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Ultrasonic extraction of Antioxidants and Anthocyanins from different species of wild Hawthorn fruit

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Abstract

The hawthorn is a fruit with antioxidant properties that make this fruit useful for the treatment of many diseases. In this study twelve hawthorn species were assayed for their antioxidant capacities evaluating total phenolic compounds, ferric reducing antioxidant power and anthocyanin content. In addition, these extraction methods (cold solvent, cold solvent plus sonication for 5 min and cold solvent plus sonication for 10 min) were investigated. The results from this study thus indicate that there is a significant difference between hawthorn species in terms of antioxidant activity, total phenolic compounds, ferric reducing antioxidant power and anthocyanin content. The *C. pentagyna* Sub sp. *pentagyna* species were known as the best species in terms of antioxidant capacities. In addition, the results demonstrated that sonication for 5 min could enhance the level of antioxidant compounds in hawthorn fruit extracts and save extraction time.

Key words : Antioxidants, Hawthorn, Phenolic compounds, Ultrasonic.

INTRODUCTION

Hawthorn (*Crataegus*) is a perennial plant and part of the rose family (Rosaceae). It is well known in traditional Chinese medicine and is an herb remedy in cultures throughout much of the world where there is a temperate climate supporting growth of the trees, especially in Europe. Hawthorn, grows as a shrub or tree 1 to 6 m in height, and is found in deciduous forests and underbrush in the regions of West of Iran. It grows in moderate, continental climate in the northern hemisphere. The leaves are 26 cm long and 25 cm broad, with 23 shallow, forward-pointing lobes on each side of the leaf. The hermaphrodite flowers are produced in corymbs of 612, each flower with five white or pale pink petals and two or three styles, and are pollinated by midges. The fruit is a dark red or yellow pome 610 mm diameter, slightly broader than long, containing 23 nutlets. Hawthorn is well known in phytotherapy for the treatment of many cardiovascular diseases; it regulates blood pressure, increases the strength of heart muscle, and is used against arteriosclerosis and angina pectoris [1].

Free radicals play an important role in degenerative diseases like cancer, cataract, immune system weakness and brain problems [2]. Free radicals can also induce nutrition and medicine deterioration [3]. Fortunately, formation of free radicals is controlled by a variety of systems which called antioxidant. Antioxidants are defined as compounds which can reduce oxidation rate considerably [4]. In recent years, researchers are trying to replace synthetic antioxidants with natural compounds such as tocopherols and flavonoids [5-6-7-8]. Fruits, vegetables and medicinal plants have been shown to have antioxidant activity by [9], so people who consume these products have a lower risk of the relevance of free radical diseases such as heart disease and neurological disorders.

In plants many polyphenolic compounds have been found to have a much stronger antioxidant activity than vitamins C, E and β-carotene within the same food [10]. Phenolics are characterized by at least one aromatic ring possessing one or more hydroxyl groups. They are divided into several groups: anthocyanins, flavonoids, catechins, phenolic acids, stilbenes, coumarins and isoflavones. Most of them arise from a common origin: the amino acids phenylalanine or tyrosine [11].

These compounds have been reported to quench free radicals derived from oxygen, giving a hydrogen atom or an electron to the radical [12]. The antioxidant properties of phenolic compounds have been demonstrated in many systems through studies *in vitro* and in human low-density lipoproteins and liposomes [13]. Literature showed only one antioxidant study on DPPH radical scavenging activity reported for *P. bleo* [14].

Among various antioxidants present in fruits and vegetables, anthocyanins have received much attention since being reported to have a positive influence on human health [15]. Anthocyanins belong to the class of phenolic compounds. They are water-soluble glycosides and acylglycosides of anthocyanidins. Many reports have been written about the antioxidant activity and anthocyanin profiles of various fruits or fruit extract [16].

Therefore, the objective of this work was to compare antioxidant capacity of twelve hawthorn species regarding the amount of total phenolic compounds, antioxidant activity, ferric reducing antioxidant power and anthocyanins in fruits. Furthermore, three methods of extraction were investigated.

MATERIAL AND METHODS

Plant materials

In order to evaluate total phenolic compounds, antioxidant activity, ferric reducing antioxidant power and anthocyanin content of twelve hawthorn species (*C. azarolus* var. pontica, *C. ambigua*, *C. songarica*, *C. microphylla* var. dolichocarpa, *C. pentagyna* Sub sp. pentagyna, *C. assadii-kurdistanica*, *C. assadii-aronia*, *C. curvisepala*, *C. szovitsii*, *C. pseudoheterophylla* Sub sp. turcomanica, *C. atrosanguinea* and *C. azarolus-aronia*) (Table 1), fruit samples were collected from West and Northwest regions of Iran. The fruits were washed with distilled water several times and pips were removed. The samples

were put into sealed plastic bags separately and stored at -18° C until further analyses.

Extraction

The fruit samples were ground with mortar and pestle after thawing for 24 h at 60° C. The samples were mixed with ethanol-HCl (85:15 v/v), 1: 8 ratio, and subjected to ultrasonic waves (Bandelin Sonorex Digitec, DT 510 H, Germany, 100 kHz) for 5 and 10 min. Solid part was separated from the mixture by passing through Whatman #1 filter paper by using Buchner funnel with vacuum. Extraction solvent was evaporated at 40-45° C. The dried samples were transferred onto glass plates and heated again at 45-50° C using benmary method. Eventually plates were wrapped with aluminium foil and placed in freezer at -18° C. Control samples were not subjected to ultrasound waves.

Antioxidant capacity (IC_{50})

Antioxidant capacity was determined by the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay according to the method reported by [8]. First, 0.006% DPPH solution was prepared by dissolving DPPH in methanol. Then 1 ml DPPH solution was added into methanol extracted samples. Samples were mixed well and left to stand for 1 h in the dark. The absorbance was then measured at 512 nm. Standard carve was plotted using different concentration of iron sulphate II. The DPPH scavenging activity was calculated as follows.

Equation 5: $$A(\%)\frac{Ac-As}{Ac}\times 100$$

Where A: scavenging activity, Ac: absorbance of control and as: absorbance of sample. The values were plotted onto graph and IC_{50} was defined as the concentration in which antioxidant is able to inhibit 50% of free radicals.

Total of total phenolic compounds (FOLIN)

The content of total phenolic was determined by using folin-ciocalteu method [17]. The sample (5 mg) was extracted in 10 ml methanol and then 2.5 ml folin-ciocalteu agent (folin-ciocalteu was dissolved in water 1:10 v/v) was added into a falcon tubes. Samples were left on the bench to start reaction. Afterwards, 5 ml of 7.5% Na_2CO_3 solution was added into aqueous phase and after 1 min the samples were diluted to 50 ml with distilled water. Each sample was allowed to stand for 24 h in dark place at room temperature and absorbance was measured at 765 nm. Standard carve was plotted using different concentration of gallic acid. Total phenolic was calculated using following equation and expressed as $mg.ml^{-1}$.

Equation 6 : $$P=\frac{Y}{W}\times 100$$

Where P: Total phenolic compounds, W: sample weight

Ferric reducing antioxidant power (FRAP)

Briefly, 25 mg sample was dissolved in 10 ml methanol. Then 90 µl of the solution was mixed into 2700 µl FRAP solution and 270 µl distilled water in test tube. Test tubes were vortexed and placed into benmary until 37° C for 5 min. At this time absorbance was recorded at 595 nm [18]. The Fe II content was calculated using following equation.

Equation 4:

Y= 1782X-9.211

Where Y: Fe II ($\mu mol\ l^{-1}$), X: absorbance at 595 nm

Anthocyanin content

Total anthocyanin content was determined according to pH differential method based on cyanidin 3-galactoside (dominant anthocyanin) and using following equation [8].

Equation 3:

Total anthocyanin content ($mg.l^{-1}$) = $A\times M_w\times DF\times 1000/\varepsilon\times L$

($A=(A_{\lambda vis\,max}\ A_{\lambda 700})^{pH=1}\_(A_{\lambda vis\,max}\ A_{\lambda 700})^{pH=4.5}$

Where A: absorbance difference between pH 4.5 and 1, M_w: cyanidin 3-galactoside molecular weight (484.8 g mol^{-1}), ε: cyanidin 3-galactoside molecular absorptivity (34300), DF: Dilution factor, L: cuvette length.

Statistical analysis

The experimental design was a completely randomized design arranged in factorial with three replications. The first factor was twelve species of hawthorn and the second factor was sonication time. Statistical analysis was conducted using MSTAT-C. Significant differences between means were determined by LSD test. Differences were considered to be significant at $P<0.05$.

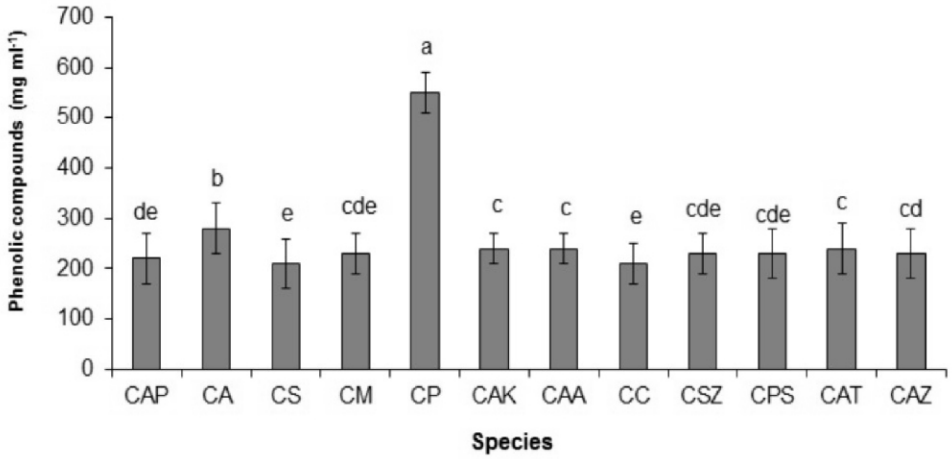

Figure 1: Individual effect of hawthorn species on fruit phenolic compounds. Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected LSD Test.

Table 1: Hawthorn species and abbreviations

Hawthorn species		Abbreviation
1	C. azarolus var. Pontica	CAP
2	C. ambigua	CA
3	C. songarica	CS
4	C. microphylla var. dolichocarpa	CM
5	C. pentagyna subsp. Pentagyna	CP
6	C. assadii kordestanica	CAK
7	C. assadii aronia	CAA
8	C.curvisepala	CC
9	C. szovitsii	CSZ
10	C.pseudoheterophyllasubsp. turcomanica	CPS
11	C. atrosanguinea	CAT
12	C.azarolus-aronia	CAZ

RESULTS

Total phenolic compounds

According to the results the highest phenolic compounds was observed in CP species (Figure 1). On the other hand, the lowest amount of phenolic compounds was observed in CS and CC species. In addition the CAK, CAA and CAT were all the same in terms of total phenolic compounds (Figure 1). The effect of extraction methods on phenolic compounds is illustrated in (figure 2). As can be seen, there was no significant difference between cold solvent and 5 min sonication treatments. However, 10 min sonication significantly decreased phenolic compounds in hawthorn extract (Figure 2). Interaction between species and extraction methods is shown in (figure 3). The highest phenolic compounds were related to CP sonicated for 5 or 10 min (Figure 3). By contrast, the lowest value was observed in CC, CSZ and CPS, sonicated for 10 min (Figure 3). According to the results plotted in figure 3 there was no considerable difference between other treatments.

Ferric ion reducing power test (FRAP)

From the results, the highest FRAP value was obtained from CP species (Figure 4). In addition, there were no significant difference between CAP and CAZ as well as between CA, CAA and CAT and also between CM, CAK and CSZ in terms of FRAP (Figure 4). On the other hand, the lowest FRAP value was observed in CS species (Figure 4). These results suggest that genotype and climate have significant effect on FRAP values in hawthorn.

The results demonstrated that the effect of sonication was not significant on FRAP value (Figure 5).

Interaction between species and extraction methods showed that the highest FRAP value was related to CP species, irrespective of extraction method (Figure 6). On the contrary, the lowest FRAP value was observed when CS species was sonicated for 5 min (Figure 6). There was no considerable difference between other species in term of FRAP (Figure 6).

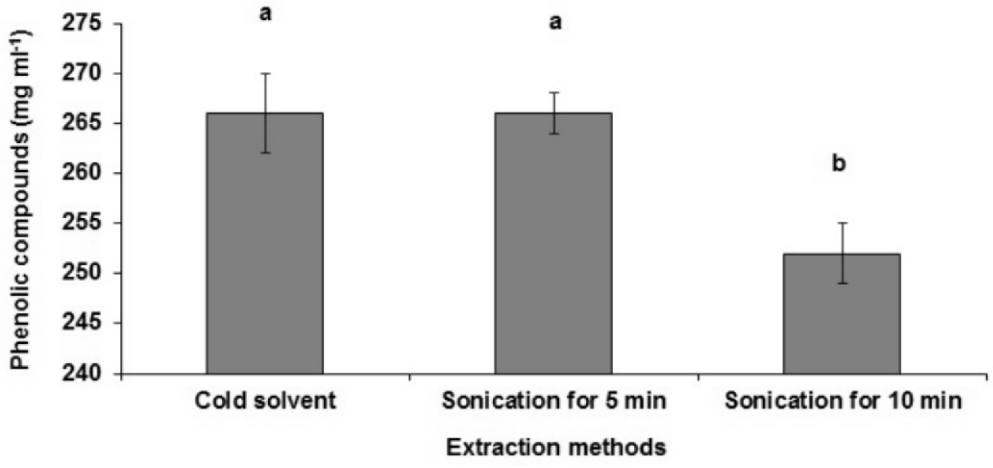

Figure 2: Individual effect of extraction methods on hawthorn phenolic compounds. Values within the each column and followed by the same letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

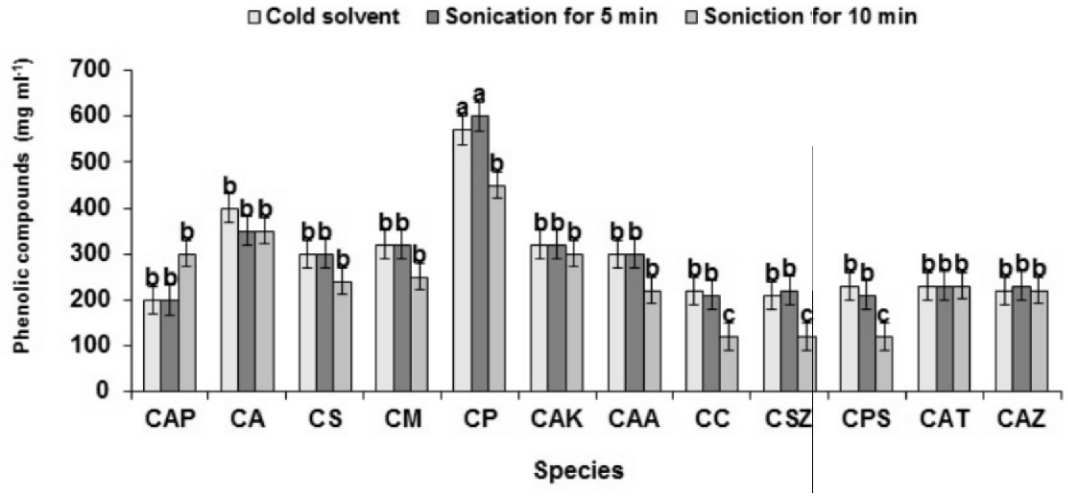

Figure 3: Interaction between species and extraction methods on hawthorn phenolic compounds. Values within the each column and followed by the same letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

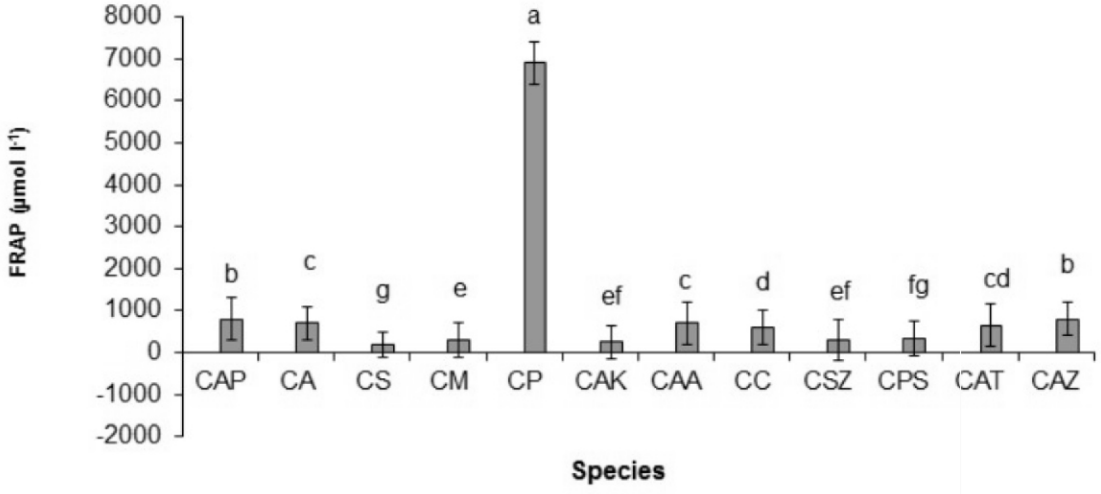

Figure 4: Individual effect of species on FRAP. Values within the each column and followed by the sae letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

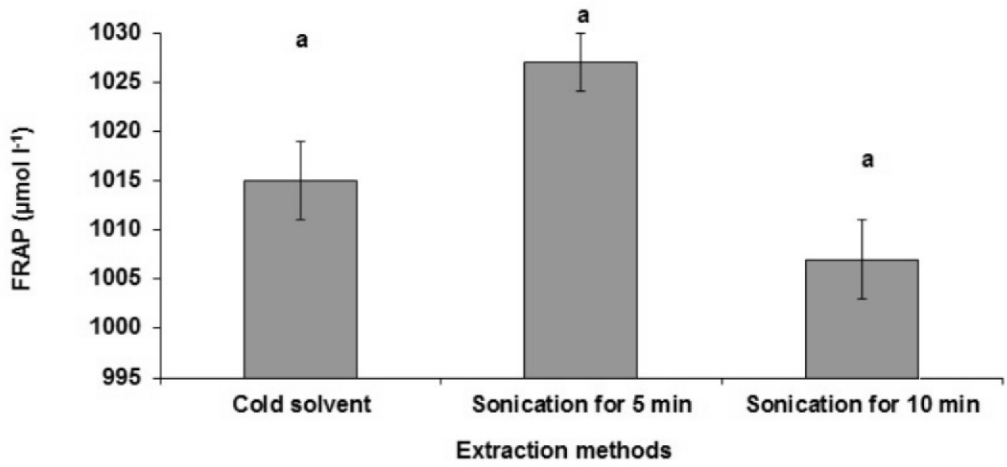

Figure 5: Individual effect of extraction methods on FRAP. Values within the each column and followed by the same letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

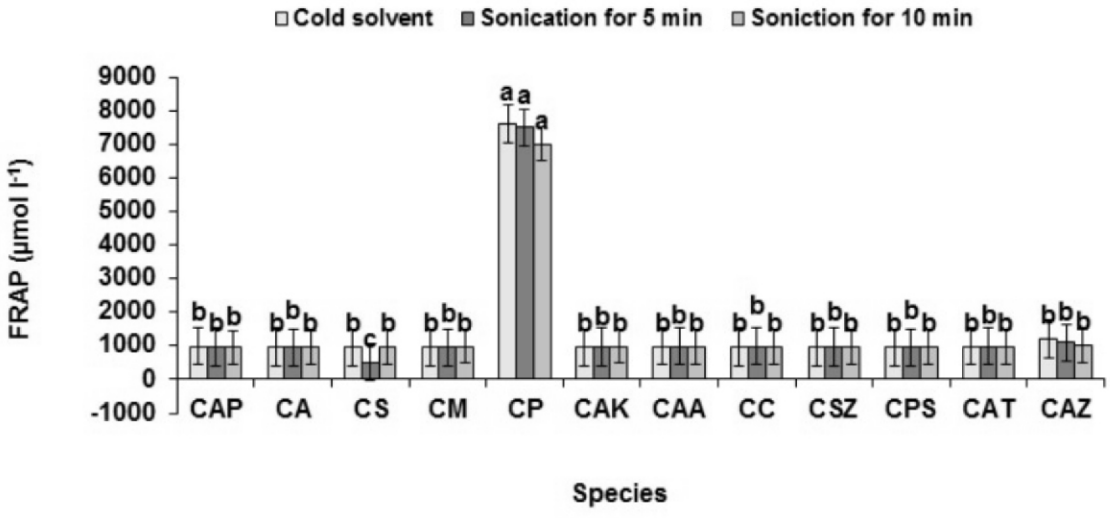

Figure 6: Interaction between species and extraction methods on FRAP. Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected LSD Test.

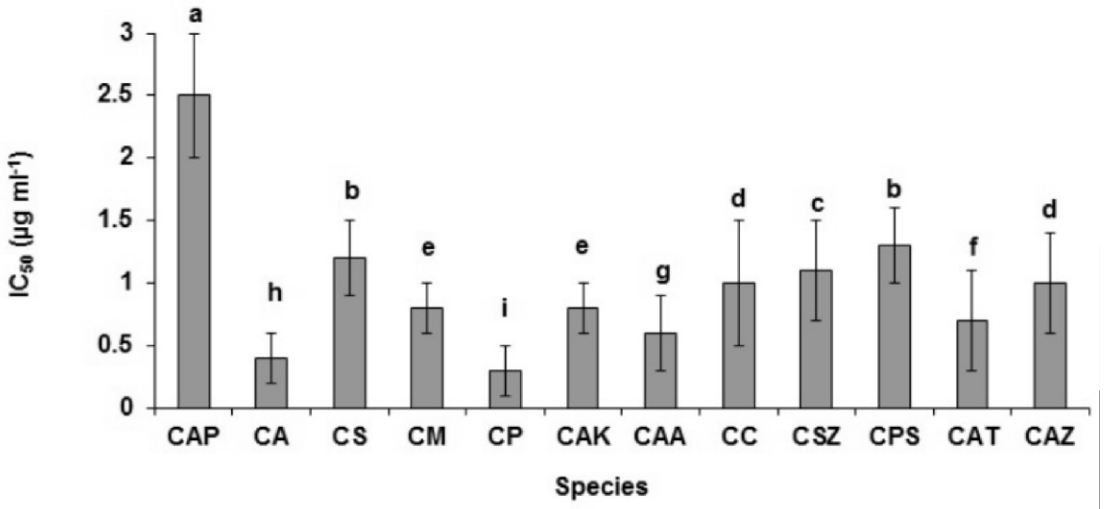

Figure 7: Individual effect of species on IC50. Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected LSD Test.

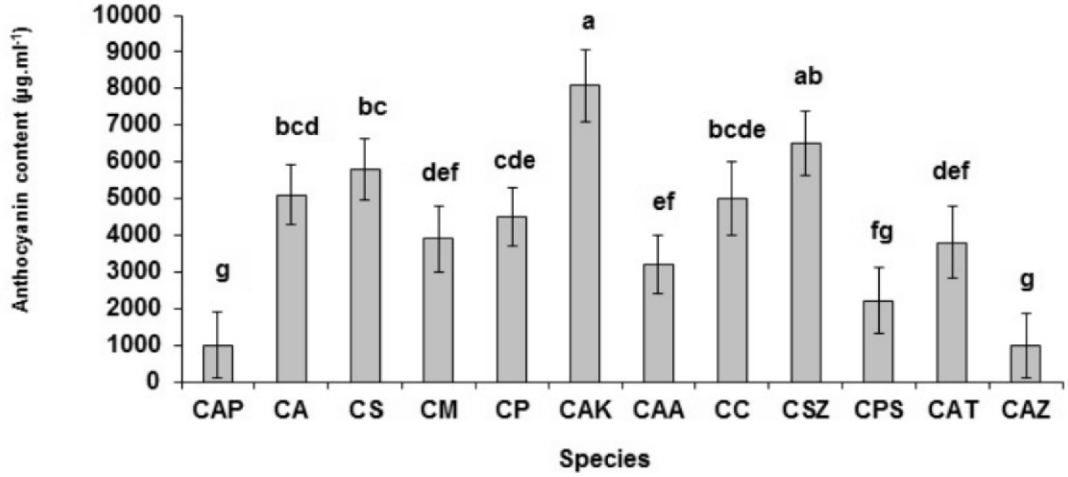

Figure 8: Individual effect of species on anthocyanin content. Values within the each column and followed by the same letter are not different at P < 0.05 by an ANOVA protected LSD Test.

Figure 9: Individual effect of extraction methods on anthocyanin content. Values within the each column and followed by the same letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

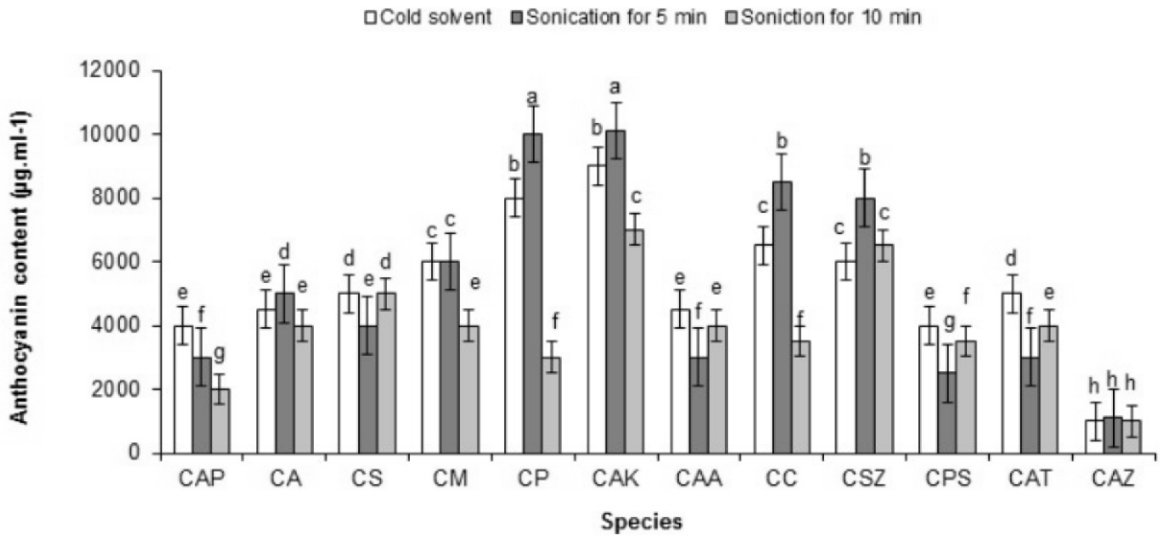

Figure 10: Interaction between species and extraction methods on anthocyanin content. Values within the each column and followed by the same letter are not different at $P < 0.05$ by an ANOVA protected LSD Test.

Antioxidant activity of Hawthorn varieties

The highest antioxidant capacity was observed in CP species, with the lowest IC_{50} (Figure 7). By contrast, CAP species, showed the lowest antioxidant capacity showing the highest IC_{50} (Figure 7). The results revealed that extraction methods had no significant effect on antioxidant capacity. Similar results were obtained in case on interaction between species and extraction methods (data was not shown).

Anthocyanin contents

The antocyanin contents of howthorn species is shown in (figure 8). From the figure, CAK showed the highest followed by CSZ while CAP, CAZ and CPS represented the lowest anthocyanin content. These findings suggest that genetic background affects anthocyanin synthesis in hawthorn plant.

Sonication for 5 min increased anthocyanin content, whereas there was no significant difference between cold solvent extraction method and sonication for 10 min (Figure 9). Interaction between species and extraction methods is given in (figure 10). The highest anthocyanin content was observed in CAK and CP species sonicated for 5 min. On the contrary, the lowest content was obtained from CAZ species.

DISCUSSION

Total phenolic compounds

It could be concluded that the interaction of plant genotype and climate conditions affects hawthorns phenolic compounds. It has been reported that antioxidant activity and phenolic compounds in leaves and stems of almond (*Prunus amygdalus* L.) have seasonal differences [19].The results indicate that although ultrasound application has no positive effect on extraction phenolic compounds, it could reduce extraction time from 2 h to 5 min and protect the extract from degradation. It can also be noted

that, in some species, sonication has reverse effect on extraction. The only positive effect of sonication is saving time. Improvement in total phenolic compounds of the henna (*Lawsonia Inermis* L.) leaves extracts due to sonication have been reported by [20] who studied the phenolic compounds of henna leaves, extracted by two different solvent extraction methods (solvent and ultrasound-assisted method). They have also indicated that extraction method has significant effect on phenolic compound and antioxidant activity of henna extract.

Ferric ion reducing power test (FRAP)

The effect of increased temperature from 110 to 160□ C on FRAP in canola (*Brassica napus* L.) was evaluated by [21], they have concluded that there is no significant difference between temperatures in terms of FRAP. Furthermore, significant interaction between temperature and solvent concentration was reported by [22] who studied these parameters on rosemary (*Rosmarinus officinalis*).

Antioxidant activity of Hawthorn varieties

Several studies suggest that the antioxidant activity of fruits is influenced by genotype, habitat conditions and ripeness of the fruits [23]. Other factors, such as altitude, light, temperature, and content of nutritive matter in the soil, can influence antioxidant activity [24].

Anthocyanin contents

Some researchers have studied the effect of ultrasound waves on red raspberry they have found that sonication at 20 and 490 kHz significantly affected the anthocyanin content of red raspberry, while 986 kHz had no significant effect. Moreover, they have reported that sonication beyond 10 min (and up to 30 min) using 20 kHz either produced no change or caused a drop in anthocyanin content. At 20 kHz, anthocyanin content increased by 17.3% and 12.6% after 10 min [25].

CONCLUSION

In conclusion our results suggested that the suitable time for extraction, using ultrasound waves, is more than 5 or 10 min. In addition, comparison of IC_{50}, FRAP and total phenolic compounds in fruits of twelve hawthorn species showed that *C. pentagyna* Sub sp. *pentagyna* are the best species in terms of antioxidant activity followed by CA and CAT were placed in the second and third place, respectively. Furthermore, CAZ and showed the highest antioxidant power. The highest anthocyanin content was related to CAA, CSZ and CS, respectively.

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