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Cytotoxic Properties of *Marrubium globosum* ssp. *libanoticum* and its Bioactive Components

Mariangela Marrelli[a], Filomena Conforti[a], Daniela Rigano[b,*], Carmen Formisano[b], Maurizio Bruno[c], Felice Senatore[b] and Francesco Menichini[a]

[a]*Department of Pharmaceutical Sciences, University of Calabria, via Pietro Bucci, I-87036 Rende (CS), Italy*
[b]*Department of Pharmacy, University of Naples "Federico II", Via D. Montesano, 49, I-80131 Naples, Italy*
[c]*Department of Biological, Chemical and Pharmaceutical Sciences and Technologies - Section Chemistry, University of Palermo, Viale delle Scienze, Parco d'Orleans II, I-90128 Palermo, Italy*

drigano@unina.it

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Marrubium globosum Montbr. et Auch. ex Benth. ssp. *libanoticum* Boiss. (Lamiaceae) is a medicinal plant used in Lebanon for the treatment of inflammatory diseases, asthma, coughs and other pulmonary and urinary problems. The goal of our study was to assess the biological activity of *M. globosum* by testing different extracts of the aerial parts for their antiproliferative activity against human melanoma cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The chloroform fraction showed the greatest activity. The compounds isolated from the extracts were also tested: the mixture of (13*S*)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone and (13*R*)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone was the most active fraction, with an IC_{50} value of 29.2 ± 0.06 μg/mL.

Keywords: *Marrubium globosum libanoticum*, Labdane diterpenoids, Phenolic compounds, Melanoma, Cytotoxicity.

The *Marrubium* genus, commonly known as horehound or hoarhound, family Lamiaceae, has more than 30 species [1]. Extracts of flowering aerial parts of *Marrubium* sp. are reported to be used in folk medicine to treat upper respiratory tract infections, cough and as a choleretic in digestive and biliary complaints. In particular, it has been used to reduce pain and spasms from menstruation and intestinal conditions [2]. Many of the activities traditionally ascribed to *Marrubium* species were confirmed by intensive modern research and clinical trials.

M. globosum Montbr. et Auch. ex Benth. ssp. *libanoticum* Boiss. is a medicinal plant called "hashiashat el kelb" in northern Lebanon. A decoction or infusion of flowers and leaves is used in traditional medicine as a hypoglycemic, febrifuge, antispasmodic and anti-inflammatory, and in external applications against snake bite and as a cicatrizant of wounds [2]. Recently, our research group has thoroughly studied this plant [3-8]. Different extracts of *M. globosum* ssp. *libanoticum* have shown a very complex metabolic pattern, containing various labdane diterpenes [4-6, 8], flavonoids, and a series of phenyl propanoid esters, together with their derivatives [5].

Previous studies demonstrated the antiproliferative activity of various *Marrubium* sp. and their constituents. Several diterpenoids isolated from aerial parts of *M. cylleneum* and *M. velutinum* showed cytotoxic effects against the human melanoma cell line FM3, the breast cancer cell line MCF-7, cervix cancer cells HeLa, and the leukemic MOLT-4, K562 and U937, and also immunomodulating potential in human peripheral blood mononuclear cells [9]. *M. alysson* extract and some isolated flavonoids (diosmetin, luteolin, and quercetin) showed *in vitro* activity against Ehrlich and human tumor cell lines U251 and MCF7 [10]. Ladanein from *M. vulgare* showed high cytotoxicity towards a dasatinib-resistant murine leukemia cell line (DA1-3b/M2BCR-ABL) and also a moderate activity against K562, K562R (imatinib-resistant), and 697 human leukemia cell lines [11].

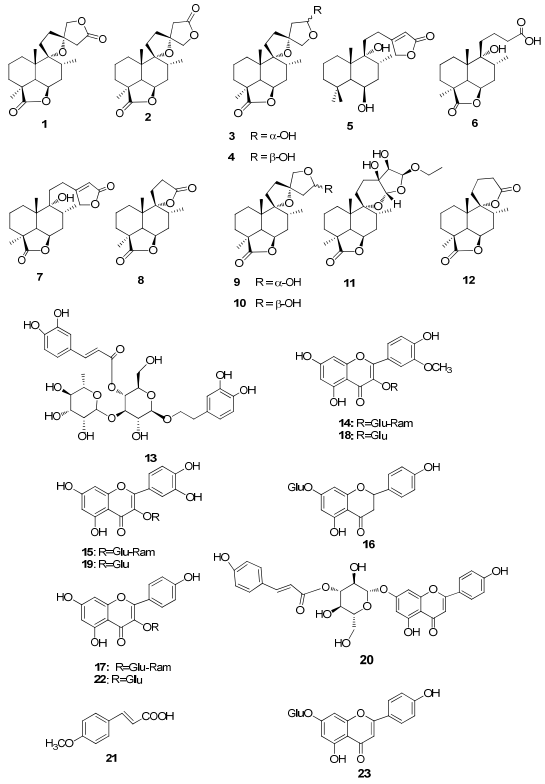

Figure 1: Structure of isolated compounds.

The crude methanol extract and different secondary metabolites of *M. thessalum* showed cell growth-inhibitory activity against four human cancer cell lines, HeLa, MCF-7, FM3 and HCT-116 [12]. Moreover, Zarai and coworkers demonstrated the effects of *M. vulgare* essential oil on HeLa cell lines [13].

Table 1: Antiproliferative activity of fractions and pure compounds isolated from *M. globosum* ssp. *libanoticum* extracts.

Compds	IC_{50} (μg/mL)	Compds	IC_{50} (μg/mL)
1, 2	29.2 ± 0.06 [a]	**14**	> 100
3, 4	> 100	**15**	n.d.
5	> 100	**16**	n.d.
6	n.d.	**17**	> 100
7	> 100	**18**	> 100
8	n.d.	**19**	> 100
9,10	41.7 ± 0.03 [b]	**20**	> 100
11	46.5 ± 0.04 [c]	**21**	> 100
12	> 100	**22**	> 100
13	> 100		

Data are expressed as mean ± SEM (n = 4). Different letters along column indicate statistically significant differences at $P < 0.05$ (Tukey's test). n.d.: not determined.

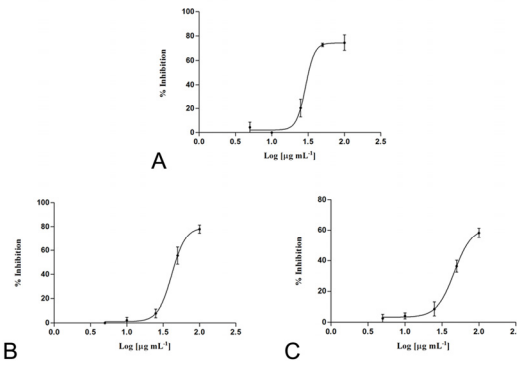

Figure 2: Antiproliferative effects exerted by fractions and pure compounds of the chloroform extract of *M. globosum* ssp. *libanoticum* on A375 cells. Data represent mean ± SEM (n = 4). A: (13S)-9a,13a-epoxylabda-6b(19),16(15)-diol dilactone (**1**) and (13R)-9a,13a-epoxylabda-6b(19),16(15)-diol dilactone (**2**); B: 13-epicyllenin A (**9**) and 13,15-diepicyllenin A (**10**); C: Marrulibacetal (**11**).

Since different extracts and metabolites such as flavonoids and diterpenoids from *Marrubium* species have been reported to exhibit antitumor activity against various human cancer cell lines [9-14], in this work, the methanol, acetone and chloroform extracts of aerial parts of *M. globosum* ssp. *libanoticum* where tested for their antiproliferative activity against human melanoma cells A375 by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The chloroform fraction showed the highest cytotoxic activity (IC_{50} = 68.2 ± 0.32 μg/mL).

Besides the extracts, all the pure compounds isolated from *M. globosum* ssp. *libanoticum* aerial parts (see Experimental) were tested for their antiproliferative activity against A375 melanoma cells. The mixture of (13*S*)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**1**) and (13*R*)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**2**) was the most active fraction, with an IC_{50} value of 29.2 ± 0.06 (Table 1, Figure 2). Among the other compounds, the mixture of 13-epicyllenin A (**9**) and 13,15-diepicyllenin A (**10**), and the pure compound marrulibacetal (**11**) also induced antiproliferative effects (IC_{50} values of 41.7 ± 0.03 and 46.5 ± 0.04, respectively). The other compounds isolated from *M. globosum* extracts did not show cytotoxic activity. The analysis of variance (Tukey's Post-hoc test) showed statistically significant differences between samples, the biological activity of compounds **1** and **2** being significantly higher than that of the other isolated labdane diterpenoids.

Morphological changes of cell line A375 were assessed after 48 h of incubation with different concentrations of bioactive molecules from *M. globosum* ssp. *libanoticum*, and significant changes in cell shape and structure were observed, while neither morphological changes nor decreases in the number of cells were observed within the control group of cells in DMEM 0.5% DMSO (A). Figure 2 shows the effects of various active fractions and pure compounds from *M. globosum.* The mixture of (13S)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**1**) and (13R)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**2**) caused a high quantitative decrease in cell number and also considerable changes in morphological shape (B).

In conclusion, we have demonstrated for the first time the cytotoxic activity of extracts from *M. globosum* ssp. *libanoticum* on the human melanoma cell line A375. The molecules responsible for such activity seem to be the mixture of (13S)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**1**) and (13R)-9α,13α-epoxylabda-6β(19),16(15)-diol dilactone (**2**) and to a lesser extent the mixture of 13-epicyllenin A (**9**) and 13,15-diepicyllenin A (**10**), and marrulibacetal (**11**). This study confirms that the genus *Marrubium* represents a potential source of many bioactive labdane diterpenoids potentially useful in the treatment of malignant melanoma and other diseases.

Experimental

Plant material: *M. globosum* ssp. *libanoticum* aerial parts were collected from flowering plants in July 2004 on Col de Cèdres, Lebanon, at 2340 m above sea level. The identification was made by Prof. N. A. Arnold, University of Saint Esprit, Lebanon, and confirmed by Prof. T. Raus, Botanische Garten, Berlin.

Preparation of the acetone extract and isolation of compounds: Dried and finely powdered aerial parts of *M. globosum* ssp. *libanoticum* (1 kg) were extracted with Me_2CO (3 X 5 L) at room temperature for 1 week. After filtration, the solvent was evaporated at low temperature (35°C) to give a gum (60 g). The diterpenoids (13*S*)-9α,13α-epoxylabda-6*β* (19),16(15)-diol dilactone (**1,** Figure 1), (13*R*)-9α,13-epoxylabda-6*β*(19),16(15)-diol dilactone (**2**), cyllenin A (**3**), 15-*epi*-cyllenin A (**4**), deacetylvitexilactone (**5**), marrulanic acid (**6**) and marrulibanoside (**7**) were isolated as described by Rigano *et al.* [5,6].

Preparation of the chloroform and methanol extracts and isolation of compounds: Dry plant inflorescences (250 g) were minced into small pieces and then sequentially extracted by cold maceration with solvents of increasing polarity [light petrol (40–60°) (3 × 2.5 L), $CHCl_3$ (3 × 2.5 L) and CH_3OH (3 × 2.5 L)]. The chloroform solution was concentrated under reduced pressure to yield 14.42 g of extract. Besides compounds **1**-**7** already present in the acetone extract, cyllenine C (**8**) 13-epicyllenin A (**9**), 13,15-diepicyllenin A (**10**), marrulibacetal (**11**), and marrulactone (**12**) were isolated, as described previously [8].

The methanol solution was concentrated under reduced pressure to yield 30 g of methanol extract. Verbascoside (**13**), isorhamnetin 3-*O*-β-D-rutinoside (**14**), quercetin 3-*O*-β-D-rutinoside (**15**), naringenin 7-*O*-β-D-glucoside (**16**), kaempferol 3-*O*-β-D-rutinoside (**17**), isorhamnetin 3-*O*-β-D-glucoside (**18**), quercetin 3-*O*-β-D-glucoside (**19**), apigenin 7-*O*-(3″-*p*-coumaryl)-glucoside (**20**), *p*-methoxy-cinnamicacid (**21**), kaempferol 3-*O*-β-D-glucoside (**22**) and apigenin 7-*O*-β-D-glucoside (**23**) were isolated, as described previously [4].

Cell line and cell culture: The human melanoma cell line A375 was grown in plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum

(FBS), 1% antibiotic/antimycotic solution (penicillin/streptomycin) and 1% L-glutamine under 5% CO_2 at 37°C. After 4-5 days, cells were removed from the 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 x 10^4 cells/well) used for experiments 24 h later. Cells were then treated with serial concentrations of samples. One hundred µL/well of each concentration was added and used in triplicate to obtain final dilutions ranging from 2.5 to 100 µg/mL for the extracts and from 5 to 50 µg/mL for pure compounds. Treatments never exceeded 0.5% of the solvent (DMSO), the percentage of solvent that was used to treat control wells. The culture plates were kept at 37°C with 5%, v/v, CO_2 for 48 h.

Evaluation of cytotoxic activity: Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously reported [15].

Cell morphology microscopy: Changes in cell morphology were visualized 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 were carried out in quadruplicate. Data were expressed as mean value ± S.E.M. The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression using GraphPad Prism Software (San Diego, CA, USA). Statistical significance was assessed using one-way analysis of variance (ANOVA) using the SigmaStat Software (Jantel scientific software, San Rafael, CA, USA). Significant differences between the means were analyzed using Tukey's multiple comparisons test. Differences of $P < 0.05$ were considered significant.

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