Phytochemical composition and bioactivities of *Crataegus aronia* as antioxidant, antibacterial and antioxidative stress in red blood cells – Is it a window of hope for children with glucose-6-phosphate dehydrogenase deficiency

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Keywords: *Crataegus aronia*; Antioxidative stress; Antibacterial; Red blood cells; Secondary metabolites

**ABSTRACT**

**Background:** *Crataegus aronia* (*C. aronia*) extracts have been used medicinally since ancient times and are often utilized in traditional Arab medicine. An extensive study has revealed that *Crataegus* species have antioxidant, antibacterial, anti-inflammatory, and hypotensive properties.

**Objectives:** This work was performed to explore the phytochemical contents of *C. aronia* extract, as well as its antioxidant and antibacterial properties, and to assess the lipid peroxidation level as an oxidative stress biomarker in erythrocytes.

**Methods:** Chemical constituents in the methanolic extract of *C. aronia* were identified by gas chromatography-mass spectrometry and their relative concentrations were determined. The antioxidant activity of *C. aronia* extract was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The effect of *C. aronia* on the concentration of malondialdehyde (MDA) in the erythrocyte hemolysates was studied. Also, the crude extract was assessed for its antimicrobial activity through agar diffusion and microbroth dilution assays.

**Key findings:** The DPPH IC\textsubscript{50} value of the extract showed that the antioxidants activity was equal to (14.3 μg/mL) and according to FRAP assay, the antioxidant activity was in the range of 33.9 μmol–82.86 μmol Fe\textsuperscript{2+}/g dw. The extract exerts a protective effect against oxidative stress in RBCs and shows a 50% inhibition of malondialdehyde (MDA) at 39.48 μg/mL extract. Minimum inhibitory concentrations were found in the range of 800–1000 μg/mL of leave extracts. The phytochemical analysis showed that the total phenols, flavonoids, and flavonols content were 494.071 mg GAE/g extract, 155.251 mg RE/g extract, and 103.2049 mg RE/g extract). *C. aronia* extract contains alkaloids, flavonoids, terpenoids, and steroids. Crude extract of *C. aronia* was more potent in inhibiting the growth of *B. subtilis*, *S. aureus* and *M. luteus* with MIC and MBC values of 800, 800 and 1000 μg/mL, respectively. According to GC-MS, 20 compounds were identified: dihydro-3-methylene-5-methyl-2-furanone (14.71%), hexanoic acid (6.57%), ethyl 3,5-ditert-butyl-4-hydroxybenzoate (6.4%), N, N-dimethylheptadecan-1-amine (4.91%), methyl 2-oxobutanoate (4.14%), glyceraldehyde (3.98%), and 2-methoxy-1-(2-nitroethenyl)-3-phenylmethoxybenzene (3.16%), were the major constituents.

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1. Introduction

Oxidative stress is simply elucidated by the imbalance between processes that produce reactive oxygen radicals and the cellular response that is exemplified by antioxidant cascades. Thus, Oxidative stress is typically enhanced by reactive oxygen species (ROS). Saturation of the antioxidant cascades leads to the assemblage of these ROS and consequent impairment of cellular biomolecules including nucleotides, lipids, and proteins, actuate loss of cell function and eventual death. ROS are implicated in numerous human diseases, such as inflammation, metabolic disorders, aging, atherosclerosis, carcinogenesis, and diabetes [1, 2, 3, 4]. Nowadays, plants’ bioactive ingredients have obtained indispensable concern, endured their medicinal importance in either being employed as prophylactic or curative supplements, whether utilizing the entire plant, extracts, or even the isolated active constituents [5].

Plants are rich in secondary metabolites including phenolic compounds that participate in the defensive role of the intracellular anti-oxidative cascades [6]. Thus, the inspection of nontoxic natural ingredients with efficient anti-oxidative potential had been recently emphasized [7, 8]. Being exceedingly disseminated in the temperate areas of the world North, there are more or less 280 species of the Crataegus genus that is commonly named hawthorn [9, 10]. Currently, hawthorn extract is alternatively used in folklore medicine for the treatment of some cardiovascular disorders like tachycardia and hypertension as well as for curing central nervous, reproductive, and immune systems [8, 11]. It also has anti-carcinogenic properties [7], diabetes and impotence, it is typically believed to be well-tolerated and safe [11, 12].

Its strong antioxidant potential is the most significant characteristic when compared with the other natural antioxidants [13, 14]. In Jordan, one of the hawthorn species is Crataegus aronia (C. aronia) which is distributed in many places such as Wadi Sir, Wadi Shosib, and Al Tafilhe. C. aronia has a prominent, long, straight, and sharp thorn, ranging from 1 to 5 in. Despite the significant medicinal efficacy of the plant, data about the species of C. aronia are still deficient, and the phytochemical profile, as well as the pharmacological activity of C. aronia leaves, have not been investigated thoroughly. Most local shops in Amman deal with other types of Hawthorn, like C. azorolu. Crataea is as much important for utilization in traditional medicine, and some parts of the plant including leaves and flower buds are being extensively utilized as remedies for some conditions like migraines, memory loss, confusion, and irritability. The plant’s leaves and flowers as well as fruits were found to have a hypotensive potential and vasodilatory action on coronary arteries. In addition, its fruit extracts are nowadays used as a dietary supplement in some developed countries [7, 8, 15].

Furthermore, some extracted active ingredients displayed antibacterial activity and this could be significantly employed as an alternative medication for some antibiotic-resistant bacterial diseases [15, 16]. In Jordan, as likely as, in other countries, herbs are commonly used in traditional medicine for the treatment of some conditions. Scientific awareness of antioxidants is significant in understanding the background of many diseases are interceded by reactive oxygen species (ROS) [17]. Many diseases including neurodegenerative like peptic ulcers, viral infection, autoimmune diseases, heart diseases, cancer, brain dysfunction, multiple sclerosis, Down syndrome, Parkinson’s disease as well as Alzheimer’s disease are influenced by ROS [18]. Several reports have described oxidative stress as being an imbalance between a promoted reactive oxygen/nitrogen species and the particular insufficient protective capability of antioxidants. Initiating from that standpoint, free radical-induced damage caused simply by oxidative stress appears to become a probable implicating factor to the particular pathogenesis of several new borne diseases, like leukomalacia, bronchopulmonary dysplasia, patent ductus arteriosus, respiratory distress syndrome, peri-ventricular necrotizing enterocolitis, retinopathy of prematurity and hemolytic anaemia due to glucose-6-phosphate dehydrogenase and favism [19, 20] as well as apoptosis induction [21].

The present study was performed to explore and assess the antioxidant and antibacterial features of C. aronia used in traditional medicine and to test their anti-oxidative stress as well as to show the possible beneficial medical applications. This study, and according to our best knowledge, was not performed before on C. aronia from Jordan.

2. Materials and methods

2.1. Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) (CAS No.: 1898-66-4), Gallic acid (CAS No: 149-91-7), FeCl₃ (CAS No: 7705-08-0), and Folin – Ciocalteu’s phenol reagent (MDL No.: MFC00132625 were obtained from Sigma-Aldrich (Germany). l-Ascorbic acid (CAS No: 50-81-7) was purchased from Janssen Chimica (Belgium). Other chemicals and solvents were used of analytical grade.

2.2. Plant material

C. aronia leaves have been collected in March/April 2017 from Wadi Shueib, Jordan (https://maps.app.goo.gl/vqEJSVP22Y1T1R3g7). Leaves of C. aronia were separated, dried under shadow at room temperature, and crushed to obtain a fine powder. C. aronia was identified by Ferial Khreesat (Assistant Professor- Department of Biology/Faculty of Sciences-Mutah University, Jordan). Voucher specimens were kept at the Laboratory of biological science, Department of Biology/Mutah University.

2.3. Extraction of plant material

The dried leaves powder of C. aronia (30 g) were soaked for 3 days with 300 mL of 99% methanol at room temperature and then filtered using Whatman paper (No. 4). The filtrates were concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator (Buchi R-215, Switzerland). The percentage yield of the extract was determined using the %yield = Weight of dry extract/weight of ground plant material × 100 and expressed as (g extract/100 g dry leaves) [22].

2.4. Preliminary phytochemical screening

2.4.1. Saponins test

Saponins test was done as described by Iqbal et al. [23]. 20 mg of each plant’s dried leaves were dissolved in 20 mL of distilled water and boiled in a water bath for 5 min. The solution was filtered through Whatman filter paper no. 4, then 10 mL of the filtrate were mixed with 5 mL of distilled water and shook vigorously until the formation of froth. Three drops of olive oil were mixed with the froth and stirred vigorously. Then,
the foaming formation which persisted was taken as evidence of the presence of saponins.

2.4.2. Terpenoids test

Terpenoids test was described as described by Iqbal et al. [23]. 5 mL of the crude extract (1 mg/mL) were separately mixed and shaken with chloroform (2 mL) followed by the addition of concentrated H2SO4 (3 mL) along the side of the test tube, a reddish-brown coloration of the interface indicates the presence of terpenoid.

2.5. Anthraquinones test

Anthraquinones were evaluated according to Auwal et al. [24]. 200 mg of each dried leaves were mixed with 6 mL of 1% HCl, the mixture was boiled and filtered. The filtrate was mixed with 5 mL of benzene, filtered and 2 mL of 10% ammonia solution was added to the filtrate. The mixture was shaken and the presence of a pink, violet or red color in the ammoniacal phase indicated the presence of free hydroxyl anthraquinones.

2.6. Cardiac glycosides test

Cardiac glycosides were evaluated according to Auwal et al. [24]. 5 mL of each extract (10 mg/mL in methanol) were mixed with 2 mL of glacial acetic acid and one drop of FeCl3 solution. One mL of concentrated H2SO4 was added to the obtained mixture. The presence of a brown ring at the interface gives a positive indication of cardiac glycoside.

2.6.1. Test for coumarins

Coumarins were evaluated according to Roghini and Vijayalakshmi [25]. 300 mg of dried leaves were transferred to a small test tube and covered with filter paper moistened with 1 N NaOH. The test tube was boiled in a water bath for a few minutes. After removing the filter paper, it was examined under UV light. Intense yellow fluorescence indicates the presence of coumarins.

2.6.2. Test for tannins

Tannins were assayed as shown by Auwal et al. [24]. 50 mg of each extract were boiled in 20 mL of distilled water and filtered. A few drops of 0.1% FeCl3 were added to the filtrate and observed for color change; brownish-green or blue-black coloration was taken as evidence for the presence of tannins.

2.6.3. Test for phlobatannins

Phlobatannins were evaluated according to Auwal et al. [24]. 80 mg of each extract were boiled in 1% aqueous hydrochloric acid; the formation of a red precipitate, thus indicated evidence for the presence of phlobatannins.

2.7. Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteau’s method as described by Singleton et al. [26]. 0.2 mL of the plant extract (0.5 mg/mL) was mixed with 1 mL of 10% Folin-Ciocalteau solution and 0.8 mL of 7.5% Na2CO3. The mixture was incubated for 1 h at room temperature in the dark. The absorbance was measured at 760 nm by using UV-Spectrophotometer (U-5100, Hitachi, Japan). All assays were carried out in triplicate. Gallic acid was used as standard; results were expressed as gallic acid equivalent per gram of extract (mg GAE/g).

2.7.1. Total flavonoid content

Total flavonoid content was measured using the modified colorimetric method of Sharma and Agarwal [27]. A total of 0.5 mL of test extract (10 mg/mL), 2 mL of distilled water, and 0.15 mL of 5% NaNO2 solution were added. After 6 min, 0.15 mL of 10% AlCl3 solution was added and the mixture was maintained in the dark for another 6 min. 2 mL of 4% NaOH solution and 0.2 mL of water were added to this reaction mixture to generate a final volume of 5 mL. The reaction mixture was thoroughly mixed and allowed to stand for 15 min before measuring absorbance at 510 nm. The total flavonoid concentration was given in mg Rutin equivalent (RE)/g extract. All measurements were made in triplicate.

2.8. Total flavonol component

The total flavonol content was estimated using the AlCl3 colorimetric method as described by Kalita et al. [28] with some modifications. 2 mL of plant extract (10 mg/mL) or rutin (standard) were mixed with 2 mL of (20 mg/L) AlCl3 and 6 mL of (50 mg/L) sodium acetate. The absorbance of the standard and sample was taken at 440 nm. The total Flavonol concentration was given in mg Rutin equivalent (RE)/g extract. All measurements were made in triplicate.

2.8.1. HPLC chromatographic conditions

The HPLC analysis of the crude extract was run on ODS column (4.6ID × 150 mm, 5 μm). The mobile phase is a mixture of phosphoric acid in water (0.1%) (solvent A) and acetonitrile (solvent B) running in a linear gradient mode. 100% (solvent A) descended to 70% (solvent A) in 40 min. Then to 40% (solvent A) in 20 min and finally to 10% (solvent A) in 2 min and stayed there for 6 min and then back to the initial conditions in 2minutes. The HPLC system was equilibrated for 5minutes with the initial acidic water mobile phase (solvent A) before injecting the next sample. All the samples were filtered with a 0.45 μm filter. The PDA wavelengths range was from 210 to 500 nm. The flow rate was 1 mL/min. Injection volume was 20 μL and the column temperature was set at 25 °C. All compounds were detected via doing a comparison of their relative retention time with the standards of mixture chromatogram.

2.9. GC-MS system and chromatographic conditions

The GC was Agilent technology type 6890 GC equipped with a Split-splitless injector and HP-5MS capillary column coated with a film of 5% phenylenedimethylpolysiloxane (30 m × 0.25 mm, 0.25 μm film thickness). This Agilent 6890 GC is equipped with a mass spectrometer type 5973C Inert MSD (Mass Spec, Mass Spectrometer, Mass Selective Detector, MS, GC-MS). The column oven temperature was programmed as follows: start temperature at 60 °C, increased to 300 °C with a ramp of 15 °C/min, the temperature was held at 300 °C for 7 min until elution was complete. After 15 s the split valves were opened for 3 min to purge the injector. All injections (1 μL) were made with a 10 μL syringe. Helium gas (purity of 99.999%) was used as the carrier gas at a flow rate of 1.0 mL/min [29, 30].

2.10. Antioxidant activity

2.10.1. Radical scavenging activity by the DPPH assay

The antioxidant potential of C. aronia for the DPPH radical scavenging has been estimated by the Khattak, Simpson, and Ihasnullah method (2008) [31]. DPPH solution (950 μL, 0.2 mM) was mixed with aliquots of 50 μL (0.1-4.0 mg/mL) of C. aronia extract, to obtain final concentrations ranged from 5 to 30 μg/mL. Then the mixture was incubated at 25 °C for 30 min in a dark area. The positive standard (ascorbic acid) was ranged from 0.3125 to 10 μM and tested by the same procedure. The absorbance was tested at 517 nm. The scavenging ability of DPPH radicals was identified according to scavenging activity (%)= (Abs (Control) – Abs (Sample)/Abs (Control)) × 100.

Where Abs control is the absorbance of the control reaction (it is containing all reagents except the plant extract or ascorbic acid), and Abs (sample) is the absorbance of the tested plant extract. Extract concentrations giving 50% inhibition (IC50) are calculated based on the plot of
inhibition (%) against extract concentration and compared with the IC50 of vitamin C value as a positive control.

2.11. Ferric reducing antioxidant power (FRAP)

The antioxidant function of the extracts was measured by the Ferric Reducing Antioxidant Potential (FRAP) assay as determined by Benzie and Strain [32]. Dilute extracts from C. aronia (100 μL) and 900 μL of freshly formulated FRAP solution to achieve final extract concentrations of 5, 10, 15, 20, 25 and 30 μg/mL (involving 25 mL of 300 mM acetate buffer of pH 3.6 plus 2.5 mL of 10 mM tripotassiumdicitraionate as a stock solution in 40 mM HCl with 2.5 mL of 20 mM FeCl3⋅6H2O) have been combined. Absorption was registered at 593 nm of the blank containing 100 μL of representing solvent after 30 min of incubation at 37 °C. The FRAP value was determined according to the calibration curve of FeSO4⋅7H2O as standard solutions, serving a concentration of 100–1000 μmol/L and defined as mmol Fe2+/g dry weight plant.

2.12. Reducing power activity

The Fe3+ reducing ability of the extracts was identified according to Oyaizu [33] with some modifications. C. aronia leave extract (0.1 mL) of (200–1000 μg/mL) was mixed with 0.25 mL of phosphate buffer (0.2 M, pH 6.6) and 0.25 mL of K3Fe(CN)6 (1% w/v). After incubating the mixture at 50 °C in a water bath for 20 min, the reaction was stopped by adding 0.25 mL of trichloroacetic acid solution (10% w/v). Then, the mixture was centrifuged at 5,000 rpm for 10 min. Subsequently, 0.25 mL of the supernatant was mixed with 0.25 mL of distilled water and 0.5 mL of ferric chloride (FeCl3) solution (0.1% w/v) for 10 min. The absorbance was immediately determined at 700 nm to measure the reducing potential. Ascorbic acid (vitamin C) had been used as a reference standard.

2.12.1. Blood sample collection and washing

Blood samples were collected from healthy donors, at the “Blood bank of Medical City Hospital” who did not receive any medication during the 4 weeks before the blood collection. Venous blood was drawn in tubes containing 15% (EDTA) and have been centrifuged at 4 °C, 5000 rpm for 10 min to separate the red blood cells and to remove the plasma and the leukocytes. The red blood cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) and resuspended at 2% in PBS [34].

2.13. Membrane lipid peroxidation determination

The malondialdehyde (MDA) levels, an indicator of the oxidative damage of cell membranes in erythrocytes were determined, using a thiobarbituric acid reagent-based assay (TBARS) according to Srour et al. [29]. The washed erythrocytes were subjected to 10% hematocrit and the C. aronia extract was suspended to 1% (EDTA) and centrifuged at 4 °C for 24 h. The total phenol, flavonoids and phenolic contents are shown in Figure 1. The bacterial strains used in this study included: two Gram-negative strains; Escherichia coli (E. coli) (ATCC 25922) and Enterobacter aerogenes (E. aerogenes) (ATCC 13048) and three Gram-positive strains; Staphylococcus aureus (S. aureus) (ATCC 43300), Bacillus subtilis (B. subtilis) (ATCC 6633) and Micrococcus luteus (M. luteus) (ATCC 10240).

2.14. Bacterial strains

The bacterial strains used in this study included: two Gram-negative strains; Escherichia coli (E. coli) (ATCC 25922) and Enterobacter aerogenes (E. aerogenes) (ATCC 13048) and three Gram-positive strains; Staphylococcus aureus (S. aureus) (ATCC 43300), Bacillus subtilis (B. subtilis) (ATCC 6633) and Micrococcus luteus (M. luteus) (ATCC 10240).

2.15. Disc diffusion method

The antibacterial activity of the crude extract (15 mg/mL) was determined by agar diffusion test dilution assay. Bacterial suspensions (100 μL, 106 CFU/mL, with CFU/mL of bacterial cells calculated by absorption at 600 nm) have spread to agar already in Petri dishes. First, impregnated sterile paper discs (6 mm in diameter, 1 mm in thickness) with 10 μL of each extract (12.5–100 μg/mL); 125–100 μg/disc is individually deposited in Petri dishes and then incubated for 24 h at 37 °C. The antimicrobial ability was measured by calculating the inhibition region surrounding the disc (mm).

2.16. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration MBC

The antibacterial activity of the crude extract was determined by the MIC and MBC assays and were determined by micro-broth dilution assay as described in Ali-Shtayeh et al. [30]. The suspension of the microorganism was prepared to achieve a final cell density of approximately 106 CFU/mL. Serial dilutions of each extract of 20–100 μg/mL were prepared using MH broth. Subsequently, 100 μL of the diluted samples are spread into the tubes. A total of 9900 μL of the various bacterial suspensions (106 CFU/mL) were added to have 200–1000 μg/mL. Tubes were then incubated at 37 °C for 24 h. The MIC was known to be the lowest concentration of drugs or substances capable of inhibiting any visible microbial development. For MBC 50 μL of each tube was spread in a new sterile Petri dish, incubated for 24 h at 37 °C, and the growth was tested.

2.17. Statistical analysis

All results are reported as means ± standard deviation (SD). All experimental data were subjected to one-way analysis of variance (ANOVA) with Tukey’s post hoc test using the Statistical Package for the Social Sciences software (SPSS, version 16). All means were considered statistically significantly different at p<0.05.

3. Results

According to the results, the yield of methanol extract of C. aronia leaves is 13.5% and revealed the qualitative contents of phytochemical compounds (Table 1).

The methanol solvent as an effective extracting solvent was used for leaves extraction for the flavonoid and the phenolic contents are shown in Figure 1.

The total phenol, flavonoids, and flavonols contents of C. aronia leaves extract are presented in Figure 1.

In this study, C. aronia leaves show a higher concentration of phenol concentration with 494.0 mg GAE/g extract, and the total flavonoids and flavonol concentrations were 155.3 mg RE and 103.2 mg RE/g extract, respectively. Also, a significant difference between total phenol, flavonoids and flavonol concentration was found (P < 0.05).
3.1. Antioxidant activity

3.1.1. Radical scavenging activity by the DPPH assay

The DPPH radical scavenging activity increased in a concentration-dependent manner at 5–25 μg/mL with 21–85% inhibition of DPPH (Figure 1). At 14.3 μg/mL C. aronia leaves were associated with 50% inhibition of the DPPH radical, whereas the ascorbic acid (as a reference) was associated with a 50% inhibition of the DPPH radical, at the 5.6 μg/mL concentration (Figure 2).

3.1.2. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay evaluates the reducing capability of an antioxidant by having it interact with a ferric tripyridyltriazine complex and produce a colored ferrous tripyridyltriazine. As shown in Figure 3, C. aronia extract at 5–30 μg/mL has FRAP values ranging from 33.9 μmol/L to 82.86 μmol/L ferric. The reducing power of the extract increases with the increase in the amount of sample. The reducing power shows good linear relation (R^2 = 0.9283). The FRAP values differ significantly at the highest concentration used, compared to the low sample concentrations (P < 0.05) (Figure 3).

3.1.3. Reducing power assay

The extracts of C. aronia demonstrate smaller power reductions than ascorbic acid. The declining strength suggests an increase in absorbance at 700 nm. Figure 4 presents the declining strength activities of aronia leaves. Fe^{3+} in the presence of an antioxidant (i.e., aronia leaf extracts) was reduced to Fe^{2+} in the reduction power assay; high absorption values suggested a good reduction power. Aronia leaf absorption improved in a concentration-dependent way, suggesting that higher extract concentrations were correlated with higher power reduction activities. The reducing power of the extracts and standard increases with the increase in the amount of sample and standard concentrations. The reducing power shows good linear relation in both standard (R^2 = 0.9991) and sample extract (R^2 = 0.9966).

3.2. Membrane lipid peroxidation determination

As shown in Figures 5 and 6, the MDA concentration in red blood cells was decreased significantly as the concentration of extracts increased. The pre-incubation of red blood cells with C. aronia extracts protected from oxidation effects of H_2O_2 as evaluated by MDA as by-product of phospholipid peroxidation. MDA values were significantly changed (p < 0.05) after treatment of blood samples with C. aronia as compared to positive control (H_2O_2) (Figure 5). Also, the was changed significant after The MDA % of inhibition were increased significantly when blood samples treated with different concentrations of C. aronia (Figure 6).

3.3. HPLC-PDA profiles of the extracts

Figure 7 shows the chromatogram of the crude extract of C. aronia at 340 nm. This wavelength was selected because the major peaks showed maximum absorption at this wavelength. The eluted compounds were detected in the range of 22–29 (Figure 7a and b) and 50–64 min (Figure 7c); indicating compounds with different polarities.

3.4. GC–MS analysis of the extract

The extracted constituents of C. aronia were investigated using GC–MS (Table 2). Each constituent in the methanolic extract was quantified and identified by comparing mass fragmentation patterns with standards such as Wiley 9 library spectral data and NIST. Twenty peaks

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Table 1. Qualitative phytochemical screening of C. aronia leaves extract.

| No. | Test          | C. aronia |
|-----|---------------|-----------|
| 1   | Saponins     | +         |
| 2   | Terpenoids   | +         |
| 3   | Anthraquinons| +         |
| 4   | Cardiac glycosides | +     |
| 5   | Coumarins    | +         |
| 6   | Tannins      | +         |
| 7   | Phlobatannins| +         |

+ present
were displayed with one compound with an area % of 14.7 and 2 peaks with an area % of around 6%, and 8 peaks with an area % from 2 to 6%, and 9 peaks with an area % less than 2%.

3.5. Antimicrobial susceptibility testing

3.5.1. Disk diffusion method

The results of the antimicrobial activity of the *C. aronia* methanolic extract are presented in Table 3. The antibacterial activities of methanolic extract of *C. aronia* against the pathogenic organisms react significantly to extract and to antibiotic.

3.6. Determinations of MIC and MBC

The effect of bacteriostatic and bactericidal activity of *C. aronia* methanolic extract is represented in Table 4.

4. Discussion

Undue formation or inadequate neutralization of cellular reactive oxygen stress could induce an accumulation of harmful free radicals that eventually cause damage to cellular biomolecules like proteins, nucleic acids, and lipids. An assemblage of ROS is a lead player in the initiation and progression of a variety of diseases. In this consensus, mitochondria are considered significant provenances and targets for such ROS. Some extracted natural antioxidants revealed potential efficacy in supporting the endogenous antioxidant protective mechanism to counteract ROS and rectify the optimal oxidant/antioxidant balance by equalizing the reactive radicals [35]. Antioxidants have the potential to prohibit the oxidation of fats, proteins, or other biomolecules by constraining the progression of oxidative cascades [13].

*C. aronia* is a naturally occurring plant in several areas of Jordan. Therefore, in our study, the efficacy of *C. aronia* methanolic leaves extract as an effective herb in the medical field, especially as an antioxidant, has been evaluated. Precursory phytochemical screening accomplished in this study displayed that *C. aronia* leaves methanolic extract encloses saponins, terpenoids, anthraquinons, cardiac glycosides, coumarins...
tannins, and phlobatannins in its methanolic extract. These phytochemical ingredients are recognized to implement diversified specific significance in herbs that, when ingested by animals, may elaborate various pharmacologic and biochemical impacts [36]. These impacts are revealed either by cell toxicity or cell-protective action. Similar phytochemical results were found in other Crataegus species, namely C. monogyna and C. laevigata [36, 37]. Phenols and flavonoids (particularly flavans) constitute two of the substantial compounds groups that primarily act as free radical scavengers. The antioxidant potential of such compounds is attributed to their ability to reduce free radicals by enduing two hydrogen atoms. Owing to their structural properties, they can serve as strong antioxidants and free radicals’ neutralizers [36, 37].

The total yield of the methanol extract was 13.5% (w/w)%. This result was consistent with previous study showing that methanol is an excellent solvent for the extraction of Pistacia phenolic components [38]. Our results showed that methanolic extracts of C. aronia leave contained a high number of phenolic compounds. The polyphenolic contents of C. aronia leaves were found to be 2.8 times greater than those of R. officinalis (results not shown) [39]. C. aronia extract is known to be a rich source of flavonoids including isoquercitrin, quercetin, epicatechin, hyperoside, chlorogenic acid, protocatechuic acid, and rutin [36, 37, 38, 39, 40, 41]. It is shown by Alirezalu et al. [36], that the overall phenol content of Crataegus fruit species was substantially variable (P < 0.001) across species, varying from 21.19 to 69.12 mg GAE/g dry weight. Our results for total phenol, flavonoid, and flavonol contents of the leaves C. aronia are comparable to or higher than total the phenol, flavonoid, and flavonol contents of the other medicinal plants [28].

At the same time, the presence of flavonoids and flavonols in the methanolic extract of C. aronia was confirmed by phytochemical screening. While HPLC confirmed the presence of polyphenolic compounds in C. aronia and polar and nonpolar compounds were detected as HPLC results chromatogram revealed. Flavonoids abundance was indicated by the UV-Vis of these compounds in the range of 210–350nm [42]. Flavonoids can suppress the production of hydroxyl radicals and superoxide ions, which are considered to be potential peroxidation agents that induce cell destruction [43, 44].

GC-MS chromatograms showed that the methanolic extract of C. aronia contains twenty peaks with retention times between 1.82 and

Figure 5. Effect of different concentrations of C. aronia methanolic leaves extract on MDA concentration. Results are means ± standard deviations of three experiments. Different letters indicate the significant differences between the control, positive control, and treated groups, respectively (P < 0.05).

Figure 6. Effects of C. aronia leaves extract on MDA percent of inhibition. Results are means ± standard deviations of three experiments. Different letters indicate the significant differences between the treated groups (P < 0.05).
Figure 7. HPLC-PDA chromatogram of crude extract of *C. aronia* at 340 nm.
Table 2. Results of chemical components of *C. aronia* analyzed by GC-MS.

| Components                          | Retention time (min) | Composition percentage % |
|-------------------------------------|----------------------|--------------------------|
| Propoxymethyl acetate               | 1.82                 | 0.87                     |
| Tetrahydrofuran (THF)               | 1.87                 | 1.45                     |
| 2-methoxy-1-(2-nitroethenyl)-3-phenylmethoxybenzene | 1.99             | 3.16                     |
| Methylisilene                       | 2.28                 | 0.81                     |
| Glycerin                            | 2.33                 | 2.32                     |
| Oxalic acid                         | 2.71                 | 2.92                     |
| Hydrogen azide                      | 2.83                 | 3.46                     |
| Alanine                             | 3.18                 | 1.22                     |
| Glyceraldehyde                      | 3.61                 | 3.98                     |
| N-methoxy-N-methylhydroxylamine     | 3.96                 | 0.732                    |
| Dihydroxyacetonate                  | 4.34                 | 2.52                     |
| Dihydro-3-methylene-5-methyl-2-furanone | 8.00           | 14.71                    |
| 5-hydroxyhex-2-enolic acid          | 10.35                | 1.59                     |
| Methyl 2-oxobutanoate               | 17.57                | 4.14                     |
| 3,5-dietert-butylphenol             | 19.07                | 0.98                     |
| Acetamide                           | 19.57                | 1.39                     |
| N, N-dimethylethylpteadecan-1-amine | 27.65                | 4.91                     |
| Hexanoic acid                       | 27.97                | 6.57                     |
| Ethyl 3,5-dietert-butyl-4-hydroxybenzoate | 28.08              | 6.40                     |
| Octadeca-9,12,15-trienol            | 31.25                | 1.32                     |

Total identified components 20

Table 3. Antibacterial activity of the *C. aronia* methanolic extract against tested bacterial strains.

| Bacterial strain | Zone of inhibition [mm ± SD] (μg/disc) | Streptomycin* (50 μg/disc) |
|------------------|----------------------------------------|---------------------------|
|                  | 125         250      500       1000       |                           |
| **Gram-positive**|                                        |                           |
| B. subtilis      | 10.0 ± 0.9  10.0 ± 1.0  11.0 ± 0.4  13.0 ± 0.6 | 31.0 ± 1.4                |
| S. aureus        | NA          NA          8.0 ± 0.7  9.0 ± 0.3  29.0 ± 1.3  |
| M. luteus        | 7.0 ± 0.5   8.0 ± 0.3  9.0 ± 0.6  10.0 ± 0.4 | 25.0 ± 1.6                |
| **Gram-negative**|                                        |                           |
| E. coli          | NA          NA          NA          25.0 ± 11.8  |
| P. aeruginosa    | NA          NA          NA          22.0 ± 0.85 |

* *Data are expressed as means ± SD (n = 3), *asterisks indicated positive control compared to tested concentrations of crude extract. Concentration was observed when there was no visible growth, NA: not active at tested concentration. Different letters are significantly different at (P < 0.05).*

Table 4. MICs, and MBCs of *C. aronia*.

| Microorganism | MICs (μg/mL) | MBCs (μg/mL) |
|---------------|--------------|--------------|
| **Gram negative** |              |              |
| E. coli (ATCC 25922) | -            | -            |
| E. aerogenes (ATCC 13048) | -            | -            |
| **Gram positive** |              |              |
| B. subtilis (ATCC 6633) | 800 ± 8.75   | 800 ± 10.5   |
| M. luteus (ATCC 10240) | 1000 ± 11.35 | 1000 ± 15.9  |
| S. aureus (ATCC 43300) | 800 ± 7.4    | 800 ± 12.9   |

* MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; Data are expressed as means ± SD (n = 3), NA: not active at the tested concentration. Different letters are significantly different at (P < 0.05).**
the cell phospholipid and protein cytoskeleton is typically enhanced by Oxidative stress in erythrocytes [49, 50, 51]. Therefore, this will cause morphological, microrheologic changes, and premature eryptosis [48, 49, 50, 51, 52, 53, 54, 55, 56]. The human body can reduce oxidative stress by operating either endogenous or exogenous antioxidants. The major functional roles of antioxidants are to constrain the overabundance of ROS, cellular protection against the harmful influence of the reactive oxygen species, and contribute to cell protection and disease prevention [57, 58].

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that has the competency to detoxify free radicals involved in the mechanisms of cell damage [58, 59, 60]. Typically, there are more than 20 various hereditary erythrocytes’ enzymes defects that have been depicted, most are linked to hemolytic anemia. Of these, only glucose-6-phosphate dehydrogenase (G6PD) deficiency happens with a frequency impacting more or less 400 million subjects worldwide. Therefore, G6PD deficiency is the most widespread erythrocyte enzyme defect. The diminution or complete deficiency of this enzyme augments RBC susceptibility to oxidative stress typically caused by exposition to some drugs or ingestion of some food including fava beans (favism) [19, 20, 59].

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Clinical manifestation of the disease is displayed mainly as severe anemia and jaundice and more than 40% of neonates admitted to hospitals with detectable neonatal jaundice were either heterozygous or homo/hemizygous for G6PD c.202, genotype variants of glucose-6-phosphate dehydrogenase deficiency [61]. Although G6PD deficiency may be substantially wavering in its clinical presentation, it should be indispensably kept in mind during the diagnosis of hemolytic anemia at any age [62].

Infection is supposedly the most prevalent cause of the hemolytic crisis [56, 60] in children with G6PD deficiency. Hence, seeking antioxidant active ingredients with anti-bacterial potential may be beneficial to those children with hemolytic crises due to glucose-6-phosphate dehydrogenase deficiency.

The antibacterial activities of the methanol leaves extract of these plants were used against two Gram-negative strains; *E. coli* and *E. aerogenes* and three Gram-positive strains; *S. aureus*, *B. subtilis*, and *M. luteus*, using the disk diffusion methods and broth macro dilution method. The effect was performed at a low concentration of the extract (125, 250, 500, and 1000 μg/mL) for disk diffusion assay; and (200, 400, 600, 800, and 1000 μg/mL) to determine MIC and MBC in broth macro dilution method. Via these concentrations, all plants have an effect against Gram-positive bacteria and no effect on Gram-negative bacteria.

In erythrocytes, which are responsible for O2 and CO2 gases transportation throughout the circulatory system, the most crucial concern is to maintain the functionality of haemoglobin that necessitates holding the haem group in ferrous (Fe2⁺) condition. Transformation of oxidized ferric ion back into ferrous in haemoglobin requires a distinct mechanism engaging the reduced form of glutathione, the enzyme glucose-6-phosphate dehydrogenase, glucose, and NADPH as a reductive power.
[60]. Over the last two decades, it has been documented that many bacterial and viral infections trigger the particular output of reactive oxygen (ROS) and nitrogen (RNS) species. Regardless of the overwhelming evidence associated with the impact of oxidative stress in the associated diseases with acute and chronic infection, the effect of the vast majority of infectious organisms on the host redox systems is not appropriately recognized [54, 55, 56, 57, 58, 59, 60].

Because severe G6PD deficiency could be a phenocopy of chronic granulomatous disease with due respect to the cellular and clinical phenotype, careful assessment of neutrophil function is likely imperative in this regard to governing the convenient preventive anti-infective measures. Estimating the level of G6PD enzyme activity should be accompanied by the analysis of ROS production to take the appropriate decision of treatment. Hence, the results of this study could help ameliorate the condition of glucose-6-phosphate dehydrogenase inadequacy by minimizing both oxidative stress and infection which negatively impact the disease sequelae [60].

5. Conclusion

C. aronia has diverse pharmacological effects, which include antioxidant, antimicrobial, hypotensive, and antithrombotic. It exerts antioxidant effect and antibacterial growth potential as well as protects red blood cells from oxidative stress.

The findings of this study demonstrate the presence of many phytochemicals in the leaf methanolic extract of C. aronia, including saponins, terpenoids, anthraquinone, coumarin, tannins, cardiac glycosides, and flavonotannins. Additionally, the extract is rich in phenols, flavonoids, and flavonols, all of which can bind to free radicals and neutralize them, delaying the aging process of our cells. As a result, the extract of C. aronia leaves exhibits antioxidant activity via inhibiting DPPH in addition to FRAP and reducing power activities. In addition, this research demonstrates that C. aronia protects erythrocytes from oxidative stress by reducing MDA, a consequence of lipid-peroxide formation when cells are exposed to it. The C. aronia have an antibacterial activity with interesting activity against B. subtilis. Dihydro-3-methylene-5-methyl-2-furanone, hexanoic acid, ethyl 3,5-dimethyl-4-hydroxybenzoate, N, N-dimethylthiopentadecan-1-amine, and methyl 2-oxobutanoate were the main substances found. 3,5-dimethylbutenophenol and 2-Methoxy-1-(2-nitroethylheptadecan-1-amine, and methyl 2-oxobutanoate were the main substances found. 3,5-dimethylbutenophenol and 2-Methoxy-1-(2-nitro-phenyl)-3-(phenylmethoxy)benzene were both found in the extract. This study may open a window of hope for children with Glucose-6-phosphate dehydrogenase disorder by possible utilization of the active ingredients of C. aronia to minimize both oxidative stress and infection which negatively impact the disease sequela.

Declarations

Author contribution statement

Ghashab Al-Mazaideh; Ahmed H. Al-Mustafa; Ibrahim Nasir-Allah; Khaled A Taranneh; Fuad Al-Rimawi: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Mohammed Ayed Huneif; Shifaa O. Alshammari; Fadel Wedian: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Khaled A Tarawneh; Fuad Al-Rimawi: Conceived and designed the experiments; Wrote the paper.

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