Plant Foods Hum Nutr (2017) 72:432–438
https://doi.org/10.1007/s11130-017-0645-y

ORIGINAL PAPER

Effect of Genotype on the Sprouting of Pomegranate (*Punica granatum* L.) Seeds as a Source of Phenolic Compounds from Juice Industry by-Products

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Published online: 2 November 2017

Abstract Pomegranate (*Punica granatum* L.) fruits are used mainly by the juice industry, for which seeds are a by-product to be disposed of, though they could potentially be a source of bioactive compounds. In this work, germination (total germination percentage, G; mean germination time, MGT; time to reach 80% of germination, TG80; seedling shoot length, fresh weight and dry matter), and nutritional value (total phenolics, TP; total flavonoids, TF; total non-tannins, TNT; antioxidant activities) of pomegranate seeds and sprouts were determined on four commercial pomegranate cultivars (Akko, Dente di Cavallo, Mollar de Elche and Wonderful). Seeds were removed from ripe fruits and incubated in plastic trays containing sterile cotton wetted with distilled water. Sprout shoots were harvested when they reached the complete cotyledon expansion, *i.e.*, the ready-to-eat stage. Akko showed the best germination performance (G = 98%; MGT = 14 days after sowing, DAS; TG80 = 16 DAS), followed by Mollar de Elche. Sprouting dramatically increased TP, TF, TNT and antioxidant activity in all genotypes, with the highest values recorded in Mollar de Elche and Dente di Cavallo. Overall, based on germination performance, Akko and Mollar de Elche would be the best cultivars for sprouting. Sprouting pomegranate seeds appears to be a suitable way of utilizing by-products of the juice industry to obtain bioactive compounds.

Keywords Sprouts · Phytochemicals · Bioactive compounds · ABTS · DPPH · FRAP

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) sodium salt
DAS	Days after sowing
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Dry weight
FRAP	Ferric reducing antioxidant power
G	Germination percentage
GAE	Gallic acid equivalent
MGT	Mean germination time
PUFA	Polyunsaturated fatty acids
TE	Trolox equivalent
TF	Total flavonoids
TG80	Time to reach 80% germination
TNT	Total non-tannins
TP	Total phenolics

Introduction

The pomegranate (*Punica granatum* L.) is an ancient fruit, widely consumed for thousands of years due to its therapeutic properties and beneficial effects against several diseases, such as microbial infections, gastro-intestinal, cardiovascular, and respiratory pathologies [1, 2]. Currently, the fruit is mainly used for juice, and the seeds are a waste-product. Pomegranate seeds are rich in nutrients (*e.g.*, a high PUFA content in the lipid fraction) and phytochemicals (*e.g.*,

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polyphenols) [3–5], *i.e.*, secondary metabolites of plants providing several benefits for human health, like antioxidant activity [6]. It has been reported that the use of pomegranate seeds as dietary supplement might prevent DNA damage [7], reduce menopausal symptoms [8], and lower the risk of obesity, type 2 diabetes [9, 10], and cancer [8]. On the other hand, the consumption of pomegranate seeds by humans is hampered by their woody texture, astringency and bitter taste. Several studies have investigated feasible and cheap ways to convert pomegranate seeds into value-added products [4, 5, 11, 12] including the production of sprouts for human consumption [13].

Sprouting is a new trend in healthy food and nutraceutics, because it provides edible seedlings with reduced anti-nutrient and increased phytochemical contents [14]. While sprouts of many herbaceous species are sold for human consumption [15], the potential for fruit-tree species has not been explored, and the seeds often represent a waste-product. Sprouting requires seeds with no dormancy and rapid germination, which is not common in fruit-tree species. Pomegranate seeds, however, germinate quite easily without any pre-treatment, as observed in control treatments (*i.e.*, non pre-treated seeds) of some experiments [16, 17]. At present, pomegranate sprouts have been studied only by Vinokur et al. [13], who reported on their high nutritional value. This suggests the potential perspectives in the use of pomegranate seeds to obtain high-value food while reducing the fruit-juice industry waste. However, the germination performance and phytochemical content of sprouts in pomegranate, as in any plant species, may depend on the genotype [18]. An adequate study should consider both common cultivars worldwide, because of their importance in the fruit-juice industry, as well as local cultivars, where lower selective pressure might have prevented the loss of high-value compounds.

The aim of this work was to study widespread and local pomegranate genotypes for germination performance and the phenolic content and antioxidant activity of sprouts.

Materials and Methods

Plant Material and Sprouting

Pomegranate (*Punica granatum* L.) seeds were obtained from ripe fruits of four cultivars (Akko, Dente di Cavallo, Mollar de Elche, and Wonderful), provided by Linoci Farm (Apulia Region, Southern Italy) in September 2015. Arils were squeezed by hand and washed with tap water. After removing residual pulp, an additional washing with distilled water was performed. Seeds were dried at 30 °C for one week and stored at 4 °C until sprouting. Some seeds from each cultivar were frozen in liquid nitrogen and stored at −80 °C until chemical analysis, which was performed in duplicate.

A preliminary germination test was performed with two replicates of 100 seeds each for any of the four cultivars to determine the total germination percentage (G), the mean germination time (MGT), and the time to reach the 80% of germination (TG80). Seeds were incubated in plastic trays containing sterile cotton wetted with distilled water. The trays were incubated in a growth chamber at 20 °C in a light:dark regime of 16:8 h. Each tray was weighed daily to measure the water loss by evaporation, considering dry matter change to be negligible. Distilled water was added to trays in order to restore initial tray weight, *i.e.*, initial water content. The number of germinated seeds was recorded daily throught 8 weeks from the start of the incubation. MGT was calculated according to Ellis and Roberts [19].

The sprouting experiment was carried out by incubating 10 g of seeds per tray with the same procedure and conditions used for the germination test. Treatments (*i.e.*, the four cultivars) were laid down according to a completely randomized design with four replicates (trays). The ready-to-eat stage of sprouts was assumed to be between the complete cotyledon expansion and just before the emission of the first true leaf (*i.e.*, a time interval of a few days), according to Vinokur et al. [13]. Since germination time varied among cultivars and within each cultivar, sprouts reached the ready-to-eat stage at different times. For chemical analysis, sprouts were harvested when each cultivar had about 30% of sprouts ready-to-eat, since this provided more the enough material. Thus, harvest dates were 17 DAS for Akko, 18 DAS for Mollar de Elche, and 29 DAS for Dente di Cavallo and Wonderful. Only shoots were collected, discarding roots. Fresh weight and length of shoots were measured on a subsample of 10 sprouts per replicate. The remaining shoots were then harvested regrouping replicates two by two. The plant material was frozen in liquid nitrogen and stored at −80 °C until the analysis, which was performed in duplicate for each sample. Oven dry weights of seeds and shoots were determined in duplicate following the guidelines of the AOAC method 967.03 [20].

Chemicals

Methanol, n-Hexane, acetone, hydrochloric acid (37% *w/v*), sodium carbonate, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) sodium salt (ABTS), potassium persulfate and 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of an analytical grade.

Extraction and Measurement of Phenolic Compounds

The extraction of phenolic compounds was performed according to Yasoubi et al. [21] with some modification. Seeds (50 mg) were ground and mixed with 10 mL of n-hexane at

room temperature. After 1.5 h the n-hexane was removed by filtration and residual defatted seed pellets were kept. The defatted seed pellet and frozen sprouts (shoots) (100 mg) were mixed with 10 mL of pure acetone and homogenized with Ultra-Turrax for 1 min in ice, alternating 30 s of homogenization and 30 s pause to prevent the material from heating. The phenolic extracts were centrifuged at 1000 *g* for 10 min and the supernatants were collected and frozen at −20 °C for all tests.

The content of total phenolics (TP) was performed following the method of Singleton and Rossi [22] with phosphomolybdic – phosphotungstic acid reagent (Folin-Ciocalteu reagent). An aliquot (0.4 mL) of phenolic extract was mixed with 2 mL of Folin-Ciocalteu reagent (1:10) and 1.6 mL of 7.5% sodium carbonate (Na_2CO_3). After two hours the absorbance was read at 765 nm.

The contents of total flavonoids (TF) and non-tannins (TNT) were calculated by subtracting from the total phenolic content (TP) the total non-flavonoid and tannic contents, respectively, both measured following the method of Floridi et al. [23]. Briefly, non-flavonoids were extracted by mixing an aliquot (1 mL) of phenolic extract with hydrochloric acid 1:4 (*v*/v) (1 mL) and formaldehyde (0.5 mL). The mixture was kept at room temperature for 24 h. Tannins were extracted by mixing an aliquot (1 mL) of phenolic extract with methylcellulose (0.2 mL), ammonium sulfate (0.4 mL) and distilled water (0.4 mL). The mixture was centrifuged at 1000 *g* for 15 min. As described above for TP, aliquots (0.4 mL) of extracts, either for non-flavonoids and tannins, were mixed with Folin-Ciocalteu reagent (1:10) and 7.5% sodium carbonate (Na_2CO_3), and the absorbance was read at 765 nm after two hours. Gallic acid was used as a standard and results were expressed as mg gallic acid equivalent (GAE) g^{-1} DW of sample.

Antioxidant Activity Measurement

Antioxidant activity describes the ability of redox molecules in foods or biological systems to scavenge free radicals, and, in this study it was measured in both seeds and sprouts by ABTS, DPPH and FRAP tests, using Trolox as a standard for the calibration curves, according to Thaipong et al. [24]. For ABTS an aliquot (150 μL) of phenolic extract was mixed with the $ABTS^{\bullet+}$ solution (2850 μL), and the absorbance was read at 734 nm after 2 h in the dark. Before use, the $ABTS^{\bullet+}$ solution was prepared by mixing equal amounts of stock solutions of 7.4 mM $ABTS^{\bullet+}$ and 2.6 mM potassium persulfate for 12 h and the solution was then diluted adding methanol to obtain an absorbance of 1.1 ± 0.01 units at 734 nm. For DPPH assay, an aliquot of phenolic extract (150 μL) was mixed with DPPH solution (2850 μL), and the absorbance was read at 515 nm after 24 h in dark. DPPH solution was prepared by dissolving 24 mg DPPH in 100 mL of methanol and the solution was then diluted adding methanol to obtain an absorbance of 1.1 ± 0.01 units at 515 nm. For the FRAP assay, the working solution was prepared by mixing 100 mL of 300 mM acetate buffer (pH 3.6), 10 mL of 10 mM TPTZ solution (2, 4, 6-tripyridyl-s-triazine) in 40 mM HCl, 10 mL of $FeCl_3 \cdot 6H_2O$ solution and 12 mL of distilled water. An aliquot (150 μL) of phenolic extract was mixed with the FRAP working solution (2850 μL) and warmed at 37 °C, at dark, for 30 min. The FRAP reaction mixture of samples was read at 593 nm. Results of all tests were expressed in μmol Trolox equivalents (TE) g^{-1} DW of sample.

Statistical Analysis

All data were analysed by one-way ANOVA. Means were compared by Fisher's least significance difference (LSD) at $P < 0.05$. The R statistical environment [25] was used for the analysis.

Results and Discussion

Germination and Sprout Growth

Germination and sprout growth are reported in Table 1. G was over 80% in three out of four cultivars, with Akko showing the highest value (98%) and Dente di Cavallo the lowest one (65%). Data on germination substantially agree with literature on pomegranate [16, 17, 26], despite using different genotypes and germination conditions aimed at obtaining edible sprouts: for example, we used cotton as substrate because it guarantees long lasting seed wetting and clean sprout harvest, is highly available, cheap and microbiologically safe (compared to vermiculite, perlite or peat). All pomegranate cultivars were able to germinate without any pre-treatment. Previous work used cold stratification [16, 17, 26], warm + cold stratification [26] or submersion of seeds in sulphuric acid [17, 26] to break non-deep physiological dormancy [26] and enhance germination. We chose to apply no pre-treatment in order to evaluate whether pomegranate seeds are suitable for home sprouting, which needs to be simple, and to avoid hazardous substances like sulphuric acid. MGT was about two weeks in Akko or three days longer in Mollar de Elche and almost 4 weeks in Dente di Cavallo and Wonderful. However, TG80 was low only in Akko (16 DAS), much higher in Mollar de Elche and Wonderful. Therefore, Akko would be the easiest to sprout, followed by Mollar de Elche, while Wonderful and particularly Dente di Cavallo appear less suitable for sprouting and would increase the risk of microbiological contamination.

Table 1 Total germination % (G), mean germination time (MGT), time to reach 80% germination (TG80), sprout shoot length, fresh weight and dry matter of four pomegranate (*Punica granatum* L.) cultivars (Akko, Dente di Cavallo, Mollar de Elche and Wonderful)

Cultivar	Germination indexes			Shoot growth indexes		
	G (%)	MGT (DAS)	TG80 (DAS)	Length (mm)	Fresh weight (mg)	Dry matter (%)
Akko	98 (1.0)	14.1 (0.14)	16.0 (2.00)	21.3 (0.05)	47.4 (0.11)	15.9 (0.18)
Dente di C.	65 (5.0)	27.5 (1.89)	not reached	20.4 (0.57)	43.4 (1.26)	19.4 (0.31)
Mollar de E.	90 (1.0)	16.5 (0.26)	34.0 (2.00)	17.2 (1.02)	39.8 (0.18)	17.7 (0.29)
Wonderful	84 (1.0)	28.2 (1.88)	43.5 (2.50)	25.3 (0.33)	51.6 (0.09)	17.4 (0.60)

Standard errors in brackets

At harvest, cultivars did not differ markedly in sprout growth: shoot length ranged from 17 to 25 mm and fresh weight from 40 to 52 mg, with Wonderful having the biggest sprouts and Mollar de Elche the smallest ones (Table 1). No literature is available about the fresh weight of pomegranate sprouts. In fact, we fixed the growth stage instead of the sprouting time, because, according to Vinokur et al. [13], the best compromise for high phytochemical content and appreciable taste is when cotyledons are fully open. Also dry matter concentration of shoots was similar among cultivars (16–19%).

Phenolic Content and Antioxidant Activity

In seeds, TP ranged from 1.84 (in Akko) to 4.67 (in Mollar de Elche) mg g^{-1} DW (Fig. 1 left). These values were generally higher than those reported by Pande and Akoh [4] and markedly lower than those reported by Elfalleh et al. [27]. Such differences should not be surprising, given the different genotypes, organic solvent and extraction method used. We analysed only the hydrophilic fraction but this is far greater than the lipophilic one (*i.e.*, that extracted with hexane) in seeds [28]. In addition, some authors expressed seed phenolic content on a fresh weight basis without reporting the dry matter concentration [3, 29]. Our choice to report results on a dry weight basis was intended to allow the comparison between seeds and sprouts, which have a very different dry matter concentration (around 94% in seeds vs 16–19% in sprouts). In sprouts, TP was on average 38-fold compared to seeds (Fig. 1 right). The highest TP was recorded in sprouts of Dente di Cavallo and Mollar de Elche. The increased phenolic content of sprouts relative to seeds was also observed by Vinokur et al. [13].

Both in seeds and sprouts, about 99% of TP was represented by flavonoids (data not shown), therefore results on TP stand also for TF. Previous work demonstrated that phenolic acids [3, 4] or phytoestrogens [5] are only a minor part (about 5%) of total phenolics in seeds. This could explain the lack of non-flavonoids we observed in sprouts. However, Vinokur et al. [13] found ellagic and gallic acids to be even higher than total flavonoids. Such a contrasting result can be only partly explained by differences in the genotype and methodology, and thus needs further investigation.

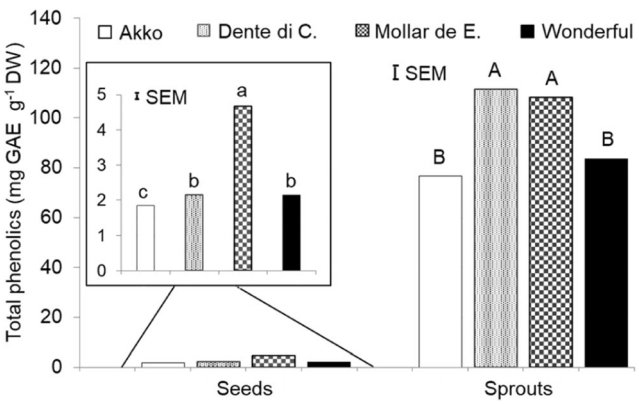

Fig. 1 Total phenolic content (mg GAE g^{-1} DW) in seeds (left) and sprouts (right) of four pomegranate (*Punica granatum* L.) cultivars (Akko, Dente di Cavallo, Mollar de Elche and Wonderful). SEM is the pooled standard error of the means. Different letters (lower case within seeds; upper case within sprouts) indicate significant differences at $P < 0.05$ (Fisher's LSD)

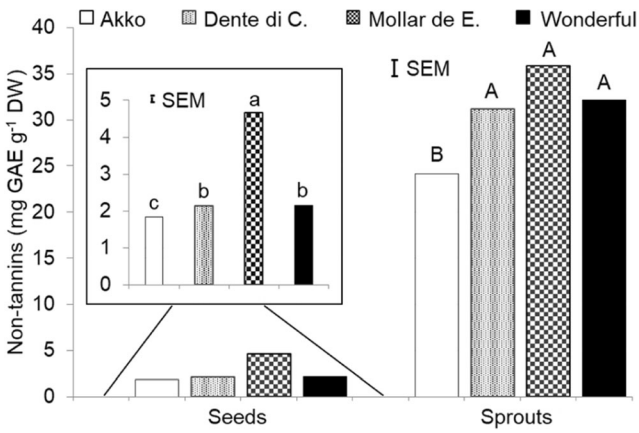

Fig. 2 Total non-tannic content (mg GAE g^{-1} DW) in seeds (left) and sprouts (right) of four pomegranate (*Punica granatum* L.) cultivars (Akko, Dente di Cavallo, Mollar de Elche and Wonderful). SEM is the pooled standard error of the means. Different letters (lower case within seeds; upper case within sprouts) indicate significant differences at $P < 0.05$ (Fisher's LSD)

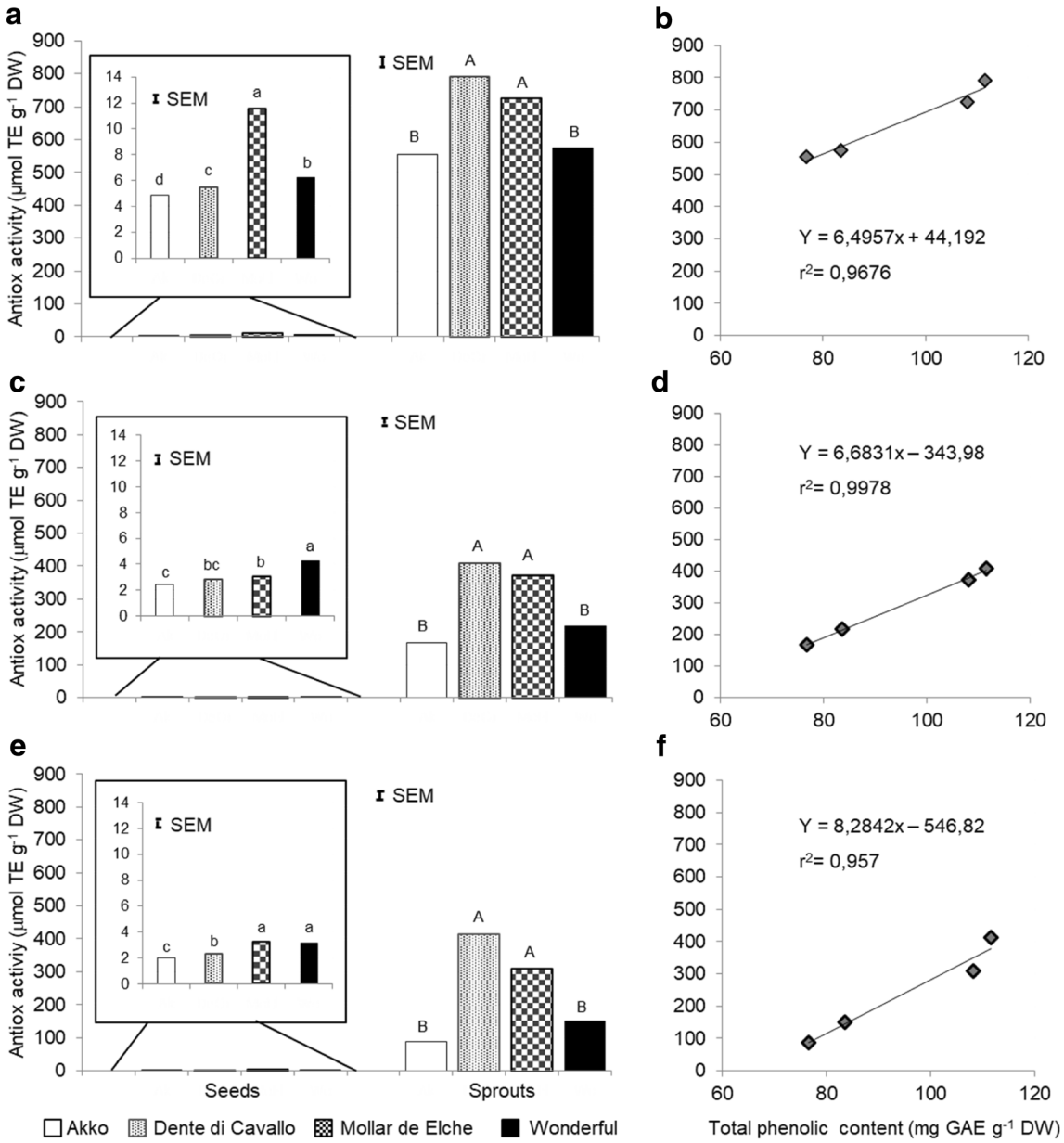

Fig. 3 Antioxidant activities measured by ABTS (**a**), DPPH (**c**) and FRAP (**e**) tests (µmol TE g^{-1} DW) in seeds (left) and sprouts (right) of four pomegranate (*Punica granatum* L.) cultivars (Akko, Dente di Cavallo, Mollar de Elche and Wonderful) and correlations with total phenolic content in sprouts (**b**, **d**, **f**, respectively). SEM is the pooled standard error of the means. Different letters (lower case within seeds; upper case within sprouts) indicate significant differences at $P < 0.05$ (Fisher's LSD)

Non-tannins in seeds represented almost all of TP (Fig. 2 left). Sprouting increased the content of non-tannins up to 12-fold but decreased their proportion in total phenolics to about one third (Fig. 2 right). Cultivars did not differ in TNT of sprouts, except for Akko, which showed a significantly lower value (Fig. 2 right). The complementary increase of tannins from nil to about two thirds might represent a means to face adverse conditions during germination and attacks from predators and microorganisms, as suggested for procyanidins (a sub-class of proanthocyanidins) [30, 31]. The presence of tannins in sprouts is not necessarily negative. Although they reduce the absorption of some metal ions, proteins and polysaccharides [31, 32], they also imply benefits for human health like antioxidant activity, protection against stomach and duodenal tumours, and anti-diarrhoea, anti-inflammatory and antiseptic properties [32]. In some previous works on pomegranate, tannins were not measured, while in other works only specific classes were measured (*e.g.*, hydrolizable tannins or condensed tannins or ellagitannins) and the methodology was different [3, 4, 13, 27, 29].

Figure 3 shows antioxidant activities as ABTS (Fig. 3a) DPPH (Fig. 3c) and FRAP (Fig. 3e) tests for seeds (left) and

sprouts (right). Antioxidant activities of seeds did not differ much between cultivars except for a higher value in the ABTS test for Mollar de Elche, but absolute values were too low to be relevant. In sprouts, the three tests showed antioxidant activities 90 to 100 times higher than in seeds, on average over the four cultivars. In all tests, Dente di Cavallo and Mollar de Elche showed the highest values. Linear correlations were found between antioxidant activities and TP, which were positive and strong for sprouts (Fig. 3b–f), much weaker for seeds (data not shown).

Among the possible approaches that can be followed to measure the antioxidant activity, we used tests involving the reduction of a coloured oxidant (ABTS and DPPH) and the electron transfer (FRAP) caused by antioxidants in the sample. The three methods were reported by Thaipong et al. [24] to give comparable results in guava fruits, and for this reason they can be discussed together. Our findings for ABTS in seeds appear in line with those of Elfalleh et al. [27], taking into account the different extraction solvent. Moreover, notwithstanding the different methods, antioxidant activities for seeds were found to be very low also in works by Pande and Akoh [4] and Jing et al. [3]. The absence of a positive linear correlation between antioxidant activities and total phenolics in seeds (data not shown) suggests that other molecules are involved. The dramatic increase of antioxidant activity from seeds to sprouts, as measured with three different methods, is probably the most meaningful finding of our work. There is no previous report of such an increase. Vinokur et al. [13] measured only ABTS and only in sprouts, but not in seeds. Differences in antioxidant activities among cultivars were important and clearly related to total phenolics, as suggested by the strong positive correlations in Fig. 3. However, total phenolics and antioxidant activity were remarkable in all cultivars, including those with the lowest values (Akko and Wonderful). Thus, for sprouting purposes, the germination performance (G, MGT and TG80) should be the principal aspect to consider. On this basis, Akko and Mollar de Elche would probably be the best choices.

Conclusions

Results indicate that pomegranate seeds have good germination characteristics, making them suitable for sprouting. Sprouting resulted in a 38-fold increase of total phenolic content and over 90-fold increase in antioxidant activity compared to seeds. Thus, pomegranate sprouts may represent a valuable source of phenolics, the content of which strongly correlated with antioxidant activity. Cultivars differed for germination characteristics, phenolic content and antioxidant activity. Based on these traits, the best cultivars for sprouting purposes were Akko (best germination and good nutritional value) and Mollar de Elche (good germination and best nutritional value).

Acknowledgements We gratefully acknowledge Mr. Silvano Locchi for technical assistance in the seed lab and Mr. Giacomo Linoci for providing the pomegranate fruits used in this study. We thank Darcy Gordon (PhD) for language editing and for critical review of the manuscript.

Compliance with Ethical Standards

Conflict of Interest Authors declare no conflict of interest.

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