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Research Paper

Antioxidant activity of hawthorn (*Crataegus monogyna*) from Morocco

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ABSTRACT

Antioxidants are tremendously important substances that possess the ability to protect the body from damage caused by free radicals induced oxidative stress. This study aims to investigate the antioxidant effect of hawthorn (*Crataegus monogyna*) from the Mid-Atlas Mountains of Morocco as a potential source of new bioactive natural compounds. Hawthorn is a medicinal plant widely used in phytotherapy for the treatment of many cardiovascular diseases. In this study, flowers, leaves, ripe and unripe fruits were analyzed. The antioxidant activity was measured by the 2,2-diphenyl-1-picrylhydrazyl(DPPH) free radical scavenging method. Then, Folin–Denis and aluminum chloride colorimetric assays were used to determine total polyphenol and total flavonoid contents of the plant extracts, respectively. The results obtained showed that all the plant parts studied expressed important antioxidant properties. Unripe fruits and flowers revealed the highest antioxidant activity with IC_{50} values of 7.3 and 8.3 µg/ml, respectively. Total polyphenol content in different plant parts ranged from 105.1 to 280.4 µg Gallic Acid Equivalent /100 mg Extract and total flavonoid from 4.7 to 70.8 µg Quercetin Equivalent/100 mg Extract. Antioxidant activity presented a significant correlation with total polyphenol content. These results indicate that *C. monogyna* extracts exhibit an important antioxidant activity and thus can present a great potential as a source of natural antioxidants.

Key words: *C. monogyna*, total polyphenol content, total flavonoid content, antioxidant activity.

INTRODUCTION

Free radicals and their precursors are parts of a reactive chemical family named reactive oxygen species (ROS) which are produced constantly by the human body during its metabolism. ROS have fundamental and positive roles in some physiological functions such as production of energy *in vivo* systems, regulation of cell growth, *inter* or *intra* cellular signal transfer, phagocytosis and synthesis of important biological compounds (Halliwell, 1991; Keser et al., 2012). Normally, the rates of generation and elimination of reactive oxygen species are in equilibrium. However, an oxidative stress may occur, resulting from a disequilibrium between pro-oxidant sources of radicals and antioxidant systems. The main source of free radicals is endogenous. These radicals are produced from enzymatic reactions mainly related to breathing and defense functions. Other exogenous factors may also contribute to their formation such as UV radiation, 8 or X-rays, smoking, alcohol, prolonged exposure to the sun and intense physical effort (Pham-Huy et al., 2008; Birben et al., 2012).

Free radicals initiate chain oxidation reactions that have a detrimental action on the body. All tissues and all their components can be affected by oxidative stress (lipids, proteins, carbohydrates and even DNA). Free radicals are implicated in more than one hundred disorders in humans (Pourmorad et al., 2006). They are reported to be involved in triggering several diseases such as cancer, diabetes, rheumatism, amyotrophic lateral sclerosis, acute respiratory distress syndrome, pulmonary edema,

restenosis, AIDS, cardiovascular diseases, neurodegenerative diseases and accelerated aging (Montagnier et al., 1998; Sohal et al., 2002). Following an oxidative attack, the body can deploy two strategies, (i) active detoxification which relies primarily on enzymes (Superoxide Dismutase (SOD), Catalase, Glutathione Peroxidase, etc…) and (ii) passive detoxification which includes all non-enzymatic antioxidants that can neutralize free radicals (Halliwell, 1991). Recently, many studies have focused on the high toxicity of synthetic antioxidants and the potential health problems that may arise from their long-term use, such as tetragenic, mutagenic and carcinogenic effects (Chavéron, 1999).

Medicinal plants have played a vital role in protecting human health for thousands of years through their richness in bioactive compounds. Natural phytochemicals from plants have been receiving increased interest from researchers and consumers for their health benefits and particularly for their lack of toxicity as compared with synthetic molecules. Hawthorn (*Crataegus monogyna*) is a shrub or small tree belonging to the Rosaceae family and wide spreading in almost all temperate zones of the northern hemisphere (Bruneton, 2009). Its flowers, leaves and fruits are used in phytotherapy for the treatment of many health problems. It is commonly known for its cardiovascular, sedative, antioxidant and antibacterial properties (Nabavi et al., 2015; Walker et al., 2002; Bouzid et al., 2011; Benmalek et al., 2013).

This study aims to determine the polyphenols and flavonoids contents of Moroccan hawthorn (*C. monogyna*) and to investigate its antioxidant activity as a potential source of new bioactive natural compounds.

MATERIALS AND METHODS

Plant material and Study area

Samples of *C. monogyna* were collected from the Middle Atlas mountain area East of the city of Azrou (Latitude N 33.42°; Longitude W 5.16°; Altitude 1680 m). This areas hosts a great diversity of spontaneous plant species collected by the local population for their medicinal vertus. The plant samples were collected on 2016/2017 season, from all sides of the tree, on a chronological sequence according to the organ concerned. Leaves and flowers are collected at full bloom (mid- may), while immature fruits (green in color) and ripened fruits(red in color) are collected in early autumn (September) and late autumn (November), respectively. Plant samples were air dried in the shade on a laboratory bench, then powdered and passed through a 1 mm sieve.

Plant extracts

Extraction was carried out by maceration in methanol, and extracts were evaporated at 35°C under reduced pressure, dissolved in methanol at 10 mg/ml and stored at 4°C for subsequent use in colorimetric assay and antioxidant activity.

Total polyphenols and flavonoids

Polyphenols

Determination of total polyphenols was carried out according to Li et al. (2007) using Folin Denis reagent instead of Folin Ciocalteu. 75 ml of distilled water, 10 g of sodium tungstate, 2 g of phosphomolybdic acid and 15 ml of Phosphoric Acid were mixed and boiled for 2 h. After cooling, the mixture was completed to 100 ml with deionised water. 200 µl of each hawthorn extract solution was mixed with 1 ml of the Folin-Denis solution diluted ten-fold. After 4 min, 800 µl of sodium carbonate (Na_2CO_3) (75 mg/ml in distilled water) was added to the solution and incubated for 2 h in the dark. Then, polyphenol absorbance was determined at 765 nm using a Shimadzu spectrophotometer. Gallic acid solutions ranging from 0 to 200 µg/ml were prepared and used to establish a standard calibration curve..

Flavonoids

Aluminum Chloride ($AlCl_3$) method was used to quantify flavonoids according to the method used by Bahorun et al. (1996). Hawthorn extract solutions, ranging from 0 to 35 µg/ml, were prepared and mixed with $AlCl_3$ (2% in methanol) (V/V). After 10 min, the absorbance was measured at 430 nm. A calibration curve was established with quercetin (0 - 35 µg/ml).

Scavenging of DPPH radicals

Antioxidant power of methanolic extracts of flowers, leaves and fruits of *C. monogyna* against DPPH radical was evaluated by spectrophotometry using the method described by Selles et al. (2012). A series of dilutions were performed in order to obtain concentrations ranging from 5 µg/ml to 0.4 mg/ml for hawthorn extracts and from 1 µg/ml to 0.4 mg/ml for ascorbic acid. A methanolic solution of DPPH 0.8mM was prepared and 0.25 ml of this solution was added to 3.75 ml of hawthorn extract solutions. The resulting mixtures were kept in the dark at room temperature for 30 min. Then absorbance was measured at 517 nm. The results were compared with the negative control (for which no plant extract was added). Ascorbic acid was used as a reference anti-oxydant.

The results were expressed as percent inhibition of DPPH radical using the formula:

Table 1: Polyphenols and flavonoids contents and antioxidant activity of *Crataegusmonogyna*plant parts(mean ± SD; n=3 , and p <0.05).

Parameter	Extraction yield (g/100g dry weight)	Polyphenols content (µg EqGA/mg extract)	Flavonoids content (µg EqQ/mg extract)	Antioxidant activity (IC-50) µg/ml
Flower	24.68 ± 1.81 b	244.26 ± 10.68 b	40.78 ± 2.02 a	06.78 ± 0.34 c
Leaf	13.79 ± 1.28 c	196.49 ± 04.57 c	38.08 ± 0.73 a	11.02 ± 0.76 b
Ripened fruit	34.31 ± 0.91 a	105.10 ± 02.09 d	04.69 ± 0.18 c	17.66 ± 1.79 a
Immature fruit	10.74 ± 1.01 c	280.36 ± 02.50 a	14.56 ± 0.46 b	06.42 ± 0.51 c
Ascorbic acid				02.02± 0.28 d

$$\% \text{ inhibition} = \frac{(\text{Abs Control} - \text{Abs Test})}{\text{Abs Control}} * 100$$

With: Abs Control=Absorbance of the negative control, Abs Test=Absorbance of the test sample.

The percent inhibition of DPPH radical was plotted against extract concentration (log scale) and the IC-50 values were determined graphically using GraphPad Prism 8.

Chemical reagents

DPPH (2-2 Diphenyl-1-picylhydrazyl), Quercetin ($C_{15}H_{10}O_7$) and Methanol (CH_3OH) were purchased from Sigma-Aldrish. Sodium Tungstate-2hydrate ($Na_2WO_4,2H_2O$) and Sodium Carbonate (Na_2CO_3) were purchased from Polysciences. Gallic Acid ($C_7H_6O_5$) was purchased from Fluka. Phosphomolybdic Acid × Hydrate ($H_3PMo_{12}O_{40}xH_2O$) was purchased from Panreac. Ascorbic Acid ($C_6H_8O_6$) was purchased from Fisher Scientific. Phosphoric Acid was purchased from Gerraw. Aluminium Chloride was purchased from Riedel-deHaën.

Statistical analysis

All tests were conducted in triplicates; the results were expressed as mean ± standard deviation. A multiple comparison of means was performed using Tukey-test with a probability level of 0.05. Correlations between different parameters were computed as Pearson's correlation coefficient (r). Statistical analyzes were performed using SPSS software version 20.0.

RESULTS

Total polyphenols

Total polyphenol content was derived using the Gallic acid calibration curve and the absorbance value of each test extract. The results obtained showed that *C. monogyna* is rich in polyphenols with significant differences among plant parts (Table 1). Immature fruits contained the highest concentration (280.36 µg GAE/mg extract), followed by flowers (244.26 µg GAE/mg extract), then leaves (196.49 µg GAE/mg extract), while ripened fruits registered the lowest value (105.10 µg GAE/mg extract).

Total flavonoids

Total flavonoid content, derived using the Quercetin calibration curve and the absorbance value of each test extract, showed significant differences among plant parts (Table 1). Flowers and leaves presented the highest values (40.78 and 38.08 µg QE/mg extract respectively), followed by immature fruits (14.56 µg QE/mg extract). Mature fruits presented the lowest value (4.69 µg QE/mg extract).

Scavenging of DPPH radicals

The principle of analyzing antioxidant activity is based on the color change of diphenyl-picrylhydrazyl (DPPH) solution from purple to yellow. The intensity of the color change is proportional to the amount of antioxidants. Figure 1 shows the antioxidant capacity of methanolic extracts of hawthorn plant parts. The antioxidant activity profiles obtained presented a dose-dependent activity. Inhibition of DPPH radical increased with increasing concentrations of plant extracts. Inhibition percentages were stabilized at varying concentrations according to the organ studied (0.1 mg/ml for immature fruit, 0.05 mg/ml for flower, leaf and ripened fruit and 0.005 mg/ml for ascorbic acid). Immature fruit reached the highest percent inhibition of DPPH radicals (89%) followed by ripened fruit, flower and leaf (83%). However, the maximum DPPH radical inhibition of ascorbic acid (76%) was relatively lower as compared with that exhibited by hawthorn plant parts.

The IC50 values were obtained graphically using GraphPad Prism 8 (Table 1).These values are defined as the inhibitory extract concentration necessary to decrease by 50% the initial concentration of DPPH and are expressed in µg/ml. The results obtained showed significant differences among different plant parts, with IC50 values ranging from 7.27 to 23.67 µg/ml against 2.83 µg/ml for ascorbic acid. Immature fruits and flowers

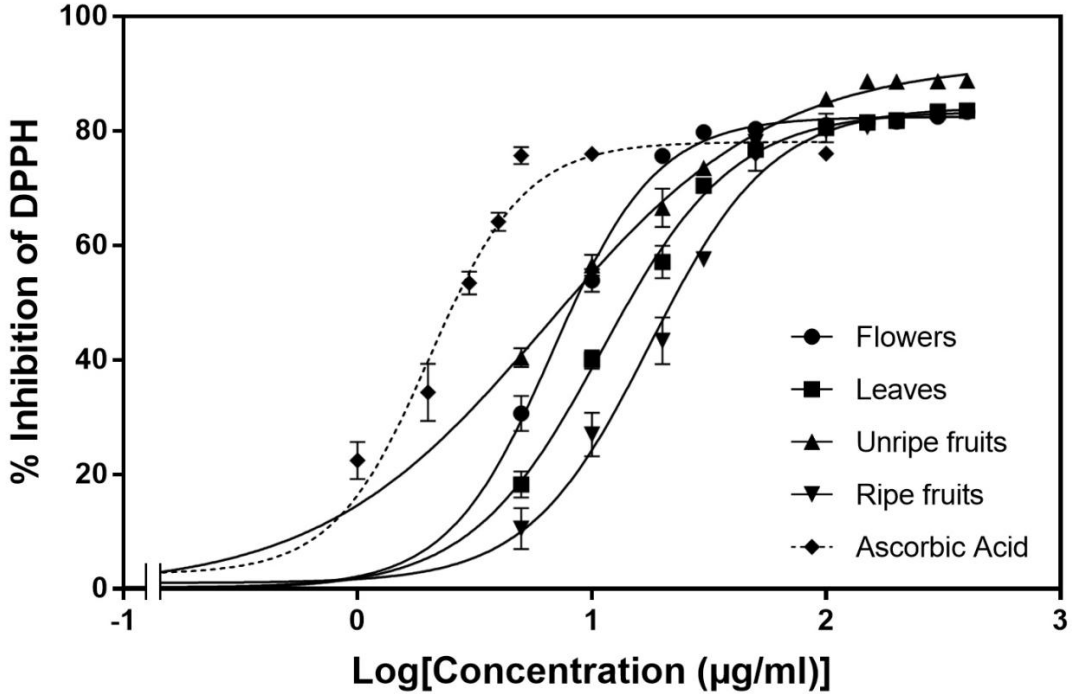

Figure 1: Percent inhibition of DPPH radical of *Crataegus monogyna* plant parts and ascorbic acid.

exhibited the lowest IC-50 values (7.27 and 8.27 µg/ml respectively), followed by leaves (15.47 µg/ml) and then ripened fruit (23.67 µg/ml).

Polyphenol content of different plant parts presented a strong and significant correlation with their corresponding antioxidant activity (r = 0.97), while flavonoid content was moderately correlated (r = 0.49). This clearly shows that antioxidant potential of hawthorn extracts is mainly associated with its polyphenols content.

DISCUSSION

Polyphenols and flavonoids contents

Plants produce accumulate a large variety of secondary metabolites along their growth and development cycle. These compounds have potential roles in signalization and protection against different biotic or abiotic stresses and represent adaptive traits that allow plants to survive in their environment. Polyphenols are an important class of secondary metabolites found ubiquitously in plants.

Our findings showed that *C. monogyna* from the mid Atlas Mountains of Morocco contains important amounts of polyphenols in its reproductive plant parts, with differential distribution among the different organs. In fact, several authors (N'Guessan, 2011; Mraihi et al., 2013; Simirgiotis, 2013) have reported unequal distribution of polyphenols in different organs of a plant.

A study conducted on *C. monogyna* collected from Portugal (Barros et al., 2011) showed that immature fruits are the richest in polyphenols, followed by flowers and finally ripened fruits, a trend similar to our findings. The levels of polyphenols content reported were much higher, especially in the case of immature fruit (701.65 µg GAE/mg extract). Similarly, another study on *C. monogyna* collected from France (Bahorun et al., 1994) showed that foliar and reproductive organs were rich in polyphenols. Their results follow perfectly the same trend as those of our study, with a maximum polyphenols content for immature fruits. However, Bouzid et al. (2011), working on *C. monogyna* ripened fruits from Algeria, reported lower polyphenol contents (21.72 µg GAE/mg extract) as compared with the finding of the present study. The high content of polyphenols reported specifically for immature fruits by all studies may be the strategy of the plant to discourage herbivores, thus avoiding early dispersal of immature seeds as reported by Barros et al. *(*2011).

Concerning flavonoids, our results showed that flowers and leaves are the richest parts of the plant, followed by fruits. Flavonoid contents obtained in the present study are very close to those of Bouzid et al. (2011), who reported that methanolic extract of ripened hawthorn fruits from Algeria recorded a flavonoids content of 3.2 µg QE/mg extract. The same trend is also reflected in the results of Bahorun et al. (1994) who expressed their results in terms of Vitexin and Hyperoside equivalent. Other studies also confirm that polyphenols and flavonoids contents of

immature fruits are much higher than those of mature fruits. This could be explained by their use as antioxidants along fruit maturity, thus resulting in a phenolic content decrease in advanced maturity stages (Simirgiotis, 2013; Barros et al., 2011).

Several factors can affect the content of phenolic compounds. The literature highlights the influence of both intrinsic and extrinsic factors as well as their interaction. Some studies suggested that the phenolic content of plants is influenced mainly by genetic factors (Atanasova and Ribarova, 2009; Bouzid et al., 2011; Kostić et al., 2012). Extrinsic factors may also influence phenylpropanoid metabolism (kostić et al., 2012), such as environment and its characteristics (altitude, temperature, light, soil nutrient content, etc.). Indeed, Kirakosyan et al. (2003) showed that hawthorn plant extracts subjected to drought and cold stress did not only give higher yields of polyphenolic compounds but also have greater antioxidant capacity as compared with control plants. In addition, several authors linked the variation of phenolic content with the intensity of the sun (Urbonaviciute et al., 2006; Atanasova and Ribarova, 2009), and concluded that solar radiation can induce their biosynthesis. Furthermore, the date of harvest, the maturity of the plant, the spatial distribution of the sample, and storage conditions may also exert an important effect on the observed variations (Proestos and Komaitis, 2008; Bouzid et al., 2011; kostić et al., 2012; Rodriguez et al., 2012; Bahorun et al., 1994; Urbonaviciute et al., 2006). In addition, the extraction methods adopted and the solvents used also exert an important effect on the yield of phenolic compounds (Ignat et al., 2013; Tahirović and Bašić, 2014; Bouzid et al., 2011).

Antioxidant activity

Various hawthorn organs have been described in the literature as antioxidants in several studies. In terms of IC_{50} values, a study conducted by Simirgiotis (2013) on different plant part of *C. monogyna* reported that: (i) samples with IC_{50} values less than 50 µg/ml possess high antioxidant activity, (ii) those with IC_{50} values ranging from 50-100 µg/ml are considered as intermediate antioxidant activity, (iii) while samples with IC_{50} value greater than 200 µg/ml are considered as no relevant antioxidant activity.

According to this classification, all hawthorn plant parts expressed a high antioxidant activity. Since IC_{50} value is inversely related to antioxidant capacity, the relative comparison of the various organs activity allows concluding that immature fruits and flowers expressed the highest antioxidant activity (IC-50 of 6.42 and 6.78 µg/ml respectively); leaves presented an intermediate activity (11.02 µg/ml), whereas ripened fruits were the lowest (17.66 µg/ml). A similar trend was reported by Bahorun et al. (1994) and Barros et al. (2011). Our study revealed greater DPPH scavenging activities as compared with hawthorn fruits collected from Serbia (IC_{50} = 52.04 µg/ml, Tadić et al., 2008). In contrast, ripened fruits (IC_{50} = 3.61 µg/ml) and leafy branches (IC_{50} = 3.34 µg/ml) of chilean *C. monogyna* presented a higher antioxidant activity as compared with the results of our study (Simirgiotis, 2013).

Our results showed that Hawthorn flowers and unripe fruits exhibited a relatively higher maximum DPPH radical inhibition activity as compared with that of ascorbic acid used as a reference antioxydant. This increased intensity could be attributed to potential synergetic effects among different compounds contained in the extract (Bernatonienė et al., 2008; Rodrigues et al., 2012; Ignat et al., 2013; Simirgiotis, 2013). In addition, the antioxidant activity of hawthorn extracts presented a strong correlation with polyphenol content (r = 0.973). This is in agreement with the reports of other researchers (Tahirović and Bašić, 2014; Mraihi et al., 2013; Bahorun et al., 1994).

Conclusion

C. monogyna collected from Morocco showed a high DPPH anti-radical scavenging activity, especially immature fruits and flowers. These findings substantiate the traditional uses of *C. monogyna* in treating various disorders and increase its interest for potential use as a natural source of antioxidants.

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