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Extraction, Identification and Quantitative HPLC Analysis of Flavonoids From Fruit Extracts of *Arbutus unedo* L from Tiaret Area (Western Algeria)

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Abstract

The aim of the current study was to evaluate the total phenolic, flavonoid content and to investigate the antioxidant capacities of the fruit extracts of *Arbutus unedo* L. that grows in Tiaret area (Western Algeria). First we have extracted the fruit by some non-polar solvent (chloroform, ethyl acetate, 1-butanol).

Total phenolic content and total flavonoid content were evaluated according to the Folin-Ciocalteu procedure, and a colorimetric method, respectively. Extracts content was determined by using a high-performance liquid chromatography (HPLC)-UV method. The total phenolic contents of *A.unedo* L. varied between 12.75±0.06 to 34.17±1.36 mg gallic acid equivalent/g of dry weight of extract. The total flavonoid varied from 2.18±0.10 to 6.54±1.14 mg catechin equivalent/g.

The antioxidant potential of all extracts was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, the IC50 of acetate ethyl was the best by 0,009 mg/ml may due to the phenolic compound, in the second was the chloroform extract by IC50=0,015mg/ml, in the third was butanol extract by IC50= 0,022 mg/ml and in the last was water extract by IC50= 0,048mg/ml. the antioxidant activity of all extracts was better than ascorbic acid. The extract obtained under optimum conditions was analyzed by HPLC and five flavonoid compounds were identified; they are catechin, apiginin, silybin, fisetine and naringin.

Keywords: antioxidant activity; *Arbutus unedo* L.; fruit; flavonoids; phenols; HPLC; Western Algeria.

Introduction

The fruits of the strawberry tree (Arbutus unedo L.) are consumed mainly as processed product, but may be a good source of antioxidants if consumed as fresh fruit (Pallauf et al, 2008).

A. unedo, the strawberry tree, belongs to the Ericaceae family, and it is native of the Mediterranean climate (Celikel et al., 2008). It grows wildly in Mediterranean basin from sea level to about 600 m above sea level (Ayaz et al., 2000).

This species have been traditionally used as food, by using the arbutus berries in the production of alcoholic beverages, jams, jellies and marmalades (Alarcao-e-Silva et al., 2001; Pallauf et al., 2008); and as phytopharmaceuticals. The fruits are well known in folk medicine as antiseptic, diuretic, and laxative, while the leaves are used as astringent, diuretic, urinary antiseptic, antidiarrheal, depurative and more recently in the therapy of hypertension, diabetes and in the treatment of inflammatory diseases (Ziyyat et al., 1997; Ziyyat and Boussairi, 1998; Mariotto et al., 2008; Afkir et al., 2008). It is therefore of considerable interest to validate the bioactivities of *A. unedo* fruits in cell/organism-based assays to assess their potential therapeutic effect against a wide range of human diseases.

Polyphenolic compounds, such as phenolic acids and flavonoids, are important constituents in many plants, and their identification and quantification can give vital information relating to antioxidant function, food quality, and potential health benefits (Vesna et al, 2004). Wild fruits have been for a long time part of the human diet. In rural areas, wild fruits are consumed because of economic reasons not only as vitamin sources but also for medicinal purposes. Recent studies have focused on the description of phenolic compounds, such as flavonoids, phenolic acids and anthocyanins, as a part of the antioxidant compositions of these fruits (Pawlowska et al., 2006; Barros., 2008).

This paper is concerned with high performance liquid chromatography (HPLC) qualitative analysis of in *A.unedo* L. extracts obtained by successive extraction with solvents of different polarities (chloroform, ethyl acetate,1-butanol). Total phenolic and flavonoid contents as well as antioxidant activity of different extracts were investigated.

Materiel and methods:

Sample preparation and extraction

Biological material

Fruits of *A.unedo* L. (Ericaceae) were collected, in November 2013, from Guezoul djebel located at the end in North of the wilaya of Tiaret (latitude 35°22'26" N, longitude 1°10'30" E, altitude 1150 m), characterized by a semi-arid climate at fresh variant.

Powder preparation

Fruits randomly collected were oven dried (<40 ° C) until a constant dry weight, then ground to a fine powder using an electric grinder and finally stored at darkness at 25 ° C.

Extraction

100 g of fine powder of the dried fruit were macerated in a hydro-alcoholic mixture (methanol / water 80/20: v/v) for 72 hours, in the dark at room temperature, with renewal of solvent every 24 hours (350 ml x 3). Macerate was filtered through filter paper to obtain a clear extract, and then solvent was evaporated under vacuum at a temperature of 40° C, 200 ml of distilled water was added to the residue, extract was kept for 48 h in a refrigerator, then decanted, and was filtered again through filter paper (Lim et al., 2012).

The liquid-liquid extraction has been then carried out with three organic solvent system of increasing polarity: 200 ml of petroleum ether, 200 ml of chloroform, 200 ml of ethyl acetate and finally 200 ml of 1-butanol **(**Benhammou et al., 2007**)**. The residue of each solvent extract was dissolved in water, frozen and lyophilized. Chloroform, ethyl acetate, 1-butanol and aqueous crude extract powder were used for investigate phytochemical compounds, determination of total phenols, flavonoids content, antioxidant screening and for HPLC.

Determination of total phenolic contents

Total soluble phenolics of each fraction (chloroform, ethyl acetate, 1-butanol, aqueous) were determined with Folin-Ciocalteu reagent according to the method of (Singleton et al, 1999) using gallic acid as a standard phenolic compound. A volume of 40 µL of each fraction and standard was transferred into separate test tubes and to each added 3.16 mL water and 200 µL of Folin-Ciocalteu's reagent. The mixture was mixed well, waited for 8 min and then added 600 µL of sodium carbonate solution with continuous stirring. The solution was allowed to stand for 1 hour at

room temperature in a dark place and the absorption was measured at 750 nm using a spectrophotometer. The concentration of total phenolic compounds of all fractions of *A.unedo* fruit was determined as milligrams of gallic acid equivalent (GAE).

Determination of Flavonoid Content

The total flavonoid content of different fractions was analyzed by using catechin as standard (Kim et al., 2003). To 1 ml of extract solution (each of 100 µg/ml concentration), 4 ml of distilled water, 300 µl of sodium nitrite and aluminium chloride were added. The mixture was incubated at room temperature for 5 min. After the completion of incubation, 2 ml of sodium hydroxide was added and final volume of solution was raised to 10 ml by addition of distilled water. The absorbance of samples was measured at 510 nm. The total flavonoid content for all the fractions was expressed in terms of catechin equivalents (mg/g).

DPPH free radical-scavenging activity assay

The DPPH radical scavenging activity of extract was measured according to the slightly modified method of Liyana-Pathirana and Shahidi (2005). Sample extracts at various concentrations were taken and the volume was adjusted to 100 µl with methanol. 900 µl of 0.1 mM methanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27 °C. The absorbance of the sample was measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: % DPPH radical scavenging activity = (control OD – sample OD/control OD) × 100.

Qualitative HPLC Analysis:

Analysis of extracts and standards were performed by Liquid chromatography was performed using a Shimadzu HPLC apparatus equipped with two LC-6A Vp pumps, an UV- VIS SPD-10A Module, an SLC-6B system controller, a Rheodyne injector 20 µL loop with RP-8 LiChroCart column (125 mm × 4.60 mm, 5 µm particle size). Chromatograms were obtained and analyzed using the software Class-VP ® (Shimadzu, Tokyo, Japan). The mobile phase consisted of a binary mixture of methanol- water (50:50 v/v) adjusted to pH 2.8 with phosphoric acid (Kuntić et al 2007) at isocratic flow rate of 1.0 mL min^{-1}. The absorbance was monitored at λ = 280 and 350 nm.

The need to know and identify flavonoidy individual compounds in the samples requires the replacement of traditional methods of separation techniques. High performance liquid chromatography is probably the most widely used analytical technique for characterizing the polyphenolic compounds (Gomez-Caravaca et al., 2006). This technique is used for the qualitative analysis of different extracts of the fruit of *A.unedo* L. taken from Tiaret area.

Statistical analysis

All tests were performed in triplicate and the results are presented as mean±standard deviation (SD). Differences with P values of <0.05 were considered significant.

Results and discussion

The yields of the extracts obtained per 100 g of dry plant material with the different solvents are given in Table 1.

Extract	Total extract (g/100 g of dry plant material)
Chloroform	0.25 ± 0.06
Aqueous	1.75 ± 0.16
1-butanol	0.34 ± 0.09
Ethyl acetate	0.44 ±0.09

The yield of aqueous extract was higher than yield of ethyl acetate,1-butanol and chloroform extract.

Total phenolic and flavonoid content

Total phenolic content of four crude extracts was determined by Folin-Ciocalteu method (Figure 1). Total phenols of *A.unedo* L. extracts were calculated according to the equation y= 0.001x + 0.0015 (r^2= 0.995) as Gallic Acid Equivalent (GAE, mg/g dry material), while total flavonoid contents were determined in accordance with the equation y= 0.002 x - 0.003 (r^2= 0.993) obtained by calibration curves as Catechin Equivalent (CE, mg/g dry material).

Total phenolic varied in different extracts was reported in Table 1: 29.46, 12.75, 34.17 and 32.84 mg Gallic Acid Equivalent /g of the dry extract in chloroform, aqueous, 1-butanol and ethyl acetate extract, respectively. Among the four crude extracts, 1-butanol extract contained the highest (34.17 mg GAE g of dry extract) amount of phenol compounds.

Figure 1: Gallic acid standard curve.

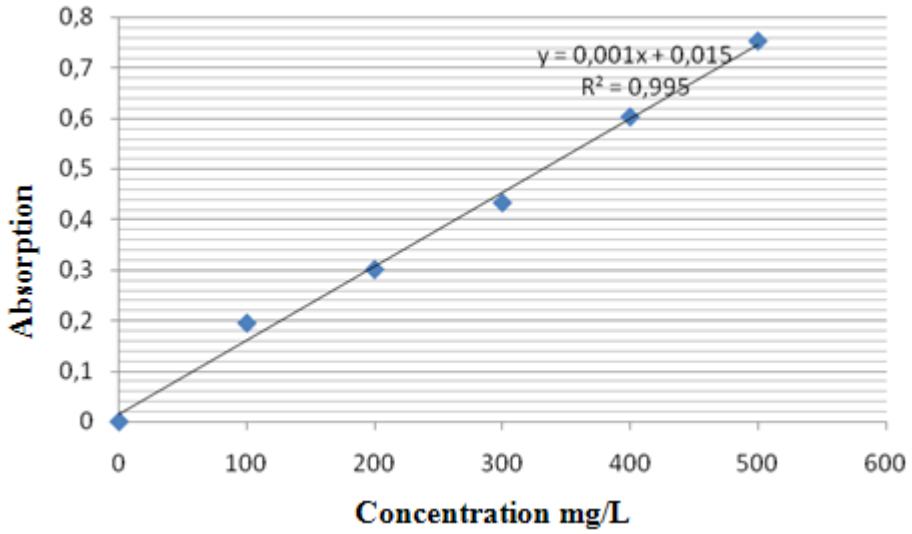


Table 1: Total phenolic content in *A.unedo* L. extracts

Extract	Total phenolic content (GAE mg/g extract)
Chloroform	29.46 ± 0.34
Aqueous	12.75 ± 0.06
1-butanol	34.17 ± 1.36
Ethyl acetate	32.84 ±0.53

In regard to the amount of flavonoids in the different crude extracts varied from 2.18 to 6.54 mg catechin equivalent/g (Tableau 2), chloroform extract contained a higher content of flavonoids (6.54 mg CE/g of the dry material), compared to ethyl acetate (5.59 mg CE/g), 1-butanol (3.61 mg CE/g) and aqueous extracts (2.18 mg CE/g) (figure 2).

Figure 2: Calibration cruve of quercetin standard.

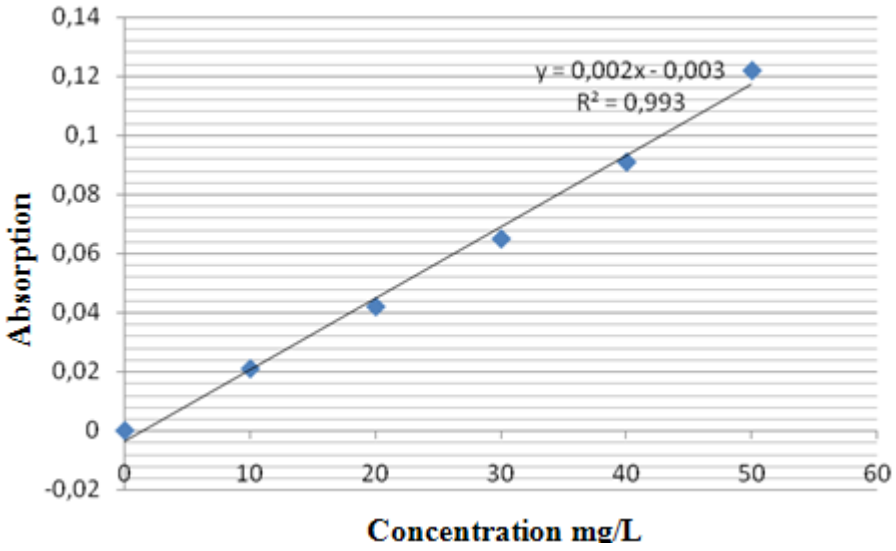
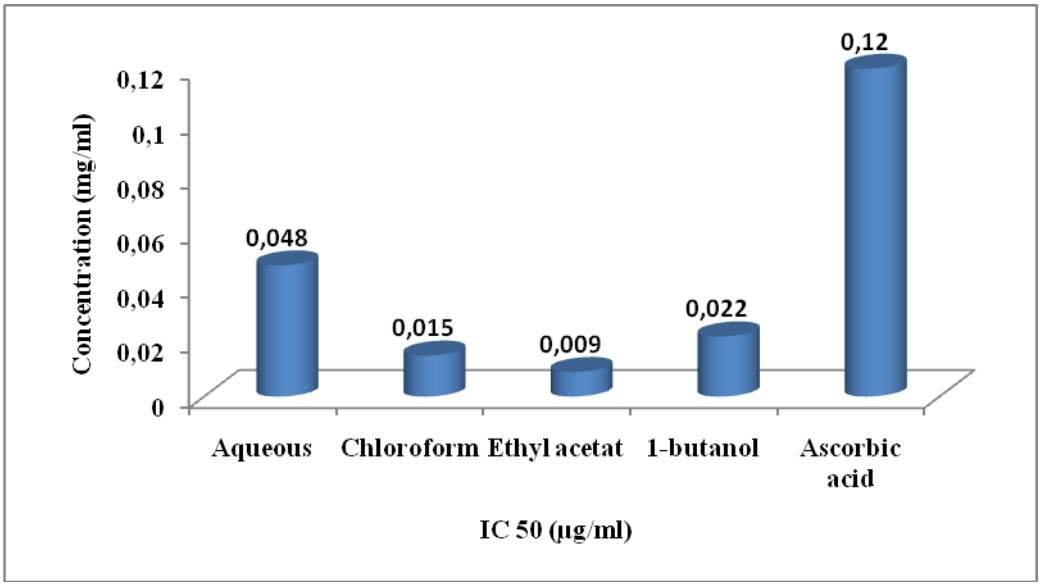


Table 2: Total flavonoid content in *A.unedo* L. extracts

Extract	Total flavonoid content (CE mg/g extract)
Chloroform	6.54 ± 1.14
Aqueous	2.18 ± 0.10
1-butanol	3.61 ± 0.59
Ethyl acetate	5.59 ± 0.82

Figure 2: IC50 (µg/mL) values of the DPPH assay in A.*unedo* L. fruit fractions



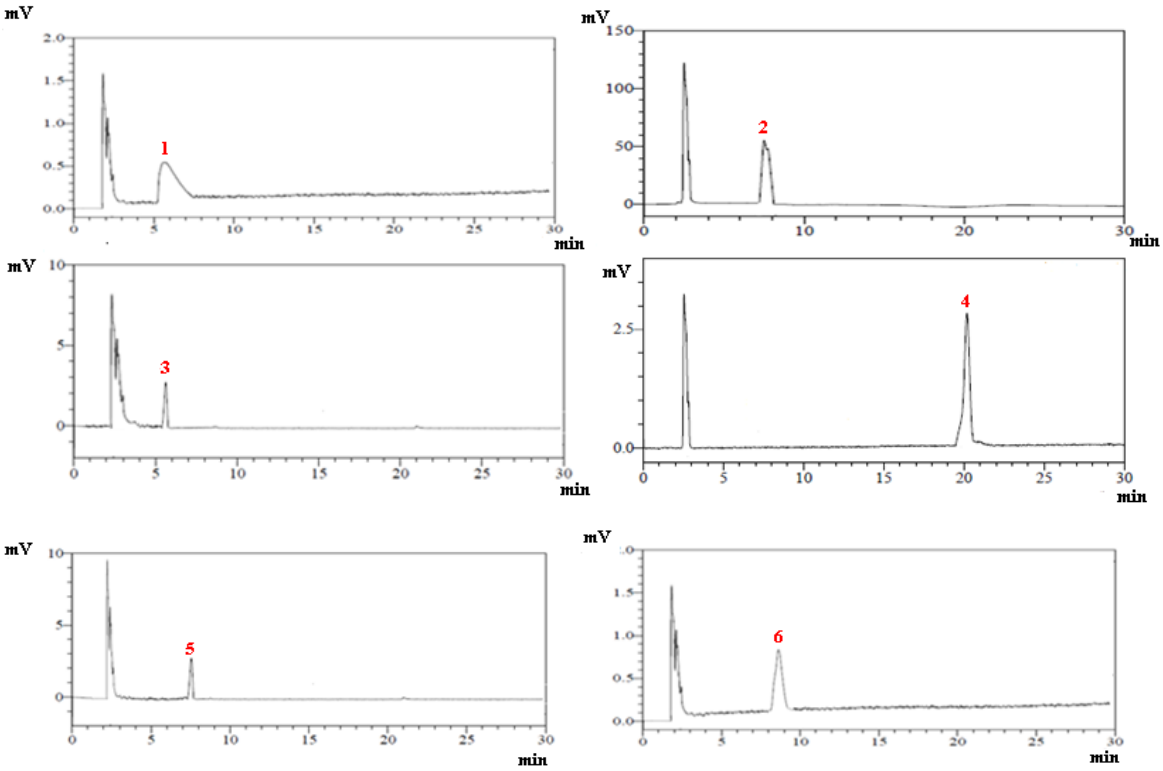
The IC50 value is a widely used parameter to measure the free radical scavenging activity. A lower IC50 indicates a higher antioxidant activity (Maisuthisakul et al., 2007). IC50 values for 1-butanol, aqueous, chloroform and ethyl acetate extract were: 0.022, 0.048, 0.015 and 0.009 mg/ml respectively, compared to the other work carried out by (Bouzid and Toumi, 2014). Several studies provide evidence to the fact that DPPH-scavenging ability is a reliable index of antioxidant

potential. The difference in the antioxidant activity of extracts may be attributed to the difference in the total phenolic and flavonoid content as well as the composition of these bioactive phytochemicals (sing et al., 2007). Reveals that fruits and vegetables have high values of important nutrients and phytochemicals which exhibit antioxidant functions (Hamza et al., 2013).

Identification of Various Phenolic Acids and Flavonoids in *A.unedo* L.

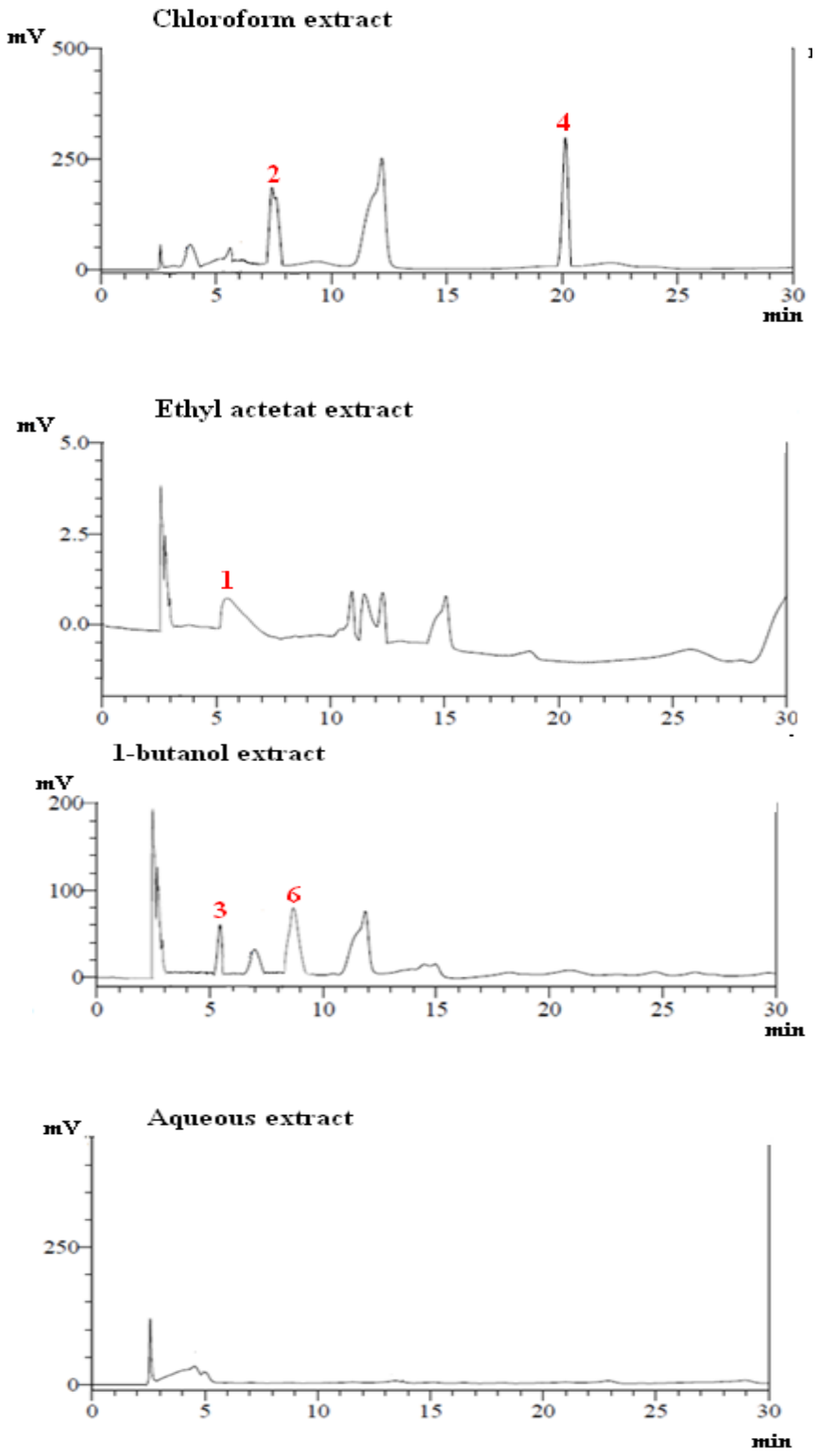
Based on these results, HPLC analysis was employed to define qualitative content of phenolic acids in A.*unedo* L extracts. HPLC Chromatograms of standards and different extracts are represented in Figure 4. (Naringin, Catechin, Apiginin, Fisetin, Taxifolin, Silybin) were identified in the investigated extracts by comparing spectra with those of standards (Figure 3).

Figure 3: HPLC chromatogram of part of phenolic standards at 280 nm



Peaks: **1**, Naringin; **2**, Catechin; **3**, Apiginin; **4**, Fisetin; **5**, Taxifolin; **6**, Silybin.

Figure 4: HPLC profiles of four fruit extract at 280 nm.



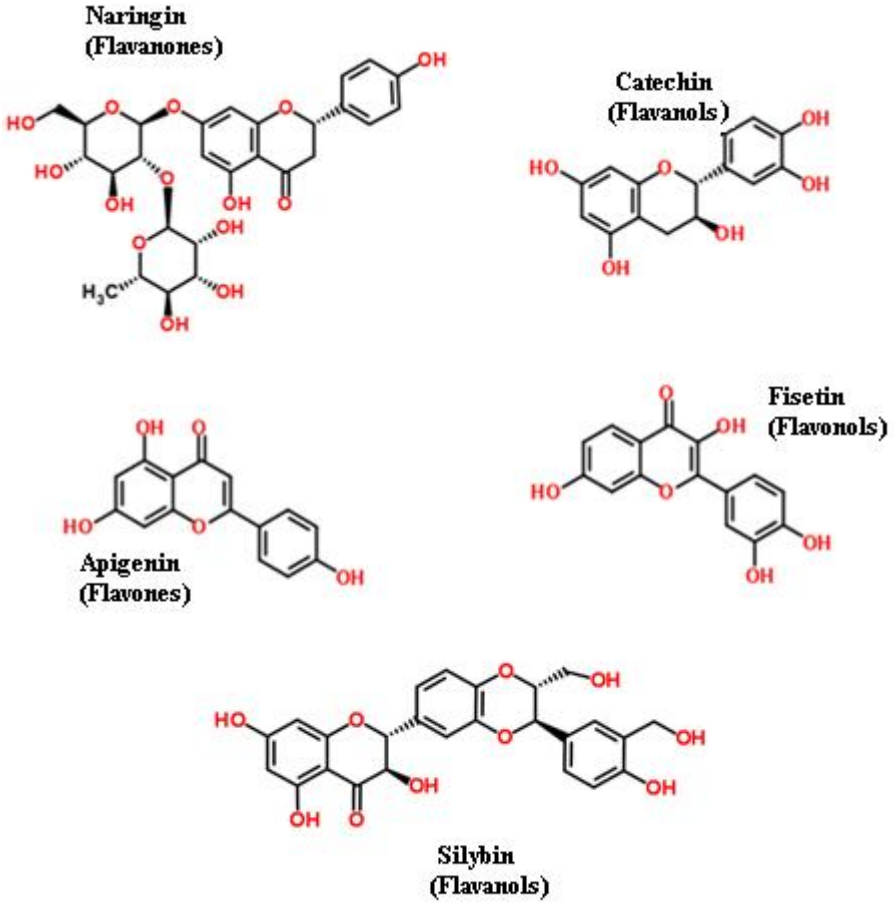
Peaks: **1**, Naringin; **2**, Catechin; **3**, Apiginin; **4**, Fisetin; **6**, Silybin

The results of qualitative HPLC analysis showed that Taxifolin was absent in all the fruit extract. Catechin and fisetin are present in chloroform extract, apeginin and silybin in 1-butanol

extract. These phenolic acids were not detected in aqueous extract, while ethyl acetate extract contained only naringin.

Peaks 1, 2, 3, 4 and 6 were positively identified as Naringin, Catechin, Apiginin, Fisetin and Silybin respectively, by comparing the retention time, absorption and mass spectra with that of standards (Figure 3 and 4). Figure 5 shows the chemical structures of phenolic compounds positively identified by comparison with standards.

Figure 5: Structures of phenolic compounds detected in fruit extracts.



Conclusion

In conclusion, this study indicates that the extracts obtained from the fruit of *A. unedo* L. have significant free radical scavenging activity on stable DPPH• and high reactive hydroxyl radical. The data suggest that aqueous, ethyl acetate and chloroform extracts of *A.unedo* L. from Tiaret area are a potential source of natural antioxidants.

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