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Antileishmania and Immunostimulating Activities of Two Dimeric Proanthocyanidins From *Khaya senegalensis*

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Abstract

Bioassay-guided fractionation of the bark of *Khaya senegalensis* (Desr.) A. Jus. (Meliaceae) led to the isolation of two dimeric proanthocyanidins, catechin-(4α,6)-catechin (**1**) and catechin-(4α,8)-catechin) (**2**), with immunostimulating activities. Their structures were determined by chromatographic and spectroscopic methods (HREIMS, EIMS, ^{1}H-NMR). Anti-*Leishmania* screening of extracts and pure compounds was conducted against *Leishmania donovani*, *L. major*, *L. infantum* promastigotes and retested against *L. donovani* amastigotes persisting in RAW macrophages as host cells. Their cytotoxicity was evaluated against three mammalian cell lines (RAW, A549 and KB). Isolated compounds (**1**) and (**2**) were not active against promastigotes (EC_{50} > 25.0 µg/ml), but exhibited significant effects when tested against amastigotes indicating an indirect immunostimulating principle (EC_{50} = 3.85 and 3.98 µg/mL, respectively) with no cytotoxicity.

Keywords: *Leishmania*, antiprotozoal, proanthocyanidins, tannins, *Khaya senegalensis*, Meliaceae, immunomodulation, *in vitro*, ethnomedicine.

Introduction

Khaya senegalensis (Desr.) A. Jus. (Meliaceae) has a wide range of applications in traditional African medicine. It is used as a bitter tonic, fever remedy, vermifuge, taenicide, emmenagogue, and as antimicrobial agent for the treatment of venereal diseases (Bever, 1986; Iwu, 1993). Its value in traditional veterinary practice has also been reported (Iwu, 1993; Kasonia & Ansay, 1994). *K. senegalensis* has been the subject of extensive phytochemical and pharmacological investigations (Bever, 1986; Iwu, 1993; Olayinka et al., 1994; Lompo et al., 1998; El-Tahir et al., 1998; Fall et al., 1999). The plant yields limonoids (Adesida et al., 1971; Olmo et al., 1996; Olmo et al., 1997; Govindachari & Kumari, 1998; Khalid et al., 1998; Govindachari et al., 1999) that constitute the bitter principle of the bark (Iwu, 1993), that also contains 2,6-dimethoxy-*p*-benzoquinone, β-sitosterol, and its β-D-glucoside, catechin, tannins, saponins, polysaccharides, and coumarins. The coumarins found in the plant have been associated with analgesic, antipyretic, and moderate anticonvulsant action. The crude aqueous alcohol extracts of the stem bark possess sedative and reduced locomotor activity, CNS depressant activity in mice (Iwu, 1993), as well as antiprotozoal activity (El-Tahir et al., 1999).

From our continued phytochemical studies on the antiprotozoal activity of *Khaya senegalensis*, we confirmed antiparasitic activity previously reported (El Tahir et al., 1999) showing antiplasmodial activity below an EC_{50} of 5 µg/mL (*P. falciparum*, strain 3D7 and Dd2). In preliminary phytochemical analysis of different fractions, limonoids have been found responsible for antimalarial activity. Although these triterpenes are well known constituents of *Khaya* spp., thus far they have only been tested against *Plasmodium falciparum*. Pursuing our search for new antileishmanial natural products from plants, we re-investigated *Khaya senegalensis* for its potential to inhibit *Leishmania* growth *in vitro*.

To our surprise we did not detect antiprotozoal activity against *Leishmania* promastigotes, as we could have expected from related malaria screening (Bickii et al., 2000). Activity was only significant when we tested the compounds

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against the amastigote forms, the stage after invading the *Leishmania* parasite in the macrophages.

The discrepancy between no direct antileishmanial effects against promastigotes on one hand and the alleged efficacy of plant extracts and specific compounds against intracellular pathogens on the other, could best be explained by an auxiliary stimulation of macrophages mediated by biological response modifiers. To assess the immunostimulating principal of the plant extracts, we isolated the active compound using bio-guided fractionation and employed an *in vitro* infection model with macrophages infected with *Leishmania donovani*. Criteria for an activating effect were dissemination of the intracellular persisting parasites combined with low macrophage toxicity. Of the multitude of functions and molecular signals involved in cellular immune response, the potential of tumour necrosis factor (TNF) induction was selected for evaluation following recent reports on immunomodulatory activities of tannins.

Materials and methods

Plant material and extraction

Collection of the plant material and preparation of the extracts were previously described (Abreu et al., 1999). A voucher specimen is on deposit at the Chemistry Department, CQFB, Faculty of Sciences and Technology, New University of Lisbon, 2825-114, Caparica, Portugal.

Isolation of compounds

The two dimeric proanthocyanidins were isolated from the crude methanolic extract (30 g). The residual aqueous phase was successively extracted with petrol ether (10 × 500 mL), $CHCl_3$ (8 × 400 mL), and EtOAc (10 × 400 mL). Evaporation of the solvent produced a brown residue in each instance (1.25, 1.71, and 2.48 g, respectively). After antileishmanial testing of the obtained phases, the bioactive ethyl acetate phase was refractionated by column chromatography on Sephadex LH-20 with MeOH/H_2O (10:90) with an increasing amount of the organic phase (10:90 to 70:30). Seventeen crude subfractions were collected and tested for their antileishmanial activity against *L. donovani* amstigotes. Biologically active fractions of the test tubes 601–675 (fraction 11) and 825–900 (fraction 15) were further rechromatographed by HPLC on Eurospher 100C18 (25 × 0.8 cm, 5 µm) using H_2O/MeOH, (4:1 to 1:1) at a flow rate of 5 mL/min with detection wave length of 275 nm over 30 min. Compound (**1**) (3.5 mg) was isolated directly from fraction 11, whereas compound (**2**) (2.8 mg) was isolated from fraction 15 by successive HPLC (H_2O/MeOH, 100:0 to 80:20). The hydrolysis of (**1**) with 5% ethanolic HCl afforded the same two catechin units, identified as cyanidin and delphinidin by comparison with authentic material. From both free phenolic proanthocyanidins, NMR spectra were recorded in CD_3OD with TMS as internal standard.

Catechin-(4α,8)-catechin (**1**): positive FAB (*m/z* %) 579 $[M+H]^+$ (44) 601 $[M+Na]^+$ (11), HREIMS: 530.46106 $[C_{30}H_{26}O_9]^+$. [calcd for ($C_{30}H_{26}O_9$) 530.46112] EIMS *m/z* (% rel.int): 290.1 (4.7), 272.0 (0.7), 256.1 (0.6), 225.0 (1.38) 151.9 (4.9), 139.0 (6.9), 123.0 (8.7), 110.0 (3.9), 61.1 (36.9), 44.0 (26.9). ^{1}H-NMR (400 MHz, CD_3OD): δ 2.40–4.80 [m, 7× heterocyclic H (C, F)], 5.8–6.15 [m, 4× aromatic H (A, D)], 6.32–7.18 [m, 6× aromatic H (B, E)].

Fisetinidol-(4α,6)-catechin (**2**): positive FAB (*m/z* %) 563 $[M+H]^+$ (2) 585 $[M+Na]^+$ (2), HREIMS: 530.46106 $[C_{30}H_{26}O_9]^+$. [calcd for ($C_{30}H_{26}O_9$) 530.46112] EIMS *m/z* (% rel.int): 290.1 (15), 256.0 (7.1), 181.0 (5.0), 169.0 (10.0), 123.0 (8.7), 110.0 (100), 91.0 (12.0), 44.0 (21.29). ^{1}H-NMR (400 MHz, CD_3OD): δ 2.25–4.75 [m, 8× heterocyclic H (C, F)], 5.8–6.15 [m, 4× aromatic H (A, D)], 6.88 [d, H-8 (A)], 6.95 [s, H-8′ (D)], 6.45–6.85 [m, 9× aromatic H (A, B, E)].

Antileishmanial assays

Experimental procedures and general data for these assays are fully described elsewhere (Kayser et al., 1999; Kiderlen & Kaye, 1990). In short, *Leishmania donovani* LV9, *L. infantum* strain D.SCH., *L. enriettii*, and *L. major* LV39 promastigotes in stationary culture phase were seeded at 1 × 10^4/100 µL/well in 96-well flat-bottom microtiter plates in *Leishmania* based on RPMI 1640 medium. Test compounds or drug standards were dissolved in DMSO and added in further 100 µL/well to give final concentrations of 50 µg/mL and serial two-fold dilutions thereof. Promastigotes were incubated over a period of 92 h in a humidified incubator at 25 °C, 6% CO_2 before MTT (20 µL/well of a 5 mg/mL PBS stock) was added for a further 6 h. MTT metabolization was stopped and produced formazan crystals solubilized with SDS. The relative absorbence, i.e., the relative amount of formazan/well produced by

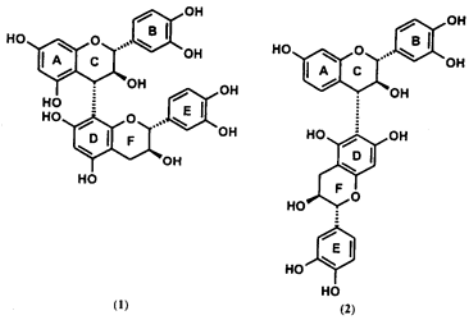

Figure 1. Catechin-(4α,8)-catechin (= proanthocyanidin B3) (**1**) and fisetinidol-(4α,6)-catechin (**2**).

viable cells, was measured photometrically at 570 nm. Leishmanicidal effects were expressed as LD_{50} values, i.e., the concentration of a compound which caused 50% reduction in parasite viability. For testing leishmanicidal activity against intracellular amastigotes, murine bone marrow culture-derived MΦ (BMMΦ) were infected *in vitro* with *L. donovani* parasites, seeded at 1×10^5 BMMΦ/100 µL/well in RPMI 1640 medium supplemented with antibiotics, 20 mM Na-pyruvate and 10% heat-inactivated fetal calf serum (herein designated R10 medium) and allowed 24 h at 37 °C for internalized *Leishmania* to transform into the amastigote form. Further 100 µL R10/well with serial dilutions of the test compounds were added and the cells incubated for 72 h when BMMΦ were lysed with SDS to release intracellular parasites. The lysates were cultured at 25 °C for 3–4 d to allow viable parasites to transform back to promastigotes and the relative number of *Leishmanial*/well was determined colorimetrically with MTT as described above.

Cytotoxicity assays

All compounds were tested against three mammalian cell lines: non-infected RAW macrophages, squamous carcinoma (KB) and lung carcinoma (A 549). Cells were exposed to linear two-fold dilutions of test compounds for 48 h directly as activity described above. MTT was added for the final 6 h and cytotoxic effects expressed as the concentration of a compound which provoked a 50% reduction in cell viability compared to cell cultures in R10 alone.

TNF analysis

TNF-α secretion was measured by modification of an ELISA, as previously described in (Ding et al., 1988). TNF-α is directly and indirectly involved in host defense against bacteria, eukaryotic parasites, and tumor cells, and a useful parameter for macrophage activation. Its quantitation in a functional bioassay has the advantage of recording only functionally active molecules. Highly pure, resting murine macrophages were obtained from bone marrow stem cell cultures and exposed to compounds for 24 h. Bacterial entotoxin (LPS) was used as a positive control for the induction of TNF-release by macrophages. The supernatants of these cultures were harvested and tested for TNF-activity by their lytic effect on murine TNF-sensitive L929 fibroblasts. For this, actinomycin D-sensitized L929 (TNF) were exposed to a dilution series of the respective test supernatant. Actinomycin-D further enhances the cytopathic effect of TNF, reducing total assay time and detection threshold. Recombinant murine TNF-α was used as a positive control. After 24 h, supernatants were replaced by a methanol/crystal violet solution, thereby fixing and staining all intact cells. Excess dye and cell lysate were removed by repeated rinsing in dd H_2O before drying the plates and then dissolving the remaining crystal violet in acetic acid. Relative optical density (OD) and thus the relative amount of viable cells/well was read at 592 nm. Units/mL TNF-activity were defined by interpolation as the dilution necessary to achieve half-maximum lysis of L929 (TNF) cells. These values were standardized relative to a laboratory TNF standard to account for fluctuations in assay sensitivity. This functional assay does not discriminate between TNF-α or -β. However, with highly pure macrophage cultures as the only source of TNF in this system, all TNF-activity detected was attibuted to TNF-α.

Results and discussion

From bio-guided screening, two tannins were isolated after tlc detection with vanilin/HCl reagent. Initial fractionation of the aqueous methanolic extract was achieved by chromatography over Sephadex LH-20 with increasing amount of methanol to furnish 17 fractions that were tested for their antileishmanial activity. Fractions 11 and 15 were most active with EC_{50} = 4.2 and 5.8 µg/ml, respectively. Both fractions were separately subjected to HPLC with a variety of solvent systems to give two proanthocyanidins (**1**) and (**2**).

Compounds (**1**) and (**2**) were differing in the mass and molecular formula determined as $C_{30}H_{25}O_{12}$ for (**1**) and $C_{30}H_{25}O_{11}$ for (**2**), as established by positive FAB. Compound (**1**) gave a red coloration (characteristic of proanthocyanidins) with the vanilin-HCl reagent. The occurrence of two flavan units in the molecule was deducted from the appearance of two flavan signals at δ 5.47 due to the respective H-2 protons whose detectable coupling constants (J = 6.73) suggested that (**1**) consists entirely of flavan-3-ol units with catechin stereochemistry. The identity of (**1**) was confirmed by comparison of the hydrolysis products of (**1**) with authentic samples of cyanidin and delphinidin. Compound (**2**) displayed chromatographic properties and colour reactions similar to those of (**1**). The presence of two flavan units was evident from the the downfield position of the aromatic A-ring proton resonances (δ 6.45–6.85) (Santos Buelga et al., 1995). Characterization, however, was preliminary based on comparison with authentic material (Santos Buelga et al., 1995).

The *in vitro* antileishmanial activity of (**1**) and (**2**) against *L. donovani*, *L. major*, *L. infantum*, and *L. enriettii* is shown in Table 1 in comparison to pentamidine-isethionate as an antileishmanial reference drug. Tested against promastigotes, no activity was detected (EC_{50} > 50 µg/ml). In contrast, when tested against amastigotes, both compounds exhibited high toxicity against intracellular persisiting pathogens (EC_{50} = 3.85 and 3.98 µg/mL, respectively). To exclude toxic effect against the host cells as possible mode of action, compounds were retested for cytotoxicity against RAW-macrophages, squamous carcinoma (KB) and lung carcinoma (A 549). Obtained effective doses (EC_{50}) indicated no toxicity up to 25 µg/mL. These data in total indicate that tested compounds act indirectly on *Leishmania* parasites. The pronounced

Table 1. Antileishmanial, cytotoxic and TNF-inducing activity of (**1**) and (**2**).

No	*L. major*[1] extra.	*L. donovani*[1] extra.	*L. infantum*[1] extra.	*L. enriettii*[1] extra.	L. donovani[1] intra.	RAW-Tox[1]	A549[1]	KB[1]	TNF [U/mL]
(1)	>25.0	>25.0	>25.0	>25.0	3.85	>25.0	>25.0	>25.0	164
(2)	>25.0	>25.0	>25.0	>25.0	3.98	>25.0	>25.0	>25.0	167
P	2.7	2.5	1.4	2.8	7.8	>25.0	n.d.	n.d.	n.d.
IFN/ LPS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	184

[1] Values indicate the effective concentration of a compound in µg/mL necessary to achieve 50% growth inhibition (EC_{50}). n.d. = not determined, P = Pentostam®, extra. = extracellular, intra. = intracellular.

effects only against amastigotes were on the first view contrary, but the reason might be explained by immunostimulating of macrophage defense mechanisms.

As integral part of the immune system macrophages play a major role for the cleaving of invading infectious pathogens. Even activated macrophages (e.g., IFNγ) show enhanced microbiocidal activity and produce reactive intermediates, e.g., nitric oxide radicals, reactive oxygen radicals and additional compounds thereof. These reactive intermediates have been identified as powerful intracellular effector molecules. As best shown for the *in vitro Leishmania* infection model, TNF-α/β-release is an essential mechanism in the activation of *Leishmania* infected macrophages. For this reason we determined the content of TNF-α/β induced by catechin-(4α,8)-catechin (**1**) and fisetinidol-(4α,6)-catechin (**2**). TNF concentration was assessed in a functional bioassay according to Ding et al. (1988) by photometrically determination of the lysis of TNF-sensitive cells. Both compounds proved to exhibit strong TNF-induction at the antileishmanial, with EC_{50}-concentrations reaching 110 and 112%, respectively, of the amount induced by IFN/LPS (= 100%). It should be mentioned that both compounds did not induce IFN release in our test system (unpublished data).

These results are in line with previously found immunostimulating effects of a series of similar tannins (Kolodziej et al., 2001) documenting the high activity of hydrolysable and condensed tannins on the TNF and IFN-release of murine bone marrow macrophages *in vitro*. It is obvious that further experiments are needed to elucidate the mode of action of *Khaya senegalensis* tannins on the antileishmanial activity, but the documented activity provide a first basis that not only limonoids and related terpenoid compounds are responsible for the claimed antiparasitic activity of the ethnomedicinally used plant.

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