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An extract of *Lannea microcarpa*: composition, activity and evaluation of cutaneous irritation in cell cultures and reconstituted human epidermis

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Abstract

Lannea microcarpa (Anacardiaceae) is a tropical tree used in African folk medicine and commercial dermopharmaceutical formulations. Fractionation and analysis of its polar extract allowed the identification of 4′-methoxy-myricetin 3-*O*-α-L-rhamnopyranoside, myricetin 3-*O*-α-L-rhamnopyranoside, myricetin 3-*O*-β-D-glucopyranoside, vitexin, isovitexin, gallic acid and epi-catechin, as the major constituents. In-vivo assay (the croton oil ear test in mice) showed that the extract had significant anti-inflammatory effect (ID50 = 900 μg cm^{-2}) but ten times lower than that of indometacin (ID50 = 93 μg cm^{-2}), the non-steroidal anti-inflammatory drug used as reference. Cytotoxicity and cutaneous irritation of the extract and its constituents were investigated. The crude extract and its major components did not affect cell viability in-vitro either in three different cultures (J774.A1, WEHI-164 and HEK-293) of cells grown in monolayers or in the reconstituted human epidermis (RHE, 3D model), nor did they cause release of pro-inflammatory mediators (IL-1α) or histomorphological modification of RHE.

Introduction

Lannea microcarpa Engl et K. Krause (Anacardiaceae, synonym: *Lannea djalonica* A. Chev.; *Lannea oleosa* A. Chev.), commonly known by the trivial name paudre de feuille, is a tropical tree used in different African regions for its medicinal properties. Traditional remedies prepared from its edible leaves are used topically against conjunctivitis, stomatitis and gingivitis, and as dressing for ulcers and wounds. Infusions from bark and roots are indicated for the treatment of skin eruptions and for stomach trouble (Irvine 1961; Burkill 1994). The fibrous bark exudating a soluble and edible gum is used internally for beriberi, schistosomiasis and haemorrhoids and externally for eye disorders (Kerharo & Adam 1974). Vapour from bark decoction is inhaled into the mouth for dental caries and other mouth infections. The acid fruit, eaten raw or dried, gives a fermented drink. The oil of the seeds is used in cosmetology for hair-care products (Malgras 1992).

Dihydroflavonols (Tofazzal & Tahara 2000), β-sitosterol, physcion and physcion anthranol B have been isolated from *Lannea coromandelica* stem bark and quercetin 3-*O*-arabinoside and ellagic acid from flowers and leaves, while its heartwood is rich in leucocyanidin (Subramanian & Nair 1971). Epicatechin and leucocyanidin have been reported in the bark of *L. grandis* (Sulochana et al 1967) and flavones in *L. acida* (Sultana & Ilyas 1986). Organic extract of *L. welwitschii*, containing alkylated hydroquinones, exhibited modest cytotoxicity against the NCI panel of 60 human tumour cell lines (Groweiss et al 1997). Free-radical scavenging dihydroalkylhexenones have been isolated from the dichloromethane extract of the bark of *L. edulis* (Queiroz et al 2003). Plants belonging to this genus produce gum exudates (gum jeol, jinghan gum) (Jefferies et al 1977; Anderson et al 1986), which are used as industrial emulsifying agents. Other species of the *Lannea* genus have been biologically and phytochemically analysed; however, such knowledge of *L. microcarpa* is limited.

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Neither phytochemical nor pharmacological investigation was found in the literature on *L. microcarpa*. This paper reports on the composition and in-vivo topical anti-inflammatory activity of its polar extract. Despite the extensive use in topical traditional remedies and dermo-pharmaceutical formulations (Pauly et al 2001), the cutaneous irritation of the crude extract and of its constituents was investigated in-vitro using both cells grown in monolayers (ML) and reconstituted human epidermis (RHE, 3D model).

Materials and Methods

Materials

For the croton oil ear test, croton oil and indometacin were Sigma products (St Louis, MO, USA). Ketamine hydrochloride was from Virbac S.r.l. (Milano, Italy). For the cell viability test, *E. coli* lipopolysaccharide (LPS) was obtained from Fluka (Milan, Italy). 3-(4,5-Dimethyl-thiazolyl-2yl) 2,5 diphenyl tetrazolium bromide (MTT), phosphate buffer solution (PBS), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and 6-mercaptopurine (6-MP) were obtained from Sigma Chemical Co. (Milan, Italy). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, HEPES, glutamine, and foetal calf serum (FCS) were from Hy Clone (Euroclone-Cellbio, Pero, Milan, Italy).

For 3D tests, a model of reconstructed human epidermis (RHE) (SkinEthic Laboratories, Nice, France) of 0.63 cm^2 was used. The model has been fully characterized (Rosdy & Clauss 1990; de Brugerolle de Fraissinette et al 1999) 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). MTT, SDS, PBS and 2-propanol were obtained from Sigma Chemical Co. (Milan, Italy). For the quantification of the IL-1α 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%), H&E (Merck) staining and light microscope (Laborlux S, Leitz, 200×) were used.

Instruments

A Bruker DRX-600 NMR spectrometer, operating at 599.2 MHz for ^1H and at 150.9 MHz for ^{13}C, using the UXNMR software package, was used for NMR measurements in CD_3OD solutions. 1D and 2D NMR experiments were obtained using the conventional pulse sequences as previously described (Aquino et al 2002). Electrospray-ionization mass spectrometry (ESIMS) were recorded using a Finnigan LC-Q Deca instrument (Thermoquest, San Jose, CA, USA) equipped with Xcalibur software. Samples were dissolved in methanol and infused in the electrospray ionization source by using a syringe pump (capillary temperature 220°C). High-resolution mass spectra (HREIMS) were recorded by a Q-Star Pulsar (Applied Biosystems) triple-quadrupole orthogonal time-of-flight (TOF) instrument, equipped with electrospray ionization source. HPLC separations were performed with a Waters 590 series pumping system (Waters Corporation, Milford, USA) equipped with a W R401 refractive index detector, and μ-Bondapack 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

Leaves of *L. microcarpa* Engl et K. Krause, were collected near Bamako, Mali, in May–July 2002 and identified by one of the authors (R. S.). A voucher sample (LM, 2002) is deposited at the Herbarium of the Faculte de Medicine, Farmacie et D'Odontostomatologie, University of Bamako, Mali.

Fractionation procedure

The dried and powdered leaves (300 g) were defatted with *n*-hexane and chloroform, and then extracted at room temperature with methanol to give 29.6 g of residue. The dried extract was partitioned between *n*-butanol and water to afford an *n*-BuOH-soluble portion (dried 12.4 g). This was suspended in water and lyophilized (extract A). A portion (6 g) of extract A was chromatographed over a 1 m × 3 cm i.d Sephadex LH-20 column (Pharmacia, Uppsala, Sweden) using methanol as eluent. Fractions (8 mL each) were collected and checked by TLC (Si-gel plates in *n*-butanol–acetic acid–water, 60:15:25, and chloroform–methanol–water 7:3:0.3). Fractions with similar R_f values were combined giving six main fractions (I–VI), which were further purified by RP-HPLC on a 30 cm × 7.8 mm i.d C_{18} μ-Bondapack column (flow rate of 2.0 mL min^{-1}), using methanol–water in different ratios as the eluent. Fraction II (150.1 mg, methanol–water 1:1) yielded pure compounds **6** (15.5 mg, $t_R = 6.5$ min), **5** (8.7 mg, $t_R = 17$ min) and **1** (16 mg, $t_R = 38$ min); Fraction III (251.7 mg, methanol–water 4:6) gave compounds **4** (23 mg, $t_R = 13$ min), **3** (12.7 mg, $t_R = 25.1$ min), **2** (14.1 mg, $t_R = 32.6$ min) and **1** (22 mg, $t_R = 55$ min); Faction IV (225 mg, methanol–water 4:6) afforded compound **2** (21 mg, $t_R = 32.6$ min), and Fraction V (56 mg, methanol–water 3:7) gave compound **7** (6 mg, $t_R = 55.0$ min).

4′-Methoxy myricetin 3-O-α-L-*rhamnopyranoside* ***(1)***
Yellow pale amorphous powder. $[\alpha]^{28}_D = -148°$ ($c = 0.2$, MeOH). HREIMS m/z 478.4086; calculated for $C_{22}H_{22}O_{12}$; ESIMS m/z 479 $[M+H]^+$, m/z 477 $[M-H]^-$. ^1H and ^{13}C NMR data were consistent with those previously reported (Naira et al 1999; Braca et al 2001).

Myricetin 3-O-α-L-rhamnopyranoside (2)
^{1}H and ^{13}C NMR data were consistent with those previously reported (Martin et al 1995); ESIMS *m/z* 465 $[M+H]^+$, *m/z* 463 $[M-H]^-$.

Myricetin 3-O-β-D-glucopyranoside (3)
^{1}H and ^{13}C NMR data were consistent with those previously reported (Takino et al 1962); ESIMS *m/z* 481 $[M+H]^+$, *m/z* 479 $[M-H]^-$.

The minor constituents were identified as vitexin (**4**) (Picerno et al 2003), isovitexin (**5**) (Picerno et al 2003), gallic acid (**6**) (Aquino et al 2002) and epi-catechin (**7**) (Foo et al 1996).

Quantitative determination of total phenols

Extract A, dissolved in methanol, was analysed for its total phenolic content according to the Folin-Ciocalteu colorimetric method (Aquino et al 2002). Total phenols were expressed as myricetin equivalents (387.97 ± 2.1 μg/mg extract).

HPLC analysis

Quantitative HPLC was conducted using a 150×3.9 mm i.d. C-18 μ-Bondapack column. The solvent were TFA 0.1% in water (solvent A) and methanol (solvent B). The elution gradient used was as follows: $0 \rightarrow 10$ min, $20 \rightarrow 30\%$ B; $10 \rightarrow 40$ min, $30 \rightarrow 40\%$ B; $40 \rightarrow 50$ min, $40 \rightarrow 50\%$ B; $50 \rightarrow 60$ min, $50 \rightarrow 100\%$ B. Analyses were carried out in triplicate, at a flow rate of 0.8 mL min^{-1}, with DAD detector set at λ 257.

Linearity
Reference standard solutions were prepared at three concentration levels and were injected (20 μL) three times. The concentration levels for **2** were within the range 600–2400 μg mL^{-1}. The standard curve was analysed using the linear least-squares regression equation derived from the peak area (regression equation $y = 2027.3x - 317.12$, $R = 0.999$, where y is the peak area and x the concentration used).

Specificity
The peaks associated with compounds **1**–**3** were identified by retention times, UV spectra and MS spectra compared with standards and confirmed by co-injections.

Analysis of the extract
Extract A was redissolved in methanol and analysed under the same chromatographic conditions. The results showed that **1** represented 3.04%, compound **2** was 6.13% and compound **3** was 1.54% w/w of the extract.

Anti-inflammatory activity

Topical anti-inflammatory activity was evaluated as inhibition of the croton-oil-induced ear oedema in mice (Tubaro et al 1985). All experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC). Male CD-1 mice, 28–32 g (Harlan-Italy, Udine, Italy), were anaesthetized with ketamine hydrochloride (145 mg kg^{-1}, i.p.). Cutaneous inflammation was induced on the inner surface of the right ear (surface: about 1 cm^2) of anaesthetized mice by application of 80 μg of croton oil dissolved in 42% aqueous ethanol (v/v) used as vehicle, also for extract and its control. Control mice received only the irritant solution, whereas the other mice received both the irritant and the test substance. At the maximum oedematous response, 6 h later, mice were sacrificed and a plug (6 mm, diameter) was removed from both the treated (right) and the untreated (left) ears. The oedematous response was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percent reduction of the oedema in treated mice compared with the control mice. As a reference, the non-steroidal anti-inflammatory drug (NSAID) indometacin was used.

Cell viability in cells grown in monolayers

J774.A1, murine monocyte/macrophage cells, were grown in adhesion on Petri dishes and maintained at 37°C in DMEM supplemented with 10% FCS, 25 mM HEPES, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. 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^{-1} penicillin and 100 μg mL^{-1} streptomycin.

Cytotoxic activity of extract A (1–1000 μg mL^{-1}), compound **1**, **2** and **3** (0.01–1 mM) in PBS solutions was evaluated in cell cultures (J774.A1, HEH-293 and WEHI-164 cell lines) by MTT assay. J774.A1, WEHI-164 and HEK-293 (3.5×10^4 cells) were plated on 96-well microtitre plates and allowed to adhere at 37°C in a 5% CO_2 atmosphere for 2 h. Thereafter, the medium was replaced with 90 μL of fresh medium and 10-μL volumes of serial dilution of Extract A, compounds **1**, **2** and **3** and then the cells incubated for 72 h. In some experiments, serial dilutions of 6-MP were added as reference drug. Cell viability was assessed through an MTT conversion assay (Autore et al 2001). Briefly, 25 μL of MTT (5 mg mL^{-1}) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilized with 100 μL of a solution containing 50% (v/v) *N*,*N*-dimethylformamide, 20% (w/v) SDS with an adjusted pH of 4.5 (Mosmann 1983). The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP was calculated as: % dead cells = 100 − (OD treated/OD control) × 100.

Cutaneous compatibility on 3D model

Extract A and compound **2** were dissolved in PBS to obtain a concentration of 10% and 1%, respectively. After topical exposure for 24 and 72 h, three complementary

end-points (Meloni et al 2002) were evaluated: cellular parameter, the cytotoxicity by MTT assay; molecular parameter, the release of IL-1α; histomorphological modifications. For each concentration of Extract A, 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 micropipette, 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 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 through the MTT assay (Mosmann 1983; Meloni et al 2002). Briefly, 300 μL of MTT (0.5 mg mL^{-1} of test medium) was added and the tissue was incubated for an additional 3 h at 37°C. The resulting dark blue formazan 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 kit (Bernard et al 2000). Results are expressed as pg mL^{-1} of interleukin IL-1α in the medium.

Histological analysis of tissues

After treatment for 24 and 72 h, tissues were fixed by immersion in formalin (10%, 1 mL) and dehydratated by multiple passages through increasing concentrations of ethanol 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.

Statistical analysis

Oedema was expressed as mean ± standard deviation of the mean. Oedema values were analysed by one-way analysis of variance followed by Dunnett's test for multiple comparison of unpaired data. $P < 0.05$ was considered as being significant. The ID50 value (dose giving 50% oedema inhibition) was calculated by graphic interpolation of the dose–effect curve.

Data on cell viability in ML and RHE are expressed as percentages of viability versus negative control (PBS treated cells or epidemis). Data on IL-1α release in RHE are expressed as pg mL^{-1} in the medium underneath. All data were calculated from the mean ± s.d. values of three independent determinations. Statistical analysis was performed by analysis of variance test and multiple comparison by Bonferroni test. All experiments in-vitro were made at least three times, each time with three or more independent observations.

Results

Phytochemical analysis

Dried leaves of *L. microcarpa* were defatted with *n*-hexane and chloroform, and then extracted with methanol. The methanol solubles were then partitioned between water and *n*-BuOH. The lyophilized *n*-BuOH-soluble fraction (extract A) was fractionated by gel filtration on a Sephadex LH-20 column and by RP-HPLC, giving compounds **1**–**3** (Figure 1), as the major components, and four additional polyphenols (compounds **4**–**7**).

The ESIMS, in the positive mode, exhibited quasi-molecular ion peaks at m/z 478 $[M+H]^+$ for compound **1**, at m/z 464 $[M+H]^+$ for **2** and at m/z 480 $[M+H]^+$ for **3**. High-resolution measurements indicated the molecular formulas $C_{22}H_{22}O_{12}$ (compound **1**), $C_{21}H_{20}O_{12}$ (compound **2**) and $C_{21}H_{20}O_{13}$ (compound **3**), respectively, in accordance with ^{13}C NMR data. 4′-Myricetin 3-*O*-α-L-rhamnopyranoside (**1**) (Braca et al 2001), myricetin 3-*O*-α-L-rhamnopyranoside (**2**) (Martin et al 1995) and myricetin 3-*O*-β-D-glucopyranoside (Takino et al 1962) structures were indicated by ^{1}H and ^{13}C NMR analysis. All the proton resonances in the spectra of **1**–**3** were assigned unambiguously by correlation with the relevant carbon atoms using the HSQC spectrum. The Homonuclear Hartmann Hahn (2D-HOHAHA and 1D TOCSY) spectra of **1–3** exhibited a spin system belonging to the respective saccharide moiety (α-L-rhamnopyranosyl for **1** and **2** or β-D-glucopyranosyl for **3**). The proton sequence within each spin system was elucidated by analysis of cross-peaks in the COSY spectra, while data arising from HMBC spectra were used to interconnect the flavonol aglycones (4′-methoxy-myricetin for **1** and myricetin for **2** and **3**) to the respective saccharide moieties. Compound **2** was the major constituent, representing 6.13% w/w of the extract, compound **1** was 3.04%, and compound **3** was 1.54% w/w of the extract as shown by HPLC analysis (see Methods section). The extract had a total phenolic content, determined by the colorimetric Folin-Ciocalteu method (Aquino et al 2002) and expressed as myricetin equivalents, equal to 387.97 ± 2.1 (μg/mg extract). Minor constituents were identified as vitexin (**4**) (Picerno et al 2003), isovitexin (**5**) (Picerno et al 2003), gallic acid (**6**) (Aquino et al 2002) and epicatechin (**7**) (Foo et al 1996), by ESIMS and NMR spectroscopic methods and by comparison with data reported in the literature.

Screening of the topical anti-inflammatory activity

The anti-oedematous effect of extract A, at doses of 100, 300 and 900 μg cm^{-2}, is reported in Table 1. The dose–activity relationship of the extract was investigated in comparison with that of the NSAID reference drug indometacin (ID50 = 93 μg cm^{-2}). The extract provoked a significant and

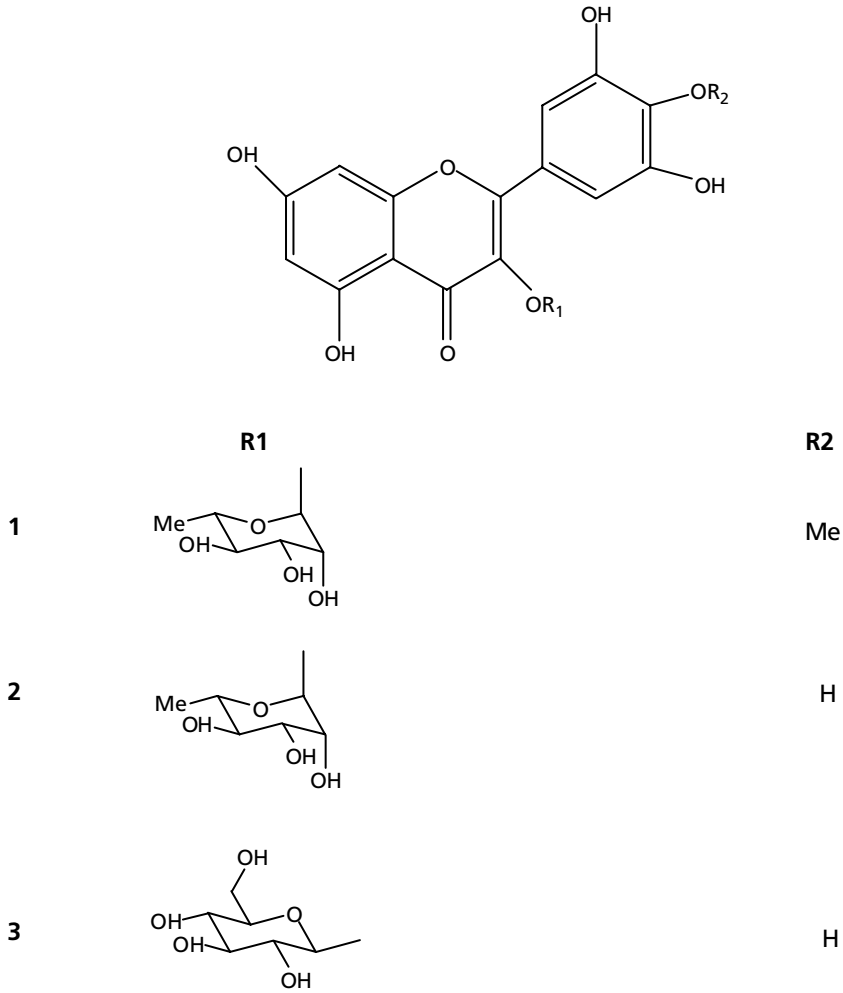

Figure 1 Structures of compounds **1**–**3**.

Table 1 Anti-inflammatory activity of *L. microcarpa* extracts after 6 h induction of croton oil mouse ear oedema

Substance	Dose ($\mu g\,cm^{-2}$)	No. of mice	Oedema (mg)	Oedema reduction (%)
Controls	—	10	7.2 ± 0.5	—
Extract A	100	10	6.2 ± 0.3*	14
	300	10	5.0 ± 0.3*	31
	900	10	3.6 ± 0.4*	51
Indometacin	100	10	2.9 ± 0.2*	60

Oedema values are expressed as mean ± s.d. *$P < 0.05$ at the analysis of variance, as compared with controls.

dose-dependent oedema inhibition with a potency ten times lower (ID50 = 900 $\mu g\,cm^{-2}$) than that of indometacin.

Cell viability on cell cultured in monolayers

To establish the effects of extract A and its main constituents on the viability of cells grown in monolayers (ML), the extract (1–1000 $\mu g\,mL^{-1}$), compound **1** (4′-methoxy myricetin 3-*O*-α-L-rhamnopyranoside, 0.01–1 mM), **2** (myricetin 3-*O*-α-L-rhamnopyranoside, 0.01–1 mM) and **3** (myricetin 3-*O*-β-D-glucopyranoside, 0.01–1 mM) were each tested on three different cell lines J774.A1 (murine monocyte/macrophage cells), WEHI-164 (murine fibrosarcoma cells) and HEK-293 (human epithelial kidney cell) by the MTT assay (Autore et al 2001). They did not show any significant cytotoxic effect (data not shown).

Cutaneous irritation assays on 3D model

Extract A (10%) and its major constituent, myricetin 3-*O*-α-L-rhamnopyranoside (**2**, 1%), in PBS were applied on the surface of a commercially available human reconstituted epidermis (RHE; Skinethic, Nice, France), in comparison with PBS as control and SDS (0.25%) as a cutaneous irritant. After topical exposure for 24 and 72 h, cell viability by MTT assay, 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 (Bernard et al 2000; Meloni et al 2002).

The epidermis viability, measured as optical density (OD) at 570 nm by MTT assay and calculated as percentage of cytotoxicity compared with the control (PBS-treated epidermis), was not reduced after topical application of either the extract or compound **2**, 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 **2**, both at 24 and 72 h, compared with 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^{-1} at 24 h and to 110 pg mL^{-1} at 72 h (Figure 2).

Finally, histological analysis of RHE treated with the extract or compound **2** (for 24 and 72 h) did not show significant modifications in the tissue morphology, at both stratum corneum and viable epidermis levels, compared with the control. In this condition the irritant reference compound (SDS 0.25%) caused tissue degeneration and necrosis. Representative photographs of tissues treated for 24 h with extract and compound **2** are shown in Figure 3C, D; tissue treated with the control (PBS) is shown in figure 3A; and histological changes after exposure to severe skin irritant SDS are illustrated in Figure 3B.

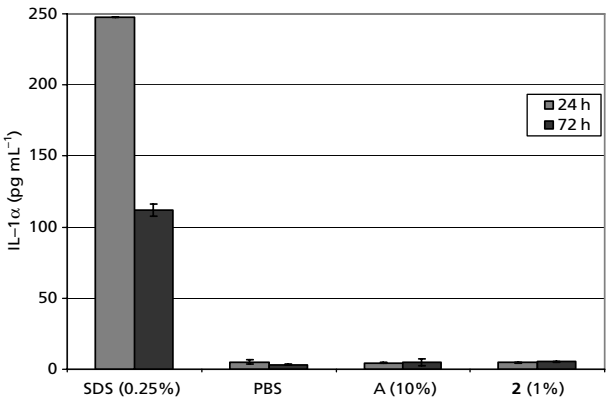

Figure 2 Effect of Extract A (10%) and compound **2** (1%), on IL-1α release (pg/mL of medium), estimated by ELISA, in the reconstituted human epidermis (RHE) after 24 and 72 h of exposure in comparison with control (PBS), and irritant reference compound SDS (0.25%). Values are expressed as mean $\pm$ s.d. of three independent experiments.

Discussion

Although the leaves of *L. microcarpa* are empirically used in African folk medicine, this is the first study on their biological properties as well on their chemical composition. This research showed that the extract of *L. microcarpa* contains 4′-methoxy myricetin 3-*O*-α-L-rhamnopyranoside (**1**), myricetin 3-*O*-α-L-rhamnopyranoside (**2**) and myricetin 3-*O*-α-L-glucopyranoside (**3**) as the major constituents, and a series of other polyphenols, such as vitexin (**4**), isovitexin (**5**), gallic acid (**6**) and epi-catechin (**7**). Polyphenols are a class of natural plant products and common components in the human diet, showing a wide range of biochemical and pharmacological effects (Laughton et al 1991; Packer et al 1999). Particularly, myricetin derivatives, vitexin and isovitexin, widely diffused in tea leaves, possess anti-oxidant, anti-inflammatory, and anti-thrombotic properties (Takino et al 1962).

Reports of biological effects of *Lannea* skin formulations (Malgras 1992; Pauly et al 2001) led us to investigate the in-vivo topical anti-inflammatory activity of the extract using the croton oil ear test in mice. The extract showed a potency (ID50 = 900 μg cm^{-2}) ten times lower than that of indometacin (ID50 = 93 μg cm^{-2}) in reducing the oedematous response.

Information about a drug's potential to 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 this contribution was also to provide a method for investigating cutaneous irritation of plant extracts and bioactive molecules using both monolayer cell cultures (ML model) and reconstituted human epidermis (RHE, 3D model). The RHE model has been reported to mimic morphologically and biochemically the living skin to a significantly higher degree than monolayer cultures. It has been used to investigate complementary parameters of the irritation mechanism by the application of products directly onto the skin surface (Bernard et al 2000; Meloni et al 2002).

Based on the observation that cytotoxic agents inhibit dehydrogenase activity of cells, a dye assay for cytotoxicity was developed by Mosmann (1983) in which a yellow tetrazolium salt (MTT) is reduced to blue formazan only by living cells. This method is usually applied with cells grown in monolayers or suspensions (ML); the tested drugs have to be added as aqueous solutions to the cell media, thus, it is impossible to test dermal formulations.

We carried out a preliminary screening of cytotoxicity by the MTT test using three different (J774.A1, HEH-293 and WEHI-164) cell cultures as general monolayer (ML) models. Cell viability was evaluated after dissolution in the cell media of 0.01–1 mM PBS solutions of pure compounds **1**–**3** and 1–1000 μg mL^{-1} of the extract. Using the RHE (3D model), higher concentrations of pure compounds (**2**, 1%) and of the extract (10%) could be tested after topical applications to the epidermis surface. To discriminate very slightly irritant products able to induce pro-inflammatory mediator release without affecting tissue integrity, we

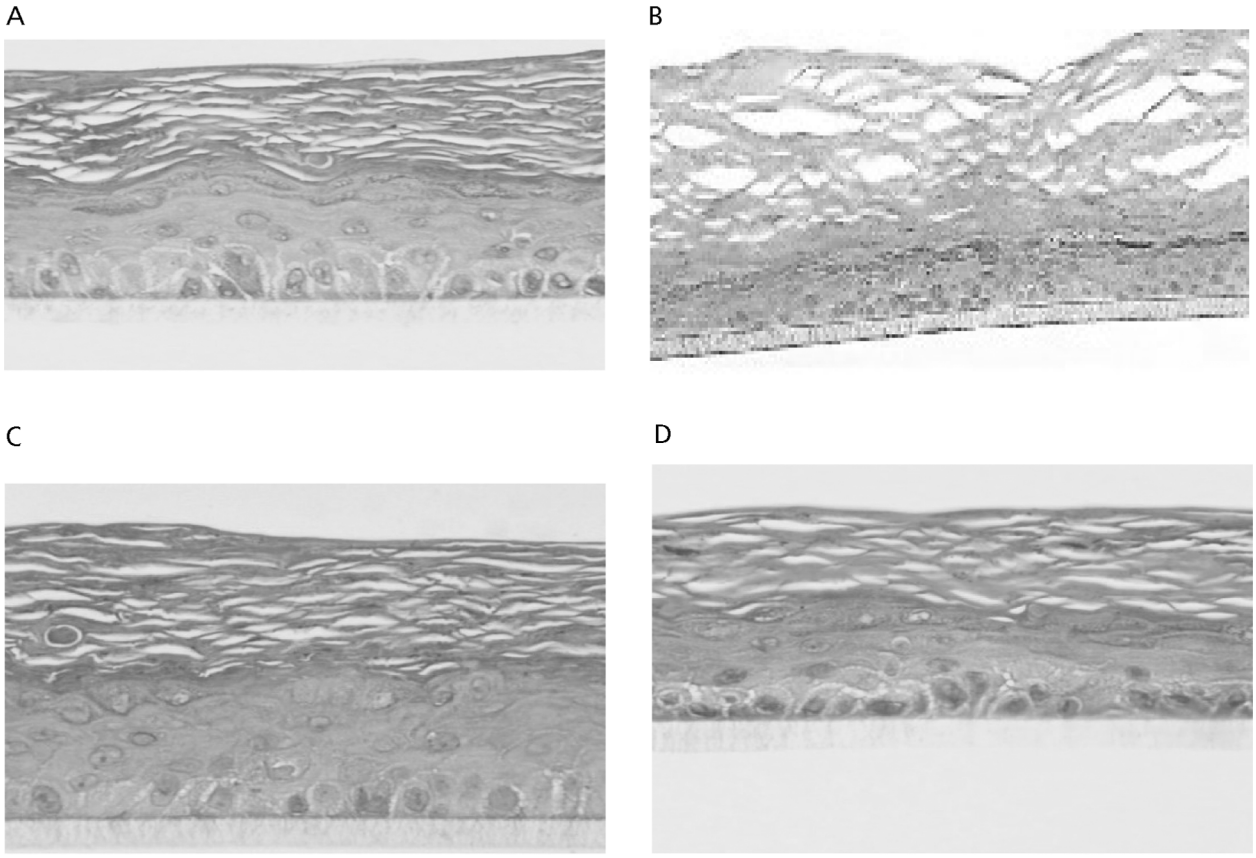

Figure 3 Histopathological examination of in-vitro reconstituted human epidermis (RHE) model samples after 24 h exposure to control (PBS) (A), irritant compound SDS (0.25%) (B), compound **2** (1%) (C) or Extract A (10%) (D) as a topical application. Note, in B, application of SDS shows severe epidermal changes with cellular necrosis.

evaluated three end-points (cell viability, release of IL-1α, histological analysis). After topical exposure to the extract and compound **2** at concentrations similar to those used in topical formulations in-vivo (10% plant extract and 1% plant bioactive molecules), the results indicated that the extract and its constituents appear to be safe for topical use.

Conclusion

This research showed that the polar extract of *L. microcarpa* leaves contains myricetin derivatives as the major constituents and a series of other phenols that may all contribute to the biological activity of the extract, providing a support to the traditional use of the plant in the folk medicine of Africa against inflammatory- and oxidative-based disorders. Extract A, showing promising anti-inflammatory activity, and its major constituent **2**, appear to be suitable for cutaneous application using a skin model at high concentrations and for exposure times tested, based on the lack of cytotoxicity both on cells cultured in monolayers (ML) and reconstituted epidemis (RHE), on the low pro-inflammatory cytokine (IL-1 α) release comparable with the control (Figure 2) and on the observation of a regular morphology in all epidermis layers at 24 and 72 h in RHE (Figure 3).

In addition, utilization of in-vitro tests using both monolayer cell cultures (ML) and 3D model for the evaluation of the irritant potential of active ingredients on the skin seem to offer several advantages: firstly, comparability of data on cell viability by MTT assay obtained both from cell cultures (ML) and RHE model; and, secondly, the possibility of evaluating in-vitro the cytotoxic effects and the cutaneous irritation of both complex plant extracts and bioactive molecules alone, at the same concentrations used in topical formulations in-vivo.

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