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Flavonoids inhibit myelin phagocytosis by macrophages; a structure–activity relationship study

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Abstract

Demyelination is a characteristic hallmark of the neuro-inflammatory disease multiple sclerosis. During demyelination, macrophages phagocytose myelin and secrete inflammatory mediators that worsen the disease. Here, we investigated whether flavonoids, naturally occurring immunomodulating compounds, are able to influence myelin phagocytosis by macrophages *in vitro*. The flavonoids luteolin, quercetin and fisetin most significantly decreased the amount of myelin phagocytosed by a macrophage cell line without affecting its viability. IC_{50} values for these compounds ranged from 20 to 80 μM. The flavonoid structure appeared to be essential for observed effects as flavonoids containing hydroxyl groups at the B-3 and B-4 positions in combination with a C-2,3 double bond were most effective. The capacity of the various flavonoids to inhibit phagocytosis correlated well with their potency as antioxidant, which is in line with the requirement of reactive oxygen species for the phagocytosis of myelin by macrophages. Our results implicate that flavonoids may be able to limit the demyelination process during multiple sclerosis.

Keywords: Multiple sclerosis; Inflammation; Macrophage; Demyelination; Flavonoid; Reactive oxygen species

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating inflammatory disease of the central nervous system (CNS), resulting in neurological deficits. It is considered as an autoimmune disease and is likely the outcome of various genetic and environmental factors. Current treatments are not very effective and may exert many side effects. The availability of a complementary non-invasive therapy, that has a long-term beneficial effect and is less harmful, is therefore, favourable for people suffering from MS.

Macrophages are the main effector cells in MS and its animal model, experimental allergic encephalomyelitis (EAE) [1]. In MS, monocyte-derived macrophages accumulate in the CNS and produce mediators that cause myelin degradation. In addition, myelin is being phagocytosed by macrophages, which leads to a further activation and production of toxic mediators by these cells. One group of factors produced are reactive oxygen species (ROS), which are involved in the pathogenesis of MS and EAE [2,3]. In MS lesions, cellular DNA of neurons and glial cells is damaged in an oxidative manner [4], which finally causes neuron degeneration and oligodendrocyte loss. In this process, various ROS are involved but in particular hydrogen peroxide (H_2O_2) seems to be responsible for many of the cytotoxic effects found. Indeed, catalase, an enzyme that converts the reactive H_2O_2 to water, markedly suppressed clinical signs of EAE [3] and protected oligodendrocytes from ROS-induced cell death [5]. In contrast, superoxide dismutase (SOD), a potential superoxide scavenger was ineffective [3]. H_2O_2 causes myelin lipid peroxidation [6] and enhances the phagocytic capacity of peritoneal macrophages [7], a process that can be blocked by catalase [8]. Besides ROS, infiltrated or resident macrophages produce other harmful inflammatory mediators, like cytokines, prostaglandins and matrix metalloproteases, which may also contribute to the pathogenesis of MS. Many

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Abbreviations: ROS, reactive oxygen species; MS, multiple sclerosis; EAE, experimental allergic encephalomyelitis; DiI, 1.1″-diotadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate; DHR, dihydrorhodamine 123; NO, nitric oxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; H_2O_2, hydrogen peroxide.

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immunotherapeutic strategies to treat MS, therefore, focus on modulating the secretion and/or activity of these macrophage products.

Flavonoids are a widespread group containing over 4000 naturally occurring compounds, mainly found in fruit and vegetables, of which many are known to have anti-inflammatory, antiviral and anticarcinogenic properties [9]. The anti-inflammatory action of many, but not all flavonoids, appears to be largely based on their antioxidant effect. They can scavenge products, like superoxide [10], H_2O_2 [11], nitric oxide (NO) [12] and hydroxyl radicals [13]. For instance, oxygen radical formation by peripheral blood monocytes is suppressed by the flavonoid catechin [14] and others, like fisetin and quercetin, have been shown to inhibit oxidative modification of low-density lipoproteins by macrophages [15]. The activation of nuclear factor-kappa B (NF-κB) is critical for the production of pro-inflammatory cytokines and NO. Flavonoids and related compounds are reported to repress NF-κB-dependent gene expression [16] and inhibit tumour nercrosis factor alpha (TNFα), interleukin-1 and NO release [17,18], illustrating their wide range of immunomodulating properties.

Since myelin phagocytosis by macrophages appears to be a crucial event in the course of MS, we here study the *in vitro* effects of flavonoids on this process. For this study, we selected flavonoids with different structural properties, and tested their capacity to affect phagocytosis of fluorescently labelled myelin by mouse RAW 264.7 cells. To study if the effectiveness of flavonoids to inhibit myelin phagocytosis related to their antioxidant capacity, their ability to influence cellular ROS levels was also tested.

2. Materials and methods

2.1. Flavonoids

Quercetin, luteolin, hesperetin and apigenin were obtained from Kaden Biochemicals, morin and fisetin from INDOFINE, and epicatechin from Sigma. Structural properties are shown in Table 1. These components were kept in the dark and were freshly dissolved in 50 mM KOH to a 15 mM stock solution prior to each experiment.

2.2. Myelin isolation

Myelin was isolated from brain tissue of adult mice by means of sucrose density-gradient centrifugation, according to the method of Norton and Poduslo [19]. Myelin protein content was determined comparing a myelin sample with a BSA standard curve. For this purpose, myelin and BSA samples were added to bicinchoninic acid (Pierce) reagent and incubated for 30 min at 37°, whereafter extinction was measured at 562 nm. Myelin was fluorescently labelled by incubating 1 mg/mL for 30 min at 37° with 12.5 μg/mL of the lipophilic dye 1.1″-diotadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate (DiI; Molecular probes). Excess DiI was removed by washing with sterile PBS (15 min at 4000 *g*). Labelled myelin was stored in small aliquots at −20° in the dark [20].

2.3. Cytotoxicity

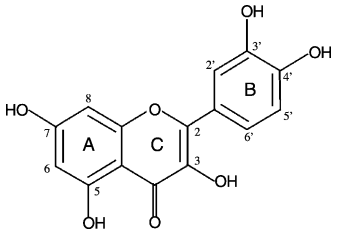
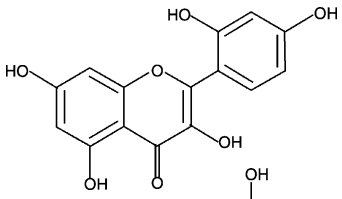
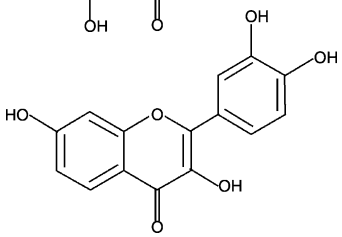
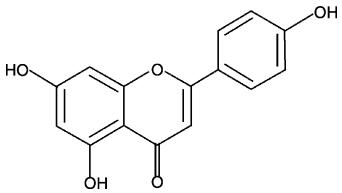
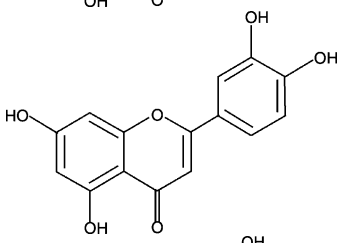
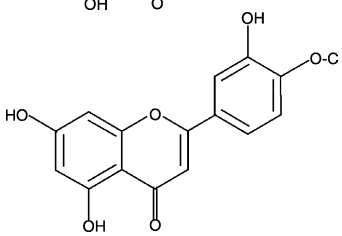
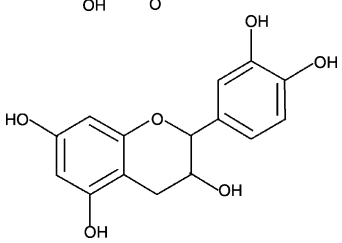
Cell viability was established using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [21]. Mouse RAW 264.7 macrophages (ATCC), screened on a regular basis for mycoplasma infections, were plated in a 96-wells culture dish in a concentration of 0.25×10^5 cells/well in RPMI-1640 supplemented with 10% foetal calf serum, penicillin (100 IU/mL), streptomycin (50 mg/mL) and glutamine (1 mM). After an overnight culture period, cells were washed with RPMI-1640 medium and subsequently, various concentrations of testing compounds and 10 μL of MTT solution (5 mg/mL in PBS) were added in a total volume of 100 μL medium. Cells were incubated for 4 hr at 37° and 5% CO_2 whereafter the supernatant was removed and 175 μL DMSO/glycine was added to each well to dissolve produced formazan crystals. The extinction was measured at 540 nm using a microplate reader. The mean value of three wells was used for calculating the viability as percentage of control. To detect significant differences, statistics were performed using a Student's *t*-test on four independent experiments.

2.4. Myelin phagocytosis assay

The myelin phagocytosis assay was performed as described before [8,20]. Briefly, RAW 264.7 cells were cultured overnight in a 24-well culture dish in culture medium at a concentration of 0.25×10^6 cells/well. The following day, non-adherent cells were removed by washing the wells twice with RPMI-1640, whereafter 0.5 mL RPMI with 2% normal mouse serum and flavonoids were added. DiI-labelled myelin was added (20 μg/well) and cells were incubated for 90 min at 37° and 5% CO_2. After phagocytosis, non-ingested and non-bound myelin was removed by washing the plates two times with RPMI-1640 and once with PBS. Cells were detached by incubation at 37° and 5% CO_2 for 15 min with PBS/1 mM EDTA, collected, washed with PBS/0.1% BSA (5 min, 340 g) and resuspended in 200 μL PBS/0.1% BSA. The amount of myelin phagocytosed was determined by measuring the cellular fluorescent intensity using a FACScan (Beckton Dickinson). Experiments were repeated at least four times, whereafter significant differences were determined using a Student's *t*-test.

Fluorescence microscopy was used to visualise myelin uptake by macrophages and the effect of flavonoids on this process. For this purpose, RAW 264.7 cells were cultured on 12-mm cover glasses, and after phagocytosis of myelin,

Table 1
The flavonoids tested including their structural properties and the IC_{50} values for inhibition of myelin phagocytosis and cellular ROS levels

Flavonoid	Class	Structure	Phagocytosis inhibition IC_{50} (μM)	Estimated IC_{50} for ROS inhibition (μM)
Quercetin	Flavonol		80	41
Morin	Flavonol		>300	243
Fisetin	Flavonol		78	60
Apigenin	Flavone		105	73
Luteolin	Flavone		21	45
Hesperetin	Flavanone		210	>300
Epicatechin	Flavanol		No effect	>300

cells were fixed in 4% paraformaldehyde for 10 min. Subsequently, cell nuclei were stained for 10 min with the Hoechtst dye (0.5 μg/mL) and cells were embedded in mounting medium. Samples were viewed using a Nikon Eclipse 800 fluorescence microscope.

2.5. ROS production

The effect of flavonoids on ROS production during myelin phagocytosis was determined using a dihydrorhodamine 123 (DHR) assay. DHR mainly reacts with H_2O_2 in

a peroxidase-like reaction to yield rhodamine 123, which localises to the mitochondria [22], although it may also react with other ROS-like superoxide [23] and NO [24]. RAW 264.7 cells were plated in a 24-wells culture dish at 0.5×10^6 cells/well, cultured overnight and washed once with RPMI-1640 medium. Quercetin, luteolin, hesperetin, fisetin, apigenin, morin and epicatechin were solved in KOH, further diluted in medium and added in a concentration of 30, 100 or 300 μM to the cells. Phorbol myristate acetate (PMA, 1 mg/mL) was used as a known stimulator of ROS production. Finally, myelin (20 μg/well) was added and cells were incubated for 90 min at 37°/5% CO_2. Non-bound myelin was removed by washing the cells twice with RPMI-1640 medium, followed by incubation for another 30 min with 0.5 μM DHR (stock solution of 5.8 mM solved in 96% ethanol). Cells were washed once with PBS and detached after incubating with 1 mM PBS/EDTA by vigorous pipetting. Cells were washed with and resuspended in PBS/0.1% BSA. Fluorescence intensity was determined in a FACScan flowcytometer. The mean fluorescence intensity of three wells was used for calculating the DHR oxidation as percentage of control. Statistics were performed on four independent experiments using the Student's *t*-test.

DHR oxidation was visualised by fluorescence microscopy by further preparing the cells as described earlier for the myelin phagocytosis assay.

3. Results

3.1. Effects of flavonoids on myelin phagocytosis

All tested flavonoids, except epicatechin, were able to dose-dependently inhibit myelin phagocytosis by RAW 264.7 cells (Fig. 1). Myelin uptake by macrophages occurred to a variable extend, which was almost completely inhibited by flavonoids, like quercetin (Fig. 1A). Luteolin was the most potent flavonoid and decreased myelin phagocytosis at concentrations of 10 μM and

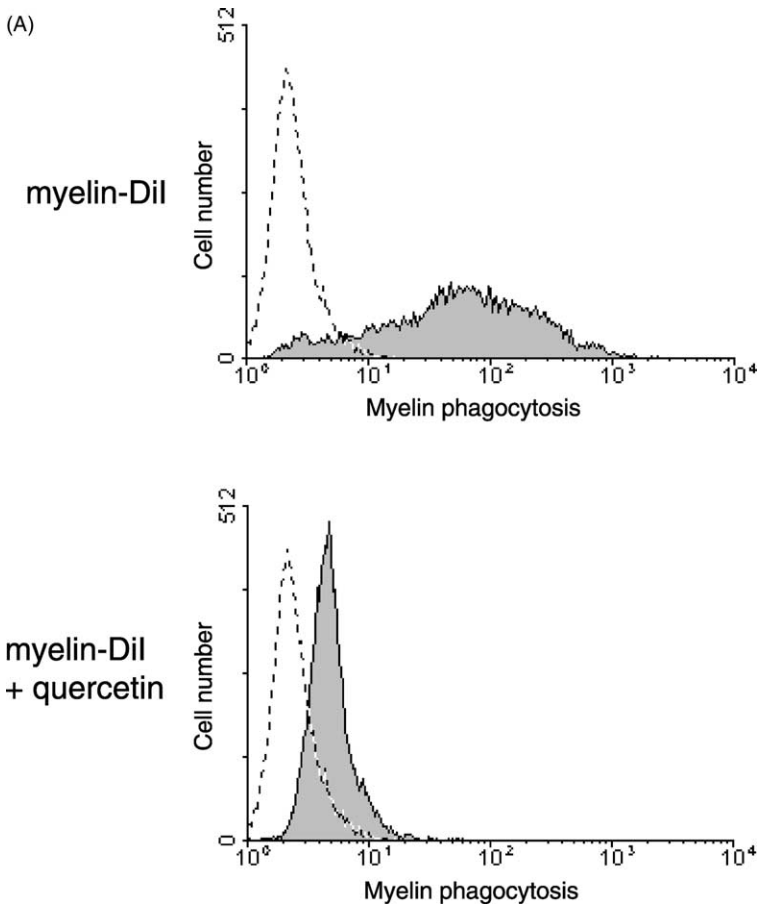


Fig. 1. Flavonoids inhibit myelin phagocytosis by RAW 264.7 cells. DiI-labelled myelin, is taken up by RAW 264.7 cells during a 90-min co-culture period, as measured by flowcytometry. (A) Phagocytosis occurred to a variable extent and could be almost completely blocked when 300 μM quercetin was added (dashed line, cells without myelin; grey, cells with myelin DiI ± quercetin). (B) Quercetin and other flavonoids dose-dependently inhibited myelin phagocytosis (●) without affecting cell viability as detected using an MTT cytotoxicity assay (□). Myelin phagocytosis by and viability of untreated cells were used as controls and set to 100%. Data are presented as percentage of control ± SEM and represent mean fluorescence intensity/extinction derived from four independent experiments. (∗) Indicates significant differences compared to control myelin phagocytosis/MTT conversion, $P < 0.05$.

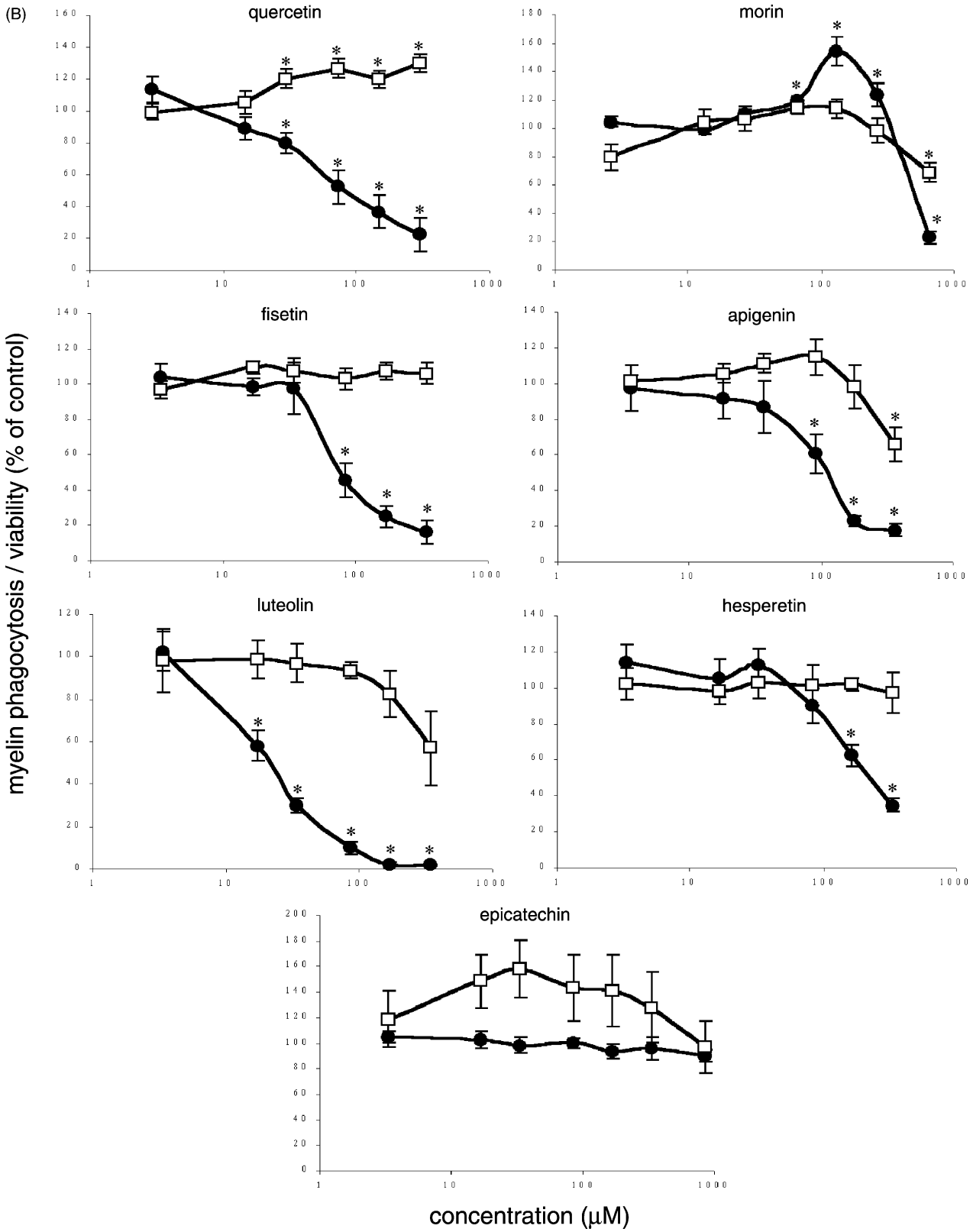


Fig. 1. (*Continued*).

higher, with an IC_{50} value of 21 μM (Table 1). Quercetin, fisetin and apigenin were less effective and inhibited myelin phagocytosis with IC_{50} values ranging from 78 to 105 μM. Hesperetin only reduced myelin phagocytosis when used in relatively high concentrations (>80 μM; IC_{50} 210 μM). In contrast, morin, although inhibiting phagocytosis at high doses (>250 μM), increased myelin uptake at lower doses of around 100 μM. Finally, epica-

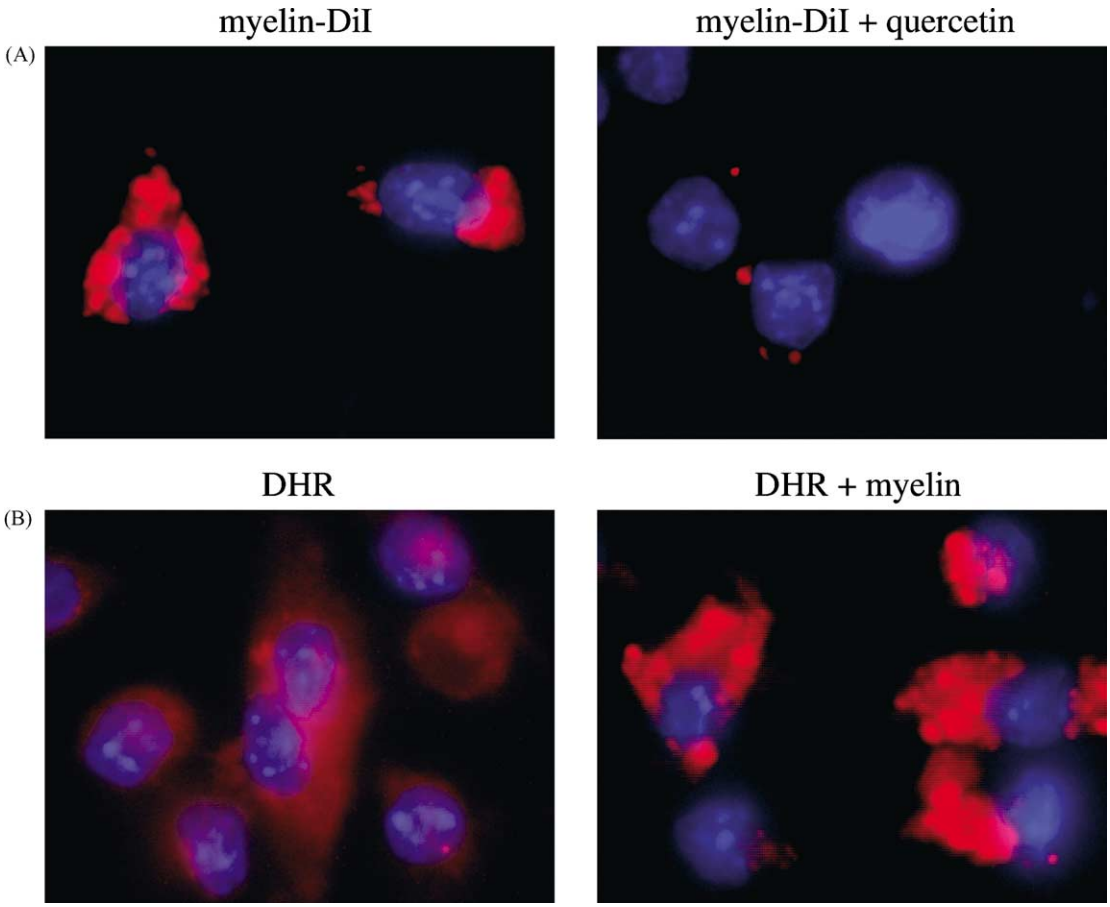


Fig. 2. Visualisation of myelin phagocytosis and ROS production. RAW 264.7 cells were incubated during 90 min with DiI-labelled myelin with or without 300 μM quercetin. (A) Binding and/or uptake of myelin is blocked by quercetin (myelin in red; nuclear staining by Hoechst dye in blue). (B) Myelin-induced ROS production, as measured by DHR oxidation, is mainly present in vesicles in contrast to the basal cellular ROS levels (oxidised DHR in red; nuclear staining in blue).

techin did not affect macrophage myelin phagocytosing capacity.

Myelin phagocytosis inhibition by flavonoids was visualised by fluorescence microscopy. Here, it was found that quercetin diminishes myelin phagocytosis by blocking both the binding and/or uptake of myelin (Fig. 2A). After a 90-min culture period with DiI-labelled myelin, only very few fluorescent myelin particles were found to be attached to and taken up by RAW 264.7 cells treated with flavonoids, whereas control cells ingested large amounts of myelin particles (Fig. 2A).

3.2. Cytotoxicity of flavonoids

Cell viability of RAW 264.7 cells was tested using the MTT assay and was not significantly affected when incubated with any of the selected flavonoids up to a concentration of 100 μM (Fig. 1, data shown up to concentrations of 350 μM). When tested in higher concentrations, morin and apigenin were cytotoxic and dose-dependently decreased cell viability. In contrast, quercetin increased cell viability. The vehicle, KOH, alone did not affect cell viability (up to 0.1 mM, data not shown).

3.3. Effect of flavonoids on ROS production during myelin phagocytosis

The amount of DHR oxidation was used as a measure for the ROS content of macrophages. The addition of myelin to RAW 264.7 cells was found to increase ROS production by macrophages in a vesicular pattern, similar to that of myelin uptake (Fig. 2B). Even without myelin cellular oxidation of DHR occurred, which is probably due to basal ROS production by these murine macrophages (Fig. 3A). Addition of myelin increased ROS production by 60% (Fig. 3A) which could be blocked by quercetin (Fig. 3B), whereas PMA stimulated ROS production by 170% (data not shown).

All tested flavonoids diminished DHR oxidation during myelin phagocytosis by RAW 264.7 cells in a dose-dependent fashion, and sometimes even reduced ROS content below basal levels (Fig. 3C). Their structure–activity relationship is depicted in Table 1. Quercetin and luteolin were most effective and were able to reduce the ROS levels at all tested concentrations with a maximum of $77 \pm 1.2\%$ and $67 \pm 4.1\%$ inhibition, respectively, at 300 μM. Fisetin, apigenin and morin were slightly less effective and

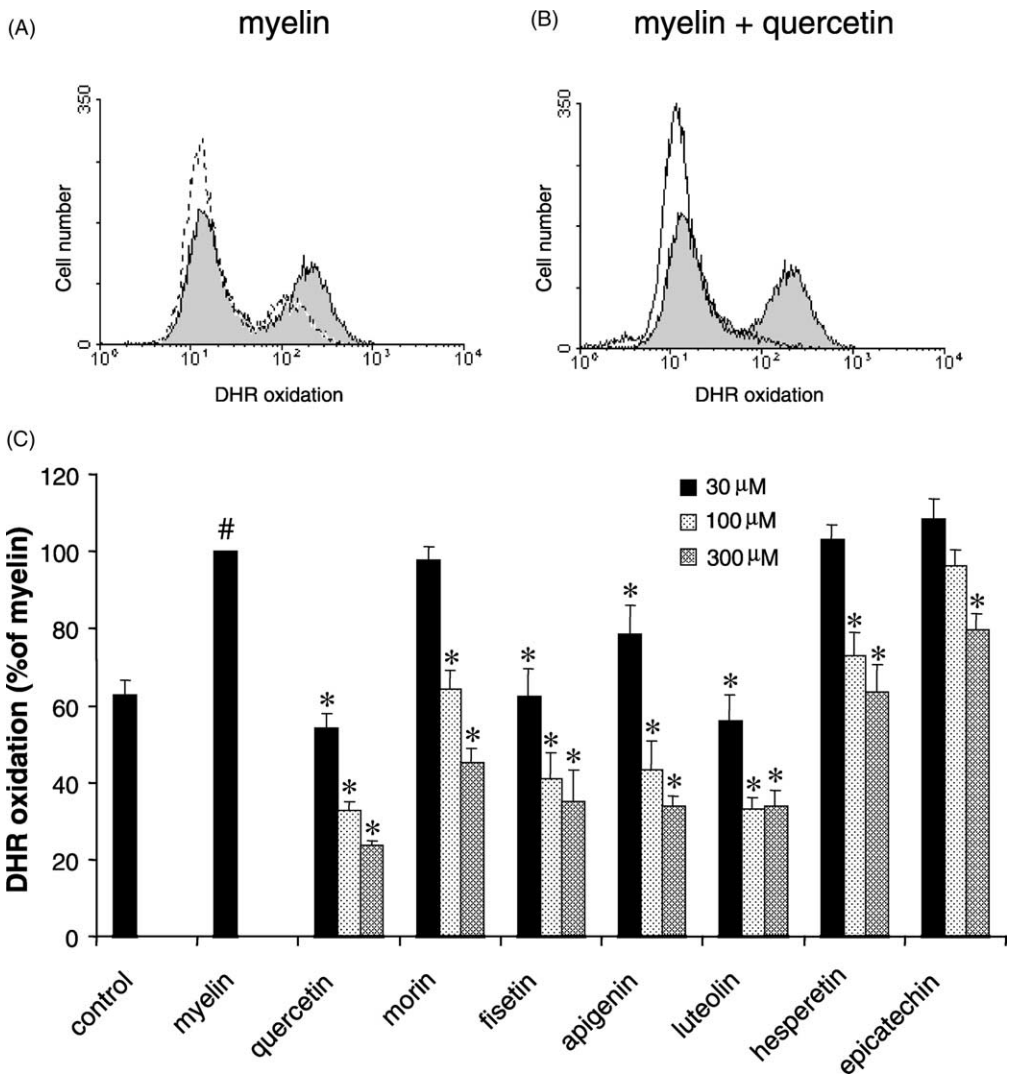


Fig. 3. Inhibition of ROS production by flavonoids during myelin phagocytosis. RAW 264.7 cells were allowed to phagocytose myelin during 90 min in the absence or presence of flavonoids. After phagocytosis of myelin, flavonoids and non-bound myelin were washed away and DHR solution was added for 30 min. (A) Myelin induces ROS production in RAW 264.7 cells as indicated by DHR oxidation, measured by flowcytometry (dashed line, cells with DHR; grey, cells with DHR and myelin). (B) Quercetin (300 μM) inhibits ROS production in myelin phagocytosing cells (grey, cells with DHR and myelin; solid line, cells with DHR, myelin and quercetin). (C) ROS production of cells treated with flavonoids is compared to ROS production of untreated phagocytosing cells as described in Fig. 2A and B, which was set to 100%. Data are derived from four independent experiments and represent mean fluorescence intensity ± SEM. (#) Indicates significant differences compared to DHR oxidation in control cells and (∗) indicates significant differences compared to DHR oxidation after myelin phagocytosis, $P < 0.05$.

decreased ROS content of RAW 264.7 cells in concentrations higher than 100 μM. Hesperetin and epicatechin were least effective and only reduced DHR oxidation at 300 μM with a maximum of 37 ± 7.1% inhibition for hesperetin.

4. Discussion

Flavonoids have been suggested to be beneficial for the course of neurodegenerative diseases, like Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis [25]. It is thought that one of the most curative effects of flavonoids is their scavenging property of ROS. Oxygen radicals also play a predominant role in the neuro-inflammatory disease MS [26,27] and its animal model EAE [3]. Here, it is reported for the first time that flavonoids are able to inhibit myelin phagocytosis, one of the crucial events in MS. Flavonoids exert this inhibitory function in a dose-dependent manner and in particular flavones and certain flavonols were found to be effective. IC_{50} values (20–200 μM) found for these flavonoids are in the same concentration range as for other reported cellular effects, like inhibition of NO production [17].

The capacity of flavonoids to diminish myelin phagocytosis can be related to their structural properties. The most important feature is the presence of a C-2,3 double bond in combination with a 4-oxo group, since all flavonoids were effective except epicatechin, a flavonoid derivative with an

identical structure as quercetin but lacking the 4-oxo group. Furthermore, the possession of a catechol structure in the B ring (hydroxyl groups at the B-3 and the B-4 positions) seems relevant for their potency, since luteolin, quercetin and fisetin are the most effective compounds. In addition, modification of the hydroxyl group at B-4 by an OCH_3 group profoundly lowered the capacity to inhibit phagocytosis 10-fold. An OH group at C-3 lowers the inhibitory capacity 4-fold (quercetin vs. luteolin), while an OH group at A-5 had little effect (quercetin vs. fisetin).

For the flavonoid antioxidant effect it has already been described that a C-2,3 double bond conjugated with a 4-oxo group (good electron delocalising capacity) is important in combination with a catechol moiety (good electron donating properties) [28]. Flavonoids containing these groups, furthermore protect neuronal cells from ROS-induced damage [29] and inhibit NO production by lipopolysaccharide-stimulated RAW 264.7 cells [17]. During myelin phagocytosis by these cells, no significant production of NO and TNFα could be detected (results not shown), and these mediators are, therefore, unlikely to be involved. Our previous data revealed a predominant role for ROS in the phagocytosing process, and scavenging of ROS in these experiments with catalase (known H_2O_2 scavenger) or mannitol (known OH scavenger) completely blocked the myelin uptake [8]. Therefore, flavonoids are likely to exert their effect on myelin phagocytosis *via* the lowering of cellular ROS levels. Flavonoids act as antioxidants for various ROS as recently reviewed by Pietta [30], by acting as their direct scavenger or *via* inhibition of ROS-producing enzymes, like xanthine oxidase [31] and NADPH oxidase [32]. Here, we used the DHR assay to study a possible correlation between the phagocytosis reducing and antioxidant capacity of flavonoids. We found that, after the addition of myelin, ROS production in macrophages was increased as described before [8]. During this time frame most of the myelin is taken up by the macrophages [20]. This uptake occurred in a lysosomal pattern as did the increase in ROS production, which indicated that ROS levels are induced locally during myelin uptake. The ability of flavonoids to inhibit ROS production correlated well with their capacity to reduce myelin phagocytosis. Similar structural properties seem to be required for acting on ROS levels as well as on myelin phagocytosis, although a hydroxyl group at the C-3 position affects myelin phagocytosis but not DHR oxidation. In contrast, others have found the C-3 and A-5 hydroxyl groups to be important for efficient radical scavenging [33]. Apart from ROS scavenging, there may be other ways *via* which flavonoids affect myelin phagocytosis. They may mediate their effect by influencing signal transduction pathways [34], enzyme systems [35], modify cytoskeletal elements [36] and reduce complement activation [37].

Our results suggest that flavonoids may be protective in MS by affecting macrophage functions. Besides this, they may exert additional protective effects, like the inhibition of monocyte and T cell activation [16,18] and protection of neurons from damaging insults [38,39]. Furthermore, flavonoids inhibit mast cell activation [40], which has been found to be involved in MS [41]. It will be interesting to test the effects of flavonoids in the EAE model, preferably administered orally, although the bioactivity of metabolised flavonoids is still largely unclear [42]. It is noteworthy in this respect that curcumin, like flavonoids a naturally occurring polyphenol, was recently found to inhibit clinical signs of EAE when injected intraperitoneally [43]. Curcumin inhibits myelin phagocytosis in a similar mode as flavonoids but with a higher cytotoxicity (results not shown). As flavonoids and related compounds are beneficial in *in vitro* and animal models for MS and other neuroinflammatory diseases, it is worthwhile to further study their potential therapeutical application.

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