SUPPLEMENTARY MATERIALS

Echinophora tenuifolia L. branches phytochemical profile and antiproliferative activity on human cancer cell lines

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Abstract: The methanolic extract of *Echinophora tenuifolia* L. branches and its fractions were evaluated for their *in vitro* cell growth inhibitory activity on different human cancer cell lines (C32, LoVo and SKBr3) and the normal BJ fibroblasts. All tested samples were effective against the melanoma cell line C32, with IC₅₀values ranging from 22.8 ± 0.8 to $78.7 \pm 1.2 \mu g/mL$, the antiproliferative activity of the dichloromethane fraction being significantly higher. This fraction was also effective against the LoVo adenocarcinoma cell line, with an IC₅₀ value of $53.0 \pm 2.1 \mu g/mL$. The ethyl acetate and dichloromethane fractions showed the highest lipid peroxidation inhibitory activity, verified by means of the β -carotene bleaching test. The phytochemical profiles of *E. tenuifolia* branches extract were established by means of GC-MS and HPTLC. Overall, branches of *E. tenuifolia* L. could represent a rich source of bioactive compounds, potentially useful in the pharmaceutical field.

Keywords: antioxidant activity; antiproliferative activity; Echinophora; extract, flavonoid content

Experimental

Chemicals

Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, phosphate buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), chlorogenic acid, quercetin, Folin-Ciocalteu reagent, aluminum chloride, ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), propyl gallate, β-carotene, linoleic acid, Tween 20, and reference compounds utilized in HPTLC analyses were purchased from Sigma-Aldrich S.p.A. (Milan, Italy). Human melanoma cancer cells C32 (American Type Culture Collection no. CRL-1585), breast cancer cells SKBr3 (ATCC no HTB-30), colorectal adenocarcinoma cancer cells LoVo (ATCC no.

CCL-229) and normal BJ fibroblast cells (no. CRL-2522) were used in this study. Normal phase glass plates 20 cm x 10 cm with glass backed layers silica gel 60 (2-10 μ m; 2 mm thickness) were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide, methanol, ethanol, *n*-hexane, ethyl acetate and dichloromethane were obtained from VWR International s.r.l. (Milan, Italy).

Plant material and extraction procedure

Branches from *E. tenuifolia* L. were collected in the province of Crotone in Calabria (Italy) in September 2010. A voucher specimen is deposited in the Herbarium of the University of Calabria (CLU 21879, leg. and det. C. Gangale, D. Uzunov). Dried sample (44.6 g) was extracted with methanol through maceration at room temperature (446 mL; 48 h x 3) and dried under vacuum (extraction yield, 6.3%). The crude extract was then suspended in methanol/water (9:1) and partitioned with *n*-hexane (yield, 0.6%, referred to dry plant material). The aqueous methanol was then evaporated under reduced pressure, suspended in distilled water and extracted successively with dichloromethane and ethyl acetate (0.2% and 0.4%, respectively). Samples were stored at -20° C until analyses.

GC-MS analysis

The apolar volatile constituents of the *n*-hexane and dichloromethane fractions of *E. tenuifolia* L. branches were identified by means of GC-MS. The phytochemical profile was acquired on a Hewlett-Packard 6890 gas chromatograph equipped with an SE-30 capillary column (100 % dimethylpolysiloxane, 30 m length, 0.25 mm in diameter, 0.25 μ m film thickness) and a selective mass detector Hewlett Packard 5973. Analyses were conducted using a programmed temperature from 60 to 280 °C (16 °C/min) with helium as carrier gas (linear velocity, 0.00167 cm/sec) (Conforti et al. 2012). The comparison of GC retention factors with those of standards, and the comparison of mass spectra with those present in the Wiley 138 library allowed the identification of compounds.

Evaluation of total phenolic and total flavonoid contents

The total phenolics and total flavonoids contents of the *E. tenuifolia* branches methanolic extract were assessed by means of spectrophotometric methods. Values were calculated from calibration curves based on the standard chlorogenic acid (analysis of phenolics) or quercetin (analysis of flavonoids); final results were expressed as mg of chlorogenic acid or quercetin equivalent *per* g of dry plant material, respectively. The Folin-Ciocalteau reagent was utilized to evaluate total phenolics (Menichini et al. 2013) with absorbance measurements at 726 nm. The flavonoids content was estimated using a colorimetric method based on the formation of a flavonoid-aluminum complex (Marrelli et al. 2015) with absorbance measurements at 430 nm. Analyses were run in triplicate.

HPTLC analysis

Qualitative and quantitative analyses of polar constituents of the AcOEt fraction of *E. tenuifolia* L. were carried out by means of High-Performance Thin Layer Chromatography (HPTLC).

The utilized apparatus consisted of a Linomat5 sample applicator connected to a TLC Visualizer (CAMAG, Muttenz, Switzerland). Normal phase glass plates 20 cm×10 cm (silica 2-10 µm; 2 µm thickness) were used. Operating conditions were the same as previously described (Marrelli et al. 2014). Plates were developed using a mixture ethyl acetate/dichloromethane/acetic acid/formic acid/water (100:25:10:10:11, v/v/v/v/v). For post-chromatographic derivatization, the plates were treated with NPR reagent (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) or anisaldehyde reagent (1.5 mL p-anisaldehyde, 2.5 mL H₂SO₄,1 mL AcOH in 37 mL EtOH; heating at 105°C for 3 min). The plates were examined under a UV light at 254 or 366 nm and under white light upper and lower (WRT) before and after derivatization. For the qualitative analysis of phenolic compounds, the AcOEt fraction of *E. tenuifolia* L. crude extract (50 mg/mL in methanol) was used for TLC fingerprinting and co-chromatography with the reference compounds chlorogenic acid, quercitrin, catechin, caffeic acid, luteolin, naringin, rutin and biapigenin. For quantitative analyses, solutions at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 mg/mL were prepared for each marker compound. Calibration curves were prepared using absolute amount (μ g/band) as independent variable (X) and the peak area of standards as dependent variable (Y). Quantification of compounds was performed using regression equations (correlation coefficients R^2 , typically > 0.98). All determinations were carried out in triplicate (three spots on the same plate).

Free radical scavenging activity (FRSA) assay

The free radical scavenging activity (FRSA) was assayed using a test based on the reduction of a purple methanolic solution of the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). 200 μ l of samples solutions at different concentrations (5-1000 μ g/mL) were added to 800 μ l of a 10⁻⁴ M methanol solution of DPPH. After 30 min in the dark, absorbances were measured at 517 nm. Ascorbic acid was used as positive control and all experiments were run in triplicate (Conforti et al. 2006).

β-Carotene bleaching-linoleic acid assay

The antioxidant activity was determined using the β -carotene bleaching test as previously reported (Conforti et al. 2010). Briefly, 1 mL of a β -carotene solution (0.5 mg/mL in CHCl₃) was added to 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20. An emulsion was prepared by evaporation of chloroform and dilution with 100 mL of water. 0.2 mL of different samples solutions (1-100 µg/mL) were added to 5 mL of the prepared emulsion that was placed in a water bath at 45 °C; absorbances were measured at 470 nm at initial time and after 30 and 60 minutes. The antioxidant activity was measured in terms of successful prevention of β -carotene bleaching. Propyl gallate was used as positive control and all experiments were run in triplicate.

Cell lines and cell cultures

Human melanoma cancer cells C32, breast cancer cells SKBr3 and colorectal adenocarcinoma cancer cells LoVo were cultured in RPMI-1640 medium and normal fibroblast cell line BJ was maintained in Dulbecco's Modified Eagle's Medium (DMEM). Culture media were supplemented with 1 % antibiotic solution (penicillin/streptomycin), 1 % l-glutamine and 10% fetal bovine serum (FBS). Cell cultures were incubated at 37°C under 5% CO₂.

Antiproliferative activity

Cells viability analyses and cell counting were performed using the trypan blue dye exclusion staining. Cell monolayers were subcultured onto 96-well culture plates $(2x10^4 \text{ cells/well})$ used for experiments 24 hours later. Cells were then exposed to different concentrations of *E. tenuifolia* methanolic extract and fractions (ranging from 2.5 to 100 µg/mL, final concentration) for 48 h. The cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Marrelli et al. 2013). Culture medium was removed and 100µL of 0.5% w/v MTT in phosphate buffered saline were added to each well. After four hours, MTT was removed and 100 µL of DMSO were added to dissolve formazan crystals and absorbances were measured at 550 nm by means of a microplate reader (GDVDV990B/V, Roma, Italy). Control and treated cells were visualized using an inverted microscope (AE20 Motic; Motic Instruments, Inc.). Images were captured on a VWR digital camera (VisiCam 3.0 USB camera, Milano, Italy). Cytotoxicity was expressed as IC₅₀ (the concentration giving 50% inhibition compared with the control untreated cells). Experiments were performed in biological quadruplicates (four plates).

Statistical analysis

Experiments were run in triplicate, with the exception of antiproliferative assay, for which four replicates were performed. Data were expressed as means \pm S.E. D'Agostino-Pearson's K2 test was used for assessing normality of data and Levene's test for homogeneity of variances. Raw data were then fitted through nonlinear regression in order to deduce the IC₅₀ parameter (Graph-Pad Prism Software, San Diego, CA, USA). Post-hoc comparisons of antiproliferative activity of samples at 100 µg/mL with the control group were performed using Dunnett's multiple comparison test. For all other experiments, statistical significance of differences among group means were estimated by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test ($P \le 0.05$) (SigmaStat Software, Jantel Scientific Software, San Rafael, CA).

References

- Conforti F, Marrelli M, Statti G, Menichini F. 2006. Antioxidant and cytotoxic activities of methanolic extract and fractions from *Senecio gibbosus* subsp. *gibbosus* (GUSS) DC. Nat Prod Res. 20:805-812.
- Conforti F, Loizzo MR, Marrelli M, Menichini F, Statti GA, Uzunov D, Menichini F. 2010. Quantitative determination of Amaryllidaceae alkaloids from *Galanthus reginae-olgae* subsp. *vernalis* and *in vitro* activities relevant for neurodegenerative diseases. Pharm Biol. 48:2-9.

- Conforti F, Marrelli M, Statti G, Menichini F, Uzunov D, Solimene U, Menichini F. 2012. Comparative chemical composition and antioxidant activity of *Calamintha nepeta* (L.) Savi subsp. *glandulosa* (Req.) Nyman and *Calamintha grandiflora* (L.) Moench (Labiatae). Nat Prod Res. 26:91-97.
- Marrelli M, Cachet X, Conforti F, Sirianni R, Chimento A, Pezzi V, Michel S, Statti GA, Menichini F. 2013. Synthesis of a new bis(indolyl)methane that inhibits growth and induces apoptosis in human prostate cancer cells. Nat Prod Res. 27:2039-2045.
- Marrelli M, Conforti F, Toniolo C, Nicoletti M, Statti G, Menichini F. 2014. *Hypericum perforatum*: influences of the habitat on chemical composition, photo-induced cytotoxicity, and antiradical activity. Pharm Biol. 52:909-918.
- Marrelli M, Menichini F, Conforti F. 2015. A comparative study of *Zingiber officinale* Roscoe pulp and peel: phytochemical composition and evaluation of antitumour activity. Nat Prod Res. 29: 2045-2049.
- Menichini G, Alfano C, Marrelli M, Toniolo C, Provenzano E, Statti GA, Nicoletti M, Menichini F, Conforti F. 2013. *Hypericum perforatum* L. subsp. *perforatum* induces inhibition of free radicals and enhanced phototoxicity in human melanoma cells under ultraviolet light. Cell Prol. 46:193-202.

Compound ^a	RT ^b	RAP ^c	
<i>n</i> -Hexane fraction			
Terpenes			
<i>o</i> -Cymene	8.523	0.2	
Cumic alcohol	11.021	0.1	
Fatty acids			
Undecanoic acid	17.885	0.3	
Myristic acid	18.350	2.9	
Palmitic acid	18.423	0.8	
10,13-Octadecadienoic acid	19.411	2.3	
Isooleic acid	19.457	0.4	
Linoleic acid	19.777	7.8	
Stearic acid	19.862	0.2	
Phytosterols			
24,26-Dimethylcholesta-5,22-dien-3β-ol	31.703	2.4	
Stigmasterol	31.912	3.8	
22,24-Dimethylcholesterol	33.751	0.7	
Stigmast-6-en-3β-ol	33.853	0.1	
Dichloromethane fraction			
α-Phellandrene epoxide	11.708	0.1	
Carvacrol	12.445	5.0	

Table S1. Chemical constituents identified in the *n*-hexane and dichloromethane fractions from *E. tenuifolia* L. crude extract.

^aCompounds listed in order of elution from SE30 MS column.

^b Retention time (as minutes).

^c Relative area percentage (peak area relative to total TIC peak area %).

Sample	IC_{50} (µg/mL)			
	DPPH test	β-carotene bleach	β -carotene bleaching test	
		30 min	60 min	
Raw extract	$360.9\pm13.2^{\circ}$	$24.4\pm1.0^{\rm c}$	50.5 ± 1.5^{e}	
<i>n</i> -Hexane	> 1000	> 100	> 100	
Dichloromethane	400.4 ± 8.3^{d}	$22.7 \pm 1.3^{\rm c}$	46.8 ± 1.5^{e}	
Ethyl acetate	$53.7{\pm}~1.5^{\text{b}}$	15.6 ± 0.3^{b}	$31.8\pm0.9^{\text{d}}$	
Ascorbic acid*	2.00 ± 0.01^{a}	-	-	
Propyl gallate*	-	$1.00\pm0.02^{\rm a}$	1.00 ± 0.02^{a}	

Table S2. IC50 values of biological activity of *E. tenuifolia* L. branches extract and fractions.

Data are expressed as mean \pm S. E. (n=3). Different letters along column (DPPH test) or between columns (β -carotene bleaching test) indicate statistically significant differences at *P* < 0.05 (Bonferroni post-hoc test). * Positive controls.

Sample		IC ₅₀ (μg/mL)			
	C32	LoVo	SKBr3	BJ	
Raw extract	$70.0\pm1.0^{\rm c}$	> 100	> 100	> 100	
<i>n</i> -Hexane	$70.2\pm1.2^{\rm c}$	> 100	> 100	> 100	
Dichloromethane	$22.8{\pm}0.8^{a}$	$53.0\pm2.1^{\text{b}}$	> 100	> 100	
Ethyl acetate	$78.7\pm1.2^{\rm d}$	> 100	> 100	> 100	

Table S3. Antiproliferative activity exerted by *E. tenuifolia* L. samples on human cancer cell lines.

Data are expressed as mean \pm S. E. (n=4). Different indicate statistically significant differences at P < 0.05 (Bonferroni post-hoc test).



Figure S1. HPTLC chromatograms of ethyl acetate fraction from *E. tenuifolia* L. branches (A) and rutin (B.) Mobile phase: ethyl acetate/dichloromethane/acetic acid/formic acid/water (100:25:10:10:11; v/v/v/v/v).



II)

Figure S2. I) Inhibition of cell proliferation induced by samples of *E. tenuifolia* L. branches at 100µg/mL on C32, LoVo, SKBr3 and BJ cell lines. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control (Dunnett's multiple comparison test); **II**) Dose depending inhibition induced by dichloromethane fraction on LoVo (A) and C32 cancer cell line viability (B); (C) C32 cell line, control, cells in DMSO (0.5% v/v), without sample; D) C32 cells incubated with dichloromethane fraction 100 µg/mL in DMSO.