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Phenolic acids of modern and ancient grains: effect on in vitro cell model

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Abstract

BACKGROUND: The gastrointestinal tract establishes a barrier between external and the internal compartment. When this barrier is disrupted, an inflammatory cascade promotes intestinal inflammation and the development of several intestinal diseases. Plant-derived polyphenols are health-promoting phytochemicals with a role in the regulation of the intestinal barrier and in the prevention of intestinal inflammatory diseases. Modern breeding programs have been primarily focused on yield improvement than in nutritional and functional wheat proprieties. Therefore, a research that aims at the characterization of the phytochemical profile of wheat varieties and on their healthy proprieties could be a new prospect for the genetic improvement of the genus *Triticum*.

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In the present work, the effects of phenolic compounds extracted from nine soft and seven durum wheat varieties were studied for their polyphenol contents and antioxidant activities. Moreover, a set of experiments were conducted to study their effects on cell proliferation and wound healing of three different cell lines: mouse fibroblasts (L929), intestinal human cells (Caco2) and human monocytes (U937).

RESULTS: The results obtained from all the analyses were gathered together and discriminant analysis evidenced differences between soft and durum wheat phenolic compounds. Among the soft varieties, it was possible to identify clusters, where ancient wheat varieties showed different properties compared to moderns, while no evident clusters where detected among durum varieties.

CONCLUSION: Taken together these results suggest that a specific selection of wheat grains based on their nutritional parameters will certainly help in designing diets with protective effects against chronic and inflammatory diseases.

Keywords: Polyphenols, ancient wheat, modern wheat, antioxidant proprieties, cell proliferation, wound healing

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Introduction

Epidemiological studies have correlated whole-grain cereal consumption to a lower risk of developing chronic diseases, such as coronary heart disease, arteriosclerosis, type-2 diabetes, and some form of cancers[1].The physiological mechanisms behind the protective effects of whole grain are unclear, but they can be linked to the action of the dietary fibers together with a wide variety of phytochemicals[2]. According to nutritional guidelines, cereals and their products are at the base of the food pyramid[3]. Recently, studies on the health benefits of wheat products have become more focused on the importance of introducing phytochemicals through the use of different varieties[4]. As a consequence, there is a renewed interest in the ancient varieties, particularly with regard to their potential nutraceutical proprieties[5,6,7,8]. A modern wheat cultivar can be considered a dwarf or semi-

dwarf registered genotype, while an old wheat one can be defined as a not dwarf and unregistered genotype. Modern genetic breeding programs have been primarily focused on yield and pest resistance improvement, instead of being targeted at the increase of nutritional and functional proprieties. As a consequence, the selection of varieties has not been done according to their nutritional proprieties. Therefore, research aiming at the characterization of the phytochemical profile of wheat varieties focusing on compounds like polyphenols may represent a new prospect for the genetic improvement of the genus *Triticum*. There is a new paradigm, where food and nutrition scientists align their efforts and approaches to generate novel research aimed at improving human health[9]. One essential step integral component of this new paradigm will be to develop "functional foods," which are foods and food components that can provide a health benefit beyond basic nutrition and can promote health and reduce disease risk[10]. According to this, it should be developed an integrated work that connects farm-and -cell in order to create the framework able to address public health issues[11] by providing an innovative food supply that translates cutting edge nutrition research into consumer-friendly foods. Different experimental approaches are commonly used for nutraceutical value assessment of foodstuff and ingredients[12].

The objective of this study was to quantify and to characterize the polyphenols extracted from 16 wheat varieties (9 soft and 7 durum), in order to highlight their possible nutraceutical effects exerted in biological systems. To this aim, polyphenols were analyzed in terms of phenolic contents and antioxidant properties. Moreover, cellular experiments were set up in order to evaluate polyphenols effects in wound healing process and on *in vitro* cell proliferation, using three types of cell lines: mouse fibroblasts, L929, human intestinal cells, Caco2, and human monocytes cells, U937.

Materials and Method

Chemicals

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Folin–Ciocalteu reagent were purchased from Sigma–Aldrich (St Louis, MO, USA). Acetic acid was of an analytical grade (assay > 99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) assay was from Life Technologies (California, USA), reagents for cell cultures as Hank's Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), L-Glutammine, Penicillin-Streptomicyn and RPMI-1640 Medium were purchased from GIBCO (Massachusetts, USA). All other chemicals and solvents were of analytical grade.

Grain samples

Wheat samples included 5 old (Andriolo, Frassineto, Gentil Rosso, Inallettabile, Verna) and 4 modern (Bilancia, Bolero, Palesio, Sagittario) soft wheat (*Triticum aestivum* L.) varieties, 4 old (Bidì, Russello, ScorsoNera and Timilia) and 2 modern (Claudio, Svevo) durum wheat (*Triticum turgidum* spp. *durum*) varieties and the KAMUT® wheat (*Triticum turgidum* spp. *Turanicum*, KAMUT® is a registered trademark of Kamut International, Ltd. and Kamut Enterprises of Europe, bvba).

Seeds from all of the investigated genotypes were grown in the same location at a biodynamic farm of the Emilia Romagna region, Italy, located at Argelato (latitude 44◦ 39′ 57″ N, longitude 11◦ 19′ 43″ E, 25mabove sea level (a.s.l.), during the growing season 20016/20017. Each genotype was grown in plots (665 m) according to strict low-input agro-technique. Weeds were hand controlled and no chemical treatments were applied. Plants were harvested at grain full ripening stage. Whole grain samples were milled into flours, immediately cooled to -20°C and kept at this temperature until analysis.

Extraction of phenolics from grains

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Preparation of wheat phenolic extracts was performed as previously described[13]. Briefly, 0.5 g of whole wheat flour was mixed with 20 mL of 80% chilled ethanol for 10 min. After centrifugation at 2500 g for 10 min, the supernatant was removed and extraction was repeated. Supernatants were pooled, evaporated at 45°C and reconstituted in 2 mL of 70% ethanol. The residue from the free phenolic extraction was subjected to alkaline and acid hydrolysis to recover the bound phenolic compounds[14]. For each wheat variety, the free and bound phenolic fractions were pooled and stored at -20°C until use.

Total polyphenol contents

The amount of total phenolics in the extracts was determined according to the Folin–Ciocalteu procedure[15]. Results are expressed as micrograms of gallic acid equivalents (GAE) per 100 gr of grain.

Antioxidant activity

The total antioxidant activity of wheat phenolic extracts was measured by ferric ion reducing power (FRAP) assay[16] and 2,2- diphenyl-1-picrylhydrazyl radical (DPPH) assay[17] with some modifications. Briefly, freshly prepared FRAP Reagent (10:1:1 Acetate buffer 300 mM pH 3.6, 10 mM 2, 4, 6-tripyridyl-striazine (TPTZ) in 40 mM HCl and 20 mM $FeCl3*6H_2O$) was mixed with the sample and allowed to stand for 60 min at room temperature before measuring absorption at 593 nm. Aqueous solutions of Fe^{2+}($FeSO4*6H_2O$) in the concentration range of 125–1250 mmol L^{-1} were used for calibration of the FRAP assay. FRAP values were expressed as micromoles of Fe^{2+} per L of sample (μmol of Fe^{2+} L^{-1}). As regards DPPH assay, results were expressed as micromoles of Trolox equivalent L^{-1}.

Cell model systems

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A mouse fibroblasts cell line, L929, human epithelial colon cancer cell line, Caco2 and a human monocytes cell line, U937 were used for the *in vitro* studies. Cells received different concentrations of wheat extracts for 24 hours after which their effects were evaluated in terms of cytotoxicity. L929 mouse fibroblasts (ATCC-CCL1) were cultured with DMEM added with 10 %, fetal bovine serum, 1mM L-glutammine and 1 % penicillin-streptomycin. Caco2 human epithelial cell line (ATCC HTB-37) obtained from Colorectal adenocarcinoma was cultured with DMEM added with 10 %, fetal bovine serum and 1 % penicillin-streptomycin. U937 is a pro-monocytic, human myeloid leukemia cell line (ATCC CRL-1593.2) were cultured in RPMI-1640 Medium added with 10%, fetal bovine serum and 1% penicillin-streptomycin.

MTT Viability Assay

In order to assess whether there is a correspondence between the *in vitro* antioxidant activity and potential healthy properties of different wheat extracts, cell lines were treated with two concentrations of wheat polyphenols (5µg or 20µg gallic acid equivalents [GAE] ml^{-1}) for 24 hours or 70% ethanol as control resuspended in DMEM alone.

Adherent cells, L929 and Caco2, cells were plated in 96-well tissue culture plate (10^5 cells $well^{-1}$) in complete medium. After treatments, proliferative cells were detected by MTT assay, according to the ISO 10993-5 International Standard procedure (ISO 10993-5, 2009). The main purpose of the ISO 10993-5 procedure is to define a scheme for testing *in vitro* cytotoxicity of different extracts according to a multi- step approach. The MTT substrate was prepared in DMEM then added to cells in culture at a final concentration of 1 mg mL^{-1} and incubated for 2h in the culture incubator at 37 C with 5% CO2. After incubation, the medium was removed by aspiration, 100 µL of isopropanol was added to each well and the formazan dye formation was evaluated by scanning multiwell spectrophotometer at 540 nm.

Suspension cells, U937 cell line, were plated 10^5 cells $well^{-1}$ in 96-well culture plates in complete medium. After treatments cells were incubated with 0.5% MTT solution in PBS for 4h at 37°C and

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then dissolved with 100 µl isopropanol with 0.04N HCl. The plate was read at 540 nm and results were expressed as percentage of viable cells with respect to untreated controls (70% ethanol).

The percentage of cell proliferation was calculated using the following formula:

(absorbance value of treated sample / absorbance value of control) X 100 = % of cell viability.

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Wound Healing assay

A function of the intestinal epithelium is to be a barrier, that regulates interactions between the luminal contents, the underlying immune system and the rest of the body, while supporting transport of nutrients. Epithelial barrier function requires a contiguous layer of cells and compromised intestinal barrier has been associated with a number of diseases, both intestinal and systemic[18]. Wound healing is a process of recovering the forms and functions of injured tissues and in general, it is a complex process that consists of three overlapping phases: inflammatory reaction, proliferation, where fibroblasts play a critical role, and remodeling phase to restore integral tissue[19]. Any alteration that disrupt the healing processes would worsen the tissue damage and prolong repair process[26]. In order to understand if polyphenols extracted from wheats could play a role in the repairing intestinal tissue damages, wound healing assay was performed with fibroblasts. A total of 20×10^4 L929 fibroblasts were plated on 24-well tissue culture plates. At 24 h after seeding, cells were washed three times in HBSS and a line for each well was drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with HBSS to remove all detached cells and incubated in DMEM with 5µg gallic acid equivalents (GAE) ml^{-1} of wheat extracts or 70% ethanol as control. Cells were monitored at 4 and 24 h from stimulation and pictures of cells were taken. The results of each experiment were expressed as the mean of migrated cells from three different areas. The final results are expressed as the mean of three different experiments

Statistical analysis

The quantification of chemical compounds and antioxidant activities was performed on triplicate samples of each wheat accession. Data of chemical analyses were represented as mean values. The cell tests were carried out with six replicates for each treatment and data were expressed as mean values of three different experiments. Statistical analysis was performed with R software[20]. Normal and homoscedastic data were analyzed with ANOVA and Tukey post-hoc tests with Bonferroni correction. Non-normal homoscedastic data were analyzed with the nonparametric Kruskall–Wallis test and Dunn's post-hoc test with Bonferroni correction. Differences were considered to be significant at a p value < 0.05. In the tables mean values followed by different letters are statistically different.

Linear discriminant analysis (LDA) was performed on the standardized matrix of the phytochemical content, antioxidant activities and cell tests, including a total of 11 variables: total polyphenols (Poly), DPPH, FRAP, MTT activities on Caco2 cell treated with 5 μg (Caco_5) and 20 μg (Caco_20) of wheat extract, on L929 fibroblasts treated with 5 μg (L929_5) and 20 μg (L929_20) of wheat extract, U937 monocytes treated with 5 μg U937_5) and 20 μg (U937_20) of wheat extract, and wound healing test after 4 (Mig_4h) and 24 (Mig_24h) hours. LDA is a multivariate technique that allowed scoring of the cases as a function of the first two roots (canonical discriminant functions) to visualize similarities and differences among the wheat genotypes[21]. LDA is also a statistical method used to find a linear combination of features (discriminant functions) that characterizes or separates two or more classes of objects or events[22]. All statistical analyses were conducted using Statistica 6·0 software (2001, StatSoft, Tulsa, OK, USA).

Results

Content and antioxidant properties of polyphenols extracted from soft wheats

The total polyphenol content of each soft wheat variety, both old and modern, is presented in Table 1 and expressed as mg of gallic acid equivalent (mg GAE $100gr^{-1}$). Inallettabile had the highest total phenolic content (249.52 mg $100gr^{-1}$), which was similar to Gentil Rosso. In contrast, Bilancia had

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the lowest value (110.72 mg $100gr^{-1}$), which was similar to Sagittario. Taken together, the mean polyphenol content of old varieties resulted to be significantly higher (224.00 mg $100gr^{-1}$) than in modern ones (140.40 mg $100gr^{-1}$). DPPH and FRAP assays were used to measure the antioxidant activity of the total phenolic extracts. DPPH allows the quantification of the hydrogen donating potency of phenolic compound[23] and the DPPH radical scavenging activities were expressed as micromoles of Trolox equivalents (TE) per gram of wheat grain. The FRAP assay measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron) giving the concentration of electron-donating antioxidants[15]. Old soft wheat genotypes showed higher antioxidant activity compared to modern ones, in both DPPH and FRAP assays. However, the lowest antioxidant ability measured by FRAP assay was observed for Bilancia (0.91 μmol Fe $100gr^{-1}$), followed by Bolero and Palesio. Conversely, the highest antioxidant capacity was measured for Verna and Gentil Rosso (both 1.81 μmol Fe $100gr^{-1}$). Regarding DPPH assay, the wheat varieties showed different antioxidant activities with values ranging from 4.09 μmol TE gr^{-1} to 2.24 μmol TE gr^{-1} and decreased in the following order: Sagittario > Inallettabile, Frassineto > Andriolo > Gentil Rosso, Verna > Bolero, Palesio > Bilancia.

Cellular effects of soft wheats polyphenols

In order to study the effects of polyphenol extracts on cell proliferation and migration, murine L929 fibroblasts cell line was used. To study the effect on cell migration, wound healing assay was performed and migrated cells were counted after 4 and 24 hours from the addition of 5 μg GAE ml^{-1} of the total polyphenol extracts (Figure 1A). All wheat varieties induced cell migration in a similar way after 4 hours, while modern genotypes resulted to stimulate wound healing more than old ones after 24 h of treatments. In particular, Verna induced the highest number of migrating cells after 4 h of treatment (76.7 mean of migrated cells), followed by Bilancia, Inallettabile, Gentil Rosso, Bolero and Palesio, with a range of mean of migrated cells from 59.4 to 52.7. The polyphenol extract of Frassineto induced the strongest effect (37.5 mean of migrated cells). Regarding the 24 h of

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treatment, Palesio had the highest effect (116.8 mean of migrated cells), followed by Bilancia, Verna, Bolero, Gentil Rosso and Sagittario. The weakest effect was observed for Frassineto both after 4 and 24 h of treatment (respectively 37.5 and 64.1 mean of migrated cells).

Total polyphenol extracts at the concentration of 5 μg GAE ml^{-1} and 20 μg GAE ml^{-1} were added to three different cell lines: mouse fibroblasts L929, human intestinal epithelial cells, Caco2, and human monocytes U937, and proliferation assay (MTT) was performed 24 h after treatments (Figure 2A and Figure 3A). Regarding L929 cells, at the concentration of 5μg GAE ml^{-1}, all polyphenols upregulated cell proliferation with percentages ranging from 157.3 (Inallettabile) to 124.3 (Bilancia) with respect to control. In contrast, all polyphenol extracts, with the exception of Inallettabile (114.3 % respect to control), reduced L929 cell proliferation at the concentration of 20 μg GAE ml^{-1} according the following order: Verna and Andriolo, Gentil Rosso and Bolero, Sagittario, Frassineto, Bilancia and Palesio. Taken together, modern varieties resulted to be more toxic (40.4 % with respect to control) than the old ones (78.0 % respect to control) on fibroblast proliferation. Regarding Caco2 cells, extracted polyphenols showed different behaviors at the concentration of 5 μg GAE ml^{-1}, with percentages ranging from 107.3 % (Inallettabile) to 76.9 % (Sagittario) with respect to control. Regarding the dose 20 μg GAE ml^{-1}, it is possible to distinguish three groups: Inallettabile, Gentil Rosso and Frassineto, that showed similar low effects on cell proliferation (from 94.7 to 89.1 % respect to control), Andriolo, Verna and Bolero (with a range from 65.1 to 57.6% respect to control) and Palesio, Bilancia and Sagittario (with a range from 46.0 to 33.8% respect to control) with the highest toxic effect. As observed for fibroblasts, old varieties resulted to be less toxic than the moderns on Caco2 cells proliferation. Regarding monocytes proliferation (U937), the highest value was obtained from Bolero treatment (112.6% respect to control) and it was immediately followed by Palesio (101.8% respect to control). All the other polyphenols reduced cell proliferation in the following order: Verna, Bilancia, Andriolo, Gentil Rosso, Inallettabile, Frassineto and Sagittario with a range from 78.6 to 54.2 % respect to control. Regarding the treatment at the concentration of 20 μg GAE ml^{-1}, from Figure 3 it is possible to

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distinguish Palesio and Bolero with a similar effect, Verna and Inallettabile and the last group with Andriolo, Gentil Rosso, Frassineto and Sagittario with the weakest effect up to 45.2 % with respect to control. Taken together all the results, polyphenols extracted from old soft varieties reduced monocytes proliferation more than the modern ones, at both the concentration of 5 and 20 μg GAE ml^{-1}.

Content and antioxidant properties of polyphenols extracted from durum wheats

The polyphenol content of the total fraction of each durum wheat variety, both ancient and modern, is presented in Table 2. Russello had high total phenolic content (160.68 mg $100gr^{-1}$), which was similar to KAMUT®, Scorsonera and Bidì. These polyphenol contents were higher than Svevo (126.28 mg $100gr^{-1}$), whereas the lowest value was observed for Claudio (93.68 mg $100gr^{-1}$). Taken together, polyphenol content obtained from old varieties resulted to be significantly higher (147.56 mg $100gr^{-1}$) than that of modern varieties (107.56 mg $100gr^{-1}$). Regarding the antioxidant potential of all analyzed durum wheat genotypes, several groups can be distinguished: Timilia resulted to have the highest antioxidant potential (4.45 μmol TE gr^{-1} and 1.56 μmol Fe^{2+} $100gr^{-1}$); Bidì and Russello showed a similar lower antioxidant activity with both DPPH and FRAP assays, followed by Claudio, Svevo and KAMUT®. By considering the mean values of DPPH and FRAP results, no significant difference was observed between old and modern durum wheat varieties.

Cellular effects of durum wheat polyphenols

Regarding fibroblasts migration, the effects of polyphenol extracts obtained from durum wheat varieties were studied in wound healing assay (Figure 1B). All wheat varieties induced cell migration in a similar way, even if the effect was more evident after 24 h of treatment. Considering the mean values, both old and modern varieties induced migration in a similar way. However, among them, several different effects could be highlighted: KAMUT® have the strongest effect (45.6 and 84.4 mean of migrated cells, respectively at 4 and 24 h), followed by Svevo and Bidì;

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Scorsonera had the weakest effect (4.5 and 19.7 mean of migrated cells at 4 and 24 hours after treatment, respectively).

Total polyphenol extracts at the concentration of 5 μg GAE ml^{-1} (Figure 2B) and 20 μg GAE ml^{-1} (Figure 3B) were added to cells and proliferation assay (MTT) was performed 24 hours after treatments. Polyphenol extracts from modern varieties resulted to be toxic for L929 cell proliferation at the concentration of both 5μg GAE ml^{-1} and 20 μg GAE ml^{-1}, while those of old varieties were toxic only at the concentration of 20 μg GAE ml^{-1}. At the concentration of 20 μg GAE ml^{-1}, KAMUT® resulted the most toxic with a value of 32.5 % with respect to control. In contrast, it resulted to have the highest positive effect on cell proliferation at the concentration of 5 μg GAE ml^{-1}. Regarding the intestinal cells Caco2, at the concentration of 5 μg GAE ml^{-1}, the most toxic effect was induced by Svevo, Scorsonera and Russello (51.2, 53.3 and 68.9 % with respect to control, respectively). Conversely, KAMUT® resulted to have a positive effect on cell proliferation (126.4% with respect to control). The mean values of modern and old varieties resulted to have a similar toxicity at the concentration of 5 μg GAE ml^{-1} on Caco2 cells. Regarding 20 μg GAE ml^{-1}, all the polyphenols were toxic for Caco2 cells in a similar way, with percentages ranging from 40.7 to 33.8 % with respect to control. Polyphenols of all modern durum wheat varieties reduced monocytes proliferation both at 5 μg GAE ml^{-1} and 20 μg GAE ml^{-1} in a more effective way than old genotypes. KAMUT® resulted to be the less toxic for monocytes at both concentrations (87.1 and 94.6%, respectively). At the concentration of 20 μg GAE ml^{-1} old varieties resulted to be less toxic (52.4 % with respect to control) than the modern ones (43.3 % with respect to control).

Linear discriminant analyses

LDA was employed to analyze the complete dataset of both soft and durum wheat genotypes. The outcome of the discriminant analysis of the eight soft wheat genotypes is represented in two dimensions by a combined-group scatter plot (Figures 4 and 5), where the x-axis plots the values of the first discriminant function (Root 1) and y-axis plots the values of the second discriminant

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functions (Root 2). The cumulative percent variance explained for the first function in the discrimination of the nine soft wheat genotype was equal to 98.9%. The adapted model allowed clear discrimination between modern (Bilancia, Bolero and Palesio) and old (Andriolo, Frassineto, Gentil Rosso, Inallettabile and Verna). The three modern varieties are closely clustered on the negative branch of the Root 1 as revealed from the values of the canonical functions standardized within variance of each variable. The described clusterization was strongly influenced by fibroblasts migration after 24h from the treatment and proliferation effect on monocytes (U937) treated with 5 μg GAE ml^{-1} of wheat extracts. In contrast, the five old soft wheat varieties were placed in the north-east and south-east quadrants of the scatterplot (Figure 4). The cluster of cases (Andriolo, Frassineto and Inallettabile) along the positive branch of the first canonical function (Root 1) was strongly influenced by polyphenol content and DPPH antioxidant activity. In addition, Gentil Rosso and Verna are clustered along the positive branch of the second canonical function (Root 2): this cluster was strongly influenced by FRAP antioxidant activity and the effect on proliferation of fibroblasts (L929) treated with 20 μg GAE ml^{-1} of wheat extracts. The Sagittario location in the cartesian plan was related to its DPPH antioxidant activity and its effects on Caco2 cell proliferation.

To explain the variability observed among the seven investigated durum wheat genotypes the LDA was carried out according to the same protocol applied for soft wheat genotypes (Figure 5). The cumulative percent variance explained for the first function in the discrimination of durum wheat genotypes was equal to 82.4%. Ancient (Bidì, KAMUT®, Russello, Scorsonera, Timilia) and modern (Claudio, Svevo) were not fully discriminated from the adopted model. Svevo, Claudio and Bidì formed a cluster strongly influenced by FRAP antioxidant activity and wound healing 4 and 24 hours after treatments. Timilia and Russello are clustered near the previous described group and their position in the scatterplot is significantly influenced by DPPH antioxidant activity. The most divergent genotypes in the scatterplot are KAMUT® and Scorsonera. The KAMUT® location in the cartesian plan was related to its stimulation on wound healing, and on Caco2 and U937 cells

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proliferation. In contrast, the Scorsonera is associated with the negative branch of the second canonical function (Root 2): this position is due to the observed strong inhibition in the wound healing test.

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Discussion and Conclusions

The study showed significant differences between soft and durum, ancient and modern, wheat polyphenol extracts in terms of polyphenol contents and antioxidant activities, as well as their effects in relation to wound healing and cell toxicity. Both old soft and durum varieties contained more polyphenols than the modern ones. This data resulted to be in line with other literature reports[24, 25]. Moreover, polyphenols obtained from old soft varieties resulted to have higher antioxidant potential, as observed from DPPH and FRAP analyses[26] while no significant differences were observed between modern and ancient durum genotypes. Despite the significantly lower content of polyphenols, the modern durum wheat varieties exhibited the same DPPH and FRAP activities observed for old genotypes indicating that their polyphenols exert a higher antioxidant activity for mass unity.

In order to avoid any effect due to differences of polyphenol concentrations, all the cell assays on different cellular lines were carried out by diluting the polyphenol extract of soft and durum wheat varieties at the same concentrations (5 and/or 20 μg GAE ml^{-1}). As a consequence, any observed significant effect in cell tests was exclusively due to the different qualitative composition of each extract.

In the present work, wound healing assay revealed that all polyphenols, both from ancient and modern varieties, were able to induce migration of fibroblasts in a similar way. In general, no evident differences were detected for soft varieties, except for Verna, which resulted to induce more migrated cells 4 hours after treatments than the others at both 4 and 24 hours after the treatments. Regarding durum wheat varieties, KAMUT® induced migrating cells more than the others, while, Scorsonera had a very low wound healing effect. As regards modern varieties, the best

performances in inducing fibroblast migration were observed for extracts of Palesio (soft) and Svevo (durum). These results were supported by other works demonstrating that the polyphenols, like gallic acid or tocopherol, might be a viable wound healing agent able to treat wounds resulting from metabolic complications[27,28].

In cell proliferation test all ancient soft varieties resulted to reduce L929 and Caco2 proliferation less than modern soft wheats. These data were particularly significant for the soft varieties at the concentration of 20 μg GAE ml^{-1} on L929 and Caco2 cell lines. Except for the concentration of 5 μg GAE ml^{-1} no significant differences were observed between modern and ancient durum accessions. In this last group, it was possible to distinguish KAMUT®, which resulted in the scatterplot (Figure 2) separated from the other durum wheat varieties, for its positive effects on Caco2 cell proliferation and its ability in inducing wound healing in fibroblasts. These results are in agreement with literature data. Active polyphenolic compounds such as proanthocyanidin, catechin, caffeic acid, and chlorogenic acid incorporated into the mouse fibroblast cell line (3T3) in a short time could accelerate the proliferative response of the cells[29].

The behavior of polyphenols extracted from soft and durum varieties in relation to proliferation of U937 resulted of particular interest. Modern soft wheat varieties resulted to reduce monocyte cell proliferation less than ancient genotypes, both at the concentration of 5 μg GAE ml^{-1} and of 20 μg GAE ml^{-1}. In contrast, the opposite trend was observed for durum wheat varieties. In fact, modern durum varieties resulted to downregulate monocyte proliferation more than old varieties at the concentration of 20 μg GAE ml^{-1}.

Taken together, all the results evidence differences between soft and durum wheat phenolic compounds suggesting a significant genotype influence on the proprieties of wheat extracts. In particular, positive effects on cells were observed for polyphenols of both modern and old varieties. In the case of soft wheat, modern and old genotypes exhibited contrasting effects on different cell lines. Combining ancient and modern varieties in specific crosses (segregating populations) could be useful for developing specific selections of wheat grains based on their nutritional parameters

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helping in designing diets with protective effects against chronic and inflammatory diseases. Although further intervention studies in humans are required, *in vitro* studies represent an essential step for health claim substantiation regarding functional properties of the wheat varieties analyzed in this study.

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Conflict of interest:

Authors state no conflict of interests

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Figure Captions

Figure 1. Effects of polyphenols extracted from soft (A) and durum (B) wheat varieties on fibroblasts (L929) wound healing. The mean number ± SD of migrated cells were counted from 3 pictures of each experiments at 4 and 24 hours after the treatments. Between brackets, different letters indicate mean values significantly different at $P<0.05$.

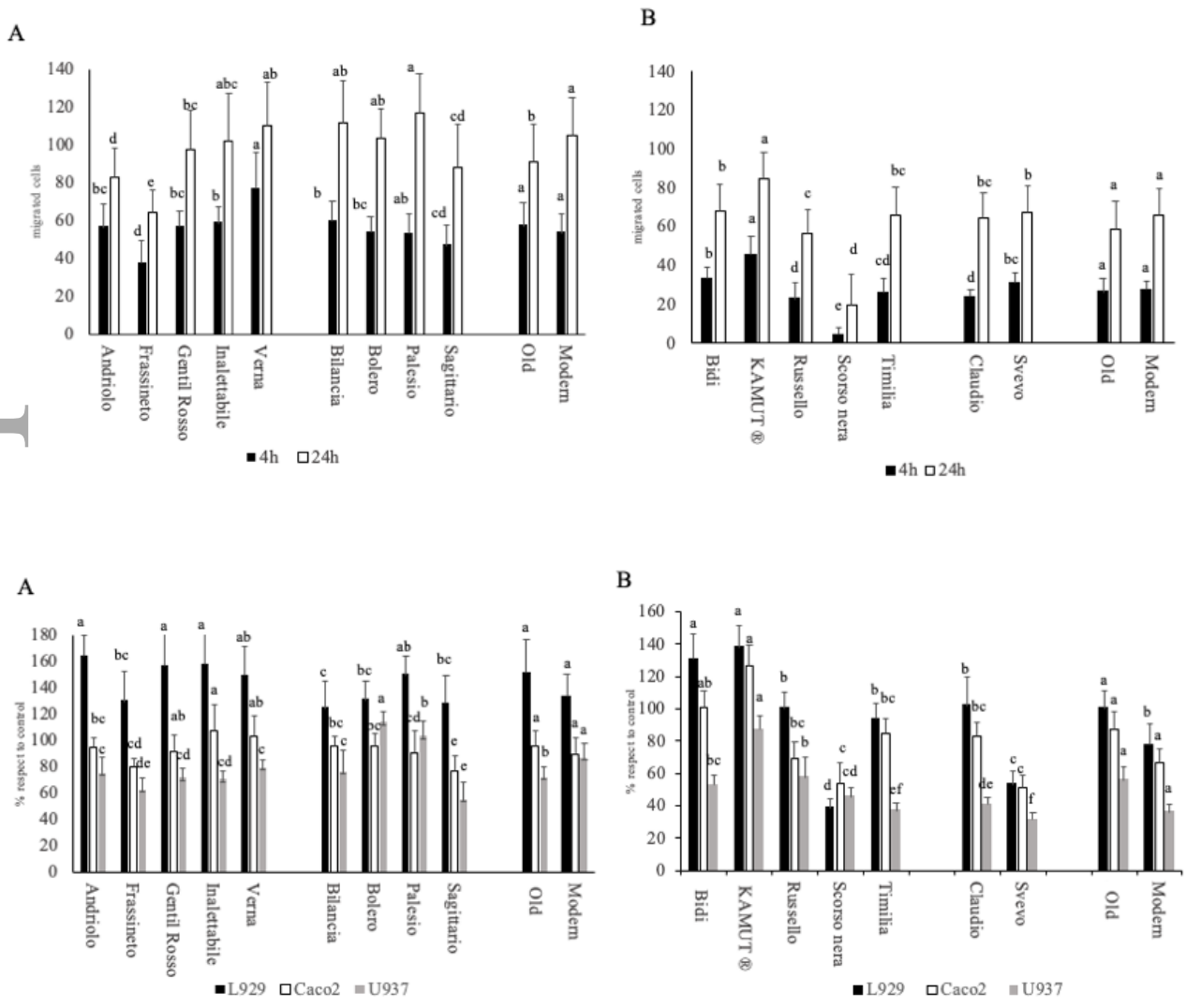
Figure 2. Effects of polyphenols at the concentration of 5µg GAE ml^{-1} extracted from soft (A) and durum (B) wheat varieties on fibroblasts (L929), intestinal cells (Caco2) and monocytes (U937) respiration test (MTT). Data are expressed as % with respect to control after the treatments ± SD and between brackets, different letters indicate mean values significantly different at $P<0.05$

Figure 3. Effects of polyphenols at the concentration of 20µg GAE ml^{-1} extracted from soft (A) and durum (B) wheat varieties on fibroblasts (L929), intestinal cells (Caco2) and monocytes (U937) respiration test (MTT). Data are expressed as % with respect to control after the treatments ± SD and between brackets, different letters indicate mean values significantly different at $P<0.05$

Figure 4. Scatter plot of the nine investigated soft wheat varieties (A) according to polyphenol content, antioxidant activities, fibroblasts migration, and MTT respiration test on L929, Caco2 and

U937 cell lines defined by the first two canonical functions of linear discriminant analysis and projection of 11 variables (as reported in Material and Methods section) on the first two dimensions (B).

Figure 5. Scatter plot of the nine investigated durum wheat varieties (A) according to polyphenol content, antioxidant activities, fibroblasts migration, and MTT respiration test on L929, Caco2 and U937 cell lines defined by the first two canonical functions of linear discriminant analysis and projection of 11 variables (as reported in Material and Methods section) on the first two dimensions (B).



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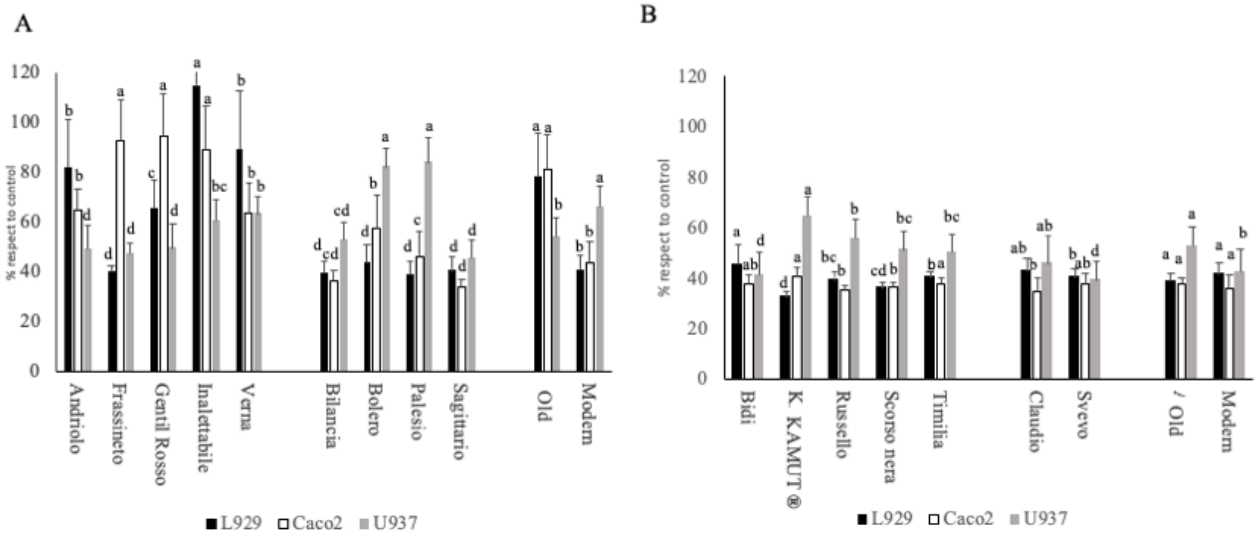

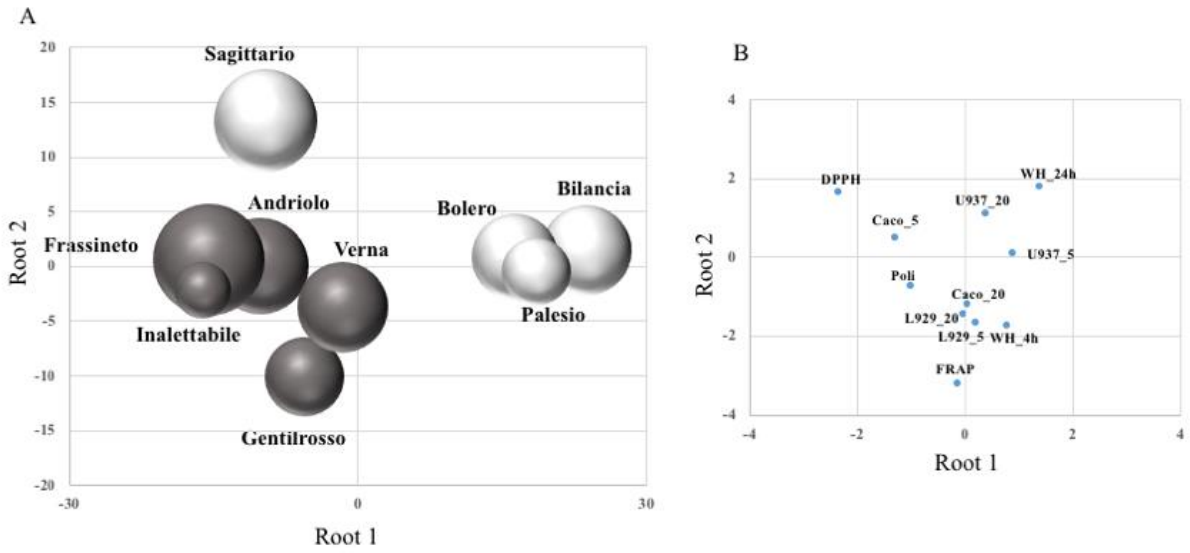

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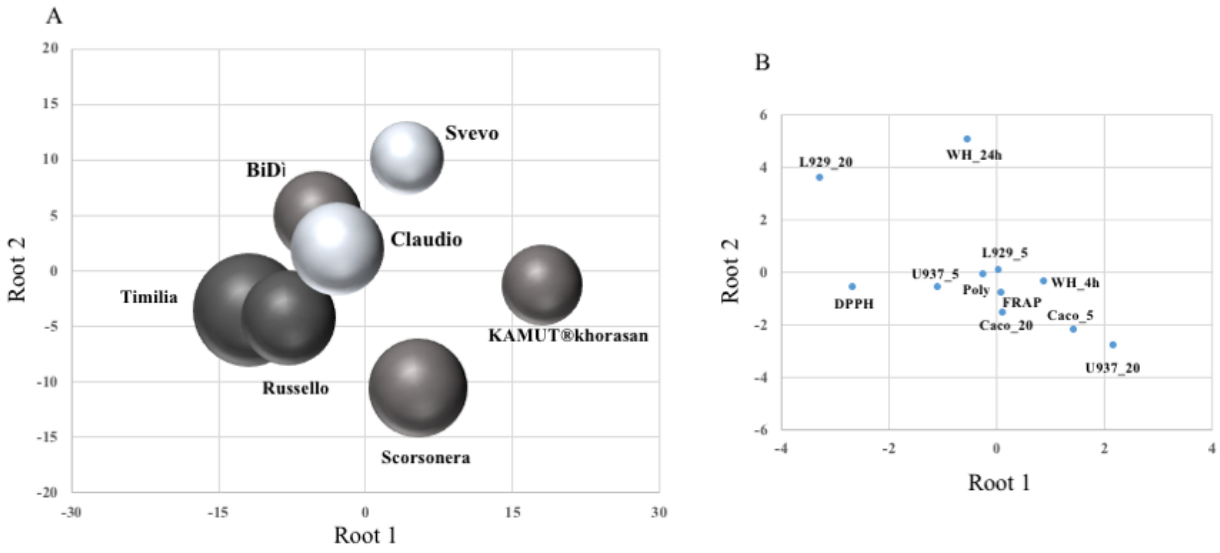

Table 1. Polyphenols of old (O) and modern (M) soft wheat varieties: mean content (mg 100gr^{-1}), DPPH (µmol Trolox gr^{-1}) and FRAP (µmol Fe 100gr^{-1}) antioxidant proprieties ± sd. Between brackets, different letters indicate mean values significantly different at P<0.05

Variety	Polyphenols (mg 100gr^{-1})	DPPH (µmol Trolox gr^{-1})	FRAP (µmol Fe 100gr^{-1})
Andriolo (O)	203.96 ± 37.93(c)	3.85 ± 0.15 (c)	1.60 ± 0.07 (ab)
Frassineto (O)	220.64 ± 0.18 (bc)	3.95 ± 0.10 (b)	1.59 ± 0.08 (ab)
Gentil Rosso (O)	233.04 ± 4.81 (ab)	3.46 ± 0.13 (d)	1.81 ± 0.01 (a)
Inallettabile (O)	249.52 ± 2.40 (a)	3.99 ± 0.05 (b)	1.45 ± 0.17 (abc)
Verna (O)	212.88 ± 5.00 (bc)	3.47 ± 0.19 (d)	1.81 ± 0.75 (a)
Bilancia (M)	110.72 ± 0.55 (f)	2.24 ± 0.18 (f)	0.91 ± 0.07 (d)
Bolero (M)	166.76 ± 1.85 (d)	2.61 ± 0.16 (e)	1.10 ± 0.09 (cd)
Palesio (M)	153.08 ± 4.07 (de)	2.55 ± 0.15 (e)	1.13 ± 0.08 (bcd)
Sagittario (M)	131.08 ± 14.61 (ef)	4.09 ± 0.22 (a)	1.37 ± 0.04 (abcd)
Old	224.00 ± 22.02(a)	3.75 ± 0.97 (a)	1.65 ± 0.32 (a)
Modern	140.40 ± 23.25(b)	2.87 ± 1.08 (b)	1.13 ± 0.18 (b)

Table 2. Polyphenols of old (O) and modern (M) durum wheat varieties: mean content (mg $100gr^{-1}$), DPPH (μmol Trolox gr^{-1}) and FRAP (μmol Fe $100gr^{-1}$) antioxidant proprieties ± sd. Between brackets, different letters indicate mean values significantly different at $P<0.05$

Variety	Polyphenols (mg $100gr^{-1}$)	DPPH (μmol Trolox gr^{-1})	FRAP (μmol Fe $100gr^{-1}$)
Bidì (O)	140.88 ± 0.74 (ab)	3.86 ± 0.12 (c)	1.29 ± 0.03(c)
KAMUT® (O)	147.72 ± 26.46 (ab)	3.13 ± 0.11 (e)	1.21 ± 0.05 (de)
Russello (O)	160.68 ± 1.67 (a)	4.12 ± 0.24 (b)	0.97 ± 0.07 (b)
Scorsonera (O)	145.48 ± 3.15 (ab)	3.30 ± 0.13 (d)	1.05 ± 0.11 (f)
Timilia (O)	134.24 ± 10.36 (b)	4.45 ± 0.09 (a)	1.56 ± 0.04 (a)
Claudio (M)	93.68 ± 2.8 (c)	3.83 ± 0.20 (c)	1.28 ± 0.05 (cd)
Svevo (M)	126.28 ± 2.04 (b)	3.35 ± 0.14 (d)	1.17 ± 0.07 (e)
Old	147.56 ± 14.12 (a)	3.81 ± 0.53 (a)	1.30 ± 0.18 (a)
Modern	107.56 ± 17.97 (b)	3.54 ± 0.30 (a)	1.23 ± 0.08 (a)

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