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Red Corn *(Zea mays L.)* Special Ultrasonic Extract Zea-Phen® Profile and Its Protective Activity against Oxidative Stress in Isolated Mouse Organs

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Abstract:

It could not be denied that both red corn anthocyanins and other phenolic compounds were shown to be great antioxidants, and some variation of their antioxidant power should be expected between different studies. The DPPH assay was used to better understand why red corn showed relatively high antioxidant activity compared to other fruits and vegetables. Red corn phenolics exhibited higher antioxidant capability and faster reaction kinetics than the same amount of blueberry phenolics, suggesting red corn phenolics to have a larger number of active hydroxyl groups and more favorable configurations to allow for better interactions with the free radicals. It has been observed in previous reports that antioxidant assays such as FRAP, DPPH, ABTS, and oxygen radical absorbance capacity exhibit a high correlation with polyphenols content. Gardner et al also reported a high correlation between total polyphenols and antioxidant activity measured by FRAP. The study observed that there is a high correlation between the antioxidant activity assays (DPPH, ABTS and FRAP) and the content of total polyphenols such as flavonoids, phenolic acids, and anthocyanins, which indicates that these classes of bioactive compounds contribute the most to the antioxidant activity of Zea-phen®. The effect of red corn extract named Zea-phen® on cellular antioxidant response in mouse organs was also investigated by treatment with added H_2O_2 to isolated mouse kidney, heart, skin and brain ex vivo. The presence of malondialdehyde (MDA) in these organs served as an indicator of cell membrane oxidative injury after applying H_2O_2. When the organs showed low MDA levels and were treated with red corn extracts, the levels of antioxidant enzymes superoxide dismutase (SOD), catalase, and total peroxidase in the organs were increased. These enzymes are believed to have the capability to eliminate reactive oxygen species and prevent oxidative damage to cells. These results suggested that certain functional compounds in red corn extract Zea-phen® can penetrate cell membranes and participate in stimulating antioxidant enzyme secretions to reduce oxidative damage to cells caused by free radicals.

Introduction

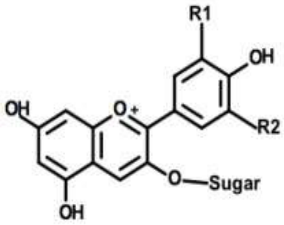
There is a large body of evidence to suggest that the inclusion of whole grains in the diet is beneficial for human health. Whole grain cereals, i.e., grains that include the bran, germ, and endosperm, are good sources of vitamins, minerals, dietary fiber, and phytochemicals. These compounds are mostly present in the outer kernel layers, i.e., the pericarp and aleurone, which are typically removed when the grain is milled and cereal flour is produced [1]. The intake of high levels of dietary fiber, vitamins and minerals, and important phytochemicals like indoles, polyphenols, carotenoids, and

phytosterols is considered to be responsible for the potential health-promoting effect of regular cereal whole grain consumption. The relationship between the regular consumption of whole meal products and a reduced risk of non-communicable chronic diseases has been previously established. The specific effects of food structure and dietary fiber content, combined with the antioxidant and anti-carcinogenic effects of numerous compounds (especially polyphenolic compounds), are well recognized actors in the risk reduction of cardiovascular diseases, obesity, diabetes, and certain types of cancer [2]. Most research has focused on the health effects of the major constituents of whole grains (e.g., dietary fiber). However, bioactive small molecules also play a role in the benefits of whole grain consumption. Polyphenols, for example, are compounds that possess one or more aromatic rings, with one or more hydroxyl groups, and can be categorized into one of five groups (flavonoids, stilbenes, phenolic acids, coumarins, and tannins). Phenolics act as singlet oxygen quenchers and free radical hydrogen donors, which have a protective effect on cell constituents against oxidative damage. Among them, anthocyanins are a group of water-soluble flavonoids and are responsible for red, violet, and blue colors in fruits, vegetables, and cereal grains. Anthocyanins have demonstrated in vitro antioxidant potential, and consumption of foods high in anthocyanins has been linked to a lower risk of chronic diseases. A very similar activity is also shown for the phenolic acids [1,3].

Chemistry of Anthocyanins and Phenolic Acids in Summary

Anthocyanins are water-soluble phenolic pigments responsible for red, purple, blue, or even black colors in fruits, vegetables, grains, flowers, and other pigmented plant tissues. All anthocyanins share the same core structure, a flavylium ion, consisting of two aromatic ring structures linked by a three-carbon heterocyclic ring that contains oxygen. The anthocyanidin (aglycone form) is the core structure of the anthocyanin. The addition of a sugar side chain results in the glycosidic form of the anthocyanidin molecule, called an anthocyanin. Anthocyanidins, as well as their glycosylated and acyl glycosylated forms, can be found in nature [4].

Over 23 anthocyanidins and 500 different anthocyanins have been isolated from plants. Anthocyanidins differ in their hydroxylation and methoxylation degree and pattern. The large diversity in anthocyanins does not only stem from the variability in the anthocyanidin core structure, they also differ in the nature and number of sugars attached to the core structure, as well as the nature and number of side chains attached to these sugar residues. Sugars are most commonly linked to the aglycone core at position 3. When multiple sugar groups are present, these additional sugar moieties are often linked to positions 5 and/or 7 of the aglycone core structure. The acylglycosidic form occurs when sugar groups are acylated with aliphatic, hydroxybenzoic, or hydroxycinnamic acids. The most commonly found phenolic acids are malonic, acetic, and caffeic acids [5].

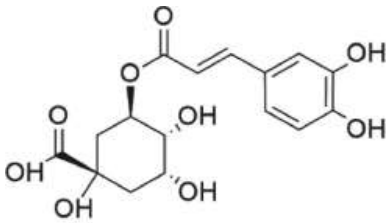


Pelargonidin-3-glucoside: R1-H R2-H sugar- glucose
Cyanidin-3-glucoside: R1-OH R2-H sugar- glucose
Delphinidin-3-glucoside: R1-OH R2-OH sugar- glucose
Peonidin-3-glucoside: R1-OCH_3 R2-H sugar- glucose
Petunidin-3-glucoside: R1-OCH_3 R2-OH sugar- glucose
Malvidin-3-rutinoside: R1-OCH_3 R2-OCH_3 sugar-rutinose
Delphinidin 3-(6-p-coumaroyl) glucoside: R1-OH R2-OH sugar-glucose + coumaric acid

The structures of six common anthocyanins and one acylated anthocyanin, with the individual ring substituents listed below the flavylium ion backbone.

Despite the wide structural variations present in anthocyanin structures, six main anthocyanidin compounds are commonly found in food products. About 50% of anthocyanins found in fruits and cereals are cyanidin derivatives, followed by pelargonidin (12%), delphinidin (12%), peonidin (12%), petunidin (7%), and malvidin (7%) derivatives. The conjugated bonds of the anthocyanidin moiety are responsible for the molecule's color. Anthocyanidin compounds vary in the degree of ring hydroxylation and methoxylation. The level of hydroxylation/methoxylation generally influences the hue of the compound as well as the molecule's stability, with increased hydroxylation increasing the anthocyanin's blueness and reducing its stability. Increased methoxylation, on the other hand, increases the anthocyanin's redness and molecular stability [4].

Chlorogenic Acid (CGA), otherwise known as 3-caffeoylquinate (3-CQA) or chlorogenate, is a biologically active polyphenolic compound that represents an entire ester-hydroxycinnamic and quinic acid group involving dicaffeoyl, caffeoyl, coumaroylquinic and feruloyl acids respectively [6].



Chlorogenic acid
Chemical formula: $C_{16}H_{18}O_9$
Molecular weight: 354.30

With regards to its health boosting attributes, CGA and the other phenolics acids are also significantly applied clinically, particularly against fibrosis and cancer and serves as the main constituent in Traditional Herbal Medicine (THM) formulations for detoxification, and heat clearance. Furthermore, the excretion, utilization and bioavailability of these acids is still yet unclear. In humans, around one-third of the phenolic acids ingested are absorbed via the small intestine, whereas absorbed in the stomach of mice through prototype. Following absorption these phenolic acids are further metabolized into metabolites of sulfate, glycosides and glucuronic acid [7-9].

A phenol is any compound that contains a hydroxyl (–OH) group attached to a benzene ring. Plants contain a huge range of phenols, including the tocopherols and tocotrienols. Most phenols found in red corn exert antioxidant effects in vitro, inhibiting lipid peroxidation by acting as chain-breaking peroxyl radical scavengers [10,11]. In addition, phenols often scavenge other reactive species such as OH , NO_2 , N_2O_3, ONOOH, and HOCl [11]. Recently, it has been proposed that the other mechanism by which phenolic phytochemicals function in counteracting the oxidative stress could be by stimulating the synthesis and/or replenishment of cellular antioxidant status or by inducing and improving the host cellular antioxidant enzyme response through the superoxide dismutase (SOD) and catalase (CAT) systems [12].

The aims of this study were to determine (1) the content of total polyphenols, flavonoids, phenolic acids, stilbenes and anthocyanins and the spectral analysis, (2) the antioxidant activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), (3) the individual polyphenols by high-performance liquid chromatography (HPLC) and (4) to evaluate changes in cellular antioxidant response in isolated mice organs using markers of oxidative stress such as SOD, CAT, and total peroxidase (TPX).

Materials And Methods

Sample

The red corn MRC20 (Zea mays L.) was cultivated near the town of Cremona, Italy. After collecting the grains were immediately grinded and then extracted with an experimental ultrasound plant (Ecotecne, Italy). We obtained three extraction samples named sample 1 (only water as solvent with 1% citric acid), sample 2 (only water as solvent) and sample 3 (water 80% and ethanol 20% as solvents), following these extraction parameters:

1) Ultrasonic power 4.000 Watt (for all the samples)

2) Frequency 25-27 KHz (for all the samples)

3) Optimal power 80% of the maximum one (for all the samples).

4) Extraction temperature 34°C (for all the samples).

5) Ratio solvent / vegetal material 10:1 for sample 1 and sample 3, and ratio solvent / vegetal material 20:1 for sample 2.

7) Duration of the extraction 60 minutes for all the samples.

After our ultrasound extraction method of this vegetal material we obtained a dry extract with 3.704,92 mg/kg total polyphenols, of whose 1.857,58 mg/kg phenolic acids, 45,44 mg/kg anthocyanins and 297,44 mg/kg stilbenes measured by HPLC. These values are referred to the sample 1.

Chemicals

The specific chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA): ABTS, 2,2′-azobis(2-amidinopropane) HCl (ABAP), DPPH, Folin–Ciocalteu reagent, p-dimethylaminocinnamaldehyde, cyanidin (for the anthocyanins), ferulic acid (for the organic acids), quercetin-3-O-rutinoside (for flavonols), (+)-catechin (for flavan-3-ols), oleuropein (for tyrosol derivatives) and resveratrol (for stilbenes), HPLC-grade acetonitrile, methanol, and formic acid were purchased from Merck KGaA (Darmstadt, Germany).

Treatment of Samples for Phenolic Profiling Analyzes by Method UHPLC-HRMS.

To process sample, 10 mL (in triplicate) was placed

in a falcon 15 mL, centrifuged at maximum speed and filtered through 0.2 syringe filters micron (RC) in UHPLC vials. For the treatment of samples, 1 gram of sample (in triplicate) was transferred to a 15 mL falcon and extracted into 10 mL of an extracting solution consisting of 80% methanol (v/v) acidified with 0.1% formic acid (v/v) by using an Ultraturrax homogenizer for 1 min at maximum power. THE samples were then centrifuged at maximum speed and incubated overnight at -18°C to promote the precipitation of any residual protein fractions. The next morning, the supernatants were filtered through 0.2 micron syringe filters (RC) in UHPLC vials.

Phenolic profiling and semi-quantification analyzes of the different classes phenolic studies were based on the use of a Vanquish UHPLC chromatographic system coupled to a mass spectrometer (nominal resolution at 200 m/z of 70,000 FWHM) Q-Exactive Focus (Thermo Scientific, Waltham, MA, USA). Major details regarding the instrumental conditions used are available in magazines international scientists in the sector [13]. In general, for analysis of the phenolic profile, the mass range 80-1200 m/z was scanned.

Polyphenols were then identified using MS-DIAL software (version 4.90) using the "Food Database" database, coupled with the database exclusive for polyphenols called "Phenol-Explorer". The raw data obtained is were then processed for semi-quantitative analyses. In particular, the abundances relatives of each compound belonging to the same phenolic class were added to calculate a total value which was then converted into a semi-quantitative value taking advantage of the calibration lines available for analytical reference standards. In particular, the following standards were used: cyanidin (for anthocyanins), catechin (for flavan-3-ols), luteolin (for other flavonoids), quercetin (for flavonols), ferulic acid (for phenolic acids), oleuropein (for tyrosol derivatives), and resveratrol (for stilbenes). Semi-quantitative results were then expressed as triplicate analytical (n = 3) in mg Equivalents/g (for grains). The analysis of the phenolic profile allowed the following to be identified in terms of numbers: 71 anthocyanins, 51 flavonols, 19 flavan-3-ols, 73 other flavonoids, 14 stilbenes, 69 phenolic acids, and 6 tyrosolic derivatives. At a qualitative level they were also identified lignans, alkylresorcinols and other low molecular weight polyphenols (not quantifiable due to the absence of standards). This extract was named Zea-phen®.

Measurement of Antioxidant Activity

DPPH assay: The method used for the DPPH radical scavenging was adapted from that of de Campos et al. [14]. The reaction occurred between 50 µL of sample and 950 µL of 100 µM DPPH. The sample concentrations were in the range of 5–500 µg/mL. The absorbance values were recorded every 30 seconds for 10 minutes. The antioxidant activity was expressed as the 50% inhibitory concentration (IC_{50}) value,[7,8] and the values were adjusted to the following models: reciprocal-Y model of Y=1/(a+[b×X]) and the logarithmic-X model of Y=a+[b×ln(X)].

ABTS assay: The total radical trapping antioxidant potential assay was developed using the ABAP/ABTS reagent [15]. The sample concentrations ranged between 125 and 1000 µg/mL. A sample aliquot of 10 µL was allowed to react with 990 µL of ABTS/ABAP reagent. The ABTS/ABAP reagent consisted of a mixture of 2.25 mM ABTS, 20 mM ABAP, and phosphate-buffered saline buffer (50 mM K_2HPO_4 and 0.9% NaCl, pH 7.4). The reaction was incubated at 70°C for 20 minutes. The absorbance was recorded every 20 seconds for 10 minutes at 734 nm. The antioxidant activity was expressed as the IC_{50} value. The absorbances were adjusted for the following models: multiplicative model of ln(Y)=a – [b×ln(X)] and reciprocal-X model of Y=a+(b/X).

FRAP assay: The total antioxidant potential was determined using the method developed by Thaipong et al [16] and Benzie and Strain [17] with some modifications. The sample concentrations ranged between 250 to 2500 µg/mL. A 50-µL sample aliquot and 50 µL of $FeCl_3$ (3 mM in 5 mM citric acid) were incubated at 37°C for 30 minutes, then 900 µL of 2,4,6-tripyridyl-s-triazine (1 mM in 0.05 M HCl) was added, the mixture was stirred strongly, and after 10 minutes of reaction the absorbance was read at 620 nm. The results were expressed in micromolar Trolox equivalents (TE)/g.

Study description: The study was designed to have two parts. The first one was conducted on human cheratinocites (HaCaTs cells) cultivated in vitro to observe the effects of Zea-phen® on these cells. The second was performed on albino rats, to study the effects of Zea-phen® on various internal organs.

Cellular antioxidant response on Human Adult Keratinocytes (HaCaTs)

Cell viability assay: The MTT assay was used to detect cell viability. HaCaTs in the logarithmic phase were counted and seeded in 96-well plate. After UVB (125 mJ/cm^2) radiation and EB (1, 10, and 100 µg/mL) treatment for 24 h, 0.1 mg/mL of MTT was added and incubated for 4 h. DMSO was used to dissolve the formazan crystals. The absorbance was read at 570 nm using a micro plate reader (Molecular Devices E09090; San Francisco, CA, USA). The experiment was repeated three times, independently.

Measurement of ROS generation: ROS production by UVB-induced HaCaT cells exposed to EB was evaluated by the 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich) assay, as previously described [18]. Following UVB (125 mJ/cm^2) irradiation and EB (1, 10, and 100 µg/

mL) treatment for 24 h, HaCaTs were stained with 30 µM DCFH-DA for 30 min at 37 °C in the dark and analyzed by a multi-mode microplate reader (Molecular Devices Filter Max F5; Sunnyvale, CA, USA) at excitation wavelength of 485 nm and detection wavelength of 535 nm. The experiment was repeated three times, independently.

Cellular Antioxidant Response in Isolated Mouse Organs

Experimental animals: Thirty albino male rats 3 months old and weighing 26–43 g, bred in the rodent vivarium of the Faculty of Veterinary Medicine, University of Turin, Turin, Italy, were included. They were then transferred to the vivarium of the Faculty of Pharmacy, University of Turin, Italy, where they were maintained in accordance with international regulations for environment moisture, food, circadian cycles, and temperature. Animals were fed with a standard rodent balanced diet in food pellets and had free access to food and water for all the duration of the study. The animals did not receive any treatment for 48 hours besides ad libitum access to food and water, because the objective was to extract different organs for measurement of in vitro cellular antioxidant response in isolated mouse organs. For this, the experimental animals were euthanized by neck truncation and the organs (kidney, skin, liver, and brain) were immediately isolated and placed in phosphate-buffered saline.

Experimental design: A part of the animals did not receive Zea-phen® and formed the placebo group. The other part of the rats received the Zea-phen® mixed with their usual diet at the daily dose of 50 mg/kg (25 mg/kg in the morning and 25 mg/kg in the evening) for 28 days. Zea-phen® was mixed in the food pellets, which have been pulverized for an optimal insertion of the Zea-phen® itself. The extract was kept in a refrigerator at 4 °C prior to treatment. The rats were monitored for 5 min following dosing to ensure that they did not regurgitate the content or were not injured.

Preparation of homogenates: The organs were homogenized using a Potter–Elvehjem glass–Teflon® (Dupont, Wilmington, DE, USA) homogenizer (Omni International, Kennesaw, GA, USA). The homogenate was clarified by centrifugation at 4,500 g for 8 minutes, and the supernatant and pellet were separated and stored at −18°C. The soluble supernatant was collected and used for the treatment. The tissue homogenates were treated using H_2O_2 (100 µM), Zea-phen® (100 mg/mL), and Zea-phen®+H_2O_2. For this, 490 µL of tissue homogenate was mixed with 210 µL of either H_2O, Zea-phen®, or Zea-phen®+H_2O_2 depending on the treatment. All samples were incubated at 37°C for 2 hours and then maintained at 0°C for a period of about 30 minutes until analysis of the different endogenous enzymes. For the different endogenous enzyme activity assays, PTX was measured by the method described by Laloue et al. [19] SOD by that of Marklund and Marklund [20] CAT by that of Beers and Sizer [21] and lipid peroxidation (thiobarbituric acid–reactive substances [TBARS] production) by that of Tamagnone et al. [22].

Statistical Analysis

Data were analyzed using Microsoft Excel data analysis tool package (Microsoft, Washington, WA, USA) and IBM SPSS Statistics Data Editor Version 24 (IBM, New York, NY, USA) All data were expressed as means ± standard error of the means (S.E.M). A p-value of less than 0.05 was considered significant. The statistical analysis was evaluated using one-way analysis of variance (ANOVA), performed with the Post-hoc Tukey test.

Results and Discussion

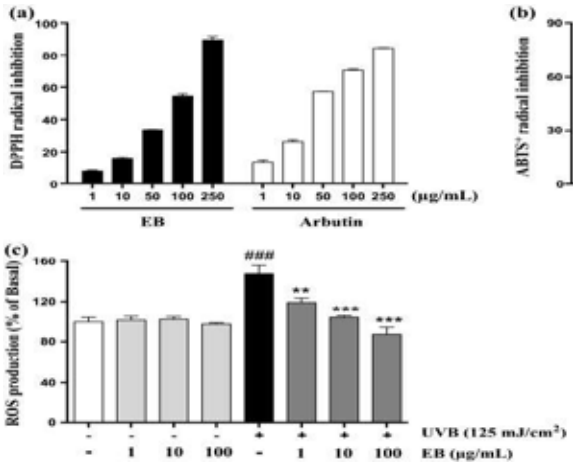
Results on the Human Keratinocytes

This study determined the skin protective effect of Zea mays L. (red corn extract) Zea-phen® in experimental skin injured by UVB radiations. HaCaTs cells, originating from human keratinocytes, were purchased from the Italian cell line bank (Italy). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin–streptomycin (Gibco BRL, Grand Island, NY, USA). Cell cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C and were incubated with various concentrations of Zea-phen® ranging between 1, 10, and 100 microg/mL and exposed or not to UVB at room temperature for 72 h. The UVB source (Bio-Link BLX-312, Vilber Lourmat, Gmbh, France) had a spectral emission at 312 nm. Cells were subjected to a UVB dose of 125 mJ/cm^2, and UVB irradiation lasted 59 s. At the end of the incubation period, the evaluation of Free-radical-scavenging capacity analysis and intracellular ROS production in EB-treated HaCaTs by using DPPH assay and ABTS assay were performed, using arbutin as a control. The results showed that above all high doses of Zea-phen® decreased ROS production and significantly ameliorated the result of ABTS and DPPH assay. No other significant changes were observed. The red corn Zea- phen® extract is the potential candidate to protect skin cells from UVB induced oxidative damage. The mechanism of action may occur via the decreased oxidative stress and the suppression of ROS production. However, further research in vivo is still essential.

These results are summarized in (Figure 1)

Increasing evidence has shown that oxidative stress and inflammation are involved in multiple processes and play crucial roles in skin photoaging. UVB-induced excessive ROS generation causes the activation of MAPK kinases, leading to NF-κB-dependent inflammatory responses in HaCaTs. Thus, the crosstalk between NF-κB

Figure 1



Free-radical-scavenging capacity analysis and intracellular ROS production in EB-treated HaCaTs. a DPPH assay; b ABTS assay; Arbutin was used as the positive control. c Effect of EB on ROS production; following UVB irradiation (125 mJ/cm2), HaCaTs were treated with or without the indicated concentration of EB (1, 10, and 100 µg/mL) for 24 h. Relative ROS generation is shown in each histogram. $P < 0.001$, compared to the Basal; $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the UVB irradiated cells. EB=extract Zea-Phen.

and MAPKs under oxidative stress has recently garnered interest [23,24]. UVB-induced inflammatory response also accelerates aging by degrading the collagen and elastin proteins in the skin. Therefore, EB attenuated UVB-induced inflammatory responses, and decreased inflammation-induced ECM breakdown. Zea-phen® thus also represents a complementary and alternative medicine for skin photoaging therapy.

Results of Antioxidant Activity on Rat Organs

The scavenger activity of free radicals by DPPH and ABTS was determined by the capacity of the red corn extracts to scavenge 50% of radicals. The FRAP method is based on the reducing power of the sample. The results indicate that the bioactive compounds present in Zea-phen® has a good activity as free radical scavengers. The measurement of the antioxidant activity using different extraction systems obtained using the DPPH, ABTS and FRAP assays exhibited significant differences for different vegetal extracts. The DPPH IC_{50} values observed with red corn extract Zea-phen® are comparable with those of other plant species with a known antioxidant activity. For instance, Benvenuti et al [18] reported DPPH IC_{50} values for blackberry (R. fruticosus L.) between 46 and 95 microg/mL, raspberry (Rubus idaeus L.) between 55 and 109 microg/mL, black currant (Ribes nigrum L.) between 10 and 42 microg/mL, red currant (Ribes rubrum L.) between 43 and 59 microg/mL, and black chokeberry (Aronia melanocarpa Elliot) of 18 microg/mL.

This table shows a clear and significant difference between the results regarding the antioxidant activity measured with DPPH, ABTS and FRAP tests in placebo fed animals and in Zea Phen fed animals. The reduction of these tests indicates a reduction of the free radicals level, which is proportional to the entity of this reduction. The scavenger activity of the free radicals measured by DPPH and ABTS tests determines the capacity of a vegetal extract, in this case the red corn extract, to scavenge the free radicals. In fact the reduction of the score of these two tests indicates a reduction of radical damage, which is proportional to the entity of this reduction. It is evident that, versus placebo, Zea Phen determines a significant reduction in the DPPH and ABTS score, so demonstrating its good capacity of scavenging the free radicals. The FRAP assay is defined as a method that measures the antioxidant capacity of a sample by evaluating its ability to reduce ferric ions (Fe 3+) to ferrous ions (Fe 2+)

Table 1: Antioxidant Activity of Red corn (Z. mays L.) Zea-phen® extract. The confrontation was performed between the organs of placebo fed animals and those of Zea-phen® fed animals and measured by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), 2,2' -Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP).

	DPPH IC50 (microg/ml)	ABTS IC50 (microg/ml)	FRAP micro M TE/g???
Placebo animals	89.0 ± 0.50^{de}	376 ± 0.40^{e}	26.3 ± 0.03^{e}
Zea-phen® animals	70.8 ± 0.30^{e}	242 ± 0.30^{f}	18.1 ± 0.03^{f}

in a redox reaction, with the degree of color change indicating the level of antioxidant activity. In this case too the Zea Phen obtained best results versus placebo, thus indicating its broad spectrum antioxidant activity.

It has been observed in previous reports that antioxidant assays such as FRAP, DPPH, ABTS, and oxygen radical absorbance capacity exhibit a high correlation with polyphenols content [16]. For instance, Gil et al [26] reported a correlation coefficient ($r>0.9$, $P\leq.05$) between the antioxidant activity (DPPH or ABTS) and total polyphenols in nectarines, peaches, and plums. Gardner et al [27] also reported a high correlation between total polyphenols and antioxidant activity measured by FRAP. The study observed that there is a high correlation between the antioxidant activity assays (DPPH, ABTS and FRAP) and the content of total polyphenols such as flavonoids, phenolic acids, and anthocyanins, which indicates that these classes of bioactive compounds contribute the most to the antioxidant activity of red corn Zea-phen®. However, there was a low correlation between the antioxidant activity assays and the flavonol content.

Cellular Antioxidant Response in Isolated Mouse Organs

Table 3 shows the activity of endogenous enzymes and TBARS production for the different treatments in isolated mouse kidney, skin, heart and brain. The results suggest that treatments with red corn extract Zea-phen® had a protective effect in maintaining cellular homeostasis through stimulation of antioxidant enzymes in isolated mouse organs. It could not be denied that both red corn phenolic acids, flavonoids, anthocyanins and stilbenes were shown to be great antioxidants, and some variation of their antioxidant power should be expected between different studies. The DPPH assay was used to better understand why red corn showed relatively high antioxidant activity compared to other fruits and vegetables. Red corn phenolics exhibited higher antioxidant capability and faster reaction kinetics than the same amount of blueberry phenolics, suggesting red corn phenolics to have a larger number of active hydroxyl groups and more favorable configurations to allow for better interactions with the free radicals [28].

Oxidative stress in isolated mouse organs was induced by H_2O_2 as a source of reactive oxygen species. Oxidation of membrane lipids had been reported to change the membrane plasticity and flexibility, which can cause an increase in membrane permeability. This increase in membrane permeability could have resulted in increased uptake of partially hydrophobic phenols in the presence of H_2O_2 [29]. The results shown in table 3 indicate that the Zea-phen®+H_2O_2 and the Zea-phen® only treatments were more effective in stimulating the production of the antioxidant enzymes SOD, CAT and TPX than the control (H_2O_2), which indicates the good functionality of the bioactive compounds present in Zea-phen®. The anti-oxidant effects of the phenolic compounds are explained by the number and arrangement of the hydroxyl groups around the nuclear structure. Anti-oxidant activity is exerted through two major modalities: phenolic compounds can act as radical scavengers that either prevent the cellular damage produced by ROS or prevent the generation of ROS in the first place. Phenolic compounds action is mediated by inhibition of the enzymes involved in ROS generation (such as glutathione S transferase, mitochondrial succinoxidase, NADH oxidase), and/or by an increase of anti-oxidant and detoxifying enzymes levels (such as glutathione peroxidase, glutathione reductase, and catalase) [30].

The H_2O_2 treatment showed a high content of malondialdehyde (MDA) formed in all the tested organs (kidney, skin, liver, and brain), indicating its pro-oxidative effect. This correlates with the fact that lipid oxidation in biological systems is carried by the action of reactive oxygen species, resulting in the formation of MDA, which it is also a metabolite of hydroperoxides and serves as a good marker of oxidation and injury to the cell membrane [31]. However, this system is closely regulated by several antioxidant enzymes such as SOD and CAT, which help to remove reactive oxygen species. As shown in Table 2, the MDA (measured by the TBARS assay) value reaches its highest levels in organs treated with H_2O_2. This is probably due to a rapid progression of secondary lipid oxidation induced by H_2O_2, which decreases the cellular antioxidant response. However, when organs are treated with Zea-Phen® low MDA values are observed, most likely due to the presence of various polyphenol substances and especially of phenolic acids, which have the ability to scavenge free radicals and to increase levels of endogenous antioxidant enzymes. When calculating the SOD ratio between treatment and the negative control (H_2O), it was observed that this ratio ranged between 1.81 and 3.42 for kidney, heart, skin and brain, indicating an increase in SOD levels after treatment with red corn extract Zea-phen. Similarly, the ratio for CAT ranged between 1.64 and 25.66, whereas the ratio for TPX ranged between 1.07 and 1.36.

This table shows that H2O2 versus placebo significantly reduces the activity of the antioxidant enzymes and that increases the production of malondialdehyde, thus causing a strong oxidative damage in the tissues. Zea Phen alone versus placebo substantially increases the activity of the antioxidant enzymes and slightly reduces the production of malondialdehyde. The most important data of this table is that Zea Phen, when used contemporarily with H2O2, significantly reduces the impairment of the antioxidant enzymes and the increase of the malondiadehyde levels induced by H2O2. This data means that Zea Phen is capable to substantially reduce the oxidative damage induced in the skin by H2O2, above all increasing the activity of the antioxidant anzymes and reducing the levels of malondialdehyde in the skin.

Table 2: Antioxidant Activity of Red corn (Z. mays L.) Zea-phen® extract. The confrontation was performed between the organs of placebo fed animals and those of Zea-phen® fed animals and measured by 1,1-Diphenyl-2-Picrylhydrazyl (DPPH), 2,2′ -Azinobis (3-Ethylbenzothiazoline-6-Sulfonic Acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP).

Organ, treatment	CAT	TPX	SOD	TBARS
Kidney				
Zea-phen®+H_2O_2	10.4±2.3[a]	67.7±1.4[a]	33.3±0.8[a]	1.19±0.03[b]
Zea-phen®	6.11±2.3[b]	62.3±1.6[b]	26.7±0.6[b]	0.18±0.03[a]
H_2O_2	1.55±0.4[c]	49.6±1.1[c]	10.5±0.2[c]	8.32±0.08[c]
Placebo	1.12±2.2	58.7±1.2	16.6±0.3	3.12±0.03
F ratio	21.4	148	1,490	18,700
P	.001**	.001**	.001**	.001**
This table shows that H_2O_2 versus placebo significantly reduces the activity of the antioxidant enzymes and that increases the production of malondialdehyde, thus causing a strong oxidative damage in the tissues. Zea Phen alone versus placebo substantially increases the activity of the antioxidant enzymes and slightly reduces the production of malondialdehyde. The most important data of this table is that Zea Phen, when used contemporarily with H_2O_2, significantly reduces the impairment of the antioxidant enzymes and the increase of the malondiadehyde levels induced by H_2O_2. This data means that Zea Phen is capable to substantially reduce the oxidative damage induced in the kidney by H_2O_2, above all increasing the activity of the antioxidant enzymes and reducing the levels of malondialdehyde in the kidney.				
Heart	CAT	TPX	SOD	TBARS
Zea-phen®+H_2O_2	22.1±4.9[b]	52.5±1.6[b]	36.2±1.5[a]	4.84±0.07[a]
Zea-phen®	35.2±3.9[a]	58.8±1.8[a]	37.2±0.6[a]	4.85±0.06[a]
H_2O_2	1.38±2.8[c]	43.5±1.3[c]	10.7±0.6[b]	24.7±0.3[b]
Placebo	18.5±2.4	33.1±1.5	24.2±1.5	14.2±0.14
F ratio	56.2	58.6	634	13,750
P	.001**	.001**	.001**	.001**
This table shows that H_2O_2 versus placebo significantly reduces the activity of the antioxidant enzymes and that increases the production of malondialdehyde, thus causing a strong oxidative damage in the tissues. Zea Phen alone versus placebo substantially increases the activity of the antioxidant enzymes and slightly reduces the production of malondialdehyde. The most important data of this table is that Zea Phen, when used contemporarily with H_2O_2, significantly reduces the impairment of the antioxidant enzymes and the increase of the malondiadehyde levels induced by H_2O_2. This data means that Zea Phen is capable to substantially reduce the oxidative damage induced in the heart by H_2O_2, above all increasing the activity of the antioxidant enzymes and reducing the level of malondiadehyde in the heart.				
Brain				
Zea-phen®+H_2O_2	18.5±1.2[a]	34.4±0.6[b]	12.2±0.3[b]	2.44±0.04[a]
Zea-phen®	16.4±0.9[b]	38.3±0.7[a]	22.1±0.5[a]	2.95±0.04[a]
H_2O_2	9.77±0.8[c]	32.1±0.6[c]	6.44±0.1[c]	34.1±0.4[b]
Placebo	10.3±0.6	34.9±0.6	8.58±0.2	2.23±0.04
F-ratio	59.5	74.9	1,61	17,900
P	.001**	.001**	.001**	.001**
This table shows that H_2O_2 versus placebo significantly reduces the activity of the antioxidant enzymes and that increases the production of malondialdehyde, thus causing a strong oxidative damage in the tissues. Zea Phen alone versus placebo substantially increases the activity of the antioxidant enzymes and slightly reduces the production of malondialdehyde. The most important data of this table is that Zea Phen, when used contemporarily with H_2O_2, significantly reduces the impairment of the antioxidant enzymes and the increase of the malondiadehyde levels induced by H_2O_2. This data means that Zea Phen is capable to substantially reduce the oxidative damage induced in the brain by H_2O_2, above all increasing the activity of the antioxidant anzymes and reducing the levels of malondialdehyde. This finding is particularly important because it shows that the antioxidant substances of Zea Phen can penetrate the blood brain barrier and reach the brain structures.				
Skin				
Zea-phen®+H_2O_2	18.5±1.2[a]	32.5±0.6[b]	11.8±0.3[b]	2.14±0.04[a]
Zea-phen®	15.2±0.9[b]	37.1±0.7[a]	20.8±0.5[a]	2.75±0.04[a]
H_2O_2	8.91±0.8[c]	31.3±0.6[c]	6.14±0.1[c]	33.1±0.4[b]
Placebo	10.8±0.6	33.4±0.6	8.18±0.2	2.14±0.04

Organ, treatment	CAT	TPX	SOD	TBARS
F-ratio	58.2	73.4	1,44	16,400
P	.001**	.001**	.001**	.001**

This table shows that H_2O_2 versus placebo significantly reduces the activity of the antioxidant enzymes and that increases the production of malondialdehyde, thus causing a strong oxidative damage in the tissues. Zea Phen alone versus placebo substantially increases the activity of the antioxidant enzymes and slightly reduces the production of malondialdehyde. The most important data of this table is that Zea Phen, when used contemporarily with H_2O_2, significantly reduces the impairment of the antioxidant enzymes and the increase of the malondiadehyde levels induced by H_2O_2. This data means that Zea Phen is capable to substantially reduce the oxidative damage induced in the skin by H_2O_2, above all increasing the activity of the antioxidant anzymes and reducing the levels of malondialdehyde in the skin.

Conclusions

The free radical-involved chain reaction is the generally accepted mechanism for degenerative oxidation in living tissue. Antioxidant capability often refers to the ability to scavenge reactive oxygen radicals: superoxide, singlet oxygen, peroxide, hydrogen peroxide, and hydroxyl radicals. It is thus believed that antioxidants can provide health protection to oxidative degradation/damage in biological systems. The antioxidant property of red corn has been comprehensively evaluated in various free radical-scavenging assays, cellular studies in vitro, and animal studies in vivo.

Consistent between studies, various red corn extracts exhibited positive antioxidant capability in all assays throughout the years. Overall, the antioxidant activity of red corn is highly correlated with the amounts of bioactive compounds such as polyphenols, and particularly of phenolic acids, flavonoids, stilbenes and anthocyanins.

The antioxidant activity of Zea-phen®, measured as the IC_{50}, ranged between 59,7 and 81.2 µg/mL (medium value 70,8 µg/mL) for the DPPH assay, 206 and 279 µg/mL (medium value 242 µg/mL) for the ABTS assay and 14.1 and 22.3 µM TE/g (medium value 18,1 µM TE/g) for the FRAP assay.

It could not be denied that red corn phenolic compounds were shown to be great antioxidants, and some variation of their antioxidant power should be expected between different studies. The DPPH assay was used to better understand why red corn showed relatively high antioxidant activity compared to other fruits and vegetables. Red corn phenolics exhibited higher antioxidant capability and faster reaction kinetics than the same amount of blueberry phenolics, suggesting red corn phenolics to have a larger number of active hydroxyl groups and more favorable configurations to allow for better interactions with the free radicals. This phenomenon could also be explained by the high content of phenolic acids in the product Zea-phen®.

The effect of red corn extract Zea-phen® on cellular antioxidant response in mouse organs was investigated by treatment with added H_2O_2 to isolated mouse kidney, heart, skin and brain ex vivo. The presence of malondialdehyde (MDA) in these organs served as an indicator of cell membrane oxidative injury after applying H_2O_2. When the organs showed low MDA levels and were treated with red corn extract Zea-phen®, the levels of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and total peroxidase (TPX) in the organs were increased. These enzymes are believed to have the capability to eliminate reactive oxygen species and prevent oxidative damage to cells. These results suggested that certain functional compounds in red corn extract Zea-phen® could penetrate cell membranes and participate in stimulating antioxidant enzyme secretions to reduce oxidative damage to cells caused by free radicals.

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Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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