

Article

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Comparative study of the antitumor effect of natural monoterpenes: relationship to cell cycle analysis

Abdeslam Jaafari,¹ Mounir Tilaoui,¹ Hassan Ait Mouse,¹ Lahcen Ait M'bark,¹ Rachida Aboufatima,¹ Abderrahmane Chait,² Michel Lepoivre,³ Abdelmajid Zyad^{1,*}

¹Laboratoire de Génie Biologique, Université Sultan Moulay Slimane, Faculté des Sciences et Techniques, Morocco,

²Laboratoire Pharmacologie Neurobiologie et Comportement, Faculté des Sciences Semlalia, Morocco,

³CNRS UMR 8619, Université de Paris-Sud, France.

Abstract: Monoterpenes have been identified as responsible of important therapeutic effects of plant-extracts. In this work, we try to compare the cytotoxic effect of six monoterpenes (carvacrol, thymol, carveol, carvone, eugenol and isopulegol) as well as their molecular mechanisms. The *in vitro* antitumor activity of the tested products, evaluated against five tumor cell lines, show that the carvacrol is the most cytotoxic monoterpene. The investigation of an eventual synergistic effect of the six natural monoterpenes with two anticancer drugs revealed that there is a significant synergy between them ($p < 5\%$). On the other hand, the effect of the tested products on cell cycle progression was examined by flow cytometry after DNA staining in order to investigate the molecular mechanism of their cytotoxic activity. The results revealed that carvacrol and carveol stopped the cell cycle progression in S phase; however, thymol and isopulegol stopped it in G0/G1 phase. Regarding carvone and eugenol, no effect on cell cycle was observed.

Introduction

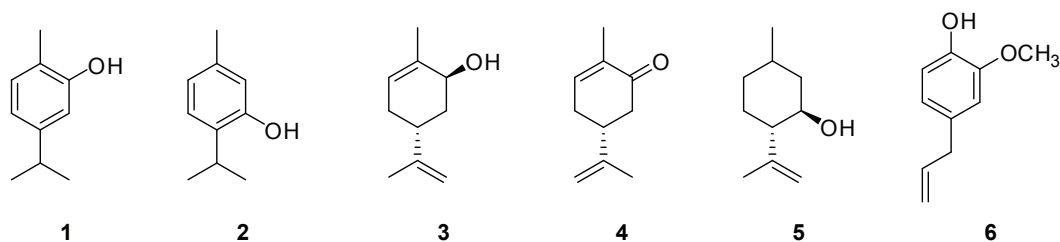
There is no doubt that medicinal plants and their extracts have important therapeutic effects. The development of techniques for chemical analysis revealed that the chemical composition of plant-extracts is very rich and diversified. Thus, a single extract may contain dozens of interactive biomolecules (Jaafari et al., 2007). Therefore, it became imperative to know those responsible of the therapeutic effect in the purpose of their purification and their industrial reproduction. Many groups of molecules, in particular monoterpenes, have been identified as responsible of pharmacological activities. These molecules showed different degrees of cytotoxicity. In fact, several biological activities of eugenol have been described in the literature (Rasheed et al., 1984; Burt, 2004) such as its *in vitro* and *in vivo* antiviral activity against human herpesvirus (Benencia & Courreges, 2000). On the other hand, when carvone promoted protection of 75%-87.5% against convulsions at 300-400 mg/kg (Zheng et al., 1992), isopulegol showed significant bactericidal and fungicidal activities (Naigre et al., 1996). Furthermore, carvacrol, a component of thyme essential oil, is one of natural products with important biological activities. It has been reported to

have an important antitumor effect (Jaafari et al., 2007, 2009; Zeytinoglu et al., 2003). The combination of these molecules with conventional drugs could have a synergistic effect (Wiseman et al., 2007; Chander et al., 1994). The aim of this work is to establish a comparative study of the *in vitro* cytotoxic activity of five monoterpenes carvacrol (1), thymol (2), carveol (3), carvone (4), isopulegol (5) and the phenyl propanoid eugenol (6) against five tumor cell lines as well as their effect on the progression of the cell cycle.

Materials and Methods

Cell lines and chemicals

Monoterpenes carvacrol (1), thymol (2), carveol (3), carvone (4), isopulegol (5), the phenyl propanoid eugenol (6) and culture medium (DMEM), foetal calf serum and methyl tetrazolim (MTT) were purchased from Sigma Aldrich (St Quentin, France). The cell lines (K-562, P-815, CEM, MCF-7 and MCF-7 gem) were gift by Dr. Michel Lepoivre (Institut de Biochimie, Orsay, France).



In vitro cytotoxic effect of the products against a panel of target cells

The cytotoxic activity was studied against the following tumor cell lines: P-815 (murine mastocytoma), K-562 (human chronic myelogenous leukemia), CEM (acute T lymphoblastoid leukemia), MCF-7 (human breast adenocarcinoma) and its counterpart resistant to gemcitabine (MCF-7 gem). Cytotoxicity was measured using the colorimetric methyl tetrazolium test (MTT) as described and modified by Tim Mosmann (Mosmann, 1983). The target cells were washed twice and placed in 96-well microtiter plates (Bioster, Italy) at a density of 1.5×10^5 cells/mL in 100 μ L/well of culture medium (DMEM supplemented with 5% FCS and 1% of penicillin and streptomycin). Then, 100 μ L of culture medium containing the specified concentration (0.05 to 1.25 μ M) of the tested compounds was added in each well. After exposure of cells to serial concentrations of tested products for 48 h at 37 °C and 5% CO₂, 100 μ L of medium were carefully aspirated from each well and replaced by 20 μ L of MTT solution (5 mg/mL of PBS). After incubation in the same conditions for 4 h, the plates were treated with a solution of HCl/isopropanol (24:1) to dissolve the blue intracellular formazan product. One hour later, the plates were read in a MicroELISA reader at 590 nm.

Synergy

Evaluation of the synergy was realised for the six monoterpenes in combination with two conventional anticancer drugs (methotrexate and cis-platin). It was performed against P-815 tumor cell line using MTT test as described above. All the molecules were used at their IC₂₀ concentration. The degree of synergism between the six monoterpenes and conventional anticancer drugs (methotrexate and cis-platin) was determined by using the combination index (CI). The last was calculated by the Chou-Talalay equation (Chou & Talalay, 1984). The general equation for the classic isobologram is given by:

$$CI = \frac{(D)_1^1 (D)_2^2}{(D_x)_1^1 (D_x)_2^2}$$

Where (D)₁ and (D)₂ are the doses of drug 1 and drug 2 required to produce x% effect in combination. (D_x)¹ and (D_x)² are the doses of drug 1 and drug 2 required to produce x% effect individually. An average CI < 1 indicates synergism, CI > 1 indicates antagonism and an average CI of 1 indicates additivity.

Cell cycle analysis

Cell cycle analysis was performed to evaluate the effect of our products on the distribution of tumor cells in G₁, S and G₂/M phases of the cell cycle. This test was performed by flow cytometry after DNA staining to reveal the total amount of DNA. Approximately, 1.5×10^6 of K-562 tumor cells were cultured in the presence of the tested products used at their respective IC₅₀. After 24 h of incubation, cells were collected, washed with PBS, fixed with cold 70% ethanol and conserved at -20 °C overnight. 100 μ L of RNase A (1 mg/mL) were then added and after 30 min of incubation at 37 °C, cells were stained with a solution containing 10 μ g/mL of propidium iodide (PI). The samples were analysed using a FACStar plus flow cytometer (Becton-Dickinson) and the WinMDI software.

Statistical analysis

Data are reported as means \pm SEM. Statistical differences were assessed by analysis of variance, with the level of significance set at $p < 0.05$.

Results

In vitro cytotoxic effect of the monoterpenes against a panel of target cells

The antitumor activity of the products was evaluated against the following five tumor cell lines: P-815, K-562, CEM, MCF-7 and MCF-7 gem. The results are summarized in Figure 1. It is shown in this figure that the cytotoxic effect depends on the nature of the products as well as on the target cell lines. In general, the effect of these products is more important on P-815 and CEM tumor cell lines compared to the other tested cells. Overall, this effect is dose-dependent. Furthermore, among the

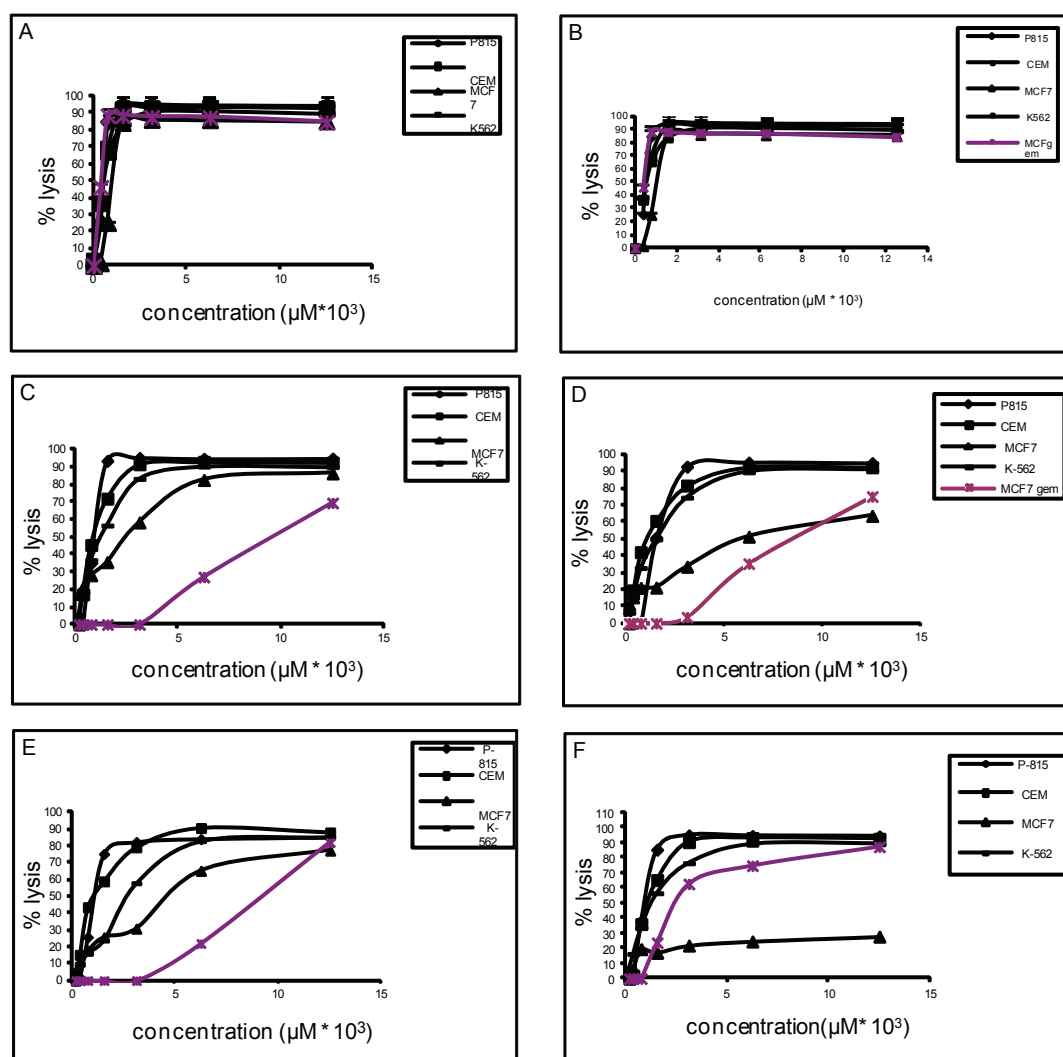


Figure 1. Cytotoxic effect of carvacrol (A), thymol (B), carveol (C), carvone (D), eugenol (E) and isopulegol (F) against different tumor cell lines: P815 (◆), CEM(■), K562(□), MCF-7(▲) and MCF-7 gem(*).

different products, the carvacrol is the most cytotoxic one and, unlike the other molecules, it acts on all the cell lines tested in the same extent. Carveol, carvone and eugenol have also an important effect, especially against P-815, K-562 and CEM tumor cell lines. The IC₅₀ values are ranging from 0.09 to 0.24 µM (Table 1). However, this effect is low against MCF-7 and very low against MCF-7 gem tumor cell lines as revealed by the IC₅₀ values ranging from 0.26 to 0.87 µM. On the other hand, comparing the effect of thymol and isopulegol on these cell lines, a differential activity can be observed from a cell line to another and it appears that P-815 is the most sensitive one; IC₅₀ 0.15 µM and 0.09 µM, respectively. Interestingly, the acquisition of resistance to gemcitabine by MCF-7 cell line was associated with a development of resistance to thymol, carveol, carvone and eugenol but not to carvacrol or isopulegol (Table 1).

Table 1. IC₅₀ (µM) of the tested monoterpenes against different target cell lines.

Product	P815	CEM	K-562	MCF-7	MCF-7/gem
Carvacrol	0.067	0.042	0.067	0.125	0.067
Thymol	0.15	0.31	0.44	0.48	-
Carveol	0.11	0.11	0.13	0.26	0.45
Carvone	0.16	0.11	0.17	0.63	0.91
Eugenol	0.10	0.09	0.24	0.41	0.87
Isopulegol	0.09	0.11	0.13	-	0.25

Synergy

These experiments were performed in order to explore a possible synergistic effect between the six natural monoterpenes and two conventional anticancer drugs (methotrexate and *cis*-platin). The results are

summarized in Table 2 and Figure 2. Our results show that the interaction of each natural monoterpene with these drugs exhibited a synergistic effect at the concentrations used (IC₂₀). This activity is statistically significant ($p < 0.05$). The percentage of lysis obtained with the different combinations varies between 53 and 62%. In addition, a slight difference which is not significant, was observed between the combinations monoterpene/methotrexate and monoterpene/*cis*-platin.

Table 2: fa and CI of molecule combinations.

Combination	fa	CI
C-MTX	54,9	0,17
C-Cis	56,6	0,01
T-MTX	61	0,14
T-Cis	57,6	0,01
Cl-MTX	53,3	0,17
Cl-Cis	57,9	0,01
Cn-MTX	51,2	0,17
Cn-Cis	58,5	0,01
E-MTX	58,6	0,15
E-Cis	55,9	0,01
I-MTX	58,5	0,15
I-Cis	62,3	0,01

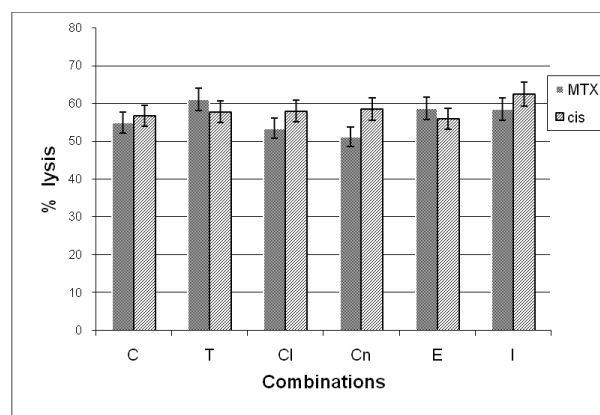


Figure 2. Synergistic effect of the six monoterpenes studied (C: carvacrol; T: thymol; Cl: carveol; Cn: carvone; E: eugenol and I: isopulegol) used in combination with two anticancer drugs (MTX: methotrexate and Cis: cis-platin)

Effect of the monoterpenes on the cell cycle

In order to investigate the molecular mechanism of the observed cytotoxic activity of our products, their effect on the cell cycle progression was examined by flow cytometry after DNA staining to reveal the cell distribution in the cell cycle phases. The results are summarized in Figure 3. The last revealed that carvacrol and carveol stopped the cell cycle progression in S phase.

However, thymol and isopulegol stopped it in G₀/G₁ phase (Figure 3). Regarding the carvone and eugenol, no effect on cell cycle was observed in these conditions.

Discussion

Monoterpenes (carvacrol, thymol, carveol, carvone, eugenol and isopulegol) have been described for their antitumor activity when studied separately. In fact, eugenol was found to induce apoptosis in mast cells (Park et al., 2005) and melanoma cells (Ghosh et al., 2005) and it has been proved not to be carcinogenic neither mutagenic (Miller et al., 1979; Stich et al., 1981). Carveol has chemopreventive activity against mammary cancer when fed during the initiation phase (Crowell, 1997). Carvone, prevent chemically induced lung and forestomach carcinoma development (Wattenberg et al., 1989). Carvacrol and thymol significantly reduced the level of DNA damage induced in K-562 cells by the strong oxidant H₂O₂ (Horvathova et al., 2007). Furthermore, carvacrol has an important *in vitro* antitumor effect against tumor cell lines like Hep-2 (Stammati et al., 1999), B16 (He et al., 1997) and A-549 (Zeytinoglu et al., 2003; Tansu Koparal & Zeytinoglu, 2003).

Comparative study of cytotoxicity induced by these monoterpenes against our panel of target cell lines revealed a differential activity from cell line to another and differential sensitivity of each cell line to these products (Table 1). As it can be observed, P-815 and CEM cell lines are the most sensitive targets to all tested molecules (Table 1). Whether the mutation of p53 in these cell lines is involved in their higher sensitivity remains to be established. Although the effects of these products are dose dependant, the carvacrol is the most cytotoxic molecule as revealed by the IC₅₀ values (Table 1). The difference of chemical structure of tested molecules could partly explain the observed difference in the cytotoxic activity between products. Indeed, the chemical structure, responsible of molecule pharmacokinetic properties, plays a fundamental role in its activity and this observation has been described in the literature. In fact, the monoterpene perillyl alcohol and its analogue, perillaldehyde induced dose- and time-dependent inhibition of proliferation in BroTo and A549 cell lines, but the IC₅₀ of the two products in 24 h were 1 and 3 mM, respectively (Elegbede et al., 2003). Interestingly, unlike carvacrol and isopulegol, the acquisition of resistance to gemcitabine by MCF7 cell line was associated with a resistance to carveol, carvone and thymol. These results suggest that these molecules may share a commune pathway. The differential sensitivity of MCF-7 and MCF-7 gem may be associated to the level of the subunit of ribonucleotide reductase R1 (Jordheim et al., 2005). In order to investigate the molecular mechanism involved in the cytotoxic activity of these monoterpenes, we have

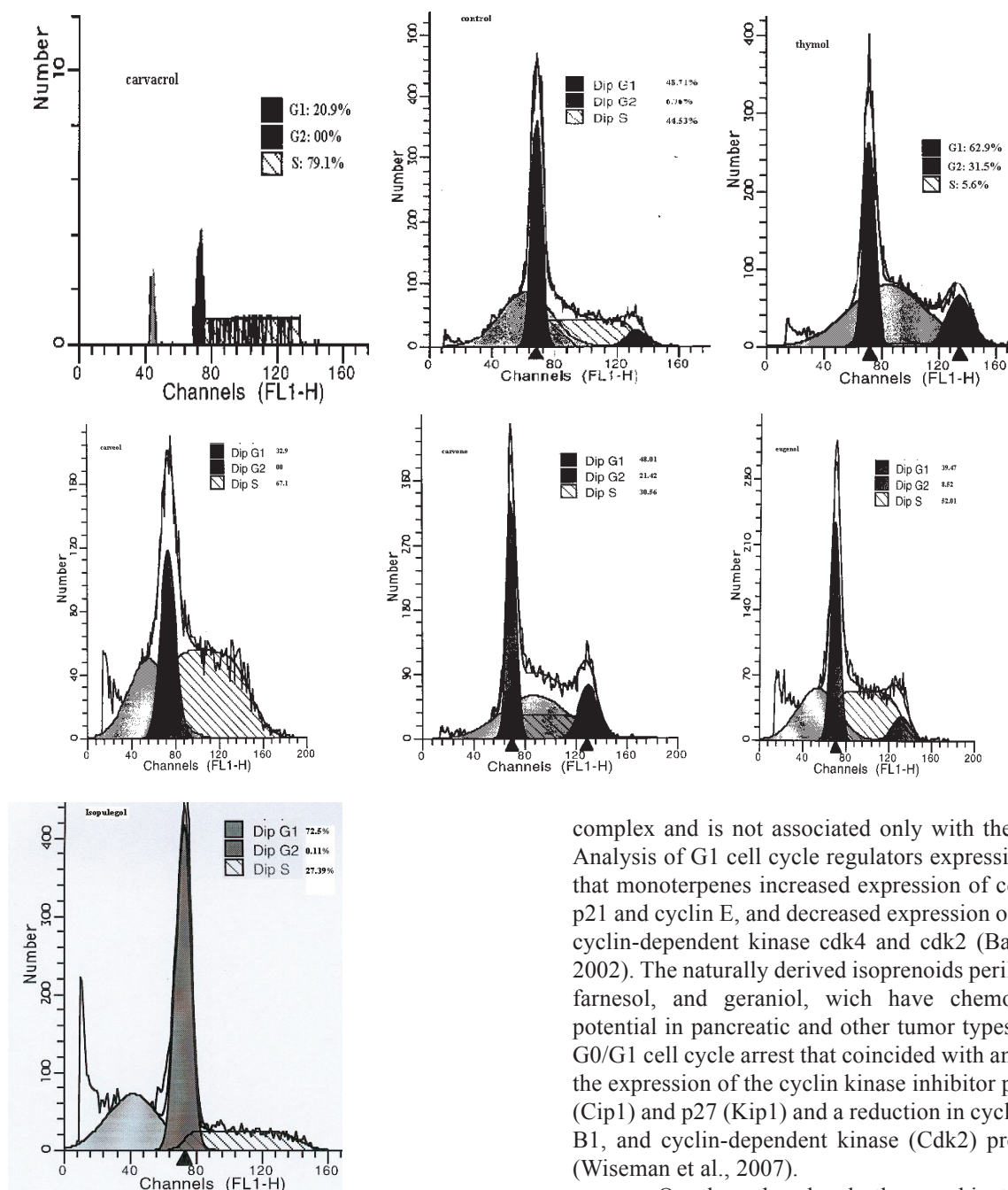


Figure 3. Effect of the tested products on cell cycle progression. The samples prepared as described in materials and methods were analysed using a FACStar plus flow cytometer and the WinMDI software. Results are the mean±SEM of two tests.

analysed their effect on the cell cycle using the K-562 cell line as target. We reported here that carvacrol and carveol caused a cell cycle arrest in the S phase when thymol and isopulegol stopped this cell cycle in G0/G1 phase. However, no effect on the cell cycle was observed for carvone and eugenol. These results suggest that the molecular mechanism of the observed cytotoxicity is more

complex and is not associated only with the cell cycle. Analysis of G1 cell cycle regulators expression revealed that monoterpenes increased expression of cdk inhibitor p21 and cyclin E, and decreased expression of cyclin D1, cyclin-dependent kinase cdk4 and cdk2 (Bardon et al., 2002). The naturally derived isoprenoids perillyl alcohol, farnesol, and geraniol, which have chemotherapeutic potential in pancreatic and other tumor types, induced a G0/G1 cell cycle arrest that coincided with an increase in the expression of the cyclin kinase inhibitor proteins p21 (Cip1) and p27 (Kip1) and a reduction in cyclin A, cyclin B1, and cyclin-dependent kinase (Cdk2) protein levels (Wiseman et al., 2007).

On the other hand, the combination of low doses of each tested monoterpene (IC20) with those of conventional anticancer drugs (methotrexate, cis-platin) showed a synergistic effect (Figure 2). This result is very important in so far that this combination could reduce the toxicity of the conventional anticancer molecules by reducing their doses. These results corroborate those of Wiseman et al. (2007), who reported that when combined to isoprenoids perillyl alcohol, farnesol and geraniol showed an additive antiproliferative activity against the human pancreatic cancer cell line MIA PaCa-2. Furthermore, Chander et al. (1994) reported that in chemotherapy of breast tumors, the combination of limonene, natural

monoterpene, and 4-hydroxyandrostenedione, inhibitor of aromatase, was more effective than each drug used alone. Interestingly, in our study we reported a synergistic effect and not an additive one suggesting that only low doses of each monoterpene combined with tolerable low doses of methotrexate or cis-platin (IC20) showed an important effect (60% lysis).

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***Correspondence**

Abdelmajid Ziad
Laboratoire de Génie Biologique, Equipe des substances naturelles et immuno-pharmacologie cellulaire et moléculaire, Faculté des Sciences et Techniques, B.P. 523, Béni-Mellal, Morocco
a.ziad@fstbm.ac.ma
Tel. 00 212 23 48 51 12/22/82 ext. 289/261
Fax: 00 212 23 48 52 01