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Pretreatment with Natural Flavones and Neuronal Cell Survival after Oxidative Stress: A Structure−Activity Relationship Study

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Quercetin shows structural features that have been related to the antioxidant potency of flavonoids and also shows neuroprotection in different models of oxidative death. Because only a few studies have focused on the flavonoid structural requirements for neuroprotection, this work evaluated the protective capacity of 13 flavones structurally related to quercetin, isolated from Kenyan plants, to rescue primary cerebellar granule neurons from death induced by a treatment with 24 h of hydrogen peroxide (150 μM). Each flavone (0−100 μM) was applied 24 h prior to the oxidative insult, and neuronal viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Results suggest that the *o*-dihydroxy substitution in the B-ring is not necessary to afford neuroprotection and could be partly responsible for neurotoxic effects. Furthermore, the hydroxy substitutions in the positions C3 (C-ring) in C5 and C7 (A-ring) would be important for neuroprotection in this model.

KEYWORDS: Flavones; neuronal protection; oxidative stress; SAR

INTRODUCTION

The lack of effective therapies against neuronal death involved in neuropathologies such as Parkinson's and Alzheimer's diseases has led to the search for new therapeutic strategies mainly related to the events of oxidative stress implicated in these pathologies. In this sense, epidemiological studies have shown a lower incidence of such illnesses in people consuming flavonoid-rich diets (*1*, *2*), although the exact mechanisms by which these compounds exert their beneficial effects are not fully understood. Beyond their classical hydrogen-donating antioxidant activity, in the past 5−10 years, some evidence has emerged from cell studies suggesting that flavonoids could influence cellular fate by other mechanisms of action: enzymatic modulation, interaction with several receptors, and modulation of intracellular signaling cascades that control cell survival, death, differentiation, and gene expression (*3*−*6*). This capacity to modify multiple cellular targets is likely the basis of the therapeutic potential of flavonoids and also their toxic effects.

Flavonoids represent the most common group of polyphenolic compounds in the human diet and are widely found in plants. They consist of two aromatic carbon rings, benzopyran (A- and C-rings) and a benzene (B-ring), and can be divided into six groups depending on the degree of oxidation of the C-ring, the hydroxylation pattern, and the substitution at the C3-position. Among these, the flavones that are based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one) are the most commonly encountered, being ubiquitous within the families and genera of the higher plants (**Figure 1**). The structural diversity of these natural compounds offers the possibility to identify the molecular substitutions required for different biological actions. In this sense, there is an agreement in the literature on some of the molecular features that determine particular activities of flavonoids. Thus, their antioxidant potency has been linked to the *o*-dihydroxy substitution in the B-ring and the presence of 2,3-unsaturation and a 4-carbonyl in the C-ring (**Figure 1**) (*7*, *8*). Their activity as metal ion chelators appears to be defined by the presence of the *o*-dihydroxy substitution in the B-ring, the 4-carbonyl in the C-ring, and a 5-hydroxy substitution in the A-ring (*9*), whereas the prooxidant properties of flavonoids would be associated with the number of hydroxyl groups present in the molecule (*10*). In particular, the cytoprotective capacity of flavonoids against different insults has been mainly attributed to their antioxidant potency (*11*, *12*). However, in a previous work (*13*), assessing the cytoprotective potency of several structurally related flavonoids against oxidative stress, we found that cytoprotection did not correlate with the antioxidation potency. Nevertheless, the antioxidant structural features described above were common to all of the cytoprotective flavonoids studied, as shown by other authors (*14*, *15*). In this context, the search for particular structural features that afford protection would be significant for the development of new neuroprotective molecules

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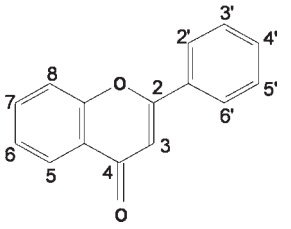


Figure 1. Molecular structure of the flavone backbone.

and for the understanding of the mechanisms of action of flavonoids.

To this aim we performed a structure−activity relationship (SAR) study focused on the protective effects of flavonoids against oxidative stress in neuronal cultures. We assessed the protective capacity of 13 natural flavones in an experimental paradigm in which an oxidative insult that leads to cell death is applied to cultured neurons after previous exposure to the flavones. The flavones were mainly isolated from Kenyan plants, and they are structurally related to quercetin, a prototypic antioxidant molecule and a potent neuroprotective compound in different experimental paradigms (*13*, *16*). To assess more accurately the potential value of the protective molecules as leading compounds to design molecules of therapeutic use, we also assessed the toxicity of the selected flavones on the cultured neurons.

The main results showed the structural requirements for neuronal cell protection, which are different from those that afford antioxidant capacity.

MATERIALS AND METHODS

General Chemicals. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Hydrogen peroxide (H_2O_2) was dissolved in distilled water and subsequently filtered before addition to the cultures

Flavones Utilized. Quercetin (3,5,7,3′,4′-pentahydroxyflavone) was acquired from Xi'an Sino-Dragon I/E Co., Ltd., China. Apigenin (5,7,4′-trihydroxyflavone), luteolin (5,7,3′,4′-tetrahydroxyflavone), and fisetin (3,7,3′,4′-tetrahydroxyflavone) were obtained from Sigma-Aldrich. The rest of the flavones studied were isolated by Prof. J. Midiwo's research group at the Department of Chemistry, University of Nairobi, from the following plants: *Dodonaea angustifolia*, *Psiadia punctulata*, *Gardenia ternifolia*, *Senecio roseiflorus*, and *Hypericum lanceolatum*. The following compounds were obtained from these plants (**Table 1**): compound **1** (5,7-dihydroxy-3,6,4′-trimethoxyflavone), compound **2** (5-hydroxy-3,7,4′-trimethoxyflavone), compound **3** (5,7-dihydroxy-3,4′-dimethoxyflavone), compound **4** (3,5-dihydroxy-7,4′-dimethoxyflavone), compound **5** (5,6,7-trihydroxy-3,4′-dimethoxyflavone), compound **6** (3,5,7-trihydroxy-4′-methoxyflavone), compound **7** (5,7,4′-trihydroxy-3-methoxyflavone), compound **8** (3,5,7,3′-tetrahydroxy-4′methoxyflavone, tamarixetin), and compound **9** (3,5,7,4′-tetrahydroxy-3′methoxyflavone, isorhamnetin).

Each molecule was dissolved in dimethyl sulfoxide (DMSO) 30%, prior to bioassay experiments.

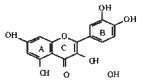
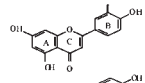
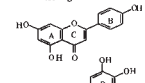
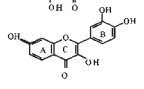
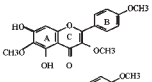
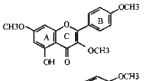
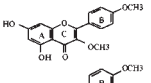
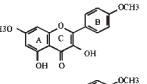
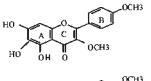
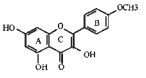
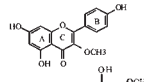
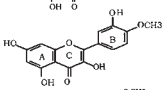
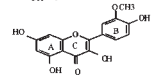
Plant Materials. The plants used and voucher specimens have been described previously (*17*, *18*).

The leaf and flowers of the plants mentioned were collected from their geographical location and handled according to established procedures.

Extraction. Extraction was performed as previously described for exudates from aerial parts (*17*, *18*); the aerial branches and leaves were dipped into organic solvent and shaken for not more than 15 s to avoid extraction of internal tissue substances indicated by the appearance of the green of chlorophyll. This wash was dried in vacuo using a rotary evaporator to give a solid gum. Other parts of the plants were dried before the powder was extracted at ambient temperature with various organic solvents, dichloromethane/methanol (1:1) followed by methanol, and solvent was removed to yield the gummy solid.

Analysis was done using analytical TLC: Merck precoated silica gel 60 F254 plates with standard previously characterized compounds to locate desired compounds in the extracts, CC on silica gel 60 (70−230 mesh).

Table 1. Lethal Concentration 50 (LC_{50}) and Effective Concentration 50 (EC_{50}) of the Flavones Studied

Compound	Structure	LC $_{50}$ (μM)	EC_{50} (μM)
Quercetin		80.3	5.7
Luteolin		79.3	-
Apigenin		76.2	-
Fisetin		63.5	-
1		29.7	-
2		26.0	-
3		25.1	-
4		41.3	-
5		24.0	-
6		72.1	2.6
7		14.4	-
8		> 100	2.9
9		85.6	5.6

EIMS: direct inlet, 70 eV, ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) run on DRX-500 (Bruker), ARX 300 (Bruker), or Varian-Mercury spectrometers using solvent resonances to calibrate the spectra.

Neuronal Culture. Primary cerebellar granule neurons (CGN) were obtained from PN6−8 Sprague−Dawley rats (*19*) and seeded in poly-L-ornithine precoated 96-well plates at a density of 200,000 cells/well. They were kept in basal medium Eagle supplemented with fetal bovine serum (PAA Laboratories, Austria) (10%), 20 mM KCl, and 25 mM glucose in a humidified chamber at 37 °C in a 5% CO_2 atmosphere. Glial growth was inhibited by addition of cytosine arabinoside (10 μM).

Experimental Treatments. For flavone neuroprotective capacity evaluation, neuronal death was induced at seven days in culture (DIC7) by adding 150 μM hydrogen peroxide (H_2O_2) for 24 h. Flavones (0−100 μM in 1.5% DMSO, final concentration) were applied 24 h before the H_2O_2 insult. Neurotoxicity assessment was done by exposing granule cell cultures to 0−100 μM concentrations of each flavone during 24 h.

Cell Viability Measurements. Neuronal mitochondrial activity, as an indicator of cell viability, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*20*).

Briefly, after experimental treatments, cells were incubated for 90 min at 37 °C with MTT (0.1 mg/mL final concentration) that was reduced by metabolically active cells to purple formazan. Formazan crystals were dissolved with DMSO, and the absorbance was measured on an MRX microplate reader (Dynex Technologies), using a reference wavelength of 630 nm and a test wavelength of 570 nm.

Results are presented as percentage of MTT reduction, assuming that absorbance of control cells was 100%.

Neuronal viability was also examined qualitatively by phase-contrast microscopy (Axiovert 25, Zeiss, Germany).

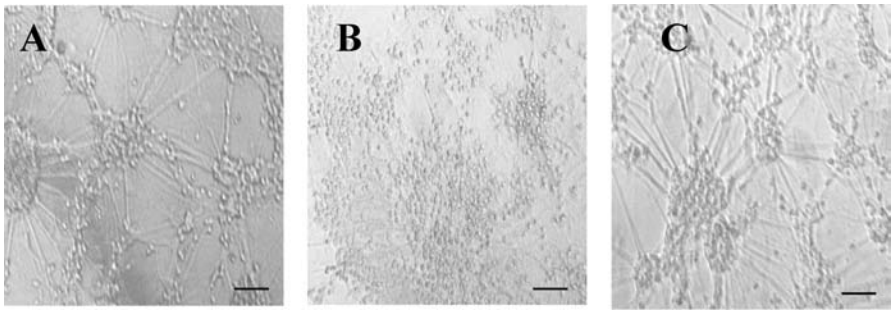


Figure 2. Phase contrast photomicrographs of neurons in culture showing neuroprotection by pretreatment with 10 μM compound **9**: (**A**) control vehicle neurons (DMSO 1.5%); (**B**) H_2O_2 vehicle neurons (DMSO 1.5% + H_2O_2 150 μM 24 h); (**C**) H_2O_2−compound **9** neurons (10 μM compound **9** 24 h + H_2O_2 150 μM 24 h). Scale bar: 35 mm.

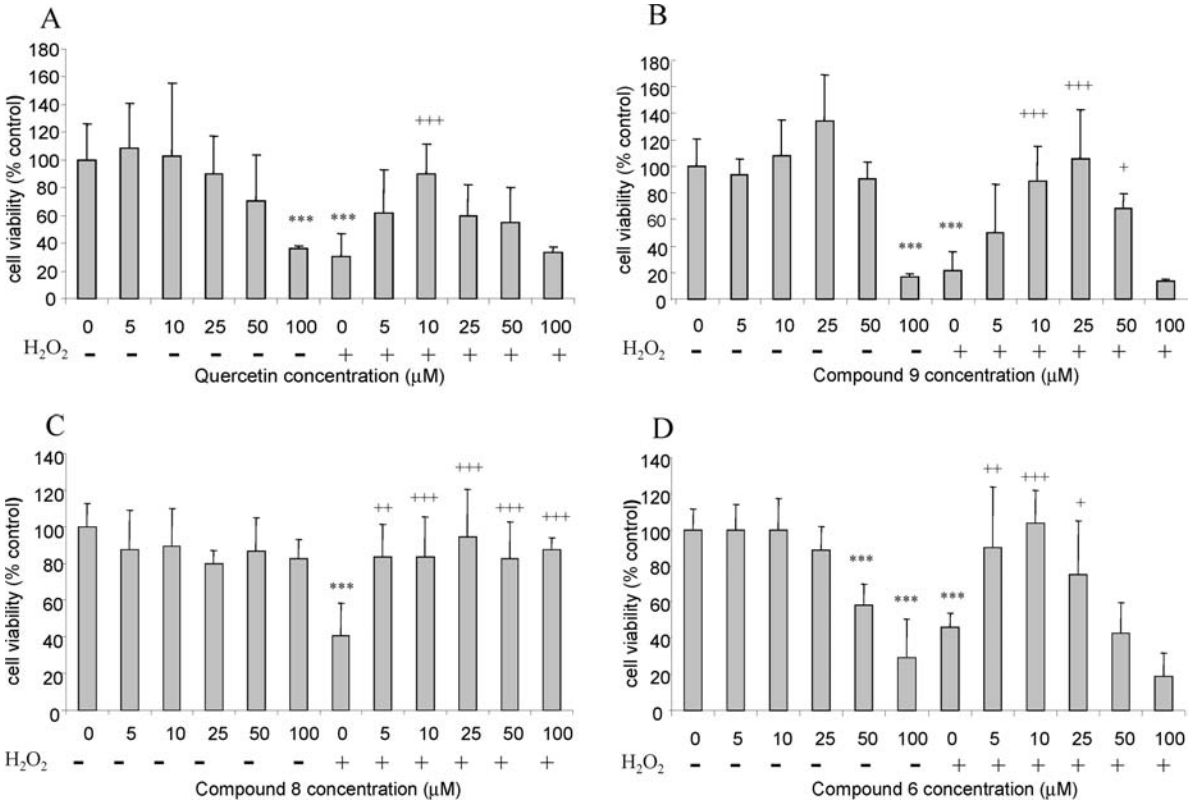


Figure 3. Flavone CGN toxicity and protection against oxidative insult: (**A**) quercetin (3,5,7,3′,4′-pentahydroxyflavone) profile; (**B**) compound **9** (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, isorhamnetin) profile; (**C**) compound **8** (5,7,3′-trihydroxy-3,4′-dimethoxyflavone, tamarixetin) profile; (**D**) compound **6** (3,5,7-trihydroxy-4′-methoxyflavone) profile. Data are presented as mean of cell viability ± SD (***, $p < 0.001$ as compared to CV; +++, $p < 0.001$ as compared to PV; +2, $p < 0.05$ as compared to PV; +, $p < 0.01$ as compared to PV, by ANOVA−Tukey multiple-comparison test). CV, control vehicle neurons; PV, peroxide vehicle neurons.

Statistical Analysis. Cell viability data are presented as mean ± SD and were analyzed by ANOVA−Tukey multiple-comparison tests.

RESULTS

From the molecules studied, compound **8** (5,7,3′-trihydroxy-3,4′-dimethoxyflavone, tamarixetin), compound **9** (3,5,7,4′-tetrahydroxy-3′-methoxyflavone, isorhamnetin), compound **6** (3,5,7-trihydroxy-4′-methoxyflavone), and quercetin (3,5,7,3′,4′-pentahydroxyflavone) prevented the H_2O_2-induced cell death, evidenced by phase contrast microscopy and MTT assays, indicating that they are neuroprotective.

A representative set of phase contrast photomicrographs of neurons in culture that were treated with 10 μM compound **9** and 24 h later exposed to the oxidative insult are shown in **Figure 2**. Treatment with H_2O_2 caused marked neuronal death (**Figure 2B**). In contrast, neurons pretreated with 10 μM compound **9** and subsequently exposed to the same oxidative insult (**Figure 2C**) appeared to be morphologically similar to control cells (**Figure 2A**).

Effective half maximal concentrations for toxicity (LC_{50}) and for protection (EC_{50}) were calculated from the dose−response curves (**Table 1**).

The toxicity profile of each neuroprotective flavone was different (**Figure 3**). Only compound **8** did not show toxicity in the concentrations tested (**Table 1** and **Figure 3**). Although the correlation between the number of OH and LC_{50} was positive, it was low ($r^2 = 0.537$, **Figure 4**). The compounds with minor LC_{50} values (compounds **7**, **1**, **2**, **3**, and **5**) show methoxy substitution in C3 (C-ring).

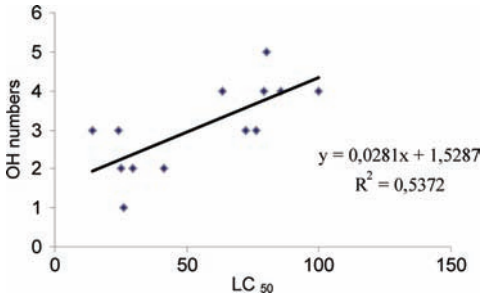


Figure 4. Correlation between OH numbers of flavones and LC_{50}.

The protective compounds **8**, **9**, **6**, and quercetin showed an EC_{50} = 2.9, 5.6, 2.6, and 5.7 μM, respectively (**Table 1**; **Figure 3**). Interestingly, apart from quercetin, the rest of the neuroprotective molecules do not present the *o*-dihydroxy substitution in the B-ring, although they have similar potencies.

In this model fisetin did not show neuroprotection (**Table 1**), although its only structural difference from quercetin is the absence of OH substitution at C5 of the A-ring. On the other hand, compound **4**, that does not show protection, possesses an OH in position C5, but has an OMe group at C7 of the A-ring (**Table 1**).

Of the other nonprotective compounds, apigenin and luteolin both have the OH substitution at C5 and C7 in the A-ring, although they lack the OH substitution at C3 (C-ring); compounds **7** and **3**, which also have an OH group in positions C5 and C7 (A-ring), present an OMe group at C3 (C-ring) and, finally, compounds **1**, **2**, and **5** have an OMe group at C3 (C-ring) and present diverse substitutions in the A-ring (**Table 1**).

In summary, the common structural characteristics of the four neuroprotective molecules are an OH substitution at the C5- and C7-positions of the A-ring, an OH substitution at the C3 position of the C-ring, and variable B-ring substitutions.

DISCUSSION

The results described above showed that only 4 of the 13 flavones studied exhibited neuronal protective activity, strongly indicating that specific structural features are related to this action. Results suggest that the hydroxy substitutions in the A-ring (C5 and C7) and at position C3 (C-ring) of the flavones would be necessary to afford neuronal cell protection. The OH substitution at position C3 (C-ring) has been previously shown as important in a wide range of biological activities such as scavenger activity and anti-inflammatory effects (*21*, *22*). This structural feature appears to be significant for the cationic divalent chelating properties (*21*), and it appears to be responsible for the increase in the molecular coplanarity that has been implicated in these biological activities (*21*, *23*).

Our results are in agreement with Kang and co-workers, who studied the neuroprotective capacity of four structurally related flavones, wogonin, chrysin, apigenin, and luteolin, by testing whether these flavones protect SH-SY5Y human neuroblastoma cells against oxidative stress-induced cell death. They found that one hydroxyl group at C3 is essential to exert protection (*14*).

Additionally, a few studies have demonstrated that both the 5-OH and 7-OH groups of the A-ring are significant for flavonoid bioactivities. Lee and co-workers have suggested that OH substitutions at C5 and/or C7 might significantly affect the anti-apoptotic properties of flavonoids (*24*). Furthermore, Van Hoorn et al. (*25*) showed the importance of these substitutions on xanthine oxidase inhibition by flavones. Because free radicals are produced by xanthine oxidase activity, its inhibition has an indirect antioxidant effect that could contribute to neuroprotection. In this case, the authors sustained the notion that 3′- and 4′-hydroxyl moieties (B-ring) strengthen this activity.

When we compare the activity profiles of the four protective molecules with the nonprotective fisetin, it can be concluded that the *o*-dihydroxy substitution in the B-ring is not needed for protection in our model. This is an important and novel contribution of our work because several studies have shown the importance of this structural feature in diverse bioactivities of flavonoids (*26*−*29*), particularly for their scavenger capacity. Thus, it is generally accepted that the positions and numbers of OH in the B-ring give the scavenger capacity of these compounds, whereas OH groups on the A-ring do not appear to be significant for this activity (*21*). Indeed, it has been suggested that OH groups on the A-ring have poor reactivity toward peroxyl radicals (*30*, *31*). Accordingly, our results showed that structural features implicated in neuronal protection are different from those that provide the free radical scavenging capacity of flavonoids. It has to be borne in mind that in the model utilized in this work, flavones are added to cells in culture 24 h before they are submitted to the oxidative aggression. This is an important difference with most utilized models that place the flavonoids and the oxidative insult in close relationship (*14*, *32*). In our model, the flavonoid molecule could interact with intracellular targets before the oxidative insult is applied, likely inducing the expression of cell signaling molecules. Further research now in progress in our laboratory aims to detect the intracellular presence of the flavones during the preteatment period, a fact already demonstrated for some flavonoids (*33*, *34*). Metabolomic studies are also being performed to identify putative active metabolites as it has been claimed in previous studies (*33*).

The toxicity studies of the flavones evidenced a positive correlation between the number of OH substitutions and the LC_{50} values. This result indicates that in neurons in culture, toxicity may be inversely related to hydrophilicity. The low correlation coefficient would suggest that besides the number of OH, their positions would also be important for toxicity. Although some studies have shown the involvement of OH and OMe groups in cytotoxicity (*35*), none has implicated the OMe substitution, particularly at C3 (C-ring), with this action. Our work shows for the first time that the OMe substitution at C3 (C-ring) would increase the neurotoxicity. This feature should be studied further to identify possible mechanisms implicated in the toxicity of flavones.

Structure−acivity studies like those reported here are a contribution to the knowledge of the pharmacophoric structures (**Figure 1**: OH substitutions at C5, C7, and C3; OH, OCH_3, or H substitutions at C3′; and OH or OCH_3 substitutions at C4′) that afford neuronal cell protection and could be meaningful for the design of new molecules for the treatment of brain pathologies that involve massive neuronal cell death.

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