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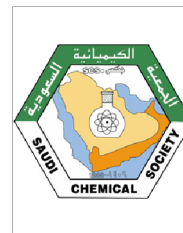

ORIGINAL ARTICLE

Composition and biological activity of the Algerian plant *Rosa canina* L. by HPLC-UV-MS

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KEYWORDS

Ethanolic extract;
HPLC-UV-MS;
Antioxidant activity;
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Bilobalide A

Abstract The present study was carried out in order to identify and characterize the compounds of *Rosa canina* fruits by HPLC-UV-MS. The total phenolic determiner by a new Fast Blue method (FBBB), which detects phenolic directly, reported an average total phenolic concentration of 1.7 folds greater than Folin-Ciocalteu (F-C), which indicates that an indirect detection method of total phenolic should be replaced in future studies by the FBBB method. TPC of the ethanolic extract was positively correlated with 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging effect. The DPPH activity of *R. canina* extract which is higher than the IC_{50} of the ascorbic acid and Butylated Hydroxytoluene (BHT), but lower than the IC_{50} of quercetin and trolox. The determination of intracellular reactive oxygen species (ROS) proved the antioxidant effect of the extract on HepG2 and SH-SY5Y cells. A concentration of 1.63 µg/ml on HepG2 cells had an oxidizing effect instead of the antioxidant effect, which is due to the existence of a tert-butyl group in sesquiterpene identified by HPLC-UV-MS method. These results indicate that the fruits of *R. canina* can be used as a natural source of antioxidants against oxidative stress and some types of cancer.
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1. Introduction

R. canina belongs to the family of Rosaceae and genus Rosa, with about 200 species spread in the temperate zone and subtropics of the Northern hemisphere, *R. canina* pseudo-fruit was traditionally used in preventive therapy and for the preparation of some foods such as jam, beverages and probiotic drinks (Montazeri et al., 2011).

Natural substances and plants in general, are an immense source of chemiodiversity, often with very original structures

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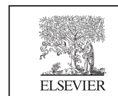

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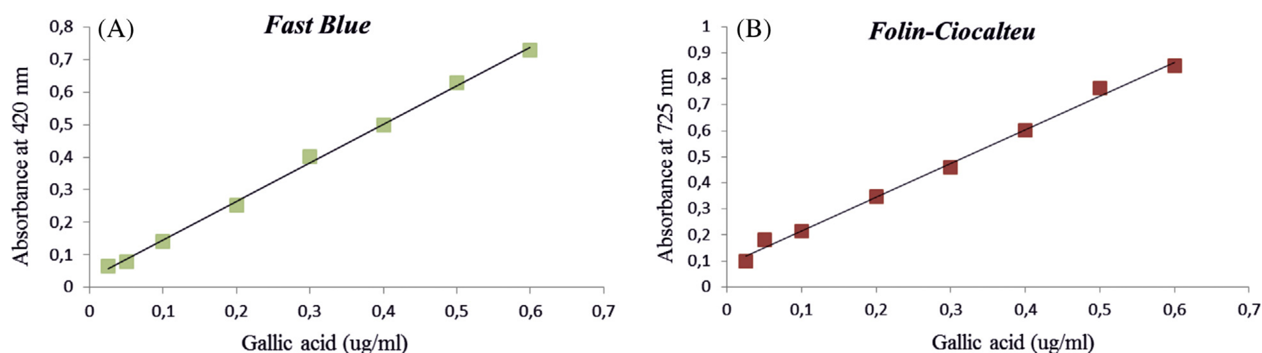

Fig. 1 Calibration curve of total phenolic content (TPC) of ethanolic extract of *Rosa canina* fruits using fast blue method (1A), and Folin-Ciocalteu (1B).

Table 1 Total phenols contents in ethanolic extract *R. canina* fruits.

Content (μg/mg DM) Total phenols	FBBB	F-C	Extraction yield (%)
30% ethanol	598.25 ± 0.49	354.46 ± 0.05	20

Data expressed in μg equivalent of gallic acid (GAE) to 1 mg of methanolic extract of Rosa fruits.

that are usually impossible to synthesize (structural complexity, stereospecificity …). Flavonoids, tannins and terpenoids are the most essential bioactive polyphenols identified in *R. canina* fruits by HPLC–UV–MS method. They are a wide range of secondary compounds distributed in various plants and play an important role in normal growth and defence. They display various structures and a broad range of biological activities (Naczk and Shahidi, 2004).

Most qualitative and quantitative analyses of phenolics are usually traditional methods such as HPLC-UV for qualitative analysis and Folin-Ciocalteu method (F-C), which is the only procedure used to measure total phenolics through the reduction capacity of the components of the extract. Whereas, the novel total phenolic method utilizing Fast Blue BB diazonium salt is based on the coupling of phenolic compounds with the diazonium. The coupling mostly occurs para to the phenolic activating group, unless the position is already occupied. For *R. canina* extract, the Fast Blue (FBBB) method had higher gallic acid equivalents (GAE) values than the standard Folin–Ciocalteu in the dosage of total phenolic (Maria et al., 2017; Medina, 2011a,b).

There are many analytical methods available to assess the antioxidant capacity such as DPPH scavenger effect, which is the method used to evaluate the antioxidant activity in vitro. Cellular antioxidant activity by determination of intracellular reactive oxygen species (ROS) is an approach used to evaluate the antioxidant activity based on cells (Yin et al., 2016). The phenolic compounds present in *R. canina* made it one of the preferred plants in phyto-therapy and in the pharmaceutical sector. *R. canina* presents a cytotoxic effect following an MTT test. This effect prevented from cancer by several mechanisms. They decrease both cell proliferation and oxidative stress, block cell cycles, and induce apoptosis (Ren et al., 2003).

The aim of the present study is to identify and characterize the main antioxidants in the ethanolic extract of *R. canina* fruits using HPLC–UV–MS. Additionally, the antioxidant and cytotoxic effects of *R. canina* extract are also explored.

2. Materials and methods

2.1. Samples

The fruits of *R. canina* were harvested from the region of Batna, Algeria, in October 2016. The samples were washed, then dried at room temperature and ground before storing at −20 °C.

2.2. Standards and reagents

All chemicals and reagents used in this study were of analytical grade and were obtained from Sigma-Aldrich chemistry (Madrid, Spain). HPLC grade solvents were purchased from Merck Darmstadt, Germany). The HPLC grade water was prepared using a Milli-Q system (Millipore Lab., Bedford, MA, USA).

2.3. Preparation of the phenolic extracts

The extraction of the polyphenols is carried out three times by mixing 50 ml of ethanol/water (70:30; v/v)/plant powder 50 mg. The extract was left in the ultrasonic bath (FALC Instruments, Italy) for 30 min after centrifugation at 5000 rpm for 20 min at 10 °C and the ethanol was removed in vacuum using a rotary evaporator Temperature below 40 °C. In order to remove the apolar molecules contained in the extract, a second extraction was performed by using 4 ml of the hexane/water mixture at a ratio of 50:50; v/v, and then mixed with the resulting ethanolic extract of the first extraction. The operation was repeated three times, and then the extract was dried under vacuum by heat applied in the Savant Speed VacThermo Scientific Concentrator SPD131DDA for 4 h. The resulting extract was lyophilized in a laboratory lyophilizer (BETA 2-8 LD plus - Martin Christ) for 24 h.

Extraction yield (%) = weight of extract obtained × 100/ weight taken of the material vegetal (Baghdikian et al., 2016).

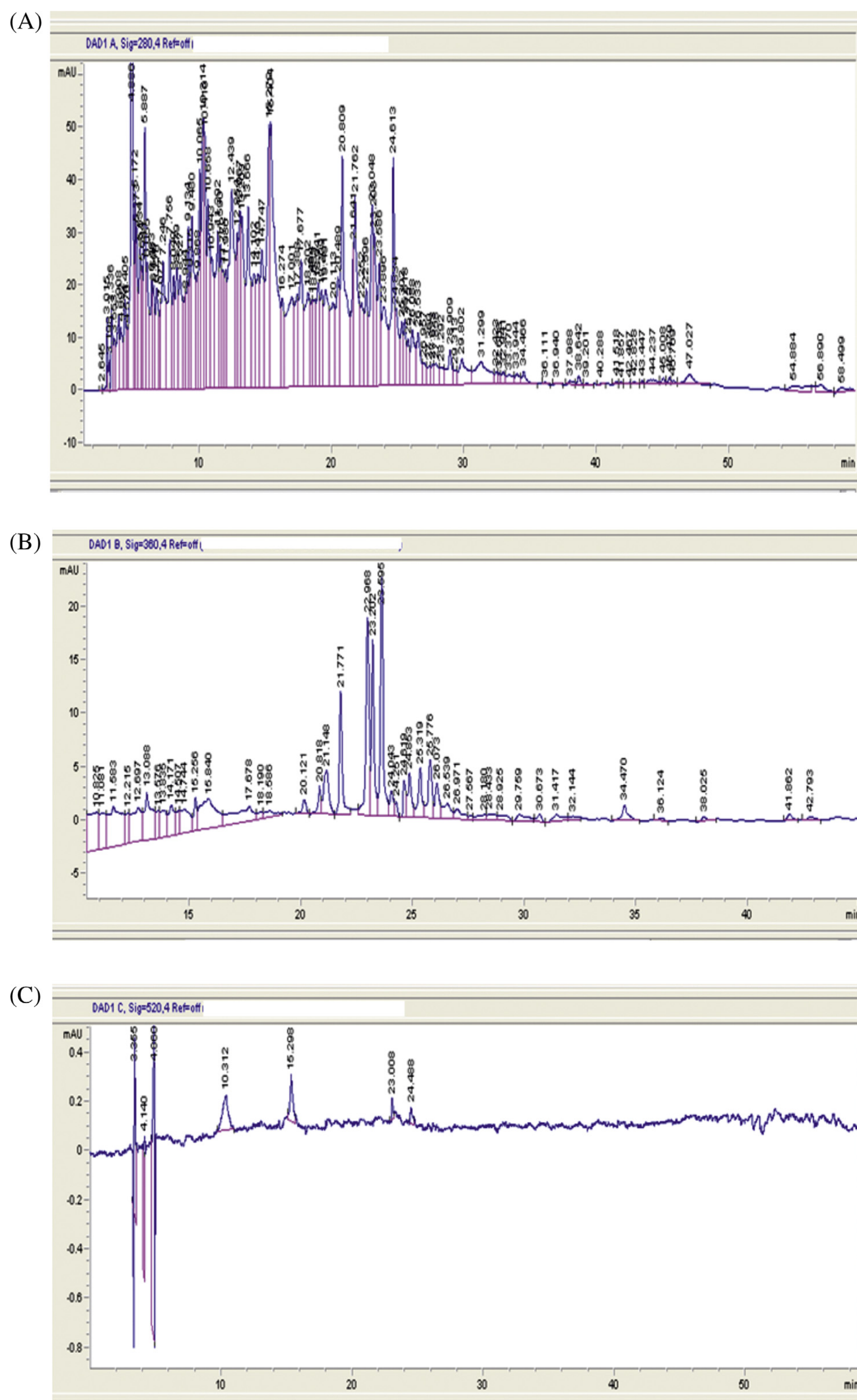

Fig. 2 HPLC-UV-DAD chromatograms of extract of *Rosa canina* fruits.

2.4. Determination of total phenols contents by Folin-Ciocalteu (F-C) and Fast Blue BB (FBBB) methods

The total phenolic content was evaluated by F-C method (Khanam et al., 2012). The ethanolic extracts were prepared at a concentration of 0.5 mg/ml. Briefly, 10 µl of the sample was transferred into a well in a 96-well plate and 150 µL of 6% Folin–Ciocalteu reagent was added and mixed, after 3 min, add 50 µl/well of saturated sodium bicarbonate solution (0.6 M) was added and mixed gently. Incubate the plate for

2 h at room temperature and in the dark and read the absorbance at 725 nm by the BioTek Synergy HT multi-mode microplate reader (BioTek Instruments Inc., Vermont, USA) and the data was acquired and processed using BioTek's Gen5TM software (BioTek Instruments Inc.).

The total phenolic content was determined by FBBB method (Lester et al., 2012). The ethanolic extracts were prepared at a concentration of 0.25 mg/ml. The reaction started by adding 150 μl/well of sample in the 96-well plate, and then mixed with 15 μl/well of Fast Blue (0.01% v/v H_2O) and 15 μl/well of NaOH (5% v/v H_2O). After incubating the plate for 120 min at room temperature, the absorbance was read at 420 nm with a BioTek Synergy HT multi-mode microplate reader (BioTek Instruments Inc., Vermont, USA), and data was acquired and processed using BioTek's Gen5TM software (BioTek Instruments Inc.).

A calibration curve was established for the two methods using a standard solution of gallic acid (0.6–0.025 mg/ml) and expressed as μg of gallic acid equivalents (GAE)/mg of dry weight (dw). For F-C method, $y = 1.2943x + 0.0877$; $R = 0.997$, and for FBBB method, $y = 1.1869 + 0.0267$; $R = 0.999$.

Where x is the total phenolic content of ethanolic extract. A control was carried out with water, which followed the same treatment as the extract.

2.5. Preparation of sample the HPLC-UV-MS analysis

50 mg of the dried paste was mixed with 10 ml of water/methanol-0.5% HCl (80:20; v/v). The mixture was placed in the ultrasonic bath for 30 min (FALC Instruments, Italy), then the extract was centrifuged at 5000 rpm for 20 min at 10 °C. The operation was repeated three times and the three successive acidified methanolic supernatants were evaporated to dryness under vacuum without heat applied to the SPV131DDA Thermo Scientific SPD131DDA Concentrator for 3 h. The samples were reconstituted with 200 μl of formic acid 0.1%, where the sample was centrifuged for 10 min at 9000 rpm, and then the supernatant was filtered and diluted (1:10) and finally stored in a flask at −20 °C until analysis (Mraihi et al., 2015).

2.6. Preparation of standard solution for HPLC-UV analysis

For the preparation of the standard calibration curves, the stock solutions of phloroglucinol, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, catechin, epicatechin, quercetin, quercetin-3-glucoside, apigenin, resveratrol and kaempferol were prepared informic

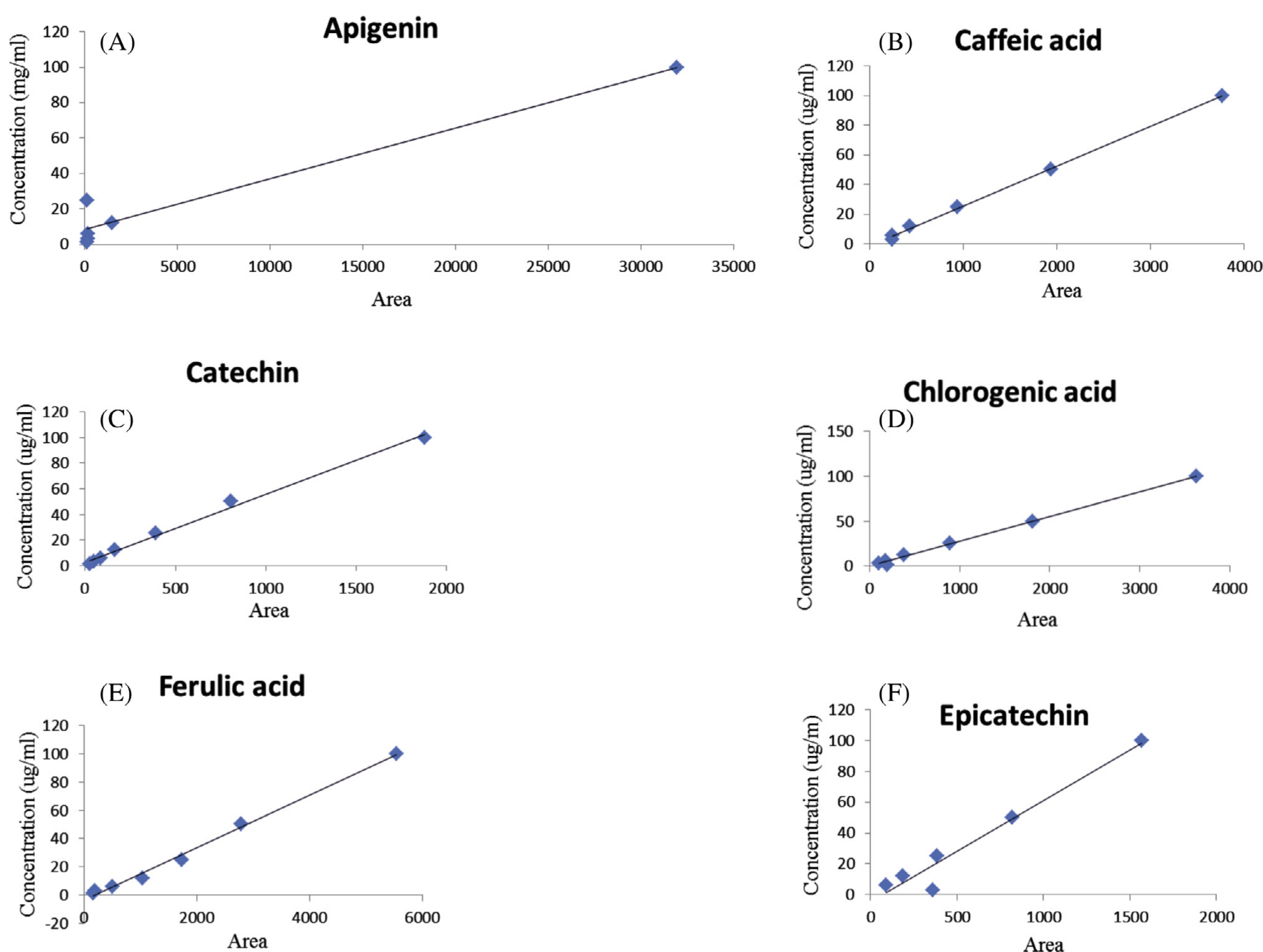

Fig. 3 HPLC-UV-DAD calibration curves of phenolic standards, Apigenin (3A), Cafeic acid (3B), Catechin (3C), Chlorogenic acid (3D), Ferulic acid (3E), Epicatechin (3F), Gallic acid (3G), Kaempferol (3H), p-coumaric acid (3I), Phloroglucinol (3J), Protocatechuic acid (3K), Quercetin (3L), Quercetin-3-glucoside (3M), Resveratrol (3N).

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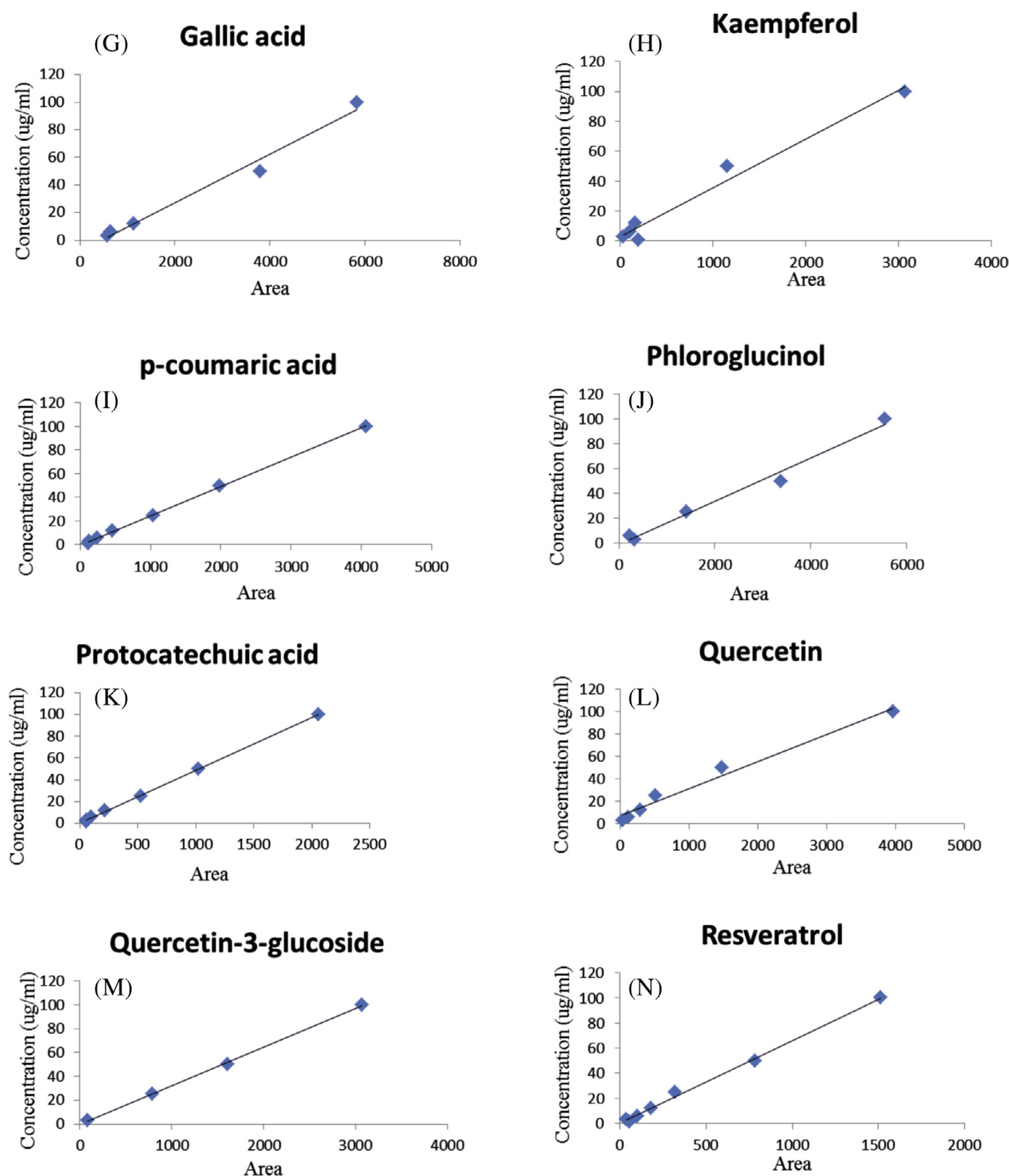

Fig. 3 (*continued*)

acid 0.1% at a concentration of 1 mg/ml. The concentrated solutions were then diluted with 0.1% formic acid to obtain 1, 3, 6, 12, 25, 50 and 100 µg/ml. All solutions were filtered through a 0.2 µm sartolon polyamide membrane filter.

2.7. Analysis of polyphenols by HPLC-UV

20 µl of the sample was placed in an Agilent 1200 Series HPLC for analysis. The Ultrabase column (C18.5 µm, 150 × 4.6 mm) was used at a flow rate of 0.5 ml/min coupled to a diode array detector (DAD) and a quaternary pump. The temperature of the column was set at 25 °C and the stopping time was 55 min. The solvents used for the analysis were formic acid 0.1% (A), acetonitrile with formic acid 0.1% (B), ultrapure water (C) and acetonitrile (D) (Pallaufa et al., 2008).

2.8. HPLC–MS analysis

In order to identify and characterize the phenolic compounds, a sample of 10 µL of extract was analyzed using HPLC with mass spectrometry detection Agilent 1200 Series liquid chromatography equipped with an electrospray atmospheric pressure (ESI) and employing an ESI (electrospray ionization), phenomenex Luna C18 (150 × 4.6 m 3 µm) was used with a flow rate of 0.5 ml/min. The capillary tension was 3 V, and the capillary temperature was 180 °C. Mobile Phase A was 0.1% Formic Acid in water, while Mobile Phase B was 0.1% Formic Acid in Acetonitrile (% B: 0 min = 10%, 30 min = 30%, 35 min = 35%, 40 min = 45%, 50 min = 10%). The spectra were recorded in negative ion mode and the MS detector was programmed

Table 2 Identification of certain phenolic compounds in the extract of *Rosa canina* L. fruits by LC-UV.

Compounds	Concentration (μg/ml)	λmax	Rt (min)	Area	Equation	Coefficient R
Phloroglucinol	100	280	5.887	505.37	y = 0.0175x – 1.2643	0.992
Gallic acid	50	280	4.886	2239.65	y = 0.0176x – 8.1344	0.992
Protocatechuic acid	50	280	10.658	546.69	y = 0.0486x + 0.1167	1.000
Chlorogenic acid	50	280	12.392	1117.06	y = 0.0277x – 0.3489	0.999
Caffeic acid	50	280	15.404	1399.72	y = 0.0268x – 1.1533	0.999
p-coumaric acid	50	280	21.762	649.97	y = 0.0248x – 0.2303	1.000
Ferulic acid	50	280	20.809	1002.28	y = 0.0185x – 3.4214	0.997
Catechin	100	280	11.392	518.73	y = 0.0533 + 2.3051	0.997
Epicatechin	100	280	13.666	716.76	y = 0.0659x – 4.7479	0.976
Quercetin	100	280	33.37	57.78	y = 0.0243 + 6.862	0.990
Quercetin-3-glucoside	100	280	19.491	508.84	y = 0.0326x – 0.7688	1.000
Apigenin	100	330	38.642	25.37	y = 0.0029 + 8.2269	0.974
Resveratrol	15	360	31.299	305.4	y = 0.0661x – 0.0455	0.998
Kaempferol	100	360	44.44	44.237	y = 0.0326 + 2.9976	0.988

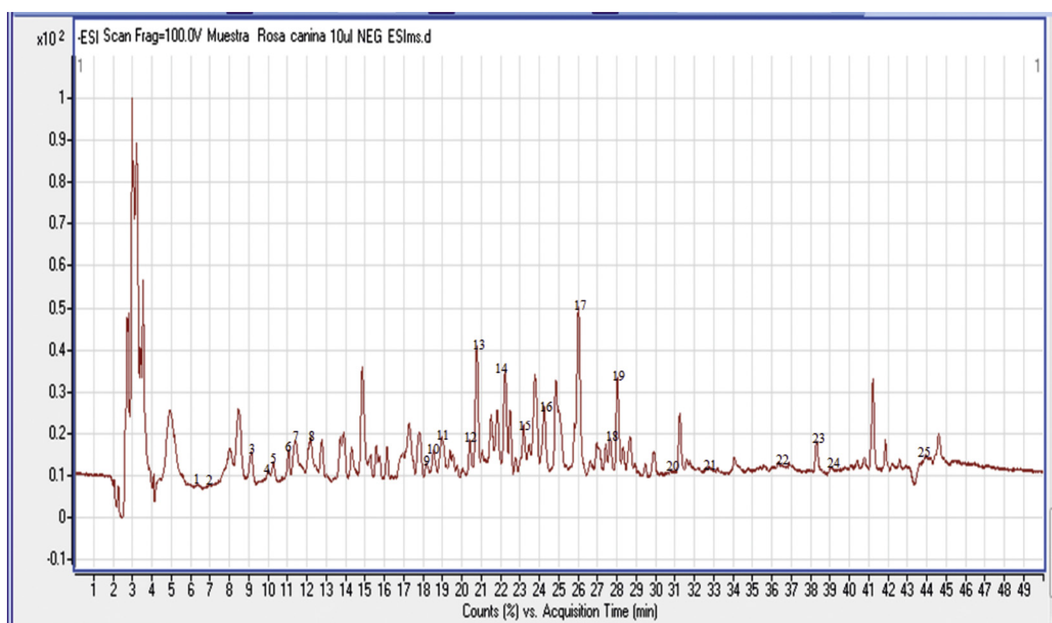

Fig. 4 HPLC-MS-DAD chromatograms of extract of *Rosa canina* fruits at-ESI mode.

to perform a consecutive scan series: extended dynamic range, low 1700 m/z (Pallaufa et al., 2008).

2.9. DPPH radical-scavenging activity

Antioxidant activity was determined by DPPH free radical scavenging effect by using quercetin, ascorbic acid, trolox and BHT as positive control, Crude extract was prepared with a range of concentrations (10–600 μg/ml). 290 μL of methanolic DPPH solution (1×10^5 M) was added and the mixture was incubated for 30 min at room temperature. The absorbance was read at 517 nm and the data were acquired and processed using BioTek's Gen5TM software (BioTek Instruments Inc.). Free radical scavenging activity was determined according to the equation % Antioxidant Activity (AA) = 100-[{(Abs_{sample}-Abs_{blank}) × 100}/$Abs_{control}$] published previously (Nwaehujor et al., 2014).

2.10. Cell culture and extract treatments

HepG2 human hepatoma cells, SH-Sy5y human neuroblastoma were seeded and cultured regularly in DMEM medium and 10% fetal bovine serum, but they were changed to serum-free medium 24 h prior to testing. Cells were treated with different concentrations of ethanolic extract (0.01–250 μg/ml) within 24 h.

2.11. Determination of ROS

Cellular oxidative stress was quantified by the dichlorofluorescence test (DCFH). DCFH becomes dichlorofluorescein (DCF) after being oxidized by intracellular oxidants, and emits fluorescence. By quantifying the fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, a fair estimate of

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the overall oxygen species generated under the various conditions was obtained by using fluorescent probe (DCFHDA) after incubation in CO_2 at 37 °C for 30 min (Granado-Serrano et al., 2006).

2.12. Statistical analysis

The data were expressed as mean ± DS. Significant differences were calculated by testing linear trends using

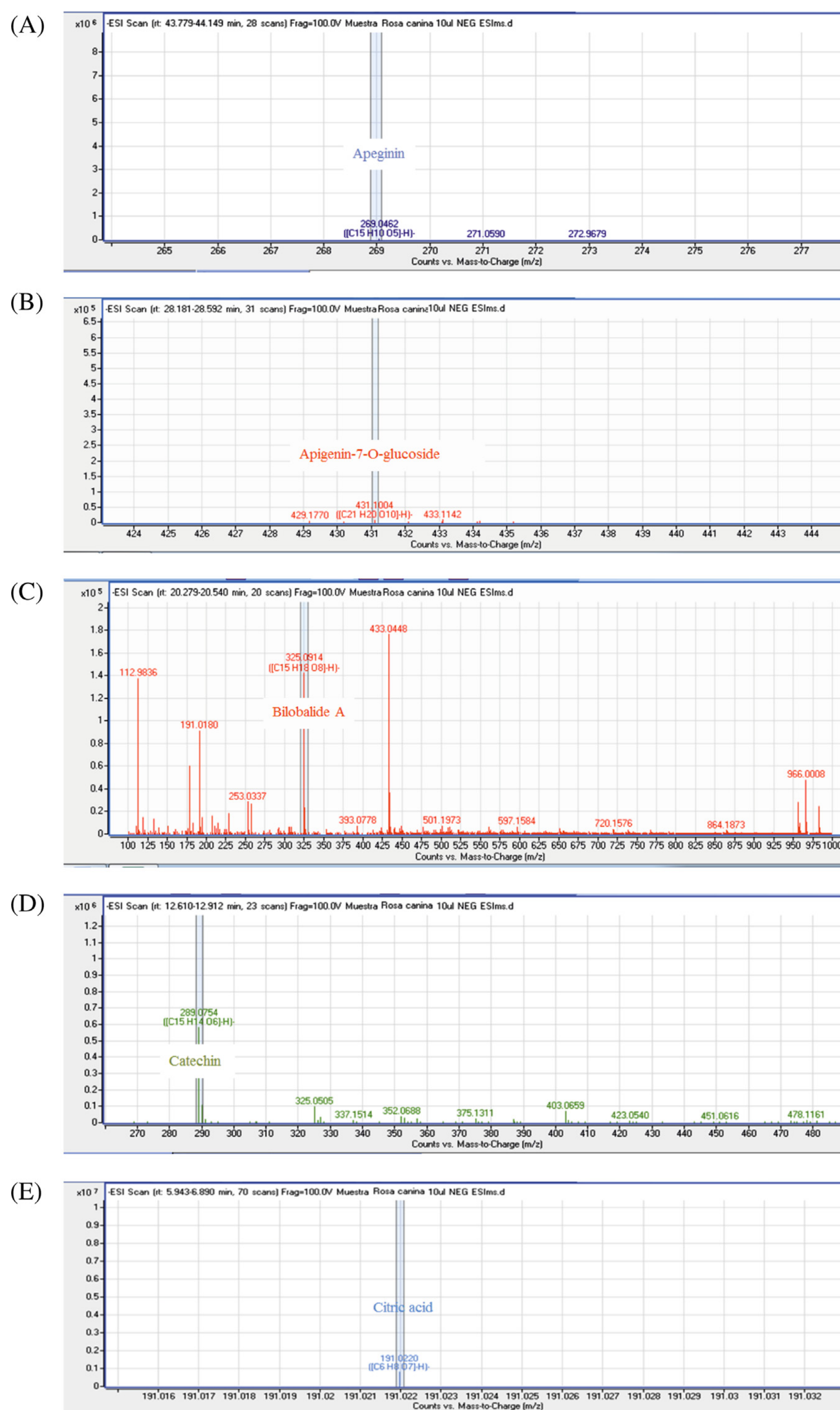

Fig. 5 MS/MS spectra of chemical compounds of extract of *Rosa canina*. Apigenin (5A), Cafeic acid (3B), Apigenin-7-O-glucoside (5B), Bilobalide A (5C), Catechin (5D), Citric acid (5E), Dihydroquercetin (5F), Ellagic acid (5G), Gallacetophenone (5H), Gallocatechol (5I), Kaempferol-7-O-glucoside (5J), Luteolin 5-methyl ether (5K), Luteolin-4′-O-glucoside (5L), Luteolin-7-O-glucoside (5M), Procyanidin B3 (5N), Procyanidin B6 (5O), Pyrogallol-2-O-glucuronide (5P), Quercetin dehydrate (5Q), Quercetin (5R), Quercetol 3-O-rutinoside (5S), Rosmarinic acid (5T).

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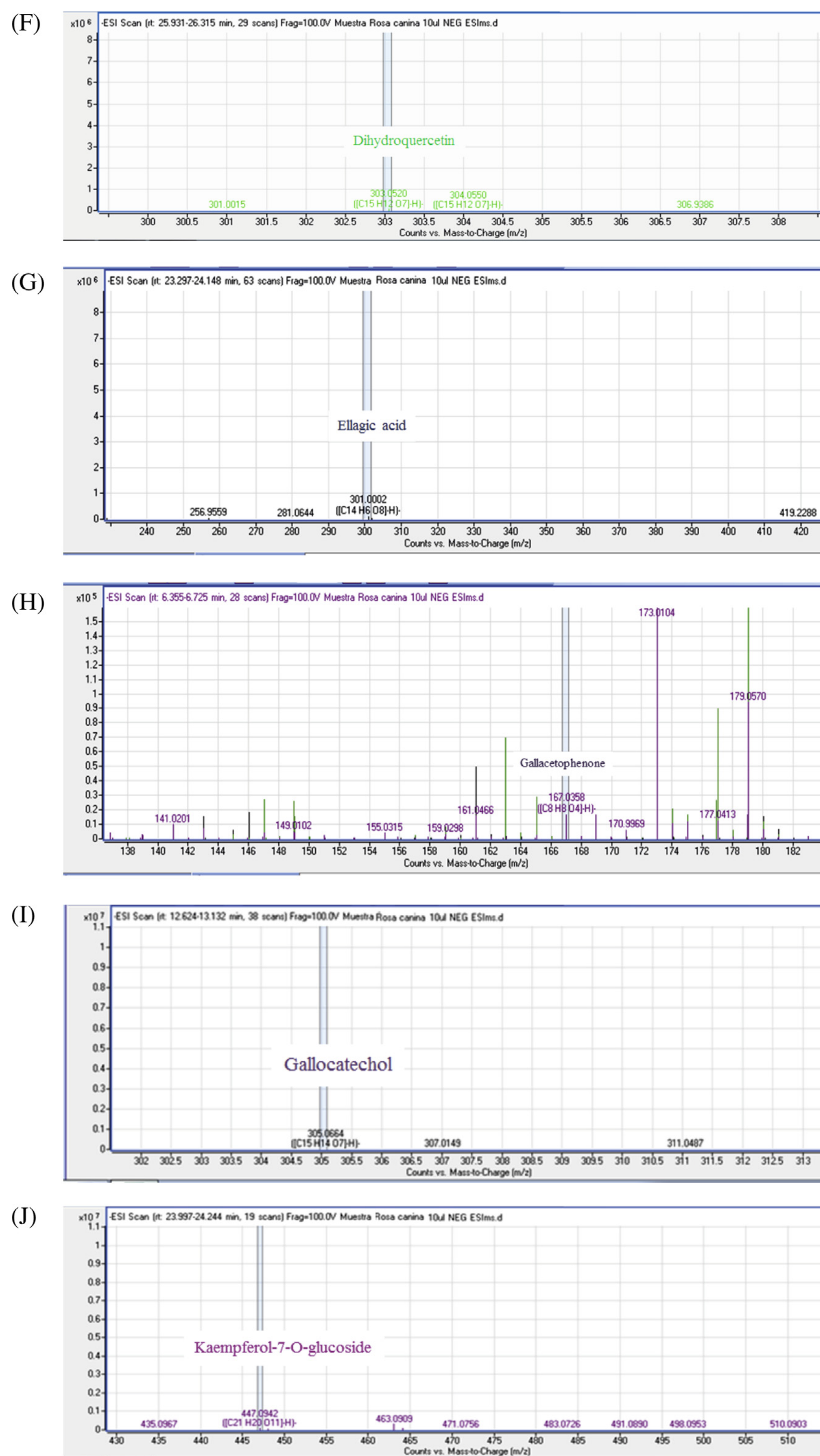

Fig. 5 (*continued*)

single-factor analysis (ANOVA), using Dunnett's multiple comparison test. The data used for the IC_{50} determination were examined using a nonlinear regression sigmoid fit using the GraphPad Prism. Differences with $P < .05$ were considered significant. The SPSS version 20.0 program was used.

3. Results and discussion

3.1. Total phenolic contents (TPC) in *R. canina* fruits

Our results indicate that the FBBB test provides a higher and more accurate estimate of total phenolic due to its direct

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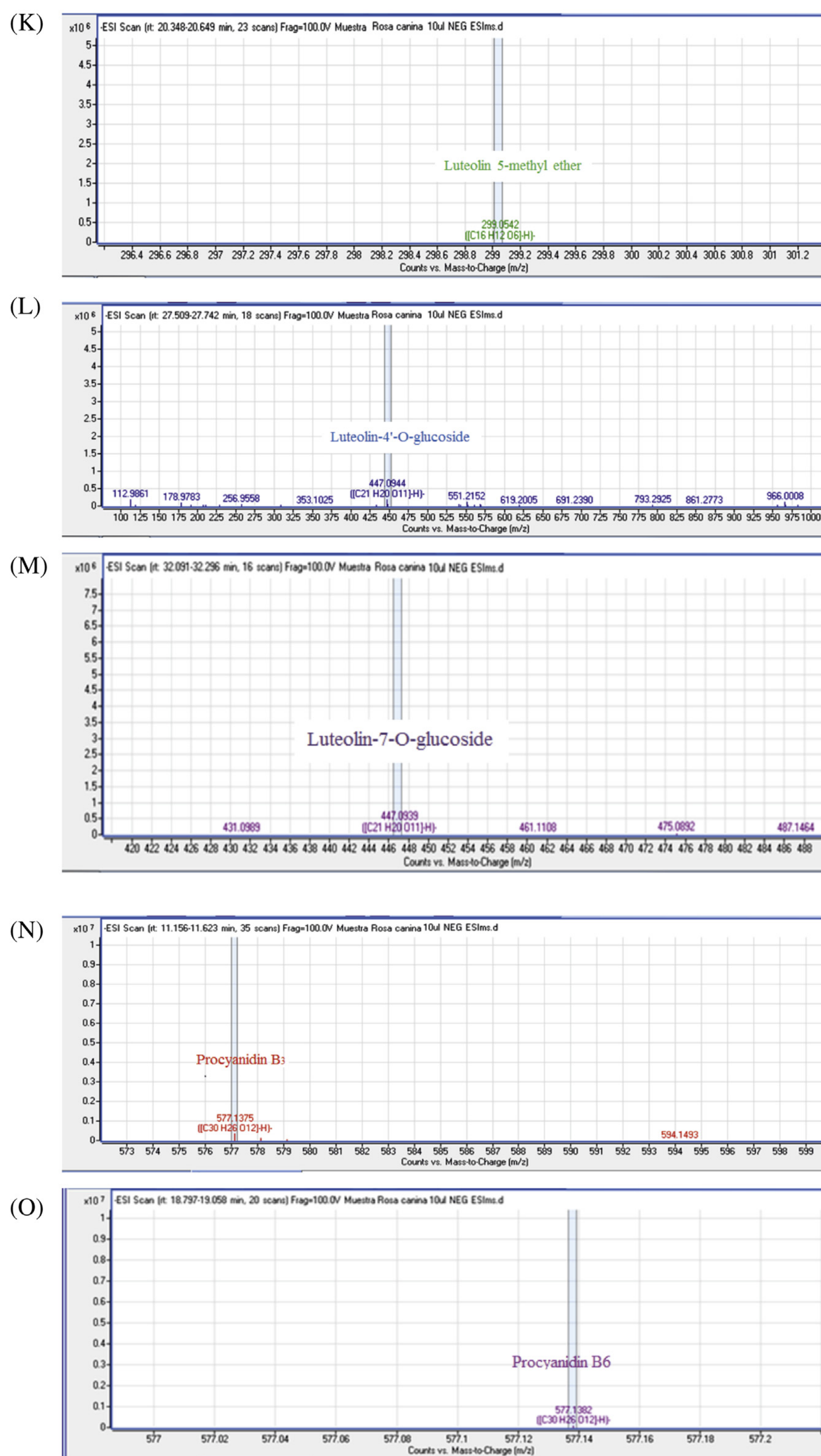

Fig. 5 (*continued*)

reaction with phenolic compounds in *R. canina* fruits as well as the FBBB reaction with gallic acid substrate was highly linear (R = 0.999). Accordingly, the F-C assay had a positive linear response to gallic acid (R = 0.997) Fig. 1. Although the total phenol concentration of F-C expressed as the value (354.46 ± 0.05 μg GAE/mg DM), these values were lower in the same fruit than in the total phenolics analyzed by FBBB assay (598. 25 ± 0.49 μg GAE/mg DM). Results are presented in Table 1. The FBBB test, which detects phenolics directly, reported an average 1.7 fold higher concentration of total phenolics than F-C. Previous studies of strawberry fruit using the F-C assay have greatly underestimated the total phenolic concentration,

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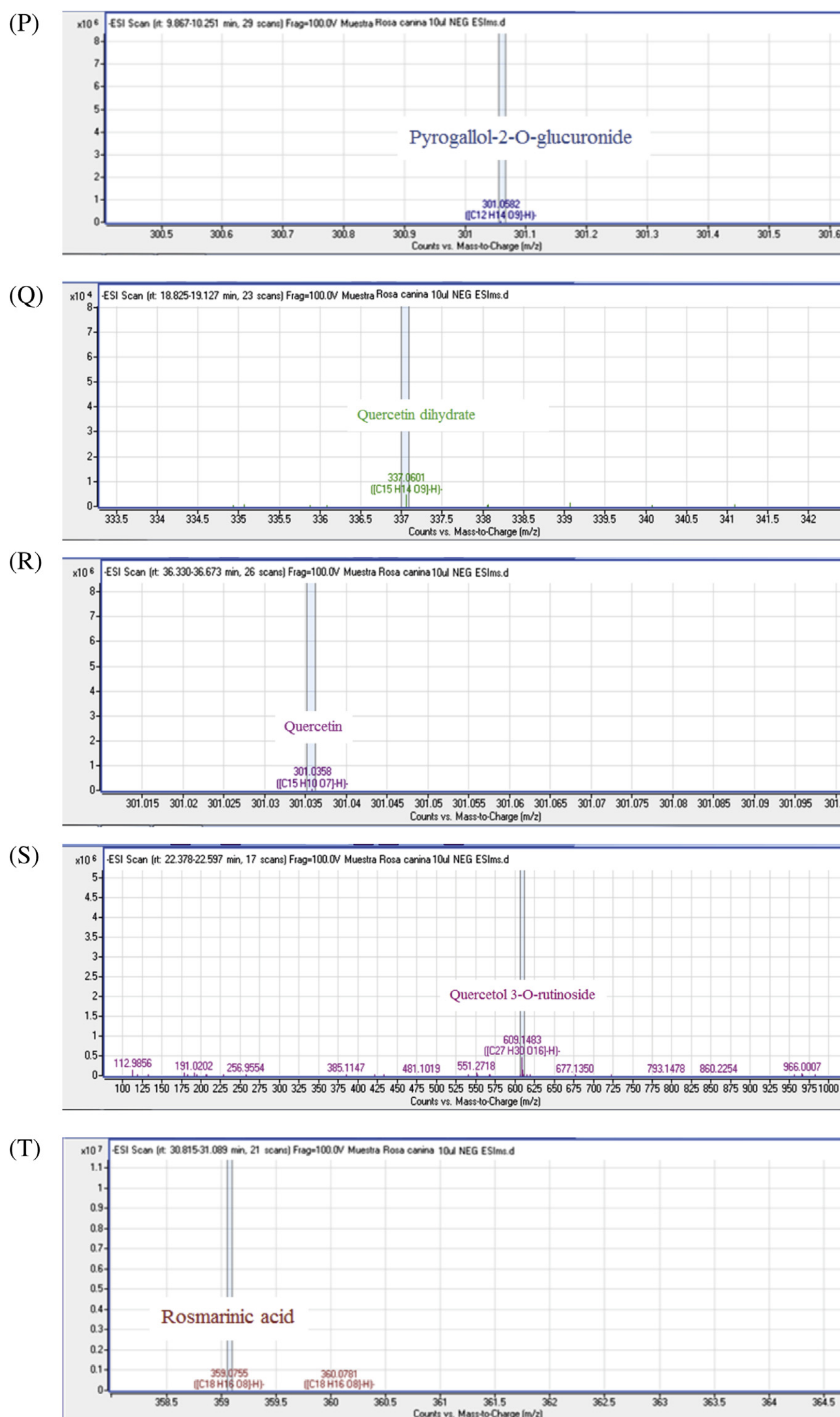

Fig. 5 (*continued*)

and this assay should be replaced in future studies by the Fast Blue BB assay (Medina, 2011a,b).

3.2. HPLC-UV analysis

Qualitative analysis of the ethanolic extract of *R. canina* fruits was tested by using HPLC-UV analysis and their chromatographic profile was compared with the retention times and absorption spectrum of reference standards (phloroglucinol, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, catechin, epicatechin, quercetin, quercetin-3-glucoside, apigenin, resveratrol and kaempferol). From the HPLC-UV profile it was observed that the ethanolic extract showed the presence of four big peaks at 280 nm. The peaks were identified as gallic acid, chlorogenic acid, caffeic acid and ferulic acid with Rt between 4.886 and 20.809 min.

Table 3 Identification of certain phenolic compounds in the extract of *Rosa canina* L. fruits by LC-MS.

No	Proposed compound	Rt (min)	Accurate mass [M-H]$^-$	MS/MS (m/z)	Peak purity (%)	Molecular formula	Nature of compound
01	Citric acid	6.41	191.022	191	79.71	$C_6H_8O_7$	Tricarboxylic acid
02	Gallacetophenone	6.56	167.0358	125, 167	77.41	$C_8H_8O_4$	Hydrolysable tannin
03	Procyanidin B2	9.13	577.1375	425, 407, 289. 287	91.66	$C_{30}H_{26}O_{12}$	Proanthocyanidin
04	2,3-Digalloylglucose	9.26	483.0815	483, 125	55.19	$C_{20}H_{20}O_{14}$	Hydrolysable tannin glucoside
05	Pyrogallol-2-O-glucuronide	10.06	301.0582	301, 125	81.95	$C_{12}H_{14}O_9$	Hydrolysable tannin glucoside
06	Procyanidin B3	11.39	577.1375	425, 407, 289, 287	91.66	$C_{30}H_{26}O_{12}$	Proanthocyanidin
07	Catechin	12.76	289.0754	289	68.36	$C_{15}H_{14}O_6$	Flavan-3-ol
08	Gallocatechol	13.02	305.0664	305, 289	71.21	$C_{15}H_{14}O_7$	Flavan-3-ol
09	Quercetin 3,7-diglucoside	18.43	625.1462	625, 463, 301	47.89	$C_{27}H_{30}O_{17}$	Flavonol glucoside
10	Procyanidin B6	18.93	577.1382	425, 407, 289, 287	87.09	$C_{30}H_{26}O_{12}$	Proanthocyanidin
11	Quercetin dihydrate	18.97	337.0601	337, 301	nd	$C_{15}H_{14}O_9$	Flavonol
12	Bilobalide A	20.48	325.0914	325	91.1	$C_{15}H_{18}O_8$	Sesquiterpene
13	Luteolin 5-methyl ether	20.50	299.0542	299, 285	67.16	$C_{16}H_{12}O_6$	Flavone
14	Quercetol 3-O-rutinoside	22.47	609.1483	609, 463, 301	93.23	$C_{27}H_{30}O_{16}$	Flavonol glucoside
15	Ellagic acid	23.46	301.0002	301	95.34	$C_{14}H_6O_8$	Hydrolysable tannin
16	Kaempferol-7-O-glucoside	24.11	447.0942	447, 285	67.18	$C_{21}H_{20}O_{11}$	Flavonol glucoside
17	Dihydroquercetin	26.16	303.0520	303	96.65	$C_{15}H_{12}O_7$	Flavonol
18	Luteolin-4′-O-glucoside	27.63	447.0944	447, 285	96.92	$C_{21}H_{20}O_{11}$	Flavone glucoside
19	Apigenin-7-O-glucoside	28.40	431.1004	431, 269	68.94	$C_{21}H_{20}O_{10}$	Flavone glucoside
20	Rosmarinic acid	30.94	359.0755	359	82.78	$C_{18}H_{16}O_8$	Hydroxycinnamic acid
21	Luteolin-7-O-glucoside	32.19	447.0939	447, 285	85.61	$C_{21}H_{20}O_{11}$	Flavone glucoside
22	Quercetin	36.48	301.0358	301	95.14	$C_{15}H_{10}O_7$	Flavonol
23	Kaempferol-3-Glucoside-2″-p-coumaroyl	38.31	593.1311	593, 463, 285, 163	98.27	$C_{30}H_{26}O_{13}$	Flavonol glucoside
24	Kaempferol	39.13	285.0411	285	97.12	$C_{15}H_{10}O_6$	Flavonol
25	Apigenin	43.88	269.0462	269	83.54	$C_{15}H_{10}O_5$	Flavone

nd: not detected.

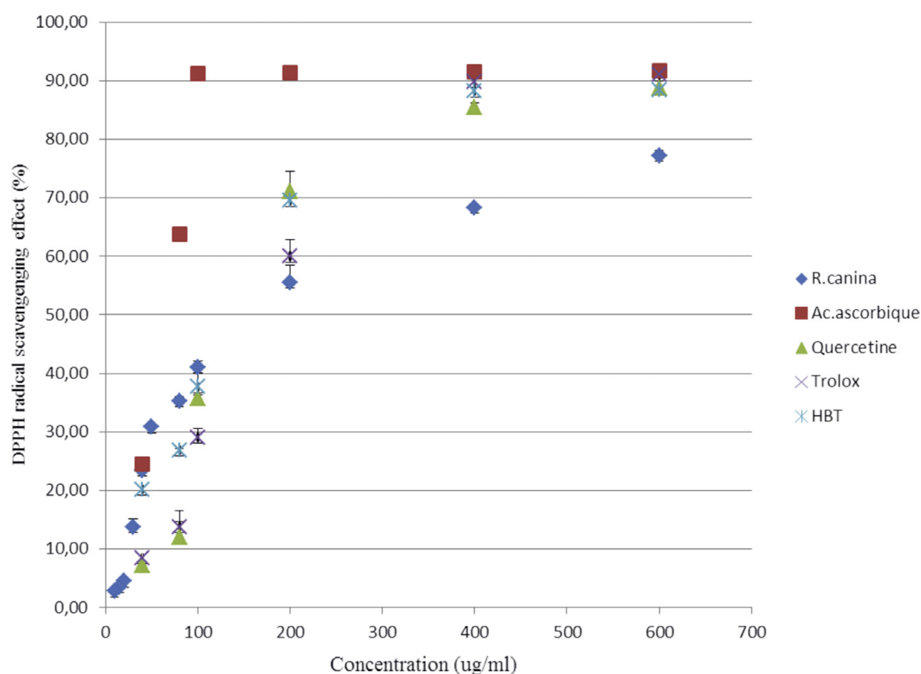

Fig. 6 Reduction indexes of the DPPH scavenging effect (%) versus concentration, each value in the graph is shown as means ± SD.

Table 4 IC_{50} values of the DPPH free radical scavenging of *Rosa canina* fruits.

Samples	Rosa extract	Ascorbic acid	Quercetin	BHT	Trolox
DPPH scavenging activity (IC_{50} μg/ml)	156.74 ± 0.56	46.74 ± 1.14	157.45 ± 2.11	132.22 ± 0.64	163.55 ± 1.25

Positive controls and Rosa extract were done in triplicate (n = 3).

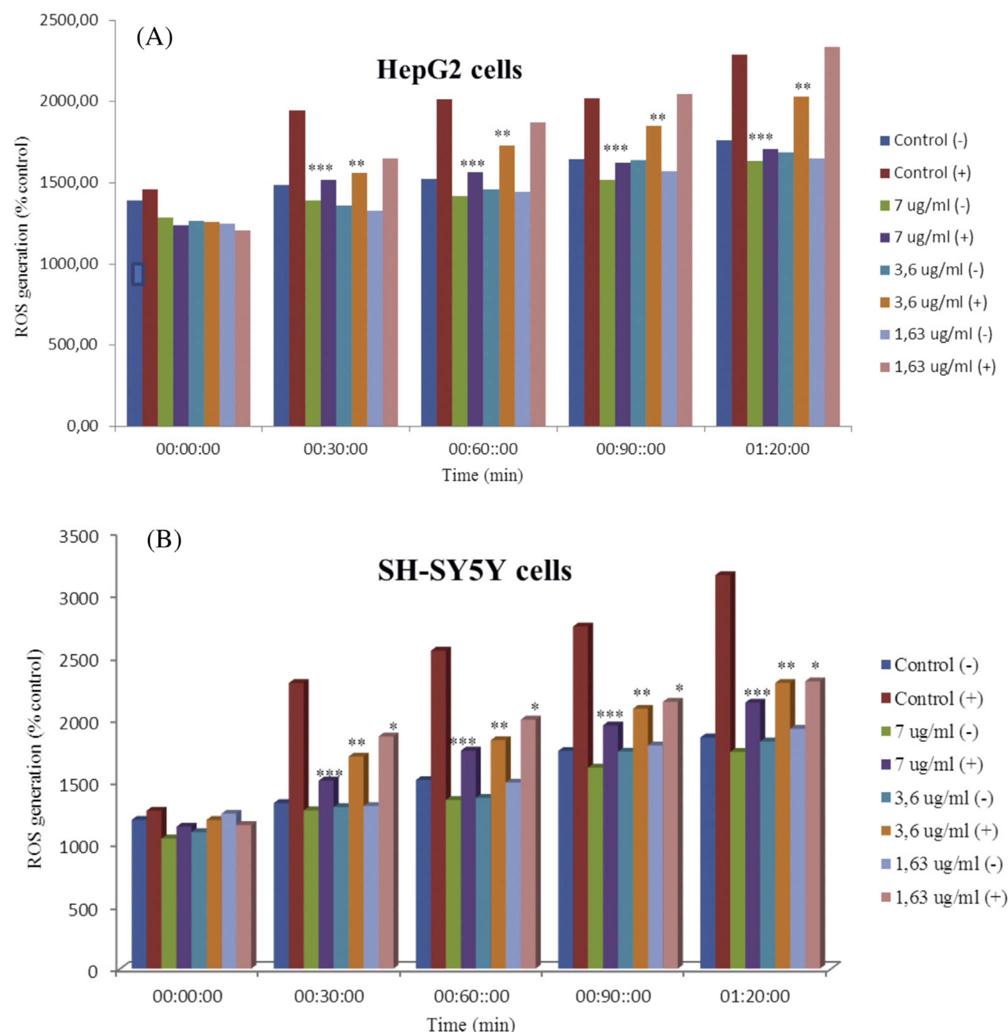

Fig. 7 Effect of ethanolic extract on intracellular ROS levels in HepG2 (7A), and SH-SY5Y cells (7B). *P < .05, ***P < .001 significantly different from negative control cells and positive control cells.

Phloroglucinol, protocatechuic acid, p-coumaric acid, Catechin, epicatechin, quercetin-3-glucoside and resveratrol were identified at 280 nm with Rt between 5.887 and 31.299 min, and traces of quercétine, apigenin and kaempferol Figs. 2 and 3 (Table 2). This analysis gives an idea on the composition of polyphenols presented in the fruits of *R. canina* (Jafri et al., 2014).

3.3. HPLC-MS analysis of phenolic compounds and peaks identification

25 phenolic compounds belonging to a variety of classes of natural products were detected in *R. canina* by HPLC-UV-MS/MS analysis using negative ionization modes, results are shown in Fig. 4, the identities, retention times (Rt min), chemical formula, pseudomolecular ions $[M-H]^-$, peak purity (%) and fragment ions MS/MS (m/z) for individual compounds were reported in Fig. 5 (Table 3).

The phenolic acids with its types (Hydroxycinnamic acid and Tricarboxylic acid) were represented by peaks 1 and 18 in the chromatogram, and they were identified as citric acid and rosmarinic acid, respectively.

Citric acid was identified at Rt 6.41 min and m/z 191.022, the important role of this acid in the Krebs cycle has drawn the attention of a number of research groups, especially for use in diagnosis and targeting of cancer. Rosmarinic acid,

which is the print of Rosaceae family, corresponded to Rt 30.94 min and m/z 359.0755 as shown in (Table 3) (Mcdunn et al., 2013; Tohma et al., 2016).

Flavan-3-ol peaks were represented by peaks 6 and 7at Rt 12.76, 13.02, and m/z 289.0754, 305.0664 respectively, which corresponded to catechin and gallogatechol. Proanthocyanidin B-type isomers were detected in three peaks (2, 5 and 9) at 9.13, 11.39 and 18.93 min and the signal at m/z 577 indicated that the dimers are mixed; two units of catechin and/or epicatechin. The obtained MS/MS (m/z) fragments of the condensed tannins were 425, 407, 289 and 287 (Friedrich and Eberhardt, 2000). The fragmentation pathway by Retro Diels-Alder in the T-unit of procyanidin B3 was the important mechanism that allowed its detection in the ions m/z 425 and 407. Hydrolysable tannins (gallotannin and ellagitannin; gallacetophenone and ellagic acid respectively) were reported by two peaks (2 and 15). Hydrolysable tannin glucosides were assigned to peaks 3 and4, which corresponded to 2,3-Digalloylglucose and Pyrogallol-2-O-glucuronide; they were identified at 6.56, 9.26, 10.06 and 23.46 min with m/z 167.0358, 483.0815, 301.0582 and 301.0002 respectively (Table 3) (Kassim et al., 2010; Ridder et al., 2012).

The chromatogram peak 11 reflected the different types of terpenoids such asbilobalide A. These compounds were identified at Rt 20.48 min and m/z 325.0914 with high peak purity 91.1% (Table 3) (Rana et al., 2014; Wang et al., 2013).

The principal subclasses of flavonoids were the abundant compounds present in *R. canina* (Table 3), in the structure portrayal, we initially judged if the flavonoid glycoside is a C-glycosylated. The carbon-carbon obligation of C-glycosyl flavonoids is impervious to burst and in C-glycosides for the most part the fracture of the sugar unit is watched. Fracture pathway of O-glycosylated flavonoids begins with the cleavage of the glycosidic bonds and disposal of the sugar moieties with charge maintenance on aglycone. In mixes containing at least two sugars to the same aglycone carbon, particles emerging from the cleavage of the glycosidic bonds between sugar units are feeble. Despite the fact that the aglycone and the glycane were altogether recognized, the exact structure of the flavonoids glycoside couldn't be constantly decided on the grounds that personality and the site of association of monosaccharide can't be controlled by LC-MS. The structures of mixes were at last distinguished by correlation with bibliographical studies (Ana Plazonić et al., 2009).

The C-glycosylated flavones were additionally found in *R. canina* fruits. Peaks 18 and 21 corresponded to two isomers, namely, luteolin-4′-O-glucoside and luteolin-7-O-glucoside (Table 3). Their identities were based on their pseudomolecular ion $[M\text{-}H]^-$ at m/z 447 and MS/MS spectra. They gave out fragments assigned to the losses of a hexosyl (glucose = −16 2 µma) to obtain the luteolin aglycone at m/z 285 and luteolin 5-methyl ether at m/z 299, and from an apigenin-7-O-glucoside at m/z 431 giving an apigenin aglycon at m/z 269 (Fernandes et al., 2014).

Flavonols are the principal compounds present in *R. canina* fruits; they differed in the number and position of phenolic hydroxyl —OH. Flavonols were frequently found in the form of heteroside in plants are potent antioxidants that serve to protect the plant from reactive oxygen species (ROS) (Nakabayashi et al., 2014), MS/MS spectra discharging fragments relating to the misfortunes of sucre, the glycosidic bond was cleaved about the mechanism of fragmentation of O-glycosylated flavonols. The releasing of monosaccharide residue is shown by the loss of sugar (Mraihi et al., 2015).

The MS/MS fragmentation mechanism of flavonolsaglycone based on cleavage of two C—C bonds of the C-ring releasing two fragment ions which give informations about the number and type of substituents (Cuyckens and Claeys, 2004), the pseudomolecular ions $[M\text{-}H]^-$ of flavonolsaglycone identified in *R. canina* at m/z 625, 609, 593, 447 and 463. Furthermore, Therefore the product ions, [aglycon–H]-, were reported at m/z 285 (Table 3), were shown fragmentations of flavonols peaks 16 and 23 (same pseudomolecular ion $[M\text{-}H]^-$ at m/z 447 and 593, respectively) corresponding to kaempferol-7-O-glucoside, kaempferol-3-Glucoside-2″-p-cou maroyl. The releasing fragments of the peaks 9 and 14 corresponded to the loss of dihexosyl (glucose and/or galactose = −162 µma) and rhamnosyl hexosyl (−146 to 162 µma) to obtain the quercetin aglycon, dihydroquercetin and quercetin dehydrate at m/z 301.303 and 337 respectively. These disaccharide should be either a diglucoside = Glc-Glc (m/z 324) or a Gal-Gal (m/z 324) and a rutinoside = Glc-Rha (m/z 308) or a Gal-Rha (m/z 308) respectively linked to the quercetin aglycone by either Glc or Gal sugar. So these peaks corresponded to quercetin 3,7-diglucoside and quercetol 3-O-rutinoside at m/z 625 and 609, respectively.

3.4. DPPH radical-scavenging activity

The antioxidant activity of the phenolic compounds depends on their chemical composition and structural conformation. These antioxidants react with DPPH, diminishing a number of DPPH molecules equivalent to the quantity of accessible hydroxyl groups (Chaouche et al., 2014; Shimada et al., 1992). The ethanolic extract and the standards (trolox, quercetin, BHT and ascorbic acid) were tested and expressed in percentage of reduction of DPPH radicals in a dose-dependent manner. For instance, At 600 and 400 µg/ml the ethanolic extract resulted in 77.22 and 68.33% reduction, respectively. Meanwhile, ascorbic acid at concentrations ranging between 600 and 400 µg/ml resulted in 91.61 and 91.49% reduction. Quercetin on the other hand displayed 88.78 and 85.39% reduction. BHT resulted in 88.44 and 88.19% reduction. While trolox displayed 90.99 and 89.80% reduction. Results are shown in Fig. 6. This extract had a high activity compared with each of the standards because it was rich in phenolic compounds such as methyl 4-O-galloylchlorogenate that showed high antioxidant activity in the DPPH free-radical assay. The high antioxidant capacity of this compound suggested that this plant was a good protection against oxidative damage (Ma et al., 2003). The genkwanin and hydroxygenkwanin exhibited significant anti-oxidative effect on DPPH (Ren et al., 2013). The DPPH radical scavenging effect was measured for some terpenoids and derivates of glycerol (Talla et al., 2016). The antioxidant activities displayed as IC_{50} values are illustrated in (Table 4). The IC_{50} value of ethanolic extract was higher than that of the ascorbic acid and BHT, but lower than that of quercetin and trolox. This difference was due to the richness of the extract in flavones (isoorientin and wogonin), glycosylated derivatives of isoflavone glucoside (daidzein and cavi-unin), and tannins that were detected by HPLC-MS. The important properties of the abundant antioxidants in *R. canina*

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fruits conferred dismutation of free radical DPPH. The mechanism by which some hydroxyl groups were connected with hydrogen atoms of phenolic compounds to become stable diamagnetic molecules (Boutennoun et al., 2017; Yang et al., 2016).

In the present study, the positive correlation between the quantity of antioxidants in *R. canina* and the DPPH free radical activity (R = 0.9962) was proved (Aksoy et al., 2013; Tchouya and Barhe, 2016).

3.5. Determination of intracellular ROS levels

HepG2 and SH-SY5Y cells are the very useful model for studying the effect of oxidative stress, it is demonstrated that the cytotoxic effect of tert-butyl-induced exogenous oxidative stress (t-BOOH) trigger deleterious effects on cell growth resulting both from a rapid inflow of the free radical generator into the cell. As a result the induction of transient increases in cytosolic Ca^{2+} to regulate the cellular process by transcription factors that regulates the expression of genes in response to oxidative stress and kept cell survival. The correlation is reversed between the high concentration of tert-butyl with the amount of cytosolic Ca^{2+} obtained during the shock oxidation and when the excessive or unregulated levels of Ca^{2+} in the cytosol can lead to cell death (Popa et al., 2010). Antioxidant scavengers of *R. canina* modulated and reduced the oxidative stress of ROS to cells (HepG2 and SH-SY5Y).

Three concentrations of ethanolic extract (7, 3.6 and 1.63 µg/ml) containing t-BOOH similar to the control positive and without t-BOOH being lower to the negative control were added for both cell types respectively. Results showed a reduction in the production of ROS in all concentrations of extract used in this test compared to positive and negative controls for SH-SY5Y cells Fig. 7 (Jiménez et al., 2016). Moreover, the concentrations of 7 and 3.6 µg/ml showed high reduction in ROS in HepG2 compared to controls. Nevertheless, a concentration of 1.63 µg/ml with tert-butyl resulted in lower reduction of ROS compared to the positive control. It is noteworthy to mention that this oxidative effect of *R. canina* has never been mentioned before. The presence of sesquiterpene (bilobalide A) confers on the one hand neuroprotective effects and, on the other hand, the presence of such a tert-butyl group remains exceptional for such a natural compound. This tert-butyl group was the major cause of the oxidative effect of *R. canina* at this concentration.

There are an association of ROS and free radicals in the pathogenesis of certain human sicknesses, including cancer, is ending progressively, in this study, the antioxidants of *R. canina* fruits were modulated the ROS in order to reduce the oxidative stress in HepG2 and SH-SY5Y cells and as a result reduced the danger of risk in injury of cancer (Al-Gubory et al., 2010; Serrano et al., 2007; Trachootham et al., 2009).

4. Conclusions

In the present study, a number of analysis using HPLC-UV-MS method were carried out to reveal the composition of *R. canina*. Afterwards, in situ essays on HepG2 and SH-SY5Y cells were carried out to further explore the antioxidative and cytotoxic effects of *R. canina*. Results indicated that the antioxidant and antiproliferative effects of *R. canina* fruits were due to the presence of flavonoids, tannins, terpenoids, xanthonoids and glycerol glucoside. The phytochemicals of *R. canina* fruits made the plant a rich source of antioxidants. The high antioxidant capacity of *R. canina* extract suggested that this plant could be used as an additive in the food industry; providing good protection against oxidative damage. It can also be used as an effective natural treatment to control several types of cancer. The beneficial effects of *R. canina* fruits can lead to its usage in diets with nutritional and healthy advantages.

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