Cytotoxic and Proapoptotic Activity of Diterpenoids from *in vitro* Cultivated *Salvia sclarea* Roots. Studies on the Leukemia Cell Lines

Marek Różalski[a,*], Łukasz Kuźma[b], Urszula Krajewska[a], and Halina Wysokińska[b]

[a] Department of Pharmaceutical Biochemistry, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland. Fax: 0484267791 30. E-mail: mrozalski@pharm.am.lodz.pl

[b] Department of Biology and Pharmaceutical Botany, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland

[*] Author for correspondence and reprint requests

Z. Naturforsch. **61 c**, 483–488 (2006); received January 31, 2006

Four diterpenoids, ferruginol, salvipisone, aethiopinone and 1-oxoaethiopinone, were isolated from transformed roots of *Salvia sclarea*. Salvipisone and aethiopinone showed relatively high cytotoxicity against HL-60 and NALM-6 leukemia cells (IC_{50} range 0.6–7.7 μg/mL which is equal to 2.0–24.7 μM), whereas 1-oxoaethiopinone and ferruginol were less active in this regard. Moreover, we have found that all four diterpenoids of *S. sclarea* had equal cytotoxic activity against parental HL-60 and multidrug-resistant HL-60 ADR cells, what indicates that they are poor substrates for transport by multidrug resistance-associated protein (MRP1). Caspase-3 activity determinations showed that salvipisone and aethiopinone were able to induce apoptosis in a time- and concentration-dependent manner. The results obtained in this study show that *S. sclarea* diterpenoids aethiopinone and salvipisone may be useful in the treatment of human cancers, especially in the case of drug resistance.

Key words: Diterpenoids, Cytotoxicity, Leukemia Cells

Introduction

Plant extracts and purified compounds of a known chemical structure have been extensively investigated in order to identify their biological activity and their therapeutic application. *Salvia sclarea* L. (clary sage) is a biennial plant naturally occurring in the Mediterranean basin region, northern Africa and Iran. The species has been widely used in folk medicine for the treatment of stomach ache, diarrhoea, sore throat swellings and headaches (Dweck, 2000). The essential oils are the major constituents present in the aerial parts of *S. sclarea* (Giannouli and Kintzios, 2000), while the roots of the plant are rich in abietane diterpenoids (Ulubelen *et al.*, 1997; Ulubelen, 2000). Abietane diterpenoids are a class of tricyclic diterpenoids and possess several pharmacological properties, such as antibacterial (Moujir *et al.*, 1996), antifungal, antileishmanial (Tan *et al.*, 2002), sedative and tranquilizing effects (Dweck, 2000). Some of them have also been used as antitumor agents (Hernandez-Perez *et al.*, 1999). Recently, we have obtained transformed roots of *S. sclarea* after infection with *Agrobacterium rhizogenes* LBA 9402. The root culture was studied for its growth and diterpenoids synthesis and four compounds with an abietane and rearranged abietane skeleton were isolated and identified as ferruginol, salvipisone, aethiopinone and 1-oxoaethiopinone (Kuźma *et al.*, 2006a). We have also reported on the bacteriostatic as well as bactericidal activity of some of these compounds towards *Staphylococcus aureus* and *S. epidermidis* strains, regardless of their antibiotic susceptibility profile. It has also been shown that salvipisone demonstrated a very interesting antibiofilm activity (Kuźma *et al.*, 2006b). In the present study we investigated the antitumor effects of the diterpenoids isolated from the transformed roots of *S. sclarea*. The cytotoxic activity of the crude diterpenoid fraction of an acetone extract from hairy roots was also determined to compare its activity with that of a fraction from natural roots of *S. sclarea*.

Experimental

Plant material

Hairy roots were initiated on sterile four-week-old shoots of *S. sclarea* by infection with *Agrobacterium rhizogenes* LBA 9402 strain carrying the pR_i 1855 plasmid. The cultures were grown in growth regulator-free liquid B5 Gamborg medium (Gamborg *et al.*, 1968) diluted to half of the nor-

mal concentration of salts, containing 30 g/L of sucrose. Hairy root cultures were maintained in Erlenmeyer flasks on a rotary shaker (100 rpm) under continuous cool white fluorescent light at 26 °C and were subcultured at 4-week intervals. The transformation of the roots was confirmed by opine detection by electrophoresis. Additionally, PCR method was used to detect the *rol* C gene of the Ri plasmid integrated into the root genome (Kuźma *et al*., 2005a). For comparison, roots from two-year-old plants of *S. sclarea* (collected in August 2004) were used. The plants were grown in the garden of the Faculty of Pharmacy, Medical University of Łódź (Łódź, Poland). The specimen was identified by Prof. J. Siciński (Department of Plant Ecology and Phytosociology, University of Łódź). Voucher specimen was deposited at the Department of Biology and Pharmaceutical Botany, Medical University of Łódź.

Preparation of fractions and diterpenoids for bioassays

The powdered samples of lyophilized transformed and natural roots of *S. sclarea* (ca. 1.4 g) were extracted with acetone (50 mL). The extracts were evaporated under reduced pressure to yield residues (ca. 140 mg from hairy roots and 120 mg from natural roots). Each of the residue was dissolved in dichloromethane and chromatographed over a silica gel column (2 × 100 cm, 70–230 mesh, 30 g) eluted with CH_2Cl_2. TLC (silica gel 60 F_{254} plates, Merck, solvent system: $CH2Cl_2$/EtOAc, 4:1, v/v, and anisaldehyde-H_2SO_4 reagent) was used for confirming the diterpenoids presence in the fractions. After evaporation of the eluates fraction F1 (18 mg) from hairy roots and fraction F2 (10 mg) from roots of field-grown plants of *S. sclarea* were obtained and used for bioassays. The major components of the dichloromethane fraction obtained from *S. sclarea* hairy roots were isolated and identified as ferruginol (**1**), salvipisone (**2**), aethiopinone (**3**) and 1-oxoaethiopinone (**4**) (Kuźma *et al*., 2006a).

HPLC analysis of dichloromethane fractions

In order to compare the chemical profiling and the amounts of diterpenoids in F1 and F2 both fractions were analyzed on a Varian 9050 HPLC system (Varian, UK). 50 μL of filtrate (0.45 μm, Millipore) were injected on a Nucleosil C18 column (4.6 mm × 250 mm, 5 μm). Separation of diterpenoids was obtained by applying a gradient system of solvent A (water acidified with conc. H_3PO_4 to pH 2.6) and solvent B (acetonitrile/methanol, 2:1, v/v). The flow rate was 1.5 mL/min and diterpenoids were detected at 270 nm. The identification of diterpenoids was done by comparing their retention time with those of standard compounds as was previously described by Kuźma *et al*. (2006a). The diterpenoid concentrations were estimated by the interpolation of the peak areas with calibration curves constructed for standard compounds over the range of 100–400 μg/mL.

Cell lines

Human leukemia lymphoblastic NALM-6, promyelocytic HL-60 and drug-resistant HL-60 ADR (adriamycin-resistant, MRP1-overexpressing) cells were used. The NALM-6 cell line was purchased from the German Collection of Microorganisms and Cell Cultures. HL-60 cell line was obtained from the Institute of Immunology and Experimental Therapy (Wrocław, Poland) and the adriamycin-resistant subline HL-60 ADR was kindly provided by Prof. Grzegorz Bartosz, University of Łódź (Łódź, Poland). Cells were cultured in RPMI 1640 medium (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated foetal bovine serum (Cytogen, Łodz, Poland) and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) at 37 °C in 5% CO_2/95% air atmosphere.

Cytotoxicity assay

Exponentially growing cells were seeded at 3×10^5/well on a 24-well plate (Nunc, Roskilde, Denmark). Cells were then exposed to the test compounds for 48 h. Stock solutions of analyzed compounds were freshly prepared in dimethyl sulphoxide (DMSO) and diluted with complete culture medium to obtain the concentration range from 0.1 to 1000 μg/mL. Cells were then exposed to the test compounds for 48 h. After incubation, the number of viable cells was counted under a microscope in a Bürker hemocytometer using the trypan-blue exclusion assay (Roper and Drewinko, 1976). IC_{50} values (the concentration of the tested compound required to reduce the cell survival fraction to 50% of the control) were calculated from dose-response curves and used as a measure of cellular sensitivity to a given treatment.

Apoptosis studies

HL-60 cells after the treatment with salvipisone, aethiopinone and ferruginol (used at two concentrations: corresponding to the calculated IC_{50} and ten times IC_{50}) were used for the determination of caspase-3 activity by the fluorometric immunosorbent enzyme assay (Roche, Mannheim, Germany). After 1, 2 and 4 h of incubation with the tested compounds, the cells were spun down, washed twice with cold 0.01 phosphate buffer (pH 7.0), containing 0.9% NaCl, and lysed with dithiothreitol. Cellular lysates were used directly for determination of the enzyme activity. Caspase-3 activity assay, based on the capture of caspase-3 from cellular lysates by a monoclonal antibody, was performed according to the manufacturer's protocol. Caspase-3 activity is proportional to the concentration of fluorochrome (amidofluorocoumarin, AFC) formed and released from the substrate [acetyl-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC)]. Generated free AFC was determined fluorometrically at λ = 505 nm (Victor-2 multifunction counter, Wallac, Turku, Finland). Enzyme activity is expressed as the concentration (in μM) of AFC released by 10^6 cells.

Statistical analysis

Results are expressed as mean values ± SD. Statistical analysis was performed by using Student's t-test. A probability level of 0.05 was used to indicate statistical significance.

Results and Discussion

Crude diterpenoid fractions used in this study were obtained by extraction of the transformed roots of *Salvia sclarea* with acetone and further by silica gel column chromatography eluted with dichloromethane. The major components of the dichloromethane fraction obtained from *S. sclarea* hairy roots, ferruginol (**1**), salvipisone (**2**), aethiopinone (**3**) and 1-oxoaethiopinone (**4**), were isolated and also used in the study. The cytotoxic activity exhibited by the dichloromethane fraction from the hairy roots (F1) and from the roots of field-grown *S. sclarea* (F2) are shown in Table I. Fraction F1 exerted a significant cytotoxic effect against both NALM-6 and HL-60 leukemia cells, with IC_{50} values of 2.1 μg/mL and 2.6 μg/mL, respectively. Since the F1 fraction is a mixture of at least four diterpenoids, the cytotoxic activity of each purified diterpenoid against leukemia cells was also measured. Among these aethiopinone (**3**), a diterpenoid with an *o*-naphthoquinone moiety, was found to be the most active compound, with IC_{50} values of 0.6 μg/mL for NALM-6 and 4.8 μg/mL for HL-60 cells. Its high cytotoxic activity was not unexpected as the strong antiproliferative properties of aethiopinone from *Salvia aethiopis* against human carcinoma KB cells have earlier been observed by Hernandez-Perez *et al.* (1999). A relatively high cytotoxicity was also observed for salvipisone (**2**), which contains a *p*-naphthoquinone group, with IC_{50} values in the range of 6–8 μg/mL for both cell lines. Two other diterpenoids tested, ferruginol (**1**) and 1-oxoaethiopinone (**4**) exhibited only a weak cytotoxic effect, with IC_{50} values over 20 μg/mL (Table I). Relatively low cytotoxicity of ferruginol (**1**) against a panel of five cell lines was also observed earlier by Ulubelen *et al.* (1999). No previous reports on the cytotoxic effect of salvipisone and 1-oxoaethiopinone have been found. Taking into account the presented data we might hypothesize that the diversified cytotoxicity of *S. sclarea* diterpenoids reflects differences in their structure (Fig. 1). Ferruginol has no quinone group which seems to be necessary for the cytotoxic activity. The oxidation of the aethiopinone molecule at C-1 position can explain the very weak cytotoxicity of 1-oxoaethiopinone.

Table I. *In vitro* cytotoxicity of crude fractions and purified diterpenoids from *Salvia sclarea* roots tested against HL-60 and NALM-6 leukemia cells.

Compound	IC_{50} [μg/mL] (± SD)[c, d]	
	HL-60	NALM-6
Ferruginol (**1**)	33.6 ± 4.7 (117.5 ± 16.4)	27.2 ± 3.4 (95.1 ± 11.9)
Salvipisone (**2**)	6.0 ± 0.4 (19.2 ± 1.3)	7.7 ± 1.1 24.7 ± 3.5
Aethiopinone (**3**)	4.8 ± 1.7 (16.2 ± 5.7)	0.6 ± 0.03 2.0 ± 0.1
1-Oxoaethiopinone (**4**)	45.6 ± 4.8 (147.1 ± 15.5)	39.2 ± 4.9 (126.5 ± 15.8)
F1[a]	2.6 ± 0.3	2.1 ± 0.1
F2[b]	45.8 ± 4.4	37.7 ± 4.9

N = 4.
[a] Dichloromethane fraction of acetone extract from hairy roots.
[b] Dichloromethane fraction of acetone extract from field-grown roots.
[c] IC_{50}-concentration of a test compound required to reduce the fraction of surviving cells to 50% of that observed in the control non-treated cells.
[d] IC_{50} values (in brackets) are expressed in μM.

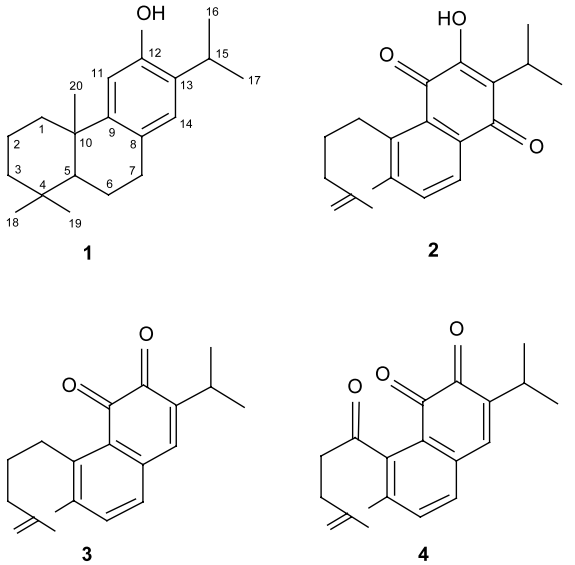

Fig. 1. Chemical structures of ferruginol (**1**), salvipisone (**2**), aethiopinone (**3**) and 1-oxoaethiopinone (**4**) isolated from hairy roots of *S. sclarea*.

Table II. Diterpenoid proportion in F1 and F2 fractions.

Compound	Content in fraction (% dry weight) (± SD)	
	F1	F2
Ferruginol (**1**)	12.8 ± 0.7	9.1 ± 1.3
Salvipisone (**2**)	12.2 ± 1.1	12.0 ± 0.6
Aethiopinone (**3**)	38.6 ± 1.8	29.1 ± 1.2
1-Oxoaethiopinone (**4**)	0.8 ± 0.1	1.0 ± 0.1

$N = 4$.

Therefore, in further experiments 1-oxoaethiopinone was excluded from bioassays. When the contents of abietane diterpenoids present in the dichloromethane fraction (F1) from *S. sclarea* hairy roots were measured by HPLC it was found that aethiopinone (**3**) was the main constituent with the value of 38.6% (Table II). It seems that aethiopinone is mainly responsible for high cytotoxicity demonstrated by this crude diterpenoid fraction. As shown in Table I, diterpenoid fraction from natural roots (F2) of *S. sclarea* was about 20-fold less cytotoxic than fraction F1 from transformed roots. Unexpectedly distinct predominance of fraction F1 in cytotoxic activity might be partly due to a higher content of the most active aethiopinone. However, since the content of total diterpenoids in fraction F1 from hairy roots was higher than

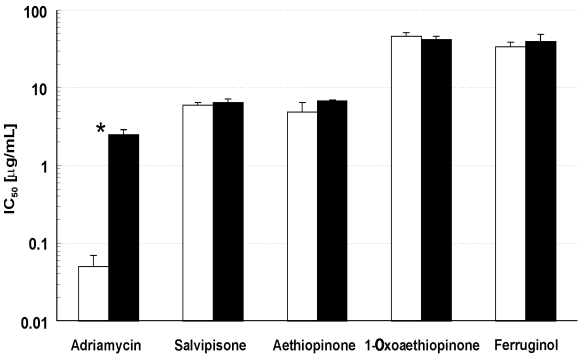

Fig. 2. Cytotoxic activity of salvipisone, aethiopinone, 1-oxoaethiopinone, ferruginol and adriamycin against parental HL-60 cells (white bars) and multidrug resistance-associated protein (MRP1)-overexpressing adriamycin-resistant HL-60 ADR cells (black bars). Averaged data ± SD from two independent experiments, each performed in triplicate. Asterisk marks statistically significant effect, $p < 0.001$.

in F2, a synergistic effect between all diterpenoids cannot be excluded. Furthermore, it is also possible that additional unknown compounds with significant activity could be present in F1. Subsequent studies on the isolation and identification of diterpenoids of *S. sclarea* hairy roots are needed to confirm this suggestion.

In the next stage of the study, cytotoxic effectiveness of four *S. sclarea* diterpenoids to maternal HL-60 cells and multidrug-resistant subline HL-60 ADR (adriamycin-resistant, MRP-1-overexpressing) was examined. Multidrug resistance (MDR) is a phenomenon found in many tumor cells. Although, resistance can be intrinsic or acquired, in either case, tumor becomes resistant to a variety of structurally diverse anticancer drugs. The best characterized mechanism of MDR is mediated by the overexpression of membrane-based proteins pumping out antitumor agents from cells, leading to a low and ineffective intracellular drug concentration (reviewed by Litman *et al.*, 2001). Thus for example, a therapeutic effectiveness of taxanes (*e.g.* paclitaxel and docetaxel), oncostatic drugs which contain a diterpene core and are used in the treatment of different kinds of human cancers (breast, lung, ovarian, bladder, and head and neck cancer), is limited by the development of drug resistance (Rowinsky, 1997; Dumontet and Sikic, 1999). Looking for new natural or synthetic compounds, which could be used effectively in the treatment of drug-resistant tumors, is therefore

still important. As shown in Fig. 2, drug-resistant HL-60 ADR cells were much less sensitive to adriamycin (doxorubicin) in comparison to maternal HL-60 cells. Interestingly, all four tested diterpenoids isolated from *S. sclarea* were equally effective against HL-60 and HL-60 ADR cells. It should be stressed out that the antitumor activity of aethiopinone (**3**) and salvipisone (**2**) was much lower than that of adriamycin (90-times), but only against the drug-susceptible HL-60 cells. In contrast, these diterpenoids were almost equally effective as adriamycin towards the drug-resistant HL-60 ADR cells. These data indicate that aethiopinone (**3**) and salvipisone (**2**) are poor substrates (if any) for transport by MRP1 pump, suggesting that they might be useful for treating drug-refractory patients.

The most cytotoxic diterpenoids, *i.e.* aethiopinone, salvipisone and ferruginol, were tested for their ability to induce apoptosis – a programmed cell death. To this end, activation of caspase-3 in HL-60 cells after treating them with the tested compounds was measured by the cleavage of the fluorometric substrate Ac-DEVD-AFC. Caspase-3 is one of the major proteases involved in apoptosis (Hale *et al.*, 1996). During an early stage of apoptosis, procaspase-3 is transformed into the active protease, and then the cascade of apoptotic process is propagated (Nagata, 2000). In our studies, cells were exposed to diterpenoids used at two different concentrations corresponding to IC_{50} and $10 \times IC_{50}$ values calculated earlier in cytotoxicity tests. The time-course of the induction of caspase-3 activity expressed as a concentration of released AFC reagent is shown in Fig. 3. The induction of caspase-3 activity began quite early, within 2 h after exposure of the cells to salvipisone and was continued in a concentration-dependent manner. In case of aethiopinone, initiation of the apoptosis process was delayed in comparison to that for salvipisone and the most significant effect was observed only at a high drug concentration ($10 \times IC_{50}$). In contrast, when HL-60 cells were exposed to ferruginol, caspase-3 activity was not induced even at high concentration (data not shown).

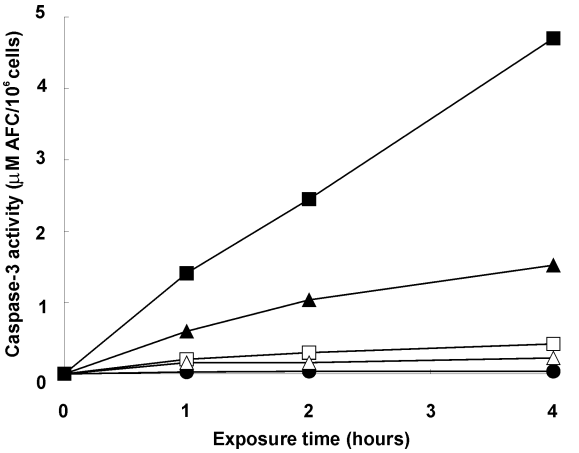

Fig. 3. Time-course of induction of caspase-3 activity in HL-60 cells by salvipisone at the concentration $1 \times IC_{50}$ (white square) and $10 \times IC_{50}$ (black square) and aethiopinone at the concentration $1 \times IC_{50}$ (white triangle) and $10 \times IC_{50}$ (black triangle). Untreated cells were used as a control (black circle). After 1, 2 and 4 h of incubation, the cells were lysed and quantified for caspase-3 activity, which is proportional to the amidofluorocoumarin (AFC) released from the labelled substrate. Generated free AFC was determined fluorometrically at λ = 505 nm. Enzyme activity is expressed as the concentration (μM) of AFC released by 10^6 cells.

In conclusion, the present study demonstrates that two diterpenoids from hairy roots of *Salvia sclarea*, aethiopinone and salvipisone, exhibit relatively high cytotoxic activity towards HL-60 and NALM-6 leukemia cells. These compounds induce programmed cell death (apoptosis) *via* the caspase-3 pathway in the concentration-dependent manner after short time exposure. Good effectiveness of *S. sclarea* diterpenoids towards drug-resistant leukemia cells suggests that they might be useful for drug-refractory patients.

Acknowledgements

This investigation was supported by research grant PBZ-KBN – 092/PO5/2003 from the State Committee of Scientific Research.

Dumontet C. and Sikic B. I. (1999), Mechanisms of action of and resistance to antitubulin agents: microtubule dynamics, drug transport and cell death. J. Clin. Oncol. **17**, 1061–1070.

Dweck A. C. (2000), The folklore and cosmetic use of various *Salvia* species. In: Sage. The Genus *Salvia*, Vol. 14 (Kintzios S. E., ed.). Harwood Academic Publ., Amsterdam, The Netherlands, pp. 1–25.

Gamborg O. L., Miller R. A., and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151–158.

Giannouli A. L. and Kintzios S. E. (2000), Essential oils of *Salvia* spp.: examples of intraspecific and seasonal variation. In: Sage. The Genus *Salvia*, Vol. 14 (Kintzios S. E., ed.). Harwood Academic Publ., Amsterdam, The Netherlands, pp. 69–79.

Hale A. J., Smith C. A., Sutherland L. C., Stoneman V. E. A., Longthorne V. L., Culhane A. C., and Williams G. T. (1996), Apoptosis: molecular regulation of cell death. Eur. J. Biochem. **236**, 1–26.

Hernandez-Perez M., Rabanal R. M., Arias A., De La Torre M. C., and Rodriguez B. (1999), Aethiopinone, an antibacterial and cytotoxic agent from *Salvia aethiopis* roots. Pharm. Biol. **37**, 17–21.

Kuźma Ł., Skrzypek Z., and Wysokińska H. (2006a), Diterpenoids and triterpenoids in hairy roots of *Salvia sclarea*. Plant Cell Tiss. Org. Cult. **84**, 152–160.

Kuźma Ł., Różalski M., Walencka E., Różalska B., and Wysokińska H. (2006b), Antimicrobial activity of diterpenoids from hairy roots of *Salvia sclarea* L.: Salvipisone as a potential anti-biofilm agent active against antibiotic resistant *Staphylococci*. Phytomedicine doi: 1016/j.phymed.2005.10.008. (in press).

Litman T., Druley T. E., Stein W. D., and Bates S. E. (2001), From MDR to MRX: new understanding of multidrug resistance systems, their properties and clinical significance. Cell Mol. Life Sci. **58**, 931–959.

Moujir L., Gutierrez-Navarro A. M., Andres L. S., and Luis J. G. (1996), Bioactive diterpenoids isolated from *Salvia mellifera*. Phytotherapy Res. **10**, 172–174.

Nagata S. (2000), Apoptotic DNA fragmentation. Exp. Cell Res. **256**, 12–18.

Roper R. R. and Drewinko B. (1976), Comparison of *in vitro* methods to determine drug-induced cell lethality. Cancer Res. **36**, 2182–2188.

Rowinsky E. K. (1997), The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. Annu. Rev. Med. **48**, 353–374.

Tan N., Kaloga M., Radtke O. A., Kiderlen A. F., Öksüz S., Ulubelen A., and Kolodziej H. (2002), Abietane diterpenoids and triterpenoic acids from *Salvia cilicica* and their antileishmanial activity. Phytochemistry **61**, 881–884.

Ulubelen A. (2000), Chemical constituents. Terpenoids in the genus *Salvia*. In: Sage. The Genus *Salvia*, Vol. 14 (Kintzios S. E., ed.). Harwood Academic Publ., Amsterdam, The Netherlands, pp. 55–68.

Ulubelen A., Sönmez U., and Topcu G. (1997), Diterpenoids from the roots of *Salvia sclarea*. Phytochemistry **44**, 1297–1299.

Ulubelen A., Topcu G., Chai H., and Pezzuto J. M. (1999), Cytotoxic activity of diterpenoids isolated from *Salvia hypargeia*. Pharm. Biol. **37**, 148–151.