

Development and Evaluation of a Topical Anti-Inflammatory Preparation Containing *Dodonaea polyandra* Extract

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ABSTRACT - Purpose: We have previously reported that the Australian Northern Kaanju (Kuuku I'yu) medicinal plant *Dodonaea polyandra* has anti-inflammatory activity. This is attributed largely to the presence of clerodane diterpenoids contained within the leaf resin. We envisaged developing a topical preparation to treat indications relating to skin inflammation. However, it was unknown whether the resin could be incorporated into a suitable dosage form while retaining the therapeutic value demonstrated in previous work. Therefore, the following study was undertaken to assess parameters of safety and efficacy for a prototype formulation containing the leaf resin extracted from *D. polyandra*. **Methods:** Using the assessment criteria of optimum appearance, tactile feeling, spreadability and odour, 78 different formulations were developed. Formulation stability was assessed using a centrifugal test with preparations displaying phase separation further modified or re-formulated. A prototype formulation containing 5% w/w plant resin was selected and subjected to in vitro release studies. This was quantified through HPLC analysis using two major bioactive diterpenoids as reference. The prototype formulation was tested for efficacy in a TPA-induced acute murine skin inflammation model as well as a 3D human skin model for irritancy/toxicity (Epiderm™). **Results:** The prototype resin cream was a chartreuse-coloured homogenous semisolid preparation that was readily spreadable upon contact with skin with no sensation of tackiness, residual greasiness, or irritation. The optimized cream showed no phase separation after 30 min centrifugation at 825 g. In the TPA-induced inflammation model, the resin formulation significantly reduced ear thickness and interleukin-1 beta levels in mouse ear tissue. The 5% w/w resin cream formulation showed no irritancy in a 3D human skin model. **Conclusions:** Our results demonstrate that bioactive resin from *D. polyandra* can be formulated into a stable and non-irritant semi-solid dosage form and reduce parameters of acute skin inflammation *in vivo*.

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INTRODUCTION

This study was undertaken as part of continued research aimed at establishing an efficacious and commercially viable product for use against inflammatory conditions of the skin, including eczema, psoriasis and dermatitis. These common skin inflammatory disorders have substantial impacts on quality of life for patients and costs to the healthcare system and society (1-3). While there are a range of anti-inflammatory drug therapies in existence the limitations of these therapies, particularly in terms of their side-effect profiles, has driven the search for new drug entities with novel mechanisms of action and improved safety profiles (2, 4).

Our pharmacological investigation of the medicinal plant *Dodonaea polyandra* Merr. &

L.M. Perry (Sapindaceae) began as part a project initiated by the Chuulangun Aboriginal Corporation. This Corporation represents Traditional Owners of the Northern Kaanju (Kuuku I'yu) homelands centred on the Wenlock and Pascoe Rivers in Cape York Peninsula, Queensland, Australia. Traditional Owners are seeking to examine opportunities for development of their natural resources such as medicinal plants in a way that could contribute to economic development on their homelands. Particular Kuuku I'yu (Northern Kaanju) families involved in the project use *Dodonaea polyandra* as a medicine for relief of pain and inflammation in the mouth. The plant is used by breaking off one of the terminal branchlets (stems) with a leaf attached.

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The plant material is applied directly to the mouth to reduce pain and inflammation. In Australia, the natural distribution of *D. polyandra* is restricted to Cape York Peninsula, particularly on the east and north coast and off-shore islands (5). The plant also grows in the Western province of Papua New Guinea (5).

Initially we showed that crude ethanolic extracts of the leaves of the plant have significant anti-inflammatory activity in the 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear oedema model for skin inflammation (6). In this model, thickening of skin tissue occurs which is indicative of a number of processes that occur during skin inflammation, including increased vascular permeability, swelling in the dermal layer, and proliferation of the epidermal cells (7). We subsequently showed that extracts of this plant species are rich in diterpenoid secondary metabolites of both the clerodane and labdane classes (8-10). In particular, two of the furanoclerodane diterpenoids, now designated polyandric acid (PA) A and B (Figure 1), were found to be the main active components of the plant extract, demonstrating potent anti-inflammatory activity in the TPA-induced mouse ear oedema model (8). These compounds can be found in the leaf resin that forms a white powder on the leaf surface once dried.

In a subsequent study (10) focussed on the clerodane PAA we demonstrated that topical application of an ethanolic solution of the compound to mouse ear tissue significantly inhibited interleukin (IL)-1 β production in the ear tissue in an acute TPA-induced inflammation model. Further, in a mouse model of chronic skin inflammation, a marked reduction in ear thickness, associated with significant reduction in myeloperoxidase accumulation (a measure of neutrophil infiltration), was observed. In order to better understand potential effects in human skin, treatment of primary neonatal human keratinocytes with PAA followed by activation with phorbol

ester/ionomycin was studied, showing a significant reduction in secretion of IL-6 but not tumour necrosis factor (TNF)- α (10).

Studies on extracts of a more widely distributed medicinal *Dodonaea* species, *Dodonaea viscosa*, have also identified a furanoclerodane diterpenoid called hautriwaic acid as an active anti-inflammatory compound (11, 12). Recently, it has been shown that this compound inhibits inflammatory cytokine release in a mouse model of knee arthritis and reduces the concentration of pro-inflammatory cytokines IL-1 β , IL-6, and TNF- α in the joint (12).

In this study we sought to develop and optimise topical formulations containing the leaf resin of *D. polyandra* that would display similar efficacy to resin delivered through an ethanol vehicle. The anti-inflammatory efficacy and toxicity of one prototype cream was evaluated using a TPA-induced acute mouse ear oedema model and a three-dimensional human skin model, respectively.

MATERIALS AND METHODS

Materials and equipment

Materials used included stearyl alcohol (Scharlan, Spain), Vaseline® (Chem-supply, Australia), Span 40 and 80 (Aldrich, USA), Tween 80 (Aldrich, USA), absolute ethanol (Chem-supply, Australia), Polychol 15 (CRODA, Australia) Carbopol 934P (Neveon, USA), liquid paraffin (Ajax Finechem, New Zealand), polyethylene glycol (PEG) 4000 (BDH, England), PEG 300 (Sigma, Germany), Cremophor EL, Pluronic F-127, methyl paraben, propyl paraben, and 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma, USA), acetone (Merck, Germany). For the HPLC mobile phase acetonitrile (HPLC grade) and formic acid (purity 98-100%) were purchased from Merck (Germany). Additional formulation excipients are listed separately in supplementary information.

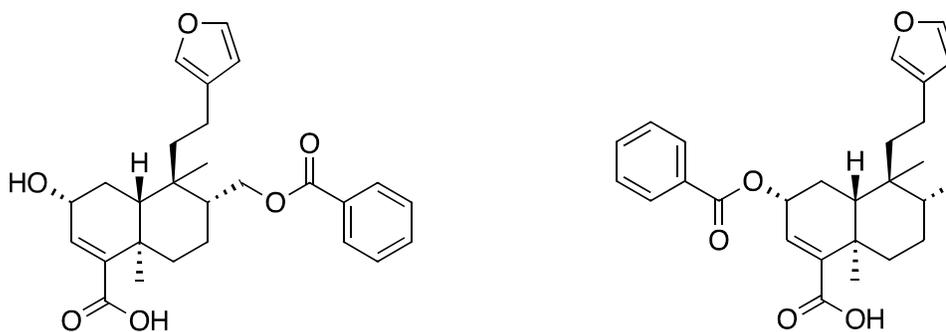


Figure 1. Chemical structures of polyandric acid A (left) and B (right).

Equipment included a homogenizer (Ultra TURRAX® T25 basic, IKA Labortechnik), digital electronic micrometer (± 0.001 mm, Mitutoyo, Japan), Cyber Scan pH 1100 (Southern Cross, Science Pty Ltd, Australia), tissue processing machine (ASP300S, LEICA), tissue embedding machine (EG1160, LEICA), microtome (HM325, LEICA), tissue homogenizer (MICCRA D-1, ART-moderne Labortechnik, Germany), water bath (HI1210, LEICA), microscope (OLYMPUS BX 40F-3, Japan), centrifuge (Sigma 1-15, SIGMA Laborzentrifugen GmbH), mechanical tumbler (Erweka AR400, Erweka Apparatebau GMBH, Germany) and rotary evaporator (Buchi Rotavap RII).

Preparation of the resin extract and quantification of active clerodane diterpenoid compounds

Leaf material from *Dodonaea polyandra* was collected in June 2011 by Northern Kaanju researchers on Kuuku I'yu Northern Kaanju homelands, Central Cape York Peninsula, Queensland, Australia. Voucher specimens and details of plant location (13.04231° S 143.02617° E) were recorded and lodged at the South Australian State Herbarium (voucher specimen number AD268969). Plant material was allowed to air dry in the shade and packed into paper bags for transportation. The leaves were stored at -20 °C until extraction. Plant material, 100 g, was placed in a mechanical tumbler for 2 h to loosen dried resin from the leaves. The resin powder was separated from leaves, which were subsequently transferred to a 2 L container and loose resin was washed from the leaves with a total of 450 mL absolute ethanol and combined with the first portion of the extract. The ethanolic extract was decanted and filtered in vacuo through Whatman No.1 filter paper (Whatman, UK) and concentrated using a rotary evaporator (40–42 °C). Dried extract was forced through a 450 μ m sieve to procure fine resin powder. In order to determine the content of active clerodane diterpenoid compounds in the extracted resin, a sample of the resin was analysed by HPLC.

Resin properties

The resin extract obtained was olive-green in colour and had a mild leafy odour. The resin possessed a granular texture and crystalline appearance. The resin was freely soluble in ethanol, although some crystalline matter was observed to precipitate upon standing. The resin was insoluble in aqueous solutions.

Quantification of active clerodane diterpenoid compounds by High Performance Liquid Chromatography (HPLC)

Analysis of active components was performed using a previously validated method (PAA: intra/inter-day accuracy -0.66/-1.32 %, intra/inter-day precision 2.59/4.01 %, linearity $r^2 = 0.9992$; PAB: intra/inter-day accuracy -0.48/-2.14%, intra/inter-day precision 2.49/3.34 %, linearity $r^2 = 0.9994$) (13). The method was conducted using a Shimadzu LC-20AD with degasser (DGU-20A3) coupled with an auto-sampler (SIL-20A HT, 200 μ L loop) and PDA detection SPD-M20A. Separations were carried out on Phenomenex Gemini C18 column with guard cartridge. The mobile phase consisted of acetonitrile (65 % v/v), Milli-Q water (35 % v/v) and formic acid (0.05 % v/v), degassed prior to use. The mobile phase flow rate was 1.4 mL/min with 30 min run time. The active components were detected using a wavelength of 225 nm. Sample injection volume was 20 μ L. Retention times for active components PAA and PAB were approximately 7 and 20 min, respectively.

Preparation of formulations and their assessment

A series of semisolid formulations were used in the initial trial for formulations. Briefly, 78 semisolid formulations were designed and prepared. They were categorised into three series, including cream, gel and ointment-based formulations according to pharmaceutical nomenclature used by the CDER Data Standards Manual Definitions for Topical Dosage Forms (14). Full details of the different formulations tested are provided in the Supplementary Information (Tables S2 to S15). Creams (Supplementary Information Tables S2, S3, S7 to S13 and S15) were classified into 10 types according to the application of different emulsifiers and surface active agents, including Tween, Span, Polychol and Cremophor. Gels (Supplementary Information Tables S4, S5 and S6) were classified into 3 types based on the gelling agents used, which were Carbopol, Polyethylene glycol (PEG) and Pluronic F-127. One ointment (Supplementary Information Table S14) was prepared, in which the major components were Vaseline® and liquid paraffin. During the formulation development phase, a range of conditions including temperature, ratio of excipients, the sequence of excipient addition and force and duration of homogenation were all investigated in order to produce the optimal product.

Physical characteristics

All formulations listed in Supplementary Information tables S2 to S15 were evaluated using standard criteria for spreadability and acceptability. Spreadability of formulations was determined according to the criteria outlined in Table 1. Vehicles rating at “0” or “1” were considered to have adequate spreadability. Acceptability was determined with subjective assessment of appearance, tactile feel, texture and odour.

In practice, preparations with insoluble sediments, poor spreadability or unpleasant smell or tactile feel were further modified or re-formulated. The pH of each formulation was also determined (Universal indicator pH 0-14, Merck).

Centrifugation test

All creams and the ointment (supplementary Tables S2, S3, S7 to S15) were subjected to centrifugation to assess accelerated deterioration based on the method by Anchisi et al. (15) with some modification. The preparations were centrifuged at 25 °C, 825 g for 30 min. Gravitational stability was assessed as ‘not stable’ (phase separation occurs) or ‘stable’ (no phase separation). Preparations with signs of phase separation were further modified or re-formulated. Gravitational stability assessment was not applied to gels.

Formulation of the prototype *D. polyandra* resin cream

The resin cream contained 5% w/w of *D. polyandra* resin, the strength of which was recognised to achieve significant anti-inflammatory effect based on unpublished preliminary studies. The optimised resin cream formulation was prepared with the following process. A 0.5 g portion of resin was dissolved in 1.2 g absolute ethanol before adding to the oil phase which consisted of 0.3 g Span 40, 1g Polychol 15, 0.6 g Vaseline, 1.2 g stearyl alcohol, 18 mg methyl paraben and 2 mg propyl paraben melted at 80°C. The aqueous phase (5.2 g purified water) was heated to 80°C and slowly added to the

oil phase under constant stirring. The mixture was homogenized for 2 min at 13,000 rpm, 80°C (Ultra TURRAX® T25 basic). The cream was allowed to cool to room temperature with continuous homogenizing at 10,000 rpm. The characteristics of the cream were then investigated using the standard criteria for physical characteristics and deterioration (by accelerated centrifugal test described above).

***In vitro* release test**

Franz diffusion cell was applied to quantify the release of active compounds from the formulation. The area for diffusion was 0.785cm², and the receptor chamber volume was 5.2 ± 0.2 mL. Hydrophilic polyvinylidene fluoride (PVDF) membranes (0.45 µm, Pall Corporation) were placed between the donor chamber, which was loaded with 22±2 mg test samples, and the receptor chamber, filled with water. The receptor fluid was kept at 32 ± 1 °C by a water jacket and constantly stirred with a magnetic stirring bar. A 150 µL volume of receptor fluid was drawn at 1, 2, 4, 6, and 8 h through a sampling port and was immediately replaced with 150 µL of fresh water. As preliminary data indicated that the active compounds were sensitive to light, experiments were conducted in the dark. Active diterpenoid compounds PAA and PAB were determined by HPLC analysis as described above.

Experimental animals

Male Balb/C mice, 7-9 weeks of age, were housed in groups at a constant room temperature (20 ± 2°C) and a 12 h normal day/night cycle (University of South Australia Animal Care Facility, Adelaide). They were kept in mouse cages and given free access to tap water and standard food. The mice were assigned randomly and kept in the laboratory for 48 h during which time their ear thickness and body weight were measured. Animal ethics approval was obtained for the experiments from the Institute for Medical and Veterinary Research (Adelaide) Animal Ethics Committee (Project # 15/11).

Table 1. Criteria used to assess spreadability of formulations

Score	Description
-2	Formulation or vehicle appears as a solid state, which cannot be spread around the application site.
-1	Formulation or vehicle has to be applied with force greater than normal to achieve a desirable spread.
0	Formulation or vehicle displays good spreadability with desirable stay-on semisolid property.
1	Formulation or vehicle displays good spreadability with a slight tendency to flow over the skin surface.
2	Formulation or vehicle has low viscosity with high tendency to flow over the skin surface.

TPA-induced mouse ear oedema model

Anti-inflammatory activity was measured by a mouse ear oedema model using topically applied 12-O-tetradecanoylphorbol 13-acetate (TPA) to induce inflammation on mice ears as described previously (6). The initial baseline ear thicknesses were recorded by a digital micrometer (± 0.001 mm). The micrometer was applied near the tip of the ear distal to the cartilaginous ridges and the thickness was recorded in mm with 3 repeated measures.

At time zero, 20 μ L TPA (2.5 μ g/20 μ L/ear) dissolved in acetone was applied topically to the right ear; an equivalent volume of acetone was applied to the left ear as control. Meanwhile, mice allocated in solvent negative control and cream vehicle negative control groups were treated with 20 μ L acetone on both ears. Thirty minutes later, cream (5% w/w resin), cream vehicle, resin solution (dissolved in absolute ethanol, 20 mg/mL) and absolute ethanol were applied to both surfaces of the right ear either in a volume of 20 μ L for solutions or liquid preparations, or in a thin layer for semisolid preparations. The left ears of mice receiving cream or cream vehicle remained untreated. A non-treated control group received TPA in acetone and was used as a measure of maximum inflammation. Twenty microliters of absolute ethanol was applied on the left ears as solvent control. Ear thicknesses were measured 6 h after TPA application. Mice were euthanized by inhalation of isoflurane followed by cervical dislocation. After excision of the ears, punch biopsies (5 mm) of each ear (one biopsy per ear) were taken. Ear tissue was homogenized in 500 μ L of Tris-HCl (50 mM, pH 7.4)/EDTA (1 mM) buffer containing protease inhibitor cocktail (Sigma, cat. no. P8340). The homogenate was incubated on ice in the presence of 1% (v/v) Triton X-100 for 15 min and subsequently centrifuged at 4°C for 15 min at 9500 g. The supernatant was removed and stored at -80 °C pending analysis by IL-1 β ELISA.

IL-1 β assay

The supernatant from ear tissue homogenates was thawed 30 min prior to assaying for the pro-inflammatory cytokine IL-1 β . The concentration of IL-1 β was determined using a Mouse IL-1 β Platinum ELISA kit according to the manufacturer's instructions (eBioscience, cat# BMS6002).

Histological analysis

Ear samples were fixed in 10% v/v neutral buffered formalin overnight, then embedded in paraffin,

sectioned and stained with haematoxylin and eosin (H&E) based on the standard procedures. Slides were observed under a light microscope (OLYMPUS BX 40F-3, Japan). A representative area was selected and inflammatory severities were subsequently qualitatively assessed based on ear thickness and inflammatory cell infiltration, indicated by purple/black stained cells.

Epiderm™ MTT viability test

To provide an estimate of dermal irritation potential, the 5% w/w resin cream, and an equivalent cream formulation without resin (cream base) were prepared and submitted for testing for toxicity using a three-dimensional human skin model – the MatTek Epiderm™ MTT viability test. Epiderm™ tissues consist of normal, human-derived epidermal keratinocytes cultured to form a multilayered and differentiated model of the human epidermis. The study was conducted by the contract research organization MB Research Laboratories, Spinnerstown, PA, USA (Project number 13-21604.19), according to standard Good Laboratory Practice (GLP) protocols (16). Briefly, EpiDerm™ human skin tissues were obtained from MatTek Corporation (Ashland, MA, USA). Tissue samples were treated with the resin cream, cream base or positive control (1% Triton®X-100, MatTek). The cream samples, 100 μ L, were applied undiluted to the top of each EpiDerm™ tissue. The treatment times were 1, 4 and 24 hours for the cream samples and 4 and 9 hours for the positive control. Cells were incubated at 37 °C in 5% CO₂. Negative controls (no treatment) samples were also tested. Each treatment with the cream or control was performed in duplicate.

Following the specified treatment periods the viability of the tissues was determined using a methylthiazole tetrazolium (MTT) uptake and reduction assay. Tissue samples rinsed with phosphate buffered saline (PBS) were transferred to a 24-well plate containing 300 μ L of an MTT solution sample (1 mg/mL MTT in Dulbecco's Modified Eagle's Medium). The tissues were incubated with MTT for three hours after which each tissue was rinsed with PBS and treated overnight with 2 mL of isopropanol as an extractant. An aliquot of the extracted formazan was transferred to a microplate and the absorbance was measured at 540 nm with a reference wavelength of 690 nm (μ Quant Plate Reader, Bio-Tek instruments, Winooski, VT). The viability was expressed as a percentage of control values and the percent viability at each time point was used to determine an ET₅₀ which represented the time point at which the tissue viability was reduced by

50% compared to the negative control tissues. The ET_{50} score was used to give an irritancy classification.

STATISTICS

Results are presented as mean \pm SEM and statistical significance between groups was determined by one-way analysis of variance (ANOVA) with Dunnett t-test post hoc analysis, where p-values < 0.05 were considered significant.

RESULTS

Quantification of active compounds

The content of active compounds PAA and PAB in the resin, as determined by HPLC is shown in Table 2. An HPLC chromatograph of the resin is shown in Figure 2.

Characteristics of the resin cream

The oil-in-water cream containing 5% w/w of the resin was a chartreuse-coloured homogenous semisolid preparation with light alcoholic odor. It was readily spread around upon contact with skin. No sensation of tackiness, residual greasiness, or irritation was detected after application. In addition, resin cream possessed the desired spreadability and pH value of 5.0 - 6.0. The prototype cream containing 5% w/w resin showed no phase separation as observed after 30 min centrifugation at 825 g.

Drug release test

The release profiles of PAA and PAB, the main active compounds of *D. polyandra*, from the 5% w/w resin cream are presented in Figure 3.

Table 2. Content of PAA and PAB in the resin

	PAA	PAB
Content in resin (mean \pm SD)	11.4 \pm 0.16 mg/g	133.3 \pm 0.6 mg/g

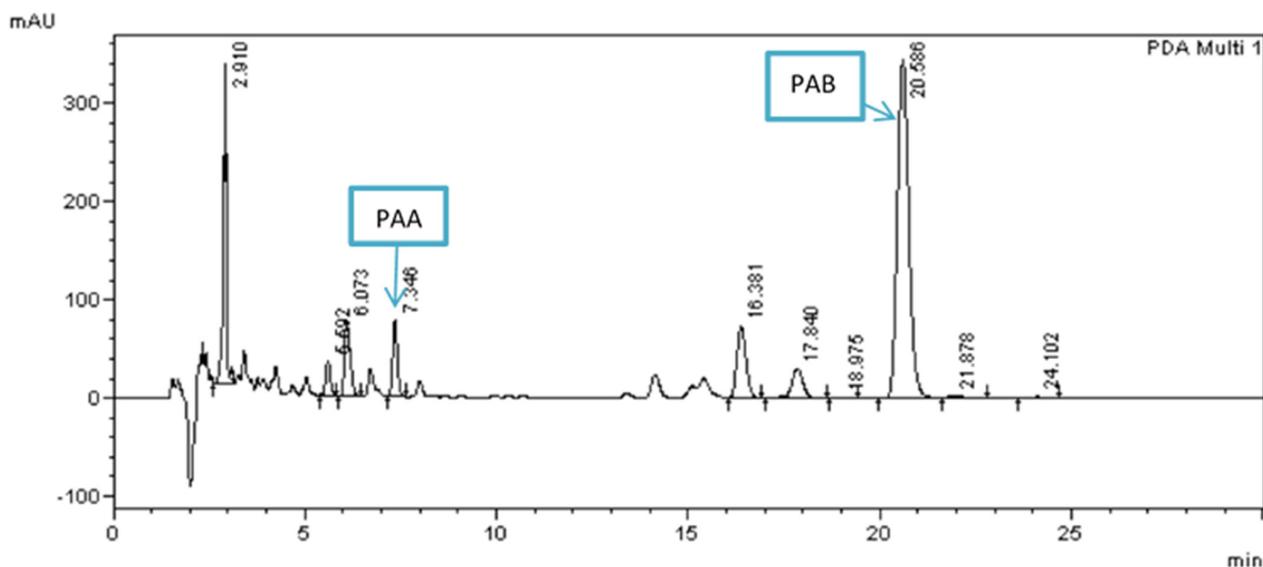


Figure 2. HPLC chromatograph of the *Dodonaea polyandra* resin used in the cream formulation. Chromatograph was run on a Shimadzu LC-20AD HPLC with PDA detection SPD-M20A. Separations were carried out on Phenomenex Gemini C18 column with guard cartridge with a mobile phase of acetonitrile (65% v/v), Milli-Q water (35% v/v) and formic acid (0.05% v/v). The mobile phase flow rate was 1.4 mL/min with 30 min run time. The active components were detected using a wavelength of 225 nm. Sample injection volume was 20 μ L. Retention times for known active components PAA and PAB were 7.35 and 20.59 min, respectively. PAA= polyandric acid A; PAB= polyandric acid B

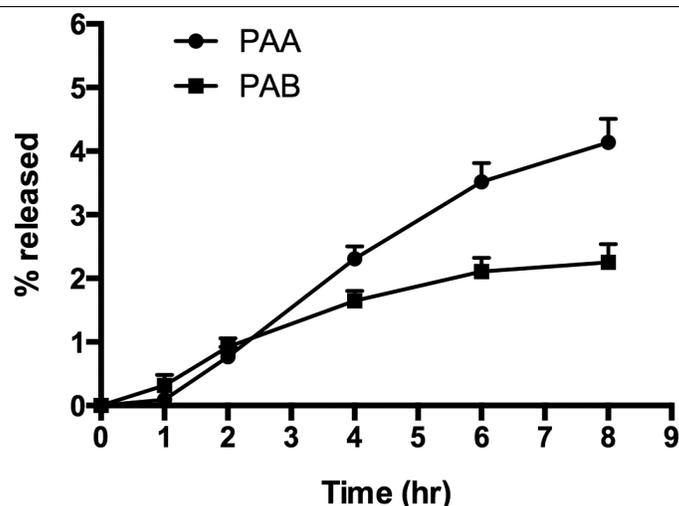


Figure 3. Release profiles of PAA and PAB from the resin cream (5% w/w) through polyvinylidene fluoride (PVDF) membranes using a Franz diffusion cell (Results are expressed as mean \pm SEM, n=6)

The release test results showed after 8h, 4.14 ± 0.9 % PAA and 2.25 ± 0.7 % PAB from resin cream were detected in receptor medium by HPLC.

Anti-inflammatory effect of cream in vivo

The anti-inflammatory effect of an optimized cream containing 5% w/w resin was tested in a TPA-induced acute mouse ear oedema model. We wanted to determine whether comparable efficacy could be achieved to that of resin delivered in an ethanol vehicle (0.4 mg/ear), as previously reported (6). Following 6 h of treatment, ear thickness difference measurements showed that the cream containing 5% resin significantly reduced

ear oedema by 25% compared to TPA-only control ($p < 0.01$), while there was no statistical difference with the resin delivered in ethanol vehicle ($p > 0.05$). The cream formulation vehicle (i.e. without resin) did not show significant interference with changes in ear oedema (Figure 4).

The anti-inflammatory effects of treatments were also assessed by quantifying the pro-inflammatory cytokine IL-1 β in ear tissue homogenates (Figure 5). The cream containing resin reduced IL-1 β by 32% compared to TPA-only control ($p < 0.001$). Interestingly, there was no statistical difference between the 20 mg/mL resin solution (0.4 mg/ear) and the TPA-only control, which is surprising given the reduction in oedema.

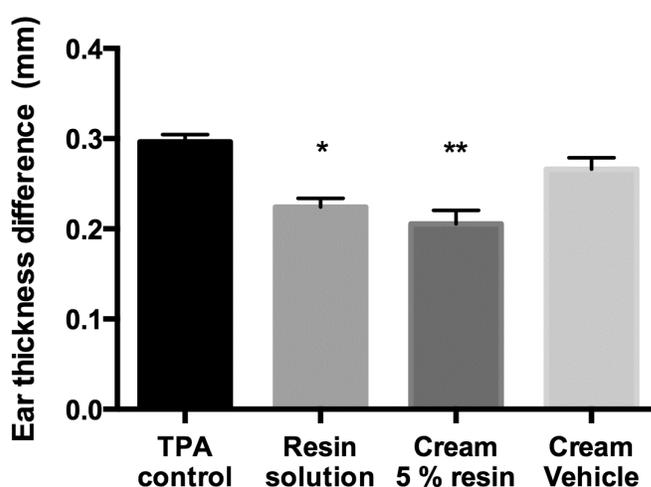


Figure 4. Effect of creams and resin solution on ear thickness in the TPA model. Increased ear thickness was represented by ear thickness difference (thickness of left ear – thickness of treated right ear). Measurement was conducted 6 h after the application of TPA. Each data represents Mean \pm SEM, n=4 mice per group; * $p < 0.05$ and ** $p < 0.01$ compared to TPA control.

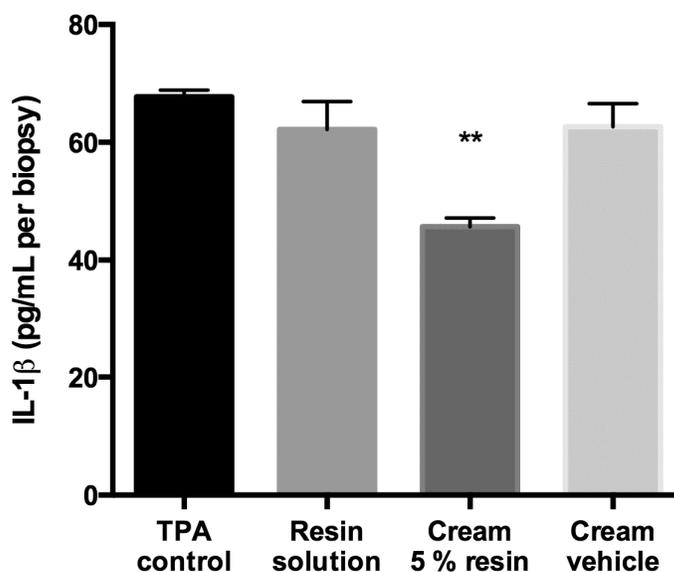


Figure 5. Effect of creams and resin solution on inflammatory cytokine IL-1 β in ear tissue homogenates in the TPA model. Data shown are from biopsies of the right ears of mice 6 h after the application of TPA. Ear tissue was homogenized and the supernatant from ear tissue homogenates was assayed for the concentration of IL-1 β using a Mouse IL-1 β Platinum ELISA kit. Each data represents Mean \pm SEM, n=4 mice per group. **p < 0.01 compared to TPA control.

Mouse ear tissue histological study

Mouse ear tissue samples were stained with hematoxylin and eosin (H&E) to examine histology of skin sections (Figure 6). In comparison with an untreated ear (Figure 6A), application of TPA resulted in a considerable increase in ear thickness (edema) and substantial inflammatory cell infiltration (Figure 6B). Treatment with the cream base only (Figure 6C) resulted in only a small reduction in the ear thickness and inflammatory cell infiltration. By contrast, treatment with the cream containing 5% w/w resin (Figure 6D) reduced ear thickness and cell infiltration to an extent that was comparable to normal ear skin tissue. Treatment with resin ethanol solution (Figure 6E) also reduced ear thickness and cell infiltration but to a lesser extent compared to the resin cream.

Skin irritancy (toxicity) assessment

The irritancy of the cream containing 5% w/w of *D. polyandra* resin and cream base was assessed in the MatTek EpidermTM MTT viability assay. Both samples were classified as non-irritating according to irritancy scoring criteria, showing ET₅₀ values of greater than 24.0 h compared to a 1.0 % Triton X-100 positive control with ET₅₀ of 6.0 h.

DISCUSSION

This study was undertaken as part of work to develop new anti-inflammatory drug therapies that may have application in the management of common skin inflammatory disorders such as dermatitis and psoriasis. Previously we had demonstrated that crude extracts of *D. polyandra* and clerodane diterpenoids PAA and PAB can inhibit markers of inflammation in acute and chronic murine models of TPA-induced skin inflammation (6, 8, 10). In our previous work we had used ethanolic solutions of the plant extracts and compounds for application to the skin in the in vivo models. This present study was undertaken to examine the feasibility of formulating a leaf resin extract of *D. polyandra* into a topical formulation at concentration that could demonstrate in vivo activity as well as acceptable physical properties for further development as a prototype formulation for pre-clinical testing.

In this study we demonstrated that the leaf resin could be formulated in an oil-in-water cream formulation at a strength of 5% w/w. At this concentration the cream formulation was able to significantly reduce ear oedema, inflammatory cell infiltration and production of the pro-inflammatory cytokine IL-1 β in the ear tissue.

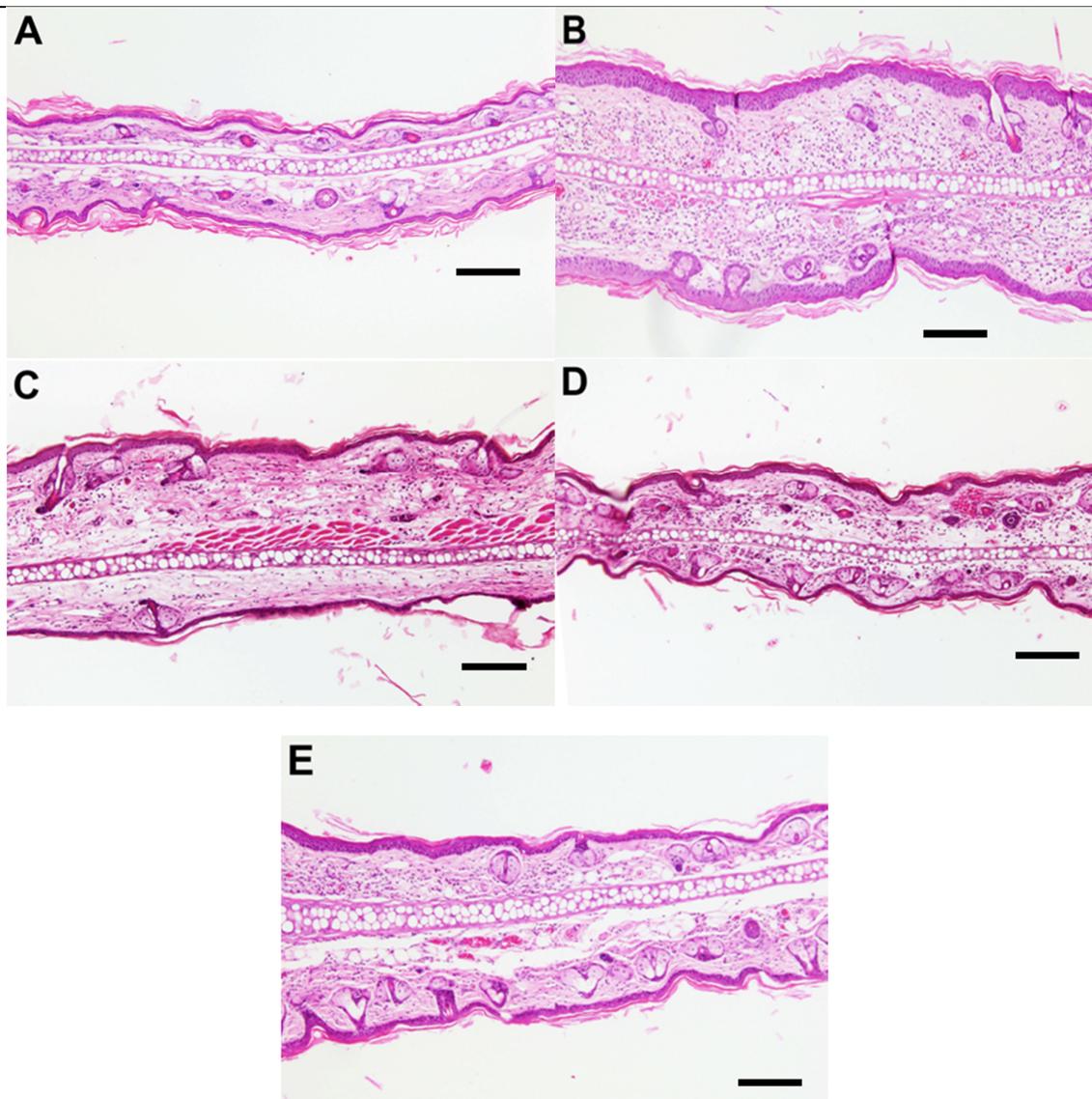


Figure 6. Histological analysis of the mouse ear tissue. Representative H&E stained transversal cuts of mice ear sections, scale bar 100 μm . (A) No treatment – normal mouse ear; (B) TPA only treatment; (C) TPA with cream vehicle; (D) TPA with 5% resin cream; (E) TPA with resin 20 mg/mL in ethanol solution (0.4 mg/ear). TPA treatment induced an increase in ear thickness with epidermal hyperplasia and inflammatory cell infiltration (indicated by purple/black stained cells) (B). The 5% resin cream (D) reduced ear thickness and the associated pathological features of inflammation to a greater extent than the cream base (C) or the resin dissolved in an ethanol vehicle (E).

The cream formulation was able to reduce these parameters of inflammation to a greater extent than a previously established dose of 0.4 mg/ear of the resin when delivered as an ethanol solution. This suggested that the cream formulation could deliver the active components of the extract to the site of action in the skin.

Increased ear thickening is often the first sign of skin irritation and local inflammation. This parameter is an indicator of number of processes that occur during skin inflammation, including increased vascular permeability, edema and proliferation of epidermal keratinocytes (7, 17, 18).

Epidermal keratinocytes release high amounts of the pro-inflammatory cytokines $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, which consequently initiate a series of cellular inflammatory responses and further exacerbation of the symptoms (19, 20). Utilizing a combination of moisturizing cream and anti-inflammatory agents could offer optimization in treating irritant and allergic contact dermatitis (21).

We also conducted an *in vitro* release test using a Franz diffusion cell with a synthetic PVDF membrane to examine the release of the diterpenoids PAA and PAB from the formulation.

In order for the intended therapeutic effects of topical preparations to be achieved, actives must first be liberated from the preparation to reach the surface of skin. The PVDF membrane is known to give reproducible results and has been recommended by the United States Food and Drug Administration (FDA) as a measure of batch consistency (22, 23). The resin cream allowed the release of both diterpenoid compounds in a reproducible manner, however the amounts released were low, with approximately 4% of PAA and 2% of PAB released after 8 hours. The amount of drug released from a semisolid preparation can be influenced by many factors including, drug physico-chemical properties, vehicle compositions and the interactions between them (24). Further studies using full-thickness human skin membranes instead of synthetic membranes (25) and over a more extended time period (up to 12 hours) are now needed to better predict in vivo release.

As the first stage in evaluating the safety and tolerability of the resin cream formulation and cream base these were assessed using a MatTek Epiderm™ MTT viability assay. This is a three-dimensional reconstructed human epidermis (RhE), constructed from human-derived non-transformed keratinocytes which can be used to assess dermal irritancy and toxicology (26, 27). This method is an Organization for Economic Cooperation and Development (OECD) Guideline approved method for assessing skin irritancy as an in vitro alternative to methods such as the Draize test in animals (28). Cell viability is determined after exposure of the RhE to the test substance and measured using an MTT viability assay. Irritant chemicals are identified by their ability to decrease cell viability below defined levels (28). In the present study the resin cream and cream base showed no reduction in cell viability after 24 hour of exposure and were classified as non-irritant. Further studies of chronic and repeated exposure, including in vivo models, are now required to further assess the tolerability and safety of the formulation.

According to USP regulations (29) on semisolid formulations, the product quality attributes should include but are not limited to the following: description, assay, uniformity, physicochemical properties and apparent viscosity. In addition, the appearance, tactile feeling, spreadability, and odour of formulations are crucial properties which closely relate to patient compliance (29). In this study, an oil-in-water cream formulation based on two emulsifiers, Span 40 and Polychol 15, was found to give the optimal

spreadability, acceptable appearance, tactile feel, texture and odour.

The cream emulsion was also assessed using a centrifugal accelerated deterioration test. This centrifuge test is widely used as means to assess the long-term stability of a cream. In terms of a typical oil-in-water (O/W) cream, the oil phase has a tendency to separate and rise to the top of the system, leading to emulsion break up which is represented by phase separation (30). For the resin cream formulation used in this study the composition was finely tailored based on the hydrophile-lipophile balance (HLB) system (31). The blending of two emulsifiers, Span 40 with a HLB value of 6.7 and Polychol 15 with a HLB value of 13.0 gave rise to a HLB value of 11.5, which theoretically supported the soundness of this O/W cream (31).

In the present study, formulations were freshly prepared for pharmacological and pharmaceutical assessments. Further studies are now required to measure the stability of the active components in the cream formulation, and to examine the influence of factors including temperature, pH and light. The use of standardized plant extracts for medical and complementary treatments has been increasingly popular. Given their complex chemical profiles, the contents of bioactive components had been recognized as a crucial criterion for natural products identification and quality control. In the case of plant *D. polyandra*, the two benzoyl ester clerodane diterpenoids PAA and PAB have been identified as most effective anti-inflammatory compounds (8). Further development of this formulation will require standardization of these compounds in different batches of plant extract.

CONCLUSION

Our early development study has demonstrated that the leaf resin from *D. polyandra* can be readily formulated into a cream-type dosage form. The physical characteristics of the prototype cream met criterion outlined in the United States Pharmacopoeia. Our formulation demonstrated efficacy in an acute mouse ear oedema model over a 6 h period. This was commensurate with a decrease in the pro-inflammatory cytokine IL-1 β . Importantly the prototype formulation lacked skin irritancy as assessed in an acute in vitro model. Overall, this study provides sound evidence that our prototype topical formulation product possesses favourable characteristics amenable to further development towards commercial application.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the content of this article.

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SUPPLEMENTARY INFORMATION
Criteria used for the determination of physical properties of trial formulations

- a) **Spreadability** of formulations was determined according to the criteria outlined in table S1. Vehicles rating at “0” or “1” were considered acceptable.
- b) **Acceptability** was determined with subjective assessment of appearance, tactile feel, texture and odour.
- c) **Gravitational stability** was assessed as **X (not stable, phase separation occurs) or √ (stable, no phase separation)**. Gravitational stability assessment was not applied to gels.

Table S1. Criteria used to assess spreadability of formulations

Score	Description
-2	Formulation or vehicle appears as a solid state, which cannot be spread around the application site.
-1	Formulation or vehicle has to be applied with force greater than normal to achieve a desirable spread.
0	Formulation or vehicle displays good spreadability with desirable stay-on semisolid property.
1	Formulation or vehicle displays good spreadability with a slight tendency to flow over the skin surface.
2	Formulation or vehicle has low viscosity with high tendency to flow over the skin surface.

Formulation ingredients and physical properties of trial formulations

The ingredients and physical properties of different series of trial formulations are summarised in Tables S2 to S15. The preservative used in formulations was an ethanolic solution of methyl paraben and propyl paraben.

Table S2. Formulation ingredients and physical properties of trial formulations (series 1-1, Cream formulations)

Series 1-1				
Cream				
Ingredients	Formulation number			
	1	2	3	4
Glycerol monostearate	1.2g	0.72g	1.2g	1.2g
Liquid paraffin	0.5g	0.3g	0.5g	0.5g
Stearic acid	0.5g	0.3g	0.5g	0.5g
Lauryl sulfate	0.03g	0.03g	0.03g	0.03g
Glycerol	0.5g	0.5g	0.5g	0.5g
Preservative	0.125mL	0.1mL	0.15mL	0.1mL
Absolute ethanol	-	-	-	0.6g
Purified water	QS to 12.5g	QS to 10g	QS to 15g	QS to 10g
In vitro physical properties				
Spreadability	0	-1	0	1
Acceptability	√	√	√	√
Gravitational stability	×	×	×	×

QS= quantum sufficiat (sufficient quantity)

Table S3. Formulation ingredients and physical properties of trial formulations (series 1-2, Cream formulations)

Series 1-2								
Cream								
	Formulation number							
Ingredients	1	2	3	4	5	6	7	8
Stearyl alcohol	1g	1g	0.8g	1g	1g	1g	1g	1g
Vaseline	2.5g	1.25g	1g	1.125g	1.125g	1.125g	1.125g	1.125g
Tween 80	0.35g	0.35g	0.35g	0.35g	0.2g	0.225g	0.15g	0.375g
Span 80	0.35g	0.35g	0.35g	0.35g	0.2g	0.075g	0.05g	0.125g
Glycerol	1g	1g	1g	1g	1g	1g	1g	0.8g
Propylene glycol	/	/	/	/	0.3g	0.275g	0.375g	0.275g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g
In vitro physical properties								
Spreadability	-2	-1	2	1	1	1	1	1
Acceptability	×	×	×	√	√	√	√	√
Gravitational stability	×	×	×	×	×	×	×	×
QS= quantum sufficiat (sufficient quantity)								

Table S4. Formulation ingredients and physical properties of trial formulations (series 1-3, based on Carbopol Hydrogel)

Series 1-3									
Carbopol Hydrogel									
	Formulation number								
Ingredients	1	2	3	4	5	6	7	8	9
Carbopol 934P	0.05g	0.25g	0.3g	0.35g	0.4g	0.3g	0.25g	0.2g	0.12g
Glycerol	0.2g	0.2g	0.2g	0.4g	0.2g	0.2g	0.25g	0.3g	0.2g
Propylene glycol	-	-	-	-	-	4g	4g	4g	3.6g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Absolute ethanol	-	-	-	-	-	2.0g	2.0g	2.0g	1.2g
NaOH	-	-	-	-	-	-	-	-	-
Purified water	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g
In vitro physical properties									
Spreadability	-2	-1	1	0	0	1	0	-1	1
Acceptability	√	√	√	√	√	×	×	×	×
QS= quantum sufficiat (sufficient quantity)									

Table S5. Formulation ingredients and physical properties of trial formulations (series 1-4, based on PEG Hydrogel)

Series 1-4 PEG Hydrogel							
	Formulation number						
Ingredients	1	2	3	4	5	6	7
PEG 4000	4.5g	3.5g	3g	3.5g	3g	3.5g	6.5g
PEG 300	4.5g	3.5g	3g	3g	3g	3g	/
Glycerol	0.6g	1g	1.2g	1g	1.5g	1.25g	1.2g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g	QS to 10g
In vitro physical properties							
Spreadability	-3	-1	1	1	0	1	1
Acceptability	×	×	×	×	√	√	×
QS= quantum sufficiat (sufficient quantity)							

Table S6. Formulation ingredients and physical properties of trial formulations (series 1-5, based on Pluronic F-127 Gel)

Series 1-5 Pluronic F-127 Gel			
	Formulation number		
Ingredients	1	2	3
Pluronic F-127	2.3g	3g	3g
Propylene glycol	0.1g	0.2g	0.2g
Polyvinylpyrrolidone K90	-	-	0.5g
Preservative	0.1mL	0.1mL	0.1mL
Absolute ethanol	1.8g	1.2g	1.2g
Purified water	QS to 10g	QS to 10g	QS to 10g
In vitro physical properties			
Spreadability	2	2	2
Acceptability	√	√	×
QS= quantum sufficiat (sufficient quantity)			

Table S7. Formulation ingredients and physical properties of trial formulations (series 2-1).

Series 2-1								
	Formulation number							
Ingredients	1	2	3	4	5	6	7	8
Stearyl alcohol	1g	1g	1g	-	-	-	-	-
Vaseline	1.2g	1.2g	1.2g	1.2g	1.2g	0.6g	0.6g	1.2g
Propylene glycol	0.5g	0.3g	0.2g	0.2g	0.2g	0.2g	0.2g	0.2g
Captex 300 EP/NF	-	-	-	1g	-	1g	1g	-
Isopropyl myristate	-	-	-	-	1g	-	-	0.5g
Glycerol	-	-	-	-	-	-	0.6g	0.5g
Paraffin oil	-	-	-	-	-	0.6g	-	-
Absolute ethanol	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g
Polawax	0.5g	1g	1.5g	1.5g	1.5g	1.5g	1.5g	1.5g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g
In vitro physical properties								
Spreadability	2	2	2	1	1	failed to form emulsion	1	1
Acceptability	×	×	×	×	×		√	√
Gravitational stability	×	×	×	√	√		√	×
QS= quantum sufficiat (sufficient quantity)								

Table S10. Formulation ingredients and physical properties of trial formulations (series 3-1), based on cream base used for fluticasone propionate cream.

Series 3-1		
Based on fluticasone propionate (FP) cream formulation		
	Formulation number	
Ingredients	FP-1	FP-2
Cetostearyl alcohol	0.7g	0.7g
Isopropyl myristate	0.5g	-
Cetareth-20	0.4g	-
Liquid paraffin	1g	-
Propylene glycol	0.4g	-
Dimethicone	-	0.5g
Span 80	-	0.4g
Polychol 40	-	1g
Absolute ethanol	1.2g	1.2g
Preservative	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g
In vitro physical properties		
Spreadability	2	1
Acceptability	√	√
Gravitational stability	×	×
QS= quantum sufficiat (sufficient quantity)		

Table S11. Formulation ingredients and physical properties of trial formulations (series 3-2), based on cream base used for alum cream.

Series 3-2	
Based on Alum Cream formulation	
	Formulation number
Ingredients	Alum-1
Cetostearyl alcohol	0.4g
Octyldodecanol	0.5g
Lanolin alcohol	0.4g
Cremophor EL	0.2g
Vaseline	0.2g
Absolute ethanol	1.2g
Preservative	0.1mL
Purified water	QS to 9.5g
In vitro physical properties	
Spreadability	2
Acceptability	×
Gravitational stability	×

QS= quantum sufficiat (sufficient quantity)

Table S12. Formulation ingredients and physical properties of trial formulations (series 3-3), based on the base of an antiseptic cream formulation

Series 3-3			
Antiseptic Cream base			
	Formulation number		
Ingredients	AtS-1	AtS-2	AtS-3
Polawax	0.5g	0.8g	0.8g
Lanolin	0.1g	0.5g	0.5g
Cetearyl alcohol	0.7g	0.3g	0.3g
Dimethicone	0.3g	0.3g	0.3g
Liquid paraffin	1.5g	-	1g
Isoeicosane	-	1.2g	-
Propylene glycol	-	-	0.4g
Absolute ethanol	1.2g	1.2g	1.2g
Preservative	0.1mL	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g	QS to 9.5g
In vitro physical properties			
Spreadability	1	1	1
Acceptability	×	√	√
Gravitational stability	√	×	√

QS= quantum sufficiat (sufficient quantity)

Table S13. Formulation ingredients and physical properties of trial formulations (series 3-4), based on the base of a baby cream formulation

Series 3-4			
Baby Cream base			
	Formulation number		
Ingredients	BbC-1	BbC-2	
Silica	0.8g	0.2g	
Polawax	1g	-	
Cetayl alcohol	0.3g	0.6g	
Lanolin	0.2g	0.3g	
Glycerol	0.5g	1g	
Ceteareth-20	-	0.4g	
Span 80	-	0.3g	
Absolute ethanol	1.2g	1.2g	
Preservative	0.1mL	0.1mL	
Purified water	QS to 9.5g	QS to 9.5g	
In vitro physical properties			
Spreadability	2	1	
Acceptability	×	√	
Gravitational stability	×	√	

QS= quantum sufficiat (sufficient quantity)

Table S14. Formulation ingredients and physical properties of trial formulations (series 3-5), based on the base of a betamethasone valerate ointment formulation

Series 3-5			
Based on betamethasone valerate ointment base			
	Formulation number		
Ingredients	BVO-1	BVO-2	BVO-3
Vaseline	5.8g	5.8g	7g
Liquid paraffin	1.5g	1.5g	1.5g
Cetostearyl alcohol	0.1g	0.1g	0.6g
Ceteareth-20	0.1g	0.1g	0.4g
Polychol 40	-	0.5g	-
Absolute ethanol	2g	1.5g	-
Preservative	0.1mL	0.1mL	0.1mL
In vitro physical properties			
Spreadability	0	0	0
Acceptability	√	×	×
Gravitational stability	×	×	×

QS= quantum sufficiat (sufficient quantity)

Table S15. Formulation ingredients and physical properties of trial formulations (series 3-6), based on a coal tar and allantoin cream formulation base.

Series 3-6				
Coal tar and allantoin cream base				
	Formulation number			
Ingredients	CTA-1	CTA-2a	CTA-2b	CTA-2c
Stearic acid	1.5g	1.2g	1.2g	1.2g
Lanolin	0.2g	0.2g	0.2g	0.2g
Triethanolamine	0.4g	0.4g	0.4g	0.4g
Glycerol	1.2g	1.2g	1.2g	1.2g
Captex 300 EP/NF	-	0.3g	-	-
Octyldodecanol	-	-	0.3g	-
Dimethicone	-	-	-	0.3g
Absolute ethanol	1.2g	1.2g	1.2g	1.2g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g
In vitro physical properties				
Spreadability	-1	1	Failed to form emulsion	1
Acceptability	×	√		√
Gravitational stability	√	√		√
QS= quantum sufficiat (sufficient quantity)				

Table S8. Formulation ingredients and physical properties of trial formulations (series 2-2).

Series 2-2													
Ingredients	Formulation number												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Glycerol monostearate	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	0.8g	1.4g	1.2g
Polychol 40	1g	1g	1g	1g	1g	1g	1g	0.5g	0.2g	-	1g	0.8g	0.6g
Glycerol	0.5g	0.5g	0.3g	-	-	-	-	-	-	-	-	-	-
Stearyl alcohol	-	0.4g	0.2g	0.5g	0.3g	-	-						
Polychol 15	-	-	-	-	-	-	-	0.5g	0.8g	1g	-	-	-
Dimethicone	-	-	-	-	-	-	-	-	-	-	0.2g	-	-
Propylene glycol	-	-	-	-	-	-	-	-	-	-	0.2g	0.4g	0.4g
Cetyl alcohol	-	-	-	-	-	-	-	-	-	-	-	0.2g	0.2g
Stearic acid	-	-	-	-	-	-	-	-	-	-	-	-	0.3g
Triethanolamine	-	-	-	-	-	-	-	-	-	-	-	-	0.1g
Tween 80	0.1g	0.2g	0.3g	0.5g	-	0.1g	0.2g	-	-	-	-	-	-
Absolute ethanol	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g
In vitro physical properties													
Spreadability	2	1	1	1	0	0	0	1	1	1	2	2	1
Acceptability	×	√	√	√	×	×	×	√	√	√	×	×	×
Gravitational stability	√	√	√	√	√	√	√	√	√	√	√	×	√
QS= quantum sufficiat (sufficient quantity)													

Table S9. Formulation ingredients and physical properties of trial formulations (series 2-3).

Series 2-3											
Ingredients	Formulation number										
	1	2	3	4	5	6	7	8	9	10	11
Span 40	0.1g	0.2g	0.3g	0.2g	0.3g	0.5g	0.5g	0.5g	0.5g	-	-
Polychol 15	0.5g	0.5g	0.5g	1g	1g	1g	1g	1g	1g	-	-
Stearyl alcohol	1.5g	1.2g	1.2g	1.2g	1.2g	1.2g	-	-	-	-	-
Cremophor EL	0.2g	0.4g	0.5g	-	-	-	-	-	-	0.3g	0.3g
Vaseline	-	0.6g	0.6g	0.6g	0.6g	0.6g	0.6g	-	-	-	-
Octyldodecanol	-	-	-	-	-	-	0.5g	-	0.8g	-	-
Lanolin alcohol	-	-	-	-	-	-	0.7g	0.4g	-	-	-
Microcrystalline wax	-	-	-	-	-	-	-	0.6g	0.6g	-	-
Isoeicosane	-	-	-	-	-	-	-	0.8g	-	-	-
Beeswax	-	-	-	-	-	-	-	-	0.4g	-	-
Cetyl alcohol	-	-	-	-	-	-	-	-	-	0.6g	0.8g
Span 80	-	-	-	-	-	-	-	-	-	0.2g	0.2g
Polychol 40	-	-	-	-	-	-	-	-	-	0.5g	0.5g
Liquid paraffin	-	-	-	-	-	-	-	-	-	0.6g	-
Stearic acid	-	-	-	-	-	-	-	-	-	0.5g	-
Triethanolamine	-	-	-	-	-	-	-	-	-	0.2g	-
Polawax	-	-	-	-	-	-	-	-	-	-	1g
Glycerol	-	-	-	-	-	-	-	-	-	-	0.5g
Absolute ethanol	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g	1.2g
Preservative	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL	0.1mL
Purified water	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g	QS to 9.5g

Table S9 - Series 2-3 (cont.)

	Formulation number										
	1	2	3	4	5	6	7	8	9	10	11
In vitro physical properties											
Spreadability	2	1	1	1	1	1	1	1	1	failed to	1
Acceptability	×	√	√	√	√	√	√	√	√	form	×
Gravitational stability	√	√	√	√	√	√	×	×	×	emulsion	×
QS= quantum sufficiat (sufficient quantity)											