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Anthocyanin-DNA copigmentation complex: mutual protection against oxidative damage

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Abstract

The absorption spectra of a cyanidin derivative showed a 15–20 nm bathochromic shift when mixed with calf thymus DNA (ctDNA), indicating formation of a cyanidin-DNA copigmentation complex. Exposure of either cyanidin or ctDNA individually to hydroxyl radicals ($OH^{\cdot}$) obtained in the Fenton reaction between ferrous ions and hydrogen peroxide caused severe oxidative damage. However, formation of cyanidin-DNA complex prior to exposure to $OH^{\bullet}$ protected both the cyanidin and ctDNA from the oxidative damage. These results suggest that cyanidin-DNA copigmentation might be a possible defense mechanism against oxidative damage of DNA and may have in vivo physiological function attributable to the antioxidant ability of anthocyanins.

Keywords: Anthocyanin; DNA; Fenton's reaction; Antioxidant; Copigmentation

1. Introduction

In aerobic organisms atmospheric oxygen serves as a terminal oxidant for respiration and several other oxidative reactions. During the reduction of O_2, reactive oxygen species (ROS) such as O_2^- and H_2O_2 are frequently generated (Halliwell, 1994). In plant cells, which consume O_2 in respiration and also generate O_2 in photosynthesis, the production of ROS is a ubiquitous process. In addition, several environmental stresses such as UV-light (Shibata, Baba & Ochiai, 1991), drought (Moran, Becana, Ormaetxe, Frechilla, Klucas & Tejo, 1994), heavy metal exposure (Luna, Gonzalez & Trippi, 1994), chilling (Wise, 1995) and mineral deficiency (Godde & Hefer, 1994) also cause ROS generation. Nevertheless, the plant cells are armed with various enzymatic and non-enzymatic antioxidant systems, which significantly attenuate the damage caused by ROS (Larson, 1988).

Though H_2O_2 is an innocuous metabolite present in cells, irradiation with UV-light breaks it down to extremely deleterious hydroxyl free radicals ($OH^{\bullet}$) (Husain, Cillard & Cillard, 1987). In addition, in presence of transition metal ions and H_2O_2 a Fenton-type reaction generates $OH^{\bullet}$ radical: $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$. Since H_2O_2 can easily diffuse through cell membranes, and several cell compartments possess transition metals, $OH^{\bullet}$ formation could be extremely deleterious to cellular constituents. Among the cellular constituents, DNA is particularly sensitive to $OH^{\bullet}$ radical-induced damage, which generates both DNA strand breakage and base hydroxylations resulting in generation of genetic alterations such as mutations or rearrangements (Wiseman & Halliwell, 1996). Moreover, if $OH^{\bullet}$-induced DNA damage is not repaired or improperly repaired, it could lead to DNA strand breaks that activates poly(ADP-ribose)polymerase, a reaction which can lead to apoptosis (Heller et al., 1995).

One of the ways plants respond to stress such as

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UV-B light is by accumulating phenylpropanoid metabolites, particularly flavonoids including anthocyanins (Hahlbrock & Scheel, 1989). It is believed that increased level of anthocyanins defends the plants against biotic and abiotic stress. Several studies indicated that in vitro, flavonoids (including anthocyanins) could act as effective antioxidants (Rice-Evans, Miller & Paganga, 1997). Various mechanisms have been proposed to account for the antioxidant ability of anthocyanins, which include their radical scavenging ability (van Acker, Tromp, Haenen, van der Vijgh & Bast, 1995), metal chelating property (Sarma, Sreelakshmi & Sharma, 1997; Somaatmadja, Powers & Hamdy, 1964) and hydrogen donating ability (Rice-Evans et al., 1997). In addition, anthocyanins can also make complexes with other molecules (copigmentation), but physiological implication of such copigmentation is not known (Brouillard, 1983). However, such complexation can protect partner compounds against oxidative damages, for example anthocyanins prevent ascorbic acid (AsA) against metal induced oxidation by forming a stable AsA-metal-anthocyanin co-ordinate complex (Sarma et al., 1997). Above complex not only protects AsA from H_2O_2 and $OH^{\bullet}$, but also protects anthocyanins from oxidative damage (Grommeck & Markakis, 1964).

In this study, we show that anthocyanin and DNA associate to form a complex, and this complexation protects both DNA and anthocyanin from the damage caused by $OH^{\bullet}$ radical generated through Fenton reaction.

2. Results and discussion

2.1. Cyanidin-DNA copigmentation complex

Though flavonoids strongly absorb UV light, they do not absorb light in visible region of spectrum and are perceived as colorless compounds, barring anthocyanins, which possess strong absorbance in the visible region of spectrum. In their natural state, anthocyanins exist in equilibrium in several isomeric forms (Strack & Wray, 1989). In aqueous solutions at lower pH, they mainly exist as flavylium cations, and often a nucleophilic attack (ROS or water molecules) on the flavylium cation results in color loss (Brouillard, 1983). Therefore, it is expected that in vivo mechanisms exist that stabilize the color. Complexation or copigmentation is one such phenomenon, wherein, the anthocyanin associates with several colorless compounds (both organic as well as inorganic), resulting in the prevention of color loss. This process not only protects anthocyanins from damaging effects of nucleophiles like, ROS, but also saves the associated copigment from destruction by the same (Sarma et al., 1997).

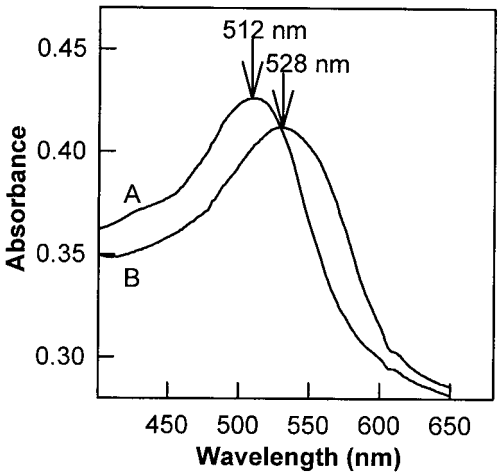


Fig. 1. Absorption spectra of cyanidin derivative (60 μM) from rice, in the absence (A) and presence of 80 μg of ctDNA (B), in 500 μl of distilled water. The absorption spectra were recorded immediately after mixing at room temperature.

We examined the possibility whether purified ctDNA molecule can make a complex and act as copigment to anthocyanin. Fig. 1 shows that adding DNA to cyanidin solution resulted in a 15–20 nm bathochromic shift in the λ_{max} of the cyanidin derivative, indicating that DNA can form a copigmentation complex with the cyanidin molecule. The formation of a cyanidin-DNA copigmentation complex is corroborated by reported interaction of DNA with quercetin and its derivative dihydroquercetin. The studies using flow-linear dichroism in aqueous solutions (Solimani, 1996) indicated that the hydrophobic segment of quercetin (benzopyran-4-one), allows the molecule to penetrate the DNA helix and to arrange its planar structure more or less parallel to the adjacent planes of the nitrogenous bases, whereas the non-planar and hydrophilic dihydroquercetin showed limited interactions. It is suggested that the planarity, hydrophobicity and hydrogen bonding are the possible responsible factors for flavonoid-DNA complex formation. Additionally, it is observed that intramolecular association of anthocyanins and several other flavonoids occurs by a stacking process that is related to the hydrophobic interactions and hydrogen bonding between the adjacent residues. It is presumed that such an interaction reduces the reactivity of the carbon-2 of the positively charged pyrylium ring with nucleophilic reactants resulting in the greater stability of the chromophore (Brouillard, 1983).

2.2. Effect of hydroxyl radical attack on cyanidin and cyanidin-DNA complex

The above possibility was examined by subjecting the cyanidin-DNA copigment complex and also cyani-

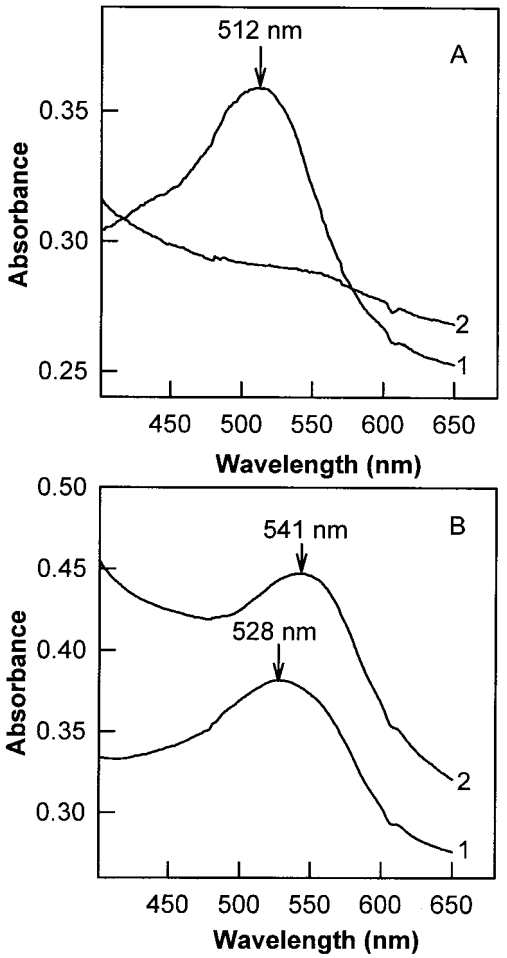


Fig. 2. Absorption spectra of cyanidin derivative (A) and cyanidin-DNA complex (B) in the absence (1) and presence (2) of Fenton reaction system (400 μM Fe^{2+} + 0.2 mM H_2O_2) in a final volume of 500 μl of distilled water. The absorption spectra were recorded immediately after mixing of contents at room temperature.

din to hydroxyl radical dependent oxidation in the presence of iron (II) and H_2O_2. Fig. 2 A shows that cyanidin is very susceptible to $OH^{\bullet}$ attack, the addition of $OH^{\bullet}$ instantaneously bleaches the chromophore. In contrast, the addition of $OH^{\bullet}$ to the cyanidin-DNA complex shows no such bleaching. On the contrary, it shows a further 10–15 nm bathochromic shift to longer wavelength (Fig. 2B) indicating a possible chelation of metal ion by the bound and intact cyanidin with the 3′-4′-dihydroxy group of its B ring. It is clearly evident from these results that once cyanidin complexes with DNA, it is no longer accessible to the nucleophilic attack by the $OH^{\bullet}$.

2.3. Effect of cyanidin-DNA copigmentation on hydroxyl radical dependent DNA damage

The investigations of $OH^{\bullet}$-mediated oxidation of free nucleotides have shown that $OH^{\bullet}$ radical attacks

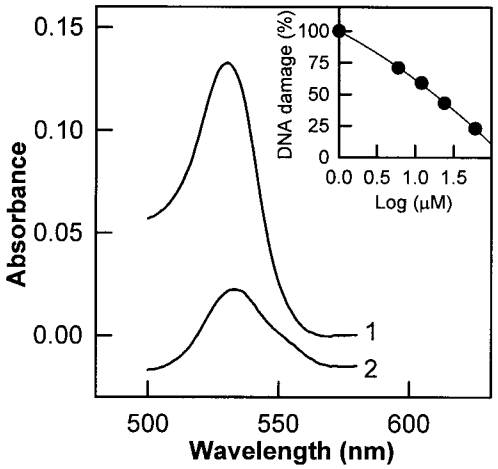
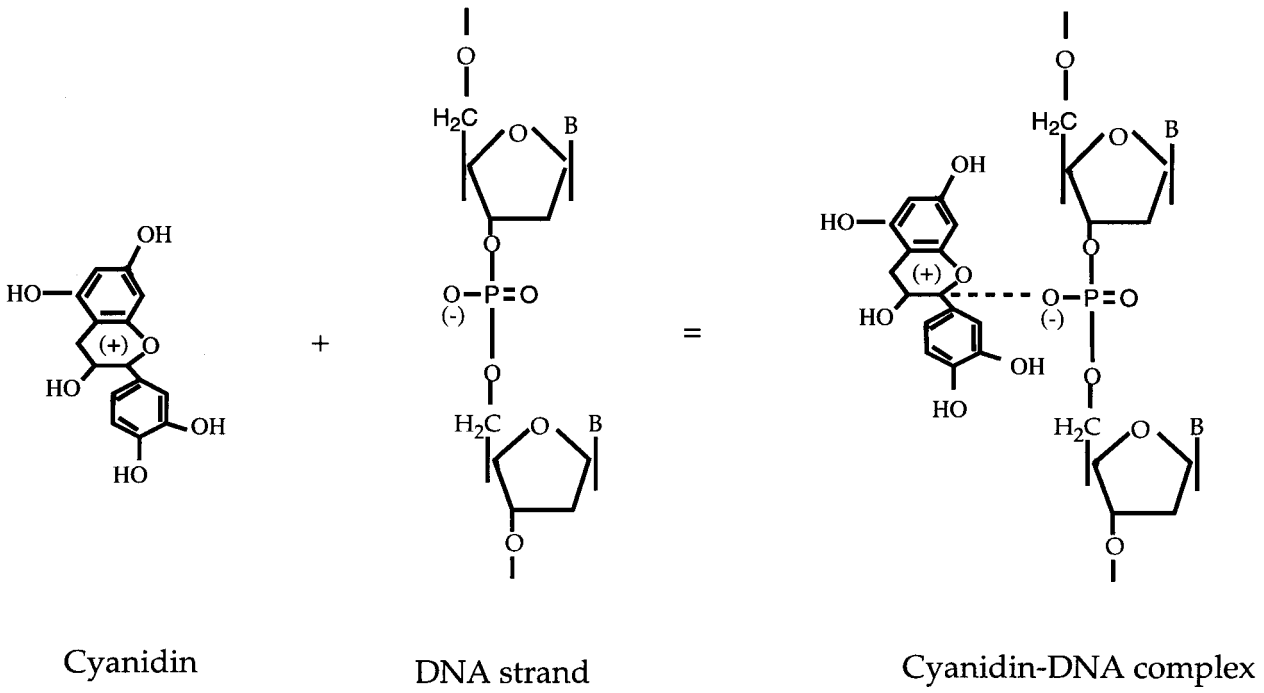


Fig. 3. Absorption spectrum demonstrating the formation of TBA reactive aldehyde formation from ctDNA exposed to Fenton reaction system in the absence (1) and presence (2) of cyanidin derivative (see material and methods for details). The *inset* shows the correlation between the decreased DNA damage (less TBA reactive compounds) with increased cyanidin concentration (Log μM). The data presented in the inset are mean of three independent experiments.

the 5,6 double bond of the nitrogenous base and also the sugar residue (Simic, Bergtold & Karam, 1989). Breakage of the sugar phosphate bond which ultimately leads to strand breakage arises from the oxygen entrapment and $OH^{\bullet}$-induced hydrogen abstraction from the ribose moiety. It is reported that often DNA binds Fe^{2+} in a manner such that $OH^{\bullet}$ formation is enhanced resulting in severe DNA damage (Floyd, 1981). The $OH^{\bullet}$-induced damage to DNA can be quantitatively assessed by monitoring increase in a thiobarbituric acid (TBA) dependent pink chromophore (λ_{max} at 528–530 nm), that results from the aldehydes produced from the damaged DNA (Halliwell & Gutteridge, 1981).

To test whether cyanidin complexed with DNA can afford protection to DNA from $OH^{\bullet}$ radical attack, the free DNA as well as the DNA complexed with cyanidin derivative was exposed to the Fenton reaction. Fig. 3 shows that the complexation of cyanidin to DNA significantly decreased the TBA-dependent chromophore formation. Evidently, the interaction between DNA and cyanidin effectively blocked the site susceptible to $OH^{\bullet}$ radical attack. This observation was further confirmed by obtaining a dose-response curve after mixing increasing concentrations of cyanidin to a fixed concentration of DNA. Fig. 3 (inset) shows that increasing concentration of cyanidin linearly decrease the damage to DNA.

It is believed that in H_2O_2-dependent cell killing, the major portion of H_2O_2 toxicity is a consequence of DNA damage caused by iron mediated Fenton reaction (Luo, Han, Chin & Linn, 1994). It was proposed that the iron bound to the phosphate backbone of



Scheme 1. Proposed mechanism for cyanidin-DNA interaction that leads to the formation of cyanidin-DNA copigmentation complex.

DNA is easily accessible to H_2O_2, thus setting off the Fenton reaction and is the major contributor to DNA damage. However, Fig. 3 shows that formation of complex of DNA, iron and cyanidin, prior to addition of H_2O_2, protects the DNA from damage. We propose that this protection of DNA from the Fenton reaction is mediated by binding of cyanidin to DNA either via the iron bound to the DNA or it directly interacts with phosphate backbone (Scheme 1). The latter possibility seems to be more likely, since complexation between cyanidin and DNA can take place even without the addition of any metal ion (Fig. 1). Additionally, this possibility is also consistent with the crystal and molecular study of a complex formed between a flavone (2,6-dimethoxyflavone) and orthophosphate (Wallet, Cody, Wojtezak & Blessing, 1993). This study indicates that such a complex involves strong hydrogen bonds that stabilize the flavone-nucleotide interaction. However, we cannot rule out the possible role of metal ion in the complex formation, since it was also suggested that metal ions stabilize the copigmentation of anthocyanin (Takeda, Kariuda & Itoi, 1985); moreover, DNA is often associated with metal ion (Wiseman & Halliwell, 1996). Nevertheless, it is reasonable to speculate that copigmentation between the cyanidin and DNA is likely protection mechanism, rather than radical scavenging or metal chelation offered by cyanidin derivative to DNA.

Several studies have shown that exposure of plants to high light irradiance, especially UV light damages DNA resulting in decrease in transcription rate (Koostra, 1994) and increased levels of cyclobutane dipyrimidine dimers (Stapleton & Walbot, 1994). The UV-B light-absorbing flavonoids are implicated as protective pigments in shoots and leaves exposed to UV-B light, and it is assumed that their specific location in epidermal layer protects internal cell layers by attenuating the impinging UV-B radiation at the epidermis (Braun & Tevini, 1993). The flavonoids mediated protection of plants from UV-B radiation is evident from studies showing that seedlings of *Arabidopsis* mutants deficient in flavonoids are hypersensitive to UV-B radiation (Li, Ou Lee, Raba, Amundsen & Last, 1993) and exhibit a lethal response, suggesting that the UV-B inducible flavonoids, play a protective role. Similarly maize cultivars with higher flavonoids (primarily anthocyanins) level show reduced UV-induced damage to DNA, compared to cultivars which are genetically deficient in flavonoid accumulation (Stapleton & Walbot, 1994).

Most of the above studies have suggested that the flavonoids protect plants and also DNA by acting as UV filters, since these compounds strongly absorb in the UV region of the spectrum, and are located in epidermal layers. The results obtained in this study indicate that, in addition to acting as sunscreen, the complexation of DNA and anthocyanin can protect DNA from oxidative damage. Since both cyanidin and DNA mutually protect each other it is likely that such

a protection mechanism may also operate in vivo. Since in the plant cell, anthocyanins are predominantly localized in the vacuole their putative role in protection of DNA or other cytosolic components has not been critically examined. Considering the fact that anthocyanins are synthesized in the cytosol and then transported to vacuole, it is likely that some amount of anthocyanin may exist in the cytosol (Marrs, Altonito, Lloyd & Walbot, 1995). In fact, a significant amount of flavonoids has been detected in chloroplast or etioplast isolated from a wide range of plants (Saunders & McClure, 1976). It is likely that some amount of anthocyanin may also be present in nuclei and organelles and may associate with DNA offering protection to DNA.

3. Experimental

3.1. Isolation of cyanidin derivative

The cyanidin derivative was isolated from seeds of a cyanic cultivar of rice (*Oryza sativa*, purple puttu). The isolation was performed as described elsewhere (Sarma et al., 1997). The final pH of the solution was adjusted to 4.0 using 0.1N HCl and the amount of cyanidin derivative was quantitatively estimated by measuring the $A_{535\,nm}$ ($\varepsilon = 31{,}623$).

3.2. Spectroscopic studies

The interaction between cyanidin derivative and the DNA molecule was monitored by, first recording absorption spectra of a 500 μl solution of cyanidin derivative (60 μM). Thereafter, to the above cyanidin solution, 80 μg of calf thymus DNA (ctDNA) was added from a 4 mg/ml stock solution. The cyanidin-DNA solution was mixed and after 1 min the absorption spectra was recorded the second time.

The oxidative damage to cyanidin in presence or absence of DNA was examined by recording absorption spectra of cyanidin derivative in presence of Fenton reaction system ($H_2O_2 + Fe^{2+}$). To a final volume of 500 μl distilled water, 60 μM of cyanidin derivative, 400 μM of $FeSO_4(NH_4)_2SO_4$, 0.2 mM H_2O_2 were added with 80 μg of ctDNA. The absorption spectra were recorded after a thorough mixing of the solutions. The control spectra were recorded after adding all of the above components without addition of ctDNA.

3.3. Measurement of TBA-reactive substances

The degradation of DNA by the Fenton reaction system generates TBA chromophore with a maximal absorption at 532 nm (Floyd, 1981). To a final volume of 500 μl reaction mixture, either varying (2–60 μM) or a fixed concentration (60 μM) of cyanidin derivative, 400 μM $FeSO_4(NH_4)_2SO_4$, 80 μg ctDNA and 0.2 mM H_2O_2 were added. Thereafter, the resultant mixture was incubated at 37° for 15 min. At the end of incubation period, 20 μl of 85% H_3PO_4 (v/v) and 1.0 ml of 1% (w/v) TBA were added and the mixture was incubated in boiling water bath for 20 min. To remove the interference due to cyanidin absorbance a parallel set of control samples for each concentration of cyanidin-DNA was run simultaneously in the absence of H_2O_2. After cooling the mixtures to room temperature, the absorbance was recorded at 532 nm for each sample against its corresponding control.

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References

Braun, J., & Tevini, M. (1993). *Photochem. Photobiol*, *57*, 318–323.
Brouillard, R. (1983). *Phytochemistry*, *22*, 1311–1323.
Floyd, R. A. (1981). *Biochem. Biophys. Res. Commun*, *99*, 1209–1215.
Godde, D., & Hefer, M. (1994). *Planta*, *193*, 290–299.
Grommeck, R., & Markakis, P. (1964). *J. Food Sci*, *29*, 53.
Hahlbrock, K., & Scheel, D. (1989). *Ann. Rev. Plant Physiol. Mol. Biol*, *40*, 347–369.
Halliwell, B., & Gutteridge, C. (1981). *FEBS Letts*, *128*, 347–352.
Halliwell, B. (1994). *Nut. Rev*, *52*, 253–265.
Heller, B., Wang, Z. Q., Wagner, E. F., Randons, J., Burkle, A., Fehsel, K., Burkart, V., & Kolb, H. (1995). *J. Biol. Chem*, *270*, 11176–11180.
Husain, S. R., Cillard, J., & Cillard, P. (1987). *Phytochemistry*, *26*, 2489–2491.
Koostra, A. (1994). *Plant Mol. Biol*, *26*, 771–774.
Larson, R. A. (1988). *Phytochemistry*, *29*, 969–978.
Li, J., Ou Lee, T. M., Raba, R., Amundsen, R. G., & Last, R. L. (1993). *Plant Cell*, *5*, 171–179.
Luna, C. M., Gonzalez, C. A., & Trippi, V. S. (1994). *Plant Cell Physiol*, *35*, 11–15.
Luo, Y., Han, Z., Chin, S. M., & Linn, S. (1994). *Proc. Nat. Acad. Sci. USA*, *91*, 12438–12442.
Marrs, K. A., Altonito, M. R., Lloyd, A. M., & Walbot, V. (1995). *Nature*, *375*, 397–400.
Moran, J. F., Becana, M., Ormaetxe, I. I., Frechilla, S., Klucas, R. V., & Tejo, P. A. (1994). *Planta*, *194*, 346–352.
Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1997). *Trends Plant Sci*, *2*, 152–159.
Sarma, D. A., Sreelakshmi, Y., & Sharma, R. (1997). *Phytochemistry*, *45*, 671–674.
Saunders, J. A., & McClure, J. W. (1976). *Phytochemistry*, *15*, 809–810.

Shibata, H., Baba, K., & Ochiai, H. (1991). *Plant Cell Physiol*, *61*, 62–67.

Simic, M. G., Bergtold, D. S., & Karam, L. R. (1989). *Mut. Res*, *214*, 3–12.

Solimani, R. (1996). *Int. J. Biol. Macromol*, *18*, 287–295.

Somaatmadja, D., Powers, J. J., & Hamdy, M. K. (1964). *J. Food Sci*, *29*, 655–660.

Stapleton, A. E., & Walbot, V. (1994). *Plant Physiol*, *105*, 881–889.

Strack, D., & Wray, V. (1989). In P. M. Dey, & J. B. Harborne, *Methods in plant biochemistry* (pp. 325–356). London: Academic Press.

Takeda, K., Kariuda, M., & Itoi, H. (1985). *Phytochemistry*, *24*, 2251–2254.

van Acker, S. A., Tromp, M. N., Haenen, G. R., van der Vijgh, W. J., & Bast, A. (1995). *Biochem. Biophys. Res. Commun*, *214*, 755–759.

Wallet, J. C., Cody, V., Wojtezak, A., & Blessing, R. H. (1993). *Antican. Drug Des*, *8*, 325–332.

Wise, R. R. (1995). *Photosyn. Res*, *45*, 79–97.

Wiseman, H., & Halliwell, B. (1996). *Biochemical Journal*, *313*, 17–29.