Tarun Kumar Dasgupta *et al*. / *International Journal of Biological & Pharmaceutical Research.* 2014; 5(12): 886-892.

e- ISSN 0976 - 3651
Print ISSN 2229 - 7480

International Journal of Biological & Pharmaceutical Research
Journal homepage: www.ijbpr.com

IJBPR

SIMULTANEOUS ESTIMATION OF ANTI-OXIDANT FLAVONOIDS OF HIGH YIELD POLYPHENOLIC EXTRACT (HYPE) OBTAINED FROM AN AGRO WASTE MATERIAL OF *P.GRANATUM* BY HPLC-DAD TECHNIQUE

Tarun Kumar Dasgupta[1], Priscilla M D'Mello[1], Deep Bhattacharya*[1]

[1]Department of Pharmacognosy and Phytochemistry, Prin.K.M.Kundnani College of Pharmacy, Colaba, Mumbai-400005, India.
[2]Department of Pharmaceutical Chemistry, Bombay College of Pharmacy, Kalina, Santacruz [E], Mumbai 400098, India.

ABSTRACT

Pomegranate is widely known to exhibit anti-viral, anti-oxidant, anti-cancer and anti-proliferative properties. The anti-oxidants like catechin, myricetin and quercetin in High Yield Polyphenolic Extract (HYPE) from agro waste of *P.granatum* with the aim of developing a rapid RP-HPLC method for detection of each and estimation of the anti-oxidants in HYPE extract. The samples were analysed using a HPLC system, standard solution and sample, a photo diode array detector, C_{18}analytical column (4.6mm φ x 25. mm, 5µm particle size).The Chromatographic analysis of HYPE sample and standards were performed using gradient elusion with acetonitrile, water and phosphoric acid. Optimal detection of quantification was set to 272nm. Calibration exhibited good linearity $R^2 \geq 0.999$,accuracy and repeatability yielded good results of R.S.D <2.2% and recovery (85.7149-99.7385%).The extract of peel powder was found to contain catechin 115mg gm^{-1} , quercetin 18.3mg gm^{-1} and myricetin 1.5 mg gm^{-1} .The method was robust in detecting the three flavonoids within 12 minutes of elusion time with good resolution. Therefore we report a report an efficient and simultaneous estimation and detection of flavonoids in HYPE extract.

Key Words: *Punica granatum*, HYPE, RP-HPLC, Catechin, Myricetin and Quercetin.

INTRODUCTION

Pomegranates (*Punica granatum L.*) have been used extensively in folk medicine of varied cultures across the world. Pomegranate peel is the main waste fraction of pomegranate fruit, which has been widely studied because it contains numerous biologically active compounds including natural antioxidants such as phenolic acids and flavonoids. It is widely reported that pomegranate exhibits anti-viral, anti-oxidant, anti-cancer and anti-proliferative activities (Kidd PM, 2009; Weichselbaum E, Buttriss JL, 2010; Ahn JG *et al*., 2004; Yunfeng Li CG *et al*., 2006). Pomegranate is consumed fresh and in processed in forms such as juices, wines, flavours and extracts. Commercial pomegranate juice has the highest antioxidant activity compared to other fruit juices, red wine and green tea. The pomegranate antioxidant activity is typically higher in commercial juices extracted from whole pomegranates than in experimental juices obtained from the arils only. This can be attributed to its high content of polyphenols such as condensed tannins and anthocyanin's in the peel. It has been reported that the peel in particular possesses relatively higher antioxidant activity than seed and pulp and therefore is a rich source of natural antioxidants (Qu W PZ *et al*., 2009; Wenjuan Qu ZP, Haile Ma, 2010; Rice-Evans CA *et al*., 1996). Phenolic compounds have

Corresponding Author

Deep Bhattacharya
Email: deep.bhattacharya1991@gmail.com

attracted more and more attention for their anti-oxidant and beneficial health promoting effects in chronic and degenerative diseases (Lodovici M GF *et al*., 2001; Riaz Ahmad Rather SC, Rajagopal K, 2010; Hee Kee Kima BSC *et al*., 1999; Srinivas KVNS *et al*., 2003). Flavonoids are polyphenolic compounds, widely found in the plant kingdom. In the recent years, scientists have carried out extensive research on biological activities of flavonoids such as antibacterial, antifungal, anti- cancer and anti-inflammatory effects. Flavonols and flavones are of particular importance as they are found to possess anti-oxidant and free radical scavenging ability(Hisashi Matsuda YP et al., 2003; Toshio Aoki TA, Shin-ichi A, 2000; Lapornik B PM, Wondra AG, 2005; Landbo AK MA, 2001). For instance, the use of agricultural wastes such as wine-making wastes as an alternative low-cost source of phenolic compounds has been on the increase. Among the flavonoids studied in our experiment, quercetin and myricetin are flavonols and catechin is flavan 3-ol and has been reported to possess several biological activities (Zu Yuan gang Fu Y-jL *et al*., 2006; Celia M *et al*., 2013; Yasunori HTI *et al*., 2006). Sample extraction procedure is often regarded as a bottleneck in analytical methods. Classical sample preparation techniques are both time and solvent consuming. Therefore, the importance of sample preparation in analytical methods should not be undermined. Isocratic elution in HPLC often increases both time and solvent consumption during analysis (Pinelo-Jiménez MM, Anne S, 2008). Complex polysaccharides from pomegranate peel have been studied and characterized. Phenols have been reported to be linked with polysaccharides by hydrophobic and hydrogen bond that creates a challenge for extraction. Cell wall degrading enzymes (cellulase and pectinase) have been reported to be used to decompose the structure of polysaccharides in order to increase the yield of extracts**.** Pectinolytic and cellulolytic preparation have been reported to increase the yield more than 30% in fruit processing industry (Jahfar KKV and Azadi P, 2003; Lee LGC *et al*., 2010; Jafri MA *et al*., 2000). In this study a new simple validated method using RP-HPLC with PDA for the simultaneous determination of flavonoids catechin, myricetin and quercetin in the High Yield Polyphenolic Extract (HYPE) of an agro waste of pomegranate obtained after soxhalation of pectinolytic and cellulolytic enzyme pre-treated material was developed. The simultaneous determination of polyphenols namely quercetin, catechin and myricetin has been used to develop an optimized technology for the High Yield Polyphenolic Extract (HYPE) of peels of *P.granatum*.

MATERIALS AND METHODS

All the biomarkers were purchased from Sigma (USA). All technical grade solvents were procured from SD fine chemicals Mumbai (India). Peels of *P.granatum* were obtained from local markets, Mumbai. All HPLC grade and technical grade solvents were procured from SD fine chemicals (India).

Standard solutions

All the standard solutions were prepared in methanol to give a concentration of 10µg/ml. All the solutions were stored in dark at 4^0C. The HPLC analysis indicated that these solutions were stable for 2 months. Standard quercetin, catechin and myricetin solution was mixed in equi-proportion (1ml each of 10µg/ml solution) to give 10µg/ml solution of standard mixture of polyphenols. The final solution of standard polyphenol mixture was made in the range of 20-15000ng/ml.

High Performance Liquid Chromatographic (HPLC) conditions

The chromatographic system (Jasco) consisting of a PDA detector was used. A reverse phase Qualisil BDS-C_{18} column (4.6mm φ x 25. mm)packed with 5µm diameter particles was used. In order to estimate the three polyphenols simultaneously a gradient phase chromatographic method was developed. In the course of experiments, different ratios of acetonitrile-water in isocratic system were studied and the effect of o-phosphoric acid to regulate the pH value was examined.

OPTIMIZATION OF EXTRACT PREPARATION

Macerate (I)

The powder of *P.granatum* (Mesh 40#) peel was stirred with methanol, filtered and concentrated under vacuum.

Pre-treated enzymes macerate (II)

The peel powder of *P.granatum* (Mesh 40#) was stirred with acetate buffer and pectinolytic and cellulolytic enzyme preparation. The solution was evaporated under vacuum. Then dried residue of powder and enzyme were stirred, filtered and concentrated under vacuum.

High Yield Polyphenolic Extract (HYPE) of *P. granatum* (III)

The peel powder of *P.granatum* (Mesh 40#)was stirred with acetate buffer and pectinolytic and cellulolytic enzyme preparation. The solution was evaporated under vacuum. Then dried residue of powder and enzyme were taken in Soxhlet apparatus, filtered and concentrated under vacuum.

RESULTS AND DISCUSSION

Monitoring for method optimization of extract preparation by gradient RP-HPLC

The critical step in the quantification of flavonoids in an agro waste of *Punica granatum* is the sample extraction. The extraction must enable the complete extraction of biomarkers to be analyzed. Three different extraction methods i.e. maceration, maceration with

enzymes cellulase and pectinase and soxhalation of pre-treated plant material with cellulolytic and pectinolytic preparation were selected for an optimum method of extraction of flavonoids from an agro waste of *Punica granatum*. The determination of the yield of all flavonoids by extraction was carried out by using a developed and validated gradient RP-HPLC. The results are shown in Fig.I. The data revealed that the yield by maceration was low and by soxhalation of pre-treated sample with cellulolytic and pectinolytic enzymes was higher.

Optimization of chromatographic condition (gradient RP-HPLC)

Effect of mobile phase

Catechin, quercetin and myricetin are polar compounds and polar mobile phase like methanol - water, acetonitrile - water system were chosen in the beginning with various proportions (A) and (B) but separation was not satisfactory. In all the isocratic systems the interference of the solvent peak was predominant and as a result the retention time was recorded up to 25-28 min. In order to separate the three polyphenols successfully within 12 min, a gradient phase of chromatographic technique was developed with the help of a PDA detector. Following mobile phases were used for the study: Phase- A = Acetonitrile, Phase -B = Acetonitrile: water (10:90) with buffer to maintain pH = 3.20 (O-phosphoric acid). (Table 1)

The presence of acid in mobile phase can significantly improve peak tailing and resolution. This also acts as a buffer. Percent ionization of an analyte can change its capacity factor and its solubility, so a pH of 3.20 was confirmed as per their retention time. Percent ionization of catechin can be calculated from its pKa value.

Effect of detection wavelength

Quantification was carried out by the integration of the peaks using external standard method.Selecting a proper wavelength is of great importance to ensure that the three polyphenols are detected precisely and to achieve the goal of maximizing the absorption and minimizing the interference. With PDA detector this problem was solved and after a scan of optimal detection 272nm was selected for quantification. Multiple wave length scanning programs were used to detect several λ_{max} of different components present in the mixture simultaneously. Figure 2 showing HPLC chromatogram of catechin, myricetin and quercetin in gradient system.

Validation of assay using gradient RP-HPLC – System suitability test

The HPLC method was validated by defining the linearity, limits of detection, identification and quantification of analytes, repeatability, precision, stability and recovery. Three polyphenol standard solutions in the concentration range 20-15000 ng/ml were prepared in methanol and diluted with mobile phase -B (Acetonitrile: water) along with buffer to maintain required pH by O-phosphoric acid. LOD and LOQ values of individual components were determined.The analytical method demonstrated excellent sensitivity.

Reproducibility, linearity, limit of quantification and detection

The peak area values were the average of the three replicate injections. The result of calibration exhibits good linearity ($R^2 \geq .999$) for all the polyphenols. The limit of detection is defined as the smallest peak detected with a signal height (signal to noise ratio) three times that of the base line while limit of quantification value is often calculated as 10 times the signal height to the base line. Various chromatograms for the experiments are given below in Table 2.

LOQ = LOD X10/3

Repeatability, Precision and Stability

The precision of the proposed method has been reported as inter-day and intra-day precision that was determined from relative standard deviation (RSD) for retention time and peak area resulting from analysis of the studied compounds. For the stability test retention time and peak area of

Recovery / Accuracy

The recovery experiments of the three polyphenols were carried out by adding the standard polyphenols to the pre-analyzed samples .The polyphenols were checked for the accuracy at concentration of 8,10,12 µg/ml. The recovery rate of the three polyphenols was within the range of 85%-99%. Table 4.

Selectivity (Specificity)

Peak purity data revealed that the peaks of catechin, quercetin and myricetin were sufficiently pure ranging from 0.8999-0.9999.

Robustness

To evaluate the robustness of the method, the influence of small and premeditated alteration of analytical parameters on the quantification of the related substances and selectivity was studied. The parameters selected were mobile phase composition (±2% of gradient composition), pH of the mobile phase (±0.05 units), flow rate (±20%) and wavelength (±2 nm). Only one parameter was changed while the others were kept unaltered. The mean and R.S.D. for each related substance were evaluated. The difference between the mean values (for all the analytes from each of the robustness parameters) from the repeatability mean results was found to be below 10.0%. The studies indicated no effect on the determination of related substances and the

selectivity. Therefore the test method is robust for the quantification of related substances.

Application of HPLC method in quantification of polyphenols

Three different extracts A, B and C were injected separately under the optimum conditions. The chromatograms showed several unidentifiable peaks. The extracts were passed through a C-18 cartridge to get a pure form. The retention time of the unidentified peaks did not overlay the peaks of the standard polyphenols. The interference of these peaks did not influence the precision and the validity of this analytical method. The sample solutions of three different extracts A, B and C of *Punica granatum* (Pomegranate) were injected directly and separated under the optimum conditions. The extract C was passed through a C-18 cartridge (solid-phase extraction) to get a fraction with reduced interference. The concentration of the three flavonoids was calculated according to their calibration equations described above and the content of these three flavonoids in dry material was calculated according to the following equation-

Q = (C x V)/W

Q (µg/g): the content of each flavonoid in dry matrices;

C (µg/mL): the concentration of each flavonoid in the sample solution;

V (mL): the volume of the sample solution;

W (g): the weight of the dry matrices.

Table 1. Gradient Programming details

Timing	Mobile phase Ratio (A,B)	Response
0-5 min	A(100): B(0)	Linear gradient
5-15 min	A (60%): B (40%)	Linear gradient
15-20 min	A (100):B(0)	Re-equilibration

Table 2. HPLC Validation parameters

Polyphenols	Linearity range µg/ml)	Calibration equation	LOD (µg/ml)	LOQ(µg/ml)	Correlation factor (R^2)
Catechin	.95-15	11.82758C + 0.46171	.288	.9504	0.99977
Quercetin	.63-15	39.17278C + 2.12210	.192	.6336	0.99988
Myricetin	.73-15	43.56501C + 2.45514	.222	.7326	0.99985

N=6; C: the concentration of flavonoids standard (µg ml^{-1}). Table-3 summarizes LOD and LOQ values of individual components and clearly indicates that the analytical method has excellent sensitivity

Table 3. Precision data for three flavonoids

Analytes	Inter-day RSD for retention time	Inter-day RSD for peak area	Intra-day RSD for retention time	Intra-day RSD for peak area
Catechin	1.93	1.12	1.10	1.94
Myricetin	.95	1.72	1.30	1.02
Quercetin	1.01	.98	1.10	1.65

Table 4. Recovery analysis of three standard polyphenols – catechin, myricetin and quercetin respectively in HYPE of Pomegranate *(Punica granatum)*

Compounds	Amount added(µg)	Amount found	% level	Recovery (%)	R.S.D
Catechin (1.15 µg)	12	11.9686	120	99.7385	0.2400
	10	9.7010	100	93.3496	0.0980
	8	6.8572	80	85.7149	2.1965
Myricetin (.015 µg)	12	11.8549	120	98.7909	0.0103
	10	9.9217	100	99.2167	0.0057
	8	7.7537	80	96.9213	0.1398
Quercetin (.138 µg)	12	11.8549	120	97.2325	0.0593
	10	9.9217	100	97.0173	0.1024
	8	7.7537	80	95.5170	1.0604

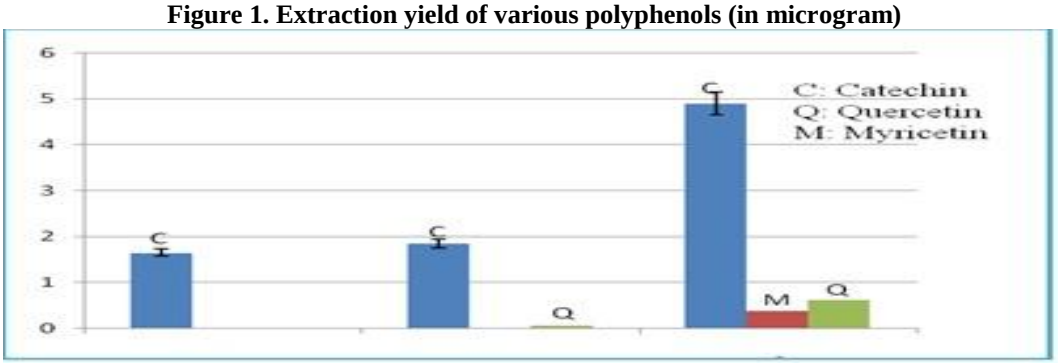
N= 3 average of three determination, value were expressed as ± RSD

Table 5. Quantitative analysis of the extracts

Polyphenols	Extract-I (10µg/ml)	Extract-II (10µg/ml)	Extract-III (10µg/ml)
Catechin	.65 µg	.85µg	1.15 µg
Myricetin	ND	ND	.015µg
Quercetin	ND	.06µg	.183µg

*ND = Not Detected

Figure 1. Extraction yield of various polyphenols (in microgram)



I= Macerate, II= Pre-treated enzymes Macerate, III= High Yield Polyphenolic Extract (HYPE) of *P. granatum*

Figure 2. HPLC chromatogram of catechin, myricetin and quercetin in gradient system

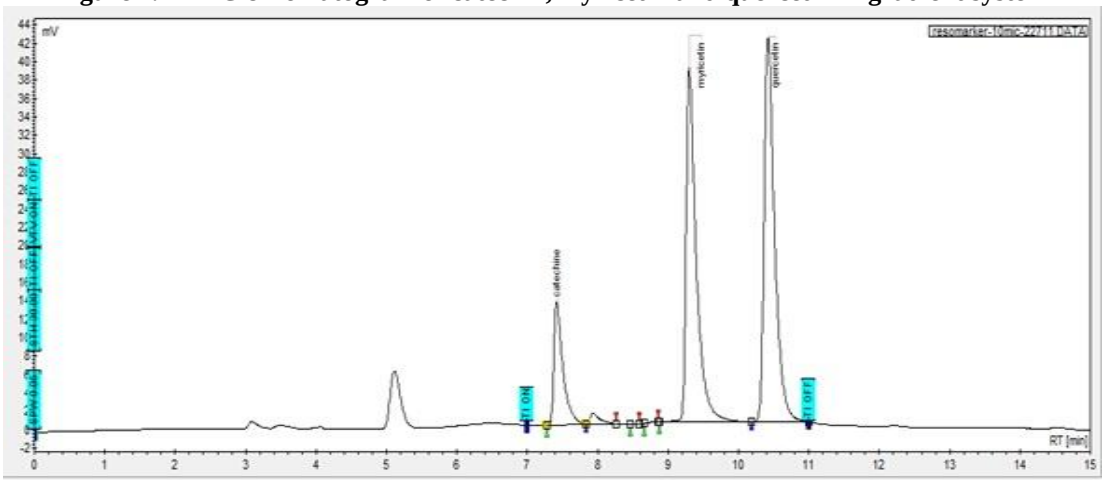
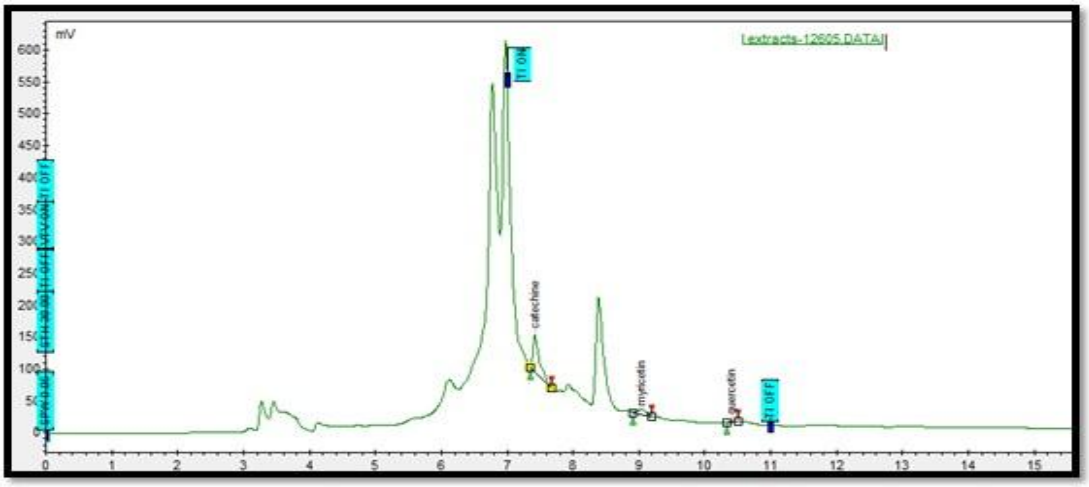


Figure 3. HPLC chromatogram of Extract III (High Yield Polyphenolic Extract (HYPE) of *P. granatum*)



CONCLUSION

This work describes a RP-HPLC method for the simultaneous quantification of three flavonoids, i.e. catechin, myricetin and quercetin in HYPE of *Punica granatum* (Pomegranate). The enzyme assisted extraction of peels of *Punica granatum* gave the highest extraction yield compared to the other two extraction techniques i.e. maceration and pre-treated enzyme maceration. The RP-HPLC method was sensitive enough for the separation and analysis of the three flavonoids. Good results were obtained with respect to repeatability (relative standard deviation (R.S.D.) <2.2%) and recovery (85.7 149-99.7385%). The amount of catechin, quercetin and myricetin present in the powder of peels of *Punica granatum* (Pomegranate) were found to be 115, 18.3 and 1.5 mg gm^{-1} of extract respectively. Validation statistics showed that this method documents good sensitivity, precision and repeatability. In Table 5 and Figure 3.

ACKNOWLEDGMENTS

The authors are thankful for the Analytical and research facilities provided by Reliable Analytical Laboratory, Mumbai, India.

REFERENCES

Ahn JG, Ingolf U, Mustapha, Azlin. Antimicrobial and antioxidant activities of natural extracts *in vitro* and in ground beef. *Journal of Food Protection*. 2004; 67(1): 148-155.

Celia M. Librán LM, Esperanza M. Garcia-Castello, Daniel Vidal-Brotons. Polyphenol extraction from grape wastes: Solvent and pH effect. *Agricultural Sciences*. 2013; 4: 56-62.

Hee Kee Kima BSC, Young Ha K, Sung Yong K, Hyun Pyo K. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure–activity relationships. *Biochemical Pharmacology*. 1999; 58(5): 759–765.

Hisashi Matsuda YP, Toshio M, Momotaro O, Masayuki Y. Gastroprotective effects of phenylpropanoids from the rhizomes of Alpinia galanga in rats: structural requirements and mode of action. *European Journal of Pharmacology*. 2003; 471(1): 59–67.

Jafri MA, Javed K, Singh S. Effect of *Punica granatum* Linn. (flowers) on blood glucose level in normal and alloxan-induced diabetic rats. *Journal of Ethnopharmacology*. 2000; 70(3): 309-314.

Jahfar KKV and Azadi P. Studies on a polysaccharide from the fruit rind of *Punica granatum*. *Research Journal of Chemistry and Environment.* 2003; 7(1): 43–50.

Kidd PM. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Altern Med Rev*. 2009; 14(3): 226-46.

Landbo AK MA. Enzyme-assisted extraction of antioxidative phenols from black currant juice press residues (Ribes nigrum). *J Agric Food Chem*. 2001; 49(7): 3169-3177.

Lapornik B PM, Wondra AG. Comparison of extracts prepared from plant byproducts using different solvents and extraction time. *Journal of Food Engineering*. 2005; 71(2): 214–222.

Lee LGC, Liang WL, Wang CC. Anti-inflammatory effects of *Punica granatum* Linne *in vitro* and *in vivo. Food Chemistry*. 2010; 118(2): 315–322.

Lodovici M GF, Meoni M, Dolara P. Effect of natural phenolic acids on DNA oxidation *in vitro*. *Food and Chemical Toxicology*. 2001; 39(12): 1205-1210.

Pinelo-Jiménez MM, Anne S. Enzyme-assisted extraction of antioxidants: Release of phenols from vegetal matrixes. *Electronic Journal of Environmental, Agricultural and Food Chemistry.* 2008; 7(8): 3217-3220.

Qu W PZ, Zhang R, Ma H, Chen X, Atungulu GG. Integrated extraction and anaerobic digestion process for recovery of nutraceuticals and biogas from pomegranate marcs. *Trans ASABE 2009.* 2009; 52(6): 1997-2006.

Riaz Ahmad Rather SC, Rajagopal K. Screening of Peel Extracts as Antioxidants, Anticancer Agents and antimicrobials. *Advances in Bioresearch*. 2010; 1(1): 29-33.

Rice-Evans CA, Miller NJ & Panganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids: A review. *Free Radical Biology and Medicine*. 1996; 20(7): 933-56.

Srinivas KVNS, Koteswara Rao Y, Mahender I, Das B, Krishna KVSR, Kishore KH and Murty USN. Flavanoids from Caesalpinia pulcherrima. *Phytochemistry*. 2003; 63: 789-793.

Toshio Aoki TA, Shin-ichi A. Flavonoids of Leguminous Plants: Structure, Biological Activity, and Biosynthesis. *Journal of Plant Research.* 2000; 113(4): 475-488.

Weichselbaum E, Buttriss JL. Polyphenols in the diet. *Nutrition Bulletin*. 2010; 35(2): 157-64.

Wenjuan Qu ZP, Haile Ma. Extraction modeling and activities of antioxidants from pomegranate marc. *Journal of Food Engineering*. 2010; 99(1).

Yasunori Hamauzu TI, Chihiro K, Miho I and Kohzy H. Antioxidant and Antiulcerative Properties of Phenolics from Chinese Quince, Quince, and Apple Fruits. *J Agric Food Chem*. 2006; 54(3): 765–772.

Yunfeng Li CG, Jijun Y, Jingyu W, Jing Xu, Shuang C. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry*. 2006; 96(2): 254–260.

Zu Yuan gang Fu Y-jL, Wei H, Chun-lian, Kong Y. Simultaneous Determination of Four Flavonoids in Pigeonpea Leaves Using RP-LC-DAD. *Chromatographia*, 2006; 63(9): 499.