ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2011; 2(6):1658-1662

ORIGINAL RESEARCH ARTICLE

Antiproliferative and Erythroid Differentiation Activities of *Cedrus libani* Seed Extracts against K562 Human Chronic Myelogenus Leukemia Cells

Antoine M. Saab[1], Ilaria Lampronti[1], Alessandro Grandini[2], Monica Borgatti[1], Alessia Finotti[1], Gianni Sacchetti[2], Roberto Gambari[1], Alessandra Guerrini*[2]

[1]*Department of Biochemistry and Molecular Biology, Section of Molecular Biology, University of Ferrara, via Fossato di Mortara 74, I-44121 Ferrara, Italy*

[2]*Department of Biology and Evolution, University of Ferrara, c.so Ercole I d'Este 32, I-44121 Ferrara, Italy*

Received 05 Aug 2011; Revised 24 Nov 2011; Accepted 01 Dec 2011

ABSTRACT

Seeds of *C. libani* have been extracted through ultrasound-assisted maceration and evaluated in order to check their antiproliferative and erythroid differentiation properties. Preliminary chemical compositions of the extracts have been also performed with the object to identify the chemical classes mainly responsible of the bioactivity. Chloroform and ethanol extracts were tested for growth inhibition and erythroid differentiation induction in K562 cells. Both the extracts were then analysed by GC and GC-MS. Chloroform extracts evidenced the predominant presence of terpenes, the monoterpenes α- and β-pinene checked as the most abundant (34.42±1.22 % and 33.28±1.08 % respectively), and most probably directly involved in the antiproliferative activity against K562 cells (IC_{50}=69.20±1.69 µg/ml). Ethanol extracts evidenced a slightly higher antiproliferative activity (IC_{50}=40.57±1.16 µg/ml) which could be mainly related to abietane diterpenoids and to the less abundant palmitic acid (0.53±0.03 %) and methyl linoleate (1.43±0.12 %). Erythroid differentiation assays showed the better performance of ethanol extracts (16.00±1.52 % at 10 µg/ml), with respect to that lower of chloroform ones (12.00 ±1.25 % at 50 µg/ml). This first report about *C. libani* seed extracts sheds a light both on the health importance of this lebanese plant specie and, in particular, on natural compounds researches for treatments of human blood related diseases.

Key words: *Cedrus libani,* antiproliferative activity, erythroid differentiation, K562 cells, GC-MS, terpenoids

INTRODUCTION

Cedrus libani L. (Cupressaceae) is widespread in the coastal mountains of Lebanon, the Alaouite Chain Mountains in Syria and in Toros mountains in Turkey, at altitudes between 1050m and 1925m above sea level. Ethnomedical, phytochemical and bioactivity studies have been performed on different parts of *C. libani* employed as human and veterinary remedies, as leaves, stems, seeds, crude oleoresins (a sort of tar) from cones, woody carved stems and roots[1]. Interesting results have been reported about antimicrobial, larvicidal and antiviral properties (*Herpes symplex* virus type 1, HSV-1) of alcoholic extracts of all *C. libani* crude drugs and of essential oils hydrodistilled from seeds: in all these reports, bioactivities have been related to the abundance of terpene compounds[3-7]. Chloroform and butanol fractions of methanol and water woody stem extracts have revealed anti-ulcerogenic properties and a promising diabetes related α-amylase inhibitory effect[2, 5].

The study about plant derived phytocomplexes, their fractions and isolated pure chemicals as possible natural tools to threat blood related disorders as complementary and/or alternative pharmaceuticals is increasing, moved both by the need to find new effective and safer drugs and by the appealing that phytopharmaceuticals meet with people as natural medicines with less side effects[6-9]. Since differentiation of K562 cells is associated with an increase in expression of embryo–fetal globin genes, such as the ζ-, ε-, and γ-globin genes, the K562 cell line has been proposed in literature as a very useful *in vitro* system to determine the therapeutic potential of new differentiating compounds or phytochemicals

***Corresponding Author:** Dr. Alessandra Guerrini, **Email:** grrlsn@unife.it, **Phone No:** +39 0532 293774

as well as for studying the molecular mechanism(s) regulating changes in the expression of embryonic and fetal human globin genes[7].

Therefore, this paper reports the results about chemical characterization, antiproliferative activity and cell differentiation induction (K562 cell lines) of different polarity extracts from *C. libani* seeds, never investigated before under this perspective as contribute to researches for new blood related disorders treatments.

MATERIALS AND METHODS

Plant material and chemicals

C. libani seeds were collected from different adult plants growing in Tanourine reserve (Lebanon) in November 2009 at the altitude 1700 m above sea level. Voucher specimens were authenticated by Prof. S. Safi, Department of Biology, Faculty of Sciences II, and deposited in the Chemistry Department Herbarium, Faculty of Sciences II of Lebanese University.

All chemicals and reagents were purchased from Sigma-Aldrich Co. and VWR (Milano, Italy). The fetal bovine serum was from CELBIO (Milano, Italy).

Sample extractions

Chloroform and ethanol extracts were obtained through ultrasound assisted maceration (20 min at 25°C) of *C. libani* dried and ground seed samples (0.9 g) employing 15 ml of each solvent. The extracted material was then centrifuged at 3000 rpm for 20 min; the supernatant was transferred into a 50 ml round bottom flask and then dried with a rotary vacuum evaporator. The extraction was performed three times.

Gas-chromatography (GC), Gas-chromatography/mass spectrometry (GC/MS)

The chloroform and ethanol extracts were analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library. A Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 µm) was used. Operating conditions were as follows: injector temperature 300°C; FID temperature 300°C, Carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55°C and then raised to 100°C at a rate of 1°C/min, then raised to 250°C at a rate of 5°C/min and finally held at that temperature for 15 minutes. One µl of each sample dissolved in CH_2Cl_2 was injected. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150°C, transfer line temperature, 300°C. The constituents of the volatile oils were identified by comparing their relative ritention time, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns with the above mentioned mass spectra libraries and with those in the literature[10]. In order to determine the Kovats index of the components, a commercial 24 aliphatic hydrocarbons mixture (Sigma Aldrich) was added to the essential oil before injecting in the GC–MS equipment and analyzed under the same conditions as above.

K562 cells bioactivity assays

Antiproliferative activity:

Human chronic myelogenous leukemia K562 cells[11] were cultured in a humidified atmosphere at 5% CO_2 in RPMI-1640 (Flow laboratories, Irvine, UK) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 mg/mL of streptomycin. The *in vitro* antiproliferative activity of *C. libani* seed extracts were assayed as follows: cell number ml^{-1} was determined by using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). Cells were plated at an initial concentration of 30000 cells/mL and the cell number/mL was determined after 4 days, when untreated cells were in the log phase of cell growth.

Erythroid differentiation induction:

Erythroid differentiation of *C. libani* extracts was evaluated by counting benzidine positives cells after suspending the cells in a solution containing 0.2% benzidine in 0.5 M glacial acetic acid, 10% H_2O_2, as elsewhere described[7,11]. Induction of differentiation was compared to that obtained using arabinose cytosine (AraC), known as strong differentiation inducer in K562 cells [11].

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± standard deviations. Differences were evaluated by one-way analysis of variance (ANOVA) test completed by Dunnett's test. Differences were considered significant at $^{**}p < 0.01$. The 50% inhibitory concentration (IC_{50}) was calculated by nonlinear regression curve with the use of Prism Graphpad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA).

IJPBA, Nov - Dec, 2011, Vol. 2, Issue, 6

© 2010, IJPBA. All Rights Reserved.

RESULTS AND DISCUSSION

Phytochemical analyses

Ultrasound assisted maceration has been performed to obtain chloroform and ethanol seed extracts with corresponding yields of 13.8±1.2 % and 35.8±2.8 % respectively. Twenty four and twenty two compounds have been checked by GC and GC-MS in chloroform and ethanol *C. libani* seed extracts respectively (**Table 1**). Ethanol extract, as most active phytocomplex, evidenced fatty acids and ethers as most abundant compounds, in particular oleic acid (17.26±1.34 %), methyl oleate (7.77±0.42 %) and ethyl oleate (5.27±0.48%). Diterpenes, as neo-abietol (11.81±0.98 %), abieta-7,13-diene (7.99±0.46 %), abieta-8(14),13(15)-diene (7.92±0.66 %), abietol (6.04±0.55 %), abietal (2.05±0.13 %) were also checked. The occurring of similar diterpenes, as abieta-7,13-diene,abieta-8(14),13(15)-diene, abieta-8,11,13-triene, has been already observed with different abundance in ethanol extracts of other *C. libani* parts (i.e. cones) [5]. In this case, instead, neo-abietol has been also detected and could be considered as peculiar of *C. libani* seeds. For what concerns chloroform extracts, terpene compounds have been exclusively detected, even if some of them were also identified in ethanol extracts (i.e. *trans*-pinocarveol, 0.71±0.04 %). The most abundant were α-pinene (34.42%), β-pinene (33.28%), verbenol (3.16%), *trans*-pinocarveol (2.82%), abieta-7,13-diene (2.58%), myrtenol (1.99%), β-phellandrene (1.98%), β-farnesene (1.89%) and abieta-8,11,13-triene(1.50%). The chemical profile of chloroform extracts, in particular for what concerns β-pinene, abieta-7,13-diene and abieta-8,11,13-triene, partially reflects what evidenced for essential oils obtained from female cones oleoresins of *C. libani* plants grown in Turkey[4, 12].

Since β-pinene exibited antiproliferative activity against cancer cells MCF-7, A375, Hep G2[13] and since α- and β-pinene have been correlated to activity detected against human leukemic K562 cells[8], we could suggest these two monoterpenes as compounds mainly involved in the growth inhibition and erythroid differentiation induction of the chloroform extract. As further element to stress this conclusion, it has been shown that monoterpenes are compounds able to exert chemopreventive as well as chemotherapeutic activities in mammary tumor models. Moreover, palmitic acid and methyl linoleate, occurring in ethanol extract as minor components, were characterized to exhibit antiproliferative activity against human leukemic K562 cells [11]. Starting from this statement, it could not be excluded that these fatty acid molecules could contribute to the activity expressed by ethanol extract, perhaps through a synergic mechanism of action involving also abietane diterpenoids, that characterize ethanol extract as one of the most abundant class of compounds, and that could be equally responsible of the bioactivity. Abietane diterpenoids, that characterize ethanol extract as class of compounds among those most abundant, could be responsible of the observed biological activity. In fact, as reported in related literature, this class of diterpene compounds was also find to be occurring in other different *Cedrus* species and responsible of strong *in vitro* antiproliferative activity against five different human cancer cell lines, i.e. MCF-7, NCI-H460, SF-268, TK-10, and AUCC-62[14].

Table 1: Chemical profile of *C. libani* seed extracts

Constituents	KI[b]	*Cedrus libani* seed extracts: Chloroform extract (%)[c]	Ethanol extract (%)[c]	ID method[a]
α-pinene	939	34.42±1.22	-	GC-MS, Co-GC
camphene	954	0.38±0.02	-	GC-MS
thuja-2,4(10)-diene	958	-	0.54±0.03	GC-MS
β-pinene	979	33.28±1.08	-	GC-MS, Co-GC
myrcene	991	1.10±0.09	-	GC-MS
p-cymene	1025	0.48±0.04	-	GC-MS
limonene	1029	1.18±0.07	-	GC-MS, Co-GC
β-phellandrene	1030	1.98±0.11	-	GC-MS
cis-limonene oxide	1137	0.46±0.03	-	GC-MS
6-camphenol	1138	0.48±0.02	.	GC-MS
trans-pinocarveol	1139	2.82±0.15	0.71±0.04	GC-MS
trans-verbenol	1145	3.16±0.89	-	GC-MS
pinocarvone	1165	0.53±0.04	-	GC-MS
borneol	1169	0.34±0.02	-	GC-MS, Co-GC
4-terpineol	1167	0.37±0.01	-	GC-MS, Co-GC
p-cymen-8-ol	1183	0.43±0.03	-	GC-MS
myrtenal	1196	0.89±0.07	0.29±0.01	GC-MS

© 2010, IJPBA. All Rights Reserved.

myrtenol	1197	1.99±0.12	-	GC-MS
verbenone	1205	1.15±0.06	0.46±0.02	GC-MS
trans-carveol	1217	0.54±0.04	-	GC-MS
bornyl acetate	1289	0.75±0.06	0.34±0.02	GC-MS, Co-GC
β-*cis*-farnesene	1443	1.89±0.15	0.41±0.02	GC-MS, Co-GC
α-bisabolene	1507	1.16±0.09	0.57±0.03	GC-MS
methyl palmitate	1922	-	0.46±0.01	GC-MS
palmitic acid	1954	-	0.53±0.03	GC-MS, Co-GC
manoyl oxide	1998	-	0.35±0.01	GC-MS
abieta-8,11,13-triene	2057	1.50±0.12	2.05±0.13	GC-MS
methyl linoleate	2096	-	1.43±0.12	GC-MS
abieta-7,13-diene	2098	2.58±0.22	7.99±0.46	GC-MS
methyl oleate	2110	-	7.77±0.42	GC-MS
methyl stearate	2125	-	0.54±0.04	GC-MS
oleic acid	2133	-	17.26±1.34	GC-MS, Co-GC
abieta-8(14),13(15)-diene	2154	-	7.92±0.66	GC-MS
ethyl oleate	2168	-	5.27±0.48	GC-MS
dehydro abietal	2275	-	1.07±0.09	GC-MS
abietal	2314	-	2.75±0.21	GC-MS
abietol	2402	-	6.04±0.55	GC-MS
neo-abietol	2469	-	11.81±098	GC-MS
total peak identified		**94.25**	**77.03**	

[a]GC-MS: gas-chromatography-mass spectrum, Co-GC:co-injection with authentic compound
[b]Retention indices calculated on a Varian VF-5ms column
[c]Relative peak area identified by GC-FID

IJPBA, Nov - Dec, 2011, Vol. 2, Issue, 6

K562 cells bioactivity assays

In order to check the antiproliferative and erythroid differentiation activities of chloroform and ethanol *C. libani* seed extracts, K562 cell lines were adopted because of their known importance as *in vitro* system to determine the interaction capacity of compounds, fractions or whole phytocomplexes with the expression of embryonic and fetal human globin genes[7-9; 11]. Both the extracts evidenced a different antiproliferative capacity (**Table 2**). In particular, the ethanol extracts showed a cell growth inhibition 41.37 % higher than that expressed by chloroform ones. Similarly, the ethanol extracts showed an higher differentiation induction capacity than that exhibited by chloroform phytocomplexes at a five fold lower concentration. If compared to the positive control, it emerges a low biological incidence of both the extracts, since AraC exhibited an IC_{50} cell growth inhibition at 250.0±0.2 nM and a 92.00±2.70 % of benzidine-positive cells, against the *C. libani* concentration samples assessed as µg/ml. However, it should be considered that each bioactivity exherted by a pure compound, even more if it is a positive control, is almost ever higher than that exhibited by a mixture of compounds, such a phytocomplex is. This is due both to the low concentration of the possible active compounds in the phytocomplex with respect to that used for the extract itself, and to the possible synergic role that the compounds in the extracts could exhert in the expression of the bioactivity[9]. These considerations, also in light of lower – but however positive – evidences, do not exclude the presence of natural compounds in *C. libani* seeds extracts as K562 cell growth inhibitors and erythroid differentiation inducers[8, 9].

Table 2: Effect of *Cedrus libani* seed extracts on K562 cell growth and differentiation (% of benzidine-positive cells after five days of culture at the indicated concentrations)

	IC_{50}	Differentiation max %
Ethanol extract	40.57±1.16 µg/ml	16.00±1.52 (10 µg/ml)
Chloroform extract	69.20±1.69 µg/ml	12.00±1.25 (50 µg/ml)
AraC	250.0±0.2 nM	92.00±2.70 (0.5 µM)

Note: IC_{50} values are mean (n = 3). Differences within and between groups were evaluated by one way analysis of variance test *** $P < 0.0001$ (F=187.6 r^2= 0.98) followed by a multicomparison Dunnett's test: **$P < 0.01$ compared with the positive control Ara C

CONCLUSION

A preliminary phytochemical investigation of chloroform and ethanol extracts of *C. libani* seeds has been here reported for the first time together with the bioactivity expressed as growth inhibition and erythroid differentiation induction on K562 cells. On the basis of previous related

© 2010, IJPBA. All Rights Reserved.

researches[8,9], the high abundance of α- and β-pinene could be related to the observed antiproliferative activity of chloroform extract, while the presence of palmitic acid, methyl linoleate and abietane diterpenoids in ethanol extracts could be related to the biological activity of this kind of phytocomplexes[15]. Even if further investigations should be performed to better characterize those compounds and their possible synergism responsible of the bioactivity, it however emerges as clear and interesting result that *C. libani* seeds could be suggested as food supplements and/or health herbal products or starting point of in-depth pharmaceutical treatments development for human chronic myelogenus leukemia.

ACKNOWLEDGEMENT

The Authors wish to thank dott.ssa Immacolata Maresca for skilful technical assistance. This research has been supported by grant of FAR 2009, University of Ferrara, Italy.

REFERENCES

1. Yusuf K, Suleyman KM, Kani I. Traditional tar production from *Cedrus libani* A.Rich on the Taurus Mountains in Southern Turkey. Econ Bot 2008; 62(4): 615-620.
2. Kizil M, Kizil G, Yavuz M, Aytekin C. Antimicrobial activity of resins obtained from roots and stems of C*edrus libani* and *Abies cilicia.* Appl Biochem Microbiol 2002; 38(2): 144-146.
3. Yilmaz N, Alma MH, Nitz S, Kollmannsberger H, Efe FT. Chemical composition of the essential oils from oleoresins on cones of *Cedrus libani*. Asian J Chem 2005; 17(4): 2300-2306.
4. Loizzo M, Saab A, Lampronti I, Gambari R, Menichini F, Cinatl J, Doerr HW. Phytochemical analysis and *in vitro* evaluation of the biological activity against herpes simplex virus type1 (HSV-1) *Cedrus libani* essential oil. Phytomedicine 2008; 15: 79-83.
5. Cetin H, Kurt Y, Isik K, Yanikoglu A. Larvicidal effect of *Cedrus libani* seeds oils on mosquito *Culex pipens.* Pharm Biol 2009; 47(8): 665-668.
6. Loizzo MR, Saab AM, Statti G, Menichini F. Phytochemical composition and in vitro α-amylase inhibitory effect of essential oils from *Cedrus libani* A.Rich. Fitoterapia 2007; 78, 323-326.
7. Bianchi N, Chiarabelli C, Borgatti M, Mischiati C, Fibach E, Gambari R. Accumulation of γ-globin mRNA and induction of erythroid differentiation after treatment of human leukaemic K562 cells with tallimustine. Br J Haematol *2001;* 113(4): 951-961.
8. Lampronti I, Saab AM, Gambari R. Antiproliferative activity of essential oils derived from plants belonging to the Magnoliophyta division. Int J Oncol 2006; 29: 989-995.
9. Guerrini A, Lampronti I, Bianchi N, Zuccato C, Breveglieri G, Salvatori F, et al. Bergamot (Citrus bergamia Risso) Fruit Extracts as γ-Globin Gene Expression Inducers: Phytochemical and Functional Perspectives. J Agric Food Chem 2009; 57: 4103-4111.
10. Adams RP. Identification of Essential Oil Components by Gas-chromatography/Quadrupole Mass Spectrometry. US, IL. Allured: Carol Stream; 2001.
11. Lampronti I, Martello D, Bianchi N, Borgatti M, Lambertini E, Piva R, Jabbar S, Choudhuri MS, Khan MT, Gambari R. *In vitro* antiproliferative effects on human tumor cell lines of extracts from the Bangladeshi medicinal plant *Aegle marmelos* Correa. Phytomedicine 2003; 10: 300-308.
12. Fleisher A, Fleisher Z. The volatiles of the leaves and wood of lebanon cedar (*Cedrus libani* A. Rich) aromatic plants of the holy land and the Sinai. Part XIV. JEOR 2000; 12 (6): 763-765.
13. Li YL, Yeung CM, Chiu LCM, Cen YZ, Ooi VEC. (2009). Chemical composition and antiproliferative acitivity of essential oil from the leaves of a medicinal herbs *Schefflera heptaphylla.* Phytother Res 2009; 23(1): 140-142.
14. Barrero AF, Quìlez del Moral JF, Herrador MM, Arteaga JF, Akssira M, Benharref A, et al. Abietane diterpenes from the cones of *Cedrus atlantica*. Phytochemistry 2005; 66: 105-111.
15. Marques CG, Pedro M, Simões MFA, Nascimento MSJ, Pinto MMM, Rodríguez B. Effect of Abietane Diterpenes from *Plectranthus grandidentatus* on the Growth of Human Cancer Cell Lines. Planta Med 2002; 68(9); 839-840.

© 2010, IJPBA. All Rights Reserved.