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Towards an understanding of bacterial metabolites prodigiosin and violacein and their potential for use in commercial sunscreens

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Synopsis

OBJECTIVES: To exploit the microbial ecology of bacterial metabolite production and, specifically, to evaluate the potential use of the pigments prodigiosin and violacein as additives to commercial sunscreens for protection of human skin. And to determine antioxidant- and antimicrobial activities (against pathogenic bacteria) for prodigiosin and violacein.

METHODS: Prodigiosin and violacein were used to supplement extracts of *Aloe vera* leaf and *Cucumis sativus* (cucumber) fruit which have photo-protective activity, and commercial sunscreen preparations. For each, sunscreen protection factors (SPFs) were determined spectrophotometrically. Assays were carried out using 96-well plates to quantify growth-inhibition of *Staphylococcus aureus* and *Escherichia coli*.

RESULTS: For the plant extracts, SPFs were increased by an order of magnitude (i.e. up to ~3.5) and those for the commercial sunscreens increased by 10-22% (for 4% w/w violacein) and 20-65%

(for 4% w/w prodigiosin). The antioxidant activities of prodigiosin and violacein were approximately 30 and 20% those of ascorbic acid (a well-characterized, potent antioxidant). Violacein inhibited *S. aureus* (IC_{50} 6.99±0.146 μM) but not *E. coli*, whereas prodigiosin was effective against both bacteria (IC_{50} values were 0.68±0.06 μM and 0.53±0.03 μM, respectively).

CONCLUSION: The bacterial pigments prodigiosin and violacein have antioxidant and antimicrobial activities, and were able to increase the SPF of commercial sunscreens as well as the extracts of the two plant species tested and so have potential value as ingredients for a new product-range of (and represent a new paradigm for) sunscreens that utilise substances of biological origin. We discussed the biotechnological potential of these bacterial metabolites for use in commercial sunscreens, and the need for studies of mammalian cells to determine safety.

Keywords- Bacterial pigments, *Escherichia coli*, Natural skin-care products, *Staphylococcus aureus*, UV-protection

Introduction

Collectively, microbes can produce an as-yet-unidentified number of secondary metabolites, all of which have antimicrobial and/or other biological activities in diverse types of microbial habitat [1-3]. Many microorganisms found in high-UV habitats (e.g. solar salterns and other shallow water) produce pigments which protect against UV damage. Furthermore, a select number of species are metabolically wired to produce some of these pigments constitutively. Prodigiosin and violacein are two such metabolites that, despite their role in UV resistance, can be produced in the dark [4]. This alludes to their additional ecophysiological roles such as the antimicrobial activities which they exert against other microbes, including both marine microalgae and Gram-positive bacteria [5-8].

Like microbial cells, human skin is highly vulnerable to UV damage which, in turn, causes immunosuppression, ageing, and diseases such cancers and, therefore, can cause mortality [9-11]. Commercial sunscreens marketed to protect human skin contain substances which can absorb, reflect or scatter UV radiation including inorganic compounds (including zinc oxide, titanium dioxide), minerals (talc and kaolin), and organic substances (such as p-amino benzoic acid and oxybenzone). Whereas mixtures of these substances are effective at protecting against UV of different wavelengths [12], there are concerns about the safety of some. For example, organic ingredients including p-amino benzoic acid and oxybenzone, can be absorbed through the skin and have been associated with adverse health effects [13-14]. Other ingredients, such as zinc oxide nanoparticles, cause lipid peroxidation and oxidative stress and are thereby genotoxic [15]. There is, therefore, a need for sunscreens which are both safer and more acceptable to the discerning consumer willing to pay a premium for products formulated using nature-derived substances.

We carried out the current study of the bacterial metabolites prodigiosin and violacein to investigate their potential for use as UV-protectants, which also have antimicrobial activity, in commercial sunscreens. The specific aims were to (i) evaluate the microbial ecology of prodigiosin

and violacein production, (ii) investigate their potential as additives to commercial sunscreens which are intended to prevent UV-induced damage to human skin, and (iii) quantify their antimicrobial activity against pathogenic bacteria found on human skin.

Materials and methods

Commercial sunscreens and plant extracts

Three commercially available sunscreens with stated sunscreen protection factors (SPFs) of 15, 24 and 40 were purchased from local market of Jalgaon, India. Plant extracts were prepared by macerating (10 g) of *Aloe vera* leaf and *Cucumis sativus* (cucumber) fruit, followed by filtration through muslin cloth (pore-size 0.7 to 1.5 mm). Each bacterial pigment, prodigiosin and violacein, was added at 4% (w/w) to samples of the commercial sunscreens and plant extracts. Commercial sunscreens, plant extracts, pigment-supplemented sunscreens, and pigment-supplemented plant extracts were used for empirical determinations of SPF as described below.

Bacterial strains and culture conditions

Serratia marcescens (NMCC 75) was obtained from North Maharashtra Culture Collection Centre (NMCC, Jalgaon, India), and *Chromobacterium violaceum,* an isolate from soil of Jalgaon, India identified by 16SrRNA gene sequencing, submitted to Gen Bank (KM226331). Cultures of both these pigment-producing species were maintained on Nutrient Agar (Hi Media, Bangalore, India) at 28 °C. *Escherichia coli* (NCIM 2139) and *Staphylococcus aureus* (NCIM 2492) were obtained from the NCIM and maintained on Nutrient Agar at 37° C. Both strains were used for bacterial inhibition assays as described below.

Microbial production of prodigiosin and violacein

Cell suspensions of *S. marcescens* and *C. violaceum,* that had been cultured in Nutrient Broth (Hi Media, India) for 24 h at 28°C, were used to inoculate media for pigment production. *Serratia marcescens* was cultured in previously optimized culture medium (Basal Salt Medium containing potato starch and casein as the carbon and nitrogen sources, respectively) [16] and *C. violaceum* was cultured in Nutrient Broth for pigment production, and incubated on a shaker at 28°C ±2 for 24 h. Cultures were then centrifuged at 7000 x *g* for 10 min, the cell pellet was re-suspended in methanol, and then centrifuged again. The pigment, located within the supernatant, was further purified using column chromatography with the column packed with silica gel. The solvent system used was n-butanol: hexane (2:1) for prodigiosin and methanol: water (7:3) for violacein. The extracts collected were then dried and quantified on dry weight basis. The prodigiosin and violacein were analysed for UV absorbance and Furrier transform infra-red (FTIR) spectroscopy (Testscan FTIR 8400, Shimadzu, Tokyo, Japan). Spectrophotometric analysis of UV absorbance was

carried out (using a UV Mini 1240, Shimadzu, Tokyo, Japan) at 536 nm and 580 nm for prodigiosin and violacein, respectively.

Spectroscopic and chromatographic characterisation of prodigiosin and violacein

Purified pigments, commercial sunscreens, and their combinations were subjected to spectroscopic and chromatographic analysis. The pigments, individually and in combination with commercial sunscreen (SPF 15), were analysed by FTIR spectroscopy using a Testscan Shimadzu FTIR 8400 at 4000 to 400 cm^{-1}.

Determination of sunscreen protection factors

Multiple samples (1 g) of each of the commercial sunscreens and plant extracts were separately added to volumetric flasks containing 100 ml ethanol. Some of these were left unaltered (controls), and 4% (w/w) of either prodigiosin or violacein was added to others. Control samples and pigment-supplemented samples were ultrasonictaed for 5 min, and then filtrated through muslin cloth (pore size, 0.7 to 1.5 mm). The first 10 ml of filtrate was discarded and the following 5 ml aliquots of the subsequent filtrate were collected by transferring to 25-ml volumetric flasks, and made up to 25 ml by addition of ethanol. Absorbance in the UV range (290 to 320 nm) of each of these solutions was then quantified at 5-nm intervals using ethanol as the blank. Three independent analyses were performed and SPFs were calculated, according to Mansur *et al*. [17], using following formula:

$$SPF = CF \times \sum_{290}^{320} [EE\ (\square]\lambda) \times I(\lambda) \times Abs\ (\lambda)$$

where CF (correction factor) = 10; EE (λ) = erythmogenic effect of radiation with wavelength λ;Abs. (λ) = spectrophotometric absorbance value of a solution; and I = solar intensity spectrum. EE (λ) $\times$ I is constant and is determined by Sayre *et al*. [18].

Ferric-reducing potential

Ferric-reducing activity was determined as a measure of antioxdiant activity as described previously [59]. In brief, 20 mg each of prodigiosin, violacein, ascorbic acid, ascorbic acid + prodigiosin (ratio 5:1 w/w), and ascorbic acid + violacein (5:1 w/w) were added to 2.5 ml phosphate buffer (pH 6.6) and 2.5 ml 1 % w/v potassium ferricyanide. The mixture was incubated for 20 min at room temperature followed by addition of 10 % w/v trichloroacetic acid (2.5 ml) and then centrifugation at 3000 rpm for 10 min (C-24 BL, REMI, India). Supernatant from the upper layer (2.5 ml) was mixed with distilled water (2.5 ml), 0.1% w/v freshly prepared ferric chloride solution (0.5 ml) was added, and the volume was made up to 100 ml by addition of distilled water. The absorbance was recorded at 700 nm using the UV–visible spectrophotometer. The percentage of ferric chloride that was reduced determined by comparison with the control [59].

Assay for antibacterial activity

Prodigiosin and violacein were assayed for inhibitory activity against *S. aureus* and *E. coli*, using a 96-well plate system. A two-fold dilution series of each pigment (100 µl) was made using Nutrient Broth and added to each of the 96 wells of each plate. Wells were also inoculated with bacterial cell suspensions (100 µL) in duplicate, to give a 0.5 McFarland density. As a positive control, the antibiotic streptomycin was used in place of prodigiosin or violacein. As a negative control, bacterial cells were suspended in Nutrient Broth without any inhibitor (neither a pigment nor antibiotic). Plates were incubated for 24 h at 37 °C and then OD_{600} nm was determined. Percentage growth inhibition was calculated by comparison with the negative control. OD_{600} nm was directly proportional to the growth of bacterial cells.

Results and discussion

Microbial ecology of prodigiosin and violacein

Prodigiosin and violacein are produced by a small number of bacteria, most of which are members of the Beta- or Gammaproteobacteria (Table I). Many of these inhabit high-UV environments and/or open habitats (Table 1) which are characterised by high microbial diversity and intense inter-species competition [2, 3, 33]. Whereas pigment production can occur independently of light, both prodigiosin and violacein have been associated with enhanced UV-resistance (Table I). These pigments also enhance competitive ability due to inhibitory/toxic activities against marine microalgae and a range of other microbial species (both eukaryotes and prokaryotes; Table I). Whereas bacterial cells can be exceptionally sensitive to changes in water activity (indeed, the sensitivity level has yet to be determined [60-61]). Prodigiosin and violacein are not sufficiently soluble to inhibit bacteria by reducing water activity [e.g. 62]. Indeed, these pigments are sufficiently hydrophobic (e.g. log $P_{octanol\text{-}water}$ for prodigiosin and violacein is 5.16 and 3.34 respectively) to suggest that their mode-of-action as antimicrobials may operate via chaotropicity-mediated stress [63-64]. Some chaotropic solutes, that destabilise macromolecular systems and are highly stressful to microbial cells at moderate temperatures [65-68], may promote growth at low temperatures by enhancing macromolecular flexibility (at < 10°C) [39, 69]. However, prodigiosin and violacein can act as potent stressors/toxicants which inhibit and even kill microbial cells, and are capable of protecting macromolecular systems under stress, regardless of temperature (Table I). More work is needed to understand the mechanistic bases of these diverse activities on biological systems.

Production and characterization of prodigiosin and violacein

Microbial synthesis, identification and chromatogenic characterization of pigments

Approximately 50 mg of each pigment was produced, and spectroscopic analyses showed single peaks for prodigiosin and violacein at 535 and 580 nm, respectively (Fig. 1). FTIR spectroscopy was used to identify functional groups via the analysis of the infra-red spectra of pigments. For

prodigiosin, there was a strong and broad absorption at 3429 cm^{-1} indicating an N-H amines stretching viabrations, 2821 cm^{-1} (C-H stretch) and 1327 cm^{-1} indicating C-N amines bend (Fig. 2) which possibly shows pyrrolenine; prodigiosin exhibits similar absorptions in $CHCl_3$ at 1630 and 1602 cm^{-1} [70]. Phenyl rings were evident within the fingerprint region, which was characterized by weak absorption intensity at 1634 cm^{-1}. Violacein also showed strong and broad absorptions at 3341 and 2966 cm^{-1}, indicating O-H stretches for phenol and/or carboxylic acid, N-H amines and C-H stretch. Absorption at 1538 cm^{-1} indicates aromatic rings, and at 1023 cm^{-1} a C-O stretch. Weak absorptions at 1387 cm^{-1} indicates C-H scissoring and bending and/or aromatic rings and 1647 cm^{-1} may represent C-N amine bend (Fig. 3).

Antioxidant activity and antimicrobial activity

The antioxidant activities of prodigiosin and violacein, tested via ferric-reducing activity, were considerable; i.e. approximately 30 and 20% that of ascorbic acid (respectively) which is a well-characterised, potent antioxidant (Table II) [71]. Moreover, the antioxidant activities of prodigiosin and violacein increased considerably (and were comparable) in the presence of ascorbic acid; i.e. 134.9 and 138.4 % relative to ascorbic acid alone (Table II). Antioxidants act by preventing the formation of free radicals, repairing the damage caused by them and/or sequestering them but chronic exposure to UV radiation can result in an imbalance between oxidant/antioxidant systems leading to excessive generation of reactive oxygen species resulting in DNA damage, protein denaturation, lipid peroxidation, and skin damage [10, 12].

Prodigiosin was highly inhibitory to both the Gram-positive and Gram-negative bacterial pathogens tested, while violacein showed weak inhibition to *E. coli*. The IC_{50} of prodigiosin for *S. aureus* was 0.68±0.06 μM and for *E. coli* was 0.53±0.03 μM. Violacein, which did not inhibit *E. coli* even at 174.7 μM, gave an IC_{50} for *S. aureus* of 6.99±0.146 μM. The IC_{50} values are consistent with those of strongly hydrophobic stressors [2, 12, 65]. Prodigiosin may cause a breakdown of the intracellular pH gradient and inhibit proton pumps [72-73] and violacein has a multi-target action and induces oxidative stress and plays role in enzyme modulation [74-75], whereas the antimicrobial modes-of-action of these pigments have not yet been fully resolved, all these characteristics are consistent with the activities of chaotropic and (chaotropicity-mediated) hydrophobic stressors [2, 63, 65, 68, 76].

Potential of prodigiosin and violacein as additives to sunscreens

Enhanced UV absorption within in range 290 to 320 nm indicates that the pigments have the potential to increase SPFs when combined with sunscreen formulations. The SPFs of the three commercial sunscreens assayed were also determined empirically. Whereas the stated SPFs were 15, 24 and 40, the empirically determined values were 16.46±0.83, 26.64±0.58 and 39.48±0.73, respectively (Table III). Addition of prodigiosin (at 4% w/w) to each of these sunscreens caused an increase of SPF to 27.72±0.66, 37.89±0.97, 47.59±0.78, respectively. Similarly, addition of violacein (at 4% w/w) to each of these sunscreens caused an increase of SPF to 19.66±0.6, 32.63±0.89,

43.79±0.86, respectively (Fig. 4; Table 3). Addition of prodigiosin resulted in 65, 42 and 20% increases in SPF values for SPF 15, 24 and 40 sunscreens, respectively. By comparison, addition of violacein showed 19, 22 and 10 % increases in SPF, respectively (Table III). Pigments alone did not show considerable SPF.

To eliminate the possibility that changes in sunscreen SPFs were an artefact of specific interactions between the microbial pigments and specific sunscreen components, this experiment was repeated for plant extracts (Table III). Extracts of *A. vera* and *C. sativus*, which are known to contain substances that protect the plant against UV [77-78] had SPF values of 0.1±0.02 and 0.2±0.03, respectively (Table III); values in agreement with those reported by Borase *et al*. [59]. Addition of prodigiosin and violacein (at 4% w/w) to *A. vera* extract caused an SPF-increase to 3.47±0.85 and 3.5±0.59, respectively. Upon addition of the pigments to *C. sativus* extract, they caused SPF increases to 3.51±0.46 (for prodigiosin) and 1.54±0.25 (for violacein) (Table III).

The increased absorption of the combinations in UVB region was responsible for increased SPF. This can be observed through absorption spectra (Fig. 4). The combination of both clearly offers better protection in UVB region and this is consistent with earlier findings [37]. Collectively, the SPF values for pigments alone (see above), their antioxidant activities (Table II), and their ability to increase the SPF of plant extracts constitutes evidence that the increase in sunscreen SPF upon addition of pigments relates to the photo-protective activities of the latter.

Comparison of the SPF values for microbial pigments, commercial sunscreens and pigment + sunscreen mixtures (Table III) suggests a synergistic effect of pigments on sunscreen protection factors. It is therefore possible that prodigiosin and/or violacein may induce structural changes in active ingredients of the sunscreen which enhance the photo-protective activities of the latter. Intriguingly, the infra-red spectrum of prodigiosin + commercial sunscreen (SPF 15) reflects strong absorption at 1547 cm^{-1} (indicating a NO_2 group and/or aromatic rings) and weak absorption at 1461 cm^{-1}, indicating scissoring-bending of C-H group (Fig. 2). This finding is consistent with synergism between prodigiosin and sunscreen ingredients whereas, for violacein, the enhanced sunscreen SPF observed (Fig. 4; Table III) may indicate an additive effect of pigment plus sunscreen.

Conclusion

The information obtained from evaluation of the microbial ecology of prodigiosin and violacein indicated biotechnological potential role as photo-protectants, it also confirmed the paradoxical and enigmatic nature of their various biological activities. The prodigiosin and violacein which were produced by *S. marcescens* and *C. violaceum* (respectively) demonstrated antioxidant and antimicrobial activities, and absorbed UV in the 290- to 320-nm range. Furthermore, these substances were able to increase the SPF of commercial sunscreens as well as the extracts of the two plant species tested and so can have value as ingredients for a new range of sunscreens utilising substances of biological origin. However, evaluation of cytotoxic properties of these

pigments against epithelial cells merits further study. Sunscreens must chemically inert, non-irritating, non-toxic, non-allergic, non-carcinogenic and photostable [79-80]. The biotechnological value of prodigiosin and violacein as additives for sunscreens will therefore depend on both consumer preference and an evaluation of their safety.

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Table I. Ecology of pigment production in prodigiosin- and violacein-producing bacteria.

Bacterial species	**Primary habitat(s)**	**Metabolic traits and ecophysiology**	**Pigment production**
Hahella chejuensis	Marine habitats such as coastal sediments [4]	Obligately halophilic: growth occurs in 1 to 8% (w/v) NaCl, and from 10 to 50°C and pH 6 to10 [4]. The Gram-negative, rod-shaped cells are facultatively anaerobic and motile. They produce a large amount of extracellular polymeric substances (EPS) which are involved in biofilm formation, and may play a role in pathogenicity against marine microalgae [19-20]. Reports of lytic activity (*in vitro* studies) and ability to produce toxins (genome-based studies) also imply pathogenicity against diverse microalgae [19-20]	Prodigiosin, one of the primary pigments in *H. chejuensis*, is produced by non-dividing, stationary-phase cells [21]. Synthesis of pigments occurs independently of light levels, as well as in the dark [4]. Prodigiosin has lytic activity towards the dinoflagellate *Cochlodinium polykrikoides* [6], and is also inhibitory to a number of Gram-positive bacteria and fungal pathogens [8]
Serratia marcescens	Soils, water, aerosols, foods, and in association with plants and animals [22]	Halotolerant: growth occurs up to 8% (w/v) NaCl, and from 0 to 44°C and pH 5 to 11 [22-24]. The Gram-negative, rod-shaped cells are facultatively anaerobic and motile. *S. marcescens* is a saprotroph that produces a number of extracellular enzymes including DNAase, lipase and gelatinase [25]; it efficiently hydrolyses chitin and casein [5, 26] and is also an opportunistic pathogen (e.g. causes hospital-acquired infections in immuno-compromised patients) [22]	Some strains are non-pigmented (including the majority of clinical isolates) but those of environmental origin are typically pigmented [27-28]. Prodigiosin levels are determined by parameters such as temperature, pH, oxygen availability, and substrate-nutrient composition [24, 29, 30], and production of this pigment occurs primarily in the stationary-phase [31]. Soil microbes are typically exposed to enormous fluctuations in water activity and microbes typically increase the production of antimicrobials under water stress [32-33]. Initial indications (for *Serratia, Streptomyces* and *Vibrio* spp.) suggest that prodigiosin production may increase at moderate

			levels of solute stress [7, 34, 35] but this has yet to be determined in relation to water activity *per se*. Prodigiosin may inhibit germination of fungal competitors [5]. Prodigiosin is hydrophobic (log $P_{octanol\text{-}water}$ 5.16) so prodigiosin-rich pigmented cells are more likely to adsorb to air bubbles within bacterial suspensions, thereby enhancing the likelihood that cells may enter the atmosphere on bubbles or aerosols [36]
Colwellia psychrerythraea (formerly *Vibrio psychroerythrus*)	Marine-associated habitats including seawater, ice, sediments, estuarine water, and eggs of marine fish [37-38]	*C. psychrerythraea* is a polyextremophile (both halophilic and psychrophilic): growth occurs only above 2.75% (w/v) NaCl and from -14 to +19°C; cells lyse above 20°C [38-39]. The Gram-negative, rod-shaped cells are non-spore forming, facultatively anaerobic and motile. Production of EPS increases at sub-zero temperatures and, in conjunction with the uptake of the compatible solute ectoine, enhances cold-tolerance [40].	Prodigiosin is also produced by diverse *Vibrio* spp. (including *V. ruber*, *V. gazogenes* and *V. rhizosphaerae*) which are found in shallow, saline waters (which, like sea-ice, are subject to UV radiation) [41-43]. Prodigiosin confers UV-resistance, which is greatest in stationary-phase cells in which pigment concentrations are highest [37]. In addition, prodigiosin inhibits other bacteria including *Bacillus* spp. and *Vibrio harveyi* [7].
Chromobacterium violaceum	Soils, water, vegetables, and in association with humans and other animals [44-45]	Halotolerant: growth occurs at up to 6.5% (w/v) NaCl, and from 10 to 44°C and pH 6 to 9 [46]. The Gram-negative, coccobacillus cells are non-spore forming, facultatively anaerobic and motile. *C. violaceum* is saprotrophic and able to efficiently utilize chitin as its sole carbon and nitrogen source [47] and is also an opportunistic pathogen of humans and animals [45].	Production of violacein, hydrogen cyanide, proteases and antibiotics is regulated by *N*-acylhomoserine lactones which are involved in quorum-sensing [48]. Violacein protects the plasma membrane against oxidative stress [49]. *C. violaceum* and other violacein-producing bacteria are highly inhibitory to bactiverous protozoans and plankton [50-51] as well as some bacteria [52]. This antimicrobial activity has been attributed to violacein [50]
Janthinobacterium lividum	Soils, water, food, and in association with animals [53-55]	Growth occurs from 4 to 30°C and from 4 to 7.5. The majority of strains do not grow in the presence of 2% (w/v) NaCl. The Gram-negative, rod-shaped cells are non-spore forming and strictly aerobic [56]. *J. lividum* is an opportunistic pathogen [57].	Violacein production depends on the type of carbon substrate(s) available [54, 58]. Correlations have been reported between intracellular violacein concentration and resistance to the antibiotic amphicillin [54].

Table II. Antioxidant activities for ascorbic acid, individual pigments, and ascorbic acid-pigment mixtures.

Substance or mixture	Ferric chloride-reducing activity (absorbance at 700 nm)	Antioxidant activity relative to ascorbic acid (%)
Ascorbic acid	0.2±0.002	100
Prodigiosin	0.061±0.007	30.5
Violacein	0.038±0.005	19
Ascorbic acid + prodigiosin (5:1)	0.193±0.05	134.9
Ascorbic acid + violacein (5:1)	0.198±0.04	138.4

Table III. Sunscreen protection factors (SPFs) for commercial sunscreen preparations and natural plant extracts before and after supplementation with bacterial metabolites prodigiosin and violacein.

Commercial sunscreen or plant extract	Ingredients	SPF according to manufacturer	Empirically determined SPF	Empirically determined SPF after addition of pigment at 4%, w/w	
				Prodigiosin	Violacein
Sunscreen 1	Water, paraffinum liquidum, titanium dioxide, stearic acid, borax, almond oil, vitamin E, benzophenone-3, isopropyl myristate, octyl methoxy cinnamate, propylene glycol, methyl and propyl paraben, EDTA, imidazolidinyl urea	15	16.46±0.83	27.72±0.66	19.66±0.6
Sunscreen 2	Water, glycerine, ethylhexyl salicylate, avobenzone, xanthan gum, potassium hydroxide, phenyl benzymidazol sulphonic acid, palmitic acid, polyethylene glycol 100 and glycol sterate, steramide AMP, lactic acid, potassium hydroxide, sodium hydroxide, dimethicone, propylene glycol, methyl paraben, propyl paraben, cetyl alcohol, lemon grass, extract of *Cucumis sativus* fruit extract	24	26.64±0.58	37.89±0.97	32.63±0.89

Sunscreen 3	Water, zinc oxide, polyethylene glycol 10, dimethecone, triethoxycaprylylsilane, dimethycone crosspolymer, cyclopentasiloxane, ethylhexylmethoxycinnamate, potassium chloride, caprylylmethicone, disteardimonium hectorite, sucrose distearate, disodium EDTA, lemon grass, extract of *Cucumis sativus* fruit extract, lactic acid, propylene glycol, idopropynyl butyl carbamate.	40	39.48±0.73	47.59±0.78	43.79±0.86
Aloe vera leaf extract	Alkaloid, tannin, saponins, steroids, etc.	NA	0.1±0.02	3.47±0.85	3.5±0.59
Cucumis sativus fruit extract	Lactic acid, Z-6-nonenol, E-2-nonenal, etc.	NA	0.2±0.03	3.51±0.46	1.54±0.25

NA – Not applicable