# Tannins and Related Compounds: Killing of Amastigotes of *Leishmania donovani* and Release of Nitric Oxide and Tumour Necrosis Factor $\alpha$ in Macrophages *in vitro*

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The antileishmanial and immunomodulatory potencies of a series of 28 polyphenols were evaluated in terms of extra- and intracellular leishmanicidal activity and macrophage activation for release of nitric oxide (NO), tumour necrosis factor (TNF) and interferon (IFN)-like properties. For this, several functional bioassays were employed including an *in vitro* model for leishmaniasis in which murine bone marrow-derived macrophages (BMMΦ) were infected with the obligate intracellular parasite *Leishmania donovani*, an extracellular *Leishmania* proliferation assay, a fibroblast-lysis assay (TNF-activity), and a biochemical assay for NO. Except for gallic acid, its methyl ester, shikimic acid and catechin ($EC_{50}$ 25.8–67.9 nM) all polyphenols tested significantly inhibited the intracellular survival of *L. donovani* amastigotes ($EC_{50}$ 0.4–13.9 nM) when compared with the clinically used agent, sodium stibogluconate ($EC_{50}$ 10.6 nM). In contrast, none of the samples proved to be directly toxic for the extracellular promastigote form of the parasite. Noteworthy, the phenolic samples showed only moderate or no cytotoxicity against the murine host cells ($EC_{50}$ 10 to >144 nM). Although NO is an important effector molecule in macrophage microbicidal activity, the inducing potential of the test compounds for its release was found to be very moderate ranging from 7–54 μM (IFN-γ/LPS 119 μM). On the other hand, inhibition of NO production had no apparent effect on intracellular leishmanicidal activity of polyphenols. Their *in vitro* TNF-inducing potential producing 50% lysis in murine L929 cells increased in the order of simple phenols and flavanols (34–48 U/ml) < A-type proanthocyanidins (53–80 U/ml) < B-type proanthocyanidins (64–200 U/ml) < hydrolyzable tannins (287–350 U/ml) at the host cell subtoxic concentration of 50 μg/ml. Furthermore, gallic acid and some hydrolyzable tannins showed appreciable IFN-like activities ( 14–23 U/ml) as reflected by inhibition of the cytopathic effect of encephalomyocarditis virus on fibroblast L 929 cells. The results provide a rational basis for the recorded anti-infectious efficacy of traditionally used herbal medicines containing tannins *in vivo*, in the light of both only moderate direct antimicrobial activities of distinct polyphenols *in vitro* and the limited knowledge on their uptake in humans.

## Introduction

Leishmaniasis is a major public health problem with many clinical manifestations in humans. In their mammalian hosts, protozoa of the genus *Leishmania* are obligate intracellular parasites of the monocyte-macrophage system. Species of the parasitic protozoa *Leishmania* are estimated to threaten some 350 million people with a broad range of diseases and to infect an estimated 1.4 million people annually world-wide (Ashford *et al.*, 1992; WHO Report, 1993). Also noteworthy is the alarming increase in *Leishmania*/HIV co-infection, especially in the Mediterranean area. *Leishmania donovani*, a haemoflagellate protozoan parasite, is the causative agent of potentially fatal visceral leishmaniasis (VL). Despite the major adverse impact of *Leishmania* parasites on human

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populations throughout the world, only a few new drugs such as allopurinol, amphotericin B formulations, hexadecyl-phosphocholin, ketoconazole and paromomyxin are currently on clinical trial since the introduction of the pentavalent antimonials, the first choice of drugs (Kayser *et al.*, 1999a). These standard therapeutics, however, have severely toxic side effects and variable efficacy especially in AIDS-related VL (Bermann, 1997). Additionally, resistance of parasite strains has arisen and is spreading. Given the limitations of the current treatments for leishmaniasis, there is clearly an urgent need for the development of new therapeutics to alleviate the human morbidity and mortality caused by these infections. In endemic countries, a number of traditional plants are commonly used to treat infectious conditions, thus providing promising sources for finding new anti-infectious lead compounds. Advances in the research of natural products for the treatment of leishmaniasis have been recently reviewed (Akendengue *et al.*, 1999). However, there are no data regarding the use of tannins to treat this severe debilitating parasitic disease. Following our recent more systematic investigation of aurones based on structure-activity relationships (Kayser *et al.*, 1999b), we now report on antileishmanial effects of some tannins and related compounds on both *Leishmania donovani* promastigotes and amastigotes, taking into account their direct toxic effects on macrophage host cells in *in vitro* cultures.

Having also in mind the relatively moderate antimicrobial activity of chemically defined tannin molecules (Kolodziej *et al.,* 1999), though polyphenols are generally regarded as quite potent antibiotics (Haslam, 1989), and the limited knowledge on their uptake in humans, we also evaluated their inducing potential for release of nitric oxide (NO), tumour necrosis factor (TNF) and interferon (IFN)-like activity in bone marrow-derived macrophages (BMMΦ). Stimulation of the non-specific immune system by ingested polyphenolic metabolites present in herbal preparations may provide a scientific basis for claimed remedial effects in infectious conditions. The aim of the present study was to assess direct anti-parasitic and immunomodulatory activities of a series of polyphenols as clues for the efficacy of traditionally used herbal medicines against various microbial infections.

## Materials and Methods

### *Test compounds*

Compounds (**1**, **2**, **6**, **8** and **9**) were isolated from *Pelargonium* species (Kolodziej, 2000), (**7** and **18**) from *Hamamelis virginiana* (Hartisch and Kolodziej, 1996), and (**19–21**) from *Aesculus hippocastanum* (Santos-Buelga *et al.*, 1995), while the remaining polyphenols were available as reference samples either in the research group of H. K. (**3**, **10–17**) or in that of D. F. (**22–28**). The samples (**4** and **5**) were kindly provided by Dr. Nishioka, Japan. The identity of the compounds was proved by MS and NMR spectroscopy. Sodium stibogluconate (Pentostam®; kindly provided by Dr Goldbach, Glaxo Wellcome, Germany) and amphotericin B (Sigma, Deisenhofen, Germany) were used for comparison as standard drugs of anti-leishmanial activity. Recombinant murine γ-interferon (rIFN-γ), expressed in *E. coli*, was produced by Genentech, San Francisco, USA and kindly provided by Bender & Co., Wien, Austria.

### *General procedures and assays for leishmanicidal and cytotoxic activity*

General experimental procedures and assays for extra- and intracellular leishmanicidal activity including cytotoxicity testing against host cells are fully described elsewhere (Kiderlen and Kaye, 1990; Kayser *et al.*, 1997; Kayser *et al.*, 1999b).

### *Assay for NO production in activated BMMΦ*

BMMΦ, cultured in R10 medium at 37 °C, 5% $CO_2$ in humidified air, were seeded at $1 \times 10^5$ / ml in 96-well microtiter plates and activated by incubating in the same medium containing 50 μg/ml of test compounds. After 48 h, the supernatants were collected as a source of secreted NO. To measure the nitrite concentration derived from NO, 100 μl of culture supernatants were mixed with 100 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylendiamine dihydrochloride/3% $H_3PO_4$) (Ding *et al.*, 1988). After the chromophore was formed at room temperature for 5 min, absorbance was determined at 550 nm. The nitrite concentration was calculated from a standard curve generated with $NaNO_2$. The experiments were performed in duplicates.

*Inhibition of iNO synthase and activation for intracellular* Leishmania *kill*

Inducible NO synthase (iNOS) from BMMΦ was prepared as described (Kiderlen, 1996). The enzyme activity after treatment of the preparation with the test compounds was determined, using an HPLC method for the determination of amino acids (Algermissen *et al.*, 1980). The assay is based on the enzymatic oxidation of L-arginine to yield citrulline and nitric oxide. Detection of citrulline was achieved by its derivatization with *ortho*-diphthalaldehyde using a fluorescence detector. Since citrulline can easily be converted into ornithine, both amino acids were quantified, using homoserine as internal standard. In parallel experiments, iNOS was blocked by addition of $N^{(G)}$-monomethyl-L-arginine (L-NMMA) (200 μM) and enzyme inhibition confirmed by the absence of its products. The survival rates of *Leishmania* parasites in sample-stimulated BMMΦ that had been pretreated with L-NMMA (inactived iNOS) was determined as described (Kayser *et al.*, 1999b).

*Assay for TNF release*

BMMΦ were incubated in R10 medium (Kayser *et al.*, 1999b) in 96-well microtiter plates. Cells were allowed to adhere overnight, then non-adherent cells were gently washed out twice with fresh medium. Test samples were added to the first wells of each row at 50 μg/ml in a final volume of 200 μl R10/well and 12 serial twofold dilutions performed across the plates for each sample and the medium control. Treatment with bacterial endotoxin (*Salmonella abortusequi*) at 100 ng lipopolysaccharide (LPS)/ml served as positive control for TNF induction. After 18 h incubation at 37 °C in 6% $CO_2$ in humidified air, the supernatants were collected and stored at −20 °C. For determining TNF activity, TNF-sensitive murine L929 fibroblasts were seeded at $3\times10^4$/100 μl R5/well (Kayser *et al.*, 1999b) in microtiter plates and incubated overnight to achieve a monolayer of cells in late logarithmic growth phase. Culture supernatants were then replaced by fresh medium containing actinomycin D to inhibit further proliferation and render L929 cells more sensitive for the cytotoxic effect of TNF. Serial twofold dilutions of samples were carefully performed over the monolayer in parallel with recombinant murine TNF-α as positive control. Media additives were calculated for final concentrations of 2 μg/ml actinomycin D and 5% fetal calf serum in all wells. Following an incubation period of 18 h, culture supernatants were replaced by methanolic crystal violet, thereby staining all intact cells. After washing the plates with distilled $H_2O$, the remaining dye crystals were dissolved in acetic acid and the preparations subjected to photometric analysis. Relative optical density, correlating with the relative amount of viable cells/well was measured at 570 nm. TNF units/ml were calculated as the reciprocal values of the TNF-containing supernatant dilution that would cause 50% lysis of L929 cells (Fraunhofer Institute of Toxicology, Hannover, Germany). These values were correlated with the TNF standard to account for fluctuation in assay sensitivity. This functional assay does not discriminate between TNF-α and -β.

*Assay for IFN induction and detection of IFN-activity*

$1\times10^5$ BMMΦ cells/200 μl R5/well were incubated for 48 h with test compounds (50 μg/ml) or medium alone before further fetal calf serum (10% final concentration) was added and the supernatants removed and stored at −20 °C. Interferon activity was assessed as capacity to protect murine L929 fibroblasts from the cytopathic effect of murine encephalomyocarditis virus (EMCV, generously provided by Lohmann-Matthes, Fraunhofer Institute of Toxicology, Hannover, Germany) following with minor modifications the procedures reported by Marcucci *et al.* (1982). In brief, a monolayer of EMCV- and IFN-sensitive murine L929 fibroblasts ($2 \times 10^4$ cell/100 μl R5/well) was incubated in R5 alone or with dilutions of the experimental culture supernatants described above. After 8 h, 100 μl of a pretested EMCV suspension was added to each well and the cells incubated for another 18–24 h. Within this period, nonprotected cells disintegrated due to the cytopathic effect of the virus. The relative number of viable cells per well was assessed colorimetrically by the crystal violet method as described above for the TNF bioassay. The cytoprotective effect of tannin-stimulated BMMΦ supernatants was compared to that of recombinant murine IFN-γ. Antiviral protection (IFN activity) was ex-

pressed in units/ml, defined as reciprocal value of the supernatant dilution that would inhibit 50% of the cytopathic effect induced by EMCV on L929 cells. These values were correlated with the IFN standard to account for any fluctuation in assay sensitivity. This functional assay does not discriminate between IFN-α, -β, and -γ.

## Results and Discussion

Tannins represent a unique group of phenolic metabolites in woody and some herbaceous plants (Porter, 1994). These metabolites exhibit a remarkably wide range of biochemical and pharmacological activities *in vitro* including antioxidant, antitumour, antiviral, antimicrobial, enzyme-inhibiting, and radical-scavenging properties, and are believed to be the principle curative and palliative agents of a variety of traditional herbal medicines (Okuda *et al.*, 1992; Haslam, 1996). In studies to identify the active principle(s) of *Pelargonium* species used for treatment of infections of the respiratory tract, we have demonstrated that phenolic constituents showed marked antileishmanial and immunoenhancement effects (Kolodziej, 2000; Kayser *et al.*, 2001). Encouraged by our recent results, we extended our studies to a series of tannins to evaluate their leishmanicidal potential, their possible role as NO-inducer in host defence, and their capabilities to stimulate release of tumour necrosis factor (TNF) as well as interferon (IFN)-like activities. Attention is given to structure-activity relationships, with emphasis on simple phenols and the two major groups of polyphenols, the hydrolyzable tannins and the proanthocyanidins.

An *in vitro* infection model with obligate intracellular *Leishmania* parasites is a valuable tool for assessing both antiparasitic and immunomodulatory potency of lead structures for potential therapeutics. Phagocytes of the monocyte/macrophage lineage (MΦ) play an essential role both as regulator and effector cells of the immune response to invading microorganisms. However, for certain microorganisms they also function as facultative or obligatory host cells. Hence, MΦ are a prime target for immunomodulatory agents and a great challenge for intracellular, antimicrobial therapy. The antileishmanial activity of compounds **1**–**28** (Figs 1 and 2) was assessed against both extracellular promastigotes and intracellular amastigotes of *Leishmania donovani*. As a parameter for antileishmanial activity, the $EC_{50}$ value, the sample concentration causing 50% reduction in survival/viability of the parasites, was used. The *in vitro* leishmanicidal activities of the test compounds are shown in Table I with amphotericin B and sodium stibogluconate (Pentostam ®) as references. As can be seen, none of these phenolic samples showed selective toxicity when tested against the extracellular *L. donovani* promastigotes ($EC_{50}$ >25.0 mg/ml). In contrast, most compounds showed considerable activity against *L. donovani* amastigotes with $EC_{50}$ values ranging from 0.4–13.9 nM (0.5–8.8 μg/ml), when compared with the $EC_{50}$ value 10.6 nM (7.9 μg/ml) of the clinically used antileishmanial drug, Pentostam®. Compounds **15**, **18** and **24** exhibited the highest relative toxicity for intracellularly persisting *L. donovani* parasites with $EC_{50}$ values of 0.4–0.9 nM (0.5–0.9 μg/ml), which also compared favourably with that of amphotericin B (Table I).

The difference in leishmanicidal activity of the phenolic samples against *L. donovani* amastigotes and promastigotes may be due to dissimilar biochemical or metabolic characters to the two stages of the parasite. On the other hand, antiparasitic polyphenols might function indirectly by activating MΦ for leishmanicidal functions as has been shown for the physiological activator MΦ IFN-γ (Kiderlen and Kaye, 1990).

Concerning structure-activity relationships, it would appear that both the presence and number of galloyl elements are crucial for marked leishmanicidal potency as is evident from the series of shikimic acid derivatives (**3**–**7**) and the flavan-3-ols (**10**–**11**), while the limited number of ellagitannins (**8**–**9**) does not provide additional structural clues in this group of compounds. For proanthocyanidins, comparison of the antileishmanial activity indicated that 4α,8-coupled dimers of the B-type (**12** and **13**) were more active than their 4ß,8-counterparts (**14** and **15**) (Table I). Conspicuously, the $EC_{50}$ values for representatives of 5-deoxy analogues (**22**–**28**) indicated higher potencies for the 4ß,8-compounds relative to their 4α,8-analogues. It also appeared that an increase in molecular weights (dimers **12**–**15** and **22**–**28** vs. trimer **16** and **17**) did not enhance antileishmanial activity, while the pronounced activity of oligomer **18** may be attributable to the presence of galloyl groups.

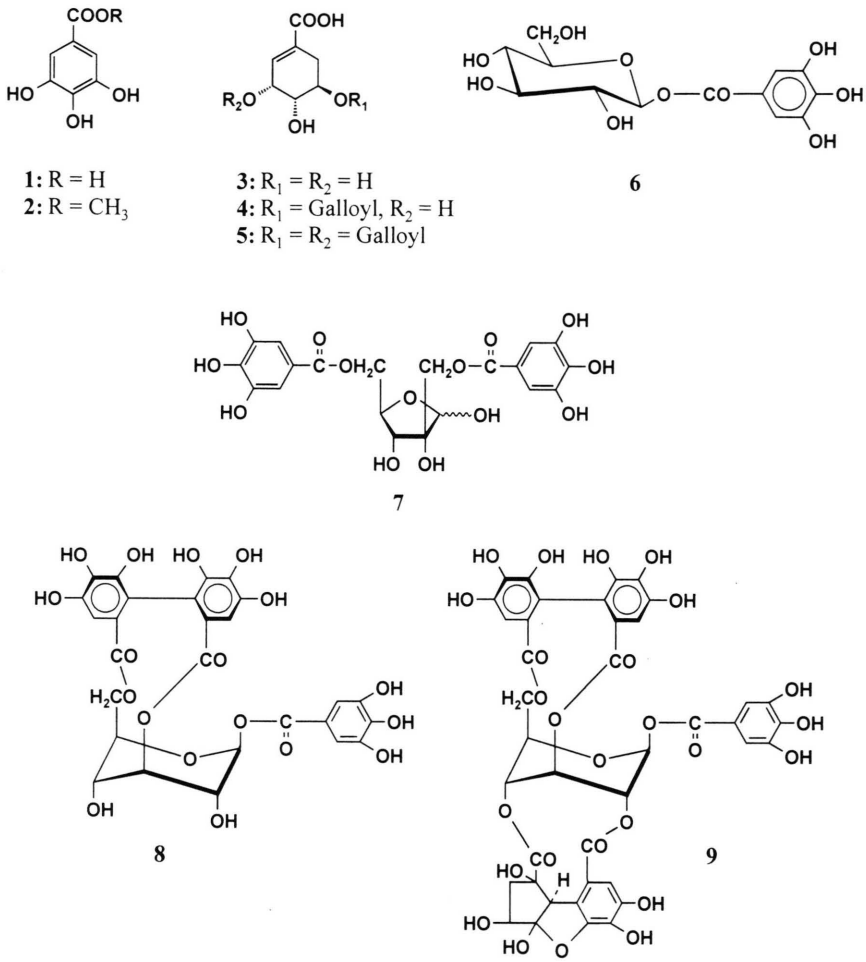



Fig. 1. Structures of the galloyl derivatives (**1–6**) and the hydrolyzsable tannins (**7–9**).

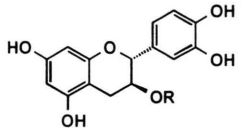
When tested against murine bone marrow-derived macrophages (BMMΦ) as a mammalian host cell control, all compounds revealed no or only moderate cytotoxicity ($EC_{50}$ 10 to > 144 nM; >25.0 μg/ml) (Table I).

Phagocytes, representing an integral part of the immune system, are known to produce reactive oxygen species that have potent antimicrobial activity. For example, there is confirming evidence that NO produced by inducible NO synthase (iNOS) from L-arginine plays an important role as effector molecule in macrophage cytotoxicity of a number of microorganisms (Moncarda *et al.*, 1991; Nathan and Hibbs, 1991; Nussler and Biliar, 1993). Compared with the IFN/LPS stimulus, the NO-inducing effect of the samples accounted only for 14%–45% at a sample concentration of 50 μg/ml (Table II). The most potent NO-inducers of the series of samples tested were gallic acid (**1**), its methyl ester (**2**) and the galloylated procyanidin oligomer (**18**) with NO release of 54.0, 32.0 and 31.4 μM, respectively.

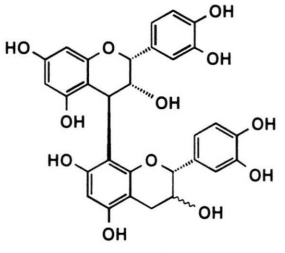
A closer reflection of the role of induced NO in killing amastigotes in sample-activated BMMΦ was obtained from the relative number of surviving organisms of *L. donovani*, following SDS lysis of the host cells. With gallic acid (**1**) as the most potent NO-inducer, the survival rate of the parasites was < 1% at subtoxic concentration ranges of 30–60 μg/ml. However, when NO-radical production itself was inhibited by addition of $N^{(G)}$-monomethyl-L-arginine (L-NMMA), this had no

effect on the antileishmanial potential of polyphenols. This phenomenon may, at least in part, be attributable to the well-known radical scavenging activities of phenolics, thus rendering extracellular NO non-detectable for the Griess assay, while the intracellular leishmanicidal activity of NO-radicals remained unaffected. This finding might also indicate the activation of additional NO-independent leishmanicidal mechanisms in activated BMMΦ. In this context it is appropriate to note that similar experiments using macrophage-like RAW cells revealed significant differences in the survival rate of *Leishmania* parasites in experiments with and without inhibitor (unpublished data). The apparent dependency of the *Leishmania* kill/gallic acid-induced NO relationship on the cell line is the subject of current research.

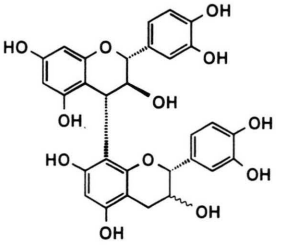
Macrophage activation usually is a polyphenotypic event, leading to enhanced effector- and regulator functions. Enhanced TNF-release is a hallmark of MΦ activation and necessarily involved in NO-mediated killing of *Leishmania* parasites in MΦ (Roach *et al.*, 1991). Here, polyphenols stimulated murine MΦ for marked release of biologically active TNF, often close to or well above positive control levels (Table II) and regularly in parallel to intracellular *Leishmania* kill. All phenolic samples showed TNF-inducing activities producing 50% lysis in murine fibroblast cells ranging from moderate to pronounced potentials (34–350 U/ml), with hydrolyzable tannins appearing to be generally more potent. Starting with simple phenols, gallic acid **(1)**, its methyl ester **(2)**, and shikimic acid **(3)** stimulated BMMΦ only moderately for TNF-release (34–39 U/ml). However, introduction of galloyl groups as reflected in **(4)** and **(5)** dramatically enhanced the amount of cytokine released (146 and 306 U/ml, respectively), indicating a strong dependency of the stimulatory activity on the degree of galloylation. This assumption is further substantiated by similarly high TNF production induced by the digalloylated compound, hamamelitannin **(7)** (350 U/ml). Enhancement in potency also applied to structurally related molecules possessing an HHDP moiety derived from two adjacent galloyl groups, as reflected by corilagin **(8)** and phyllantusiin C **(9)** with inducing po-



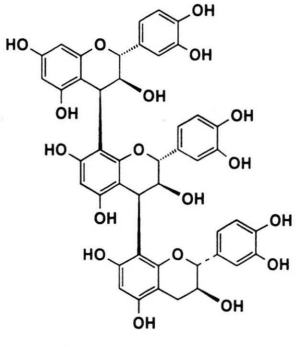
**10**: R = H
**11**: R = Galloyl



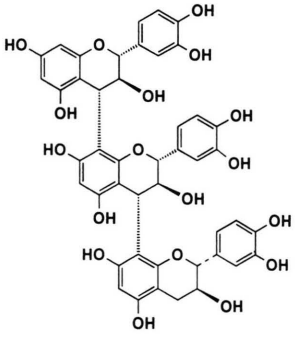
**12**: ⦚ = |
**13**: ⦚ = ⦙



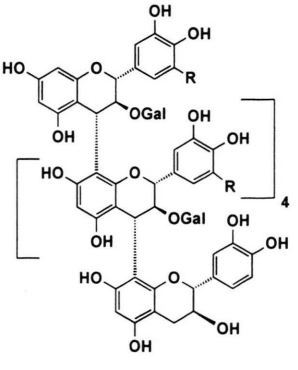
**14**: ⦚ = |
**15**: ⦚ = ⦙



**16**



**17**



**18**: R = H or OH

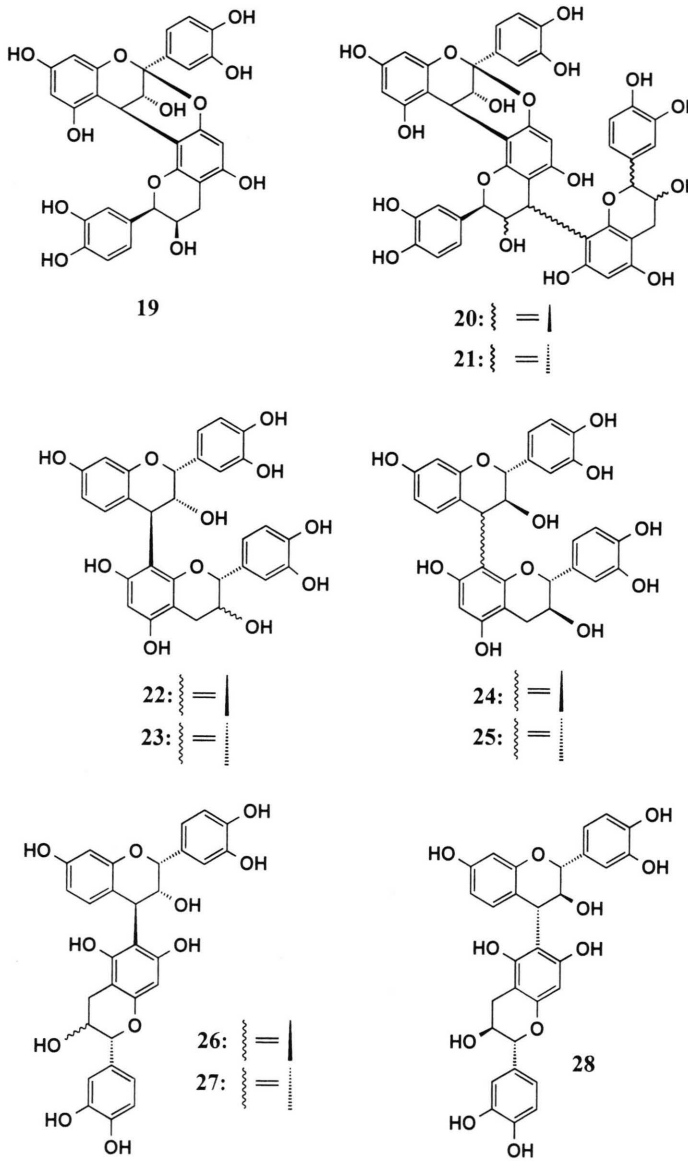



Fig. 2. Structures of the flavan-3-ols (**10–11**) and the proanthocyanidins (**12–28**).

tentials of 287 and 350 U/ml, respectively. The presence of two phenolic rings (galloyl group, HHDP moiety) linked by a spacer which might place them in a position suitable for binding to cellular signaling elements, could be an important structural requirement for an effective polyphenolic TNF inducer in this group of polyphenols.

In the series of flavanol-3-ols, catechin **(10)** showed a similar moderate stimulatory potential as indicated above for simple phenols. Again, the presence of a galloyl group as reflected in (**11**) significantly enhanced TNF release (Table II), in analogy to recent reports on IL-1 stimulating activity of epicatechin, epigallocatechin and their 3-O-galloyl analogues (Sakagami *et al.*, 1992a and 1992b).

Within the group of proanthocyanidins, most of the B-type members showed similar TNF-inducing capabilities (Table II) irrespective of the degree of polymerisation, indicating that an increase in the

Table I. Antileishmanial activity (*L. donovani*) and host cell (BMM Φ) cytotoxicity of compounds **1–28** ($EC_{50}$ values in nM; μg/ml in parentheses).

| Compound | Leishmanicidal activity | | | | BMMΦ Toxicity | |
|---|---|---|---|---|---|---|
| | Extracellular | | Intracellular | | | |
| *Simple phenols* | | | | | | |
| **1** | >47.0 | (>25.0) | 25.8 | (4.4) | 91.8 | (15.6) |
| **2** | >35.8 | (>25.0) | 67.9 | (12.5) | 96.7 | (17,8) |
| **3** | >143.7 | (>25.0) | 38.5 | (6.7) | >43.7 | (>25.0) |
| **4** | >76.7 | (>25.0) | 11.3 | (3.7) | 32.8 | (10.7) |
| **5** | >52.3 | (>25.0) | 1.9 | (0.9) | >52.3 | (>25.0) |
| **6** | >75.3 | (>25.0) | 8.1 | (2.7) | >75.3 | (>25.0) |
| *Hydrolyzable tannins* | | | | | | |
| **7** | >51.6 | (>25.0) | 6.0 | (2.9) | >51.6 | (>25.0) |
| **8** | >39.4 | (>25.0) | 13.9 | (8.8) | >39.4 | (>25.0) |
| **9** | >27.0 | (>25.0) | 8.1 | (7.5) | >27.0 | (>25.0) |
| *Flavan-3-ols* | | | | | | |
| **10** | >86.2 | (>25.0) | 50.3 | (14.6) | 67.9 | (19.7) |
| **11** | >56.6 | (>25.0) | 10.6 | (4.7) | >56.6 | (>25.0) |
| *Proanthocyanidins* | | | | | | |
| *B-types* | | | | | | |
| **12** | >43.2 | (>25.0) | 7.1 | (4.1) | >43.2 | (>25.0) |
| **13** | >43.2 | (>25.0) | 5.0 | (2.9) | >43.2 | (>25.0) |
| **14** | >43.2 | (>25.0) | 1.9 | (1.1) | >43.2 | (>25.0) |
| **15** | >43.2 | (>25.0) | 0.9 | (0.5) | >43.2 | (>25.0) |
| **16** | >28.9 | (>25.0) | 6.7 | (5.8) | >28.9 | (>25.0) |
| **17** | >28.9 | (>25.0) | 9.8 | (8.5) | >28.9 | (>25.0) |
| **18**[a] | >9.8 | (>25.0) | 0.4 | (0.9) | >9.8 | (>25.0) |
| *A-types* | | | | | | |
| **19** | >43.4 | (>25.0) | 2.2 | (1.3) | >43.4 | (>25.0) |
| **20**) | >28.9 | (>25.0) | 1.4 | (1.2) | >28.9 | (>25.0) |
| **21** | >28.9 | (>25.0) | 1.7 | (1.5) | >28.9 | (>25.0) |
| *5-Deoxy analogues* | | | | | | |
| **22** | >44.5 | (>25.0) | 2.1 | (1.2) | >44.5 | (>25.0) |
| **23** | >44.5 | (>25.0) | 5.5 | (3.1) | >44.5 | (>25.0) |
| **24** | >44.5 | (>25.0) | 1.9 | (1.1) | >44.5 | (>25.0) |
| **25** | >44.5 | (>25.0) | 0.9 | (0.5) | >44.5 | (>25.0) |
| **26** | >44.5 | (>25.0) | 5.9 | (3.3) | >44.5 | (>25.0) |
| **27** | >44.5 | (>25.0) | 6.6 | (3.7) | >44.5 | (>25.0) |
| **28** | >44.5 | (>25.0) | 1.4 | (0.8) | >44.5 | (>25.0) |
| References | | | | | | |
| Na stibogluconate | 3.5[b] | (2.6[b]) | 10.6 | (7.9) | n.d.[c] | n.d.[c] |
| Amphotericin B | 0.03 | (0.03) | 0.3 | (0.3) | n.d.[c] | n.d.[c] |

[a] Calculated for a 1:1 ratio of procyanidin/prodelphinidin extender units.
[b] the activity against promastigotes is probably due to the presence of the preservative, chlorocresol (Kayser *et al.*, 1999b).
[c] n.d., not determined.

flavanyl chain length did not necessarily enhance induction of TNF-release. Noteworthy is the fairly high potency of (4,8)-linked dimers with flavan-3-ol units of 'mixed' stereochemistry **(12, 15** and **22)** relative to those containing either 2,3-*trans* **(14** and **24)** or 2,3-*cis* configurated moieties **(13** and **23)** exclusively. Within this group of 'mixed' representatives, it also appeared that TNF-release increased in the presence of 2,3-*cis* configurated chain extender units **(12** and **22)** when compared to the 2,3-*trans* analogue **(15)**. The significance of the associated 4β-orientation of the interflavanyl linkage is reflected by the higher stimulatory activity of fisetinidol-4β,8-catechin **(25)** relative to that of its 4α,8-analogue **(24)**. Unlike the conventional (4,8)-linked proanthocyanidins **(12–18)**, prominent TNF production was observed for (4,6)-coupled 5-deoxy compounds possessing upper and lower flavanyl moieties of the same relative 2,3-configuration **(27, 28)**. A further group of com-

Table II. NO-, TNF- and IFN-inducing potential of compounds **1–28** in BMM Φ.

| Compound | Nitric oxide [a] | Tumour necrosis factor [b] | Interferon [c] |
|---|---|---|---|
| *Simple phenols* | | | |
| **1** | 54.0 | 39 | 17.9 |
| **2** | 32.0 | 35 | n.d. |
| **3** | 18.9 | 34 | n.d. |
| **4** | 17.4 | 146 | n.d. |
| **5** | 19.7 | 306 | n.d. |
| **6** | 20.4 | 37 | n.d. |
| *Hydrolyzable tannins* | | | |
| **7** | 21.6 | 350 | n.d. |
| **8** | 20.8 | 287 | 14.4 |
| **9** | 21.7 | 350 | 22.4 |
| *Flavan-3-ols* | | | |
| **10** | 17.5 | 48 | n.d. |
| **11** | 18.9 | 256 | n.d. |
| *Proanthocyanidins* | | | |
| *B-types* | | | |
| **12** | 21.4 | 175 | n.d. |
| **13** | 19.7 | 118 | n.d. |
| **14** | 17.8 | 84 | n.d. |
| **15** | 22.4 | 153 | n.d. |
| **16** | 17.0 | 148 | n.d. |
| **17**) | 25.8 | 183 | n.d. |
| **18** | 31.4 | 185 | n.d. |
| *A-types* | | | |
| **19** | 17.6 | 80 | n.d. |
| **20** | 24.3 | 53 | n.d. |
| **21** | 22.4 | 74 | n.d. |
| *5-Deoxy analogues* | | | |
| **22** | 24.7 | 134 | n.d. |
| **23** | 21.1 | 64 | n.d. |
| **24** | 19.9 | 64 | n.d. |
| **25** | 18.9 | 125 | n.d. |
| **26** | 21.3 | 64 | n.d. |
| **27** | 22.1 | 200 | n.d. |
| **28** | 24.7 | 167 | n.d. |
| IFN (100 U/ml) + LPS (10 ng/ml) | 119.0 | 184 | – |

[a] μM; assessed as nitrite.
[b] U/ml, calculated as the reciprocal of the values of the macrophage supernatant dilution that would cause 50% lysis of the L929 cells.
[c] U/ml, calculated as the reciprocal of the values of the macrophage supernatant dilution that would cause 50% inhibition of the cytopathic effects of EMC virus on L929 cells.
n.d., not detectable.

pounds possessing doubly-linked units **(19–21)**, showed only moderate TNF-inducing capability (53–80 U/ml). This observation indicated that conformational rigidity imposed by the presence of two interflavanyl linkages is not favourable in this respect.

Regarding possible interferon-like activity of the tested polyphenols, only gallic acid **(1)** and the hydrolyzable tannins **(8)** and **(9)** inhibited the cytopathic effect of murine EMC virus on IFN- and virus-sensitive L929 fibroblasts, as revealed by means of the MTT assay with *ca* 70–95% maximum cell viability relative to the positive control. The interferon-like activity of these compounds producing 50% inhibition of the cytopathic effect of murine EMC virus on L929 cells ranged from 14–23 U/ml (Table II). All other samples exhibited only negligible protective effects at subtoxic concentrations up to 25 μg/ml. In order to differentiate direct anti-virus-protection from an indirect activity where the sample first stimulates the target cells to produce IFN which in turn mediates

protection from EMC virus, L929 cells were incubated with polyphenols. Supernatants were collected and supplemented with albumin to preclude possible virus-polyphenol interactions by precipitating traces of polyphenols still present in the supernatants. These supernatants were added to fresh L929 cells for standard IFN-detection. Again, only culture supernatants of fibroblasts treated with compounds **(1)**, **(8)** or **(9)** showed prominent IFN-activity. This finding clearly indicates that the IFN-like activity of distinct polyphenols did not result from an IFN-activity *per se*, but rather from the induction of IFN in L929 fibroblasts. These findings are presently being followed up in detail.

From these limited data, conclusions regarding structure-activity relationships are difficult to make. It may be postulated that the presence of galloyl moieties significantly contributes to inhibition of cytopathic effects. However, there are a few compounds, for example (**7**), that exhibited only low cytoprotective properties, despite the presence of galloyl groups. On the other hand, gallic acid itself showed significant inhibitory activity of cytopathic effects. The possibility that gallic acid was released from parent derivatives was ruled out by careful examination of incubations for co-occurring traces of this phenol. IFN induction then seems to be correlated with the whole structure of each polyphenolic compound, but distinct structural features may be identified by extending the range of hydrolyzable tannin samples.

In conclusion, these data not only support previously reported immunomodulatory activities of tannins and related compounds, but also provide first evidence for their NO-, TNF-, and possibly IFN-inducing properties including some structure-activity relationships. Although not yet confirmed by *in vivo* data, the demonstrated immunological responses provide a sound basis for the recorded efficacy of polyphenolic herbal medicines for the treatment of infectious conditions. The general emerging picture is that anti-infectious protection by certain polyphenols may be attributed to both direct antimicrobial effects, whenever possible, and stimulation of the nonspecific immune system. However, since several immunological factors are associated with the immune response, further experiments are needed to understand the roles of polyphenols in the stimulation of immune reactions.

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