***Hypericum perforatum* L. subsp. *perforatum* induces inhibition of free radicals and enhanced phototoxicity in human melanoma cells under ultraviolet light**

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Abstract

Objectives: Our interest continues in discovering phytocomplexes from medicinal plants with phototoxic activity against human melanoma cells; thus the aim of the present study was to assess antioxidant, anti-inflammatory and phototoxic activity of *Hypericum perforatum* L. subsp. *perforatum*, and relate these properties to the plant's chemical composition.

Materials and methods: Components of *H. perforatum* subsp. *perforatum* were extracted by hydroalcoholic solution and chemical profiles of preparations (HyTE-3) performed by HPTLC. Linoleic acid peroxidation and DPPH tests were used to assess antioxidant activity, while MTT assay allowed evaluation of anti-proliferative activity with respect to A375 human melanoma cells after irradiation with UVA dose, 1.8 J/cm². Inhibition of nitric oxide production of macrophages was also investigated.

Results: HyTE-3 indicated better antioxidant activity with β -carotene bleaching test in comparison to DPPH assay (IC₅₀ = 0.89 μ g/ml); significant phototoxicity in A375 cells at 78 μ g/ml concentration resulted in cell destruction of 50%. HyTE-3 caused significant dose-related inhibition of nitric oxide production in murine monocytic macrophage cell line RAW 264.7 with IC₅₀ value of 342 μ g/ml.

Conclusions: The *H. perforatum* subsp. *perforatum*-derived product was able to suppress proliferation

of human malignant melanoma A375 cells; extract together with UVA irradiation enhanced phototoxicity. This biological activity of antioxidant effects was combined with inhibition of nitric oxide production.

Introduction

Use of certain plants and light for treatment of human skin diseases was already practised in the Middle East and in India, thousands of years ago (1). Extracts of seeds of *Psoralea corylifolia* (Fabaceae) (2), figs *Ficus carica* (Moraceae) (3) and *Amni majus* (Apiaceae) (4) have also demonstrated this specific activity. Potent photosensitization and melanin stimulation of 8-methoxypsoralen derivatives have been discovered in these plants by clinical testing. Furanocoumarins, a group of natural photoactive compounds, have been identified as constituents of numerous photoactive plants, mainly in the Apiaceae, such as *Apium graveolens*, *Petroselinum sativum*, *Angelica archangelica* and others (5). Photosensitizing activity of these compounds can be elicited using UV light (320–360 nm). A further group of photoactive compounds found in plants, the thiophenes, mainly occur in the Asteraceae (6,7).

Hypericum perforatum L. (Hypericaceae = Guttiferae), commonly known as St. John's wort, is native to relatively dry temperature zones of Europe and North America. Although its ancient use in traditional and popular medicine is well documented (8), only quite recently, has it become a popular herbal remedy; use of the crude extract of this plant as an antidepressant also is popular today (9). The market for *H. perforatum* exceeds 210 million US dollars within the USA and 570 million US dollars worldwide, annually (10). The active reagent consisting of dried flowering tops or aerial parts

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of the plant. *H. perforatum* contains biologically active secondary metabolites belonging to at least ten different classes, with prevalence of naphthodianthrone (hypericin and pseudohypericin), phloroglucinols (hyperforin), flavonoids (rutin, hyperoside, isoquercitrin, quercitrin, quercetin, amentoflavone) and phenylpropanoids (chlorogenic acid) (11). However, great variations in contents have been reported for wild populations worldwide from Australia (12) to Turkey (13). Also, morphologically, the species is phenotypically varied: in Italy, two subspecies have been reported, *perforatum* and *veronense* (14). Initially, hypericin was considered to be the antidepressant active compound of *H. perforatum*, but according to latest studies, hyperforin has emerged to be the antidepressant active compound of this plant (15). Official analytical methods utilized for identification of components depend on presence of naphthodianthrone and phloroglucinols, as marker compounds (16), but other constituents also may contribute to the antidepressant action (17); consequently, metabolome studies are now reported for herbal supplement preparations (18).

High efficacy and tolerability of the herb's extract is unquestionable, while its clinical activity is comparable to other antidepressants (19). *H. perforatum* does not cause major side effects, making it a safe herbal therapeutic agent. Daily dose of the herb of around 500 mg extract, corresponds to a total dose of 1–2 mg hypericins, the main photosensitizers of the herb. These doses of hypericin, administered orally, do not provoke skin phototoxicity in patients (20). It exhibits high fluorescence quantum yield, and its presence in tissues can easily be visualized. Due to selective accumulation of hypericin in bladder carcinomas, the compound is now used as a fluorescent diagnostic tool for superficial bladder cancer (21). However, hypericin is also a photosensitizer with potent photocytotoxic activity. Taken together, these data indicate that hypericin could be used for whole bladder wall PDT of superficial bladder tumours (21).

Despite these encouraging clinical indications, photosensitizing properties of *H. perforatum* for use in photochemotherapy in malignant melanomas, with UVA radiation, have never been examined before. Malignant melanomas, the most aggressive form of skin cancer, arise from transformation and hyperproliferation of melanocytes. Incidence of this tumour and mortality from it, have been steadily increasing in almost all countries, those with fair-skinned populations in particular. In Europe, according to the National Cancer Institutes, in the order of 62 000 cases have been diagnosed each year with malignant melanoma and around 16 500 individuals die of it (22).

Free radicals are generated in tissues by normal physiological processes, including aerobic metabolism and the inflammatory response, but may inflict cell

damage when generation is increased and antioxidant defence mechanisms are overwhelmed. Important findings support the free radical hypothesis in skin carcinogenesis, which provide a promising rationale for development of powerful new antioxidant strategies in prevention and therapy of skin cancers (23).

Our continuing interest is in discovery of phytochemicals with phototoxic activity to human melanoma cells. The aim of the present study was to assess antioxidant activity on linoleic acid peroxidation and free radical-scavenging activity [with 2,2-diphenyl-1-picrylhydrazyl (DPPH)], anti-inflammatory activity on macrophages, and phototoxic activity to A375 melanoma cells, of a hydroalcoholic extract of *H. perforatum* subsp. *perforatum* of determined chemical composition.

Material and methods

Reagents

Beta-carotene, quercetin, chlorogenic acid, NaCl, ascorbic acid, propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, Tween 20, Folin-Ciocalteu reagent, RPMI 1640 medium, foetal 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-naphthyl) ethylenediamine dihydrochloride in 2.5% H₃PO₄), L-NAME (N⁵-[imino(nitroamino)methyl]-L-ornithine, methyl ester) were obtained from Sigma-Aldrich S.p.a. (Milan, Italy). Melanoma A375 cells were purchased from ATCC No.: CRL-1619, UK. All other reagents, of analytical grade, were Merck products (VWR International, Milan, Italy). Methanol for chemical analysis was purchased from Sigma-Aldrich and Carlo Erba (Milan, Italy). Reference substances in HPTLC analysis were from Sigma-Aldrich S.p.a., with the exception of pseudohypericin, which had been isolated in a previous piece of our research. Purities of standards were checked by NMR spectroscopy as >98.5%.

Plant material

Flowering aerial parts of *H. perforatum* L. subsp. *perforatum* were collected in Calabria (Italy) (lat: 39° 6' 32"; long: 16° 7' 59") and specimens were deposited at the Herbarium CLU (no. 18749) of the Natural History Museum of University of Calabria.

Extraction

Fresh raw material (61.28 g) was extracted using a maceration technique, with hydroalcoholic solution (70%

ethanol, 2 l) at room temperature. Extraction was repeated three times over 48 h. Hydroalcoholic solutions were combined and dried under vacuum to obtain preparation HyTE-3, that was successively diluted in hydroalcoholic solution to obtain specific concentrations for chemical and biological investigation. Resultant preparations were stored at 4 °C.

Determination of total phenolic content

Amounts of total HyTE-3 phenolics were determined using the Folin-Ciocalteu method (24). Preparations (100 µl–2 mg/ml) were mixed in 0.2 ml Folin-Ciocalteu reagent (Sigma-Aldrich) and 2 ml distilled water, and flasks were shaken vigorously. Subsequently, 1.0 ml of 15% sodium carbonate solution was added and mixtures were mixed thoroughly once more. Mixtures were allowed to stand for 2 h, protected from light. Absorbance of blue reaction produced was measured using a spectrophotometer (UV-Vis Jenway 6003) at 765 nm and levels of total phenolic contents were determined in triplicate. Chlorogenic acid was used as standard and total phenolic contents were expressed as chlorogenic acid equivalents in mg/g⁻¹ fresh material.

HPTLC analysis

HPTLC system (CAMAG, Muttenz, Switzerland) consisted of (i) Linomat 5 sample applicator using 100 µl 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. Normal phase glass plates 20 × 10 cm (Merck, Darmstadt, Germany) with glass-backed layer silica gel 60 (2 µm thickness). Before use, plates were pre-washed in methanol and carefully dried for 3 min at 100 °C. Filtered solutions were applied with nitrogen flow and operating conditions were: syringe delivery speed, 10 s/µl⁻¹ (100 nl/s⁻¹); injection volume, 2 µl; band width, 8 mm; distance from bottom, 15 mm. HPTLC plates were developed in automatic and reproducible developing chamber ADC 2, saturated with identical mobile phase ethyl acetate:dichloromethane:acetic acid:formic acid:water (100:25:10:10:11; v/v/v/v), for 20 min at room temperature. Developing solvents (types and ratios) were carefully optimized before analyses were performed. Length of chromatogram run was 70 mm from point of application and developed layers were allowed to dry on Camag TLC Plate Heater III for 5 min before being derivatized with a selected solution, including Natural Product Reagent (NPR) (1 g diphenylborinic acid aminoethylester in 200 ml of ethyl acetate) and anisaldehyde (1.5 ml *p*-anisaldehyde, 2.5 ml

H₂SO₄, 1 ml AcOH in 37 ml EtOH). All treated plates were allowed to dry on Camag TLC Plate Heater III (Muttenz, Switzerland) for 5 min, 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 of HyTE-3 were prepared and stored at room temperature for 3 days then applied on the same HPTLC, plated and chromatogram evaluated for additional bands. Similarly, band stability was checked by retaining resolved peaks and inspecting at intervals of 12, 24 and 49 h. HPTLC allowed good separation and visualization of the constituents. Sample solutions of HyTE-3 were found to be stable at 4 °C for at least 1 month and for at least 3 days on HPTLC plates. Repeatability was determined by running a minimum of three analyses and RF values for main selected compounds varied less than 0.02%. Effects of small changes in mobile phase composition, mobile phase volume or duration of saturation, were minute, and reduced by direct comparison.

Free radical scavenging activity assay

Free radical scavenging activity assay (FRSA) was rapidly executed using a TLC screening method based on reduction of the coloured free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH), in methanolic solution. After application of hydroalcoholic solution of extract, developing (mobile phase CHCl₃/MeOH 8:2) and drying, TLC plates were sprayed with 0.2% DPPH solution in MeOH, and examined after 30 min. Samples showed antioxidant activity when yellow spots against a purple background appeared. Positive samples were assayed for their radical scavenging potency as described previously (25). Two hundred microlitre of test samples solutions (1–1000 µg/ml) were added to 800 µl of 10⁻⁴ M ethanolic solution of DPPH; reaction mixtures were vigorously shaken and kept in the dark for 30 min; absorbance of resulting solutions were measured in 1 cm cuvettes using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer (Milano, Italy), at 517 nm, against blank (without DPPH). All tests were run in triplicate and mean values were calculated. Ascorbic acid was used as positive control.

Beta-carotene bleaching-linoleic acid assay

Antioxidant activity was determined using the β-carotene bleaching test (25). β-carotene solution (1 ml of 0.2 mg/ml in chloroform) was added to 0.02 ml linoleic acid and 0.2 ml 100% Tween 20. Successively, emulsion was prepared after evaporation of chloroform and dilution with 100 ml water. Emulsion (5 ml) was

transferred to the different test tubes containing 0.2 ml of sample in 70% ethanol, at different concentrations. Tubes were then gently shaken and placed at 45 °C in a water bath for 60 min. Absorbances of samples, standard and control were measured at 470 nm using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer against blank, consisting of emulsion without β -carotene. Measurement was carried out at initial time ($t = 0$) and successively at 30 and 60 min. Antioxidant activity was measured in terms of successful prevention of β -carotene bleaching by using 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 absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and A_t° are absorbance values measured in samples/standard and control, at $t = 30$ and 60 min, respectively.

Cells

To determine cytotoxic effects of HyTE-3 against melanoma, A375 human melanoma cell line, obtained from the American Type Culture Collection (ATCC), was used (Sigma-Aldrich). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 1% L-glutamine, 1% of penicillin/streptomycin and 10% foetal calf serum, at 37 °C in 5% CO₂ and fully humidified atmosphere.

The murine monocytic macrophage cell line RAW 264.7 (European Collection of Cell Cultures, London, UK) was used to determine inhibition of nitric oxide production. Cells were grown in plastic culture flasks in DMEM with L-glutamine supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (penicillin/streptomycin) under 5% CO₂ at 37 °C.

Light source

Irradiation was sourced from HPW 125 Philips lamps, each being used in single line operation, at 365 nm, to culture plates containing the cells. Spectral irradiance of source was measured, at the sample level, by a Cole-Parmer Instrument Company radiometer (Niles, IL, USA), equipped with a 365-CX sensor.

Cell phototoxicity

Phototoxicity testing was adapted from Barraja *et al.* (26) with modification. Individual wells of a 96-well tissue culture microtitre plate (Sigma-Aldrich S.p.a., Milan, Italy) were inoculated with 100 μ l of complete medium

containing 1×10^4 A375 cells. Plates were incubated at 37 °C in a humidified 5% incubator for 24 h prior to experiments. After medium removal, 100 μ l of preparation, dissolved in hydroalcoholic solution and diluted with Hanks' balanced salt solution (HBSS, pH 7.2), was added to each well and incubated at 37 °C for 30 min, then irradiated at 365 nm for 1 h. Spectral irradiance of source was 0.3 mW cm^{-2} . After irradiation, solution was replaced with medium, and plates were incubated for 48 h. Cell viability was assayed using the MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide)] test.

Inhibition of nitric oxide production

Here, murine monocytic macrophage cell line RAW 264.7 was used. After 4–5 days, cells were removed from culture flasks by scraping and centrifugation at 250 g for 10 min. Medium was then removed and cells were resuspended in fresh DMEM. Cell counts and viability assessment were performed using standard trypan blue cell counting. Cell density was adjusted to 1×10^6 cells/ml in the same medium and in the region of 100 μ l of suspension was cultured in 96-well plates for one day, to become almost confluent. Concentration (1 mg/ml) of the samples was prepared from stock solutions in EtOH 70% by dilution in DMEM to provide volume of 100 μ l added to each well of a microtitre plate (96-well). Then cells were cultured with the concentrations and positive control (2 μ g/ml) for 24 h; after addition of LPS (final concentration of 1 μ g/ml) for anti-inflammatory tests.

Presence of nitrite, a stable oxidized product of NO, was determined in cell culture media using the Griess reagent (1% sulphanamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H₃PO₄) as previously described (27). In the region of 100 μ l cell culture supernatant was combined with 100 μ l Griess reagent in 96-well plates followed by spectrophotometric measurement at 550 nm, using a microplate reader (GDV DV 990 B/V, Rome, Italy). Nitrite concentration in supernatants was determined by comparison to a sodium nitrite standard curve. Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT; Sigma) assay.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means $\pm$ SEM. Concentration yielding 50% inhibition (IC₅₀) was calculated by non-linear regression with the use of Prism Graphpad version 4.0 for Windows (GraphPad Software, San Diego, CA,

USA). Dose–response curve was obtained by plotting percentage inhibition *versus* concentration.

Results

HyTE-3 was obtained from *H. perforatum* subsp. *perforatum* fresh raw material, 26.65% (16.33 g from 61.28 g of plant). Total phenolic content of HyTE-3, measured by spectrophotometry, was 51.47 ± 0.54 mg CAE equivalent/g fresh material.

Phytochemical analysis

As shown in a number of cases, multiingredient composition is essential for multiple activities of botanical supplements; plant extracts, containing hundreds of natural products, constitute a real analytical challenge. Furthermore, in the case of *H. perforatum*, great possible variability in chemical composition of the raw material must be considered, as has been shown in several studies. We chose HPTLC to be the modern analytical tool specially tailored for analysis of complex plant mixtures. Thus, it was possible, in a single analysis, to check presence of many active principles in HyTE-3, with exception of hyperforin, which needed a different and special approach. Results of HPTLC analysis of HyTE-3, compared to selected standards, are reported in Fig. 1, while those of

hyperforin, determined and confirmed by densitometry appear in Fig. 2.

Antioxidant activity

Agents that produce reactive oxygen species (ROS) can trigger mitochondrial outer membrane permeabilization and apoptosis by overwhelming antioxidant defence of mitochondria, hence causing excessive oxidative damage to mitochondria. Therapeutic benefits of medicinal plants is often attributed to their antioxidant properties (28). That of *H. perforatum* extracts is well known (29,30) and is to be expected, due to high content of phenolic compounds. Although several publications have focused on this topic, there are very different results in function between zones of the plants, from which specimens were collected (31). In this regard, antioxidant activity of HyTE-3 was evaluated using two methods based on free radical scavenging capacity, namely DPPH radical scavenging assay and β -carotene bleaching test. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable radical often used in assessment of antioxidant activity. Free radical DPPH has characteristic absorption at 517 nm (purple), which decreases significantly when exposed to radical-scavengers (due to hydrogen atom-transfer from antioxidant to DPPH). Lower absorbance of 517 nm indicates higher radical-scavenging activity of the extract. In the assay described here, ability of investigated HyTE-3 to act as donor of hydrogen atoms or electrons, in transformation of DPPH radical into its reduced form DPPH-H, was investigated. In the β -carotene bleaching test, antioxidant capacity was determined by measuring inhibition of volatile organic compounds and conjugated diene hydroperoxides, arising from linoleic acid oxidation. The results show that HyTE-3 had concentration-dependent antioxidant activity (Fig. 3); IC_{50} values of the preparation, compared to standard ascorbic acid, are reported in Table 1.

HyTE-3's free radical scavenging activities on DPPH were in the region of 7 times lower than those reported for ascorbic acid. In particular, the preparation was shown to possess more remarkable antioxidant activity in the β -carotene bleaching test (Fig. 3), IC_{50} value of 10 μ g/ml after 30 min incubation, compared to 14 μ g/ml in the DPPH assay, as shown in Table 1. The relatively high antioxidant activity observed may be directly correlated to high phenolic content of the preparation and can be attributed to hydrogen-donating ability of its components (32). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and donate hydrogen from phenolic hydroxyl groups, thereby forming a stable end product, which does not initiate nor propagate further oxidation of lipids.

Figure 1. HPTLC analysis of phenolic constituents in *H. perforatum* extract HyTE-3 with selected standards. Mobile phase: ethyl acetate: dichloromethane: acetic acid: formic acid: water 100:25:10:10:11. Derivatisation: NPR. Visualization: UV 366 nm. Tracks: 1 HyTE-3, 2 hypericin, 3 quercetin, 4 isoquercetin, 5 rutin, 6 biapigenin, 7 chlorogenic acid, 8 polyphenols. Pseudohypericin is visible as the red spot at lower R_f to hypericin. Polyphenols are present near the starting point owing to the low solubility in the mobile phase.

Figure 2. HPTLC analysis of hyperforine in *H. perforatum* extract HyTE-3. Mobile phase: toluene: dichloromethane 4:1. Derivatization: NPR. Visualization: (a). UV 366 nm; (b). white light. Tracks: 1 HyTE-3, 2 hyperforine at 4 µl, 3 hyperforine at 8 µl; (c). conversion of tracks of (b).

Figure 3. Antioxidant activities of HyTE-3: (a) free radical scavenging activity on DPPH; (b, c) lipid peroxidation inhibition activity using β -carotene-linoleic acid system after 30 and 60 min of incubation, respectively.

Table 1. IC₅₀ values of biological activities of HyTE-3

Test	IC ₅₀ (µg/ml)	
	HyTE-3	Positive control
<i>FRSA assay</i>		Ascorbic acid
Radical scavenging	14.43 ± 0.01	2 ± 0.01
<i>β-Carotene bleaching test</i>		Propyl gallate
Inhibition peroxidation		
30 min	10.89 ± 0.04	1 ± 0.02
60 min	13.96 ± 0.03	1 ± 0.02
<i>Anti-inflammatory assay</i>		L-NAME
Inhibition no	342.60 ± 0.12	45.86 ± 0.37
Cytotoxicity	605.00 ± 1.86	>1000
<i>Cellular phototoxicity</i>		Bergaptene
Irradiated cells	78.10 ± 0.20	0.0416 ± 0.008
Unirradiated cells	>100	>100

Data are expressed as mean ± SEM (*n* = 6). L-NAME = N5-[imino (nitroamino)methyl]-L-ornithine, methyl ester.

Phototoxicity

Phototoxicity of HyTE-3 was investigated for the first time on this human tumour cell line, A375 (melanoma). Figure 4 shows extent of cell survival expressed as IC₅₀, the concentration that induces 50% inhibition of cell population growth, after irradiation with a specific UVA dose (1.8 J/cm²) of hydroalcoholic extract. Control experiments with UVA light, and a parallel group of treatment for non-irradiated cells, were carried out without significant cytotoxic effects. The photosensitizer exhibited significant phototoxicity to the human melanoma cell line at concentration of 78 µg/ml, resulting in 50% cell destruction. These results were compared to results obtained with the same cell line, under the same conditions with a known photosensitizer, bergaptene (Table 1).

The preparation contained polyphenolic compounds, such as flavonoids and naphthodianthrone, but individual components and their concentrations are important findings to explain its biological activity. Huang *et al.* (2007) came to similar conclusions when they studied effects of extract of black raspberries, strawberries and blueberries, on UV-induced damage to a mouse epidermal cell line (33). We cannot exactly say which compound(s) is/are responsible for the protective effect; the modern approach in chemoprevention is to formulate a mixture of active compounds or prepare extract/fractions from plant material that has protective activity. A mixture of various phytochemicals has a number of advantages, for example, extracts/fractions of a mixture are cheaper and easier to prepare, individual components may provide additive and/or synergistic effects, and lower concentration of constituents reduces possible toxic effects.

Figure 4. Effect *H. perforatum* extract on UVA-induced A375. Cells were pre-treated (30 min) with samples (2.5–100 µg ml⁻¹). For irradiation the dose of 1.8 J/cm² was used. Irradiated and non-irradiated control cells were incubated with DMSO (0.5%, v/v) under the same conditions.

Inhibition of nitric oxide production

Nitric oxide is known to play an important role in maintenance of tissue homeostasis; it is produced by nitric oxide synthase, whose inducible isoform (iNOS) is known to be implicated in several pathological conditions and inflammation. NO produced by iNOS kills infectious pathogens, but overproduction of NO results in damage to tissues and, eventually, destruction of tissue homeostasis (34). Thus, iNOS expression and NO production might be a good target for research into disturbed inflammatory conditions. Macrophages can release inflammatory mediators, such as prostaglandins,

cytokines and nitric oxide in response to lipopolysaccharide (LPS) stimulation, validating use of LPS-treated macrophages as a model of inflammation. Here, HYTE-3 caused significant dose-related inhibition of nitric oxide production in the murine monocytic macrophage cell line RAW 264.7 used, with IC_{50} of 342 $\mu\text{g/ml}$ (Fig. 5 and Table 1).

Discussion

High mortality and increased chemoresistant levels in people suffering from malignant melanoma, have spurred interest in sourcing natural products, as potential anticancer agents. Thus, in this study, we explored the possibility of using a *H. perforatum* subsp. *perforatum*-derived product HyTE-3, for treatment of malignant melanoma by examining its anti-proliferative activity on A375 human malignant melanoma cells.

Prior to the anti-cancer activity study, HyTE-3 was subjected to chemical analysis (HPTLC) to identify its polyphenolic components. Results of this revealed that the preparation consists mainly of flavonoids (rutin, quercetin, quercetrin, biapigenin), naphthodianthrones (hypericin and pseudohypericin), acylphloroglucinols (hyperforin) and phenylpropanes (chlorogenic acid). Effects of flavonoids against cancer have been extensively explored for many years and it has been proven that certain flavonoids are selectively toxic to malignant

cells. Previous studies revealed that flavonoids at micromolar concentrations were toxic to several cancer cell lines *in vitro* such as human prostate cancer cell lines, human breast cancer cell lines and lung carcinoma cells. The primary objective of this study was to identify anti-proliferative properties of *H. perforatum* subsp. *perforatum*-derived product HyTE-3, to human skin malignant melanoma and to determine whether UVA irradiation could enhance its phototoxicity. We demonstrated for the first time that HyTE-3 had a concentration- and time-dependent anti-proliferative activity on A375 cells in culture (Fig. 4).

Investigation of Bernd *et al.* (20) confirmed phototoxic properties of *Hypericum* extract using UVA, UVB and visible light, but high concentrations were necessary in both cases. This finding confirmed results of Kubin *et al.* (35) who showed *in vivo*, that hypericin is a potent photosensitizer only within UV and green light ranges, after application of very high hypericin concentrations (100 $\mu\text{g/ml}$, administered subcutaneously). One of the important criteria for potential anti-cancer agents is their ability to selectively kill cancer cells, but not normal cells. Currently, few drugs are known to target cancer cells without affecting normal ones (36); conventional anti-cancer drugs including doxorubicin, cisplatin and paclitaxel inhibit expansion of both cancer and normal cell populations, causing side effects that limit their therapeutic application.

Chronic inflammatory states are associated with pathogenesis of major chronic diseases, specially cancer (37). Various studies have demonstrated the ability of *H. perforatum* to inhibit inflammatory mediators (38). It is well known that hyperforin has potent antibacterial activity and pronounced anti-inflammatory properties (39). *In vitro* studies have confirmed that flavonoids are able to inhibit nitric oxide production, and expression of iNOS, but their strength depends on structure or subclass of flavonoids (40). Oskoueian *et al.* (41) reported the role of phenolic compounds in antioxidant activity and their ability to act as free radical and NO scavengers, leading to formation of phenoxyl radicals. Recently, Kazłowska *et al.* (42) suggested that inhibition of iNOS in RAW 264.7 cells, is due to NO suppressing action of flavonoid and phenolic compounds such as rutin and catechol.

In conclusion, use of natural products, which absorb UV photons and exhibit wide ranges of biological activities such as being antioxidant, anti-inflammatory, immunomodulatory and/or antitumour, is of great interest in UV skin protection.

Our data suggest that *H. perforatum* subsp. *perforatum* preparation, HyTE-3 has a definitive ability to suppress proliferation of human malignant melanoma A375

Figure 5. Effect *H. perforatum* extract on nitric oxide production in lipopolysaccharidestimulated RAW 264.7 macrophages.

cells. The extract, together with UVA irradiation, shows high phototoxicity. However, additional studies are needed to verify these data in other types of human malignant melanoma cells to establish broader implication of HyTE-3. In addition, further investigations to determine molecular basis of HyTE-3 activity on human malignant melanoma, as well as its efficacy *in vivo*, are crucial to support its effectiveness as an anti-cancer agent.

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