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ORIGINAL ARTICLE

Hypericum perforatum: Influences of the habitat on chemical composition, photo-induced cytotoxicity, and antiradical activity

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Abstract

Context: Hypericin, isolated from *Hypericum perforatum* L. and about another 300 *Hypericum* species (Guttiferae), is one of the most powerful photosensitizers found in nature.
Objective: The aim of this study was to assess the variability of chemical composition and biological activities of four *H. perforatum* samples, collected at different altitudes in the South Apennine of Italy.
Materials and methods: MTT assay was used to evaluate the antiproliferative activity of different samples concentrations (0.6–100 μg/mL) after irradiation at 365 nm. The inhibition of nitric oxide production was evaluated after 24 h of incubation using the macrophage cell line RAW 264.7 and sample solutions ranging from 12.5 to 1000 μg/mL. Antioxidant activities were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and β-carotene bleaching test (ranges were 12.5–1000 and 1–400 μg/mL, respectively). Chemical composition was evaluated through HPTLC, and different contents of hypericin and rutin have been observed.
Results: The most phototoxic sample was collected from Zumpano (no. 1 at 370 m), with IC_{50} values of 24.61 ± 0.02 μg/mL. Sample no. 1 showed also the best radical scavenging activity (IC_{50} value of 9.18 ± 0.03 μg/mL) and the best antioxidant activity (IC_{50} value of 10.04 ± 0.03 μg/mL after 30 min of incubation). Best activity of extract no. 1 was well in accordance with chemical data, including the phenolic total content and particular metabolome profile.
Discussion and conclusion: This paper confirms the usefulness in maintaining the exploration of *H. perforatum* activities, in order to confirm its potentiality as a multipurpose plant.

Keywords

Anti-inflammatory, antioxidant, HPTLC, hypericin, melanoma, phenolic compounds, phototoxicity

History

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Introduction

Hypericum perforatum L. is a perennial herb, belonging to Hypericaceae (former Clusiaceae or Guttiferae) (Carine & Christenhursk, 2010), known as ''Erba di S. Giovanni'' in Italy and ''St. John's Wort'' in Anglo-Saxon countries, because the flowers bloom around St. John's Day (June 24). It is commonly found in the wild flora of the temperate zones throughout Eurasia, Northern Africa, and naturalized in the USA. It has been used since antiquity for its putative medicinal properties (Fernie, 1897; Pickering, 1879). Nowadays, it is a well-known medicinal plant (Commission Nationale de Pharmacopée France, 1982; Kommission Deutsche Arzneimittel-Codex, 1986), being the most widely prescribed antidepressant in mild state conditions, in the UE (Germany, Italy) and USA. Uses in traditional medicine includes wound-healing, being very common the familiar use of the aerial flowering portion in the olive oil to treat every kind of burns, injuries, or wounds (Upton et al., 1997).

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In regard to active principles, initially attention has been paid to hypericin and pseudohypericin, two naphthodianthrones considered responsible for biological activities, including St. John's worth-induced photosensitization (Brockmoller et al., 1997). After hypericins, phloroglucinols, such as hyperforin and pseudohyperforin, have been proposed to be most relevant for the antidepressant activity (Chatterjee et al., 1998; Muller et al., 1998; Witte et al., 1995). However, these are not the only biologically active compounds (Nahrstedt & Butterweck, 1997) nor the most abundant ones; flavonoids account for 2–4% (Upton et al., 1997). Several factors, including subspecies, habitat and growing conditions, as well harvesting treatments and conservation of the drug could have a significant impact on the chemical composition (Southwell & Bourke, 2001). As a matter of fact, several studies reported the variability of morphological characters, giving rise to several subspecies, and the changes in composition, also in populations of restricted areas. Therefore, in exploring biological activities, it is necessary to assess carefully the origin of the raw material and determine the total chemical composition if possible.

In this paper, we report the photoinduced activity of four populations of *H. perforatum* collected in the South Apennine

of Italy, in connection with the chemical composition. The Sila Massif consists of different Hercynian metamorphic, plutonic rock composed of medium–high, medium–low, and low metamorphic grade. Its natural landscape is mainly mountainous and forest with extensive plateau from 1200 to 1500 m high and several mountains over 1600 m. Part of the Sila is preserved in the integral reserves of Sila National Park (73 695 ha) dominated by a very important forest in terms of biodiversity and ecological conservation. Proposed boundaries include a rich variety of eco-region endemism, namely plants and insects, and the site is included in the centers of plant diversity (WWF-IUCN) in the Mediterranean basin (Apennines and the Apuan Alps), being recognized as the only one plant diversity hotspot area in southern Europe, and among priority regions according to Global 200 Eco-regions by WWF (European–Mediterranean mountain mixed forests). It also represents a unique geological setting, where the Apennine chain is superseded by the Alps.

Hypericin is one of the most powerful photosensitizing agents in nature (Skalkos et al., 2006). Therefore, the ingestion of *H. perforatum* causes a kind of sensitivity to sunlight known as hypericism (Barnes et al., 2001). Hypericin showed both *in vitro* and *in vivo* antineoplastic activity upon irradiation at 595 nm (Agostinis et al., 2002). The structure of this compound is characterized by the presence of extended *p*-orbital electrons that can be excited by light causing the production of radical species and leading to cytotoxicity (Barnes et al., 2001). Molecular membranes are considered to be the major cellular target of hypericin, as it accumulates in the membranes of various organelles, including lysosomes, mitochondria, the endoplasmic reticulum, and Golgi complex (Theodossiou et al., 2009). Conducted clinical trials confirmed the potential of hypericin in photodynamic therapy for the treatment of basal and squamous cell carcinoma and cutaneous T cell lymphoma (Alecu et al., 1998). While the photosensitizing properties of this compound are well documented (Agostinis et al., 2002; Theodossiou et al., 2009), only a restricted number of studies focused on the phototoxic properties of the whole extract of *H. perforatum*, although a fluorescence spectrum of the extract similar to that of hypericin, with two main bands at 595 and 640 nm, has been observed (Upton et al., 1997). The aim of the present study was to assess the photobiological properties of four hydroalcoholic extracts from different samples of *H. perforatum* subsp. *veronense* collected in diverse localities of Sila Massif. The *in vitro* antioxidant and anti-inflammatory activity of samples was also analyzed. The differences in the chemical composition and biological activity were discussed and related to the different sites of collection of the plants with the aim to characterize the species through the determination of biovariability.

Materials and methods

Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, β-carotene, propyl gallate, linoleic acid, Tween 20, Folin–Ciocalteu reagent, chlorogenic acid, RPMI 1640 medium, fetal bovine serum, L-glutamine, penicillin/streptomycin, Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hanks' Balanced Salt Solution, Griess reagent (1% sulphonamide and 0.1% *N*-(1-naphtyl) ethylenediaminedihydrochloride in 2.5% H_3PO_4), L-NAME were obtained from Sigma-Aldrich S.p.a. (Milano, Italy). Melanoma A375 cells and the murine monocytic macrophage cell line RAW 264.7 were purchased from ATCC no. CRL-1619 and no. CRL-2278, Reed, UK. Methanol for chemical analysis was purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). Reference substances in HPTLC analysis are from Sigma-Aldrich S.p.a. (Milano, Italy), with exception of pseudohypericin isolated in previous research. Purities of standards were checked by NMR spectroscopy as >98.5%. All other reagents, of analytical grade, were Merck products (VWR International, Milan, Italy).

Plant material and extraction procedure

Flowering aerial parts of *H. perforatum* L. subsp. *veronense* were collected in June 2011 in Calabria (Italy). The sites of collection together with other information of four samples are shown in Table 1. Fresh materials were extracted using maceration technique with EtOH 70% (2 L) at room temperature. Extraction was repeated three times for 48 h. The hydroalcoholic solutions were combined and dried under vacuum to obtain the extracts that were successively diluted in EtOH 70% in order to obtain the specific concentrations for chemical and biological investigation (Table 1).

Determination of total phenolic content

The amount of total phenolics of *H. perforatum* extracts was determined by the Folin–Ciocalteau method (Menichini et al., 2012). A solution of each samples (2 mg/mL) in extraction solvent (acetone:methanol:water:acetic acid, 40:40:20:0.1) was heated at 60 °C for 1 h. About 200 µL of samples were mixed with 1 mL of Folin–Ciocalteau reagent (Sigma-Aldrich, Milan, Italy) and 1 mL of sodium carbonate solution (7.5%). The mixtures were allowed to stand for 2 h. The absorbance of the blue color produced was measured at 726 nm with a Perkin Elmer Lambda 40 UV/VIS spectrophotometer (Milano, Italy). Experiments were conducted in triplicate. Chlorogenic acid was used as a standard and the total phenolic content was expressed as chlorogenic acid equivalents in mg/g of fresh material.

HPTLC analysis

The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of (I) Linomat 5 sample applicator using 100 µL

Table 1. Sites of collection and yield of extraction.

Sample	Collection site	Altitude (m a.s.l.)	Fresh weight (g)	Extract (g)	Yield (%)[a]
1	Zumpano (cultivation area)	370	79.06	17.54	22.19
2	San Pietro in Guarano (mountain base)	840	29.72	7.24	24.36
4	Righio (mountain area)	1650	53.34	14.06	26.36
3	Righitano (area of pastures)	1320	80.46	15.95	19.82

[a]Yield in % (w/w) based on the fresh weight of plant material.

syringes and connected to a nitrogen tank; (II) chamber ADC 2 containing twin trough chamber 20 × 10 cm; (III) Camag TLC Visualizer; (IV) Camag TLC scanner 3 linked to winCATS software (Muttenz, Switzerland). Normal phase glass plates 20 cm × 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 μm thickness). Before use, plates were prewashed with methanol and carefully dried for 3 min at 100 °C. Filtered solutions were applied with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s/μL (100 nL/s); injection volume, 2 μL; band width, 8 mm; distance from bottom, 15 mm. The HPTLC plates were developed in the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at room temperature. The developing solvents (i.e., type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 70 mm from the point of application. The developed layers were allowed to dry on Camag TLC Plate Heater III for 5 min and then derivatized with a selected solution, including natural product reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and Gorin reagent. All treated plates were allowed to dry on Camag TLC Plate Heater III for 5 min and then inspected under a UV light at 254 or 366 nm or under white light upper and lower (WRT), respectively, at a Camag TLC visualizer, before and after derivatization. Sample solutions were prepared and stored at room temperature for 3 d and then applied on the same HPTLC plated and the chromatogram evaluated for additional band. Similarly band stability was checked by keeping the resolved peaks and inspecting at intervals of 12, 24 and 49 h. HPTLC allowed a good separation and visualization of the constituents. Sample solutions were found to be stable at 4 °C for at least 1 month and for at least 3 d on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. RF values for main selected compounds varied less than 0.02%. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison.

Free radical scavenging activity assay

Radical scavenging activity was determined using a TLC screening method based on the reduction of a methanolic solution of the colored free radical DPPH. After application of *H. perforatum* extracts, TLC plates were developed (mobile phase $CHCl_3$/MeOH 8:2), sprayed with a 0.2% DPPH solution in MeOH and examined after 30 min. The samples showed antioxidant activity as they yielded yellow spots against a purple background. Extracts were then assayed for their radical scavenging potency as described previously (Conforti et al., 2012). Briefly, 200 μL of test samples solutions (0.3–1000 μg/mL in methanol) were added to 800 μL of a 10^{-4} M methanol solution of DPPH. Reaction mixtures were vigorously shaken and kept in the dark for 30 min. Absorbance of resulting solutions was measured in using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at 517 nm against blank (MeOH). All tests were run in triplicate. Ascorbic acid was used as a positive control.

β-Carotene bleaching-linoleic acid assay

Antioxidant activity was determined using the β-carotene bleaching test (Conforti et al., 2012). β-Carotene solution (1 mL of 0.5 mg/mL in CH_2Cl_2) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. An emulsion was prepared after evaporation of chloroform and dilution with 100 mL of water. Successively, 5 mL of emulsion were transferred into different test tubes containing 0.2 mL of samples in methanol at different concentrations. The tubes were then gently shaken and placed at 45 °C in a water bath for 60 min. The absorbance was measured at 470 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against a blank, consisting of an emulsion without β-carotene. Measurements were carried out at initial time ($t = 0$) and successively at 30 and 60 min. Propyl gallate was used as a positive control. The antioxidant activity was measured in terms of successful prevention of β-carotene bleaching as shown in the following equation:

$$\text{Antioxidant activity} = [1 - (A_0 - A_t)/(A_0^\circ - A_t^\circ)] \times 100$$

where A_0 and A°_0 are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and A_t^o are the absorbance values measure in the samples/standard and control, at $t = 30$ and 60 min, respectively.

Cell line and cell culture

The human melanoma cell line A375 was grown in plastic culture flask in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotic solution (penicillin/streptomycin) at 37 °C and in 5% CO_2 and fully humidified atmosphere. After 4–5 d, cells were removed from culture flask and centrifuged at 1500 rpm for 10 min. The medium was then removed and cells resuspended with fresh DMEM. Cells counts and viability were performed using a standard Trypan blue cell counting technique. Cell monolayers were subcultured onto 96 well culture plates (2×10^4 cells/well) used for experiments 24 h later.

The murine monocytic macrophage cell line RAW 264.7 was used to determine the inhibition of nitric oxide production. The cells were grown in plastic culture flask in DMEM with 1% L-glutamine supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin/streptomycin) under 5% CO_2 at 37 °C. Cell monolayers were subcultured onto 96 well culture plates (1×10^5 cells/well) used for experiments 24 h later.

Cellular photo-induced cytotoxicity

After medium removal, 100 μL of samples, dissolved in EtOH 70%, and diluted with Hanks' Balanced Salt Solution (HBSS, pH 7.2), were added to each well. Treatments never exceeded 0.5% of the solvent, the same that was also used in control wells. Cells were incubated at 37 °C for 30 min and then irradiated at 365 nm at a dose of 1.08 J/cm^2. After irradiation, the solution was replaced with medium and the plates were incubated for 48 h. Cell viability was assessed by the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide)] test, as previously described (Marrelli et al., 2012). Briefly, medium was removed and 100 µL of 0.5% w/v MTT (Sigma, Milan, Italy), dissolved in phosphate buffered saline, were added to each well. Plates were incubated for a further 4 h and 100 µL of DMSO were then added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as IC_{50} which is the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

Light source

Cells were irradiated by means of a HPW 125 Philips (Amsterdam, The Netherlands) lamps mainly emitting at 365 nm. The spectral irradiance of the source was 0.3 mW/cm^2 as measured by a Cole-Parmer Instrument Company radiometer (Niles, IL), equipped with a 365-CX sensor.

Inhibition of nitric oxide production

The murine monocytic macrophage cells RAW 264.7 were cultured with different concentrations of extracts for 24 h, after addition of lipopolysaccharides (LPS, final concentration of 1 µg/mL) for anti-inflammatory tests. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media 24 h later by the Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphtyl) ethylenediaminedihydrochloride in 2.5% H_3PO_4) as previously described (Conforti et al., 2011). About 100 µL of cell culture supernatant were combined with 100 µL of the Griess reagent in a 96-well plate followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, Italy) assay.

Cell morphology microscopy

Changes in cell morphology were visualized and assessed using an inverted microscope (AE20 Motic; Motic Instruments, Inc., VWR, Milano, Italy). Images were captured on a VWR digital camera (VisiCam 3.0 USB camera, Milano, Italy).

Statistical analysis

All experiments for the evaluation of antioxidant activity were carried out in triplicate, while six replicates were conducted to determine the phototoxic and anti-inflammatory properties. Data were expressed as mean value ± SEM. Concentration providing 50% inhibition (IC_{50}) was calculated by non-linear regression using GraphPad Prism Software (San Diego, CA). Statistical significance was assessed with a one-way analysis of variance (ANOVA) using SigmaStat Software (Jantel Scientific Software, San Rafael, CA). Significant differences among means were analyzed using Tukey's multiple comparisons test. Differences were considered significant at $P < 0.05$.

Results and discussion

Hydroalcoholic extracts were obtained from *H. perforatum* subsp. *veronense* fresh raw material through maceration. Collection details and yield of extractions are reported in Table 1. The first two samples were collected in the localities Zumpano and San Pietro in Guarano (Cosenza District, Italy) in the territory of Presila, at lower heights. The other two samples, nos. 3 and 4, were collected at altitudes of about 1300 m a.s.l. The best extraction yield was obtained for sample no. 3 (26.36% w/w), while the extract no. 4 was obtained with the lowest yield (19.82% w/w).

Total phenolic content

The total phenolic contents of *H. perforatum* extracts, expressed as chlorogenic acid equivalent in mg/g of fresh material, was significantly different ($p < 0.05$). Sample nos. 2 and 1 showed the highest contents (41.77 ± 0.29 and 35.83 ± 1.02 mg/g). Extract no. 3 contained the minor amount of phenolics (27.87 ± 1.34 mg/g) (Table 2).

Phytochemical analysis

In order to obtain as much information as possible on secondary metabolites composition, the HPTLC analysis was selected. HPTLC, the last evolution of planar chromatography, is a practical solution to characterize the complex mixtures of substances present in natural products (Nicoletti, 2011a, 2012). HPTLC is performed in two steps (Nicoletti, 2011b). First, the qualitative analysis consists into a track, the individual fingerprint, showing the extract constituents separated at different R_f values and identified by comparison with selected standards. Further on, the track is converted, by densitometry, into a series of peaks, for the quantitative determination of each. In Figure 1, the fingerprints of the four extracts are reported, together with the standards of hypericin and selected flavonoids. Also chlorogenic acid was inserted. This constituent is ubiquitary and well present in advanced plants, being connected with the main biogenetic pathways of aromatic constituents.

The fingerprints were very similar, with chlorogenic acid as the most prominent spot. However, intensities of the spots are very different, including those of hypericins, with the lowest quantity in extract no. 1. A relevant presence of rutin can be detected, but only in tracks 3 and 4. In track 1, a relevant fluorescent spot at high R_f is present, so far not identified. A further analysis was focused on hyperforins and related compounds. As evident in Figure 2, in this analysis, the track 1 resulted very different from the other ones, owing to the presence of several additional spots.

Table 2. Total phenolic content of *H. perforatum* extracts.

Sample	Fresh plant (mg/g)
1	35.83 (± 1.02)^b
2	41.77 (± 0.29)^a
3	27.87 (± 1.34)^d
4	31.37 (± 2.00)^c

Total phenolics are expressed as chlorogenic acid equivalents per g of fresh material. Data are expressed as mean ± SD ($n = 3$). Different letters along columns indicate statistically significant differences at $p < 0.05$ (Tukey's test).

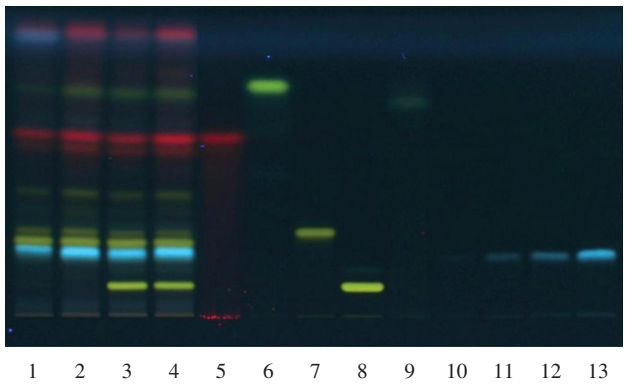


Figure 1. HPTLC analysis focused on hypericin and selected standards. Mobile phase: AcOEt/CH_2Cl_2/CH_3COOH/HCOOH/H_2O (100:25:10:10:11); derivatization: NP reagent. Visualization: 366 nm. Tracks: 1–4 the four extracts of *H. perforatum* collected in the South Apennine of Italy, 5 hypericin, 6 quercetin, 7 quercitin, 8 rutin, 9 biapigenin, 10–12 chlorogenic acid at different concentrations.

Antioxidant activity

Several reports in the literature recognized the antioxidant activity of *H. perforatum* extract (Radulović et al., 2007; Sagratini et al., 2008), due to the high content of phenols. However, reported results largely differ in function of the zone of collection of plants (Conforti et al., 2005), as expected in consideration of the chemical variability of the species. In this regard, the antioxidant activity of the four extracts was evaluated using two methods: the DPPH radical scavenging assay and the β-carotene bleaching test. DPPH is a stable radical often employed in the evaluation of the antioxidant activity. The free radical DPPH possesses a characteristic absorption at 517 nm (purple in color), which decreases significantly when exposed to radical-scavengers that act as donors of hydrogen atoms or electrons in transformation of DPPH radical into its reduced form DPPH-H. In DPPH test the extract no. 1 showed the best radical scavenging activity, with an IC_{50} value of 9.18 ± 0.03 μg/mL (Figure 3). Statistical analysis (Tukey's

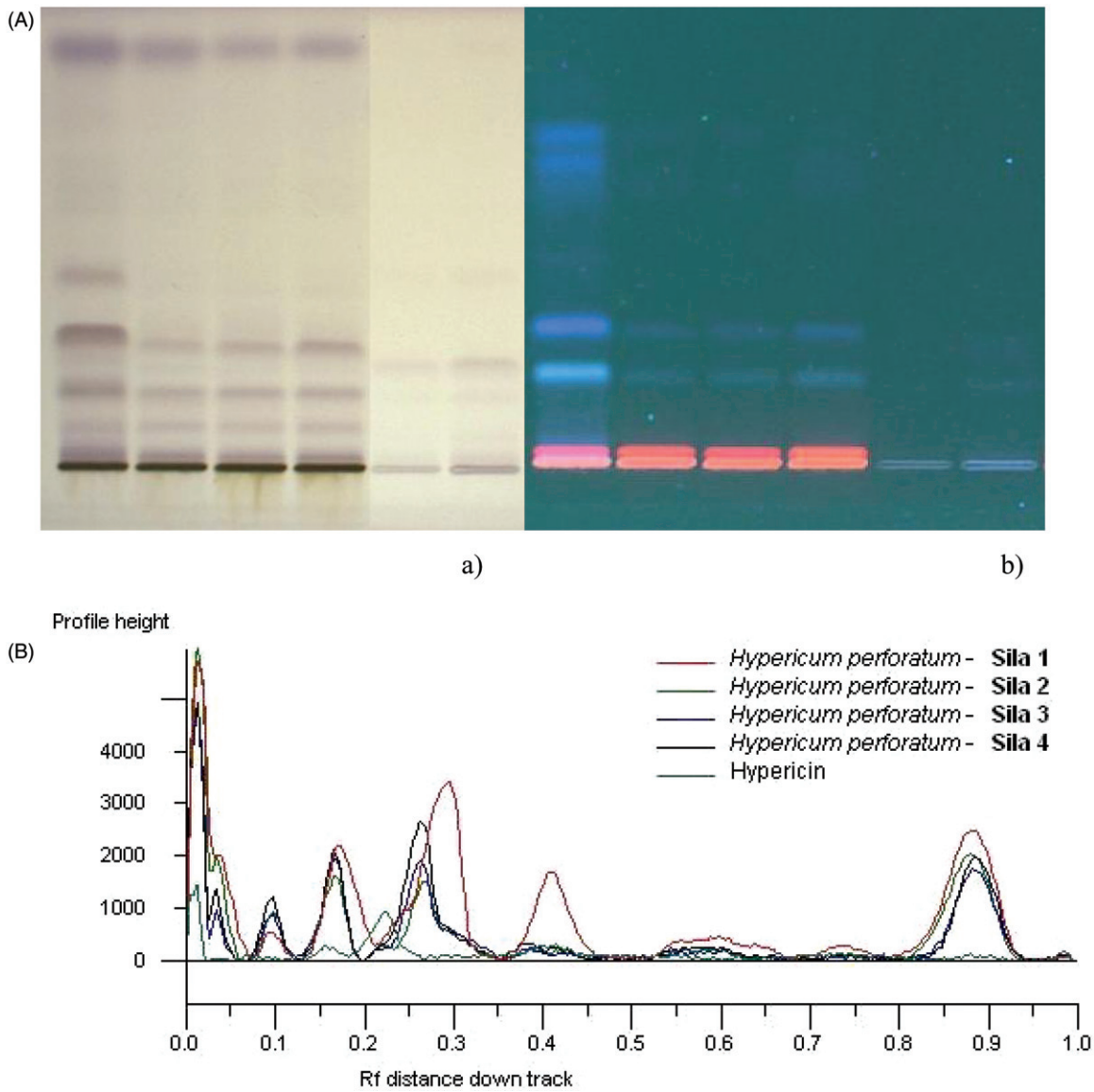


Figure 2. (A) HPTLC analysis focused on hyperforin and related products. Mobile phase: toluene, CH_2Cl_2 (4:2); derivatization: Godin reagent. Visualization: plate a white light, plate b 366 nm. Tracks: 1–4 the four extracts of *H. perforatum*; (B) densitometric analysis of plate A.

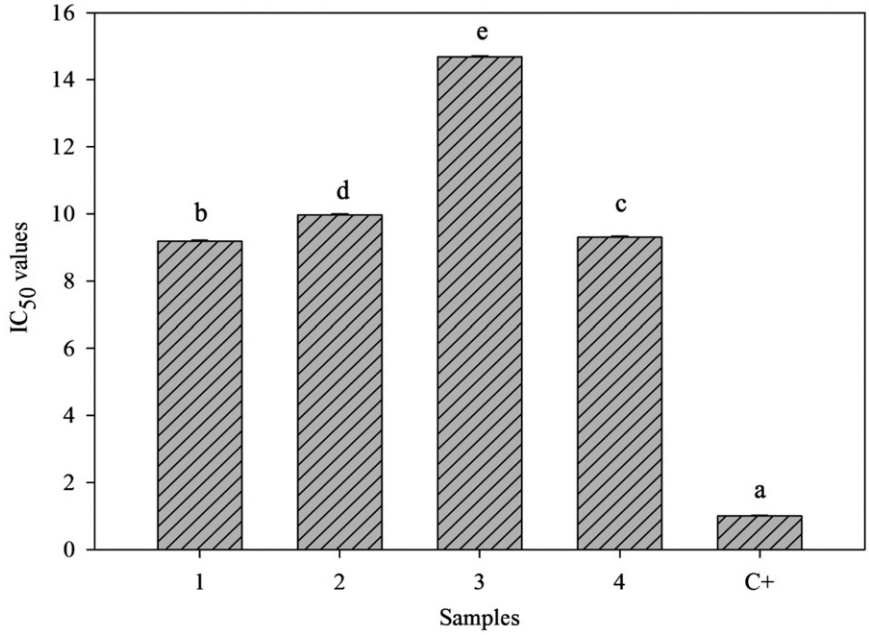


Figure 3. Free radical scavenging activity on DPPH of *H. perforatum* extracts. Data are expressed as mean ± SEM ($n = 3$). Different letters indicate significant differences at $p < 0.05$ (Tukey's test). Ascorbic acid (C^+, IC_{50} value of 2.00 ± 0.01 μg/mL) was used as a positive control.

test) demonstrated a significant difference between the biological activity of these samples and extracts nos. 4 and 2 (9.30 ± 0.03 and 9.97 ± 0.03 μg/mL, respectively). Sample no. 3, which also contained a minor amount of phenolics, showed the lowest activity ($IC_{50} = 14.69 \pm 0.02$ μg/mL) in agreement with several studies that have reported the relationships between the phenolic content and the antioxidant activity. The correlation is shown in Figure 4.

A similar trend was observed in the β-carotene bleaching test, in which the antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated dienehydroperoxides arising from linoleic acid oxidation. As a matter of fact, sample no. 1 showed again the best antioxidant activity after 30 min of incubation, with an IC_{50} value of 10.04 ± 0.03 μg/mL (Figure 5). Also, in this experiment, sample no. 3 showed the lowest activity ($IC_{50} = 106.50 \pm 0.63$ μg/mL after 30 min of incubation). The correlation is shown in Figure 4.

Cellular photo-induced cytotoxicity

The phototoxic properties of the whole extract of *H. perforatum* extract are not exhaustively documented, while a great number of studies focus on the phototoxic properties of hypericin (Agostinis et al., 2002; Theodossiou et al., 2009). Kapsokalyvas and coworkers (2005) tested the potential application of *H. perforatum* extracts as photosensitizer in autologus bone marrow transplantation, assessing the effects of the methanolic extract on leukemic cell line HL-60 and normal cord blood hemopoietic progenitor cells. Results showed a good activity at a concentration of 50 μg/mL, with irradiation at 532 nm with a dose of 74.87 J/cm^2. Unluckily, the results of this study showed that normal cells were also susceptible to *Hypericum* extract mediated photoirradiation, demonstrating a non-selective uptake by leukemic cells. The efficacy of the polar fraction of *H. perforatum* was also tested *in vitro* against urinary bladder carcinoma, utilizing the T24 and RT4 human bladder

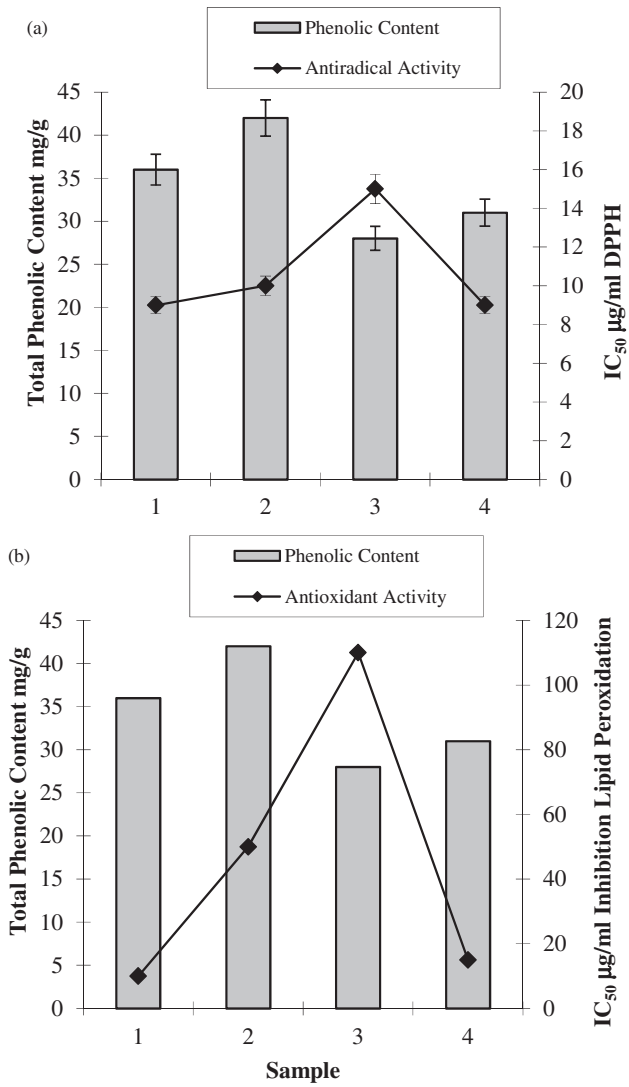


Figure 4. Correlation between phenolic content and antiradical (A) and antioxidant (B) activity.

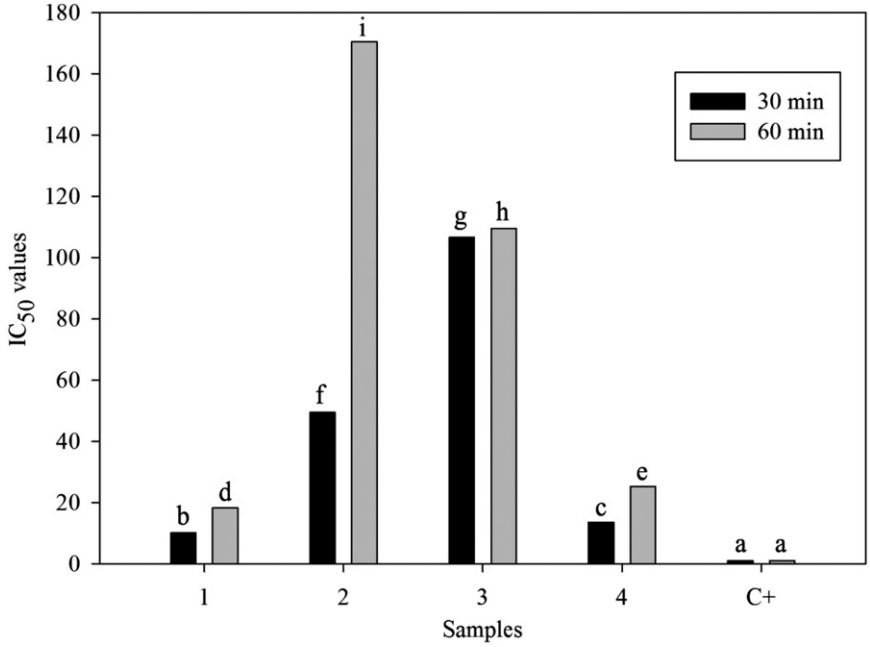


Figure 5. Lipid peroxidation inhibition activity using β-carotene–linoleic acid system after 30 and 60 min of incubation of *H. perforatum* extracts. Data are expressed as mean ± SEM ($n = 3$). Different letters indicate significant differences at $p < 0.05$ (Tukey's test). Propyl gallate (C^+, $IC_{50} = 1.00\,\mu g/mL \pm 0.02$) was used as a positive control.

Table 3. Phototoxic effects exerted by *H. perforatum* extracts on UVA-induced A375 cells.

Sample	IC_{50} (μg/mL)	
	Irradiated cells	Unirradiated cells
1	24.61 (± 0.02)[b]	30.58 (± 0.07)[d]
2	25.62 (± 0.209)[c]	39.94 (± 0.03)[g]
3	33.05 (± 0.03)[f]	>100
4	25.38 (± 0.07)[c]	31.20 (± 0.05)[e]
Bergaptene*	0.0416 (± 2.00)[a]	ND

Cells were pre-treated (30 min) with samples (0.6–100 μg/mL). For irradiation, the dose of 1.08 J/cm^2 was used. Irradiated and non-irradiated control cells were incubated with EtOH 70% (0.5%, v v-1) under the same conditions. Data are expressed as mean ± SEM ($n = 6$). Different letters along columns indicate statistically significant differences at $p < 0.05$ (Tukey's test). ND, not detectable.
*Positive control.

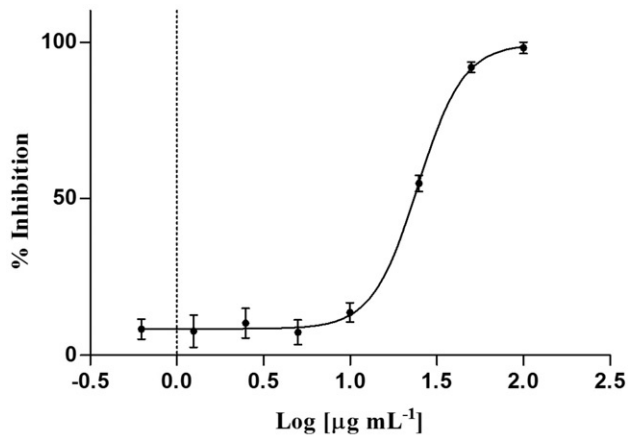


Figure 6. Effect of *H. perforatum* extract no. 1 on UVA-induced A375. Cells were pre-treated (30 min) with samples (2.5–100 μg/mL) and irradiated at 365 nm with a dose of 1.8 J/cm^2.

cancer cells (high-grade metastatic cancer and primary low-grade papillary transitional cell carcinoma, respectively). The extract showed a good phototoxicity against both cell lines at a concentration of 60 μg/mL, when irradiated at 630 nm with a light dose of 4–8 J/cm^2. Results demonstrated that this effect was related to the induction of apoptosis (Stavropoulos et al., 2006). In the present study, the photo-induced cytotoxicity of *H. perforatum* extracts were tested against human melanoma cells A375. All samples showed cytotoxic effects, with extract no. 1 being the most active one ($IC_{50} = 24.61 \pm 0.02\,\mu g/mL$, Table 3, and Figure 6). A very interesting activity was observed for sample no. 3, which showed a biological activity significantly lower than other samples ($IC_{50} = 33.05 \pm 0.03\,\mu g/mL$) but, in contrast, did not induce cytotoxic activity in the absence of UV irradiation ($IC_{50} > 100\,\mu g/mL$). Statistical analysis revealed no significant differences among the other two samples, nos. 2 and 4, with IC_{50} values of 25.62 ± 0.09 and $25.38 \pm 0.07\,\mu g/mL$, respectively. Figure 7 shows changes in treated cells morphology that were visualized using an inverted microscope and captured on a VWR digital camera. The incubation of cell cultures in the presence of a concentration of 100 μg/mL of each sample significantly affected cell viability.

Inhibition of nitric oxide production

Nitric oxide is an important factor in the maintenance of tissue homeostasis and the protection against infectious pathogens. NO is produced by nitric oxide synthase, which inducible isoform (iNOS) is known to be implicated under several pathological conditions including inflammation. An overproduction of NO results in damage to tissues and eventually destruction of tissue homeostasis and for this reason the NO production and iNOS expression represent good targets of research (Kröncke et al., 1998). The inhibition of NO production was here evaluated using the murine monocytic macrophage cell line RAW 264.7. Macrophages can release inflammatory mediators, such as prostaglandins, cytokines, and NO in response to LPS stimulation, validating

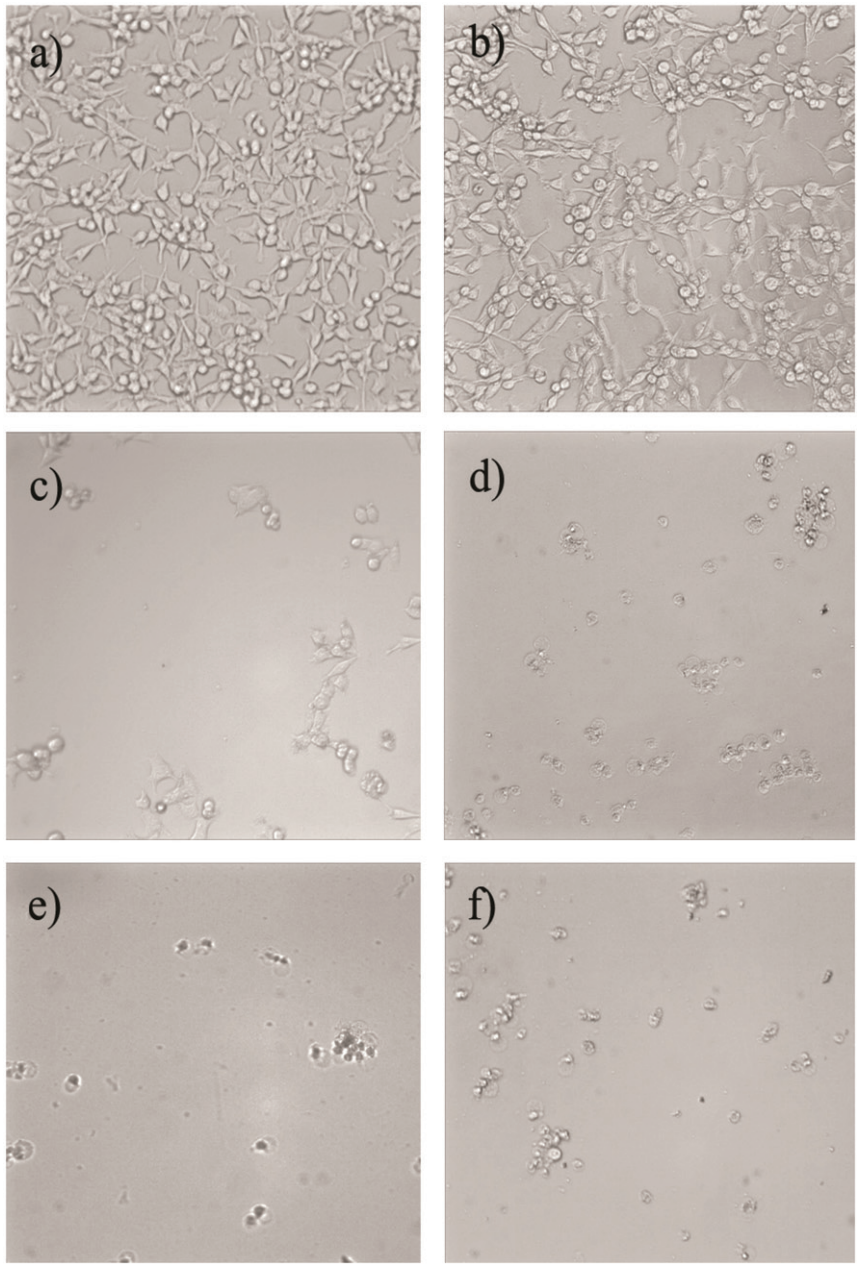


Figure 7. Morphological changes in A375 cells. (a) Control without irradiation, cells in EtOH 70% (0.5%, v/v), without sample; (b) control upon irradiation (365 nm, a dose of 1.08 J/cm^2); (c) *H. perforatum* no. 1, 100 µg/mL upon irradiation; (d) *H. perforatum* no. 2, 100 µg/mL upon irradiation; (e) *H. perforatum* no. 3, 100 µg/mL upon irradiation; (f) *H. perforatum* no. 4, 100 µg/mL upon irradiation.

Table 4. Inhibition of nitric oxide production in lipopolysaccharide stimulated RAW 264.7 macrophages.

	IC_{50} (µg/mL)	
Sample	NO inhibition	Cytotoxicity
1	169.10 (±0.10)^{d}	264.4 (±0.10)^{g}
2	126.20 (±0.07)^{c}	185.10 (±0.04)^{e}
3	330.10 (±0.30)^{h}	331.80 (±0.27)^{i}
4	67.59 (±0.08)^{b}	260.80 (±0.04)^{f}
Indomethacin*	58.00 (±0.37)^{a}	N.D.

Data are expressed as mean ± SEM ($n = 6$). Different letters along columns indicate statistically significant differences at $p < 0.05$ (Tukey's test). ND, not detectable. *Positive control.

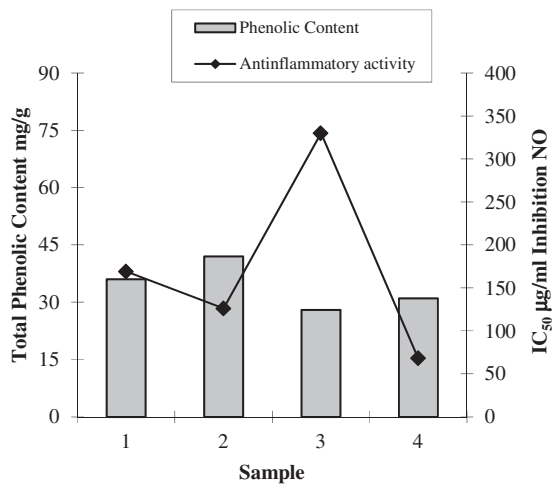


Figure 8. Correlation between phenolic content and anti-inflammatory activity.

the use of LPS-treated macrophages as a model of inflammation. A good anti-inflammatory activity has been demonstrated for *H. perforatum* oil extract obtained by maceration with 96% ethanol followed by extraction with sunflower oil by heating on a water bath (Šavikin et al., 2007; Zdunić et al., 2009) demonstrated also a good anti-inflammatory activity for the ethanol extract of *H. perforatum*, using the carrageenan-induced rat paw edema test.

In the present study, sample no. 4 caused a significant dose-related inhibition of nitric oxide production in the murine monocytic macrophage cell line RAW 264.7 with an IC_{50} value of 67.59 ± 0.08 µg/mL (Table 4). The IC_{50} value of cytotoxic activity against RAW 264.7 cells was significantly different (260.8 ± 0.04 µg/mL). The correlation between inhibition of nitric oxide and total phenolic content (Figure 8) showed a partial correlation evidencing that the sample (no. 3) with lower phenolic content showed also the higher IC_{50} value. This fact may be explained in numerous ways, in fact, the total phenolic content does not incorporate all the antioxidants. In addition, the synergism between the antioxidants in the mixture makes the antioxidant activity not only dependant on the concentration, but also on the structure and the interaction between the antioxidants.

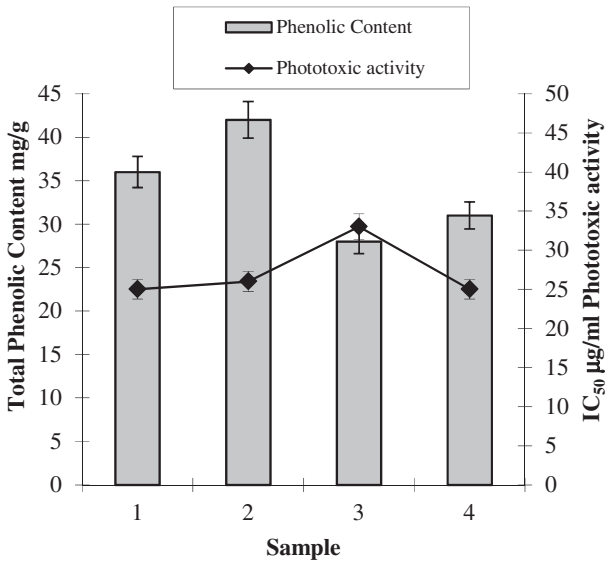


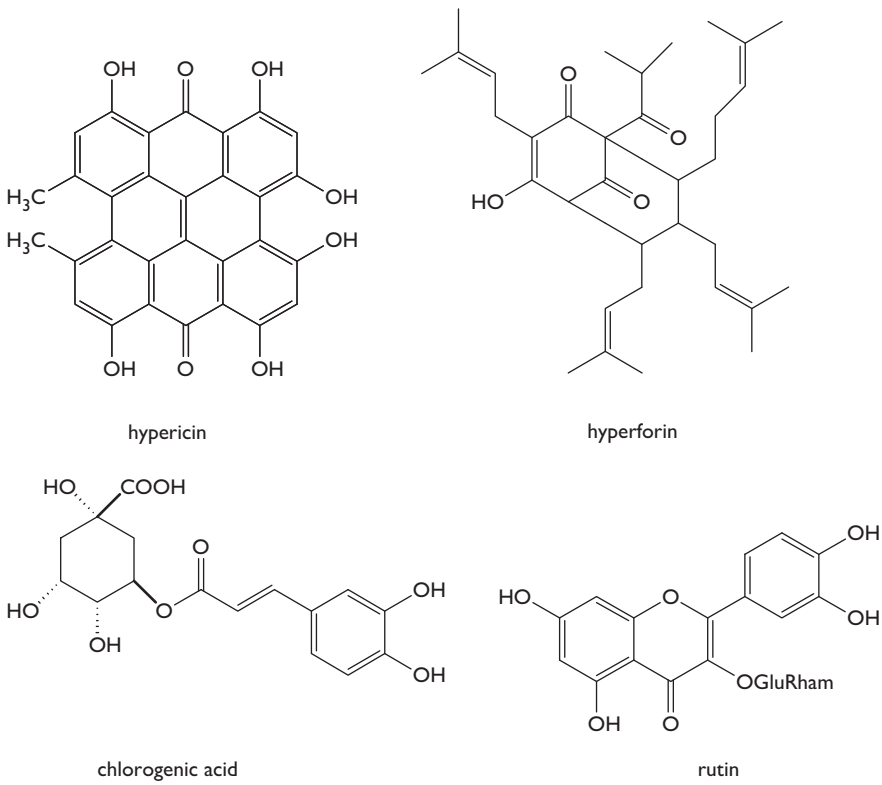
Figure 9. Correlation between phenolic content and phototoxic activity.

Conclusions

Nowadays *H. perforatum* extracts are utilized for the treatment of mild and moderate depression, but the daily oral dose did not provoke skin phototoxicity. However, the photoactive properties of the whole extract are not yet exhaustively documented, as well as other putative activities.

The present study confirms some of the previous phytochemical data: composition of *H. perforatum* is very complicated and variable, also populations of this species collected in near places present evident differences. We focused on the differences in chemical composition and above all photobiological properties of four extracts of different populations of *H. perforatum* collected in the South Apennine of Italy (Sila Massif). The better photosensitizing effects as well as others biological activities were showed by sample no. 1, collected in Zumpano, in the territory of Presila, at an altitude of 429 m a.s.l. Best activity of extract no. 1 obtained from pharmacological experiments were well in accordance with chemical data, like the phenolic total content and the particular metabolome profile (Figure 9).

Figure 10. Principal constituents of *H. perforatum* utilized in HPTLC analysis.



Usually in medicinal plant remedies, a single constituent is considered responsible of the reported activity, but, as already observed (Wagner, 2004), in the case of *H. perforatum* a complex composition must be considered, including compounds not considered so far, as evidenced by the hypericin/hyperforin story. Although further experiments are necessary to explain the different biological effects of the various studied samples, to exclude possible toxic effects of extracts and active components (Figure 10), and to clarify their phototoxic activity *in vivo*; this paper confirms the usefulness and potentiality of *H. perforatum* as a multipurpose plant, inherent to the multitarget and multicomponent nature of its herbal preparations.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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