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Comparison of different aqueous extraction methods for optimum extraction of polyphenols and *in-vitro* anti-oxidant activity from pomegranate peel

Sarbaswarup Ghosh, Jayanta Kumar Chatterjee, Banti Chalkroborty and Alok Kumar Hazra

Abstract
Pomegranate (*Punica grantum* L.) peel is the source of vast array of bioactive polyphenols that may be safely extracted using water as a solvent. Water based extraction is not only safe but also suitable for animal consumption. Objective of the present study was to compare three different aqueous extraction techniques (Continuous shaking extraction, maceration, hot water infusion) used for extraction of polyphenols and to evaluate their *in-vitro* antioxidant activity. Results demonstrated that hot water infusion method gives significant ($P<0.05$) level of antioxidant activity over others. It could be concluded that hot water infusion method of extraction is a simple, cheap and convenient method for polyphenol extraction from pomegranate peel and it might be used for further *in-vivo* antioxidant testing in animal models.

Keywords: Antioxidant, continuous shaking extraction, gallic acid, hot water infusion, hptlc, maceration, pomegranate peel

1. Introduction
Pomegranate (*Punica grantum* L.) is an ancient fruit and widely used in Indian subcontinent as traditional and folk medicine [1]. Pomegranate peel (PP) contains diverse groups of polyphenols with strong anti-oxidant activities (Malviya *et al*., 2014) [2]. In fact, PP showed much more pronounced anti-oxidant activity than its aril part (Hasan *et al*., 2018; Jalal *et al*., 2018) [3-4] and hence pomegranate peel extract (PPE) is now commercially used as ingredients in health drink and other functional foods for human consumption. PPE has also beneficial role on animal productivity and poultry meat quality (Saleh *et al*., 2017) [5]. Bioactive roles of PP are believed to be due to presence of hydrolysable tannins (HT) present in it. Earlier reports (Al-Rawahi *et al*., 2014) [6] suggested that ellagi-tanins, gallic acid and ellagic acid are mostly responsible for the remarkable *in-vitro* antioxidant activity of PP. Various authors (Wang *et al*., 2011; Singh *et al*., 2014; Masci *et al*., 2016) [7-9] attempted to optimize extraction methods of bioactive molecules from PP using water, methanol, ethanol, hydro-alcohol or hexane as solvent in hot or cold conditions. However, alcohols or hydro-alcohols are costly and toxic in nature and thus should not be used during extraction meant for use as feed additives. Water as a solvent is a good alternative for green extraction and various authors (Dar *et al*., 2015; Oleforuh-Okoleh *et al*., 2014) [10-11] described different methods for aquous extraction from peel wastes and herbs. Presently there was no comparable data to identify most simple, less labor intensive method that gives optimum yield and antioxidant activity from PP out of the different aquous extraction protocols. Therefore, present study aims to compare different aquous extraction methods using both hot and cold condition for optimum extraction of polyphenols from PP. Three methods *viz*. continuous shaking extraction (Upadhya *et al*., 2015) [12], masceration (The Ayurvedic Pharmacopoeia of India) [13] and hot infusion (Oleforuh-Okoleh *et al*., 2014) [11] were compared for yield %, total phenolic and flavonoid content, gallic acid content and antioxidant activities.

2. Materials and Methods
2.1 Collection of samples
Peel wastes of pomegranate were collected from a jelly and juice manufacturing centre located at Narendrapur situated in Kolkata, India. These waste materials were collected in sterilized plastic bins during the month of May-June'2018. Average moisture contents of the peel wastes were recorded during collection with a moisture meter (HE53, Metler Toledo, USA) at the day of collection.

2.2 Preparation of samples for extraction
After collection, peels were washed with distilled water and then air-dried under shed for five days. The peels were chopped into small pieces with a sharp scissor and then made it into coarse powder using an electrical grinder. The dried powder of pomegranate peels (PP) was packed into air-tight containers in refrigerated condition (4°C) for preparation of extracts.

2.3 Extraction methods
a) Continuous shaking extraction (T_1)
This extraction method was earlier described by Upadhya *et al.*, (2015) [12]. Briefly, 5 gram of dried PP was extracted with 100 ml of deionized water in 25°C. The mixtures were kept in an orbital shaker for 6 hours with 110 rpm. The extracts were filtered through Whatman No.1 filter paper. The filtrate was placed in dark bottles in freezer (-20°C) for further analysis.

b) Maceration (T_2)
Maceration method [10,13], widely used for the preparation of herbal drugs, was done with minor modifications. Briefly, 5 gram of dried PP was soaked with 100 ml of deionized water in a glass container for 48 hours in 25°C with occasional shaking using a glass rod. The extracts were filtered through Whatman No 1 filter paper and placed in dark bottles in freezer (-20°C) for further analysis.

c) Hot Infusion (T_3)
Preparation of PP extract by hot infusion method was done as described by Oleforuh-Okoleh *et al.*, (2014) [11] with minor modifications. Briefly, 5 gram of dried PP was placed in conical flak and 100 ml hot water (initial temperature: 70°C) was poured on it. Conical flask was tightly plugged by cotton. The extracts were filtered through Whatman No 1 filter paper after 12 hours of soaking and the filtrate was placed in dark bottles in freezer (-20°C) for further analysis.

2.4 Percentage yield of extracts
Twenty five mL extract was pipetted out to a pre-weighed petri dish and kept in the hot-air oven for at 100°C until the weight of the petri dish become constant. The weight of the petri dish was then measured. The difference in weight of the petri dish gave the yield of extract in 25 mL.

2.5 Estimation of total phenolic and flavonoid content
Total phenolic content (TPC) was measured by using Folin-Ciocalteu method [14] with slight modifications. The total phenolic content was measured against the serially diluted standard curve of gallic acid in a spectrophotometer (Shimadzu 1800-UV, Japan) and expressed in terms of gallic acid equivalent (mg of GAE/g of dry weight).Total flavonoids content (TFC) was measured according to Pal *et al*. [15] Results were expressed in mg of quercetin equivalent (mg of QE/g of dry weight).

2.6 Determination of free-radical scavenging activity by DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) test
In-vitro anti-oxidant activity was determined by DPPH assay following Szabo *et al.* [16] Radical Scavenging Activity (RSA %) was calculated by using the following equation:

Radical Scavenging Activity% = [1- (Absorbance of sample/Absorbance of blank)] X 100

RSA % values were used to calculate Inhibition Concentration at 50% (IC_{50}) values that denote the effective concentration of a sample required to decrease the absorbance at 517 nm by 50%. All measurements were performed in triplicate.

2.7 Determination of Ferric Reducing Antioxidant Power (FRAP)
The Ferric Reducing Antioxidant Power (FRAP) of PP extracts was performed based as per Benzie and Strain [17], with slight modification. Absorbance of serially diluted standard $FeSO_4$, $7H_2O$ (0.001M) was recorded after incubating it with 2ml of the FRAP solution for 30 min at 37◦C in dark chamber. Absorbance of the blue colour product (ferrous tri pyridyl triazine complex) was taken at 593 nm using a spectrophotometer (Shimadzu 1800-UV, Japan). FRAP values of peel waste extracts were obtained from the standard curve and were expressed as μM Fe (II)/mg dry material.

2.8 Determination of Total Antioxidant Capacity by ABTS (2, 2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) test
Total antioxidant capacity of the PP extracts was evaluated in tandem by ABTS assay using the Antioxidant Assay Kit (CS0790, Sigma-Aldrich) according to manufacturer's instructions. Trolox was used as the antioxidant standard. The radical scavenging activities of extracts were expressed in mM of Trolox Equivalent Antioxidant Capacity.

2.9 Quantification of gallic acid by HPTLC
Gallic acid of the samples was estimated by high performance thin layer chromatographic (HPTLC) analysis described by Khanvilkar and Chalak, (2016) [18]. The test was carried out on a HPTLC plate pre-coated with silica gel. Samples (10µl) and standards (10-25 µl) were applied on plates by Linomat 5 applicator (Camag, Switzerland). The plates were developed to a distance of 90 mm in Camag twin- trough chamber with mobile phase of Toluene: Ethyl Acetate: Formic Acid (4.5:4:0.5, *v/v/v*) in 27°C for 15 min. Afterwards, the plates were scanned for densitometry analysis in CAMAG TLC scanner (Camag, Switzerland) at λmax =280 nm. The R_f values of gallic acid was found to be 0.42. The chromatograms were finally integrated using Win CATS 4.0 computer programme.

2.10 Statistical Analysis
Data of all the parameters from the extracts (n=3 for each extraction method) were analyzed for test of significance at 5% levels by ANOVA [19]. Multiple comparisons of means were measured by Duncan Multiple Range Test using SPSS v. 19 (IBM, USA).

3. Result and Discussion
3.1 Extraction Yield
The yield of PP in different aquous extracts is shown in Table 1 where continuous shaking extraction method (T_1) had the highest extraction yield followed by hot infusion (T_2) and maceration (T_3). Significant ($P<0.05$) variation was observed among three extraction techniques.

3.2 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)
TPC was significantly ($P<0.05$) high in T_2 and it was in the order of $T_2>T_1\geq T_3$. High TPC is perhaps due to more soaking time involved in T_2 than T_1 and T_3. More soaking time invariably helped in releasing more soluble phytochemicals.

Wang *et al.* (2011) [7] reported effect of increasing temperature on extraction of total phenolic. In the present study, however, hot infusion method of extraction (T3) produced less phenolic than cold methods. Yan *et al*. (2017) [20] reported phenolic profile of six Chinese pomegranate varieties and TPC ranged from 57.66 to 155.88 mg GAE/g DW. This large variation may be attributed to many factors like variation of cultivars, ripening stage of fruit, solvent used in the extraction process, time and temperature of the extraction process, solvent-solute ratio etc.

Pomegranate peels are rich source of flavonoids like catechin, rutin, kaempferol, quercetin etc. with good nutraceutical values (Middha *et al*., 2013; Rahmani *et al*., 2017) [21-22]. In the present study, there was significant (P<0.05) variation in TFC among different extraction methods and highest flavonoid content was observed in the continuous shaking extraction method (T1) whereas least flavonoid content was observed in hot infusion method (T3). Overall, TFC results of PP in the present experiment were in agreement with previous study [23] using Indian pomegranate varieties.

Table 1: Yield%, TPC and TFC of aqueous extracts of pomegranate peel by different methods

	T_1	T_2	T_3
Yeild %	3.5±0.008^a	2.38±0.04^c	2.53±0.012^b
TPC (mg GAE/g DW)	27.66±0.176^b	30.04±0.087^a	27.55±0.023^b
TFC (mg QE/g DW)	15.23±0.153^a	12.13±0.083^b	10.92±0.09^c

Values are means ± SEM, n = 3 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the DUNCAN test.

3.3 *In vitro* anti-oxidant assays of peel waste extracts

3.3.1 DPPH Assay

The DPPH radical scavenging activity (RSA %) of T_1, T_2 and T_3 was presented in figure 1. It was evident that hot water infusion method (T_3) of extraction showed better antioxidant activity than others in higher concentrations. Antioxidant activity of PP is mainly contributed by presence of ellagi-tannins (Al-Rawahi *et al.*, 2014) [6]. Present study revealed that hot water infusion extraction method may be more capable to extract ellagi-tanins and other phenolic acids responsible for *in-vitro* antioxidant capacity of PP and it was superior green extraction method over other aqueous extractions. IC_{50} values (concentration to scavenge 50% of free radicals) of PP in different extractions were derived from regression curves of DPPH radical scavenging activities at different concentrations and presented in Table 2. Lowest IC_{50} value gives highest antioxidant activity. The hierarchical order of IC_{50} values were: $T_3>T_1>T_2$.

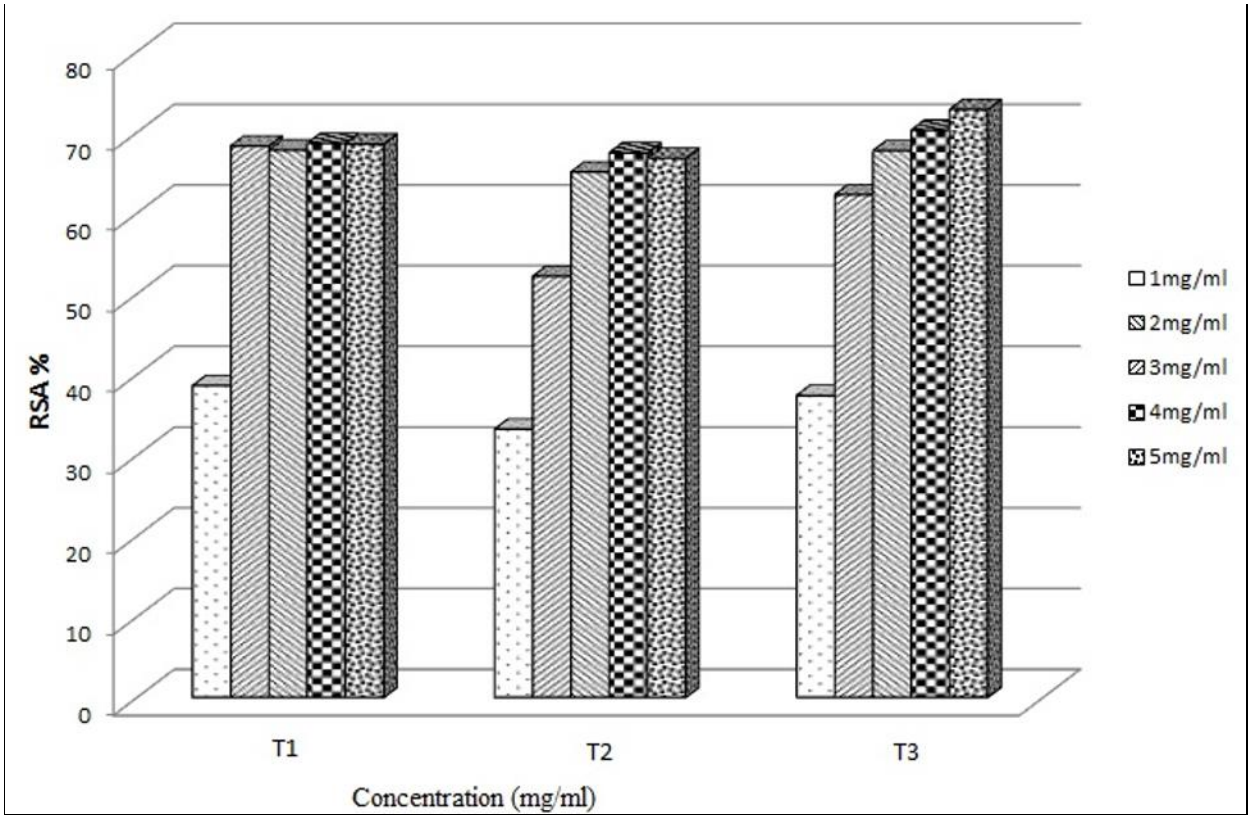

Fig 1: Evaluation of antioxidant properties by DPPH assay for different aquous extracts

3.3.2 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay is a REDOX type reaction in which a single electron is donated by anti-oxidants to reduce the colorless ferric (Fe3) ion to blue colored ferrous (Fe2) ion. FRAP assay (Table 2) revealed that antioxidant activity of T_3 was significantly (P<0.05) higher T_2.

3.3.3 Total Antioxidant Capacity by ABTS

Significant variation (P<0.05) in Total Antioxidant Capacity was observed among different extraction methods. Total Antioxidant Capacity (Table 2) was in the order of: $T_3>T_2>T_1$. The higher antioxidant potential of hot water infusion method may be due to the fact that hot water permeates into cell wall more efficiently and releases thermostable ellagi-tanins and other phenolic in the infusion.

Table 2: Antioxidant activities of aquous extracts of pomegranate peel by different methods

	T_1	T_2	T_3
DPPH IC50 (mg/ml)	1.41 ±0.026[b]	2.11±0.052[a]	0.959±0.009[c]
FRAP (μM Fe (II)/mg dry material)	194 ± 1.91[a]	159 ± 1.1[b]	195 ± 1.83[a]
Total Antioxidant Capacity (mM Trolox Equivalent)	0.952 ± 0.006[c]	1.05 ± 0.001[b]	1.07 ± 0.003[a]
Gallic acid content (% *w/w*)	2.32 ± 0.003[b]	2.46 ± 0.01[a]	2.03 ± 0.003[c]

Values are means ± SEM, n = 3 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the DUNCAN test.

3.4 Gallic acid estimation by HPTLC

Gallic acid (3,4,5-trihydroxy benzoic acid) is a major polyphenol in pomegranate peel [2] with strong antioxidant activity. In the present study, the band of gallic acid in the extracts was confirmed by comparing Rf values (0.42) and spectra (Fig 2). Quantification of gallic acid was done by five point standard curve (Y= 244.336+11.3X; r^2= 0.996). Densitometry analyses (Fig 3) showed gallic acid peaks in extractions. Gallic acid in the extracts (Table 3) was observed in the order of: T2>T1>T3 with significant variation (P<0.05). Despite the benefits of gallic acid, hydrolysable tannin, it has some limitations for application in animal model like poor absorption from the intestine, growth limiting effect etc. (Shahrzad *et al.*, 2001) [24]. Hot water infusion method (T_3) produced least gallic acid and thus might be better suitable for using it in animal trial for further *in-vivo* research.

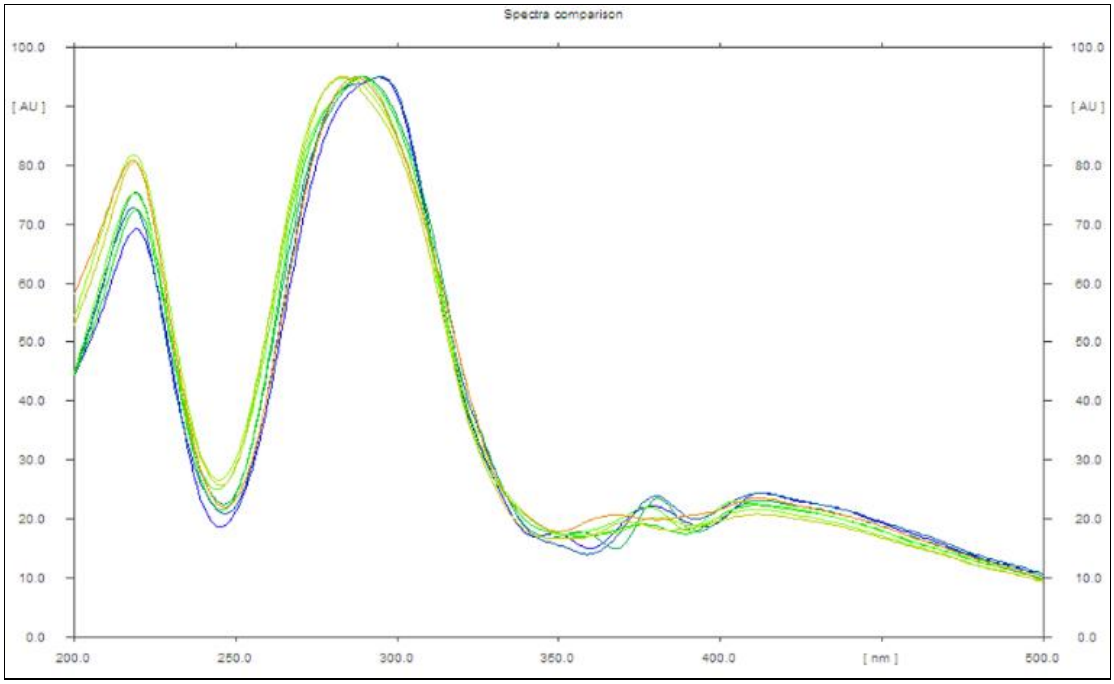

Fig 2: Spectra of standard gallic acid and other extracts

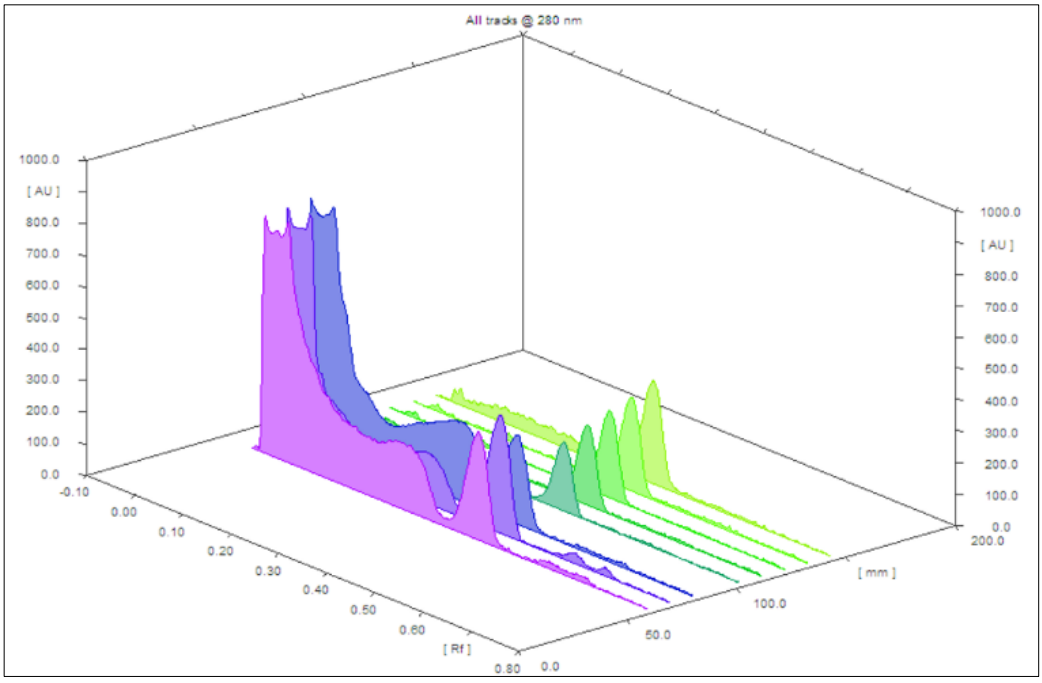

Fig 3: HPTLC densitogram at 28nm in the order of T_1, T_2 and T_3 (tracks 1-3) and standard gallic acids (tracks 4-8)

Table 3: Gallic acid content (% *w/w*) of aquous extracts of pomegranate peel by different methods

	T_1	T_2	T_3
Gallic acid content (% *w/w*)	2.32 ± 0.003^b	2.46 ± 0.01^a	2.03 ± 0.003^c

Values are means ± SEM, n = 3 per treatment group. Means in a row without a common superscript letter differ (P<0.05) as analyzed by one-way ANOVA and the DUNCAN test.

4. Conclusions

In recent years polyphenolic extraction modeling from pomegranate peel has attracted considerable research worldwide. Many authors identified methanol or hydro-alcoholic solvents as most efficient for phenolic extraction from pomegranate peel (Shiban *et al.*, 2012; Malviya *et al.*, 2014; Masci *et al.*, 2016) [25, 2, 9]. However these solvents are toxic and hence not suitable for animal consumption. Present study showed that hot water infusion method of polyphenol extraction from pomegranate peel is a better choice in terms of antioxidant activity, simplicity, cost effectiveness and animal consumption.

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