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Scavenging Effect of Various Extracts of the *Gymnema sylvestre* R. Br. and Antioxidant Activity of the Isolated Triterpenes

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Abstract: *Gymnema sylvestre* has been used in Asian traditional medicine for its anti-microbial, anti-hypercholesterolemic, hepatoprotective and sweet suppressing properties and activities. *G. sylvestre* has also been used extensively in chewing gum, as a health food for preventing obesity and diabetes, and as a tea. This study has evaluated the total phenolic content and antioxidant activity of the aqueous and organic *G. sylvestre* extracts and their sub-fractions for the initial characterization of the biological properties of the isolated compounds. An *in vivo* cell model was used to calculate the concentration inhibiting cell growth by 50% and the ability to exert antioxidant activity. All compounds inhibit cell growth in a dose-dependent manner, with an IC_{50} value ranging between 29 and 1462 μM. The effects on intracellular ROS levels are extremely variable, but it is of interest that some of the compounds appear to display an antioxidant effect.

Keywords: *Gymnema sylvestre*; antioxidants; gymnemic acids; herbal drug; DCFH-DA assay.

1. Introduction

Gymnema sylvestre R. Br. belongs to the family Asclepiadaceae, it is a perennially growing woody creeping herb native to India that is distributed in China, Japan, tropical Africa and Australia, and in dry forests of low-altitude hills. In India, it is normally known as ‘Gur-ma,’ which means "destroyer of sugar" and is used as a medicinal plant in folk medicine [1].

The herb, showing a broad range of therapeutic effects, is used in the treatment of asthma, eye complaints, arthritis, anaemia, osteoporosis, hypercholesterolemia, cardiopathy, constipation, dyspepsia, urinary complaints, haemorrhoids, microbial infections, indigestion, inflammation, and in the treatment of snakebites, stomach problems, chronic cough, breathing difficulty, and colic pain [2–3]. In addition, *G. sylvestre* possesses hepatoprotective and sweet suppressing activities [4–6].

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Furthermore, *G. sylvestre* is able to act against the caterpillar *Prodenia eridania*, to counteract the cariogenic action of –*Streptococcus mutans* and serves as an ingredient in skin cosmetics [7].It has been proven that the leaves of *G. sylvestre* cause hypoglycaemia in laboratory animals and if used in herbal medicine they help in the treatment of adult onset diabetes mellitus. In addition to a series of compounds such as alkaloids, flavones, anthraquinones, resins, stigmasterols and dammarenes, *G. sylvestre* leaves contain oleanane triterpene saponins, which are generally known as gymnemic acids [8–9]. Gymnemic acids have anti-diabetic [10], anti-sweet [11] and anti-inflammatory activities. They have also been found useful against obesity for their ability to delay glucose absorption in the blood and to inhibit intestinal sugar absorption and insulin secretion; these properties have led to the use of *G. sylvestre* leaves to obtain tea-type beverages or their extracts for chewing gum and for the prevention of obesity and diabetes [12–13]. Although commonly misidentified due to the hundreds of articles on *gymnemic acid* describing its isolation and the determination of its biological activities, *gymnemic acid* is a non-existent molecule to which we should never refer [14]. Following a guided fractionation approach, based on a classic test set (DPPH, Fenton method, Folin-Ciocalteau), we explored the antioxidant behaviour of the metabolites contained in the most active extracts, by the DCFH-DA assay. DCFH-DA test was chosen because it takes into account the solubility and the cellular uptake of the tested compounds, which are important variables when assessing their potential use in pharmacological approaches.

2. Materials and Methods

2.1. General Experimental Procedures

1D and 2D NMR experiments were performed on a Varian INOVA-500 FT NMR spectrometer ^{1}H at 499.710 MHz and ^{13}C at 125.663 MHz, in $CDCl_3$ or CD_3OD solutions, at 25°C, δ in ppm, J in Hz. Optical rotations were measured in MeOH or CH_2Cl_2 with a Perkin-Elmer 141 polarimeter. The IR spectra were recorded on a Jasco FT/IR-430 instrument. HPLC was performed on a Shimadzu LC-10AD by using a refractive index detector Shimadzu RID-10A. A semipreparative HPLC was performed using RP-18 LiChrospher (10 μm, 250 x 10 mm i.d, Merck) column with a flow rate of 1.2 mL/min. Column chromatography was carried out on Merck Kieselgel 60 (230−400 mesh) and preparative TLC was performed on silica gel (UV−254 precoated) plates with 0.5 and 1.0 mm thickness (Merck).

2.2. Plant Material

G. sylvestre was purchased from Mother Herbs Ltd., 13 Street, Madhu Vihar, Patpadganj, Delhi - 110 092, India, E-mail: info@motherherbs.com and identified by Prof. *Antonino Pollio* of the Department of Biology, University Federico II of Naples, Italy. A sample specimen (HERBNAWY 124) has been deposited at the herbarium of the same University

2.3. Extract Preparation

Dried and powdered aerial plant material (7.0 kg) was extracted with H_2O (25 L for 1 d) and successively with CH_2Cl_2 (20 L for 4 d). The CH_2Cl_2 extract was filtered, evaporated to complete dryness (350 g, percentage yield 5%) and fractionated into acidic and neutral fractions with aqueous 2N NaOH solution. The neutral fraction was debated with H_2O until to neutral pH, evaporated to complete dryness and chromatographed on silica gel with petroleum ether (PE), CH_2Cl_2, EtOAc, Acetone, MeOH and H_2O.

2.4. Determination of Total Phenolic Content

Aliquots of all extract or fractions (10 mg/mL) were added to a series of test tubes and the volume was adjusted to 3 mL with distilled water. Folin-Ciocalteau reagent (0.5 mL) was added to each tube and incubated for 3 min at 25°C. Sodium carbonate 20% (W/v) solution (2 mL) was added, mixed thoroughly, and incubated in the tubes for 1 min in a boiling water bath. The absorbance was

measured at 720 nm against a reagent blank. A standard curve using different concentrations of standard gallic acid was prepared (80 μg/mL). From the standard curve, the concentration of phenols in the test samples was in terms of gallic acid equivalents (GAE, mg/g of plant material). All determinations were performed in triplicate. The total phenolic content (TPC), in gallic acid equivalents, was calculated by the following equation:

$$TPC = (GAC \times W)/V$$

where is the concentration of gallic acid established from the standard curve (μg/mL), W is the weight of crude plant extract, and V is the volume of plant extract.

2.5. Estimation of DPPH· Scavenging Activity

Different concentrations (100, 200, 500 and 1000 μg) of extract and BHA were added to test tubes and the volume was adjusted to 0.5 mL with methanol. To these tubes, 0.1 mM methanolic DPPH· solution (5 mL) was added and shaken vigorously. A control sample without the test compound, but with an equivalent amount of methanol, was maintained. The tubes were allowed to stand at 25°C for 30 min. The absorbance of each supernatant was measured at 517 nm. All determinations were performed in triplicate. ŧThe following formula:

$$\% \text{ radical scavenging activity} = 100 \times (A_{control} - A_{sample}/A_{control})$$

provides the percent scavenging of DPPH·.

2.6. Estimation of ·OH Radical Scavenging

Various concentrations (100, 200, 500 and 1000 μg) of the extract were taken in different test tubes, and the volumes were adjusted to 250 μL with 0.1 M phosphate buffer. To these tubes, iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA; 1 mL), EDTA (0.018%, 0.5 mL) and dimethyl sulphoxide (0.85% in 0.1 M phosphate buffer, v/v, pH 7.4; 1 mL) were added, and the reaction was initiated by adding 0.22% ascorbic acid (0.5 mL). These reaction mixtures were incubated at 25°C for 20 min. The reaction was terminated by adding ice-cold TCA (17.5%, w/v, 1 mL). Next, Nash's reagent (150 g of ammonium acetate, 3 mL of glacial acetic acid and 2 mL of acetyl acetone; 3 mL) was mixed and adjusted to a volume of 1 L with distilled water, after which this solution was added to all tubes and left at 25°C for 15 min for colour development. The intensity of the yellow colour that formed was measured spectrophotometrically at 412 nm against blank reagent. All determinations were performed in triplicate. The ·OH radical scavenging activity was calculated using the following formula:

$$\% \text{ scavenging activity} = 100 \times (1 - A_{sample}/A_{control})$$

2.7. Statistical Analysis

All determinations were done in triplicate for each sample to be analyzed and IC_{50} values were calculated by using the equation of line. The results are given as mean ± Standard Deviation (SD). Student's *t*-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$.

2.8. Isolation and Identification of the Main Components

The neutral fraction was filtered on silica gel, and the CH_2Cl_2 eluate was fractionated by silica gel column chromatography (CC) using a MeOH/CH_2Cl_2 gradient system (100 mL for each fraction) as eluent.

The fractions eluted with CH_2Cl_2 were further purified by preparative TLC using CH_2Cl_2/Me_2CO (85 : 15, v/v) to give triterpene **1**. The fractions eluted with CH_2Cl_2/MeOH (95 : 5, v/v) were purified by silica gel CC, and the fractions eluted with CH_2Cl_2/MeOH (95 : 5, v/v) were

further purified by RP-18 HPLC with MeOH/MeCN/H_2O (20 : 70 : 10, v/v/v) to yield triterpenes **3**, **4**, **9,** and **13**. The fractions eluted with CH_2Cl_2/MeOH (90 : 10, v/v) were purified by RP-18 HPLC with MeOH/MeCN/H_2O (30 : 40 : 30, v/v/v) to yield triterpene **6** and with MeOH/MeCN/H_2O (20 : 20 : 10, v/v/v) to give triterpenes **8** and **12**. The fractions eluted with CH_2Cl_2/MeOH (10 : 90, v/v) were purified by flash silica gel CC, and the fractions eluted with Me_2CO were purified by RP-18 HPLC with MeOH/MeCN/H_2O (20 : 30 : 50, v/v/v) to give triterpene **11** and with MeOH/MeCN/H_2O (20 : 50 : 30, v/v/v) to give triterpenes **2**, **5**, **7** and **10** (Scheme).

2.9. Cell Culture

American Type Tissue Culture Collection provided the RAT-1 immortalised rat fibroblasts that were cultured in MEM medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

2.10. Cytotoxicity and Cell Proliferation Assays

MTT assay was used to evaluate the cytotoxicity, as an indicator of the metabolic competence of the cells [16]. Briefly, $3x10^4$ cells/well were seeded in 24-well culture plates, grown for an additional 24 h and then incubated in medium containing increasing amounts of tested compounds (from 0 to 1 mM). After 48 h of incubation, the medium was removed, and the cultures were incubated with medium containing 1 mg/mL MTT (1 mg/mL) for 2 h at 37°C. The medium was then discarded and 250 μL of (0.04 N HCl in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes that convert MTT to an insoluble purple formazan in living cells. The plates were then kept at 25° and shaken for 15–20 min, and the level of the coloured formazan derivative was determined on a multi-scan reader at a wavelength of 540 nm (reference wavelength 630 nm).

2.11. Determination of Cellular ROS

The ROS-fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to detect endogenous ROS levels. The cells (2×10^4) were plated in 96-well plates, and after 36 h, the medium was replaced with fresh medium supplemented with each compound. After 1, 16 and 48 h, the cells were washed once with HBSS (Hanks' Balanced Salt Solution) and incubated in the same buffer containing 10 μM DCFH-DA for 45 min at 37°C. The intracellular fluorescence was detected using a SPECTRAmax GEMINI spectrofluorometer (San Diego, California). H_2O_2 was used at 100 μM in the last 15 min of DCFH-DA incubation to evaluate the effect of pre-treatment with the tested compounds for the prevention of intracellular ROS generation. The data shown are the mean of three independent experiments performed on triplicate samples. The SD values were <20% for each of the tested conditions and compounds.

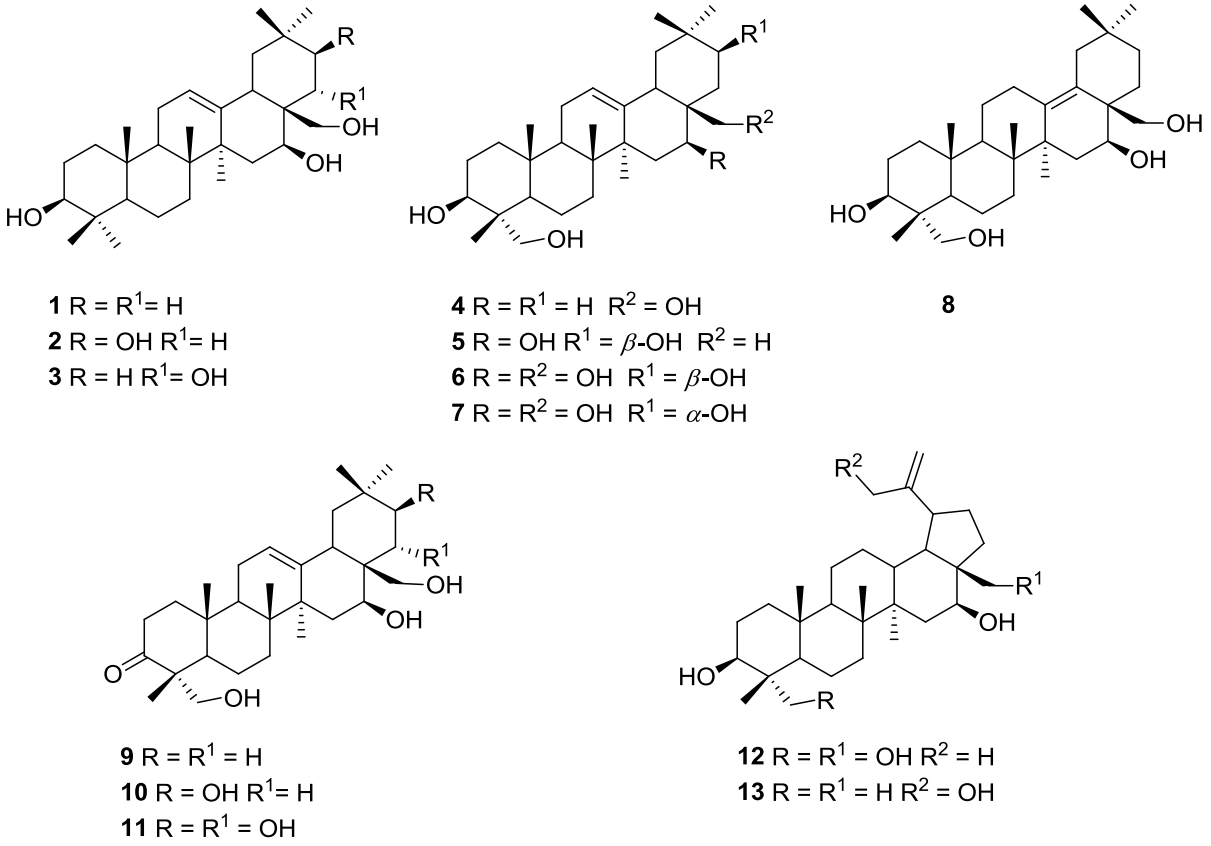
3. Results and Discussion

The dried and powdered aerial parts of the *G. sylvestre* were extracted with H_2O and, subsequently, with CH_2Cl_2. The aqueous phase was reduced in volume and lyophilised, while the organic phase was evaporated under reduced pressure. Based on the phenolic content and antioxidant activities, the extract with CH_2Cl_2 showed the best evidence of active phytochemical content, and it was decided to continue the study of this one sample, fractionating it into acidic and neutral fractions with aqueous 2N NaOH solution.

The content of the acidic fraction was extracted with EtOAc after the addition of 2N HCl to pH 3; then, both fractions were washed with H_2O and, finally, the samples were evaporated under reduced pressure to remove the solvent.

The data showed that the neutral fraction was the most promising; thus, this fraction was purified by silica gel column chromatography with solvents of increasing polarity. The CH_2Cl_2 extract

showed the highest DPPH· and OH scavenging activities and from its investigation we have isolated thirteen triterpenes (**1-13**, Figure 1) [15].



1 R = R^1= H
2 R = OH R^1= H
3 R = H R^1= OH

4 R = R^1 = H R^2 = OH
5 R = OH R^1 = β-OH R^2 = H
6 R = R^2 = OH R^1 = β-OH
7 R = R^2 = OH R^1 = α-OH

8

9 R = R^1 = H
10 R = OH R^1= H
11 R = R^1 = OH

12 R = R^1 = OH R^2 = H
13 R = R^1 = H R^2 = OH

Figure 1. Structures of the oleane (**1**–**11**) and lupane-type triterpenes (**12**–**13**).

3.1. Phenolic Content of Extracts and Fractions

The total phenolic contents of the two extracts (H_2O and CH_2Cl_2) and the two fractions (acidic and neutral) were measured by the Folin-Ciocalteau method, with a calibration curve developed using gallic acid. The results are shown in Figure 2A. Based on the solution absorbance measurements of the standard samples, the following regression equation was obtained:

$$y = 0.005\ x,\ R^2 = 0.996$$

where the total phenolic content of the extracts and fractions was determined as mg gallic acid equivalent (GAE) per gram of plant material.

Of the two extracts, the organic fraction showed the highest phenol content (15.6 mg GAE/g plant material) compared to the 9.2 mg GAE/g plant material measured in the aqueous fraction. In particular, the neutral fraction of the organic extract showed a phenolic content equal to 10.8 mg GAE/g plant material, more than twice the content of the acidic fraction (4.3 mg GAE/g plant material).

3.2. Estimation of DPPH· and ·OH Radical Scavenging Activities of Extracts and Fractions

The free-radical scavenging capabilities of both extracts and of the acidic and neutral fractions were evaluated by the DPPH· scavenging assay (Figure 2B), a rapid, sensitive, and reproducible procedure that is commonly employed for screening of plant extracts. The samples were also tested for their ability to scavenge OH radicals (Figure 2C), which are generated by the Fenton reaction and

produced in the human body under certain physiological conditions. Butylated hydroxyl anisole (BHA) was used as standard antioxidant.

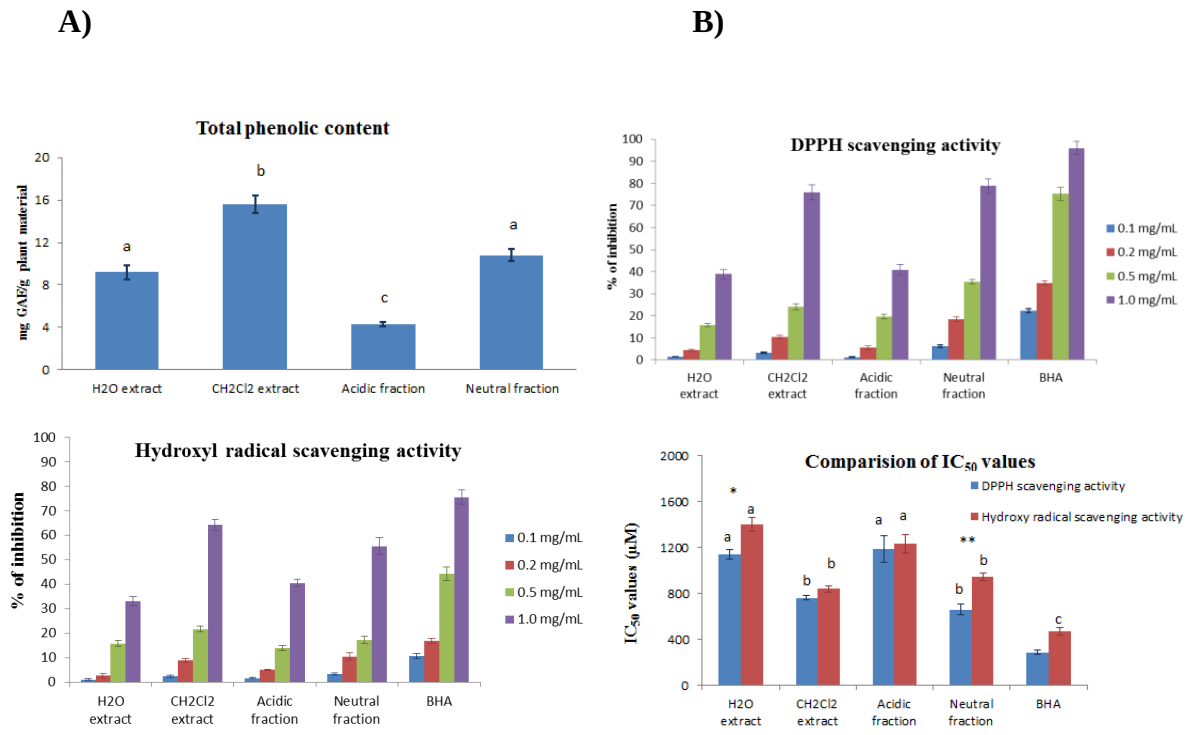


Figure 2. **A)** Total phenolic content (in gallic acid equivalents) in the extracts of the *G. sylvestre* aerial parts and their fractions; **B)** *In vitro* concentration dependent percentage inhibition of the DPPH radical by the extracts of the *G. sylvestre* and their fractions; **C)** *In vitro* concentration dependent percentage inhibition of the hydroxyl radical by the *G. sylvestre* extracts and their fractions; **D)** Comparison of the IC_{50} values of the *G. sylvestre* extracts and their fractions. Asterisk indicates significant within scavenging activity,*: $p<0.05$, **: $p<0.01$. Different letters indicate significant difference among treatments

All fractions exhibited potent DPPH·radical scavenging activity. Between the two extracts, the fractions obtained with CH_2Cl_2 were more active than those obtained with H_2O, almost in the ratio of 2 to 1 at the same concentration. Of the two organic fractions, the neutral sample was more active (6.2, 18.5, 35.4 and 78.8%) than the acidic sample (1.3, 5.6, 19.6 and 40.8%) at the four concentrations considered (100, 200, 500 and 1000 µg/mL, respectively). The neutral fraction showed the highest ·OH radical scavenging activity, ranging between 3.2 and 55.5% at the same concentrations previously tested. The acidic fraction was less active, with a maximum of 40.5% activity at the highest concentration (1.0 mg/mL). Between the two extracts, the CH_2Cl_2 extract was once again the most active, with percentages ranging between 2.4 and 64.3% at the lowest and highest concentrations, respectively; the CH_2Cl_2 extract was much more active than the H_2O extract, which exhibited a maximum activity of 33.0% at the highest concentration.

The results of the DPPH·and OH radical scavenging activities were also reported in terms of IC_{50} values (Figure 2D). The IC_{50} value for the DPPH assay of the H_2O extract was 1138 µg/mL, while the IC_{50} of the CH_2Cl_2 extract was 757 µg/mL. IC_{50} values of 1181 and 655 µg/mL were obtained for the acidic and neutral fractions, respectively, while the IC_{50} value for BHA was 283 µg/mL. Higher IC_{50} values of 1397 and 834 µg/mL were obtained for the ·OH radical scavenging activity of the H_2O and CH_2Cl_2 fractions, respectively, and the IC_{50} values for the acidic, neutral, and BHA fractions were 1229, 939, and 466 µg/mL, respectively.

The data showed that between the two fractions, the contents of the neutral fraction were the most promising; thus, this fraction was further purified by silica gel with PE, CH_2Cl_2, EtOAc, Me_2CO,

MeOH, and H_2O (Scheme 1). The phenolic content and the results of the estimated DPP and OH scavenging activities are reported in Figures 3A-D, respectively, which highlight how the fraction eluted with CH_2Cl_2 was the most promising among all the samples analysed.

3.3. Phenolic Content of Neutral Fractions (Figure 3A)

Significant differences were observed amongst the total phenolic content values of different extracts. The values varied from 12.7 mg GAE/g plant material, relative to the most active fraction eluted with CH_2Cl_2, to 3.9 mg GAE/g plant material for the least active fraction eluted with petroleum ether. The phenolic content of the other four fractions did not differ notably, exhibiting values of approximately 6.9-8.3 mg GAE/g plant material, which was slightly higher in the case of the two fractions eluted with MeOH and H_2O and was lower for the other two fractions eluted with EtOAc and Me_2CO.

3.4. Estimation of DPPH and OH Radical Scavenging Activities of Neutral Fractions

In Figures 3B and 3C, the results of the DPPH and OH radical scavenging activity assays are reported, respectively. The activities are in good agreement with the concentrations at which the fractions were tested, with values ranging from 0.5 to 86.5%. The most potent DPPH radical scavenging activity was exhibited by the sample eluted with CH_2Cl_2 (2.5, 9.0, 22.5 and 86.5% at concentrations of 100, 200, 500 and 1000 µg/mL, respectively), and the less active samples were those eluted with PE (0.5, 2.3, 6.9 and 26.5% at the 4 tested concentrations). The samples eluted with Me_2CO were moderately active compared to the others, but their activity did not exceed 50.0% at the highest concentration. The eluate isolated with CH_2Cl_2 also displayed the highest ·OH radical scavenging activity (2.0, 6.3, 12.5 and 66.4%), although to a lesser extent, and with a trend that was not perfectly correlated to the tested concentrations. The other eluates were much less active, among which the most active was the aqueous sample, with OH radical scavenging activity equal to 1.0, 4.3, 8.5 and 45.7% at the 4 tested concentrations.

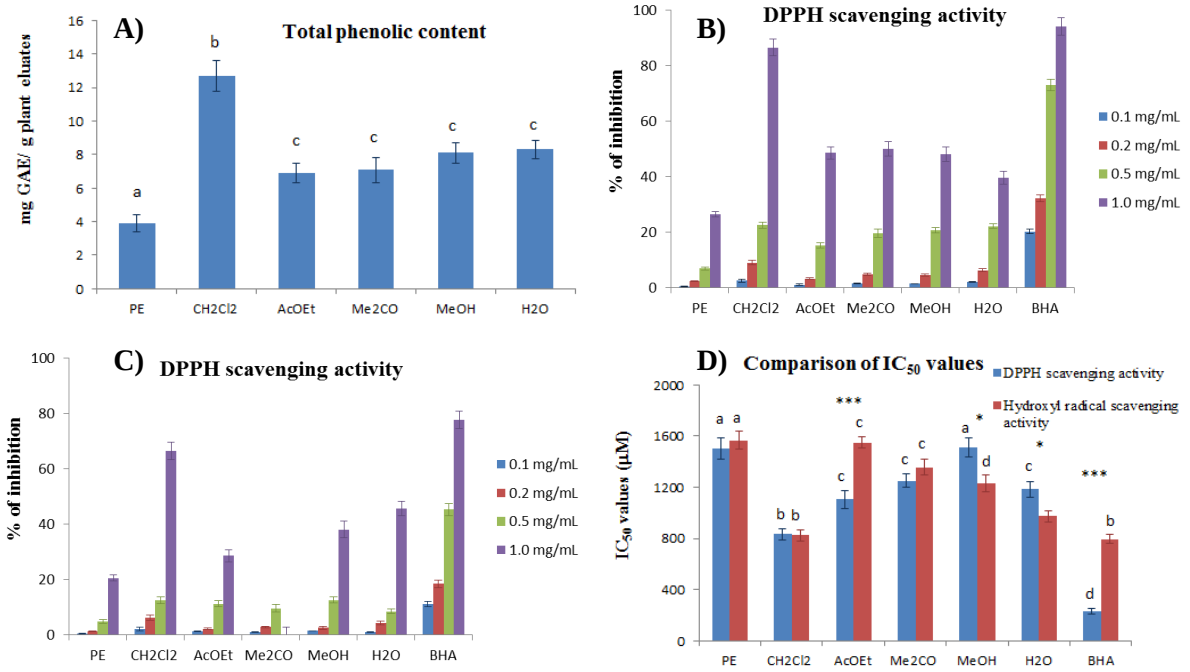


Figure 3. A) Total phenolic content of the gallic acid equivalent in different eluates of the CH_2Cl_2 extract of the *G. sylvestre;* **B)** *In vitro* concentration dependent percentage inhibition of the DPPH radical by different eluates of the CH_2Cl_2 extract of the *G. sylvestre*; **C)** *In vitro* concentration dependent percentage inhibition of the hydroxyl radical by different eluates of the CH_2Cl_2 extract of the *G. sylvestre*; **D)** Comparison of the IC_{50} values

of different eluates of the CH_2Cl_2 extract of the *G. sylvestre*. Asterisk indicates significant within scavenging activity. *: p<0.05, ***: p<0.001. Different letters indicate significant difference among treatments.

The IC_{50} values (Figure 3D) clearly show that the eluate isolated with CH_2Cl_2 was the most active sample in both the DPPH and OH radical activity assays (836 and 825 μg/mL, respectively), whereas the other eluates were less active, in particular PE (1505 and 1569 μg/mL, respectively), MeOH (1514 and 1228 μg/mL, respectively), H_2O (1185 and 973 μg/mL, respectively), and EtOAc (1105 and 1549 μg/mL, respectively).

The fraction eluted with CH_2Cl_2 contained compounds **1**-**13**. Triterpenes **1**-**4**, **6** and **13** were identified as 3*β*,16*β*,28-trihydroxyolean-12-ene (**1**) [17], 3*β*,16*β*,21*β*,28-tetrahydroxyolean-12-ene (**2**) [18], 3*β*,16*β*,22*α*,28-tetrahydroxyolean-12-ene (**3**) [19], 3*β*,23,28-trihydroxyolean-12-ene (**4**) [20], 3*β*,16*β*,21*β*,23,28-pentahydroxyolean-12-ene (**6**) [21], 3*β*,16*β*,30-trihydroxylupane (**13**) [22], and triterpenes **5**, **7**-**12** [23-25] were identified as 3*β*,16*β*,21*β*,23-tetrahydroxyolean-12-ene (**5**), 3*β*,16*β*,21*α*,23,28-pentahydroxyolean-12-ene (**7**), 3*β*,16*β*,23,28-tetrahydroxyolean-13,18-ene (**8**), 16*β*,23,28-trihydroxyolean-12-en-3-one (**9**), 16*β*,21*β*,23,28-tetrahydroxyolean-12-en-3-one (**10**), 16*β*,21*β*,22*α*,23,28-pentahydroxyolean-12-en-3-one (**11**) and 3*β*,16*β*,23,28-tetrahydroxylupane (**12**).

For an initial characterisation of the biological properties of the isolated compounds, and in view of their potential clinical use, we initially utilised an *in vitro* cell model to calculate the concentration inhibiting cell growth by 50% (IC_{50}) and to evaluate whether they were able to exert any antioxidant activity. Rat diploid immortalised fibroblasts represent one of the simplest available and widely used cellular models for toxicological assays because rat and human cells exhibit good metabolic similarity, and the results obtained with rat cells are typically confirmed in humans. Cytotoxicity assays were performed using the MTT test, as previously described [16, 23, 26-30].

Exponentially growing cultures of rat fibroblasts were exposed to increasing concentrations of each compound (0-1 mM) and cell viability was assessed after 48 h. A dose-dependent decrease in viable cells was observed with all tested compounds showing an IC_{50} rangof 29-and 1462 μM (Table 1).

Table 1. IC_{50} values for the triterpenes **1**–**13** using MTT assay.

Triterpene	IC_{50} (μM)[a]	Triterpene	IC_{50} (μM)[a]
1	1462 ± 201	**8**	29 ± 3
2	245 ± 33	**9**	96 ± 11
3	62 ± 9	**10**	43 ± 5
4	40 ± 6	**11**	209 ± 31
5	193 ± 30	**12**	103 ± 16
6	207 ± 29	**13**	208 ± 31
7	120 ± 18		

[a] concentration inhibiting cell growth by 50%.

To assess the potential antioxidant activity of the compounds, we decided to use a dose of 30 μM for all of them since the majority of the compounds did not exert an evident toxic effect at such a dose. Intracellular reactive oxygen species (ROS) levels were measured by the oxidative conversion of stable, non-fluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) occurring in the presence of ROS.

This assay, known as the DCFH-DA test, was preferred over the traditional *in vitro* assays because it takes into account the solubility and the cellular uptake of the tested compounds, which are important variables when assessing their potential use in humans. Moreover, the test differentiates

between the different types of ROS, such as ·OOH, ·OH and H_2O_2, which are all relevant in an *in vivo* setting. Therefore, the fluorescence detected is a sensitive indicator of intracellular ROS.

We evaluated the effect of ROS exposure to the tested compounds for variable times (1, 16 and 48 h) on the basal intracellular ROS level and on its increase when induced by a strong oxidative stimulus, such as H_2O_2. As shown in Figure 4, we did not detect any significant effect on the basal or stimulated intracellular levels of ROS, and most of the compounds (i.e, compounds **4**, **8** and **13**) induced an increase, rather than a decrease, in the basal level of ROS. Compounds **5**, **10**-**12** showed a slight antioxidant activity.

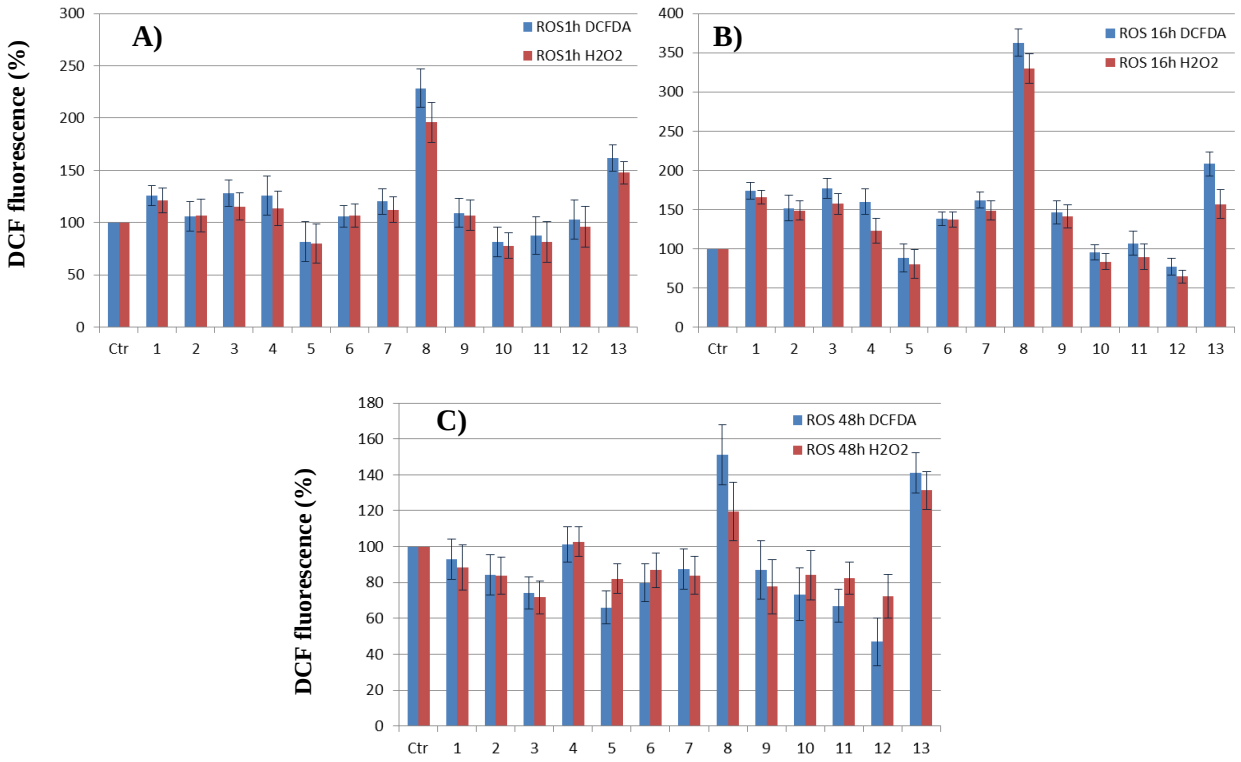


Figure 4. Effects of tested compounds on intracellular ROS production in Rat-1 cells, using DCF (10 M) as the fluorescent probe. DCF fluorescence was measured at basal conditions (blu bars) or following cell exposure to 100 μM H_2O_2 for 15 min (red bars) and a 1, 16 or 48 h (**A**, **B** and **C,** respectively) pre-treatment with the indicated compounds. The values are expressed as % compared to the fluorescence value observed in untreated control cells, which was set at 100. Experiments were performed three times on triplicate samples. The SD was <20% for all tested conditions.

After 16 h, the less active compounds appeared to further diminish their activity (**1**-**4**, **6**-**9**, **13**), while the inhibitory activity of the most active compounds was relatively constant (**5**, **10**-**12**). After 48 h, most compounds displayed activity below the threshold value of the control sample, with the exception of compounds **4**, **8** and **13**. The most active samples were again compounds **5** and **10**-**12**. In particular, compound **12** under basal conditions showed inhibitory activity of at least 60%. In all cases, compounds **8** and **13** were the exception with respect to antioxidant activity. Curiously, compounds **12** and **13** were respectively the most active and least active while having only a difference in the positions of the OH groups, which have equivalent oxidation states. However, the presence of two methyl groups at carbon C-4 seems to be advantageous.

These compounds were able to reduce the basal endogenous levels of ROS, but most importantly, they were also able to prevent an H_2O_2-induced generation of intracellular ROS. These findings warrant further studies to better characterize the properties of these compounds and their effects on intracellular ROS which, as shown for well-known antioxidants such as lycopene and *β*-

carotene, might be affected by the concentration used, as well as several other cellular variables [31-32].

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