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Evaulation of Antioxidant Activity of *Grewia asiatica* Berry Using 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) and *N,N*-Dimethyl-*p*-phenylenediamine Radical Cations Decolourization Assays

MUHAMMAD NADEEM ASGHAR, ISLAM ULLAH KHAN*,
LUBNA SHERIN and MUHAMMAD ASHFAQ
Department of Chemistry, GC University, Lower Mall, Lahore-54000, Pakistan
Fax: (92)(42)6376085; Tel: (92)(42)111-000-010 Ext: 263
E-mail: iukhan@gcu.edu.pk

Antioxidant activity and phenolic contents of fresh and stored samples of seed, peel and pulp of *Grewia asiatica*, a black berry, was evaluated using 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and modified *N,N*-dimethyl-*p*-phenylenediamine (DMPD) decolourization assays. Results showed that when *G. asiatica* is stored at 0 ºC for duration of one month, the polyphenols are partly degraded and/or transformed into other products. 2-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-yl)ethanoic acid (Trolox) equivalent antioxidant capacity (TEAC) values showed that polyphenols in fresh *G. asiatica* samples have a potent *in vitro* antioxidant activity which may be related to its medicinal properties against diseases like diabetes mellitus, hepatitis, *etc*. The highest values of antioxidant activity were obtained for peel followed by pulp and seeds. Extraction solvents of different polarity were employed in a bid to extract maximum phenolic contents. Total phenolic contents for peel, pulp and seed were estimated spectrophotometrically using Folin-Ciocalteu reagent with gallic acid as standard. Statistically positive correlation was found between phenolic content and antioxidant activity.

Key Words: Antioxidant activity, Polyphenols, Folin Ciocalteu reagent, Degradation, Berries, Spectrophotometry.

INTRODUCTION

Nevertheless, all aerobic organisms including human beings have antioxidant defences that protect them against oxidative damages caused by reactive oxygen species (ROS) produced during metabolic reactions[1-4]. However, under stressed conditions, this natural defence may be insufficient and too weak to combat the adverse effects of ROS and their products and hence additional dietary intake of antioxidant compounds is essential to keep the health quality in tact[5-7]. Besides their implication in more than 100 diseases, including malaria, acquired immuno-deficiency syndrome,

heart disease, stroke, arteriosclerosis, diabetes and cancer[8-11], ROS can also cause lipid peroxidation in stored food products, thus affecting the quality of these products and economy of the country[12,13].

Grewia asiatica (Phalsa, Urdu) is an indigenous dark purple coloured small fruit which is found throughout South East Asia. In the family Tiliaceae, only one genus Grewia, yields edible fruit. The only specie of any importance is *G. subinaequalis* which has been long referred as *G. asiatica* L in literature. The flavour of the *G. asiatica* fruit is pleasantly acid, somewhat grape-like. It has been reported that fruits of *G. asiatica* have positive effects in the medical treatment of various conditions including inflammation and respiratory, cardiac and blood disorders, as well as in fever. In addition, the fruits are eaten fresh as dessert, are made into syrup and are extensively employed in the manufacture of soft drinks. Although, Gupta *et al.*[14] have recently reported antioxidant activity of successive extracts of leaves of *G. asiatica*, yet, there is no report concerning the quantitative antioxidant potential of *G. asiatica* berry. The main objective of the present study is to investigate the antioxidant activity of fresh and stored *G. asiatica* in different extraction systems and to correlate phenolic-content with its antioxidant activity.

EXPERIMENTAL

N,N-Dimethyl-*p*-phenylenediamine (DMPD), 2,2'-azino*bis*-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin-Ciocalteu reagent and gallic acid were purchased from Fluka (Switzerland). Potassium persulphate was obtained from Merck (Germany). All chemicals were of HPLC grade and type 1 reagent grade deionized water was used.

Spectrophotometric measurements were recorded by using CECIL CE 7200 UV-Visible spectrophotometer (England) and quartz cells with 10 mm path length, under temperature controlled conditions.

Extraction of samples: Fruit was collected from three different local markets. After washing with distilled water, skin, pulp and seeds were separated manually and were allowed to dry under shade. All the three fragments were homogenized separately in a fine blender. Equal amounts of each part (1 g) were mixed with equal amounts (6 mL) of different solvents (70 % acetone, deionized water, 60 % methanol, dichloromethane and ethyl acetate) and after 1 h of gentle shaking the samples were centrifuged at 1800×g for 15 min. The whole extraction procedure was done at room temperature.

***N,N*-Dimethyl-*p*-phenylenediamine (DMPD) radical cation decolourization assay:** A modified $DMPD^{\bullet+}$ decolourization assay was employed to determine the antioxidant activity of different parts of *G. asiatica* berry[13]. Briefly, a 100 mM solution of DMPD was prepared by dissolving 209 mg

of DMPD dihydrochloride salt in 10 mL of deionized water. 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer (pH 5.6/1 mM final concentration) with 500 µL of potassium persulphate (0.4 mM) at 25 ºC. Oxidation of DMPD commenced immediately but the absorbance was not maximum and stable until more than 3 h had elapsed. The radical was stable in this form for more than 2 h at 25 ºC. An optical density of 0.800 ± 0.100 absorbance units was obtained and it represents the uninhibited signals.

Trolox stock solution was prepared by dissolving 0.01 g of Trolox in 100 mL of methanol. Different concentrations of Trolox were prepared in the range of 1-15 µm from the stock solution. 10 µL of Trolox (standard antioxidant) or appropriately diluted fruit samples were added in quartz cuvette containing 3.49 mL of the working solution of DMPD. The decrease in absorbance was monitored for 5 minutes at 25 ºC under continuous stirring at 517 nm. The buffered solution was placed in reference cuvette each time.

A dose response curve of Trolox was obtained by plotting a graph between the absorbance at 517 nm as % inhibition and the concentration of the standard antioxidant. % Inhibition is calculated according to the following equation:

$$\text{Inhibition of } A_{(517)}\ (\%) = (1 - A_f/A_o) \times 100$$

where A_o = uninhibited absorbance and A_f = absorbance measured for 5 min after the addition of sample and standard antioxidants.

Antioxidant ability of samples was expressed as trolox equivalent antioxidant capacity (TEAC) according to Miller *et al.*[12] using the calibration curve plotted with different amounts of Trolox. Each measurement is the mean of five determinations of each sample within the range of dose-response curve with Trolox.

2,2'-Azino*bis*-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay: ABTS decolourization assay was employed as the standard reference assay. ABTS radical cation was prepared by mixing ABTS stock solution (7 mM in water) with potassium persulphate solution (2.45 mM final concentration)[15]. The mixture was allowed to stand for 12-16 h in the dark at room temperature until it reached a stable oxidative state. ABTS solution was diluted with methanol to an absorbance of 0.700 ± 0.01 at 745 nm. For the spectroscopic assay, 3.49 mL of the ABTS solution and 10 µL of standard or sample were mixed and the absorbance was determined at 745 nm after 5 min of mixing. The absorbance was corrected for the absorbance of an ABTS blank.

Total phenolic content: The total phenolic phytochemical concentration was measured using Follin-Ciocalteu method. Briefly, 20 µL of appropriately diluted samples or standard solutions of gallic acid were added to 1.58 mL

water. Reagent blank using deionized water was prepared. 100 µL of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 300 µL of a 7 % Na_2CO_3 solution was added with mixing. After incubation for 0.5 h at 40 ºC, the absorbance relative to that of prepared blank at 750 nm was measured using a spectrophotometer. The total phenolic contents of the samples were determined in milligrams per grams of gallic acid equivalents (GAE) by way of comparison with standard calibration curve formed using gallic acid as standard antioxidant. All samples were prepared in three replications.

RESULTS AND DISCUSSION

Solvent extraction has been the most common method in fruit sample preparation. Previously, phenolic compounds were extracted from ground, dried, freeze-dried fruit samples or by macerating the fresh sample with the extracting solvents such as aqueous mixtures with ethanol, methanol and acetone[16,17]. In the present studies, five different solvent systems were tried with the aim to have best extraction of the phenolic compounds with the maximum antioxidant potential. The standard $ABTS^{\bullet+}$ decolourization method and modified $DMPD^{\bullet+}$ decolourization method[13] were employed to determine the antioxidant activity of different parts of the *G. asiatica* by using five different extraction systems. Employing ABTS and DMPD assays, 70 % aqueous mixture of acetone gave the most active extract in case of peel and pulp while ethyl acetate extract of seeds showed the highest antioxidant activity amongst the other solvent systems. It is quite evident from the Tables 1 and 2 that changing the polarity of the extraction solvent from water to ethyl acetate through acetone, there is a gradual increase in the antioxidant activity of seed with both ABTS and DMPD assays. This gradual increment in the activity may be attributed to an increase in the total phenolic content (TPC) of seed from aqueous to ethyl acetate solvents (Table-3). It is worth noticed that whatever extraction system was employed, the seed of the fruit showed a considerable antioxidant activity. Thus the addition of ground seed instead of their discarding as waste during the manufacture of syrups and drinks, may add to the dietary uptake of antioxidants. For peel and pulp of the fruit, lower values were obtained with ethyl acetate and dichloromethane as solvents and this may be ascribed to the presence of large amount of water soluble polyphenols[18].

The data obtained through both the assays is clearly suggestive of dependence of antioxidant activity and extraction of phenolic contents on the nature of extraction system. The results depict that no single solvent could be used to obtain the maximum antioxidant activity and TPC for all the three parts of *G. asiatica*. Aqueous mixture comes out to be more efficient than water or aqueous methanol in extracting phenolics in *G. asiatica* which is in agreement with the findings of Heinonen *et al.*[18].

Seeds of *G. asiatica* showed maximum polyphenolic content and antioxidant activity in ethyl acetate (Tables 1-3) whereas antioxidant activity and total phenolics of peel and pulp were maximum in 70 % acetone (Tables 1-3). A dramatic decrease in the antioxidant power and phenolic contents of the seed was observed after storage for 1 month at 0 ºC. This indicates that degradation of polyphenolic components of *G. asiatica* had occurred during storage (Table-3).

TABLE-1
ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF FRUIT OF *G. asiatica* USING ABTS ASSAY

Solvents	70 % Acetone	Water	60 % Methanol	Dichloro-methane	Ethyl acetate
TEAC (μ mol/g) of Fresh samples					
Seed	19.6±0.6	28.56±0.42	49.0±0.62	25.3±0.02	55.8±0.36
Peel	107.2±2.4	91.4±1.30	87.8±1.12	69.4±2.10	37.4±0.69
Pulp	60.9±1.8	54.0±0.80	56.1±1.20	29.6±0.06	25.3±1.22
Total TEAC (μ mol/g)	187.7	173.96	192.9	124.3	118.5
TEAC (μ mol/g)of Stored samples					
Seed	8.7±0.04	15.6±0.07	19.3±0.88	11.5±0.07	24±0.18
Peel	56.8±1.73	53.39±2.80	33.13±1.02	24.90±0.76	14.80±0.02
Pulp	29.5±0.90	20.89±90.1	25.8±1.00	15.8±0.04	14.7±0.73
Total TEAC (μ mol/g)	95.07	89.88	78.23	52.2	50.74

The data are presented as mean value ± SD (n = 5).

TABLE-2
ANTIOXIDANT ACTIVITY OF DIFFERENT PARTS OF FRUIT OF *G. asiatica* USING DMPD ASSAY

Solvents	70 % Acetone	Water	60 % Methanol	Dichloro-methane	Ethyl acetate
TEAC (μ mol/g) of Fresh samples					
Seed	6.6±0.01	9.8±0.42	10.1±0.09	8.25±0.33	11.25±0.21
Peel	29.4±0.71	24.0±1.10	18.6±1.01	17.7±0.69	10.5±0.52
Pulp	15.8±0.69	11.0±0.83	10.9±0.19	9.4±0.15	8.04±0.61
Total TEAC (μ mol/g)	51.8	45.1	39.6	35.35	29.78
TEAC (μ mol/g)of Stored samples					
Seed	1.8±0.01	1.3±0.04	n.d.	n.d.	n.d.
Peel	6.1±0.16	3.6±0.08	n.d.	3.0±0.14	n.d.
Pulp	5.7±0.18	5.0±0.01	3.7±0.02	n.d.	n.d.
Total TEAC (μ mol/g)	13.6	9.9	3.7	3.0	n.d.

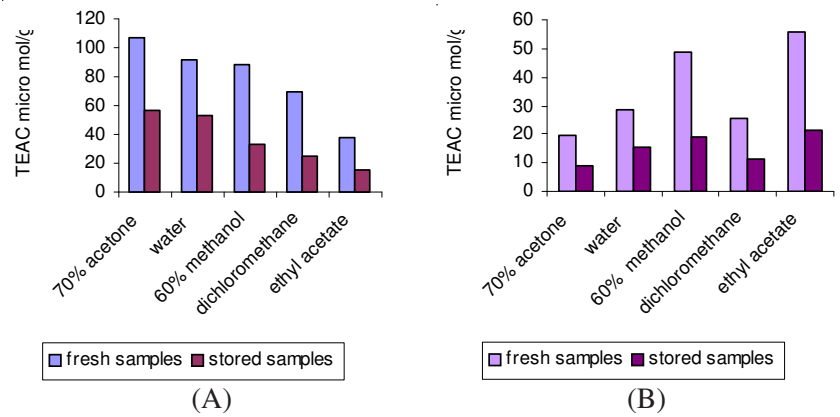
The data are presented as mean value ± SD (n = 5); n.d. = not detected.

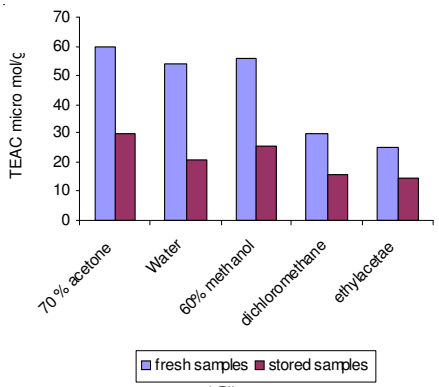
TABLE-3
TOTAL PHENOLICS OF SEED, PEEL AND PULP OF *G. asiatica* USING FOLIN-CIOCALTEU METHOD

Solvents	70 % Acetone	Water	60 % Methanol	Dichloro-methane	Ethyl acetate
Seed	1020±99	991±32	920±83	880±22	2060±65
Peel	5080±102	3990±120	2852±92	1980±79	1094±71
Pulp	2060±46	2050±56	1261±42	1000±38	895±55

The total phenolics are measured as mg of GAE/100 g using different solvent systems. The data are presented as mean value ± SD (n = 3).

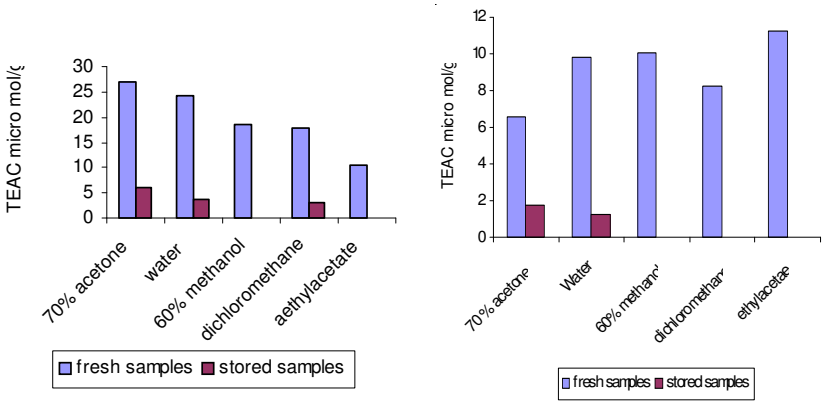
The antioxidant activity values of different parts of *G. asiatica* are shown in Figs. 1 and 2 which are quite higher than the values reported in literature for most of the other small-coloured fruits. The order of antioxidant power for different parts of *G. asiatica* was found to be peel > pulp > seed which remained unchanged for all the solvent systems used for extraction. The high antioxidant activity of *G. asiatica* may be due to its phenolic content. To verify this, the Trolox equivalent antioxidant capacity (TEAC) values were graphed against the respective total phenolic contents (TPC) values of peel, pulp and seed. Using 70 % acetone extract, the mean TPC values were found to be 90.1, 49.2 and 23.1 mg gallic acid equivalent (GAE) per 10 g of peel, pulp and seed, respectively. A positive correlation, between antioxidant activity and TPC, with a few deviations was found for all the parts of *G. asiatica* fruit (Fig. 3). The minor deviations can be attributed to synergistic effects of different antioxidant compounds particularly the ascorbic acid which is reportedly present in 7.4 mg/100 g of *G. asiatica*[20]. The values of antioxidant capacity and TPC are comparable to those reported for dark coloured berries like chokeberry, elderberry, blackberry and black currant[21].



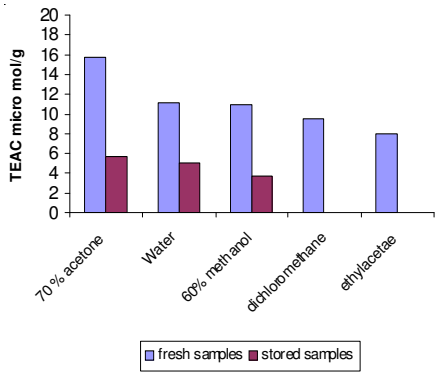


(C)

Fig. 1. Comparison of antioxidant activity of fresh and stored samples of (A) peel (B) seed (C) pulp of *G. asiatica* in different extraction systems using ABTS$^{\bullet+}$ decolourization assay



(A) (B)



(C)

Fig. 2. Comparison of antioxidant activity of fresh and stored samples of (A) peel (B) seed (C) pulp of *G. asiatica* in different extraction systems using modified DMPD$^{\bullet+}$ decolourization assay

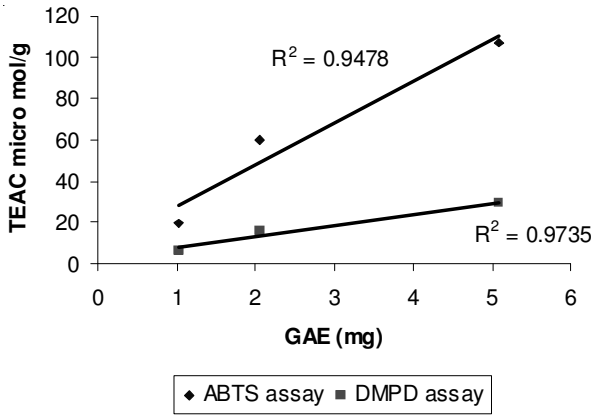


Fig. 3. Correlation between antioxidant activity and total phenolic content of 70 % acetone extracts of seed, peel and pulp of *Grewia asiatica*

G. asiatica has been reported to be rich in different anthocyanins and polyphenols, amongst which delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin 3,5-diglucoside, cyanidin, quercetin, quercetin-3-O-β-D-glucoside, tannins, catechins and luecoanthocyanins are more significant[18,22]. Anthocyanidin levels (mg/100 g fresh weight) range from 0.25 in the pear to 500 in the blueberry[19]. Cyandin-3-glucoside (C-3-G) and other anthocyanins are powerful *in vivo* antioxidants which not only suppress the changes caused by hepatic ischemia-reperfusion in rats but also lower the susceptibility to further lipid peroxidation[23-26]. These studies indicate that anthocyanins are much effective as *in vivo* antioxidants when included in amounts 20-40 mg/d, a much higher amount than found in the typical diet of humans. In addition, Wu *et al.*[20] and Mazza *et al.*[26] demonstrated that the plasma antioxidant capacity increases markedly after supplements of blueberries which are rich in anthocyanins. Similarly, flavonoids and other phenolics have also been shown to modify eicosanoid biosynthesis (antiprostanoid and antiflammatory responses), protect low-density lipoprotein (LDL) from oxidation (prevention of atherosclerotic plaque formation), prevent platelet aggregation (antithrombic effects) and promote relaxation of cardiovascular smooth muscle (antihypertensive, antiarrhythmic effects). Moreover, these flavonoids show antiviral and anticarcinogenic properties[29]. Cao *et al.*[29] found that following the consumption of a single meal of phenolic compounds, serum antioxidant capacity increased in humans as assessed by 3 different methods: ORAC, TEAC and FRAP[14,30]. In brief, there is a growing body of evidence that the diets rich in both anthocyanins and other phenolic contents function as strong antioxidants *in vivo* and may prove better arsenals against the degenerative diseases if taken in routine diet. The results of the present study show *G. asiatica* as a powerful natural source of these vital

antioxidants which are essential for maintaining health standard and combating the imminent risks of degenerative disease.

Conclusion

This paper reports useful data about the antioxidant activity and phenolic contents of seed, peel and pulp of *G. asiatica*. The antioxidant activity of peel and pulp of *G. asiatica* is considerably higher in comparison of many small fruits and may be compared with chokeberry and blackberry. Although 70 % acetone extract of *G. asiatica* exhibits the best antioxidant activity, but a comparable activity can also be obtained by using deionized water as the extraction solvent which enhances the value of *G. asiatica* in food and medicine industry. The storage studies on *G. asiatica* show a sharp decrease in its antioxidant and TCP values, which may be ascribed to the degradation or transformation of phenolic components into other products.

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