ABSTRACT

Objective: The aim of this study was the isolation and identification of secondary metabolites from *Crataegus azarolus* (L.) and the antioxidant evaluation of its extracts and compounds.

Methods: The air-dried powdered parts of the plant were extracted with 70% methanol and fractionated by chloroform, ethyl acetate, and n-butanol. The n-butanol extract was separated using polyamide SC6 column and silica gel TLC. In addition, a fraction of silica gel column of the CHCl₃ extract was analyzed by gas chromatography–mass spectrometer (GC–MS). The total phenolic and total flavonoid contents of CHCl₃, n-butanol extracts, and two flavonoids were evaluated according to five different methods.

Results: Eight compounds were identified in CHCl₃, and n-butanol extracts, among them, five volatile compounds identified by GC–MS for the 1st time from the species, as well as three known flavonol glycosides identified by spectral analysis (ultraviolet, 1H-nuclear magnetic resonance [NMR], and 13C-NMR) and by comparison with literature data. The n-butanol extract showed the higher content of polyphenols (307.33 ± 2.33 mg (gallic acid equivalents)/g extract) and flavonoids (143.0 ± 2.12 mg QE/g extract) and it proves the highest antioxidant activity with all assays used.

Conclusion: Five volatile compounds were identified for the 1st time from the *C. azarolus* and the antioxidant potential of plant extracts was measured using five different methods.

Keywords: *Crataegus azarolus* (L.), Volatile compounds, Flavonol glycosides, Phenolic contents, Antioxidant activity.

INTRODUCTION

*Crataegus* species (Rosaceae) known as "Hawthorn" are shrubs or small tree usually with thorns with about 200 species commonly distributed throughout the Northern temperate region [1]. *Crataegus* extracts from leaves, flowers, and fruits are generally used for heart diseases such as hypertension, angina, arrhythmia, and congestive heart failure. In traditional Arabic medicine, the leaf and fruit extract of *Crataegus aronia* are used for cardiovascular diseases, sexual weakness, cancer, and diabetes treatment [2]. Some *Crataegus* species are currently officially listed as herbal drugs in the pharmacopoeia of many countries such as France, China, England, and Germany [3-5]. The species *Crataegus azarolus* (L.) is a fruit tree cultivated in the Mediterranean region [6], it is known in the east of Algeria as "Zaour" and its fresh fruits are consumed during the autumn season.

Previous phytochemical studies on *C. azarolus* have revealed the presence of polyphenols including flavonoids from the species collected from Tunisia [7-10] and volatile oils from the species from Algeria [11]. The present paper aimed at the exploration of the richness of secondary metabolites of *C. azarolus* as a possible source of compounds having beneficial biological activities as well as the evaluation of its antioxidant activity. As a result, eight compounds were identified from the plant, where five volatile compounds are reported for the first time from *Crataegus* genus with three flavonol glycosides, also the total phenolic content (TPC), total flavonoid content (TFC), and the antioxidant activity were evaluated.

METHODS

Plant material

Aerial parts of *C. azarolus* were collected from Mila region in the east of Algeria during flowering period in 2013. A voucher specimen was deposited under number CA 022 in our laboratory.

Extraction and isolation

Air-dried powdered aerial parts (300 g) of *C. azarolus* were extracted 3 times with a solution of 70% methanol during 24 h. The combined extracts were evaporated under reduced pressure and temperature. The residue was dissolved in hot distilled water (300 ml), and left in cold overnight. After filtration, the aqueous extract was successively extracted by solvents with increasing polarity and evaporated under reduced pressure and temperature. The residue was dissolved in hot distilled water (300 ml), and left in cold overnight. After filtration, the aqueous extract was successively extracted by solvents with increasing polarity and evaporated under reduced pressure to yield chloroform (0.8 g), ethyl acetate (1.2 g), and n-butanol (8 g). The n-butanol extract (4 g) was subjected to a polyamide SC6 column chromatography using H₂O-MeOH as the system of elution to give 100 fractions of 100 ml each. Similar fractions were combined according to their TLC (cellulose) properties using 15% HOAc (H₂O-acetic acid, 85:15) and BAW (n-butanol-acetic acid-water, 4:1:5, upper phase) as eluent to give 12 main fractions (A-L). Compounds 1 and 2 were separated from fraction G by silica gel TLC using CH₃Cl₂-MeOH (9:1) as eluent, while compound 3 was obtained from fraction L as a yellow solid by repeated crystallization from methanol.

Gas chromatography–mass spectrometer (GC–MS) analysis

The chloroform extract (0.8 g) was fractioned by a silica gel column, eluted with a gradient of cyclohexane-ethyl acetate with increasing...
polarity to give 10 main fractions (1–10). The fraction 3 (eluted with 20% ethyl acetate) was subjected to GC–MS analysis using a gas chromatograph VARIAN 3900 with CP-8400 autoinjector coupled to MS (VARIAN Saturn 2000 MS-MS). A Factor Four VF-5 ms fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm, Varian) was used. Oven temperature was programmed at 5°C/min. From 45°C to 280°C, the oven was maintained at 280°C for 5 min. The injector temperature was set at 230°C and 1 μl aliquots were injected with a split ratio of 1:10. Helium was used as carrier gas with a flow rate of 1 ml/min. The ion trap MS was operated in EI mode at 70 eV. The analyses were performed with a filament–multiplier delay of 9 min and data acquisition was done in the range 50–650 m/z in full scan mode. The transfer line was maintained at 250°C, the manifold and trap temperatures were set at 80°C and 150°C, respectively. Instrument control and MS data were managed by a personal computer running the Saturn GC–MS Workstation software (6.30 version). Identification of the chromatographic peaks of compounds (4–8) was realized by comparison of their mass spectra with those present in literature including a comparison with National Institute of Standards and Technology library (NIST).

Biological studies

TPC

Folin-Ciocalteu assay was carried out by the procedure [12], with minor modifications. Gallic acid solution at various concentrations was used for calibration (Table 1). The TPC of samples was expressed as gallic acid equivalents (mg GAE/g extract) by means of a dose–response curve for gallic acid (Y=0.0051 x+0.1731, (R²: 0.9964)).

TFC

The TFC was based on the complexation with Al³⁺. Quercetin at various concentrations was used for calibration [13] (Table 1). The TFC of samples was expressed as quercetin equivalents (mg QE/g extract) by means of a dose–response curve for quercetin (Y=0.0664x–0.01586, (R²: 0.9972)).

Antioxidant activity

Free radicals are regularly generated in the body during the metabolic process. Once the production of these free radicals in the body exceeds its antioxidant mechanism, they cause wide damage to the cells may lead to many degenerative diseases [14]. It is also known that plants extracts rich in polyphenols are having varying antioxidant activity and there are many methods to determine antioxidant capacity. These methods differ in terms of their assay principles and experimental conditions [15]. Therefore, the antioxidant activities of C. azarolus extracts, as well as two pure compounds were evaluated using five assays.

Free radical scavenging activity by 2,2-diphenyl-picrylhydrazyl (DPPH) assay

The DPPH assay was determined by the method described previously with some modifications [16,17]. A solution of 40 μl of the sample (extracts, pure compounds, and standards) at various concentrations was added to 160 μl of the methanolic solution of DPPH (0.1 mM). Blanks were prepared using the solvent in addition to the DPPH reagent. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm using a microplate reader. Butyl hydroxytoluene (BHT) and butyl hydroxyanisole (BHA) were used as standards for comparison of the activity. The free radical scavenging activity of each solution was calculated as percent inhibition according to the following equation:

\[
\%\text{Inhibition} = \left[\left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right)\right] \times 100
\]

Where, Ablank and Asample are the absorbance of initial and remaining concentrations of the DPPH in the presence of samples and standards, respectively. The extract concentration providing 50% radical scavenging activity half maximal inhibitory concentration (IC₅₀) was calculated from the graph of DPPH radical scavenging effect percentage against extract concentration (Table 2).

2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation decolorization assay

ABTS decolorization assay was determined by the method described previously [18]. ABTS⁺ solution was prepared by mixing 7 mM of ABTS⁺ in water with 2.45 mM potassium persulphate. The mixture was left in the dark at room temperature for 12 h before use. The dilution of the obtained solution was adjusted to obtain the absorbance of 0.70 ± 0.02 at 734 nm. Then, to 40 μl of the sample solution in methanol at different concentrations was added 160 μl of ABTS⁺ solution. The mixture was left at ambient temperature for 10 min and then the absorbance was measured at 734 nm using a 96-well microplate reader. The percentage inhibition was calculated for each concentration relative to a blank absorbance (methanol). The scavenging capability of ABTS⁺ was calculated using the following equation:

\[
\%\text{Scavenging effect} = \left[\left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right)\right] \times 100
\]

Where, Ablank is the initial concentration of the ABTS⁺ and Asample is the absorbance of the remaining concentration of ABTS⁺ in the presence of the sample. The extract concentration providing 50% radical scavenging activity (IC₅₀) was calculated from the graph of ABTS⁺ scavenging effect percentage against sample concentrations. BHT and BHA were used as antioxidant standards (Table 2).

Capric reducing antioxidant capacity (CUPRAC)

The CUPRAC was determined according to the method described previously with minor changes [17,19]. To 50 μl of Cu (II) solution (10 mM) was added 50 μl of neocuproine solution (7.5 mM) and 60 μl of NH₄Ac buffer (1 M, pH 7.0) solution. To the above mixture was added, 40 μl of the sample solutions (extracts and pure compounds) at different concentrations. After 60 min, the absorbance at 450 nm was recorded after a reagent blank using a 96-well microplate reader. The results were given as A₅₃₀ (μg/ml) corresponding to the concentration indicating 50% absorbance intensity and were compared with those of the standards BHA and BHT (Table 2).

Reducing power assay

The reducing power was measured with small changes according to [17,20]. Briefly, a solution (10 μl) of the sample at various concentrations was mixed with 40 μl of phosphate buffer (0.2 M, pH 6.6) and 50 μl of potassium ferricyanide (10 mg/ml). The obtained solution was incubated at 50°C for 20 min. Then, the solution was mixed with 50 μl of trichloroacetic acid solution (100 mg/ml) 10 μl of ferric chloride solution (1.0 g/l), and finally diluted with 40 μl of distilled water. The absorbance was measured at 700 nm using a 96-well microplate reader. Ascorbic acid, tannic acid, α-tocopherol, BHA, and BHT were used as standards. The results were given as A₅₃₀ (μg/ml) corresponding to the concentration indicating 50% absorbance intensity (Table 2). The increase of reducing power by the extract and standard was calculated as follows:

\[
\%\text{Reducing power} = \left[\left(\frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{blank}}}\right)\right] \times 100
\]

Where, Atest and ABlank are absorbance of sample and blank solutions, respectively.

β-carotene bleaching assay

The assay was carried out as described previously with minor modifications [17,21]. A stock solution of β-carotene/linoleic acid was initially prepared by mixing a solution of β-carotene (5 mg) in chloroform (1 ml) with linoleic acid (25 μl) and Tween 40 emulsifier (200 mg). The mixture was evaporated under vacuum to remove chloroform. Then, 100 ml of water saturated with oxygen was added to the above mixture by agitation. A volume of 40 μl of the samples (in ethanol) at different concentrations was mixed with 160 μl of the above
Table 1: Total phenolic and flavonoid contents of *C. azarolus* extracts

| Extract (1 mg/ml) | Phenolic content (mg GAE/g extract)* | Flavonoid content (mg QE/g extract)* |
|------------------|------------------------------------|--------------------------------------|
| Chloroform       | 118.0±1.52                         | 7.33±0.47                            |
| n-butanol        | 307.3±2.33                         | 143.0±2.12                           |

*Values were expressed as mean±SD. (n=3). SD: Standard deviation, *C. azarolus*: Crataegus azarolus, GAE: Gallic acid equivalent.

Table 2: Antioxidant activities of chloroform, n-butanol extracts, and two compounds from *C. azarolus*

| Extract/Compound | DPPH* IC50 µg/ml | ABTS* IC50 µg/ml | CUPRAC* A0.50 µg/ml | Reducing power* A0.50 µg/ml | β-carotene* IC50 µg/ml |
|------------------|------------------|------------------|---------------------|-----------------------------|-----------------------|
| Chloroform       | 54.4±0.77        | 1.389±0.03       | 29.67±0.17          | 96.9±2.65                   | 6.80±0.01             |
| n-butanol        | 11.1±0.22        | 4.99±0.06        | 8.95±0.03           | 42.3±0.31                   | 5.29±0.04             |
| Compound 2       | 9.1±0.00         | 11.4±0.00        | 20.87±0.64          | 45.67±3.30                  | /                     |
| Compound 3       | 6.7±0.09         | 10.6±0.12        | 15.0±0.47           | 22.87±0.29                  | /                     |
| Standards        |                  |                  |                     |                             |                       |
| BHT (1 µg/µl)    | 22.3±1.19        | 1.29±0.30        | 9.62±0.87           | >50                         | 1.05±0.01             |
| BHA (1 µg/µl)    | 5.7±0.41         | 1.8±0.10         | 3.64±0.19           | 8.4±0.67                    | 0.90±0.02             |
| Ascorbic acid (1 µg/µl) | 9.0±1±0.46     | 6.7±1.15         | 5.39±0.91           | 34.93±2.38                  |                       |
| Tannic acid (4 µg/µl) |                  |                  |                     |                             |                       |
| α-tocopherol (4 µg/µl) |                  |                  |                     |                             |                       |

*Values were expressed as mean±SD. Of three parallel measurements, (p<0.05). SD: Standard deviation, *C. azarolus*: Crataegus azarolus, BHT: butyl hydroxyl toluene, BHA: Butyl hydroxyl anisole, DPPH: 2,2-diphenyl-picrylhydrazyl, ABTS: 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid, IC50: Half maximal inhibitory concentration.

**RESULTS AND DISCUSSION**

**Phytochemical contents**

The structures of the flavonoid glycosides 1, 2, and 3 (Fig. 1) isolated from the n-butanol extract were identified by spectral analysis (ultraviolet [UV], H, and 13C-nuclear magnetic resonance [NMR]), acid hydrolysis and by comparison with literature data:

- Quercetin-3-O-rutinoside (3): Yellow powder, UV (λ, nm): MeOH 257 269±360, +NaOH 272 329±413, +HCl 269 364±401, +NaOAc 274 322±390, +H2O 264±390. 1H-NMR (250 MHz, CD3OD, δ ppm): 7.87 (1H, d, 1H, 1.5 Hz, H-2'), 7.60 (1H, d, 1.5 Hz, H-5'), 6.9 (1H, d, 1.5 Hz, H-6), 6.9 (1H, d, 1.5 Hz, H-5'), 6.4 (1H, d, 1.5 Hz, H-8), 6.2 (1H, d, 1.5 Hz, H-6), 5.1 (1H, d, 1.5 Hz, H-1'), 4.5 (1H, brs, H-1'''), 3.5 (1H, m, protons of glucose and rhamnose).

- Compounds 1-3 were obtained as a yellow powder and their chromatographic behavior indicated that are flavonoid glycosides. The UV-visible spectra in MeOH and with reagents revealed the presence of quercetin skeleton as aglycone for the three compounds by the presence of free OH groups at positions 5, 7, 3', and 4' and the presence of a substituted hydroxyl group in position 3.

The nature of glycosidic moiety of the compounds was determined by acid hydrolysis with 2N HCl at 100°C for 30 min of compounds 1 and 2 which gave galactose and glucose, respectively, while compound 3 gave glucose and rhamnose [22-24]. The sugars were identified by TLC on silica gel with authentic samples, using aceton-H2O (9:1) as solvent and aniline malonate as detection reagent. The 1H-NMR spectra of the three compounds showed the existence of five aromatic protons: H2' (1H, d, J=8.5 Hz), H6' (1H, d, J=8.5 Hz), 2H-2 for A-ring and two meta-coupled protons H6 (1H, d, J=2 Hz) and H8 (1H, d, J=2 Hz) for A-ring, characteristic of a quercetin aglycone.

In addition, the 1H-NMR spectrum of compounds 1 showed the presence of an anomic proton H1'' (1H, d, J=7.6 Hz) at δ 6.29 and a bulk of six other protons between 3.1 and 3.9 ppm confirmed the presence of galactosyl moiety. Finally, the structure of compound 1 was confirmed by 81C-NMR spectrum that gave characteristic peaks of quercetin-3-O-galactoside compared to literature data [25,26]. The 1H-NMR spectrum of compound 2 revealed the presence an anomic proton H1'' (1H, d, J=7.5 Hz) at δ 6.51 attributed to glucosyl moiety and a bulk of six other protons between 3.3 and 4.0 ppm which confirm the structure of compound 2 as quercetin-3-O-glucoside [26]. The 1H-NMR spectrum of compound 3 showed the existence of two anomic protons, the first (1H, d, J=7.8 Hz) at δ 6.51 attributed to glucose (H1') at position 3 and the second (1H, br. S) for rhamnose (H1'') at δ 4.5 with the...
characteristic signal (3H, d, J=5.7 Hz) at 6.1.1 of their methyl (H6′′). The upfield of H1′′ indicated that rhamnose is a terminal sugar. The linkage of rhamnose to position 6 of glucose was deduced by comparison of our data with the literature [24,26]. The structure of compound 3 was confirmed to be quercetin-3-O-rutinoside.

Moreover, the compounds 4-8 were identified by comparison of their mass spectra obtained by GC/MS analysis with literature data (NIST library) to be 2,4-bis (1,1-dimethyl ethyl)-phenol (4), tridecanolic acid 12-methyl-methyl ester (5), pentadecanoic acid 14-methyl-methyl ester (6), 8-octadecanoic acid methyl ester (7), and isobutyl nonyl phthalate (8) as shown in Fig. 1. Compounds 4-8, which are volatile compounds, are reported for the first time from the Crataegus genus.

**Total phenolic and TFC**

The estimation of TPC and TFC using methods described above indicates that _C. azarolus_ was very rich in phenolic and flavonoid compounds (Table 1). The TPC and TFC of the n-butanol extract reached 30.7% (expressed as GAE) and 14.3% (expressed as QE) of the weight of extract, respectively, and were largely higher than chloroform extract contents. Furthermore, the TPC and TFC values, in this study, are well above the values reported in other studies [7,8] about the species, which can be explained by the difference in the period and region of plant collection and the method of extraction.

**Antioxidant activity**

Fruits and vegetables are highly potent antioxidants, easily available, inexpensive, and more reliable than any synthetic marketed medicament do not have side effects and possess a high safety index. Many research studies have confirmed the importance of antioxidants from plants [27]. In this study, it was necessary to evaluate _C. azarolus_ extracts through several tests to get more informative results on its antioxidant potential. It was necessary to evaluate its extracts through several tests. Consequently, the antioxidant activity was carried out using five different assays: DPPH free radical scavenging, ABTS cation radical decolorization, CUPRAC, reducing power activity, and β-carotene bleaching activity. The results in Table 2 revealed that the n-butanol extract was more potent than the CHCl3 extract because there is a close relationship between the phenolic and flavonoid contents and the antioxidant activity. The results in Table 2 confirmed that compound 3 was the most active compared to compound 2 with almost used methods. In addition, IC50 values for the n-butanol extract and compound 3 were in generally close to those of used standards. This finding is in agreement with other studies about other species of _Crataegus_ genus collected in other countries [7,9,28,29].

**CONCLUSION**

We report, herein, for the first time, the identification of five volatile compounds (4-8) from the aerial parts of _C. azarolus_ (Rosaceae) by GC-MS. From obtained results, the highest level of TPC and TFC was found in the n-butanol extract. The antioxidant activities of the chloroform, n-butanol extracts, and the pure compounds 2 and 3 have been determined using five assays. The n-butanol extract shown a powerful antioxidant activity, which can be ascribed to its richest in phenolic compounds. However, further studies are required to isolate other phytochemical constituents and subjecting it to biological evaluation.

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**CONFLICTS OF INTERESTS**

All authors have none to declare.

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