Thieme

Phytochemistry and Antioxidant Activity of Aerial Parts of *Phagnalon sordidum* L.[1]

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ABSTRACT

One new natural monoterpene, 5-*O*-*β*-D-glucopyranosyl-2-hydroxy-*p*-cymene (**1**), and 11 known compounds were isolated through a biologically oriented approach from the aerial parts of *Phagnalon sordidum* L. The most active extract and fractions were selected using 3 complementary antioxidant activity assays. Results and the different methods were compared by relative antioxidant capacity index. In addition, the most active extract of *P. sordidum* was subjected to liquid chromatography coupled with electrospray ionization hybrid linear ion trap quadrupole Orbitrap mass spectrometry to quantify secondary metabolites. Antioxidant activities of ethyl acetate extract, and purified 3,4-dihydroxyacetophenone (**3**) and nebrodenside A (**7**) were demonstrated by *in vitro* cell free model assays, and their protective effect against H_2O_2-induced oxidative stress in a HepG2 (human hepatocellular carcinoma) cell line was established.

Introduction

In recent decades, the medicinal value of plant species has gained more interest due to the discovery of new secondary metabolites and evidence that they are effective as antioxidants [1]. Antioxidants are vital substances with the ability to protect the body from damage caused by free radical–induced oxidative stress, such as in atherosclerosis, dementia, inflammation, coronary heart disease, ageing, and cancer [2, 3].

Natural sources rich in antioxidant phenolic compounds have been attracting increasing attention since they represent an important source of food supplements, beverages, and natural remedies for several ailments. Nowadays, it is important to discover new natural sources of safe and inexpensive antioxidants [3, 4], since some synthetic antioxidants show potential health risks and toxicity [5]. The genus *Phagnalon* includes annual and perennial herbaceous plants belonging to the family Asteraceae, including about 200 species spread all over the world [6, 7]. *Phagnalon* spp. have found wide use, mainly in folk medicine, and in particular for

[1] Dedicated to Professor Dr. Cosimo Pizza on his 70th birthday in recognition of his outstanding contribution to natural product research.

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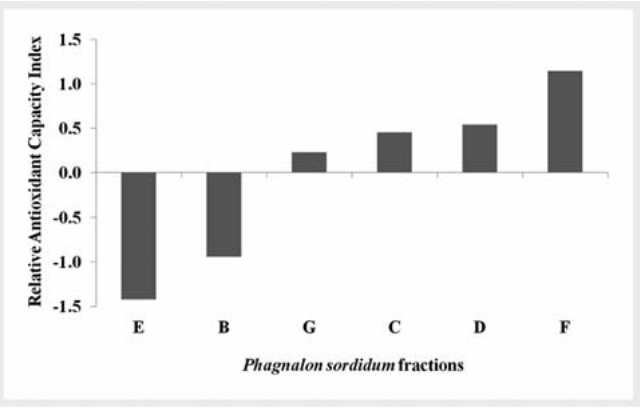
the treatment of hypertension and respiratory and gastric diseases [8, 9]. Biological studies have also reported several other properties, such as antibacterial and antifungal, expectorant and antitussive, insect antifeedant, anti-hypouricemic, antidiabetic, antioxidant, anti-inflammatory, and cytotoxic activity [6]. Previous phytochemical investigations of the genus have led to the identification of about 125 chemical constituents, including flavonoids, anthraquinones, phytosterols, sesquiterpenes, diterpenes, triterpenes, and caffeoylquinic acid derivatives [6].

Phagnalon sordidum L. is a perennial plant species distributed throughout the Mediterranean region, growing in rocky environments [10]. Phytochemical profiling of *P. sordidum* has shown the presence of several secondary metabolites, such as terpenes and flavonoids and particularly caffeoylquinic chlorogenic acid derivatives [11, 12]. In this study, an antioxidant-oriented approach was carried out, leading to the isolation and chemical characterization of 1 new natural compound, 5-*O*-*β*-D-glucopyranosyl-2-hydroxy-*p*-cymene (**1**), together with 11 known phenolic derivatives (**2**–**12**), from *P. sordidum* aerial parts. In order to more fully characterize the phytochemical profile of *P. sordidum*, liquid chromatography-high resolution electrospray ionization mass spectrometry (LC-HRESIMS) and tandem MS (LC-HRESIMS/MS) analyses were also performed. The antioxidant activity of extracts, fractions, and pure compounds was tested using 3 different complementary assays: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and inhibition of lipid peroxidation by the *β*-carotene bleaching (BCB) test. In addition, the relative antioxidant capacity indexes (RACI) were calculated; as dimensionless values they were useful in comparing outcomes from the different assays. The protective effect of extracts and pure compounds against H_2O_2-induced oxidative stress in the HepG2 (human hepatocellular carcinoma) cell line was also evaluated.

Results and Discussion

In this study the aerial parts of *P. sordidum* were extracted using solvents of increasing polarity. All extracts were subjected to the ABTS, DPPH, and BCB assays to screen for antioxidant activity. ABTS radical cation decolorization test and DPPH (neutral radical) test were both spectrophotometric methods, applicable for both lipophilic and hydrophilic compounds, and widely used for the assessment of radical scavenging activity. Results from the ABTS and DPPH assays were expressed as milligrams of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents per gram of extract/fraction (mg TE/g). In addition, the BCB test measured the effectiveness of antioxidant compounds present in the samples in binding the radical derived from linoleic acid and preventing the destruction of the conjugated system. *β*-carotene was used for comparative purposes, with results being expressed as percentage of antioxidant activity (%AA) at a final sample concentration of 0.1 mg/mL.

The petroleum ether and chloroform extracts were inactive (data not shown). By contrast, the ethyl acetate (EtOAc) extract showed antioxidant activity (ABTS assay, 1485.00 ± 36.78 mg TE/g; DPPH assay, 999.02 ± 3.76 mg TE/g; and AA in BCB assay 18.89 ± 0.29 %) and was a good source of phenolics followed by the *n*-butanol extract (**Table 1S**).



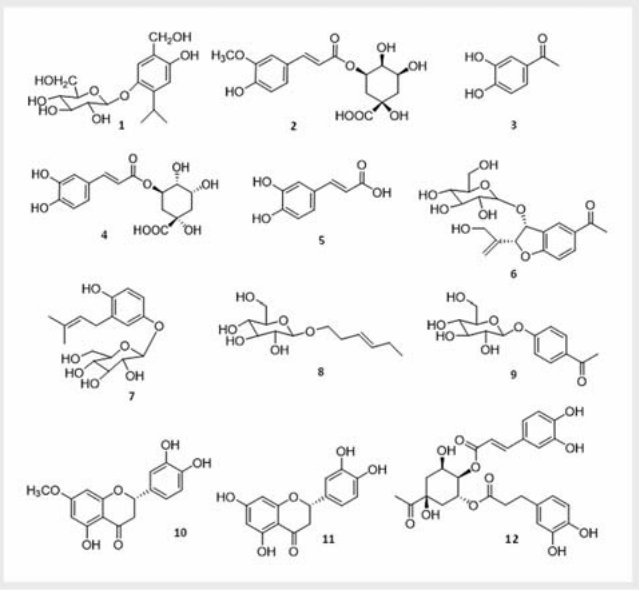
▶ **Fig. 1** RACI of fractions derived from the EtOAc extract of *P. sordidum*.

As the EtOAc extract showed the strongest antioxidant activity, it was further analyzed. Sephadex LH-20 column chromatography (CC) was used for the preliminary fractionation, and 7 major fractions (**A**–**G**) were collected and evaluated for their antioxidant activities (▶ **Fig. 1**, **Table 1S**). The fraction A was inactive in the antioxidation assays (data not shown). In the ABTS assay, fractions F and C were the most active, with the highest radical-scavenging activity (**Table 1S**). These results correlated with those of the DPPH method. In fact, fractions D and F showed the highest radical-scavenging activities against the DPPH radical, followed by fraction C (**Table 1S**). These results suggested that the most active samples with regard inhibiting lipid peroxidation were fractions F, G, D, and C (**Table 1S**). The lowest activity was found in fraction E for all the assays.

The data from the ABTS, DPPH, and BCB assays were used to calculate RACI. This recently introduced statistical approach was used to compare antioxidant capacities measured by different chemical methods, which, as in this study, were often expressed in different units [13]. This dimensionless index measured the distance between the average of the results obtained and the raw data expressed in standard deviation units. The interpretation of the values obtained with the RACI is presented in ▶ **Fig. 1**, with higher values representing greater antioxidant capacity.

Fraction F showed the highest anti oxidation rate (1.14), followed by fractions D (0.54) and C (0.45). By contrast, fraction E presented the lowest index (− 1.42) and, therefore, the smallest antioxidant activity. The results identified *P. sordidum* fractions B–G as active for antioxidation, and these were further analyzed. Reverse phase HPLC was used for the isolation of pure compounds, which might possibly be responsible for the antioxidant activity of the fraction (▶ **Fig. 2**, **Table 1**).

Compound **1** was obtained as a yellow powder. IHRESIMS analysis exhibited a molecular ion at *m/z* 367.1360 $[M + Na]^+$, indicating a molecular formula $C_{16}H_{24}O_8$. A product ion detected by its ESIMS spectrum at *m/z* 205 $[M + Na - 162]^+$ suggested the presence of a hexose unit. The ^1H NMR, ^{13}C NMR spectra (▶ **Table 1**) of compound **1** indicated, besides signals attributable to 1 sugar moiety, the presence of an 1,2,4,5 tetrasubstituted aromatic ring, and 4 sp^3 carbons, including 2 methyls, 1 hydroxymethylene,



▶ **Fig. 2** Chemical structure of pure compounds **1**–**12**.

and 1 methine carbon. Double-quantum filtered correlation spectroscopy, selective 1D-TOCSY, and heteronuclear single quantum coherence spectroscopy (HSQC) analysis of compound **1** established the presence of an isopropyl group showing connectivities, H-7—Me-9 and H-7—Me-8, indicative of an aromatic monoterpenoid. The cymene skeleton was confirmed by the presence in the ^{1}H NMR spectrum of signals at δ 3.50 (1H, m,), 1.19 (3H, d, J = 6.5 Hz), and 1.20 (3H, d, J = 6.5 Hz), characteristic of a isopropyl ring [14]. Chemical shifts, signal multiplicities, and J-values in the ^{1}H NMR spectrum and ^{13}C NMR chemical shifts indicated the presence of a β-glucopyranosyl moiety. The sugar moiety was determined to be a D enantiomer by hydrolysis, trimethylsilylation, and GC analysis [15]. The chemical shift assignments of the carbon atoms were established from the HSQC and HMBC spectra. Key HMBC correlations were observed between H-7—C-1, H-7—C-1, H-7—C-9; H-3—C-1, H-3—C-5; H-6—C-10, H-6—C-1; H-6—C-4; and H-1$_{glc}$—C-1 and located the glucopyranosyl moiety at C-1. Therefore, the structure of compound **1** was 5-*O*-β-D-glucopyranosyl-2-hydroxy-*p*-cymene, a new natural product. In addition, 3-*O*-feruloyl quinic acid (**2**) [16], 3,4-dihydroxyacetophenone (**3**) [17], 3-*O*-caffeoyl quinic acid (**4**) [18], caffeic acid (**5**) [19], gnaphaliol 3-*O*-β-D-glucopyranoside (**6**) [20], nebrodenside A (**7**) [21, 22], ergiside B (**8**) [23], picein (**9**) [24], eryodictiol-7-*O*-methyl ether (**10**) [25], eryodictiol (**11**) [25], and 3,4-*O*-dicaffeoyl quinic acid (**12**) [25] were isolated and characterized.

Analyses of the liquid chromatography coupled with electrospray ionization hybrid linear ion trap quadrupole Orbitrap mass spectrometry (LC-ESI/LTQOrbitrap/MS) profile of the metabolites identified in the initial EtOAc extract of *P. sordidum* is reported in ▶ **Table 2**. This revealed the presence of 12 compounds (**1**–**12**), identified by comparing their *m/z* values in the total ion current with those described in literature [26–29]. ▶ **Table 3** reports the content of phenolic compounds (mg/kg) found in the EtOAc extract of the aerial parts of *P. sordidum*.

▶ **Table 1** ^{1}H and ^{13}C NMR data of compound **1** (MeOH-d_4, 600 MHz)[a].

Position	1	
	δ_H	δ_C
1	–	147.9
2	–	139.4
3	6.70; s	113.0
4	–	150.6
5	–	125.5
6	7.12; s	116.0
7	3.50; m	26.0
8	1.19; d (6.5)	23.0
9	1.20; d (6.5)	23.0
10	4.60; s	60.0
1′	4.76; d (8.0)	104.0
2′	3.42; br t (8.5)	74.0
3′	3.37; t (9.0)	77.0
4′	3.42; t (9.0)	70.0
5′	3.46; m	78.8
6a'	3.72; dd (11.5, 5.0)	61.4
6b'	3.92; dd (11.5, 3.0)	

[a] Data assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

All pure compounds were investigated using the same antioxidation assays described above; however, the newly identified compound **1** was not tested as it was isolated in very small amounts. Compound **11** in fraction G presented the highest RACI value (1.03), followed by compounds **10**, **5**, **12**, and **7** (▶ **Fig. 3**). These results explained the antioxidant activity of their original fractions. Compounds **10** and **12** came from antioxidant fraction F, which had the highest activity, while compound **7** came from fraction D and compound **5** from fraction C, consistent with previous results [21, 30, 31]. By comparison, compound **9** had the lowest index (− 1.28) and came from the fraction with the lowest activity, E.

With its phytochemical profile and antioxidant activity, the EtOAc extract of *P. sordidum* was assessed in a cell model. There was no cytotoxicity for a HepG2 cell line (▶ **Fig. 4a**), even at the highest concentration of the EtOAc extract after 48 h of treatment. Moreover, to confirm the results obtained from the *in vitro* antioxidation assays, intracellular levels of reactive oxygen species (ROS) were measured. For this test, 2′,7′–dichlorofluorescin diacetate (DCFH-DA), a nonfluorescent cell permeable probe, was used. In the presence of intracellular ROS, DCF-DA was oxidized, with conversion to DCFH, the florescence of which was proportional to the concentration of ROS in the cells. H_2O_2 treatment significantly increased ROS in HepG2 cells; however, treatment with the EtOAc extract decreased their levels to approximately those of the control (CTRL, ▶ **Fig. 4b**).

Most of the identified compounds had previously been demonstrated to have antioxidant activity on cells [30, 32–39]; however, the current study was the first report of the effect of compounds **3**

▶ **Table 2** Metabolites identified in *P. sordidum* EtOAc extract by LC-ESI/LTQOrbitrap/MS and LC-ESI/LTQOrbitrap/MS/MS analysis.

n	Retention time (min)	Molecular Formula	Precursor ion (*m/z*)	Polarity	Product ions (*m/z*)	Identity
1	1.24	$C_{14}H_{18}O_7$	297.2687	Negative	279.06; 177.01	picein (**9**)
2	1.83	$C_{16}H_{24}O_8$	367.1360	Positive	205.14	5-*O*-*β*-D-glucopyranosyl-2-hydroxy-*p*-cymene (**1**)
3	2.75	$C_9H_8O_4$	179.0389	Negative	161.3; 135.1; 133.2; 116.91; 97.02	caffeic acid (**5**)
4	2.86	$C_{17}H_{20}O_9$	367.1718	Negative	349.11; 323.10; 291.17; 193.02; 132.99	3-*O*-feruloyl quinic acid (**2**)
5	4.48	$C_{17}H_{24}O_7$	339.2287	Negative	320.95; 177.06; 160.99	nebrodenside A (**7**)
6	5.4	$C_{16}H_{18}O_9$	353.2336	Negative	191.02; 179.05; 173.03; 160.6;134.9	3-*O*-caffeoyl quinic acid (**4**)
7	5.47	$C_{25}H_{24}O_{12}$	515.1786	Negative	497.10; 353.10; 191.01; 178.98; 161.12	3,4-*O*-dicaffeoyl quinic acid (**12**)
8	8.31	$C_{12}H_{22}O_6$	261.1952	Negative	n. s.	ergiside B (**8**)
9	8.6	$C_8H_8O_3$	151.0666	Negative	n. s.	3,4-dihydroxyacetophenone (**3**)
10	8.66	$C_{15}H_{12}O_6$	287.2121	Negative	269.02; 243.01; 202.89; 161.08; 150.92; 106.89	eriodictyol (**11**)
11	8.92	$C_{16}H_{14}O_6$	301.1706	Negative	283.01; 239.02; 193.02; 178.94; 150.94	eriodictyol-7-methyl ether (**10**)
12	10.36	$C_{19}H_{26}O_{10}$	413.2795	Negative	n. s.	gnaphaliol 3-*O*-*β*-D-glucopyranoside (**6**)

n. s.: no signal

and **7** on HepG2 cells. Compounds **3** and **7** were tested at different concentrations (▶ **Fig. 5a** and **Fig. 5b**, respectively). Only the highest concentration of the tested pure compounds showed cytotoxicity after 48 h of treatment. Notably, HepG2 treated with these pure compounds also showed a significant decrease in intracellular ROS (▶ **Fig. 5c**). For *in vitro* antioxidant activity, compound **7** had a higher RACI than compound **3** (▶ **Fig. 3**). The full procedure for the analysis of antioxidation in extracts from the aerial parts of *P. sordidum* is given in ▶ **Fig. 6**. Taken together, the data indicated that fractions and pure compounds from *P. sordidum* might prevent the formation of ROS in cells.

^{1}H NMR, HSQC, HMBC, 1D-TOCSY, and HRESIMS spectra of compound **1**, the LC–MS profile of the EtOAc extract, and antioxidant activities of *P. sordidum* extracts, fractions, and pure compounds are available as Supporting Information.

▶ **Table 3** Compound contents (expressed as mg/kg) in the EtOAc extract from *P. sordidum*.

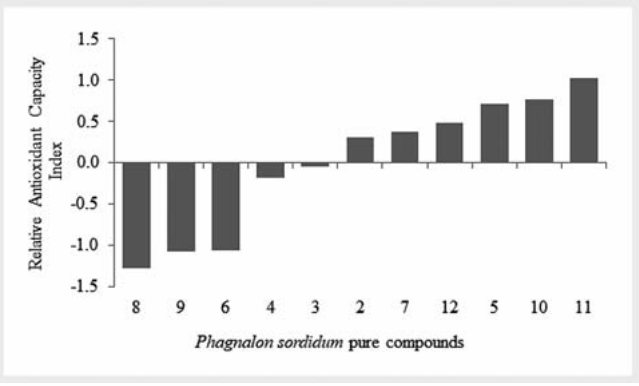
Compound	Mean ± SD
picein (**9**)	78.4 ± 3.3
5-*O*-*β*-D-glucopyranosyl-2-hydroxy-*p*-cymene (**1**)	27.3 ± 1.1
caffeic acid (**5**)	81.8 ± 2.6
3-*O*-feruloyl quinic acid (**2**)	215.8 ± 11.2
nebrodenside A (**7**)	179.1 ± 9.1
3-*O*-caffeoyl quinic acid (**4**)	121.7 ± 4.8
3,4-*O*-dicaffeoyl quinic acid (**12**)	868.4 ± 13.1
ergiside B (**8**)	55.5 ± 2.7
3,4-dihydroxyacetophenone (**3**)	35.6 ± 1.5
eriodictyol (**11**)	33.0 ± 1.4
eriodictyol-7-methyl ether (**10**)	29.8 ± 1.1
gnaphaliol 3-*O*-*β*-D-glucopyranoside (**6**)	trace[a]

[a] Minimum value not calculated.

Materials and Methods

Chemicals and reagents

Solvents used for LC-ESI/LTQOrbitrap/MS, and for the extractions were purchased from VWR, while acetonitrile and formic acid were purchased from Merck. Chloroform, MeOH, EtOAc, *n*-butanol, isopropanol, DMSO, and orthophosphoric acid were pur-



▶ **Fig. 3** RACI of pure compounds derived from *P. sordidum*.

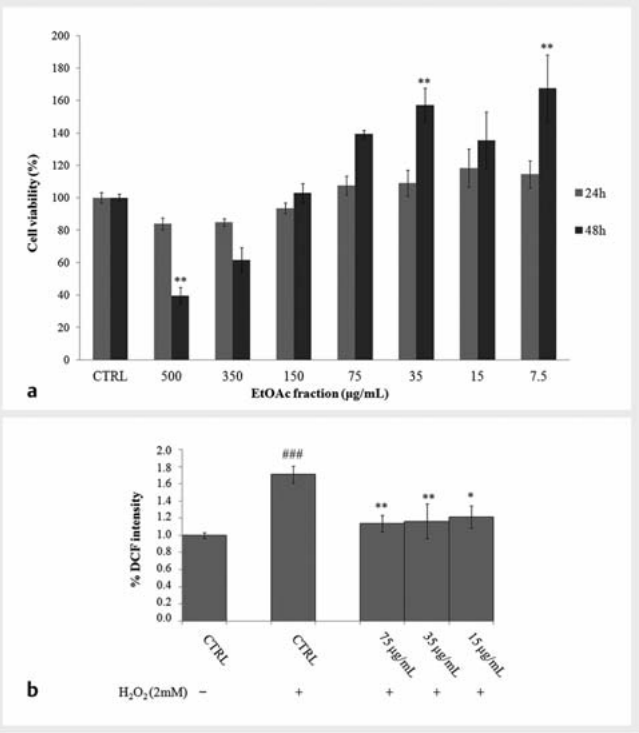
chased from Carlo Erba Reagents. The following were purchased from Sigma-Aldrich: ABTS, potassium persulfate, DPPH, β-carotene, linoleic acid, Tween 20, DCFH-DA, DMEM, glutamine, penicillin, streptomycin, hydrogen peroxide, PBS, MTT, Trolox, ascorbic acid, butylhydroxytoluene (BHT), D-glucose, FBS, and 1-(trimethylsilyl)imidazole and pyridine.

General experimental procedures

Optical rotations were measured on an Atago AP-300 digital polarimeter equipped with a sodium lamp (589 nm) and a 1-dm microcell. Briefly, NMR spectroscopy involved recording at 300 K in CD_3OD on a Bruker DRX-500 spectrometer (Bruker BioSpin GmbH) [19]. HRESIMS was performed in a positive ion mode on a quadrupole time-of-flight premier mass spectrometer (Waters). ESI-MS spectra were obtained from an LCQ Advantage Thermo-Finnigan spectrometer (ThermoFinnigan), equipped with Xcalibur software. CC was carried out over Sephadex LH-20 (40–70 µm; Pharmacia). Reverse-phase HPLC (RP-HPLC) was conducted on a Shimadzu LC-8A series pumping system, equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C-18 Luna column (250 × 10 mm, 10 µm; Phenomenex). TLC was with silica gel 60 F_{254} (0.20 mm thickness) plates (Merck) and cerium sulphate (Sigma-Aldrich) as the spray reagent [19]. LC-ESI/LTQOrbitrap/MS analyses were performed on a Accela 600 HPLC system (Thermo Scientific) coupled to a LTQ Orbitrap XL mass spectrometer (Bremen). Separation was achieved using a Luna 2.50 µm C18 (100 mm × 2.10 mm) column (Phenomenex). HepG2 cells were analyzed on a FACS Canto II flow cytometer (BD Pharmingen). All spectrophotometric measurements were taken in 96-well microplates on a SPECTROstar^{Nano} UV/Vis spectrophotometer (BMG Labtech), and each reaction was performed in triplicate.

Plant material

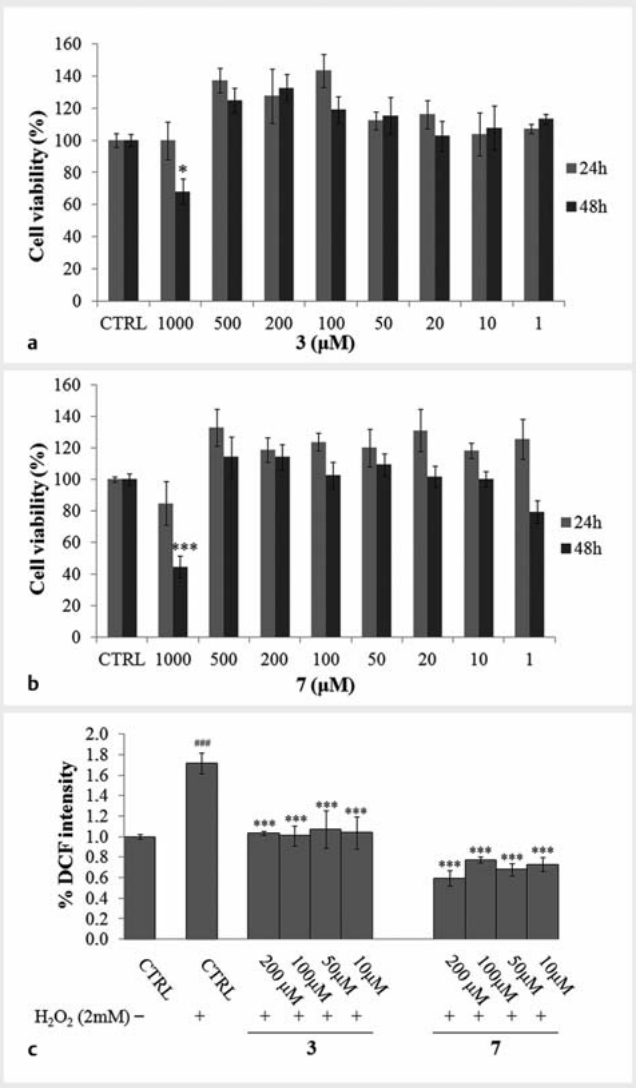
The aerial parts of *P. sordidum* were collected in May 2016 from Guelma (eastern Algeria). The plant was authenticated by Mr. Kamel Kabouche. A voucher specimen (LOST Phs05/16) was deposited at the herbarium of the Laboratory of Therapeutic Substances (LOST), Faculty of Sciences, Université des Frères Mentouri-Constantine 1, Algeria.



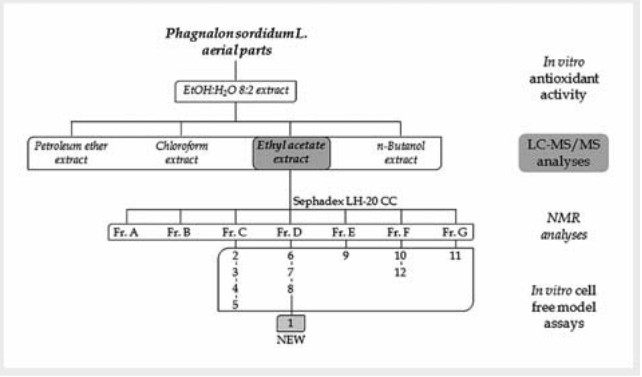
▶ **Fig. 4** **a** Cell viability, evaluated by MTT assay, of HepG2 cells treated for 24 and 48 h with different concentrations of EtOAc extract from *P. sordidum* aerial parts. Data are expressed as the mean ± SD of 3 independent experiments (n = 3). $^{**}p < 0.01$ vs. CTRL. **b** Effect of EtOAc extract on H_2O_2-induced intracellular ROS generation in HepG2 cells stained with DCFH-DA and analyzed by flow cytometry. Data are expressed as the mean ± SD of 3 independent experiments (n = 3). $^{###}p < 0.001$ vs. CTRL; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. H_2O_2-treated cells.

Extraction and isolation

The powdered dried aerial parts (1.3 kg), dried at room temperature, of *P. sordidum* were macerated at room temperature in EtOH and water (8 : 2). The extract was concentrated under low pressure, and the residue (380.0 g) was dissolved with water (1 L). It was then filtered and successively extracted with petroleum ether, chloroform, EtOAc, and *n*-butanol (3 × 300 mL each) to yield 0.8, 1.8, 11.0, and 20.0 g of the respective residues. The EtOAc extract (2.5 g) was then submitted to a Sephadex LH-20 CC, using MeOH as eluent, obtaining 7 fractions (A–G) grouped by TLC. Fraction C (320.1 mg) was purified by RP-HPLC using MeOH-H_2O (30 : 70) to give compounds **2** (1.9 mg, t_R 14.0 min), **3** (1.4 mg, t_R 20.0 min), **4** (2.0 mg, t_R 42.0 min), and **5** (1.6 mg, t_R 49.0 min). Fraction D (815.0 mg) was subjected to RP-HPLC on a C-8 Luna column (250 × 10 mm, 10 µm) with MeOH-H_2O (2 : 3) as eluent to give compounds **1** (1.2 mg, t_R 16.5 min), **6** (2.1 mg, t_R 31.0 min), **7** (7.2 mg, t_R 45.0 min), and **8** (2.1 mg, t_R 70.0 min). RP-HPLC of fraction E (289.1 mg) on a C-8 Luna column (250 × 10 mm, 10 µm), with MeOH-H_2O (3 : 7) as eluent yielded compound **9** (4.2 mg, t_R 45.0 min). Fraction F (163.6 mg) underwent RP-HPLC on a C-18 Luna column (250 × 10 mm, 10 µm) with MeOH-H_2O (3 : 7) as eluent to give compounds **10** (2.5 mg, t_R 25.0 min) and



▶ **Fig. 5** **a** Cell viability, evaluated by MTT assay, of HepG2 cells treated for 24 and 48 h with different concentrations of pure compound **3** from *P. sordidum* aerial parts. Data are expressed as the mean ± SD of 3 independent experiments (n = 3). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. CTRL. **b** Cell viability, evaluated by MTT assay, of HepG2 cells treated for 24 and 48 h with different concentration of pure compound **7** from *P. sordidum* aerial parts. Data are expressed as the mean ± SD of 3 independent experiments (n = 3). $^{*}p < 0.01$ vs. CTRL. **c** Effects of pure compounds, **3** and **7**, on H_2O_2-induced intracellular ROS generation in HepG2 cells stained with DCFH-DA and analyzed by flow cytometry. Data are expressed as the mean ± SD of 3 independent experiments (n = 3). $^{###}p < 0.001$ vs. CTRL; $^{***}p < 0.001$ vs. H_2O_2-treated cells.



▶ **Fig. 6** Procedure followed to analyze the aerial parts of *P. sordidum*.

12 (1.8 mg, t_R 35.0). Finally, fraction G (30.0 mg) did not undergo further fractionation and was identified as compound **11**.

Compound ***1***: yellow amorphous powder; $[\alpha]_D$ − 23.1 (*c* 0.1, MeOH); ^1H and ^{13}C NMR data, see ▶ **Table 1**; ESIMS *m/z* 367 $[M + Na]^+$, 205 $[M + Na - 162]^+$; HRESIMS *m/z* 367.1360 $[M + Na]^+$ (calcd. for $C_{16}H_{24}O_8Na$, 367.1368).

Acid hydrolysis of compound **1** was carried out as previously described [40]. D-Glucose was identified as the sugar moiety by comparison of retention time with an authenticated sample of D-glucose (Sigma Aldrich), after treatment with 1-(trimethylsilyl) imidazole in pyridine.

LC-ESI/LTQOrbitrap/MS

To analyze the EtOAc extract of *P. sordidum*, an in-house HPLC method coupled with a mass spectrometer, associated with linear trap quadrupole and an Orbitrap mass analyzer, was used. LC-ESI/LTQOrbitrap/MS analyses were performed in positive and negative ion mode using an Accela 600 HPLC system (Thermo Scientific) coupled to an LTQ Orbitrap XL mass spectrometer. Separation was achieved using a Luna 2.5 µm C18 (100 mm × 2.10 mm) column (Phenomenex).

The mobile phases used were water + 0.1 % formic acid (solvent A) and acetonitrile (solvent B). The flow rate was 0.200 mL/min and the gradient was 20 % of B from 0 min to 1 min, 80 % at 21 min, 95 % at 22 min until 30 min, returning at 20 % of B at 31 min to 35 min.

For MS in positive ion mode, source voltage was 3 kV, capillary voltage 49 V, and tube lens voltage120 V; while in negative ion mode, source voltage was 5 kV, capillary voltage − 48 V, and tube lens voltage − 176.47. Capillary temperature for both positive and negative ion modes was 280 °C. MS spectra were acquired by full range acquisition covering *m/z* 150–1000.

Data were acquired using Xcalibur software version 2.1, and for fragmentation studies a data dependent scan experiment was carried out selecting precursor ions as the most intensive peak in LC-MS analysis. Identification of compounds was based on retention times, accurate mass measurements, MS/MS data, exploration of specific spectral libraries and public repositories for MS-based metabolomic analysis [27, 29], and comparison with data reported in the literature [21, 22, 26, 28, 41].

Antioxidant activity

DPPH assay

The radical scavenging activity of the samples was evaluated *in vitro* using the neutral radical DPPH and Trolox as a standard. Fifty microliters of different dilutions of sample or standard were added to 200 µL of a methanolic solution of DPPH in a 96-well plate. After 30 min in the dark at room temperature, the absorb-

ance of the mixtures was measured at 515 nm. The results were expressed as milligrams of Trolox equivalents per gram of dried extract (mg TE/g) [42].

ABTS assay

The antioxidant capacity of samples was also studied using the cationic 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt) ($ABTS^{\bullet}$) radical [42]. Fifteen microliters of each sample were added to 235 µL of $ABTS^{+\bullet}$ solution, and the mixture was incubated in the dark for 2 h. Absorbance was then measured at 734 nm. Trolox was used as a standard, and results were expressed as milligrams of Trolox equivalents per gram of dried extract (mg TE/g).

Inhibition of lipid peroxidation

The BCB method [13] was used to evaluate the inhibition of lipid peroxidation. To an Eppendorf tube containing 50 µL of 0.1 mg/mL sample, 950 µL of *β*-carotene emulsion was added. From this mixture, 250 µL was transferred into a 96-well plate and incubated at 50 °C for 3 h. Absorbance was measured at 470 nm at 0′, 30′, 60′, 90′, 120′, 150′, and 180′ of incubation, with BHT being used as a positive control. Results were expressed as percentage of antioxidant activity (%AA) on the basis of BCB inhibition.

Cell culture

The HepG2 cell line has been used as a cellular model for testing the effectiveness of extracts or pure compounds. Cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin in a humidified 5% CO_2 incubator at 37 °C. The EtOAc extract and pure compounds **3** and **7** were dissolved in DMSO and different concentrations of each were tested. The final DMSO concentration in the samples (< 1%) had no effect on HepG2 cell viability. DMSO-treated cells were used as a control (CTRL) in all the experiments.

Cell viability analysis

HepG2 cell viability was evaluated by MTT assay, a colorimetric assay based on the conversion of the yellow tetrazolium salt MTT into purple insoluble formazan by the succinate dehydrogenase enzyme of viable cells. The EtOAc extract and pure compounds **3** and **7** were dissolved in DMSO and different concentrations of each were tested. The final DMSO concentration in the samples (1%) had no effect on HepG2 cell viability. DMSO-treated cells were used as a control (CTRL) in all the experiments.

HepG2 cells were seeded in 96-well plate (10^4 cells/well), incubated overnight, and treated with different sample concentrations for 24 and 48 h. After removal of the medium, the cells were washed with PBS and incubated with 0.75 mg/mL MTT in PBS for 4 h [43]. The solution was then removed and the cells were lysed using a solubilization solution of DMSO : isopropanol (1 : 1). The solubilized formazan product was spectrophotometrically quantified at 560 nm using a Multiskan GO (Thermo Scientific).

Measurement of intracellular ROS

Intracellular ROS levels were measured with DCFH-DA as previously described [43, 44]. Briefly, HepG2 cells were plated at a density of 2×10^5 cells/well in 24-well plate. They were incubated with different concentrations of fractions and pure compounds **3** and **7** for 24 h and then treated with 2 mM H_2O_2 for 1 h. Finally, the cells were stained with 10 µM DCFH-DA for 30 min at 37 °C in the dark, and fluorescence was measured with a FACSCanto II flow cytometer (BD Pharmingen) at an excitation wavelength of 485 nm and emission wavelength of 515–540 nm.

Statistical analysis

Data was subjected to analysis of variance (one-way analysis of variance) followed by Tukey's test, using GraphPad Prism 5 Software. A p-value ≤ 0.05 was considered statistically significant. All assays were performed in triplicate as 3 independent experiments. Data were reported as means ± standard deviation (SD). The R^2 values for all calibration curves were over 0.99.

Supporting Information

^{1}H NMR, HSQC, HMBC, 1D-TOCSY, and HRESIMS spectra of compound **1**, the LC–MS profile of the EtOAc extract, and antioxidant activities of *P. sordidum* extracts, fractions, and pure compounds are available as Supporting Information.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

[1] Sulaiman M, Tijani HI, Abubakar BM, Haruna S, Hindatu Y, Mohammed JN, Idris A. An overview of natural plant antioxidants: analysis and evaluation. Adv Biochem 2013; 1: 64–72

[2] Pratheeshkumar P, Son YO, Korangath P, Manu KA, Siveen KS. Phytochemicals in cancer prevention and therapy. Biomed Res Int 2015; 2015: 324021

[3] Kaur C, Kapoor HC. Antioxidants in fruits and vegetables – the millennium's health. Int J Food Sci Tech 2001; 36: 703–725

[4] Mangoni O, Imperatore C, Tomas CR, Costantino V, Saggiomo V, Mangoni A. The new carotenoid pigment moraxanthin is associated with toxic microalgae. Mar Drugs 2011; 9: 242–255

[5] Safer AM, al-Nughamish AJ. Hepatotoxicity induced by the anti-oxidant food additive, butylated hydroxytoluene (BHT), in rats: an electron microscopical study. Histol Histopathol 1999; 14: 391–406

[6] Zheng X, Wang W, Piao H, Xu W, Shi H, Zhao C. The genus *Gnaphalium* L. (Compositae): phytochemical and pharmacological characteristics. Molecules 2013; 18: 8298–8318

[7] Funk VA, Susanna A, Steussy TF, Robinson HE. Classification of Compositae. In: Funk VA, Susanna A, Stuessy TF, Bayer RJ, eds. Systematics, Evolution, and Biogeography of Compositae. Vienna: International Association for Plant Taxonomy (IAPT); 2009: 171–189

[8] Guan PC, Liu HC, Luo GY. The classification and utilization of wild vegetables resources in Guangdong. J South China Agric Univ 2000; 21: 7–11

[9] Li GP, Liu JQ. The exploration and utilization of wild vegetable resources in Fujian province. J Southwest Agric Univ 1999; 21: 433–437

[10] Kirschnerová L, Kirschner J. A nomenclatural and taxonomic account of *Willemetia* (Compositae, Lactuceae, Crepidinae). Taxon 1996; 45: 627–630

[11] Brunel M, Vitrac C, Costa J, Vitrac X, Muselli A. Subcritical water extraction of antioxidant compounds from *Phagnalon sordidum* (L.). 14th European Meeting on Supercritical Fluids. Marseille, France; 2014

[12] Epifano F, Marcotullio MC, Menghini L. Constituents of *Phagnalon sordidum*. Chem Nat Compd 2002; 38: 204–205

[13] Dekdouk N, Malafronte N, Russo D, Faraone I, De Tommasi N, Ameddah S, Severino L, Milella L. Phenolic compounds from *Olea europaea* L. possess antioxidant activity and inhibit carbohydrate metabolizing enzymes *in vitro*. Evid Based Complement Alternat Med 2015; 2015: 684925

[14] Castillo UF, Wilkins AL, Lauren DR, McNaughton DE, Smith BL. Structure elucidation of 1, 4-dihydroxy-2-iso-propyl-5-methylphenyl-1-*O*-*β*-glucopyranoside, a constituent of *Pteridium aquilinum* var. *caudatum*. J Nat Prod 1995; 58: 1889–1891

[15] Dal Piaz F, Vera Saltos MB, Franceschelli S, Forte G, Marzocco S, Tuccinardi T, Poli G, Nejad Ebrahimi S, Hamburger M, De Tommasi N, Braca A. Drug affinity responsive target stability (DARTS) identifies laurifolioside as a new clathrin heavy chain modulator. J Nat Prod 2016; 79: 2681–2692

[16] Spreng S, Hofmann T. Activity-guided identification of *in vitro* antioxidants in beer. J Agric Food Chem 2018; 66: 720–731

[17] Trimpin S, Lu IC, Rauschenbach S, Hoang K, Wang B, Chubatyi ND, Zhang WJ, Inutan ED, Pophristic M, Sidorenko A, McEwen CN. Spontaneous charge separation and sublimation processes are ubiquitous in nature and in ionization processes in mass spectrometry. J Am Soc Mass Spectrom 2018; 29: 304–315

[18] Galani JHY, Patel JS, Patel NJ, Talati JG. Storage of fruits and vegetables in refrigerator increases their phenolic acids but decreases the total phenolics, anthocyanins and vitamin C with subsequent loss of their antioxidant capacity. Antioxidants 2017; 6: 59

[19] Aquino R, Peluso G, De Tommasi N, De Simone F, Pizza C. New polyoxypregnane ester derivatives from *Leptadenia hastata*. J Nat Prod 1996; 59: 555–564

[20] Sahakitpichan P, Disadee W, Ruchirawat S, Kanchanapoom T. 3-Hydroxydihydrobenzofuran glucosides from *Gnaphalium polycaulon*. Chem Pharm Bull 2011; 59: 1160–1162

[21] Muhammad A, Tel-Çayan G, Öztürk M, Duru ME, Nadeem S, Anis I, Ng SW, Shah MR. Phytochemicals from *Dodonaea viscosa* and their antioxidant and anticholinesterase activities with structure-activity relationships. Pharm Biol 2016; 54: 1649–1655

[22] Muhammad A, Anis I, Ali Z, Awadelkarim S, Khan A, Khalid A, Shah MR, Galal M, Khan IA, Choudhary MI. Methylenebissantin: a rare methylene-bridged bisflavonoid from *Dodonaea viscosa* which inhibits *Plasmodium falciparum* enoyl-ACP reductase. Bioorg Med Chem Lett 2012; 22: 610–612

[23] Góngora L, Giner RM, Máñez S, del Carmen Recio M, Ríos JL. New prenylhydroquinone glycosides from *Phagnalon rupestre*. J Nat Prod 2001; 64: 1111–1113

[24] Dommisse RA, Van Hoof L, Vlietinck AJ. Structural analysis of phenolic glucosides from *Salicaceae* by NMR spectroscopy. Phytochemistry 1986; 25: 1201–1204

[25] Milella L, Milazzo S, De Leo M, Vera Saltos MB, Faraone I, Tuccinardi T, Lapillo M, De Tommasi N, Braca A. *α*-Glucosidase and *α*-amylase inhibitors from *Arcytophyllum thymifolium*. J Nat Prod 2016; 79: 2104–2112

[26] Aversano R, Contaldi F, Adelfi MG, D'Amelia V, Diretto G, De Tommasi N, Vaccaro C, Vassallo A, Carputo D. Comparative metabolite and genome analysis of tuber-bearing potato species. Phytochemistry 2017; 137: 42–51

[27] Horai H, Arita M, Kanaya S, Nihei Y, Ikeda T, Suwa K, Ojima Y, Tanaka K, Tanaka S, Aoshima K, Oda Y, Kakazu Y, Kusano M, Tohge T, Matsuda F, Sawada Y, Hirai MY, Nakanishi H, Ikeda K, Akimoto N, Maoka T, Takahashi H, Ara T, Sakurai N, Suzuki H, Shibata D, Neumann S, Iida T, Tanaka K, Funatsu K, Matsuura F, Soga T, Taguchi R, Saito K, Nishioka T. MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom 2010; 45: 703–714

[28] Tuberoso CIG, Serreli G, Montoro P, D'Urso G, Congiu F, Kowalczyk A. Biogenic amines and other polar compounds in long aged oxidized Vernaccia di Oristano white wines. Food Res Int 2018; 111: 97–103

[29] Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, Sayeeda Z, Lo E, Assempour N, Berjanskii M, Singhal S, Arndt D, Liang Y, Badran H, Grant J, Serra-Cayuela A, Liu Y, Mandal R, Neveu V, Pon A, Knox C, Wilson M, Manach C, Scalbert A. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 2018; 46: D608–D617

[30] Walker J, Reichelt KV, Obst K, Widder S, Hans J, Krammer GE, Ley JP, Somoza V. Identification of an anti-inflammatory potential of *Eriodictyon angustifolium* compounds in human gingival fibroblasts. Food Funct 2016; 7: 3046–3055

[31] Hong S, Joo T, Jhoo JW. Antioxidant and anti-inflammatory activities of 3, 5-dicaffeoylquinic acid isolated from *Ligularia fischeri* leaves. Food Sci Biotechnol 2015; 24: 257–263

[32] Joo TW, Hong SH, Park SY, Kim GY, Jhoo JW. Antioxidant effects of eriodictyol on hydrogen peroxide-induced oxidative stress in HepG2 cells. J Korean Soc Food Sci Nutr 2016; 45: 510–517

[33] Mokdad-Bzeouich I, Mustapha N, Sassi A, Bedoui A, Ghoul M, Ghedira K, Chekir-Ghedira L. Investigation of immunomodulatory and anti-inflammatory effects of eriodictyol through its cellular anti-oxidant activity. Cell Stress Chaperones 2016; 21: 773–781

[34] Taguchi N, Hata T, Kamiya E, Kobayashi A, Aoki H, Kunisada T. Reduction in human hair graying by sterubin, an active flavonoid of *Eriodictyon angustifolium*. J Dermatol Sci 2018; 92: 286–289

[35] Baeza G, Amigo-Benavent M, Sarriá B, Goya L, Mateos R, Bravo L. Green coffee hydroxycinnamic acids but not caffeine protect human HepG2 cells against oxidative stress. Food Res Int 2014; 62: 1038–1046

[36] Góngora L, Giner RM, Máñez S, del Carmen Recio M, Schinella G, Ríos JL. Effects of caffeoyl conjugates of isoprenyl-hydroquinone glucoside and quinic acid on leukocyte function. Life Sci 2002; 71: 2995–3004

[37] Peluso G, De Feo V, De Simone F, Bresciano E, Vuotto ML. Studies on the inhibitory effects of caffeoylquinic acids on monocyte migration and superoxide ion production. J Natural Prod 1995; 58: 639–646

[38] Nakajima Y, Tsuruma K, Shimazawa M, Mishima S, Hara H. Comparison of bee products based on assays of antioxidant capacities. BMC Complement Altern Med 2009; 9: 4

[39] Granado-Serrano AB, Angeles Martín M, Izquierdo-Pulido M, Goya L, Bravo L, Ramos S. Molecular mechanisms of (−)-epicatechin and chlorogenic acid on the regulation of the apoptotic and survival/proliferation pathways in a human hepatoma cell line. J Agric Food Chem 2007; 55: 2020–2027

[40] De Leo M, Peruzzi L, Granchi C, Tuccinardi T, Minutolo F, De Tommasi N, Braca A. Constituents of *Polygala flavescens* ssp. *flavescens* and their activity as inhibitors of human lactate dehydrogenase. J Nat Prod 2017; 80: 2077–2087

[41] Cottiglia F, Bonsignore L, Casu L, Deidda D, Pompei R, Casu M, Floris C. Phenolic constituents from *Ephedra nebrodensis*. Nat Prod Res 2005; 19: 117–123

[42] Faraone I, Rai DK, Chiummiento L, Fernandez E, Choudhary A, Prinzo F, Milella L. Antioxidant activity and phytochemical characterization of *Senecio clivicolus* Wedd. Molecules 2018; 23: 2497

[43] Russo D, Miglionico R, Carmosino M, Bisaccia F, Andrade PB, Valentão P, Milella L, Armentano MF. A comparative study on phytochemical profiles and biological activities of *Sclerocarya birrea* (A. Rich.) Hochst leaf and bark extracts. Int J Mol Sci 2018; 19: 186

[44] Miglionico R, Ostuni A, Armentano MF, Milella L, Crescenzi E, Carmosino M, Bisaccia F. *ABCC6* knockdown in HepG2 cells induces a senescent-like cell phenotype. Cell Mol Biol Lett 2017; 22: 7