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Review

Antileishmanial activity and immune modulatory effects of tannins and related compounds on *Leishmania* parasitised RAW 264.7 cells

Herbert Kolodziej [a,*], Albrecht F. Kiderlen [b]

[a] *Freie Universität Berlin, Institute of Pharmacy, Pharmaceutical Biology, Königin-Luise-Str. 2+4, D-14195 Berlin, Germany*
[b] *Robert Koch-Institut, Department of Infectious Diseases, Nordufer 20, D-13353 Berlin, Germany*

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Abstract

The antileishmanial and immunomodulatory potencies of a total of 67 tannins and structurally related compounds were evaluated in terms of extra- and intra-cellular leishmanicidal effects and macrophage activation for release of nitric oxide (NO), tumour necrosis factor (TNF) and interferon (IFN)-like activities. Their effects on macrophage functions were further assessed by expression analysis (iNOS, IFN-α, IFN-γ, TNF-α, IL-1, IL-10, IL-12, IL-18). With few exceptions, e.g., caffeic acid derivatives, these polyphenols revealed little direct toxicity for extracellular promastigote *Leishmania donovani* or *L. major* strains. In contrast, many polyphenols appreciably reduced the survival of the intracellular, amastigote parasite form in vitro. Upon activation, e.g., by immune response mediators such as IFN-γ, macrophages may transform from permissive host to leishmanicidal effector cells. Our data from functional bioassays suggested that the effects of polyphenols on intracellular *Leishmania* parasites were due to macrophage activation rather than direct antiparasitic activity. Gene expression analyses not only confirmed functional data, they also clearly showed differences in the response of infected macrophages when compared to that of noninfected cells. Conspicuously, infected macrophages showed augmented and prolonged activation of host defense mechanisms, indicating that parasitised macrophages were exquisitely predisposed or "primed" to react to activating molecules such as polyphenols. This promotive effect may be of special benefit, e.g., stimulation of the non-specific immune system selectively at the site of infection and when needed. Although these data provide the basis for an immunological concept of plant polyphenols for their beneficial effects in various infectious conditions, in vivo experiments are essential to prove the therapeutic benefits of polyphenolic immunomodulators.

Keywords: *Leishmania*; Polyphenols; Hydrolysable tannins; Proanthocyanidins; Macrophages; Antileishmanial activity; Immunomodulatory activity; Nitric oxide; Cytokines; Gene expression analysis

Contents

* Corresponding author. Tel.: +49 30 838 53731; fax: +49 30 838 53729.
E-mail address: kolpharm@zedat.fu-berlin.de (H. Kolodziej).

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1. Introduction

The leishmaniases comprise a group of diseases with extensive morbidity and mortality in most developing countries. They are caused by species of the genus *Leishmania* (Sarcomastigophora, Kinetoplastida) and range from self-healing cutaneous leishmaniasis (CL) to progressive mucocutaneous infections (MCL) to fatal disseminating visceral leishmaniasis (VL). While CL poses basically cosmetic problems, and MCL leads to painful disfiguration, social stigmatisation and often severe secondary infections, VL is generally lethal if left untreated. According to the World Health Organization, leishmaniasis currently affect some 12 million people and there are 2 million new cases per year and with growing tendency. Moreover, it is estimated that approximately 350 million people live at risk of infection with *Leishmania* parasites (Ashford et al., 1992). Leishmaniases are prevalent in 88 countries throughout the world in tropical to Mediterranean climate zones, including 22 in the New World and 66 in the Old World; of these, 72 are developing countries (Desjeux, 1996). CL is endemic in Iran, Saudi Arabia, Syria, Afghanistan and in some South American countries. More than 90% of the VL cases worldwide are registered in India, Bangladesh, Indonesia and Sudan. In Mediterranean Europe, poor-health communities and certain risk groups such as intravenous drug abusers sharing needles and immunodeficient persons (e.g., AIDS-patients) are strongly affected. *Leishmania*/HIV co-infections have increased in Mediterranean countries, where up to 70% of potentially fatal VL cases are associated with HIV infection, and up to 9% of AIDS cases suffer from newly acquired or reactivated VL (Alvar et al., 1997).

Protozoa of the genus *Leishmania* are obligate intracellular parasites of mononuclear phagocytes of vertebrate hosts (Alexander and Russell, 1992). The pathogen requires two different hosts to complete its biological cycle: an insect vector (sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World), and a vertebrate host (e.g., humans, rodents, dogs). To survive successfully and multiply within these two disparate biological environments, the parasites must undergo profound biochemical and morphological adaptations (Alexander et al., 1999). Within the insect vector, the parasite exists as extracellular, motile flagellate in the gut. During a blood meal, promastigotes are discharged in the bloodstream and are rapidly phagocytized by Langerhans cells, macrophages, monocytes, or, transiently, by neutrophils. Within these cells they reside in compartments originating from the plasma membrane, the phagosomes, and transform into nonmotile amastigotes. Lysosomes readily fuse with the phagosomes, but *Leishmania* amastigotes not only resist phagolysosomal enzymes, they also thrive and multiply within the acidic, hydrolase-rich parasitophorous vacuole. Massive amastigote multiplication leads to host cell disruption and release of amastigotes to infect newly recruited host cells. Though the parasite is sensitive to humoral defense mechanisms such as antibodies or the complement system, its intracellular habitat offers almost complete protection. Only if the macrophage is activated, the parasites may be killed and then degraded by the host cell. Ingestion of infected peripheral monocytes during the blood meal by a female sandfly completes the biological cycle.

Macrophage activation, i.e., conversion of a host to an effector cell, occurs both during natural (innate) and specific (adaptive) immune reactions to the infection in an immunocompetent host. It is induced by interferon (IFN)-γ, a cytokine that is released mainly by appropriately stimulated natural killer or T cells. Activation of microbicidal mechanisms in macrophages may also be achieved by their exposure to immunomodulating agents. Immunomodulatory activities have been shown for a number of plant extracts and natural products, providing a rational explanation for their medicinal

application. With respect to leishmaniasis, an ideal drug should have both direct and selective leishmanicidal effects as well as the ability to properly activate the patient's immune system.

The recommended drugs for leishmaniasis are the pentavalent antimonials sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime). Both drugs have been used for over 50 years, the history and use of antimonials (Berman, 1988) as well as the pharmacology of leishmaniasis (Balana-Fouce et al., 1998) have being excellently reviewed. Although these antimonials are successfully employed worldwide, severe side effects and a dramatic increase in the number of cases that do not respond to these drugs represent critical limitations in the treatment of these parasitic diseases (Kayser et al., 2002). Alternative treatments include the polyene antibiotic amphotericin B, the aminoglycoside paromomycin, the alkylphospholipid miltefosine, and ketoconazole. Limited advances in the chemotherapy of leishmaniasis have been the introduction of new dose regimens or new formulations of old drugs, such as encapsulated preparations of amphotericin B, although costs of such treatment render them useless for third world countries. Similarly, the topical or systemic application of recombinant IFN-γ, alone or in combination with, e.g., antimonial drugs, is of little practical use due to high costs, though highly efficient in otherwise treatment-resistant cases. Except for Miltefosine, no new drugs have been developed for the treatment of leishmaniasis since the introduction of the antimonials more than 80 years ago. These, however, have variable efficacy and severe side effects.

The leishmaniases, traditionally considered rather exotic diseases of tropical areas, are beginning to have a major impact on human populations of the developed world and is compounded by more ready access to international travel and the carelessness of people, while the expansion of both the insect vector and the parasites due to global warming is similarly crucial (Maier et al., 2003). Taking into account the annual incidence of about 2 million new cases and the deficiencies in current drug therapy, there is an urgent need for new and innovative antileishmanial drugs.

In endemic countries, a number of traditional plants are commonly used to treat infectious conditions, thus providing promising sources for finding new antiinfectious lead compounds. The renewed interest in plant products with antileishmanial activities has been stimulated, at least in part, by the identification of licochalcone A as a potential drug against *Leishmania, Trypanosoma*, and *Plasmodium* parasites (Chen et al., 1994). Advances in the research of natural products for the treatment of leishmaniasis have been recently reviewed (Akedengue et al., 1999; Corona et al., 2000; Chan-Bacab and Pena-Rodriguez, 2001; Kayser et al., 2002). However, there are no data regarding the use of tannins and related compounds to treat this severe debilitating or potentially lethal parasitic disease.

Table 1
Antileishmanial activity (EC_{50} values in μM[a]) and NO-, TNF- and IFN-inducing potential of classes of polyphenols (sample concentration 50 μg/ml) in RAW 264.7 cells[b]

Class of polyphenol	Antileishmanial activity[c]	NO[d] (μM)	TNF[e] (U/ml)	IFN[f] (U/ml)
Simple phenols (**1**,**2**,**56**–**58**)	2–250	17–54	35–310	0–18
Hydrolysable tannins				
Gallotannins (**3**–**8**)	<1–8	ca. 20	ca. 20	0–20
Ellagitannins (**9**–**13**)	<1–14	ca. 20	0–25	0–38
Dehydroellagitannins (**14**–**18**)	<1–2	ca. 20	270–350	5–65
C-Glucosidic ellagitannins (**19**–**21**)	<1–3	ca. 20	80–320	14–35
Oligomers (**22**–**29**)	<1–3	ca. 20	n.d.	n.d.
Flavan-3-ol derivatives (**30**–**35**)	3–50	8–25	0–140	0–40
Proanthocyanidins				
A-types (**49**–**51**)	1–3	ca. 20	50–80	n.d.
B-types (**36**–**41**)	1–10	ca. 20	30–70	0–10
5-Desoxy analogues (**42**–**48**)	1–7	ca. 20	60–200	n.d.
Oligomers (**52**–**55**)	1–4	ca. 20	0–30	0–40
Caffeic acid derivatives (**59**–**65**)	4–175	ca. 20	25–120	n.d.
Miscellaneous (**66**–**67**)	1–50	ca. 20	n.d.	ca. 5
Reference				
Pentostam®	10	–	–	–
IFN-γ (100 U/ml)/LPS (10 ng/ml)	–	119	184	–

n.d. = not detectable.

[a] Some previous data were erroneously given in nM, and apparently also by others, e.g., Germonprez et al. (2004), regarding Pentostam.

[b] Kolodziej et al. (2001a, 2001b); Kiderlen et al. (2001); Radtke et al. (2003).

[c] Activity against amastigotes.

[d] Assessed as nitrite.

[e] U/ml, calculated as the reciprocal of the values of the macrophage dilution that would cause 50% lysis of the L929 cells.

[f] U/ml, calculated as the reciprocal of the values of the macrophage dilution that would cause 50% inhibition of the cytopathic effect of EMCV on L929 cells.

2. Antileishmanial activity

Tannins represent a unique group of phenolic metabolites in numerous woody and some herbaceous higher plant species (Porter, 1994). These secondary products exhibit a remarkably wide range of biochemical and pharmacological activities in vitro including antioxidant, antitumour, antiviral, antimicrobial, enzyme-inhibiting, and radical scavenging properties. Their presence in fairly high concentrations has commonly been used to explain the claimed curative and palliative efficacy of a variety of traditional herbal medicines and the reports of profound health-beneficial effects of certain foodstuffs (Haslam et al., 1989; Okuda et al., 1992; Haslam, 1996). Treatments of a broad spectrum of infectious conditions with polyphenolic herbal medicines prompted our interest in the evaluation of polyphenols as promising antileishmanial agents.

A series of 67 tannins and structurally related compounds (**1**–**67**) including simple phenols (**1**, **2**, **56**–**58**), hydrolysable tannins (**3**–**29**), flavan-3-ols (**30**–**35**), proanthocyanidins (**36**–**55**), caffeic acid-derived metabolites (**59**–**65**), and miscellaneous polyphenols (**66**–**67**) was tested for activity against promastigotes and amastigotes of *Leishmania donovani* (agent of VL) and *L. major* (agent of CL). As a parameter for antileishmanial activitiy, the EC_{50} value, the sample concentration causing 50% reduction in survival/viability of the parasites was used, while the first-line clinical antileishmanial drug, Pentostam®, served as a positive control.

In vitro studies on the susceptibility of *Leishmania* promastigotes to the polyphenols revealed that none of these compounds showed selective toxicity for the extracellular form (EC_{50} > 25 μM) except for some caffeic acid derivatives (Radtke et al., 2003). Following incubation of the promastigotes with the samples for 72 h, the tested polyphenols did not show any antiprotozoal effects.

In contrast, pronounced effects against amastigotes were found for a number of phenolic samples in our in vitro model for leishmaniasis (Table 1). For this, macrophages were infected with *Leishmania* promastigotes, allowed 24 h for transformation into amastigotes, and were then further incubated with the phenolic samples. The host cells were lysed and the relative number of viable *Leishmania* organisms determined using a standard MTT assay (Kiderlen and Kaye, 1990). With EC_{50} values mostly in the range of 1–2 μM, hydrolysable tannins proved to be considerably leishmanicidal when compared with the EC_{50} value 10 μM of Pentostam®. Pronounced activities were observed for the gallotannins pentagalloylglucose (**5**) and tannic acid (**6**), the ellagitannin hippophaenin A (**13**), and most of the members of the dehydroellagitannin group (**14**–**18**) (EC_{50} 0.4 μM) (Kolodziej et al., 2001b). It would therefore appear that the number of galloyl groups were crucial for marked leishmanicidal potency of gallotannins as is also evident from the series of shikimic acid derivatives (**56**–**58**) (EC_{50} 2–38 μM), while the presence of a DHHDP moiety enhanced the activity of ellagitannins.

Compared to hydrolysable tannins, the antileishmanial activity of flavan-3-ol derivatives (**30**–**35**) (EC_{50} 3–50 μM) and proanthocyanidins (**36**–**55**) was generally less pronounced (EC_{50} 1–10 μM). Again, the marked leishmanicidal potency of flavan-3-ols was apparently associated with the presence of 3-*O*-acyl groups (EC_{50} 3–10 vs 5 μM). For proanthocyanidins, no significant differences in antileishmanial activity were evident, irrespective of the type of compound, the constituent flavanyl moiety and the molecular weight, i.e., A-types (EC_{50} 1–3 μM), B-types (EC_{50} 1–10 μM), 5-deoxy derivatives (EC_{50} 1–7 μM).

Within the group of miscellaneous compounds tested, pseudotsuganol (**66**) showed marked antileishmanial activity. Since 5,5′-bisdihydroquercetin (**67**) was inactive, this finding implicated that the crucial structural element for activity of **66** may be the pinoresinol moiety rather than the taxifolin unit, consistent with reports on the antiprotozoal activity of lignans (Barata et al., 2000; Bastos et al., 1999). Conspicuously, caffeic acid (**59**) representing the essential building block of a series of sage phenolics (**60**–**65**) exhibited the relatively strongest activity among these test compounds (EC_{50} 6 vs 15–175 μM), demonstrating that interesting biological activities are not confined to exotic molecules.

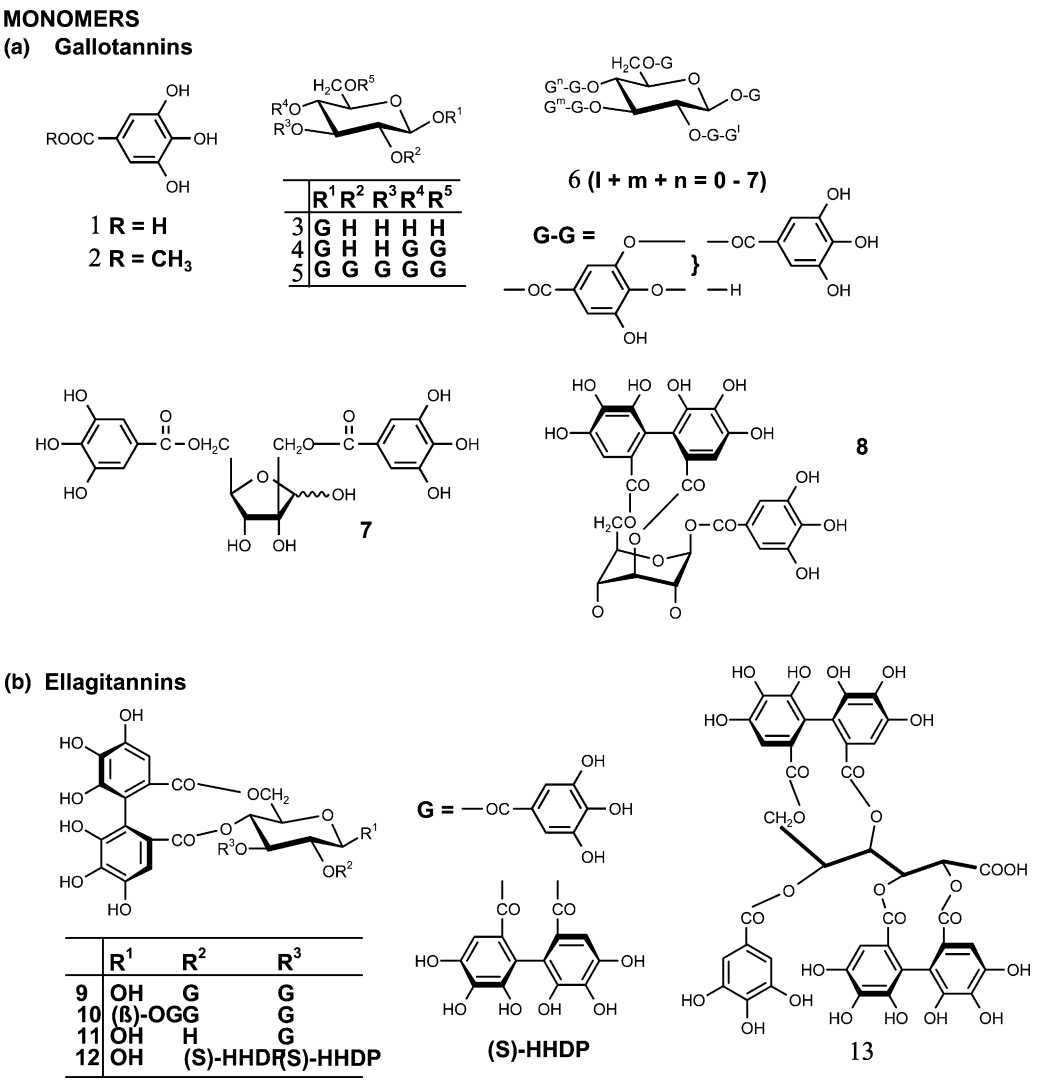
When tested against RAW 264.7 cells as a mammalian host cell control, most compounds revealed no or only moderate cytotoxicity (EC_{50} > 25 μM) except for tannic acid (**6**) (EC_{50} 0.8 μM), 1,4,6-trigalloylglucose (**4**) (EC_{50} 0.8 μM), and oligomeric hydrolysable tannins (**22**–**29**) (EC_{50} ca. 1 μM), thus rendering these candidates less suitable as leishmanicidal agents.

3. Immunomodulatory activity

The differences in leishmanicidal activity of the phenolic samples against *Leishmania* promastigotes and amastigotes may be due to dissimilar biochemical or metabolic characters to the two stages of the parasite. Besides a direct effect on amastigotes, this finding may also be indicative of activating leishmanicidal macrophage functions. For the assessment of immune modulatory effects on macrophage functions, several functional bioassays were employed including a biochemical assay for nitric oxide (NO), a fibroblast-lysis assay for release of tumour necrosis factor (TNF), and a cytopathic effect inhibition assay for interferon (IFN)-like properties.

3.1. Induction of nitric oxide release

Phagocytes, representing an integral part of the innate immune system, are known to produce reactive



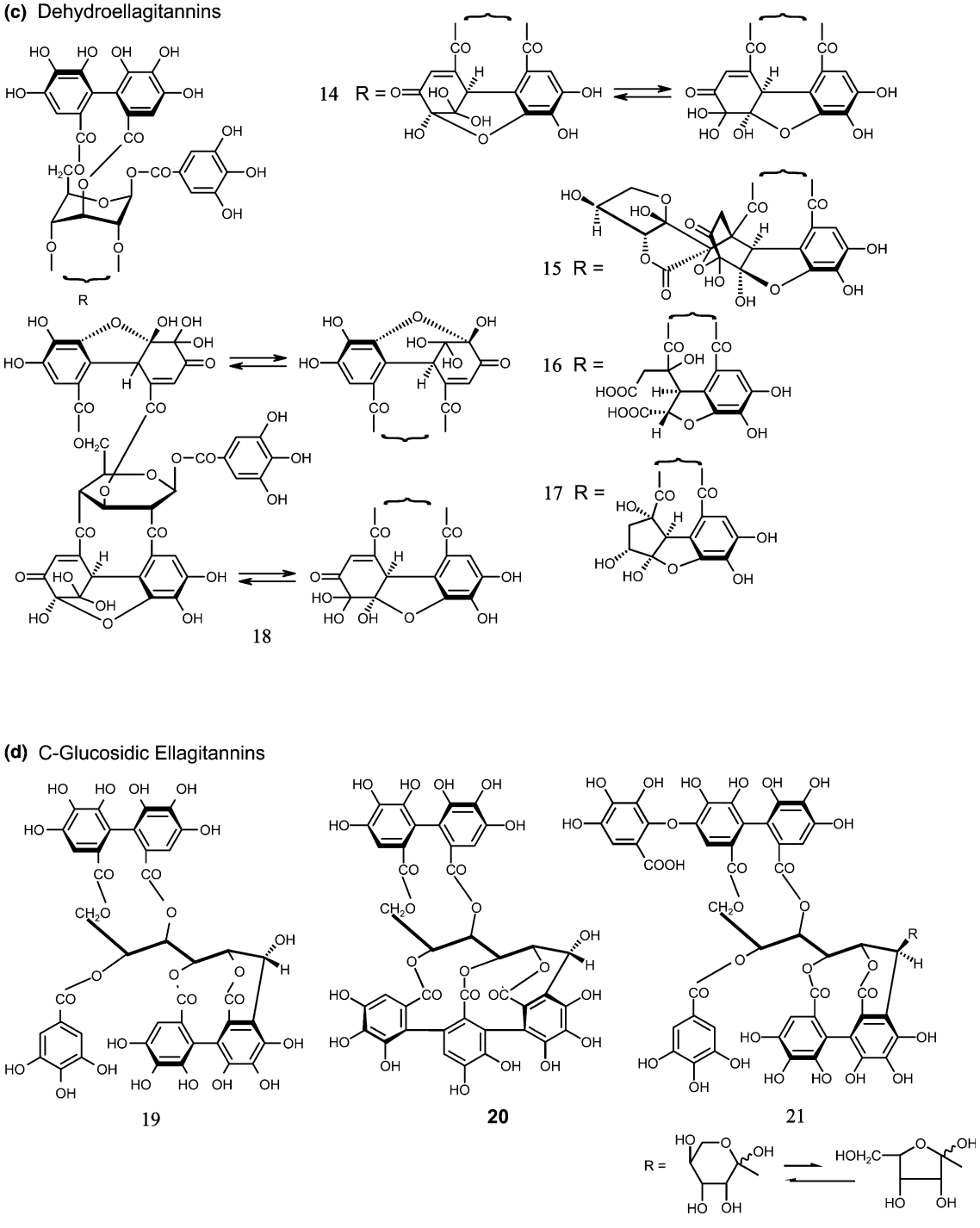
oxygen and nitrogen species that have potent antimicrobial activity. For example, there is ample evidence that NO produced by inducible NO synthase (iNOS) from L-arginine plays a decisive role as microbicidal effector molecule in macrophage cytotoxicity of a number of microorganisms (Nathan and Hibbs, 1991; Moncada et al., 1991; Nussler and Biliar, 1993; Mauel and Ransijn, 1997; Bogdan et al., 2000).

Compared with the stimulus rIFN-γ (100 U/ml) plus lipopolysaccharide (LPS) (10 ng/ml) for activation standard (119 μM NO), the NO-inducing effect of all the samples was found to be only moderate (Table 1), ranging from 8 to 54 μM as assessed in murine RAW 264.7 cells using the Griess assay (Ding et al., 1988; Kiderlen and Kaye, 1990). Of the series of test compounds, the simple phenols **1** and **2** proved to be the relatively most potent NO-inducers, with inducing effects accounting for 45 (54 μM) and 27% (32 μM), respectively, relative to that of IFN-γ + LPS at the subtoxic sample concentration of 50 μg/ml. Higher concentrations had an opposite effect by slightly decreasing the intracellular NO production. Apparently the NO-inducing potentials of nearly all samples, being in the range of ca. 20 μM, reflect some degree of nonspecificity. Conspicuously, the intracellular *Leishmania* kill did not correlate with NO levels, when compared to the kill/NO relationship in IFN-γ + LPS-stimulated RAW 264.7 cells. One possible explanation is that polyphenols acted as scavengers for extracellular NO radicals, thus rendering this amount non-detectable for the Griess assay, while the intracellular leishmanicidal activity remained unaffected. In order to further assess the role of iNOS, experiments using **1** as a potent NO-inducer were carried out in parallel with and without the inhibitor $N^{(G)}$-monomethyl-L-arginine (L-NMMA) and the survival rate of parasites was determined (Kolodziej et al., 2001b). This comparative study indicated significant differences in the leishmanicidal effects in the assay with and without inhibitor (survival rate of parasites ca. 1% and 60%, respectively). Although the involvement of additional cytotoxic defense mechanisms in gallic acid-stimulated RAW 264.7 cells such as increased cytokine production can not be

excluded, this finding provided strong evidence for the crucial role of NO as toxic effector molecule in the host defense to microbial infections.

3.2. Induction of tumour necrosis factor (TNF)-α release

Macrophage activation usually is a polyphenotypic-event, leading to enhanced effector- and regulatory-functions. Augmented TNF-release is a hallmark of macrophage activation and necessarily involved in NO- mediated killing of *Leishmania* parasites and various other pathogens (Beutler and Cerami, 1989; Roach et al., 1991; Zidek et al., 1998). TNF-release was assessed in a functional assay, in which TNF-sensitive L929 fibroblasts treated with supernatants of sample-activated RAW 264.7 cells were rapidly lysed



in its presence. Surviving cells incorporate crystal violet dye and their relative number was spectrophotometrically determined (Roach et al., 1991).

At the host cell subtoxic concentration of 50 μg/ml, the TNF-inducing potential of the polyphenols producing 50% lysis in the murine L929 cells varied greatly, ranging from inactive to pronounced actions (Table 1). In the series of hydrolysable tannins, this particular biological activity increased in the order of oligomeric ellagitannins (>5 U/ml) < gallotannins and monomeric ellagitannins (>5–40 U/ml) < C-glucosidic ellagitannins and dehydroellagitannins (80–350 U/ml). It should be noted that members of the latter two groups stimulated RAW 264.7 cells for marked release of biologically active TNF, being consistently well above positive control levels (184 U/ml for IFN-γ + LPS) and regularly in parallel to intracellular *Leishmania* destruction. Concerning structure–activity relationships, conspicuous dependency of TNF-inducing capabilities of polyphenols on both the conformation of the glucose core and the nature of their galloyl-derived substituents was apparent. For example, the highly potent dehydroellagitannins **14**–**18** share a 3,6-bridging HHDP group on a $^1C_4$ glucose core, while the powerful C-glucosidic ellagitannins **17** and **19** have a C-5 galloyl group in common. Another notable feature for pronounced activity of hydrolysable tannins is represented by the presence of both an HHDP and a DHHDP unit in the same molecule, as evident from the low potential of members with either just HHDP (**9**–**13**) or DHHDP (**18**) groups.

In the series of flavan-3-ol derivatives, members possessing 3-*O*-galloyl groups were more active (100–250 U/ml) than nongalloylated analogues (ca. 50 U/ml), though gallic acid itself exhibited a similar moderate stimulatory potential (39 U/ml). Likewise, shikimic acid stimulated RAW 264.7 cells only moderately for TNF release (34 U/ml). However, introduction of galloyl groups as reflected in its 3-*O*-galloyl and 3,5-di-*O*-galloyl analogues (**57** and **58**, respectively), remarkably enhanced the amount of cytokine released (146 and 306 U/ml, respectively).

Within the group of proanthocyanidins, dimers and trimers showed similar moderate capabilities (30–70 U/ml), while an increased degree of polymerisation was apparently less favourable, as evidenced by the absence of effects of the polymers on TNF-induced cytotoxicity in murine L929 fibroblasts. In this context it is appropriate to note that similar experiments using bone marrow-derived macrophages revealed significant differences in the TNF-inducing potentials of proanthocyanidins, as reflected by an approximately doubled TNF release (Kiderlen et al., 2001). The apparent dependency of the TNF-inducing capability of phenolic samples on the cell line is the subject of current research and has not been observed to this extent for the NO production and IFN-like activities.

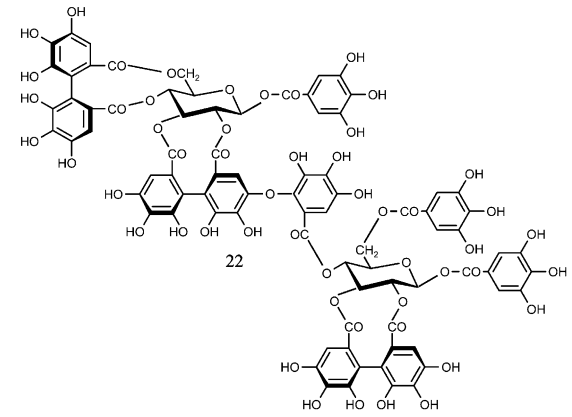
3.3. Induction of interferon-like activity

Macrophage functions are intimately related to the IFN system (Billiau, 1996). Numerous lines of evidence indicated that induction of protective immunity against leishmaniasis was mediated, among others, through IFN-γ production (Liew and O'Donnell, 1993; Reiner and Locksley, 1995; Liew et al., 1997; Alexander et al., 1999). The release of IFN-α by *Leishmania*-infected, activated macrophages also seems to play an important role in antileishmanial defense (Müller et al., 1997). Accordingly, attention was given to possible IFN-like activities of polyphenols induced in RAW 264.7 cells. For this, supernatants of sample-activated RAW cells were analysed for their capacity to protect IFN-sensitive murine L929 fibroblasts from the cytopathic effect (CE) of encephalomyocarditis virus (EMCV), using a recombinant murine IFN-γ standard (100 U/ml) as positive control for IFN-mediated cytoprotection (Kolodziej et al., 2001a,b; Kiderlen et al., 2001; Radtke et al., 2003). The relative number of protected, i.e., viable cells was determined spectrophotometrically using crystal violet as cell stain. The antiviral effect (IFN activity) was expressed in U/ml, defined as reciprocal value of the supernatant dilution that would inhibit 50% of the CE induced by EMCV on L929 cells. It should be noted that this functional assay does not discriminate between IFN-α, -β, and -γ. Keeping in mind that polyphenols are known to possess antiviral activities themselves, differentiation between direct and indirect cytoprotective effects appeared crucial. Thus, supernatants of incubations were in parallel treated with fetal calf serum for polyphenol binding prior to their transfer to L929 cells for the detection of IFN-like activity. The similar results observed in untreated and FCS-treated supernatants from polyphenol-exposed RAW 264.7 cells clearly indicated that the IFN-like activities had been released by the cell line rather than resulting from the polyphenols themselves.

The IFN-like activity induced in RAW 264.7 cells by the tested phenolic compounds ranged from below detection limits to 63 U/ml (Table 1). In general, hydrolysable tannins were more active (>5–65 U/ml) when compared with flavan-3-ol derivatives and proanthocyanidins (>5–38 U/ml). Within the group of hydrolysable tannins, measurements of IFN-like activity revealed that all compounds including gallic acid (**1**) but gallotannins (**3**–**8**), ellagitannins lacking a galloyl group at C-2 of the $^4C_1$ or open glucose core (**11**–**13**), and most of the oligomers (**22**–**29**), possessed the capability to stimulate IFN release (15–65 U/ml). The absence of IFN-like induction by oligomers may be attributed to their enhanced cytotoxicity against

host cells. Of the series of flavan-3-ol derivatives and proanthocyanidins tested, only phylloflavan (**35**) and the hexameric prodelphinidin **54** (38 and 36 U/ml, respectively), inhibited the CE. Galloylated flavan-3-ols and the procyanidin **52** exhibited moderate (ca. 10 U/ml), the remaining samples only negligible cytoprotective effects.

DIMERS



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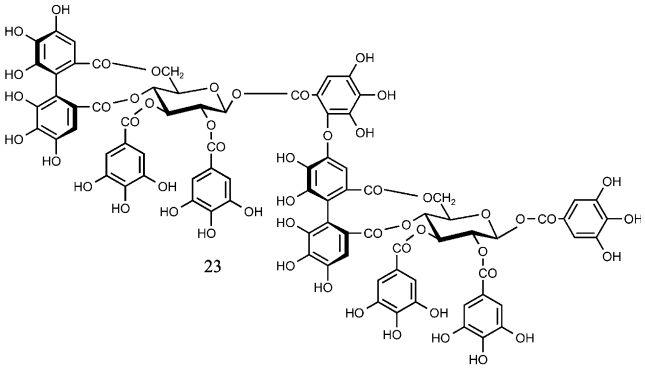

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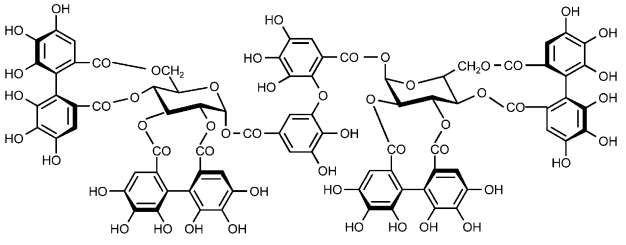

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3.4. Gene expression experiments

To gain insight into the underlying molecular mechanisms of the demonstrated immune modulatory activities of tannins and related compounds, we recently embarked on a set of experiments for the expression of transcripts of iNOS and the cytokines interleukin (IL)-1, IL-10, IL-12, IL-18, IFN-α, IFN-γ and TNF-α (Fig. 1) using reverse-transcription polymerase chain reaction (RT-PCR) (Radtke et al., 2004; Trun, 2004). The authors' and their co-workers recent findings of interesting expression profiles induced by some polyphenol-containing plant extracts (Fig. 2) and polyphenols (Figs. 3 and 4) not reported previously have also been included in this review (see Section 5 for experimental details).

The experiments were performed in parallel in non-infected and in *Leishmania*-infected RAW 264.7 cells and the expression profiles were compared with those mediated by IFN-γ plus LPS. The PCR products were separated on agarose gels and quantified by densitometric analysis. Density of hypoxanthin-guanine-phosphoribosyl transferase (HGPRT) mRNA in the same sample was used to normalize the expression of each gene.

Starting with non-infected cells, the conspicuous lack of major up-regulations defined the response at the chosen time-point of measurement (3–4 h) in most experiments. The phenolic samples moderately up-regulated the gene expressions without affecting expression of the housekeeping gene (HGPRT). Compared to the stimulus IFN-γ + LPS, gene expressions were less prominent (Figs. 2–4). Although this result reflected a developing immune response, time course studies with both IFN-γ + LPS and gallic acid revealed short term effects only.

Activation of parasitised cells with IFN-γ + LPS induced strongly the production of iNOS, TNF-α, IL-1, and IL-12 mRNA, and transiently that of IL-18 mRNA (Fig. 2). The gene expression of the latter cytokine mRNA can therefore not conclusively be related to the activation status of the cells in these experiments. Transcripts of IL-10, a cytokine associated with downregulatory functions (Mocellin et al., 2003), were clearly expressed later (10 h). Also worthy of mention is that the *Leishmania* infection per se induced the expression first of IL-1 and TNF-α mRNA, followed by that of IL-10 transcripts (not shown), reminiscent of the response of non-infected cells stimulated with IFN-γ + LPS.

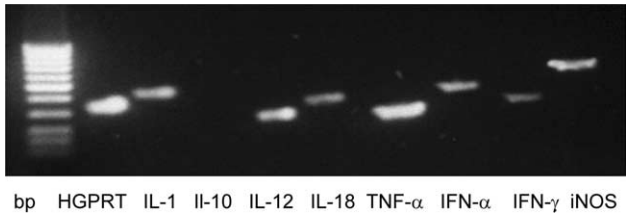
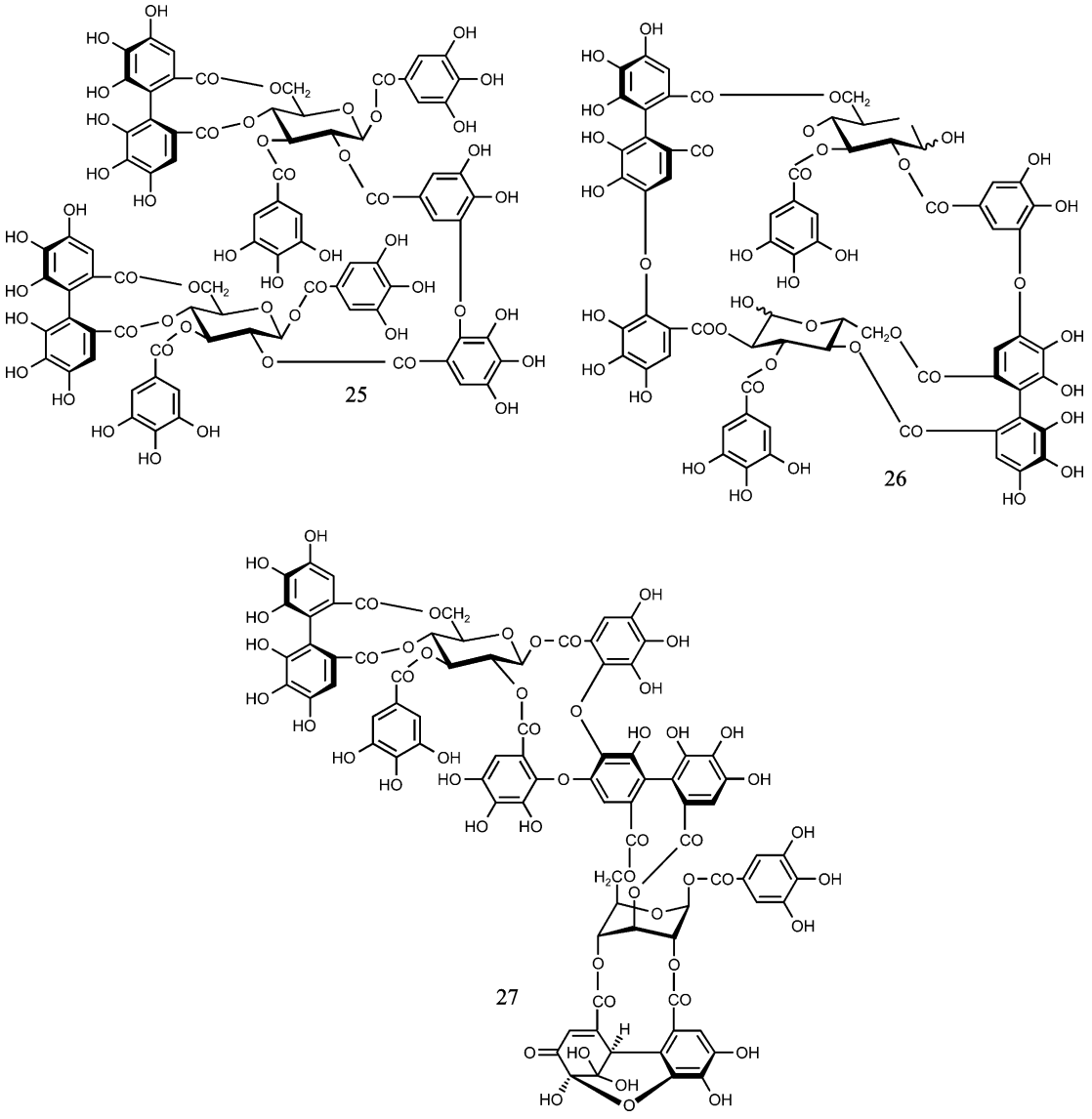


Fig. 1. Representative gel photograph of the expression of iNOS and cytokine transcripts (4 h of incubation; IL-10 mRNAs appearing at ca. 10 h).



Next, the effect of some plant extracts on the expression of these genes was investigated (Fig. 2). The selection of the plants was primarily based on firmly established immune modulatory activities of their extracts or constituents and on the phenolic composition (Kayser et al., 2001; Radtke et al., 2003). While simple phenolic acids and polymeric proanthocyanidins occur in the roots of *Pelargonium sidoides* DC (Geraniaceae) (Kolodziej, 2000), the characteristic constituents of the aerial parts of *Phyllanthus amarus* (Euphorbiaceae) (Foo, 1995) and *Salvia officinalis* L. (Lamiaceae) (Lu and Foo, 2002) are members of the hydrolysable tannins and caffeic acid-derived metabolites, respectively. For comparison, similarly prepared 70% aqueous acetone extracts of the plants were used for the gene expression experiments, although the lack of quantitative data of plant constituents limited the evaluation of the effects of the various phenolic plant types. This protocol, however, represented a valuable tool for the screening of plant extracts and the detection of gene activating principles. Kinetic studies with the stimulus IFN-γ + LPS revealed that gene expressions were maximal within 4-6 h of stimulation and declined rapidly over the next 2–6 h in parallel with a conspicuous increase of IL-10 transcripts, thus deciding on a 4 h incubation period for screening purposes. As shown (Fig. 2), all extracts (50 μg/ml) were capable of enhancing the iNOS and cytokine mRNA levels in parasitised cells when compared with those in non-infected conditions, consistent with the previously demonstrated NO- and TNF-inducing potentials as well as IFN-like activities of constituents at functional levels. Among the phenolic plant extracts tested, the material of *P. sidoides* considerably enhanced the respective mRNA levels. An additional remarkable feature of the expression profile induced by the *P. sidoides* extract, and also

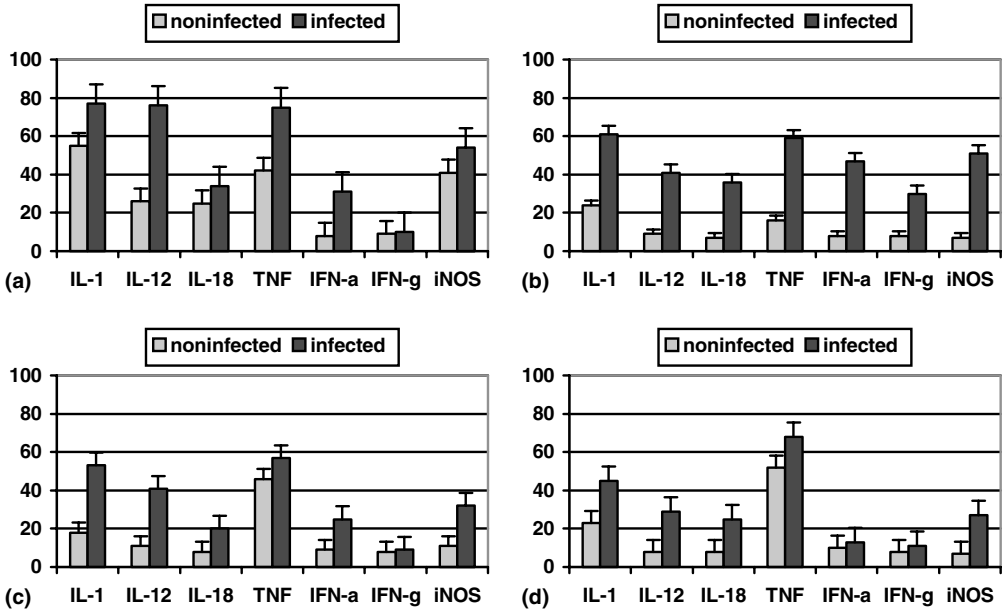


Fig. 2. Expression of iNOS and cytokine transcripts in RAW 264.7 cells stimulated with (a) LPS/IFN-γ, (b) *Pelargonium sidoides* extract, (c) *Phyllanthus amarus* extract, (d) *Salvia officinalis* extract. Sample concentration of extracts was 50 μg/ml. Results are shown relative to HGPRT, defined as 100%.

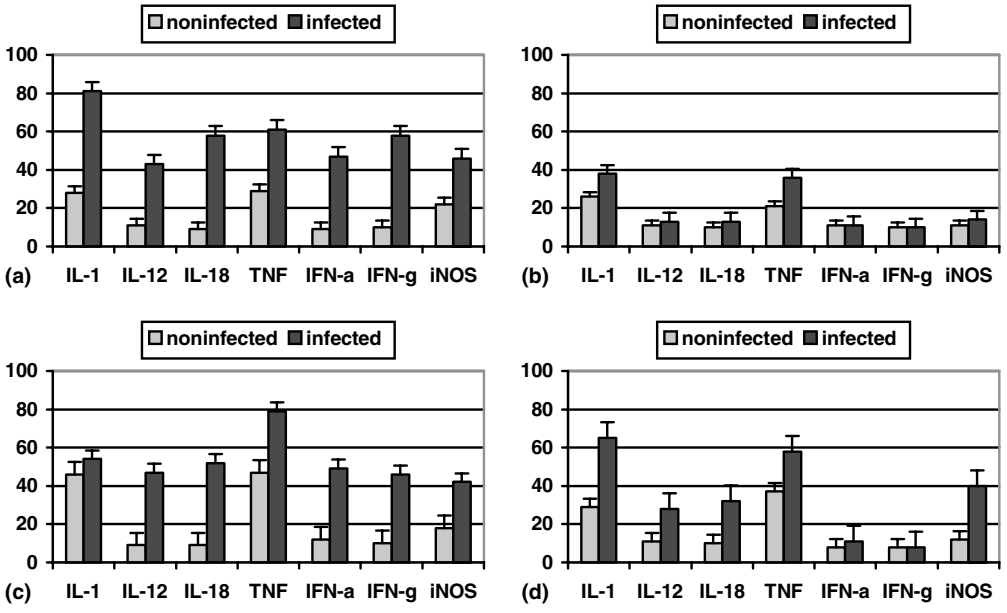
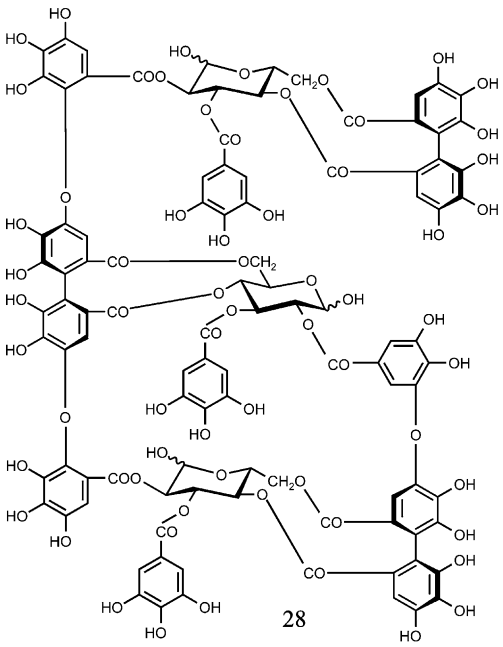


Fig. 3. Expression of iNOS and cytokine transcripts in RAW 264.7 cells stimulated with (a) gallic acid **1** (250 μM), (b) 3-*O*-galloyl shikimic acid **57** (15 μM), (c) corilagin **8** (13.5 μM), (d) hexamer **55** (0.4 μM). The sample concentrations corresponded to those of the EC_{50} values. Results are shown relative to HGPRT, defined as 100%.

in contrast to activation by IFN-γ + LPS was the production of IFN-γ transcripts. Although it is known that macrophage functions are intimately related to the IFN system (Billiau, 1996), these cells are commonly considered to produce IFN-α and to represent only targets for IFN-γ induced activation. The production of IFN-γ itself has only been noted under certain conditions (Mogensen and Virelizier, 1987; Gessani and Belardelli, 1998). It should also be emphasised that the plant extracts only enhanced the gene expression of iNOS as well as that of the critical regulators IL-1 and TNF-α (Liew and O'Donnell, 1993; Liew et al., 1997) in *Leish-*

TRIMERS



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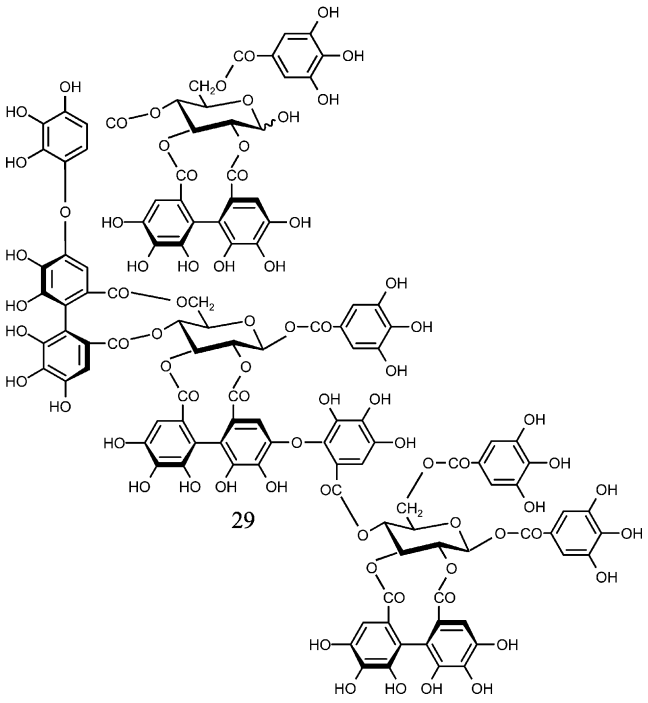

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mania-infected RAW 264.7 cells, albeit with differing potentials.

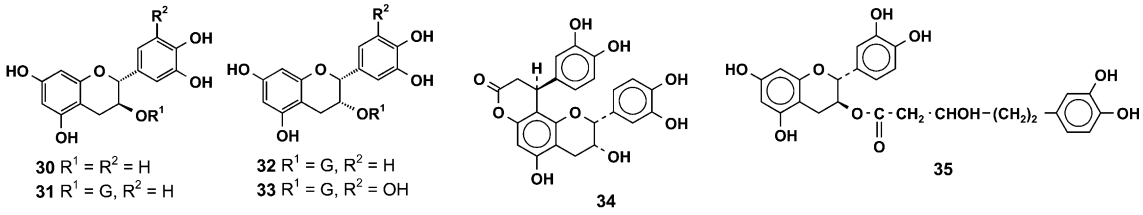
From *P. sidoides*, gallic acid (**1**) was subsequently identified as a gene activating constituent. At a concentration of 250 μM corresponding to that of its antileishmanial EC_{50} value, it induced low levels of iNOS and cytokine mRNAs in non-infected cells, but it clearly enhanced gene expressions in *Leishmania*-parasitised cells (Fig. 3) (Radtke et al., 2004). Most notably, **1** apparently represented the distinctive compound of the extract capable of enhancing the expression of IFN-γ mRNA. Furthermore, kinetic studies revealed that exposure of infected cells to gallic acid not only augmented iNOS and the cytokine mRNA levels but also prolonged their expressions up to 4 h when compared to just parasitised cells.

The detection of **1** as an IFN-γ mRNA inducing plant constituent prompted the evaluation of the structurally related **57** and **58**, the ellagitannin **8**, and the oligomeric procyanidin **55** for their expression profiles (Fig. 3). While the shikimic acid derivatives **57** and **58** (not shown) moderately enhanced the expression of the IL-1 and TNF-mRNA levels merely, compound **8** considerably up-regulated the iNOS and all cytokine transcript levels in infected RAW 264.7 cells. The distinctly lower increase of TNF mRNA compared with **1** in the early response to infection may be rationalised by the relatively pronounced but temporarily level of this cytokine already expressed in non-infected cells. Similar but less pronounced effects were found for **55**. Although hydrolysable tannins appeared more potent, this finding gave no decisive information regarding the IFN-γ inducing structural requirements.

The range of gene expression experiments was therefore extended to **33** and some hydrolysable tannins, being representative of C-glucosidic ellagitannins (**13**) and dehydroellagitannins (**14**) (Fig. 4). Compound **33** up-regulated the iNOS and cytokine transcript levels in infected RAW 264.7 cells similar to those of **1** except for IFN-γ. Since **14** showed similar expression profiles in infected and non-infected RAW 264.7 cells, the presence of an additional DHHDP unit in the molecule of ellagitannins may be less favourable for the transcripts expressions. However, further studies are needed to support such structure–activity relationship, taking into account also dose- and time-dependent effects.

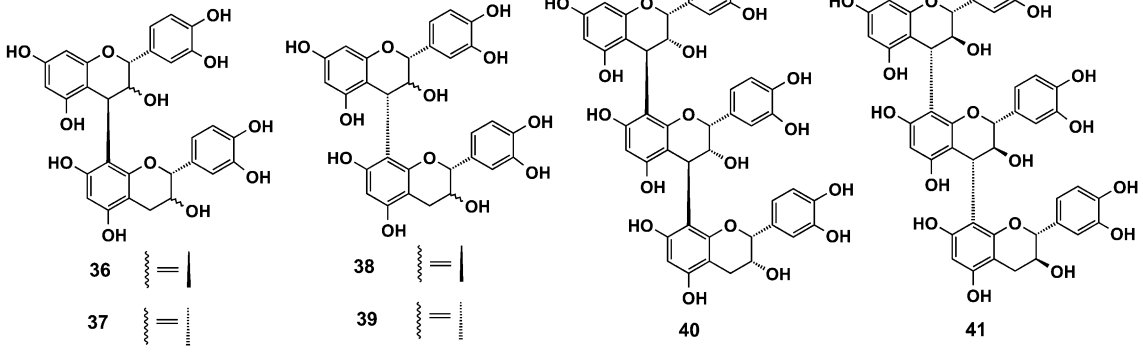
The initial gene expression experiments clearly demonstrated that polyphenols have the potential to activate macrophage functions when taken in conjunction with the functional bioassays. Independent support was also obtained from measurements of IFN-α (Radtke et al., 2004) present in the supernatants of cell incubations, ranging from 6 to 160 pg/ml (Fig. 5). Furthermore, the measured IFN-α levels were within the range of antiviral units as assessed in our cytopathic effect inhibition assay that does not differentiate between the various types of interferons. For this, the cytoprotective effects (U/ml) were determined as a function of the amount of IFN-α (1 U/ml correlated to 2.5 pg/ml). Detailed studies including ELISA for IL-1 and TNF are currently in progress for more information at the protein level.

Flavan-3-ol derivatives

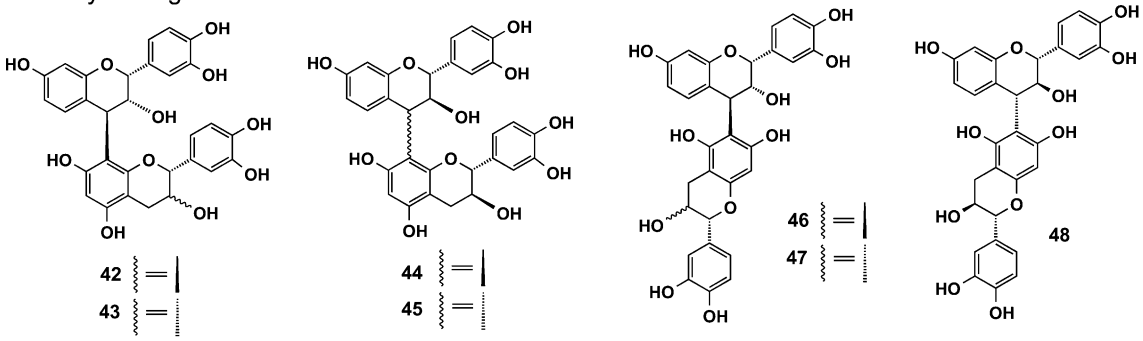


PROANTHOCYANIDINS

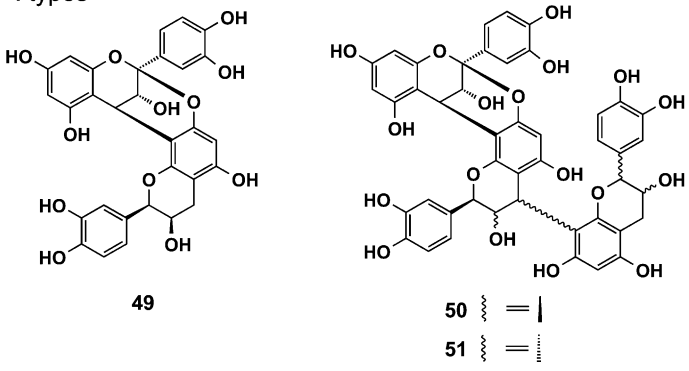
B-Types



5-Deoxy analogues



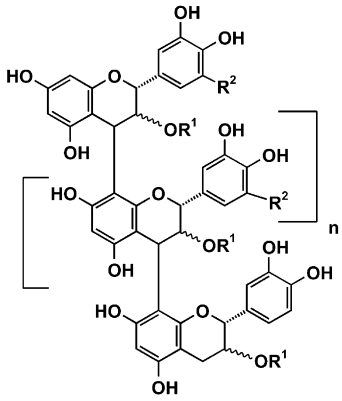
A-types



4. Concluding remarks

A number of papers reported beneficial effects of polyphenols in infectious conditions that may be due to immunomodulatory actions, though the mode of action remains to be clarified in vivo. In particular, little information is available on the bioavailability of polyphenols in a human body, giving rise to debate on the significance of in vitro studies. On the other hand, polyphenols have also been found to inhibit NO production (Virgili et al., 1998; Kim et al., 1999; Ishii et al., 1999; Cheon et al., 2000) and secretion of cytokines such as TNF (Okabe et al., 2001; Chiu et al., 2002; Park et al., 2000) or IL-1 (Cho et al., 2000). TNF-α release is an

Oligomers



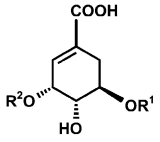
52 $R^1 = R^2 = H$; n = 9

53 R^1 = H or G, R^2 = H or OH; n = 4

54 R^1 = H or G, R^2 = H; n = 4

55 R^1 = H or G, R^2 = H or OH; n = 4

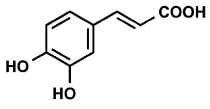
Miscellaneous



56 $R^1 = R^2 = H$

57 $R^1 = G$, $R^2 = H$

58 $R^1 = R^2 = G$



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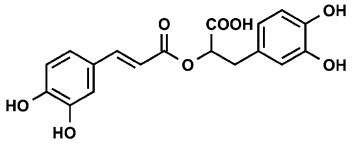

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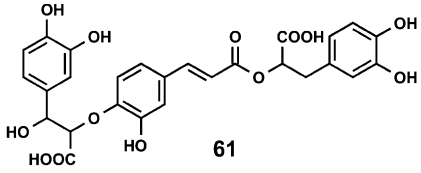

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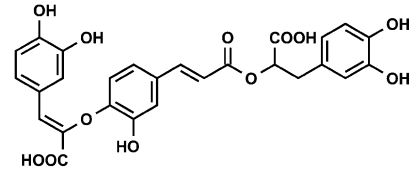
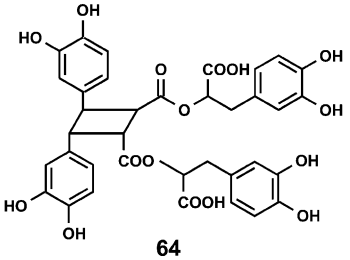


62 R = H

63 R = CH_3



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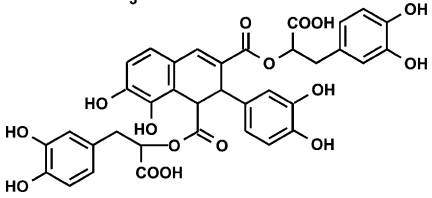

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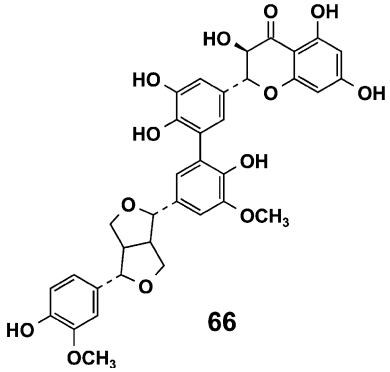

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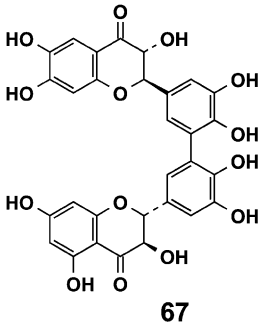

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essential early step in a signalling cascade leading to production of antimicrobial iNO (Roach et al., 1991). However, overproduction may be harmful, as dramatically shown in septic shock. Accordingly, TNF-inhibitors also have a strong therapeutic potential in certain diseases (e.g., rheumatoid arthritis, septic shock, cerebral malaria). This apparent controversy, being reflective of complex regulatory mechanisms, may be rationalised by differences in the response of infected macrophages when compared to that of non-infected cells.

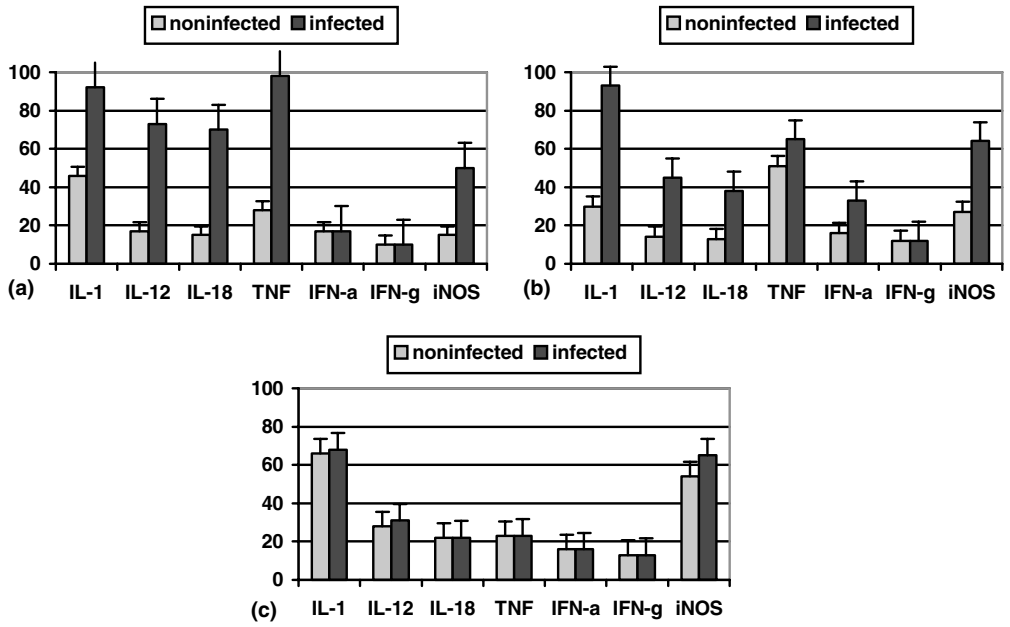

Fig. 4. Expression of iNOS and cytokine transcripts in RAW 264.7 cells stimulated with 50 μg/ml of: (a) EGCG **33** (109 μM), (b) casuarinin **13** (52 μM), (c) geraniin **14** (52 μM). Results are shown relative to HGPRT, defined as 100%.

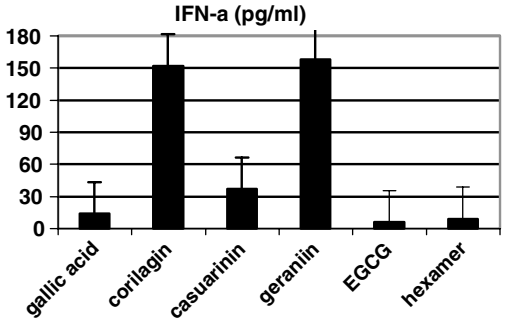

Fig. 5. Effect of tannins on IFN-α production in infected RAW 264.7 cells as assessed in a sandwich immunoassay. Cells were treated with the samples for 24 h, the supernatant was collected for IFN-α ELISA kit.

Conspicuously, infected macrophages showed augmented and prolonged activation of host defense mechanisms as concluded from our gene expression experiments, while inhibitory effects of polyphenols on NO and cytokine production have been reported for activated but non-infected macrophages. This promotive effect may be of special benefit as the human nonspecific immune system is not permanently activated but only sensitised to react effectively when needed, e.g., during infectious conditions. Although these data provide the basis for an immunological concept of plant polyphenols for their beneficial effects in various infectious conditions, in vivo experiments are essential to prove the therapeutic benefits of polyphenolic immunomodulators. Furthermore, the transcription factors involved in the molecular mechanism remain to be defined towards a better understanding of the regulatory principles.

5. Experimental

5.1. Phenolic samples

All compounds tested were available in our research group and their origin is cited in recent publications (Kolodziej et al., 2001a,b; Kiderlen et al., 2001; Radtke et al., 2003). Their identity and purity were determined on the basis of MS and NMR spectroscopic data as well as chromatographic analyses. The samples were first subjected to assays for endotoxin contamination (*Limulus* amoebocyte lysate method), which may stimulate immune cells, of which we found no evidence.

5.2. General procedures, antileishmanial activity and functional immunomodulatory assays

General experimental procedures including cell cultures, parasites, in vitro infection of macrophages with *Leishmania* parasites, assays for leishmanicidal and cytotoxic activity and functional assays (induction of cytokine release and assays for NO release, TNF and IFN-activity are fully described (Kolodziej et al., 2001a,b; Kiderlen et al., 2001; Kiderlen and Kaye, 1990)).

5.3. Gene expression analysis

Details of the treatment of non-infected and *Leishmania*-infected RAW 264.7 cells with gallic acid, RNA isolation, RT-PCR and densitrometric analysis are presented elsewhere (Radtke et al., 2004). Application of the principles and practical tools outlined in this reference to a number of plant extracts and polyphenols

afforded the results shown in Figs. 2–4 at the indicated sample concentrations.

Acknowledgements

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Herbert Kolodziej graduated from the Westfälische-Wilhems University of Münster, Germany in 1976. He was trained in pharmacy before obtaining a Dr. rer. nat. (1980) in Pharmaceutical Biology (isolation and characterisation of polyphenols). In 1980 he was appointed as Scientific Assistant in the Pharmaceutical Biology Department at University of Münster and progressed to the ranks of Academic Lecturer and Senior Lecturer in 1983 and 1986, respectively. In 1988, he obtained his habilitation (spectroscopic analyses of proanthocyanidins) at Münster and accepted in 1991 a professorship in Pharmaceutical Biology at the Freie Universität Berlin, Germany. Sabbatical leaves were spent with the late Professor David G. Roux and Professor Daneel Ferreira, University of Orange Free State, Bloemfontein, South Africa. His research program ranges from phytochemistry, chemotaxonomy, pharmacology and molecular biology to infectious diseases.

Albrecht F. Kiderlen studied zoology, fresh-water sciences and parasitology at the Albert Ludwigs-Universität, Freiburg, Germany where he graduated in 1982. As Ph.D.-student, he worked on the role of macrophages in the defense against intracellular pathogens, first at the Max Planck Institute for Immunobiology, Freiburg, then at the Fraunhofer Institute for Toxicology, Hannover and received his Dr. rer. nat. 1986. Subsequently, he worked as a post-doc, first in Hannover, then at the London School of Hygiene and Tropical Medicine mainly on immunological defense mechanisms against *Leishmania* parasites. Since 1990, Kiderlen has a permanent position as research scientist at the Robert Koch-Institut in Berlin. His research interests range from natural immunity to opportunistic pathogens, to infection biology of pathogenic amoebae, and novel plant-derived pharmaceuticals against infectious agents.