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Pomegranate iron (III) reducing antioxidant capacity (*iRAC*) compared to ABTS radical quenching

Hau Ching Wan [1], Richard Owusu-Apenten [1,3]*, Poonam Singh Nigam[1], and Bushra Sultana [2]

[1] School of Biomedical Sciences, Faculty of Life and Health Sciences, University of Ulster, Cromore Road, Coleraine, BT52 1SA, UK.

[2] Department of Chemistry and Biochemistry, University of Agriculture, Agriculture University Road, Faisalabad, Zip Coad 38000, Pakistan

[3] Department of Clinical Sciences and Nutrition, Faculty of Medicine, Dentistry and Life Sciences, University of Chester, Parkgate Road, Chester, CH1 4BJ, UK; owusuapenten@yahoo.com

* Correspondence: owusuapenten@yahoo.com

Abstract: Pomegranate juice (PJ) has total antioxidant capacity (TAC) which is reportedly higher compared to other common beverages. This short study aimed to evaluate the TAC of commercial PJ and pomegranate fruit in terms of a newly described iron (III) reducing antioxidant capacity (*iRAC*) and to compare with ABTS free radical quenching activity. Commercial PJ, freeze-dried pomegranate, and oven dried-pomegranate were analyzed. The total phenols content (TPC) was also assessed by the Folin-Ciocalteu method. The calibration results for *iRAC* were comparable to ABTS and Folin-Ciocalteu methods in terms of linearity (R2 > 0.99), sensitivity and precision. The TAC for PJ expressed as trolox equivalent antioxidant capacity (TEAC) was 33.4 ± 0.5 mM with the *iRAC* method and 36.3 ± 2.1 mM using the ABTS method. For dried pomegranates, TAC was 89–110 mmol/100g or 76.0 ± 4.3 mmol/100 g using *iRAC* and ABTS methods, respectively. Freeze-dried pomegranate had 15% higher TAC compared with oven-dried pomegranate. In conclusion, pomegranate has high TAC as evaluated by the *iRAC* and ABTS methods, though variations occur due to the type of cultivar, geographic origin, processing and other factors. The study is relevant for attempts to refine food composition data for pomegranate and other functional foods.

Keywords: Pomegranate; Fruit Juice; Total Antioxidant Capacity; ABTS; *iRAC*; Total Phenols Content; Folin-Ciocalteu; Food Composition; Databases

1. Introduction

Pomegranate (*Punica granatum* L.) is an ancient food used as a traditional remedy against a variety of conditions including microbial infections. Pomegranate is perceived as a "superfood" due to its high antioxidant capacity [1-8]. Current databases show pomegranate juice (PJ) possesses total antioxidant capacity greater than many other beverages [9-12]. Although the total antioxidant capacity for pomegranate from different countries were reported, only few publications deal with commercial PJ as sold in the market [9, 13]. The effect of drying on pomegranate seed, arils and peels were examined [14, 15], but oven-drying and freeze-drying effects on the total antioxidant capacity of whole pomegranate fruit has not been compared.

The aims of this short study were, to reevaluate the total antioxidant capacity of pomegranate fruit and commercial PJ using a newly described method for assessing iron (III) reducing antioxidant capacity (*iRAC*) [16] and to compare results with the ABTS method [17].

Total phenol content (TPC) was evaluated also as another well characterized antioxidant method [18]. The study is significant for current attempts to refine food composition data for pomegranate and other functional foods for improved nutrition applications, product development or international trade [19].

2. Materials and Methods

2.1. Preparation of samples and antioxidant standard

Pomegranate fruit (Hicaz variety, Turkey) and commercial PJ (POM Wonderful 100% PJ; POM Wonderful LLC, UK) were purchased from a large supermarket in the United Kingdom (UK). Unpeeled pomegranate was washed, diced using a stainless steel knife and divided into two portions. One portion of pomegranate was oven dried at 80°C overnight and another was frozen at -80 °C for 48 hrs., then freeze-dried for 48hrs using the HETO Power Dry PL6000 instrument (ThermoFisher, Ltd., UK). The dried pomegranate samples were ground using a blender (DeLonghi Coffee Grinder; Type KG40 EXA) and the resulting powders (5 g) were extracted by stirring with 100ml of solvent (40:60 v/v methanol: water) for 2 hours. The pomegranate solvent extract was centrifuged using a microcentrifuge (@11, 000rpm for 5min) and the supernatant stored at -18 °C. The solids content for PJ was determined by drying a known volume and weighing the residue. Gallic acid and trolox reference compounds were prepared as 1000 µM solution and diluted to 500 µM, 250 µM, 125 µM, and 62.5 µM daily before use. Pomegranate extract and PJ were diluted (25-100 fold) before analysis.

2.2. Iron (III) Reducing Antioxidant Capacity (iRAC) Assay

The *iRAC* reagent comprised 20 mg of ferrozine dissolved with 18ml of Tris buffer (0.1M, pH 7.0) or potassium acetate buffer (0.1m, pH 4.5) and mixed with 8mg of ferric (III) ammonium sulphate (8 mg) dissolved with 2 ml of deionized water. Typically, the final *iRAC* working solutions was prepared after the sample array to be analyzed was ready; 20 µL of pomegranate extract, PJ, or reference compound (gallic acid or trolox) were added to a 96-well microplate followed by 280 µL of the *iRAC* reagent. The reaction mixtures were incubated for 30 minutes at 37°C. Absorbance was read at 562 nm (A562) using a microplate reader (VersaMax model reader; Molecular devices, Sunnydale, California, USA). Several (25, 50, 100-fold) diluted samples were analyzed to determine the optimum dilution necessary for sample absorbances to fall linear range for analysis. Final samples were analyzed on two separate occasions suing (n =) 12 – 16 wells of a microplate. For time-course measurements A562 readings were recorded at 2 minutes for 30 minutes.

2.3. ABTS Assay

The ABTS was performed as described by Walker and Everette [17] with modifications. ABTS (27.4mg) was added to 90 ml PBS buffer. Sodium persulfate (20mg/1ml PBS) was prepared separately, added to ABTS stock solution, and both were made up to 100 ml using PBS buffer. The mixture was stored in the dark for 16 hours. The ABTS·+ solution was diluted with PBS buffer to obtain an absorbance of 0.85 at 734 nm (A734) using a 1-cm conventional spectrophotometer (Ultrospec 2000 UV/Visible spectrophotometer, Pharmacia Biotech. Ltd, Sweden). Thereafter, 20 µL of samples or reference compounds (trolox) were added to 96-well microplate followed by 280 µL ABTS·+ solution. The plates were incubated in the dark for 30 minutes at 37°C and A734 was recorded using a microplate reader. Pre-diluted samples were analyzed on two separate occasions using (n=) 12 – 16 wells of a microplate.

2.4. Folin-Ciocalteu Assay for Total phenols

The Folin-Ciocalteu method of Singleton et al. [18] was used for TPC determination, with minor modification. Antioxidant standards or samples (50 µL) of were added to microcentrifuge tubes with 100 µL Folin-Ciocalteu reagent and 850 µL of sodium carbonate solution. The samples were vortexed briefly and incubated for 20 minutes at 37-40°C. Thereafter, 200 µL of the reacted

samples were transferred to a 96-well microplate (x4 200 µL per sample) and absorbance was read at 760 nm (A760) using a microplate reader.

2.5. Data analysis and statistical analysis

Microplate readouts were transferred to Excel for calculations and graphing. Calibration graphs for *iRAC*, ABTS or Folin-Ciocalteu assays were generated by plotting absorbances changes (ΔA) corrected for the sample-blank (B1) and zero-reagent blank (B2), .e.g. ΔA = A-B1+B2) on the y-axis. The concentration of analyte (mol/l) in the assay vessel was plotted on the graph x-axis. For the ABTS assay ΔA is A760 for ABTS reagent minus A734 for antioxidant samples. Calibration parameters (e.g. molar absorptivity, the minimum detectable concentration, upper limit of detection, regression coefficient) were determined by fitting a straight lines of (y=mx) to the data, where m is the slope. The total antioxidant capacity for samples were determined from absorbance changes (ΔAs) using Beer's relations (Eq. 1-3) ;

$$\text{TAC (mg-EqAC/ 100)} = \frac{\Delta As}{m} * \frac{V_a}{S_V} * \left(\frac{V_{ex}}{W}\right) * D_F * 10^5 * F_W \quad (1)$$

$$\text{TAC (mmol –EqAC)/ 100g} = \frac{\Delta As}{m} * \frac{V_a}{S_V} * \left(\frac{V_{ex}}{W}\right) * D_F * 10^5 \quad (2)$$

$$\text{TAC (mmol –EqAC)/ liter} = \frac{\Delta As}{m} * \frac{V_a}{S_V} * D_F * 10^6 \quad (2)$$

where, TAC = total antioxidant capacity, m = slope for the trolox calibration graph, Va = assay volume (µl; x10^{-6}L), S_V = samples sip volume assayed (µl; x10^{-6}L), DF = dilution factor for samples before analysis (1 if undiluted), Vex = total volume of pomegranate extract, FW = formula weight for the reference antioxidant (g/mole), W = dry weight of food sample (g). For the PJ samples W/Vex is the solid content as determined by drying.

Statistical significance was tested by using one-way ANOVA with Turkey post-hoc testing for separation of means. Significant differences were noted with $P<0.05$. All analyses were carried out using IBM SPSS Statistics 24.

3. Results

3.1. Calibration results of, iRAC, ABTS and Folin-Ciocalteu methods

The assay time was fixed at 30 minutes based on the time-course of A562 readings for the *iRAC* procedure (Figure 1); the other assays were also conducted over 30 minutes. Calibration responses for *iRAC,* ABTS and Folin-Ciocalteu assays (Table 1) were linear with the regression coefficient (R^2) >0.99. Other calibration parameters for *iRAC* and ABTS assays were broadly similar with respect to, lower limit of detection (LLD) and upper limits of detection (ULD), but the assay sensitivity (slope) and the precision (CV %) were higher in the former case (Table 1).

Table 1. Calibration parameters for *iRAC,* ABTS and Folin-Ciocalteu assays

Method*	Slope(M^{-1} cm^{-1})⊥	R^2	LLD-ULD(µM)	CV (%)
iRAC (TX)	53397±667	0.9993	2.8-1000	4.0
ABTS (TX)	25466±378	0.9993	5.0-250	9.8
Folin (GA)	17207±315	0.9986	0.8-1000	3.1

⊥ Calibration slopes adjusted for an optical pathlength of 0.7 cm. Data shows means ±SD for triplicate experiments, with n = 16 data points.*Trolox (TX) or gallic acid (GA). LLD = lower limit of detection, ULD = upper limit of detection, CV = coefficient of variation.

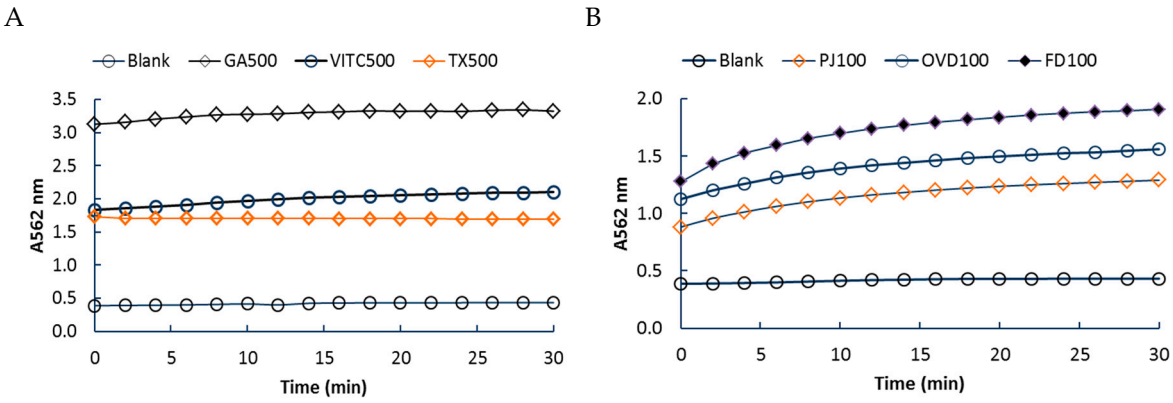

Figure 1. Time-course for absorbance readings at 562 nm (A562) for iron (III) reducing antioxidant capacity (*iRAC*) method; (A) Gallic acid (GA500), vitamin C (VITC500) or trolox (TX500) were reference compounds (500uM), B. Timed response for pomegranate samples, PJ100 = pomegranate juice, OVD100= oven dried sample, FD100 = freeze dried sample extracts diluted 100x before analysis.

3.2. Total antioxidant capacity for pomegranate samples

The total antioxidant capacity for PJ was 33.4±0.5 mM or 24.5±0.7mM (mol trolox equivalents per liter of PJ) determined by the *iRAC* method at pH 7.0 and pH 4.5, respectively. The ABTS assay for PJ at pH 7.0 showed a total antioxidant capacity was 36.3±2.1 mM (Figure 2A).

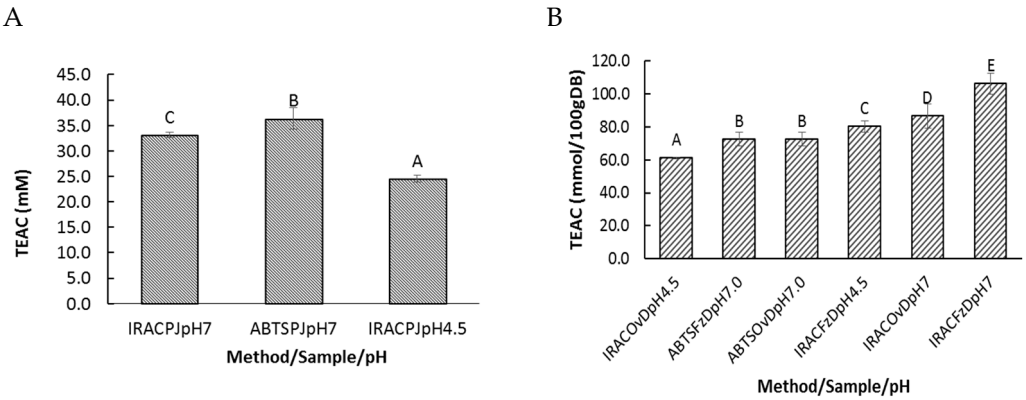

Figure 2. Total antioxidant capacity for pomegranate samples. (A) Pomegranate Juice (PJ) was diluted 100x and analyzed by *iRAC* method at pH 7.0 & 4.5 (IRACPJpH7, IRACPJpH4.5) or ABTS method (ABTSPJpH7.0). (B) Whole pomegranate fruit was freeze dried (FzD) or oven-dried (OvD) and blended to powder, extracted with methanol/water (60/40v/v) and analyzed (iRACOvDpH4.5, ABTSFzDpH7.0 etc.). Data shows total antioxidant capacity (mean ±SD) for n = 12 points. Bars with different letters show significant differences ($p<0.05$).

The method of drying and type of assay affected values for total antioxidant capacity (Figure 2B). Freeze-dried pomegranate showed a higher *iRAC* response compared with oven-dried pomegranate, but no differences were observable using the ABTS assay. The order of total antioxidant capacity for whole pomegranate fruit was, freeze-dried pomegranate > oven-dried pomegranate and also *iRAC* (pH 7.0) ≥ ABTS (pH7.0) > *iRAC* (pH 4.5).

3.3. Total phenols content of pomegranate samples by Folin-Ciocalteu assay

Values for the TPC ranged from 5.8% to 6.9% GAE for dried pomegranate (Table 2). The order of decreasing values for TPC was, freeze-dried pomegranate > oven-dried pomegranate > PJ

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on per dry weight basis. A one-way ANOVA test showed the TPC for freeze-dried and oven-dried pomegranate samples were significantly different ($p<0.05$). Expressed on as is basis the TPC for PJ was 250±12 mg GAE/100ml.

Table 2. Total phenol content for pomegranate samples per dry weight basis⊥

Sample	TPC(mg GAE/100g DB)
Oven dried Pomegranate	5830 ± 356 (A)
Freeze dried Pomegranate	6916 ± 200 (B)
POM Wonderful 100% PJ	1559 ±74 (C)

⊥ DB = Dry weight basis for powders extracted by methanol/water (40:60) and analyzed Juice was analyzed after diluting x100 fold. TPC is mean± SD for 3-replicate experiments, with n= 18 data points. Letters in different rows shows significant differences ($p<0.05$)

4. Discussion

The health benefits of PJ are attributed partly to its high antioxidant capacity and TPC [1-8]. Currently, pomegranate is listed as one the highest sources of dietary antioxidants amongst many beverages including red wine, green tea, grape, apple, orange or cranberry juices [9-12]. Nonetheless, published total antioxidant capacity values for pomegranate vary considerably (Table 3). In this paper, we examined total antioxidant capacity for pomegranate in terms a newly described iron (III) reducing antioxidant capacity [16] and compared values with the ABTS method [17]. As per AOAC guidelines, total antioxidant capacity values were expressed as trolox equivalent antioxidant capacity (TEAC) to enable comparisons [21]. The recommended units for TEAC are mmol/l (mM) for liquids (PJ) or mmol/100g for solid samples [21]. The Folin-Ciocalteu assay was applied also as another well-standardized assay for total phenols and antioxidants from plant derived foods [18].

Table 3. Reported total antioxidant capacity and TPC values for Pomegranate juice ↕

Sample	TAC (mM)	TPC (mg GAEC/ 100 ml)	Ref.
POMW100% PJ*	41.6±1.8	380±20	[10]
PJ (From frozen arils)	10.0-20.0	140-212	[9]
PJ (8 Cultivars)	12.89±0.31	272±46	[24]
PJ (From frozen arils)	5.6±1.17	150±2.5	[23]
PJ (15 cultivars)	10.6-18.30	139-948	[25]

↕. TAC = total antioxidant capacity (mM) determined by ABTS method. TPC by Folin-Ciocalteu method, TS = this study; PJ = Pomegranate juice. *From US.

4.1. Total antioxidant capacity and TPC of pomegranate juice

The basic principles behind the *iRAC* method is that an excess amount of iron (III) is reduced to iron (II) by antioxidants. The concentration of iron (II) is then detected with ferrozine as a complexing agent [16]. The *iRAC* method is a modification of the FRAP method [22] which is performed at pH 7.0 rather than pH 3.6; the *iRAC* method was also useable at pH 4.5 (Fig 2). Interestingly, PJ total antioxidant capacity values were ~8% lower using the *iRAC* method compared with the ABTS method, whilst the former was ~20% higher overall after the dried pomegranate samples are also considered (see below).

The total antioxidant capacity for commercial PJ in this study (33-34 mM) was higher than values [24] cited for PJ obtained from eight pomegranate cultivars (Table 3). However, our sample for POMW 100%PJ manufactured in the UK had 50% lower total antioxidant capacity compared

another POMW 100%PJ brand produced in California (USA) 10 years ago [10]. The former PJ contained 120mg vitamin C per liter (0.7mM) which is ~2% of the total antioxidant capacity.

Total phenols content values for commercial PJ (250±12 mg GAE/100ml; this study) were within the range reported previously (Table 3). In general, TPC for PJ prepared from whole fruit is higher than the TPC for PJ extracted from frozen arils or peeled pomegranate (Table 3). Processing whole fruit led to the transfer of hydrolysable tannin from pomegranate peels to the PJ [9]. An estimated 29% of TPC for pomegranate was associated with PJ compared with 69% associated with pomegranate peel [26]. Significant process losses for TPC (and antioxidant capacity) were reported also when manufacturing pomegranate nectar from whole fruit [20]; under such circumstances about 37% TPC was associated with pasteurized PJ compared with 47% associated with peel [20]. No TPC differences were reported for PJ extracted using organically grown versus conventionally grown pomegranate fruits [27]. Clearly, total antioxidant capacity and TPC for PJ may considerably as a result of processing factors.

4.2. Total antioxidant capacity and TPC for Pomegranate fruit

There is less data available on the total antioxidant capacity and TPC for *whole* pomegranate fruit as compared with PJ [20, 25]. In this study, whole pomegranate fruit was pretreated by dicing, freezing/ oven drying, blending to form powers, and then extracting with methanol: water (40:60%) prior to analysis. The observed total antioxidant capacity and TPC values are for whole fruit and values are also moderated by drying and the efficiency of the extraction. In other studies, fresh whole pomegranates were homogenized or macerated directly with solvent and the extract subjected to analysis before the data were adjusted for moisture content [20, 26 29]. There is been no concerted investigation to to examine whether two alternative sample treatment regimens affect the final results materially. Sometimes, whole pomegranates were also separated as, rind, flesh (core and arils) or seeds prior to analysis [4].

The total antioxidant capacity for pomegranate fruit using *iRAC* method ((72-106.3 mmol/100g DB; Figure 2B) agreed closely with values from ABTS analysis (this study) and ABTS results reported previously as 122.9 mmol/100g DB [20]. Past studies showed that total antioxidant capacity of pomegranate was strongly correlated TPC, tannins and flavonoids [4, 28].

The TPC for pomegranate samples (this study) were comparable to values reported previously (Table 1S; Supplementary data) in spite of differences in the cultivars used and processing factors (Section 4.1). Freeze dried pomegranate fruit had 15% higher TPC and 18% higher total antioxidant capacity compared with oven drying. However, past studies showed that moderate drying temperatures (55-75 °C) had no effect on TPC [15]. Some general difference in the values for TPC were noted (Table 1S; Supplementary data) with different cultivars, fruit parts (Whole fruit > Peel >>Seeds or arils) and extraction solvent choice (Methanol, Methanol: Water > Water solvent [26, 30]. The Hicaz variety of pomegranate had a high TPC but comparisons with other varieties are not possible owing to the various experimental approaches used. The TPC for pomegranate varieties declined with increasing maturation and ripening [28].

5. Conclusions

The iron (III) reducing antioxidant capacity (iRAC) for pomegranate and **juice** was similar to values for ABTS free radical quenching capacity, both expressed as TEAC units. Both the *iRAC* and ABTS assays confirm previously reported high total antioxidant capacity values for PJ. Some differences in the TAC and TPC values for pomegranate and PJ were evident due to varying cultivars and processing factors. Such results have relevance for attempts to refine food composition data for pomegranate and other functional foods.

Author Contributions

Conceptualization, ROA, PSN & BS.; Methodology, ROA, BS & PSN; Software, ROA.; Formal Analysis, HWC. Investigation, WHC. ; Data Curation, HWC.; Writing-Original HWC/ ROA, Writing, Review & Editing, ROA.; Visualization, HWC/ ROA; Supervision, ROA.; Project Administration, ROA.

Supplementary Materials: The following are available online, Table S1: Total phenol content of pomegranate samples.

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