Mol. Nutr. Food Res. 2008, *52*, 427 – 438 DOI 10.1002/mnfr.200700180

Research Article

Plant-derived polyphenols attenuate lipopolysaccharide-induced nitric oxide and tumour necrosis factor production in murine microglia and macrophages

Kirubakaran Shanmugam[1], Lina Holmquist[1], Megan Steele[1], Grant Stuchbury[1], Katrin Berbaum[1], Oliver Schulz[2], Obdulio Benavente García[3], Julián Castillo[3], Jim Burnell[1], Vernon Garcia Rivas[1], Geoff Dobson[4] and Gerald Münch[1]

[1] Biochemistry and Molecular Biology, James Cook University, Townsville, Australia
[2] Eurochem Feinchemie, Groebenzell, Germany
[3] Furfural Español-Nutrafur, Alcantarilla, Spain
[4] Tropical & Veterinary Sciences, James Cook University, Townsville, Australia

Lipopolysaccharides released during bacterial infections induce the expression of pro-inflammatory cytokines and lead to complications such as neuronal damage in the CNS and septic shock in the periphery. While the initial infection is treated by antibiotics, anti-inflammatory agents would be advantageous add-on medications. In order to identify such compounds, we have compared 29 commercially available polyphenol-containing plant extracts and pure compounds for their ability to prevent LPS-induced up-regulation of NO production. Among the botanical extracts, bearberry and grape seed were the most active preparations, exhibiting IC_{50} values of around 20 μg/mL. Among the pure compounds, IC_{50} values for apigenin, diosmetin and silybin were 15, 19 and 12 μM, in N-11 murine microglia, and 7, 16 and 25 μM, in RAW 264.7 murine macrophages, respectively. In addition, these flavonoids were also able to down-regulate LPS-induced tumour necrosis factor production. Structure-activity relationships of the flavonoids demonstrated three distinct principles: (i) flavonoid-aglycons are more potent than the corresponding glycosides, (ii) flavonoids with a 4′-OH substitution in the B-ring are more potent than those with a 3′-OH-4′-methoxy substitution, (iii) flavonoids of the flavone type (with a C2=C3 double bond) are more potent than those of the flavanone type (with a at C2-C3 single bond).

Keywords: Bacterial meningitis / Flavonoids / Inflammation / Lipopolysaccharide / Septic shock

Received: May 19 2007; revised: August 2, 2007; accepted: August 21, 2007

1 Introduction

The fight against bacterial infection represents one of the high points of modern medicine. The development of antibiotics in the 1940s offered physicians a powerful tool against bacterial infections that has saved the lives of millions of people. The goal of these drugs is to kill invading bacteria without harming the host. Gram-negative bacterial cell walls contain LPS, a highly inflammatory chemical that provokes an immune response in the human body, particularly by treatment of infected patients with beta-lactams and other cell-wall active antibiotics, which kill bacteria but release massive amounts of their wall components [1, 2]. LPS is responsible for triggering the overreaction of the host immune system, which results in the release of oxygen and nitrogen species, cytokines, and other proinflammatory mediators.

During bacterial infections, when there is a large quantity of LPS present, cytokines, in particular tumour necrosis factor (TNF), the majority released by macrophages, cause

Correspondence: Dr. Gerald Münch, Dept. Biochemistry and Molecular Biology/Comparative Genomics Centre, James Cook University, Townsville, Australia
E-mail: gerald.muench@jcu.edu.au
Fax: + 61-7-4781-6078

Abbreviations: **BM**, bacterial meningitis; **COX,** cyclooxygenase; **FCS**, foetal calf serum, **IκB,**: inhibitor of NF-κ!Unicode:3BA>B; **IL**, interleukin; **iNOS**, inducible nitric oxide synthase; **M-CSF**, macrophage-colony stimulating factor; **NADPH**, nicotinamide-adenine dinucleotide phosphate; **NF-κB**, nuclear factor kappaB; **NO**, nitric oxide; **NR**, neutral red; **PPAR**, peroxisome proliferator-activated receptor; **ROS**, reactive oxygen species; **SNP**, sodium nitroprusside; **TNF**, tumour necrosis factor

increased vasodilation, leading to hypotension, impaired oxygen distribution and myocardial depression [3]. Approximately one-third of cases result in death due to multiple organ failure or secondary infection or other complications [4]. Currently, treatment of septic shock involves fluid resuscitation to restore blood pressure and tissue reperfusion, as well as administration of antibiotics to eliminate the causative organism. The third key strategy is to interfere with the pathogenic signalling cascades mediating septic shock. Due to the significant contribution of pro-inflammatory cytokines to the progression of septic shock, a much-researched approach for treatment is the use of anti-inflammatory agents. For example, prostacyclin and pentoxifylline, which are inhibitors of leukocyte and platelet activation as well as potent vasodilators, have been tried with limited success [5]. Alternative approaches include anti-endotoxin antibodies, TNF receptor fusion proteins, anti-TNF antibodies, interleukin (IL)-1 receptor antagonist, or cyclooxygenase (COX) inhibitor, to name a few [6].

When bacteria have penetrated the blood-brain-barrier and infected the brain, an inflammation of the leptomeninges, the tissues that surround the brain and spinal cord (bacterial meningitis, BM) can occur. Inflammation in the subarachnoid space, involving the resident macrophages of the brain, the microglia, quickly causes damage to the brain. Diffuse inflammation causes pressure around the brain, leading to compression of vessels and decreased blood flow. This, as well as bacterial and white blood cells toxins, can very quickly cause brain damage. The bacterial products that elicit responses are peptidoglycan and/or LPS. Injection of either one of these into cerebrospinal fluid (CSF) can cause inflammatory response, *e.g.* the expression of TNF, IL1, IL6 and IL8 and the production of nitric oxide.

LPS initiates signalling events resulting in the up-regulation of inflammatory mediators upon binding to the Toll-like receptor 4 [7]. Among other pathways, signalling can involve the activation of the pro-inflammatory transcription factor (TF), nuclear factor κB (NF-κB), which controls the transcription of cytokines such as IL1ß, 6, 8 and 12 and TNF, as well as adhesion molecules and enzymes, COX 2 and inducible nitric oxide synthase (iNOS) [8]. In addition, activation of nicotinamide-adenine dinucleotide phosphate (NADPH)-oxidase in macrophages and microglia results in a "respiratory burst", generating reactive oxygen species (ROS), first the superoxide anion (O_2^-), which is then converted to hydrogen peroxide (H_2O_2) [9].

A vast amount of evidence suggests that glia-derived NO plays a role in the pathophysiology of BM. First, in an experimental rat model of pneumococcal BM, treatment with an iNOS inhibitor attenuated a number of early acute events associated with BM, such as increases in regional blood flow, intracranial pressure, brain water content, and leukocyte (white blood cells) pleocytosis. Secondly, co-incubation of rat astrocytes in primary culture with pneumococci stimulated NO production and was inhibited by NO synthase inhibitors. Thirdly, a significantly increased concentration of nitrite (a breakdown product of NO synthesis) in the cerebrospinal fluid (CSF) of a small number of patients with meningococcal meningitis was documented. This increased CSF nitrite levels occurred in the absence of an increase in nitrite in serum. Finally, a small study showed increased of CSF nitrite in seven patients with meningitis [10]

As the involvement of NO in the development of the complications of bacterial infection was recognised, its attenuation or modulation became a new treatment strategy in therapeutic research, but side effects were a consistent problem [11 – 13]. NO is a ubiquitous signalling molecule involved in an array of cellular processes, including inflammation [14]. In sepsis, it is released in large quantities from circulating immune cells, due to the activation of the enzyme iNOS by LPS [15]. The increased production of NO by endothelial cells is thought to contribute to vasodilation, as the deletion of iNOS prevented LPS-induced vasodilation [16]. NO is a radical scavenger, and can react with superoxide to form the highly reactive peroxynitrite ($ONOO^-$), which causes damage to membrane lipids and proteins, contributing to organ and tissue damage in septic shock [17]. As iNOS is under the control of NF-κB, suppression of NF-κB activity may contribute to regaining vascular tension in a pro-inflammatory setting. Oxidative stress, or high levels of ROS, contributes significantly to tissue damage, as well as augment the inflammatory process by increasing the activity of NF-κB [18]. Because NF-κB is redox-regulated, radical-scavenging antioxidants, such as N-acetyl cysteine (NAC) can reduce its activity [19]. NAC is the most extensively studied antioxidant for the treatment of sepsis, and it has been found to decrease TNF and IL8 production as well as inhibit platelet aggregation and leukocyte infiltration, however, more research is required to evaluate its potential clinical use [5].

Polyphenols are powerful antioxidants found in plants, which have long been the focus of interest and research for their apparent beneficial health effects. Flavonoids are a major group of polyphenolic compounds that are well known for their potent antioxidant and peroxisome proliferator-activated receptor (PPAR) agonistic properties.

Due to the large number of plant polyphenols available and their varying efficacy, we have taken a comparative approach to determine, *in vitro*, the most probable candidates for use as anti-inflammatory drugs or assisting treatment of LPS-mediated inflammatory processes. Using NO and TNF as indicators of inflammation, the aim of this study was to compare the activity of 29 plant preparations or pure plant-derived bioflavonoids. Furthermore, we attempted to identify the most important structural features determining their anti-inflammatory activities by structure-activity relationships.

2 Materials and methods

2.1 Cell maintenance

RAW264.7 cells (obtained from Ian Cassidy, University of Queensland, Australia) and N11 microglia (obtained from the University of Tübingen, Germany) were grown in 175-cm^2 cell culture flasks on DMEM containing 5% foetal calf serum (FCS), supplemented with penicillin (200 U/mL), streptomycin (200 μg/mL) and Fungizone (2.6 μg/mL). N11 cells were grown in the same manner. Both cell lines were maintained at 37°C in a humid environment containing 5% CO_2.

2.2 Activation of macrophages and microglia with LPS

After cells had grown to confluence in culture flasks, they were removed using a rubber cell scraper. Use of trypsin was avoided as it can catalyse the removal of membrane-bound receptors. Cells were concentrated by centrifugation for 3 min at 900 rpm, resuspended in a small volume of fresh DMEM (5% FCS), cell densities were estimated using a Neubauer counting chamber and cell densities adjusted to 10^6 cells/mL. Cells (100 μL) were dispensed into the 60-inner wells of 96-well plates. Sterile distilled water was added to the outer row of wells. Plates were incubated at 37°C for 24 h with 5% FCS DMEM to allow growth to confluence. The media was removed by aspiration and cell were the grown in 0.1% FCS containing media for 18 h.

Samples to be tested were dissolved in DMSO, 95% ethanol, ddH_2O or 1 M NaOH to concentrations of 100 mg/mL for complex extracts or 200 mM for 'pure' compounds (minimum 95%) (Table 1). Dilutions were then made in media from these concentrates, so that the maximum solvent content did not exceed 0.05% of the final well volume. Final concentrations of complex extracts ranged from 5 to 50 μg/mL and those of pure compounds ranged from 10 to 100 μM. All stock solutions were stored at −20°C and dilutions in media were stored at 4°C for no longer than 1 week.

After the cells had been in media for 18 h, the media were removed by aspiration. For assays with extracts and pure compounds, the dilutions in media were added 1 h prior to addition of LPS. In assays without extracts, LPS dilutions in media were added and the total volume adjusted to 100 μl with media. Plates were incubated at 37°C (5% CO_2) for 24 h. Every plate contained three wells that only contained media as a negative control, while 10 μg/mL LPS was used as a positive control. Vehicle controls were initially present, where all solvents used were tested alone at 0.05% of total volume. Once it had been established that this quantity of solvent did not have a significant effect on cells in terms of activating ability or effect on viability, vehicle controls were no longer included.

2.3 Determination of NO-scavenging activity of polyphenols

In a 96-well plate, 50 μL of a freshly dissolved 5 mM sodium nitroprusside solution in 20 mM sodium phosphate solution, pH 7.4, 0.09% NaCl was added to 50 μL of a solution of apiginin, diosmetin or silymarin in various concentrations ranging from 5 μg/mL to 5 mg/mL to for 1 h at 50°C. Since all polyphenol stock solutions were dissolved in DMSO and DMSO itself turned out to act as an NO scavenger, a solution of 20 mM sodium phosphate solution, pH 7.4, 0.9% NaCl with the respective amount of DMSO, corresponding to the DMSO in the polyphenol solution, was set to 100%. Nitrite formed was analyzed with the Griess reagent as described below.

2.4 NO determination

NO production was monitored by measuring the concentration of nitrite in the media using the "Griess reagent". Conditioned media (50 μL) from each well were transferred to a fresh 96-well plate and 25 μL of Reagent 1 (1%w/v sulfanilamide in ddH_2O) and 25 μL of Reagent 2 (0.1%w/v naphthyethylene-diamine in 5% HCl) were added and the absorbance at 540 nm determined using a plate reader (Multiskan *Ascent* with Ascent software v2.4, Labsystems).

2.5 Determination of TNF in cell culture supernatant by ELISA

The concentration of TNF, following 24 h of incubation of cells with LPS was determined by a Sandwich ELISA, according to the manufacturer's manual (Peprotech). Briefly, capture antibody was used at a concentration of 1 μg/mL in PBS (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl) (pH 7.4). Serial dilutions of TNF standard from 0 to 10 000 pg/mL in diluent (0.05% Tween-20, 0.1% BSA in PBS) were used as internal standard. TNF was detected with a biotinylated second antibody and an Avidin peroxidase conjugate with ABTS as detection reagent. Colour was determined at 405 nm in a 96-well plate reader.

2.6 Cell viability assays

Cell viability was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide), which measures the level of respiration in a cell. DMEM (50 μL) containing 1 mg/mL MTT was added to each well and incubated for 1 h at 37°C (5%CO_2). Media was then removed and 100 μL of 95% ethanol added. Following shaking for at least 30 min, the absorbance at 595 nm was measured. Cell viability was also assessed using Neutral Red (NR) uptake. Following removal of media of 50 μL of DMEM containing 25 μg/mL NR was added to the wells and incubated for 1 h at 37°C (5% CO_2). The media were then removed and

100 µL of the cell lysis solution (50% ethanol, 10% acetic acid) was added to each well. Plates were then shaken for 30 min and the absorbance at 540 nm was measures with a 96-well plate reader.

2.7 Graphical presentation and statistical analysis

Graphical data presented in this report was generated using Graphpad Prism 3.0 software. The same program was used to perform statistical analysis of results. Datasets with statistically significant differences from controls or reference values were determined using a one-way ANOVA, followed by Tukey's test for multiple comparisons. Datasets with P values less than 0.01 were considered statistically different.

3 Results

3.1 LPS-induced NO production in RAW264.7 and N11 cells

We have used murine RAW264.7 and N11 cells as model cell lines for macrophages and microglia, respectively. LPS was applied at concentrations ranging from 0.1 to 50 µg/mL. Figure 1 demonstrates that basal production of NO in cells in medium is negligible with respect to that induced by LPS. After 24 h, NO production was estimated by measuring the nitrite concentration. LPS induced concentration-dependent NO production in a hyperbolic fashion in both RAW264.7 macrophages and N11 microglia (Fig. 1). The maximum rate of NO production was very similar between the two cell lines, but the EC_{50} value for LPS in the macrophages was lower than in the microglia (2 µg/mL *vs.* 10 µg/mL). The concentration of LPS chosen for assays with plant extracts was 10 µg/mL, as this generated an intermediate response, which would allow apparent inhibition as well as margin for potential augmentation.

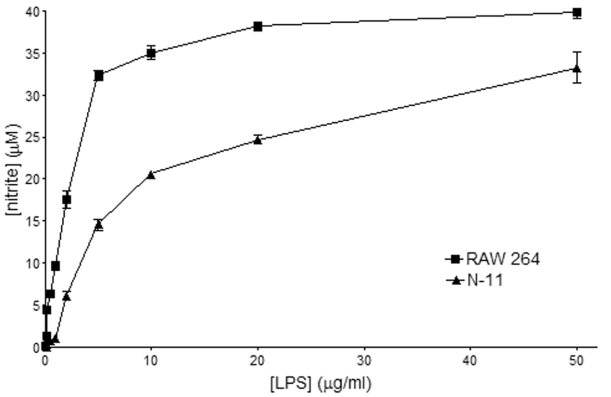


Figure 1. Nitric oxide production by RAW264.7 macrophages and N11 microglia upon activation with LPS. Nitric oxide production was measured by determination of nitrite present in the cell culture media after 24 h of incubation with LPS (*E. coli* serotype 0127:B8). Data points represent mean values of three replicates from two separate experiments, bars represent SEM.

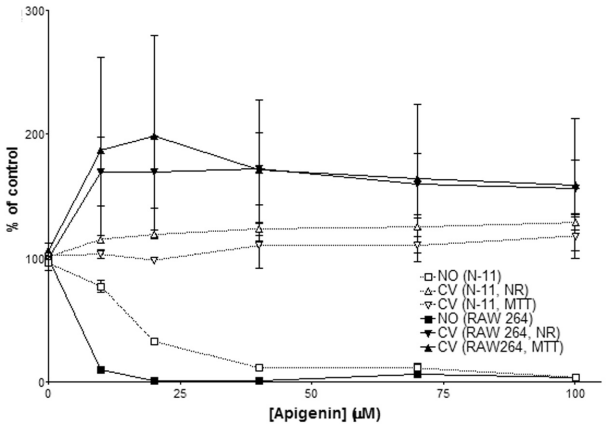


Figure 2. Dose-dependent effect of apigenin on LPS-induced NO production by N11 microglia and RAW264.7 macrophages. Cells were incubated with apigenin for 1 h, before they were activated with 10 µg/mL LPS for 24 h. In addition to nitrite, cell viability was also determined after 24 h by Neutral Red uptake (NR) and MTT reduction assays. Bars represent SEM.

3.2 Effect of plant extracts and pure compounds on LPS-induced NO production

Twenty-nine plant extracts/compounds were tested on LPS-activated N11 microglia and RAW264.7 macrophages (Table 1). Cell viability was monitored by two assays, the MTT reduction assay, which measures mitochondrial function, and the NR assay, which correlates with phagocytic activity. Results (mean values) from assays with N11 microglia and RAW264.7 macrophages with all 29 extracts or pure compounds are summarized in Tables 2 and 3, respectively. Among the botanical extracts, bearberry (containing arbutin, a glycosylated hydroquinone), grape seed (containing procyanidins) were among the most active preparations, exhibiting IC_{50} values of around 20 µg/mL. The pure compounds, which most effectively inhibited LPS-induced NO production in both cell types were apigenin (96% purity), diosmetin (96% purity), and silymarin (81% silybin + 9% isosilybin = 90% total flavanolignans).

Apigenin inhibited LPS-induced NO production in a dose-dependent manner in N11 microglia with an IC_{50} value of approximately 15 µM (Fig. 2, Table 2). In RAW macrophages, apigenin reduced LPS-induced NO production even more dramatically, reaching an IC_{50} at 7 µM (Fig. 2, Table 3). In both N11 microglia and RAW264.7 macrophages (Fig. 3), diosmetin dose dependently down-regulated LPS-induced NO production, with IC_{50} values of 19 and 16 µM, respectively (Tables 2 and 3). In N11 microglia (Fig. 4), the silymarin extract effectively decreased NO production in response to LPS, with an IC_{50} value of 8 µg/mL,

Table 1. Characteristic data of the plant extract and their active ingredients

	Solvent	Concentration of stock solution	Active compound(s) in preparation	% Active compound in preparation	Approximate concentration of major active compound in stock solution (μM)
Botanical extracts					
Bearberry (Uva ursi)	DMSO	100 mg/mL	Arbutin (phenolic)	20	60
Bitter orange	ddH2O	100 mg/mL	Flavanones	50	90
Chamomile	1M NaOH	100 mg/mL	flavones	5	9
Echinacea purpurea 4%	ddH2O	100 mg/mL	Caffeoyl-tartaric acids	5	11
Grape seed	95% Ethanol	100 mg/mL	procyanidins	95	90
Grape skin	1M NaOH	100 mg/mL	Anthocyanins	30	100
Grapefruit	DMSO	100 mg/mL	Flavanones	50	90
Green Olive leaf (20%)	DMSO	100 mg/mL	Oleuropeosides + flavones	35	70
Labiatae (Sage, Thyme & Rosemary)	DMSO	100 mg/mL	Rosmarinic acid + flavones	10	30
Rosemary (20%)	DMSO	100 mg/mL	Diterpenes	10	30
Sage	DMSO	100 mg/mL	Rosmarinic acid + flavones	10	30
Thyme	DMSO	100 mg/mL	Rosmarinic acid + flavones	10	30
Valerian	1M NaOH	100 mg/mL	Valerenic acids	1	3
Flavonoids					
Apigenin	DMSO	200 mM	Apigenin	95	200
Diosmetin	DMSO	100 mg/mL	Diosmetin	93	310
Diosmin	1M NaOH	200 mM	Diosmin	93	200
Hesperetin	DMSO	200 mM	Hesperetin	90	200
Hesperidin	DMSO	200 mM	Hesperidin	93	200
Naringenin	95% Ethanol	200 mM	Naringenin	92	200
Naringin	DMSO	200 mM	Naringin	90	200
Naringin water-soluble	DMSO	100 mg/mL	Naringin	50	90
Water-soluble citrus bioflavonoids	DMSO	100 mg/mL	Flavanones + flavones	50	90
Polyphenols (single compounds and combinations)					
Antimicro	DMSO	100 mg/mL	Rosmarinic acid	20	60
Carnosic acid 70%	95% Ethanol	100 mg/mL	Carnosic acid	80	235
Citroflavan-3-ol	1M NaOH	100 mg/mL	Flavanones + procyanidins	70	80
Citrolive	DMSO	100 mg/mL	Oleuropeosides + flavones + flavanones	40	110
Dental Care	DMSO	100 mg/mL	Flavonoids + procyanidins + rosmarinic acid	50	90
Hydroxytyrosol	DMSO	100 mg/mL	Hydroxytyrosol	10	70
Silymarin compounds (Milk thistle)	DMSO	100 mg/mL	Silybin and isosylibin	70	150

corresponding to 12 μM silybin. In macrophages (Fig. 4), the silymarin also reduced LPS-induced NO production, with an IC_{50} value of approximately 15 μg/mL, which corresponds to approximately 25 μM silybin. In contrast to the other flavonoids, the silymarin exhibited a significant degree of cytotoxicity with IC_{50} values of around 40 to 70 mM for both cell lines (Tables 2 and 3).

3.3 Direct NO-scavenging effects of apigenin, diosmetin and silymarin

It could be argued that the effective polyphenolic compounds in the experiments scavenge NO directly and that this reactivity is the main reason for their inhibitory effects on nitrite levels as it has been described for quercetin [20]. To investigate if direct NO scavenging is the cause for lower nitrite levels in the presence of apigenin, diosmetin or silymarin in our experiments, a control experiment in a cell-free system was performed. Here, NO was released by the NO donor sodium nitroprusside (SNP), which releases NO in a reaction that obeys first-order kinetics [21]. NO in aqueous solution containing oxygen is oxidized primarily to NO^{2-} [22], which allows the Griess assay to be used to indirectly measure NO production similar to the cellular assays. Of a 5 mM solution of freshly dissolved SNP, 50 μL was added to 50 μL of serially diluted solutions of apigenin, diosmetin and silymarin. Oxyhemoglobin reacts with NO under physiological conditions and was used as a positive control [23]. After incubation of the NO generating system with the potential scavengers for 1 h at 50°C, Griess reagent

Table 2. Effect of plant extracts on NO production and cell viability in microglia (N11)) activated with LPS. Cell viability is expressed as percentage of control at the highest concentration of plant extracts or compounds tested (50 μg/mL or 100 μM). In extracts, which contain at least 80% of a single active compound, concentrations of the major active compounds are expressed in μM and μg/mL. In plant extracts, which are complex mixtures or combinations of compounds, concentrations are expressed only in μg/mL

N11		NO production			Cell viability	
		Max inhibition	IC_{50}		NR	MTT
No	Extract	%	μg/mL	μM	%	%
1	*Uva ursi*	95	16	CE	110	95
2	Bitter orange	10	NA	NA	110	100
3	Chamomile	0	NA	NA	ND	90
4	*Echinacea purpurea*	0	NA	NA	ND	100
5	Grape seed	90	26	CE	80	80
6	Grape skin	20	NA	NA	110	60
7	Grapefruit	20	NA	NA	90	ND
8	Green olive leaf	50	20	CE	90	90
9	Labiatae	50	40	CE	90	90
10	Carnosic acid	95	6	18	20	ND
11	Rosemary	20	NA	NA	100	ND
12	Sage	10	NA	NA	100	ND
13	Thyme	20	NA	NA	90	ND
14	Valerian	10	NA	NA	110	ND
15	Apigenin	90	5	15	125	125
16	Diosmetin	90	6	19	120	140
17	Diosmin	60	58	90	ND	100
18	Hesperetin	50	32	100	90	80
19	Hesperidin	20	NA	NA	ND	100
20	Naringenin	70	12	40	60	80
21	Naringin	40	43	70	70	60
22	Naringin water-soluble	30	NA	NA	105	110
23	Water-soluble citrus bioflavonoids	40	NA	NA	100	110
24	AntimicRO	30	NA	NA	100	115
25	Citroflavan-3-ol	30	NA	NA	110	100
26	Citrolive	0	NA	NA	80	60
27	Dental Care	0	NA	NA	100	100
28	Hydroxytyrosol	0	NA	NA	100	60
29	Silybin (active compound in Silymarin)	100	8	12	40	80

NA: 50% inhibition of NO not achieved at the highest concentration tested; ND: No data; CE: complex extract, an IC_{50} cannot be expressed as a concentration.

was added and the concentration of nitrite was determined. Under these assay conditions, approximately 50 μM nitrite was formed. Whereas haemoglobin was effective as a NO scavenger in a dose-dependent manner, apigenin, diosmetin and silymarin did not scavenge NO to the same degree as observed in the cellular assays, indicating that direct NO scavenging is not the major mode of action for the tested polyphenols (Fig. 5).

3.4 Effect of apigenin, diosmetin and silymarin on LPS-induced TNF production

Further evidence for the interference of the selected polyphenols with signalling rather than unspecific NO scavenging was demonstrated by using TNF release as a second readout besides NO. For this purpose, RAW264.7 macrophages were pre-incubated with the selected polyphenols for 1 h before addition of 10 μg/mL LPS. After 24 h of incubation, TNF was determined by ELISA with samples of the conditioned media. Apigenin was most efficient at decreasing TNF production, with an IC_{50} of approximately 5 μg/mL, which corresponds to roughly 20 μM. Diosmetin also significantly reduced TNF production, down to 50% at approximately 25 μg/mL, which corresponds to roughly 80 μM. Silymarin decreased TNF production significantly, nearly reducing it to 50% at 35 μg/mL (Fig. 6). These data support the hypothesis that the flavonoids inhibit intracellular pro-inflammatory signalling pathways rather than scavenging NO directly.

4 Discussion

Both RAW264.7 macrophages and N11 microglia are known to produce NO in response to LPS through activation of iNOS [24–27]. NO production is easy to quantify,

Table 3. Effect of plant extracts on NO production and cell viability in macrophages (RAW 264.7) activated with LPS. Cell viability is expressed as percentage of control at the highest amount/volume or concentration of plant extracts or compounds tested (50 μg/mL or 100 μM). In extracts, which contain at least 80% of a single active compound, parameters were related to concentrations of this compound and expressed in μM. In plant extracts, which are complex mixtures or combinations of compounds, parameters were related to weight/volume and expressed in μg/mL

RAW264.7		NO production			Cell viability	
		Max inhibition	IC_{50}		NR	MTT
No	Extract	%	μg/mL	μM	%	%
1	*Uva ursi*	30	NA	NA	100	100
5	Grape seed	90	45	CE	80	50
8	Green olive leaf	40	NA	NA	110	ND
9	Labiatae	0	14	CE	120	ND
15	Apigenin	90	2	7	130	ND
16	Diosmetin	80	5	16	100	ND
29	Silybin (active compound in Silymarin)	80	15	25	80	ND

NA: 50% inhibition of NO not achieved at the highest concentration tested; ND: No data; CE: complex extract, an IC50 cannot be expressed as a concentration

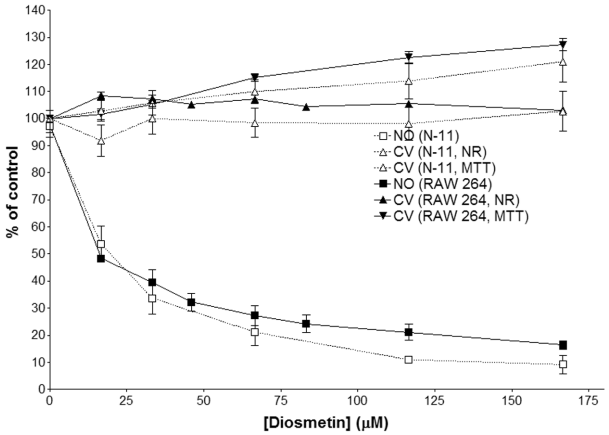


Figure 3. Dose-effect of diosmetin on LPS-induced NO production by N11 microglia and RAW264.7 macrophages. Cells were incubated with diosmetin for 1 h, before they were activated with 10 μg/mL LPS for 24 h. In addition to nitrite, cell viability was also determined after 24 h by Neutral Red uptake (NR) and MTT reduction assays. Bars represent SEM.

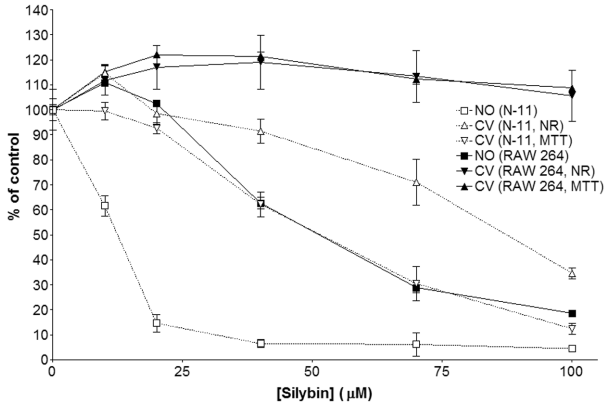


Figure 4. Dose-effect of silybin (silymarin extract) on LPS-induced NO production by N11 microglia and RAW264.7 macrophages. Cells were incubated with silymarin extract for 1 h, before they were activated with 10 μg/mL LPS for 24 h. In addition to nitrite, cell viability was also determined after 24 h by Neutral Red uptake (NR) and MTT reduction assays. Bars represent SEM.

and generally correlates with induction of inflammatory gene expression, hence it is a good indicator of cellular activation and production of other NF-κB regulated pro-inflammatory cytokines [14]. Thus, a reduction in NO production suggests that the inflammatory response is down-regulated, and that the production of other pro-inflammatory mediators would be reduced. Because NO also has cytotoxic properties, its elimination in an inflammatory setting would reduce the damage to cells and thus the extent of tissue damage.

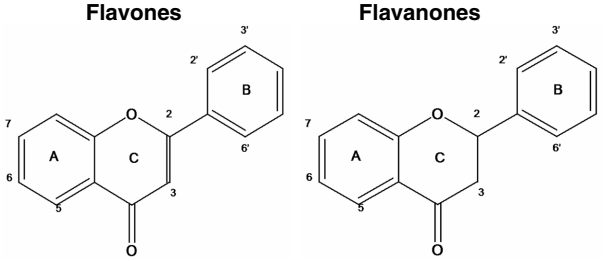
The screen of the 29 different plant extracts revealed that not all polyphenols are capable to reduce LPS-induced NO production. From the results of this screen, the three pure compounds apigenin, diosmetin and silybin, emerged as the most potent inhibitors of inflammation, based on their ability to reduce levels of NO. Interestingly, they all belong to the family of flavones characterized by a double bond in the C-ring (Table 4).

The flavonoid apigenin is found in many green herbs, but in high concentrations in chamomile [28]. It has been studied extensively and has been shown to inhibit LPS-induced inflammatory responses in many different settings. For example, iNOS and COX-2 expression in LPS-activated RAW264.7 cells was reduced by 50% at a concentration of 15 μM, which corresponds well with the results herein [29]. In addition, the results from the TNF assay strongly indicated that TNF production in response to LPS was decreased by apigenin in a dose-dependent manner. Apigenin has also been found to inhibit cytokine-induced expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin, as well as inhibit prostaglandin synthesis and IL6 and IL8 production [30]. In that particular study, apige-

Table 4. Chemical structures of polyphenolic compounds

		Substituents				
		3	5	7	3′	4′
Flavones	Apigenin	H	OH	OH	H	OH
	Diosmetin	H	OH	OH	OH	OCH_3
Flavone glycosides	Diosmetin	H	OH	OR	OH	OCH_3
Flavanones	Nariginin	H	OH	OH	H	OH
	Hesperetin	H	OH	OH	OH	OCH_3
Flavanone glycosides	Naringin (R: β-Neohesperidose)	H	OH	OR	H	OH
	Hesperidin (R:β-Rutinoside)	H	OH	OR	H	OCH_3

Flavones **Flavanones**



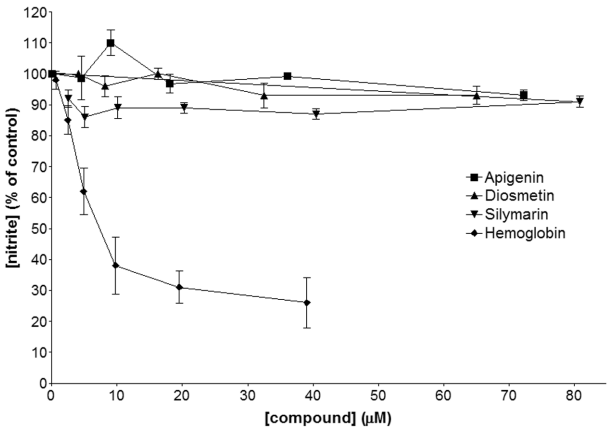


Figure 5. Direct NO scavenging by apigenin, diosmetin and silymarin. NO production was measured as the concentration of nitrite present in the media after 1 h of incubation of the polyphenolic compounds with 5 mM SNP at 50°C. The known NO scavenger haemoglobin was used as a positive control. Data points were expressed as % of control and represent the mean of three replicates, bars represent SEM.

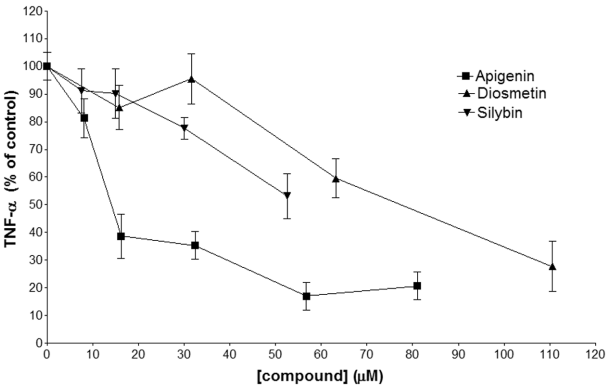


Figure 6. TNF production by LPS-activated RAW264.7 macrophages in the presence of selected polyphenols. TNF was measured by a commercial sandwich-ELISA (Peprotech) using media from cells incubated with LPS and various concentrations of apigenin, diosmetin and silymarin for 24 h. Data points represent the mean of three replicates, and bars represent SEM.

nin did not inhibit TNF-induced NF-κB nuclear translocation, suggesting its mechanism involves decreased NF-κB transcriptional activity.

Further properties of apigenin include antagonism at the thromboxane A_2 receptor. When the effects of several flavonoids on platelet aggregation, serotonin release and TxA_2 generation were investigated, apigenin abrogated arachidonic acid and collagen-induced platelet responses, such as aggregation and secretion, with a less substantial effect on TxA_2 synthesis. It was identified as a specific and potent ligand of the TxA_2 receptor in the μM range, which accounts for anti-platelet effects related to stimulation with those agonists [31].

Diosmetin is a bioflavonoid, which is highly abundant in citrus [32]. It proved to be as potent as apigenin in the inhibition of NO production, which could be expected, considering their structural similarity. Diosmetin also reduced TNF production in response to LPS [33]. Studies on the metabolism of bioflavonoids have shown that oral diosmin is readily converted to its aglycone; diosmetin, which was not metabolised and thus remained in the plasma for at least 24 h [34]. Based on its similarity to apigenin in structure, the mechanism of its anti-inflammatory activity might be similar to that of apigenin.

The active constituents of milk thistle are flavonolignans including silybin, silydianin, and silychristine, collectively known as silymarin. Silybin is the component with the greatest degree of biological activity, and milk thistle extracts are usually standardized to contain 70 – 80% silybin. Silymarin extract has been shown to effectively inhibit NO production [35]. The activity of this extract is assumed to be mediated by silybin [36]. Cell viability, however, was particularly reduced in N-11 microglia by silymarin. A possible explanation for this might have been inhibition of the retroviral driven expression of macrophage-colony stimulating factor (M-CSF), which is a growth factor for N11 cells [37]. M-CSF expression is partially under NF-κB control, which implies that silybin down-regulated NF-κB activity, consistent with previous studies [35, 38, 39].

Regarding the mechanisms of anti-inflammatory activity of bioflavonoids, the down-regulation of NF-κB activity by antioxidants is possible because it is a redox-sensitive transcription factor [40]. Its activity is regulated by the inhibitor of NF-κB (IκB), the release from which allows translocation into the nucleus [41]. The reduction of ROS by the antioxidants may have resulted in reactivation of protein tyrosine phosphatases that inhibit the kinases responsible for IκB phosphorylation. The presence of apigenin has been shown to result in inhibition of IκB kinase activity [42]. Protein kinase C (PKC), which is involved in mediating the assembly of NADPH oxidase, as well as activation of IκB kinases, can be activated by oxidative stress, through oxidation of the cysteine-rich catalytic domain [43, 44]. Thus, inhibition of ROS-mediated PKC activation by antioxidants may be an alternative mechanism in the reduction of ROS as well as NF-κB activity.

Previously, four structural groups have been implicated in determining their radical scavenging and/or antioxidant capacity: (i) the *O*-dihydroxy (catechol) structure in the B-ring of the flavonoid skeleton, (ii) the 2,3-double bond in conjugation with 4-oxo function, which are responsible for electron dislocation from the B-ring, (iii) the presence of both 3-(a)- and 5-(b)-hydroxyl groups for maximal radical scavenging capacity and strongest radical absorption, and (iv) other elements such as the presence or absence of glycosidic moieties in the flavonoid skeleton, the glycosylation site, and number and position of the free and sterified hydroxyl groups [45].

Analysis of structure-activity relationship from our study is mostly supportive of these previous results and showed three distinct principles (Table 4). IC_{50} values for statistical analysis below were used from the experiments with N11 cells.

(i) Flavonoid-aglycons (without glucose-rhamnose) are significantly more potent than the corresponding glycosides: diosmetin (IC_{50}= 6 μM) versus diosmin (IC_{50}= 58 μM); naringenin (IC_{50} = 12 μM) versus naringin (IC_{50} = 43 μM) and hesperetin (IC_{50} = 32 μM) versus hesperidin (IC_{50} > 100 μM), ($p > 0.05$ for all 3 pairs). One of the reasons for the higher potency of the non-glycosylated compounds could be their cell membrane permeability.

(ii) The flavonoids with only a 4′-OH substitution in the B-ring are more potent than those with a 3′-OH-4′-methoxy substitution: apigenin (IC_{50} = 15 μM) versus diosmetin (IC_{50} = 19 μM); naringenin (IC_{50} = 40 μM) versus hesperetin (IC_{50} = 100 μM) and naringin (IC_{50} = 70 μM) versus hesperidin (IC_{50} > 100 μM), ($p > 0.05$ for all three pairs). Apigenin, the most potent compound, does not possess a catechol structure in the B-ring, suggesting that a 4′-OH group in the B-ring is sufficient for a potent anti-inflammatory effect.

(iii) The flavonoids of the flavone type (with a $C_2 = C_3$ double bond) are more potent than those of the flavanone type (with a single bond at $C_2 – C_3$) (apigenin (IC_{50} = 15 μM) versus naringenin (IC_{50} = 40 μM) and diosmetin (IC_{50} = 19 μM versus hesperetin (IC_{50} = 100 μM), ($p > 0.05$ for each of the two pairs).

Now, it can be speculated that the “anti-inflammatory” potencies of flavonoids are based on a combination of their anti-oxidant and structural properties, *e.g.* interference with other signalling systems. Recent research on antagonism of compounds such as apigenin suggests the thromboxane A2 receptor and subsequent inhibition of thromboxane-mediated platelet aggregation and that the “anti-inflammatory” effect of the most potent flavonoids in this study might be related to their structural properties at the “receptor level”, not purely on their general antioxidant activity [31]. One anti-inflammatory target could be the nuclear PPAR receptor, a transcription factor, which can also modulate the transcriptional activity of non-PPRE-containing genes via transrepression. The best-documented mechanism by which PPAR can transrepress non-PPRE-containing genes is its ability to physically interact with the p65 subunit of NF-κB, which inhibits NF-κB-dependent transactivation [46, 47].

In respect to potential therapeutic applications, the efficacy of orally applied plant-derived polyphenols as anti-inflammatory drugs might be limited by low bioavailability and loss of function due to hepatic metabolic processing [48]. For example, flavonoid supplementation of 500 mg to 2 g/day is necessary to yield plasma levels of approximately 5 – 10 μM [49, 50]. However, the use of plant derived polyphenols as treatment for septic shock or meningitis is sub-

ject to slightly different conditions. Bioavailability would not be an issue as they could be administered intravenously prior to, or in conjunction with, antibiotics and fluid resuscitation. The main issue would rather be toxicity. The results herein show that the most potent polyphenols, namely apigenin, diosmetin and silymarin (silybin), were non-toxic at active concentrations in our cell culture system, although the therapeutic window is smaller with the silymarin extract. If the sudden high level of oxidative stress and secretion of pro-inflammatory cytokines ("cytokine storm") in septic shock can be prevented or rapidly eliminated by the tested flavonoids, then much of the damage to tissues caused by hypoperfusion and organ failure may be avoided, thus significantly improving the patient's chances of survival.

First animal studies appear to support the validity of this approach. For example, baicalein, a major bioactive flavonoid component from dried roots of *Scutellaria baicalensis* Georgi (Huang qin) was shown to have beneficial effect on circulatory failure and vascular dysfunction during sepsis induced by LPS (10 mg/kg, i.v.) in anesthetized rats. Treatment of the rats with baicalein (20 mg/kg, i.v.) significantly attenuated the deleterious hemodynamic changes of hypotension and tachycardia caused by LPS and significantly inhibited the elevation of TNF. Baicalein also decreased levels of iNOS and the overproduction of NO and superoxide anions caused by LPS. It also increased the survival rate of treated mice challenged by LPS (60 mg/kg) [51]. In conclusion, there is accumulating evidence that bioflavonoids are potent antioxidant and anti-inflammatory compounds and are likely to be safe for human use [52 – 56]. Long-term oral supplementation may aid the body's detoxification of oxygen radicals, thus reducing damage to cellular membranes, as well as protect organs from the harmful effects of chronic inflammatory conditions. Herein, we suggest an additional application, namely that plant-derived antioxidants could be used to aid in the treatment of LPS-induced complications of bacterial infections, by reducing levels of ROS and NO and thus down-regulating NF-κB-driven cytokine production. By reducing cellular damage and inhibiting the rampant inflammatory response, organ failure may be prevented, thus significantly improving the patient's chances of survival. Screening these bioflavonoids in animal models of septic shock, *e. g.* induced by administration of a high dose of LPS into mice would be an appropriate next step to compare their efficacy and safety for this application [57].

Microglial activation also plays an important role in various neurodegenerative diseases such as Alzheimer's and Parkinson's diseases as well as cerebral inflammatory diseases such as multiple sclerosis, whereby other pro-inflammatory inducers – often yet unknown – assume the role of LPS. Therefore, the inhibition of microglial activation by the compounds tested might have significant meaning. For example, Alzheimer's disease (AD) is characterized by a chronic inflammatory process around amyloid plaques, activation of micro- and astroglia and increased levels of radicals and pro-inflammatory cytokines [58]. iNOS, IL1ß, IL6 and TNF have been detected in amyloid plaques and adjacent microglial and astroglial cells [59]. Activated glia are a further source of ROS (such as superoxide and NO) through the activation of NADPH oxidase and iNOS, respectively [60, 61]. ROS act as second messengers in pro-inflammatory signal transduction and intracellular acting antioxidants such as flavonoids including apigenin and diosmetin can thus act as anti-inflammatory drugs for these diseases. In a recent meta-analysis of 187 retrospective studies, garlic extract, curcumin, melatonin, resveratrol, Ginkgo biloba extract, green tea, vitamin C and vitamin E were identified as agents that show promise in helping to prevent AD [62].

We thank A. Baxter and H. Korner for inspiring and stimulating discussions. We are also very thankful to Sheryl Robertson, David Miller and Lubna Ukani for donations of chemicals. This work was supported by Furfural Español-Nutrafur, the JO & JR Wicking Trust (to GM), by Alzheimer's Australia and an ANZ Ph.D. fellowship (to GS).

The authors have declared no conflict of interest.

5 References

[1] Nau, R., Eiffert, H., Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. *Cli. Microbiol. Rev.* 2002, *15*, 95 – 110.

[2] Nau, R., Eiffert, H., Minimizing the release of proinflammatory and toxic bacterial products within the host: a promising approach to improve outcome in life-threatening infections. *FEMS Immunol. Med. Microbio.* 2005, *44*, 1 – 16.

[3] Bridges, E. J., Dukes, S., Cardiovascular aspects of septic shock: pathophysiology, monitoring, and treatment. *Crit. Care Nurse* 2005, *25*, 14 – 16, 18 – 20, 22 – 14 passim; quiz 41 – 12.

[4] Angus, D. C., Linde-Zwirble, W. T., Lidicker, J., Clermont, G., *et al.*, Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit. Care Med.* 2001, *29*, 1303 – 1310.

[5] Del Sorbo, L., Zhang, H., Is there a place for N-acetylcysteine in the treatment of septic shock? *Crit. Care* 2004, *8*, 93 – 95.

[6] Arndt, P., Abraham, E., Immunological therapy of sepsis: experimental therapies. *Intensive Care Med.* 2001, *27 (Suppl 1)*, S104 – 115.

[7] Beutler, B., TLR4 as the mammalian endotoxin sensor. *Curr. Top. Microbiol. Immunol.* 2002, *270*, 109 – 120.

[8] Baeuerle, P.A., Pro-inflammatory signaling: last pieces in the NF-kappaB puzzle? *Curr. Biol.* 1998, *8*, R19 – 22.

[9] Adami, C., Sorci, G., Blasi, E., Agneletti, A.L., *et al.*, S100B expression in and effects on microglia. *Glia* 2001, *33*, 131 – 142.

[10] Buster, B. L., Weintrob, A. C., Townsend, G. C., Scheld, W. M., Potential role of nitric oxide in the pathophysiology of experimental bacterial meningitis in rats. *Infect. Immun.* 1995, *63*, 3835 – 3839.

[11] Wolfe, T. A., Dasta, J. F., Use of nitric oxide synthase inhibitors as a novel treatment for septic shock. *Ann. Pharmacother.* 1995, *29*, 36 – 46.

[12] Thiemermann, C., Selective inhibition of the activity of inducible nitric oxide synthase in septic shock. *Prog. Clin. Biol. Res.* 1995, *392*, 383 – 392.

[13] Feihl, F., Waeber, B., Liaudet, L., Is nitric oxide overproduction the target of choice for the management of septic shock? *Pharmacol. Ther.* 2001, *91*, 179 – 213.

[14] Korhonen, R., Lahti, A., Kankaanranta, H., Moilanen, E., Nitric oxide production and signaling in inflammation. *Curr. Drug Targets Inflamm. Allergy* 2005, *4*, 471 – 479.

[15] Liu, S., Adcock, I. M., Old, R. W., Barnes, P. J., *et al.*, Lipopolysaccharide treatment *in vivo* induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem. Biophys. Res. Commun.* 1993, *196*, 1208 – 1213.

[16] Gunnett, C. A., Chu, Y., Heistad, D. D., Loihl, A., *et al.*, Vascular effects of LPS in mice deficient in expression of the gene for inducible nitric oxide synthase. *Am. J. Physiol.* 1998, *275*, H416 – 421.

[17] Szabo, C., Cuzzocrea, S., Zingarelli, B., O'Connor, M., *et al.*, Endothelial dysfunction in a rat model of endotoxic shock. Importance of the activation of poly (ADP-ribose) synthetase by peroxynitrite. *J. Clin. Inves.* 1997, *100*, 723 – 735.

[18] Zhang, H., Slutsky, A. S., Vincent, J. L., Oxygen free radicals in ARDS, septic shock and organ dysfunction. *Intensive Care Med.* 2000, *26*, 474 – 476.

[19] Haddad, J. J., Science review: Redox and oxygen-sensitive transcription factors in the regulation of oxidant-mediated lung injury: role for nuclear factor-kappaB. *Crit. Care* 2002, *6*, 481 – 490.

[20] Lopez-Lopez, G., Moreno, L., Cogolludo, A., Galisteo, M., *et al.*, Nitric oxide (NO) scavenging and NO protecting effects of quercetin and their biological significance in vascular smooth muscle. *Mol. Pharmacol.* 2004, *65*, 851 – 859.

[21] Roncaroli, F., van Eldik, R., Olabe, J. A., Release of NO from reduced nitroprusside ion. Iron-dinitrosyl formation and NO-disproportionation reactions. *Inorg. Chem.* 2005, *44*, 2781 – 2790.

[22] Ignarro, L. J., Nitric oxide-mediated vasorelaxation. *Thromb. Haemost.* 1993, *70*, 148 – 151.

[23] Blomberg, L. M., Blomberg, M. R., Siegbahn, P. E., A theoretical study on the binding of O(2), NO and CO to heme proteins. *J. Inorg. Biochem.* 2005, *99*, 949 – 958.

[24] Nathan, C., Xie, Q. W., Regulation of biosynthesis of nitric oxide. *J. Biol. Chem.* 1994, *269*, 13725 – 13728.

[25] Dukic-Stefanovic, S., Gasic-Milenkovic, J., Deuther-Conrad, W., Munch, G., Signal transduction pathways in mouse microglia N-11 cells activated by advanced glycation end-products (AGEs). *J. Neurochem.* 2003, *87*, 44 – 55.

[26] Sumi, D., Ignarro, L. J., Regulation of inducible nitric oxide synthase expression in advanced glycation end product-stimulated raw 264.7 cells: the role of heme oxygenase-1 and endogenous nitric oxide. *Diabetes* 2004, *53*, 1841 – 1850.

[27] Schmidt, H. H., Warner, T. D., Nakane, M., Forstermann, U. *et al.*, Regulation and subcellular location of nitrogen oxide synthases in RAW264.7 macrophages. *Mol. Pharmacol.* 1992, *41*, 615 – 624.

[28] Smolinski, A. T., Pestka, J. J., Modulation of lipopolysaccharide-induced proinflammatory cytokine production *in vitro* and *in vivo* by the herbal constituents apigenin (chamomile), ginsenoside Rb(1) (ginseng) and parthenolide (feverfew). *Food Chem. Toxicol.* 2003, *41*, 1381 – 1390.

[29] Liang, Y. C., Huang, Y. T., Tsai, S. H., Lin-Shiau, S. Y., *et al.*, Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. *Carcinogenesis* 1999, *20*, 1945 – 1952.

[30] Gerritsen, M. E., Carley, W. W., Ranges, G. E., Shen, C. P., *et al.*, Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. *Am. J. Pathol.* 1995, *147*, 278 – 292.

[31] Guerrero, J. A., Lozano, M. L., Castillo, J., Benavente-Garcia, O., *et al.*, Flavonoids inhibit platelet function through binding to the thromboxane A2 receptor. *J. Thromb. Haemost.* 2005, *3*, 369 – 376.

[32] Manthey, J. A., Grohmann, K., Guthrie, N., Biological properties of citrus flavonoids pertaining to cancer and inflammation. *Curr. Med. Chem.* 2001, *8*, 135 – 153.

[33] Comalada, M., Ballester, I., Bailon, E., Sierra, S., *et al.*, Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: analysis of the structure-activity relationship. *Biochem. Pharmacol.* 2006, *72*, 1010 – 1021.

[34] Villa, P., Cova, D., De Francesco, L., Guaitani, A., *et al.*, Protective effect of diosmetin on *in vitro* cell membrane damage and oxidative stress in cultured rat hepatocytes. *Toxicology* 1992, *73*, 179 – 189.

[35] Manna, S. K., Mukhopadhyay, A., Van, N. T., Aggarwal, B. B., Silymarin suppresses TNF-induced activation of NF-kappa B, c-Jun N-terminal kinase, and apoptosis. *J. Immunol.* 1999, *163*, 6800 – 6809.

[36] Kosina, P., Kren, V., Gebhardt, R., Grambal, F., *et al.*, Antioxidant properties of silybin glycosides. *Phytother. Res.* 2002, *16 (Suppl 1)*, S33 – 39.

[37] Righi, M., Pierani, A., Boglia, A., De Libero, G., *et al.*, Generation of new oncogenic murine retroviruses by cotransfection of cloned AKR and MH2 proviruses. *Oncogene* 1989, *4*, 223 – 230.

[38] Wang, M. J., Lin, W. W., Chen, H. L., Chang, Y. H., *et al.*, Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *Eur. J. Neurosci.* 2002, *16*, 2103 – 2112.

[39] Saliou, C., Rihn, B., Cillard, J., Okamoto, T., *et al.*, Selective inhibition of NF-kappaB activation by the flavonoid hepatoprotector silymarin in HepG2. Evidence for different activating pathways. *FEBS Lett.* 1998, *440*, 8 – 12.

[40] Kabe, Y., Ando, K., Hirao, S., Yoshida, M., *et al.*, Redox regulation of NF-kappaB activation: distinct redox regulation between the cytoplasm and the nucleus. *Antioxid. Redox Signal* 2005, *7*, 395 – 403.

[41] Regnier, C. H., Song, H. Y., Gao, X., Goeddel, D. V., *et al.*, Identification and characterization of an IkappaB kinase. *Cell* 1997, *90*, 373 – 383.

[42] Lin, W. J., Yeh, W. C., Implication of Toll-like receptor and tumour necrosis factor alpha signaling in septic shock. *Shock* 2005, *24*, 206 – 209.

[43] Lee, S. R., Kwon, K. S., Kim, S. R., Rhee, S. G., Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J. Biol. Chem.* 1998, *273*, 15366 – 15372.

[44] Curnutte, J. T., Erickson, R. W., Ding, J., Badwey, J. A., Reciprocal interactions between protein kinase C and components of the NADPH oxidase complex may regulate superoxide production by neutrophils stimulated with a phorbol ester. *J. Biol. Chem.* 1994, *269*, 10813 – 10819.

[45] Benavente-Garcia, O., Castillo, J., Marin, F., Ortuno, A., *et al.*, Uses and properties of citrus flavonoids. *J. Agric. Food Chem.* 1997, *45*, 4505 – 4515.

[46] Delerive, P., De Bosscher, K., Besnard, S., Vanden Berghe, W. *et al.*, Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. *J. Biol. Chem.* 1999, *274*, 32048 – 32054.

[47] Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., *et al.*, A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* 2005, *437*, 759 – 763.

[48] Duthie, G. G., Gardner, P. T., Kyle, J. A., Plant polyphenols: are they the new magic bullet? *Proc. Nutr. Soc.* 2003, *62*, 599 – 603.

[49] Nakagawa, K., Okuda, S., Miyazawa, T., Dose-dependent incorporation of tea catechins, (-)-epigallocatechin-3-gallate and (-)-epigallocatechin, into human plasma. *Biosci. Biotechnol. Biochem.* 1997, *61*, 1981 – 1985.

[50] Nielsen, I. L., Chee, W. S., Poulsen, L., Offord-Cavin, E., *et al.*, Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J. Nutr.* 2006, *136*, 404 – 408.

[51] Cheng, T. J., Wang, Y. J., Kao, W. W., Chen, R. J., *et al.*, Protection against arsenic trioxide-induced autophagic cell death in U118 human glioma cells by use of lipoic acid. *Food Chem. Toxicol.* 2007, *45*, 1027 – 1038.

[52] Halliwell, B., Rafter, J., Jenner, A., Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr.* 2005, *81*, 268S – 276S.

[53] Kim, H. P., Son, K. H., Chang, H. W., Kang, S. S., Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.* 2004, *96*, 229 – 245.

[54] Middleton, E., Jr., Effect of plant flavonoids on immune and inflammatory cell function. *Adv. Exp. Med. Biol.* 1998, *439*, 175 – 182.

[55] Gerritsen, M. E., Flavonoids: inhibitors of cytokine induced gene expression. *Adv. Exp. Med. Biol.* 1998, *439*, 183 – 190.

[56] Havsteen, B., Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol.* 1983, *32*, 1141 – 1148.

[57] Takahashi, K., Morikawa, A., Kato, Y., Sugiyama, T., *et al.*, Flavonoids protect mice from two types of lethal shock induced by endotoxin. *FEMS Immunol. Med. Microbiol.* 2001, *31*, 29 – 33.

[58] Eikelenboom, P., Bate, C., Van Gool, W. A., Hoozemans, J. J., *et al.*, Neuroinflammation in Alzheimer's disease and prion disease. *Glia* 2002, *40*, 232 – 239.

[59] Griffin, W. S., Sheng, J. G., Roberts, G. W., Mrak, R. E., Interleukin-1 expression in different plaque types in Alzheimer's disease: significance in plaque evolution. *J. Neuropathol. Exp. Neurol.* 1995, *54*, 276 – 281.

[60] Shimohama, S., Apoptosis in Alzheimer's disease – an update. *Apoptosis* 2000, *5*, 9 – 16.

[61] Wong, A., Dukic-Stefanovic, S., Gasic-Milenkovic, J., Schinzel, R., *et al.*, Anti-inflammatory antioxidants attenuate the expression of inducible nitric oxide synthase mediated by advanced glycation endproducts in murine microglia. *Eur. J. Neurosci.* 2001, *14*, 1961 – 1967.

[62] Frank, B., Gupta, S., A review of antioxidants and Alzheimer's disease. *Ann. Clin. Psychiatry* 2005, *17*, 269 – 286.

