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1.0 Introduction

1.1 Wound

A wound can be defined as an injury or disruption to anatomical structure and function resulting from simple or severe break in structure of organ such as the skin and can extend to other tissues and structures such as subcutaneous tissue, muscles, tendons, nerves, vessels and even to the bone (Gonzalez, 2016). Wound can be accidental (e.g. burns, abrasion, skin tears ect.), surgical, occur because of underlying disease for example diabetic and vascular ulcer and some skin conditions may also develop into a wound for example eczema or psoriasis. Wound can be open or closed. Open wounds have exposed body tissue in the base of the wound. Closed wounds have damage that occurs without exposing the underlying body tissue.

Wounds generally fall into two categories: acute wound and chronic wound. Chronic wounds are acute wounds have not progressed through the stages of healing normally. They may heal at a much slower rate, heal only partially or reoccur after or complete healing. These chronic wound are almost associated with underlying chronic disease that affect either the blood supply or how the cells function at the wound site (Sen CK, et. al., 2017). There are different types of chronic wounds such as pressure injuries, diabetic ulcers, leg ulcers etc. Disruptions in the integrity of the skin that heals uneventfully with time re considered acute wounds (Sen CK et. al., 2017). Surgical and traumatic wounds, abrasion, or superficial burns are generally considered acute wound.

A burn is tissue damage caused by heat, steam, fires, gases, chemicals, electricity, sunlight, radiation. Burns are defined by how large an area they cover. Burns can cause swelling, blistering, scarring and in serious cases shock and even death. The y also can lead to infections because they damage skin's protective barrier. Types of burns include:

i. First-degree burns damage the outer layer (epidermis) of the skin. These burns usually heal within a week. A common example is sunburn.

ii. Second-degree burns damage epidermis and dermis. These burns might need a skin draft, natural or artificial skin to cover and protect the body while it heals and they may leave a scar.

iii. Third-degree burns damage or completely destroy both layers of skin including hair follicles and sweat glands and damage underlying tissues. These burns always required skin graft.

iv. Fourth degree burns extend into fat, fifth degree burns into muscle, and sixth degree burns to bone.

The significance of wound management can be described by following facts:

- According to retrospective analysis of medicare beneficiaries (2018), about 8.2 million people had different types of wounds. Patients suffering from diabetes and obesity are at a high risk of developing chronic wounds. Chronic wound are mostly seen in the elderly people. In US, 3% of the population > 65 years of age have open wound. Serena TE (2015) mention in report that the US government estimates that the elderly population will be over 55 million, suggesting that chronic wounds will continue to be an increasingly persistent problem in this population by 2020. About 2% of total population of US is estimated to be affected from chronic wound (Jarbrink et. al., 2017). Report published by Settipalli S (2015) said that the annual cost for wound care was projected to rise up to 3.5 billion in 2021 form an average of $2.8 billion in 2014 globally.
- The need for treatment strategies for treating chronic wounds is urgent. Diabetic wounds that do not heal are the most severe type of chronic wounds, affecting millions of people annually. Foot ulcers are common in diabetic patient and individuals with compromised blood circulation (Amin et. al, 2016). Many of the roughly 350 million diabetes patients in the world develop foot ulcers, and around 10-15 million cases this ultimately leads to amputation.
- In India, a recent study estimated a prevalence rate of chronic wounds at 4.5 per 1000 population. The incidence of acute wounds was at 10.5 per 1000 population which is more than double form chronic wound.
- Burns is a potential public health problem in the world. As per the Global burden of disease study (2013), there were 33.5 million thermal burn injuries reported in all over the world, resulting 237500 deaths. 90% burn deaths occur in low/middle-income countries (Peck M. D., 2017). According to National program for

prevention of Burns injuries, approximately 7 million people sustain burn injuries in each year in India, out of which 0.7 million need hospitalization, 0.25 million get crippled, and 0.14 million succumb (Singh AK. 2018). According to global Health data (2017), over 61,000 deaths in 2015 due to burns in India.

1.2 Wound Management:

Wound healing is a complex process involving several inter-related biological and molecular activities for achieving tissue regeneration. The main physiological events include coagulation, inflammation, removal of damaged matrix components, followed by cellular proliferation and migration, angiogenesis, matrix synthesis and deposition, re-epithelization, and remodeling (Gonzalez et al., 2016). Wound healing is a global medical concern with several challenges including the increasing incidence of obesity and diabetes, an ageing population and the requirement for more effective dressings. Infection is the greatest risk from non-healing wounds. Each of us is exposed every day to common bacteria such as staphylococcus and pseudomonas. These bacteria are present and thus settle easily on our skin. Wound increases the risk for infection, which can lead to serious health problems. Traditional Wound Care, comprised mostly of gauze-based dressings such as woven and non-woven sponges, conforming bandages, non-adherent bandages. Also topical formulations like solution, suspension, cream, gel are used for wound healing (Dhivya et al., 2015).

Ideal wound dressing: It is widely accepted that a warm, moist wound environment encourages healing and prevents tissue dehydration and cell death. These conditions also allow the interaction of the cells and growth factors involved in the in the healing process. Therefore, ideal wound dressing should:

- Maintain a moist environment at the wound surface
- Provide thermal insulation
- Provide mechanical protection and protect against secondary infection
- Be non-adherent and easily removed without trauma
- Leave no foreign particle in the wound
- Remove excess exudates

- Be cost effective and offer effective pain relief

Over the past few years an ever-expanding list of dressing has come onto the market in an attempt to meet these conditions. Among them are the transparent film dressing, hydrogel, hydrophilic foams, alginates, hydrocolloids, antimicrobial dressing and biological dressing. There is however no magic "one-size-fits-all" dressing. Absorbable haemostats and hydrogel dressings are the part of modern wound management systems which help in haemostasis and moist wound healing respectively.

Treatment of burn injury: Burn treatment varies depending on the cause and severity. In preliminary treatment of first degree burns, burns area are gently wash with cool water and some lotion or cream applied on it. Antibiotic ointments or creams are often used to prevent or treat infections in patients with second-degree burns. Using these ointments may require the use of bandages. There are many advanced wound care products includes impregnated gauzes, foams, honey dressing, hydrogel, silver dressings for the burn treatment. Larger are of third degree burns are treated with different types of skin grafts such as sheet grafts, meshed grafts, full-thickness grafts.

1.3 Some Biomaterials used in wound care systems

Biomaterials like Gelatin, Sodium hyaluronate, Sodium alginate, Collagen, Chitosan, Growth factors, plasminogens, fibrin, honey, turmeric, calendula, aloe vera are able to interact actively with the wound 'microenvironment', stimulating its healing, and are defined as 'bioactive medications'.

1.3.1 Collagen

Collagen is a biodegradable and biocompatible protein mostly found in connective tissue. One key component of chronic wounds is an elevated level of matrix metalloproteinases (MMPs). At elevated levels, MMPs not only degrade nonviable collagen but also viable collagen. In addition, fibroblasts in a chronic wound may not secrete tissue inhibitors of MMPs (TIMPs) at an adequate level to control the activity of MMPs. These events prevent the formation of the scaffold needed for cell migration and

ultimately prevent the formation of the extracellular matrix (ECM) and granulation tissue. Collagen based wound dressings are uniquely suited to address the issue of elevated levels of MMPs by acting as a 'sacrificial substrate' in the wound. It has also been demonstrated that collagen breakdown products are chemotactic for a variety of cell types required for the formation of granulation tissue. In addition, collagen based dressings have the ability to absorb wound exudates and maintain a moist wound environment (David Brett, 2008).

The first medical usage of collagen in humans was reported by Knapp et al. (1977) and was used to provide co-reaction of contour deformities. Bovine collagen was used as suture and hemostatic agents after years. In 1980, Zyderm 1 was released, a suspension form containing sterilised fibrillar bovine collagen that was used for injecting under the dermis in wounds. Today, collagen is used in numerous biomedical applications. These include collagen suspensions for dermal injection, topical haemostatic agents, wound dressing materials, collagen suture and catguts, collagen gels for periodontal reconstruction, collagen sponges for the hemostasis and coating of joint, and collagen rich pig skin wound dressing materials.

1.3.2 Hyaluronic acid :

Hyaluronic acid is a natural biopolymer that alternately consists of D-glucuronic acid and 2- acetamido-2-deoxy-D-glucose and is generally found in mammal's bond tissues and synovial fluids. It has been reported that hyaluronic acid interacts with proteins, proteoglycans, growth factors and tissue components called biomolecules which has vital importance in healing of various types of wounds. This interaction plays an important role in acceleration of tissue repair and wound healing. Hyaluronic acid and its derivatives also play a role in the protection of the injured area against microorganisms due to their bacteriostatic activity (Joshua boating et al., 2015).

Hyaluronan binds through three major classes of surface receptor: CD44 (specific adhesion molecule), RHAMM (Receptor for Hyaluronan Mediated Motility) and ICAM-1 (IntraCellular Adhesion Molecule 1), involved in the modulation of tissue repair and in various other functions.CD44 is widely distributed in the organism and is considered the main surface receptor for hyaluronan. It is believed that the two main functions of CD44

in the skin are to regulate the proliferation of keratinocytes in response to extracellular stimuli and to maintain local homeostasis of hyaluronan. The interaction between hyaluronan and CD44 affects cell proliferation, motility and adhesion (Benedetti et al., 1993).

The expression of RHAMM on cell surface is associated with cell motility and has been identified in a wide range of migrating cells (fibroblasts, etc.). RHAMM is an adhesion molecule that is not localised only at the level of the cell surface but also into the cell, at the level of mitochondria, cytoskeleton and nucleus.The hyaluronan-RHAMM surface complex plays a key role in activating intracellular signalling pathways that, as in the case of CD44, affect cell proliferation and migration (Benedetti et al., 1993).

The binding of hyaluronan to ICAM-1 can affect its relation with other receptors (such as LFA-1/Mac-1) that, mediating the interaction between endothelial cells and leukocytes, can contribute to controlling the inflammatory response mediated by ICAM-1(Benedetti et al., 1993)

1.3.3 Pot Marigold (*Calendula officinalis L.*)

Calendula officinalis L. or pot marigold is a common garden plant belonging to the compositae family. The flowers are the part of the herb used medicinally. The main compounds within calendula are the triterpenoids which are claimed to be the most important anti inflammatory and antiedematous components within the plant (Zitterl-Eglseer et al., 1997; Parente at al., 2012). Other constituents identified in calendula such as the saponins, micronutrients, flavonoids, and polysaccharies, may also be responsible for the antiedematous, anti-inflammatory, antioxidant, and wound healing effect of the plant (Preethi et al., 2009). Clinical study was conducted in between May 2012 to Dec 2013 on 109 patients by Marcelo buzzi et al in Brazil to evaluate the effect of *Calendula officinalis* hydroglycolic extract on the healing rate of Diabetic foot ulcers in patients with diabetes until complete wound closure and during a 30 week follow up period to monitor the long –term effect of the treatment (Marcelo buzzi et. al., 2016). The study findings suggested that *Calendula officinalis* extract is safe and has a beneficial effect on Diabetic foot ulcers healing (Leila maria leal parente et al., 2012). It has a significant anti-inflammatory (Parente et al., 2012), antioxidant (Bernatoniene et al., 2011), wound

healing (Buzzi et al., 2016), antibacterial (Faria et al., 2011), and antiviral effect (Kalvatchev et al., 1997).

1.5 Selected formulations for effective wound healing.

1.5.1 Scaffolds / Films

Hybrid scaffolds comprised of polymeric substrates coated with bioactive materials, collagen, silk fibroin, as well as advanced tissue engineered substrates impregnated with endothelial progenitor cells, and nanomaterial-based scaffolds are used as advanced wound dressings to initiate and expedite wound healing (Waghmare et al., 2018; Atul et al., 2016). These pharmaceutical dosage forms, which are available in thickness ranging from μm to mm, are prepared by different methods using one or more polymers. Films and scaffolds are the ideal dressing materials and available commercially (Ajay et al., 2016). Films/membranes with a homogeneous polymeric network structure are used to treat the damaged area and generally protect the wound and burn area against external factors. Dermal scaffold would mimic the tissue's natural extracellular matrix. Scaffolds are manufactured using natural or synthetic polymer like chitosan, collagen, gelatin, PLGA, hyaluronate. In the case of major burns where injury damages the deep dermis and no sources of cells for regeneration remain; there is a requirement to provide a dermal scaffold to fill in the deep wound (Bakhshyayesh et al., 2017; Ajay et al., 2016).

1.5.2 Hydrogel

Hydrogel is a three dimensional network of hydrophilic polymers. Hydrogels consist of swellable hydophilic materials such as starch, cellulose; synthetic polymers like polyvinyl alcohol, polyacrylic acid, Polyvinylpyrrolidone, poly(methacrylates) or other plant or animal derived polysaccharides and contain up to 96% water. Hydrogels are capable of absorbing large volumes of water because of the presence of hydrophilic chains, which allow them to swell extensively without changing their gelatinous nature. They can be used on dry, sloughy, or nectrotic wound.

The advantages of hydrogels in wound and burn treatment can be listed as follows

- Bioadhesion of gels to the surface of the wound is high and this also eases the treatment due to increased contact with the wound
- Their structures facilitate the moisture and water vapour permeability necessary to heal the wound area. Moist treatment of wound is considered to be the gold standard of therapy for acute open wounds as well as chronic wounds.
- Difficulties that are particularly related to the application to open wounds are not seen in these preparations
- They can easily be removed from the application site when adverse events are seen

2.0 AIMS AND OBJECTIVES

2.1 Aims: The aims of the current project are as follow:

2.2.1 : To develop safe and effective hydrogel sheet containing calendula flower extract for wound management

2.2.2 : To develop safe and effective calendula flower extract loaded biodegradable collagen film for wound management.

2.2 Objectives

- ❖ Overall:
- Development and evaluation of biomaterial based formulations containing *Calendula officinalis* flower extract for wound management
- Perform safety and efficacy study of developed formulations

.

- ❖ Specific:

i. Development of calendula flower extract loaded biodegradable film and hydrogel sheet

ii. Optimization of formulations using Design of Experiment Techniques

iii. *In-vitro* and *In-vivo* performance of the formulations for the evaluation of safety and efficacy.

2.3 Hypothesis:

- Biodegradable film containing biomaterial such as collagen and sodium hyaluronate would mimic the tissue's natural extracellular matrix which plays an active role in normal wound healing process and tissue regeneration. Calendula flower's constituents like triterpenoids and flavanoids which are responsible for its significant therapeutic activity such as anti-inflammatory, antioxidant, anti-odemetaous etc., will give synergistic effect in wound healing with collagen and sodium hyaluronate.
- Hydrogel sheet are easy to remove, easy to apply, have good moisture donating and absorbing capacity and water vapor permeability which helps in wound healing process. By incorporating *Calendula officinalis* flower extract into hydrogel sheet, there will be improved and increased would healing efficacy of hydrogel sheet due to anti-inflammatory, antioxidant, anti-odemataous activity of *Calendula officinalis* flower extract.

3.0 METHODOLOGY

3.1 ANALYTICAL TECHNIQUES

3.1.1 **Determination of total Phenolic content:** Total phenolic content of *Calendula officinalis* flower extract was determined by colorimetric method using Folin-Ciocalteau reagent against gallic acid as a reference standard (Singleton et. al., 1965). The absorbance was measured at 765 nm using UV-Visible spectrophotometer (Shimadzu, Japan). The technique was used for developed formulation evaluation.

3.1.2 Determination of total flavonoid content: Total flavonoid content of *Calendula officinalis* flower extract was determined by colorimetric method (Ordon Ez et. al., 2006). Samples were analyzed using UV Visible spectrophotometer (Shimadzu, Japan) and the total flavonoids content was calculated using quercetin

as a reference standard. The technique was used for developed formulation evaluation.

3.1.3 **Estimation of antioxidant activity:** The radical scavenging capacity of *Calendula officinalis* flower extract was examined by the colorimetric assay using 2,2-diphenl-1-picryllhydrazyl (DPPH) (Blois M.S., 1958). The technique was used for developed formulation evaluation.

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3.2 PRE-FORMULATION STUDIES

DSC, FT-IR, Colorimetric methods for active constitutes quantification, karl fischer titration and TLC were used for calendula flower extract characterization. The selection of excipients were carried out based on compatibility with CFE. DSC and FT-IR study were used to check compatibility between CFE and selected excipients.

3.2 FORMULATIONS DEVELOPMENT, OPTIMIZATION AND CHARACTERIZATION

3.2.1 HYDROGEL SHEET

Hydrogel sheet was prepared by gamma irradiation technique (Razzak et al., 1999). In one vessel, aqueous solution of polymer and preservatives were prepared by dissolving them into distilled water with slowly stirring at 60° - 90° C. In another vessel solidifying polymer, CFE, and biomaterial were dissolved in distilled water with constant striring at 40° – 60° C. After that, both solutions were mixed properly and the resulting hot solution (40° – 60° C) was poured into the plastic molds for solidification at room temperature. After solidification of solution, plastic molds were irradiated with gamma rays for sterilization and crosslinking. Optimizations of various formulation variables were done based on DoE based approach using Box-Behnken design. Prepared hydrogel sheets were evaluated for physicochemical parameters such as %water absorption (%A), %Gel fraction (%GF) and Hardness. They were also evaluated for pH, FT-IR, DSC, total phenolic content, total flavonoid content, antioxidant activity and sterility.

3.2.2 BIODEGRADABLE FILM:

Biodegradable film was prepared using solvent evaporation method and optimized using OVAT method. Firstly, Collagen slurry was made by homogenization of collagen dough into distilled water. Subsequently, in another vessel other materials such as CFE, sodium hyaluronate, plasticizer were dissolved into distilled water which was added to the collagen slurry. After homogenous mixing, resulting slurry was poured into the moulds and kept for air drying in dark under a laminar air flow at room temperature. The dried films were packed into the pouch and sterilized by gamma irradiation process. The prepared films were evaluated for dimension, folding endurance test, water absorption capacity, mechanical strength, %moisture content, water vapor permeability, pepsin digestion time, FT-IR, DSC, Atomic Force Microscopy, antioxidant activity, total phenolic content, total flavanoid content, gel electrophoresis and sterility.

3.5 STABILITY STUDIES

The developed formulations were evaluated for stability under ambient temperature (for real time stability study, 25ºC±2º C, 60%RH±5% RH) and accelerated condition (40ºC±2º C, 75%RH±5% RH) for 6 months as per ICH guidelines. Samples were withdrawn after 1, 2, 3 and 6 month for evaluating stability indicating parameters.

3.6 CELL LINE STUDIES

3.6.1 MTT study

MTT study (Cell viability study) was carried out using Swiss 3T3 fibroblasts cell line. The toxicity profile comparison between growth media, CFE, placebo formulation and formulations containing CFE by MTT assay showed that the formulations were non-toxic in nature (Faria et al., 2011).

3.6.2 Scratch assay

To evaluate the wound healing potential of prepared formulation, scratch assay was performed in which artificial injured cell by scratch were treated with developed formulations and CFE. Cell migration into the scratches area was observed. The spreading and migration capabilities of Swiss 3T3 fibroblasts were assessed using a

scratch wound assay which measures the expansion of a cell population on surfaces (Fronza 2009).

3.7 IN-VIVO STUDIES

The experimental protocol for the study was evaluated and approved by the Institutional Animal Ethics Committee, Faculty of Pharmacy, The MSU of Baroda, Gujarat, India.

Following two animal models are used for study:

- Burn Wound model on Rat (Khazaeli et al., 2014) and
- Cutaneous excision wound healing model on Rat (Millán et al., 2016).

Male Sprague-Dawley (SD) rats were randomly allocated to groups that contained 6 rats in each group as follows: Group I (Normal control without any treatment), Group II (Placebo treatment), Group III (Treatment with developed formulation 1), Group IV(Treatment with Developed formulation 2) and Group V(Marketed product). The rats of all groups were anesthetized by injection of 0.8 cc Ketamine (5%) and 0.2 cc xylazine/diazepam intramuscularly in the hamstring muscles. The dorsal aspect of the cervical or cervical to lumbar area was shaved and washed with a scrub solution of povidone-iodine. Under sterile conditions, a skin incision / Burn was made in a square shape (2x2 cm) in the cervical region. All wounds in the treatment groups were dressed with either marketed product or formulation followed by transparent adhesive dressing as a secondary dressing.

Wound area was observed and measured using proper scale during 21 days. After 21 days post injury, the rats of all groups were anesthetized and under sterile conditions, a skin incision was made to get skin sample (about 3cm x 3cm) for Histopathological and Biochemical studies.

4.0 RESULTS & DISCUSSIONS

4.1 Pre-formulation study: CFE and excipient were authenticated by FT-IR graph. Compatibility study between CFE and excipient were studied using DSC and IR. It was found that the there was no incompatibility between them.

4.2 Formulation development and evaluation of Hydrogel sheet containing calendula flower extract:

After preliminary trials of hydrogel sheet, it was found that the PVA content, CFE content, solidifying agent content and gamma irradiation dose most significantly affected the properties of hydrogel sheet like %water absorption, %gel fraction and hardness. The formulation was further optimized using Box-Behnken design using DesignExpert® 7.0 (Stat-Ease Inc., MN). The prepared hydrogel sheet containing calendula flower extract was transparent, flexible, non sticky and golden yellow in color. % Gel fraction were reduced significantly with incorporation of CFE, decreased slightly with the increase of Solidifying agent and increased with irradiation dose. The swelling capacity decreased with increased in %GF because increased in crosslinking of polymer. Higher irradiation dose enhance the crosslinking density and reduce the degree of swelling of hydrogel. The hardness of hydrogel sheet increased with irradiation dose because chemical crosslinking in the PVA by gamma irradiation. pH value of aqueous extract of developed formulation was in acceptable range for wound dressing application. Water vapor transmission rate of hydrogel sheet was within 80 – 120 g/m2/h which is acceptable for wound dressing application. Total polyphenols and flavonoids content were found to be 7 to 10 mg GAE/g extract and 0.6 to 0.9 mg QUE/g extract respectively. Developed hydrogel sheet was good barrier against the microbes because there was no any microbial growth was found on surface of hydrogel sheet in microbe penetration test.

4.3 Formulation development and evaluation of Collagen film containing calendula flower extract: Collagen films were prepared by solvent evaporation method. Concentration of collagen, concentration of plasticizer, speed of homogenizer and drying method were optimized using OVAT method. Prepared film was 2-3 mm thin, transparent, flexible and slight yellowish in color. It had about 15 -25 g/g water absorbency and about 4 hr pepsin digestion time. It had less than 8% moisture content.

pH of the prepared film was in between 3 to 4. It was not brittle as its folding endurance was more than 250 times. Total polyphenols and flavonoids content were found to be 7mg to 9 mg GAE/g extract and 0.6 to 0.9 mg QUE/g extract respectively. Collagen of prepared film after sterilization was in native form which was confirmed by AFM study and gel electrophoresis study.

4.4 Stability study: The samples did not show any significant changes in any stability indicating parameters during stability study. Hence developed formulations were stable.

4.5 Cell line study

4.5.1 MTT assay: The toxicity profile comparison between growth media, CFE, placebo formulation and formulations containing CFE by MTT assay showed that the formulations were non-toxic in nature (Faria et al., 2011). The CFE and formulations containing CFE significantly stimulated the cell proliferation as number of cells markedly increased after the treatment with formulations compare to placebo and growth media sample

4.5.2 Scratch assay: Wells were treated with formulations had more population of fibroblast cells in the scratched area compared to non-treated wells and placebo formulations treated wells which confirms usefulness of developed formulations for wounds. *In-vitro* scratch assay showed that the developed formulations were promoted cell migration and proliferation better than placebo in artificial wound.

4.6 *In-vivo* study: *In-vitro* scratch assay showed that the developed formulations were promoted cell migration and proliferation better than placebo in artificial wound. Developed formulations were significantly enhanced wound healing activity in by increasing cellular proliferation, formation of granulation tissue, neovascularization, synthesis of collagen epithelization and early histological maturation in rat. There was significant increase in the collagen amount in the treated group compared to non treated and placebo group. This indicates a positive role of the extract on the cutaneous wounds healing process. The triterpene faradiol palmitic ester stimulated the proliferation and migration of fibroblasts which stimulates the fibroplasias.

5.0 CONCLUSIONS

The methodology utilized in the present work have produced potentially useful stable formulations which were able to improve the wound healing process. *In-vivo* study indicated that calendula flower extract containing formulation showed significantly better wound healing activities compared to placebo formulations. We concluded that *Calendula officinalis* extract containing biodegradable film and hydrogel sheet stimulated wound healing process and increased the cell proliferation better than placebo formulation due to enhanced bio-activity of hydrogel sheet and biodegradable film by *Calendula officinalis* flower extract.

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