



## RESEARCH PAPER

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## Influence of fruit ripening stages on antioxidant enzymes in *Rubus hyrcanus* Juz

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### Abstract

The purpose of this study was to evaluate effects of ripening stages on antioxidant enzymes and antioxidant activity of fruits of *Rubus hyrcanus* Juz., grown in Northwest of Iran. The activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and antioxidant compounds, total phenols, flavonoids, ascorbic acid and anthocyanins along three growth stages of raspberry fruit were determined in this work. DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging capacity, assay was used to screen the antioxidant properties of extracts. Significant variations in antioxidant properties and involved compounds were observed at three different growth stages. Enhanced fruits ripening were reflected by decreased values for, phenol and flavonoid contents and increased concentration of total anthocyanins and ascorbic acid. The highest activities of SOD, CAT was at the unripe fruit. The antioxidant activity of raspberries was directly related to the total amount of phenolics and flavonoids. Our results support the use of unripe fruit of raspberry as sources of antioxidant compounds.

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## Introduction

Raspberries (*Rubus* sp) are a member of the Rosaceae family, grown as a perennial crop. Raspberries are a compound fruit made up of many drupelets and a hollow center where the fruit detaches from the receptacle. Raspberries are soft, juicy with a distinct aroma and are a good source of natural antioxidants. In addition to vitamins and minerals, raspberries are also rich in anthocyanin, phenolic acids, and other flavonoids (Wang and Lin., 2000).

Antioxidants mainly include a group of oxidative enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) etc. and phenolic compounds of various chemical structures (e.g. catechins, flavonoids, anthocyanins) and vitamin (C, E and A) (Ferreyra *et al.*, 2007). They can neutralize harmful free radicals to protect the cell against the attack from the free radicals and reduce oxidative damage, thus preventing a critical step in the onset of carcinogenesis (Meyskens and Szabo., 2005). Fresh raspberries are aromatic fruits, appreciated by consumers, and contain healthy dietary compounds with antioxidant activity (Ancos *et al.*, 2000; Wang and Lin, 2000).

Genetic and environmental factors, such as cultivar, maturity and harvesting method, play an important role in berry composition (Parr and Bolwell., 2000). It is well-known that levels of phenolics and the antioxidant activity of raspberries are influenced by maturity (Wang and Lin., 2000). Studies related to changes of phytochemicals with fruit maturity have often been carried out over a wide range of development stages (Beekwilder *et al.*, 2005; Vincente *et al.*, 2006; Ferryra *et al.*, 2007). However, the influence of narrow but different maturity stages at harvest on the health promoting quality of raspberries has not yet been thoroughly investigated in Iran.

*Rubus hyrcanus* Juz. Is a very important raspberry in Iran, few works have described its antioxidant compounds and properties during ripening. Considering the importance of antioxidant and

antioxidant activity for functional benefit of raspberry, the aim of this study was to determine effect of maturity stage on the content of total phenolics, vitamin C, anthocyanins and flavonoids as well as antioxidant activity of *Rubus hyrcanus* Juz in order to have a clear understanding of antioxidant metabolic changes, phytochemical accumulation and make the best use of the different botanical stages of fruits to extract for dietary supplements.

## Materials and methods

### Fruit materials

Raspberries that were evaluated in this study (*Rubus hyrcanus* Juz.) were collected from the northwest (Heyran- Ardebil province) of Iran. Fruits were separated into three maturity stages representing unripe, semi-ripe and ripe. All fruits were transported to lab within 30 min after harvest and damage free were selected, immediately treated with liquid nitrogen and stored at -80 °C until extraction.

### Ascorbic Acid (Vitamin C)

Total ascorbic acid content was determined using the dinitrophenylhydrazine (DNPH) method (Terada *et al.*, 1978). Five grams of homogenized fruit tissue was added to 100 ml of a mixture of 6% metaphosphoric acid in 2 mol<sup>-1</sup> acetic acid. The mixture was centrifuged at 17,000 × g for 15 min at 4°C and supernatant was filtered through Whatman filter paper. One milliliter aliquot of the supernatant was mixed with 0.05 ml of 0.2% 2, 6-dichlorophenolindolphenol (DCIP) and the solution was incubated at room temperature for 1 h. After that, 1 ml of 2% thiourea in 5% metaphosphoric acid and 0.5 ml of 2% DNPH in 4.5 mol<sup>-1</sup> sulfuric acid were added to the solution, and then incubated at 60°C for 3 h. The reaction was stopped by placing the tubes in an ice bath and slowly adding 2.5 ml of cold 90% sulfuric acid. Total ascorbic acid was measured by absorbance at 540 nm using a standard curve. The concentrations were expressed as ascorbic acid on a fresh weight basis, mg per 100 g of fruit.

### Total anthocyanin content

Some of frozen tissue was ground to a fine powder

under liquid nitrogen by cold mortar and pestle and 1g of the resultant powder was added to 10 ml of methanol containing HCl (1%, v/v) and held at 0°C for 10 min (Cordenunsi *et al.*, 2003). The slurry was centrifuged at 17,000× g for 15 min at 4 °C and then the supernatant was used. Total anthocyanins content was measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extracts were measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid-sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 29,600 (cyaniding-3- glucoside).

$$\text{Absorbance (A)} = (A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5}$$

Results were expressed as mg cyaniding 3-glucoside equivalent per 100g of fresh weight.

#### Total phenolic content

Total phenol in the methanol extracts was determined with Folin-Ciocalteu reagent by the method of Slinkard and Singleton (1972). Gallic acid (GAE) was used as a standard and results were expressed as mg gallic acid equivalents per 100 g fresh weight.

#### Total flavonoid content

Some of frozen tissue was ground to a fine powder under liquid nitrogen by cold mortar and pestle. One gram of the resultant powder was added to 10 ml of methanol containing HCl (1%, v/v) and held at room temperature for 24 h (Cordenunsi *et al.*, 2003). The slurry was centrifuged at 4000× g for 15 min at 4°C, and the supernatant was used. The total flavonoid contents were determined by a colorimetric assay (Yanping *et al.*, 2004). One milliliter aliquot of appropriately diluted sample was added to a 15 ml tube containing 4ml of deionized water. Then 0.3 ml of 5% NaNO<sub>2</sub> was added to this mixture, which was allowed to stand for 5 min at room temperature, and 0.6 ml of 10% AlCl<sub>3</sub>.6H<sub>2</sub>O was added. The mixture was allowed to stand for 6 min at room temperature, and 2 ml of 1 mol l<sup>-1</sup> NaOH was added, and the total was made up to 10 ml with deionized water. The absorbance of the solution was measured

immediately at 510 nm. Quercetin was used as a standard compound for the quantification of total flavonoid.

#### SOD and CAT activities

SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activity were assayed by the determination kit (Nanjing Jiancheng Bioengineering Institute). One unit of SOD activity was defined as the amount of enzyme required for 1 g tissue in 1 mL of reaction mixture SOD inhibition rate to 50% as monitored at 550 nm. One unit of CAT activity defined as the amount of enzyme required for 1 mg tissue protein decomposed 1 μmol H<sub>2</sub>O<sub>2</sub> in 1 min.

#### Antioxidant activity by DPPH radical scavenging method

The antioxidant capacity of the raspberry fruits were evaluated by free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methods. For the determination of free radical scavenging capacity, raspberry samples were extracted with methanol. Then, they were centrifuged (Sigma 3K30, Germany) at 15,000× g for 10 min. The supernatants were concentrated under reduced pressure at 40° C. The dried extracts were dissolved in methanol. Free radical scavenging activity was measured according to the principle of Nakajima *et al.* (2004) with some modifications reported by Chiou *et al.* (2007). Fifty microliters of the diluted extracts (concentrations 2-20 mg ml<sup>-1</sup>) were added to 1 ml of 6× 10<sup>-5</sup> mol l<sup>-1</sup> DPPH (free radical, 95%, sigma-Aldrich Chemie GmbH, Steinheim, Germany) in methanol. The mixture was shaken and left at room temperature for 30 min; the absorbance was measured spectrophotometrically at 515 nm. Methanol was used as an experimental control. The percent of reduction of DPPH was calculated according to the following equation

$$\% \text{ inhibition of DPPH} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

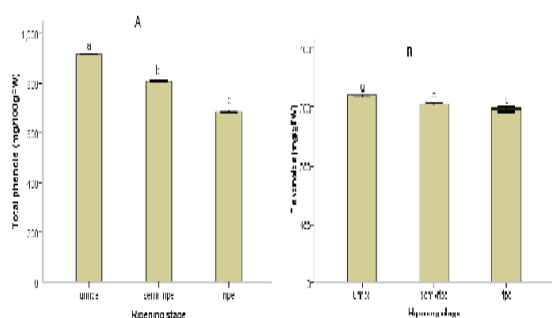
#### Statistical analysis

Statistical analyses were performed using the SPSS for Windows version 16.0 (SPSS Inc.,USA).

Differences between means were first analyzed by ANOVA test and then least significant difference (LSD) test ( $P < 0.05$ ). Pearson's correlation coefficient was used to estimate the relationship between the contents of phenolics.

## Results

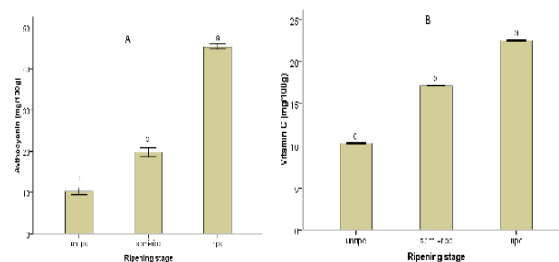
Total phenol and flavonoid of each development stage were statically different and showed a declining trend with advancing maturity. Total phenol and flavonoid contents decreased during raspberry development. Total phenol content in fruits unripe was 916.5 mg gallic acid /100 g FW. Flavonoid contents also decreased from 321.5 to 296.5 mg Quercetin / 100g FW from unripe to ripe stage.



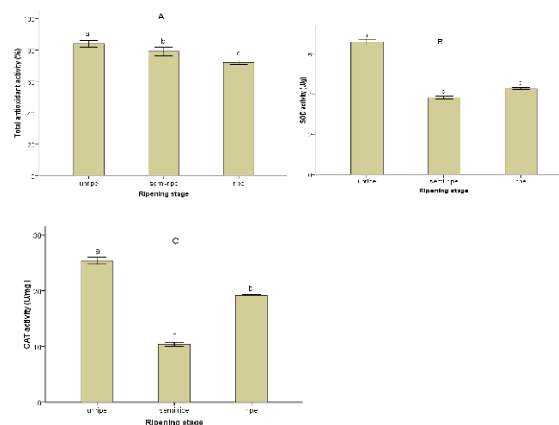
**Fig. 1.** Variation of total phenol (A) and flavonoid (B) contents during ripening of *Rubus hyrcanus* Juz. Mean followed by the same letters are not significantly different for  $p=0.05$ .

Anthocyanin content increased gradually during maturation (Fig) which was a typical maturation process. Along the ripening stages anthocyanins were detected only from unripe stage, the content was 10.35 mg cyaniding-3-glucoside /100 g FW and values were very low up to the semi ripe stage. From the stage on, the content of anthocyanins increased sharply and reached 45.36 mg cyaniding-3-glucoside / 100 g FW at ripe stage. In the experiment, the ascorbic acid content showed an increasing trend with advancing maturity (Fig). The contents were 22.58 mg AA / 100 g Fw at ripe stage. In this experiment, SOD activities decreased sharply from unripe to semi ripe stage, from semi ripe to ripe stage SOD activities increased slightly. Comparing unripe and ripe stage, SOD activities decreased from 60.57 to 4.28 U.g<sup>-1</sup>FW

(Fig). The activities of CAT showed a rapid decreased and then increased a little (Fig ).



**Fig. 2.** Variation of anthocyanin (A) and ascorbic acid (B) contents during ripening of *Rubus hyrcanus* Juz. Mean followed by the same letters are not significantly different for  $p=0.05$ .



**Fig. 3.** Variation of DPPH inhibition ratio (A) and SOD (B) and CAT (C) activities during ripening of *Rubus hyrcanus* Juz. Mean followed by the same letters are not significantly different for  $p=0.05$ .

The antioxidant activity results using DPPH method in ripening stages of raspberries are shown in Fig. A statistical significant difference ( $P < 0.05$ ) was found among ripening stages. Fruits harvested at ripe stage the lowest DPPH value. The unripe fruits had stronger antioxidant activity compared to ripe and semi ripe fruit, which was demonstrated by higher DPPH values and total phenol and flavonoid content. There was a positive correlation between DPPH with the total phenolic content at harvest for unripe, semi ripe and ripe with  $R=0.538$ .

## Discussion

### Ripening effects on ascorbic acid

In the present study ascorbic acid content of the

raspberries was affected by the enhanced ripening stage. Ascorbic acid content increased during maturation. According to our knowledge, there is no information available about the ascorbic acid content of *Rubus hyrcanus* Juz. at different maturity stages in Iran. Erica Kruger *et al.*, (2011) found an increase of ascorbic acid from the unripe to the ripe stage in raspberry and also Liagat Ali *et al* (2011) have reported an increase in the ascorbic acid level of raspberry during fruit development.

#### *Ripening effects on anthocyanins*

*The different ripening stages of Rubus hyrcanus* Juz. *Significantly affected the total anthocyanin concentration only, with ripe fruits showing the highest concentration.*

The increase of total anthocyanins during raspberry fruit development and ripening is well known and described in the literature, (Wang and Lin., 2000). Anthocyanins as red pigments are synthesized during the last stages of maturity when fruit become red. Anthocyanins seem to contribute to the antioxidant activity of raspberry fruit (Mullen *et al.*, 2002, Wang and Lin., 2000), their other health benefits seem unclear. Mullen *et al.*, (2002) suggests that raspberry anthocyanins provide only little cardio protective vasodilator effects, and their role in the inhibition of tumor cells may also be less important than that of other phytochemicals in raspberry (Liu *et al*, 2002).

#### *Ripening effects on total phenols and flavonoid content*

It is well-known that phenolic compounds contribute to fruit quality and nutritional value by modifying color, taste, aroma and flavor and also by providing beneficial health effects. In this experiment, total phenol and flavonoid contents showed a decreasing trend with advancing maturity. According to Wang and Lin, (2000), the content of total phenolics increased in black and red raspberry from the pink to the ripe stage whereas for other berry species like strawberry and blackberry, less ripe fruit have higher contents of total phenolics than fully ripe berries. Also, Shin *et al.*, (2008) reported decreased total phenol contents in strawberries with enhanced

ripening. In contrast, Siriwoharnet *al.*, (2004) found no marked changes in the content of total phenols with maturity of blackberry. Ferreyra *et al.*, (2007) showed that the content of total phenolics in strawberry remained almost constant from the white to purple red fruit. In the present study, phenol and flavonoid content decreased with progress of fruit ripening stages.

#### *Ripening effects on antioxidant activity*

The antioxidant activity of raspberry fruit is constituted by their high content of anthocyanins, polyphenolics and ascorbic acid. Ripening had an important influence on antioxidant activity in experiment (DPPH). The DPPH scavenging activity decreased significantly during ripening compared to unripe, not ripe fruit. This change was contributed the decreasing contents of total phenolics and total flavonoids. Fruits harvested at their unripe stages consistently yielded higher antioxidant activity. This may be due to an abundant in procyanidin content in the unripe fruit. Wang and Lin (2000) showed that, blackberries, raspberry and strawberries had the highest ORAC values and total phenolic content during the green stages. Whereas fruit harvested at pink stage (50% maturity) had the lowest ORAC and DPPH values (Shiow Y.Wang *et al.*, 2009). In contrast Erika Kruger *et al.*, (2011) showed no effect of the different ripening stages on the antioxidant capacity of cv. Tulameen.

#### **Conclusions**

The concentration of phenolic compounds within the berries is important for their beneficial effects and quality. Raspberries contained several flavonoids with potent antioxidant properties. The amount of phenolic compounds significantly changed during the maturation process. Anthocyanins increased during ripening, but total phenol, flavonoid and antioxidant activity significantly decreased during fruit ripening. Thus, the content of individual health promoting compounds in raspberry could vary significantly according to their developmental stage. Raspberries represent a diverse source of potentially healthy antioxidants and thus can provide a useful

component in our daily diet.

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