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# Neuroprotective properties of the natural phenolic antioxidants curcumin and naringenin but not quercetin and fisetin in a 6-OHDA model of Parkinson's disease

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**Abstract**
Although the cause of dopaminergic cell death in Parkinson's disease (PD) remains unknown, oxidative stress has been strongly implicated. Because of their ability to combat oxidative stress, diet derived phenolic compounds continue to be considered as potential agents for long-term use in PD. This study was aimed at investigating whether the natural phenolic compounds curcumin, naringenin, quercetin, fisetin can be neuroprotective in the 6-OHDA model of PD. Unilateral infusion of 6-OHDA into the medial forebrain bundle produced a significant loss of tyrosine hydroxylase (TH)-positive cells in the substantia nigra (SN) as well as a decreased of dopamine (DA) content in the striata in the vehicle-treated animals. Rats pretreated with curcumin or naringenin showed a clear protection of the number of TH-positive cells in the SN and DA levels in the striata. However, neither pretreatment with quercetin nor fisetin had any effects on TH-positive cells or DA levels. The ability of curcumin and naringenin to exhibit neuroprotection in the 6-OHDA model of PD may be related to their antioxidant capabilities and their capability to penetrate into the brain.

**Keywords:** *Curcumin (Curcuma longa), naringenin, antioxidant flavonoids, 6-hydroxydopamine, neuroprotection, Parkinson's disease*

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by a primary loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) resulting in a reduction in striatal dopamine (DA) concentrations. Although the precise mechanism of nigral cell death in PD remains unknown, oxidative stress has been strongly implicated [1,2]. Postmortem studies in humans have shown that nigral cell death in PD is associated with increased lipid peroxidation [3], decreased reduced glutathione (GSH) levels [4], enhanced superoxide activity in SN [5] and increased levels of iron in SN [6]. At present, DA replacement with levodopa or DA agonists is the most effective treatment in PD. Although such drugs are effective in the early stages of the disease, long-term therapy has been associated with serious side effects. Thus, a therapeutic approach of PD treatment could include the modulation of oxidative stress. Phenolic antioxidants including flavonoids are an extensive group of naturally occurring compounds that are widely distributed in plants as constituents of various fruits, nuts and leaves etc. [7,8]. Flavonoids are potent antioxidants and free radical scavengers, with efficacy suggested to exceed the antioxidant capacity of vitamins C and E [9–12]. Flavonoids are capable of chelating metal ions, modify the activity of cellular antioxidants

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and antioxidant enzymes such as catalase and GSH [13], modulating NO production, tumour necrosis factor α secretion and nuclear factor kB (NFκB) dependent gene expression *in vitro* [14], and have anti-inflammatory properties, inhibiting the activities of lipoxygenase and cyclooxygenase [15]. Since they show very little toxicity even in long-term studies, they may be appropriate for long term therapies in PD to possibly slow down the rate of nigral cell loss.

Curcumin is abundant in turmeric (*Curcuma longa*), a food plant that has been used in India for centuries as a food preservative and medicinal agent. Naringenin, the predominant flavanone in grapefruit, protects against oxidative processes associated with chronic degenerative diseases [16]. Quercetin abundant in apple, onions, tea, berries, in medicinal botanicals including Ginkgo biloba, Sambucus Canadensis etc. has a demonstrated antioxidant [17,18] and anti-inflammatory [17–20] properties. Fisetin, a flavonol is abundant in citrus fruit and has Fe chelating properties [18,21]. In this study, we sought to determine whether the sub-chronic use of different natural phenolic compounds provides a neuroprotection in the unilateral 6-hydroxydopamine (6-OHDA) rat model of PD. The 6-OHDA induces a lesion in the nigrostriatal dopaminergic pathway that results in a progressive loss of dopaminergic neurons in the SNpc by increasing the oxidative stress. Both the integrity and functionality of the nigrostriatal pathways were assessed by quantifying the number of dopaminergic neurons in the SNpc and DA and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) concentrations in the striata.



## Materials and methods

### *Materials*

Curcumin (98–99% of purity) was from Indo-World Trading Company (New Delhi, India), naringenin, quercetin, fisetin, 6-hydroxydopamine and all the chemicals used were purchased from Sigma-Aldrich (Dorset, UK), polyclonal TH antibody was obtained from Chemicon (Harrow, UK), and the ABC immunostaining kit from Vector Laboratories (Peterborough, UK).

### *Animals*

Adult Sprague-Dawley male rats (Harlan, UK), of initial body weight ~200 g were used. The animals were kept under standard laboratory conditions (temperature 21 ± 1°C; relative humidity 50 ± 10%, 12–12 h light-dark cycle) and had free access to drinking water and a standard pellet diet. All scientific procedures were carried out with the approval of the Home Office, UK. Rats were handled daily and allowed one week to acclimatise after arrival before any treatment.

### *Experimental design*

The animals were randomly allocated to 5 experimental groups of six rats each: Group 1: control group (drug vehicle, 10% Cremophor, p.o); Group 2: curcumin (50 mg/kg dissolve in 10% of Cremophor; p.o); Group 3: quercetin (20 mg/kg dissolve in 10% of Cremophor, p.o); Group 4: naringenin (50 mg/kg dissolve in 10% of Cremophor; p.o); Group 5: fisetin (20 mg/kg dissolve in 10% of Cremophor; p.o). Rats were treated with the flavonoids at the concentration as above or drug vehicle by gavage, daily for 4 days prior to lesioning. The dose of each of these compounds was based on either the study with tangeretin [22] or from the literature.

### *Animal treatment*

On the 4th day of treatment, one hour after final dosing, rats received an unilateral infusion of 6-OHDA (12 μg dissolved in 4 μl 0.1% ascorbic acid/saline solution) into the medial forebrain bundle (stereotactic co-ordinates: 2.2 mm anterior, 1.5 mm lateral from bregma and 7.9 mm ventral to dura with ear bars 5 mm below incisor bars [23] under isofluorane anaesthesia. One week after surgery, animals were killed by cervical dislocation, brains rapidly removed and cut at the level of the infundibular stem to separate the hindbrain block (containing the SN) and the forebrain block (containing the striata).

### *TH-immunohistochemistry*

Tyrosine hydroxylase (TH) is the rate limiting enzyme in DA synthesis and was used as a marker for DA neurons. Hindbrain was fixed for 7 days in 4% paraformaldehyde, then cryoprotected with 30% sucrose solution. Free-floating 20 μm sections cut on a bright cryostat at −22°C were blocked in 20% normal serum in PBS and then processed for TH immunohistochemistry using a polyclonal rabbit anti-TH antibodies followed by biotinylated anti-rabbit IgG. Sections were then incubated with Vector ABC kit and finally visualized with 3-3′-diaminobenzidine and $H_2O_2$. TH-positive cells were visualized under bright field illumination using a Nikon Eclipse E800 microscope and counted manually at region B (−5.1 mm with respect to Bregma) of SNpc [24]. Due to the ease of identification of the dopaminergic neurons within the SNpc, low numbers of cells involved that can be counted in their entirety without operator bias, non-stereological counting techniques were employed, as with most studies in the field.

*HPLC analysis*

Both striata were dissected out from the forebrain block, flash frozen and stored separately at −80°C until analysis. Striatal DA and DOPAC and HVA concentrations were quantified by HPLC-ECD, as described elsewhere [25]. Briefly, striata were individually weighed and homogenized in 0.5 ml of ice-cold buffer (50 mM trichloroacetic acid, 0.5 mM, EDTA, 0.5 pmol/μl 3,4-dihydroxybenzylamine hydrobromide as an internal standard) for 20s using ultrasonicator tissue disrupter (Soniprep, Sanyo, UK). After keeping on ice for 10 min for extraction, the samples were centrifuged (Heraeus, UK) at 1000*g* for 10 min, at 4°C. Supernatants were filtered (0.45μm, Whatman, UK) and loaded onto an autosampler (Gina 50, Gynkotek, UK) kept at 5°C on-line with HPLC system. The analytes were separated on a Altex 3μm ODS column (4.6 mm × 7.5 cm, Beckman, UK) by using a mobile phase consisting of 0.1 mM $KH_2PO_4$, 0.1 mM EDTA, 1 mM octyl sodium sulfonate, 10% methanol V/V (pH 2.75 adjusted with orthophosphoric acid; flow rate 0.9 ml/min) and Coulochem-II detector with electrode one set at −0.20 V and electrode two at +0.34 V with respect to palladium reference electrode (ESA Analytical).

*Statistical analysis*

Percentage was calculated by comparing lesioned and non lesioned sides of the brain. The effect of the drug treatment was analyzed by comparing both the loss of TH-positive cells in the different flavonoid-treated groups vs. control group and the loss of DA concentrations and its metabolites in the striata. Data are expressed as means ± s.e.m. Data were analyzed by student's *t*-test (two-tailed). *P* value less than 0.05 was considered as significant.

## Results

*TH-positive cells in the SNpc*

The cytoplasm and fibers of dopaminergic neurons were specifically stained following TH-staining. Following injection of 6-OHDA into the medial forebrain bundle, ~50% of dopaminergic neurons were lost in the lesioned SNpc in control group when compared to the unlesioned side of the brain (Figure 1A). The loss of TH-positive cells was significantly decreased following treatment with flavonoids curcumin and naringenin, indicating a neuroprotective effect. Indeed, pretreatment with curcumin and naringenin significantly reduced the mean percentage TH-positive cell loss (expressed as the percentage of the number of TH-positive cells in the intact SNpc) when compared to control group; in the curcumin-treated group (21 ± 3% vs. 50 ± 3%; $p < 0.005$), in the naringenin-treated group (30 ± 3% vs.50 ± 3%; $p < 0.02$) for drug-treated vs. control

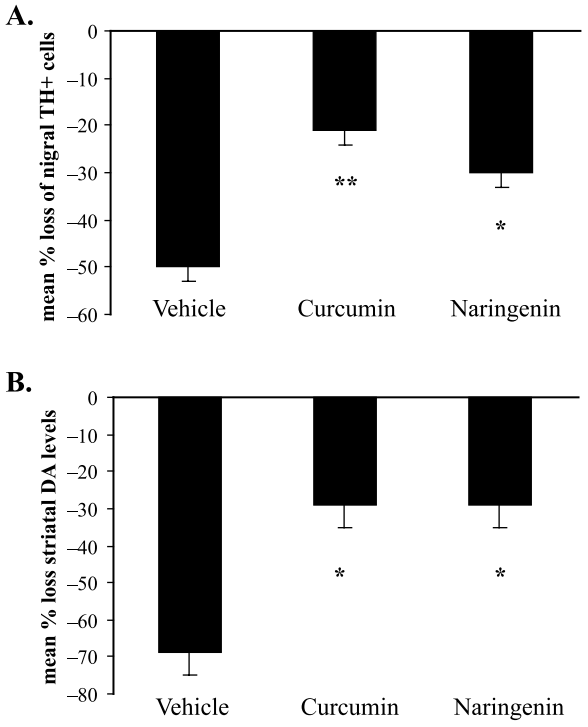


Figure 1. Pretreatment with curcumin and naringenin (50 mg/kg, 4 days, p.o.) reduces the mean percentage loss of nigral TH-positive cells and striatal DA levels after 6-OHDA injection. Histogram represents the percentage loss of TH-positive cells in the SNpc (A) and DA levels in the striata (B). The mean percentage loss of TH-positive cells and DA levels in curcumin and naringenin treated animals was compared to that of vehicle-treated group. Data shown are expressed as the mean cell loss as a percentage of cells in the lesioned SNpc compared to unlesioned side ± s.e.m. * $p < 0.02$ and ** $p < 0.005$ vs vehicle-treated group after unpaired Student's *t*-test; n = 6 rats/group.

animals respectively (Figures 1 and 2). However, administration of quercetin or fisetin failed to prevent this loss of TH-positive cells (Table I). There was, however, a significant decrease in the number of TH-positive cells in the lesioned SNpc compared to the unlesioned side in quercetin and fisetin-treated group (~50%; $p < 0.02$) but there was no difference when compared to control group (~55%; unlesioned vs. lesioned side, respectively) (Table I).

*Striatal concentration of DA and its metabolites DOPAC and HVA*

The injection of 6-OHDA onto the medial forebrain bundle caused a marked reduction in the concentration of DA in the striatum (by 70 ± 7%), (Figure 1B). This is consistent with the significant loss of TH-positive cells observed in the SNpc. Treatment with naringenin or curcumin significantly attenuated the loss of DA after 6-OHDA administration (29 ± 6% and 29 ± 3% respectively; $p < 0.05$ vs. control group) (Figure 1B) whereas, after quercetin or fisetin administration there was no

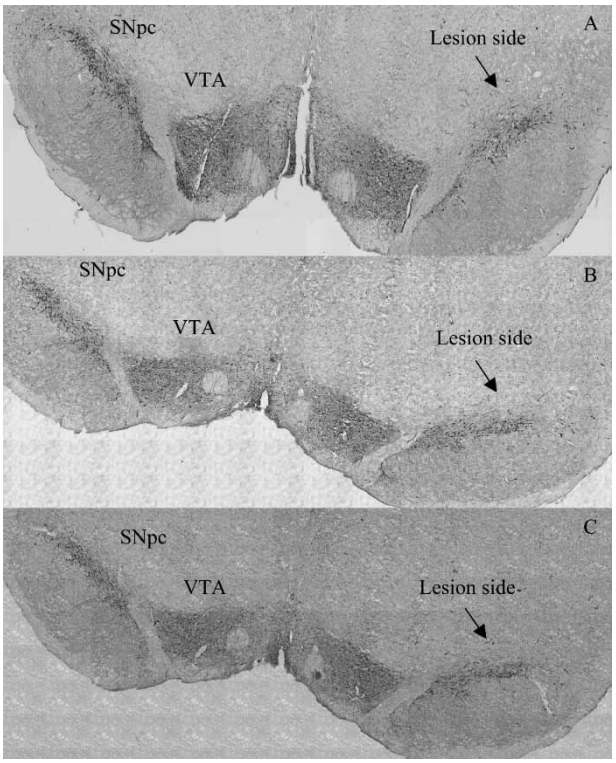


Figure 2. Micrographs of rat substantia nigra pars compacta sections immunostained for tyrosine hydroxylase. A. Vehicle treatment. B. Curcumin treatment. C. Naringenin treatment.



significant difference on the concentration of DA in the striata in relation to control group (Table I). In relation to DOPAC and HVA concentration, analysis of control animals showed that administration of 6-OHDA produced a significant loss of striatal DOPAC and HVA (from 2.44 ± 0.41 to 0.73 ± 0.12; $p < 0.02$ and from 0.88 ± 0.02 to 0.46 ± 0.08, respectively; $p < 0.02$ vs. unlesioned side of the brain). Treatment with curcumin and naringenin attenuated this loss of DOPAC (Table II). After treatment with curcumin and naringenin there was no significant difference in the concentration of DOPAC in the unlesioned striata vs. lesioned one (2.06 ± 0.14 vs 1.20 ± 0.33 and 2.64 ± 0.36 vs. 1.78 ± 0.19, respectively) but a significant difference was observed compared to control group ($p < 0.01$). Curcumin slightly reduced the loss of HVA in the striata compared to control group in a non significant way. Neither quercetin nor fisetin had any effect on the concentration of striatal DA and its metabolites. Finally, no changes were observed in the DA turnover (expressed as the concentration ratio of dopamine relative to its primary metabolites DOPAC and HVA in each sample) in the lesioned side of the brain compared to the unlesioned side neither in control groups nor in curcumin, naringenin, quercetin and fisetin-treated animals. This indicates that the restoration of DA concentrations observed was due to neuroprotective effects rather than compensatory effects by remaining TH-positive cells after 6-OHDA lesioning.

## Discussion

Sub-chronic administration of naringenin or curcumin significantly attenuated the loss of dopaminergic neurons in the SNpc and the decrease in striatal DA concentrations observed after the 6-OHDA lesion. However, quercetin and fisetin were unable to attenuate the loss of TH-positive cells in the SNpc or the loss of DA levels in the striatum in our *in vivo* model.

The 6-OHDA model of nigral injury has been utilized for many years as a classical experimental model of Parkinsonism [26]. The unilateral stereotaxic injection of 6-OHDA into the medial forebrain bundle of the rat leads to a progressive death of the dopaminergic nerve cells and a corresponding depletion of DA in the corpus striatum [27,25]. 6-OHDA is thought to produce toxicity through the generation of oxidative stress and the production of ROS [28,29], since 6-OHDA can auto-oxidize to semiquinone and superoxide radical ($O_2^{\cdot -}$). Subsequent reactions may result in the formation of the more cytotoxic OH radicals through Fenton chemistry involving $H_2O_2$. The number of nigral TH + cells in the SNpc and the striatal DA loss due to 6-OHDA injection was significantly decreased by pretreatment with naringenin or curcumin, indicating a possible scavenging of hydroxyl radicals.

Tea extracts exhibit neuroprotection against 6-OHDA-induced human neuroblastoma (NB) SH-SY5Y and pheochromocytoma (PC12) cells

Table I. Effect of quercetin and fisetin on nigral TH-positive cells and striatal DA levels after 6-OHDA lesioning.

| Treatment | TH-positive cells in the SNpc | | Striatal DA level (ng/mg of tissue) | |
|---|---|---|---|---|
| | Unlesioned SNpc | Lesioned SNpc | Unlesioned striata | Lesioned striata |
| Control | 135 ± 6 | 60 ± 8 ** | 10.7 ± 0.49 | 3.9 ± 1.2 ** |
| Quercetin | 140 ± 9 | 72 ± 12 ** | 13.7 ± 0.98 | 4.0 ± 1.2 ** |
| Fisetin | 121 ± 7 | 62 ± 7 ** | 10.9 ± 0.49 | 3.8 ± 1.6 ** |

Data show the number of TH-positive cells in the lesioned and unlesioned SN following treatment with quercetin and fisetin (20 mg/kg, 4 days, p.o.) and control group and the levels of DA in both striata. The number of TH-positive cells in the lesioned SNpc was compared to its unlesioned side for each group of animals and to control group. ** $p <$ 0.02 vs vehicle-treated group after unpaired Student's *t*-test. Each result represents the mean ± s.e.m; n = 6 rats per group.

Table II. Effect of curcumin and naringenin on striatal levels of DA, DOPAC and HVA after 6-OHDA lesioning.

| | Striatal level (ng/mg of tissue) | | | |
|---|---|---|---|---|
| | DOPAC | | HVA | |
| Treatment | Unlesioned striata | Lesioned striata | Unlesioned striata | Lesioned striata |
| Control | 2.44 ± 0.41 | 0.73 ± 0.12 ** | 0.88 ± 0.02 | 0.46 ± 0.08 ** |
| Curcumin | 2.06 ± 0.14 | 1.20 ± 0.33 # | 0.76 ± 0.05 | 0.52 ± 0.12 |
| Naringenin | 2.64 ± 0.36 | 1.78 ± 0.19 # | 0.75 ± 0.05 | 0.62 ± 0.04 * |

Data represent levels of DA and its metabolites in the both striata following treatment with curcumin and naringenin (50 mg/kg, 4 days, p.o.). * $p < 0.05$ and ** $p < 0.02$ vs. unlesioned side; # vs. vehicle-treated group after unpaired Student's *t*-test. Each result represents the mean ± s.e.m; n = 6 rats per group.



damage culture [30,31]. Similarly, treatment of rats with tangeretin [22] or with the flavonoid rich EM-X drink [32] were protective in this model.

The mechanisms by which curcumin can exert a neuroprotective effect are not fully defined. Curcumin is both a potent antioxidant [33,34] and an effective anti-inflammatory agent [35,36]. Since PD has been linked to increased oxidative damage and anti-inflammatory process, curcumin may be effectively used in the treatment of this disease. Structurally, curcumin does not have the typical ring structure of polyphenol compounds, possessing a diketone group and two phenol rings that act as electron traps to prevent $H_2O_2$ production and to scavenge OH· and superoxide radicals. Besides, both the hydroxyl groups and the β-diketone moiety of curcumin are involved in metal–ligand complexation [37]. Curcumin can chelate $Fe^{2+}$ (known to be increased in PD) needed for Fenton reaction for generating OH radicals [38]. Thereby, an alternative possibility is that by chelating Fe, curcumin may inactivate any toxic effects of this metal. Curcumin is several times more potent than vitamin E as a free radical scavenger [39], protects the brain from lipid peroxidation [40], inhibits the nuclear factor κB-mediated transcription of inflammatory cytokines [41], inducible NO synthase [42] lipoxygenase and cyclooxygenase 2 [43]. Dietary supplementation of curcumin to mice enhanced the activity of glutathione peroxidase, glutathione reductase and catalase in liver and kidney [44]. A significant increase in reduced GSH levels, superoxide dismutase and catalase activities were also observed in the brain in rats simultaneously treated with curcumin and lead [45]. It has also been reported that curcumin protects PC12 and HUVEC cells from Aβ (1–42) insult [46], inhibits formation of β-amyloid fibrils [47] suppresses indices of inflammation and oxidative stress in the brains of APPs mice, factors implicated in Alzheimer's disease [48,49] and significantly attenuated MPTP-induced striatal DA depletion in mice [50]. Due to its anti-tumor activity, relative safety, and its long history of use, curcumin is currently being developed for clinical use as a cancer chemopreventive agent [48]. Studies have also shown that curcumin is relatively nontoxic and has few side effects [51,52]. Toxicity studies with very high dose of curcumin (2000 mg/kg), which greatly exceeds the low dose used in this study (20 mg/kg), revealed non-toxic effects and a low ulcerogenic index [53].

Naringenin was moderately nuroprotective against 6-OHDA-induced toxicity. This protection although significant was moderate. This lower antioxidant activity could be related to its structure. The presence of the 2, 3-double bond in conjugation with a 4-oxo group in the structure naringenin has been suggested to be important for its antioxidant activity. Naringenin has neuronal protective effect against oxidative cell death induced by Aβ peptide in the PC12 cells [54], can partially suppress the Fenton reaction characteristic of Fe-ATP [55]. Naringenin or its glycoside naringin are reported to increase in the enzyme activities of superoxide dismutase and catalase activities and to an up-regulate the expression of the genes for superoxide dismutase, catalase and glutathione peroxidase in high cholesterol-fed rabbits and rats [56,57]. The introduction of naringenin into α-tocopherol-deficient microsomes has been shown to restore the GSH-dependent protection against lipid oxidation, providing evidence that naringenin can assume the role of α-tocopherol as a chain-breaking antioxidant [58]. Besides, it has been suggested that naringin has a poor blood-brain barrier (BBB) penetration whereas, naringenin exhibited high permeability across the *in vitro* and *in situ* BBB models [59,60]. The uptake of naringenin into the cerebral cortex and the striatum [61,62] suggests that naringenin should afford neuroprotection within the CNS. The pharmacokinetics and metabolism of dietary flavonoids is widely reviewed and that of Manach and Donovan [63] is worthwhile to the reader. Quercetin and fisetin did not protect dopaminergic neurons after 6-OHDA, a result that may have been determined by variable metabolism by the intestinal flora and systemic availability and ability to cross the blood brain barrier [63]. Thus phenolic compounds such as curcumin and naringenin

conferred neuroprotection against 6-OHDA-induced toxicity *in vivo*. This indicates the possibility that dietary compounds can be potential candidate for consideration as a dietary supplement in the treatment of PD. Indeed studies indicate that there is a reduced age-adjusted prevalence of Alzheimer's disease in India [16,20], as well as a lower prevalence of PD [17,21]. Further studies need to be done in order to evaluate the molecular mechanisms of neuroprotection of these compounds.

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