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Biflavonoids from Brazilian pine *Araucaria angustifolia* as potentials protective agents against DNA damage and lipoperoxidation

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Abstract

A biflavonoid fraction (BFF) obtained from *Araucaria angustifolia* needles was effective to quench singlet oxygen ($^1O_2$), to protect plasmid DNA against single strand break (ssb) caused by $^1O_2$ or Fenton reaction and to inhibit Fenton or UV radiation-induced lipoperoxidation in phosphatidylcholine liposomes. The activity of the biflavonoid fraction (BFF) was compared with quercetin, rutin (flavonoids), ginkgetin, amentoflavone (biflavonoids), α-tocopherol and Trolox®. The BFF displayed a higher quenching rate constant compared to flavonoids and biflavonoids and protected against ssb induced by $^1O_2$. Although the BFF was not as efficient as either flavonoids, α-tocopherol or Trolox® in protection against ssb induced by Fenton-reaction or lipoperoxidation, these scavenging properties suggest that BFF is still an excellent candidate for successful employment as an antioxidant and photoprotector.

Keywords: *Araucaria angustifolia*; *Araucariacea*; Biflavonoids; Ginkgetin; Amentoflavone; Singlet oxygen; Plasmid DNA; Lipoperoxidation; UV radiation; Photoprotection

1. Introduction

The generation of reactive nitrogen and oxygen species (RNOS) has been shown to occur in multiple physiological processes, resulting in oxidation of several biological targets, including proteins, enzymes, lipids and DNA (Sies, 1991; Martinez et al., 2003). Singlet molecular oxygen ($^1O_2$) is an electronically excited species involved in mammalian cells under both normal and pathological conditions (Halliwell and Gutteridge, 1998; Pierlot et al., 2000). Due to its relatively long half-life in aqueous systems, $^1O_2$ is able to move through appreciable distances in the cellular environment affecting different targets (Piette, 1991; Ravanat et al., 2000; Miyamoto et al., 2003; Martinez et al., 2002).

There is a much evidence indicating that RNOS are involved in in situ DNA damage, contributing to the aetiology of several human diseases (Helbock et al., 1999). Thus the search for new compounds able to prevent oxidative damage is an important strategy aimed at preventing injuries (Sies, 1993). Several studies have revealed antioxidant activities of compounds, such as lycopene (Di Mascio et al., 1989a, 1992), curcumin (Subraminian et al., 1994), flavonoids (Williams et al., 2004), and related polyphenols (Arteel and Sies, 1999; Klotz and Sies, 2003) as inhibitors of RNOS-induced damage to biomolecules.

Flavonoids are widely distributed secondary metabolites in the plant kingdom and have several important roles as pigments, bactericides, fungicides, etc. Besides, they have other characteristic, properties, such as anti-inflammatory, antibacterial, anticarcinogenic and oestrogenic agents (Middleton and Kandswami, 1986). Additionally, flavonoids can inhibit some enzymes, such

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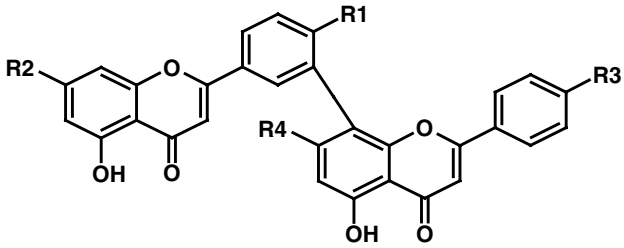
Table 1
Identification and characterization of six major compounds of BFF by HPLC co-elution of standards and mass spectrometry analyses

Compounds	Retention times (min)	Molecular ions $[M + H]^+$	Structures
			
1	22	539	R1 = R2 = R3 = R4 = OH amentoflavone
2	26	553	Mono-*O*-methylamentoflavone
3	30	567	Di-*O*-methylamentoflavone
4	33	567	R1 = R2 = OH; R3 = R4=OMe ginkgetin
5	36	581	Tri-*O*-methylamentoflavone
6	41	595	Rl = R2 = R3=R4 = OMe tetra-*O*-methylamentoflavone

as cyclooxygenase, lipoxygenase and phospholipase A_2 (Lee et al., 1996; Kim et al., 1998; Saponara and Bosisio, 1998), and are also able to chelate metals, which play an important role in metal-induced free radical reactions (Deng et al., 1997). Biflavonoids are dimers of flavonoids, linked by a C–O–C or C–C bond, their biological properties include anti-inflammatory and antiarthritic activites in animals (Harborne, 1967). Such capacities were associated with an ability to suppressing reactive species and to inhibit cyclooxygenases as well (Kim et al., 1999).

The needles of *Araucaria angustifolia*, an endemic conifer in southern Brazil, contain several amentoflavone-type (C8″–C3′) biflavones (Fonseca et al., 2000), which differ from each other in the number and position of methoxyl groups (Table 1).

The major aim of this study were: to determine the kinetic parameters of $^1O_2$ quenching of *A. angustifolia* biflavonoids, using a time-resolved near infrared luminescence technique; to examine the biflavonoid effect on DNA damage induced by a clean source of $^1O_2$ generated chemically by a naphthalene endoperoxide derivative and by Fenton reaction in plasmid pBluescript and PUC 19. The protector capacity of these biflavonoids against UV radiation-induced lipoperoxidation was also determined.

2. Results and discussion

2.1. Identification of biflavonoids

The BFF, precipitated from MeOH–H_2O (3:7) solution was analyzed by HPLC/MS and contained six major biflavonoids (Table 1). The determination of a common 3′-8″-interflavonoid linkage (amentoflavone type) between the flavonoid units was based on the analysis of ^1H and ^{13}C NMR spectroscopic on data of the per-methylated product of BFF. Spiking with authentic samples of biflavonoids from *Ginkgo biloba* allowed the identification and characterization of amentoflavone (**1**, 2.4 % of relative abundance in BFF) and ginkgetin (**4**, 27.1 %), eluted at 22 and 33 min, respectively. The compounds tetra-*O*-methylamentoflavone (**6**, 16.4 %), mono-*O*-methylamentoflavone (**2**, 11.4 %), di-*O*-methylamentoflavone (**3**, 26.3 %) and tri-*O*-methylamentoflavone (**5**, 14.5 %) (Fig. 1) were also identified by HPLC/MS; estimation of molarity was based on the content of major biflavonoids, ginkgetin and di-*O*-methylamentoflavone.

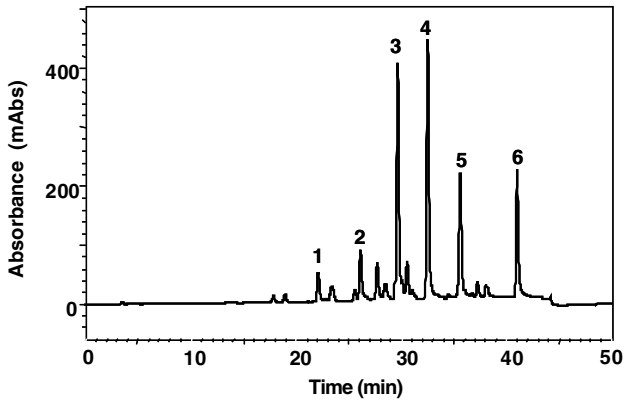


Fig. 1. HPLC profile of BFF obtained from *A. angustifolia* needles. Compounds **(1)** amentoflavone, **(2)** mono-*O*-methylamentoflavone, **(3)** di-*O*-methylamentoflavone, **(4)** ginkgetin, **(5)** tri-*O*-methylamentoflavone and **(6)** tetra-*O*-methylamentoflavone.

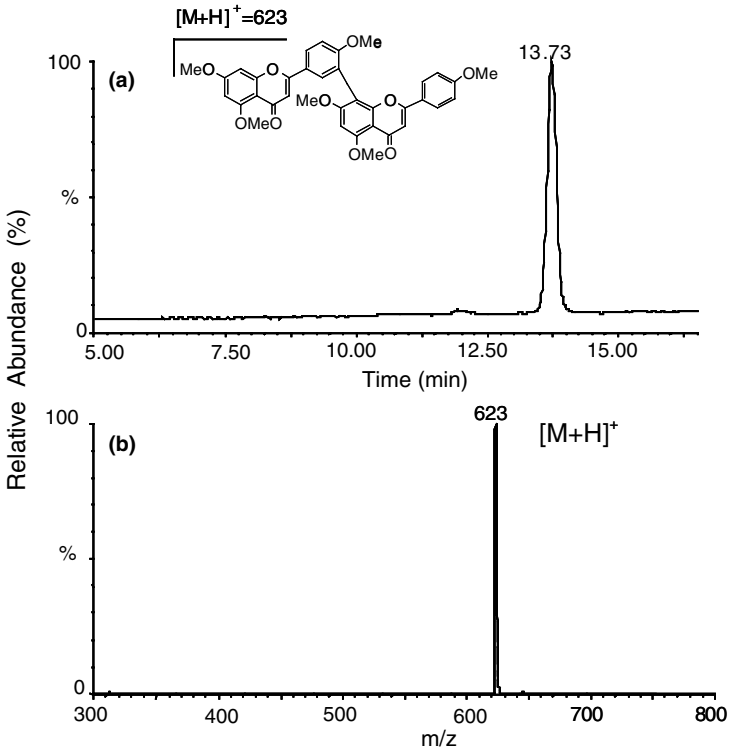


Fig. 2. HPLC/MS analyses of *per*-methylated product **(7)** of BFF (hexa-*O*-methylamentoflavone). (a) UV chromatogram (330 nm) of per-methylated BFF, (b) hexa-*O*-methylamentoflavone identified by MS spectrum of the peak at 13.73 min.

2.2. Per-methylation of BFF

Since there has been a number of biflavonoids with different interflavonoid linkages, the BFF was subjected to per-methylation in order to determine the presence of other types of biflavonoids in *A. angustifolia.* The major product of the BFF reaction with iodomethane was established as hexamethyl amentoflavone (**7**), based on detection of the pseudo-molecular $[M + H]^+$ ion at *m/z* 623 (Fig. 2) in the HPLC/MS and following analysis of the ^1H NMR spectroscopic data. The ^1H NMR spectrum of compound **7** showed only two *meta*-coupled proton signals at δ 6.29 and 6.43 (2*d*, $J = 2.0$ Hz), which were assigned to H-6 and H-8, respectively. Since no similar doublet was observed for H-6″ and H-8″, the linkage for the biflavonoids should involve either C6″ or C8″. The coupled system H-2‴/H-6‴–H-3‴/H5‴ appeared as two distinct doublets at δ 7.33 (*d*, $J = 8.9$ Hz) and at δ 6.72 (*d*, $J = 8.9$ Hz), respectively. Furthermore the presence of two singlets [δ 6.62 (H-3, s) and 6.56 (H-3″, s)] assignable to H-3 and H-3″ excluded the possibility of any linkage between the flavone moieties at C-3″, C-2‴, C-3‴, C-5‴ or C-6‴ with C-3′. According to these results, compound **7** was determined as a biflavonoid with a C-3′-C-8″ interflavonoid linkage, corresponding to the amentoflavone group in accordance with previous data (Fonseca et al., 2000).

2.3. Stern–Volmer analysis of the $^1O_2$ luminescence quenching

Physical detection of $^1O_2$ is based on monomol or dimol light emission. The determination of $^1O_2$ quenching rate constant can be performed by measuring the monomol light emission at 1270 nm detected by a sensitive infra-red photomultiplier. Single exponential fitting was used to determine the intercept of the Stern–Volmer plot which gave the value of $1/\tau = k_{obs}$ (τ is the lifetime of $^1O_2$), while the value of k_t was derived from the slope of the linear plots (Table 2 and Fig. 3). The τ obtained in acetone was ~53 μs, according to the value reported in the literature (Monroe, 1985).

Table 2
Singlet molecular oxygen total quenching constants $k_t = (k_q + k_r)$ of flavonoids and biflavonoids in acetone

Compounds	$\tau\ ^1O_2$ μs	k_t (10^6 M^{-1} s^{-1})
Quercetin	53.45	9
Ginkgetin **4**	53.16	9
Amentoflavone **1**	53.16	30
BFF (**1**–**6**)	52.94	50

The k_t values, derived from titration, such as shown in Fig. 3, were obtained from time-resolved luminescence (Fig. 3, inset). For comparison, the k_t, value of quercetin in CD_3OD_3 was 2.4×10^6 M^{-1} s^{-1} (Tournaire et al., 1993).

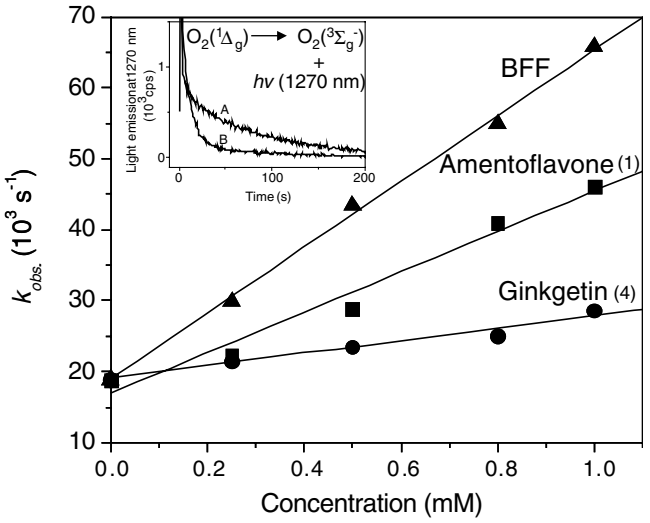


Fig. 3. Stern–Volmer plot of BFF, amentoflavone and ginkgetin, with overall quenching calculated according to Stern–Volmer plots using $k_d^{-1} = 53.17$ μs in acetone. Conditions: see Section 4. Inset: Observed near-infrared emission of $^1O_2$, after pulse excitation of 1-H-phenalen-1-one ($\lambda_{exc.} = 355$ nm) in normally aerated acetone. (A) pure solvent, (B) 1 mM BFF.

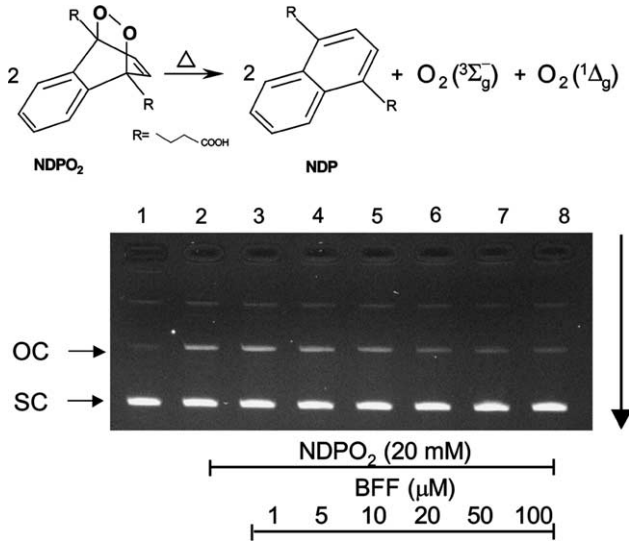


Fig. 4. Agarose gel electrophoretic pattern, showing protection by BFF against $^1O_2$, induced strand break in plasmid pBluescript after 100 min of incubation. 1, plasmid pBluescript control; 2, plasmid + $NDPO_2$ 20 mM; 3, plasmid + BFF 1 μM; 4, plasmid + $NDPO_2$ 20 mM + BFF 5 μM; 5, plasmid + $NDPO_2$ 20 mM + BFF 10 μM; 6, plasmid + $NDPO_2$ 20 mM + BFF 20 μM; 7, plasmid + $NDPO_2$ 20 mM + BFF 50 μM; 8, plasmid + $NDPO_2$ 20 mM + BFF 100 μM. OC and SC are open circle and supercoiled forms, respectively. Inset: Generation of singlet molecular oxygen by the thermodissociation of the endoperoxide 3,3′-(l,4-naphtylene)-dipropionate ($NDPO_2$).

In a previous report (Tournaire et al., 1993), the rate constant of physical quenching of the flavonoids was stronger than the chemical reaction, mainly in compounds having a catechol moiety in the ring C, such as quercetin. The BFF showed a higher k_t among all tested compounds. The amentoflavone was more efficient than ginkgetin, probably due to presence of a methoxyl group in ginkgetin, which reduces the efficiency of physical quenching.

2.4. Cleavage of plasmid pBluescript or PUC DNA in the presence of $^1O_2$ generated by $NDPO_2$ thermodecomposition and biflavonoid protection

The damage in plasmid DNA (pBluescript and PUC 19) was analyzed, using gel electrophoresis on 1% agarose, measuring the amount of induction of single-strand break (ssb), which results in the formation of open circular forms (OC) and decreases supercoiled (SC) forms in plasmid DNA.

Singlet oxygen, generated by incubating the thermodissociable endoperoxide disodium 1,4-naphthalenedipropanoate ($NDPO_2$) at 37 °C (Di Mascio et al., 1989b), significantly increased the ssb in plasmid pBluescript DNA, from 8% (Line 1, Fig. 4) of OC form in the control to 25% in the treated sample (Line 2, Fig. 4). All the compounds examined inhibited the formation of ssb. The BFF (100 μM) decreased the OC form of pBluescript plasmid DNA caused by 20 mM of $NDPO_2$ ($\sim 10^{-12}$ M of $^1O_2$ in steady state) (Di Mascio et al., 1989b) more efficiently than quercetin. The percentage of protection afforded by addition of 100 μM of BFF was 78%, which was similar to that observed for rutin (72%). In case of quercetin, the percentage of protection (44%) was almost half of that observed for BFF and rutin (Fig. 5). Trolox®, a water-soluble α-tocopherol derivative, which is considered a lipoperoxidation suppressor, provided only 21% of protection, at concentration of 100 μM (Fig. 5).

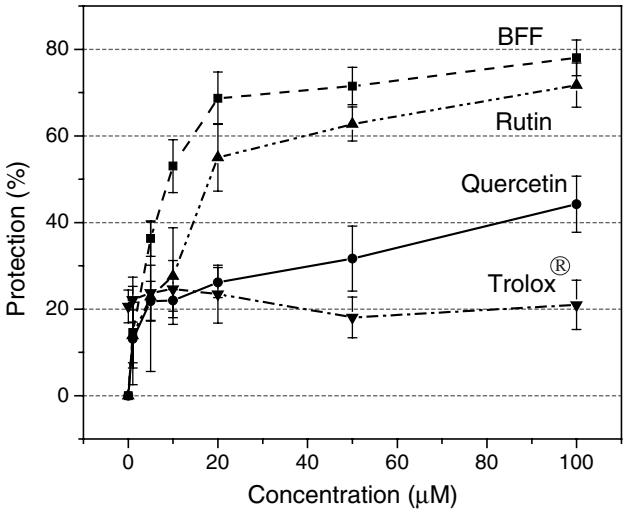


Fig. 5. Concentration-dependent pattern of inhibition of OC formation by BFF, quercetin, rutin, and Trolox® against ssb formation induced by $^1O_2$ generated by $NDPO_2$ on pBluescript plasmid DNA. Values were obtained by scanning electropherograms and represent mean ± SE from three independent experiments.

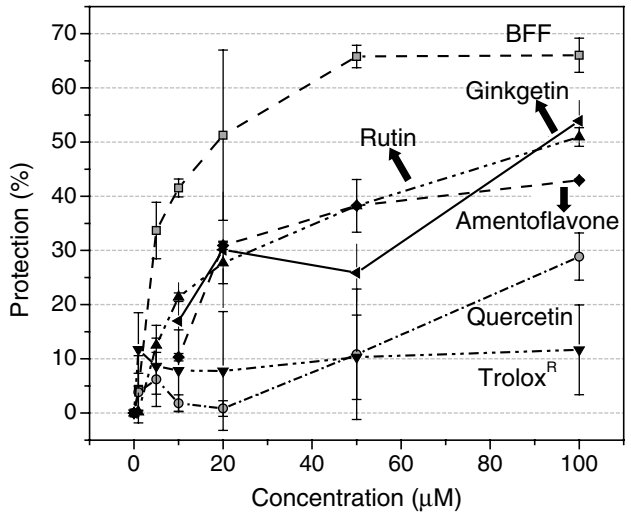


Fig. 6. Concentration-dependent protection provided by BFF, rutin, quercetin, ginkgetin, amentoflavone and Trolox® (1–100 (μM)) against $^1O_2$-generated by $NDPO_2$ induced single strand breaks in plasmid PUC 19 DNA. Values plotted are mean ± SE derived from scanning of gel electrophoresis from three independent experiments.

The dose–response curve of plasmid PUC 19 exposed to $NDPO_2$ and different concentrations of BFF, quercetin, rutin, Trolox®, amentoflavone and ginkgetin were evaluated (Fig. 6), using 100 μM, the BFF protected 66%, followed by ginkgetin (53%), rutin (51%) and amentoflavone (42%). The inhibition pattern shown by BFF and rutin differed considerably from that displayed by quercetin and Trolox®. In case of BFF, a protection of 70% was observed at low concentration of 20 μM (Figs. 5 and 6). Nevertheless, a five-fold increase of the concentration from 20 to 100 μM failed to significantly increase the suppressor ability. Similar results were also observed with rutin, in which 20 μM gave 55% of protection, whereas 100 μM yielded about 72% of protection; hence, the protection was not fully dose-dependent (Fig. 5). Amentoflavone (50%) and ginkgetin (40%), displayed similar inhibition profile although lower than BFF (Fig. 6).

The mechanism by which these compounds protect the genetic material against $^1O_2$ may involve physical and/or chemical quenching. The chemical quenching is based on the chemical reaction of $^1O_2$ with an appropriate substrate, resulting in a product (Eq. (1)). In the case of the BFF, amentoflavone, ginkgetin and quercetin the efficacy of physical quenching greatly exceeds that of chemical quenching, considering the linear curve obtained in Stern–Volmer plots (Fig. 3). The physical quenching involves two types of mechanisms: electron transfer and/or energy transfer (Foote, 1979) (Eq. (2)). Carotenoids (e.g., β-carotene, lycopene) (Di Mascio et al., 1992; Yamaguchi et al., 1999) and nickel complexes (Rabek, 1985) have been suggested as quenchers of $^1O_2$ by energy transfer, with the remaining energy dispersed by heat. Phenols, sulfides and azides can quench $^1O_2$ by a charge-transfer mechanism (Khan et al., 1992; Yamaguchi, 1985)

$$Q + {}^1O_2 \rightarrow QO_2 \quad (1)$$

$$Q + {}^1O_2 \rightarrow Q + {}^3O_2 + \text{heat} \quad (2)$$

2.5. Fenton reaction

The effect of BFF, flavonoids (quercetin and rutin), biflavonoids (amentoflavone and ginkgetin) and tocopherol derivative (Trolox®) on hydroxyl radical-mediated plasmid DNA cleavage is summarized in Fig. 7. The Fenton reaction increased the cleavage of the plasmid DNA from 8% in the control to 40% in the treated sample. Flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism with the formation of less reactive flavonoid phenoxyl radicals (Cotele et al., 1992; Hanasaki et al., 1994). Alternatively, these compounds may inactivate metals ions through complexation (Deng et al., 1997), thereby suppressing the superoxide-driven Fenton reaction. Despite some complex of compounds with metal ions can cause free-radical damage to biomolecules, such as cytochrome *c*-bond haem iron.

Quercetin displayed the higher antioxidant properties at 1 μM 75% of protection against the formation of ssb. The rutin was also extremely effective in this system, providing 75% protection at 5 μM (Fig. 7). Both quercetin and rutin evidenced stronger antioxidant activities than Trolox®. Albeit not as strong as the others, BFF also demonstrated high antioxidant activity, providing 72% protection with 100 μM.

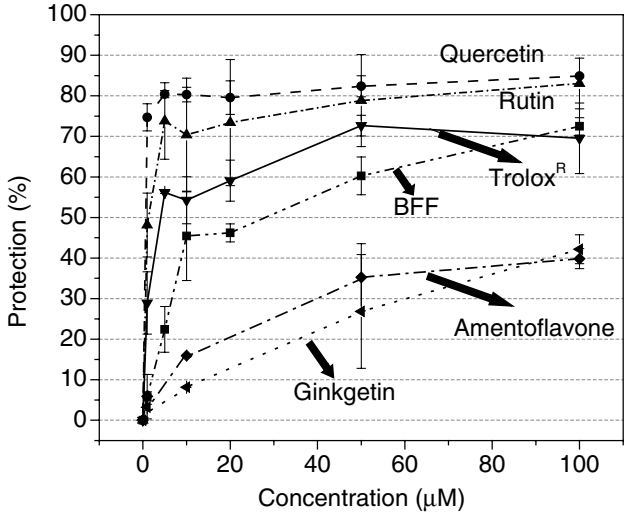


Fig. 7. Inhibitory effects of BFF, flavonoids quercetin and rutin, biflavonoids amentoflavone and ginkgetin and Trolox® on ssb formation induced by Fenton-type reaction. The ssb formation was monitored by OC formation in plasmid pBluescript DNA. Values plotted are mean ± SE after DNA.

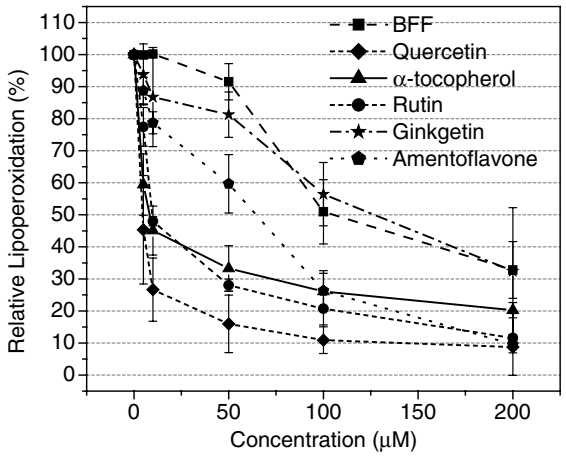


Fig. 8. Inhibition of UV radiation-induced peroxidation on phosphatidylcholine liposomes by increasing concentrations of quercetin, rutin, BFF, α-tocopherol, amentoflavone and ginkgetin. Antioxidant activity was determined by measuring the formation of TBARS. Results are expressed as mean ± SD, $n = 3$.

Among the flavonoids, the presence of 3-OH in the ring C and the catechol moiety in the ring B increase their capacity to scavenge hydroxyl radical ·OH, such as quercetin (Rice-Evans, 1995) and possibly, explain the moderate effect of BFF, amentoflavone and ginkgetin in which these functions are absent.

2.6. Protection against UV-induced peroxidation

The quercetin, rutin, BFF, amentoflavone, ginkgetin and α-tocopherol were evaluated as protector of phosphatydylcholine (PC) peroxidation induced by UV light in the liposomal bilayer. After one hour of exposure of PC liposomes to radiation a significant increase in the thiobarbituric acid reactive species (TBARS) production was observed. The addition of the compounds gave in a dose-dependent manner a reduction of TBARS production.

The BFF, quercetin and rutin (Fig. 8) were able to protect liposomes against peroxidative degradation caused by UV-irradiation. Since there is considerable evidence relating reactive oxygen species with UV phospholipid degradation, the protective effect of quercetin and rutin observed were resulting from their well-known scavenger property against ·OH, peroxyl radicals (ROO^{-}) and superoxide anions ($O_2^{\bullet -}$). Since the ·OH formed in this reaction is a very reactive species, it is a very short-lived radical that reacts with most organic compounds at nearly diffusion-controlled rates, thus, ·OH can react rapidly with the solvent, resulting in secondary free radicals that can be suppressed by flavonoids.

3. Conclusions

Araucaria angustifolia needles contain six major biflavones amentoflavone, mono-*O*-methylamentoflavone, di-*O*-methylamentoflavone, ginkgetin, tri-*O*-methylamentoflavone and tetra-*O*-methylamentoflavone (BFF). Since the per-methylation of BFF produced hexamethylated amentoflavone, determined by analysis of MS, ^1H, and ^{13}C NMR data, all components in the BFF were identified as derivatives of amentoflavone-type biflavonoids.

A biflavonoids fraction obtained from *A. angustifolia* needles showed a stronger $^1O_2$-quencher rate than individual amentoflavone, ginkgetin and quercetin. The BFF was the strongest protector, as indicated by inhibition of ssb formation in plasmid DNA through $^1O_2$ generated by $NDPO_2$, confirming the results observed in the quenching rate analysis. The low efficiency of quercetin as $^1O_2$ quencher has previously been demonstrated (Tournaire et al., 1993; Devasagayam et al., 1995). On the other hand, the BFF did not protect plasmid DNA against ssb formation by Fenton reaction as effectively as quercetin and rutin.

The BFF, quercetin and rutin were able to protect liposomes against peroxidative degradation caused by UV-irradiation.

The UV radiation is believed to be largely responsible for the greatest damage to skin by the effects of sunlight. In fact, the skin exposure to UV light gives rise to the formation of reactive oxygen intermediates, such as ·OH, $O_2^{-\bullet}$ and $^1O_2$ (Fucks and Packan, 1991; Mefferth et al., 1976), and the exposition to UV-C irradiation promotes progressive degradation of membrane fatty acids (Dumontet et al., 1992). Several natural antioxidants, such as genistein, an isoflavonoid from soybeans (Wei et al., 2002), and carotenoids (lycopene, β-carotene) (Stahl and Sies, 2002) are known to provide protection against UV irradiation damage in biomolecules. These data indicated that the fraction of biflavonoids in *A. angustifolia* is efficient to protect against oxidative damage to important biological molecules.

4. Experimental

4.1. General

NMR analyses were performed in a Bruker DRX 500 MHz using deuterated DMSO as solvent and TMS as internal standard.

The 1-*H*-phenalen-1-one was purchased from Acros (NJ, USA), whereas the hydrogen peroxide was supplied by Peróxidos do Brasil (Curitiba, Brazil). The biflavonoids, ginkgetin and amentoflavone from *Ginkgo biloba*, were kindly provided by Dr. Nicola Fuzzati from Indena Chemicals (Milano, Italy). Fresh needles of *A. angustifolia* (Bert) O. Kuntz were collected from a tree growing in the garden of Instituto de Química, Universidade de São Paulo, Brazil.

The HPLC/MS analyses of the BFF were performed using a Shimadzu 10A*vp* chromatograph with a diode array detector, with detection at 270 and 330 nm. A Synergi 250 mm × 4.6 mm, 5 μm, (Phenomenex, CA, USA) analytical column was used, with a mobile phase of (A) MeOH:(B) CH_2O + 1% formic acid) in a linear gradient mode. The gradient profile A:B (3:1 → 9:1) was from 0 to 20 min; then from 20 to 30 min (A:B, 9:1 → 100% A), held at 100% A for 5 min, then from 35 to 45 min for A to A:B (3:1), with the latter held for 5 min/40–45 min. The electrospray ionization (ESI) mass spectrometry measurement was achieved in the positive mode with in a Quattro II triple quadrupole MS/MS mass spectrometer (Micromass, Altricham; UK); the flow of 1 ml/min derived from HPLC was reduced using a splitter to deliver the mobile phase MeOH:H_2O + 1% of formic acid at a final flow rate of 20 μl/min. Sample of BFF 1 mg/ml in MeOH was injected through a 10 μl Rheodyne loop (Rheodyne, CA, USA). The source temperature was kept at 100 °C, and the flow-rates of the drying and nebulizing gas (nitrogen) were optimized to 250 and 30 l/h, respectively. The cone voltage was held at 70 V, and the capillary and HV-electrode potentials were kept at 3.00 and 0.69 kV, respectively. Full-scan data were acquired over a mass range of 100–800 Da. The data were processed and transformed into molecular masses on a mass scale, using version 3.2 of the MassLynx NT™ data system (Micromass, Altricham, UK).

The monomol light emission of the $^1O_2$ in the near infrared region was detected at right angles, using a sensitive photomultiplier (R 5509 PMT, Hamamatsu Photoniks KK, Japan) cooled to −80 °C through the refrigerator system using gas and liquid N_2 (S600 Photocool™, PC176TSCE 005 cooler, Products for Research Inc., MA) connected to a high tension source (High voltage DC power supply Model C3360, Hamamatsu Photoniks KK, Japan) and a monochromator for scanning IR spectra (M 300, Edinburgh Analytical Instruments, UK). The irradiance was measured with a radiometer (IL 1700, International light, MA, USA).

4.2. Extraction and isolation of biflavonoid fraction

Dried and powdered *A. angustifolia* needles (500 g) were exhaustively extracted with MeOH (4 × 500 ml) at room temperature. The resulting crude extract was suspended in MeOH:H_2O (1:4) and extracted with hexane (3 × 500 ml). Addition of more water (20% by volume) to the hydroalcoholic phase yielded an amorphous yellow precipitate, which was separated and dried, yielding a 0.5% by weight fraction of biflavonoids (BFF). Determination of the major compounds were achieved by HPLC/MS analysis. The biflavonoids, ginkgetin and amentoflavone, were identified by comparison with authentic standards.

4.3. Per-methylation of the BFF

An aliquot of the BFF (10 mg, 0.017 mM), suspended in CH_2Cl_2/acetone, was methylated overnight with iodomethane (0.025 mM) in the presence of K_2CO_3 under a nitrogen atmosphere, in the dark. The crude product was analyzed by HPLC/MS, using a Supelcosil LC 18, 250 mm × 4.6 mm, 5 μ (Supelco, PA, USA) column, where mobile phase was MeOH:H_2O + 1% formic acid, in gradient mode as described above; detection was carried out using a diode array detector (Shimadzu, Kyoto, Japan).

4.4. Hexa-O-methylamentoflavone (7)

UV (MeOH) λ_{max} 270 and 330 nm; MS *m/z* 623 (100), ^{1}H NMR 500 MHz (DMSO): δ 6.29 (*d*, 2.0 Hz, 1H, H-6), 6.43 (*d*, 2.0 Hz, 1H, H-8), 6.48 (*s*, 1H, H-6″), 6.56 (*s*, 1H, H-3″) 6.62 (*s*, 1H, H-3), 6.72 (*d*, 8.9 Hz, 2H, H-3‴/H-5‴), 7.08 (*d*, 8.7 Hz, 1H, H-5′), 7.33 (*d*, 8.9 Hz, 2H, H-2‴/H-6‴), 7.79 (*d*, 2Hz, 1H, H-2′), 7.89 (*dd*, 8.7 and 2.0 Hz, 1H, H-6′), 3.70, 3.71, 3.79, 3.85, 3.89, 4.02 (6 OMe); ^{13}NMR 125 MHz (DMSO) δ: 90.8 (C-8), 91.8 (C-6), 95.2 (C-6″), 105.7/106.9 (C-3/C-3″), 105.7/105.9 (C-10/C-10″), 106.9 (C-8″), 110.1 (C-5′), 113.3 (C-3‴/C-5‴) 121.3 (C-1‴), 122.7 (C-1′), 126.3 (C-3′), 126.6 (C-2‴/C-6‴), 129.5/129.6 (C-2′/C-6′), 155.2, 158.9, 159.1, 159.5, 159.7, 159.9, 160.0, 160.9, 161.0, 162.9 (C-2/C-5/C-7/C-9/C-4′/C-2″/C-5″/C-7″/C-9″/C-4″), 176.6/177.0 (C-4/C-4″).

4.5. Time-resolved infrared luminescence

Singlet oxygen was generated by light excitation of a sensitizer (l-*H*-phenalen-one, λ_{ex} = 355 nm) to determine the rate constants of $^1O_2$ quenching by quercetin, amentoflavone, ginkgetin and BFF, respectively. The 1-*H*-phenalen-1-one solution (0.2 OD) in acetone was excited by a Nd:YAG pumped dye laser (Nonell and Braslavsky, 2000). Several concentrations (0.1–1 mM) of quercetin, amentoflavone, ginkgetin and BFF dissolved in acetone were added to the sensitizer before excitation. The precise determination of lifetimes is an easy and accurate method in which to calculate quantum yields and quenching constants using Stern–Volmer plots (Eqs. (3)–(6)) (Wilkinson et al., 1993)

$$\tau_0/\tau = 1 + (k_q + k_r)k_d^{-1}\,[Q] \quad (3)$$

$$\tau_0/\tau = 1 + k_t\tau_0\,[Q] \quad (4)$$

$$1/\tau = 1/\tau_0 + k_t\,[Q] \quad (5)$$

$$k_{obs} = k_d + k_t\,[Q] \quad (6)$$

where τ is the life time of $^1O_2$ in the absence (τ_0) or presence (τ) of the quencher (Q) k_q is the physical and k_r the chemical quenching rate constant of $^1O_2$ by the

quencher (total quenching is $k_t = (k_q + k_r)$), and k_d is the $^1O_2$ decay constant. The value of the $^1O_2$ decay lifetime ($\tau = 1/k_d = 53.18 \pm 0.21$ μs) in acetone was in agreement with the value reported by Monroe (1985).

4.6. Preparation of plasmid pBluescript and PUC 19

The *Escherichia coli* HB 101 strain was transformed with a plasmid pBluescript and PUC 19, using the $CaCl_2$ procedure (Hanahan, 1993). Bacteria were plated on ampicillin agar plates and then incubated overnight at 37 °C. Transformed *E. coli* cells were grown in LB medium. The plasmid pBluescript and PUC 19 DNA were purified using the Clontech Maxi Prep Kit (Clontech, CA, USA). The preparation was constituted by 92% SC form and 8% of OC form.

4.7. Chemical generation of singlet oxygen

Singlet oxygen was generated by thermal dissociation of ionic disodium 1,4-naphthalenedipropanoate ($NDPO_2$), yielding the parent compound 3,3′-(1,4-naphthylene)-dipropionate (NDP), $^1O_2$ (yield ~50 %) and triplet molecular oxygen (Fig. 4, inset) (Di Mascio and Sies, 1989).

4.8. Treatment of pBluescript and PUC 19 plasmid DNA with singlet oxygen or Fenton reaction

The plasmid DNA (1 μg) was incubated in phosphate buffer (50 mM, pH 7.4) with 20 mM $NDPO_2$ at 37 °C for 100 min. The biflavonoids were analyzed using 1,5,10, 20, 50 and 100 μM (phosphate buffer, 50 mM, pH 7.4 and 25% v/v MeOH). For comparison, freshly prepared Trolox®, quercetin, rutin, amentoflavone (**1**), and ginkgetin (**4**) (Table 1) in the same solution in concentrations of (1–100 μM) were used. Damage in plasmid DNA was analyzed, using gel electrophoresis on 1% agarose, DNA bands were stained using ethidium bromide, then viewed under UV light and quantified with an Image Master VDS densitometer (Pharmacia Biotech, CA, USA).

Fe^{2+} salts were used to induce damage in plasmid DNA, resulting in a Fenton-type reaction with H_2O_2 derived reactive hydroxyl radicals. The pBluescript plasmid DNA (1 μg) was incubated with 50 μM of freshly prepared Fe^{2+} and 50 μM of H_2O_2 for 15 min at 37 °C. The sample was then immediately analyzed by electrophoresis, using 1% of agarose gel stained with ethidium bromide. The quercetin, rutin, Trolox®, amentoflavone, ginkgetin and BFF (1–100 μM) were added to the plasmid solution before Fe^{2+} and H_2O_2 and their capacity to prevent damage in plasmid DNA promoted by Fenton reaction was analyzed.

4.9. UV radiation-induced peroxidation in liposomal membranes

The protective effect of BFF, quercetin, rutin, α-tocopherol, amentoflavone and ginkgetin against UV-induced peroxidation was evaluated on phosphatidylcholine (PC) liposomes (Mandal and Chatterji, 1980) prepared with PC (2.5 mg) dissolved in EtOH. Each antioxidant compound (5, 10, 50, 100 and 200 μM dissolved in MeOH) were added to the PC and then dried under nitrogen. The mixtures were dissolved in (4 μl) EtOH and 25 mM Tris–HCl (5 μl, pH 7.4) and individually heated to 60 °C for 2 min. The liposome suspension was produced by adding 25 mM Tris–HCl, pH 7.4 to a final volume of 100 μl and followed by sonication for 1 min. This suspension was exposed for 1 h to UV-radiation from a germicidal lamp (254 nm) placed 10 cm away from the sample. The reaction was then stopped with 35% $HClO_4$ (10 μl) (Saija et al., 1995). To analyze the amount of TBARS, the samples were heated to 100 °C with TBA (200 μl) reagent (HC1 0.25 M, 0.375% of thiobarbituric acid (TBA) and 2 mM deferoxamine for 15 min. The TBARS were extracted with (400 μl) *n*-BuOH and analyzed using a fluorescence spectrophotometer (λ_{exc} 515 and λ_{emis} 550 nm) (Hitachi F-2000, Japan).

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