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Identification of phenolic compounds from medicinal and melliferous plants and their cytotoxic activity in cancer cells

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Abstract — The aim of this work is to carry out a phytochemical analysis and biological screenings of vegetable extracts from *Sida acuta* and *Malva sylvestris* leaves, *Castanea sativa* and *Eucalyptus camaldulensis* pollen. Chemical analyses was focused on secondary metabolites, particularly phenolic compounds, which have several roles in the plant physiological processes and had demonstrated significant capacity in the prevention and care of human health diseases. Solid phase extraction (SPE) and analyses with liquid chromatography and mass spectrometry (HPLC-MS) allowed the identification of 5,7-dimethoxycoumarin, kaempferol, quercetin, genistein, apigenin and myricetin. Moreover, the *M. sylvestris* and *S. acuta* extracts demonstrated a cytotoxic activity on murine and human cancer cell lines by using a MTT assay.

Key words: antineoplastic activity, phenolic compounds, phytochemical analysis, plant extracts.

INTRODUCTION

The plants are used in the treatment of human diseases all the time. From the last century, a scientific interest for phytotherapy increased in several medical fields such as immunology, oncology, haematology and the use of plants in medicine has affected the identification of natural compounds: cocaine, morphine, vinblastine, taxolo, codeine are some examples (Balunas and Kinghorn 2005).

The aim of this work is to carry out a phytochemical analysis of vegetable extracts and their secondary metabolites, particularly phenolic compounds. These molecules have several roles in the plant physiological processes, as protection from UV, defense against pathogens and phytophagous, pollination and dissemination, symbiosis and allelopathic interactions (Buchanan *et al.* 2000). Epidemiological evidences have supported the role that intake of antioxidants, including phenolic compounds, plays in the prevention of several chronic diseases such as cardiovascular disease, cancer, diabetes, bacterial and parasitic infections (Murakami *et al.* 1994; Sherman and Billing 1999). Two medicinal plants, *Sida acuta* Burm. F. (Malvaceae) and *Malva sylvestris* L. (Malvaceae); and two melliferous plants, *Castanea sativa* Mill. (Fagaceae) and *Eucalyptus camaldulensis* Dehnh (Myrtaceae) were selected.

S. acuta is a shrub distributed in tropical regions of the world. In traditional medicine, the leaves are used in the care of asthma, renal inflammation, colds, fever, headache, ulcers, worms and malaria (Coee and Anderson 1996). Biological screenings have shown a significant antimalarial activity of plant extracts (Karou *et al.* 2003); recently, cryptolepine and quindoline were identified in alkaloid extracts of the leaf (Banzouzi *et al.* 2004; Karou *et al.* 2005) and they are considered the major metabolites of the plant having antimalarial activity. *M. sylvestris* is an herbaceous plant used in phitotherapy and widely distributed in Italy (Guarrera 2005): the leaves are used as emollient, laxative and cough medicine (Gonda *et al.* 1990). Terpenoids, phenolic acids and anthocyanins were identified in water leaf extract of the plant (Cutillo *et al.* 2006). *C. sativa* is an arboreal plant very common in Mediterranean scrub. Its leaf extracts contain a high level of total phenolic compounds and showed a significant antioxidant potential with antiproliferative activ-

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ity on B16 cells (CALLISTE *et al*. 2001; 2005) and an *in vitro* antibacterial effect. Moreover, the seeds of the plant are used in treatment of gastroenteritis and in coeliac disease; in the relative extracts, alkaloids have been identified (HIERMANN *et al*. 2002). *E. camaldulensis* is a tree indigenous of Australia and it is the species of *Eucalyptus* more common in Italy; antioxidant activity of its leaf extract are known (EL-GHORAB *et al*. 2003).

The high pressure liquid chromatography-mass spectrometry (HPLC-MS) has been used to identify and to quantify the molecules in the plant extracts, obtained using the solid phase extraction (SPE). This extraction method allows to concentrate and to clean up the extracted sample before chromatographic injection, through the separation of interesting molecular classes from interferences of biological matrix.

Moreover, some analysed extracts have been screened about their antineoplastic activity on murine B16 melanoma and human A375 melanoma cell lines.

MATERIALS AND METHODS

Plant material - 300 g sample of *Sida acuta* L. (Malvaceae) from West Cameroun and of *Malva sylvestris* L. (Malvaceae) leaves from Italy were collected in March 2006; samples were collected and identified by Prof. A. Canini. A voucher specimen of this raw material is deposited at Herbarium of University of Rome "Tor Vergata". Moreover, pollen of *Castanea sativa* Mill. (Fagaceae) and *Eucalyptus camaldulensis* Dehnh (Myrtaceae) was collected in italian "Appia Antica" Park in May 2006.

Reagents - Standard reference compounds used for qualitative and quantitative analyses, as well as, methanol, hydrochloric acid (HCl), trifluoroacetate (TFA) and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard reference compounds are 5,7-dimethoxycoumarin, kaempferol (3,4',5,7-tetrahydroxyflavone), quercetin (3,3',4',5,7-pentahydroxyflavone), genistein (4',5,7-trihydroxyisoflavone), apigenin (4',5,7-trihydroxyflavone) and myricetin (3,3',4',5,5',7-hexahydroxyflavone). All solvents and reagents used in this study were of analytical grade purity.

Extraction of phenolic compounds - The extraction of phenolic compounds from the leaves and pollen was carried out as follows. Plant material (10 g) was reduced to a fine powder and extracted in a Soxhlet extractor (90°C for 24 h) with 200 ml of 70% aqueous methanol (v/v) acidified to pH 2 with some concentrated HCl drops.

The filtered extract was used for solid phase extraction of phenolic compounds. After methanol evaporation in a rotary evaporatory, the resulting aqueous solution was adjusted to pH 2 and extracted using C-18 cartdrige (Sigma-Aldrich): the stationary phase of the column was conditioned with methanol and TFA 0,1%, then the sample was transferred into the column and phenolic components were eluited with methanol solvent for chemical analyses.

Preparation of the standard solutions - Methanolic solutions of 5,7-dimethoxycoumarin, kaempferol, quercetin, genistein, apigenin and myricetin were prepared for qualitative and quantitative analyses of leaf extracts. In HPLC-APCI analyses, 5 μL of each standard solution were injected in the column.

Analytical protocol - Chemical analyses were performed using an HPLC instrumentation (Waters), coupled to a mass spectrometry at triple-quadrupole model TSQ 7000 (Finnigan, U.S.A.). The HPLC was equipped with a Supelco C-18 column. In the mobil phase the solvent was (A) H_2O-formic acid (99,9:0,1) and (B) methanol; it was set to a pressure of 1 ml/min and used in isocratic conditions as follows, solvent (A) 30%, solvent (B) 70%. The mass spectrometer was operated in APCI (atmospheric pressure chemical ionization) mode, detection was performed in scanning mode and peaks identified using target ions. For the samples analysed, the chromatogram peak identification was carried out with the comparison of the retention time (rt) and mass spectra of the compounds in the extract to those of analysed standards.

Cell culture - B16 (murine melanoma), A375 (human melanoma) and CHP100 (human neuroblastoma) cell lines were cultured in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum (FCS), 1% L-glutamine (v/v), 100 U penicillin and 100 μg/ml streptomycin. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

MTT assay - Cytotoxicity of *M. sylvestris* and *S. acuta* extracts was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cell suspensions (100 μl) were

seeded into sterile 96-well plates at a density of $1x10^3$ cells/well for B16 and of $2x10^3$ for A375 and CHP100; 100 µl of methanolic solutions of extract were added in cellular culture media over the final dilutions. The plates were incubated in 5% CO_2 at 37°C for a period of 48 and 72 h. After incubation time, cellular survival was determined according to the method described by MOSMANN (1983).

RESULTS AND DISCUSSION

The standard solutions and the plant methanolic extracts were analyzed in HPLC and APCI scanning mode. For each standard molecule analysed, the relative retention time (rt) was obtained. In Table 1, the rt and the characteristic molecular ion for each compound are shown. The results of chemical analyses were compared with those of standard solutions analyses, as explained in the experimental section. For each plant extract, the secondary metabolites identified are shown in Table 2: they are quercetin (rt 2.94 min), 5,7-dimethoxycoumarin (rt 3.04), kaempferol (rt 3.42 min), apigenin (rt 3.49 min), myricetin (rt 3.51 min) and genistein (rt 3.90 min). The 5,7-dimethoxycoumarin is a compound studied not much and few data are available on it, but it is a member of a very large class of molecules widely distributed in nature (EGAN *et al*. 1990). This coumarin was identified in some species, among which *Euodia borbonica* var. *borbonica* L. (VALENCIENNES *et al.* 1999), *Citrus limon* L. (SALVATORE *et al*. 2004) and *Citrus medica sarcodactylis* (GAO *et al.* 2002). It is a phototoxic coumarin (SALVATORE *et a*l. 2004); lately, some preliminary works about its anti-cancer activity have been carried out. (KAWAII *et al.* 1999). Kaempferol has antiviral (MITROCOTSA *et al.* 2000), antioxidant and antiprotozoic (CALZADA *et al*. 1999) activities; quercetin is the major flavonoid in the human diet and has been reported to exhibit antioxidative (CHOPRA *et al*. 2000), anticarcinogenic (PEREIRA *et al*. 1996), anti-inflammatory (FERRY *et al*. 1996), anti-aggregatory (PIGNATELLI *et al*. 2000) and vasodilating (PEREZ-VIZCAINO *et al*. 2002) effects. Apigenin showed to possess antitumor and a strong antioxidant activities (CÁRDENAS *et al*. 2006), as well as genistein (JEUNE *et al*. 2005; RUFER and KULLING 2006) and myricetin (VALDEZ *et al*. 2004; MAGGIOLINI *et al.* 2005). In this work, for the first time, some of active compounds responsable of vegetable activity in animal models have been isolated and identified both in *S. acuta* and *M. sylvestris*. *S. acuta* extracts showed a cancer chemopreventive activity (JANG *et al*. 2003); *M. sylvestris* extracts are reported for their radical scavenging effect (EL and KARAKAYA 2004) as well as *E. camaldulensis* and *C. sativa*; the later demonstrated also antineoplastic activity in B16 cells (CALLISTE *et al*. 2001). Surely, the identified metabolites cooperate to these effects.

Table 1 — Secondary metabolites analysed using HPLC-MS: for each, retention time obtained from chromatogram and molecular ion from mass spectrum are shown.

Number	Compound	rt (min)	Molecular ion
1	quercetin	2.94	302
2	5,7-dimeth-oxycoumarin	3.04	206
3	kaempferol	3.42	286
4	apigenin	3.49	270
5	myricetin	3.51	318
6	genistein	3.90	271

Table 2 — Identification of secondary metabolites in plant extracts analysed (l=leaf; f=flower; p=pollen).

Compound	*M. sylvestris*	*C. sativa*	*Eucalyptus*
quercetin	+ (l)	-	+ (p)
5,7-dimeth-oxycoumarin	+ (l)	-	+ (p)
kaempferol	+ (l, f)	-	+ (p)
apigenin	+ (f)	-	+ (p)
myricetin	+ (l)	+ (p)	-
genistein	+ (f)	-	-

Fig. 1 shows the results of MTT assay by using *Malva sylvestris* extract. This extract showed a cytotoxic effect for B16 and A375 cell lines. In B16 cells, the extract showed an antiproliferative activity of 61% and 97% respect to the control for 1:200 and 1:40 dilution, respectively. For A375 cells, the 1:10 dilution produced a significant reduction of the cell proliferation, 58% respect to the control; the 1:40 dilution showed a lower effect with a reduction of 85%. The biological assay showed, for the first time, that *M. sylvestris* extracts reduces proliferation of cancer cell lines. For B16 cells, the greater effetc is produced by 1:40 dilution in which the sample is more concentrated; instade, in A375 cells the lower concentartions analysed, 1:40, showed a major activity respect to 1:10. These data showed that each line reacts specifically to the same treatment, both the vegetable extracts and the methanol. *S. acuta* methanolic extract demonstrated, after 48h incubation, a dose-dependent antiproliferative effect on CHP100 cells with a cell viability reduction of

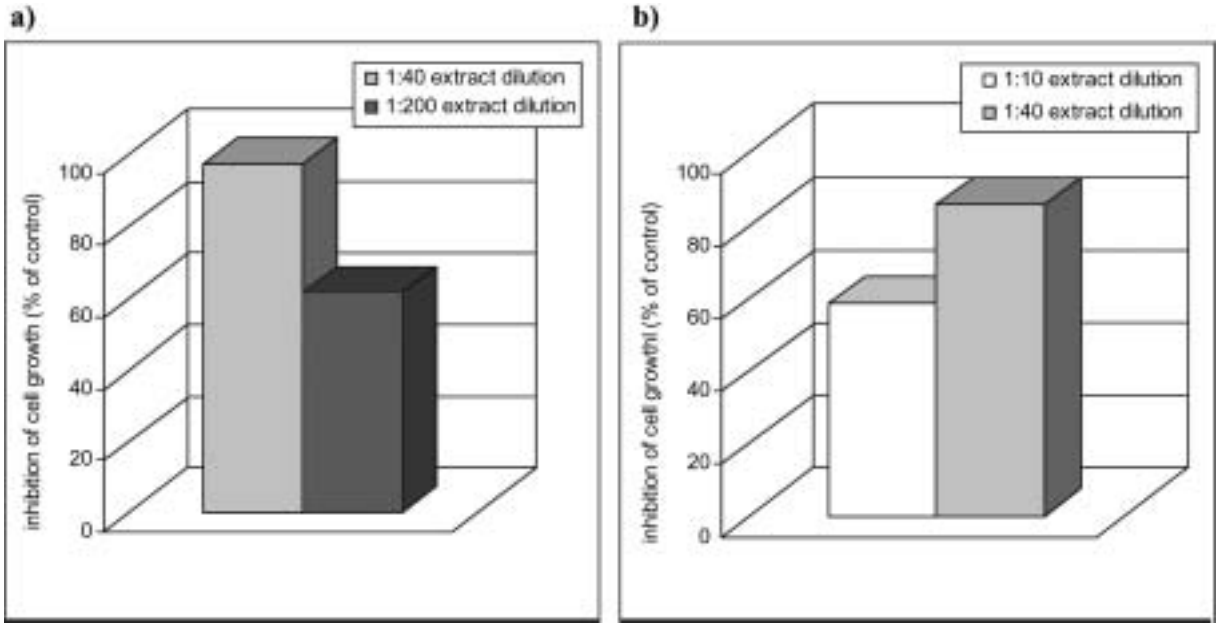

Fig. 1 — Antiproliferative activity of *M. sylvestris* extract in a) B16 and b) A375 cell lines as detected by the MTT assay. The cells were incubated with different concentrations of extract for 72 h, as reperted in the section of Materials and Methods.

7%, 9%, 20% and 82% for 0,1 μg/ml, 1μg/ml, 10μg/ml and 50μg/ml, respectively (Fig. 2). For *S. acuta*, chemopreventive activity is shown in literature (Jang *et al*. 2003) and this preliminary *in vitro* experiment showed a cytotoxic activity of metabolites from plant extract in cancer cells, which after treatment reduced significatively cellular growth and survival. Further investigations are necessary to demonstrate an antineoplastic activity of the screened extracts and to understand the molecular mechanism triggered in the cell by them until the final antiproliferative result.

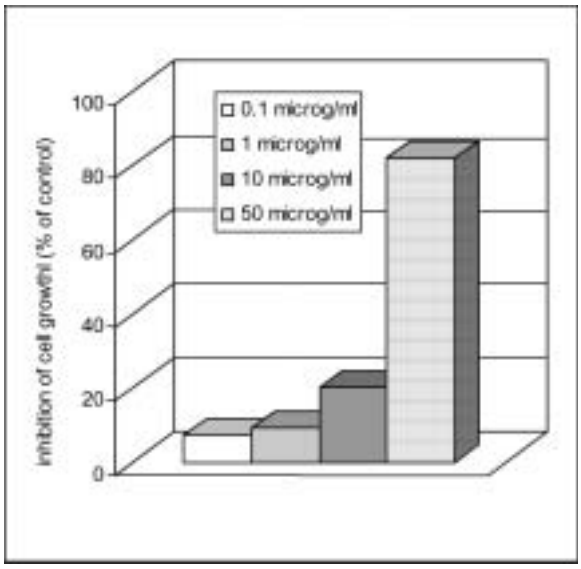

Fig. 2 — Antiproliferative activity of *S. acuta* methanolic extract in CHP100 cell line as detected by the MTT assay. The cells were incubated with different concentrations of extract for 72 h, as reperted in the section of Materials and Methods.

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