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Development of Facial Cosmetic Formulations Using Microbial Levan in Association with Plant-Derived Compounds Using Simple Lattice Design

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HIGHLIGHTS

- A levan-based formulation composed of Aloe vera and avocado oil was development.
- Biocosmetic showed antioxidant activity, retention capacity and good spreadability.
- Levan-based formulation was efficiently modeled within the simplex design space.
- Cosmetic formulations showed great stability and pH compatible with skin.

Abstract: The cosmetics industry searches for new products with sustainable, natural and non-toxic characteristics. Levan is a microbial exopolysaccharide composed of fructose units with β-2,6-glycoside bonds. Due to its biological properties, levan has a wide range of applications in cosmetics and pharmaceuticals industry. The effects of levan can be improved by its combination with biocompatible actives such as plant-derived compounds and vitamins resulting in a multifunctional cosmetic formulation. Thus, the aim of this study was to develop a new biocosmetic gel-anionic type containing 1 % of levan and plant-derived compounds, such as Aloe vera extract and avocado oil and vitamin E, using simplex lattice mixtures design. According to the statistical analyses, the concentrations to obtain a cosmetic with maximum antioxidant activity (76%), hydration capacity (98.37%) and spreadability (767.30 mm²), would be 10% (0.1 mL) of Aloe vera, and 90% (0.9 mL) of avocado oil. Cosmeceutical formulations showed good stability and did not show phase separation or visible changes being classified as normal. The development of innovative and sustainable cosmetic formulations, with natural, vegan and non-toxic molecules is of great importance for cosmetic industry.

Keywords: Levan; *Aloe vera*; Avocado oil; Antioxidant; Simplex lattice design.

INTRODUCTION

The growing concern about the health skin has been reflected in a great demand for new biotechnological products of natural origin, as they cause fewer side effects and do not harm the environment [1]. Cosmetic formulations including natural active ingredients are in evidence due to their sustainable characteristics and biological properties. According to Brazilian Association of the Personal Hygiene, Perfumery and Cosmetic Industry, Brazil has a market demand for natural cosmetics that corresponds to the expectation of growth of 5 to 10% and is projected to reach USD 25.11 billion by 2025 [2].

Molecules from biotechnological source are very studied due to their biological properties, non-toxicity and renewable characteristics [3]. Levan is an extracellular polysaccharide that has several interesting properties such as biocompatibility, biodegradability, renewability, flexibility, and eco-friendliness [4, 5]. Levan is a polysaccharide consisting of fructose units linked with β2,6-glycoside bonds in its main chain and β-2,1 in its branches [6, 7]. Several microorganisms are reported as levan producers, such as *Erwinia herbicola* [8], *Zymomonas mobilis* [9,10], *Microbacterium laevaniformans* [11], *Bacillus subtilis* [12,13] and *Bacillus subtilis natto* [12,14]. Alternatively, levan can be produced by enzymatic synthesis using levansucrase. Levansucrase (EC.2.4.1.10) belongs to the family of glycoside hydrolases (GH68) that catalyze sucrose hydrolysis and the transfer of fructosyl units to acceptor molecules. An interesting aspect of levansucrase is its specificity in the formation of high-molecular-mass polymers, such as levan [15,16]. Levan exhibits several dermocosmetic properties [17] such as antioxidant action, moisture retention activity [18], cell proliferation and skin irritation relief [19]. In addition, levan is biocompatible with other ingredients of cosmetic formulations [18].

Plant-derivate ingredients are also widely used in cosmetic formulations due to their fragrance and a myriad of properties as antioxidant, anti-inflammatory, antimicrobial, photo protective, wound healing action and emollient characteristics [20, 21]. Avocado oil (*Persea americana*), is generally found in various subtropical countries and are recognized as a healthy food supplying of proteins and monounsaturated fats [22]. *Aloe vera* (*Aloe barbadensis*) is a traditionally medicinal plant used in several countries such as Greece, China, and Mexico to cure many infection and skin lesions. *In vitro* and *in vivo* investigations have showed that *Aloe vera* is able to improve the wound healing and to reduce the inflammation process [23]. Vitamins have also been added in cosmetic formulations. Vitamin E (alpha-tocopherol) is frequently used in cosmetics due to its antioxidant [24], anticarcinogenic and photoprotective properties [25], and has great potential to be incorporated into biocosmetics.

Therefore, the association of levan, avocado oil, *Aloe vera* extract and vitamin E could result in a multifunctional, sustainable and non-toxic cosmetic formulation. Different ingredients can be combined using statistical methods as the simplex lattice method in order to optimized the formulations. This statistical tool associated with the software is able to analyze the effects of the interactions between the different variables studied [26]. This methodology is common used to optimize the concentrations of the ingredients in mixture, combining mathematical theory, statistical analysis and experimental design [26]. In this study we describe the development of a multifunctional biocosmetic using levan combined with Aloe vera and avocado oil with high spreadability level, retention capacity and antioxidant activity. The development of innovative and sustainable cosmetic products, with natural ingredients and multifunctional molecules is of great importance for cosmetic industry.

MATERIAL AND METHODS

Microorganism, Growing Conditions, and plant-derived compounds

B. subtilis natto CCT7712 was isolated from fermented soybeans, a Japanese food called “natto” at the Department of Biochemistry and Biotechnology of Londrina State University (Brazil) and identified by André Tosello Foudation (Campinas-Brazil). The strain was cultivated for 48 h and maintained at 37 °C on a medium containing (g L^{-1}): peptone 50, meat extract 30, and agar 20, stored at 4 °C. The avocado oil (*Persea americana*), Aloe vera extract and Vitamin E (alpha-tocopherol) were purchased from Mapric (São Paulo, Brazil).

Synthesis of levan by levansucrase

The exopolysaccharide levan was produced by levansucrase from *B. subtilis* natto. The enzyme was produced using a medium containing 420.7 g/L sucrose at 150 rpm for 24 h and the supernatant was used as the enzyme source [27]. The levansucrase presented 23.9 U/mL of enzymatic activity. Levan production medium was composed by 200 mL of sucrose solution (350 g/L), 50 mL of the levansucrase (sterilized with UV for 2 h) in citrate buffer (0.1 M), pH 6.0 at 35 °C for 36 h [27]. The reactions were stopped by incubating at 100 °C for 15 min. Levan concentration was estimated by precipitation with absolute ethanol (1:3 v/v) for 12 h following by centrifugation at 9000×g for 20 min at 4 °C [28]. Levan was dialyzed against distilled water for 48 h and lyophilized.

Development of cosmetic formulation

The Simplex Lattice Design model was used to optimized the concentrations formulations ingredients. Thirteen formulations were developed varying the components x1 (*Aloe vera* extract), x2 (vitamin E) and x3 (avocado oil). The maximum concentrations of the components were 0.5 mL, 0.5 mL and 1.0 mL for variables x1, x2 and x3, respectively (Table 1). The responses were spreadability, antioxidant activity and moisture retention capacity. The cosmetics formulations were prepared by mechanical stirrer, where phase 1 contained: glycerin and distilled water and phase 2: Ammonium acryloyldimethyltaurate/VP copolymer, and phase 3: cyclomethicone, preservative blend (methyl ethyl propryl paraben phenoxyethanol), levan (1%), *Aloe vera* extract, vitamin E and avocado oil. Phase 1 was heated to 70 °C and phase 2 was sprayed under phase 1 until anionic gel was formed. Then, it was cooled to 40 °C and cyclomethicone and the preservative blend were added. The other ingredients in phase 3 were added according to Table 1. The pH values of cosmetic formulations were adjusted in a pH range of 4.5-5.5 with citric acid solution.

Characterization of cosmetic formulations

Stability

Stability of the formulations was verified by phase separation. Five grams of each formulation was centrifuged at 3000 rpm, for 30 minutes at room temperature. After 24 hours, the macroscopic visualization was interpreted as: normal (without phase separation), phase separation (coalescence) and creamy [29]. The pH of each formulation were performed in triplicate after 24 hours.

Spreadability

To determine the spreadability, samples was placed in a graph paper supported by a circular glass plate. The formed perpendicular diameters were measured to define the comprehensive surface, at temperature of 25 ± 2ºC [30]. Samples of 2.0, 4.0, and 10 g were used in one-minute intervals. The tests were performed in triplicates. The spreadability was calculated using the Equation 1:

$$\mathbf{Ei} = \frac{(\mathbf{d^2} \cdot \boldsymbol{\pi})}{\mathbf{4}} \qquad (1)$$

Where:

Ei: spreadability of the sample for a given weight in square millimeter (mm^2); d: average diameter in millimeter (mm).

Antioxidant activity

The antioxidant activity was evaluated by the DPPH method (2,2-diphenyl-1-picryl-hydrazil). For the assay, 1 mL of the sample (formulation at 2.5 mg mL^{-1}) and 0.3 mL of the DPPH solution were incubated in the dark for 30 min at room temperature followed by reading at ƛ = 517 nm. The blank solution was composed of 1 mL of the formulation and 0.3 mL of ethanol. Control sample was composed of 1 mL of ethanol and 0.3 mL of the DPPH solution [31]. The inhibition rate (%) of the free radical was calculated according to equation 3:

$$\%\ \mathbf{inhibition} = [(\boldsymbol{Absorbance\ control} - \boldsymbol{Absorbance\ sample}))/\ \boldsymbol{Absorbance\ control}]\boldsymbol{x}\ \mathbf{100} \qquad (2)$$

Moisture retention capacity

This moisture retention capacity was examined gravimetrically according to Zhao and coauthors [32]. The samples were placed in a saturated K_2CO_3 chamber which relative humidity (RH) was 43%. The moisture retention capacity (Ru) was evaluated by the percentage of residual water samples:

$$\mathbf{Ru}\,(\%) = \frac{\mathbf{Pt}}{\mathbf{P0}}\,\mathbf{x\;100} \tag{3}$$

Where, P0 is the weight of distilled water added to the samples and Pt is the weight of water after the tested times.

Statistical analysis

Data analysis were performed by one-way ANOVA using the software Statistica version 7.0.

RESULTS AND DISCUSSION

Levan is an exopolysaccharide very attractive for cosmetic formulations due to its great biological activity as antioxidant, antimicrobial, immunomodulatory, ability to form biofilms, biocompatibility and moisturizing capacity [33]. Previous studies also showed that levan stimulated human fibroblasts and keratinocyte proliferation, and shown no-toxicity for skin or eyes. In addition, levan was found to be non-cytotoxic in a normal human dermal fibroblast cell line and non-hemolytic on human erythrocytes suspension [34]. These results revealed that levan might be useful and safe in cosmetics and biomedical fields.

In this study, the effect of the interaction between *Aloe vera* extract, vitamin E and avocado oil on the spreadability, antioxidant activity and moisture retention capacity of levan-based biocosmetic was efficiently modeled within the simplex design space. Simplex lattice design is well documented in the pharmaceutical literature. In these models, parameters (independent variables) are varied according to a specific design, and their effect on the product properties (dependent variables) are determined. From the resulting, information predictor polynomial equations can be created, which are used to define the formulation composition [34]. This model was chosen for this study since it permits the mixtures of the compounds to be designed in a constrained space, named as the simplex space.

Stability

The stability test showed that the 13 cosmetic formulations presented good stability and did not show phase separation or visible changes being classified as normal, after 24 hours. The pH values of all formulations were close to 6.0, indicating that the biocosmetic are compatible with the facial skin (pH 4.6 to 5.8).

Spreadability

Spreadability indicates the area to which a topical formulation spreads on the skin in mm^2. According to the results (Table 1), all runs showed good spreadability. The run 1 which has only *Aloe vera* extract present the lower spreadabilidy value. In the other hand, central points (runs 11, 12 and 13) reached the highest spreadability, with average of 774.92 mm^2. In other study, the spreadability of the anionic gel formulation increased by addition of levan from *B. subtilis* natto [35].

Table 1. Simplex Lattice Design for spreadability, antioxidant activity and moisture retention capacity of levan-containing cosmetic formulations in different concentration of x1 (*Aloe vera* extract), x2 (vitamin E) and x3 (avocado oil).

Factors levels (%)				Variable responses		
Run	x_1	x_2	x_3	Spreadability (mm²)	Antioxidant activity (%)	Moisture retention capacity (%)
1	100	0.0	0.0	680.85	31	94.86
2	0.0	100	0.0	706.50	57	91.31
3	0.0	0.0	100	770.87	68	100
4	50	50	0.0	771.91	61	96.88
5	50	0.0	50	738.94	84	95.53
6	0.0	50	50	771.91	71	95.52
7	66.6	16.6	16.6	722.98	58	93.34
8	16.6	66.6	16.6	740.51	57	91.83
9	16.6	16.6	66.6	769.36	69	92.22
10	33.3	33.3	33.3	750.07	61	90.51
11	33.3	33.3	33.3	772.90	60	88.68
12	33.3	33.3	33.3	774.01	63	91.11
13	33.3	33.3	33.3	777.87	64	89.40

The response surface and the prolife of predict value and desirability for sreadability are showed in Figure 1. According to statistical analyses, the formulation composed of 0.0% of *Aloe vera* extract, 25.0% of vitamin E and 75.0% of avocado oil would be ideal for obtaining greater spreadability of the product. These results are consistent with the literature that report that vitamin E and avocado oil are composed of emollients substances, being ingredients responsible for spreading and consistency of formulations [36]. In fact, the samples that spread better have a pleasant feel on the skin, as less of the product can be applied to a larger region of the body. Consumer acceptance of the cosmetic product is given by the sensation of the initial contact with the skin, appearance, spreadability and residual oiliness after application [2].

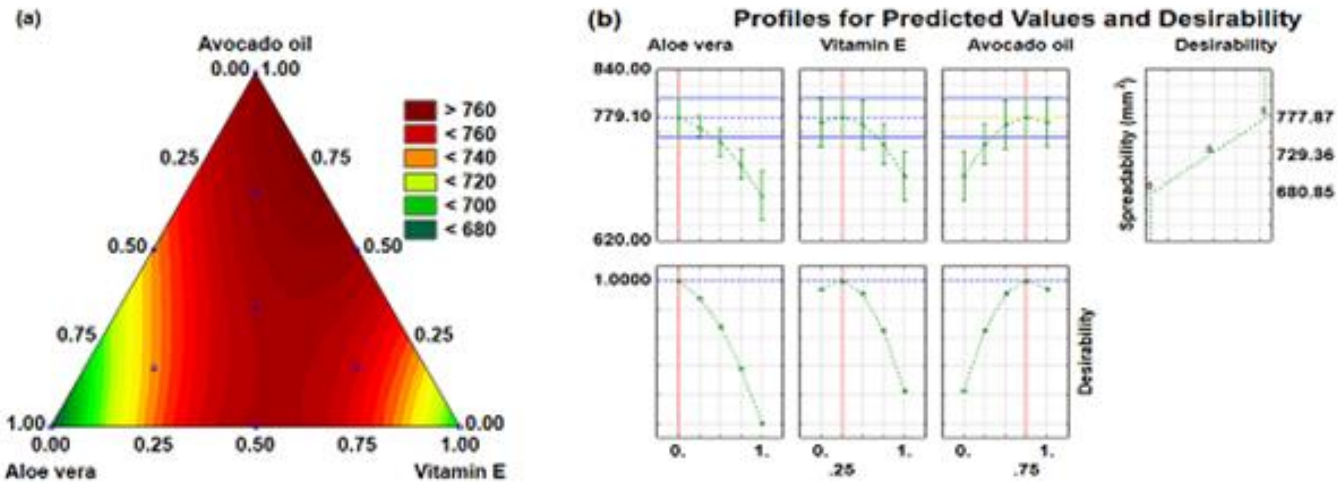

Figure 1. Response surface (a) and optimization plots (b) for the spreadability (mm²) of cosmetics formulations.

Table 2. Estimates of the model parameters for cosmetic formulations spreadability from Simplex Lattice Design.

Factors	**Estimate**	**Standard error**	***T-value***	***p-value***
Aloe vera extract (x_1)	677.83	12.85	52.74	0.000*
Vitamin E (x_2)	702.93	12.85	54.70	0.000*
Avocado oil (x_3)	772.31	12.85	60.10	0.000*
x_1x_2	299.77	64.71	4.63	0.003*
x_1x_3	49.18	64.71	0.76	0.476
x_2x_3	128.66	64.71	1.98	0.093
$x_1x_2x_3$	-159.37	336.98	-0.47	0.652

R^2 = 0.9057; $R^2_{ajusted}$ = 0.8113. Lack-of-fit: p = 0.435. *p<0.05

Aloe vera extract, Vitamin E and avocado oil were significant for spreadability (p<0.05), and showing a positive effect (Table 2). The interaction between Aloe vera and Vitamin E present greater positive effect (299.77) on spreadability of formulation. The ANOVA of the model obtained to describe the spreadability showed determination coefficient (R^2) of 0.90 and it was significant at the 5% significance level (Table 2).

Antioxidant activity

The highest antioxidant activity was obtained using 50% of Aloe vera, 0.0% of vitamin E and 50% of avocado oil, reaching 84% of activity (Table 1, run 5). The antioxidant activity of levan was already been described [18, 19]. So, the combination of levan with plant-derived compounds, can upturn the antioxidant activity of formulation resulting in potent antioxidant formulation. The antioxidant activity of levan produced by *Bacillus subtilis* AF17 was studied by Bouallegue and coauthors [37] that showed the ability of levan in to scavenge free radicals by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, with reducing power. Free radical scavenging increases when levan concentrations ranged from 0.1 mg/mL to 2 mg/mL, reaching between 28% and 58%, respectively. Recently, our researcher group have development a cosmeceutical formulations based on ammonium acryloyldimethyltaurate / VP copolymer with levan at 1% and cinnamon oil at 0.5%, 1% and 2%. The results showed that levan and cinnamon oil exhibited a maximum antioxidant activity of 58% and 21% respectively, at a concentration of 1% for both ingredients [18]. The antioxidant action is important to neutralize free radicals formed in the skin, which are unstable atoms or molecules which disrupt the functioning of the skin layer and cause premature aging. They are responsible for inhibiting and reducing damage caused by free radicals in cells [38]. Consequently, actives that present great antioxidant activity are very desired by the cosmetic industry.

The antioxidant activity of avocado oil may be due to phenolic compounds that may act through radical scavenging by donation of hydrogen atom and chelation of transition metals [38]. Most studies correlated the antioxidant activity of avocado oil to tocopherols, phytosterols, and carotenoids [22]. In the cosmetics industry, avocado oil is used in skincare products, due to its rapid absorption into the skin and sun screening properties [22].

Aloe vera extract is also widely used in the cosmetic, pharmaceutical industries and in the food industry, because it contains antioxidants [39]. Aloe vera extracts hold potent radical-scavenging activity, and several antioxidative compounds have been isolated. The antioxidant activity of a diversity of polyphenol compounds as anthraquinones and chromones has been reported [40, 41]. Aloin A, an anthraquinona, is a pharmacologically active compound obtained from several Aloe species [23].

The response surface and the prolife of predict value and desirability for antioxidant activity are showed in Figure 2. According to the results, the formulation with high antioxidant activity was archiving when 30% of *Aloe vera* extract, 0.0% of vitamin E and 70% of avocado oil was used (Figure 2).

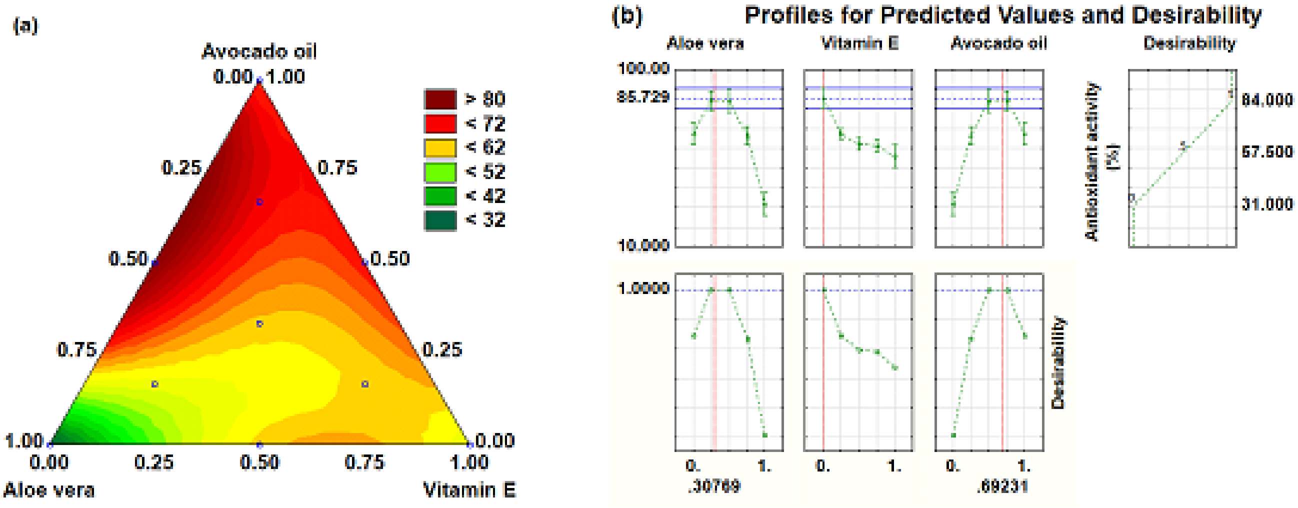

Figure 2. Response surface (a) and optimization plots (b) for the antioxidant activity of cosmetics formulations.

Aloe vera extract, Vitamin E and avocado oil were significant ($p<0.05$), and showed a positive effect for antioxidant activity (Table 3). The interaction between Aloe Vera and avocado oil present greater positive effect (137.91). For antioxidant activity a negative effect for x1x2x3 (-449.34) was detected. This result is in agreement with the negative effect observed for spreadability that also showed a negative effect of interaction of x1, x2 and x3 (Table 2). The ANOVA of the model obtained to describe the antioxidant activity showed determination coefficient (R^2) of 0.97 and it was significant at the 5% significance level (Table 3).

Table 3. Estimates of the model parameters for cosmetic formulations antioxidant activity from Simplex Lattice Design.

Factors	Estimate	Standard error	*t-value*	*p-value*
Aloe vera (x_1)	31.61	2.36	13.34	0.0001*
Vitamin E (x_2)	56.16	2.36	23.70	0.0000*
Avocado oil (x_3)	67.34	2.36	28.41	0.0000*
x_1x_2	67.55	11.93	5.66	0.0013*
x_1x_3	137.91	11.93	11.55	0.0000*
x_2x_3	31.09	11.93	2.59	0.0407*
$x_1x_2x_3$	-449.34	62.14	-7.23	0.0003*

R^2 = 0.9786; $R^2_{adjusted}$ = 0.9572. Lack-of-fit: p = 0.225. *$p<0.05$

Moisture retention capacity

The results showed that the formulation using 100 % of avocado oil showed the best moisture retention capacity (Table 1, run 3), suggesting that this oil can be a promising ingredient for skin hydration. Thus, the combination of avocado oil with levan can result in a highly hydrating formulation. Domżał-Kędzia and coauthors [33] measured the moisture retention capacity of levan from *B. subtilis natto* KB1 and also reached 100%. According to authors, levan has the potential to hold a large amount of water in its hydrogen bonds [34]. Interestingly, the moisture retention ability of levan was superior to glycerol and comparable to hyaluronic acid, indicating the great potential application in cosmetics formulations [36].

The response surface and the prolife of predict value and desirability for moisture retention capacity are showed in Figure 3. According to the results, the formulation with high moisture retention capacity was archiving when 100% of avocado oil was used.

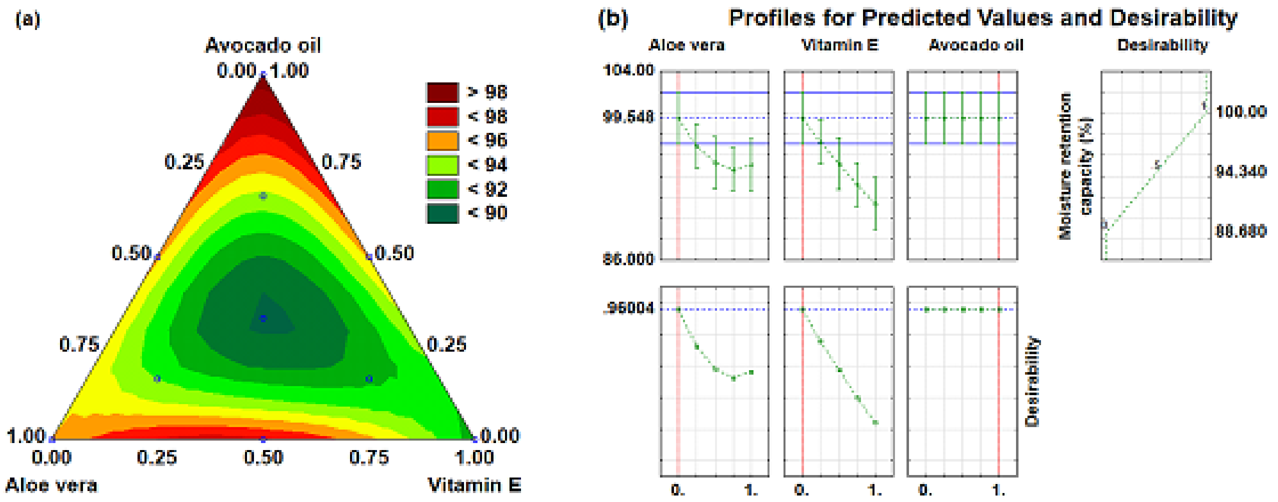

Figure 3. Response surface (a) and optimization plots (b) for the moisture retention capacity of cosmetics formulations.

Aloe vera extract, Vitamin E and avocado oil were significant ($p<0.05$), and showed a positive effect for moisture retention capacity (Table 4), with greater positive effect for $x3$ (avocado oil) this explains 94% of the data obtained. The ANOVA of the model obtained to describe the Moisture retention capacity showed determination coefficient (R^2) of 0.94 and it was significant at the 5% significance level (Table 4).

Table 4. Estimates of the model parameters for moisture retention capacity of cosmetic formulations from Simplex Lattice Design.

Factors	Estimate	Standard error	*t*-value	*p*-value
Aloe vera extract (x_1)	95.57	1.01	93.55	0.0000*
Vitamin E (x_2)	91.41	1.01	89.97	0.0000*
Avocado oil (x_3)	99.54	1.01	97.97	0.0000*
x_1x_2	15.79	5.11	3.08	0.0214*
x_1x_3	-8.11	5.11	-1.58	0.1639
x_2x_3	-1.22	5.11	-0.23	0.8184
$x_1x_2x_3$	-168.56	26.64	-6.32	0.0007*

R^2 = 0.9474; $R^2_{adjusted}$ = 0.8948. Lack-of-fit: p = 0.548. *$p<0.05$

The optimization of spreadability, antioxidant activity and moisture retention of levan-based biocosmetic from Simplex-lattice varying the concentrations of Aloe vera, vitamin E and avocado oil are showed in Figure 4. According to the statistical analyses, the concentrations to obtain a biocosmetic with maximum antioxidant activity (76%), hydration capacity (98.37%) and spreadability (767.30 mm^2), would be 10% (0.1 mL) Aloe vera, 0.0% vitamin E and 90% (0.9 mL) avocado oil.

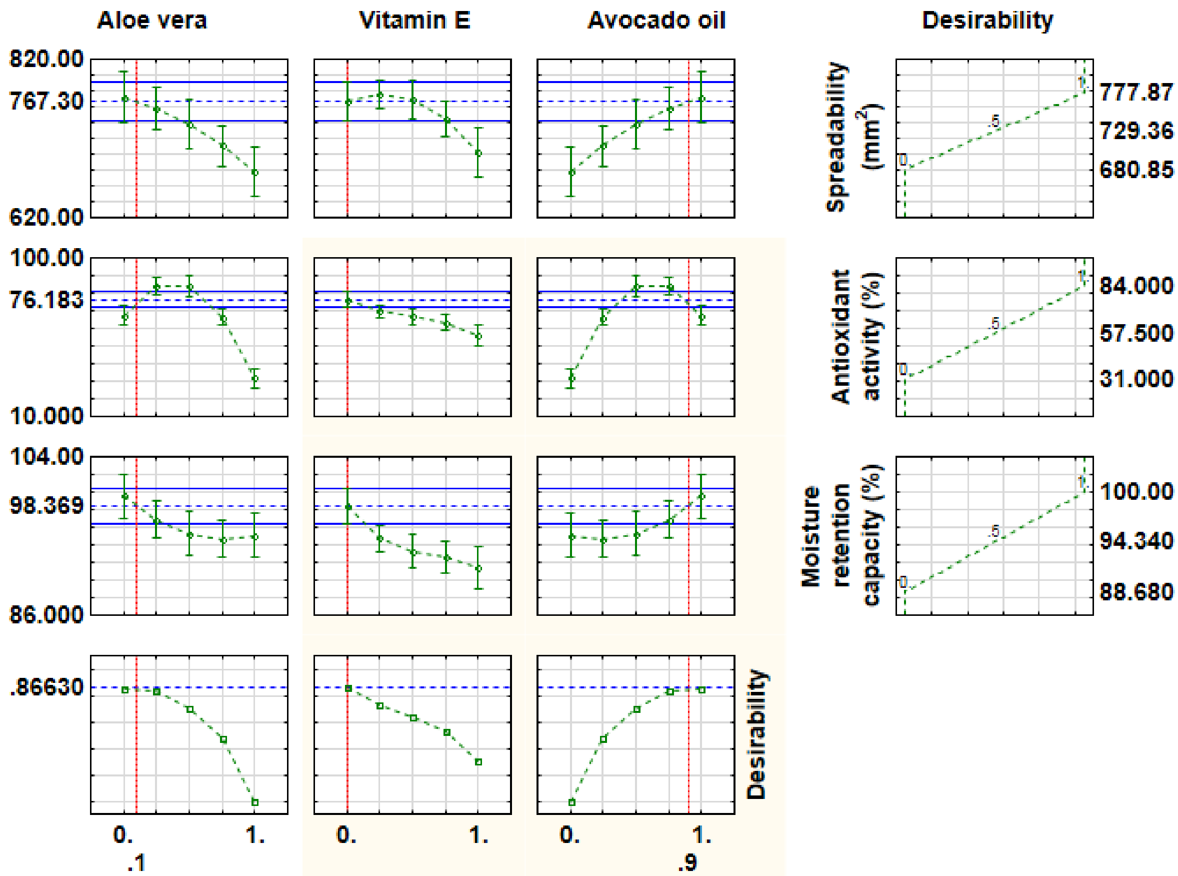

Figure 4. Optimization of spreadability, antioxidant activity and moisture retention of levan-based cosmetic formulations from Simplex-lattice varying the concentrations of Aloe vera, vitamin E and avocado oil.

Avocado oil is an exceptional source of enrichment for dry and damaged skin. Then, many investigations have been directed on the action of topical administration of avocado extract on wound models in rats, showing faster re-epithelization of wounds [42]. Besides, topical application of avocado oil in rats has also been shown to increase of collagen synthesis and decrease of inflammatory cells during the wound healing process [43,44]. Then, avocado is frequently consumed fresh, but the current demand for avocado oil has improved significantly due to its potential use in cosmetic industries, and its beneficial effects on human health.

CONCLUSION

Taken together the results showed the development of a multifunctional cosmetic formulation composed of microbial levan in combination of *Aloe vera* extract and avocado oil with exhibit antioxidant activity, retention capacity and good spreadability. The development of innovative and sustainable cosmetic formulations, with natural, vegan and non-toxic molecules is of great importance for beauty industry that searches for active biocompatible ingredients.

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Conflicts of Interest: The authors declare no conflict of interest.

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