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Research Article
Extraction and Estimation of Anthocyanin Content and Antioxidant Activity of Some Common Fruits

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Abstract

Background and Objectives: Anthocyanins are phenolic compounds which are water-soluble pigments. They are responsible for imparting a variety of colors to the plants like orange, red, pink, blue and purple. It is well known that anthocyanins possess antioxidant activity and have different other pharmacological properties. The objectives of this study were to extract and conduct an estimation of Anthocyanins content and to evaluate antioxidant activity of apple, blueberry and plum crude extracts. **Materials and Methods:** The pH differential method was used to determine the anthocyanin content and the absorbance was measured using UV- spectrophotometer. The results were expressed as cyanidin-3-glucoside equivalents. Evaluation of the antioxidant activity was done by DPPH method and the results were expressed as a percentage of inhibition of DPPH radicals. **Results:** The highest concentration for anthocyanins was obtained from plum peels (60.45 mg/100 g), followed by blueberry (22.38 mg/100 g) while apple peels had the lowest concentration (16.39 mg/100 g). The three fruits had a very good antioxidant activity. For instance, for a concentration of 500 μg mL^{-1}, the percentage of inhibition for plum peels was 99.23%, blueberry 98.85% and apple peels 98.84%. **Conclusion:** Among the three types of fruits, the highest anthocyanin content was found in the plum peels and the lowest was found in the apple peels. It was observed that plum peels and blueberry showed higher antioxidant activity when compared to apple peels.

Key words: Anthocyanin, antioxidant, *Malus domestica*, *Vaccinium myrtillus*, *Prunus domestica*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anthocyanins are water-soluble pigments that based on the pH may impart blue, red or purple color. Examples of plants rich in anthocyanins are blueberry, bilberry, red raspberry, red cabbage, cherry eggplant peel and dark grapes[1,2]. Anthocyanins are known to possess antioxidant activity and for the quantification, separation, identification and purification of anthocyanins, many methods are used. The UV-V is spectrophotometry and High-Performance Liquid Chromatography (HPLC) are mainly used for the quantification[3].

Apple (*Malus domestica*) which belongs to the family Rosaceae, is a very popular fruit that is cultivated widely among different regions in the world[4]. It contains a wide variety of constituents that carries health benefits such as; vitamins, dietary fibers and sugars. The *Malus* genus contains about 30-35 species of shrubs or small deciduous trees[5]. It was proved that apple has antioxidant activity which is mainly due to the presence of phenolic compounds like flavonoids, phenolic acids and anthocyanins[6]. Some anthocyanins components were identified in an apple peel like cyanidin-3-galactoside, cyanidin-3-arabinoside, cyanidin-3-glucoside, cyanidin-3-xyloside and cyanidin-3-rutinoside[7].

Blueberry fruits (*Vaccinium myrtillus*) are from the family Ericaceae and belong to the genus *Vaccinium*. These fruits have shown their health benefits by exerting anti-cancer, anti-inflammatory and anti-diabetic properties[8]. In addition, due to its antioxidant capacity which is high against reactive species or free radicals, it is known as "longevity fruit" and this mechanism contributes to reducing the risk of developing noncommunicable diseases. Like other berries, blueberry is rich in phenolic acids, flavonoids and tannins[9]. It was found that blueberry has health benefits in improving memory and learning. This is mediated by the protein kinases like Extracellular-signal Regulated Kinase (ERK) and Mitogen-Activated Protein Kinase (MAPK)[10].

Plum (*Prunus domestica*) is a drupe fruit that belongs to the family Rosaceae and it is available in different sizes and a wide variety of colors like red, white, green and yellow. A dusty white coating indicates a mature plum which gives a glaucous appearance. Plums are rich in anthocyanins as presented in Table 1, phenolic acids, minerals, pectins and carotenoids. The phenolic compounds present in plums are: cryptochlorogenic acid (956 mg kg^{-1}), neochlorogenic acid (85-1300 mg kg^{-1}) and chlorogenic acid (13-430 mg kg^{-1})[11].

Table 1: Anthocyanin content in plum fruits

Anthocyanin content	Quantity range of total anthocyanin content (%)
Cyanidin-3-sophoroside	-
Peonidin-3-glucoside	0.0-0.4
Peonidin-3-rutinoside	6.5-37.9
Cyanidin-3-glucoside	1.8-18.4
Cyanidin-3-rutinoside	52.6-73.0
Cyanidin-3-xyloside	4.7-7.80

Source: Birwal *et al.*[11], Castaneda-Ovando *et al.*[12] and Usenik *et al.*[13]

Anthocyanins can be used industrially as natural colors and can be used for a wide variety of foods, cosmetics and drugs. As a natural product, anthocyanins are good for health due to their antioxidant properties and may have a role in immunity by boosting our immune system. In addition, it has a role in disease prevention and maintaining health.

It was found that the percentage of anthocyanins estimated varies in different plant species. This has an impact on the individual intake of anthocyanins which is influenced by climate, cultivation, cultural and educational issues[14]. In a study conducted to assay the antioxidant activity: Anthocyanin characterization, total phenolic quantification and antioxidant features of some Chilean edible berry extracts by Brito *et al.*[15], it was shown that the amount of anthocyanins and the antioxidant capacity were different in six types of berries.

A study was conducted to identify the antioxidant activity and the total anthocyanin content in apple, The total anthocyanin contents of peel extracts determined by pH differential method were 95% while the pulp extract contained a limited amount of anthocyanin contents[16] i.e., 0.003%. The major flavanols and their corresponding polymers present in apples are procyanidins, epicatechin, gallocatechin and catechin[17]. It was found that the phenolic compounds are mostly concentrated in the peel when compared to the pulp of the apple fruit and the major compounds present in the peel are proanthocyanidins and anthocyanins[18,19].

Blueberry is reached with anthocyanin which is believed to be the reason for many health benefits of blueberry. There are different types of anthocyanin present in blueberry and it was found that malvidin predominate. Pelargonidin, delphinidin, petunidin and cyanidin were identified in similar concentrations. It was reported in researches for the quantification of anthocyanin in blueberry that the total mean content of anthocyanin is 199 mg/100 g and a range[20] of 57-503 mg/100 g.

It was observed in studies that the anthocyanin content in plums is directly proportional to the intensity of the red color[21]. In some studies, it was demonstrated that plum fruit has a higher antioxidant capacity when compared to apple.

It was proved that plums have a considerable scavenging activity against hydroxyl and peroxyl radicals which are oxygen-derived free radicals[22]. It was proved in this study that the three fruits contain a considerable amount of anthocyanins and possess high antioxidant activity due to the presence of phenolic compounds.

The main purpose of this study was to increase the awareness to the people regarding the importance of anthocyanins. Another purpose is to do advanced researches in the future to determine a possible way to contribute the antioxidant activity exhibited by these fruits in pharmacy researches and drug discovery. This study was done to extract and estimate the Anthocyanins content in apple, blueberry and plum.

MATERIALS AND METHODS

Apparatus and equipment: The pH meter, Sonicator, Magnetic stirrer, Visible spectrophotometer, Rotary evaporator (Yamato Rotary evaporator), Buchner apparatus, Filter papers, Test tubes, Volumetric flasks, Beakers, Pipette.

Reagents: The pH 1.0 buffer (0.025 M potassium chloride), pH 4.5 buffer (0.4 M sodium acetate), Ethanol, Methanol, DPPH, petroleum ether, 0.1 M HCl

Study area: This study was started in September, 2018 and ended in March, 2019. It was performed in School of Pharmacy Research Laboratory and Natural and Medical Sciences Research Center, University of Nizwa, Sultanate of Oman.

Procedure

Collection of the plant materials: Red apple (*Malus domestica*), blueberry (*Vaccinium myrtillus*) and plum (*Prunus domestica*) fruits were collected from the supermarket, Oman. Fresh fruits were used for the estimation of anthocyanin content in the three fruits and dried fruits extracts were used for the determination of antioxidant activity.

Determination of anthocyanin content in fresh fruits

Extraction of anthocyanins from fresh fruits: First, apples, blueberries and plums were washed to eliminate any waste. Then using an analytical balance the following quantities were measured and used: 150 g of apple peel, 200 g of blueberries and 200 g of plum peel. Each fruit was then taken and blended using a normal kitchen blender to get a soft puree.

A 1000 mL solution of 0.1 M HCl (hydrochloric acid) was prepared. Each fruit puree was extracted with ethanol solvent: 0.1 M HCl (85:15%, v:v). The ratio used between the fruit puree and the solvent for extraction was 1:2. The following quantities of ethanol and HCl were mixed together and then added to the fruit puree of each type: apple (Ethanol = 255 mL, HCl = 45 mL), blueberry (Ethanol = 340 mL, 0.1 M, HCl = 60 mL) and plum (Ethanol = 340 mL, HCl = 60 mL). After that, a magnetic stirrer was used to mix each fruit mixture separately for 1 h. Then, the different fruits mixtures were filtered using vacuum filtration by a Buchner apparatus to collect supernatant solutions. The extraction procedure was done in triplicate.

Preparation of buffers: The pH 1.0 buffer (0.025 M potassium chloride) was prepared by using an analytical balance to weigh 1.86 g of KCl in a 1000 mL beaker. After that, 980 mL of distilled water was added and mixed. The pH was measured using the pH meter and adjusted to pH 1.0 using HCl. The solution was transferred to a 1000 mL volumetric flask and the volume was made up to 1000 mL using distilled water.

The pH 4.5 buffer (0.4 M sodium acetate) was prepared by using an analytical balance to weigh 54.43 g of $CH_3CO_2Na{\cdot}3H_2O$ in a 1000 mL beaker. After that, 980 mL of distilled water was added and mixed. The pH was measured using the pH meter and adjusted to pH 4.5 using HCl. The solution was transferred to a 1000 mL volumetric flask and the volume was made up to 1000 mL using distilled water[23].

Preparation of test solutions: The dilutions were done by using the volumetric pipette to measure and add the test portion in a 50 mL volumetric flask. The dilution factor was measured by diluting the test portion of each fruit by using pH 1.0 buffer until the absorbance of the spectrophotometer measured at 520 nm was within the linear range (0.2-1.4 AU). The test portion added was not more than 10 mL which means that 1 test portion was added to 4 parts of the buffer to ensure that the buffer capacity of the reagents was not exceeded. Using the calculated dilution factor 2 test solutions of each fruit were prepared, the first one with a pH 1.0 buffer and the other test solution with pH 4.5 buffer.

Determination: The absorbance was measured within 20-50 min of preparation for the 2 test solutions pH 1.0 and 4.5 using a spectrophotometer at 520 nm. Absorbance is also measured at 700 nm for the correction of haze. The diluted test solution that was found to be excessively turbid was filtered before measuring the absorbance using filter papers with $\leq$1.2 mm pore size to ensure that anthocyanins were not be absorbed. The blank used was distilled water and the test solutions were read versus it.

Calculations: The anthocyanin pigment concentration (as cyanidin-3-glucoside equivalents) was calculated using the following equation[24]:

$$\text{Anthocyanin pigment} = \frac{A.\,MW.\,DF.\,10^3}{\varepsilon.\,l}$$

Where:

A = $(A_{520\,nm}-A_{700\,nm})\,pH_{1.0}-(A_{520\,nm}-A_{700\,nm})\,pH_{4.5}$
MW = 449.2 g moL^{-1} for cyanidin-3-glucoside
DF = Dilution factor
l = Path-length (cm)
ϵ = 26,900 molar extinction coefficient, in L/mol cm for cyanidin-3-glucoside
10^3 = Factor for conversion from g to mg

Determination of the antioxidant activity

Extraction of dried plants materials: Apple peel, blueberries and plum peel were dried naturally not using any instrumental method. After drying, using an analytical balance 43 g of blueberries, 63 g of plums' peel and 47 g of apples' peel were obtained and measured. The dried plants' materials were crushed using a kitchen blender to get a powder form. For the elimination of lipids and chlorophyll, the fruit powder of each type was socked in petroleum ether for 1 day (24 h). After that, the different mixtures were filtered by vacuum filtration using Buchner apparatus and the powder of blueberries, plums and apples were collected and spread to dry for 1 h. Then the powder material of each fruit was soaked in ethanol for the extraction of anthocyanin and was kept for 3 days. The solvents were then filtered under vacuum using a Buchner apparatus to obtain clear solutions for the three fruits. Ethanol was then evaporated using a rotary evaporator and crude extracts were obtained. The weight of the crude extract obtained as ethanolic extract was determined and the percentage yield was calculated.

Antioxidant activity measurement: Brand Williams method with some modifications was used to determine the antioxidant activity of the different extracts[25]. From each crude extract of the different fruits, 1 g of the crude extract was taken and mixed with 1 mL of methanol. For proper mixing, a sonicator was used. The solution prepared was further diluted to prepare different concentrations (62.5, 125, 250 and 500 μg mL^{-1}). For the preparation of methanol solution of 0.3 mM DPPH (2,2-diphenyl-1-1-picrylhydrazyl) a 3.3 mg of DPPH powder was taken and mixed with 100 mL of methanol. After that using a volumetric pipette 3 mL of each sample was measured and mixed with 1 mL of the DPPH solution in a test tube. The prepared mixture was strongly shaken and then covered and kept in a dark place for 30 min at room temperature. Then the absorbance of DPPH and the different mixtures were measured at 517 nm against a blank consisting of methanol and the control consist of DPPH and methanol. This process was done in triplicate. To calculate the radical scavenging activity that was estimated as an inhibition percentage the following formula was used[25]:

$$\text{RSA}\ (\%) = \frac{A_o - A_s}{A_o} \times 100$$

where, A_o is absorbance of control and A_s is absorbance of test sample.

RESULTS

Using the pH differential method the total anthocyanins content in apple peel, blueberry and plum peel were determined. It was found that the anthocyanin content in the peels of plum contained the highest amount of anthocyanin when compared to blueberry and the peels of apple (Fig. 1). The anthocyanin content determined in the peels of plum fruits was 60.45 mg/100 g. Blueberry with a determined anthocyanin content of 22.38 mg/100 g and then peels of the apple came with a total anthocyanin content of 16.39 mg/100 g.

Ethanolic extracts of the dried plants: The amount of the crude extract obtained as an ethanolic extract from the dried plants material of apple peel, blueberry and plum peel was 8.69, 8.40 and 8.74 g, respectively. The percentage yield calculated for apple it was 18.49% and for blueberry was 19.53% while for plum it was 13.87% (Table 2).

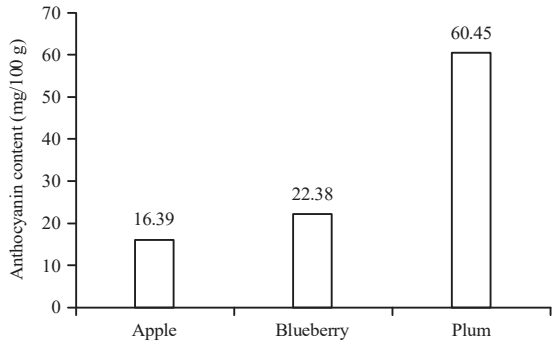

Fig. 1: Anthocyanin content in mg/100 g

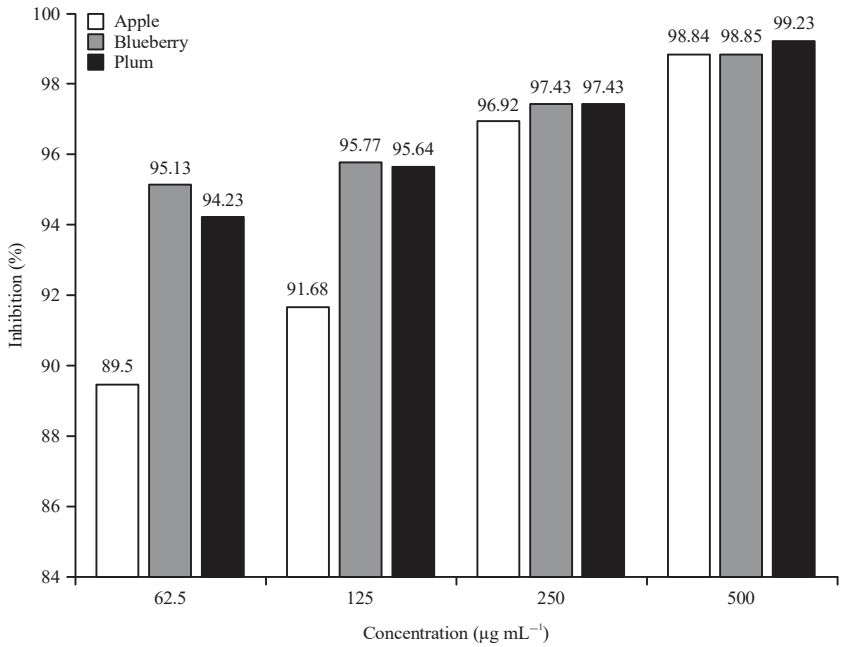

Fig. 2: Radical scavenging activity of apple, plum and blueberry

Table 2: Amount and the percentage yield of ethanolic crude extracts

Fruit	Amount of crude extract (g)	Yield (%)
Apple	8.69	18.49
Blueberry	8.40	19.53
Plum	8.74	13.87

Antioxidant activity: The results for the antioxidant activity showed that blueberry, plum and apple have high antioxidant activity against DPPH. It was observed that the higher the concentration of the test sample, the lower the absorbance and thus higher the inhibition percentage. It was shown that the concentration is proportional to the percentage of inhibition of free radicals (Fig. 2). Blueberry and plum had the highest antioxidant activity when compared to apple. For instance, for a concentration of 62.5 µg mL^{-1} blueberry had a percentage of inhibition of 95.13%, plum had 94.23%, while apple had a lower percentage of inhibition of 89.5%.

DISCUSSION

The red color of the apple peels is due to the presence of anthocyanins. The results of this study (16.39 mg/100 g) matched the range reported by Wolfe *et al.*[26] in a study conducted to determine the antioxidant activity in apple peels 2.1-33.3 mg/100 g. A much lower concentration than our result was reported in Italy by a study conducted by Contessa and Botta[18] (8.22 mg/100 g) expressed as cyanidin-3-glucoside. This result also matches what was reported by Wu *et al.*[27] for the anthocyanin concentration in 3 types of apple with a range of 1.8-17 mg/100 g, with red delicious type having the higher anthocyanin concentration of 17 mg/100 g. The result of our study is higher than what was reported by Hirsch and Martins[28] for anthocyanin concentration (1.7 mg/100 g) of red delicious apple.

When these results were compared to anthocyanin concentration in blueberries (22.38 mg/100 g) with other international studies it was found variation in anthocyanin concentration. This study results agree with a study conducted by Sellappan *et al.*[29] for determining anthocyanin concentration in blueberries: "Total anthocyanins ranged from 12.70-197.34 mg/100 g". A less amount (8.28 mg/100 g) was found by Corona *et al.*[30] for determining anthocyanin content in some of the common fruits. The result of the present study was higher than what was stated by Stevenson and Scalzo[20] of anthocyanin mean content of 15.8 mg/100 g and a range of 5.2-39.1 mg/100 g expressed as cyanidin-3-glucoside equivalent.

Anthocyanins impart the dark color in the peels of plum. The result for anthocyanin content in peels of plum (60.45 mg/100 g) expressed as cyanidin-3-glucoside was compared with other studies to observe the differences. The anthocyanins concentration determined are within the range of a study conducted in the USA by Cevallos-Casals *et al.*[31] (33-173 mg/100 g). This result also agrees with the range reported by Wu *et al.*[27] of the concentration of plum determined as cyanidin-3-glucoside (12.5-82.2 mg/100 g).

A lower concentration compared to this study was reported by Horbowicz *et al.*[32] for anthocyanin content expressed as cyanidin-3-glucoside (39.7 mg/100 g). Another lower range was reported by a study conducted in Serbia. "The total anthocyanins content ranged from 5-57 mg/100 g, expressed as cyanidin-3-glucoside equivalents, on a fresh weight basis"[22].

The variations of the results among different studies for apple, blueberry and plum may be due to many reasons. The method used for quantification and separation of anthocyanin may affect the results. Technical errors and accuracy in measuring the solvents and chemicals used play a role in determining the final result. Another thing that may contribute to the variation in the results is the cultivation methods that differ in different countries. The use of organic substances to supply plants with nutrients and the use of other chemicals could affect the synthesis of natural compounds in the plant. The climate and temperature differences around the world can affect the synthesis of anthocyanins and the other natural products that the plants produce. In addition, the amount of sunlight that reaches the plant and the pH of the soil also play a role in anthocyanins synthesis and productions. When the individual anthocyanins are not known and quantified the anthocyanin concentration is calculated and expressed as cyanidin-3-glucoside equivalents which are a standard. Further studies are needed to determine the concentration and types of individual anthocyanins presented in blueberry, plum and apple.

The antioxidant activity of apple expressed as a percentage of inhibition was determined. Apple showed higher inhibition percentages when compared to other studies. In a study done in India for the determination of antioxidant activity in apple peels using DPPH method and methanolic extracts the results for the concentrations: 62.5, 125, 250 and 500 μg mL^{-1} were 41.39, 44.83, 51.73 and 68.97%, respectively[33].

The antioxidant activity that was measured in this study and other studies could be due to the presence of many compounds in the apple peels such as phenolic compounds, epicatechin, chlorogenic acid, cyanidin glycoside, procyanidin and quercetin glycoside[34,35].

When the antioxidant activity of blueberry was compared with other studies, variations in the radical scavenging activity percentage were found. At a concentration of 500 μg mL^{-1}, it was reported[36] that the percentage of inhibition of blueberry acting as an antioxidant is 40.6%, while in this study it was 98.85%. In another study[37] to measure the antioxidant activity of blueberry at a concentration of 62.5 μg mL^{-1}, the percentage of inhibition was 74.58% while current results was 95.13%.

The antioxidant activity of blueberry is due to the presence of phenolic compounds and anthocyanins. For instance, it was reported in other studies for the quantification of individual anthocyanins that blueberry contains different types of anthocyanins like: (Cyanidin-3-glucoside, Delphinidin-3-glucoside, Malvidin-3-glucoside, Peonidin-3-glucoside, Petunidin-3-glucoside)[38]. It was also reported that blueberry contains different types of phenolic compounds like Gallic acid, Chlorogenic acid and Flavan-3-ols[39].

Plum showed a high antioxidant activity which is higher than what was reported by other studies. For instance, in a study[40] conducted for determining the antioxidant activity of Korean plum, a concentration of 250 μg mL^{-1} had a percentage of inhibition of 53% while in this study it was 97.43%. In another study[41], a concentration of 125 μg mL^{-1} had a percentage of inhibition of 62.5%. In a study conducted in Egypt for determination of antioxidant activity of 2 plums species (Santa Rosa and African Rose) using DPPH method but in ethanolic extract instead of methanolic extract which is used in this study, it was found that for a concentration of 125 μg mL^{-1} Santa Rosa plum had percentage of inhibition of 60.89% while African Rose plum[42] had 76.2%.

The antioxidant properties of plum are probably due to the presence of naturally occurring compounds such as phenolic compounds, carotenoids, anthocyanins and flavonoids[43]. It was reported that plum contains compounds that have antioxidant properties such as cyanidin-3-O-glucoside, chlorogenic acid, flavan-3-ols, neochlorogenic acid and quercetin[44,45].

CONCLUSION

Among the three types of fruits, the highest anthocyanin content was found in the plum peels and the lowest was found in the apple peels. The antioxidant activity was measured using the DPPH method. It was observed that plum and blueberry showed similar results while apple peels had the lowest antioxidant activity. The presence of anthocyanins and the antioxidant activity in the three plants should be investigated more to contribute this in the future and have a role in the treatment of diseases caused by free radicals.

SIGNIFICANCE STATEMENT

This study discovers the anthocyanin content and antioxidant activity of apple, blueberry and plum that can be beneficial for health to fight against many diseases. This study will help the researcher to uncover the critical areas of the antioxidant activity exhibited by the three fruits that many researchers were not able to explore. Thus a new theory on how to incorporate these results for drug discovery in the future may be arrived at.

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