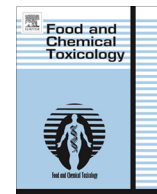




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journal homepage: www.elsevier.com/locate/foodchemtoxAllergenicity evaluation of fragrance mix and its ingredients by using *ex vivo* local lymph node assay–BrdU endpointsQ1 Ozge Cemiloglu Ulker^{a,*}, Yesim Kaymak^b, Asuman Karakaya^a^a Ankara University, Faculty of Pharmacy, Department of Toxicology, 06100 Ankara, Turkey^b Gazi University Health Center, Ankara, Turkey

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ABSTRACT

The present studies were performed to compare the differences between sensitization potency of fragrance mix and its ingredients (oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal), by using *ex vivo* LLNA–BrdU ELISA. The SI and EC₃ values were calculated and potency classification was found for the mixture and for each ingredients. T_H1 cytokines (IL-2, IFN- γ) and T_H2 cytokines (IL-4, IL-5) releases from lymph node cell culture were also investigated as contact sensitization endpoints. The EC₃ values were calculated and the potency of contact sensitization were classified for fragrance mix, oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal respectively: 4.4% (moderate), 3.4% (moderate), 0.88% (strong), 16.6% (weak), 1.91% (moderate), 9.77% (moderate), 13.1% (weak), 17.93% (weak), 7.74% (moderate). According to our results it should be concluded that exposure to fragrance mix does not constitute an evidently increased hazard compared to exposure to each of the eight fragrance ingredients separately. Cytokine analyses results indicate that both T_H1 and T_H2 cytokines are involved in the regulation of murine contact allergy and can be considered as useful endpoints.

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1. Introduction

Allergic contact dermatitis affects approximately 1–10% of the general population (Kimber et al., 2002). The incidence of allergic contact dermatitis has a tendency to increase in the industrialized countries parallel to the increase in the number of potential sensitizers. On the other hand current population are widely exposed to fragrances because of the increased usage of the cosmetic products. Fragrance mix is an important diagnostic marker in the European baseline series based on patch test results (Johansen, 2003). It is considered as the main indicator and screening test for perfume allergy (Trattner et al., 2004). Fragrance mix was developed at the beginning of the 1970s by Walter Larsen as a new diagnostic test for fragrance allergy. Fragrance mix has 8 ingredients, namely, 7 synthetic fragrances (cinnamal, cinnamyl alcohol, geraniol, isoeugenol, eugenol, hydroxycitronellal, alpha amyl cinnamal) and 1 natural fragrance (oak moss absolute). The initial concentration of each ingredients was 2% but this was reduced to 1% in 1984 (Larsen, 1985).

The Local Lymph Node Assay (LLNA) is an alternative to guinea pig models for the assessment of the contact sensitization potential. The contact sensitization of an allergen is characterized by lymphocyte proliferation in the lymph nodes (Basketter et al., 2007). The assay provides information on a substance's ability to induce sensitization, and importantly, delivers quantitative data that enable dose response assessment.

The use of standard LLNA is difficult in any laboratory because such radioisotope-based method requires special facilities and handling procedures. Several authors have been conducting investigations for the development of an alternative non-radioactive method for performing LLNA (Ehling et al., 2005a,b; Idehara et al., 2008; Lee et al., 2002; Takeyoshi et al., 2001, 2005, 2006; Kojiima et al., 2008).

The method, the LLNA:BrdU-ELISA proposed by Takeyoshi et al. (2001) and refined during an interlaboratory validation study (Kojima et al., 2008) was approved by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM, 2010). Although the validation of this assay was conducted, ICCVAM recommends the future studies to characterize the limitations of the LLNA:BrdU-ELISA test method (ICCVAM, 2010, 2011). By considering the limitation of this method recently we had developed the following *ex vivo* non-radioactive LLNA (*ex vivo* LLNA–BrdU ELISA) and we had also investigated formation/release of interleukins (IL)-2, -4, and -5, and interferon

Abbreviations: AALAC, Association for Assessment and Accreditation of Laboratory Animal Care; AOO, Acetone; Olive Oil; EC₃, estimated concentration; FBS, Fetal Bovine Serum; ICCVAM, Interagency Coordinating Committee on the Validation of Alternative Methods; IFN, interferon; IL, interleukin; LLNA, Local Lymph Node Assay; PHA-L, phytohemagglutinin-L; SI, Stimulation Index.

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(IFN)- γ from isolated lymph node cells as contact sensitization endpoints of this method. (Ulker et al., 2011, 2013).

The current study has been conducted with three aims. First is to investigate the sensitization potency of fragrance mix by using *ex vivo* LLNA–BrdU ELISA. Second is to examine any difference in sensitization potency for the ingredients arising from their exposure in a mixture. Third is to investigate T_H1 cytokines (IL-2, IFN- γ) and T_H2 cytokines (IL-4, IL-5) releases from lymph node cell culture as contact sensitization endpoints.

2. Materials and methods

2.1. Mice

Female 8–12-week-old female Balb/c mice were obtained from Gulhane Military Medical Academy (Ankara, Turkey) and then housed at a temperature of 23 °C and relative humidity of 55% and with a 12 h light/dark cycle. Mice were provided mousechow (Optima, Kırklareli, Turkey) and water *ad libitum*. All animal procedures were conducted in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility under an animal protocol approved by the Ankara University Animal Experiments Ethics Board. Before the experiment, mice were weighed, randomized and marked for identification.

2.2. Chemicals

Fragrance mix and the ingredients were applied to the animals at different concentrations in Acetone: Olive Oil (4:1 v/v) (AOO) (Sigma). Fragrance mix was prepared by mixing eight constituents at the same volume. These constituents were alpha amyl cinnamal (RTC, USA), cinnamal (RTC, USA), cinnamic alcohol (Merk, Germany), eugenol (Acros organics, ABD), geraniol (Fluka, Germany), hydroxycitronellal (Acros organics, ABD), isoeugenol (Acros organics, ABD) and oak moss absolute (Brial, Germany).

2.3. *Ex vivo* BrdU incorporation

Five groups of mice ($n = 4/\text{group}$) were exposed topically on the dorsum of both ears to 25 μl of different concentration of chemicals and vehicle (AOO) alone daily for three consecutive days. The mice in this study were only rested on Day 4. On Day 5, all mice were killed by cervical dislocation and their auricular lymph nodes were excised and weighed. Excised right and left lymph nodes were pooled and homogenized, and the released cells suspended in 15 ml physiological saline. After counting, some cells from the suspension were seeded into 96-well culture plates (at 10^5 cells/well, in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum [FBS] and 1% penicillin–streptomycin (all from Bio-chrom, Berlin, Germany). After culture had occurred for 48 h at 37 °C, BrdU (10 μl of a 10 μM BrdU labeling solution; final concentration = 1 μM BrdU) was then added to the wells for a 24 h labeling period. The cells in the wells were then recovered by aspiration and the extent of BrdU incorporation measured by ELISA (Roche, Penzberg, Germany), according to manufacturer instructions. The absorbance was measured at a wavelength of 450 nm (OD_{450}) with a reference wavelength of 620 nm, using an ELISA reader (SpectraMAX, Molecular Devices Inc., Sunnyvale, CA) microplate reader; these values were used to define the BrdU labeling index.

2.4. Calculations of stimulation indices (SI)

Stimulation Index (SI) was calculated as the ratio of the mean *ex vivo* BrdU incorporation (labeling index) for each treatment group vs that of the vehicle control group.

2.5. Calculation of EC_3 value

The EC_3 – a measure of relative skin sensitizing potency of a substance – is expressed as the estimated concentration of chemical necessary to produce a 3-fold increase in proliferation in draining lymph nodes compared with the spontaneous proliferation that occurs with cells from vehicle-treated control hosts. The method represents a simple linear interpolation of the points in the dose–response curve which lie immediately above and below the classification threshold, i.e., a stimulation index of 3. If the datapoints lying immediately above and below the SI value of 3 have the co-ordinates (a, b) and (c, d), respectively, then the EC_3 value may be calculated using the following equation (Basketter et al., 2007):

$$\text{EC}_3 = c + [(3 - d)/(b - d)](a - c)$$

2.6. Culture of lymph node cells and cytokine determinations

Harvested lymph node cells from the *ex vivo* protocols outlined above were seeded in a 24 wellculture plate (at 1×10^6 cells/well) in 1 ml of RPMI 1640 medium supplemented with 10% FBS and 1% penicillin – streptomycin. In the case of

the *ex vivo* method, the cells used here were obtained from the pooled cells from Day 4-rested hosts prior to removal of aliquots for use in BrdU staining/analyses (see above). Following the seeding steps, the wells were then supplemented with 5 $\mu\text{g}/\text{ml}$ of phytohemagglutinin-L (PHA-L; Biochrom). PHA-L was selected as the mitogen here (as opposed to concanavalin A) in that it has been widely used for mitotic stimulation of T-lymphocytes. After 72 h of culture in a 37 °C incubator containing 5% CO_2 , supernatants were collected and stored at –80 °C until analyzed for levels of IL-2, IFN- γ , IL-4, and IL-5. The levels of each cytokine in the culture supernatants were measured using commercially available ELISA kits (BenderMed Systems, Burlingame, CA), according to the manufacturer's instructions.

2.7. Statistical analyses

Means and standard errors of mean (S.E.M.) were calculated for the cytokine levels of vehicle group and the groups exposed by topical application with the different chemicals. The differences in cytokine levels between vehicle group and each treatment group were statistically analyzed with Mann–Whitney U test and $p < 0.05$ was taken as the level of significance.

3. Results

3.1. Lymph node-related parameters and *ex vivo* LLNA–BrdU results

In these studies, five groups of Balb/c mice for *ex vivo* studies were exposed topically (on dorsum of both ears) to different concentration of fragrance mix, and the ingredients of fragrance mix or to AOO vehicle alone on three consecutive days (i.e., Days 1–3). In the “*Ex vivo* LLNA–BrdU ELISA” studies, mice received no BrdU injection but were rested on Day 4. One day later (i.e., 2 d after final application, Day 5), all mice were sacrificed. The auricular lymph node weights, lymph node cell counts were elevated in a manner reflecting a clear dose-related trend.

The auricular lymph node weights, lymph node cell counts, the data for all OD_{450} , SI, and EC_3 values from *ex vivo* BrdU labeling studies are also presented in Table 1. The EC_3 values were calculated and the potency of contact sensitization were classified for fragrance mix, oak moss absolute, isoeugenol, eugenol, cinnamal, hydroxycitronellal, geraniol, cinnamic alcohol, alpha amyl cinnamal respectively: 4.4% (moderate), 3.4% (moderate), 0.88% (strong), 16.6% (weak), 1.91% (moderate), 9.77% (moderate), 13.1% (weak), 17.93% (weak), 7.74% (moderate). The EC_3 results were also compared with the result of previous standard LLNA studies and were shown in Table 2.

3.2. Cytokine determinations

As described in the method part, on Day 5, auricular lymph nodes were excised from each mouse in the various treatment groups and lymph node cell suspensions were prepared. These cells were then cultured in medium in the presence of PHA-L mitogen to assess background and inducible formation of select T_H1 (IL-2, IFN- γ) and T_H2 cytokines (IL-4, IL-5) associated. The levels of IL-2, IFN- γ , IL-4, and IL-5 present in the lymph node cell culture supernatants are provided in Figs. 1–9. Both T_H1 and T_H2 cytokines, were increased by applied chemicals and mixtures in a dose-dependent manner with statistical significance ($p < 0.05$).

4. Discussion

The standard LLNA has several advantages since it is quicker and cheaper, providing a quantitative endpoint. However to use the assay is difficult in any laboratory because such radioisotope-based method requires special facilities and handling procedures. On the other hand the nonradioactive method which was investigated by Takeyoshi et al., 2001 (LLNA:BrdU-ELISA) to eliminate this disadvantage, appears to be less sensitive to yield lower EC_3 values than the standard LLNA.

Table 1Lymph node weight, cell count, OD₄₅₀, SI and EC₃ values after treatment of mice with different concentrations of fragrance mix and ingredients of fragrance mix in AOO.

Chemical	Vehicle	Concentration (w/v%)	Lymph node weight (g)	Cell count (10 ⁶ /ml)	OD ₄₅₀	SI	EC ₃ (%)
Fragrance mix	AOO	–	0.017 ± 0.0003	6.40 ± 0.22	0.127	1	4.44
		0.5	0.031 ± 0.0009	7.60 ± 0.36	0.238	1.87	
		5	0.038 ± 0.0002	10.00 ± 0.57	0.401	3.16	
		25	0.047 ± 0.0012	12.03 ± 1.36	0.626	4.93	
		50	0.067 ± 0.0016	16.00 ± 2.06	1.107	8.72	
Oak moss absolute	AOO	–	0.015 ± 0.0002	10.60 ± 0.36	0.117	1	3.40
		0.5	0.019 ± 0.0004	12.00 ± 1.27	0.177	1.50	
		1	0.021 ± 0.0007	16.00 ± 2.00	0.274	2.34	
		5	0.027 ± 0.0010	18.00 ± 1.26	0.403	3.44	
		10	0.031 ± 0.0007	20.00 ± 2.02	0.537	4.58	
Isoeugenol	AOO	–	0.016 ± 0.0008	8.00 ± 0.25	0.117	1	0.88
		0.5	0.020 ± 0.0009	13.30 ± 0.21	0.223	1.91	
		1	0.024 ± 0.0006	17.60 ± 1.21	0.388	3.32	
		5	0.026 ± 0.0010	20.80 ± 1.15	0.519	4.44	
		10	0.038 ± 0.0005	23.20 ± 1.23	0.728	6.22	
Eugenol	AOO	–	0.015 ± 0.0002	10.60 ± 0.36	0.117	1	16.60
		2.5	0.023 ± 0.0008	10.50 ± 0.42	0.173	1.48	
		10	0.033 ± 0.0010	13.00 ± 0.56	0.223	1.9	
		20	0.035 ± 0.0009	13.00 ± 0.25	0.371	3.17	
		50	0.047 ± 0.0015	18.00 ± 1.25	0.532	4.54	
Cinnamal	AOO	–	0.016 ± 0.0008	8.00 ± 0.25	0.117	1	1.91
		0.5	0.020 ± 0.0005	11.20 ± 0.23	0.217	1.85	
		1	0.028 ± 0.0005	13.60 ± 0.86	0.305	2.6	
		5	0.030 ± 0.0008	17.60 ± 0.79	0.511	4.36	
		10	0.035 ± 0.0010	25.60 ± 1.52	1.076	9.19	
Hydroxycitronellal	AOO	–	0.016 ± 0.0008	8.00 ± 0.25	0.117	1	9.80
		2.5	0.015 ± 0.0007	9.00 ± 0.79	0.202	1.70	
		10	0.019 ± 0.0009	10.00 ± 0.58	0.356	3.04	
		20	0.025 ± 0.0012	12.00 ± 0.42	0.447	3.82	
		50	0.032 ± 0.0012	14.00 ± 0.52	0.674	5.70	
Geraniol	AOO	–	0.016 ± 0.0008	8.00 ± 0.25	0.117	1	13.10
		2.5	0.024 ± 0.0007	10.00 ± 0.56	0.232	1.98	
		10	0.027 ± 0.0003	15.00 ± 0.82	0.264	2.25	
		20	0.032 ± 0.0008	16.00 ± 0.98	0.388	3.31	
		50	0.038 ± 0.0012	18.00 ± 1.02	0.483	4.12	
Cinnamyl alcohol	AOO	–	0.015 ± 0.0002	10.60 ± 0.36	0.117	1	17.90
		2.5	0.027 ± 0.0003	12.40 ± 0.81	0.205	1.75	
		10	0.030 ± 0.0005	14.00 ± 0.75	0.243	2.08	
		20	0.033 ± 0.0012	15.00 ± 0.79	0.379	3.24	
		50	0.037 ± 0.0017	18.00 ± 0.99	0.505	4.32	
Alpha amyl cinnamal	AOO	–	0.016 ± 0.0008	8.00 ± 0.25	0.117	1	7.7
		2.5	0.018 ± 0.0006	9.20 ± 0.49	0.251	2.14	
		10	0.025 ± 0.0008	10.60 ± 0.52	0.395	3.37	
		20	0.029 ± 0.0011	14.00 ± 0.79	0.463	3.96	
		50	0.033 ± 0.0015	16.00 ± 0.82	0.723	6.18	

Table 2Comparison of the EC₃ results of our study with the EC₃ results of standard LLNA.

Chemical	EC ₃ (our study)	EC ₃ (standard LLNA)	Refs.
Fragrance mix	4.4% (moderate)	No result	
Oak moss absolute	3.4% (moderate)	4.4% (moderate)	Chapdelaine, 2004
Isoeugenol	0.88% (extreme)	1.3% (strong)	Kimber et al., 2003
Eugenol	16.6% (weak)	13% (weak)	Kimber et al., 2003
Cinnamal	1.91% (moderate)	1.7% (moderate)	Basketter et al., 2007
Hydroxycitronellal	9.77% (moderate)	23% (weak)	Basketter et al., 2007
Geraniol	13.1% (weak)	25.6% (weak)	Basketter et al., 2005
Cinnamyl alcohol	17.93% (weak)	21% (weak)	Gerberick et al., 2005
Alpha amyl cinnamal	7.74% (moderate)	11% (weak)	Smith and Hotchkiss, 2001

By considering the disadvantages and limitations of the standard LLNA and LLNA:BrdU-ELISA methods we previously made some modifications of LLNA:BrdU-ELISA test to make the assay more sensitive (*ex vivo* LLNA-BrdU ELISA) (Ulker et al., 2011, 2013). In our modified method we used an SI ≥ 3 as the cut-off classification of chemicals as sensitizers, EC₃ values were also calculated for use in comparisons against studies that utilized the standard radioactive LLNA. We also aimed to improve the welfare of the test animals- albeit slightly (i.e., they still had to be treated and ultimately euthanized)- by having isolated lymphocytes labeled with BrdU *ex vivo* (by adding BrdU at the 48-h point in lymphocyte cell culture) instead of having this done *in vivo* (in situ) (i.e., labeling by intraperitoneal BrdU injection). This method would allow for the avoidance of the additional pain and distress to a host that would occur due to the intraperitoneal BrdU injection step.

In the present study, we investigated the sensitization potency of fragrance mix and its ingredients by using *ex vivo* LLNA-BrdU ELISA. We also examined whether there was any difference in sensitization potency between them. It was also investigated the

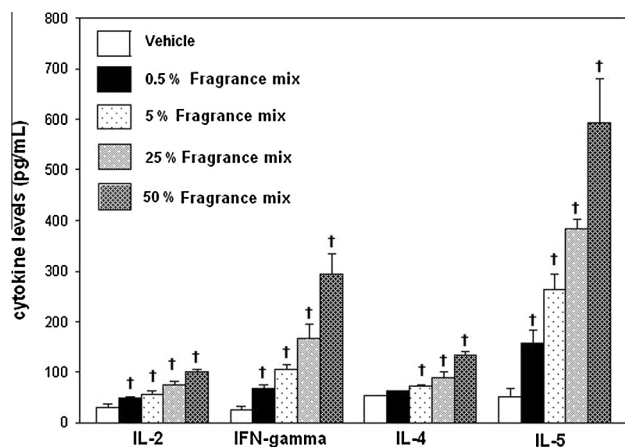


Fig. 1. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with fragrance mix. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

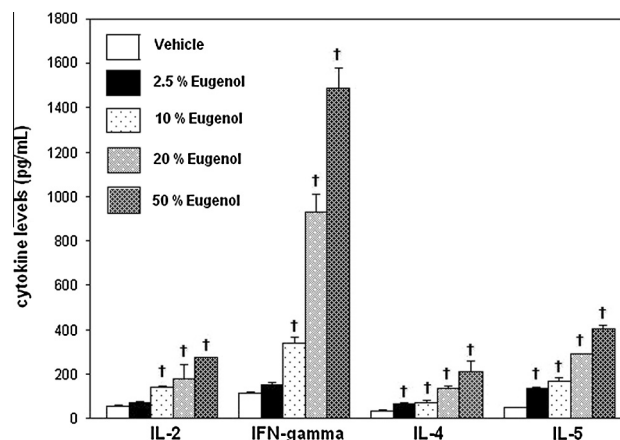


Fig. 4. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with eugenol. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

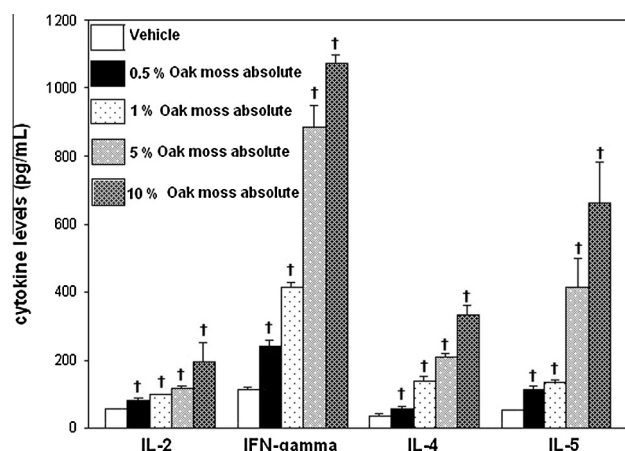


Fig. 2. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with oak moss absolute. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

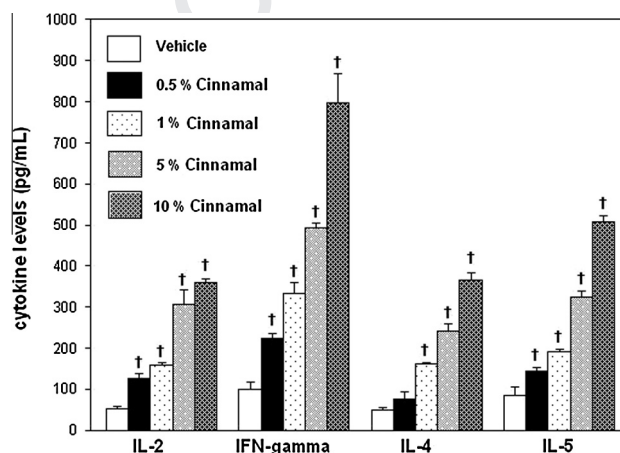


Fig. 5. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with cinnamal. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

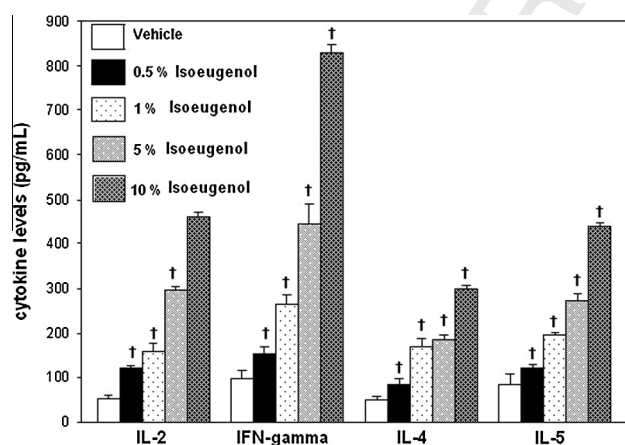


Fig. 3. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with isoeugenol. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

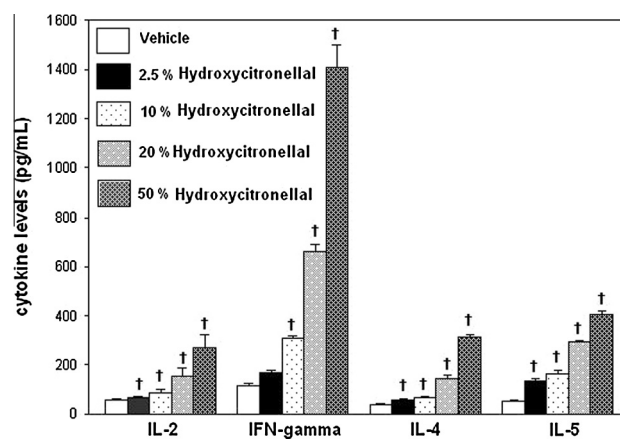


Fig. 6. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3d) with hydroxycitronellal. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

formation/release of IL-2 and IFN- γ (T_H1 cytokines) and IL-4 and IL-5 (T_H2 cytokines) from isolated lymph node cells as contact sensitization endpoints. The concentration 4.44% of fragrance mix

produced a threefold stimulation of proliferation in draining lymph nodes compared with concurrent vehicle control group. According to our EC_3 value, fragrance mix can be classified as a moderate sensitizer. Although ICCVAM reported as LLNA may not be suitable

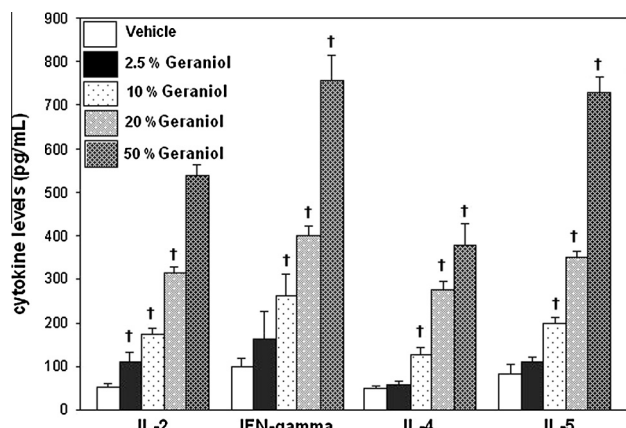


Fig. 7. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3 d) with geraniol. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

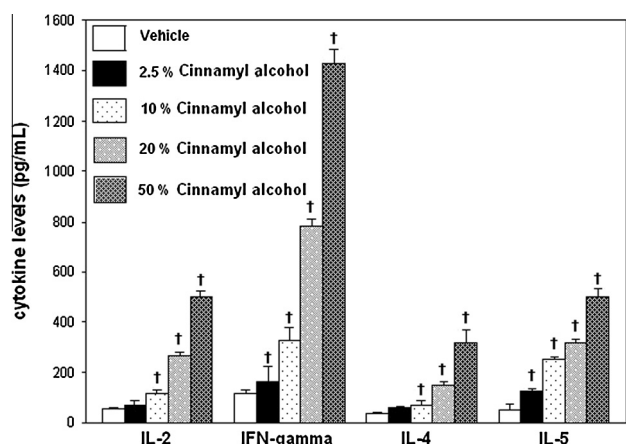


Fig. 8. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3 d) with cinnamyl alcohol. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

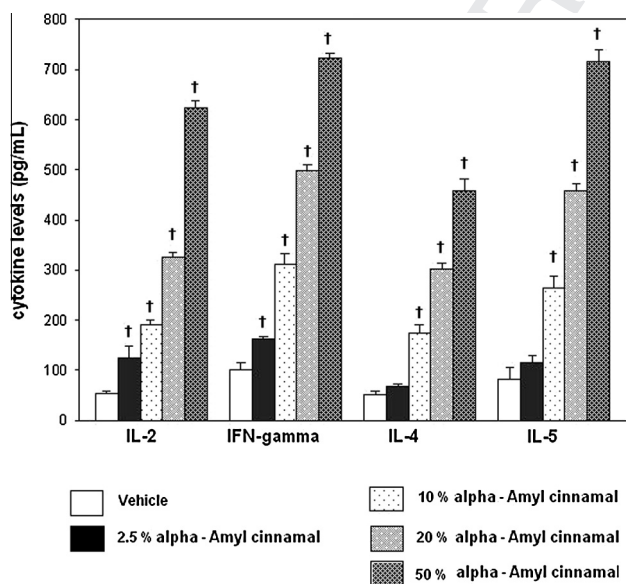


Fig. 9. Cytokine levels in cultures of auricular lymph node cells from mice treated daily (3 d) with alpha amyl cinnamal. The values are in terms of mean $\pm$ S.E.M. ($n = 4$). $^{\dagger}p < 0.05$.

for use with mixtures, there are some studies which suggested that the LLNA could be used in investigating the overall sensitizing capacity of a mixture. (Lalko and Api, 2006; Craig et al., 2004; Anderson et al., 2009; Auttachoat et al., 2011). The EC₃ values of the ingredients were compatible with standard LLNA results. On the other hand the potency of fragrance mix differed from some of its individual ingredients. It should be concluded that exposure to fragrance mix does not constitute an evidently increased hazard compared to exposure to each of the eight fragrance ingredients separately although some ingredients' sensitizing potency were found to be extreme or strong.

Many studies have tried to characterize the chemical sensitizers according to the cytokine profiles they provoke (Dearman et al., 1996a,b; Dearman et al., 2003; Dearman and Kimber, 1999; Vandebriel et al., 2003). Changes (i.e. increases) in cytokine levels are related to T-helper cell proliferation that plays important role in the induction and elicitation of contact sensitivity. In our study it was assessed the release of IL-2 and IFN- γ (T_H1 cytokines) and IL-4 and IL-5 (T_H2 cytokines) from isolated lymph node cells of mice exposed to fragrance mix and its ingredients.

IL-2 is secreted by activated T cells and is essential for the expansion of antigen-selective T-cell clones, contributing to both T_H1 and T_H2 cell responses (Ku et al., 2008). Some studies demonstrated that IL-2 may be a useful marker for detection of sensitizers (Azam et al., 2005; Ku et al., 2008; Jung et al., 2012). IFN- γ production by lymph node cells following sensitization is considered important for the development of effector T cells, which is critical for the accomplishment of contact dermatitis (He et al., 2001). IL-4 is a well characterized cytokine product of T_H2 cells. Ku et al. (2008) reported that both IFN- γ and IL-4 were significantly up-regulated in draining lymph node (LN) cells by the sensitizers and it was also found out that IL-4 plays a positive role in elicitation of contact sensitivity. Traidl et al. (1999) found that IL-4-deficient mice sensitized to DNCB showed a significant reduction in magnitude and duration of their contact hypersensitivity responses in comparison to those observed with their wild-type counterparts. It was reported that IL-5 plays an essential role in elicitation of contact sensitivity (Itakura et al., 2006). These researchers showed that contact sensitization responses were impaired in IL-5-deficient mice.

We found that there was a dose related increase in formation / release of T_H1 (IL-2, IFN- γ) and T_H2 (IL-4 and IL-5) cytokines. There is a growing body of published evidence that both T_H1 and T_H2 cytokines are involved in the regulation of murine contact allergy (Asada et al., 1997; Ulrich et al., 2001; Nagai et al., 2000; Ku et al., 2008; Ulker et al., 2011, 2013). According to our results it can be suggested that not only T_H1 cytokines such as IL-2 and IFN- γ but also T_H2 cytokines such as IL-4 and IL-5 can play important roles in elicitation of contact sensitivity. Among these cytokines, IFN- γ was strongly elevated in a manner reflecting a clear dose-related trend.

In conclusion, the sensitization potency of fragrance mix found to be moderate, it can be suggested that exposure to fragrance mix does not constitute an evidently increased hazard compared to exposure to each of the eight fragrance ingredients separately. Cytokine analyses results indicate that both T_H1 and T_H2 cytokines are involved in the regulation of murine contact allergy and can be considered as useful endpoints.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency documents associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2013.12.036>.

5. Uncited references

(Hayashi et al., 2001; Manetz and Meade, 1999; Manetz et al., 2001; Plitnick et al., 2002; Van Och et al., 2002).

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References

- Anderson, S.E., Brown, K.K., Butterworth, L.F., Fedorowicz, A., Jackson, L.G., Frasch, H.F., Beezhold, D., Munson, A.E., Meade, B.J., 2009. Evaluation of irritancy and sensitization potential of metalworking fluid mixtures and components. *J. Immunol.* 6, 19–29.
- Asada, H., Linton, J., Katz, S.I., 1997. Cytokine gene expression during the elicitation phase of contact sensitivity; regulation by endogenous IL-4. *Invest. Dermatol.* 108, 406–411.
- Attachaoat, W., Germolec, D.R., Smith, M.J., White Jr., K.L., Guo, T.L., 2011. Contact sensitizing potential of annatto extract and its two primary color components, cis-bixin and norbixin, in female balb/c mice. *Food Chem. Toxicol.* 49, 2638–2644.
- Azam, P., Peiffer, J.L., Ourlin, J.C., Bonnet, P.A., Tissier, M.H., Vian, L., Fabre, I., 2005. Qualitative and quantitative evaluation of a local lymph node assay based on ex vivo interleukin-2 production. *Toxicology* 206, 285–298.
- Basketter, D.A., Clapp, C., Jefferies, D., Safford, B., Ryan, C.A., Gerberick, F., Dearman, R.J., Kimber, I., 2005. Predictive identification of human skin sensitization thresholds. *Contact Dermatitis* 53, 260–267.
- Basketter, D.A., Gerberick, F., Kimber, I., 2007. The local lymph node assay and the assessment of relative potency: status of validation. *Contact Dermatitis* 57, 70–75.
- Chapdelaine, J.M., 2004. Local Lymph Node Assay. Calvert Laboratories. Report no: 0787MI02.013.
- Craig, A.M., Karchesy, J.J., Blythe, L.L., Gonzales-Hernandez, M.P., Swan, L.R., 2004. Toxicity studies on western juniper oil (*Juniperus occidentalis*) and Port-Orford cedar oil (*Chamaecyparis Lawsoniana*) extracts utilizing local lymph node and acute dermal irritation assays. *Toxicol. Lett.* 154, 217–224.
- Dearman, R.J., Kimber, I., 1999. Cytokine fingerprinting: characterization of chemical allergens. *Methods* 19, 56–63.
- Dearman, R.J., Basketter, D.A., Kimber, I., 1996a. Characterization of chemical allergens as a function of divergent cytokine secretion profiles induced in mice. *Toxicol. Appl. Pharmacol.* 138, 308–316.
- Dearman, R.J., Moussavi, A., Kemeny, D.M., Kimber, I., 1996b. Contribution of CD4⁺ and CD8⁺ T-lymphocyte subsets to the cytokine secretion patterns induced in mice during sensitization to contact and respiratory chemical allergens. *Immunology* 89, 502–510.
- Dearman, R.J., Betts, C.J., Humphreys, N., Flanagan, B.F., Gilmour, N.J., Basketter, D.A., Kimber, I., 2003. Chemical allergy: considerations for the practical application of cytokine profiling. *Toxicol. Sci.* 71, 137–145.
- Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A.O., Van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R., Vohr, H.W., 2005a. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: first round. *Toxicology* 212, 60–68.
- Ehling, G., Hecht, M., Heusener, A., Huesler, J., Gamer, A.O., van Loveren, H., Maurer, T., Riecke, K., Ullmann, L., Ulrich, P., Vandebriel, R., Vohr, H.W., 2005b. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: 2nd round. *Toxicology* 212, 69–79.
- Gerberick, G.F., Ryan, C.A., Kern, P.S., Schlatter, H., Dearman, R.J., Kimber, I., Patlewicz, G.Y., Basketter, D.A., 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 16, 157–202.
- Hayashi, M., Higashi, K., Kato, H., Kaneko, H., 2001. Assessment of preferential TH1 or TH2 induction by low-molecular-weight compounds using a reverse transcription-polymerase chain reaction method: comparison of two mouse strains C57BL/6 and BALB/c. *Toxicol. Appl. Pharmacol.* 177, 38–45.
- He, B., Munson, A.E., Meade, B.J., 2001. Analysis of gene expression induced by irritant and sensitizing chemicals using oligonucleotide arrays. *Int. Immunopharmacol.* 1, 867–879.
- Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Report March 2010. ICCVAM Test Method Evaluation Report on the Murine Local Lymph Node Assay: BrdU-ELISA A Nonradioactive Alternative Test Method to Assess the Allergic Contact Dermatitis Potential of Chemicals and Products. NIH Publication No. 10-7552. Research Triangle Park, NC: National Institute of Environmental Health Sciences. <<http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm>>.
- Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) Test Method Evaluation Report 2011: Usefulness and Limitations of the Murine Local Lymph Node Assay for Potency Categorization of Chemicals

- Causing Allergic Contact Dermatitis in Humans. NIH Publication No. 11-7709. Research Triangle Park, NC: National Institute of Environmental Health Sciences. <<http://iccvam.niehs.nih.gov/methods/immunotox/llna-ELISA/TMER.htm>>.
- Idehara, K., Yamagishi, G., Yamashita, K., Ito, M., 2008. Characterization and evaluation of a modified local lymph node assay using ATP content as a non-radio isotopic endpoint. *J. Pharmacol. Toxicol. Methods* 58, 1–10.
- Itakura, A., Kikuchi, Y., Kouro, T., Ikutani, M., Takaki, S., Askenase, P.W., Takatsu, K., 2006. Interleukin-5 plays an essential role in elicitation of contact sensitivity through dual effects on eosinophils and B-1 cells. *Int. Arch. Allergy Immunol.* 140, 8–16.
- Johansen, D.J., 2003. Fragrance contact allergy. *Am. J. Clin. Dermatol.* 4, 789–798.
- Jung, K.M., Jang, W.H., Lee, Y.K., Yum, Y.N., Sohn, S., Kim, B.H., Chung, J.H., Park, Y.H., Lim, K.M., 2012. B cell increases and ex vivo IL-2 production as secondary endpoints for the detection of sensitizers in non-radioisotopic local lymph node assay using flow cytometry. *Toxicol. Lett.* 209, 255–263.
- Kimber, I., Basketter, D.A., Gerberick, G.F., Dearman, R.J., 2002. Allergic contact dermatitis. *Int. Immunopharmacol.* 2, 201–211.
- Kimber, I., Basketter, D.A., Butler, M., Gamer, A., Garrigue, J.L., Gerberick, G.F., Newsome, C., Steiling, W., Vohr, H.W., 2003. Classification of contact allergens according to potency: proposals. *Food Chem. Toxicol.* 41, 1799–1809.
- Kojima, H., Takeyoshi, M., Omori, T., Sozu, T., Arima, K., Idehara, K., et al., 2008. Inter-laboratory validation study on LLNA-BrdU. *Toxicologist* 102, 216.
- Ku, H.O., Jeong, S.H., Kang, H.G., Pyo, H.M., Cho, J.H., Son, S.W., Kim, H.R., Lee, K.J., Ryu, D.Y., 2008. Intracellular expression of cytokines and granzyme B in auricular lymph nodes draining skin exposed to irritants and sensitizers. *Toxicology* 250, 116–123.
- Lalko, J., Api, A.M., 2006. Investigation of the dermal sensitization potential of various essential oils in the local lymph node assay. *Food Chem. Toxicol.* 44, 739–746.
- Larsen, W.G., 1985. Perfume dermatitis. *J. Am. Acad. Dermatol.* 12, 1–9.
- Lee, J.K., Par, J.H., Park, S.H., Kim, H.S., Oh, H.Y., 2002. A non-radioisotopic endpoint for measurement of lymph node cell proliferation in a murine allergic contact dermatitis model, using bromodeoxyuridine immunohistochemistry. *J. Pharmacol. Toxicol. Methods* 48, 53–61.
- Manetz, T.S., Meade, B.J., 1999. Development of flow cytometry assay for the identification and differentiation of chemicals with the potential to elicit irritation, IgE-mediated, or T-cell-mediated hypersensitivity responses. *Toxicol. Sci.* 48, 206–217.
- Manetz, T.S., Pettit, D.A., Meade, B.J., 2001. The determination of draining lymph node cell cytokine mRNA levels in BALB/c mice following dermal sodium lauryl sulfate, dinitrofluorobenzene, and toluene diisocyanate exposure. *Toxicol. Appl. Pharmacol.* 171, 174–183.
- Nagai, H., Ueda, Y., Ochi, T., Hirano, Y., Tanaka, H., Inagaki, N., Kawada, K., 2000. Different role of IL-4 in the onset of hapten-induced contact hypersensitivity in BALB/c and C57BL/6 mice. *Br. J. Pharmacol.* 129, 299–306.
- Plitnick, L.M., Loveless, S.E., Ladics, G.S., Holsapple, M.P., Selgrade, M.J., Sailstad, D.M., Smialowicz, R.J., 2002. Cytokine profiling for chemical sensitizers: application of the ribonuclease protection assay and effect of dose. *Toxicol. Appl. Pharmacol.* 179, 145–154.
- Smith, C.K., Hotchkiss, S.A.M., 2001. Allergic Contact Dermatitis. Chemical and Metabolic Mechanisms. Taylor and Francis, London, New York.
- Takeyoshi, M., Yamasaki, K., Yakabe, Y., Takatsuki, M., Kimber, I., 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. *Toxicol. Lett.* 119, 203–208.
- Takeyoshi, M., Iida, K., Shiraishi, K., Hoshuyama, S., 2005. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. *J. Appl. Toxicol.* 25, 129–134.
- Takeyoshi, M., Noda, S., Yamasaki, K., Kimber, I., 2006. Advantage of using CBA/N strain mice in a non-radioisotopic modification of the local lymph node assay. *J. Appl. Toxicol.* 26, 5–9.
- Traidl, C., Jugert, F., Krieg, T., Merk, H., Hunzelmann, N., 1999. Inhibition of allergic contact dermatitis to DNCB but not to oxazolone in IL-4-deficient mice. *J. Invest. Dermatol.* 112, 476–482.
- Ulker, O.C., Atak, A., Ates, I., Karakaya, A., 2011. Evaluation of auricular lymph node cell lymphocyte proliferation and cytokine production as non-radioactive endpoints during murine contact allergy. *J. Immunol.* 8, 131–139.
- Ulker, O.C., Atak, A., Ates, I., Karakaya, A., 2013. Evaluation of non-radioactive endpoints of ex vivo local lymph node assay-BrdU to investigate select contact sensitizers. *J. Immunol.* 10, 1–8.
- Ulrich, P., Grenet, O., Bluemel, J., Vohr, H.W., Wiemann, C., Grundler, O., Suter, W., 2001. Cytokine expression profiles during murine contact allergy: T helper 2 cytokines are expressed irrespective of the type of contact allergen. *Arch. Toxicol.* 75, 470–479.
- Van Och, F.M., Van Loveren, H., De Jong, W.H., Vandebriel, R.J., 2002. Cytokine production induced by low-molecular-weight chemicals as a function of the stimulation index in a modified local lymph node assay: An approach to discriminate contact sensitizers from respiratory sensitizers. *Toxicol. Appl. Pharmacol.* 184, 46–56.
- Vandebriel, R.J., De Jong, W.H., Hendriks, J.J., Van Loveren, H., 2003. Impact of exposure duration by low molecular weight compounds on interferon- γ and interleukin-4 mRNA expression and production in the draining lymph nodes of mice. *Toxicology* 188, 1–13.