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Anti-inflammatory Activity of Verminoside from *Kigelia africana* and Evaluation of Cutaneous Irritation in Cell Cultures and Reconstituted Human Epidermis

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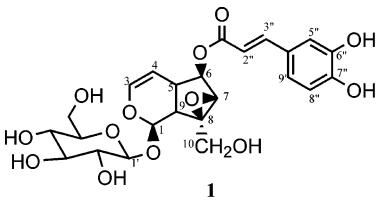
Kigelia africana is a plant used in Africa for anti-inflammatory, anti-microbial, and anti-skin-aging effects. Various papers have reported on the composition and biological activities of its CH_2Cl_2 extracts and dermal formulations. Chemical analysis of a polar extract of fruit from *K. africana* indicated the presence of verminoside (**1**), an iridoid, as a major constituent, and of a series of polyphenols such as verbascoside (**2**). In vitro assays showed that **1** had significant anti-inflammatory effects, inhibiting both iNOS expression and NO release in the LPS-induced J774.A1 macrophage cell line. Cytotoxicity and cutaneous irritation of the extract and of compounds **1** and **2** were investigated. The crude extract and **1** did not affect cell viability in vitro either in cells grown in monolayers (ML) or in the reconstituted human epidermis (RHE, 3D) model; neither caused release of pro-inflammatory mediators or histomorphological modification of RHE.

Kigelia africana (Lam.) Benth (syn *Kigelia pinnata* DC., Bignoniaceae) is a tropical tree used in African folk medicine for its medicinal properties. Traditional remedies prepared from crushed dried fruits are used for emollient, anti-eczema, anti-psoriasis, and skin-firming properties and as dressing for ulcers and wounds. Remedies from root bark are also used for the treatment of venereal disesases, haemorrhoids, and rheumatism.[1,2] Fresh fruit cannot be eaten, as it has been shown to be purgative and causes blisters in the mouth and on the skin. Steroids, iridoids, and coumarins have been isolated from the root bark[3] and flavonoids and iridoids from the fruit and leaves.[4] CH_2Cl_2 extracts from the root and stem bark of *K. africana* containing naphthoquinones[5] showed anti-trypanosomal[6] and anti-microbial activities[7] and cytotoxicity against melanoma and renal carcinoma cells.[8] Since psoriasis is a condition where the rate of skin turnover is faster than normal, a constituent that retards overactive cell growth could explain the anti-psoriasis effect attributed to this plant. The bark and its apolar extracts have been pharmacologically and phytochemically analyzed;[6−8] however, such knowledge of the fruit and of polar extracts is limited. The present paper reports on the composition of a polar extract of the fruit and on the effects of its major constituents in mediating inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) biosynthesis in J744.A1 macrophage cells stimulated for 24 h with LPS. Despite the wide use of *K. africana* extracts in commercial skin formulations,[9−14] the cutaneous irritation of the extract and of its constituents was also investigated in vitro using both cells grown in monolayers (ML) and reconstituted human epidermis (RHE, 3D) model.

Results and Discussion

Dried fruit of *K. africana* was defatted with petroleum ether and $CHCl_3$ and then extracted with MeOH. The MeOH solubles were then partitioned between H_2O and *n*-BuOH. The dried *n*-BuOH-soluble fraction was fractionated by gel filtration on a Sephadex LH-20 column and by RP-HPLC, giving compound **1**, as the major component, and four additional polyphenols.



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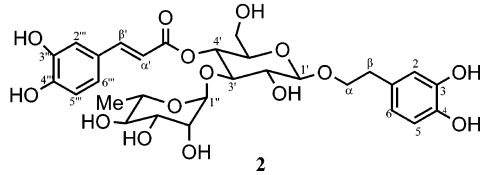

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The ESIMS of compound **1** in the positive mode exhibited a quasi-molecular ion peak at *m/z* 525 $[M + H]^+$, and a high-resolution measurement indicated the molecular formula $C_{24}H_{28}O_{13}$. The ^1H and ^{13}C NMR data of **1** led to its identification as verminoside.[3] The homonuclear Hartmann−Hahn (2D-HOHAHA and 1D TOCSY) spectra of **1** exhibited distinct spin systems belonging to the iridoid moiety, to the saccharide (*β*-D-glucopyranosyl) unit, and to the caffeoyl residue. The proton sequence within each spin system was elucidated by analysis of cross-peaks in the COSY spectrum, while data arising from the HMBC spectrum were used to interconnect the partial structures. Compound **1** was the major constituent, representing 2.1% w/w of the extract. Leaves and fruit are generally characterized by higher concentrations of polyphenols; therefore, we also analyzed the polyphenol fraction. The extract had a total phenolic content, determined by the colorimetric Folin−Ciocalteu method[15] and expressed as caffeic acid equivalents, equal to 185 ± 2.1 (*μ*g/mg extract). Verbasco-

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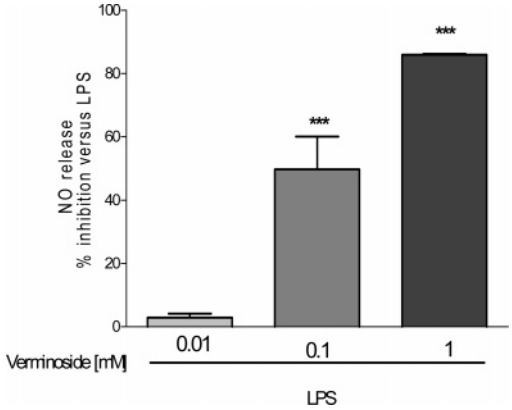

Figure 1. Effect of verminoside (**1**) (0.01−1 mM) in vitro on nitrite (NO) release on LPS-stimulated J774.A1 macrophages. Values, mean ± SD, are expressed as % inhibition of at least 3 independent experiments with 3 replicates each. Comparisons were performed using a one-way ANOVA test. ***P < 0.001 calculated versus LPS alone. No significant inhibition was observed at 0.01 mM.

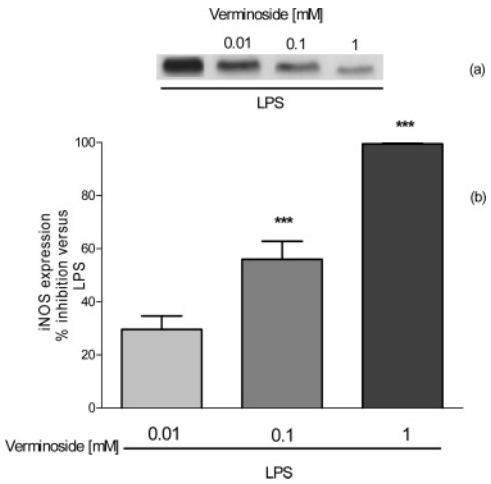

Figure 2. (a) Representative Western blot of iNOS expression. (b) Densitometric analysis of concentration-dependent effect of verminoside (0.01−1 mM) on LPS-induced iNOS expression in J774.A1 macrophages. Verminoside (**1**) (0.01−1 mM) was added 1 h before and simultaneously with LPS challenge. Values, mean ± SD, are expressed as % inhibition of at least 3−6 independent experiments with 3 replicates each. Comparisons were made using a one-way ANOVA test. ***P < 0.01 calculated versus LPS alone. No significant inhibition was observed at 0.01 mM.

side (**2**), caffeic acid, *p*-coumaric acid, and caffeic acid methyl ester were identified by ESIMS and NMR spectroscopic methods and by comparison with data reported in the literature.[16−19] HPLC analysis showed that the total caffeic acid derivatives isolated, expressed as caffeic acid equivalents, was 1.55% and that the concentration of caffeic acid was 1.05% w/w of dried extract.

The reports[9,12] of anti-inflammatory effects of *K. africana* skin formulations led us to investigate the in vitro activities of the extract and compound **1** on inducible nitric oxide synthase expression (iNOS) and on nitric oxide (NO) biosynthesis in the J744.A1 macrophage cells. Cytotoxicity was also investigated using both cells grown in monolayers [J774.A1 (murine monocyte/macrophage), WEHI-164 (human epithelial kidney), and HEK-293 (murine fibrosarcoma) cells] and in reconstituted human epidermis (RHE, 3D) model.

NO release in the cellular medium of LPS-stimulated J774.A1 macrophages, incubated with compounds **1** (0.01−1 mM) and **2** (0.01−1 mM), was evaluated 24 h after LPS (6 × 10^3 u/mL) challenge. Results were expressed as % of inhibition calculated versus controls.[20] As shown in Figure 1, verminoside (**1**) (0.01−1 mM), added 1 h before and simultaneously with LPS, inhibited NO release significantly (P < 0.001) and in a concentration-related manner. Verbascoside (**2**) (0.01−1 μM) had a significant effect on iNOS activity only at the highest concentration (1 mM). To establish whether the inhibitory effect of **1** on NO release was related to a modulation of iNOS induction, iNOS expression was evaluated by Western blot analysis on cell lysates obtained by J774.A1 incubated with **1** (0.01−1 mM), 1 h before and simultaneously with LPS. Compound **1** showed significant and concentration-related inhibition of iNOS expression at 0.1 mM (P < 0.001) and 1 mM (P < 0.001) (Figure 2).

To establish the effects on viability of cells grown in monolayers (ML), the extract (1−1000 μg/mL), compound **1** (verminoside) (0.01−1 mM), and compound **2** (verbascoside) (0.01−1 mM) were each tested on J774.A1, WEHI-164, and HEK-293 cells using the MTT test.[21,22] Only verbascoside (**2**) showed significant cytotoxicity (EC_{50} of 4.9 × 10^{-4} M) on the J774.A1 macrophage cell line. In addition, cytotoxicity and cutaneous irritation of the extract and its major constituent **1** were assessed in reconstituted human epidermis (RHE) in vitro. Usually commercial skin preparations contain from 0.1 to 1% of *Kigelia* extract;[9−11] for this reason tests on RHE were carried out using 1−3% (extract) and 0.25−1% (compound **1**) solutions in PBS (phosphate buffer solution). Increasing concentrations of the extract (1, 2, 3%) and compound **1** (0.25, 0.5, 1%) in PBS were applied on a commercial RHE model (Skinethic), in comparison to PBS as control and SDS (sodium dodecyl sulfate 0.25%) as a cutaneous irritant. After topical exposure for 24 and 72 h, cell viability, release of the major mediator of cutaneous irritation (interleukin IL-1α) in the medium underneath, and histomorphological analysis to evaluate superficial and deeper morphological modifications of the tissue were determined.[23,24] The epidermis viability, measured as optical density (OD) at 570 nm by MTT assay and calculated as percentage of cytotoxicity compared to the control (PBS-treated epidermis), was not reduced after application of either the extract or compound **1**, both at 24 and 72 h (data not shown). No increased release of interleukin IL-1α was observed in tissues treated with the extract or compound **1** at all concentrations tested, at both 24 and 72 h, compared to the control. Using the same test conditions, the irritant reference compound SDS (0.25%) caused an elevation of the pro-inflammatory mediator release to about 250 pg/mL at 24 h and to 110 pg/mL at 72 h (Figure 3). Finally, histological analysis of in vitro reconstituted epidermis treated with the extract and compound **1** (for 24 and 72 h) did not show significant modifications in the tissue morphology, at both *stratum corneum* and viable epidermis levels, compared to the control. In this condition the irritant reference compound (SDS 0.25%) caused tissue degeneration and necrosis. Representative photographs of tissues treated with **1** are shown in Figure 4c−e; tissue treated with the control (PBS) is shown in Figure 4a, and histological changes after exposure to severe skin irritant SDS are illustrated in Figure 4b.

The present research showed that the polar extract of *K. africana* fruit contains verminoside (**1**)[3] as the major constituent and a series of other phenols[17−19] that may all contribute to the anti-inflammatory activity of the extract. It is well known that in inflammatory disease NO production is elevated by both the constitutive and the inducible nitric oxide synthase, and NO produced by iNOS is an important inflammatory mediator. The present results

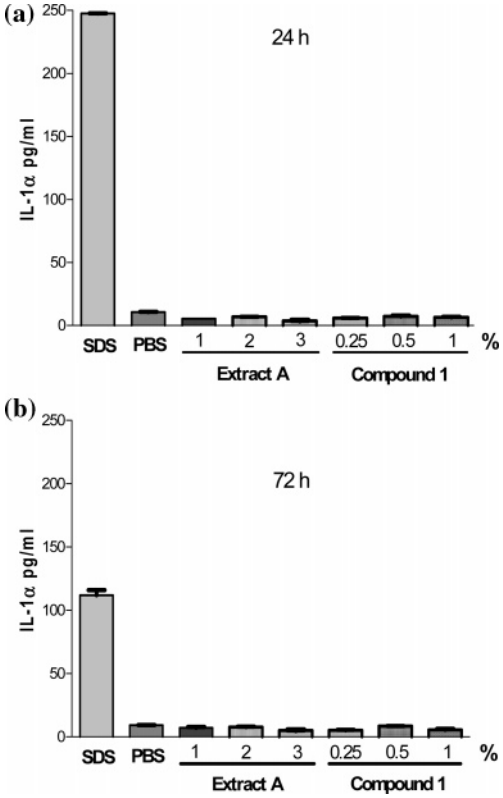

Figure 3. Effect of the extract (1, 2, 3%) and compound **1** (0.25, 0.5, 1%) on IL-1α release (pg/mL of medium), estimated by ELISA, in reconstituted human epidermis (RHE) after 24 h (a) and 72 h (b) of exposure in comparison to control (PBS) and irritant reference compound SDS (0.25%). Values are expressed as mean ± SD of 3 independent experiments.

indicate that **1** inhibits, significantly and in a concentration-related manner, both in vitro NO release and iNOS expression, providing scientific support for the traditional uses of *K. africana*.[25,26] Information on chemicals that cause skin irritation is important for general safety assessment purposes, and the detection of acute skin irritation is included in international regulatory requirements for the testing of chemicals. Thus, the aim of the present study was also to provide a method for investigating cutaneous irritation of plant extracts and bioactive molecules using both cells grown in monolayers (ML) and reconstituted epidemis (RHE). The RHE model has been reported to mimic morphologically and biochemically living skin to a more significant degree than monolayer cultures. It has been used to investigate complementary parameters of the irritation mechanism by the application of products directly on the skin surface.[23,24] To discriminate very slightly irritant products able to induce pro-inflammatory mediator release without affecting tissue integrity, we evaluated three endpoints (cell viability, release of interleukin IL-1α in the medium, and histological analysis) after topical exposure to the extract and compound **1** for 24 and 72 h, at concentrations similar to those used in topical formulations in vivo.

The polar extract and verminoside (**1**) show promising anti-inflammatory activity and appear to be suitable for cutaneous application using the skin model at all concentrations and exposure times tested, based on the lack of cytotoxicity, on the low proinflammatory cytokine (IL-1 α) release comparable to the control (Figure 3), and on the observation of a regular morphology at all epidermis layers at 24 and 72 h (Figure 4). Thus, the extract and compound **1** appear to be safe for topical use.

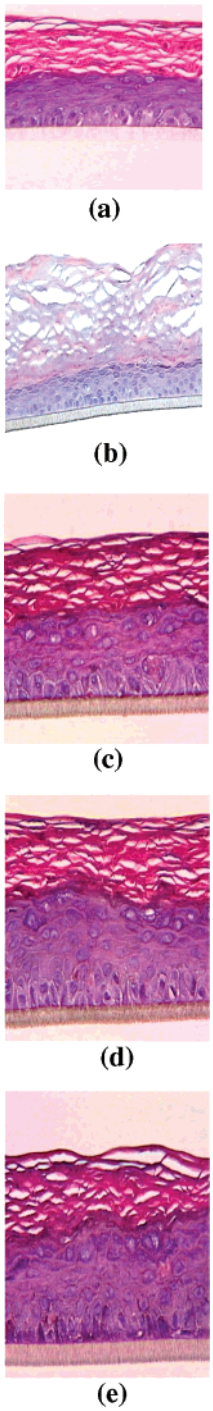

Figure 4. Histopathological examination of in vitro reconstituted human epidermis (RHE) model samples after 24 h exposure to (a) control (PBS), (b) irritant reference compound SDS (0.25%), and (c) compound **1** (0.25%), (d) (0.5%), and (e) (1%) as topical application. In part b application of SDS shows severe epidermal changes with cellular necrosis.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer model 192 polarimeter (Norwalk, CT) equipped with a sodium lamp (589 nm) and a 10 cm microcell in MeOH solution. Solubility of **1** was evaluated spectrophotometrically in H_2O at room temperature, according to a USP method, by a Shimadzu UV-2101PC (Italia srl, Milan, Italy). For NMR experiments, a Bruker DRX-600 NMR spectrometer was used, operating at 599.2 MHz for ^1H and at 150.9 MHz for ^{13}C. Electrospray-ionization mass spectra (ESIMS) were recorded using a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA) equipped with Xcalibur

software (capillary temperature 220 °C). High-resolution mass spectra were recorded using a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument equipped with electrospray ionization source. HPLC separations were performed on a Waters 590 series pumping system equipped with a W R401 refractive index detector and a 300 × 7.8 mm i.d., 10 μm μ-Bondapak C_{18} column. HPLC analyses were carried out on an Agilent 1100 series system equipped with a model G-1312 pump and a DAD G-1315 A detector.

Plant Material. Fruit of *Kigelia africana* (Lam.) Benth was collected near Bamako, Mali, in May−July 2002 and identified by one of the authors (R.S.) A voucher sample (KA, 2002) is deposited at the Herbarium of the Faculté de Medicine, Farmacie et D'Odontostomatologie, University of Bamako, Mali.

Extraction and Isolation Procedure. The dried and powdered fruit (200 g) was defatted at room temperature with *n*-hexane and $CHCl_3$ and then extracted by maceration at room temperature with MeOH to give 44 g of residue. A portion of the dried extract (31 g) was partitioned between *n*-BuOH and H_2O to afford the *n*-BuOH-soluble portion (11 g). This was suspended in H_2O and lyophilized. An aliquot (6 g) of this extract was chromatographed over a 1 m × 3 cm i.d. Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) using MeOH as eluent at a flow rate of 0.5 mL/min. Fractions (8 mL each) were collected and checked by TLC [Si gel, *n*-BuOH−AcOH−H_2O (60:15:25), $CHCl_3$−MeOH−H_2O (7:3:0.3)] Fractions with similar R_f values were combined, giving six major fractions (I−VI), which were further purified by RP-HPLC (30 cm × 7.8 mm i.d. C_{18} μ-Bondapack column, flow rate of 2.0 mL/min), using MeOH−H_2O (3.5:6.5) as the eluent. Fraction II (121.1 mg) yielded pure **2** (24.5 mg, t_R = 29.5 min); fraction III (76 mg) gave compound **1** (106 mg, t_R = 40.6 min); fraction IV (725 mg) afforded caffeic acid methyl ester (6.7 mg, t_R = 23.5 min), caffeic acid (12.7 mg, t_R = 16.0 min), and **1** (53 mg), and fraction V (56 mg) yielded caffeic acid methyl ester (6 mg) and *p*-coumaric acid (5 mg, t_R = 20.0 min).

Verminoside (1): pale yellow amorphous powder; solubility 144 g/L (H_2O, 25 °C); $[\alpha]^{25}_D$ −165.1° (*c* 0.5, MeOH); ^1H and ^{13}C NMR were consistent with those previously reported;[3] ESIMS (electrospray-ionization mass spectrometry) *m/z* 525 $[M + H]^+$, *m/z* 523 $[M - H]^-$; HREIMS (high-resolution electron impact mass spectrometry) *m/z* 525.4060; calcd for $C_{24}H_{28}O_{13}$, 525.4091.

Verbascoside (2): ^1H and ^{13}C NMR data were consistent with those previously reported;[16] ESIMS *m/z* 625 $[M + H]^+$, *m/z* 623 $[M - H]^-$.

Caffeic Acid: ^1H and ^{13}C NMR data were consistent with those previously reported;[17] ESIMS *m/z* 181 $[M + H]^+$, *m/z* 179 $[M - H]^-$.

***p*-Coumaric acid:** ^1H and ^{13}C NMR data were consistent with those previously reported;[18] ESIMS *m/z* 165 $[M + H]^+$, *m/z* 163 $[M - H]^-$.

Caffeic acid methyl ester: ^1H and ^{13}C NMR data were consistent with those previously reported;[19] ESIMS *m/z* 195 $[M + H]^+$, *m/z* 193 $[M - H]^-$.

Quantitative Determination of Total Phenols. The extract, dissolved in MeOH, was analyzed for its total phenolic content according to the Folin−Ciocalteu colorimetric method.[15] Total phenols were expressed as caffeic acid equivalents (185 ± 2.1 μg/mg extract).

HPLC Analysis. Standard solutions with a concentration range of 30−120 μg/mL for **1** and 12.5−50 μg/mL for caffeic acid were prepared. Quantitative HPLC was conducted using a 150 × 3.9 mm i.d. C-18 μ-Bondapack column. The solvents were TFA 0.1% in H_2O (solvent A) and MeOH (solvent B). The elution gradient used was as follows: 0 → 20 min, 20 → 30% B; 20 → 25 min, 30 → 40% B; 25 → 35 min, 40% B (isocratic); 35 → 60 min, 40 → 100% B. Analyses were carried out in triplicate, at a flow rate of 1 mL/min. Calibration graphs were plotted showing a linear relationship between concentration and peak areas for both reference compounds. The extract was redissolved in MeOH and analyzed under the same chromatographic conditions. Chromatographic peaks were identified on the basis of the retention times and confirmed by co-injections. The results showed that **1** represented 2.1% and caffeic acid was 1.05% w/w of the extract. The total concentration of the caffeic acid derivatives, expressed as caffeic acid equivalents, was 1.55% w/w of the dried extract A.

Pharmacological and Toxicological Methods. Materials. *E. coli* lipopolysaccharide (LPS) was obtained from Fluka (Milan, Italy). 3-(4,5-Dimethylthiazolyl-2-yl) 2,5-diphenyltetrazolium bromide (MTT), phosphate buffer solution (PBS), bovine serum albumin (BSA), 6-mercaptopurine (6-MP), and 2-propanol were obtained from Sigma Chemical Co. (Milan, Italy). Kodak X-Omat film, ECL detection system, and Hybond polyvinyldene difluoride membrane were from Millipore (Bedford, MA). Materials for Western blot analysis of iNOS[20] were obtained from Transduction Laboratories (Lexington, KY), and the peroxidase secondary antibody was purchased from Jackson (West Grove, PA). Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, HEPES, glutamine, fetal calf serum (FCS), and horse serum were from Hy Clone (Euroclone-Cellbio, Pero, Milan, Italy).

For 3D tests, the model of reconstructed human epidermis (RHE) (SkinEthic Laboratories, Nice, France) of 0.63 cm^2 was used. The epidermal model has been fully characterized[27,28] as terminal differentiation of NHK (normal human keratinocytes) cells grown in chemically defined medium on inert polycarbonate filter substrates at the air−liquid interface JID; a fully differentiated epidermis is formed after 17 day of air-lift culture of NHK from skin biopsies in a chemically defined medium (modified MCDB 153 medium, SkinEthic Laboratories). For quantification of the IL-1 α release in the medium underneath, an Elisa Kit Quantikine DLA-50 (R&D System, San Diego, CA) and a Biotek Instruments EL311SX microplate autoreader were used; for histological analysis, formalin (10%, Sigma Chemical Co.) and H&E (Merck) staining were used.

Cells. J774.A1, murine monocyte/macrophage cells, were grown in adhesion on Petri dishes and maintained at 37 °C as previously described.[20] WEHI-164 (murine fibrosarcoma cells) and HEK-293 (human epithelial kidney cells) were maintained in adhesion on Petri dishes with DMEM supplemented with 10% heat-inactivated FCS, 25 mM HEPES, 100 u/mL penicillin, and 100 μg/mL streptomycin.

Cytotoxic Activity. Cytotoxic activity of the extract (1−1000 μg/mL), compound **1**, and compound **2** (0.01−1 mM) in PBS solutions was evaluated in cell cultures (J774.A1, HEH-293, and WEHI-164 cell lines) by MTT assay as previously reported.[20,29]

Analysis of Nitrite. Monolayers of J774.A1 cells were routinely harvested by gentle scraping with a Teflon cell scraper, diluted in fresh medium, and cultured to confluency at 37 °C. Prior to each experiment cells were harvested and plated to a seeding density of 1.5 × 10^6 in P60 well plates. After cell adhesion, the extract (1−1000 μg/mL), verminoside (**1**) (0.01−1 mM), and verbascoside (**2**) (0.01−1 mM) in PBS solutions were added to the culture medium 1 h before and simultaneously to LPS (6 × 10^3 u/mL/24 h). Nitrite accumulation, an indicator of NO release, was measured in the culture medium by Griess reaction[30] 24 h after LPS challenge, according to Marzocco et al.[20] The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results are expressed as percentages of inhibition calculated versus cells treated only with LPS.

Western Blot Analysis for iNOS. After 24 h of incubation with LPS, medium was removed, cells were lysed, and Western blot analysis was performed according to Marzocco et al.[20]

Cutaneous Compatibility on 3D Model. The extract and compound **1** were dissolved in PBS to obtain concentrations of 1, 2, 3% and 0.25, 0.5, 1%, respectively. After topical exposure for 24 and 72 h, three complementary endpoints were evaluated: (i) cellular parameter, the cytotoxicity by MTT assay; (ii) molecular parameter, the release of IL-1 alpha; (iii) histomorphological modifications.[24,27,28] For each concentration of the extract, compound **1**, and controls, the tests were made in triplicate.

Cell Viability in Reconstituted Human Epidermis. PBS solutions (50 μL) of the test compounds were applied in duplicate, using a micropipet, to the surface of epidermis in a single well treated with 300 μL of its specific maintenance medium. Tissues were incubated at 37 °C in a 5% CO_2 atmosphere for 24 or 72 h. The culture medium was changed daily. The same volume of PBS at pH 7.2 and sodium dodecyl sulfate (SDS 0.25%) were applied to control wells. After 24 and 72 h of incubation, the wells were washed with PBS (1 mL), three times, and viability was assessed using the MTT assay.[24,28] Briefly, 300 μL of MTT (0.5 mg/mL of test medium) was added, and the tissue was incubated for an additional 3 h at 37 °C. The resulting dark blue product was extracted from the epidermis using 1000 μL of 2-propanol. Solutions (200 μL) were transferred to Elisa microplates, and the OD was read at 570 nm against a blank of 2-propanol. Results are expressed in % viability relative to the control (PBS).

Release of Interleukin IL-1α in Reconstituted Human Epidermis. Underlying culture media were collected 24 or 72 h after product application in triplicate. The release of interleukin IL-1α in the medium of each well was quantified by an Elisa Quantikine DLA-50 (R&D Systems) kit.[27,28] Results are expressed as pg/mL of interleukin IL-1α in the medium.

Histological Analysis of Tissues. After treatment at 24 and 72 h, tissues were fixed by immersion in formalin (10%, 1 mL) and dehydratated by multiple passages through increasing concentrations of EtOH and finally toluene. Samples were embedded in paraffin and then cut to obtain 5 μm vertical sections for H&E (Merck) staining observed under light microscopy (Laborlux S-Leitz, 200×).

Statistical Analysis. Data for NO accumulation and iNOS expression are expressed as percentages of inhibition versus cells treated with LPS alone. Data on cell viability in ML and RHE are expressed as percentages of viability versus negative controls (PBS-treated cells or epidermis). Data on IL-1α release in RHE are expressed as pg/mL in the medium underneath. All data were calculated from mean ± SD values of three independent determinations. Statistical analysis was performed by an ANOVA test and multiple comparison by a Bonferroni test.[20] All experiments in vitro were made at least three times, each time with three or more independent observations.

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