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Antigenotoxic effects of three essential oils in diploid yeast (*Saccharomyces cerevisiae*) after treatments with UVC radiation, 8-MOP plus UVA and MMS

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Abstract

Essential oils (EOs) extracted from medicinal plants such as *Origanum compactum*, *Artemisia herba alba* and *Cinnamomum camphora* are known for their beneficial effects in humans. The present study was undertaken to investigate their possible antigenotoxic effects in an eukaryotic cell system, the yeast *Saccharomyces cerevisiae*. The EOs alone showed some cytotoxicity and cytoplasmic petite mutations, i.e. mitochondrial damage, but they were unable to induce nuclear genetic events. In combination with exposures to nuclear mutagens such as 254-nm UVC radiation, 8-methoxypsoralen (8-MOP) plus UVA radiation and methylmethane sulfonate (MMS), treatments with these EOs produced a striking increase in the amount of cytoplasmic petite mutations but caused a significant reduction in revertants and mitotic gene convertants induced among survivors of the diploid tester strain D7. In a corresponding rho^0 strain, the level of nuclear genetic events induced by the nuclear mutagens UVC and 8-MOP plus UVA resulted in the same reduced level as the combined treatments with the EOs. This clearly suggests a close relationship between the enhancement of cytoplasmic petites (mitochondrial damage) in the presence of the EOs and the reduction of nuclear genetic events induced by UVC or 8-MOP plus UVA. After MMS plus EO treatment, induction of these latter events was comparable at least per surviving fraction in wildtype and rho^0 cells, and apparently less dependent on cytoplasmic petite induction. Combined treatments with MMS and EOs clearly triggered switching towards late apoptosis/necrosis indicating an involvement of this phenomenon in EO-induced cell killing and concomitant decreases in nuclear genetic events. After UVC and 8-MOP plus UVA plus EO treatments, little apoptosis and necrosis were observed. The antigenotoxic effects of the EOs appeared to be predominantly linked to the induction of mitochondrial dysfunction.

Keywords: Essential oils; *Origanum compactum*; *Artemisia herba alba*; *Cinnamomum camphora*; *Saccharomyces cerevisiae*; Cytotoxicity; Mutation; Mitotic gene conversion; Cytoplasmic petite mutations; Antigenotoxic effects; Apoptosis; Necrosis

1. Introduction

Antimutagenic effects of natural compounds in pro- and eukaryotic cell systems are often associated with their antioxidant and antimetabolic activities [1,2]. Indeed, after treatments with mutagens, most protec-

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tive agents react either directly with the mutagen or they interfere with the free radicals or reactive oxygen species produced [3–5], they inhibit cytochrome P450-mediated metabolism or inactivate active metabolites [1,2]. Essential oils (EOs) used as ingredients in foods, perfumes, pharmaceuticals and natural medicine are susceptible to exert such activities. Some ingredients of EOs have antimutagenic (anticarcinogenic) properties in bacterial and eukaryotic cells [6–9].

Recently, we showed that the three EOs extracted from the medicinal plants *Origanum compactum*, *Artemisia herba alba* and *Cinnamomum camphora* were able to induce oxidative stress, cytotoxic effects and expression of DNA-responsive genes in the yeast *Saccharomyces cerevisiae* [8]. The EOs were unable to induce nuclear genetic events such as ILV^+ point mutations and TRP^+ mitotic gene conversion, however, they clearly induced cytoplasmic petite mutations. This mitochondrial damage appeared to be somewhat linked to cytotoxicity and the absence of nuclear mutations.

In view of the beneficial effects of these EOs in humans [6–8] we were interested in the present study to assess the possible antigenotoxic activities of these EOs and to identify the mechanisms involved using the diploid yeast strain D7 of *S. cerevisiae* and known nuclear mutagens. We used three typical genotoxic agents inducing distinct types of lesions in DNA: UVC inducing mainly intrastrand crosslinks (pyrimidine dimers and 6-4 photoproducts) [10], 8-methoxypsoralen (8-MOP) plus UVA inducing mainly monoadducts and interstrand crosslinks [11,12] and the direct acting monofunctional alkylating agent methylmethane sulfonate (MMS) producing mainly N7 and N3 alkylations of purines (7-methylguanine and 3-methyladenine) and heat-labile sites in DNA [13,14]. UVC, 8-MOP plus UVA and MMS are effective inducers of nuclear genetic events in strain D7 [15,16]. We determined the relationship between these nuclear genetic events and mitochondrial damage induced in the presence and in the absence of EOs, by analysing the induction of point mutation revertants, mitotic gene convertants and cytoplasmic petite mutations in the surviving population, as well as the induction of apoptosis and/or necrosis using annexin V-FITC and propidium iodide staining and cytometric analysis [17].

We show here that in eukaryotic cells (yeast) incubation with EOs from *O. compactum*, *A. herba alba* and *C. camphora* causes a significant reduction in the nuclear genetic events induced by the three genotoxic agents. Depending on the agent and the type of damage induced, the antigenotoxic effect of the EOs appears to be triggered by mitochondrial damage and/or the initiation of apoptosis and necrosis.

2. Materials and methods

2.1. Essential oils

We used three essential oils extracted from flower heads of *O. compactum* and *A. herba alba* of Morocco and from leaves of *C. camphora* of Madagascar. The oils were chemotyped by PRANARÔM (B-7822 Ghislenghien, Belgium) [8].

2.2. Genotoxic agents

For the induction of mutagenic and recombinogenic events, we used several DNA-damaging agents: 254-nm ultraviolet radiation (UVC) from a low-pressure Philips lamp at a dose rate of $2\,J\,m^{-2}\,s^{-1}$, the bifunctional furocoumarin 8-methoxypsoralen (CAS 298-81-7, Sigma–Aldrich, USA) plus 365-nm UVA radiation at a dose rate of $12\,kJ\,m^{-2}\,s^{-1}$ from an HPW125 Philips lamp, and the chemical mutagen methylmethane sulfonate (CAS 66-27-3, Sigma–Aldrich).

2.3. Yeast strains and growth media

For the detection of cytotoxic effects, the induction of cytoplasmic petite mutations, ILV^+ point mutations, TRP^+ mitotic gene convertants and mitotic crossing-over involving the *Ade* 2 gene we used the diploid tester strain D7 (*a*/α, *Ade*2-40/*Ade*2-119, *Trp*5-12/*Trp*5-27, *Ilv*1-92/*Ilv*1-92) of the yeast *S. cerevisiae* [18]. To verify the importance of the lack of mitochondrial functions, we employed a rho^0 derivative of D7 isolated after growth of the strain D7 in the presence of 10 μg/mL ethidium bromide and selected by resistance to lycorine. For cell growth and the detection of nuclear and cytoplasmic genetic events we used the same media as before [8,15].

2.4. Treatments with UVC, 8-MOP plus UVA and MMS, alone or in combination with essential oils

Cells were grown in YEPD for 48 h at 30 °C up to the stationary phase of growth. The cells were washed once and resuspended at 2×10^7 cells/mL in distilled water.

For each treatment dose of UVC, cell suspensions were irradiated in petri dishes. Four milliliters were then transferred into erlenmeyer flasks for immediate incubation in the dark at ambient temperature for 90 min with and without the EOs using 4 μL/mL for *C. camphora* and *A. herba alba* and 0.3 μL/mL for *O. compactum*. At these concentrations EOs show moderate cytotoxicity and are not mutagenic [8]. Since the stock solutions of EOs were prepared in ethanol we added to the samples without EOs the corresponding amount of ethanol (final concentration, 1.25%). Experiments were performed under dim light to avoid possible photoreactivation of UV-induced damage.

For treatments with 8-MOP plus UVA, cells were incubated for 30 min in the dark in the presence of 5×10^{-5} M 8-MOP.

Cell suspensions were then exposed to different doses of UVA radiation and transferred to erlenmeyer flasks for immediate incubation with and without EOs under the same conditions as described above. To avoid unwanted photoreactions of 8-MOP, experiments were performed under dim light.

For treatments with methylmethane sulfonate, measured amounts were added to 4 mL of the cell suspensions. Immediately thereafter, the different samples were incubated with and without EOs as described above.

After incubation, cells were plated on appropriate media and cultured for 5–7 days at 30 °C as described before [8]. Clonogenic survival (cytotoxicity) and the frequencies of cytoplasmic petite mutations, mitotic intergenic recombinants involving the *ade* 2 locus, *ILV*$^+$ gene revertants and *TRP*$^+$ mitotic gene convertants were also determined as described previously [8]. The spontaneous frequencies of *ILV*$^+$ revertants and *TRP*$^+$ convertants ranged from 4 to 8 revertants/10^6 survivors and from 5 to 12 convertants/10^5 survivors, respectively.

Independent experiments were performed at least in quadruplicate, and standard errors were calculated.

2.5. Flow cytometric analysis of apoptosis and necrosis in diploid yeast (D7)

To test for the induction of programmed cell death (apoptosis) and necrosis we used the method described by Markkanen et al. [17]. Treatments with the three genotoxic agents, with and without the three EOs, were performed as described above. For each sample, i.e. the control and the highest dose of the mutagen with and without EO, 2-mL aliquots at 2.5×10^6 cells/mL were washed once in distilled water and once in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$). Cell walls were digested by incubation of the pelleted cells in 2 mL of binding buffer containing 50 U/mL lyticase (Sigma–Aldrich) for 2 h at 30 °C. The resulting spheroplasts were washed once with binding buffer, resuspended in 195 μL of the same buffer and 5 μL of Annexin-Fluos (Cat. No. 1 828 681, Roche) and incubated for 10 min in the dark at room temperature. Cells were then washed in binding buffer, resuspended in 190 μL of the same buffer, and 10 μL of a 20 μg/mL solution of propidium iodide (Sigma–Aldrich) was added. After 15 min incubation at room temperature in the dark, 300 μL of buffer was added. The cell suspension was submitted to flow assisted cell sorting (FACS) analysis using a Becton Dickinson FACSCAlibur cytometer (Becton Dickinson, San José, CA) with an argon-ion laser (excitation wavelength, 488 nm) and Cell QuestTM 3.3 software. Amounts of apoptotic and necrotic cells were determined at flow rates of 200–500 particles per second, and 10,000 cells were analysed per sample. Experiments were performed in triplicate.

2.6. Statistical analysis

Data were analysed using the statistical and graphical functions of Kaleidagraph version 3.6. The significance of differences between samples with and without EO treatments was assessed by use of the Student's *t*-test. The basic significance level was set at $p < 0.05$. In the group *t*-test for multiple sample analysis, *p*-values of <0.05, <0.01 and <0.001 indicated statistically significant differences.

3. Results

With the aim to identify the most suitable conditions for the detection of possible antimutagenic effect of the EOs, diploid yeast cells were incubated with the EO from *O. compactum* before and after treatment with the mutagen UVC. In both cases, similar antimutagenic effects were obtained that were more important after pre-incubation (data not shown). The same was true for the treatments with 8-MOP plus UVA with and without the EO. However, to avoid artifacts due to either thorough washing of cells to remove the EOs before irradiation or additional cell sensitization due to unwanted EO photoreactions or photodegradation, we chose to use EO post-treatment incubations after UVC and 8-MOP plus UVA treatments.

3.1. Cytotoxic effects of UVC, 8-MOP plus UVA and MMS, alone and in combination with treatments with three EOs in diploid yeast (D7)

As shown in Fig. 1A–C, incubation with the three EOs from *C. camphora* (CIN), *A. herba alba* (ART) and *O. compactum* (ORI) only slightly modulates the colony-forming ability (survival) of D7 cells treated with UVC radiation or 8-MOP plus UVA, for which there is a tendency to resistance, especially with ART. However, the oils cause a significant decrease in survival in the case of MMS treatment. Knowing that *O. compactum* is much more cytotoxic than *C. camphora* and *A. herba alba* at equal concentrations, we tested in the combined treatments the EOs at approximately equitoxic concentrations (ORI (0.3 μL/mL) $53.6 \pm 6.3\%$, CIN (4 μL/mL) $59.7 \pm 8.7\%$, ART (4 μL/mL) $37.1 \pm 8.7\%$ survival after 90 min incubation).

3.2. Induction of ILV$^+$ revertants and TRP$^+$ mitotic gene convertants by UVC, 8-MOP plus UVA, MMS alone and in combination with three EOs as a function of dose in diploid yeast (D7)

In order to evaluate the protective effects of the three EOs on the induction of nuclear genetic events amongst the surviving cell population we plotted the data obtained on the induction of *ILV*$^+$ revertants and *TRP*$^+$ convertants with and without EOs as a function of dose after treatments with UVC, 8-MOP plus UVA and MMS.

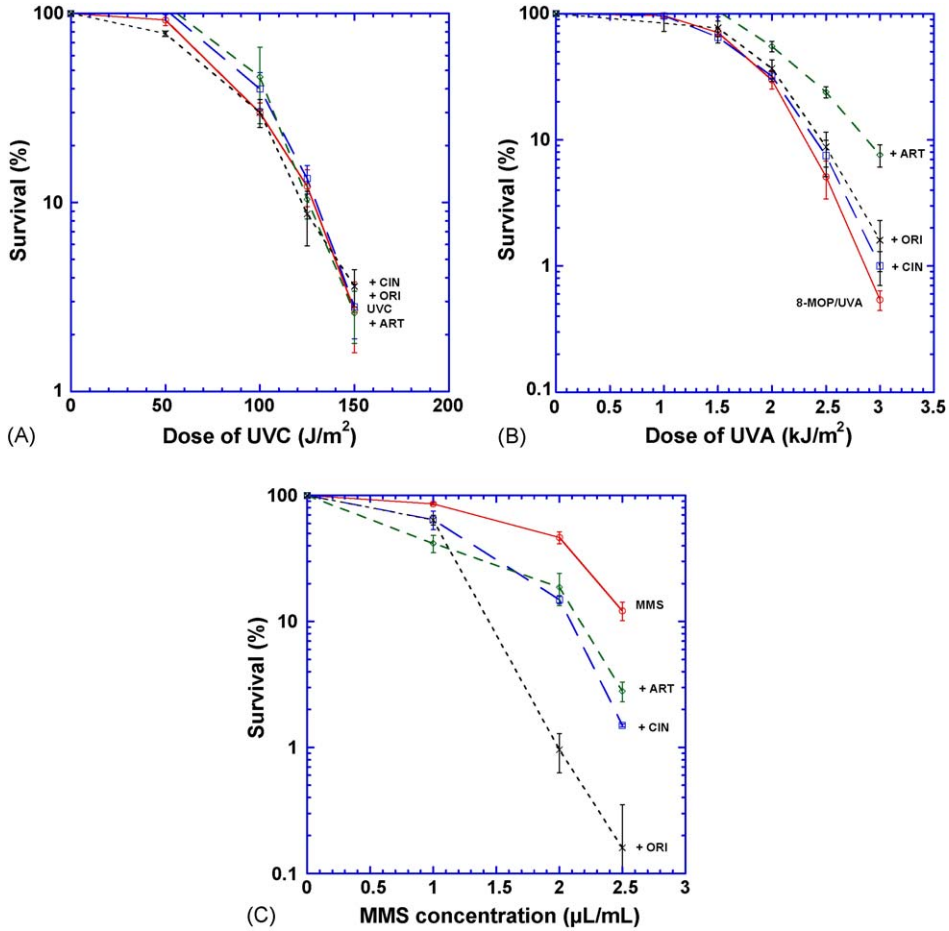

Fig. 1. Survival of diploid yeast cells (D7) in stationary phase of growth after treatments with UVC (A), 8-MOP/UVA (B) or MMS (C) alone and in combination with the EOs CIN, ART or ORI. Surviving fractions were calculated by taking the zero control for the mutagens and the EOs control for the combined treatments mutagens/EOs. According to the Student's *t*-test the differences between UVC and UVC + EOs are not significant; the differences between 8-MOP/UVA and 8-MOP/UVA + EOs are significant only with ART for the last four doses ($p < 0.001$) and the differences between MMS and MMS + EOs are significant with the three EOs for the last two doses ($p < 0.001$).

After UVC exposure and treatment with 8-MOP plus UVA, post-treatments with the three EOs clearly result in significant decreases in *ILV*$^+$ revertants (Fig. 2A and B) and *TRP*$^+$ gene convertants (Fig. 2D and E). Comparable reductions of mutagen-induced genotoxic events by EOs are observed as a function of survival (Fig. 3A, D, B and E), without decrease in survival with EOs, but even with a slight enhancement in the case of 8-MOP plus UVA combined with ART.

In the case of MMS treatment, the protective effect by the EOs against the induction of *ILV*$^+$ revertants (Fig. 2C) and *TRP*$^+$ gene convertants (Fig. 2F) appears to be less, but it is still significant for the highest dose. However, when plotting the results as a function of survival, all three EOs show a clear protective effect (Fig. 3C and F) with decreasing survival. Concerning the induction of mitotic intergenic recombination involving the *Ade2* locus, a decrease after EO treatments is also observed as a function of dose and survival (data not shown).

Thus, a certain protection against nuclear genetic effects is obtained by incubation with EOs. Interestingly, the protective effects of the EOs are similar, irrespectively whether taking into account the effect of EOs alone or not. It is clear that treatment with the EOs strongly interferes with the effects of the nuclear mutagens, giving rise to statistically significant reductions of nuclear genetic events, especially in the range of high doses and low survival. The results indicate that the EOs may exert a specific inhibitory effect on cells undergoing mutagenic or convertogenic modifications, possibly by a common mechanism.

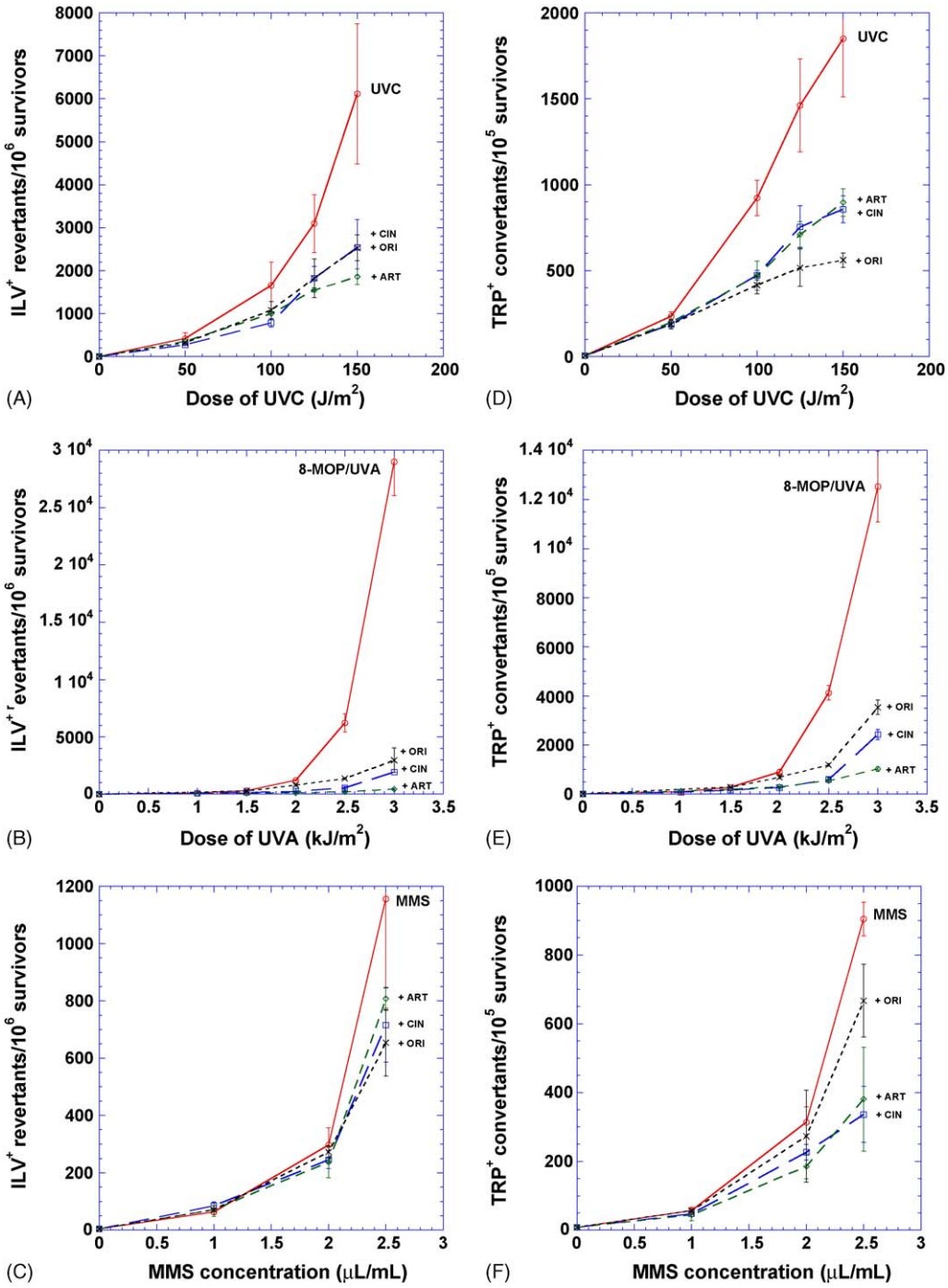

Fig. 2. Induction of *ILV*$^+$ revertants and *TRP*$^+$ mitotic gene convertants by UVC (A and D), 8-MOP/UVA (B and E) and MMS (C and F) alone and in combination with the EOs CIN, ART or ORI in diploid yeast (D7) as a function of dose or concentration. The spontaneous levels of revertants and gene convertants induced by the mutagens or the EOs alone have not been subtracted in the plots for the mutagens alone and the combined treatments. The differences between UVC and UVC + EOs are significant with the three EOs for the last three doses ($p < 0.05$ for *ILV*$^+$, $p < 0.01$ for *TRP*$^+$), the differences between 8-MOP/UVA and 8-MOP/UVA + EOs are significant with the three EOs for the last three doses ($p < 0.001$ for *ILV*$^+$ and *TRP*$^+$) and the differences between MMS and MMS + EOs are only significant with CIN and ART for the last dose ($p < 0.05$ for *ILV*$^+$ and *TRP*$^+$).

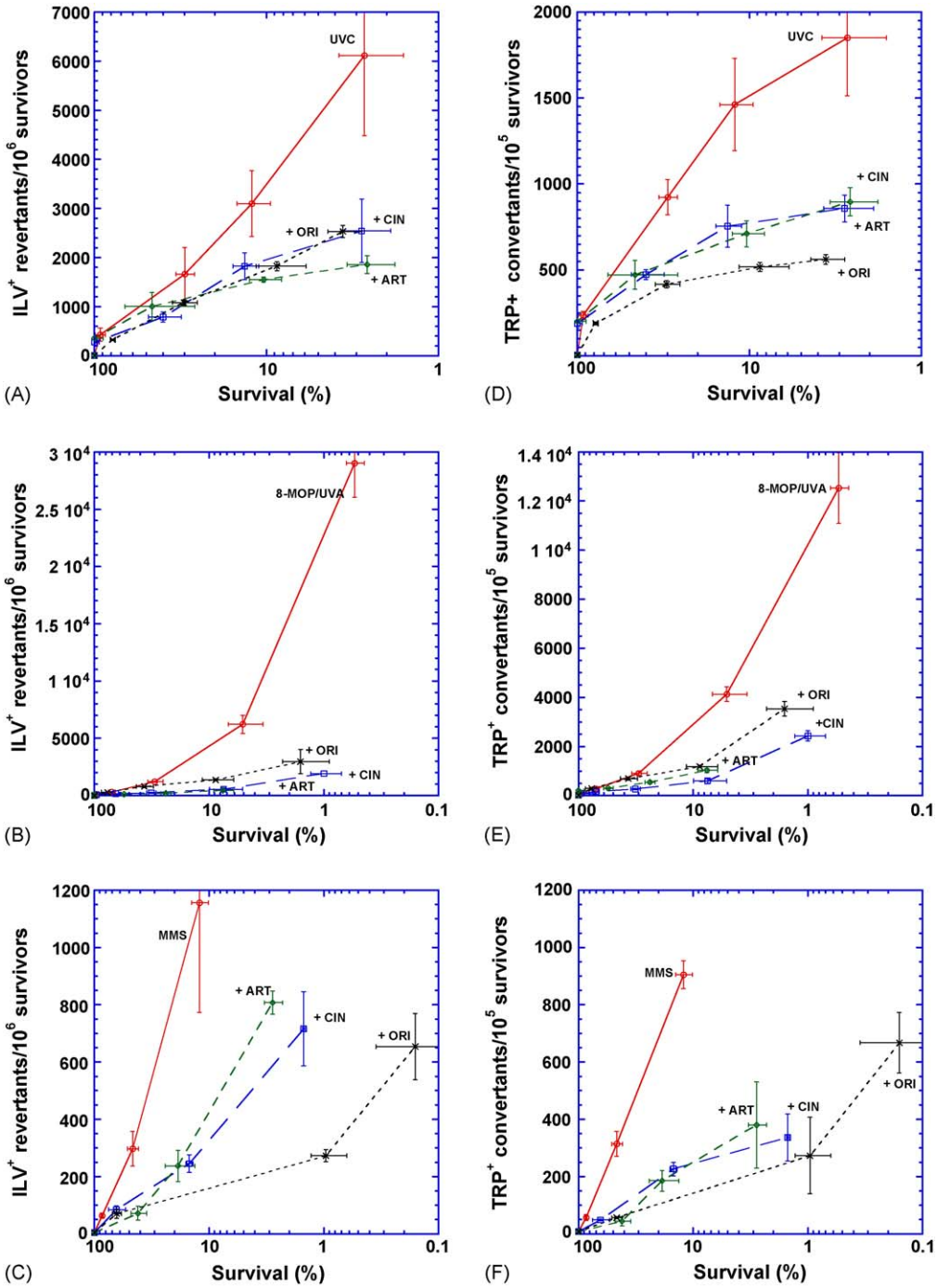

Fig. 3. Induction of *ILV*$^+$ revertants and *TRP*$^+$ mitotic gene convertants by UVC (A and D), 8-MOP/UVA (B and E) and MMS (C and F) alone and in combination with the EOs CIN, ART or ORI in diploid yeast (D7) as a function of survival. The spontaneous levels of revertants and gene convertants induced by the mutagens or the EOs alone have not been subtracted in the plots for the mutagens alone and the combined treatments.

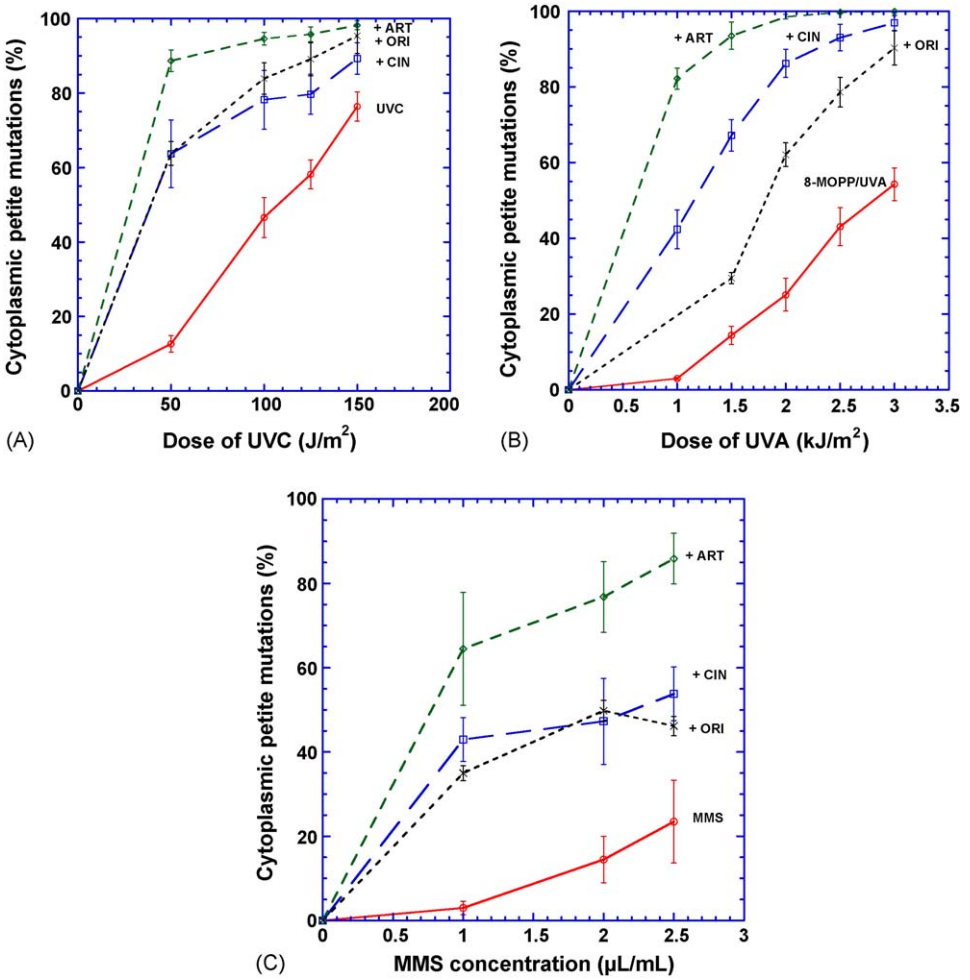

Fig. 4. Induction of cytoplasmic petite mutations by UVC (A), 8-MOP/UVA (B) and MMS (C) alone and in combination with the EOs CIN, ART or ORI in diploid yeast (D7) as a function of dose or concentration. The spontaneous level of cytoplasmic petite mutations has been subtracted. The differences between UVC and UVC + EOs are significant for all doses ($p < 0.01$), the differences between 8-MOP/UVA and 8-MOP/UVA + EOs are significant with CIN and ART for the last four doses ($p < 0.001$) and with ORI for the last three doses ($p < 0.001$), the differences between MMS and MMS + EOs are significant with CIN and ORI for all doses ($p < 0.01$) and with ART for all doses ($p < 0.001$).

3.3. Induction of cytoplasmic petite mutations by UVC, 8-MOP plus UVA and MMS, alone and in combination with treatments with EOs as a function of dose in diploid yeast (D7)

As seen in Fig. 4A–C, the presence of the three EOs after treatments with UVC, 8-MOP plus UVA or MMS, leads to a striking increase in the frequency of cytoplasmic petite mutations as a function of dose. Knowing that the three EOs are moderate inducers of cytoplasmic petite mutations at the concentrations and incubation time used [8] (ORI (0.3 μL/mL) 20.3 ± 6.6%, CIN (4 μL/mL) 21.2 ± 4.7%, ART (4 μL/mL) 47.1 ± 4.4% cytoplasmic petites after 90 min of incubation), it is clear that there is a synergistic interaction of UVC, 8-MOP plus UVA and MMS with subsequent EO treatments. As a function of survival, this synergism is even more important (data not shown).

After treatments with UVC (Fig. 4A) or 8-MOP plus UVA (Fig. 4B), the EOs appear to increase the frequency of cytoplasmic petites in the surviving population without causing a decrease, if not a slight increase in survival, whereas the increase in cytoplasmic petites after MMS treatment (Fig. 4C) is accompanied by a considerable increase in cell killing in the surviving population.

The data are presented without subtracting the effects of the EOs alone on cytoplasmic petite induction. Evidently, both the mutagenic and the latter EO effects add up in the whole surviving cell population.

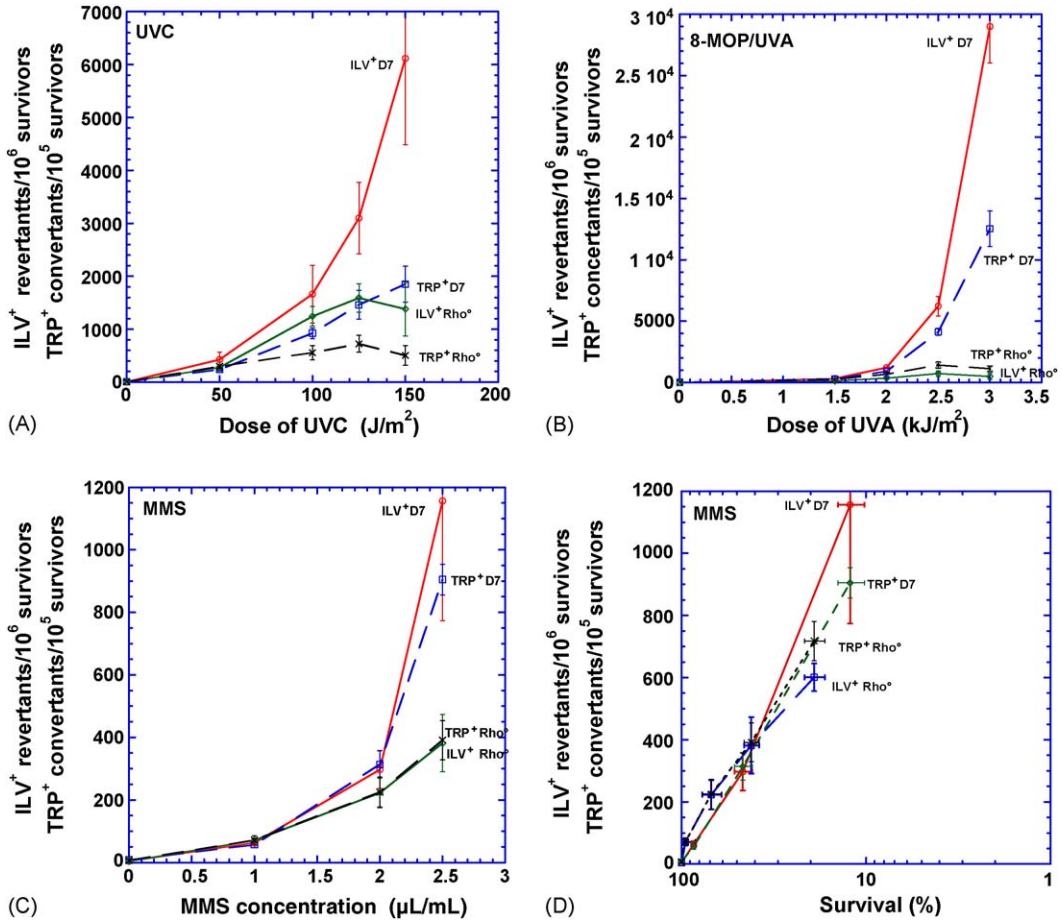

Fig. 5. Induction of *ILV*$^+$ revertants and *TRP*$^+$ mitotic gene convertants by UVC (A), 8-MOP/UVA (B) and MMS (C and D) in D7 (Rho$^+$) and a rho^0 derivative of D7 as a function of dose or concentration, and survival for MMS. With UVC, the differences between D7 and rho^0 for *ILV*$^+$ and *TRP*$^+$ are significant for the last two doses ($p < 0.01$); with 8-MOP/UVA, for *ILV*$^+$ the differences are significant at all doses ($p < 0.001$) and for *TRP*$^+$ they are significant for the two last doses ($p < 0.001$); with MMS, the differences are significant for the last two doses for *ILV*$^+$ and *TRP*$^+$ ($p < 0.05$).

3.4. Induction of ILV$^+$ *revertants and TRP*$^+$ *mitotic gene convertants by UVC, 8-MOP plus UVA and MMS as a function of dose in a rho*^{0} *derivative of D7 (D7rho*^{0}*)*

Knowing that the antimutagenic and anticonvertogenic effects of the EOs after UVC, 8-MOP plus UVA or MMS treatments are accompanied by a drastic synergistic increase in the frequency of cytoplasmic petites (mitochondrial damage), we tested whether the induction of *ILV*$^+$ revertants and *TRP*$^+$ convertants by these genotoxic agents would be the same in the wildtype strain D7 as in a derived rho^0 strain lacking mitochondria. Interestingly, in comparison with the wildtype D7 strain that carries fully functional mitochondria, the rho^0 derivative exhibits a clearly reduced capacity for the induction of *ILV*$^+$ mutations and *TRP*$^+$ convertants by treatments with UVC, 8-MOP plus UVA and MMS (Fig. 5A–C). Moreover, the decrease observed in the induction of these nuclear events is approximately the same as that observed in the D7 wildtype cells after treatments with UVC, 8-MOP plus UVA and MMS in combination with EOs. As a function of survival, the same results are obtained in the case of UVC and 8-MOP plus UVA, but after MMS treatment (Fig. 5D) the frequencies of *ILV*$^+$ revertants and *TRP*$^+$ mitotic gene convertants in rho^0 cells are quite similar to those of D7 wildtype cells.

3.5. Induction of apoptosis and necrosis in diploid yeast (D7) by combined treatments with UVC, 8-MOP plus UVA or MMS and the three EOs

We asked the question whether phenomena such as apoptosis and necrosis were also involved in the observed diminution of mutants and gene convertants. For this,

Table 1
Relative amounts of early apoptosis and late apoptosis/necrosis induced after treatments with UVC, 8-MOP/UVA and MMS alone and in combination with the EOs CIN, ART or ORI as determined by the annexin V-FITC-propidium iodide method

	Early apoptosis (%)	Late apoptosis/ necrosis (%)
Control	1.7 ± 0.7	1.6 ± 0.4
UVC	1.8 ± 1.0	1.7 ± 0.3
8-MOP/UVA	1.8 ± 0.9	1.5 ± 0.3
MMS	6.6 ± 1.8	5.1 ± 1.0
CIN	5.4 ± 2.0	4.9 ± 1.7
ART	7.1 ± 1.8	9.7 ± 2.4
ORI	4.0 ± 2.1	4.0 ± 1.3
UVC + CIN	5.0 ± 1.4	4.9 ± 1.1
UVC + ART	8.5 ± 2.1	9.6 ± 1.4
UVC + ORI	2.3 ± 0.6	2.8 ± 0.5
8-MOP/UVA + CIN	6.1 ± 0.2	5.0 ± 0.7
8-MOP/UVA + ART	7.5 ± 2.4	10.1 ± 2.7
8-MOP/UVA + ORI	3.4 ± 1.2	2.8 ± 0.3
MMS + CIN	15.3 ± 3.4	22.8 ± 4.0
MMS + ART	10.0 ± 5.6	46.7 ± 14.0
MMS + ORI	11.0 ± 4.7	27.0 ± 8.5

For early apoptosis, the differences between the mutagens and the mutagens + EOs are not quite significant ($p > 0.05$) for most values. On the contrary, for late apoptosis/necrosis, the differences are significant ($p < 0.05$).

we determined the amount of cells undergoing apoptosis using the annexin V-FITC method and the amount of necrotic cells by staining with propidium iodide, following the same exposure conditions as above. The results show that at the doses used, UVC and 8-MOP plus UVA do not significantly induce apoptosis or necrosis, whereas MMS induces both events (Table 1). At the concentrations used, the three EOs induce early stage apoptotic cells and late-stage apoptotic/necrotic cells, according to the definition of Markkanen et al. [17], as much as MMS. Combined exposures to UVC or 8-MOP plus UVA and the EOs do not induce more early stage apoptosis and late-stage apoptosis/necrosis than the EOs alone. In the case of MMS treatment, there is a very slight apoptotic effect with the three EOs with respect to early stage apoptosis, but a clear synergistic effect concerning late-stage apoptosis/necrosis (see Table 1).

4. Discussion

As shown before [8], the EOs from *C. camphora*, *A. herba alba* and *O. compactum* are themselves cytotoxic and are able to effectively induce cytoplasmic petite mutations, but they do not lead to nuclear genetic effects in yeast cells. These EOs were used together with genotoxic agents, i.e. UVC [10] 8-MOP plus UVA [12] and MMS [13,14], inducing different kinds of DNA lesions, in order to detect possible antigenotoxic effects.

Our results clearly indicate that EOs strongly enhance the amount of cytoplasmic petite mutations and concomitantly decrease the nuclear mutations and mitotic gene convertants induced by UVC, 8-MOP plus UVA or MMS treatments, suggesting that the induction of mitochondrial damage may be somehow selectively linked to the reduction in nuclear genetic events.

After treatment with the nuclear mutagens, the three EOs affect cell survival in different ways, not significantly in the case of UVC, slightly protective in the case of 8-MOP plus UVA, but clearly sensitizing in the case of MMS.

Our data clearly indicate that treatments with the EOs cause a decrease in the frequency of nuclear genetic events as a function of dose and surviving fraction without important changes in survival (if not some slight tendency to resistance) in the case of UVC or 8-MOP plus UVA. The situation appears to be different in the case of MMS, where co-treatment with the EOs appears to cause a significant decrease in survival without important decrease in ILV^+ and TRP^+ induction. This suggests that the mechanisms of the antimutagenic and anticonvertogenic effects produced by the three EOs are different according to the genotoxic agent used, i.e. the type of lesion. Thus, the type of protection appears to depend on the mutagen used and not on the EO used, the protective effect of the three EOs being always somewhat comparable.

The EOs affect both nuclear genetic endpoints to a similar extent, indicating that their action is of a rather global nature, and they do not specifically interfere with one or the other induction process.

This antigenotoxic effect is accompanied by a synergistic enhancement of the frequency of cytoplasmic petite mutations, i.e. mitochondrial damage in the surviving population. Indeed, the additional treatment with EOs efficiently transforms respiratory-competent cells without mitochondrial damage into respiratory-deficient cytoplasmic petites with highly damaged mitochondria. This suggests that it is the loss of mitochondrial functions induced by the EOs in interaction with the nuclear events induced by the mutagens that causes the decrease in induced revertants and mitotic gene convertants, even more so, because there are no substantial concomitant changes in survival in the case of UVC and 8-MOP plus UVA.

Undoubtedly, the capacity of EOs to induce cytotoxic and mitochondrial damage is an important factor for

the antimutagenic and anticonvertogenic effects reported here. For example, the EO from *Helichrysum italicum* is known to be relatively inefficient in the induction of cytotoxic and cytoplasmic damage [8] and had no effect on the induction of nuclear genetic events by UVC (data not shown). Moreover, the antigenotoxic effects decrease with decreasing EO incubation time and concentration (data not shown).

In contrast, MMS induces more early stage apoptosis and late-stage apoptosis/necrosis than the other mutagens, and incubation with EOs slightly enhanced these effects for early stage apoptosis but had a strong effect on late-stage apoptosis/necrosis. This suggests that it is mainly the decrease in cell survival by late-stage apoptosis/necrosis that causes the decline in nuclear genetic events, above all as a function of survival.

Interestingly, in rho^0 cells that lack functional mitochondria, the frequency of point mutations and gene convertants induced by UVC and 8-MOP plus UVA was as low as that caused by post-treatment with the three EOs in Rho^+ cells. After UVC and 8-MOP plus UVA treatments, post-treatment by EOs caused transformation of respiratory-competent cells into respiratory-deficient cells with mitochondrial dysfunction. This transformation does not much affect survival, but reduces the expression of nuclear genetic events to the same extent as that found in rho^0 cells, which are also somewhat more resistant than Rho^+ cells in the case of 8-MOP plus UVA, but not of UVC. This confirms our conclusion that in these cases the induction of mitochondrial damage and dysfunction by EOs is likely to be directly linked to the antimutagenic and anticonvertogenic effects observed.

There is a certain type of mitochondrial dysfunction that may inhibit error-prone repair of DNA lesions. EOs post-treatments may drive mutagen-damaged cells that would otherwise produce mutation and gene conversion not to continue the error-prone process but to follow an error-free type of mechanism.

Apart from their role in cellular signal transduction and in the release and management of reactive oxygen species such as superoxide, $OH^{\bullet}$ and H_2O_2 [19,20], mitochondria constitute the powerhouse that produces energy in the form of adenosine 5′-triphosphate (ATP) by oxidative phosphorylation [21]. This implies that it may be the reduced energy supply caused by the induction of mitochondrial damage and respiratory deficiency which inhibits the expression of revertants and gene convertants (intragenic recombinants). The functions of several proteins involved in DNA replication in yeast are dependent on ATP and many ATPases are involved in DNA repair (BER, NER, mismatch repair and recombination) [21].

Thus, a reduced energy supply due to mitochondrial dysfunction may slow down the processing of lesions induced by UVC and 8-MOP plus UVA and just favour an error-free instead of an error-prone pathway. The fact that the survival is rather stable or only slightly enhanced suggests that DNA-repair systems are not much involved, because if they were, the decrease of mutations would be accompanied by a substantial increase in survival. Therefore, DNA-repair systems may play a minor role.

Indeed, the antimutagenic effect of EO from *O. compactum* after UVC treatment was found in a nucleotide excision repair-deficient mutant (*D7rad3*) to be as strong as in the wildtype with respect to gene conversion, and much more drastic than in the wildtype with regard to point mutation ILV^+ (data not shown). It was also accompanied by a synergistic induction of cytoplasmic petite mutations. Thus, the EO antimutagenic effects appear to be rather independent of the NER system.

Furthermore, it seems possible that the lack of normal mitochondrial functions in rho^0 cells preferentially affects one of the two translesional synthesis pathways, i.e. the error-prone translesional synthesis by Pol ζ, and not the error-free translesional synthesis by Pol η [22–24].

In the case of MMS, incubation with the three EOs causes, as a function of dose, a significant reduction in both types of nuclear events, much more drastic as a function of survival. In fact, this reduction is accompanied by an additional decrease in survival. Thus, EOs affect the expression of nuclear genetic events as well as cell survival. This differs from the response to UVC or 8-MOP plus UVA treatment and may be explained by the induction of specific DNA lesions by MMS, i.e. a-basic sites and alkylated bases, which elicit another type of repair processing by base excision repair and homologous recombination.

Since rho^0 cells, lacking functional mitochondria, produce after MMS both nuclear genetic events as much as wildtype Rho^+ cells as a function of survival, these results suggest that the combined action of MMS with the EOs is promoting cell death mainly in the form of late-stage apoptosis/necrosis. Furthermore, as shown here in the case of MMS treatment of diploid yeast, the induction of mitochondrial damage plays, as is also the case in mammalian cells [21], an important part in cellular signaling towards apoptosis and necrosis [25]. In combined MMS and EO treatments, the EOs do not so much interfere with the expression of nuclear genetic events but clearly promote cell death by late-stage apoptosis/necrosis.

Our results with annexin V-FITC and propidium-iodide staining show that after UVC and 8-MOP plus UVA, post-treatment with the three EOs does not change the levels of apoptosis and necrosis compared with those seen with the EOs alone. Thus, the decrease in nuclear mutation is nearly exclusively due to the transformation of Rho^+ cells into rho^0 cells. On the contrary, MMS treatment itself induces apoptosis and necrosis approximately to the same extent as do the EOs alone. Moreover, in combination with EOs there is principally a synergistic enhancement of late-stage apoptosis/necrosis, which correlates well with the decrease in nuclear genetic events with decreasing survival. The prooxidant mode of action of the essential oils and the induction of rho^0 cells is likely to induce a switch from apoptosis to necrosis [26].

As shown here, the EOs belong to a new category of antimutagens and antigenotoxins characterized by their interference with normal mitochondrial function. The results may fit a general mechanism that underlies the antigenotoxic activity of EOs. The complex mixtures as a whole (EOs) or some of their active components cause, through a pro-oxidant activity, strong oxidative stress and mitochondrial dysfunction in yeast, which inhibit the expression of nuclear genetic events (due to the lack of energy and interference with normal processing of mutagenic lesions) after exposure to nuclear mutagens, either by promoting cytoplasmic petite induction or apoptotic/necrotic cell death. This might evoke a somewhat comparable mechanism to the cancer chemopreventive action of some known prooxidants [27,28].

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