# Antioxidant Activity of Wheat Sprouts Extract *In Vitro*: Inhibition of DNA Oxidative Damage

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**ABSTRACT: Wheat sprouts contain a remarkable level of various antioxidants. A fraction containing high amounts of powerful antioxidant glycoside molecules has been isolated. In a dose-dependent manner, this fraction reduces the lucigenin-amplified chemiluminescence produced by the superoxide anion generated from the xanthine/xanthine oxidase system, thus indicating a superoxide-scavenging activity. A protective effect of this wheat sprouts fraction on the oxidative damage of pBR322 plasmid DNA induced by Fenton reaction ($Fe^{2+}/H_2O_2$) was subsequently demonstrated. Moreover, the results reported here show that the amount of antioxidant compound strongly increases during the germination phase, while scantly present in the wheat germ, and virtually absent in the young wheat plant.**

**Keywords: wheat sprouts, antioxidants, chemiluminescence, DNA damage**



## Introduction

REACTIVE OXYGEN SPECIES (ROS) ARE CONTINUOUSLY PRODUCED *in vivo*, promoting tissue damage and disease. An imbalance in the oxidant/antioxidant status of the cell is associated with oxidative stress, and this determines a cascade of events in the cell which lead to the loss of structural and functional integrity at the membrane level (Bast and others 1991; Gey 1994). The primary defense system against ROS is constituted by the following enzymes: superoxide dismutase (SOD, the enzyme that catalyzes the dismutation of superoxide radicals), catalase (converts $H_2O_2$ to $H_2O$ and $O_2$), and glutathione peroxidase (metabolizes $H_2O_2$ as well as lipid peroxides). The secondary defense system is provided by vitamin E, vitamin C, β-carotenes, urates, bilirubin, and others which all have scavenging properties against an overproduction of free radicals that may incidentally occur during a cell's life cycle (Muggli 1993).

In the last decade, a great deal of research has been devoted to the study of natural products with antioxidant activity. There is currently much interest in the antioxidant role of natural products. The beneficial effects of fruits, vegetables, and the Mediterranean diet are very likely due to many of their components such as dietary fiber, micronutrients, and antioxidants (Niwa and others 1998; Papas 1999). Our interest in this field has focused on wheat buds.

The main constituents of the wheat kernel are bran, germ, and endosperm. It is the starchy endosperm that yields high-quality white flour, and constitutes over 80% of the wheat kernel. Consumed for millennia as part of whole wheat (despite their lower commercial value), the remaining components are rich in antioxidant compounds such as phenolic acids, alkylresorcinols, aminophenols, and aminobenzoic acids. These exist in a diversity of free and bound forms (Dexter and Wood 1996), and are powerful antioxidants. They also possess potent pharmacological properties, which could make them desirable ingredients in the emerging market of "functional food" for health.

In order to identify and isolate the antioxidant substances produced in the wheat kernel during germination, the total aqueous extracts of the wheat germ, sprouts, and young plant were processed. The extracts were analyzed by thin-layer chromatography (TLC), using phosphomolybdic acid as the total antioxidant detector (Simonovska and Vovk 2000; Baricevic and others 2001). This test is a preliminary assay useful to screen reducing activity. In the present study, the antioxidant activity of a purified fraction of wheat sprouts extract was evaluated by using a chemiluminescence technique. This method is based on the principle that the superoxide anion generated by the xanthine/xanthine oxidase system reacts mainly with lucigenin, giving rise to chemiluminescence (Faulkner and Fridovich 1993). Therefore, the level of chemiluminescence indicates the presence of $O_2^-$ in the medium and any compound capable of reacting with superoxide will reduce the degree of chemiluminescence. The antioxidant capacity of the purified extract was also investigated by evaluating the protective effect on plasmid DNA damage induced by reactive oxygen species (ROS), generated by the Fenton reaction.

## Materials and Methods

ALL REAGENTS WERE OF PURE ANALYTICAL GRADE. 5-AMINO-2,3-DIhydro-1,4 phthalazinedione, xanthine, xanthine oxidase from bovine erythrocytes (E.C.1.1.3.22), phosphomolybdic acid, Sephadex G25/G10 resins, and plasmid pBR322 were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. *Triticum aestivum* wheat variety from Umbrian organic agriculture was utilized for the experiments.

### Wheat sprouts extraction

After germination of 3 to 5 d, the wheat sprouts were dried and ground into a powder. This wheat sprouts powder (20 g) was then homogenized in 400 mL water for 2 min and centrifuged at 10000 × *g* for 30 min at 4 °C. The supernatant was lyophilized, extracted twice with 50 mL ethanol, and centrifuged at 10000 × *g* for 30 min at 4 °C. The supernatant was then evaporated and resuspended in 15 mL water, followed by lyophilization of the extract.

### Gel filtration

The dry extract was resuspended in 2 mL water and chromatographed on a Sephadex G25 column (2 × 60 cm) which was equilibrated and eluted with 1% acetic acid. The main phosphomolybdic acid-positive fraction was further chromatographed on a Sephadex G25-G10 column (1.8 × 150 cm), the lower part filled with G10 (50 cm) and the upper part with G25 (100 cm). The column was equilibrated and eluted with 50 mM ammonium acetate contain-

ing 0.1% acetic acid (flow rate: 1 mL/min). The eluates were then exposed to the phosphomolybdic acid assay after TLC.

The fractions characterized by the highest content in antioxidants were further purified by cation exchange chromatography (carboxymethyl cellulose), or by affinity chromatography (concanavalin A-sepharose).

### Electrophoresis

High-voltage electrophoresis of the phosphomolybdic acid-positive fractions was performed on silica gel plates at 600 V for 1 h. The running buffer was 30 mM ammonium acetate-acetic acid, pH 6.5.

### Thin-layer chromatography (TLC)

Thin-layer chromatography was performed on silica gel plates using different solvent systems. After drying, the plates were sprayed with 10% phosphomolybdic acid solution in ethanol (w/v) and heated at 120 °C until spot formation was obtained. The phosphomolybdic acid in the presence of reducing substances is transformed into molybdenum blue, which is visible on the TLC sheet as a blue spot.

In some experiments, the plates were sprayed with orcinol-ferric chloride-sulfuric acid and *p*-anisaldehyde-sulfuric acid reagents to show the presence of mono- and oligosaccharides (Krebs and others 1969).

### Phosphomolybdic acid reaction in the liquid phase

To obtain a quantitative determination of the antioxidant species contained in wheat sprouts extracts, an analysis based on the reaction with phosphomolybdic acid in the liquid phase at different temperatures was carried out.

Wheat sprouts extract (300 μL) was added to 10 mL of 10% phosphomolybdic acid solution in ethanol (w/v). The solution was subsequently brought to 80 °C for 30 min and the optical density was measured at 600 nm.

An aliquot of the wheat sprouts antioxidant fraction of highest purity grade, obtained from concanavalin A-sepharose chromatography, was weighed and different amounts were subjected to reaction with phosphomolybdic acid. This was performed in order to obtain a calibration curve relating the amount of antioxidant substance to the absorbance at 600 nm.

### CL assay

Chemiluminescence measurements were performed using lucigenin as the chemiluminogenic probe, and the superoxide radical was produced by the xanthine/xanthine oxidase system (Gabbianelli and others 1994). The chemiluminescence (CL) was measured in an Autolumat LB53 (Berthold Co., Wilbard, Germany), in a reaction mixture of 1 mL, containing 0.9 U/mL xanthine oxidase, 150 μM lucigenin in physiological solution, and different amounts of the purified wheat sprouts extract fraction ranging from 5 to 50 μL.

The reaction was started by injecting xanthine at a final concentration of 50 μM and followed for 60 s. The values are expressed as counts per second (cps).

### Induction and analysis of DNA single-strand breaks

The experiments were carried out by incubation of plasmid pBR322 DNA (100 ng) in 100 mM sodium phosphate buffer, pH 7.4, at 35 °C for 1 h, containing $Fe^{2+}$ 10 μM and $H_2O_2$ 100 μM alone, or in the presence of wheat sprouts extract in the range 5 to 0.1 μL. The final volume of reaction mix was 12 μL.

Two μL of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol) were added to the reaction mixture, and an aliquot (10 μL) was loaded onto a 0.7% agarose gel and electrophoresed at 85 V for 1 h. The percentages of supercoiled (form I) and relaxed (form II) forms were calculated using an imaging densitometer, BIO-RAD Chemidoc (Hercules, Calif., U.S.A.). From these values, the average number of single-strand breaks (SSB) per pBR322 DNA molecule was calculated, according to Epe and Hegler (1994) and Epe and others (1996). It was taken into account that when stained with ethidium bromide, the relaxed form (form II) gives a fluorescence intensity 1.4-fold higher than the supercoiled form (form I), and that relaxation is caused by 1 SSB per DNA molecule. The following equation was used to calculate the numbers of SSB:

$$SSB = -\ln (1.4 \times \text{form I}) / (1.4 \times \text{form I} + \text{form II})$$

The results are expressed as the numbers of SSB per $10^4$ DNA bp (base pairs).

## Results and Discussion

THE SOLUBILIZED WHEAT GERM EXTRACT WAS CHROMATOGRAPHED on a Sephadex G25 column in 1% acetic acid and 5 fractions were obtained (I, II, III, IV, V). TLC analysis was conducted on the chromatographic products in propanol 70%-water 30%, and the total antioxidant content was detected by using phosphomolybdic acid. Fraction III (data not shown) was characterized as having the highest antioxidant content, thus this fraction was further chromatographed on a Sephadex G25-G10 column and the 4 eluates ($III^{c1}$, $III^{c2}$,$III^{c3}$, $III^{c4}$) were exposed to the phosphomolybdic acid assay after TLC. Fraction $III^{c3}$ turned out to be the only fraction with antioxidant activity. Therefore, it was then used for studying its superoxide-scavenging activity by chemiluminescence. Figure 1 shows the kinetics of the lucigenin-amplified chemiluminescence measured in the presence and in the absence of different amounts of wheat sprouts extract (from 5 to 50 μL). The data obtained show that the extract causes a significant reduction in chemiluminescence, thus indicating a probable superoxide-scavenging activity. Regarding the interaction of the wheat sprouts extract with the superoxide radical, we tested the ability of this purified fraction in protecting plasmid DNA from single-strand breaks induced by hydroxyl radicals formed via the Fenton reaction. Figure 2 shows the result of agarose gel electrophoresis of plasmid DNA exposed to ROS in the presence and in the absence of wheat sprouts extract. Two bands are detectable; the lighter one corresponds to relaxed DNA following oxidation-dependent single-strand breaks, while the darker band corresponds to native plasmid DNA in its native supercoiled form. Control sample (lane 1) shows DNA almost only in the supercoiled form, whereas in lane 2 and 3, a maximum conversion of form I (supercoiled form) to form II (relaxed form) can be observed following exposure to $Fe^{2+}$ and $Fe^{2+}/H_2O_2$, respectively. In lane 4 through 8, DNA was co-incubated with decreasing amounts of wheat sprouts extract. A potent protective effect toward DNA damage is evident after addition of 5 μL of wheat sprouts extract. The extent of protection is less in the presence of 2 μL and 1 μL of wheat extract and becomes irrelevant at lower amounts. A quantitative analysis of the gel is reported in Figure 3, which shows a dose-dependent inhibition of SSB by the wheat extract. Co-incubating DNA with 5 μL of the extract yielded 23% form II, corresponding to about 0.26 SSB per $10^4$ bp.

Thin-layer high-voltage electrophoresis in 30 mM ammonium acetate-acetic acid (pH 6.5) showed that the phosphomolybdic acid-positive main fraction had a positive charge. Biochemical characterization of this fraction revealed the presence of a glycoside structure. Accordingly, the fraction gave a positive reaction also with orcinol-ferric chloride-sulfuric acid and *p*-anisaldehyde-sulfuric

acid reagents (Krebs and others 1969). In addition, the aglycon structure shows a sharp absorption spectrum with a well-defined maximum at 264 nm and a shoulder at 310 nm. In agreement with these results, we tried to further purify the wheat germ antioxidant substance(s), using carboxymethyl cellulose or concanavalin A-sepharose. These 2 chromatographic steps produced significant progress in the fractionation of the antioxidant molecules, but recovery of the active compounds was very poor. Nonetheless, the antioxidant fraction obtained from concanavalin A-sepharose chromatography was used to obtain a relationship between weighed amounts of this fraction and the reaction with phosphomolybdic acid in the liquid phase (see "Materials and Methods"). This may provide a method to normalize the antioxidant glycoside in any extract.

The results of this analysis show that 1 g of the dried wheat sprouts powder contained about 3.5 mg of the isolated antioxidant glycoside substance, and the fraction utilized for the *in vitro* biological assays herein reported contained 1 μg of this compound(s) per μL.

In addition to the research work cited above, it was of interest to analyze whether a relationship exists between the synthesis of this substance and the germination process of the wheat seed, or the subsequent differentiation of the wheat plant.

For this purpose, 20 g of dried wheat germ, wheat sprouts, and the young wheat plant (15 to 20 cm) were extracted by utilizing the procedure reported in "Materials and Methods." The analysis of the obtained fractions with the phosphomolybdic acid reaction in the liquid phase shows that the wheat sprouts had the highest content of total antioxidant compound(s) (about 3.5 mg/g powder), compared to wheat germ (about 1.5 mg/g powder), and the young wheat plant (about 0.7 mg/g powder). Very similar results have been obtained by measuring the reducing power of wheat sprouts extracts, which utilizes potassium ferricyanide as reagent (Yen and Chen 1995). TLC analysis of these extracts showed that the antioxidant glycoside substance represented by the spot with Rf = 0.75 (line a), as indicated by chromatography of a purified fraction from wheat sprouts, is scantly present in the germ but strongly increases during the germination phase (3 to 5 d of germination) and subsequently, almost completely disappears in 10 to 15-cm-high plants (Figure 4). The wheat germ extract shows another antioxidant compound with a different Rf = 0.8 (line b). Quantitative data, evaluated by densitometric analysis of the TLC spots, are reported in Table I.

Here we discuss the superoxide scavenging ability of a chromatographic fraction ($III^{c3}$) obtained from solubilized wheat germ extract and its effect on the oxidative damage of pBR322 plasmid DNA.

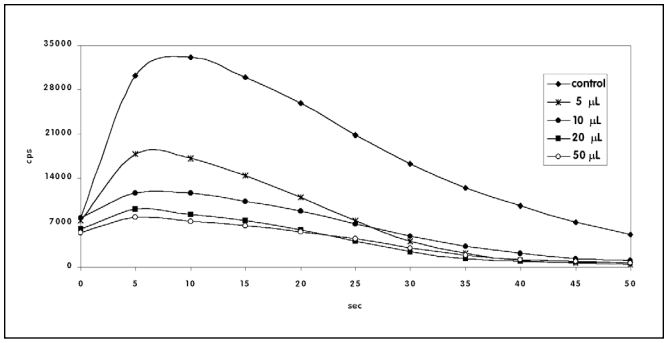


**Figure 1—Kinetics of the lucigenin-amplified CL measured in the presence and in the absence of wheat sprouts extract (WE). CL was measured in the presence of 0.9 U/mL xanthine oxidase, 150 μM lucigenin; the reaction was started by injecting xanthine at a final concentration of 50 μM in physiological solution. Chemiluminescence was measured as counts per seconds (cps). Control (◆), 50 μL WE (○), 20 μL WE (■), 10 μL WE (●), 5 μL WE (*)**

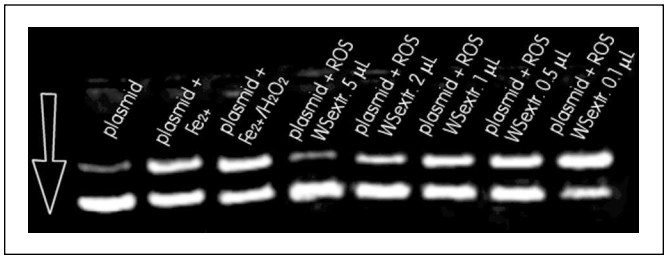


**Figure 2—Agarose gel electrophoresis of pBR322 after exposure to the Fenton reaction in the presence and in the absence of wheat sprouts extract. Controls: DNA alone (lane 1), plasmid DNA in the presence of $Fe^{2+}$ and $Fe^{2+}/H_2O_2$ (lanes 2, 3). After exposure to ROS: in the presence of 5 μL (lane 4), 2 μL (lane 5), 1 μL (lane 6), 0.5 μL (lane 7), 0.1 μL (lane 8) wheat sprouts extract**

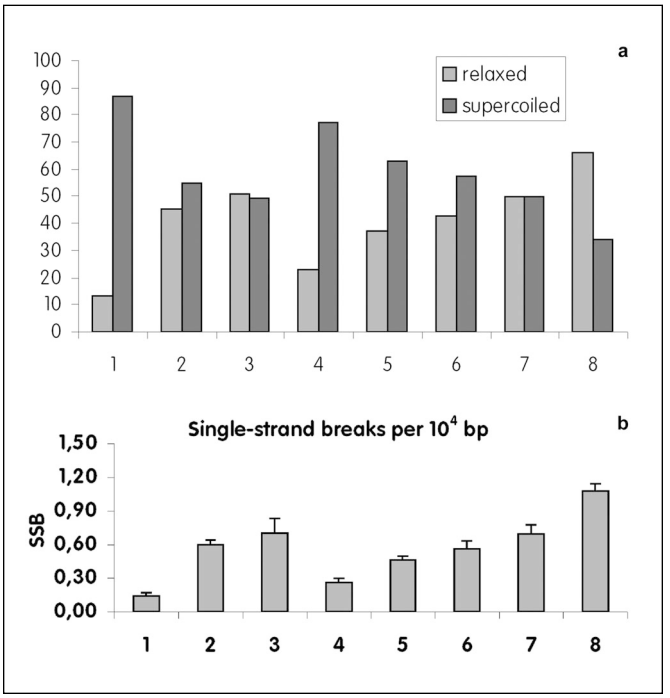


**Figure 3—Protective effect of wheat sprouts extract on strand breakage induced by the Fenton reaction. Plasmid pBR322 (100 ng) was incubated in 100 μM sodium phosphate buffer, pH 7.4, containing 10 μM $Fe^{2+}$ and 100 mM $H_2O_2$ alone, or in the presence of wheat sprouts extract in the range 5 to 0.1 μL. (a) Percentage of DNA in the native supercoiled form (form I) and relaxed form (form II); (b) SSB per $10^4$ bp induced under the conditions described above. The number of SSB was determined by the ratio of supercoiled form (form I) to the relaxed form (form II), according to Epe and others (1994, 1996). DNA alone (1); DNA in the presence of $Fe^{++}$ and $Fe^{++}/H_2O_2$ (2, 3). After exposure to ROS: in the presence of 5 μL (4), 2 μL (5), 1 μL (6), 0.5 μL (7), 0.1 μL (8) wheat sprouts extract. Data represent the mean value of triplicate experiments (± SEM).**

Chemiluminogenic probes that amplify the signal without interfering with the reaction system are now increasingly used. Different information can be obtained, depending on the type of probe utilized (Murphy and Sies 1990). Lucigenin is a probe sensitive to the level of superoxide anion radical ($O_2^-$) and permits the evaluation of superoxide-scavenging reactions (Cotelle and others 1992). The inhibition of superoxide-induced chemiluminescence by the aforementioned extracted fraction ($III^{c3}$) can be clearly observed in Figure 1. Among the different lesions that free radicals induce in DNA, single- and double-strand breaks are also included (Lloyd and Phillips 1999). In the present study, we assayed for strand breaks using the plasmid-nicking assay. As Figure 2 shows, our fraction protects against hydroxyl radical attack on plasmid DNA, and this protection is dependent upon the amount of wheat sprouts extract.

## Conclusions

IN CONCLUSION, THE DATA REPORTED IN THIS PAPER SEEM TO INDICATE that during wheat seed germination, there is synthesis of high amounts of powerful low-molecular-weight antioxidant molecules. These results may be related to the biochemical and functional properties of vegetable products reported by several authors (Graf 1983; Minamiyama and others 1996), which may be referenced for their antioxidant activities.

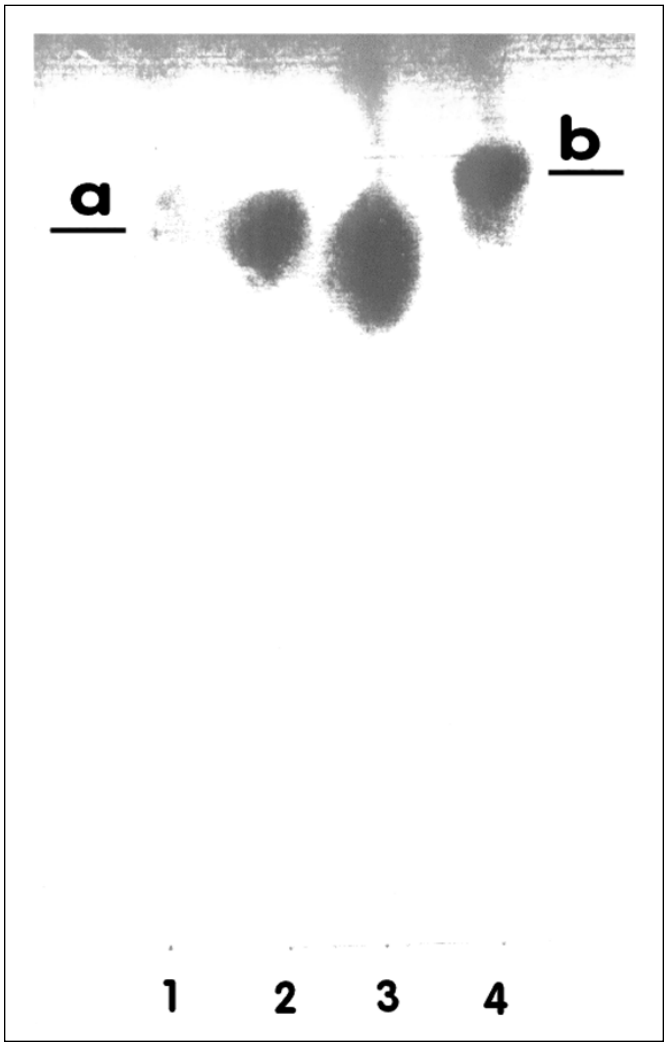


**Figure 4—Ascendant TLC chromatography on silica gel plates (10 × 20 cm) of the fractions extracted from young wheat plant (1), sprouts (3), and germ (4). (2): Co-chromatography of the glycosidic antioxidant fraction extracted from wheat sprouts and purified by gel filtration and affinity chromatography. Solvent system: propanol-water 70/30 (v/v). The spots were stained with phosphomolybdic acid.**

**Table 1—Densitometric analysis of the TLC spots shown in Figure 4. The densitometric analysis was performed by utilizing Image-Master® Software (Pharmacia). OD peak = maximum of the optical density measured in the spot; OD = average of the optical density measured in the spot; trace density = band quantification in OD × mm²**

| Sample | Rf | OD peak | OD | Trace density |
|---|---|---|---|---|
| 1 | a | 0.17 | 0.08 | 0.443 |
| 2 | a | 1.59 | 0.80 | 7.003 |
| 3 | a | 1.96 | 1.16 | 14.542 |
| 4 | a | 0.91 | 0.39 | 1.649 |
| | b | 2.00 | 1.41 | 7.786 |

It has been reported that aqueous extracts from wheat sprouts inhibited the mutagenic effect induced by benzo[a]pyrene in strain TA98 of *Salmonella typhimurium* (Peryt and others 1988, 1992). Moreover, through induction by benzo[a]pyrene of sperm abnormalities in mice, these abnormalities were found to diminish after oral administration of wheat sprout extract (Tude and others 1988). Likewise, Brussels sprouts contain bioactive substance(s) with a potential for reducing the physiological, as well as oxidative stress-induced oxidative DNA damage in rats and humans (Verhagen and others 1995; Deng and others 1998).

The results reported in this paper show that the antioxidant molecules isolated from wheat sprouts inhibits DNA oxidative damage *in vitro*. Preliminary biochemical characterization suggests that these molecules probably belong to the family of antioxidant glycosides. Further research is in progress to demonstrate whether the fraction isolated from wheat sprouts can be utilized to protect cells from damage caused by oxidative stress *in vivo*.

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