Research Article

Phenolic Composition and Antioxidant and Antimicrobial Activities of Extracts Obtained from *Crataegus azarolus* L. var. *aronia* (Willd.) Batt. Ovaries Calli

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Objective. Plant cell culture is an innovative technology to produce a variety of substances. Numerous plants synthesize among their secondary metabolites phenolic compounds which possess antioxidant and antimicrobial effects. Hawthorn (*Crataegus*) is one of these plants which has long been used in folk medicine and is widely utilized in pharmaceutical preparations mainly in neuro- and cardiosedative actions. **Methods and Results.** The production of polyphenol by fifty-two-week-old *Crataegus azarolus* var. *aronia* calli was studied in relation to growth variation and antioxidant and antimicrobial capacity within a subcultured period. The DPPH and ABTS⁺ assays were used to characterize the antioxidant actions of the callus cultures. Antimicrobial activity was tested by using disc diffusion and dilution assays for the determination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) values of each active extract. High TEAC_{DPPH}, TEAC_{ABTS}, and antimicrobial activity was observed when maximal growth was reached. An optimum of total phenol, proanthocyanidins, flavonoid, (–)-epicatechin, procyanidin B2, chlorogenic acid, and hyperoside was produced during this period. **Conclusion.** Antioxidant and antimicrobial activities were strongly correlated with total phenols and total flavonoids. *Crataegus azarolus* var. *aronia* cells culture represents an important alternative source of natural antioxidants and antimicrobials.

1. Introduction

In recent years considerable attention has been devoted to medicinal plants with antioxidant and antimicrobial properties. The antioxidant properties are commonly postulated

to play an important role in preventing diseases caused by oxidative stress, such as cancer, coronary arteriosclerosis, and the ageing processes [1]. Phenolic compounds are known to possess different pharmacological activities among which antioxidant and antimicrobial effects have

recently received more attention. There is much literature concerning the antioxidant and antimicrobial properties of many species from genus *Crataegus* L. (hawthorn). The genus *Crataegus*, known as “Zaarour” in Tunisia, is represented by two species in the flora of Tunisia: *C. oxyacanthus* ssp. *monogyna* (Jacq.) Rouy and Camus and *Crataegus azarolus* L. [2]. *Crataegus azarolus* is represented by two varieties: *Crataegus azarolus* var. *aronia* (Willd.) Batt. and *C. azarolus* var. *eu-azarolus* Maire; they differ by the color of fruit: yellow fruits for the former and red ones for the latter.

The chemistry and pharmacology of hawthorn is well documented [3–13]. Hawthorn preparations have been used for their sedative actions [14] to treat the early stages of congestive heart failure [15, 16] and to reduce blood pressure and total plasma cholesterol [17]. Oral administration of standardized extracts induces a significant decrease in mortality after ischemia reperfusion in animals [15]. The pharmacological effects were ascribed to its polyphenolics among which (–)-epicatechin, proanthocyanidins (procyanidin dimers B1, B2, and B5, trimer C1, oligomers, and polymers), and flavonoids (hyperoside, vitexin-2''-O-rhamnoside, vitexin-4'-acetyl-2'-rhamnoside, spiraeoside, rutin, isoquercitrin, and vitexin) are the most prominent constituents [3–10, 18]. Many of these phenolics have already been studied for their antioxidant functions [3, 4, 6, 9, 18–21] and antimicrobial effects [22–24].

Cell cultures represent an alternative source for producing natural antioxidants and antimicrobial compounds. Plant tissue cultures produce a variety of secondary metabolites, sometimes in higher percentage than the original plant, and particularly in the polyphenolic class, high yields have sometimes been obtained [25]. Previous studies showed an interesting polyphenolic production in *Crataegus monogyna* Jacq. callus and cell suspension cultures [3, 26–30] in *Crataegus × sinaica* Boiss [31]. Despite the extensive use of the different species of hawthorns, there are no studies on the polyphenolic production in *Crataegus azarolus*. The aim of this work was the production of phenolics in tissue culture of *Crataegus azarolus* L. var. *aronia* ovaries and the investigation of the antioxidant capacity and antimicrobial effects of those metabolites.

2. Materials and Methods

2.1. Chemicals. ABTS 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) and DPPH 2,2-diphenyl-picrylhydrazyl were purchased from Sigma (St. Louis, MO, USA), Trolox C (6-hydroxy-2,5,7,8-tetramethylchroma-2-carboxylic acid) was purchased from Sigma-Aldrich (Deisenhofen, Germany), and potassium sulphate (di-potassium peroxodisulfate), HPLC grade of (–)-epicatechin, procyanidin dimer B2, chlorogenic acid, hyperoside, rutin, spiraeoside, and isoquercitrin were obtained from Sigma-Aldrich (Taufkirchen, Germany). HPLC grade of cyanidin chloride was obtained from Ex-trasynthèse (Genay, France). HPLC solvents were of HPLC grade. All other reagents used were of analytical grade.

2.2. Plants Material and Sterilization. Floral buds were collected in March 2005 from *Crataegus azarolus* var. *aronia* native trees spreading out in the region of Hammam-Sousse (Tunisian Center-Est, semi-arid climatic conditions). A voucher specimen was deposited in the Higher Institute of Agronomy of Chott-Meriem, Botanic Laboratory Herbarium, and was assigned a corresponding number R412 [18]. The explants were washed in soapy water, rinsed under running tap water for approximately 10 min, and subsequently stirred in mercury chloride (0.1%) for 5 min and in sodium hypochlorite (0.5%) for 20 min. Finally floral buds were rinsed with sterilized distilled water three times under laminar flow hood. Excess water was removed with sterile filter paper; floral buds were deprived of sepals, petals, and stamens. The upper part of the gynoecium constituted by 2 to 3 carpels was decapitated and the under part (ovaries) was placed in sterile Petri dishes (90 mm diameter) containing 25 mL nutrient medium solidified by 7 g/L agar.

The culture medium contained B₅ Gamborg mineral solution elements [32], sucrose (30 g/L), myo-inositol (100 mg/L), pyridoxine (0.5 mg/L), nicotinic acid (0.5 mg/L), thiamine (0.5 mg/L), kinetin (0.5 mg/L), and 2,4 D (2 mg/L) [3, 33] for callus initiation. The pH was adjusted to 5.6 before autoclaving. Calli were initiated in the dark for 6 weeks at 23 ± 2°C. Experiment was performed with three replications (4 explants/Petri dishes) total number of explants for each replication was 32.

2.3. Establishment of the In Vitro Callus Cultures. Calli initiated were subcultured (6 subcultures) on the same medium under 16:8 light/dark period under a light intensity of 15 W·m^{–2} (fluorescent light tubes “Deluxe Cool White”) with intervals of 4 weeks. The red color of callus was the qualitative parameter for better production of polyphenols [3]. So, red induced calli were subcultured (7 subcultures) in the same medium under permanent light with intervals of 4 weeks. Growth and production of polyphenols by the fifty-two-week-old *Crataegus azarolus* var. *aronia* calli have been studied within 40 days subculture period. A sample of calli was harvested every 4 days for 40 days, gently pressed on filter paper to remove excess water and their fresh weights (FWs) were recorded. After that they were dried in 80°C and their dry weights (DWs) were recorded.

2.4. Preparation of Methanolic Extracts. 2 g of fresh calli, harvested at days 4, 8, ..., and 40, was transferred to a vial and 30 mL of methanol was added at room temperature and left to macerate for 3 days to obtain the methanolic extracts, from which the solvent was evaporated using a rotary vacuum evaporator and stored at 4°C.

2.5. Colorimetric Analysis

2.5.1. Analysis of Total Phenols. The concentration of total phenols in each extract was measured by UV spectrophotometry (Jenway 6300) based on a colorimetric oxidation/reduction reaction. The oxidizing agent used was Folin-Ciocalteu reagent (Merck) [34]. To 50 µL of diluted extract

(1 mg/1 mL of methanol) was added, in screw-capped test tubes, 750 μ L of distilled water-Folin-Ciocalteu solution (28/2 v/v). After 3 min, 200 μ L of sodium carbonate (Na_2CO_3) (200 g/L) was added and the test tubes were properly shaken before incubating in boiling water bath for 1 min. The tubes were then allowed to cool in the dark. The absorbance was measured at 765 nm and results were expressed in mg of gallic acid/100 g dry weight (DW) using appropriate standard curve. For a control sample, 50 μ L of methanol was used.

2.5.2. Analysis of Proanthocyanidins. The proanthocyanidins were determined by UV spectrophotometry method based on acid hydrolysis and color formation. The HCl/butan-1-ol assay was used to quantify the total proanthocyanidins [35]. One mg of the extract was dissolved in 1 mL of methanol. To 0.25 mL of this solution was added 3 mL of a 95% solution of *n*-Butanol/HCl (95/5, v/v) in glass stoppered test tubes followed by addition of 0.1 mL of a solution of $\text{NH}_4\text{Fe}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ in 2 M HCl (0.2%, w/v). The tubes were incubated for 40 min at 95°C. For a control sample, 0.25 mL of methanol was used. After incubation, the samples were cooled and analyzed by measuring absorbance at 540 nm. The results were expressed as mg of cyanidin chloride/100 g DW.

2.5.3. Analysis of Total Flavonoids. The AlCl_3 method [36] was adapted for the purpose of determining the total flavonoid content of the ethyl acetate fractions. 0.5 mg of the extract was dissolved in 1 mL of methanol. To 0.5 mL of this solution was added equal volumes of a solution of 2% $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (2 g in 100 mL methanol). The mixture was thoroughly mixed and incubated for 10 min. After incubation, the samples were cooled and analyzed by measuring absorbance at 367.5 nm. The results were expressed in mg rutin equivalents/100 g DW.

2.5.4. High Performance Liquid Chromatography Analysis. HPLC analysis of extracts was carried out using a Hewlett Packard 1500 series (Waldbronn, Germany) liquid chromatography system equipped with a vacuum degasser, quaternary pump, autosampler, thermo stated column compartment, and diode array detector. After filtration on millipore filter paper (0.22 μ m) (Whatman), 20 μ L methanol extract was injected on a Spherisorb ODS2 RP18 (5 μ m) reversed phase, and C18 column (4.6 mm i.d. $\times$ 150 mm) (Sigma-Aldrich, Taufkirchen, Germany) was eluted by an acidified acetonitrile-water gradient. Elution with a flow rate of 0.7 mL/min at 25°C was as follows: 0–5 min, 0–7.5% B in A; 5–13 min, 20% B in A; 13–20 min, 80% B in A; 20–25 min, 100% B in A (solvent A: acetonitrile/water, 1/9 v/v, pH 2.5; solvent B: acetonitrile/water, 9/1 v/v, pH 2.5).

Phenolic compounds in the methanolic extract were identified by comparison with authentic standards at 360 nm and at 280 nm. The wavelength changes automatically in 10 minutes.

2.6. Colorimetric Radical Scavenging Tests

2.6.1. DPPH Radical Scavenging Activity. Twenty μ L of diluted callus extract (1 mg/mL using methanol) was added to 980 μ L of DPPH radical (90 μ M in methanolic solution) in a test tube. Methanol was used in the place of antioxidant solution as a blank. The solution was immediately mixed vigorously for 10 s by a vortex mixer and transferred to the cuvette holder of the spectrophotometer against the blank, which did not contain the extract. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 515 nm. All experiments were performed in triplicate.

2.6.2. ABTS Radical Scavenging Activity. ABTS radical scavenging activity was measured using a modified Re et al. 1999 method [37]. ABTS radical cation (ABTS^+) was produced by reacting 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration). The reaction mixture was allowed to stand in the dark at room temperature for 12–16 h prior to use.

ABTS^+ solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. To a diluted ABTS^+ solution (980 μ L) was added 20 μ L of the extract solution (1 mg/mL of methanol). The solution was immediately mixed vigorously for 10 s by a vortex mixer and transferred to a cuvette. The absorbance was monitored at 734 nm after 6 min.

2.6.3. Radical Scavenging Expression. For the two tests (DPPH and ABTS), Trolox, a water-soluble analogue of α -tocopherol, (vitamin E) was served as a standard. A concentration-response curve, for ABTS^+ (734 nm) and for DPPH (515 nm), as a function of different Trolox concentrations was prepared. The decrease in absorption of tested samples was used for calculating the TEAC (Trolox Equivalent Antioxidant Capacity) ($\text{TEAC}_{\text{ABTS}}$ and $\text{TEAC}_{\text{DPPH}}$). All experiments were performed in triplicate. Results were expressed in μ mol Trolox/100 g DW.

2.7. Antimicrobial Activity

2.7.1. Antibacterial Activities

Bacterial Strains. Both cocci Gram-positive and Gram-negative rods bacterial species were selected as test microorganisms according to their pathologic origin. *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (NCIMB 8853), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Micrococcus luteus* (NCIMB8166), and hospital *Salmonella typhimurium* were used.

Preparation of Inoculums. Mueller-Hinton (M-H) broth was inoculated aseptically with the appropriate microorganisms, 24 h before testing. This is to ensure that bacteria are perfectly suited to the broth and reached the stationary phase of growth. The inoculated bacterial strains were incubated at 37°C during 18–24 h, the inoculum suspension containing

approximately 105 colony forming units (CFU)/mL of bacteria.

Antibacterial Activity Test. The qualitative and quantitative antibacterial assay of extracts was carried out by the disc diffusion method [38]. Five hundred μ L of the inoculums were spread over plates containing sterile M-H agar (pH 7.2) and the paper filter discs (5 mm) were impregnated with 20 μ L of extract (10 mg/mL methanol) dried and placed on the surface of the media. The plates were incubated at 37°C for 18 h. The inhibition zone around the disc was measured. Antibacterial tests were performed in triplicate. Two controls were also included in the test. The first was a control with methanol involving the presence of microorganisms but the absence of the test material and the second is a standard antibiotic (Ampicillin; 10 μ g/disc) which was used in order to control the sensitivity of the tested microorganisms. The estimation of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was carried out by the broth dilution method [39]. Dilutions of the extracts were prepared as follows: 5, 10, and 15 mg/mL of methanol. The M–H broth employed for sample dilution was supplemented with tween 80 (Merck, Germany) at a concentration of 5% [40, 41] to enhance extract solubility. MIC values were taken as the lowest extract concentration that prevent visible bacterial growth after 24 h of incubation at 37°C, and MBC as the lowest concentration that completely inhibited bacterial growth. Each experiment was repeated three times. To confirm the results of MBC, 10 mL of the experimental suspensions was subcultured in Trypto-Caseine-soja (TCS) agar plates which were incubated at 37°C for 18–24 h [42].

2.7.2. Antifungal Activity

Test Organisms. Four phytopathogenic fungal species were used for the antifungal testing, namely, *Fusarium oxysporum*, *Aspergillus niger*, *Penicillium* sp., and *Alternaria* sp. These were obtained from the laboratory of phytopathology at the Regional Pole of Agricultural Research and Development in the eastern centre of Chott Mariem, Sousse, Tunisia.

Determination of Antifungal Activity of Extracts. The disc diffusion method was used for antifungal screening [43]. Fungal broth culture aliquots adjusted to 104–105 CFU/mL were added to Potato Dextrose Agar medium and distributed uniformly in 9 cm Petri plates. Under aseptic conditions, paper discs (6 mm, Whatman no. 1 filter paper) were impregnated with 20 μ L of extract at 5 mg/mL (100 μ g/disc) and placed on the culture plates after removing the solvent by evaporation. The antifungal agent, Carbendazine (0.5 mg/mL), was used as a positive control and methanol as a negative control. The diameter of the zone of inhibition (mm) around the disc was measured after incubation at 28°C for 4 days and compared with control. The test was performed in triplicate.

2.8. Statistical Analysis. Simple regression analysis was performed to calculate the dose-response relationship of standard solutions used for calibration as well as test samples.

FIGURE 1: Total phenols, total flavonoids, and total proanthocyanidins production by *Crataegus azarolus* var. *aronia* ovaries callus cultures versus growth increase.

Linear regression analysis was performed, quoting the correlation coefficient r_{xy} between antioxidant and antibacterial activities and phenolic classes and compounds. All results are expressed as mean value $\pm$ standard deviation of three parallel measurements. The results were processed using Microsoft Excel 2007 and the data were subjected to one way analysis of variance (ANOVA) and the significance of differences between sample means was calculated by Duncan multiple range test using SPSS for Windows (Standard Version 12.0 SPSS Inc., Chicago, IL.); P values ≤ 0.05 were regarded as significant and $P \leq 0.01$ as very significant.

3. Results

3.1. Polyphenols of *Crataegus azarolus* var. *aronia* Callus Cultures. Callus cultures initiated from ovaries after 6 weeks were maintained over a period of 52 weeks by 13 subcultures. To study the optimal period for polyphenol synthesis in relation with growth, stabilized callus cultures were analyzed within a culture period of 40 days with every 4 days analysis.

The production of polyphenols in relation to growth is presented in Figure 1. Optimum growth increase was observed from days 16 to 40 (190 ± 10 – 210 ± 11 mg DW) with the highest mass production at day 24 (240 ± 12 mg DW). The highest level of total phenols appeared at day 24 with 3500.75 ± 25.3 mg/100 g DW. Maximal total proanthocyanidin production was recorded between the 24th and 36th day of culture (1845.69 ± 35.3 and 1085.69 ± 25.6 mg/100 g DW). Rates in flavonoids production are almost identical between the 8th and the 40th day of culture (857.0 ± 23.4 – 765.5 ± 25.36 mg/100 g DW). Maximal production of total flavonoids was recorded in the 24th day of culture with 1100.25 ± 12.3 mg/100 DW.

HPLC data (Figure 1) shows that the fifty-two-week-old callus cultures produced mainly chlorogenic acid, (–)-epicatechin, proanthocyanidins dimer B2, hyperoside, rutin, spiraeoside, and isoquercitrin. All compounds were at high levels in the beginning of the culture. The highest level was for (–)-epicatechin, followed by chlorogenic acid, procyanidin dimer B2, hyperoside, rutin, spiraeoside and isoquercitrin. Those levels decreased at day 4, after that all levels increased until day 24. Production of the polyphenols compounds decreased again.

The HPLC analysis of individual compounds (Table 1) shows a relation between the respective yields in the major catechin produced: (–)-epicatechin and procyanidin B2 dimer, as both have the same production profiles with maximum amounts at day 24, (–)-epicatechin: 687.69 ± 13 mg/100 g DW, procyanidin B2: 288.69 ± 6 mg/100 g DW. In cases, a similar decrease and increase was recorded of both compounds in the same age of culture. A similar observation was previously made [3, 29], showing consistency in the phenolic synthesis for the same type of calli. This correlation between dimer B2 and (–)-epicatechin production is consistent with literature data showing that (–)-epicatechin-rich plant organs generally contain procyanidin B2 as the major dimer [3, 29].

Chlorogenic acid, the main phenolic acid, reached a peak level at day 24 (400.58 ± 12.7 mg/100 g DW dry weight) before failing to 265.89 ± 11.3 mg/100 g DW dry weight at the end of the culture. As shown in Figure 1 and Table 1, the main flavonoid compounds detected were hyperoside, rutin, spiraeosid, and isoquercitrin with maximum amounts at day 24, 136.5 ± 6 mg/100 g DW, 94.6 ± 3.6 mg/100 g DW, 95.57 ± 7 mg/100 g DW, and 58.7 ± 4 mg/100 g DW, respectively.

3.2. Antioxidant Capacity of *Crataegus azarolus* var. *aronia* Callus Extracts. The DPPH and ABTS⁺ assays were used to characterize the antioxidant capacity of the callus cultures. The TEAC_{DPPH} and TEAC_{ABTS} were investigated within 40 days culture period with four-day-analysis interval (Table 2). A decrease in TEAC_{DPPH} and TEAC_{ABTS} values was observed at the early stages of culture (days 0–16). The antioxidant capacity increased after this period to attain its highest value at day 24 (3226.02 ± 11.7 μmol/100 g DW of TEAC_{DPPH} and 6741.12 ± 11.4 μmol/100 g DW of TEAC_{ABTS}). The day 24 to day 36 period corresponds to the maximal production stage of total phenols, flavonoids, proanthocyanidins, (–)-epicatechin, procyanidin B2 dimer, hyperoside, rutin, spiraeosid, and isoquercitrin which could positively influence the observed activities. These results are consistent with those obtained previously by [3, 29] which demonstrate a high TEAC of *Crataegus monogyna* callus culture.

In rationalizing the antioxidant potential of the calli in terms of phenolic classes or individual phenolic compounds, it became clear from the regression coefficient data (Table 3) that the antioxidant capacity based on TEAC_{DPPH} and TEAC_{ABTS} was associated more strongly with total phenol ($r_{\text{DPPH}} = 0.98$, $r_{\text{ABTS}} = 1.00$) and with total flavonoids ($r_{\text{DPPH}} = 0.97$, $r_{\text{ABTS}} = 0.96$) and, to a lesser extent, with total proanthocyanidins ($r_{\text{DPPH}} = 0.83$, $r_{\text{ABTS}} = 0.80$).

Hyperoside ($r_{\text{DPPH}} = r_{\text{ABTS}} = 0.86$), rutin ($r_{\text{DPPH}} = r_{\text{ABTS}} = 0.76$), spiraeosid ($r_{\text{DPPH}} = 0.76$, $r_{\text{ABTS}} = 0.83$), isoquercitrin ($r_{\text{DPPH}} = 0.77$, $r_{\text{ABTS}} = 0.76$), and chlorogenic acid ($r_{\text{DPPH}} = 0.75$, $r_{\text{ABTS}} = 0.72$) strongly influenced antioxidant efficacy of ovaries calli extracts. (–)-Epicatechin ($r_{\text{DPPH}} = 0.62$, $r_{\text{ABTS}} = 0.67$) and procyanidin B2 dimer ($r_{\text{DPPH}} = r_{\text{ABTS}} = 0.69$) influenced lesser antioxidant efficacy. This was reflected by the high correlation coefficients in the TEAC_{DPPH} and TEAC_{ABTS} assays (Table 3). Both TEAC_{DPPH} and TEAC_{ABTS} assays show similar trend in antioxidant potentialities.

3.3. Antibacterial Capacity of *Crataegus azarolus* var. *aronia* Callus Extracts. Table 4 shows the antibacterial potentialities of *Crataegus azarolus* var. *aronia* callus extract against *Staphylococcus aureus* (ATCC 29213), *Staphylococcus epidermidis* (NCIMB 8853), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27853), *Micrococcus luteus* (NCIMB8166), and hospital *Salmonella typhimurium*. Antimicrobial activity was observed when maximal growth was reached (Days 24–28) against only the cocci Gram positive bacteria with an inhibition diameter varying between 10.9 (against *Staphylococcus aureus*) and 14.5 mm (against *Micrococcus luteus*) and a MIC = MBC = 625 μg/mL for all bacteria. This higher resistance among Gram negative bacteria could be due to the differences in the cell membrane of these bacterial groups. Indeed, the external membrane of Gram negative bacteria renders their surfaces highly hydrophilic [44], whereas the lipophilic ends of the lipoteichoic acids of the cell membrane of Gram positive bacteria may facilitate penetration by hydrophobic compounds [45, 46]. According to [39, 47–50], aromatic compounds group were known with their important antibacterial activity. The latter has been found to inhibit production of amylase and protease by *Bacillus cereus*, deteriorate cell wall, and cause cell lysis [51].

3.4. Antifungal Activity. No antifungal activity was observed against the four pathogenic fungi. This result could be due to the low concentration of extract used. Therefore, we recommended the use of higher concentrations of extracts in order to obtain a more potent effect against all microorganisms.

4. Discussion

The production of polyphenols by calli cultures varied with age. Polyphenols production by two-year-old callus culture has been studied in relation to growth variation within a 40-day subculture period by [3]. Period of maximum production was found between 24 and 36 days of culture. Maximum contents in total phenol (5.89 g/100 g DW), total proanthocyanidins (2.96 g/100 g DW), and total anthocyanins (0.288 g/100 g DW) were found to be more important than in our results. On the other hand, maximal contents in (–)-epicatechin (0.838 g/100 g DW), procyanidin B2 dimer (0.321 g/100 g DW), hyperoside (0.143 g/100 g DW), and isoquercitrin (0.020 g/100 g DW) were detected. Chlorogenic acid was the main phenol acid synthesized by *Crataegus monogyna* callus culture with an optimal content equal to 0.769 g/100 g DW (Table 5).

TABLE 1: Individual phenolic content measured by HPLC in methanolic ovaries calli extracts. Amounts of compounds are given as mean values $\pm$ standard deviation ($n = 3$). Integration of area of individual peaks was compared to their corresponding standard (four points standard curve, R^2 presented in the table) to calculate the individual phenolic concentration expressed in mg/100 g DW.

Culture day	Chlorogenic acid ($R^2 = 0.9995$)	(-)-Epicatechin ($R^2 = 0.9989$)	Procyanidin dimer B2 ($R^2 = 0.9995$)	Hyperoside ($R^2 = 0.9974$)	Rutin ($R^2 = 0.9981$)	Spiraeoside ($R^2 = 0.9924$)	Isoquercitrin ($R^2 = 0.9996$)
0	360.25 $\pm$ 11.2 ^{h*}	653.48 $\pm$ 12 ^g	280.65 $\pm$ 12 ⁱ	120.36 $\pm$ 5 ^h	90.36 $\pm$ 4 ⁱ	90.25 $\pm$ 5 ^h	50.63 $\pm$ 4 ^g
4	142.23 $\pm$ 5.2 ^a	365.78 $\pm$ 10 ^{bc}	56.24 $\pm$ 12 ^a	50.36 $\pm$ 3.5 ^a	45.12 $\pm$ 3 ^a	63.47 $\pm$ 3 ^b	25.68 $\pm$ 2 ^a
8	150.23 $\pm$ 10.2 ^b	387.45 $\pm$ 13 ^{de}	75.68 $\pm$ 6 ^b	78.63 $\pm$ 2 ^b	50.78 $\pm$ 4 ^c	70.58 $\pm$ 4 ^c	30.86 $\pm$ 2 ^b
12	164.58 $\pm$ 11.3 ^c	400.58 $\pm$ 17 ^c	85.69 $\pm$ 6 ^c	80.69 $\pm$ 9 ^c	48.69 $\pm$ 3 ^b	77.63 $\pm$ 5 ^d	33.56 $\pm$ 2 ^c
16	180.69 $\pm$ 8.6 ^d	369.56 $\pm$ 11 ^{ed}	90.47 $\pm$ 6 ^d	88.69 $\pm$ 5 ^d	55.48 $\pm$ 3 ^d	89.45 $\pm$ 6 ^f	30.47 $\pm$ 3 ^b
20	265.89 $\pm$ 6.8 ^e	356.78 $\pm$ 10 ^b	99.58 $\pm$ 6 ^e	90.56 $\pm$ 4 ^e	68.74 $\pm$ 5 ^e	90.86 $\pm$ 6 ^g	36.87 $\pm$ 3 ^c
24	400.58 $\pm$ 12.7 ⁱ	687.69 $\pm$ 13 ^h	288.69 $\pm$ 6 ^j	136.54 $\pm$ 6 ⁱ	94.58 $\pm$ 3.6 ^j	95.57 $\pm$ 7 ^h	58.69 $\pm$ 2 ⁱ
28	369.451 $\pm$ 10.8 ⁱ	647.56 $\pm$ 13 ^k	230.58 $\pm$ 9 ^h	96.58 $\pm$ 5 ^g	77.48 $\pm$ 4.5 ^h	90.34 $\pm$ 7 ^g	55.36 $\pm$ 4 ^h
32	300.58 $\pm$ 11.4 ^g	452.36 $\pm$ 12 ^f	120.45 $\pm$ 8 ^g	92.36 $\pm$ 3 ^f	73.89 $\pm$ 5 ^g	80.56 $\pm$ 6 ^e	50.68 $\pm$ 4 ^g
36	280.69 $\pm$ 12.2 ^f	364.69 $\pm$ 12 ^{bc}	110.36 $\pm$ 8 ^f	88.75 $\pm$ 5 ^d	70.58 $\pm$ 3.6 ^f	70.43 $\pm$ 7 ^c	44.36 $\pm$ 4 ^f
40	265.89 $\pm$ 11.3 ^e	256.47 $\pm$ 10 ^a	100.45 $\pm$ 6 ^{ef}	80.96 $\pm$ 4 ^e	55.45 $\pm$ 3 ^d	50.36 $\pm$ 7 ^a	35.36 $\pm$ 4 ^d

* In the same column, values with the same letters are not significantly different at $P \leq 0.01$.

TABLE 2: Antioxidant activity (μ mol Trolox/100 g DW) of ovaries calli extracts of *Crataegus azarolus* var. *aronia* within days of culture.

Day culture	DPPH	ABTS
0	2750.95 $\pm$ 9.23 ^{g*}	6576.71 $\pm$ 12.75 ^h
4	1139.52 $\pm$ 7.41 ^a	3888.02 $\pm$ 11.17 ^a
8	2179.72 $\pm$ 11.24 ^b	3984.53 $\pm$ 12.37 ^b
12	2361.66 $\pm$ 12.36 ^c	4708.20 $\pm$ 12.45 ^c
16	2669.44 $\pm$ 11.47 ^f	5682.30 $\pm$ 11.11 ^d
20	2732.54 $\pm$ 8.23 ^g	5959.34 $\pm$ 10.47 ^e
24	3226.02 $\pm$ 11.74 ⁱ	6741.12 $\pm$ 11.42 ^{ij}
28	3000.89 $\pm$ 11.23 ^h	6412.09 $\pm$ 14.23 ^g
32	2765.37 $\pm$ 10.47 ^g	6290.97 $\pm$ 11.32 ^f
36	2280.04 $\pm$ 11.42 ^d	6708.89 $\pm$ 9.36 ⁱ
40	2215.31 $\pm$ 11.34 ^{bc}	6412.30 $\pm$ 8.47 ^g

* Data shown are the mean $\pm$ standard deviation; in the same column, values with the same letters are not significantly different at $P \leq 0.01$.

Kartnig et al., 1993 [27], studies of flavonoid and procyanidin contents of two-month-old callus cultures from *Crataegus monogyna* shoot tips cultured in MS medium supplied with NAA/kinetin, showed a more complex and interesting flavonoid pattern with the isolation of vitexin, vitexin-2''-orhamnoside (0.00456 g/100 g DW), rutin (0.002 g/100 g DW), and hyperoside, the latter being dominant (0.01258 mg 100 g/dry wt). Maximum total proanthocyanidins content was 0.017 g 100 g/DW (Table 5).

Rakotoarison et al., 1997 [28], analyzing the five-year-old callus culture initiated by [3] within a 30-day subculture, found that contents in polyphenolic and in the individual compounds identified decreased. Maximal contents (between 24 and 28 days of culture) in total phenols, flavonoids, and proanthocyanidins reached 2.33, 1.3, and 0.92 g/100 g DW,

TABLE 3: Correlation coefficients between TEAC_{DPPH}/TEAC_{ABTS} and phenolic contents of *Crataegus azarolus* var. *aronia* ovaries callus culture.

	TEAC _{DPPH}	TEAC _{ABTS}
Total phenols	0.98	1.00
Total flavonoids	0.97	0.96
Total proanthocyanidins	0.83	0.80
Chlorogenic acid	0.75	0.72
Hyperoside	0.86	0.86
Rutin	0.76	0.76
Isoquercitrin	0.77	0.76
Spiraeoside	0.76	0.83
Epicatechin	0.62	0.67
Procyanidin B2	0.69	0.69

respectively. (-)-Epicatechin (0.675 g/100 g DW), procyanidin B2 (0.5142 g/100 g DW), hyperoside (0.528 g/100 g DW), and chlorogenic acid (0.235 g/100 g DW) were also found (Table 5).

Bahorun et al., 2003 [29], analyzed the polyphenolic composition of ten-year-old callus culture (same initiated culture) within a 45-day subculture. Compared to previous study [3], a quantitative and qualitative different polyphenolic composition was found. Generally lower content in total phenols (4.74 g/100 g DW), total proanthocyanidins (2.081 g/100 g DW), (-)-epicatechin (0.177 g/100 g DW), hyperoside (traces), isoquercitrin (absent), and chlorogenic acid (0.111 g/100 g DW) was carried. Higher anthocyanins content was detected (0.618 g/100 g DW).

Maharik et al., 2009 [31], demonstrate that stem callus culture of *C. sinaica* produce a high content in anthocyanins equal to 157.98 μ g/g FW when culture medium contained 2 mg/L BA and 1 mg/L NAA.

TABLE 4: Antibacterial activity (ID, MIC, and MBC) of methanolic extract of ovaries calli of *Crataegus azarolus* var. *aronia*.

Culture days	Gram (+) bacteria			Gram (-) bacteria			Pseudomonas aeruginosa		
	<i>Staphylococcus epidermis</i>	<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Salmonella typhimurium</i>	<i>Escherichia coli</i>		ID ¹	MIC	MBC
	ID ¹ (mm)	MIC (µg/mL)	MBC (µg/mL)	ID ¹ (mm)	MIC (µg/mL)	MBC (µg/mL)	ID ¹ (mm)	MIC (µg/mL)	MBC (µg/mL)
0-20	0.0	>2500	>2500	0.0	>2500	>2500	0.0	>2500	>2500
24	13.5	625.0	625.0	14.5	625.0	625.0	0.0	>2500	>2500
28	11.0	625.0	625.0	13.5	625.0	625.0	0.0	>2500	>2500
32-40	0.0	>2500	>2500	0.0	>2500	>2500	0.0	>2500	>2500
Amp ²	18.0			18.0			10.0		

Amp: Ampicilline; ID: inhibition diameter; MIC: minimal inhibitory concentration; MBC: minimal bactericidal concentration.

¹Tested in de 150 µg/disque concentration.

²Tested in de 10 µg/disque concentration.

TABLE 5: Comparative of maximal phenolic production (g/100 g DW) in *Crataegus* sp. callus culture within literature.

References	Kartnig et al., 1993 [27]	Bahorun et al., 1994 [3]	Rakotoarison et al., 1997 [28]	Bahorun et al., 2003 [29]	Maharik et al., 2009 [31]
<i>Crataegus</i> species	<i>C. monogyna</i>	<i>C. monogyna</i>	<i>C. monogyna</i>	<i>C. monogyna</i>	<i>C. sinaica</i>
Five explants used	Shoot tips	Bud flower	Bud flower	Bud flower	Stem
Mineral medium used	MS	Gamborg B5	Gamborg B5	Gamborg B5	MS
Hormones used (mgL ⁻¹ /mgL ⁻¹)	NAA/kinetin (6/1)	2.4D/kinetin (2/0.5)	2.4D/kinetin (2/0.5)	2.4D/kinetin (2/0.5)	BAP/NAA (2/1)
Callus old	Two months	Two-year-old	Five-year-old	Ten-year-old	6 weeks
Period of maximum production	—	24–36	24–28	25–35	40–42
Total phenols	Not determined	5.890 (day 28)	2.330 (day 28)	4.740 (day 35)	Not determined
Total flavonoids	Not determined	Not determined	1.300 (day 28)	0.701 (day 35)	Not determined
Hyperoside	0.0125	0.140 (day 36)	0.528 (day 24)	Traces	Not determined
Isoquercitrin	Not detectable	0.020 (day 36)	Not determined	Absent	Not determined
Quercetin	Not detectable	Absent	Not determined	Present	Not determined
Chlorogenic acid	Not detectable	0.770 (day 24)	0.235 (day 24)	0.111 (day 25)	Not determined
Anthocyanins	Not detectable	0.288 (day 28)	Not determined	0.619 (day 35)	0.016 (mg/100g FW)
Total proanthocyanidins	0.017	2.960 (day 32)	0.920 (day 28)	0.2018 (day 35)	Not determined
Procyanidin dimer B2	Not determined	0.320 (day 24)	0.514 (day 24)	0.339 (day 25)	Not determined
(–)-Epicatechin	Not determined	0.840 (day 28)	0.675 (day 28)	0.177 (day 25)	Not determined
Vitexin-2''-o-rhamnoside	0.00456	Not determined	Not determined	Not determined	Not determined
Rutin	0.002206	Not determined	Not determined	Not determined	Not determined

Comparing our results to previous studies [3, 27–29] on *Crataegus monogyna* and *Crataegus sinaica* callus cultures [31], we can note that polyphenolic pattern differs both qualitatively and quantitatively. In fact, the polyphenolic composition, the optimum production of phenolic classes and individual constituents within their respective subculture periods, is different and shows fluctuations. These differences can be explained by difference in the *Crataegus* species, *Crataegus* organs explants, culture medium, growth regulators used, laboratory culture condition, and the age of callus culture.

Despite the relative difference in contents of phenolic compounds, we constate that our calli produces appreciable amounts of phenolic compounds in comparison to literature data [3, 27–29, 31]. Our calli cultures from *Crataegus azarolus* var. *aronia* ovaries remain one of the highest polyphenol producing cell lines on an important time scale (52 weeks).

Concerning antioxidant activity, we have observed rapid and strong inhibition of both DPPH and ABTS radicals after the addition of methanolic extracts from *Crataegus azarolus* var. *aronia* ovaries callus showing a high antioxidant activity of those extracts. This is in accordance with [52] studies, where authors demonstrate that hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), and superoxide anion (O₂^{•-}) scavenging potency remained low during the early growth period. After day 8 the IC₅₀ values decreased to reach minimal values in the three systems at day 28 with a respective IC₅₀ in H₂O₂, HOCl, and O₂^{•-} of 17.59, 44.52, and 77.22 mg DW/L.

Rakotarison et al., 1997 [28], demonstrate too that the lowest IC₅₀ was found at the period of the maximal total phenolic production with a respective IC₅₀ in HOCl and H₂O₂ of 48.22 and 13.26 mg DW/L at 28 days of culture. Reference [29] suggests that high TEAC (3.66 μmol/g DW) and FRAP (208.19 μmol Fe²⁺/g DW) values were observed when maximal growth was reached (days 30–35). The TEAC values were strongly associated with total flavonoids and to a lesser extent with total phenols, anthocyanins, and total proanthocyanidins.

Antioxidant activity is correlated with phenolic composition and this seemed to be influenced by phenolic composition and it is in accordance with previous studies [18, 53, 54], who demonstrated a significant correlation between phenolic composition and antioxidant activity. Reference [53] demonstrates also that coefficient correlation is respectively equal to 0.79 and 0.78 between antioxidant activity and total phenolic and flavonoids composition of Algerian plant extracts. Reference [54] demonstrates a correlation between procyanidins and antioxidant activity while [55] demonstrates that hyperoside and quercetin were antioxidant molecules.

For the antimicrobial activity, antibacterial activity against Gram positive bacteria was observed only when maximal growth was reached (days 24–28). There is no report for the antimicrobial activity of *Crataegus* sp. callus culture, but other studies demonstrate the antimicrobial activity of *Crataegus* plant organs extract. Reference [56] demonstrates that ethanolic extract of *C. cuneata* has an MIC equal to

50 mg/mL against *E. coli*. Reference [57] demonstrates the antibacterial activity of ethyl acetate extract of *C. tanacetifolia* (Poir.) Pers. against 28 bacteria. Reference [24] demonstrates that ethanolic extracts of *C. monogyna*, *C. pseudoheterophylla*, and *C. azarolus* present an MIC between 128 and 8 µg/mL against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other bacteria. In our study this activity seemed to be influenced by phenolic composition and this is in accordance with previous studies that demonstrate a significant correlation between phenolic composition and antimicrobial activity. In fact [22] demonstrates that quercetin is an antibacterial molecule that can inhibit bacteria lipase production [58] and inhibit d-alanine ligase activity which occurs in peptidoglycans production [59]. Other phenolic compounds are antibacterial such as rutin [60], (-)-epicatechin [61], and procyanidin B2 [62]. Some research indicate that antifungal activity is correlated with phenolic compound such as rutin [63], with ursolic acid [64].

5. Conclusion

In conclusion, *Crataegus azarolus* var. *aronia* ovaries callus culture can be used as a source of natural antioxidant and antimicrobial polyphenolic compounds and it is a new plant source, independent of the season and climate conditions. Further assays should be conducted with a focus on the use of different precursors (shikimic acid, gallic acid, and phenylalanine) to optimize the polyphenolic production by this calli culture and the realization of suspension culture tissue.

Conflict of Interests

The authors declare that there is no conflict of interests.

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