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Tannins and related compounds induce nitric oxide synthase and cytokines gene expressions in *Leishmania major*-infected macrophage-like RAW 264.7 cells

Herbert Kolodziej,[a,*] Anne Burmeister,[a] Weronika Trun,[a] Oliver A. Radtke,[a] Albrecht F. Kiderlen,[b] Hideyuki Ito,[c] Tsutomu Hatano,[c] Takashi Yoshida[c] and Lai Yeap Foo[d]

[a]*Institute of Pharmacy, Pharmaceutical Biology, Freie Universität Berlin, Königin-Luise-Str. 2+4, D-14195 Berlin, Germany*
[b]*Robert Koch-Institut, Department of Infectious Diseases, Nordufer 20, D-13353 Berlin, Germany*
[c]*Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan*
[d]*New Zealand Institute for Industrial Research, Gracefield Road, PO Box 31-310, Lower Hutt, New Zealand*

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Abstract—Some polyphenol-containing extracts (*Pelargonium sidoides*, *Phyllanthus amarus*) and representatives of simple phenols (shikimic acid 3- and 5-*O*-gallate), flavan-3-ols (epigallocatechin 3-gallate), proanthocyanidins (a hexamer) and hydrolysable tannins (corilagin, casuariin, geraniin) were studied for gene expressions (iNOS, IL-1, IL-10, IL-12, IL-18, TNF-α, IFN-α/γ) by RT-PCR. All extracts and compounds were capable of enhancing the iNOS and cytokine mRNA levels in parasitised cells when compared with those in non-infected conditions.

1. Introduction

The polyphenols belonging to the tannin family constitute one of the most ubiquitous groups of all plant phenolics. Their wide occurrence in the plant kingdom and their presence in significant amounts in the wood, bark, leaves and fruits of many plants of predominantly woody habit may be rationalised, at least in part, by their apparent ecological function as defense compounds, that is, the protection of plants from herbivores, microbes, viruses or competing plants.[1] This implies the interference with the physiology and biochemistry of herbivores and invading microorganisms, as evident from a large body of pharmacological and toxicological papers. Interactions with pharmacological targets make such metabolites as well as defined plant extracts interesting for medicinal applications to treat human ailments and diseases. Polyphenols that exhibit a remarkably wide range of biological activities, including antimicrobial, antitumour and enzyme-inhibiting properties,[2–4] have attracted a considerable amount of attention in the fields of nutrition, health and medicine. The potential beneficial effects of polyphenols on human health have been attributed mostly to their powerful free radical scavenging and strong antioxidant activities in vitro.[5–9]

Although numerous lines of evidence indicate that various plant extracts rich in polyphenols and polyphenolic constituents possess immunomodulatory activities,[10–15] much less attention has been given to these potential health beneficial principles. The immune system plays a crucial role in the defense against invading pathogens and in killing of cancer cells. Recent developments have been initiated by the growing realisation that polyphenols may be credited for cancer prevention and anticancer.[3,16–19]

Treatments of a broad spectrum of infectious conditions with polyphenolic herbal medicines in various medical systems has prompted our interest in the evaluation of polyphenols as promising antileishmanial drugs.[20–23]

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* Corresponding author. Tel.: + 49 30 838 53731; fax: +49 30 838 53729; e-mail: kolpharm@zedat.fu-berlin.de

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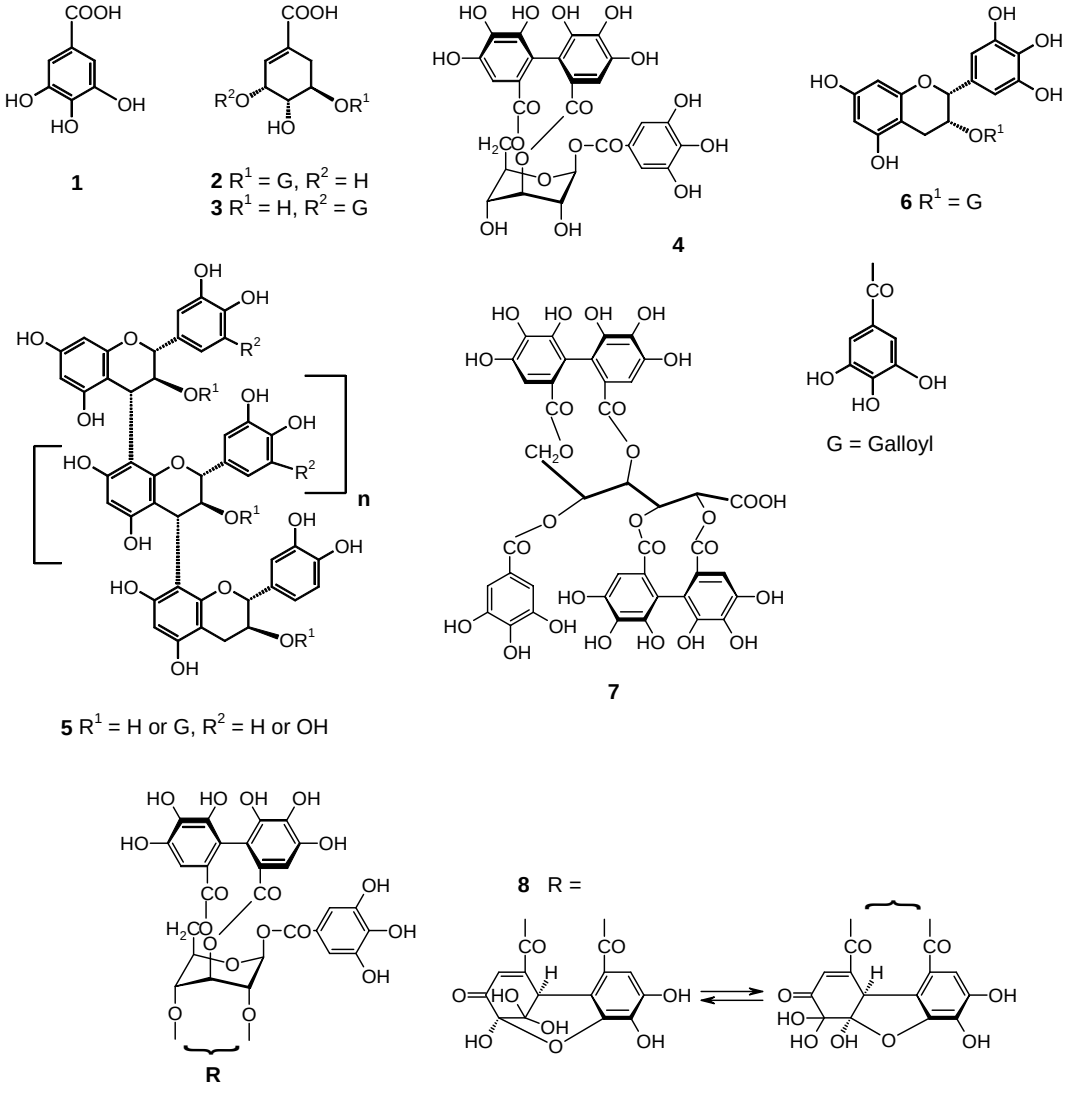
In these recent studies we have demonstrated significant immunomodulatory (NO-, TNF- and IFN-release) capabilities of caffeic acid-derived metabolites, hydrolysable and condensed tannins associated with antileishmanial activities. The mechanisms of immunological control of *Leishmania* infection include a response of the cell-mediated Th1-type, leading to macrophage activation and elimination of the intracellular parasites,[24] thus representing an attractive alternative or complement to conventional chemotherapy of infectious conditions. 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. The molecular mechanism, which may be responsible for this intracellular killing of *Leishmania* parasites is so far not characterised. We now disclose results of relevance to the expression of inducible nitric oxide synthase (iNOS) and a range of cytokine genes in non-infected and *Leishmania major*-parasitised RAW 264.7 cells induced by some polyphenolic containing plant extracts and chemically defined compounds.

2. Results and discussion

2.1. Gene expressions

The main function of the immune system is the defense against infectious agents and malignant tumours. Macrophages and other phagocytes play a key role in immune responses in that they are capable of producing toxic effector molecules such as reactive oxygen and nitrogen species that significantly contribute to the control of microbial pathogens and tumours.[25] Besides the production of nitric oxide (NO), cytokines play a major role in the immunopathology associated with intracellular pathogens.[24–29] For the assessment of immune modulatory effects on macrophage functions, we have employed several functional bioassays 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.[20–23] Since the functional assays did not provide any information regarding the underlying molecular mechanisms of the demonstrated immune



modulatory activities of the polyphenols tested, we have performed gene expression analysis using gallic acid as model compound.[30] This representative plant polyphenol not only induced the expression of transcripts of inducible nitric oxide synthase (iNOS) and the cytokines interleukin (IL)-1, IL-10, IL-12, IL-18, TNF-α and interferon (IFN)-γ, but also enhanced and prolonged the respective mRNA expressions in *Leishmania*-parasitised RAW 264.7 cells when compared to the gene expression profile induced in non-infected cells.

Encouraged by our recent results, we extended our studies to a series of tannins and related compounds including members of simple phenols [shikimic acid 3- (**2**) and 5-*O*-gallate (**3**)], flavanol-3-ols [epigallocatechin 3-gallate (**6**)], proanthocyanidins [hexamer (**5**)] and hydrolysable tannins [corilagin (**4**), casuariin (**7**), geraniin (**8**)]. Having in mind the structure of the model compound, gallic acid (**1**), a common feature of the candidates was the presence of galloyl entities in their molecules to gain insight into structure–activity relationships. The effects of compounds **2**–**8** on iNOS and IL-1, IL-10, IL-12, IL-18, TNF-α and IFN-γ gene expressions were evaluated by reverse-transcription polymerase chain reaction (RT-PCR). 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 + LPS that served as positive control. Separation of the PCR products was achieved by gel electrophoresis and their quantification by densitometric analysis. Density of hypoxanthin–guanine–phosphoribosyl transferase (HGPRT) mRNA in the same sample was used to normalise the expression of each gene between different sets of experiments.

2.2. Non-infected cells

In non-infected RAW 264.7 cells, the noticeable lack of major up-regulations characterised the response at ca. 4 h after incubation in most experiments. In this context it is appropriate to note that kinetic studies[30] with the stimulus IFN-γ + LPS and gallic acid (**1**) revealed that gene expressions were in general maximal within 4–6 h of stimulation, followed by a decline over the next 2–6 h in parallel with a conspicuous increase of transcripts of the down-regulating cytokine IL-10.[31] We thus decided on a 4 h incubation period for screening purposes. Compounds **2**–**8** moderately up-regulated the gene expressions, producing mRNA levels below those induced by the stimulus IFN-γ + LPS (Figs. 1 and 2). Although this finding reflected a developing immune response, recent kinetic studies with both IFN-γ + LPS and gallic acid (**1**) revealed short term effects only.[30]

2.3. Infected cells

Stimulation of *L. major*-infected RAW 264.7 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 (Figs. 1 and 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. Conspicuously, the *Leishmania* infection *per se* induced low and transient levels first of IL-1 and TNF-α mRNA, followed by the expression of IL-10 transcripts, reminiscent of the response of non-infected cells stimulated with IFN-γ + LPS.[30]

With reference to the polyphenols tested, the initial set of experiments included the shikimic acid derivatives **2** and **3**, the ellagitannin**4** and the oligomeric procyanidin **5** for their expression profiles (Fig. 1). For simple correlation to antileishmanial activity, the sample concentrations here corresponded to those of their EC_{50} values.[21] While the shikimic acid derivatives **2** and **3** (not shown) moderately enhanced the expression of the IL-1 and TNF-mRNA levels in the early response to infection merely, compound **4** considerably up-regulated the iNOS and all cytokine transcript levels in infected RAW 264.7 cells. Similar but less pronounced effects were found for **5**. Interestingly, and in contrast to activation by IFN-γ + LPS, corilagin (**4**) also stimulated *Leishmania*-infected RAW 264.7 cells to produce IFN-γ mRNA. This observation was reminiscent of that of gallic acid.[30] Although it is known that macrophage functions are intimately related to the IFN system,[32] 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 in cells of monocytic lineage has only been noted under certain physiological and pathological conditions.[33,34] Our findings that some polyphenolic-treated infected RAW 264.7 cells expressed IFN-γ mRNA lend support to these reports.

Besides the noted induction of IFN-γ, the up-regulation of IL-12 and IL-18 mRNA levels is also worthy of mention in that both cytokines are critical to host defense against a variety of (intracellular) pathogens. A key function of IL-12 is the induction and maintenance of Th1 responses, also representing an important link between host innate and adaptive immunity.[35,36] IL-18 is considered to have biological functions similar to those of IL-12, acting as strong co-factor for IFN-γ production from Th1 and NK cells.[27]

The detection of gallic acid (**1**) and corilagin (**4**) as IFN-γ mRNA inducing plant constituents prompted a closer look to polyphenols regarding this particular capability. The range of gene expression experiments was therefore extended to **6** and some hydrolysable tannins, being representative of *C*-glucosidic ellagitannins (**7**) and dehydro-ellagitannins (**8**) (Fig. 2). Since contemporary studies were set up to evaluate plant extracts (vide infra), the sample concentration was constantly 50 μg/ml in these experiments. Compound **6** up-regulated the iNOS and cytokine transcript levels in infected RAW 264.7 cells similar to those observed for corilagin except for IFN-α/γ. The introduction of an additional DHHDP unit in the molecule of ellagitannins may be less favourable for the transcripts expressions, as concluded from the remarkable inducing potential of **4** in parasitised cells compared with similar moderate expression profiles observed for **8** in infected and non-infected conditions.

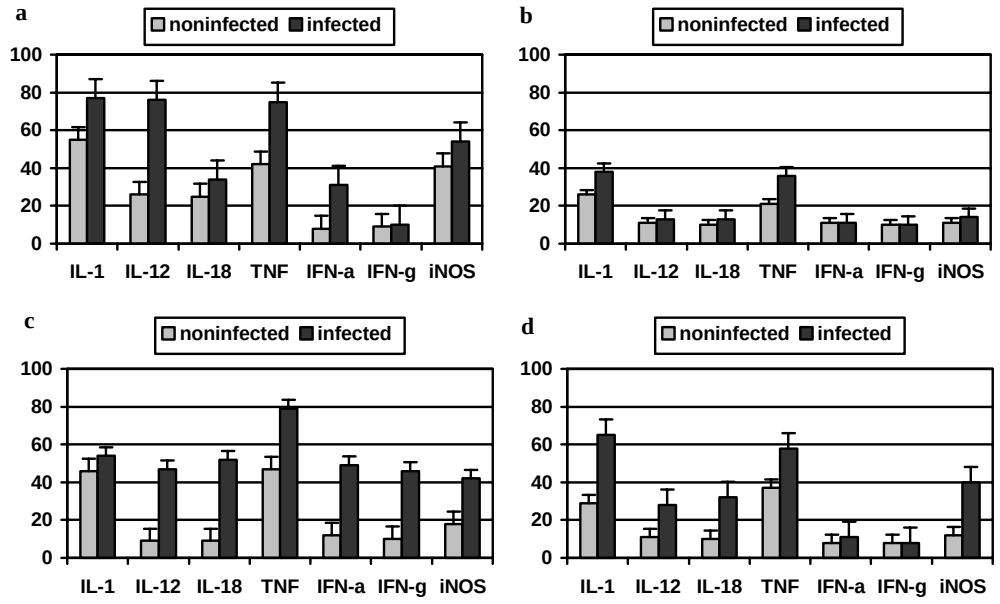


Figure 1. Expression of iNOS and cytokine transcripts in RAW 264.7 cells stimulated for 4 h with (a) IFN-γ + LPS, (b) 3-*O*-galloyl shikimic acid **2** (15 μM), (c) corilagin **4** (13.5 μM) and (d) hexamer **5** (0.4 μM). The sample concentrations corresponded to those of the EC_{50} values. Results are shown relative to HGPRT, defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

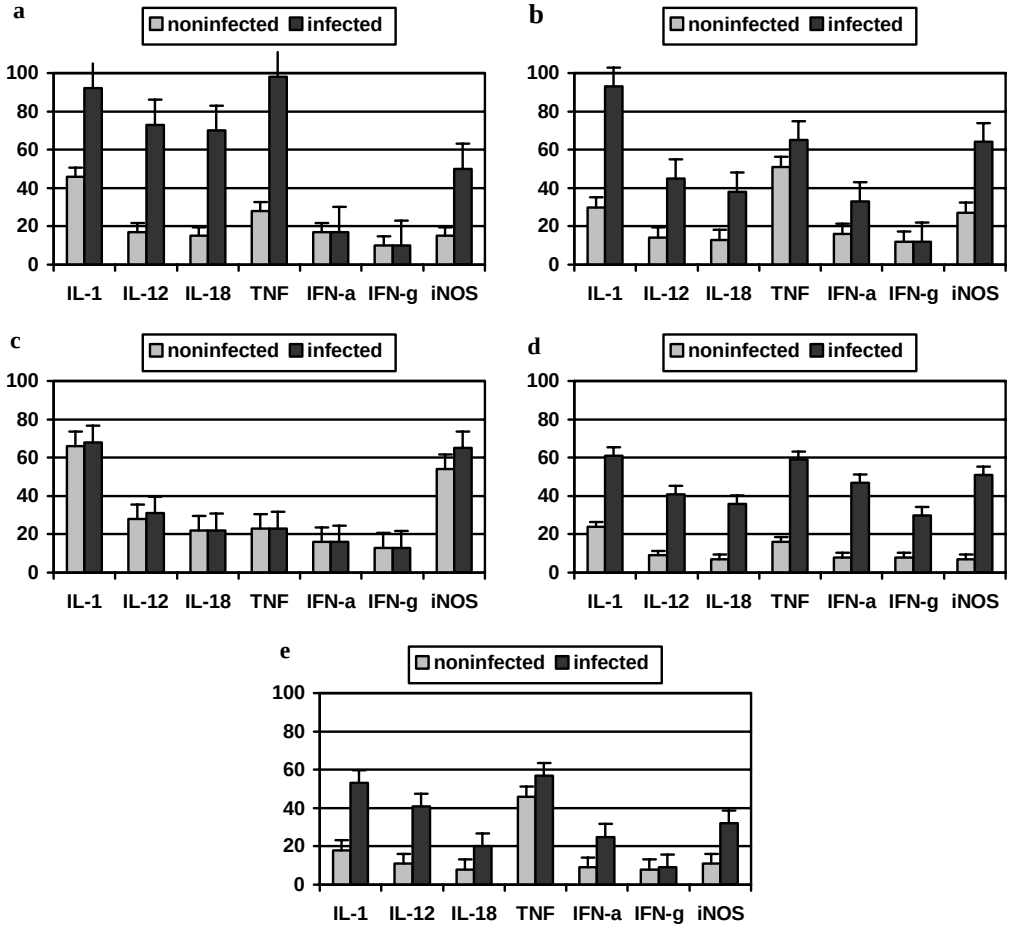


Figure 2. Expression of iNOS and cytokine transcripts in RAW 264.7 cells stimulated for 4 h with 50 μg/ml of (a) EGCG **6** (109 μM), (b) casuarinin **7** (52 μM), (c) geraniin **8** (52 μM), (d) *Pelargonium sidoides* extract (EPs 7630), (e) *Phyllanthus amarus* extract and (f) *Salvia officinalis* extract. Results are shown relative to HGPRT, defined as 100%. The values (mean ± SEM) are derived from three independent experiments.

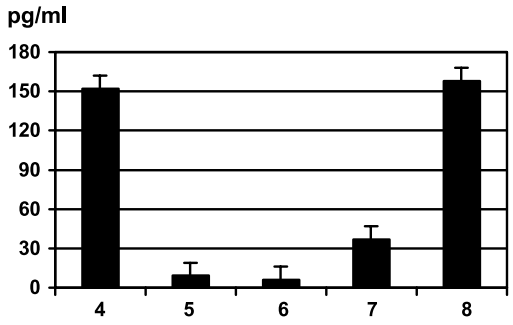


Figure 3. Effect of compounds **4**–**8** on IFN-α production in infected RAW 264.7 cells as assessed in a sandwich immunoassay. Cells were treated with the samples for 24 h, the supernatant was collected for IFN-α ELISA kit. The values (mean ± SEM) are derived from three independent experiments.

However, further studies are needed to support such structure–activity relationship, taking into account also dose- and time-dependent effects. Although hydrolysable tannins appeared more potent, the present set of expression analyses gave no decisive information regarding the IFN-γ inducing structural requirements.

In order to confirm protein production, the concentration of IFN-α present in the supernatants of cell incubations was exemplary determined by ELISA,[37] ranging from 6 to 160 pg/ml (Fig. 3). In contrast, the culture medium of analogously treated non-infected cells did not contain any detectable IFN-α proteins. Conspicuously, geraniin (**8**) apparently produced high amounts of IFN-α protein, though only low mRNA levels were detected. This conflicting finding may be rationalised by the different time points of measurement, that is, gene expression analysis and ELISA at 4 and 24 h, respectively. Although our functional bioassays already indicated cytokine productions,[20–23] the present data provided direct evidence for the capability of the test compounds to increase IFN-α levels. Furthermore, the measured IFN-α levels were within the range of antiviral units (<5–59 U/ml) as assessed in our cytopathic effect inhibition assay that does not differentiate between the various types of interferons. For this, the cytoprotective effects, calculated in U/ml, were determined as a function of the amount of IFN-α (1 U/ml correlated to 2.5 pg/ml).[37] Detailed studies including ELISA for IL-1 and TNF-α are currently in progress for more information at the protein level.

2.4. Plant extracts

This protocol represented a valuable tool for the screening of plant extracts and the detection of gene activating principles. The selection of the plants was primarily based on firmly established immune modulatory activities of their extracts or constituents and on the phenolic composition.[23,38] While simple phenolic acids including the recently demonstrated IFN-γ inducer gallic acid and the tested shikimic acid gallates **2** and **3** as well as polymeric proanthocyanidins occur in the roots of *Pelargonium sidoides* DC (Geraniaceae),[39] the characteristic constituents of the aerial parts of *Phyllanthus amarus* L. (Euphorbiaceae) are members of the hydrolysable tannins,[40] with corilagin (**4**) and geraniin (**8**) as known metabolites.[41] For comparison, similarly prepared 70% aqueous acetone extracts of the plants except for *P. sidoides* (see Experimental) 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. 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 in contrast to activation by IFN-γ + LPS was the production of IFN-γ transcripts.

3. Conclusion

The recorded uses of traditional herbal medicines rich in polyphenols as effective antiseptive drugs may be due, at least in part, to their immunomodulatory actions, though the mode of action remains to be clarified in vivo. Supporting evidence is available from a number of papers reporting beneficial effects of polyphenols in infectious conditions. On the other hand, polyphenols have also been found to inhibit NO production[42–45] and secretion of cytokines such as TNF[46–48] or IL-1.[49] While TNF-α release is an essential early step in a signalling cascade leading to production of antimicrobial NO and its congeners,[50] 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. Conspicuously, infected macrophages showed augmented and prolonged activation of host defense mechanisms as concluded from our gene expression experiments,[24] while inhibitory effects of polyphenols on NO and cytokine production have been reported for activated but non-infected macrophages. That this promotive effect on gene expressions and macrophage activation was conspicuously evident in just parasitised cells may be of special benefit, indicating that the sensitised non-specific immune system reacts more effectively when needed, for example, 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. In addition, further studies are needed to unravel exactly the cellular and molecular mechanisms that underlie these effects.

4. Materials and methods

4.1. Test compounds, plant extracts and chemicals

The polyphenols used were available as reference samples in the research groups of the authors. Their identification and purity were determined on the basis of MS and NMR data, while the characterisation of the proanthocyanidin hexamer has previously been reported.[51] Preparation of the *Phyllanthus* plant extracts is fully described elsewhere,[40] while that of *P. sidoides* is a proprietary extract of the roots, EPs 7630 (Umckaloabo®, Licensee: Iso-Arzneimittel, Ettlingen, Germany). All samples were first subjected to assays for endotoxin contamination (*Limulus* amoebocyte lysate method), which may stimulate immune cells, of which we found no evidence. Recombinant murine interferon-γ (Genentech, San Francisco, USA), expressed in *E. coli*, was kindly provided by Bender & Co., Wien, Austria. Trizol RNA isolation reagent was obtained from Invitrogen (Karlsruhe, Germany), reverse transcriptase (murine lymphoma virus RT RNAseH-), random hexamer primers and Taq polymerase, expressed in *E. coli*, and RT and PCR buffers were supplied by Promega, Madison, USA. The oligonucleotides used as primers were synthesised by TiBMolbiol, Berlin, Germany.

4.2. General procedures

General experimental procedures including cell cultures, parasites and in vitro infection of macrophages with *Leishmania* parasites are described elsewhere.[22]

4.3. Treatment of *Leishmania*-infected RAW 264.7 cells with samples

After an 18 h resting period, infected RAW 264.7 cells were activated by incubating in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (R10) containing 50 μg/ml of the samples for 48 h at 37 °C. Treatment of cells with IFN-γ (100 U/ml) + LPS (10 ng/ml) served as positive control for cell activation. In parallel cultures, non-infected cells were similarly treated with or without samples as well as the control stimuli. Total RNA was extracted from independent cultures after 4 h of exposure. Incubations were stopped by discarding the culture supernatant and freezing the cell monolayer until analysis.

4.4. RNA isolation and complementary DNA synthesis

Total RNA was extracted from RAW 264.7 cell preparations with Trizol reagent following the supplier's instructions. In brief, 5×10^5 cells were lysed in 1 ml of Trizol and then transferred into Eppendorf tubes. Following addition of 200 μl of chloroform, the suspension was centrifuged for 15 min at 12,000*g* at 4 °C. The upper hydrophilic layer was recovered, mixed with 500 μl of isopropyl alcohol, again centrifuged and the supernatant discarded. The pellet was washed twice with 75% ethanol, dissolved in H_2O, adjusted to 1 mg RNA/ml and stored at −80 °C. Reverse transcription was performed at 37 °C for 1 h in a total volume of 25 μl in RT buffer [7 mM $MgCl_2$, 50 mM Tris–HCl (pH 8.3), 75 mM KCl], using 10 mM dNTPs, 10 mM DTT, 50 U M-MLV reverse transcriptase, 2 μg random hexamer primers and 3 μg total RNA. Parallel preparations without reverse transcriptase were included for the detection of DNA contamination.

4.5. Polymerase chain reaction (PCR) and analysis of PCR products

Three μg of cDNA were added to the respective oligonucleotide primers (1 nM each) and Taq polymerase (2.5 U/ml). The reaction volume was adjusted to 50 μl using PCR buffer to reach the final concentrations of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, dNTPs (dATP, dGTP, dCTP, dTTP, 200 μM each) and 5% DMSO. After an initial denaturation for 69 s at 95 °C, 36 cycles of amplification (93 °C for 55 s, 61 °C for 45 s, 72 °C for 40 s) for cytokine and HGPRT (hypoxanthine–guanine–phosphoribosyltransferase as internal standard) cDNAs were performed followed by a 100 s step for elongation at 72 °C. Amplification cycles (30 cycles) for iNOS cDNA comprised of a 60 s step at 95 °C for denaturation, a 1-min step at 72 °C for annealing and synthesis and a final 100 s step for elongation at 72 °C. The following primers were used: HGPRT (362 bp), sense 5′-GTT GGA TAC AGG CCA GAC TTT GTT G-3′; antisense 5′-GAG GGT AGG CTG GCC TAT AGG CT-3′; IL-1 (400 bp) sense 5′-GCA ACT GTT CCT GAA CTC A-3′; antisense 5′-CTC GGA GCC TGT AGT GCA G-3′ IL-10 (256 bp) sense 5′-TAC CTG GTA GAA GTG ATG CC-3′ antisense 5′-CAT CAT GTA TGC TTC TAT GC-3′; IL-12 (312 bp) sense 5′-CGT GCT CAT GGC TGG TGC AAA G-3′ antisense 5′-CTT CAT CTG CAA GTT CTT GGG C-3′; IL-18 (440 bp) sense 5′-ACT GTA CAA CCG GAG TAA TAC GG- 3′; antisense 5′-AGT GAA CAT TAC AGA TTT ATC CC-3′; TNF-α (276 bp) sense 5′-ATG AGC ACA GAA AGC ATG ATC-3′ antisense 5′-TAC AGG CTT GTC ACT CGA ATT-3′; IFN-γ (405 bp) sense 5′-TAC TGC CAC GGC ACA GTC ATT GAA-3′; antisense 5′-GCA GCG ACT CCT TTT CCG CTT CCT-3′; IFN-α (692 bp) sense 5′-AAC AGC CCA GAG GAC AAA CAG CAT CTT CAA G-3′; antisense 5′-AAA TCA TGC ACA AAT GAC TGA TAT TTT TG-3′; iNOS (798 bp) sense 5′-CAT GGC TTG CCC CTG GAA GTT TCT CTT CAA AG-3′; antisense 5′-GCA GCA TCC CCT CTG ATG GTG CCA TCG-3′. For analysis of PCR products, 8 μl aliquots from each reaction were electrophoresed in a 1.5% agarose gel containing 0.2 μg/ml ethidium bromide.

4.6. Densitometric analysis

PCR reactions were set up as described above. Once each RT-PCR reaction had been terminated, the PCR products were electrophoresed (vide supra) and their relative amounts determined by densitometric analysis using a FLA-2000G IP/Fluorescent image analyser (Fujifilm Ltd, Tokyo, Japan) according to the instructions of the manufacturer. Gels were dried, deposited on the glass side of the FLUOR STAGE in the main unit's Stage Loading Unit, and exposed to the excitation light source at 473 nm.

Fluorescent light was detected using the light-receiving filter O580. The intensities of the bands were scanned (Image Reader 1.5; Fujifilm Ltd, Tokyo, Japan; Image Gauge 3.0 software). The RT-PCR levels of the transcripts were expressed as a percentage of the internal standard, HGPRT, defined as 100%.

4.7. Immunoassay for production of IFN-α

The amount of IFN-α in sample-stimulated RAW 264.7 cells after incubation for 24 h was determined using a sandwich immunoassay,[37] according to the instructions of the manufacturer (PBL Biomedical Laboratories). In brief, supernatants of stimulated RAW cultures (24 h) were serially diluted with dilution buffer. Ninety-six-well microtitre plates were incubated with 100 μl of these sample preparations for 1 h, then the supernatants were removed, wells rinsed with Wash Solution and the inverted microtitre plates tapped to dry on lint-free absorbent paper. Antibody Solution (100 μl) was added to each well and plates were incubated for 24 h at 24 °C and processed as above. Then 100 μl of HRP (horseradish peroxidase) Conjugate Solution was added to each well. After 1 h of incubation, wells were again rinsed with Wash Solution and tapped to dry before 100 μl of tetramethyl-benzidine was added to each well for another 15 min incubation at 24 °C in the dark. After the addition of the Stop Solution, the absorbance at 450 nm was determined using a microplate reader. The experiments were performed in duplicates. These values were correlated with the murine IFN-α standard curve, generated in parallel, to account for fluctuation in assay sensitivity.

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