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Research Article

OREOCALLIS GRANDIFLORA PHOTOPROTECTIVE EFFECT AGAINST ULTRAVIOLET B RADIATION-INDUCED CELL DEATH

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ABSTRACT

Objective: The aim of this research was to evaluate the photoprotective effect of *Oreocallis grandiflora* hydroalcoholic extract (OGHE) against ultraviolet (UV)B-induced cell death model on a strain of *Escherichia coli* (ATCC 25922) and to determine the sun protection factor (SPF) using the equation proposed by Mansur.

Methods: OGHE was obtained from leaves of *O. grandiflora*, following a standardized methodology. In short, *O. grandiflora* leaves were extracted with ethanol 70% v/v and defatted with n-hexane, hydroalcoholic fraction was concentrated under controlled conditions through rotary evaporator, and finally, the residue was freeze drying to obtain OGHE. The photoprotective effect was carried out using *in vitro* UVB-induced cell death model on a strain of *E. coli* (ATCC 25922), like a first approach to study its potential application on cosmetics.

Results and Conclusions: From results, *O. grandiflora* is an important resource to produce new cosmetic products. However, the safety of OGHE is necessary to a rational development in that sense. OGHE shows advantages in relation to conventional active compounds of commercial sunscreens used in this research (2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino)benzoate) at the concentration of 2 mg/mL, on survivor time (with OGHE until 120 min), range of inactivation of *E. coli* caused by UVB (OGHE K value minor against to positive controls), and high SPF (13.56±0.21).

Keywords: Photoprotective, Ultraviolet-B, *Oreocallis grandiflora*, Ultraviolet-B-induced cell death, *Escherichia coli*.

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INTRODUCTION

Throughout the first decade of the 21st century, a large number of scientific papers and reports have been published on the impact of climate change on human health and specifically on the skin. The impact of stratospheric ozone depletion on human health has also been independently investigated. However, only recent research is being done on how climate change is affecting the recovery of the ozone layer and its environmental and public health implications [1].

The maximum solar ultraviolet (UV) exposure index (IUV) for humans, according to the World Health Organization, is 11 IUV. On the Ecuador, the Ecuadorian Civil Space Agency, through its Hyperion report, indicated that at Guayaquil and Quito cities at noon this index averages 14 and 24 IUV, respectively, such values are alarming because there is an increased risk of skin and ocular lesions [2].

The harmful effects of solar radiation are caused predominantly by the UV region of the electromagnetic spectrum, which can be divided in three regions: UVA - from 320 to 400 nm; UVB - from 290 to 320 nm; and UVC - from 200 to 290 nm. UVC radiation is filtered by the atmosphere before reaching earth. UVB radiation is not completely filtered out by the ozone layer and is responsible for the damage due to sunburn. UVA radiation reaches the deeper layers of the epidermis and dermis and provokes the premature aging of the skin. Oxidative stress and inflammatory responses induced by UV radiation can cause a variety of harmful effects in skin, including premature photoaging and the induction of immunosuppression and skin carcinogenesis [3], sun radiation, and specificity. Its components UVA (320–400 nm) and UVB (290–320 nm) have been described as skin damage agents irreversible [4,5].

The necessity to provide high sun protection factor (SPF) and screening efficiency against both UVA and UVB wavelengths have led to the development of sunscreen formulations with multiple added sunscreen chemicals. Plants produce a variety of antioxidants against molecular damage from reactive oxygen species, and phenolic composes the major class of plant-derived antioxidants. Among the various phenolic compounds, the flavonoids are perhaps the most important group. Besides scavenging UV-induced radicals, flavonoids might provide their protective effect against UV radiation by acting as strong UV-absorbing screens [3].

The Proteaceae family is one of the most prominent families of the southern hemisphere and consists of 79 genera and over 1700 species. It has a main diversity center in Australia and secondary centers in South Africa, New Caledonia, Southeast Asia, Madagascar, New Zealand, and South America. This family, apparently of tropical origin, is characterized by a wide spectrum of variation in vegetative morphology (to which the name "Proteaceae" refers, from the Greek god Proteus, who changed his shape). However, the classification of genera in this family is based, as traditional in vascular plants, on the structure of flowers and fruits. According to a latest revision of this family, eight genera of Proteaceae can be found in South America, all within the subfamily Grevilleoideae Engl.; six of them are endemic to South America and the other two occur also in Australia and Tasmania [6].

Oreocallis grandiflora is vegetal species belonging to the family Proteaceae, and it is native of Ecuador. The common names which are known at Ecuador are as follows: Cucharillo (at Loja and Zamora provinces), cucharilla (along to the Sierra region of Ecuador), gañal (at Bolívar province), and algil (at Chimborazo province). This plant is widely used for gastric ulcer and liver (complaints), injuries, and kidneys in association with *Equisetum* sp. by the indigenous communities of Ecuador, in accordance to the information of useful plants of Ecuador compendium made by Rios *et al.* 2007 [7], and

also, according to ethnobotanical information, it has been established that it may be useful to treat: Hernias, liver diseases, intestinal tract infections, cholesterol, nephritis, diabetes, gastric ulcer, inflammations, eye problems, and influenza [8]. Another studies mention its usefulness in the treatment against flu, strong sensation of cold, after hard work in the sun, liver problems, bath against cold [9] vaginal bleeding and ovary/uterus inflammation [10]. In addition, Ecuadorian central and western indigenous communities (they situated at high altitude 2700–3700 meters above sea level) use O. grandiflora to treat sunstroke after their agricultural work at the farm fields under the sun exposure and for diuretic purposes.

The aim of this research was to evaluate photoprotective activity and solar protection factor (FPS) in vitro of *Oreocallis grandiflora* hydroalcoholic extract (OGHE), as a way of approaching its potential utility as a photo protector, to rescue and validate the ancestral wisdom of the indigenous communities.

METHODS

Plant material

O. grandiflora sample was collected in Ecuador, Chimborazo province, Colta town, sector S 01° 49' 0.012'' W 078° 52' 0.12'', at 3602 m above sea level. The plant material was taxonomically identified by the botanist Jorge Caranqui at Escuela Superior Politécnica de Chimborazo, and a specimen was deposited at Herbarium. *O. grandiflora* leaves were collected, dried at 50°C in a forced convection oven for 24 h, and it was ground in a knife mill until the particle size of 2–3 mm.

Reagents

Absolute ethanol Ph. Eur., USP grade, was purchased from Pan Reac Appli Chem (ESP), Mueller-Hinton agar was purchased from Himedia Laboratories (IND), Heart-brain broth Bioxon BD (USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Aldrich S.L. (USA), and deionized water was used in all experimental procedures. All other reagents were of analytical or high-performance liquid chromatography grade as appropriate.

Equipment

Heidolph Hei-VAP advantage rotary evaporator (Germany), MicroModulyo Freeze dryer - 1.5 L thermo (USA), Branson 2510DTH Sonicator, Vortex Gemmy VM300 (Taiwan), Thermo scientific evolution 201 UV-visible spectrophotometer, UVB Philips Broadband TL 40w/12 RS Lamp, and standard quartz cuvette B0631009, thickness 1 mm, capacity 4 mL, Perkin Elmer (USA) were used.

Hydroalcoholic extract obtention

Ultrasound-assisted extraction is a rapid technique that can also be used with mixtures of immiscible solvents. The hexane phase concentrated less polar compounds, while the aqueous alcohol phase concentrated polar flavonoids [11].

OGHE was obtained from leaves following a standardized methodology [12]. Ten grams of aerial parts of O. *grandiflora* were extracted with ethanol 70% v/v, sonicated for 15 min, followed by vortex for 15 min, and finally, defatted with n-hexane. Hydroalcoholic fraction was concentrated under controlled conditions (50°C, −0.5 bar) in a rotary evaporator, and finally, the aqueous residue was freeze drying to obtain OGHE.

Preliminary phytochemical screening test for *O. grandiflora*

Phytochemical screening of the plant extract was carried out to investigate the presence of secondary metabolites such as flavonoids, terpenoids, saponins, tannins, and phenols using standard procedure [13].

Total flavonoids determination

Total flavonoids were measured by a colorimetric assay based on the ability of aluminum chloride to react with flavonoids and develop a colored complex. 50 µL aliquot of the diluted sample (OGHE) or standard solution of quercetin (20, 40, 60, 80, and 100 mg/L) was added to a 10 mL volumetric flask containing 4 mL of H_2O. At zero time, 0.3 mL of $NaNO_2$ (5%, w/w) was added to the flask. After 5 min, 0.3 mL $AlCl_3$ (10% w/w) was added. At 6 min, 2 mL of NaOH (1M) was added to the mixture. Immediately, the reaction flask was diluted to volume with the addition of 2.4 mL of H_2O and thoroughly mixed. The absorbance of the mixture, characterized by a pink color, was determined at 510 nm compared to a water control [14]. Total flavonoids were expressed as mg quercetin equivalents (QE)/100 g of plant. The assay was performed in triplicate. For quercetin, calibration curve of absorbance versus concentration (20, 40, 60, 80, and 100 µg/mL) was described by following equation, which had a correlation coefficient value of R^2=0.995.

$$A = 0.001C+0.0015 \quad (1)$$

Total phenolics determination

Total phenol content was determined using the phenol reagent, with few modifications in the sample volume. 2 mL aliquot of an adequate dilution of OGHE was assayed with 500 µL of phenol reagent and 500 µL of aqueous sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 mL. After incubation for 30 min at room temperature, the absorbance was measured at 765 nm [14]. Total phenols were expressed as mg gallic acid equivalents (GAE)/100 g of plant, using a calibration curve of a freshly prepared gallic acid solution. For gallic acid, the calibration curve of absorbance versus concentration was described by following equation, which had a correlation coefficient value of R^2=0.9991.

$$A = 0.0512C+0.0286 \quad (2)$$

Scavenging of DPPH$^{\bullet}$

Solutions of OGHE in ethanol 70% v/v were prepared at concentrations in the range of 10–1000 µg/mL. 100 µL of each concentration was mixed with 2.9 mL of 60µM DPPH$^{\bullet}$ in methanol, at room temperature and in the dark. The samples were kept in the dark for 30 min, and only after that the absorbance was measured at 515 nm in UV-visible spectrophotometer [15]. The blank solution was composed by methanol. The negative control solution was prepared by mixing 2.9 mL of 60 µM DPPH solution with 100 µL of ethanol 70% v/v. Similar solutions in the same medium (ethanol 70% v/v) of gallic acid in the range of 10–100 µg/mL and quercetin in range of 1–250 µg/mL were prepared and tested for scavenging activity.

The experiments were repeated 3 times to confirm the reproducibility of the data. The antioxidant activity was expressed as the percentage of DPPH radical inhibition. The EC_{50} was calculated by means of logarithmic regression of the curves obtained by plotting the results of percentage the DPPH inhibition. On these plots, the abscissa represents the concentration of OGHE, and the ordinate represents the antioxidant activity [16].

In vitro UVB photoprotection

The evaluation of the *in vitro* UVB photoprotection was carried using the spectrophotometric method, according to the Mansur equation [17], to calculate SPF and relationship between the erythematogenic effect and the intensity of the radiation at each wavelength. The stock solutions of OGHE, 2-ethylhexyl 4-methoxycinnamate, and 2-ethylhexyl 4-(dimethylamino)benzoate (10 µg/mL in ethanol 70% v/v, of each one) were prepared to investigate the SPF profile. These solutions were reading in triplicate on UV-visible spectrophotometer. The readings were made at 290–320 nm [16] and ethanol 70% v/v was used as blank.

$$FPS = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad (3)$$

Where CF is a correction factor (equal to 10); EE (λ) erythematous effect of radiation of wavelength λ; I (λ) = intensity of sunlight at wavelength

λ; Abs (λ) = spectrophotometric absorbance reading for preparing the solution at a wavelength (λ) [16]. The values of EE×I are constant (Table 1) [18].

Photoprotection using a bacterial model

A strain of *Escherichia coli* (ATCC 25922) was grown in heart–brain broth until the culture reached a concentration of 10^7–10^8 cells/mL (O.D.= 0.3 read at 550 nm). The bacteria were centrifuged 10 min at 6000 rev/min, suspended in Ringer PBS (pH 7.0), and transferred into quartz cuvettes. OGHE was dissolved in ethanol 70% v/v (2 mg/mL) and placed in a quartz cuvette. A cuvette containing bacteria was placed behind the cuvette containing the photoprotective substance, thus forming one experimental unit. The experimental units were irradiated with a UVB lamp (290–315 nm, Philips TL 40W/12 RS SLV/25 UVB Broadband, AT) and an irradiation dose of 0.60 J/cm^2. The number of surviving bacteria was detected in accordance with the dilution method at different time periods. The positive controls were 2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino) benzoate (2 mg/mL in ethanol 70% v/v), and the negative control was ethanol 70% v/v. The photoprotective activities of all substances were evaluated in parallel. Tests were repeated in three independent experiments, and the assays were performed in triplicate. The results are expressed by plotting the logarithm of the number of survivors versus the irradiation time. The mortality rate (K) was calculated by linear regression analysis [19].

Statistical analysis

All values are expressed as mean±SD. A value of $p<0.05$ was considered to indicate a significant difference. ANOVA and Tukey's test were performed to establish significant differences for multiple comparison and Student's *t*-test was used to compare control group and the individual better performance treatment. The EC_{50} was calculated by means of logarithmic regression.

RESULTS AND DISCUSSION

The plant material was subjected to successive extraction with three solvents of increasing polarity (diethyl ether, ethanol, and water), obtaining three subextracts, on which tests of identification of relevant phytochemical groups were carried out. According to the results of phytochemical screening, the presence of a moderate amount of triterpenes and steroids, as well as relatively small amounts of oils, lactones, and coumarins, was evidenced in the ethereal subextract. At the ethanolic subextract was remarkable the presence of a large number of phenolic compounds, mainly flavonoids and anthocyanins; in addition, moderate amounts of triterpenes, steroids, reducing sugars, saponins, and a low content of catechins and quinones were verified. The aqueous extract showed the presence of phenolic compounds, especially flavonoids.

Based on a literature search carried out at SciFinder®, three important references were found associated with *O. grandiflora*, which deal with various topics such as: The impact of nectar theft on the behavior of pollinators and the reproduction of *O. grandiflora* [20], dating phylogenetically basal eudicots using rbcL sequences and multiple fossil reference points [21], and an invention patent named "Proteins and nucleotide sequences of proteins having β-glucosidase activity and uses thereof" [22]. At the present time, there are no published phytochemical studies of *O. grandiflora*. However, research on *Grevillea robusta* shows that the species of the family Proteaceae possesses large amounts of flavonoids [23], a fact that agrees with the results obtained for *O. grandiflora*. Flavonoids are related to possess antioxidant, anti-inflammatory, immunomodulatory, anticancer, and especially, photoprotective properties against UVB solar radiation [24].

From 10 g of dried and ground plant drug, 2.1592 g of OGHE was obtained as a homogeneous fine powder of yellow color. The extraction yield corresponds to 21.59%. The total flavonoid content expressed like QE/100 g of OGHE was 53.57% ± 2.47 and expressed like QE/100 g of plant corresponding to 11.57%±0.941. The total phenolics content of OGHE expressed like GAE/100 g of the plant was 13.969%±0.312. From these results is evident the high amount of flavonoids content in the OGHE, corresponding to approximately at more than 50% with respect to the dry weight of the OGHE.

The evaluation of the ability of OGHE to sequestrate the DPPH• radical is described in Table 2. The quercetin (a flavonoid) and gallic acid were used as positive controls (Tables 3 and 4) on the test. The control substances (quercetin and gallic acid) are enable to sequestrate 88.38% and 84.70% of the DPPH• radical formed at the concentrations of 250 μg/mL and 100 μg/mL, respectively. The result from quercetin is comparable to that obtained in the study by Silva *et al.*, [16], with the difference that flavonoid rutin (90.01%±1.393) was used as a reference in that study. The OGHE sequestrated 89.10% at the concentration of 1000 μg/mL. The EC_{50} found in OGHE was 292.37±9.37 μg/mL value that is greater than those found in *Malpighia glabra*, L. hydroalcoholic extract (308.07±0.75 μg/mL) [25], but not in the EC_{50} values obtained from the quercetin and gallic acid positive controls, which were 89.98±2.75 μg/mL and 35.19±0.11 μg/mL, respectively.

Table 1: Relationship between the erythematogenic effect and the intensity of the radiation at each wavelength (16)

λ (nm)	EE(λ) × I (λ)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180
∑	1.0000

Table 2: Antioxidant activity of OGHE

Concentration (μg/mL)	Inhibition (%)
OGHE	
10	3.04±0.085
50	10.90±0.096
100	18.27±0.676
200	29.01±0.576
500	58.29±0.916
1000	89.10±1.153

EC_{50}=292.37±9.37 μg/mL, OGHE: *Oreocallis grandiflora* hydroalcoholic extract

Table 3: Antioxidant activity of gallic acid

Concentration (μg/mL)	Inhibition (%)
GA	
10	19.71±0.513
20	27.96±0.914
40	36.94±0.754
60	70.91±0.668
80	77.96±0.777
100	84.70±0.711

EC_{50}=35.19±0.11 μg/mL, GA: Gallic acid

Table 4: Antioxidant activity of quercetin

Concentration (μg/mL)	Inhibition (%)
Quercetin	
1	9.58±0.365
10	10.99±0.241
25	15.69±0.594
50	23.08±0.459
125	54.01±1.113
250	88.38±0.717

EC_{50}=89.98±2.75 μg/mL

The traditional point of view based on correlating the content of phenols and flavonoids with antioxidant activity is a fact that should be investigated rigorously. However, some authors have advanced in suggesting that the characterization of the active compound structure is important and necessary since the locations and amounts of hydroxyl groups can influence the effectiveness of the antiradical capacity [16].

In vitro sun protection factors (SPF) were determined by replacement of absorbance values from each sample on Mansur equation [16] of OGHE,2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino) benzoate. The summation of all partial values at each wavelength (λ) corresponds to FPS value associated to OGHE, 2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino) benzoate against UVB radiation. The results are summarized in Table 5.

The *in vitro* SPF values corresponding to the positive controls obtained in this assay are very close to those found in the literature in which FPS values of 5.48±0.62 and 12.09±1.20 are reported to 2-ethylhexyl 4-(dimethylamino)benzoate and 2-ethylhexyl 4-methoxycinnamate, respectively [26].

A SPF value for a sunscreen above 2 is considered as having good sunscreen activity [18]. According to the European Cosmetic and Perfumery Association (COLIPA) classification [27], OGHE at 10 ppm provides a "high" SPF, which is relatively better than the SPF average provided from the positive controls at the same concentration. According to the above and statistical analysis performed (ANOVA and Tukey test), the photoprotective capacity of the compounds present at *O. grandiflora* leaves exceeds to the UVB chemical filters photoprotective capacity ($p<0.05$) which were used on conventional sun protection products.

Table 5: *In vitro* SPF

SPF	
O. grandiflora hydroalcoholic extract	13.56±0.21
2-ethylhexyl 4-methoxycinnamate	11.82±0.11
2-ethylhexyl 4-(dimethylamino) benzoate	6.21±0.15

SPF: Sun protection factor, *O. grandiflora: Oreocallis grandiflora*

Comparing the *in vitro* SPF of OGHE to similar studies performed plant species, it is confirmed that with a lower concentration (10 ppm) OGHE exhibits a higher SPF and even better antioxidant activity with respect to other species [3].

Experiments investigating the protective effect against UVB-induced cell death were evaluated using *E. coli* as a cell model [19]. Results are shown in Fig. 1.

The results showed that the OGHE possesses a pronounced photoprotective activity compared to the negative control. According to results, the bacterial population (1.24×10^8 cells/mL) without protection reached cell death at 4.9 min approximately that is comparable to that obtained by García-Bores *et al.* [19], with a mortality rate (K) of −1.3393. The bacterial population protected by the OGHE did not reach cell death until 120 min of irradiation with UVB and had a mortality rate of K=−0.0552.

K indicates the range of inactivation of *E. coli* caused by UVB. The exposure of a bacterial culture to UVB produces a rapid decline in the population due to damage to the DNA [19]. The K and time to death of the population treated with the OGHE were better than those obtained to 2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino) benzoate, the active compounds of commercial sunscreens (90 min; K=−0.0689 and 90 min; K=−0.0742). Likewise, under the experimental conditions, the maximum time in which surviving bacteria could be verified due to the protective action of these conventional photoprotectors was 90 min. The OGHE K (−0.0552) was 25.22-fold below the K without protection (−1.3393).

CONCLUSION

From results, *O. grandiflora* could be an important resource to produce

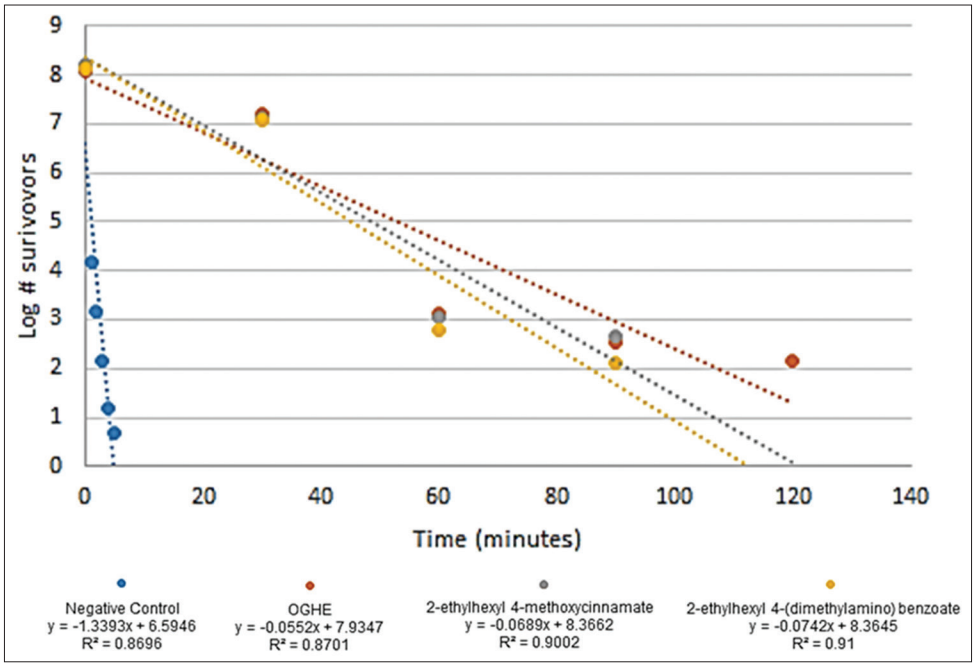

Fig. 1: Protective effect against ultraviolet (UV)B-induced cell death of *Escherichia coli*. Ethanol 70% v/v: Negative control without protection (K=−1.3393, R^2=0.87); with protection 2-ethylhexyl 4-methoxycinnamate: (K=−0.0689, R2=0.90), 2-ethylhexyl 4-(dimethylamino)benzoate: (K=−0.0742, R^2=0.91), hydroalcoholic extract of *Oreocallis grandiflora* (K=−0.0552, R^2=0.87). Where: y - Log of bacteria survivors, x - exposition time of UVR, K - mortality rate, and R^2 - coefficient of determination

new cosmetic products. However, the safety of OGHE is necessary to a rational development in that sense. OGHE shows advantages in relation to conventional active compounds of commercial sunscreens used in this research (2-ethylhexyl 4-methoxycinnamate and 2-ethylhexyl 4-(dimethylamino) benzoate) at the concentration of 2 mg/mL, on survivor time (with OGHE until 120 min), range of inactivation of *E. coli* caused by UVB (OGHE K value minor against to positive controls), and high SPF (13.56±0.21) tested at 10 µg/mL.

Indigenous population from the Andes of Ecuador use *O. grandiflora* to treat their illness. The research carried out is a contribution to appreciate the ancient use of this resource, currently underutilized. This study could become an opportunity for the local development of communities through planting and conserving this species, and in this way, improve their economy.

Further research needs to be focused on the identification of the compounds present in hydroalcoholic extract of *O. grandiflora* and to study their activity/safety on cellular models (3T3 neutral red uptake phototoxicity assay), dermal irritation, and corrosivity, as well as *in vivo* (using an adequate animal model i.e., IAF Hairless Guinea Pig Crl: HA-*Hr*^{hr}), and other tests with the purpose of collecting the necessary information to develop new safe and efficacious cosmetic products.

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REFERENCES

1. López Figueroa F. Dermatological implications of climate change and the reduction of the ozone layer. Actas Dermosifiliogr 2011; 102:311-5. Available from: http://www.elsevier.es/es-revista-acta-otorrinolaringologica-espanola-102-pdf-90014922-S300.
2. Ecuatoriana AE. Informe Hiperión; 2008. Available from: http://www.exa.ec.
3. Oliveira Junior RG, Araujo CS, Souza GR, Guimaraes AL, Oliveira AP, Lima-Saraiva SR, et al. In vitro antioxidant and photoprotective activities of dried extracts from Neoglaziovia variegata (Bromeliaceae). J Appl Pharm Sci 2013;3:122-7. Available from: http://imsear.li.mahidol.ac.th/bitstream/123456789/151521/1/japs2013v3n1p122.pdf.
4. Saija A, Tomaino A, Trombetta D, Pasquale A De, Uccella N, Barbuzzi T, *et al. In vitro* and *in vivo* evaluation of caffeic and ferulic acids as topical photoprotective agents. Int J Pharm 2000;199:39-47. Available from: http://www.sciencedirect.com/science/article/pii/S0378517300003586?via%3Dihub.
5. Mishra A, Mishra A, Chattopadhyay P. Assessment of *in vitro* sun protection factor of calendula officinalis L. (Asteraceae) essential oil formulation. J Young Pharm 2012;4:17-21.
6. Magnin A, Grosfeld J, Barthélémy D, Puntieri J. Bud and shoot structure may relate to the distribution area of South American *Proteaceae* tree species. Flora Morphol Distrib Funct Ecol Plants 2012;207:599-606.
7. Rios M, Koziol MJ, Borgtoft H, Granda G. Plantas útiles del Ecuador aplicaciones, retos y perspectivas. Quito, Ecuador: Ediciones Abya-Yala; 2007. p. 652.
8. Alejandro M, Jaramillo X, Ojeda S, Malagón O, Ramírez J. Antioxidant and antihyperglycemic activity of the medicinal species Oreocallis grandiflora (Lam.) R. Br., South of Ecuador. Bol Latinoam Caribe Planta Med Aromat 2013;12:59-68. Available from: http://www.redalyc.org/articulo.oa?id=85625709007.
9. Gonzales M, Baldeón S, Beltrán H, Jullian V, Bourdy G. Hot and cold: Medicinal plant uses in Quechua speaking communities in the high Andes (Callejón de Huaylas, Ancash, Perú). J Ethnopharmacol 2014;155:1093-117.
10. Monigatti M, Bussmann RW, Weckerle CS. Medicinal plant use in two Andean communities located at different altitudes in the Bol??var Province, Peru. J Ethnopharmacol 2013;145:450-64.
11. Andersen OM, Markham KR. Flavonoids. Chemistry, Biochemistry and Applications. Angewandte Chemie International Edition., Vol. 45. Boca Raton: CRC Press; 2006. p. 1237.
12. Vinueza D, López E, Acosta K, Abdo S. Assesment of anti-inflammatory activity and cytotoxicity of freeze dried hydroalcoholic extract of Bidens andicola on isolated neutrophils. Asian J Pharm Clin Res 2017;10. Available from: https://www.innovareacademics.in/journals/index.php/ajpcr/article/view/17574/11445.
13. Evans WC. Trease and Evans Pharmacognosy. 16th ed. London: Elsevier Health Sciences; 2009. p. 603.
14. Boukhris M, Simmonds MS, Sayadi S, Bouaziz M. Chemical composition and biological activities of polar extracts and essential oil of rose-scented geranium, pelargonium graveolens. Phyther Res 2013;27:1206-13. Available from: http://www.onlinelibrary.wiley.com/doi/10.1002/ptr.4853/pdf.
15. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol 1995;28:25-30. Available from: http://www.sciencedirect.com/science/article/pii/S0023643895800085.
16. Silva RV, Costa SC, Branco CR, Branco A. *In vitro* photoprotective activity of the *Spondias purpurea* L. peel crude extract and its incorporation in a pharmaceutical formulation. Ind Crops Prod 2016;83:509-14.
17. Mansur JS, Breder MN, Mansur MC, Azulay RD. Determination of Sun Protection Factor by Spectrophotometry. An Bras Dermatol 1986; 61: 121-4. Available from: http://www.anaisdedermatologia.org.br/detalhe-artigo/421/Determinacao-do-fatorde-protecao-solar-por-espectrofotometria.
18. Imam S, Azhar I, Mahmood ZA. In-Vitro evaluation of sun protection factor of a cream formulation prepared from extracts of *Musa accuminata* (L.), *Psidium gujava* (L.) and *Pyrus communis* (L.). Asian J Pharm Clin Res 2015;8:8-11. Available from: https://www.innovareacademics.in/journals/index.php/ajpcr/article/download/5539/2464.
19. García-Bores AM, Espinosa-González AM, Reyna-Campos A, Cruz-Toscano S, Benítez-Flores JC, Hernández-Delgado CT, *et al.* Lippia graveolens photochemopreventive effect against UVB radiation-induced skin carcinogenesis. J Photochem Photobiol B Biol 2017;167:72-81.
20. Hazlehurst JA, Karubian JO. Nectar robbing impacts pollinator behavior but not plant reproduction. Oikos. 2016;125:1668-76. Available from: http://www.onlinelibrary.wiley.com/doi/10.1111/oik.03195/epdf.
21. Anderson CL, Bremer K, Friis EM. Dating phylogenetically basal eudicots using rbcL sequences and multiple fossil reference points. Am J Bot 2005;92:1737-48. Available from: http://www.amjbot.org/content/92/10/1737.full.pdf+html.
22. Schooneveld-Bergmans M, Heijne W, Damveld R, De Jong R. Polypeptide Having Beta-Glucosidase Activity and Uses Thereof. (WO2012/000890 A1); 2012. Available from: https://www.patentscope.wipo.int/search/en/detail.jsf;jsessionid=DD2C36C6F6DFAA9A5E07D2132C264411.wapp2nA?docId=WO2012000890.
23. Wang H, Leach DN, Forster PI, Waterman PG. Secondary metabolites from *Grevillea robusta*. Biochem Syst Ecol 2008;36:452-3. Available from: http://www.sciencedirect.com/science/article/pii/S0305197807002256?via%3Dihub.
24. Shashank K, Abhay K. Review article chemistry and biological activities of flavonoids: An overview. Sci World J 2013;4:32-48. Available from: http://www.downloads.hindawi.com/journals/tswj/2013/162750.pdf.
25. Sousa MS, Vieira LM, Lima AD. Total phenolics and in vitro antioxidant capacity of tropical fruit pulp residues. Braz J Food Technol 2011;14:202-10. Available from: http://www.ital.sp.gov. br/bj/artigos/html/busca/PDF/v14n3461a.pdf.
26. El-Boury S, Couteau C, Boulande L, Paparis E, Coiffard LJ. Effect of the combination of organic and inorganic filters on the Sun Protection Factor (SPF) determined by *in vitro* method. Int J Pharm 2007;340:1-5. Available from: http://www.sciencedirect.com/science/article/pii/S0378517307004589.
27. Stengel FM. Nueva Clasificación FPS Productos Europeos-Límite Superior recomendación COLIPA # 11, Junio 2002. su aplicación obligatoria 2006. Arch Argent Dermatol 2006;56:167-8. Available from: https://www.google.com.ec/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0ahUKEwjSg5PZ9dHUAhWDOyYKHeNMCPIQFggkMAA&url=http%3A%2F%2Fwww.archivosdermato.org.ar%2FUploads%2F56%2520167-168%2 C%25202006.pdf&usg=AFQjCNExJaec2MpeXTT7gDgxl7O2G491uw.