>22.88±0.56</td>
</tr>
<tr>
<td>Povidone iodine</td>
<td>8.88±0.56</td>
<td>12.88±0.56</td>
<td>21.88±0.56</td>
</tr>
<tr>
<td>Wound control</td>
<td>7.35±0.57</td>
<td>11.88±0.56</td>
<td>12.88±0.56</td>
</tr>
</tbody>
</table>

Values are mean ±S.D (N=4). Mean values with the same superscript are significantly different (P<0.05)

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Tensile strength (N/cm2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>06.12± 2.51</td>
</tr>
<tr>
<td>Standard</td>
<td>13.24± 1.21</td>
</tr>
<tr>
<td>Apigenin-loaded ointment</td>
<td>11.01± 1.63</td>
</tr>
</tbody>
</table>

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measurements were made in triplicate. The ointment's viscosity was measured to be between 32.22 and 34.5 centipoises (CPS), which was in accordance with the specifications for topical formulation. The evaluation of the skin irritation research revealed that no skin allergies or irritations were discovered. The developed topical formulations' physiochemical characteristics underwent testing and were confirmed to be good.

The results of the triplicate measurements showed that the pH ranged from 5.5 to 6.5. It was found to be good spreadability like 5 seconds.

The ointment's viscosity was determined to be between 32.22 to 34.5 centipoises (CPS), which was in compliance with the topical formulation specifications.

The evaluation of the skin irritation research revealed that no skin allergies or irritations were observed. The developed topical formulations' physiochemical characteristics underwent testing and were confirmed to be good. F6's drug content was discovered to be 98.1%.

Anti-inflammatory activity

F6, an improved ointment composition, may have anti-inflammatory effects. The amount of F6 edoema that was inhibited was discovered to be 59.97%. When compared to the control group, the inflammation of the paw edoema was dramatically reduced 3 hours after the carrageenan injection. The bioactive molecule apigenin, it has potential antibacterial properties. It was a crucial component in creating the anti-inflammatory properties. Possible inhibitors of cyclooxygenase, lipoxygenase, and nitric oxide synthase activity, as well as antioxidants, contributed to the potential anti-inflammatory impact. This gel's potential efficacy in treating skin conditions such as boils, abscesses, burns, and eczema was also confirmed.

Wound healing activity

The rate of wound contraction ranged from 21.5% to 68.2% from days 4 to 12 and 80.5% to 97.8% from days 14 to 20. In these rats, the shedding of eschar without leaving any trace of a raw wound took an average of 12.7 days, and the mean scar area after full healing was 98.7 mm². Rats treated topically with 25 mg/kg of apigenin showed an increase in wound contraction from 32.1% on day 4 to 85.6% on day 12 and 92.2% to 100% from day 14 to day 20, whereas rats treated with standard drugs showed an increase from 35.4% on day 4 to 87.7% on day 12 and 94.2% to 100% from day 14 to day 20. With topical ointments containing apigenin, the average epithelization time and scar size were 10.3 days and 74.2 mm², respectively.

The hydroxyproline content and tensile strength increased by 62.03% and 11.03%, respectively, following Apigenin loaded ointment treatment. In comparison to the control and standard, the data reported above showed that the chloroform fraction had better wound healing activity.

The animal group that received apigenin ointment demonstrated an increase in breaking strength, which may be attributable to the stability of the fibres and rise in collagen concentration. At the site of the wound, produced collagen molecules are deposited and cross-linked to create fibres. Collagen remodelling and the creation of durable intra- and intermolecular crosslinks both contribute to increased wound strength. According to the results of the dead space wound study, the animal group treated with apigenin ointment showed better breaking strength. Animals treated with apigenin ointment had an improvement in breaking strength, which may be explained by an increase in collagen concentration and fibre stabilisation. The produced collagen molecules are deposited at the site of the wound, where they undergo cross-linking to create fibres. Collagen remodelling and steady intra- and intermolecular crosslink production both contribute to the development of wound strength. Greater breaking strength was observed in the animal group treated with apigenin, which may have resulted from increased collagen production as seen in the dead space wound investigation.

The hydroxyproline concentration in the apigenin-loaded ointment therapy was 62.03%. In comparison to the control and conventional treatments, the aforementioned findings demonstrated that Apigenin had greater wound healing activity.

In the Apigenin loaded ointment and standard treated
groups, the histology of an excision biopsy of a skin wound at day 10 revealed healed skin structures with normal epithelization, restoration of adnexa, and fibrosis within the dermis, whereas the control group lags behind the treated group in formation of the amount of ground substance in the granulation tissue, as seen in tissue sections.

Under a light microscope, sections were evaluated qualitatively for fibroblast proliferation, collagen production, angiogenesis, and epithelialization.

On the control rat's skin, white arrows indicate areas of ulceration and edema, yellow arrows indicate early epithelization, and black arrows indicate areas of granulation tissue and an abundance of mononuclear inflammatory cells. Rats treated with apigenin ointment exhibit substantial fibrosis, a large amount of granulation tissue, a modest number of mononuclear inflammatory cells, and the repair of the adnexa. Rats treated with iodine ointment displaying healed skin structures with well-formed, nearly normal-appearing epidermis, restored adnexa, and significant fibrosis and collagen tissue within the dermis.

**Conclusion**

The Apigenin loaded ointment formulation ability to speed up wound healing was tested using in vivo method. All of these evaluation results showed good wound healing activity and the potential for wound healing and anti-inflammatory effects when compared to conventional medications. Finally, apigenin, a bioactive compound, can stimulate angiogenesis, collagen synthesis, and tensile strength, all of which are essential for effective wound healing.

**References**


