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COMPARISON OF ANTIOXIDATIVE POTENTIAL AND PUNICALAGIN CONTENT OF POMEGRANATE PEELS

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ABSTRACT

Pomegranate peel has been traditionally used as folk medicine owing to rich source of polyphenolic compounds. Aim of present research was to assess total phenolics (TPC), total flavonoids (TFC) & punicalagin contents (PC) of pomegranate peels obtained from three different varieties for their application as nutraceutical ingredient in food preservation industry. Phytogenic rich fractions were extracted from *Punica granatum* L. (pomegranate) peel using solvent methanol. Moreover, methanolic extracts were characterized *in vitro* to evaluate their antioxidative potential using DPPH assay. Kandhari peel polyphenols inhibited 78.23% free radicals; though extracts of Badana peel displayed least antioxidant potential. Maximum phenolics and flavonoids were documented in Kandhari peel extracts as 289.40 mg/g GAE and 58.63 mg/g RE, correspondingly, however punicalagin (118.60 mg/g) was the most predominant ellagitannin quantified. Significant correlation ($r = 0.999$, 0.987; $N = 3$) was noticed amongst total phenolics, total flavonoids and antioxidative capacity of experimented varieties. Correlation analysis ($r = 0.975$; $N = 3$) proposed that punicalagin might be the bioactive compound responsible for antioxidant potential of extracts. Conclusively, the study illuminated the therapeutic and nutraceutical prospective & potential use of pomegranate peel as a cache of natural antioxidant.

Keywords: Phytogenic compounds, Antioxidants, Punicalagin, High performance liquid chromatography, Nutraceuticals.

INTRODUCTION

Dietary habits vitally influence human health. The human diet comprises diversified range of naturally occurring compounds having a vast range of functionality such as anti-mutagenic or anti-carcinogenic activities. Some examples of bioactive dietary ingredients are dietary fiber, flavonoids, polyphenolic compounds, tocopherols, ascorbic acid and isoflavones (Negi *et al*., 2003). Mutagenic compounds are generally associated with the production of reactive oxygen species (ROS) which are directly or indirectly linked with UV radiation, environmental hazards and several metabolic activities. Generation of ROS is claimed to be responsible for various health related ailments. In addition, ROS are also considered as potential initiator of several degenerative processes such as mutation and DNA damage which are allied to different lifestyle related disorders including aging, cancer and cardiovascular maladies (Seifried *et al*., 2007). Consumption of dietary functional ingredients plays a dynamic role to protect against the adverse effects of ROS and prevention of diet dependent diseases (Gorinstein *et al.,* 2009). Dietary intake of endogenous functional ingredients particularly antioxidants, is helpful in improving the body's defense system against carcinogenic compounds (Butt and Sultan, 2009). Studies have revealed that less consumption of fruits and vegetables increases the risk of mutagenicity so the incorporation of this food group would be a beneficial step to curtail the risks of carcinogenicity (Orak *et al*., 2012).

Polyphenols from plant origin such as ellagic acid, gallocatechins, chlorogenic, sitosterol, ferulic acids and catechins are thought to be potent anti-carcinogenic or anti-mutagenic regimes (Reddy *et al*., 2007; Zahin *et al*., 2014). It is evident from the previous studies that tea extracts have potential to reduce the menace of mutation, cancer and heart diseases (Middha *et al*., 2013).

Pomegranate peel comprises of a plentiful amount of polyphenolic compounds such as tannins, gallic acid and ellagic acid & has been used in various food and non-food formulations (Viuda-Martos *et al*., 2010; Shiban *et al.,* 2012). Additionally, pomegranate juice is also reported to possess a substantial amount of antioxidants (Gil *et al*., 2000). In a trial conducted by Aviram *et al*. (2000), pomegranate juice was employed in human and mice diet against atherogenicity and atherosclerosis respectively. Findings of the investigation concluded that pomegranate juice has the potential to combat these disorders owing to its antioxidative properties. Consequently, the current project was designed with the aim to determine total phenolics, antioxidant potential and punicalagin contents of pomegranate peel and to explicate its role as a natural preservative and nutraceutical agent.

MATERIALS AND METHODS

Chemicals and reagents: 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent, gallic acid, rutin, Tri-fluoroacetic acid (TFA), tri-chloroacetic acid (TCA), aluminium chloride ($AlCl_3$) were purchased from Sigma. Sodium nitrite ($NaNO_2$), sodium hydroxide (NaOH) and sodium carbonate (Na_2CO_3) were from Merck. HPLC grade punicalagin and methanol were also supplied by Sigma Chemical Company (Germany).

Procurement of raw material and sample preparation: Mature and healthy pomegranate fruits of three varieties namely Kandhari, Desi and Badana without any visible bruising were procured from the market of Faisalabad. Respective peels were obtained by separately peeling each pomegranate manually. Pomegranate peels were then dried in cabinet dryer at 60°C for 24 hours. The dried peels were ground to fine powder and passed through a No. 30 mesh sieve. Finally prepared powders were stored in plastic jars at ambient temperature (25±5ºC) till further analysis.

Extraction of polyphenols: The antioxidant extracts of pomegranate peel powder samples of all three varieties were obtained by treating them with methanol (50%). For this purpose, prepared mixtures were subjected to orbital/mechanical shaker for seven hours trailed by centrifugation for 15 minutes at 12,000 rpm (Viuda-Martos *et al*., 2011). Resultant pomegranate peel raw extracts were vacuum-filtered (Rusak *et al*., 2008).

Determination of Total Phenolic Contents (TPC): Total phenolic contents in each pomegranate peel extracts (PPEs) were assessed by adopting the protocol of Singleton *et al*. (1999). Accordingly, extract measuring 125 µL was added in 500 µL distilled H_2O along with addition of Folin-Ciocalteu reagent @ 125 µL and this mixture was held-up for 5 minutes. After standing time, 1.25 mL of 7% Na_2CO_3 solution was added in the mixture. Final volume was maintained up to 3 mL by adding distilled water. After standing time of 90 minutes, the absorbance was measured using UV-Visible Spectro-photometer (CECIL,CE7200) at 765 nm. Total phenolics were determined & expressed as mg Gallic acid equivalent (GAE)/g.

Determination of Total Flavonoid Contents (TFC): Total flavonoid contents (TFC) were determined by aluminium chloride calorimetric method (Chang *et al*., 2002). For this purpose, 50 µL of each pomegranate peel extract were raised up to 1 mL by adding methanol, mixed with 4 mL of distilled water followed by addition of 0.3 mL of 5% $NaNO_2$ and 0.3 mL of 10 % $AlCl_3$ after 5 min of incubation. Then resultant mixture was placed for further 6 minutes. Afterwards, 2 mL of 1 M NaOH solution was added and volume was raised up to 10 mL by adding distilled water. For all the samples the absorbance was measured at 510 nm by using UV/vis Spectro-photometer (CECIL CE7200). All findings were stated as mg rutin equivalents (RE)/g.

Measurement of in vitro antioxidant potential (DPPH): The antioxidant capacity of resultant peel extracts of all three varieties was calculated in terms of free radical scavenging potential by using DPPH-assay (Brand-Williams *et al*., 1995). Briefly, each peel extract (4 mL) were placed in the cuvette followed by the addition of 1 mL of DPPH methanolic solution. The resultant mixture was allowed to stand for 30 minutes at 25^0C. The absorbance of resultant mixture was noticed at 520 nm through UV-Vis Spectro-photometer (CECIL CE7200). Percent inhibition was measured using following formula:

$$Reduction\ in\ absorbance\ (\%) = [[AB(s) - AB(e)] / AB(s)] \times 100$$

$$AB(s) = absorbance\ of\ blank\ sample\ (t = 0\ min)$$

$$AB(e) = absorbance\ of\ tested\ extract\ solution\ (t = 30\ min)$$

Punicalagin quantification (HPLC-UV): Quantification of punicalagin was performed using HPLC (Perkin-Elmer, Series 200, U.S.A.) having C_{18} column (dimensions: 250 mm x 4.6 mm, particle size: 5 µm). A sample of 10×10^{-6} L was taken up by auto sampler and 30^0C temperature of column was ensured throughout the analytical process. Throughout analysis; mobile phase comprised of Methanol (eluent-A) and 0.1% (vol/vol) Trifluoroacetic acid in HPLC grade water (eluent-B). Chromatographic conditions (Gradient): 0–10 minutes, 5%–20% A in B; 10–20 minutes, 20–40% A in B; 20–26 minutes, 70% A in B. These protocols were trailed by re-equilibrium for 10 minutes. The flow rate was adjusted at 1 mL min^{-1} and punicalagin was quantified at 378 nm wavelength using UV-Vis detector (Lu *et al*., 2011).

Statistical Analysis: Data were statistically analyzed using one-way ANOVA under CRD and significant difference ($P < 0.05$) was measured by Tukey's HSD test. All results were expressed in triplicate as means ± SD.

RESULTS AND DISCUSSION

Knowledge about polyphenolic composition and antioxidant potential of fruit peel extracts prior to their application in food preservation industry is critically important. Spectrophotometer and high performance liquid chromatography (HPLC-UV) were used as analytical tools to collect a data base that would validate the utilization of pomegranate peel from various varieties as an abundantly and economically available agro-waste for preparation of phytogenic rich extract possessing potent antioxidative and nutraceutical properties.

Total phenolics and flavonoids in pomegranate peel extracts: Phenolics from plant origin typically possess therapeutic potential, such as prevention from cancers,

antioxidant agents and potent antibacterial (Adnan *et al*., 2011).Results of present investigation for total phenolic and flavonoid contents of pomegranate peel extracts (PPEs) of three respective varieties are demonstrated in Table 1. The total phenolic contents (TPC) of PPEs ranged from 255.35±11.89 to 289.40±12.75 mg/g GAE in different varieties with Kandhari variety showing the highest content and Badana exhibiting the least. The statistical analysis revealed that varieties had significant ($P < 0.05$) effect on total phenolics in obtained PPEs. The outcomes further illustrated that variation in phenolic composition was due to difference in affinity of pomegranate peel polyphenols towards used solvent *i.e.* methanol. Means for total phenols in Kandhari pomegranate peel extracts (289.40 ± 12.75 mg/g GAE) were 5.56% &11.76% higher as compared to Desi variety peel extracts (273.30 ± 9.30 mg/g GAE) and Badana PPEs (255.35 ± 11.89 mg/g GAE). Similarly, average amount of total flavonoids (TFC) in peel extracts obtained from different varieties of pomegranate revealed that the maximum content was observed in Kandhari (58.63±3.41 mg/g RE) trailed by Desi (55.21 ± 2.65 mg/g RE) and minimum concentration (50.86 ±3.69 mg/g RE) was documented in Badana peel extract.

Table 1. Total phenolic and flavonoid contents of Pomegranate peel extracts from different varieties

Varieties	Total Phenolic Contents*	Total Flavonoid Contents**
Desi	273.30^{ab} ± 9.30	55.21^{ab} ± 2.65
Kandhari	289.40^{a} ± 12.75	58.63^{a} ± 3.41
Badana	255.35^{b} ± 11.89	50.86^{b} ± 2.91

* Expressed as mg/g Gallic acid equivalent (GAE)
** Expressed as mg/g Rutin equivalent (RE)
Each value is expressed as means ± SD

The mean flavonoid contents extracted from variety Kandhari were almost 5.83% and 13.25% more in comparison to those extracted from Desi and Badana (Fig. 1). Likewise, Singh *et al*. (2002) concluded that solvent methanol have maximum phenolics yield, primarily due to the ascribed polarity differences among the solvent used for extraction and nature of poly-phenolic compounds to be extracted. These phytogenic molecules are chiefly recognized for their free radical quenching ability that eventually inhibits lipid peroxidation (Noda *et al*., 2002).

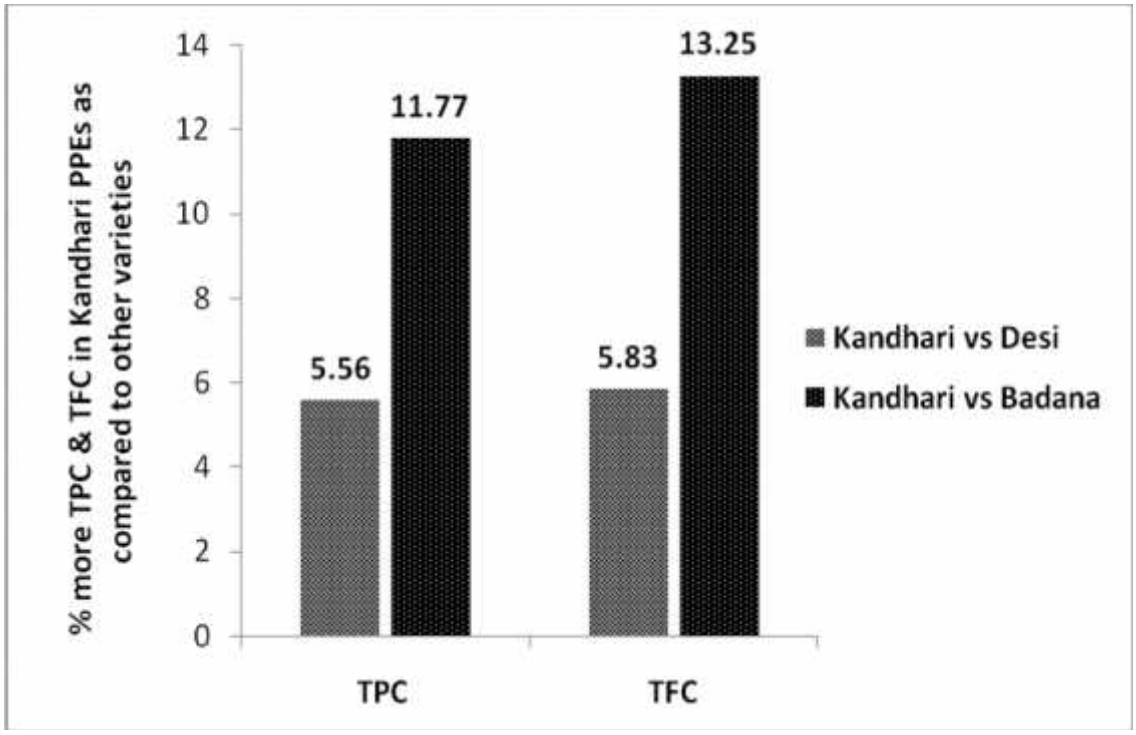

Figure 1. Extraction efficiency of Kandhari variety over Desi and Badana extracts. The results are presented as mean ± SD for PPEs obtained from three varieties

DPPH Free Radical Scavenging Activity of the different PPEs: To evaluate *in vitro* antioxidative properties of PPEs from different varieties; DPPH assay relied on quantifying the discoloration of DPPH-radical at 520 nm after its reaction with antioxidant compound present in prepared extracts. DPPH assay is the most commonly adopted procedure to assess the antioxidant capacity of corresponding samples mainly due to its short rum time, simplicity &stability during whole experimental process. Principally, the antioxidant potential of respective samples are analyzed depending upon their potential to reduce DPPH• by donating hydrogen atom that can also be authenticated spectrophotometrically due to loss of dark violet color of the tested solution (Brand-Williams *et al*., 1995).

Among all pomegranate peel extracts, Kandhari PPEs were the most effectual in demonstrating strong free radical scavenging activity (DPPH). Significant correlations (Pearson's correlation coefficients $r = 0.999$, 0.987; $N = 3$) were recorded among total phenolics, flavonoids and DPPH free radical scavenging potential of PPEs from respective varieties. The results helps in inferring that pomegranate peel antioxidant extracts retard food deterioration which mainly initiates due to production of free radicals. It is quite obvious from Fig. 2 that Kandhari variety extracts revealed highest free radical scavenging effect with highest antioxidant activity as compared to Desi and Badana peel extracts. The mean values for different varieties established that Kandhari extracts significantly ($p < 0.05$) scavenged highest DPPH free radicals (78.23±4.11%) followed by variety Desi (70.38±3.32%) and Badana (63.36±3.20%).

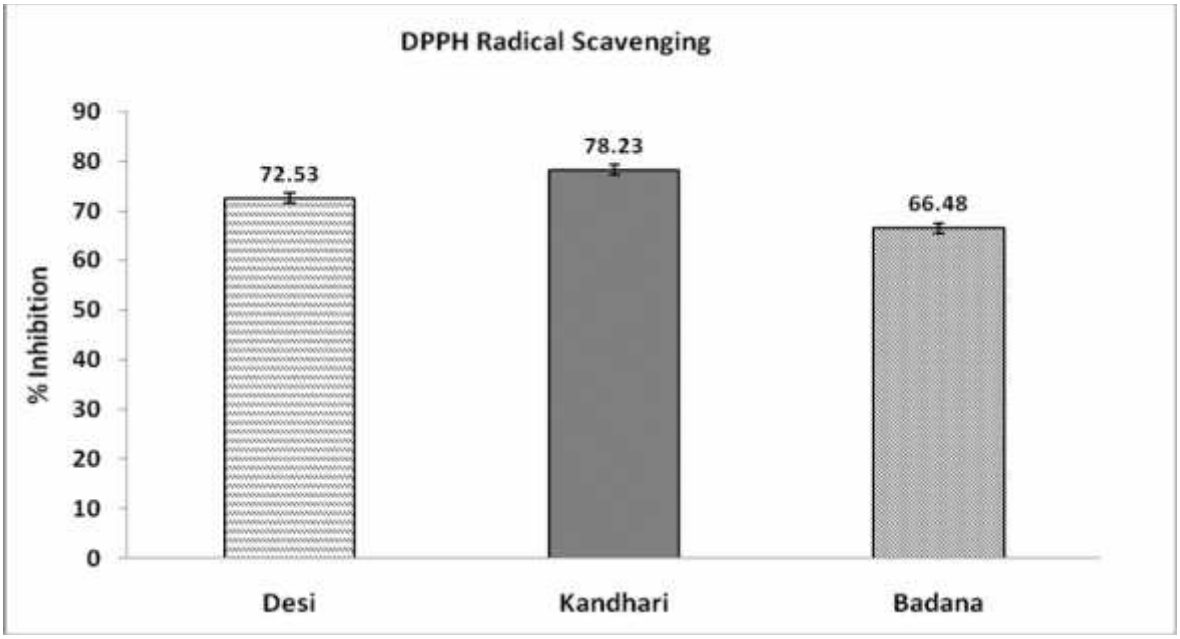

Figure 2 DPPH radical scavenging properties phytogenic extracts from different varieties of *Punica grantum* L. peels. The results are presented as mean ± SD for PPEs obtained from three varieties.

High free radical scavenging potential is usually associated with the amount of total phenolics present in polyphenolic extracts (Orak *et al*., 2012). Hence, antioxidative potential of pomegranate peel from different varieties was observed highest in methanolic Kandhari extracts, generally due to the existence of high concentration of polyphenols, such as phenolics & flavonoids, showing ability to donate hydrogen atom resulting in quenching of free radicals (Middha *et al*., 2013). Previously Brand-Williams *et al.* (1995) recognized that free radical scavenging capacity of phenolics is related with their structural properties and the resultant antioxidative potential was directly associated with the number of present hydroxyl-groups (-OH). All previously stated activities may be linked to several poly-phenolic complexes present in peels of pomegranate, including α, β-punicalagin, ellagic acid (EA) & some flavonoids (rutin, quercetin & catechin). *In Vitro* studies validate that these complexes are known for their free radical scavenging & lipid-peroxidation inhibitory potential (Gil *et al*., 2000).

HPLC quantification of Punicalagin content: Punicalagin content in pomegranate peel extracts (PPEs) from different varieties were quantified and compared with pure standard of punicalagin thorough HPLC-UV system (Fig. 3). Acquired results exhibited in Table 2 were expressed as mg/g punicalagin content and were significantly affected by the variety used.

Table 2. Total Punicalagin content in extracts of Pomegranate peel obtained from different varieties

Varieties	Total Punicalagin Content*
Desi	$110.00^{a} \pm 5.10$
Kandhari	$118.60^{a} \pm 5.26$
Badana	$98.70^{b} \pm 2.21$

* Expressed as mg/g
Each value is expressed as means ± SD

Amongst different peels extract, major portion of punicalagin was quantified in extract of Kandhari peel *i.e.* 118.60 mg/g, followed by Desi and Badana extracts with 110.00 and 88.70 mg/g dry weight extract, correspondingly. Pearson's correlation coefficients ($r = 0.975$; $N = 3$) showed substantial correlation between total punicalagin content and free radical scavenging activity of experimented pomegranate peels. The present results are in accordance with previous findings of Lu *et al*. (2008); they publicized punicalagin content in pomegranate husk ranged from 44.9-121.5 mg/g in 14 different tested varieties. Punicalagin, most abundantly present in pomegranate peel is predominantly responsible for its potent antioxidant activity (Cam and Hisil, 2010).

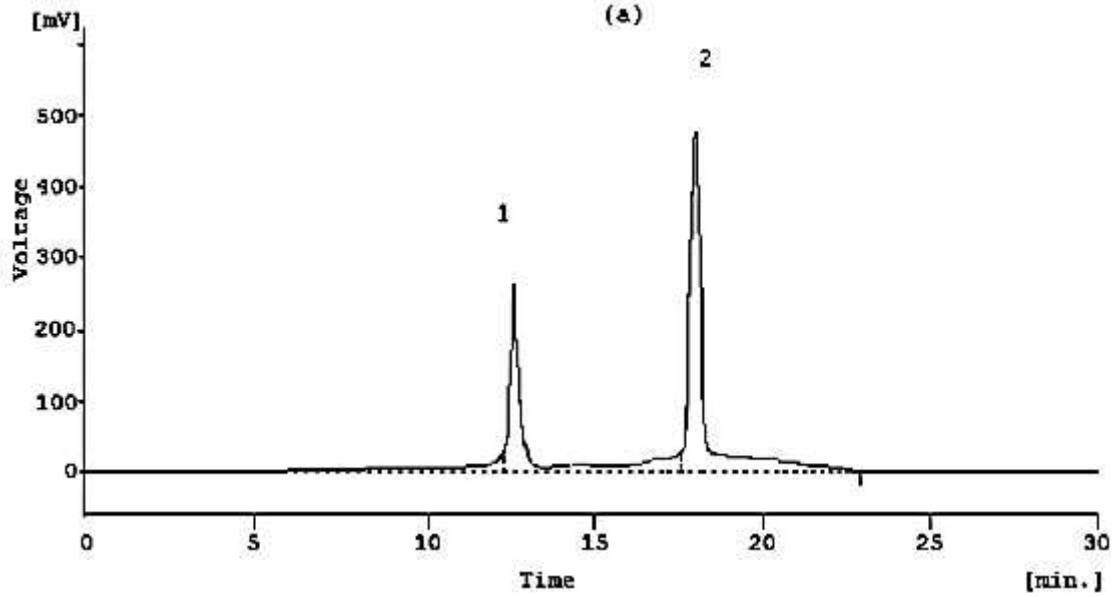

Figure 3. HPLC chromatograms of PPEs, punicalagin standard: α-punicalagin (1) and β-punicalagin (2).

Conclusion: Outcomes of present investigation provide sufficient evidence that pomegranate peel extracts from different varieties have variation in antioxidant potential depending upon the affinity of phytogenic complexes enclosed in matrix of pomegranate peels. Total phenolics and flavonoids were highest in extracts of Kandhari peel (289.40 & 58.63 mg/g RE) as compared to Desi and Badana varieties making it the most promising variety in terms of polyphenolic content. Significant correlation between phenolics and antioxidative properties was evaluated in this trial. PPEs revealed significantly strong antioxidative capacity when evaluated using DPPH assay, validating its therapeutic role. Kandhari peel established highest free radical scavenging ability *i.e.* 78.23% that varied significantly ($p < 0.05$) from Desi (72.53%) and Badana peel extracts (66.48%). Punicalagin was quantified to be the major bioactive compound present in Kandhari peel extracts (118.60 mg/g) among all experimented varieties. In a nutshell, the outcomes of this study could be helpful in developing a baseline data for fruits & vegetable processors in preparation of functional and nutraceutical designer foods.

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