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Antioxidant properties of 4-quinolones and structurally related flavones

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ABSTRACT

Neurodegenerative disorders are frequently associated with increased oxidative damage to the brain as a result of free radicals produced by cellular respiration. The onset and progression of neurodegeneration may therefore be curbed by exogenous hydrogen-donating antioxidant moieties such as the naturally occurring flavonoids. A series of 2-phenylquinolin-4(1*H*)-ones was synthesised and displayed moderate to high antioxidant activity when compared to structurally related flavones and quinolines. Activity of the hydroxy-2-phenylquinolin-4(1*H*)-ones (**8–10**) was established in reducing ferrous ions and diminishing hydrogen peroxide and hydroxyl radical production, in the FRAP (1.41–97.71% Trolox® equivalents), ORAC (9.18–15.27 μM Trolox® equivalents at 0.001 mM) and TBARS (0.05–0.72 nmol MDA/mg tissue) assays, respectively. The results indicated that the additional hydrogen donating groups on the synthesised 2-phenylquinolin-4(1*H*)-one series increased antioxidant activity.

1. Introduction

Oxidative stress is a common occurrence in neurodegenerative disorders, such as Alzheimer's[1] and Parkinson's disease[2] and takes place prior to the onset of neurodegeneration, leading to, or exacerbating deterioration.[3–5] The cellular oxidative status is regulated by antioxidant enzymes responsible for neutralising free radicals. With increasing age the enzymes are overwhelmed by the amount of radicals requiring deactivation,[6] leading to decreased protection by the body's antioxidant systems. In addition, the mitochondria produce more reactive oxygen species at the cost of producing less ATP.[7–9] These natural by-products of cellular respiration act to injure the mitochondria and cell structures containing lipids, proteins and DNA,[8,10] and serve to decrease the lifespan of the cell.[11,12] Cellular respiration is at the origin of this cascade of events, yielding, amongst others, destructive reactive oxygen species as by-products of energy production by the mitochondria.[7] Molecular oxygen (O_2) is reduced to water (Eq. 1) during respiration and yields superoxide anions, hydrogen peroxide and hydroxyl radicals as a result.[13]

$$O_2 \rightarrow HO_2^{\cdot} \rightarrow H_2O_2 \rightarrow HO^{\cdot} + H_2O \quad (1)$$

The most detrimental of the reactive oxygen species is the hydroxyl radical,[11] generated in the Fenton reaction (Eq. 2), when hydrogen peroxide is catalytically oxidised by ferrous iron (Fe^{2+}).[1] The ferric ions (Fe^{3+}) produced in the Fenton reaction further act as catalyst in the Haber–Weiss reaction (Eq. 3), producing more hydroxyl radicals that may induce lipid peroxidation.[1]

$$Fe^{2+} \rightarrow H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^- \quad (2)$$

$$O^{2-} + H_2O_2 \rightarrow O_2 + OH^- + HO^{\cdot} \quad (3)$$

With increased age the expression of antioxidant enzymes decrease[6,14] and the ability to maintain the antioxidant/pro-oxidant equilibrium diminishes, causing decreased antioxidant activity and increased neuronal damage. The brain contains high concentrations of oxidisable substrate and catalysts that enable oxidation as well as low concentrations of antioxidant enzymes.[10] Therefore free radical damage and apoptosis[8] in the brain can contribute to irreversible neurodegeneration. This deterioration is only diagnosed when damage to the brain is sufficient to induce disability and irreversible neuronal damage.

Enhancing safe metabolism of oxygen to water by introducing exogenous antioxidants could therefore prolong neuronal lifespan and antioxidants may be useful in decreasing brain damage caused by free radicals, curbing the progression of neurodegenerative disease and prolonging the lifespan and quality of life of the patient.

Antioxidants may act according to one of two mechanisms: prevention of initiation of oxidation, or as chain breaking antioxidants. Prevention of initiation of oxidation occurs by inhibiting superoxide anion production, degrading hydrogen peroxide and chelating or reducing metal ions, while chain breaking antioxidants act by scavenging radicals, mostly hydroxyl radicals, thereby inhibiting the chain of oxidative events that leads to damage of lipid membranes, proteins and DNA.[15] In order to scavenge radicals the antioxidant must act as a hydrogendonor able to reduce the radical and thereby quench it. The antioxidant should form a stable radical in order not to oxidise biological material itself.

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Polyphenols are chain-breaking antioxidants[16] that function by scavenging radicals. The phenol moiety[17] of hydroxyl substituted aromatic compounds (ArOH), act as electron donors driving radical reduction, while the aromatic ring acts to stabilise the phenolic oxygen radical produced (ArO**.**), thereby increasing reducing ability of the antioxidant.[18] Flavonoids (Fig. 1) are a well-known class of natural compounds that possess radical scavenging properties[19,20] due to the ability to form stable radicals.[21] Qin et al. indicated that flavonoids with three hydroxyl substitutions presented increased antioxidant activity,[22] while Khlebnikov et al. indicated that hydroxyl groups on either positions 3, 7 or 8 enhanced scavenging activity.[23] It was therefore hypothesised that structurally similar synthetic compounds containing extra hydrogen donating functionalities, such as an amine in position 1 of the quinolone structure, might improve the antioxidant activity compared to flavonoids.

Fluoroquinolones (Fig. 1) are antimicrobial drugs that are able to infiltrate the cerebrospinal fluid and are used to treat infections such as meningitis. Park et al. indicated that ciprofloxacin reduced ischemia and increased cell survival in a rat focal cerebral ischemic animal model and that a modified structure of ciprofloxacin improved neuroprotection while decreasing antimicrobial activity.[24]

Naturally occurring 4-quinolones are structurally similar to the flavonoids and have been found to show antioxidant activity.[25]2-Phenylquinolin-4(1*H*)-one might therefore possess similar or improved antioxidant activity to flavones when the structure–activity relationships of Park et al. are applied.[24] The aim of the study was therefore to investigate the in vitro antioxidant properties of a series of compounds including quinolines, flavones and 2-phenylquinolin-4(1*H*)-ones structurally related to the selected flavones (Fig. 2). Prior to these studies the oral bioavailability, blood–brain barrier permeability and toxicity profiles of the proposed series we re-evaluated in silico, utilising predictive computational software to determine the feasibility of these compounds as neuroprotective agents.

Since polyphenols are reported to act via the hydrogen donor mechanism of action,[26] the ability of the selected compounds to scavenge the peroxyl radical in the oxygen radical absorbance capacity (ORAC) assay and the hydroxyl radical in the lipid peroxidation thiobarbituric acid reactive substances (TBARS) assay, was experimentally evaluated. The ferric reducing antioxidant power (FRAP) of the test series was also assessed.

Quinoline structurally resembles the core of the 2-phenylquinolin-4(1*H*)-one structure and derivatives thereof were included in the test series (Fig. 2) to establish the activity of the basic structure, while the flavones were included as reference antioxidants and to establish the effect of the oxygen and the protonated amine groups on hydrogen donating ability. As 8-hydroxyflavone was not commercially available, 8-hydroxyquinoline (**3**) was included in the series to compare to the structurally related8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**). These chemical and biological evaluations provided a wide range of data enabling determination of the key antioxidative reactions involved.

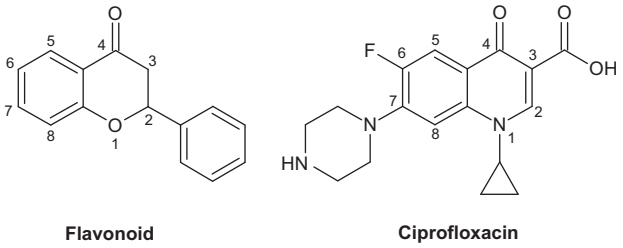


Figure 1. Basic flavonoid structure[21] and a fluoroquinolone antibiotic.[24]

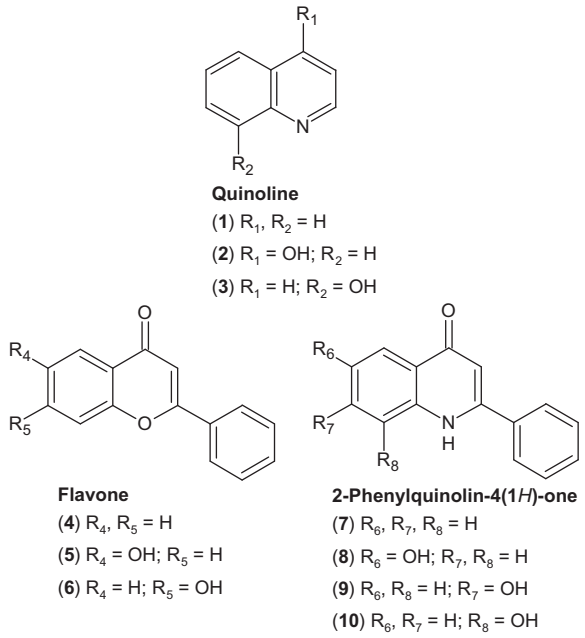


Figure 2. The quinoline, flavone and synthesised 2-phenylquinolin-4(1*H*)-one test series.

1.1. In silico bioavailability studies

The test compounds were evaluated for their oral bioavailability, physicochemical properties and theoretic ability to cross the blood–brain barrier byutilising Osiris Property Explorer, Chemsilico Property Predictor for Drug Discovery, Accelrys DS ViewerPro 5.0 and Advanced Chemistry Development Chemsketch computational software to elaborate on the potential of the derivatives as potential neuroprotective drug candidates (Table 1).

The test serieswas found to comply with the oral bioavailability standards as set by Lipinski's rule of five[27,28] since the *c*Log*P* values, the amount of H-bond donors and rotatable bonds were less than 5, the molecular weights were less than 500 g/mol and H-bond acceptors less than 10. This indicated a strong probability that the compounds would display favourable oral bioavailability.

Table 1 collates several computed physicochemical properties for prediction of brain permeation. An examination of the calculated properties indicated that effective blood–brain barrier permeability is to be expected for all test compounds as their *c*Log*P* values are between 2 and 5,[29] the sum of nitrogen and oxygen (N+O) atoms are less than 5[30]and according to the 'Norinder rule', *c*Log*P* minus (N+O)-values are greater than 0, except for 8-hydroxyquinoline (**3**).[30] The molecular weights are below 450 g/mol[31]and pK_a values were found to be between 4 and 10.[32–34] The in silico predicted LogBB values were greater than 0,[35,36]with several compounds (**1**, **4**, **5**, **6**, **8**, **9** and **10**) displaying values near the optimal level for effective permeation, at 0.3.[42] Taking the above physicochemical properties into consideration, we predict that the test compounds would allow for effective oral absorption, distribution and permeation of the brain.

Predictive toxicities were studied using Osiris Property Explorer software and indicated that the hydroxyflavones (**5**, **6**) and 2-phenylquinolin-4(1*H*)-ones (**7**, **8**, **9**, **10**) would be free of mutagenicity, tumorigenicity, reproductive side effects and irritation, while the quinolines (**1**, **2**, **3**) and flavone **4** had some potential toxicities (Table 1). These findings suggest that the toxicity decreased with the addition of the 2-phenyl moiety, substitution of the oxo in position 1 of the flavone with the secondary amine of the 2-phenylquinolin-4(1*H*)-one, and the addition of hydroxyl groups, with

Table 1
In silico physicochemical properties for oral bioavailability and blood–brain barrier permeability of the test compounds as evaluated utilising computational predictive software

Test compound	*c*Log *P*[a,d]	H-bond donors[b]	Molecular weight[a]	H-bond acceptors[b]	Rotatable bonds[b]	pK_a[a]	LogBB[a]	N+O count	*c*Log *P*- (N+O)	Drug likeliness[c]	Drug score[c]	Toxicity[c]
1	2.08	0	129	1	0	4.67	0.29	1	1.08	−1.62	0.12	Mutagenic tumorigenic irritant
2	2.45	1	145	2	0	5.28	0.08	2	0.45	−1.87	0.26	Mutagenic tumorigenic
3	1.87	1	145	2	0	5.24	0.09	2	−0.13	1.55	0.12	Mutagenic tumorigenic irritant
4	3.56	0	222	2	1	7.59	0.48	2	1.56	1.85	0.44	Mutagenic
5	3.72	1	238	3	1	8.15	0.27	3	0.72	1.35	0.74	None
6	3.32	1	238	3	1	8.14	0.27	3	0.32	−0.6	0.56	None
7	4.70	1	221	1	1	7.54*	0.49	2	2.70	2.52	0.78	None
8	4.04	2	237	2	1	8.30*	0.26	3	1.04	1.88	0.79	None
9	4.35	2	237	2	1	8.31*	0.26	3	1.35	2.20	0.80	None
10	4.42	2	237	2	1	8.76*	0.32	3	1.42	2.43	0.81	None

Values obtained using the following computational software:
[a] Chemsilico Property Predictor for Drug Discovery.[37]
[b] Accelrys DS ViewerPro 5.0.[38]
[c] Osiris Property Explorer.[39]
[d] Advanced Chemistry Development Chemsketch.[40,41]
* pK_a Value of the basic nitrogen (NH^+).

the following sequence of decreasing toxicity: quinolines > flavones > 2-phenylquinolin-4(1*H*)-ones (Table 1). The 2-phenylquinolin-4(1*H*)-oneswere also indicated to be the most likely of the test compounds to illustrate drug activity when compared to a comprehensive database of known drugs, according to their drug score and drug likeliness.[39]

This data has significance in that it indicate that the 2-phenylquinolin-4(1*H*)-ones should have improved biological activity with regards to interaction on enzyme systems, penetration through cell membranes and the blood–brain barrier as well as favourable properties during drug metabolism.[43]

1.2. Chemistry

The general synthetic route used to synthesise the 2-phenylquinolin-4(1*H*)-ones (**7**–**10**) was the traditional one pot Conrad–Limpach synthesis described by Somanathan and Smith, where a β-keto-ester was cyclisised with an aniline derivative using *para*-toluene sulfonic acid as a catalyst (Scheme 1).[44] Dehydration under Dean–Stark conditions for an extended period of 19 h, using benzene as a solvent, maintained conversion of reactants to the intermediate[45] as the reaction was halted in the presence of the by-product ethanol. Cyclisation of the generated intermediate to the product was accomplished by addition of diphenyl ether after removal of benzene, to enable reflux at a temperature of 265 °C for 30 min. Identification of synthesised compounds where performed using NMR, MS and IR.

1.3. Biological assays

1.3.1. ORAC assay

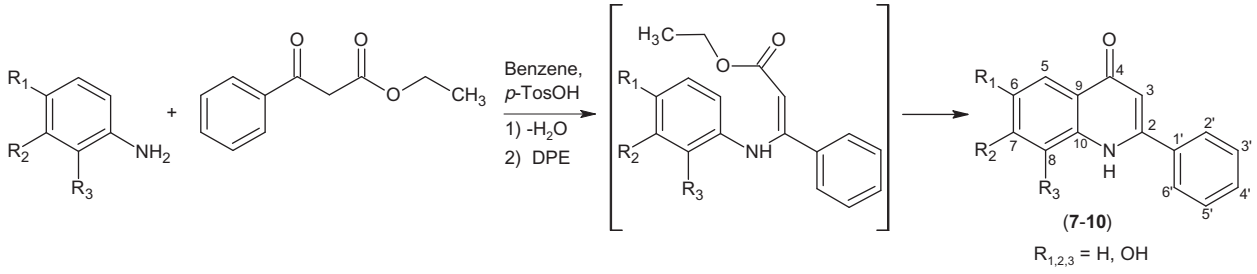
The oxygen radical absorbance capacity (ORAC) assay assessed the ability of compounds to scavenge peroxyl radicals, one of the contributors to lipid peroxidation. The assay was performed according to Ou et al. using fluorescein to measure fluorescent decay caused by the presence of peroxyl radicals, induced by AAPH (2,2′-azobi(2-amidinopropane) dihydrochloride).[26]Inhibition of fluorescent decay therefore indicated the ability of the test compounds to scavenge peroxyl radicals, protecting against fluorescent decay of fluorescein.

The underlying reaction in the ORAC assay is a hydrogen transfer reaction,[26] causing a phenolic antioxidant to donate a hydrogen atom,[46] terminating the lipid peroxidation chain reaction.[16]A Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard curve was generated, allowing for the expression of values as micromoles Trolox® equivalents utilising the formulae described by Cao et al.[47]

1.3.2. FRAP assay

The chemical ability of the test compounds to reduce ferric iron was assessed in the ferric reducing/antioxidant (FRAP) assay to indicate a measure of inhibition of hydroxyl radical production through the Haber–Weiss reaction (Eq. 3, thereby reducing oxidative stress. The assay determined the ability of the test compounds to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to its blue, ferrous form (Fe^{2+}-TPTZ). This reaction is a one electron exchange transfer reaction leading to a second electron transfer and producing a stable antioxidant radical (ArO**.**).[16]

The method described by Benzie and Strain, was used with the addition of a Trolox® standard to enable expression of data as percentage of 0.1 mM Trolox® equivalents, as this concentration of Trolox® yielded the highest ferrous-TPTZ absorbance value at the fixed end time.[48] The assay was performed over a period of 33 min,[49] to allow the reaction to reach an endpoint, since not all antioxidants are equally rapid reductants.[48]



Scheme 1. General scheme for Conrad–Limpach synthetic method with *p*-TosOH (*para*-toluene sulfonic acid) and DPE (diphenyl ether).

1.3.3. TBARS assay

Lipid peroxidation of polyunsaturated fatty acids produces aldehydes[50] that lead to the breakdown of lipid membranes and increased levels of the bio-marker malondialdehyde (MDA).[1,2,51,52] The aldehydes produced are thiobarbituric acid-reactive substances (TBARS) able to form complexes with thiobarbituric acid (TBA) to produce a pink-coloured complex, spectrophotometrically measured at a wavelength of 532 nm.[53]TBARS results are expressed as the amount of free MDA equivalents[51] per 1 mg tissue[53] through the calibration curve generated from 1,1,3,3-tetramethoxypropane.[51] The TBARS method was adapted from Ottino and Duncan and evaluated the extent of lipid peroxidation[53] by measuring the amount of free malondialdehyde equivalents produced after incubation with the toxin-solution, consisting of hydrogen peroxide, iron(III)chloride and vitamin C.[54] The hypothesised hydrogen donor mechanism of action was investigated in this assay.[55]

2. Results and discussion

Evaluation of the antioxidant activity of the synthesised 2-phenylquinolin-4(1*H*)-ones compared to structurally related flavones was done using chemical and in vitro antioxidant evaluations (Table 2). Whole rat brain homogenate and DMSO, as solvent for all test compounds, was used in these assessments and Trolox®, a water soluble derivative of Vitamin E was used as a positive control. According to Prior et al., DMSO may act as an antioxidant and was therefore included in the blank to correct for this effect.[56] Test compound concentrations of 1 mM were found to be overly concentrated in the ORAC and FRAP assays and the compounds

Table 2
Data obtained in the ORAC, FRAP and TBARS assays

Test compound (mM)		ORAC-value[e] (μM Trolox® equivalents) ± SEM	FRAP-value[f] (% Trolox® equivalents) ± S.D.	TBARS-value (nmol MDA/mg tissue) ± SEM
Blank homogenate[a]		—	—	0.27 ± 0.08
Toxin[b]		—	—	1.09 ± 0.07
DMSO[c]		—	—	0.93 ± 0.09
Trolox®[d]	0.01	—	—	0.17 ± 0.01
	0.1	—	—	0.04 ± 0.01
	1	—	—	0.03 ± 0.004
1	0.001	1.40 ± 0.09	1.97 ± 0.21	—
	0.01	N/A	2.42 ± 0.57	0.80 ± 0.04
	0.1	N/A	0.98 ± 0.11	0.77 ± 0.05
	1	—	—	0.73 ± 0.03
2	0.001	1.50 ± 0.26	2.82 ± 0.36	—
	0.01	N/A	1.69 ± 0.53	0.93 ± 0.03
	0.1	N/A	0.23 ± 0.61	0.92 ± 0.05
	1	—	—	0.77 ± 0.04
3	0.001	10.23 ± 2.44	2.65 ± 0.04	—
	0.01	30.46 ± 0.03	3.68 ± 0.78	0.93 ± 0.06
	0.1	55.42 ± 2.17	15.41 ± 0.25	0.83 ± 0.07
	1	—	—	0.07 ± 0.02
4	0.001	N/A	0.20 ± 0.14	—
	0.01	1.51 ± 0.39	3.73 ± 0.36	0.78 ± 0.02
	1	2.40 ± 1.33	2.72 ± 0.14	0.83 ± 0.04
	0.1	—	—	0.87 ± 0.04
5	0.001	21.44 ± 1.04	1.84 ± 0.32	—
	0.01	37.07 ± 1.07	2.47 ± 0.07	0.82 ± 0.07
	0.1	59.02 ± 4.14	0.61 ± 0.36	0.79 ± 0.05
	1	—	—	0.63 ± 0.07
6	0.001	N/A	2.04 ± 0.04	—
	0.01	N/A	2.52 ± 0.43	0.42 ± 0.07
	0.1	N/A	15.13 ± 1.14	0.48 ± 0.09
	1	—	—	0.91 ± 0.04
7	0.001	N/A	0.53 ± 0.68	—
	0.01	N/A	1.06 ± 0.07	0.91 ± 0.04
	0.1	1.91 ± 1.26	4.79 ± 1.00	0.87 ± 0.05
	1	—	—	0.87 ± 0.06
8	0.001	12.28 ± 0.57	2.14 ± 0.32	—
	0.01	29.01 ± 1.29	9.53 ± 0.07	0.48 ± 0.08
	0.1	58.29 ± 1.27	58.88 ± 0.39	0.31 ± 0.07
	1	—	—	0.05 ± 0.02
9	0.001	15.27 ± 0.91	−3.48 ± 0.57	—
	0.01	26.41 ± 0.83	−1.26 ± 2.28	0.69 ± 0.09
	0.1	57.51 ± 3.34	1.41 ± 0.21	0.71 ± 0.09
	1	—	—	0.72 ± 0.07
10	0.001	9.18 ± 0.45	3.56 ± 1.11	—
	0.01	26.07 ± 0.56	25.49 ± 0.04	0.58 ± 0.12
	0.1	65.39 ± 0.91	97.71 ± 0.39	0.49 ± 0.09
	1	—	—	0.27 ± 0.04

[a] Rat brain homogenate was used in TBARS.
[b] Toxin solution induced lipid peroxidation in the TBARS assay.
[c] 10% DMSO blank was used in the TBARS assay. Blank DMSO values were deducted from ORAC and FRAP values.
[d] Trolox® Standard Curves were used in the FRAP and ORAC assays, whereas Trolox® was assessed as a compound in the TBARS assay.
[e] Negative ORAC-values indicated the absence of peroxyl scavenging activity and were obtained when the blank AUC had a greater value than that of the sample AUC. These values were omitted from further calculations (Section 2).
[f] Negative FRAP-values indicated the absence of ferric reduction activity.

were therefore assessed at concentrations of 0.1 mM, 0.01 mM and 0.001 mM.

The ORAC assay was performed to establish the ability of the test series to scavenge peroxyl radicals (Fig. 3). The 2-phenylquinolin-4(1*H*)-ones demonstrated moderate activity at 0.001 mM, with 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) observed to be the best of this group. 6-Hydroxyflavone (**5**) however, performed the best of the complete test series. In the FRAP assay the chemical ability to reduce ferric iron was evaluated (Fig. 4). In this case the 2-phenylquinolin-4(1*H*)-ones showed the best activity of the test compounds, with the performance of 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) comparable to that of Trolox®, followed by 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) at 0.1 mM. In the TBARS assay the ability of the compounds to scavenge the hydroxyl radical was assessed (Fig. 5). In this assay 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) performed the best of the 2-phenylquinolin-4(1*H*)-ones, with the 1 mM concentration performing better than 0.01 mM Trolox®. The compound displaying the best activity in the TBARS assay was 4-hydroxyquinoline (**2**).

In the ORAC assay the 0.01 mM and 0.1 mM concentrations of 8-hydroxyquinoline (**3**), 6-hydroxyflavone (**5**) and 6-, 7- and 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**–**10**) did not mathematically allow the necessary standard decrease in fluorescence to 5% of the initial value (Fig. 3)—causing a calculation[47] error of the ORAC-value at 0.01 mM and 0.1 mM—indicating that these concentrations of the test compounds were overly concentrated and that AAPH, the radical generator was overpowered. This caused a near continuous protection of fluorescein and suggested potent peroxyl scavenging activity. This observation led to the conclusion that these test compounds were superior antioxidants compared to the other compounds, which demonstrated normal decay at the same concentrations. It was however possible to

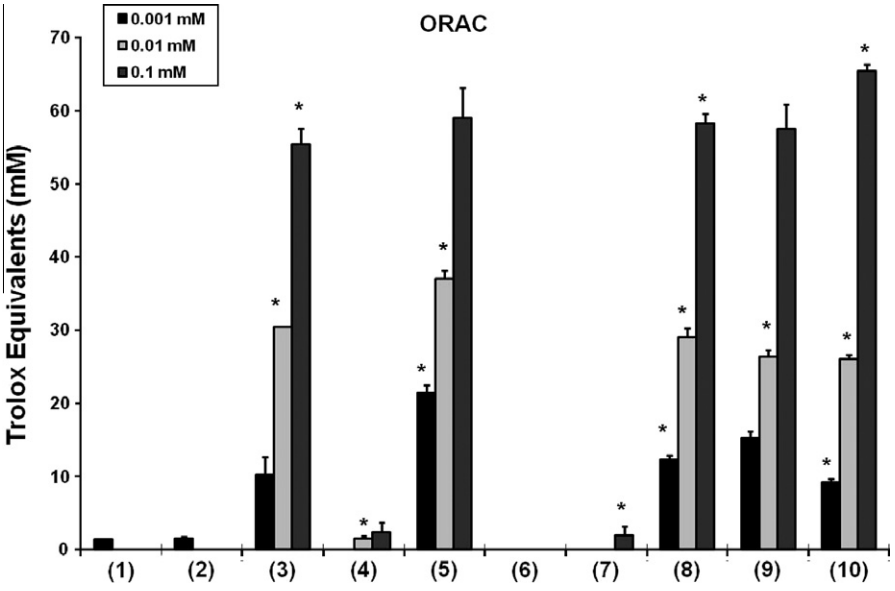


Figure 3. ORAC-values obtained for all the test compounds at three concentrations expressed as Trolox® equivalents per litre sample. Each bar represents the mean ± S.D.; *R.S.D. <5%; $N = 3$.

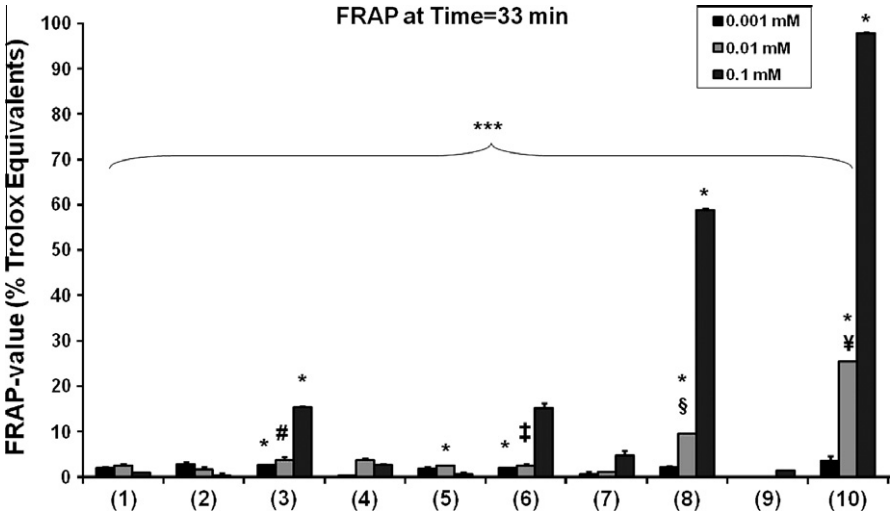


Figure 4. FRAP-values obtained for all test compounds in three concentrations at $t = 33$ min. Each bar represents the mean ± S.D.; *R.S.D.<5%; $N = 3$; ***$p < 0.0001$ versus 0.1 mM Trolox®; #$p = 0.001$ versus 0.1 mM 8-hydroxyquinoline (**3**); ‡$p = 0.0035$ versus 0.1 mM 7-hydroxyflavone (**6**); §$p = 0.0002$ versus 0.1 mM 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**); ¥$p < 0.0001$ versus 0.1 mM 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) (Paired t-test).

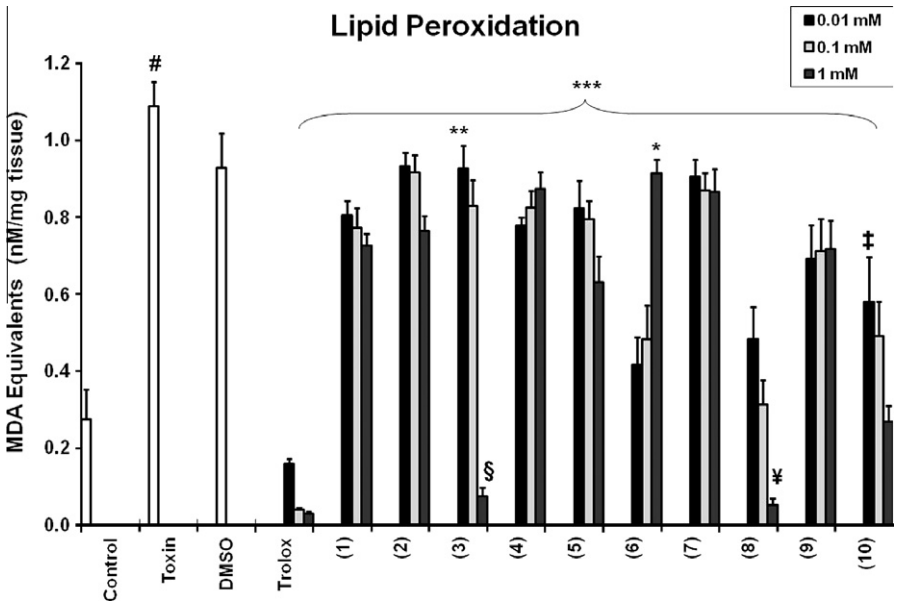


Figure 5. Lipid peroxidation inhibition of all test compounds at three concentrations. Each bar represents the mean ± S.E.M.; $N = 10$; $^{\#}p < 0.0001$ versus blank; $^{***}p < 0.0002$ versus Toxin; $^{**}p = 0.0014$ versus Toxin; $^{\S}p = 0.0217$ versus blank; $^{*}p = 0.1902$ versus Toxin; $^{¥}p = 0.0104$ versus blank; $^{‡}p = 0.0013$ versus Toxin.

compare the 0.001 mM concentration of all test compounds (Fig. 3), as well as the 0.1 mM concentration of flavone (**4**) and 2-phenylquinolin-4(1*H*)-one (**7**) as they allowed appropriate decay. At 0.001 mM concentration, better protection of fluorescein was observed

for the 2-phenylquinolin-4(1*H*)-ones than for the quinolines. 6-Hydroxyflavone (**5**), however showed the best activity. Flavone (**4**) also performed slightly better than 2-phenylquinolin-4(1*H*)-one (**7**) at a concentration of 0.1 mM. The peroxyl radical quenching ability was increased in a concentration dependent manner for most of the compounds, illustrating increased activity at higher concentrations.

In general, hydroxyl substitution increased peroxyl scavenging activity at 0.001 mM in this experiment (Fig. 3, Table 2). The increased activity of the 8-hydroxyl substituted quinoline (**3**) indicated increased scavenging activity in comparison to the other quinolines (**1** and **2**). For substitution of 2-phenylquinolin-4(1*H*)-one, the activity increased from 6-hydroxyl (**8**) to 7-hydroxyl (**9**) substitution, with the 7-hydroxyl (**9**) performing second best of all the test compounds. This trend was ascribed to the stabilising effect of the C-4 carbonyl of 7-hydroxy-2-phenylquinolin-4(1*H*)-one (**9**), which enhanced the proton donating ability of the hydroxyl group. Weaker activity was observed for the quinolines and 2-phenylquinolin-4(1*H*)-ones compared to 6-hydroxyflavone (**5**) at 0.001 mM, possibly due to the pK_a of the amine groups in the basic environment of this assay. It is possible that the phenolic compounds were hindered from donating protons in this hydrophilic assay, since hydrogen bonds might have interfered with hydrogen atom transfer.[57,58]

In the FRAP assay the 2-phenylquinolin-4(1*H*)-ones showed prominent activity when compared to Trolox® while the quinolines and flavones had low activity in reducing ferric-TPTZ to the blue ferrous complex (Fig. 4). The highest activity was observed for 8- and 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**10** and **8**), followed by 8-hydroxyquinoline (**3**) and 7-hydroxyflavone (**6**). Activity comparable to that of 0.1 mM Trolox® was observed for 8-hydroxy-2-phenylquinolin-4(1*H*)-one (**10**) at the same concentration at the fixed end time.

For all three structural groups, the best reducing activity was generally observed for the hydroxyl substituted structures. 7-Hydroxy-2-phenylquinolin-4(1*H*)-one (**9**) and 6-hydroxyflavone (**5**) were the exceptions in this case with very modest activity, the exact opposite of what is observed in the ORAC assay. However, the 8-hydroxyl substituted compounds (**3** and **10**) had the highest scavenging activity in their structural groups in both the ORAC and FRAP assays (Figs. 3 and 4).

In general the TBARS assay showed that hydroxyl substitution of compounds increased their hydroxyl radical scavenging activity by lowering the absorbance values of MDA (Fig. 5). The 6-hydroxy-2-phenylquinolin-4(1*H*)-one (**8**) performed best of the 2-phenylquinolin-4(1*H*)-ones, followed by the 8-hydroxyl (**10**) derivative, with the 7-substituted compound (**9**) demonstrating the lowest scavenging activity, as was observed in the FRAP assay. Of the flavones, 7-hydroxyflavone (**6**) had better activity than 6-hydroxyflavone (**5**), as in the FRAP assay, indicating that the pH of the assay environment as well as the type of radical affected the hydrogen donating ability of the compounds in solution.

MDA equivalent production was reduced to below that of the blank brain homogenate by 8-hydroxyquinoline (**3**) and the 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** and **10**), indicating complete inhibition of the toxin-induced peroxidation at 1 mM. Inhibition of hydroxyl radical production caused by the Haber–Weiss reaction, as evaluated in the FRAP assay, decreased lipid peroxidation further down the oxidative chain, as simulated in the TBARS assay. As postulated from the FRAP assay results, the 6- and 8-hydroxy-2-phenylquinolin-4(1*H*)-ones (**8** and **10**) displayed high activity in the lipid peroxidation assay, indicating that the FRAP assay correlated well with the data obtained in this biological assay. Not all test compounds illustrated dose dependant activity, indicating that other factors might be involved in the functioning of these compounds in living systems, at different concentrations. Further studies need to be conducted to elaborate on this finding.

3. Conclusion

Free radicals contribute to neurodegenerative disorders by causing damage to cell membranes, proteins and DNA. Antioxidants may be useful in preventative therapy and may slow the onset and progression of neurodegeneration. The data obtained

in this study confirmed the antioxidant activity of a series of 4-quinolones and demonstrated the potential for employing these structures in the development of antioxidant approaches to neurodegenerative disease.

From the biological evaluations it is possible to establish that 2-phenylquinolin-4(1*H*)-ones act as chain breaking antioxidants, with a hydrogen donor mechanism of action. It is clear that hydroxyl substitution leads to an increase in antioxidant activity, with the 8- and 6-hydroxyl substitution of the 2-phenylquinolin-4(1*H*)-ones (**10** and **8**) enhancing antioxidant activity in the FRAP and TBARS assays and the 7-substitution (**9**) in the ORAC assay. This can be attributed to the effect of the C4-carbonyl group, stabilising the anion of the 7-substituted compound (**9**), leading to increased hydrogen donation and antioxidant activity in the ORAC assay. The addition of an 8-hydroxyl substitution in the 2-phenylquinolin-4(1*H*)-one (FRAP) and in particular the quinoline series (ORAC and TBARS) indicated an increase in antioxidant activity in their separate structural groups.

Overall, the acidity of the protonated amine in the synthesised series when in solution, proved to be advantageous compared to the basic amine of the quinolines, especially in the acidic FRAP assay. The hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones (**8–10**) outperformed the flavones, with exception of 6-hydroxyflavone (**5**) in the ORAC assay, indicating that under certain conditions the hydroxyl substituted 2-phenylquinolin-4(1*H*)-ones may inhibit radical mediated damage better than the flavones and therefore show promise as possible neuroprotective agents. This indicates that the acidic, protonated amine was able to donate a proton while this was not possible in the flavone series. The type of radical present in the assay was also found to affect the ability of the test compound to donate hydrogen to neutralise or scavenge it.

Further studies into the iron chelating potential and in vitro and in vivo blood–brain barrier permeability and anti-apoptotic activity of these compounds need to be conducted. Compounds displaying favourable activity might prove useful as possible lead compounds or therapeutic agents in combating neurodegenerative disease resulting from oxidative damage.

4. Experimental

4.1. In silico physicochemical studies

The physicochemical properties of the test compounds, that is, molecular weight, *c*Log*P*, H-bond acceptor, H-bond donor, pK_a, ionisation potential, drug score, drug likeness and LogBB values were calculated using the online Osiris Property Explorer (http://www.organic-chemistry.org/prog/peo/),[39] Chemsilico Property Predictor for Drug Discovery (http://www.chemsilico.com),[37] Advanced Chemistry Development Chemsketch®[40,41] and Accelrys DS ViewerPro 5.0[38] software for prediction of drug oral bioavailability, toxicity and blood–brain barrier permeability.

4.2. Chemistry: General procedures

All reagents were obtained commercially and used without further purification. TLC Silicagel 60 F_{254} aluminium sheets from Merck, Darmstadt, Germany and Merck®Silica 60 (0.063 0.200 mm) were used as stationary phases for, respectively, thin layer and column chromatography, with the appropriate mobile phase constituted volumetrically immediately before use. R_f-values were observed under UV-light at 254 nm and 366 nm wavelengths. ^{1}H, ^{13}C, HSQC and COSY NMR spectra were obtained from a Bruker Advance 600 Spectrometer, in a 14.09 Tesla magnetic field, using tetramethylsilane as a point of reference and measuring all chemical shifts in parts per million (ppm). ^{1}H NMR and ^{13}C NMR were recorded at frequencies of 600 MHz and 150 MHz, respectively with ^{1}H NMR signal multiplicity denoted as s (singlet), br s (broad singlet) d (doublet), dd (doublet of doublets), t (triplet), q (quartet) or m (multiplet). Appropriate deuterated solvents were used. The uncorrected melting points were established using a Stuart melting point SMP10 apparatus with glass capillary tubes. Infrared spectra (IR) were obtained at 4000–450 cm^{-1} from a Nicolet Nexus 470-FT-IR Spectrometer, using KBr pellets and the diffuse reflectance method. A Thermo Electron LXQ ion trap mass spectrometer (MS) with atmospheric pressure chemical ionisation (APCI) source set at 300 °C was used to obtain low resolution APCI, with capillary voltage at 7.0 V and Corona discharge at 10 μA, while a Thermo Electron DFS magnetic sector mass spectrometer at 70 eV and 250 °C was used to obtain high resolution electron ionisation (HREI) spectra. Samples were introduced by a heated probe and perfluorokerosene was used as reference compound.

4.3. Synthesis

The Conrad–Limpach method for the synthesis of 4-quinolones was followed as described by Somanathan and Smith (1981).[44] The reaction was stirred in benzene at reflux temperature for 19 hours, with *para*-toluene sulfonic acid (50 mg, 2.629 mol) as catalyst. The removal of diphenyl ether in the work-up was achieved with a silica column, eluting over several hours with petroleum ether until all diphenyl ether was eluted from the product mixture.

4.3.1. 2-Phenylquinolin-4(1*H*)-one (7)

Aniline (279.39 mg, 0.003 moles) was cyclisised with ethyl benzoylacetate (576.66 mg, 0.003 moles). In vacuo removal of benzene yielded a yellowish oil, which was refluxed with 15 ml diphenyl ether for 30 min. The product was purified by column chromatography, initially eluting with petroleum ether, to remove diphenyl ether, then with 97% DCM, 3% MeOH (R_f = 0.115) to obtain the crude product, which was suspended in toluene and filtered to obtain the pure product, as an off-white amorphous solid (Yield: 203 mg, 0.917 mmol, 30.58%). R_f = 0.115 (97% DCM, 3% MeOH); $C_{15}H_{11}NO$; Mp = 259 °C; APCI-MS (300 °C, 7.0 V, 10 μA) *m/z*: 221.64 (M^+); HREI-MS: calcd. 221.08352, exp. 221.08371; ν_{max} (KBr, cm^{-1}): 3200.0, 3150.0, 3120.0, 3100.0, 3080.0, 3052.7, 1733.8, 1683.6, 1635.6, 1582.2, 1547.7, 1501.3, 1473.5, 1449.9, 1432.8, 2971.4, 800.5; δ_H (600 MHz, DMSO-d_6): 6.34 (s; 1H; H-3), 7.331 (m; 1H; H-6), 7.576 (s; 3H; H-8; H-3′; H5′), 7.664 (m; 1H; H-7), 7.761 (d; 1H; *J* = 7.96 Hz; H-4′), 7.83 (d; 2H; *J* = 2.9 Hz; H-2′, H-6′), 8.094 (d; 1H; *J* = 7.47 Hz; H-5), 11.732 (br s; 1H; NH-1); δ_C (150 MHz, DMSO-d_6): 107.24 (C-3), 118.80 (C-4′), 123.25 (C-6), 124.65 (C-5, C-1′), 127.37 (C-2′, C-6′), 128.96 (C-3′, C-5′), 130.39 (C-8), 131.74 (C-7), 134.25 (C-2), 140.54 (C-9), 150.05 (C-10), 176.90 (C-4).

4.3.2. 6-Hydroxyl-2-phenylquinolin-4(1*H*)-one (8)

Ethyl benzoylacetate (576.66 mg, 0.003 mole) was refluxed with 4-aminophenol (327.39 mg, 0.003 mol). Removal of benzene after 19 h yielded a dark yellow oil, which was refluxed with diphenyl ether. The petroleum ether column was flushed with ethyl acetate after removal of diphenyl ether and the crude product precipitated from toluene. The crude was further diluted in ethyl acetate (EtOAc) and extracted with water at pH 12. The water phase was acidified to pH 3 and extracted with EtOAc. The deep brown crude product was dried and resuspended in toluene and filtered. Further purification was performed with a Discovery Solid Phase Extraction Tube, from SUPELCO, Sigma–Aldrich, with positive pressure (97% DCM, 3% MeOH). The retained product was flushed from the tube with methanol to yield a dark brown amorphous solid (Yield: 83 mg, 0.35 mmole, 11.66%). R_f = 0.71 (EtOH); $C_{15}H_{11}NO_2$; mp = 151 °C; APCI-MS (300 °C, 7.0 V, 10 μA) *m/z*: 237.67 (M^+); HR-MS: calcd. 237.07843, exp. 237.07833; ν_{max} (KBr, cm^{-1}):

3300.0, 3230.0, 3200.0, 3130.0, 3100.0, 3070.0, 2972.1, 2950.0, 1704.6, 1592.6, 1580.0, 1560.0, 1510.6, 1490.0, 1449.7, 832.8; δ_H (600 MHz, DMSO-d_6): 6.22 (s; 1H; H-3), 6.40 (d; 1H; J = 8.03 Hz; H-7), 6.45 (d; 1H; J = 8.03 Hz; H-8), 6.66 (d; 2H; J = 8.08 Hz; H-2′, H-6′), 7.17 (m; 2H; J = 8.08 Hz; H3′; H5′), 7.41 (m; 1H; H-4′), 7.55 (s; 1H; H-5), 9.71 (s; 1H; OH-6); 11.61 (br s; 1H; NH-1); δ_C (150 MHz, DMSO-d_6): 105.77 (C-3), 107.23 (C-4′), 115.14 (C-2′, C-6′), 115.22 (C-7), 115.52 (C-8), 120.17 (C-3′), 120.60 (C-6), 121.98 (C-5′), 128.21 (C-1′), 128.98 (C-5), 131.47 (C-2), 140.67 (C-9), 148.21 (C-10), 186.23 (C-4).

4.3.3. 7-Hydroxyl-2-phenylquinolin-4(1*H*)-one (9)

Ethyl benzoylacetate (576.66 mg, 0.003 mol) was refluxed with 3-aminophenol (327.39 mg, 0.003 mol). Evaporation of benzene yielded an amber coloured oil, which was refluxed with diphenyl ether for 30 min. After removal of diphenyl ether the product was purified by extraction with basic water from EtOAc. The water phase was brought to pH 3 and extracted with EtOAc to yield the crude product, which was further purified using silica column chromatography (97% DCM, 3% MeOH; R_f = 0.61, EtOH). Suspending the product firstly in toluene, then in ethanol yielded the pure product by filtration as a light brown crystalline solid (Yield: 178 mg, 0.75 mmol, 25.0%). R_f = 0.61 (97% DCM, 3% MeOH); $C_{15}H_{11}NO_2$; mp = 267 °C; APCI-MS (300 °C, 7.0 V, 10 μA) m/z: 237.62 (M^+); HR-MS: calcd. 237.07843, exp 237.07854; ν_{max} (KBr, cm^{-1}): 3200.0, 3100.0, 3086.0, 3050.0, 3010.0, 2960.0, 2925.8, 2900.0, 1598.6, 1578.5, 1529.7, 1510.0, 1500.0, 1480.0, 1449.9, 1436.1, 1417.2, 852.6; δ_H (600 MHz, DMSO-d_6): 6.38 (s; 1H; H-3), 6.57 (d; 1H; J = 5.01 Hz; H-6), 7.151 (d; 1H; J = 5.01 Hz; H-5), 7.50 (s; 1H; H-8), 7.58 (m; 3H; H-2′, H-4′, H-6′), 7.83 (m; 2H; H3′; H5′), 12.16 (s; 1H; OH-7), 14.52 (br s; 1H; NH-1); δ_C (150 MHz, DMSO-d_6): 105.86 (C-3), 107.50 (C-5), 108.02 (C-6), 127.34 (C-1′), 127.83 (C-3′, C-5′), 129.17 (C-2′, C-6′),131.02 (C-14), 133.58 (C-2), 133.99 (C-8), 141.39 (C-9), 151.76 (C-10), 161.0 (C-7), 182.15 (C-4).

4.3.4. 8-Hydroxyl-2-phenylquinolin-4(1*H*)-one (10)

Ethyl benzoylacetate (576.66 mg, 0.003 mol) was refluxed with 2-aminophenol (327.39 mg, 0.003 mol) for 19 h, the solvent evaporated and the reaction heated at reflux with diphenyl ether. Removal of diphenyl ether was done as described above and the brown crude eluted from the column with ethanol. After acid-base extraction, as described above, precipitation from ethanol yielded the pure product as a brown amorphous solid (Yield: 87 mg, 0.37 mmol, 12.2%). R_f = 0.767 (EtOH); $C_{15}H_{11}NO_2$; mp = 272 °C; APCI-MS (300 °C, 7.0 V, 10 μA) m/z: 237.64 (M^+), HR-MS: calcd. 237.07843, exp. 237.07957; ν_{max} (KBr, cm^{-1}): 3293.7, 3110.0, 3100.0, 3050.0, 2970.0, 2900.0, 2550, 1781.6, 1620.0, 1573.4, 1514.4, 1482.7, 1447.8, 1409.3, 1350, 849.9; δ_H (600.17 MHz, DMSO-d_6): 6.54 (s; 1H; H-3), 7.10 (dd; 1H; J = 7.25 Hz; H-7), 7.19 (t; 1H; J = 7.25 Hz; H-6), 7.51 (m; 3H; H-2′, H-4′, H-6′), 7.56 (dd; 1H; J = 7.25 Hz; H-5), 7.88 (m; 2H; H3′; H5′), 10.57 (s; 1H; OH-8); δ_C (150 MHz, DMSO-d_6): 106.54 (C-3), 114.02 (C-5), 114.24 (C-7), 123.94 (C-6), 125.06 (C-1′), 127.54 (C-3′, C-5′), 128.18 (C-8), 128.78 (C-2′, C-6′), 130.06 (C-4′), 135.36 (C-2), 148.43 (C-9), 150.87 (C-10), 181.08 (C-4).

4.4. Biological evaluation

4.4.1. Materials

All chemicals were purchased commercially from Sigma–Aldrich (St. Louis, MO, USA and Steinham, Germany), Aldrich Chemical Company, (Milwaukee, WIS, USA), BHD (Midrand, South Africa and Darmstadt, Germany), Saarchem (PTY) Ltd (Wadeville and Muldersdrift, South Africa), Merck (Darmstadt, Germany) and Alpha Pharmaceuticals (Durban, South Africa). All the chemicals used in the FRAP and ORAC assays, save for DMSO and the test compounds, were generously donated by the Department of Biochemistry, NWU, Potchefstroom Campus.

4.4.2. ORAC assay

4.4.2.1. Reagents. Phosphate buffer, 75 mM, was prepared by diluting 1 M K_2HPO_4 with 1 M KH_2PO_4 aqueous solutions in a 61.6:38.9 v/v ratio and further diluting with Milli-Q water to 75 mM. The pH was adjusted to 7.4 and the buffer stored in the fridge. A 265 mM fluorescein stock solution was prepared and further diluted with buffer before each assay to yield a 112 nM working solution. The 72 mM AAPH solution was prepared with buffer immediately before use and kept on ice, while a sufficient quantity of the 250 μM Trolox® standard solution was prepared before each assay, dissolving Trolox® in DMSO by tip-sonication for 2 min. The water insoluble test compounds were dissolved in DMSO to yield final concentrations of 0.1 mM, 0.01 mM and 0.001 mM in the well-plate in 10% DMSO. All reagents were commercially available.

4.4.2.2. Instrumentation. The ORAC assay was performed at 485 nm excitation and 530 nm emission wavelengths in static mode on a BioTek FL600 plate reader, using KC4 software.

4.4.2.3. Method. The Trolox® standard curve was generated by diluting the Trolox® working solution with DMSO to yield 20 μl standard solutions in a final 10% DMSO concentration in the first row of an opaque 96-well plate. Concentrations of 0, 2.5, 5, 10, 15 and 20 μM were tested in duplicate, in wells next to each other. To each well 80 μl of fluorescein solution (112 nM) was added with a multi-channel pipette. The sensitivity of the well plate was determined before initiation of the reaction, in order to ascertain that emission values fell within the measurable range of the plate reader. The sensitivity was set at 146 and the relative fluorescence units (RFU) established to be approximately 65,000. The reaction was started on adding 100 μl of 72 mM AAPH solution, yielding a final 36 mM concentration in the well. The fluorescent decay was measured at 5 min intervals over a period of 3 h. A second order polynomial slope was obtained for the standard curve, enabling the use of the area under the curve (AUC) to determine the Trolox® equivalents of the assayed compounds. The test compounds were diluted to give 20 μl volumes with final concentrations of 0.1 mM, 0.01 mM and 0.001 mM in 10% DMSO, and were placed in the opaque well plate in triplicate, in wells next to each other.

4.4.2.4. Data collection. Theformulae utilised by Cao and Prior,[47] were applied to obtain the ORAC-values as micromoles Trolox® equivalents. The ORAC-values indicated the relative protection of fluorescein by the sample antioxidants compared to that afforded by Trolox®, by integrating into the Trolox® regression equation (Fig 3).

4.4.2.5. Statistical analysis. Themean of three values was expressed as the ORAC-value ± S.E.M. Statistical acceptability was determined at a relative standard deviation of less than 5%. Further statistical analysis was performed using a Paired Student–Newman Keuls t-test on GraphPad Prism.

4.4.3. FRAP assay

4.4.3.1. Reagents. Acetate buffer (300 mM) was prepared by dissolving 1.55 g $NaAc{\cdot}3H_2O$ in 8 ml glacial acetic acid and diluting to 500 ml with double distilled water, adjusting the pH to 3.6. A 40 mM HCl solution was prepared with double distilled water and used to prepare a 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) solution. A 20 mM $FeCl_3{\cdot}6H_2O$ solution was prepared with acetate buffer and the FRAP reagent subsequently constituted of 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3{\cdot}6H_2O$ solution,

directly before addition to the well-plate, given that crystallisation takes place rapidly. The Trolox® standard, as well as the test compounds was prepared in DMSO to yield final concentrations of 0.1 mM, 0.01 mM and 0.001 mM, in a final 10% DMSO concentration. All reagents were purchased commercially.

4.4.3.2. Instrumentation. Absorbance values were measured at 595 nm with a BioTek Synergy HT Reader, using Gen5 1.05 software.

4.4.3.3. Method. The 0.1 mM, 0.01 mM and 0.001 mM Trolox® standards were prepared in a 1:9 ratio with double distilled water to yield a volume of 100 μl in each well of a transparent 96-well plate. The wells containing the same concentration were placed next to each other in triplicate. The FRAP reagent was constituted and added with a multi-channel pipette; 125 μl was added to all wells, followed by a second 125 μl addition, yielding a final 250 μl reagent per well. Care was taken to start the Gen5 1.05 computer protocol at exactly 1.5 min after the first row of the well plate was filled with the first 125 μl of FRAP reagent. Time dependence was implemented to enhance the reproducibility of the assay. A Lag time of 3 min at 37 °C ensued before the first absorbance measurement was taken at 595 nm, followed by measurements at 6 min intervals for a period of 33 min to illustrate the reduction kinetics of the test compounds. The absorbance mean of 0.1 mM Trolox® at 33 min was converted to the 100% Trolox® equivalent FRAP-value since a three to fourfold increase in activity was seen for the 0.1 mM series ($^{***}p < 0.0001$) to 0.01 mM and 0.001 mM.

4.4.3.4. Data collection. The absorbance values for all test compounds were expressed as percentage Trolox® equivalents, employing the 0.1 mM Trolox® standard value at the fixed time of 33 min as a 100% reference. The blank corrected for the absorbance of the well plate, solvent and all other reagents.

4.4.3.5. Statistical analysis. The assay was performed in triplicate over a period of six time intervals and was analysed statistically against the 0.1 mM Trolox® standard series in a Paired Student–Newman Keuls *t*-test on GraphPad Prism with statistically significant difference indicated at $p < 0.05$. Standard deviation (S.D.) and relative standard deviation (R.S.D.) were calculated and were statistically acceptable at R.S.D. <5%.

4.4.4. TBARS assay

4.4.4.1. Animals. The rats used in the TBARS assay were bred and maintained in a controlled environment in the North-West University Laboratory Animal Centre and the assays approved by the North-West University Ethics Committee. Adult Sprague Dawley rats, weighing between 200 g and 250 g, were decapitated with a guillotine and the brains removed into phosphate buffer solution and held on ice. The 10% (w/v) rat brain homogenate was formulated with PBS with the aid of a manual glass–Teflon homogeniser.

4.4.4.2. Instrumentation. The absorbance values were measured with a Labsystems Original Multiscan RC plate-reader and Genesis software.

4.4.4.3. Reagents. A phosphate buffer solution (PBS) was prepared and stored in the fridge with 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4 in double distilled water and the pH adjusted to 7.4. A 0.5% solution of BHT in methanol and 10% solution of trichloroacetic acid (TCA) in double distilled water was made and stored in the refrigerator. The 0.33% thiobarbituric acid (TBA) solution was made afresh every day, dissolved in double distilled water, in an amber bottle. The test compounds were dissolved in DMSO to achieve final concentrations of 1 mM, 0.1 mM and 0.01 mM in 10% DMSO. To establish the effect of the solvent 10% DMSO was assessed in parallel.

4.4.4.4. Method. A calibration curve was generated by plotting the absorbance of the TBA/MDA-complex against the concentration MDA. A standard solution of 50 nmol/l 1,1,3,3-tetramethoxypropane (TMP) in double distilled water was used in the calibration curve as a standard, and diluted with phosphate buffer solution to achieve TMP concentrations ranging between 0 and 25 nmol/l with 5 nmol/l intervals. Before incubation at 60 °C, 2,6-di-*tert*-butyl-4-methylphenol, trichloroacetic acid, and thiobarbituric acid (TBA), solutions were added. After cooling on ice, a butanol extraction was completed by centrifugation. From the top butanol layer, 200 μl was transferred into a 96-well plate and the absorbance measured at a wavelength of 530 nm. Butanol was used as a blank. The assay was performed as for the TMP standard curve. The aqueous lipid peroxidation-inducing toxin solution consisted of 0.5 mM hydrogen peroxide solution, 0.488 mM iron(III)chloride solution and 0.14 mM ascorbic acid solution in a ratio of 2:1:1. The final concentration of the positive control was 1 mM Trolox® in 10% DMSO. A series of 1 mM, 0.1 mM and 0.01 mM drug concentrations in 10% DMSO solutions were assayed. To ascertain its effect on inhibition of lipid peroxidation, 10% DMSO was tested in parallel and treated exactly as the test compounds. The absorbance values were converted to nanomoles MDA equivalents produced per 1 mg tissue.

4.4.4.5. Statistical analysis. This experiment was repeated five times each on two rat brains. A toxin group was included in every assay and used as a point of reference. Graphpad Prism was used to statistically analyse data in the Unpaired Student–Newman Keuls *t*-test and to determine the standard error of the means (S.E.M.). Results were expressed as the mean value ± S.E.M of the 10 runs. Significant differences were obtained when $p < 0.05$.

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