***Original Research Article***

***Augmented Hybrid Sunscreen Incorporating Myrothamnus Flabellifolius and Citrullus lanatus- A Pre-Clinical Investigation***

**Abstract**

The sun emits radiation with different ionising energies in a wide spectrum of wavelengths, frequencies and energies inflicting various forms of actinic damage to dermal structures. Damage can either be direct DNA damage through alteration of gene expression and compromised inflammatory responses or indirectly through the generation of free radicals, causing tissue damage through protein oxidation, deactivation and lipid peroxidation. Sunscreens and physical barriers are the main forms of protection from actinic damage. However, recent studies confirm that chemical sunscreens have serious bio safety issues while, physical sunscreens which are deemed safer have limited use due to unesthetic white casts. To mitigate these issues, we aimed to develop safer, amplified photoprotection through augmenting nanometric physical sunscreens with oil extracts from natural photoprotective tropical plants; *Myrothamnus flabellifolius* and *Citrullus lanatus*. First, we evaluated *in-vivo,* the oils comedogenic potential as well as their genotoxic and mutagenicity potential through the Ames reverse mutation test. We further evaluated the skin sensitivity and irritation potential of the augmented sunscreen using Draize tests and human patch testing. We also investigated *in vitro* the emulsion SPF, UVAPF, photoprotective efficiency and critical wavelength (ƛC). Our findings show that photoprotective oils investigated are non-comedogenic. Neither concentration of the tested oils is genotoxic or mutagenic with or without metabolic activation. Skin sensitivity and irritation tests confirmed that the resulting augmented sunscreen does not sensitise or irritate skin in basic topical cosmetic use. The augmented sunscreen had a high SPF of 30, UVAPF of 14, UVAPFeff and SPFeff both above 97% and ƛC of 383. Based on the foregoing we concluded that the development of an amplified, broad-spectrum and biologically safe augmented sunscreen incorporating *C Lanatus* and *M flabellifolius* oils is feasible and heralds’ new perspectives in formulation of efficacious nonconventional photoprotective products.

**Key words**: Photoprotection, comedogenesis, mutagenicity, Augmented sunscreen*, C Lanatus, M flabellifolius,*

# INTRODUCTION

## Ultraviolet Radiation (UVR), photo damage protection.

The sun emits radiation with different ionising energies in a wide spectrum of wavelengths, frequencies and energies. This electromagnetic spectrum has wavelengths from as low as Xray’s (0.01–10 nm) with corresponding frequencies up to 30 exahertz and high corresponding energies which can reach 100 keV, to low-energy high high-wavelength radio frequencies (1 mm to 100 km) with frequencies as low as 3 Hz[1]. With regards to electromagnetic radiation, energy is inversely proportional to wavelength and the shorter wavelength’s have devastating health consequences due to their high energies [1]. Convenient for the protection of all life forms on earth, our atmosphere has various layers, including troposphere, stratosphere, mesosphere, thermosphere and the exosphere, which through different mechanisms and components can regulate the amount and type of radiation incident on earth. The ozone layer which sits in the stratosphere between 15 km and 30 km above the earth and is concentrated between 20 and 25km shields humans and other life forms from potential actinic damage caused by the sun’s harmful radiation [2].  This filtration system is so efficient that much of the lower wavelength radiation below the ultraviolet radiation (UVR) range part of the spectrum, with high energies, does not reach the earth.

Actinic damage summarises all the photo damage caused by this radiation from conditions as benign as freckles to malignant melanoma. Among the many bands of the solar electromagnetic spectrum, UVR, is of much interest to solar photoprotection due to the fact that much of the actinic damage observed in humans carry photo damage endpoint signatures associated with this radiation range [2,3]. To protect underlying body structures, the human skin employs two mechanisms: melanisation and keratinization with capacities to filter, neutralise or block the harmful rays. Skin actinic damage occurs through two mechanisms, which correspond to different ranges of wavelengths in the UVR spectrum. Damage can either be direct DNA damage through alteration of gene expression and compromised inflammatory responses or can be through the generation of reactive oxygen species (ROS), which cause tissue damage through protein oxidation and deactivation, lipid peroxidation as well as indirect DNA damage [3]. As shown in figure 1,UVR, is composed of three different energy bands: UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). Radiation in the highest energy UVC band is blocked entirely by the ozone layer and does not reach the Earth. UVB is partially blocked by the ozone layer, however, the little radiation reaching the earth, which is approximately 5% of all UVR, has sufficient energy to damage DNA directly. UVB incidents is highest in the tropical and subtropical summer within a 4-hour window around the solar noon, it depends on the solar zenith angle and weather patterns [4]. UVA, which comprises up to 95% of all UVR reaching the earth, on the other hand, is not dependent on season or weather conditions. It is present all year round and its prevalence is only related to altitude, notably observed for its absence at the Dead Sea, which is 400m below sea level. UVA pierces directly through the earth’s ozone layer and crosses the skin outer epidermis layer, damaging underlying skin tissue structures, including DNA, primarily through the production of reactive oxygen species (ROS) [5]. Human beings are a cosmopolitan species with numerous phenotypes resident in equally numerous geographical habitats. Therefore, actinic damage by all forms of UVR varies among individuals and the majority of the human species relies on augmenting the natural photoprotection through the use of sunscreens.

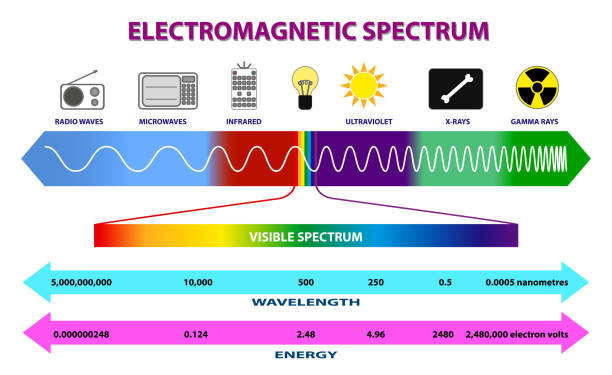


Figure 1: The electromagnetic spectrum (https://www.gettyimages.com/photos)

## Commercial sunscreen materials

Human beings have employed various materials as sun filters to protect themselves from actinic damage for thousands of years. The ancient sunscreens were mostly derived from plant oils and gels as well as clays and minerals [6]. The modern commercial sunscreens were heralded by the marketing of the chemical sunscreens, benzyl salicylate and benzyl cinnamate in the 1920s [7]. The basic materials compounded and the mechanisms of photoprotection may have changed over time, but the age-old need for sun protecting materials has not. Materials used as sunscreens are basically grouped as inorganic (physical) sunscreens, organic (chemical) sunscreens as well as plant derivatives. In the USA, through monograph MO20, the FDA recognizes 16 materials as commercial sunscreens (14 chemical and 2 physical) [8]. In the European Union COLIPA recognizes 20 materials as approved for sunscreen use. The Asian market recognizes more materials with China and Japan endorsing 25 and 27 materials as safe and effective sunscreens respectively [9]. The physical, inorganic sunscreens recognized by all regulatory authorities are the minerals namely TiO2 and ZnO. These materials, which are generally deemed as safer than chemical sunscreens and work by absorbing, scattering and reflecting UVR have numerous aesthetic drawbacks. Their opaqueness leaves cosmetically unappealing white casts on users. Their high usage levels of up to 25% to be effective also pose a challenge in formulating aesthetically pleasing and safe sunscreens [8]. Almost 70 organic materials have been scientifically validated as possessing commercially acceptable sun filtration capacities. However, due to safety and technical considerations, regulations have restricted worldwide commercial use to fewer than 30 materials. Various studies have found most organic sunscreens to exhibit skin irritation, carcinogenic, photosensitisation and phototoxicity potential [9]. Recent studies have also found traces of these sunscreens, especially Avobenzone, the most widely used sunscreen agent, in the blood plasma of users. Their bioaccumulation has also been reported in coral reefs ecosystems, which led to the FDA passing non-GRAS (generally recognisable as safe and effective) status on all commercial sunscreens in 2020 [10]. Due to these perceived weaknesses the need to supplant both classes of sunscreens with newer, safer and more effective materials or at least to lower their usage through augmentation with other materials is imperative.

## Augmented Sunscreens for photoprotection

Plant secondary metabolites with aromatic rings especially consisting of polyphenolic groups, have demonstrated broad UVR absorption capacity covering the wavelength of actinic damage concern on the electromagnetic spectrum. Botanical metabolites of polyphenolic origin, flavonoids and tannins with UVR filtration capacity also usually exhibit strong antioxidant properties [11]. These naturally occurring compounds which are not recognized under the regulatory definition of sunscreen materials are apparently more advantageous as UVR filters because of their effectiveness and safety and are widely regarded as the future of sunscreens. After the validation of these properties and capabilities, the emerging utilisation of plant-derived polyphenolic metabolites in augmenting the sun filtering capacity of skin care products due to their proven photoprotective attributes is predictable and realistic [12]. It is scientifically noted that no sunscreen material can offer total UVR protection under all exposure scenarios, therefore, the potential to augment sunscreen activity of commercial products with ‘‘non-sunscreen’’ agents to boost protection against exposure to UVR makes good sense. It is assumed that augmenting sunscreens with polyphenols can help increase their UVR filtration capacity and offer greater photoprotection to patients, however, there is very little structured preclinical, efficacy and safety research relating to specific plants. This study therefore, sought to provide the evidence by investigating the benefits of using specific plants in augmenting the safety and efficacy of a typical physical sunscreen formulation.

## *Myrothamnus flabellifolius* and resurrection plants

*Myrothamnus flabellifolius* (*M. flabellifolius*) belongs to a unique family of higher species of plants with vegetative desiccation tolerance found in most harsh plant habitats of the world but more prevalent in Southern Africa. These plants are referred to as resurrection plants due to their ability to withstand drastic decreases in normal plant water content to a virtually dry condition for extended periods only to recover fast to fully green, viable condition from this vegetative state when water is available again [13]. These remarkable plants, which can be found on every continent especially in arid to desert habitats, belong to various botanical families. Interestingly, their mechanisms and strategies to survive through virtually dry plant states seem predetermined and constitutive due to the prevalence of unique polyphenolic compounds among their secondary metabolites. The plants have an ability to induce and dictate dynamic changes in the constitution of their primary and secondary metabolites in response to environmental stress and threats. Resurrection plants are able to accumulate and maintain relevant and valuable bioactive metabolites in extremely elevated concentrations when in the desiccated state [13, 14]. *Myrothamnus flabellifolia* (figure 2) is a woody small shrub which can grow up to 2m in height, prevalent in shallow soils on rocky outcrops of Southern Africa. For a small shrub with small nutrient reserves to be able to thrive in these unimaginable conditions and to survive desiccation especially the long dry winters and the extreme UVR onslaughts, like other resurrection plants, *M. flabellifolia* has unique secondary metabolites and has developed highly adaptive specialized survival techniques [14]. Many studies confirm that to avoid apoptosis or necrosis of plant cells during the desiccated state and consequent revitalization after the availability of water, the plant utilises advanced forms of physical and metabolic adaptation and histological and morphological ingenuity. The mechanisms include folding of leaves and restructuring branches to a perpendicular arrangement to preserve chlorophyll and a surprisingly high accumulation of unique polyphenolic compounds including 3,4,5-tri-O-galloylquinic acid (theogalin) [13,14]. In Zimbabwean traditional medicine, there are numerous uses of the plant extracts against life-threatening conditions as well as in skin photoprotection, wound management and treatment of various bacterial ailments [15].

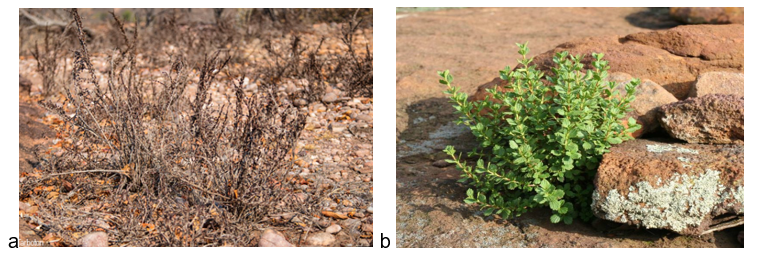


Figure 2: *M flabellifolius* thriving in very harsh low nutrient semi-arid environment in Zimbabwe (a) in the desiccated state (b) in the revitalized state

## *Citrillus lanatus*

*Citrillus Lanatus* (*C. lanatus*) also known as the Kalahari melon is a tropical vine, native to Southern African climes and thrives especially well in arid to desert conditions. The *Citrullus* genus belongs to the *Cucurbitaceae* family which is commonly referred to as the *Cucurbits* or the gourd family. *C. lanatus* and its products, which have recently found attention in the modern cosmetic industry have been the cornerstone of traditional dermaceutical applications for various indigenous groups including the Khoisan, Shona and Ndebele groups of Zimbabwe for aeons [16]. Archaeological remains of the Kalahari melon in Southern Africa, confirm the existence of the plants in the region around 5000 years ago. In his writings, the famed 19th century explorer David Livingstone extolled the virtues of the fleshy ‘melon’ fruit from the vine describing at as the most remarkable plant species from the Southern African deserts central to the adaptation of the Kalahari bushmen to their hot dry habitat [16]. There is evidence that this desert vine was grown for various medicinal uses in ancient Egypt around 2000 BCE, as well as India and China from 800 to 1000CE [17]. The Moors are believed to have introduced the Kalahari melon to Europe around the same time. The small wild watermelon has oil bearing seeds embedded in the sweet spongy flesh, and it is this oil that has been scientifically validated as a dermaceutical panacea. Highly priced in the cosmetic industry, this oil has some of the highest alpha linoleic acid levels (greater than 60%) in its fatty acid profiles compared to other naturally obtained fixed oils [16]. Various studies have confirmed that the Kalahari melon seed oil has many benefits to skin structures and function. The seed oil has demonstrated reduced TEWL and increased hydration and moisturisation and improved water retention in cosmetic applications [18]. These and many other attributes are the reasons behind the recent hype and interest in its use in global cosmetics. In our present formulations however, it is its photoprotection use by the indigenous peoples of Zimbabwe that has prompted us to use it to augment a sunscreen formulation. The seed oil has been used by present and ancient Zimbabweans as well as he Kalahari bushmen to provide skin UVR protection as well as to treat other skin conditions for time immemorial [18,19]. The seeds are traditionally chewed and moistened to produce a dermatological paste that has various uses including UVR protection by the local hunter gatherers and the peasant farmers. The oil has always traditionally been used as an after-sun care to treat sunburn, inflammation and other forms of actinic damage as well as for skin rejuvenation and moisturisation [19].

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Figure 3: *C lanatus* vine with a fleshy melon fruit thriving in hostile semi-arid conditions in Western Zimbabwe

In the present study an augmented sunscreen was developed incorporating the photoprotective active extracts of *C. lanatus* and *M flabellifolius* to boost UVR protection factors of typical sunscreen formulations, as well as the UVR sunscreen efficiency.

# MATERIALS AND METHODS

For the evaluations with human subjects, study protocols and the informed consent forms were approved by the institutional review board of the Joint University of Zimbabwe and Parirenyatwa hospital research ethics committee and the studies were conducted under the ATRIC Biosciences laboratories according to the guidelines from the Declaration of Helsinki (2013) and the International Conference on Harmonization of Technical requirements for Registration of Pharmaceutics for Human Use Guidance for good clinical practice.

## Plant oils

The organic *M Flabellifolius* essential oil and *C Lanatus* fixed oilswere both obtained from KAZA natural oils. The *M Flabellifolius* essential oilwas extractedthrough steam distillation using Clevenger type apparatus and the *C lanatus* fixed oil was extracted through cold expression. Both oils were used in their crude form, unpreserved and unstabilized. The plant sources of the oils were taxonomically authenticated by the Zimbabwe national Herbarium prior to the oil extractions.

## Comedogenesis evaluation of *M flabellifolius* and *C Lanatus* oils

### Testing procedure for human comedogenesis potential evaluation

In this present evaluation, 10 human subjects of both sexes, aged between 16 and 42 years old, with seborrheic skin biotype and comedones were selected for this test. The participants were briefed on the study procedures and signed written informed consent was obtained from all subjects prior to conducting procedures. The participants were put into 2 groups of 5 people each for each oil evaluation. In the first round of tests, each group of 5 received a specific plant oil product (either *M flabellifolius* or *C Lanatus* oils) to apply twice daily, to the intermammary cleft zone and faces for 4 weeks. At the conclusion of round 1 at the end the 4-week test period the participants only returned for round 2 of the trials with a different oil than the one used in the first round after a 2-week break.

### Comedogenesis evaluation test procedure

For the induction of microcomedones on the participants, two zones were selected: the intermammary cleft zone, and the facial zone were patched every day during the 4-week study period. A dermatological skin assessment was carried out, and the image of the selected zone was captured and registered in a file before launching the study and at the end of the four-week test period in the corresponding zone. The corresponding instrumental measurements for skin profiling were also carried out. The participants were observed for an increase of the existing follicular ostium size and increase of comedones numbers in the zone. Sebum in the skin was also assessed and the size of comedones and follicular ostiums monitored over the study period. Emerging micro-comedones were estimated by the cyanoacrylate follicular biopsy technique at 0 and after the 4 weeks period. The quantitative image analysis methodology developed by Pagnoni and Kligman (1982) was used as a guide [20]. According to the method, briefly, a drop of cyanoacrylate was applied to the chosen site and spread out under a plastic slide. The polymer was left to harden for two minutes, and the slide was gently lifted off, carrying with it a thin sheet of the outer horny layer with attached vellus hairs. Those hairs that were encased by horny casts observed under the microscope were considered to be microcomedones. The density of microcomedones was expressed as the number observed per square centimetre (table 1).

Table 1: Scale of Comedogenesis potential

|  |  |  |
| --- | --- | --- |
| Score | Description | Oil Attributes |
| 0 | Non-comedogenic | Won’t clog pores at all |
| 1 | Minimally comedogenic, | Very low likelihood they will clog pores |
| 2 | Moderately low comedogenic, | Moderately low likelihood |
| 3 | Moderately comedogenic, | Moderate likelihood |
| 4 | Strongly comedogenic, | Fairly high likelihood |
| 5 | Very Strongly comedogenic, | high likelihood of clogging pores |

## Mutagenicity/Genotoxicity potential assessment of *M flabellifolius* and *C Lanatus* oils

Mutagenicity potential of the 2 oils was evaluated using a modified Ames assay. The protocol proposed by Maron and Ames (1983) [21] supported by OECD 471 technical Guidelines [22] and opinions from Annex 1 to Directive 67/548/EEC [23] with some adaptations was carried out to investigate the potential of the 2 oils to induce genetic mutation in the *Salmonella Typhimurium (S. Typhimurium)* TA97, TA98, TA100, TA102, and TA1535 strains. The preincubation technique was used, both in the absence and presence of the metabolic activation system (4% S9 mix, Aroclor-preinduced). In both analyses, the negative control was 10% DMSO while known mutagens were used as the positive control substances. The positive controls without the S9 mix were as follows: 4-nitroquinoline 1-oxide (4-NQO) (5 μg per plate) for TA97 and TA98; sodium azide (SA) (10mg per plate) for TA100; mitomycin C (MMC) (1 μg per plate) for TA102; and methyl methane sulfonate (MMS) (200 μg per plate) for TA104. The positive controls with the S9 mix were 2-amineanthracene (2-AA) (10.0 μg per plate) for TA97, TA98, and TA1535 and benzo(α)pyrene (B[α]P) (50.0 μg per plate) for TA100 and TA102. A dose-finding test was carried out with and without the metabolic activation system (S9 mix) for each tester strain. For each oil, a total of eight concentrations diluted in DMSO were tested from 0 to 100 μL/mil. For the assays without metabolic activation, 0.5mL of 0.1 mol/L sodium-phosphate buffer (pH 7.4) was added, and for the assays in the presence of metabolic activation, 0.5mL of the S9 mix was mixed with 0.1mL culture medium (2 × 109 cells/mL) plus 0.1mL of each compound solution (0.02 to 100 μL/plate). The mixtures were incubated in a shaker at 37°C for 30 minutes in the dark and then added to and mixed with 2mL top agar containing 0.05 mol/L L-histidine and D-biotin for the S. *Typhimurium* strains. Each of these was then spread on a minimum glucose agar plate. After the top agar had solidified, the plates were incubated at 37°C for another 72 hours. Each tester strain was assayed in triplicate, and the number of revertant colonies was counted for each tester strain and treatment group. The results were judged to be positive when the average number of revertant colonies in each treated group increased with increase in the compound concentration, reaching at least twice the number in the negative control group. In order to determine the cytotoxic effects, after 30 minutes preincubation, the assay mixtures were diluted (1: 105) in 0.9% NaCl (w/v) and a suitable aliquot of the final dilution (100 μL) of this suspension was then plated on nutrient agar. The plates were then further incubated at 37°C for 10 hours and the colonies were counted. All the experiments were done in triplicate.

## Augmented sunscreen prototype formulation

The materials used in preparing the cream were cosmetic Ingredient Review (CIR) approved ingredients as follows: Stearic acid, Ceto stearyl alcohol, GMS and cetyl alcohol were obtained from Savanna Chemicals (South Africa). Engen South Africa supplied liquid paraffin and petrolatum. Triethanolamine was obtained from Merck Chemicals South Africa. EDTA, Carbopol 940®, Glycerin, MPG, methyl paraben and propyl paraben were obtained from the University of Zimbabwe pharmaceutics lab. *C lanatus and M flabellifolius* oils were supplied by KAZA natural oils, Zimbabwe. Para Amino Benzoic Acid (PABA) was obtained from ATRIC Biosciences laboratories.

### Method for preparing the emulsion sunscreen

In preparing the emulsion, published methods by Chifamba and Chifamba (2024), were used as a guide [24].

**Step 1:** A water phase was homogenized in a stainless steel thermal jacketed heating vessel, it contained 75% of the required amount of deionized water, EDTA, glycerin, Carbopol 940®, MPG and methyl paraben. The materials were homogenized by a Silverson mixer and heated to 85oC for 5 minutes. The ZnO and TiO2 nanoparticles were dispersed into this water phase.

**Step 2**: In a separate vessel, the oil phase was prepared simultaneously by adding all the required amounts of the following materials and heating them to 90oC for 5 minutes. The materials included propyl paraben Petrolatum, GMS, cetyl alcohol, castor oil and stearic acid.

**Step 3:** The clear hot oil phase from step 2, was added into the water phase vessel from step 1 while consistently agitating with an emulsifying mixer at 3000rpm. Triethanolamine was then added to the newly formed emulsion.

**Step 4:** The buffered emulsion was then cooled down naturally by removing sources of heat to 50oC. The PABA which was first dissolved in mono propylene glycol was then incorporated at this point. The remaining 25% of the deionized water was also added in to make up the required volume.

### Method for preparing the augmented sunscreen

The same procedure in preparing the nanometric emulsion sunscreen above was followed in preparing the augmented sunscreen however, in step 2 above, the incorporation levels of the emollients (petrolatum and castor oil) were lowered, and the difference was substituted by *C. lanatus* and *M. flabellifolius*. These augmenting photoprotective oils were however added in step 4 respectively.

## Skin sensitivity tests

### Animal skin sensitivity tests

The animal skin sensitivity tests conducted on the sunscreen formulation were guided by OECD technical guideline 404 with minor amendments using 3 adult male New England breed white laboratory rabbits, weighing between 1.3-1.8 kgs. The rabbits were checked for their suitability for the study over a 7-day acclimatization period. The rabbits were kept in a rodent facility in a limited access facility. They fed on a typical commercial rabbit diet and had unlimited access to drinking water. Prior to the test, the backs of the rabbits were shaved by depilatories, and the shaved area was divided into three marked parts measuring 25cm2 each. The first marked area was used for the application of the test cream, and the second and third demarcated areas were used as the positive and negative control testing sites for the skin irritation assessments according to OECD technical guideline 404. [25]

#### Cream application

To the test areas, 5ml of the test cream, negative control (de-ionised water) and positive control (Sodium laurel ether sulphate) were applied by a syringe and spread evenly over the 25cm2 demarcated shaved area of each animal. The application sites were covered by gauze, and the areas were lightly covered by non-sticky bandages. The treated rabbits were then returned to their respective cages and observations were made to the sites at 24, 48, 72 and 96 hours. Any sensitivity or reactions to the treatments were evaluated by the following criteria (Table 2) as per the documented method. [25]

### Human skin irritation potential patch testing of the augmented sunscreen

In this study, 3 healthy subjects (aged 19,30 and 44years) were recruited. Each participant tested all 3 test materials, using a different arm for each of the creams ( base cream and augmented sunscreen). In this test de ionised water was replaced by the base cream as the negative control. The participants were briefed on the study procedures, and a signed written informed consent was obtained from all subjects prior to conducting the procedure

#### Irritancy patch test procedure

Panellists were tested with the 3 test substances in double blind trials according to standard testing procedures. Using an occlusive patch [FinnChamber on Scanpore (Epitest)] the test material was applied to the volar forearm for 4 x 24-hour periods.

The augmented sunscreen cream (5 µl), basic cream (negative control) and 1% sodium lauryl sulphate (positive control) were applied topically using white litmus paper (5 mm × 10 mm) and placed on the skin in Finn chambers and kept in position. After a 24-hour interval, the test specimen was thereafter washed off by tap water and observed for any visible changes such as erythema and oedema. The mean erythemal scores were recorded (ranging from 0 to 4 as in table 2) according to Draize techniques [25,30]. A new patch was applied to the same area, and the procedure was repeated daily for the next 4 days, the test areas were kept dry for the duration of the trial. The cumulative irritancy was calculated daily and averaged over the 4 days. The visual assessment was carried out using a 2 X magnifying lamp for visual scoring and a Minolta Cr400 Chromameter was used to assess the surface colour based on the tristimulus analysis of the reflected xenon light pulse using the lamp system. To quantify the skin response for each test site compared to the baseline, the estimate values were calculated at 0, 24, 48, 72 and 96h post application of test materials.

### Score of primary irritation index (SPI)

Table 2: Observed irritation classification

|  |  |  |
| --- | --- | --- |
| **Reaction** | **Observation** | **Score** |
| Erythema | No erythema | 0 |
| Very slight erythema | 1 |
| Well-defined erythema | 2 |
| Moderate to severe erythema | 3 |
| Severe erythema to eschar formation | 4 |
| Oedema | No oedema | 0 |
| Very slight oedema | 1 |
| Well defined oedema | 2 |
| Moderate oedema (raising 1mm) | 3 |
| Severe oedema (raised more than 1 mm and extending beyond area of exposure | 4 |
| **Total score for primary irritation** | | **8** |

The Score of Primary Irritation (SPI) was calculated by the following equation 1 for both the treated and the control sites.

*Equation 1*

### Primary irritation index (PII)

The Primary Irritation Index (PII) was derived from the differences between the summed SPI scores for the treated site and the control sites. The PII was calculated by the following equation 2:

*Equation 2*

The degree of irritation was then categorized according to the Draize irritation response categories in Table 3.

Table 3: Irritation response categories

|  |  |
| --- | --- |
| **Category** | **Primary irritation index (PII)** |
| Negligible irritation | 0-0.4 |
| Slight irritation | 0.5-1.9 |
| Moderate irritation | 2-4.9 |
| Severe Irritation | 5-8 |

## *In-Vitro* photoprotection efficacy determination of the augmented sunscreen

The SPFs for both the basic and augmented sunscreens were evaluated using directives on sunscreen testing and labelling of products prescribed by the FDA and COLIPA [26] guided by published methods by Chifamba and Chifamba 2024 [24]. As mandated by both guidelines, the *in vitro* SPF determinations were carried out using a Spectrophotometer equipped with two photodiode array spectrographs and coupled to an integrating sphere. (Ultraviolet Transmittance Analyzer (UV-2000S, Labsphere, USA). The spectrophotometer xenon flash lamp supplied energy in the spectral range between 290–450 nm while emitting the required continuous peak-less spectrum of radiation. The incremental step was 1nm and the irradiance was conveniently kept low so as not to introduce potential photo stability to the sample. The formulated sunscreens were applied at a rate of 2mg/cm2 to square Polymethylmethacrylate (PMMA) plates which were roughed on one side (Helioplate™ HD6, HelioScreen, France) and was spread evenly over the plates with a fingertip covered in a vinyl glove. The individual sunscreen, 50mg in total per plate, was directly weighed and applied in droplets onto the plates. Three plates were prepared for each sample, and the filmed plates were kept in the dark to equilibrate for 20 minutes at 25oC degrees. The equilibrated plates were then subsequently mounted onto the light-path of the Ultraviolet Transmittance Analyzer (UV -2000S). The UV radiation transmittance patterns through the mounted samples were measured using the equipment settings above at 6 different sites of the plates. The built-in equipment software used the recorded transmission patterns to calculate and determine In-vitro UVA/B photo protection efficacy accordingly: The UVR photo protection efficacy of the sunscreens was determined through the calculation of the UVB protection efficacy (SPF) and the UVA protection efficacy (UVAPF), the UVA/UVB ratio and the critical wavelength ƛc.

The *in vitro* SPF is evaluated as per the following equation 3.

*Equation 3*

A math equations and formulas

Description automatically generated with medium confidence

Where-

*E (λ): Is the erythema action spectrum,*

*I (λ): Stands for spectral irradiance,*

*A0 (λ): Means monochromatic absorbance before UV exposure,*

*Dλ: Is the wavelength step (1 nm).*

The whole experiment procedure was repeated with a COLIPA SPF 30 standard, as an assaying control sample.

# RESULTS AND DISCUSSION

## Comedogenesis evaluation of *M flabellifolius* and *C Lanatus* oils

According to the 5-point scale used to determine the comedogenicity of cosmetic products, the average results for *C lanatus*, a fixed oil was 0 (table 4 and 5) which means that the oil is noncomedogenic and will not cause or increase skin outbreaks. Corresponding results (table 4 and 5) for the *M flabellifolius* which is an essential oil show a rating of 1, which means that the oil is minimally comedogenic. Our evaluation followed a prospective design of the experimental protocol based on the publication from Mills & Kligman‘s procedure [27]. The principle behind this *in-vivo* test involving humans is to determine the oils’ potential to cause or increase comedones by applying a uniform amount of test materials on a small area mounted on patches on the intermammary cleft zone and faces of participants who have skin susceptible to comedones. Sebaceous follicles are then assessed by the non-invasive “follicular biopsy” technique [27]. This was then used to assess the sebaceous follicles and follicular contents removed by the quick setting, cyanoacrylate glue. An analysis of the biopsies by an experienced dermatologist determined the number of follicles and microcomedones per unit area. The average grades of all the participants indicated the mean rate of comedogenicity for each tested cosmeceutical ingredient or product. It was therefore imperative to enroll only participants that are susceptible to acne and skin breakouts as comedogenicity is associated with this skin type.

Table 4: Clinical dermatological assessment according to the scale of comedogenic potential

Summary of the dermatological assessment in volunteers after 4-week applications of the oils

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Participant | Age | Sex | Final Dermatological Assessment  (4 Weeks face application) | |
| ***M flabellifolius*** | ***C Lanatus*** |
| 1 | 16 | F | 1 | 1 |
| 2 | 19 | F | 0 | 0 |
| 3 | 18 | F | 2 | 1 |
| 4 | 33 | F | 0 | 0 |
| 5 | 24 | F | 0 | 0 |
| 6 | 33 | F | 0 | 0 |
| 7 | 39 | F | 1 | 0 |
| 8 | 42 | F | 1 | 0 |
| 9 | 28 | F | 1 | 0 |
| 10 | 27 | F | 1 | 1 |

Table 5: Average Comedogenesis assessment of *M flabellifolius* and *C Lanatus oils*

|  |  |  |  |
| --- | --- | --- | --- |
| Oil | Comedogenic rating | Contributing constituents’ possible reasons | Skin type most suitable for oil |
| *C Lanatus* | 0 (non-comedogenic) | High in Linoleic Acid (62%) which is non comedogenic | All Skin Types, best for oily/Acne-Prone/Sensitive skin |
| *M flabellifolius* | 1 (minimally-comedogenic) | Composed mostly of cyclic terpenoids including pinocarvone and trans-pinocarveol. Essential oils themselves are generally not comedogenic and do not clog pores. However, the carrier oils used to stabilize essential oils can have varying levels of comedogenicity which could have contributed to this rating | Most skin types |

The possibility of comedogenicity and irritancy in skin care products has been well documented. Cosmetics usually contain possible follicular and surface epithelial irritating ingredients which cause breakouts. These ingredients fall into several chemical classes. By understanding the relationship between chemical moiety and ingredient characteristics, it is possible to avoid potential comedogenic ingredients and formulate nonirritating, noncomedogenic moisturizers, sunscreens, and general cosmetics. The results above confirmed that both oils will not be expected to cause any break outs in users of cosmetics that contain them at ordinary usage levels. Our findings also point out that Comedogenicity is not directly related to oiliness; the latter is a physical attribute and not a chemical entity. Some of the most potent comedogenic substances, dioxin for example, are non-oily. On the other hand, many classical oils, notably vegetable oils, are usually non-comedogenic. From a cosmetic application perspective, it is paramount to keep in mind that the foregoing evaluations and analysis were done on neat (pure) oils and therefore represent the worst possible case scenario in terms of comedogenesis for the specific oils, however, in general practice and in our sunscreen formulation in particular, the oils are most likely used diluted and mixed with other ingredients, therefore their comedogenesis contribution in final formulations is even more limited and negligible.

## Mutagenicity/Genotoxicity potential assessment of *M flabellifolius* and *C Lanatus* oils

In the Reverse mutation assays using *S. Typhimurium* strains, both oils were not mutagenic toward *S. Typhimurium* TA97, TA98, TA100, TA102 and TA104 either with or without S9 mix at doses up to 5 mg per plate (Table 6). Both oils were non-toxic to *S .Typhimurium* even at the highest dose. *C lanatus and M flabellifolius* oils were both not mutagenic in *S. Typhimurium* TA1535, TA1537 and *E.coli WP2 wvM/pKM101* "in the presence of the S9 mix.

To evaluate the genotoxic activities of the oils, we carried out six *in-vitro* mutagenicity assays for each oil. The results suggested that (both) oils have no mutagenic or clastogenic potential in bacteria or cultured mammalian cells either with or without S9 activation. We could not find any conclusive previously published evaluations on the genotoxicity of either of the oils in public literature searches to use as a comparative guide. We therefore used published results on the major phyto-constituents of the oils as comparative references. Our results are therefore consistent with the previous reports that the main constituents of the oils, linoleic acid, Oleic acid and Palmitic acid for *C lanatus* and cyclic terpenoids for *M flabellifolius* do not cause DNA damage or alterations. Even though some studies indicate that fatty acids can amplify the mutagenicity potential of genotoxic agents, there are no known genotoxins in *C lanatus* and *M flabellifolius* oils from routine analysis which could have been amplified. Other studies by De Kok et al (2003) [28] also corroborate the finding that linoleic acid and other fatty acids do not contribute to any DNA damage or chromosomal aberrations with or without metabolic activation in mutagenicity tests using bacteria, cultured mammalian cells or insects. Our findings are consistent with the expert panel conclusion in the ‘Final Report on the Safety Assessment of Oleic Acid, Lauric Acid, Palmitic Acid, Myristic Acid, and Stearic Acid’ as documented by the American college of toxicology [29] which concluded that on the basis of available data from studies using animals and humans, these fatty acids are safe in present practices of use and concentrations in cosmetics.

Table 6: Mutagenicity evaluation of C *Lanatus* and *M Flabellifolius*

|  |  |  |  |
| --- | --- | --- | --- |
| Oil | Toxicity to *S .Typhimurium* | Mutagenicity to *S. Typhimurium* TA97, TA98, TA100, TA102 and TA104 | Mutagenicity to *S. Typhimurium* TA1535, TA1537 and E.coli WP2 wvM/pKM101 |
| C Lanatus | Non-Toxic | Non-mutagenic | Non-mutagenic |
| M flabellifolius | Non-Toxic | Non-mutagenic | Non-mutagenic |

## Augmented sunscreen prototype formulation

Our formulation yielded an aesthetically pleasing cream based on the following formulation given in table 7 with a pH of 5.7 (Table 8) . The pH of 5.7 was deliberately targeted for the cream because in previous studies, Chifamba and Chifamba (2024) determined that generally, their panel of participants, which was predominantly African skin types, had higher skin pH (ranging from 5.7 to 5.9) than ordinary Caucasians average of 5.5. Matching cream pH to average users skin pH minimises irritation potential on application and reduces the risk of adverse skin creations to cosmetics. The sunscreen was not fragranced so as to avoid allergic reactions commonly associated with cosmetic fragrances and volatile organic components. Liquid paraffin was completely avoided, and the petrolatum was limited so as keep mineral-based oils and fats at a minimum since they are alleged to clog pores leading to skin outbreaks. The *Myrothamnus flabellifolius* extract was added in step 4 after cooling the emulsion due to the fact it is a volatile essential oil.

Table 7: prototype Augmented sunscreen formulation

|  |  |
| --- | --- |
| **Material** | **% Incorporation** |
| Stearic acid | 2 |
| Cetyl alcohol | 1 |
| Glycerol monostearate | 1 |
| Petrolatum | 1 |
| Castor oil | 1 |
| *C Lanatus* seed oil | Q.S |
| *Myrothamnus flabellifolius* | Q.S |
| Glycerin | 3 |
| Monopropylene glycol | 1 |
| Carbopol 940® | 0.07 |
| EDTA | 0.2 |
| Methyl hydroxybenzoate | 0.2 |
| Propyl hydroxybenzoate | 0.2 |
| Triethanolamine | 0.4 |
| PABA | Q.S |
| Distilled Water | Q. S |

Table 8: Treatment Cream Analytical Report

|  |  |
| --- | --- |
| **Parameter** | **Result** |
| Description | White smooth viscous cream, esthetically pleasing slippery feel when rubbed between two fingers. |
| pH | 5.70 |
| Odor | Characteristic of un-fragranced base cream |

## Animal and Human skin irritation potential patch testing of the augmented sunscreen

Cosmetic ingredients in general and sunscreens in particular are capable of causing adverse effects on skin, either skin irritations or allergic reactions due to various factors. One of the most skin common responses is adverse irritant contact dermatitis which causes redness, itching, stinging, or burning sensations. Allergic contact dermatitis on the other hand results in more intense itching, oedema and rashes. The most common culprits inducing adverse responses include chemical UV filters and other ingredients like fragrances. Since a number of skin care ingredients can be regarded as irritants based on the concentration in the product and the duration of the application contact time, the body site that will be exposed to the potential irritant must be considered along with the skin permeability and dermal sensitivity profile of the irritant ingredient[30]. It is the basis upon which cosmetics are labeled with directions to the application site, e.g. facials, hand and body cosmeceuticals because different anatomical sites have different responses to potential irritants. The assessment of the irritancy potential of sunscreens and other skin care products is therefore necessary and prescribed by regulatory bodies . The OECD technical guidelines 404 and 406, COLIPA as well as the FDA monographs recommend the rabbit skin irritancy evaluation based on the established methods documented by Draize (Draize H: 1944) employed in our previous published studies [31].

For ethical reasons, before any *in-vivo* animal test for skin irritation, the OECD technical guidelines recommend decision-making protocols based on the product technical specifications and rheological parameters which were adhered to in this study. For instance, the guidelines prescribe that substances with harsh pH specifications (below 2 or above 11.5) should never be tested on animals due to the obvious corrosive reactions and effects on animals and that any substances previously found to have adverse effects any other sensitivity test protocol as described under Annex 1 of Dir. 67/548/EEC should never be included in animal tests and researchers have to find suitable *in-vitro* alternatives. In our investigations, all the ingredients’ used are Cosmetic ingredient review (CIR) approved, and the plant extracts have known safe dermal toxicity profiles. Our formulated product technical specifications and the subsequent laboratory analytical report and pH in this present case justified the use of the Draize animal sensitivity tests.

## Skin sensitivity tests

### Animal tests

### **Erythema**: After the mandated test period of 96 hours, the skin irritation score for erythema in all the tested animals were low for both the augmented sunscreen under test and the base cream (control). No animals exhibited any signs of erythema.

### **Oedema:** After the 96-hour test period, the results for the test sunscreen and the base cream were also identical. No animal exhibited any signs of oedema formation.

### **Primary irritation index**: For both the sunscreen and the control, for all animals, the Primary irritation index, was found to be below 0.5. According to the test scoring criteria, the augmented sunscreen is therefore classified as a ‘negligible skin irritant because it does not pose any skin sensitisation potential based on the Draize method on test animals. The experiments were concluded as per the guidelines (table 9).

**Table 9: Primary irritation of the augmented cream on rabbits**

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | Draize Rabbit sensitivity screening | Human Primary irritation index | Conclusion |
| Augmented sunscreen | No lasting erythema and oedema after 96 hours | 0.30 | Negligible irritation |
| Base cream | No lasting erythema and oedema after 96 hours | 0.30 | Negligible irritation |
| De ionized water (negative control) | No lasting erythema and oedema after 96 hours | 0.28 | Negligible irritation |
| SLES (positive control) | Observed erythema and oedema during testing | 5.80 | Negligible irritation |

### Human patch testing

At the conclusion of the human patch testing assessments, the primary irritation index for the augmented sunscreen was very low, thereby classifying the sunscreen as a negligible irritant.

Comparative visual assessment of the positive control (sodium lauryl sulphate) revealed that it had adverse irritancy potential to human skin, as expected, while the negative control (de-ionised water) had displayed similar results to the augmented sunscreen and the base cream with low erythema scores and no visual adverse effects. The visual grading scores of the base cream (negative control) were similar to the augmented sunscreen (Table 9).

In the investigations the *In-Vivo* human testing protocols were imperative since there are differences in the morphology, biochemical and structural components of human skin and animal models. Therefore, animal-based tests, *in-vitro* or other alternative *ex-vivo* methods may have limited utility with respect to their predictive approximation of potential adverse exposure in human applications. These human panel confirmatory tests are not only necessary scientifically but ethical as well in guaranteeing biosafety. Just as in the animal tests above, the human tests can only proceed if and only if the toxicological profile of all ingredients and their resultant interactions based on animal or alternative methods are available and as long as a justifiably high degree of safety is guaranteed. Confirmatory tests of cosmeceuticals and cosmeceutical ingredients in humans is therefore limited to assessments where no irreversible damages to dermal structures are to be expected for the participants and where the study objectives are reasonably achievable with a limited population of participants. According to the World Medical Doctors Association Declaration of Helsinki in its current revision, human testing should be conducted and monitored under the direction of certified expert health personnel to ensure the health and well-being of test participants.

## *In-Vitro* photoprotection and photo stability potential of the augmented sunscreen

The photoprotection efficacy, through SPF and UVAPF determinations for the augmented sunscreen were carried out *in vitro* as described according to the harmonized Testing guidelines using UV/Vis LAMBDA Spectrophotometers”[33]. The protocols are based on agreed specified procedures by the FDA monographs, OECD and the EU guideline method M389/EN through council directive 76/768/EEC of 27 July 1976 [24]. Technically, photoprotection approximations can only be done through either of 2 mandated methods; The first is the *in-vivo* techniques based on visually appraising the effects of increased erythemal doses of UVR on human subjects and the second is the *in-vitro* spectrophotometric methods. In this study, the *in-vitro* testing protocols using Optometric 290S were employed.

Sunscreen products are hierarchically categorized based on their photoprotective efficacy which according to regulations must be visibly outlined on the labels. Based on these criteria commercial sunscreens are available to users in 4 categories: either as low, moderate, high or very high SPF as depicted in table 10. In consensus, all the regulatory bodies have set an SPF of 6 as the minimum mandated SPF for a cosmeceutical that is offered to the public as a commercial sunscreen. The consideration is that at efficacies below this agreed minimum SPF, the objectives of photo protection cannot be realized.

Table 10: The four protection classes for SPF labelling by COLIPA 2011/FDA Final Rule 2011

|  |  |
| --- | --- |
| Label SPF | Protection class |
| 6 | low |
| 10 | low |
| 15 | moderate |
| 20 | moderate |
| 30 | high |
| 50 | high |
| 50+ | very high |

For commercial sunscreens to be labelled for broad spectrum protection, the testing guidelines for sunscreen photoprotection go on to further require proof of UVAPF (UVA protection factor) which numerically should approximately be equal to or above a third of the determined SPF. The harmonized protocol guidelines also mandate an *in-vitro* Critical Wavelength (ƛC) figure greater than 370 nm [24].

Table 11: *In vitro* photo protection and photo stability of the augmented Sunscreen

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Parameter |  | Time, minutes | |  |  |
|  | **0** | **30** | **60** | **90** | **120** |
| SPF | 31.00±0.30 | 30.95±0.25 | 30.60±0.15 | 30.28±0.40 | 30.20±0.60 |
| UVAPF | 14 | 14 | 14 | 14 | 14 |
| ƛC | 383 | 383 | 382 | 379 | 375 |
| %SPFeff | - | 99.85 | 98.74 | 97.76 | 97.42 |
| %UVAPFeff | - | 100 | 100 | 100 | 100 |

Our photoprotection investigation results in Table 11 confirm that the augmented sunscreen has an SPF above 30 and is therefore regarded as high SPF, and a UVAPF of 14 and a critical wavelength (ƛC) of 383. The UVAPF is higher than 1/3 of the SPF and the ƛC is higher than 370 and in conformance with regulatory requirements, the sunscreen is regarded as broad spectrum. The *in-vitro* photoprotective efficacy percentages: %SPFeff and %UVAPFeff are all above 95% over the 2-hour exposure period which signify excellent photostability. In the experiments we preferred the *in-vitro* method over the *in-vivo* methods due to mounting ethical and technical reservations about envisaged inadequacies and questionable utility of the *in vivo* testing protocols [24]. Currently, industry and regulatory authorities are engaged in unresolved debates over the controversies and the validity of the Fitzpatrick skin classification which is the very basis of *in-vivo* SPF testing methods [34].

# CONCLUSION

The comedogenesis evaluation concluded that *C lanatus* and *M flabellifolius* oils are non-comedogenic ingredients which are substances that do not clog pores and have no potential to cause breakouts in users. Our genotoxicity and mutagenicity evaluations further suggest that neither concentration of the 2 oils is mutagenic in *S. Typhimurium TA* strains and *E.coli WP2 wvM/pKMlOl* either with or without metabolic activation. According to the data presented therefore in this investigation, we concluded that both oils do not present any mutagenicity, cytotoxic or genotoxic, properties. Our results with the Draize animal skin sensitivity tests and the human skin irritation potential patch tests confirm and supplement a series of previous studies showing that these oils and the resulting augmented sunscreen incorporating various ingredients do not sensitise or irritate skin in basic topical cosmetic use. The photoprotection efficacy was high at SPF 30 and the photoprotection efficiency and critical wavelength were high. On the basis of the available data from these studies both *in vitro* and *in vivo*, we conclude that formulation of a broad-spectrum high SPF, and biologically safe augmented sunscreen incorporating low level chemical sunscreens augmented by *C Lanatus* and *M flabellifolius* oils and emollients is feasible and herald’s new perspectives in formulation of safe, stable and efficacious nonconventional photoprotective products.

# DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

# COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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