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Dose-response assessment of the dermal toxicity of Virginia Cedarwood Oil in F344/N rats and B6C3F1/N mice

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1. Introduction

Virginia cedarwood oil (hereafter referred to as cedarwood oil) is extracted from *Juniperus virginiana* trees by steam distillation (Ullmann's, 1985). Cedarwood oil is commonly found in fragrance formulations and production for this purpose exceeds 100,000 pounds per year (RIFM, 1974; Ullmann's, 1985). Cedrene, thujopsene, and cedrol are cedarwood oil components that are commonly isolated and often acetylated in the production of fragrance materials such as cedryl acetate and cedryl methylether (Ullman's, 1985). These extracts can be found in many cosmetic products (i.e., perfumes, lotions, soaps) that are often applied to most parts of the body, and may come into contact with the ocular and nasal mucosae. Frequent application of these products on a daily basis leads to long-term chronic dermal and inhalation exposure. In addition to its use as a fragrance ingredient, cedarwood oil alcohols and terpenes have been used as food additives for flavor enhancement or adjuvants, and are considered by the United States Food and Drug Administration (FDA) to be Generally Recognized as Safe (GRAS) (Ullmann's, 1985; USEPA, 2009). The use of cedarwood oil terpenes as food additives in 1987 was estimated to be 166,500 pounds (USEPA, 1993; FDA, 2002; Clydesdale, 1997). Cedarwood oil has been used as a pesticide in sunscreens, pet collars, and in animal bedding (USEPA, 2009). Cedarwood oil has also been investigated as a topical mosquito repellent as an alternative to DEET; however, it was found to be ineffective against the multiple mosquito species that were tested (Curtis *et al.*, 1987; Barnard, 1999).

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A 1981 to 1983 National Occupational Exposure Survey (NIOSH, 1990) reported widespread use of cedarwood oil in 7,990 occupational facilities, which included industrial settings, medical fields, and esthetics and beauty care (NIOSH, 1990). Although the cosmetics industry no longer reports concentration of use values to the FDA, the 1984 product formulation data indicate that the maximum use concentration ranges for Juniper Extract and Juniper Tar in cosmetics were 0.1% to 1%, and 1% to 5%, respectively (CIR, 2001; *Fed. Regist.*, 1992).

Animal studies testing the potential for skin irritation and sensitivity following acute exposure to low concentrations of cedarwood oil have resulted in conflicting data (RIFM, 1974; Roe and Field, 1965). The majority of these studies did not find sensitization or irritancy, but a few have shown that in rabbits, topical application of full strength (undiluted) cedarwood oil for 24 hours resulted in moderate skin irritation (RIFM, 1974). In humans, several short-term studies (24 hours to 4 days) have shown limited skin irritation effects following topical application of cedarwood oil at concentrations ranging from 0.2% to 20% (Frosch *et al.*, 1995; Fujii *et al.*, 1972; RIFM, 1974; Abifadel *et al.*, 1992). Acetyl cedrene, a common constituent of cedarwood oil, was also reported to have limited toxicity when evaluated in human irritation tests at concentrations up to 30% (Belsito *et al.*, 2013).

Disposition studies in rats with α -cedrene, a component of cedarwood oil, resulted in slow absorption with a $T_{\max}$ between 2.8 and 4.4 hours following oral gavage dosing (Hong *et al.*, 2013; Kim *et al.*, 2015). In female rats, $C_{\max}$ and AUC values increased proportionally to the dose, and α -cedrene was extensively distributed to tissues with tissue:plasma ratios greater than 1 for all tissues; the highest ratio was present in lipid (Kim *et al.*, 2015). The oral LD_{50} value for cedarwood oil is greater than 5 g/kg body weight in rats, and the dermal LD_{50} in rabbits is also greater than 5 g/kg (RIFM, 1974).

Due to the widespread and increasing human exposure to cedarwood oil and lack of overall toxicological information, the National Cancer Institute nominated cedarwood oil for toxicity studies by the National Toxicology Program. To address the lack of toxicological information following cedarwood oil exposure, 3-month dermal studies in male and female F344/N rats and B6C3F1/N mice were conducted. The concentrations used in these studies resulted in extensive skin lesions with no No-Observed-Effect-Level (NOEL) achieved in either the mice or the rats. Benchmark dose (BMD) modeling was performed to put these skin lesions, in addition to the final mean body weights, into perspective for risk assessment applications. However, due to the maximum effect level achieved for skin lesions in the mice at the lowest applied concentration, BMD modeling of the skin lesions could only be performed in the rats.

2. Materials and Methods

2.1 Chemicals

Virginia cedarwood oil was obtained from Texarome, Inc. (Leakey, TX) in a single lot. The chemical was identified as cedarwood oil using infrared (IR) and proton nuclear magnetic resonance (NMR) spectroscopy, and the purity was determined based on a chromatography profile of the major components obtained by gas chromatography (GC) with flame

ionization detection (FID). For purity, five compounds (α -cedrene, β -cedrene, thujopsene, cuparene, and cedrol) were selected as marker compounds and quantitated using standards obtained from Sigma-Aldrich Corporation (St. Louis, MO). Weight percent concentrations of the five selected marker compounds were determined to be α -cedrene, 27.01%; β -cedrene, 6.05%; thujopsene, 19.64%; cuparene, 0.96%; and cedrol, 21.83%. Ethanol (95%; USP Grade) was obtained from Spectrum Chemicals & Laboratory Products (Gardena, CA) in a single lot. The cedarwood oil dose formulations were stable under simulated animal room conditions for up to 3 hours and for at least 42 days when stored in amber glass containers with Teflon®-lined lids at room temperature.

2.2 Dose Selection

Due to the lack of human exposure level information in the literature, concentrations of 0% (vehicle control), 6.25%, 12.5%, 25%, 50%, and 100% Virginia cedarwood oil in 95% aqueous ethanol were selected for the 90 day dermal application study, because these concentrations would encompass the range of all possible percentages of cedarwood oil components that could be utilized in products. The dermal route of exposure was selected for these studies because the primary use of cedarwood oil is as a fragrance material in many personal products, as well as the anticipation that cedarwood oil could eventually replace DEET in insect repellents, and naphthalene in mothballs. An untreated control group was included in addition to the 0% vehicle control group, because the 100% cedarwood oil dosing solution was applied neat (undiluted) without the dosing vehicle (95% ethanol).

2.3 Study Design

Male and female F344/N rats and B6C3F1/N mice were obtained from Taconic Farms, Inc. (Germantown, NY) at 4 to 5 weeks of age, and were tail tattooed for identification. Following a quarantine period, treatment was initiated when the rats were 6 to 7 weeks old and the mice were 5 to 6 weeks old. The animals were housed individually and provided irradiated NTP-2000 open formula wafer feed (Zeigler Brothers, Inc., Gardners, PA) and water *ad libitum* for the duration of the study. A 12-hour light/dark cycle was maintained in the animal room, with at least 10 room air changes per hour. The animal room environment was maintained at $72 \pm 3^{\circ}\text{F}$ with relative humidity at $50 \pm 15\%$. Animal care and use was in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines. Additionally, studies were conducted according to federal good laboratory practice (GLP) regulations and specifications defined by the National Toxicology Program.

Each dose group consisted of 10 randomly assigned rats and mice of each sex. The dose groups consisted of 0% (vehicle control), 6.25%, 12.5%, 25%, 50%, or 100% (undiluted) cedarwood oil in 95% aqueous ethanol, as well as an untreated control for comparison of the 100% dose group. Dose formulations were applied 5 days per week for 14 weeks, at a volume of 0.5 mL/kg body weight (rats) or 2.0 mL/kg (mice), to a shaved dorsal area that

extended from posterior to the scapulae to the base of the tail. Equivalent dose ranges for mice and rats were approximately 125 to 2,000 mg/kg bodyweight and 31.25 to 500 mg/kg bodyweight, respectively. These dose formulations resulted in the mice receiving higher doses of cedarwood oil than rats with respect to the equivalent dose in mg/kg. Dosing was completed during a consistent time period in the morning. The animals were monitored twice daily for signs of moribundity and mortality. Body weights and clinical findings were recorded initially, weekly, and at the end of the studies.

2.4 Gross Pathology and Histology

All major organs/tissues and gross lesions were evaluated microscopically in the rats and mice; the heart, right kidney, liver, lung, right testis, and thymus were weighed. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except for the eyes which were fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned at a thickness of 4 to 6 μm , and stained with hematoxylin and eosin (H&E). Complete histopathological evaluations were performed on rats in the untreated control, vehicle control, and 100% groups and on mice in the untreated control, vehicle control, 50%, and 100% groups. The bone marrow, kidney (males only), liver, salivary gland (females only), skin at the site of application (SOA), and the thyroid gland were identified as target tissues and examined to a no-observed-effect-level in the rats. The bone marrow, liver, lymph nodes, skin at the SOA, and spleen of mice were examined in the surviving dosed groups. Where applicable, non-neoplastic lesions were graded for severity using standard procedures on the following scale: a score of 1 (minimal) represented a lesion not likely to have clinical significance; a score of 2 (mild) represented a lesion likely to be subclinical and/or to have minor functional significance; a score of 3 (moderate) represented a lesion likely to have clinical significance; and a score of 4 (marked) represented a lesion approaching maximal extent for the lesion in the organ. The diagnoses and severity grades of treatment-related lesions were established by a group of National Toxicology Program pathologists and underwent external peer review by a Pathology Working Group. Although other tissues were evaluated in these studies, the focus of this manuscript is on the primary dermal lesions and the BMD modeling (NTP, 2016).

2.5 Statistics

The vehicle control group was used to compare responses at applied concentrations up to 50%; whereas, the untreated control group was used to compare responses in the 100% group due to this group not containing any vehicle control. Additional comparisons were made for rat data between the untreated and vehicle control groups when there were lesion incidences in either control group. Differences between the control groups and their respective treatment groups were considered statistically significant if $p < 0.05$.

Fisher's exact test (Gart et al., 1979) was used for determination of significant incidences of lesions between dosed and untreated control or vehicle control animals. For continuous data, Jonckheere's test (Jonckheere, 1954) was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnnett's or Dunn's test). Organ and body weights, which are typically

normally distributed, were analyzed with the parametric multiple comparison procedures of Dunnett (1955) or Williams (1971, 1972). Implausible values were eliminated prior to statistical analysis, with identification of extreme values through the outlier test of Dixon and Massey (1957).

In rats, comparisons between the 100% (neat) group and the untreated control groups were made using a t-test to determine significant differences in organ and body weight data.

2.6 Benchmark dose modeling

Benchmark doses (BMD) and the lower 95% confidence limits (BMDL) were calculated using the Environmental Protection Agency Benchmark Dose Software (version 2.6.0.1 with BMDS Wizard 1.10; <http://www.epa.gov/ncea/bmds>). In the BMDS Wizard for continuous data with standard deviation, the final mean body weights and the doses of cedarwood oil administered dermally were used in the exponential M2, exponential M3, exponential M4, exponential M5, Hill, linear, power, polynomial 2°, polynomial 3°, and polynomial 4° models to determine the best-fit model to determine the best fit of the data. The non-neoplastic skin lesions incidences and the doses of cedarwood oil administered dermally were used in the Dichotomous BMDS Wizard with the gamma, logistic, log-logistic, dichotomous-Hill, probit, log-probit, Weibull, multistage 2°, multistage 3°, multistage 4°, and quantal-linear models to determine the best fit of the data. A goodness of fit p-value (GOF) of 0.2 was used to determine the best-fit model for identification of the most sensitive endpoints in male and female rats. When more complex models reduced to a simpler form as a result of one or more parameters reaching the defined parameter bound, only the data from the more parsimonious output models were reported in Tables 4 (e.g., the Weibull model can become the quantal-linear model). Where the data for models appear to be equivalent in Table 4, there were differences in digits that were not displayed in the BMDS Wizard output tables and thus both models were included in the table. The BMD for both the dichotomous and continuous data was defined as the dose that caused a 10% excess risk of the specified adverse effect over that observed in the appropriate control group.

3. Results

3.1 Survival and Body Weights

Groups of 10 male and 10 female mice and rats were administered 0% (untreated control), 0% (vehicle control), 6.25%, 12.5%, 25%, 50%, or 100% cedarwood oil in 95% ethanol by dermal application for 13 weeks. All of the rats survived until the end of the study, with the exception of two males in the 100% group that were euthanized due to severe skin lesions (Table 1). All of the male and female mice in the 100% group were euthanized at week 10 of exposure as a result of the severity of skin lesions at the SOA. This was followed by euthanasia of one male and one female in the 50% group in week 11 and one male in the 12.5% group in week 12 (Table 1). All other male and female mice survived until the end of the study. In both rats and mice, treatment related clinical observations in the skin at the SOA included dermal irritation, thickened skin, and ulceration. These findings were noted in most of the male rats at applied concentrations of 25% or more, in most of the female rats at applied concentrations of 12.5% or more, and in the majority of the mice at all applied

concentrations. Skin lesions were first noted on day 8 of cedarwood oil application in both rats and mice in the highest dose group.

The final mean body weights of the 100% male and female rats were significantly less than those of the respective control groups at the end of the study (13% and 10% less, respectively). Additionally, the mean body weight gain of the male rats from the 25% group was significantly less than the vehicle control group (Table 1). In the mice, the final mean body weights of the 25% and 50% male dose groups (10% and 14% less, respectively) and the 12.5%, 25%, and 50% female dose groups (12%, 19%, and 13% less, respectively) were significantly less than the vehicle control groups. This also corresponded to decreased mean body weight gains in comparison to the controls, in both the male and female mice at doses of 12.5% and above (Table 1).

3.2 Gross Pathology and Histopathology

Treatment-related gross lesions were observed mainly in the skin at the SOA in the majority of the rats and mice, and included irritation, thickened skin, and ulceration (Table 2). The time to first incidence decreased, while incidence and severity of epidermal ulcers increased with increasing dose in male and female rats and mice. Ulcerations presented earlier and were more severe in both sexes of the mice at all the doses tested (6.25% and above) (Tables 2 and 3) when compared to rats, where ulcerations were noted in both sexes at doses of 12.5% and greater, albeit with lower incidences and severities than what was seen in the mice (Tables 2 and 3).

Microscopically, dermal application of cedarwood oil resulted in a spectrum of skin lesions at the SOA. These skin lesions were morphologically similar between the two species and included epidermal hyperplasia, hyperkeratosis, and ulcers accompanied by chronic active inflammation, hyperplasia of the hair follicle and sebaceous gland, and dermal fibrosis (Figures 1 and 2). The incidences and severity of these lesions increased in a dose-dependent manner, with exacerbation of the effects in the mice (Table 3). With the exception of a few rats, both sexes of rats and mice exposed to 50% or 100% cedarwood oil had all of the skin lesions listed above. Following administration of 25% cedarwood oil, male and female rats had lower significant incidences of most skin lesions (with the largest decrease seen in the number of epidermal ulcers) when compared to mice. The decrease in the incidences of skin lesions in the rats extended down to the 12.5% dose group, where only 14% of the males and 37% of the females developed skin lesions. This is in stark contrast to the male and female mice that retained almost 100% incidence of all the skin lesions at doses of 12.5% and 25% cedarwood oil (Table 3). At 6.25% cedarwood oil, there were low incidences of minimal epidermal hyperplasia and chronic active inflammation in the male rats, and hyperplasia and hyperkeratosis of the epidermis in the female rats. Again, the mice appeared to be more sensitive to application of 6.25% cedarwood oil, with 74% of the male mice exhibiting significant incidences of most of the skin lesions apart from epidermal ulcers and dermal fibrosis. In the female mice at this dose, there were significant increased incidences of epidermal and sebaceous gland hyperplasia accompanied by chronic active inflammation, however, there were occurrences of the full spectrum of skin lesions at this dose.

The dose response in histology of the skin lesions is shown in Figures 1B - 1F and 2B - 2F. The normal mouse epidermis is thin consisting of one to two layers of epithelial cells (Figs. 1A and 2A). In comparison to normal skin, epidermal hyperplasia was characterized by increased thickness of the epidermis due to an increase in the number of epithelial cell layers (Figures 1B - 1F and 2B - 2F). The severity grades for epidermal hyperplasia were based on the following criteria: increase in the thickness to three epithelial cell layers was minimal, four cell layers was mild, five cell layers was moderate, or greater than five cell layers marked. Epidermal hyperplasia was generally accompanied by epidermal hyperkeratosis (orthokeratotic- and parakeratotic), which consisted of increased thickness of the epidermal keratin layer (Figs. 1D - 1F and 2C - 2F). Severity was subjectively based on the thickness of the keratin layer compared to the respective controls. Frequently, there were focal, dense accumulations of degenerate neutrophils within the keratin layers that were consistent with epidermal pustules (Figs. 1D and 2C). The most severe lesions of hyperkeratosis occurred as increased layers of keratin diffusely mixed with dense accumulations of neutrophils (Figs. 1E and 2E).

Epidermal ulcers were characterized by focal, complete loss of the epidermis, which was often associated with necrosis of the underlying superficial dermis and filling of the epidermal defect with degenerate and/or necrotic cellular debris and inflammatory cells (Figs. 1F and 2C, 2D and 2F). The severity of the ulcers was based on their size and the number of skin section areas that were affected.

Chronic active inflammation was characterized by infiltrates of neutrophils and macrophages that increased in number with increasing dose throughout the dermis, with infrequent extension into the hypodermis (Figs. 1C - 1D and 2B - 2F). Focal infiltrates of primary neutrophils mixed with low numbers of macrophages were occasionally found within the epidermis, hair follicles, and less frequently sebaceous glands. Dermal fibrosis was often associated with inflammatory cell infiltrates and was characterized by increased amounts of collagenous or fibrous connective tissue in the dermis (Figs. 1D and 1E and 2C - 2F).

Hair follicle hyperplasia was characterized by thickening of the follicular epithelium (that was an extension of the hyperplastic response in the epidermis), elongation of the follicles and increased numbers of cross-sectional hair follicle profiles in the subcutaneous layers of the skin (Figs. 1D - 1F and 2B - 2F). Severity grading of follicular epithelial hyperplasia (minimal to moderate) was subjectively based on the degree of thickening of the epithelium and numbers of follicular profiles within the subcutis. Sebaceous gland hyperplasia was characterized by prominently enlarged sebaceous glands due to increases in the size and number of alveolar cells (Figs. 1C, 1D, 1F and 2C, 2D and 2F).

In direct response to the inflammation and ulcerations at the site of cedarwood oil application, there were also histopathological changes in the hematopoietic system (bone marrow, spleen, and regional lymph nodes) that was most prominent in mice. The increased occurrences of these hematopoietic system lesions were considered to be secondary to the lesions observed in the skin at the SOA. Increased liver and kidney weights and increased incidences of kidney non-neoplastic lesions were also observed following dermal cedarwood oil exposure. Exposure-related decreases in absolute and relative thymus weights of rats and

mice were also seen, with the male mice also displaying thymocyte depletion in the cortex of the thymus. The thymus is the most sensitive of the lymphoid organs in response to stress (Everds, 2013), and the thymus effects observed in these studies are consistent with stress-related organ effects. These additional organ weight changes and lesions are discussed more extensively within the National Toxicology Program's Toxicity Report for Cedarwood Oil and are not the main focus of the present discussion (NTP, 2016). However, these data indicate that in addition to the SOA, there was a systemic inflammatory response to the skin lesions and the kidney and liver could also be potential targets for topical cedarwood oil exposure.

3.3 Benchmark Dose (BMD) Calculations

BMD modeling was performed on the final mean body weights for mice and rats (Table 4). The lower 95% confidence limits of the BMD (BMDL) values for the final mean body weights in female and male rats ranged from 44.7 to 71.8% and 20.2 to 52.2%, respectively. In the mice, the BMDL values for the final mean body weights ranged from 5.0E-14 to 13.9% and 13.3 to 27.3% in the female and male mice, respectively.

BMD modeling was also conducted on incidences of skin lesions induced by cedarwood oil (epidermal hyperplasia, hyperkeratosis, and ulcers; sebaceous gland hyperplasia; hair follicle hyperplasia; dermal fibrosis; chronic active inflammation; Table 5 and Supplemental Data). In female rats the most sensitive endpoint was epidermal hyperplasia BMDL values of 0.65 to 2.1%, followed by epidermal hyperkeratosis (1.0 to 4.4% cedarwood oil), and sebaceous gland hyperplasia (1.3 to 5.9% cedarwood oil). In male rats the most sensitive endpoints where models could be fit to the data were chronic active inflammation with BMDL values of 1.2 to 4.4% cedarwood oil, followed by epidermal hyperkeratosis (5.3 to 9.0% cedarwood oil), and dermal fibrosis (6.0 to 17.1% cedarwood oil). In male and female mice the lowest dose of cedarwood oil applied produced a maximum effect for most of the skin lesions, which was not suitable for BMD modeling.

4. Discussion

In the present study, three months of dermal exposure to cedarwood oil resulted in reduced survival, with the early termination of two male rats and all mice in the 100% dose groups. These rats and mice had extensive exposure related skin lesions at the site of application (SOA), as well as limited adverse systemic effects (NTP, 2016). Treatment-related lesions in the skin of rats and mice at the SOA included epidermal hyperplasia, hyperkeratosis, and ulcers, hair follicle and sebaceous gland hyperplasia, dermal fibrosis, and chronic active inflammation. Marked epidermal hyperplasia, epidermal hyperkeratosis, and ulcerations are indicative of a hyper-proliferative reaction that is consistent with chronic contact irritant dermatitis (Bruner 1991, Berardesca and Distant 1994). The mice appeared to be more sensitive to the dermal application of cedarwood oil than rats, indicated by reduced survival of the 100% dose group, reduced final mean body weights, decreased time to first lesions, and increased incidences and severities of skin lesions. The difference in response between the mice and rats is also demonstrated with lower 95% confidence limits of the BMD (BMDL) values for final mean body weights. However, the apparent increased sensitivity of

the mice is most likely attributable to the application of a greater amount of test-article that resulted in an overall higher equivalent mg/kg bodyweight dose.

Most data reported in the literature from cedarwood oil induced skin irritation or sensitization studies are conflicting and limited, due in part to the limited reporting of the doses applied and the low numbers of animals or humans evaluated. The majority of sensitization or irritation studies from acute cedarwood oil exposure in animals, including mice and guinea pigs, did not result in sensitization or irritation (CIR, 2001; Roe and Field, 1965). However, full strength cedarwood oil applied to the skin of rabbits for 24 hours under occlusive patches resulted in moderate skin irritation (RIFM, 1974). Similarly, data in humans is also conflicting. Skin irritation or sensitization was not observed up to several days following application of doses ranging from 1 to 20% cedarwood oil (Frosch et al., 1995; Fujii et al., 1972; Abifadel et al., 1992), whereas irritation and sensitization occurred within 24 hours in a few subjects where either 2 or 5% cedarwood oil was applied (Ishihara, 1977; Rudzki et al., 1976). These reported differences in cedarwood oil dermal sensitivity, in comparison to the present studies, might be due to the number of subjects evaluated, the length of exposure, application of a wider dose range, and in part, species differences in transdermal absorption rates (Godin and Tuitou, 2007; Bronaugh et al., 1982).

While the doses tested in these studies were not low enough to determine a no-observed-effect-level (NOEL), benchmark dose modeling (BMD) of the skin lesions identified epidermal hyperplasia as the most sensitive endpoint in female rats with BMDL levels of 0.65 to 2.1% cedarwood oil and chronic active inflammation as the most sensitive endpoint in male rats with BMDL levels of 1.2 to 4.4% cedarwood oil. Because the lowest applied concentration of cedarwood oil produced a maximum effect in male and female mice, most of the dermal lesions in the mice were not suitable for BMD modeling. However, BMD modeling was performed for the final mean body weights in the mice, which resulted in BMDL levels ranging from 5.0E-14 to 13.9% and 13.3 to 27.3% in the female and male mice, respectively. The Food and Drug Administration (FDA) no longer requires the cosmetics industry to report concentration of use values on cosmetic product labels; however, 1984 product formulation data indicate that the maximum use concentrations for Juniper extract and Juniper tar in cosmetics were 0.1 to 1% and 1 to 5%, respectively (CIR, 2001; *Fed. Regist.*, 1992). Although these reported data are not current, these levels appear to be consistent with products we have identified as currently available on the market (e.g., an insect repellent product marketed as all natural that contains 1.5% cedarwood oil). Given the reported data and what is available on the market, the BMDL levels determined for cedarwood oil in the present studies overlap with those that were permitted for use in cosmetics.

In addition to its use as a fragrance in cosmetics and household products, cedarwood oil is also used as a pesticide and its registration with Environmental Protection Agency (EPA) is no longer required. In the past, several insecticide formulations were registered with the EPA with cedarwood oil concentrations in these products ranging from 0.48% in ready to use liquids to 2 to 8% in wooden blocks (USEPA, 1993). These levels of cedarwood oil within insecticides also coincide with the BMDL levels determined for the present studies, which were 0.65 to 2.1% and 1.2 to 4.4% (approximately equivalent to 13 to 42 mg/kg and 24 to 48

mg/kg, respectively) cedarwood oil for the most sensitive endpoint of epidermal hyperplasia in female rats and chronic active inflammation in male rats, respectively. However, there are currently no established exposure limits, and no standards or guidelines set by the National Institute for Occupational Safety and Health (NIOSH) or the Occupational Safety and Health Administration (OSHA) for occupational exposure to or workplace allowable levels.

In conclusion, dermal application of cedarwood oil for three months resulted in exposure-related skin lesions in both sexes of rats and mice. Skin irritation resulted in a systemic inflammatory response that was mild in the rats in the higher dose groups and more pronounced in the mice in nearly all the dose groups, reflecting the increased severities of the skin lesions in the mice. BMD modeling of these skin lesions in the rats resulted in BMDL levels for cedarwood oil that overlap with previously reported use levels and indicate concern for human exposure. Sensitization is a key early event in the development of allergic contact dermatitis and is a parameter currently used in both hazard and risk assessments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Dermal cedarwood oil exposure results in extensive skin lesions at the application site in rodents.
- Benchmark dose modelling (BMD) was performed for these skin lesions in rats.
- Calculated BMD levels coincide with reported use levels in cosmetics and household products.
- BMD levels calculated here can be used in human risk assessment of cedarwood oil.

Figure 1.

Hematoxylin and Eosin stained histologic sections showing the histopathology of lesions in the skin (site of application) from F344/N rats dermally administered 0%, 6.25%, 12.5%, 25%, 50% or 100% Virginia cedarwood oil for 3 months. **A.** Normal skin, vehicle control (95% ethanol) female. The epidermis (arrowheads) is composed of a 1 to 2 layers of epithelial cell; hair follicles (short arrows); sebaceous glands (sg); dermis (de); subcutaneous tissue (sc). **B.** Minimal epidermal hyperplasia (thickening) of the epidermis (arrowheads). **C.** Minimal hyperkeratosis (arrows); minimal sebaceous gland hyperplasia. Note that there is also mild epidermal hyperplasia. **D.** Moderate hair follicle hyperplasia (short arrows); moderate sebaceous gland hyperplasia (sg). Note that there is also moderate epidermal hyperplasia and also infiltrates of inflammatory cells within the dermis. **E.** Marked hyperkeratosis (arrows); marked hair follicle hyperplasia (short arrows); marked sebaceous gland hyperplasia. Note that there is also marked epidermal hyperplasia and marked fibrosis

throughout the dermis (de). **F.** Marked hyperkeratosis (arrows); focal ulcers (ul); moderate sebaceous gland hyperplasia. All images, 5x original objective.

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Figure 2.

Hematoxylin and Eosin stained histologic sections showing the histopathology of lesions in the skin (site of application) from B6C3F1 mice dermally administered 0%, 6.25%, 12.5%, 25%, 50% or 100% Virginia cedarwood oil for 3 months. **A.** Normal skin, vehicle control (95% ethanol) female. Epidermis (arrowheads); dermis (de); subcutaneous tissue (sc). **B.** Mild epidermal hyperplasia (thickening) of the epidermis (arrowheads) and mild hair follicle hyperplasia (short arrow). **C, D, E & F.** Moderate epidermal hyperplasia (arrowhead) and hyperkeratosis (arrows); moderate hair follicle hyperplasia (short arrow); mild sebaceous gland hyperplasia (sg); moderate dermal (de) fibrosis; focal ulcer (ul). All images, 5x original objective.

Table 1

Survival and body weights of F344/N Rats and B6C3F1/N Mice in the 90 day dermal study of cedarwood oil.

Dose (%)	Survival ^a	Mean bw (g)			Final weight relative to controls (%) ^b
		Initial	Final	Change	
Female Rats					
Untreated Control	10/10	102 ± 1 ^c	200 ± 4	98 ± 4	
Vehicle Control	10/10	103 ± 2	197 ± 2	94 ± 2	
6.25	10/10	102 ± 2	199 ± 4	97 ± 3	101
12.5	10/10	103 ± 1	194 ± 5	91 ± 4	98
25	10/10	102 ± 1	193 ± 4	91 ± 3	98
50	10/10	102 ± 1	182 ± 4 [*]	80 ± 3 ^{**}	92
100	10/10	102 ± 2	179 ± 3 ^{††}	78 ± 3 ^{††}	90
Male Rats					
Untreated Control	10/10	113 ± 2	323 ± 4	209 ± 3	
Vehicle Control	10/10	114 ± 2	329 ± 4	215 ± 4	
6.25	10/10	114 ± 2	318 ± 5	204 ± 4	97
12.5	10/10	113 ± 2	316 ± 6	203 ± 5	96
25	10/10	114 ± 2	308 ± 10	195 ± 8 [*]	94
50	10/10	113 ± 2	301 ± 8 [*]	188 ± 8 ^{**}	92
100	8/10 ^d	115 ± 2	279 ± 7 ^{††}	166 ± 7 ^{††}	87
Female Mice					
Untreated Control	10/10	18.1 ± 0.2	34.9 ± 0.9	16.8 ± 0.9	
Vehicle Control	10/10	17.8 ± 0.3	34.7 ± 1.1	16.9 ± 1.0	
6.25	10/10	18.0 ± 0.4	34.0 ± 0.9	16.1 ± 0.7	98
12.5	10/10	17.9 ± 0.2	30.5 ± 0.7 ^{**}	12.6 ± 0.7 ^{**}	88
25	10/10	17.8 ± 0.5	28.1 ± 0.6 ^{**}	10.3 ± 0.6 ^{**}	81
50	9/10 ^e	18.3 ± 0.4	30.2 ± 0.4 ^{**}	11.9 ± 0.5 ^{**}	87
100	0/10 ^f	---	---	---	---
Male Mice					
Untreated Control	10/10	21.9 ± 0.4	39.7 ± 1.3	17.8 ± 1.1	
Vehicle Control	10/10	21.7 ± 0.3	36.9 ± 0.8	15.2 ± 0.6	
6.25	10/10	21.7 ± 0.3	36.0 ± 0.6	14.4 ± 0.5	98
12.5	9/10 ^d	22.1 ± 0.4	35.1 ± 1.1	13.1 ± 0.9 [*]	95
25	10/10	22.1 ± 0.5	33.1 ± 0.9 ^{**}	11.0 ± 0.7 ^{**}	90

Dose (%)	Survival ^a	Mean bw (g)			Final weight relative to controls (%) ^b
		Initial	Final	Change	
50	9/10 ^e	22.1 ± 0.4	31.9 ± 0.7 ^{**}	9.7 ± 0.6 ^{**}	86
100	0/10 ^f	---	---	---	---

* Significantly different (P 0.05) from the vehicle control group by Williams' or Dunnett's test

** P 0.01

^{††} Significantly different (P 0.05) from the untreated control group by a *t*-test

^a Number of animals surviving at 14 weeks/number initially in group

^b Dosed groups are compared to the vehicle control groups, except 100% groups are compared to the untreated control groups

^c Weights and weight changes are given as mean ± standard error

^d Week of death: 12

^e Week of death: 11

^f Due to severity of skin lesions, all 100% mice were euthanized during week 10

Table 2

Time to first incidence of clinical observations at the skin site of observation in F344/N rats and B6C3F1/N Mice in the 90 day dermal study of cedarwood oil.

Endpoint	0%	6.25%	12.5%	25%	50%	100%
Female Rats						
Irritation			Day 50	Day 22	Day 15	Day 8
Thickened			Day 43	Day 15	Day 15	Day 15
Ulcer			Day 63	Day 15	Day 15	Day 15
Ulcer (multiple) ^a			Day 85	Day 29	Day 15	Day 15
Male Rats						
Irritation				Day 8	Day 8	Day 8
Thickened			Day 71	Day 15	Day 8	Day 8
Ulcer				Day 15	Day 15	Day 15
Ulcer (multiple)				Day 36	Day 15	Day 15
Female Mice						
Irritation		Day 36	Day 8	Day 8	Day 8	Day 8
Thickened		Day 36	Day 22	Day 15	Day 8	Day 8
Ulcer		Day 36	Day 36	Day 29	Day 22	Day 22
Ulcer (multiple)			Day 93	Day 36	Day 22	Day 22
Male Mice						
Irritation		Day 15	Day 8	Day 8	Day 8	Day 8
Thickened		Day 36	Day 29	Day 22	Day 8	Day 8
Ulcer			Day 36	Day 36	Day 22	Day 22
Ulcer (multiple)			Day 64	Day 43	Day 22	Day 22

^a Number of multiple ulcers (> 1 ulcer) present in the same animal

Table 3

Incidence and severity of non-neoplastic lesions at the Skin Site of Application in F344/N Rats and B6C3F1/N Mice in the 90 day dermal study of cedarwood oil.

Dose	Epidermal hyperplasia	Epidermal hyperkeratosis	Epidermal Ulcer	Chronic Active Inflammation	Hair follicle hyperplasia	Sebaceous gland hyperplasia	Dermal Fibrosis
Female Rats							
0 (UC)	0	0	0	0	0	0	0
0 (VC)	0	0	0	0	0	0	0
6.25	4 [*]	1	0	0	0	0	0
12.5	7 ^{**}	5 [*]	2.0	7 ^{**}	1	4 [*]	1
25	10 ^{**}	10 ^{**}	3.0	10 ^{**}	9 ^{**}	9 ^{**}	7 ^{**}
50	10 ^{**}	10 ^{**}	2.3	10 ^{**}	10 ^{**}	10 ^{**}	8 ^{**}
100	10 ^{††}	10 ^{††}	3.1	10 ^{††}	10 ^{††}	10 ^{††}	10 ^{††}
Male Rats							
0 (UC)	0	0	0	0	0	0	0
0 (VC)	0	0	0	0	0	0	0
6.25	2	0	0	1	0	0	0
12.5	4 [*]	1	1.0	4 [*]	0	0	0
25	10 ^{**}	7 ^{**}	3.0	9 ^{**}	9 ^{**}	9 ^{**}	7 ^{**}
50	10 ^{**}	10 ^{**}	2.6	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
100	9 ^{††}	10 ^{††}	3.0	10 ^{††}	9 ^{††}	10 ^{††}	10 ^{††}
Female Mice							
0 (UC)	0	0	0	0	0	1	0
0 (VC)	0	0	0	0	0	0	0
6.25	10 ^{**}	2	3.0	10 ^{**}	3	4 [*]	2
12.5	10 ^{**}	6 ^{**}	3.7	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
25	10 ^{**}	9 ^{**}	4.0	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
50	10 ^{**}	10 ^{**}	4.0	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}

Dose	Epidermal hyperplasia	Epidermal hyperkeratosis	Epidermal Ulcer	Chronic Active Inflammation	Hair follicle hyperplasia	Sebaceous gland hyperplasia	Dermal Fibrosis
100	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺
Male Mice							
0 (UC)	0	0	0	0	0	0	0
0 (VC) ^b	0	0	0	0	0	0	0
6.25	10 ^{**}	7 ^{**}	2	10 ^{**}	10 ^{**}	10 ^{**}	3
12.5	10 ^{**}	10 ^{**}	8 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
25	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
50	10 ^{**}	10 ^{**}	9 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}	10 ^{**}
100	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺	10 ⁺⁺⁺

* Significantly different (P 0.05) from the vehicle control group by Fisher's exact test

** P 0.01

⁺⁺⁺ Significantly different (P 0.01) from the untreated control group by Fisher's exact test

^a Average severity grade of lesions in affected animals: 1=minimal, 2=mild, 3=moderate, 4=marked

^b Number examined n=9, for all other endpoints n=10

Table 4

Benchmark dose modeling (% cedarwood) of the final mean bodyweights of male and female F344 rats and B6C3F1/N mice dermally administered cedarwood oil for 13 weeks.

Species	Sex	Model	AIC ^a	GOF ^b	BMD _{10RD} ^c	BMDL _{10RD} ^d
F344N Rats	Female	Exponential (M2)	360.5	0.575	95.3	69.9
		Exponential (M4)	361.5	0.601	89.1	44.7
		Exponential (M5)	362.3	0.699		
		Linear	360.7	0.547	96.2	71.8
		Polynomial 2 ^b	360.7	0.547	96.2	71.8
	Male	Exponential (M2)	417.1	0.307	68.3	49.6
		Exponential (M4)	415.8	0.694	49.6	22.2
		Exponential (M5)	415.8	0.694	49.6	20.2
		Hill	417.4	0.524	53.5	22.3
		Power	417.4	0.282	70.2	52.2
		Linear	417.4	0.282	70.2	52.2
B6C3F1/N Mice	Female	Exponential (M2)	296.7	0.762	31.7	11.4
		Exponential (M3)	296.7	0.762	31.7	11.4
		Exponential (M4)	298.0	0.796	9.06	0.0273
		Exponential (M5)	299.7	0.695	11.2	0.0497
		Hill	299.7	0.686	11.0	5.00E-14
		Power	296.8	0.750	34.1	13.9
		Linear	296.8	0.750	34.1	13.9
	Male	Exponential (M2)	141.2	0.656	34.7	25.8
		Exponential (M4)	141.9	0.845	25.8	13.3
		Exponential (M5)	143.6	0.835	24.7	13.7
		Hill	143.6	0.769	25.2	13.4
		Linear	141.4	0.597	35.8	27.3

^a AIC – Akaike information criterion

^b GOF – goodness of fit p-value

^c BMD – benchmark dose (% cedarwood oil)

^d BMDL – lower 95% confidence limit of benchmark dose (% cedarwood oil)

Table 5

Benchmark dose modeling best-fit models (% cedarwood) of the most sensitive non-neoplastic dermal lesions in male and female F344 rats dermally administered cedarwood oil for 13 weeks.

Sex	Endpoint	Model	AIC ^a	GOF ^b	BMD _{10Pct} ^c	BMDL _{10Pct} ^d
Female	Hyperplasia, Epidermis	Gamma	30.3	0.976	2.9	0.70
		Logistic	31.5	0.849	2.1	2.1
		LogLogistic	31.1	0.902	3.6	1.30
		Dichotomous-Hill	31.1	0.902	3.6	1.30
		Probit	31.1	0.893	3.4	2.0
		LogProbit	30.7	0.943	3.6	1.22
		Weibull	30.1	0.986	2.6	0.73
		Multistage 2°	30.0	0.992	1.4	0.74
		Multistage 3°	29.8	0.999	1.6	0.76
		Multistage 4°	29.7	1.000	2.1	0.75
		Quantal-Linear	29.6	0.944	1.0	0.65
	Hyperkeratosis, Epidermis	Gamma	24.9	0.983	6.8	3.8
		Logistic	24.7	0.995	7.1	4.3
		LogLogistic	25.6	0.930	7.1	4.4
		Dichotomous-Hill	25.6	0.930	7.1	4.4
		Probit	24.5	0.999	6.7	4.0
		LogProbit	25.4	0.947	6.8	4.4
		Weibull	24.5	1.000	6.6	3.5
		Multistage 2°	23.8	0.967	4.6	2.3
		Multistage 3°	24.4	1.000	6.4	2.3
		Multistage 4°	26.4	1.000	6.3	2.0
		Quantal-Linear	30.5	0.347	1.5	1.0
	Hyperplasia, Sebaceous gland	Gamma	24.8	0.966	8.6	5.2
		Logistic	26.2	0.799	8.4	5.3
		LogLogistic	24.7	0.975	8.8	5.8
		Dichotomous-Hill	24.7	0.975	8.8	5.8
		Probit	25.9	0.833	8.4	5.1
		LogProbit	24.4	0.988	8.8	5.9
		Weibull	25.5	0.910	7.9	4.3
		Multistage 2°	24.6	0.918	5.8	3.2
		Multistage 3°	25.5	0.882	8.3	3.4
		Multistage 4°	25.5	0.882	8.3	3.1
		Quantal-Linear	32.1	0.265	1.9	1.3
Male	Chronic Active Inflammation	Gamma	30.5	1.000	6.5	3.1
		Logistic	31.1	0.980	7.0	4.4
		LogLogistic	30.9	0.987	7.0	4.1

Sex	Endpoint	Model	AIC ^a	GOF ^b	BMD _{10Pct} ^c	BMDL _{10Pct} ^d
		Dichotomous-Hill	30.9	0.987	7.0	4.1
		Probit	30.9	0.989	6.7	4.2
		LogProbit	30.8	0.991	6.8	4.1
		Weibull	30.5	1.000	6.2	2.8
		Multistage 2°	28.6	1.000	5.6	2.3
		Multistage 3°	30.5	1.000	6.0	2.0
		Multistage 4°	32.5	1.000	6.0	1.9
		Quantal-Linear	33.8	0.528	1.8	1.2
	Hyperkeratosis, Epidermis	Gamma	22.8	1.000	12.8	8.3
		Logistic	23.2	0.991	13.8	9.0
		LogLogistic	23.1	0.993	13.1	8.8
		Dichotomous-Hill	23.1	0.993	13.1	8.8
		Probit	23.0	0.998	13.3	8.5
		LogProbit	22.9	0.999	12.8	8.8
		Weibull	22.9	0.999	13.0	7.8
		Multistage 2°	23.6	0.865	8.2	5.3
		Multistage 3°	21.2	0.998	11.4	6.7
		Multistage 4°	22.9	0.999	13.1	6.7
	Fibrosis, Dermis	Gamma	14.8	1.000	16.2	11.6
		Logistic	16.2	1.000	23.1	12.7
		LogLogistic	14.2	1.000	21.1	12.3
		Dichotomous-Hill	14.2	1.000	21.1	17.1
		Probit	16.2	1.000	21.4	12.2
		LogProbit	16.2	1.000	20.4	12.1
		Multistage 2°	20.9	0.568	8.6	6.0
		Multistage 3°	17.2	0.886	12.0	8.5
		Multistage 4°	15.7	0.976	14.0	10.1

^aAIC – Akaike information criterion

^bGOF – goodness of fit p-value

^cBMD – benchmark dose (% cedarwood oil)

^dBMDL – lower 95% confidence limit of benchmark dose (% cedarwood oil)