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Fragrance chemicals lyral and lilial decrease viability of HaCat cells' by increasing free radical production and lowering intracellular ATP level: Protection by antioxidants

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ABSTRACT

We investigate in this study the biochemical effects on cells in culture of two commonly used fragrance chemicals: lyral and lilial. Whereas both chemicals exerted a significant effect on primary keratinocyte(s), HaCat cells, no effect was obtained with any of HepG2, Hek293, Caco2, NIH3T3, and MCF7 cells.

Lyral and lilial: (a) decreased the viability of HaCat cells with a 50% cell death at 100 and 60 nM respectively; (b) decreased significantly in a dose dependant manner the intracellular ATP level following 12-h of treatment; (c) inhibited complexes I and II of electron transport chain in liver sub-mitochondrial particles; and (d) increased reactive oxygen species generation that was reversed by *N*-acetyl cysteine and trolox and the natural antioxidant lipoic acid, without influencing the level of free and/or oxidized glutathione. Lipoic acid protected HaCat cells against the decrease in viability induced by either compound. Dehydrogenation of lyral and lilial produce α,β-unsaturated aldehydes, that reacts with lipoic acid requiring proteins resulting in their inhibition. We propose lyral and lilial as toxic to mitochondria that have a direct effect on electron transport chain, increase ROS production, derange mitochondrial membrane potential, and decrease cellular ATP level, leading thus to cell death.

1. Introduction

Aromatherapy is a kind of folk medicine in which natural products, essential oils, extracted from plants are used in treatment of certain ailments and health problems such as: depression, nervous tension, insomnia, headaches, burns, fungal infection, asthma and fever (Brodhead, 2005; Holmes and Ballard, 2004; Gibb, 2006). They are characterized by their pleasant odors and volatility that facilitate their way to the brain through the olfactory system or via absorption through the skin (Bridges, 2002).

Essential oils that are extensively used in perfume formulation, and cosmetics, involve extraction of huge amount of plants, and flowers which becomes a costly operation. However, chemists in the current new era of modern perfumery have identified the odoriferous active ingredients composing essential oils and introduced the term fragrance chemicals. Subsequently, they examined the structure/odor relationship which allowed the economical chemical synthesis of artificial fragrance chemicals related to essential oils (Fortineau and Kauffman, 2004; Gibb, 2006; Holmes and Ballard, 2004).

Correlating the chemical structure of essential oils with their odor, chemist identified from lily of the valley the following chemicals odorants, all with a characteristic aldehydic group, including: lyral, lilial, cyclamen aldehyde, and bourgeonal. Modification of the aldehydic group resulted in the loss of the lily of the valley odor confirming its importance (Fortineau and Kauffman, 2004). Recent studies defined structural alerts of fragrance chemicals to include aldehydes, ketones and α,β-unsaturated aldehydes/ketones or esters aromatic and terpenes (Langton et al., 2006).

The absorption of fragrance chemicals is influenced by many external factors such as: temperature, humidity, contact time and concentration. In addition, absorption is affected by the chemical nature of the fragrance into hydrophilic hence slow penetrators, or lipophilic thus quickly absorbed (Bridges, 2002). The ability of lipophilic fragrances to cross cell membranes, would facilitate their interaction with various intra-cellular and or intra-organelle sites (Griffiths, 2005; Sikkema et al., 1995) underlying thus some of

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their reported side effects such as contact dermatitis and sensitization (Schnuch et al., 2007; Hagvall et al., 2007; An et al., 2005; Pease et al., 2003).

Following their entry into the cell, fragrance chemicals form a covalently linked conjugate with skin proteins involving Schiff base or via Michael addition reaction (Langton et al., 2006; Pease et al., 2003; Rastogi et al., 1998). Alternative fate involves biotransformation by cytochrome P450 into more polar products, which regardless of their relative toxicity compared to the original substances; they can reach the blood circulation and eventually excreted by the kidneys (Bridges, 2002). Typical examples include: cinnamic alcohol, linalool, limonene and geraniol (Smith et al., 2000; Hagvall et al., 2008) that cannot bind directly to skin proteins but are metabolized to more reactive species by xenobiotic metabolizing enzymes present in the epidermis of human skin (Smith et al., 2000).

Despite the widespread consumption of perfumes, there are minimal studies monitoring their adverse effects (Bridges, 2002) at the cellular or sub-cellular level. Previous studies (Griffiths, 2005; Uribe et al., 1984) described the effects of perfume oils, eau de parfum, eau de toilette, and fragrance chemicals on mitochondrial energetic implicating mitochondria as a potential target for their mode of action; more specifically as inhibitors of complexes I/II; disrupting membrane potential, (Griffiths, 2005; Ka et al., 2003) that eventually lead to necrosis or apoptosis.

Although in one of the recent reports by the International Fragrance Association a maximal concentration for the usage of various fragrance chemicals was recommended, they are still being used by many companies. Rarely a full listing of the ingredients is being given; instead they are summed up as perfume or fragrance, regardless of their effects (Bickers et al., 2003; IFRA, 2003).

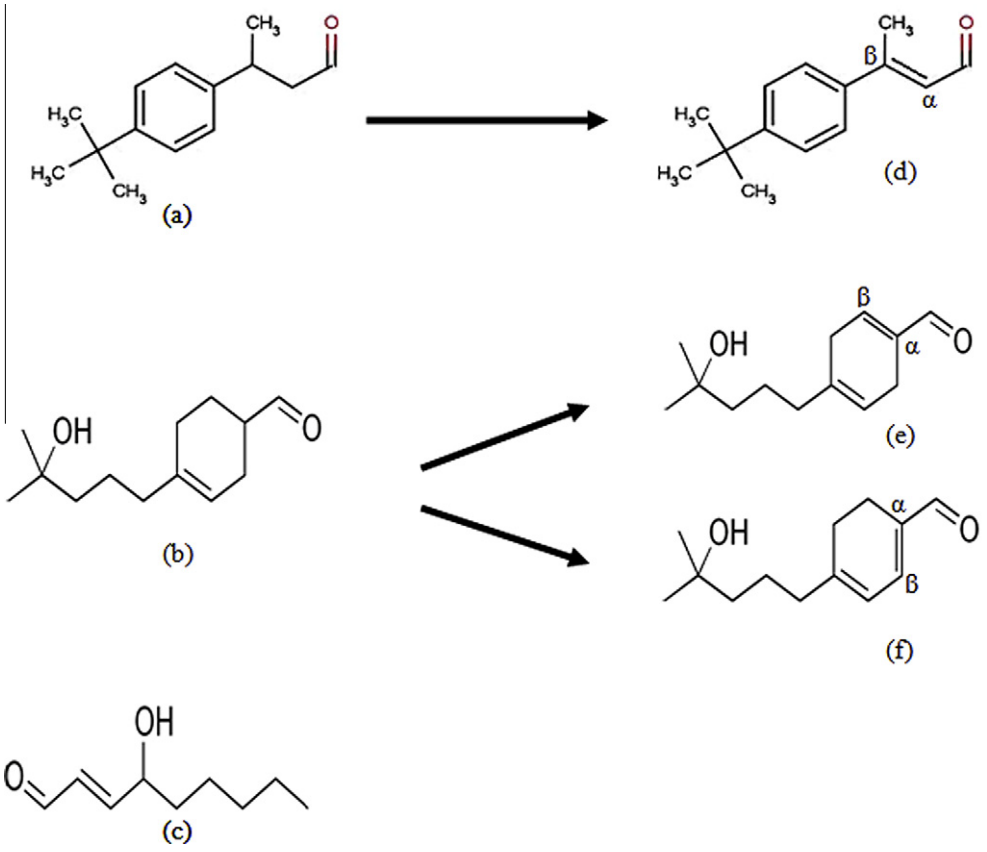
Among the commonly identified and widely used fragrance chemicals in perfume industry are lyral and lilial (Scheme 1) the trade names for: Hydroxyisohexyl-3-cyclohexene carboxaldehyde and p-t-butyl-α-methylhydrocinnamic aldehyde respectively (Fischer et al., 2009; Rastogi et al., 1998). Both chemicals have characteristic floral scent thus have been introduced in many of our personal care products such as: perfumes, creams, shampoos and household scented products used for homes, cars and public buildings (Perivier, 2005; Arnau et al., 2000). Both compounds have been clinically associated with skin sensitization causing contact dermatitis (Frosch et al., 1999; Rastogi et al., 2000; Schnuch et al., 2007; Bruze et al., 2008).

We investigate in this study the biochemical effect of lyral and lilial on tumor cell lines and HaCat cells, a human non-tumoral keratinocyte(s). The most significant effect exerted by lilial and lyral was on HaCat cells. Both compounds decreased significantly the viability of HaCat cells, increased reactive oxygen species (ROS), had no effect on glutathione level, decreased ATP level and inhibited mitochondrial complexes I and II. The decrease in viability was completely reversed by pretreatment of HaCat cells with antioxidants lipoic acid, *N*-Acetyl cysteine (NAC) and Trolox. We report lyral and lilial as compounds toxic to mitochondria.

2. Materials and methods

2.1. Materials and instruments

All chemicals and compounds were purchased from Sigma Chemical Company (St Louis, MO, USA). Fragrance chemicals were the generous gift of Dr. David Griffiths, University of Warwick,



Scheme 1. Chemical structures of (a) lilial, (b) lyral, (c) HNE and products of a and b dehydrogenations: (d) and (e or f) respectively.

Coventry UK, originally purchased from Sigma–Aldrich UK. The following cell lines were originally purchased from American type Culture collection, Manassas, VA, USA: HepG2 (cat# HB-8065), Caco2 (cat# HTB-37), MCF7 (cat# HTB-22), Hek293 (cat# CRC-1573), NIH3T3 (Cat# CRL-1658). HaCat Cells were the generous gift of Professor Nadine Darwiche from Biology Department of the American University of Beirut. Fetal bovine Serum (FBS), antibiotics, Penicillin–Streptomycin mixture and various culture reagents were purchased from Gibco (Grand Island, NY).

Spectrophotometric assays were performed using a Shimadzu UV–VIS scanning spectrometer (UV-2101 PC). Bioluminescence was measured using Ascent FL Fluroscan, Thermo Lab systems, Elisa reader, Thermo Electron Corporation Multiskan Ex.

2.2. Cell viability/cytotoxicity assays

2.2.1. MTT assay

The effect of lyral and lilial on cell viability was estimated using 3-(4-5 dimethyl thiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) assay as per instruction manual (Roche).

Cells including: HepG2, NIH3T3, Caco2, MCF7, HeK293 and HaCat cells were seeded in 96 well plates at a density of 1×10^4 cells/well/100 μl media, cultured for 24 h in a humidified incubator with a 5% CO_2 at 37 °C. The effect of lyral and lilial on these cells was evaluated initially over a concentration range (10–100 μM) and compared to a control of cells treated with the vehicle ethanol not exceeding 0.5% final concentration. Following the 24 h treatment with the chemicals the relative viability was determined as described by the Kit manual and absorbance was quantified at 595 nm using an ELISA microplate reader.

Further investigations: a time study (2–30 h) and concentration study (0.006–10 μM) were carried on using the most sensitive cell to determine the EC50 of lyral and lilial and the time at which significant cell death occurs. EC50 is defined as the concentration of lyral or lilial required to decrease the viability of HaCat cells by 50%.

2.2.2. Lactate dehydrogenase (LDH) release assay

Cytotoxicity of lyral and lilial on HaCat cells was assessed using LDH assay. The release of LDH from the cytosol into media indicates damaged and lysed cells. HaCat cells (1×10^4 /well/100 μl media) were seeded in 96 well plates, and treated for 24 h with each of the fragrance chemicals at their EC50, and at lower (6 nM) and higher (100 μM) concentration. LDH release was estimated using the cytotoxicity detection kit^{plus} (LDH) Roche (cat# 04744934001), following the instructions manual and compared to three controls with no treatment: cell free control; low control (reflecting spontaneous release of LDH under experimental conditions); and high control (indicating maximum LDH release following addition of lysis buffer). Released LDH in culture media was estimated coupled to an enzymatic assay that converts tetrazolium salt into red formazan. The intensity of the color formed was measured at 490 nm using an ELISA microplate reader. % Cytotoxicity or release of LDH was estimated as follows:

$$\frac{\text{Abs of treated cells} - \text{Abs of low control}}{\text{Abs of high control} - \text{Abs of low control}} \times 100$$

2.3. Intracellular GSH level

The intracellular glutathione level was determined in control and treated cells as per described kit instruction manual using ApoGSH^{TM} the colorimetric Kit (catalog# K261-100) purchased from Biovision Research Products, Mountain View, CA, USA.

HaCat cells (2×10^6) cultured in petri dish were treated with lyral and lilial for 24 h at their respective EC50 values. Cells were collected, lysed and the resulting supernatant was used to determine the level of GSH. For total GSH (GSH + GSSG) glutathione reductase and NADPH regenerating system were added to cell lysate, incubated for 10 min followed by 20 μl of kit glutathione substrate that generates yellow product, the intensity of which was measured using Elisa reader at 405 nm.

2.4. Intracellular ATP level and its variation with time

The level of intracellular ATP was determined in both control and treated HaCat cells as described by the ATP Bioluminecence assay Kit (Roche). Cells (5×10^5) were cultured and treated for 24 h with lyral and lilial at their EC50, 0.006 and 10 μM each. Cells were collected using the kit cell lysis reagent, and then 50 μl were transferred to white MTP plates followed by the addition of luciferase reagent. Bioluminecense of treated cells was measured, compared to control, using a luminometer.

To determine if variations of ATP level precede cell death we treated HaCat cells with either of the fragrance chemicals each at its corresponding EC50 value for different time intervals: 12, 24 and 30 h following which ATP level was determined as described.

2.5. Intracellular reactive oxygen species (ROS) using the nitroblue tetrazolium reduction assay

Nitroblue tetrazolium salt (NBT) conversion into turquoise blue color formazan was used to estimate the level of ROS. In brief, NBT (100 μl of 1 mg/ml) was added to control and fragrance chemical treated HaCat cells. This was followed by the consecutive addition of KOH (120 μl), DMSO (140 μl) to the formazan formed. The absorbance of the developed color was read immediately at 645 nm comparing lyral/lilial-treated to non treated HaCat cells. A decrease in the absorbance of the turquoise color indicates less formazan formed and more ROS generated.

We further investigated the effect of antioxidants on ROS generation. HaCat cells were pretreated (100 μM), for 2 h, with each of the following: lipoic acid, Trolox, and NAC followed by lyral or lilial treatment (24 h) each at its respective EC50. NBT assay was then performed comparing induced ROS generated by lyral/lilial in HaCat cells pretreated with antioxidants versus non-treated cells

2.6. Effect of reduced lipoic acid, on viability of lyral and lilial HaCat treated cells

The effect of 2 h pretreatment of HaCat cells (1×10^4/well, 96 wells plate) with 100 μM of reduced lipoic acid prior to lyral and lilial treatment, each at its EC50, was also investigated. Cell viability using MTT assay was determined comparing lipoic to non-lipoic acid pretreated cells.

2.7. Mitochondrial assays

2.7.1. Activity of mitochondrial complexes I and II in rat liver submitochondrial particles

2.7.1.1. Preparation of sub-mitochondrial particles (SMP). Preparation of SMP was achieved using isolated rat liver mitochondria following standard procedures (Gellerfors and Nelson, 1979; Darley-Usmar et al., 1987). Briefly rat liver was excised, washed and homogenized (1 g/20 ml) with ice cold Sucrose–HEPES–EDTA (SHE, 250 mM–10 mM–50 mM, pH 7.4). The homogenate was centrifuged for 15 min, at 3000 rpm (1000*g*) using SS34 rotor, and then the resulting supernatant was re-centrifuged for 15 min at 10000 rpm (9000*g*). The mitochondrial pellet was washed and resuspended in SHT (250 mM sucrose, 10 mM HEPES, 50 mM Tris–Cl) buffer pH 7.4, at protein concentration ranging between 20 and 30 mg/ml for SMP preparation.

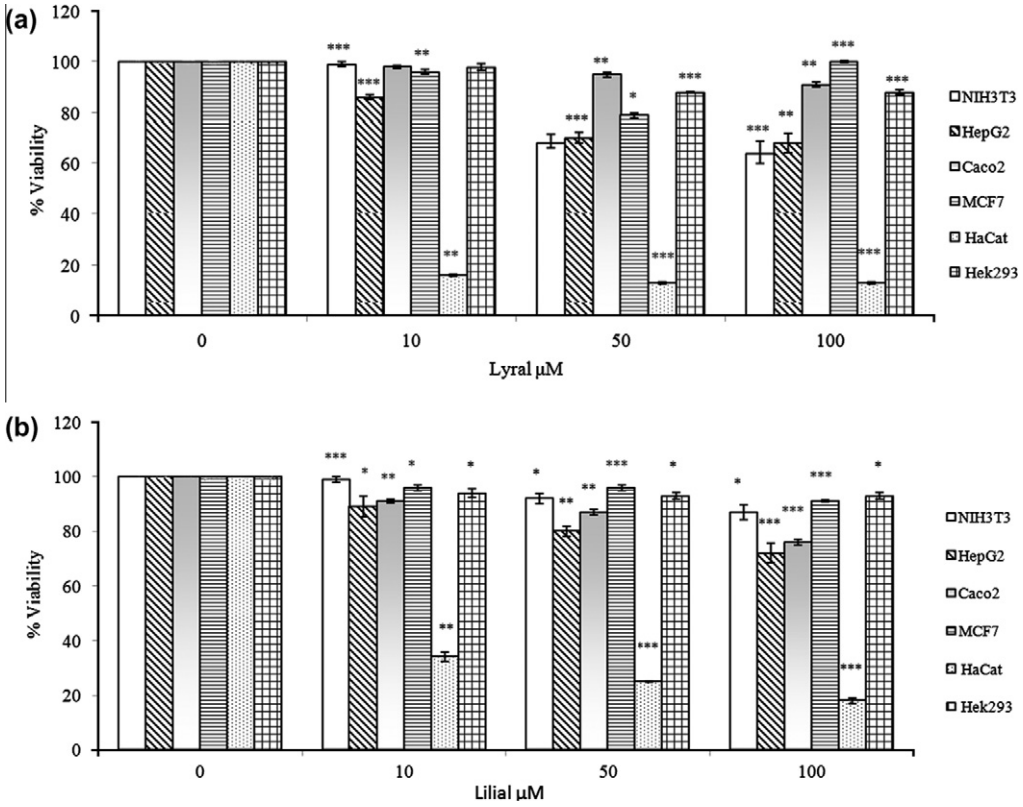


Fig. 1. Effect of fragrance chemicals (a) lyral and (b) lilial on viability of different cell lines. Cells were cultured under optimal conditions and treated with either compounds (10–100 µM) for 24 h. Viability of cells was then determined using MTT assay. Each value is an average ± SEM of four different-independent experiments and a total number of determinations $n = 9$ compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

For SMP preparation, the isolated mitochondria (20–30 mg/ml) were suspended in SHT containing ATP, and $MgCl_2$ at a final concentration of 5 and 2 mM respectively. The suspension was sonicated for 12 × 10 s intervals with intermittent cooling of 20 s, the sonicated suspension was consecutively centrifuged for 15 min at 15000 rpm (26000*g*, SS34 rotor) then ultra-centrifuged for 1 h at 40000 rpm (150000*g*, T880 rotor). The pellet comprising SMP was re-suspended in STMGA buffer (250 mM sucrose, 10 mM Tris, 2 mM $MgCl_2$, 2 mM GSH, 2 mM ATP, pH 7.4) stored as aliquots in the −80 °C. Protein concentration was determined using the Biorad assay (Bradford reagent).

2.7.1.2. Activity of complex-I. NADH Dehydrogenase. SMP (30 µg) were added to a microcuvette containing 800 µl of 4 mM Tricine, 36 mM K_2HPO_4, 1 mM EDTA buffer, pH 7.7. The reaction was initiated by adding 4 µl of 30 mM NADH. Activity of complex I was monitored following the decrease in the absorbance for 10 min at 340 nm. Enzyme activity was expressed as ΔAbs/mg/h compared to a control of alcohol considered as 100%.

2.7.1.3. Activity of complex-II. Succinate Dehydrogenase. SMP (50 µg) were introduced in a microcuvette containing in a final volume of 800 µl: KCN (9 mM), Tris–Cl (90 mM), and sodium succinate (93 mM). The reaction was initiated by the addition of $K_3Fe(CN)_6$ (75 µl of 10 mM) the reduction of which was monitored for 30 min at 400 nm. Enzyme activity was expressed as variation in absorbance ΔAbs/mg/h compared to a control of alcohol considered as 100%.

The effect of lyral and lilial on respiratory chain complexes I and II were determined using spectrophotometric assays. Different concentrations of each were added to SMP for 5 min prior to enzyme activities assay. The effect of either compound was expressed as: $\%\text{recovery of complex I /II activity} = \frac{\Delta\text{Abs/mg/h of lilial or lyral treated SMP}}{\Delta\text{Abs/mg/h of control SMP}} \times 100$

2.7.2. Mitochondrial membrane permeability

To examine whether lyral and lilial exert their effect by influencing the mitochondrial membrane potential and increasing its permeability we used the Mito PT™ JC-1 kit (Cat# 924, Immunochemistry Technologies LLC, MN, USA) that stains normal and actively coupled mitochondria red–orange while inactive ones with dissipated membrane potential will stain green. HaCat cells were seeded at a density of 1×10^5 over a cover slip introduced in 12 well plates and were treated with lyral (EC50: 100 nM, and 10 µM) and lilial (EC50: 60 nM and 10 µM) for 24 h. Media were then aspirated from wells of both control and treated cells then were consecutively incubated with 300 µl of 1× Mito PT™ JC-1 stain solution for 15 min at 37 °C, washed with 1 ml of 1× assay buffer for 5 min, and finally the Mito PT™ JC-1 stain was aspirated. The cover slips were then mounted on glass slides and were examined (20× magnification) using a fluorescent microscope (Olympus BH2-RFCA).

2.8. Statistical analysis

Results were reported as averages ± SEM of 3–4 different and independent experiments. Each experiment represents an average of 2 or 3 determinations. The total number of determinations is referred to as [*n*] in the figure legends and under the method section. Statistical significance was tested by one way analysis of variance followed by a Tukey–Kramer multiple comparison test. *P*-values <0.05 was considered significant. Data presented were normalized to the control value and averaged. The variation in control experiments was always <1.5%

3. Results

3.1. Fragrance chemicals lyral and lilial reduce significantly the viability of HaCat cells

Cytotoxicity of each fragrance chemical was initially screened between 10 and 100 μM on different cell lines including: HepG2, NIH3T3, Hek293, Caco2, MCF7 and HaCat cells.

Lyral (Fig. 1a) exerted a significant decrease (85%) in HaCat cells at 10 μM; whereas 20–30% decrease in viability of HepG2 and NIH3T3 was obtained at 50–100 μM lyral. No significant effect was obtained with MCF7, Caco2, or Hek 293 cells. Similarly lilial (10 μM) reduced significantly (65%) the viability of HaCat cells; whereas 23% decrease in viability of HepG2 and Caco2 cells was obtained at high concentration (100 μM), with no effect on NIH3T3, MCF7 or Hek293 cells (Fig. 1b). To sum up, among all the tested cells, HaCat cells were found to be the most sensitive to lyral and lilial, hence were used in all subsequent experiments.

To determine the EC50, HaCat cells, were treated for 24 h, with each of the chemicals at varying concentrations (2–100 nM). Around 50% decrease in the viability of HaCat was obtained at 60 and 100 nM with lilial and lyral respectively (Fig. 2a). Treatment of HaCat cells with the fragrance chemicals, each at its EC50, for different time intervals showed that the significant decrease in viability (35%) occurred following 16 h of treatment (Fig. 2b).

3.2. Cytotoxicity of lyral and lilial on HaCat cells: lactate dehydrogenase release assay

A dose response effect of lyral and lilial on LDH release by HaCat cells was obtained (Fig. 2c). A significant release in LDH, of 30%, and 70%; occurred with lyral and lilial respectively each at its EC50; which approached 85% at 100 μM. Our results indicate that both lilial and lyral are cytotoxic causing LDH release from cells.

3.3. Lyral and lilial decreased intracellular level of ATP

Intracellular ATP level in lyral and lilial treated HaCat cells, was significantly decreased by 69% and 40% at their respective EC50 concentration. A further decrease in ATP level by 82% was obtained with an increase in the concentration of both at 10 μM (Fig. 3a).

Examining the variation of ATP with time of treatment our data show a significant drop (35%) in ATP level following 12 h treatment of HaCat with both chemicals, at their corresponding EC50, reaching a maximal decrease of 80% and 60% with lyral and lilial respectively following 30 h of treatment (Fig. 3b). Since ATP is mainly synthesized at the mitochondria, our data implicate, derangement in mitochondrial functions induced by fragrance chemicals that lead to a decrease in ATP level and which precede cell death.

3.4. Lyral and lilial inhibited complexes I and II of rat liver submitochondrial particles

To examine if the decrease in ATP level and consequently the induced cell death is due to inhibition of respiratory chain complexes we examined the effect of varying fragrance chemical concentrations on activities of complexes I and II (NADH dehydrogenase, and Succinate dehydrogenase). Both complexes were significantly inhibited, in concentration dependent manner by lyral and lilial.

Lyral exerted a 50% inhibitory effect of complex I and complex II activities at 125 and 400 μM respectively (Fig 4a). On the other hand lilial exerted a 50% inhibitory effect of complex I and complex II activities at 5 and 80 μM respectively (Fig 4b). Activities were compared to a control activity of 152 ± 17 Δabs/mg/h for complex I and of 53 ± 11 Δabs/mg/h for complex II considered as 100% assayed in presence of alcohol.

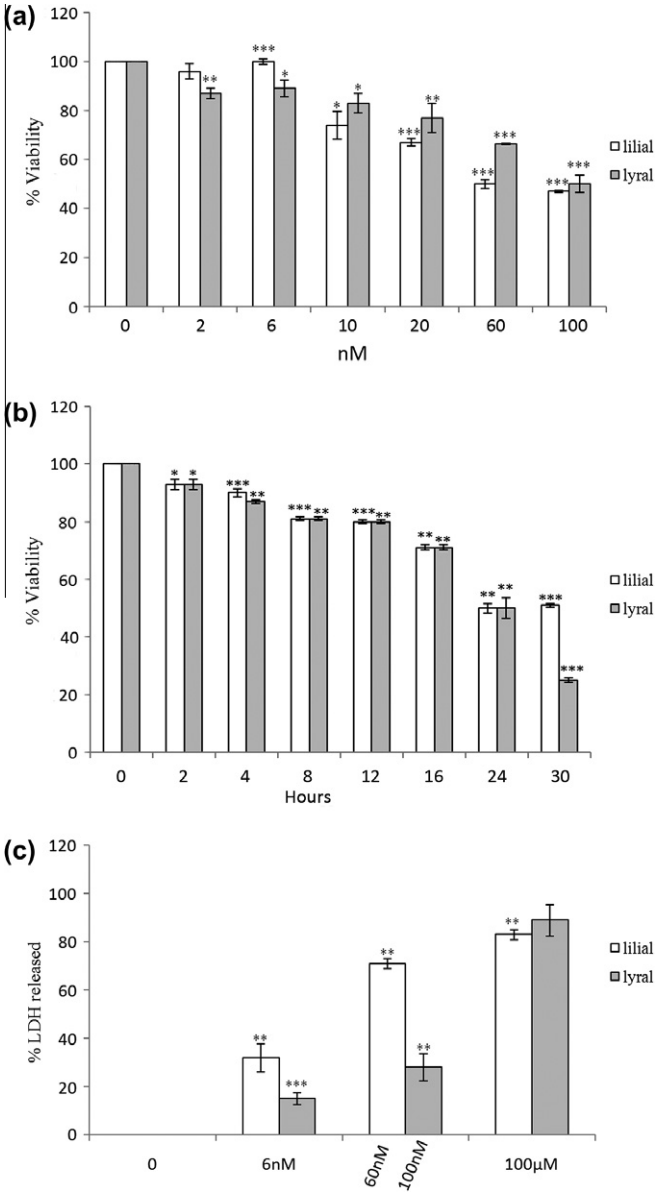


Fig. 2. (a) Concentration dependent effect of lyral and lilial on viability of HaCat cells; (b) effect of treatment time on viability of HaCat cells; (c) effect of lyral and lilial on LDH release. Each value is an average ± SEM of three different-independent experiments and a total number of determinations $n = 9$ compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

3.5. Lyral and lilial increased ROS generation without varying GSH level

Lyral and lilial exerted a dose-dependent decrease in NBT reduction (Fig. 5a) of 50% each at its corresponding EC50 and a maximal decrease of 62% and 65% at 100 μM respectively indicating an increase in ROS level. As this would favor oxidation of GSH, we examined if treatment with the fragrance chemicals will have an effect on the level of reduced and oxidized GSH. Free GSH and total glutathione level (free GSH + oxidized GSSG estimated following reduction of GSSG), were determined in control and treated cells. In control (lyral/lilial untreated cells), the concentration of GSH increased 10-fold following the enzymatic reduction of GSSG.

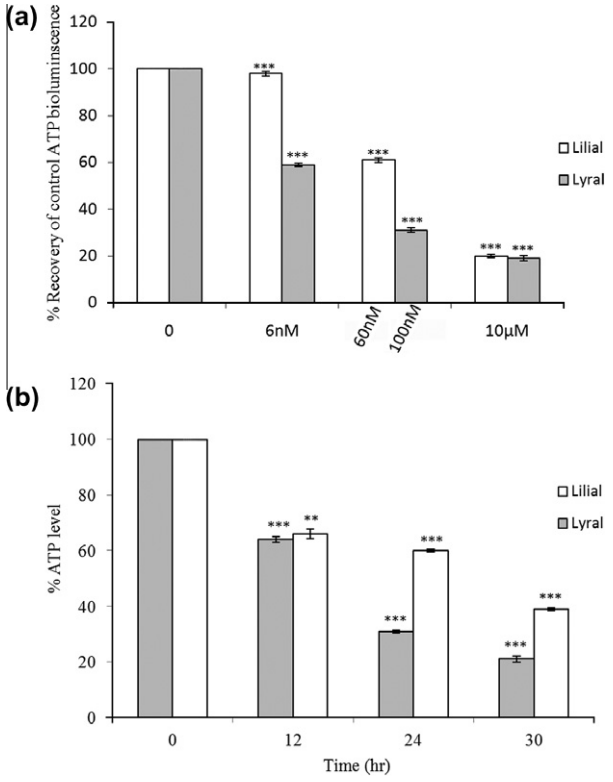


Fig. 3. Variation in ATP level in HaCat cells treated with lyral or lilial: effect of concentration; (b) effect of treatment time of HaCat with lilial (60 nM) and lilial (100 nM) Each value is an average ± SEM of three different-independent experiments and a total number of determinations $n = 6$. Compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

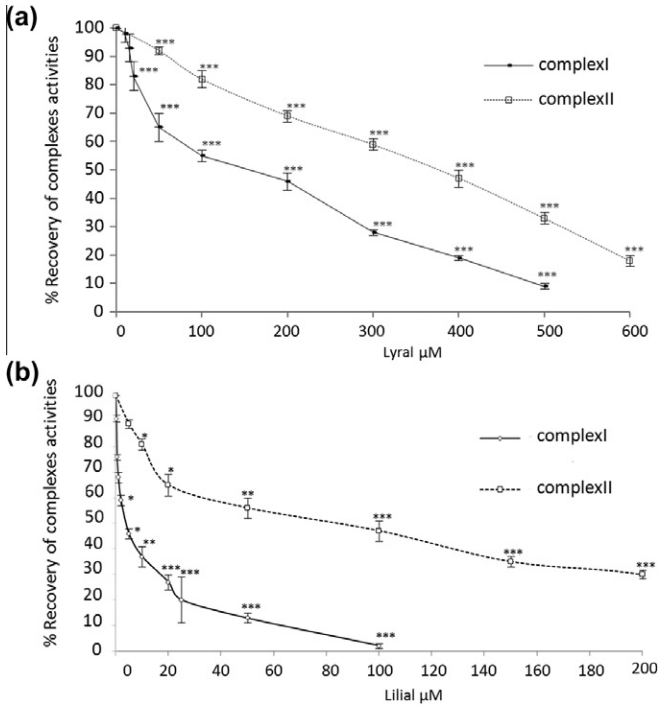


Fig. 4. Effect of (a) lyral; and (b) lilial on the activities of ETC complexes I and II of rat liver SMP. Activities were expressed as Δabs/mg/h and were compared to a control value (100%) of 152 ± 17 Δabs/mg/h for complex I and 53 ± 11 Δabs/mg/h for complex II. Each value is an average ± SEM from three different-independent experiments and a total number of determinations $n = 3$ compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

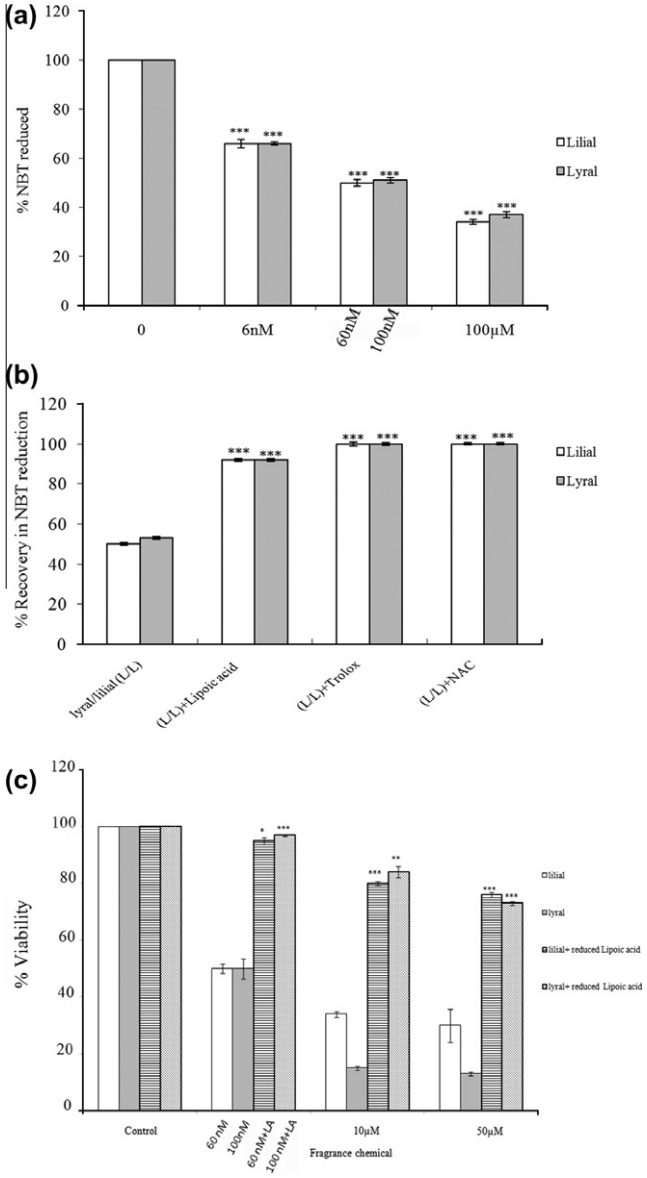


Fig. 5. Effect of lyral and lilial on: (a) NBT reduction in HaCat cells; (b) NBT reduction in HaCat cells pretreated with antioxidants (100 μM) NAC, trolox and lipoic acid; (c) Viability of HaCat cells pretreated with lipoic acid. Each value is an average ± SEM from three different-independent experiments and a total of determinations $n = 6$. Compared to control: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered significant.

Treatment with either chemical followed a similar pattern as untreated cells, indicating that the glutathione depletion is not the underlying cause of the obtained decrease in HaCat viability (Data not shown).

3.6. Antioxidants lipoic acid, NAC and trolox suppressed ROS generation by lyral or lilial

The decrease of NBT reduction by both lyral and lilial indicates an increase in ROS level. As there was no variation in GSH/GSSG upon treatment we thought of testing the effect of antioxidants on ROS. All antioxidants significantly inhibited the obtained effect of lilial or lyral (each at their EC50) on NBT reduction (decreased ROS). A significant restoration, of approximately 100% was obtained with all antioxidants (Fig 5b). Similar effect was also

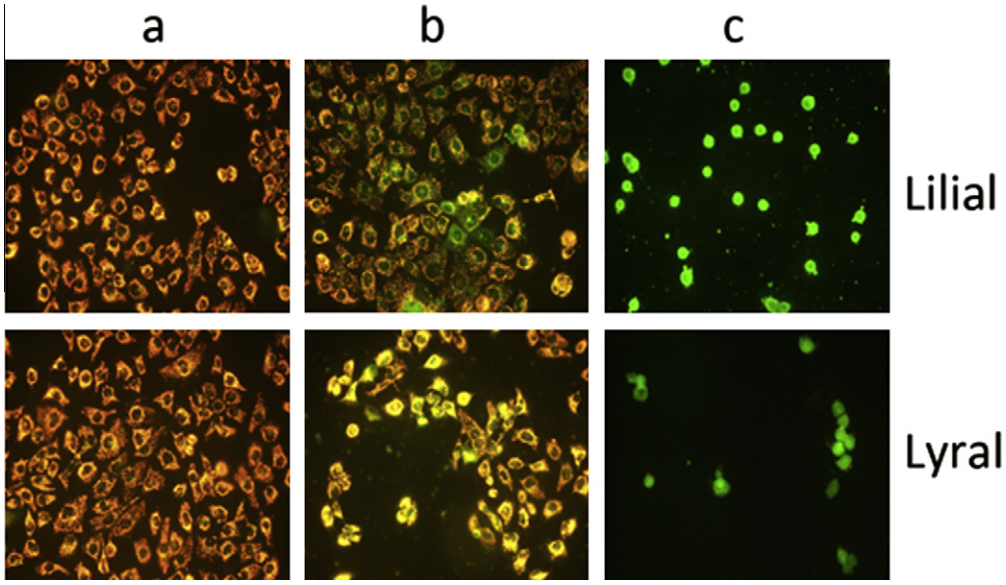


Fig. 6. Effect of lyral and lilial on mitochondrial membrane potential. HaCat cells were treated with lilial and lyral then assessed using MitoPT JC-1 kit for dissipation of mitochondrial membrane potential: (a) control; (b) EC50: lilial:60 nM; lyral:100 nM and (c) 10 μM each.

obtained with all antioxidants when higher concentration of either fragrance chemicals (50 uM) was used (Data not shown).

Lipoic acid is a natural oxidant and cofactor present in many key mitochondrial enzymes. We examined the effect of lipoic acid in protecting viability of HaCat cell. Pre-treatment for 2 h with Lipoic acid (100 μM) significantly protected the cells from the decrease in viability by lyral/lilial even at concentrations (50 uM) much higher than their corresponding EC50 (Fig 5c). This suggests that lipoic acid can protect cells against the toxic effect of either chemicals.

3.7. Lyral and lilial affect mitochondrial membrane potential

Membrane potential was influenced by both lyral and lilial. Whereas control cells were stained red–orange (Fig 6a) indicating viable cells and coupled mitochondria, treated cells at EC50 of: lyral (100 nM) and lilial (60 nM) have mitochondria stained both green and red–orange (Fig 6b) indicating initiation of membrane dissipation and formation of pores. However, cells treated at concentration (10 μM) that results in 100% decrease in viability, were mostly detached while all detected cells stained green (Fig 6c) indicating a collapse in the electrochemical gradient across the mitochondrial membrane, decreasing thus viability of cells.

4. Discussion

Fragrance chemicals, have been widely investigated and were reported to cause dermal sensitization (Hagvall et al., 2007; Schnuch et al., 2007), and respiratory irritation (Larsen et al., 2000). Few studies however, have examined their effects at cellular or sub-cellular level (Griffiths, 2005; Ka et al., 2003).

We investigate in this study the biochemical effect of two commonly used fragrance chemicals, lyral and lilial on HaCat cells. Both compounds increased ROS generation but exerted no effect on the glutathione level (oxidized and reduced), reduced cellular ATP level, inhibited the activity of mitochondrial respiratory chain complexes I and II, and induced cell death that was protected by the antioxidants NAC, trolox and lipoic acid. We hereby report the chemicals lyral and lilial as toxic to mitochondria.

Skin is one of the entry points into cells (Bridges, 2002) that provides a barrier and a line of defense, protecting the cell against the deleterious effect of some environmental factors and chemicals. Among the many various factors are the different fragrance chemicals used in household detergents and perfumes. The lipophilic nature of the fragrance chemicals enables them to cross the cell membranes including intracellular organelle membrane such as mitochondria (Griffiths, 2005). HaCat cells, are human non-tumoral keratinocyte cell lines that in terms of growth and differentiation behave phenotypically like normal keratinocyte(s) (BouKamp et al., 1988; Savini et al., 2000). Using MTT assay, the effect of lyral and lilial on different cell lines was initially tested. Whereas both compounds significantly decreased the viability of HaCat cells at nM concentrations, they exerted no effect on MCF7, Caco2, HepG2, HeK293 and NIH3T3 at concentrations as high as 100 μM. Screening the levels of lilial and lyral, in scented products, was found to vary in percentage (Ezendam et al., 2009) but have been estimated, to be present in the mM range (Griffiths, 2005). A surveillance of 26 fragrances (Schnuch et al., 2007) have categorized them into allergens belonging to: group I: the use of which should be restricted or banned (lyral is an example); Group II: the sensitizing properties of which, may be controlled or prevented by product labeling (lilial is an example); and group III: that require no labeling or restriction as sensitization may occur after their substantial oxidation. Moreover, the Scientific Committee on Consumer Safety has recently reported lyral as unsafe even at concentration as low as 200 ppm (SCCP, 2011).

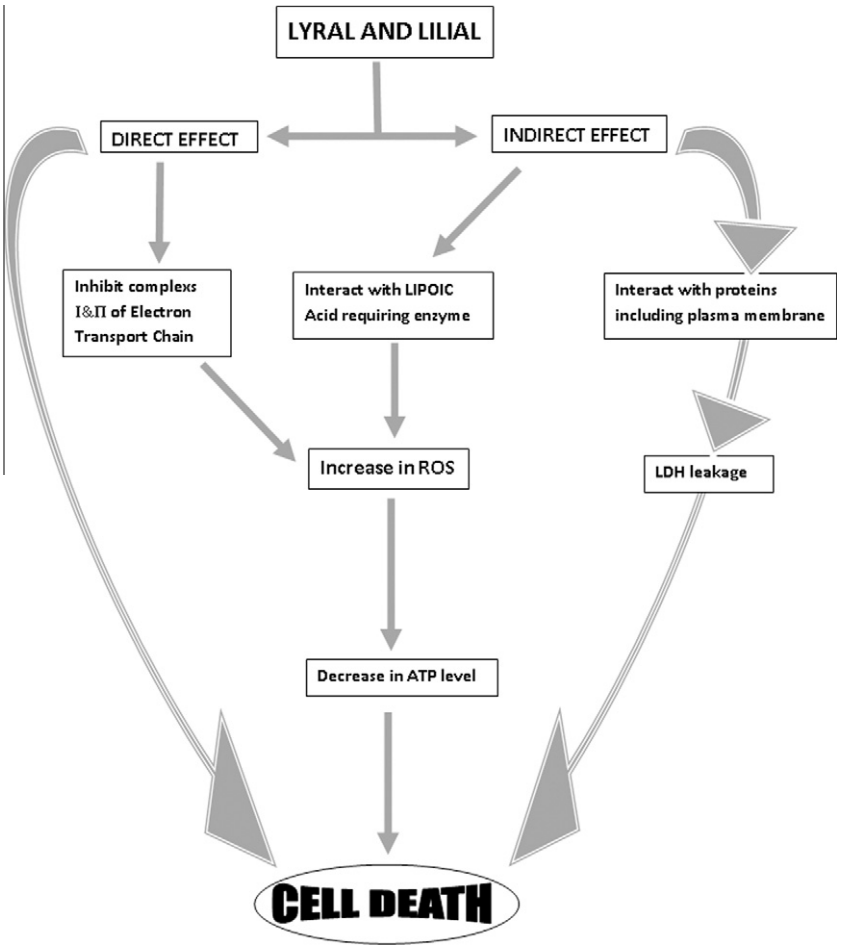
The cytotoxicity of lyral and lilial in HaCat cells was concentration dependant resulting in 50% cell death at 100 and 60 nM respectively. MTT assay assesses the ability of intact and actively coupled mitochondria to reduce MTT into formazan. A mitochondrial-toxin that impairs mitochondrial function and/or ATP production would therefore, lead to cell death. Treating HaCat cells with lyral or lilial each at its EC50, decreased ATP level after 12 h of treatment. Several studies have associated the decrease in ATP level with impaired mitochondrial electron transport chain activity and derangement in membrane potential (Duchen, 2004; Cadenas, 2004; Birch-Machon, 2000). Changes in the mitochondrial membrane potential, $\Delta\psi$, is indicative of apoptosis; which can be detected by positively charged dyes such as JC-1 (Smiley et al., 1991) that easily permeate active and negatively charged mitochondria, aggregate and fluoresce red–orange. However, in apoptotic or mitochondrial membrane compromised cells the JC-1 dye

will disperse as monomeric compound throughout the cells with green fluorescence (Cossarizza et al., 1993). Treatment of HaCat cells with lyral and lilial at their corresponding EC50 revealed red–orange and green stained fluorescent cells indicating, initiation of apoptotic events by depolarizing the mitochondria. Upon further increase in the concentration of either compound (10 μM), the entire cells had fluorescent green stain indicating dissipation of $\Delta\psi$, yielding apoptotic cells.

To elucidate further the underlying mechanism causing the decrease in ATP level, we investigated the direct effect of these chemicals on mitochondrial bioenergetics. Both complex I (NADH dehydrogenase) and complex II (succinate dehydrogenase) provide the main entry site of reducing equivalents (NADH, FADH2) into the electron transport chain. As these equivalents are oxidized, electrons are transferred coupled to pumping of protons from the matrix into the inter-membrane space. Reentry of hydrogen ions back into the mitochondrial matrix through the F_0 of F_0F_1 ATPase drives ADP phosphorylation into ATP (Griffiths, 2005; Hatefi, 1985). Inhibitors of complexes I and II would therefore inhibit electron flow through the respiratory chain, which ultimately leads to a decrease in ATP level (Nicholls and Ferguson, 1992). To examine the effect of lyral and lilial on the activity of complexes I and II, we opted to use sub-mitochondrial particles (SMP) isolated from rat liver, instead from HaCat cells for the following reasons: (1) being time and cost effective; whereas one rat liver provides enough mitochondria to prepare SMP and carry on the enzymes activity assays, 200 million cells of cultured HaCat cells would barely allow assay of one enzyme; and (2) overcoming the permeability barrier of the mitochondrial membrane by exposing complexes I and II directly to either fragrant chemicals.

Lyral and lilial exerted a direct inhibitory effect on activities of both complexes I and II, with lilial being more potent. A 50% inhibition in the oxidation of NADH and $FADH_2$ was obtained at 5 and 80 μM with lilial, versus 125 and 400 μM with lyral respectively. The discrepancy in the concentration of lyral and lilial required to decrease the viability of HaCat cells (nM range) versus that needed to inhibit complexes I and II (μM) may be attributed to: (a) higher level of complexes in isolated SMP and (b) possible metabolism of both compounds into more toxic products, that may occur in whole cells (HaCat) but not with isolated SMP.

In a recent study, commercial perfume products were screened and were found to exert an inhibitory effect on complex I of the electron transport chain (Griffiths, 2005). Defects in complex I have been associated with severe pathologies leading to: depolarization of $\Delta\Psi$, lower ΔpH, increase in NADH levels, increase in ROS, increase in lipid peroxidation, and ultimately reduction in ATP level (Koene et al., 2011; Distelmaeir et al., 2009; Leonard and Schapira, 2000). Under normal conditions, ROS generated as a consequence of oxidative phosphorylation is usually balanced by oxygen radicals' scavenging systems. Production of ROS observed in cultured cells from patients with complex I deficiency, had oxidative stress that eventually lead to cell injury and death (Raha and Robinson, 2001; Verkaart et al., 2007). Depletion of cellular GSH has also been implicated in loss of cell viability, whereas depletion of mitochondrial GSH increased sensitivity to peroxide-induced cellular damage (Wullner et al., 1999; Muyderman et al., 2004)



Scheme 2. Summary of lyral and lilial targets in HaCat cells.

Using NBT assay we obtained a significant increase in ROS level following HaCat treatment with either of the fragrant chemicals. Surprisingly however, neither lyral nor lilial had an effect on the level of free/total glutathione levels. The level of GSSG was 10-fold that of free GSH in control cells. No change was observed in lyral/lilial treated cells. However the protective effect exhibited by lipoic acid (natural antioxidant), NAC and trolox indicate the involvement of ROS in mediating the induced effect. Furthermore pretreatment with lipoic acid restored control viability protecting HaCat cells from lyral and lilial induced cell death.

Lipoic acid is an essential cofactor in the mitochondrial dehydrogenases, and a contributor in energy production (Shay et al., 2009; Loeffelhard et al., 1995). It is a natural antioxidant both in vitro and in vivo (Shay et al., 2008), that acts as a strong reductant, free radical scavenger, metal chelator and activator of antioxidant enzymes (Podda et al., 2001; Arivazhagan et al., 1999). Previous studies reported that NADH–lipoic acid and NADPH–thioredoxin dependent enzymes play a primary role in maintaining reductive power in HaCat cells, whereas the GSH–NADH dependant system has a secondary role (Savini et al., 2000, 2003) which are in line with our findings.

On the other hand, the intracellular metabolism of lilial and lyral into more biologically reactive metabolites may not be ruled out. Structurally, Lilial and lyral (Scheme 1: compounds *a* and *b*) are characterized by an aldehydic group. Dehydrogenation of either compound would lead to compound *d* from lilial where the new double bond is conjugated to the phenyl ring; whereas lyral dehydrogenation may produce either one of the following compounds *e* and *f*. The products of dehydrogenation (*d*, *e*, and *f*) belong to the general class of α,β-unsaturated aldehyde a prototype of which is 4 hydroxynonenal (HNE) (Scheme 1 compound *c*)

HNE, an alken-al, is a mediator of free radical damage in the cell and among the lipid peroxidation products that is produced in large amounts. Mitochondrial complexes are one target of HNE that are protected by thiols (Korotchkina et al., 2001). In addition, HNE biological toxicity includes: inhibiting enzymes, DNA, and protein synthesis; inactivating proteins by modifying key amino acid residues (Cys, His, Lys); stimulating phospholipase C that stimulates the opening of the calcium channels leading to ionic disturbance, imbalance, plasma membrane damage and cell death (Esterbauer et al., 1991; Echtay et al., 2003).

It is plausible therefore to propose that the oxidation of lyral and lilial into the corresponding α,β-unsaturated aldehyde intermediates disturb membrane integrity resulting in cellular injury, a biomarker of, is leakage of LDH (Fig 2c). In addition, several studies have attributed toxicity of alkenals to their reactivity towards certain protein cofactors such as lipoic acid, leading to their inactivation (Witz, 1989; Humphries and Szweda, 1998; Petersen and Doorn, 2004; Sheline and Wei, 2006).

Whereas lilial is less frequently reported as contact allergen (Schnuch et al., 2007), lyral has been clearly referred to as frequent contact allergen (SCCP, 2011). It is important however to note that the in vitro effects of these compounds result from direct treatment of cells with either chemicals. Extrapolating this in vivo, may not be as toxic as taking into consideration the skin barrier and volatility of these compounds. Our findings however may provide a plausible explanation of early aging of the cell resulting from the cumulative effect of chronic exposure, though low level, of skin cells to chemicals.

We propose (Scheme 2) that lyral and lilial have toxic effect on mitochondria decreasing viability of HaCat cells by: targeting respiratory chain complexes, inhibiting complexes I and II, depolarizing and dissipating the mitochondrial membrane potential, increasing ROS, decreasing ATP level and leading to death of HaCat cells. The implications of lyral and lilial effects', whether direct or indirect (upon their biotransformation) on pro/antiapoptotic mediators, metabolic state and aging remain to be investigated.

Conflict of Interest

The authors declare no conflict of interest with any organization or person.

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