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ARTICLE

A natural broad-spectrum sunscreen formulated from the dried extract of Brazilian *Lippia sericea* as a single UV filter

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H. C. Polonini,[a,b] M. A. F. Brandão[a] and N. R. B. Raposo[a]

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Despite the challenges in producing plant-derived photoprotective products, novel natural, broad-spectrum sunscreens have gained momentum in the cosmetic industry in the past few years. Traditionally, most of these products have been derived from plants producing polyphenolic and flavonoid compounds. In this study, we focused on plants belonging to the genus *Lippia*. The study aimed to develop a natural broad-spectrum sunscreen from a *Lippia* species that could be used as a single UV filter. Initially, UV transmission of sunscreens (10%, w/w) derived from four different *Lippia* species was determined to estimate their sun (UVB) protection factor (SPF). Next, the extract from species showing the best result was subjected to *in vivo* SPF and *in vitro* UVA radiation protection factor (UVAPF) determination using diffuse transmittance spectroscopy. The natural sunscreens obtained *in vitro* SPF values ranging from 1.7 to 7.6. The higher PSF was obtained with *L. sericea* species; the *in vivo* SPF of this sunscreen was 7.5 and its UVAPF was 2.97 when used as a single UV filter in a lotion. The photoprotective activity of this sunscreen was found to be related to the total polyphenolic content of the plant and not to its flavonoid content or antioxidant capacity. Thus, the results of this study suggested that the natural sunscreen from *L. sericea* has the potential to be used commercially.

Introduction

It is well known that ultraviolet radiation (UVR) from the sun triggers skin problems, notably skin cancer and photoageing (Iannacone et al., 2014). It has also been well established that sunscreens are the gold standard treatment for protecting the skin from UVRs. Sunscreens should primarily be used to prevent erythema formation after exposure to the sun (Federman et al., 2014). Seventy-eight percent of non-melanoma skin cancers can be prevented using sunscreens properly (Polonini et al., 2013). Although relatively new, the use of natural products (raw or derived) in sunscreens has been gaining momentum in the cosmetic industry, and studies are ongoing to develop innovative and effective products for patients (Chiari et al., 2014). The correct term here would be a 'rediscovery' of natural products for skin protection because in ancient times, Tibetans, Guyana Indians and other ancient civilizations have used diverse mixtures of plant extracts for cosmetic purposes and protection against solar radiation (Urbach, 2001).

The trend in the world market of using natural products lies in their enormous acceptance by the population and the support by the media who portray them as safe and ecologically and politically acceptable (Amer and Maged, 2009). Natural sunscreens (and natural products in general) are believed to be associated with reduced side effects and less aggressive towards the environment (Che, 2009). Therefore, synthetic UV filters are being increasingly used in smaller amounts and being replaced by natural organic filters that provide broad-spectrum protection (Velasco et al., 2008b).

To date, studies have focussed on plant species known to possess compounds containing chromophores and aromatic rings and compounds with antioxidant properties, particularly polyphenols (Vessechi et al, 2007; Nichols and Katyiar, 2010). In fact, polyphenols are structurally similar to synthetic UV filters (Schroeder and Krutmann, 2010). Moreover, it has been suggested that the primary function of polyphenols is to not prevent herbivory but to protect plant leaves from photo-oxidative damage through their antioxidant activity (Close and McCarthur, 2002).

For this reason, we focussed on the genus *Lippia*, one of the 41 genera belonging to Verbenaceae family that comprises approximately 100 species of herbs, shrubs and small trees and is widely distributed in Brazil (O'Leary et al., 2012). Polyphenolic acids and flavonoids are the main compounds present in *Lippia* species (Pascual et al., 2001). However, to the best of our knowledge, no study has determined the photoprotective activity of extracts from *Lippia* species.

Therefore, the present study aimed to verify the hypothesis that the extracts from four Lippia species (*L. brasiliensis, L. rotundifolia, L. rubella and L. sericea*) can absorb solar radiation at wavelengths within the range of UVR, thus suggesting their use them in preparing innovative natural broad-spectrum sun-care products.

Results and discussion

Raw material quality control

The results of the tests determining the quality of raw material (i.e. loss on drying, water content, residue on ignition and acid-insoluble

ash) are expressed in percentage (w/w; Table 1). Loss on drying was determined using gravimetric method; this method determines the concentration of water as well as other volatile substances. However, volumetric Karl Fischer method is more specific because it determines only the concentration of water (USP, 2014). Plant materials having high water content promote the growth of microbes and plant insects and are prone to degradation by chemical and enzymatic constituents (Hubinger et al., 2009). [This results in the spoilage and wastage of the product and hence affects its shelf life. Both the tests showed similar results for the same species, suggesting that most of the volatile material was water. These data were used to appropriately store the samples. Because the species have approximately 6%–10% water content, it is necessary to store the material judiciously to avoid degradation of the substances present in the material. In this study, material from all the species was stored in airtight containers under temperature (15°C–30°C) and humidity control (40%–70% relative humidity).

Table 1. Results of the raw material quality control tests.

Species	LD (%)	Water (%)	RI (%)	Acid-insoluble ash (%)
L. brasiliensis	9.94	9.43	10,.7	6.54
L. roduntifolia	7.49	7.19	4.80	2.92
L. rubella	8.54	7.15	5.49	3.05
L. sericea	6.98	6.45	4.65	2.88

LD: Loss on drying. RI: Residue on ignition.

The other two parameters were amount of total ash (residue on ignition) and acid-insoluble ash present in the species. The test that determines the residue on ignition aims to quantity the amount of inorganic material such as carbonates; phosphates; chlorides and oxides of silicon, magnesium, calcium, potassium, aluminium, iron and sodium. Acid insoluble ash corresponds to the amount of silica and siliceous material present in the samples. Thus, these two parameters indicate adulteration and/or contamination by foreign materials such as dirt and sand, use of fertilisers and appropriate preparation and storage such as collection and inadequate cleaning of equipment. The ash contents were low, indicating that the extracts were obtained carefully and prudently (Budel et al., 2004), which produced raw materials of appropriate macroscopic purity for use in the study (Table 1).

Total polyphenolic and flavonoids contents and antioxidant activity

The total polyphenolic and flavonoid content is shown in Table 2 (see Supplementary Material for standards curve's ANOVA). Polyphenolic and flavonoid content was assayed because the plants used for determining the photoprotective capacity contain large percentage of polypolyphenolic compounds (and consequently flavonoids because they are a type of polyphenols). The inherent ability of polyphenols and flavonoids to absorb UVRs and prevent their absorption by the skin makes them great components of sunscreens for reducing radiation damage (Nichols and Katiyar, 2009). Flavonoids can reduce oxidative damage caused by UVR, particularly that caused by radiations having short wavelengths between 280 and 315 nm (Burchard et al., 2000), and prevent the formation of reactive oxygen species (ROS) and penetration of UV rays (Agati and Tattini, 2010). This information is supported by the fact that in plants, these molecules are found at sites that are directly exposed to sunlight (such as the cell wall) and where there is production of free radicals. In addition, some flavonoids are found within plant cells, suggesting that they also protect plant DNA from oxidative stress (Feucht et al., 2004).

Large discrepancy was observed between results for *L. sericea* and those for others species . To the best of our knowledge, no other study has quantified polyphenolic and flavonoid compounds in this species. Therefore, because of the uniqueness of the study, the results obtained for this study cannot be compared with those of other studies. However, these results can be compared with those obtained for other species of the same genus. The concentration of polyphenols is 14.8 mg mL^{-1} in *L. javanica* and 14.5 mg mL^{-1} in *L. wilmsii* (Shikanga et al., 2010). The aqueous extract of *L. graveolens* contains 656 mg g^{-1} dry weight of polyphenols (Lecona-Uribe et al., 2006). Studies have shown that the concentration of polyphenols varies depending upon the type of treatment and the plant used, thus making it difficult to compare species.

Table 2. Total polyphenolic and flavonoid contents and antioxidant activity of Lippia species.

Species	TPC (g $100g^{-1}$)	TFC (g $100g^{-1}$)	AA * (IC_{50}, µg mL^{-1})
L. brasiliensis	12.91 ± 0.51	5.17 ± 3.03	14.11
L. rotundifolia	16.56 ± 0.51	7.56 ± 3.02	9.94
L. rubella	12.66 ± 0.51	3.62 ± 3.04	15.96
L. sericea	36.39 ± 0.14	4.27 ± 3.02	18.67

TPC: total polyphenolic content. TFC: total flavonoid content. Results expressed as mean (n=3) ± measurement uncertainty. *ascorbic acid reference substance value = 2.70

Initial analysis of data showed a large difference in the concentrations of phenolic and flavonoid compounds, indicating that the latter is probably not a major class of phenolic compounds in these species. The method used to quantify polyphenolic compounds has high affinity for tannins with high molecular weight because the spectrophotometric reading is performed at a wavelength (λ = 760 nm) absorbed by these compounds (Singleton, 1981). Therefore, it can be suggested that the difference between the results may be explained by the high concentrations of these compounds; for example, hydrolysable tannins and other polyphenols containing a molecule of gallic acid in their structure, especially in *L. sericea*, which presented results above other species.

The correlation between these two groups of substances was negative and weak (Pearson's r = −0.187), suggesting that the amount of flavonoids is not related to the amount of polyphenols in these species. This suggests that the chemical composition of the four species is varied. For example, the concentration of polyphenols in *L. sericea* was almost 3-times higher than that in other species but the concentration of flavonoids was equal to or less than that in other species.

The antioxidant activity of the herbal extracts was expressed as minimum inhibitory concentration 50% (IC_{50}), i.e. the amount of extract required to reduce the oxidising action of DPPH by 50%. IC_{50} values of each species are shown in Table 2. The DPPH method is a quick and simple approach to evaluate antioxidants, and it has been widely used worldwide for screening purpose (Mensor et al., 2001). It is the most employed method in the cosmetic industry to determine the antioxidant capacity of active pharmaceutical ingredients and their products. Although it is a classical method, the signal (absorbance) after the reaction of the radical with the test compound decreases because of factors such as light, oxygen, pH and type of solvent (Ratz-Lyko et al., 2012), which can lead to the overestimation of results. Yet, there is steric inaccessibility and the narrow linear range (absorbance vs. concentration) do not always is adequate for the measurements (Apak et al., 2007). For this reason, alternative *in vitro* methods have been developed such as ferric reducing antioxidant power method, which is based on the reduction

of Fe(III) complex to Fe(II) by 2,4,6-tris(2-pyridyl)-s-triazine (the cosmetic industry uses Fe(III) complex with ferroin); cupric reducing antioxidant capacity method, which is based on the reduction of Cu(II) to Cu(I) and an advantageous method that uses 2,2′-azino-di-(3-etylobenzo-tizoline-6-sulfonate) and expresses the antioxidant activity in terms of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents as Trolox equivalent antioxidant capacity (Ratz-Lyko et al., 2012). Nonetheless, several researches still use the DPPH method to estimate the antioxidant activity of chemical compounds and natural products.

All the species included in this study showed some scavenging activity against DPPH radical. Their IC_{50} values were higher than those of ascorbic acid, indicating lower antioxidant activity than that of pure substance (reference standard). Nevertheless, these results are still greater than those obtained for majority of plant species. For example, Mensor et al. (2001) studied 71 extracts from 16 species of Brazilian plant belonging to five families by using the DPPH method. IC_{50} values of all the species were higher than that of *L. rotundifolia*, and IC_{50} values of only two extracts were comparable to those of four species studied here. On the other hand, the above study showed that ethanol extracts of plant leaves from Verbenaceae family had higher antioxidant capacity (lower IC_{50}) than other plant extracts, thus corroborating the positive results obtained in the present study for the four species belonging to this family.

Flavonoids and polyphenolic compounds reduce the concentration of free radicals. The correlation coefficient (r) for total polyphenolic compounds and the IC_{50} values of the extracts was 0.604 while that for flavonoids was (-0.831), both with 95% confidence. The higher the IC_{50} value, the lower the ability of sequestering substances; therefore, the interpretation of these coefficients must be reversed. In our study, flavonoids showed a strong negative correlation with the IC_{50} value, i.e. higher the flavonoid content, the lower the IC_{50} value and greater the antioxidant capacity of the molecules in the plant extract. These data confirm that both polyphenolic compounds and flavonoids possess scavenging activity, with the latter presenting a higher potential.

The use of the present method can be justified based on the reduction of DPPH, which is positively correlated with the presence of hydroxyl groups in substances present in plant extracts (Mensor et al., 2001). The extracts contain considerable amounts of polyphenolic substances, including flavonoids that posses hydroxyl groups. Polyphenolic compounds act as radical scavengers, metal chelators, reducing agents, hydrogen donors and singlet oxygen quenchers (Elzaawely et al., 2007). Polyphenols comprise large groups of substances that are found in all known vegetal species. These compounds contain one or more aromatic rings linked to one or more hydroxyl groups (Solovchenko, 2010). Flavonoids are a specific group of polyphenols. They contain two aromatic rings joined by three molecules of carbon and one molecule of oxygen, thus closing the heterocyclic ring (Angelo and Jorge, 2007). These substances, mainly flavonoids, possess strong antioxidant properties (Bonina et al, 1996) and can absorb UVR and prevent their absorption (Nichols and Katiyar, 2009). Flavonoids specifically mitigate ROS and penetration of UV rays (Agati and Tattini, 2010).

Evaluation of the antioxidant activity is justified when one wants to study the potential of these compounds for use in sunscreens because UVA radiation exerts phototoxic effect by producing free radicals and ROS that damage DNA, proteins and cell membranes (Herrling et al., 2007). Although the human body is capable of protecting itself through its enzymatic and non-enzymatic natural antioxidants, this capacity is not enough in case of chronic or excessive exposure to UVRs. One of the reasons for the introduction of hexogen antioxidants in sunscreens and cosmetics is to decrease or prevent the damage caused by ROS (Gregoris et al., 2011).

Substances with antioxidant properties are commonly added to sunscreens to provide wider spectrum of protection (González et al., 2008). Therefore, natural products/substances with antioxidant properties are promising candidates for developing novel sunscreens that provide enhanced photoprotection (Reis et al., 2014). There is a growing interest in their use because they provide combined protection against ROS and UVR (Ebrahimzadeh et al., 2014), thus providing products with a high end value (Karim et al., 2014).

In vitro assay to determine the Sun Protection Factor

Sun protection factor (SPF) of the plant extracts was evaluated in topical lotions. This lotion was formulated such that the final product had pseudoplastic/thixotropic characteristics, i.e. reduced viscosity during application onto the skin because of the shear/application of external pressure. This facilitated the application of the product because the product became more fluid when the consumer exerted pressure with his/her fingers while applying it on the skin. Moreover, when the pressure was withdrawn, the lotion recovered the original characteristic viscosity, which prevented dripping once the product was applied on the skin.

The main constituent of the base formulation was acrylates/C10-30 alkyl acrylate crosspolymer (Pemulen® TR1, $C_3H_4O_2$), a water-soluble emulsifier polymer. It is stable even when subjected to repeated cycles of freezing and thawing, which provides long shelf life to the product containing it. Potassium cetyl phosphate (Amphisol® K, $C_{16}H_{34}O_4P.K$) is also an emulsifier whose structure is similar to that of phospholipids present in the human skin. It is primarily used in complex photoprotective cosmetic formulations and topical products. Ceteareth-20 (ethoxylated cetostearyl alcohol) is a non-ionic surfactant containing ethoxylated fatty acids. It has high emulsifying capacity and is ideal for products containing plant extracts. Antaron® WP-660 (copolymer triacontane-polyvinylpyrrolidone) is an excellent film former and confers sunscreens the ability to resist water and moisture. Similarly, silicones are added to sunscreen formulations to generate a waterproof product; in addition, silicones impart softness to the skin.

SPF of lotions containing 10% of the plant extracts was determined *in vitro*. The results of this *in vitro* assay are shown in Table 3. Higher SPF value (~8.0) was obtained for the lotion containing extracts from *L. sericea*.

Table 3. *In vitro* assay to determine the SPF of lotions containing 10% extracts from *Lippia* species.

λ (nm)	**Species**			
	L. brasiliensis	*L. rotundifolia*	*L. rubella*	*L. sericea*
290	0.241	0.192	0.166	1.274
295	0.247	0.180	0.155	1.155
300	0.235	0.178	0.165	0.938
305	0.225	0.175	0.162	0.705
310	0.232	0.182	0.171	0.545
315	0.230	0.186	0.182	0.447
320	0.244	0.197	0.200	0.418
SPF	2.3	2.8	1.7	7.6

Strong positive correlations were observed between *in vitro* SPF and total polyphenolic content (r = 0.970) and antioxidant activity (r = 0.721). However, negative correlation was observed between *in vitro* SPF and flavonoid content (r =

−0.322). These values indicate that at 95% confidence interval, the amount of polyphenolic compounds was correlated with the sunscreen activity determined *in vitro*. For antioxidant activity, the correlation was determined using the IC_{50} value, with sequestering ability inversely proportional to this value (SPF increases with lower IC_{50}).

In the past years, studies have been conducted in Brazil (Souza et al, 2005; Ferrari et al., 2007; Violante et al., 2009) and elsewhere (Kale et al., 2010; Khazaeli and Mehbarani, 2008) to develop sunscreens containing natural products. However, most of these studies did not achieve satisfactory results to suggest the production of innovative products containing plant extracts. Therefore, the results found in this study were considered promising. To confirm whether this SPF value was retained in the *in vivo* study, we selected species with the greatest potential (*L. sericea*) for efficacy trials in humans and determined the UVAPF of the extracts by using diffuse transmittance spectrophotometry.

***In vivo* assay to determine the SPF**

During the study, all the recruited 10 volunteers completed the tests (n_{final} = 10). The characteristics of the skin types of the study participants are shown in Table S6. The individual results of DEM and SPF for protector standard (control) and for natural products are shown in Table S7. The final SPF results are shown in Table 4.

Table 4. *In vivo* assay to determine the SPF of control skin care product and skin care product containing 10% extracts from *L. sericea*.

Sunscreen	$\bar{x}$	*s*	*t*	*A*	$CI_{95\%}$	
					$\bar{x} - A$	$\bar{x} + A$
Control	14.5	2.1	2.26	1.5	13.0	16.0
L. sericea	7.5	1.7	2.26	1.2	6.3	8.7

$\bar{x}$ = average SPF (n=10). *s* = standard deviation. *n* = volunteers number (=10). t = Student's t value. $A = t \times s/\sqrt{n}$. CI = confidence interval.

In vivo SPF assay showed the same results as the *in vitro* assay (8.0) To statistically confirm that the values were similar, non-parametric Mann–Whitney *U* test was used (Shapiro-Wilk normality test: *in vivo* SPF $w_{calculated}$ = 0.900, higher than $w_{critical}$ (v = 10) = 0.842, therefore the data are normally distributed; *in vitro* SPF $w_{calculated}$ = 0.356, below the $w_{critical}$ value (v = 3) = 0.767). By using 95% confidence interval, we found that the distribution of values in the two groups was not statistically different (Mann–Whitney *U* test = 12.0, n_1 = 3, n_2 = 10, *p*-value = 0.606, bilateral). Thus, it was confirmed that the two assays (*in vitro* and *in vivo*) produced statistically similar results. It was also concluded that the *in vitro* assay, in this case, could predict the value for the *in vivo* assay because there were no physical filters in the final product that reflected or scattered the incident radiation. In such cases, the spectrophotometric method described by Mansur et al. (1986) can provide good results.

UVAPF

UVAPF of the lotion containing 10% *L. sericea* extract was determined using the method described by Colipa (2011). Four polymethylmethacrylate plates were used for fitting the acceptance criteria. Figure 1 shows the mean absorbance spectra obtained for all the plates in the pre- and post-irradiation steps. Table S8 shows the diffuse transmittance SPF values, C constant and UVA_0 calculated for individual plates. The C constant was calculated to adjust the *in vivo* SPF value of 7.5 that was determined previously. After irradiating the product plate with a calculated dose of UVA (Figure 1), the final UVAPF was calculated as shown in Table 5. For the final result, the three plates having nearly similar results were used. Thus, the UVAPF was 2.97 ± 0.07 (mean ± standard deviation), with a coefficient of variation of 2.5%, and the average critical wavelength was 375 nm.

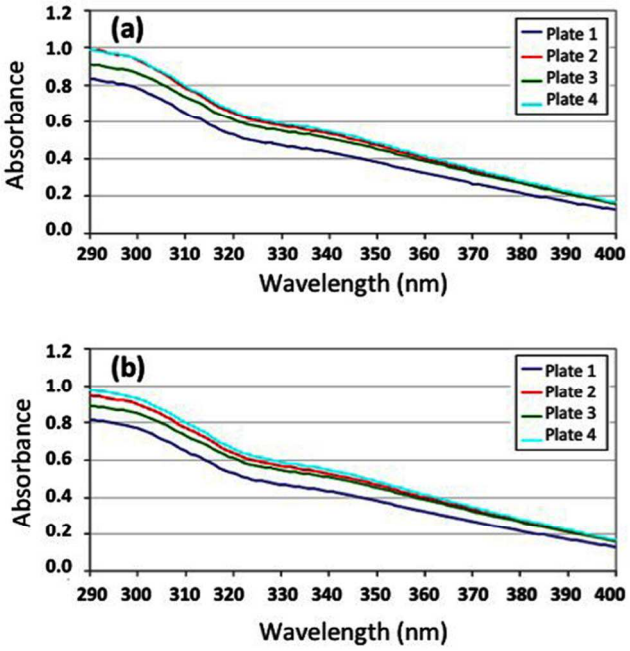

Fig. 1. Mean absorbance spectra for the UVAPF determination: (a) pre- and (b) post-irradiation.

Many studies have focussed on UVR because it has hazardous effects on the human body. It penetrates more deeply into the epidermis and reaches the basal layer, which is responsible for the proliferation of epidermal cells (Delgado et al., 2006). In addition, UVR is responsible for the immediate tanning reaction (immediate pigment darkening reaction); new melanogenesis, a phenomenon related to the oxidation of melanin present in melanocytes, and consequent increase in the synthesis of melanin 48 hours after the exposure (Masnec and Poduje, 2008). It also leads to the formation of direct products of photochemical reactions within DNA (Jiang et al., 2009) and increases the 'network of cytokines induced by UV', which in turn increases the expression of collagenase-1, a metalloproteinase, and loss of interstitial collagen due to photoaging (Honda et al., 2008). A sunscreen having the same SPF but different UVAPF protects differently, with lower values of UVA protection increasing the risk of carcinomas (Seite et al., 2000).

The ideal situation regarding the broad-spectrum protection is a SPF/UVAPF Ratio ≈ 1.0. Our results showed that this ratio in the formulation containing *L. sericea* extract was approximately 2.5. However, plants rich in flavonoids have limited activity at these wavelengths (Choquenet et al., 2008).

Critical wavelength is another parameter for measuring the degree of protection against UVA. It is defined as the wavelength at which integral partof the area under the absorption spectrum of the sample reaches 90% of the total absorption at 290 to 400 nm (Colipa, 2011). Thus, the

protection spectrum is measured (sunscreens with λc values near 400 nm are considered broad spectrum). However, classification of the spectrum can be different according to the adopted reference. For instance, the US Food and Drug Administration updated its classification method in 2011 (FDA, 2011). At present, it considers broad-spectrum test as a qualifying (pass/fail) test based on the critical wavelength value of 370 nm. Under such a classification, extracts from *L. sericea* can be considered a broad-spectrum filter.

Table 5. Final *in vitro* UVAPF, SPF/UVAPF ratio and critical wavelength of the sun care product containing 10% extract of *L. sericea*.

Plate	UVAPF	SPF/UVAPF	λ_c (nm)
1	2.89	2.6	375
2	2.94	2.6	375
3	3.05	2.5	375
4	3.02	2.5	375
Final	2.97	2.6	375

UVAPF: UVA Protection Factor. SPF: Sun Protection Factor. λ_c: Critical wavelength.

The finding that a natural extract can serve as a filter has great potential in the current cosmetic market. To a great extent, commercial sunscreens contain inorganic filters that have a high ability to absorb UVRs (Chiari et al., 2014). However, a trend has been growing in using less amounts of synthetic compounds is cosmetics, improving quality and safety without impairing the performance (Butt and Christensen, 2000). It is still undeniable that these chemicals represent the standard for protection against UVR. To illustrate this, our results for *L. sericea* were compared with those obtained for the same concentrations of synthetic filters approved for use in Europe (Table 6). These data were obtained from BASF Sunscreen Simulator (https://www.sunscreensimulator.basf.com), which is a large and reliable database of sunscreens used worldwide. Some filters cannot be used at 10% concentration; therefore, we focused on the filters that were safe for use at this concentration. Our results for exposure indicated that inorganic filters were in general more effective than natural filters, mostly in terms of SPF. However, in terms of UVAPF and critical wavelength, extracts from *L. sericea* provided results similar to the synthetic filters and in some cases, are better.

Based on the better performance of inorganic-based sunscreens and the current trend of using natural extracts in sunscreens, another trend, i.e. using a combination of these two substances, is gaining momentum (Chiari et al., 2014). In this, the amounts of the chemicals are reduced but the performance is the same and has a 'green appeal'. This can be done via direct combination of active compounds and by including nanotechnology (for example, using phytosomes; Chanchal and Swarnlata, 2008), which provides the opportunity to use a wide range of approaches to increase the capacity of sunscreens to absorb sunlight.

The lack of research on plants from Zona da Mata Mineira justified the study of plant species found in this region and their potential in developing products with commercial applicability. The search for new natural pharmaceuticals has been the focus of the current market to offer products that are safe and environmentally sustainable and have low environmental impact (Velasco et al., 2008b). In addition, we highlight the role of innovative products that use regional raw materials as a tool for local socioeconomic development..

Table 6. Comparison of *Lippia sericea* phtoprotection with inorganic-based filters approved for use in Europe, at the same concentration (10%).

Filter	SPF	UVAPF	λ_c
Natural filter			
L. sericea	7.5	2.9	375
UVB / UVA II filters			
Benzophenone-3 (Uvinul M40)	14.3	3.6	358
Diethylhexyl Butamido Triazone	12.4	1.1	325
Ethylhexyl Methoxycinnamate (Uvinul MC80)	12.4	1.5	337
Ethylhexyl dimethyl PABA	11.9	1.2	328
Homomenthyl Salicylate	4.9*	1.2	336
Isoamyl p-Methoxycinnamate	13.9	1.6	340
Octocrylene (Uvinul N539T)	11.0	2.3	354
Polysilicone-15	5.8*	1.3	337
Titanium Dioxide (nano), water phase	15.1	4.1	376
Tris Biphenyl Triazine (nano), Tinosorb A2B, active	32.4	6.7	374
Broad-spectrum / UVA I filters			
Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine (Tinosorb S Aqua)	22.5	21.4	381
Diethylamino Hydroxybenzoyl Hexyl Benzoate (Uvinul A Plus)	6.9	44.5	379
Disodium Phenyl Dibenzimidazole Tetrasulfonate	11.5	24.9	375
Drometrizole TriSiloxane	12.0	6.7	369
Methylene Bis-Benzotriazolyl Tetramethylbutylphenol (nano), Tinosorb M, active	14.6	18.0	383
Terephthalylidene Dicamphor Sulfonic Acid	12.8	22.1	372

SPF: Sun Protection Factor (*in vivo*). UVAPF: UVA Protection Factor. λ_c: Critical wavelength. *Rating: fail.

Experimental

Plant material

The plants leaves were collected at Estação Experimental de Cultivo e Manutenção de Plantas da Universidade Federal de Juiz de Fora (UFJF), Minas Gerais, Brazil, in june 2010, after three days without rain. They were identified by the botanist Dr Fátima Regina Gonçalves Salimena and their exsiccates were deposited in the Herbarium Leopoldo Krieger - UFJF, with the respective voucher numbers: 56951 (*L. brasiliensis*), 56946 (*L. rotundifolia*), 56942 (*L. rubella*), and 56344 (*L. sericea*). Specimens' leaves (50 g each) were cleaned with water and dried in a circulating air oven at 21±2.0 ºC for four days. Subsequently, the material was ground in a knife mill (TA-2, Metvisa, Brazil) and 5g of each of these materials were used for the assays: loss on drying, water determination, residue on ignition and acid-insoluble ash. The remaining material was macerated in ethanol through static maceration process for three days at room temperature (15g/300mL). The ethanol extracts filtrates were subjected to slowly rotary evaporator (R-114 - Buchi, Switzerland) under reduced pressure at 40°C until the complete removal of the solvent. These extracts were used for the assays: total phenolic content, total flavonoids content and antioxidant activity.

Raw material quality control

The amount of volatile substances in the ground leaves was determined by gravimetric loss on drying. About 1g of the materials were dried in an oven (Quimis, Brazil) for 5 hours at 105°C.

The water determination of 100 mg of the materials was determined by direct volumetric method using a Karl Fischer titration with electrometric endpoint (KF-1000 Analyser, Brazil). The method is based on the quantitative reaction between the water sample and an anhydrous solution of iodine and sulfur dioxide (Karl Fischer reagent) in the presence of methanol.

The determination of non-volatile residual substance after ignition was determined in 3 g of the materials in a porcelain crucible calcined in a muffle furnace at maximum temperature of 450°C (Q-318 521, Quimis, Brazil), so that all organic material has been completely eliminated.

The determination of silica and siliceous constituents in materials (acid-insoluble ash) was conducted using the previous obtained ignited residues. They were boiled for 5 minutes with 25 mL of hydrochloric acid (70 g L^{-1}), filtered, washed with hot water until neutralization of the filtrate, and then ignited at 500°C until constant weight was obtained.

Total phenolic content

The determination of total phenolic content was performed using Folin-Ciocalteu reagent in alkaline medium (saturated sodium carbonate). Gallic acid (Sigma-Aldrich, USA) was used as standard, and its ethanolic solutions (25-700 µg mL^{-1}) were used to built-up a standard curve for determining the extracts phenolic content. The extracts were dissolved in ethanol (10 mg mL^{-1}, n=3). Aliquots of the samples solutions (50 µL) were mixed in test tubes with 250 µL of Folin-Ciocalteu reagent, 500 µL of 20% aqueous sodium carbonate and 4.2 mL of water. The test tubes were incubated protected from light at room temperature for 30 minutes, and then the absorbance of the solutions was read at 760 nm (UV Mini 1240, Shimadzu, Japan).

Total flavonoids content

Rutin (Sigma-Aldrich, USA) was used as standard, and its aqueous solutions (2-30 µg mL^{-1}) were used to built-up a standard curve for determining the extracts flavonoid content. The extracts were dissolved in ethanol (10 mg mL^{-1}, n=3). In conical centrifuge tubes, 2.5 mL of the extract solutions were mixed with 1 mL of chloroform and 1.5 mL of water and then centrifuged for 3 minutes at 2,465 × g, at 25°C (5810 R, Eppendorf, Germany). The microplates were prepared by adding the following solutions, in this order: 99 µL of water; 25 µL of 8% methanolic aluminum chloride hexahydrate solution; 100 mL of pyridine:methanol (2:8, v/v) solution; 6 µL of glacial acetic acid; and 20 µL of the supernatant of the centrifuged solution of the plant extract (or 20 µL of each rutin solution). The plate was brought to stirring in a microplate shaker (SI- 0400, Scientific Industries, USA) for 2 minutes and incubated in the dark for 15 minutes, always capped to prevent evaporation. After incubation, the absorbance was recorded in a microplate spectrophotometric microplate reader (Microplate Reader SpectraCount, Packard, USA) at a fixed wavelength of 405 nm.

Antioxidant activity

The antioxidant activity was evaluated using the 2,2- diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, USA) assay, described by Sreejayan and Rao (1996), with minor modifications. This method is based on the DPPH reduction in the presence of an antioxidant (AH) and a proton donor (H^+) for a non-radical (DPPH-H). An aliquot of 150 µL of a DPPH ethanolic solution of 0.05 mM was added to 50 µL of ethanolic solutions of the extracts at a concentration range of 0.97-250 µg mL^{-1} in 96-well plates. Ascorbic acid was used as a standard, at the same concentrations. Reactions elapsed at room temperature for 30 minutes in the dark and then the absorbance was read in a spectrophotometer (λ= 510 nm). Inhibition of DPPH radical was calculated as $\%_{inhibition} = 100 \times (A_0 - A_S)/A_0$, being Ao absorbance of the negative control and As the absorbance of test samples.

The IC_{50} value was calculated from the line in the equation of the linear dispersion graph. All tests were performed in triplicate.

Natural sunscreens

Sun-care systems were developed by incorporation of the dried extracts of the *Lippia* species through a cold process, with the composition described in Table 6. The following ingredients were used: Acrylates/C10-30 alkyl acrylate cross-polymer (Pemulen® TR1), Potassium cetyl phosphate (Amphisol® K), Ceteareth-20 (Ceteareth® 20), Tricontanyl PVP (Antaron® WP-660), Cyclopentasiloxane (Silicone DC 245®), Dimethicone (and) trimethylsiloxysilicate (Silicone DC 593®), Phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben (Phenova®).

Table 6. Qualitative and quantitative composition of the natural sun-care systems.

Components (INCI)	Composition (%, w/w)
Phase A	
Acrylates/C10-30 alkyl acrylate cross-polymer	20
Potassium cetyl phosphate	0.8
Phase B	
Ceteareth-20	1
Tricontanyl PVP	2
Butylated hydroxytoluene (BHT)	0.05
Cyclopentasiloxane	2
Dimethicone (and) trimethylsiloxysilicate	2
Aditives	
dl-alpha-tocopherol	0.5
Phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben	0.5
2-amino-2-methyl-1-propanol (AMP) solution	0.7
Lippia extract	10
Distilled water, quantity sufficient (qsp)	100

The phases were mixed at 60°C. After emulsification, the base was taken to a mechanical stirrer with propeller (Fisatom, Brazil) to cool (5 minutes at 450 rpm; then 5 min at 780 rpm; then 10 min at 1050 rpm). The alpha-tocopherol and the phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben were only added when the emulsion was at 40 °C. The pH was corrected to 5.8 with the 2-amino-2-methyl-1-propanol (AMP) solution. The base was left to stand for 24 hours and then stored in a refrigerator. The natural sunscreens were obtained by adding the extracts at 10% (w/w) in the emulsions. These sunscreens were used for the sun protection factor (SPF) *in vitro* assay, and the product with best result in this assay was subjected to the *in vivo*

SPF and protection factor against UVA radiation (UVAPF) determinations.

In vitro SPF screening

The *in vitro* determination of the SPF followed a protocol described by Mansur et al. (1986) with modifications (n=3 for each sunscreen). Solutions of the sunscreens were obtained by proper dilutions with ethanol, to a final concentration of 200 µg mL^{-1}. These solutions were read in a spectrophotometer (Cary 50 probe Varian, USA) in the wavelength range of 290-320 nm, with 5-nm increments. The obtained absorbance values were weighted using the values of Table S1. The SPF value was calculated as $SPF_{in\ vitro} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda)$, where CF is the correction factor (=10) and $\sum_{290}^{320} EE(\lambda) \times I(\lambda) \times abs(\lambda)$ is the sum of absorbances at the specified interval, weighted with the values of Table S1.

In vivo SPF determination

The *in vivo* determination of SPF was performed following the European Cosmetic, Toiletry and Perfumery Association (Colipa, 2006) protocol. This study followed The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Ethics Committee of Universidade Federal de Juiz de Fora, Brazil (protocol no. 25/2010).

The study for SPF determination was a randomized, single-blinded, parallel-controlled clinical trial, conducted with 10 volunteers (8 females and 2 males), aged between 27 and 55 years (mean = 41 years). General inclusion criteria were: (i) healthy volunteers; (ii) intact skin in the test region (infra-scapular dorsal region); (iii) agreement to adhere to the procedures and requirements of the test and to attend the institute on certain days and times for evaluations; (iv) writing capacity to consent to participate; (v) age between 18 and 60 years; (vi) volunteers of both sexes; (vii) skin type I, II and III, according to Fitzpatrick scale (Table S2), determined using a colorimeter (MiniScan XE Plus, Hunter Lab, USA) (Chardon et al., 1991). General exclusion criteria were: (i) pregnancy or lactation; (ii) cutaneous pathology in the area of application of the product; (iii) diabetes mellitus type I or gestational complications (retinopathy, nephropathy, neuropathy); (iv) use of insulin; (v) presence of skin diseases related to diabetes mellitus (plantar ulcer, necrobiosis lipoidica, granuloma annulare, dermatophytosis, deep mycoses, bacterial infections, opportunistic infections); (vi) history of hypoglycemia, diabetic ketoacidosis and/or hyperosmolar coma; (vii) immunological failure; (viii) use of systemic corticosteroids or immunosuppressants; (ix) skin diseases: psoriasis, vitiligo, lupus, atopic dermatitis; (x) history of reaction to the category of the product tested; (xi) other diseases or medications that may interfere directly in the study or endanger the health of the volunteer; (xii) history of phototoxic or photoallergic reactions; (xiii) personal or family history of skin cancer; (xiv) presence of sunburn, tan, uneven skin tone, blemishes, moles, seborrheic keratosis or hair excess on the test site. In addition to these inclusion and exclusion criteria, during the study the volunteers were allowed to: (i) use any product on the body, except in the test region; and (ii) wash the irradiated sites with lukewarm water, without rubbing. On the other hand, they were not allowed to: (i) expose the test region to the sun; (ii) scratch or rub the irradiated sites; and (iii) use anti-inflammatory agents.

With the volunteer lying on a stretcher, two areas measuring 30 cm^2 each were staked in his back. In one of the areas the natural sunscreen was applied, and the control (formulation available in Table S3) in the other, both in quantity of 60 ± 1.5 mg to satisfy the amount of 2.0 mg cm^{-2}, spread evenly with the aid of a finger cot. After a waiting period of 15 to 30 minutes, the two treated areas and a third area of bare skin were irradiated, using a solar simulator with a xenon arc lamp and six outputs of independent radiation (Multiport 601, Solar Light Company, USA). Six doses series of UV radiation were employed, with a 25% increment between each dose. Doses were focused on expected Minimal Erithema Dose (MED) values, according to previously measures, and calculated as $D = 1.25^n \times MED_{prov} \times SPF$, where D = erythema-effective UV dose irradiated, n = integers 2, 1, 0, -1, -2 and -3 to the doses from 1 to 6, respectively; MED_{prov} = provisional individual MED, previously determined; SPF = theoretical SPF of the tested products.

Over a period of 16 to 24 hours after irradiation, the erythema formed was evaluated. The MED was defined as the lowest dose of irradiated UV rays able to produce a minimally perceptible and unambiguous erythema response. The individual SPF (SPF_i) value was determined as the ratio of the MED on product protected skin (MEDp) to the MED, on unprotected skin (MEDu) of the same subject: $SPF_i = MED_{pi}/MED_{ui}$. The SPF for the product was defined as the arithmetic mean of all valid individual SPF_i values obtained from all subjects in the test, expressed to one decimal place.

UVAPF Determination

The *in vitro* determination of UVAPF was performed using diffuse transmittance spectroscopy, and following the European Cosmetic, Toiletry and Perfumery Association (Colipa, 2011) protocol.

The samples were directly weighed on polymethylmethacrylate (PMMA) plates (1.3 mg cm^{-2}) and spread in an even manner on their roughened surface. The plates were kept protected from light exposure in a dark chamber at room temperature (≈ 20ºC) for 15 minutes. The blank was prepared using the HD6 plates covered with 15 µL of glycerin. After this period, they were placed in the light-path of a UV-2000S Ultraviolet Transmittance Analyzer (Labsphere, USA). The transmission of UV radiation through the sample was measured from 290 to 450 nm at 1 nm intervals on 9 different sites of each plate. After that, the plates were UV-irradiated and then new transmission measurements were conducted for acquisition of the second UV spectrum; the final UVAPF, the UVA/UVB Ratio and the Critical Wavelength (λc) were calculated. Theoretical background and detailed protocol can be found in Supplementary Material.

The verification of the validity of the results was obtained using the Cosmetics Europe Reference Sunscreen S2 (determined SPF = 18 ± 1.5, UVAPF = 12 ± 1.1, λ_c = 381 nm, and UVA/UVB ratio = 0.88). All results were expressed as a mean of 27 determinations (3 plates, 9 readings each, at different sites).

Data analysis

Descriptive analyzes of the data were performed using position measurements (average ± standard deviation) for the assyas of total phenolic and total flavonoids contents, *in vitro* SPF and UVAPF. For the first two tests, analysis of variance (ANOVA) of the standard curves were also performed to verify the validity of the regression and adjusting the statistical model and the measurement uncertainty were also calculated (equations available as Supplementary Material) (Ellison et al., 2000). For the *in vivo* SPF, the confidence interval was also determined as $CI_{95\%} = \bar{x} \pm A$, being $A = t \times s/\sqrt{n}$, $IC_{95\%}$ = lower and upper limits of the 95% confidence interval, n = number of measurements, s = standard-deviation, and t = value of Student's t distribution, bilateral, for "n - 1" degrees of freedom and 95% confidence. For the study to be considered valid, the average FPS should be in the range of expected values and the value of A could not exceed 17% of the average SPF, both for the product and for the control.

The correlation between the assays was evaluated by calculating the Pearson's coefficient of correlation, using the Statistical Package for Social Sciences (SPSS) version 13.0. The calculated coefficients (r) between 0 and 0.3 were considered to indicate a poor correlation;

between 0.3 and 0.7, moderate correlation; and between 0.7 and 1.0, strong correlation. The tests were conducted at the 95% level of confidence. For the species subjected to the *in vivo* study, the relationship between the result and the *in vitro* SPF was obtained by statistical nonparametric Mann-Whitney test, using the same software (limit of significance $p < 0.05$).

Conclusions

Extracts from the genus *Lippia*, notably *L. sericea*, showed interesting photoprotection properties. Incorporation of extracts from this species in a cosmetic lotion base produced a novel sunscreen containing natural ingredients having proven effectiveness in lieu of or in addition to synthetic actives.

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Notes and references

[a] Núcleo de Pesquisa e Inovação em Ciências da Saúde (NUPICS), Universidade Federal de Juiz de Fora, Campus Universitário, 36036-900, Juiz de Fora, MG, Brazil.

[b] Chemical and Food Engineering Department, Federal University of Santa Catarina, Florianópolis, 88040-090, SC, Brazil.

Electronic Supplementary Information (ESI) available: Tables, Theoretical background for UVAPF and uncertainty of measurement, and supplementary results. See DOI: 10.1039/b000000x/

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