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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

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Abstract

The essential oil extracts of western juniper oil (*Juniperus occidentalis*) and Port-Orford-cedar oil (*Chamaecyparis lawsoniana*) were evaluated for possible dermal toxic effects on mice and rabbits. Mice were tested for their response to both extracts utilizing a local lymph node assay. Western juniper oil extract at 0.5% and 5% concentrations did not show a stimulation index (SI) greater than normal (3.0); however, a 50% concentration did show a positive response at 3.3. Port-Orford-cedar oil extract did not show a positive response at concentrations of 0.5%, 5% or 50%. An acute dermal irritation study using rabbits had a primary irritation index (PII) of 3.3 with 100% Port-Orford-cedar oil extract. This was reduced to a PII of 0.625 when diluted 1:1 with olive oil. Undiluted western juniper oil extract had a PII score of 2.7. While a 5.0% solution had a PII score of 0.3, a 0.5% solution of western juniper oil was a non-irritant. It would appear that animals bedded on wood shavings have contact with essential oils at concentrations far less than the 2% maximum by weight obtained by steam distillation extraction. These concentrations did not elicit a hypersensitivity response.

Keywords: Western juniper (*Juniperus occidentalis*); Port-Orford-cedar (*Chamaecyparis lawsoniana*); Acute toxicity; Shavings; Essential oil extracts; Horses; Dogs; Laboratory animals

1. Introduction

Sawmills produce a large amount of waste. It is not uncommon for 40–50% of a log by weight to become

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processing residue even for commodity products, such as dimensional lumber. An even higher percentage of waste is generated by the limited number of manufacturers who process logs into high value-added products, such as millwork (e.g., flooring and paneling) and sporting equipment (e.g., wooden arrow shafts). Manufacturing residues of many aromatic cedars, such as western juniper (*Juniperus occidentalis*) and Port-Orford-cedar (*Chamaecyparis lawsoniana*), can be distilled for their essential oils, thereby extracting value from what traditionally has been discarded or burned and improving processing economics.

An added impetus to investigate valued-added uses for western juniper is because this species, similar to other *Juniperus* spp. in the western United States, has greatly increased in acreage and density over the last century causing loss of site productivity, decrease in forage, loss of wildlife habitat and overall decrease in biodiversity (Gedney et al., 1999; Miller and Wigand, 1994; Miller and Rose, 1995; Miller et al., 2000). The costs of removing western juniper to improve rangeland and watershed conditions are high compared to the value of the land. Given this situation, many landowners and land managers are highly interested in investigating potential markets for western juniper products to partially defray costs of management (Swan, 2001). The need for additional toxicity studies was identified by the wood products industry because unresolved questions were being raised about the use of western juniper, Port-Orford-cedar and other aromatic cedar products for horse, dog and laboratory animal bedding, as well as for fragrance products and topical applications for humans.

Few studies exist on juniper woods or their extracts (Gross and Ezerietis, 2003; Meding et al., 1996). There are no toxicological studies on western juniper (*J. occidentalis*). A pharmacological screening of different *Juniperus oxycedrus L*. extracts found low acute toxicity and significant anti-inflammatory and analgesic activity as well as inhibition of rat paw edema induced by carrageenin (Moreno et al., 1998). *Juniperus communis* L. “berries” have been found to have a variety of pharmacodynamic effects including diuretic, carminative, antiseptic, abortive and anti-diabetic activity (de Medina et al., 1993). In addition, antitumor activities were found with a crude extract of *Juniperus chinensis* leaves (Ali et al., 1996). *J. communis* wood was tested for its use as an implant material in rabbits with concurrent toxicity studies on both oral and intravenous administrations. It was found that the low concentrations of the oil that would be released were tolerated without any detrimental effects (Gross and Ezerietis, 2003). An acute dermal LD50 for juniper berry oil in rabbits has been reported >5 g/kg (final report in the safety assessment of *J. communis* extract, *J. oxycedrus* extract, *J. oxycedrus* tar, *J. phoenicea* extract and *J. virginiana* extract, 2001). Oral gavage of common juniper needles (*J. communis*) caused abortion in late term pregnancies similar to pine needle induced abortion (Gardner et al., 1998). In a study of multiple juniper species extracts used in fragrance and biological additives in cosmetic formulations, there was little toxicity of the oil or tar in animals. Irritant effects on skin were not found with the oils; however, there was some evidence of sensitization to the tar (final report in the safety assessment of *J. communis* extract, *Juniperus oxycedrus* extract, *Juniperus oxycedrus* tar, *Juniperus phoenicea* extract and *Juniperus virginiana* extract, 2001). A juniper (*Juniperus* sp.) oil-based phytomedicine was tested for nephrotoxicity in Sprague–Dawley rats by oral administration of varying doses and all were found to be non-toxic (Schilcher and Leuschner, 1997). No studies could be found on the skin irritation or possible hypersensitizing effects of western juniper oil.

Toxicity differs between the aromatic cedar species (Hausen, 1981; Mitchel and Rook, 1979; Ohmann, 1984; Woods and Calnan, 1976). Most literature focuses on western-red-cedar wood (*Thuja plicata)* as an allergen in occupational asthma (Horne et al., 2000; Lin et al., 1996; Noertjojo et al., 1996). Few studies exist on Port-Orford-cedar (*C. awsoniana*). Commercial products of Port-Orford-cedar oil for use in pet care products to repel fleas and ticks are available (Rose City Archery Inc., Myrtle Point, OR). In two pilot studies at Oregon State University, no toxicity was found in dogs and horses bedded for 6.5 months and for 8 months, respectively, on western juniper shavings (Blythe et al., 2001).

This study on western juniper oil (*J. occidentalis*) and Port-Orford-cedar oil (*C. lawsoniana*) extracts utilizing local lymph node and acute dermal irritation assays was specifically undertaken to define potential toxicity of their essential oils. The hypothesis tested was that the application of the oil extracts to the dermis at levels found in shavings would not cause inflammation or skin pathology. Essential oils from western juniper

and Port-Orford-cedar were tested for their capacity to induce a hypersensitivity response in mice as measured by the proliferation of lymphocytes in the local draining lymph nodes and in a acute dermal irritation study in rabbits.

2. Materials and methods

2.1. Extraction and analyses

Steam distilled essential oils were prepared from western juniper heartwood shavings from live trees harvested in Eastern Oregon and Port-Orford-cedar wood shavings from standing dead and down logs collected in Coos County, Oregon using protocols previously described (Tucker et al., 2000 and Adams, 1987) (oil extract obtained from western juniper supplied from Karchesy Laboratory, College of Forestry, Oregon State University, and Port-Orford-cedar from Rose City Archery Inc., Myrtle Point, OR). The extracted oils were then analyzed by GC/MS as described by Tucker et al. (2000) and Adams (1987) to determine and reaffirm presence of the major chemical components. Mass spectra were recorded with 5970 mass selective detector (MSD) (Hewlett-Packard, Palo Alto, CA) coupled to a 5890 gas chromatograph (GC) (Hewlett-Packard, Palo Alto, CA) using a DB-5 column (100 m) for western juniper oil and a 50 m × 0.2 mm fused silica column (Hewlett-Packard, Palo Alto, CA) coated with 0.33 μm FFAP (crosslinked) for Port-Orford-cedar oil. The GC was operated under the following conditions: injector temperature at 250 °C, oven temperature programmed to 60 °C and held for 1 min and progressively to 115 °C at 2.5 °C/min, 210 °C at 1 °C/min and held for 30 min; the injection size was 1 ml split 1:10. The MSD ei was operated under the following conditions: electron impact source 70 eV, 250 °C. Identification of peaks was made by retention indices and library searches of the GC/MS instrument library supplemented with searches of the National Institute of Standards and Technology (NIST) (Boulder, CO), and Wiley libraries (Wiley Publishers, Hoboken, NJ). The components of both oils are reported in Fig. 1 and Table 1, respectively.

Table 1
local lymph node assay results for western juniper oil (*J. occidentalis*) and Port-Orford-cedar oil (*Chamaecyparis lawsoniana*) extract toxicity study

Constituent	±S.D. (%)
α-Pinene	6.53 ± 0.02
Limonene	2.69 ± 1.07
Fenchone	4.67 ± 0.18
Camphor	5.94 ± 1.05
α-Fenchol	5.51 ± 1.06
α-Terpineol	14.33 ± 5.80
α-Muurolene	4.23 ± 1.56
δ-Cadinene	8.17 ± 1.75
τ-Cadinol	3.42 ± 1.18
α-Cadinol	5.30 ± 1.77

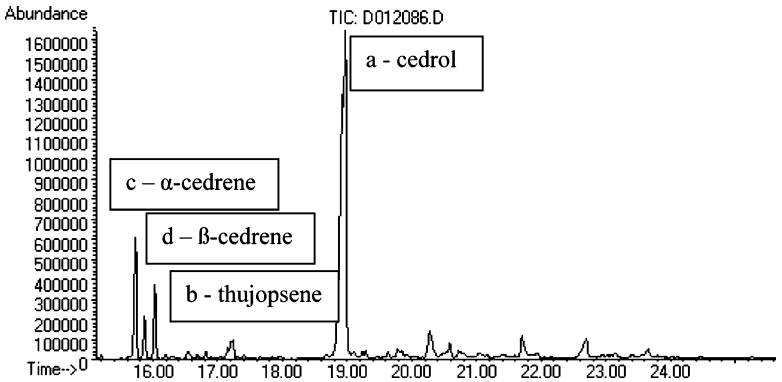

Fig. 1. GC/MS chromatogram of the oil extract from western juniper (*J. occidentalis)* heartwood and the percentage composition of the major components on a dry weight basis. Cedrol (a) was 38.9%; thujopsene (b) was 18.9%; α-cedrene (c) was 8.8% and β-cedrene (d) was 2.6%. The relative percent of yield of components (sum × percent) on a dry matter basis was 1.68%.

2.2. Animals

The local lymph node assay to test an oil's capacity to induce a hypersensitivity response as measured by the proliferation of lymphocytes in the local draining lymph nodes was conducted in mice for both western juniper oil and Port-Orford-cedar oil extracts. Two groups of 25 nine-week-old female CBA/J mice from Jackson Laboratories (Bar Harbor, ME) were selected for either the western juniper or Port-Orford-cedar extract study. The number used is the minimum number recommended (NIH Publication No. 99-4494, 1999). Only mice considered suitable for use were placed on the study. Prior to treatment initiation, all mice were weighted. The weight ranges were from 19 to 24 g. The mice were assigned to treatment groups using a computer-generated randomization method based on body weight. Mice were given identification numbers and identified by tail marks. Mice were housed (grouped five per cage) in compliance with the National Research Council "Guide for the Care and Use of Laboratory Animals". Calvert Preclinical Services Inc. (Olyphant, PA) is a USDA registered and fully accredited AAALAC facility. The animal room environment was controlled (target conditions: temperature 18–26 °C, relative humidity 30–70% and a 12 h artificial light/dark cycle). Temperatures and relative humidity were monitored daily. All animals had access to Certified Rodent Diet #7012C (Harlan Teklad, Indianapolis, IN) or equivalent ad libitum, unless otherwise specified. The lot numbers and specifications of each lot of all animals used are archived at Calvert Preclinical Services Inc.

The rabbit is a standard species used in dermal irritation studies and is acceptable to regulatory agencies. The number of animals used in this study was the minimum number necessary to properly perform this type of study (Gad, 1994). Six male and six female 20- and 24-week-old New Zealand White rabbits (HM:(NZW)fBR) from Covance (Provinceton, NJ) were used for testing each oil extract. Prior to testing, each rabbit was assessed as to their general health and acclimated/quarantined for a minimum of 5 days. Rabbits were placed on the study based upon sex, body weight, and apparent good health. All rabbits were housed individually and identified by ear tag numbers. The housing environment was the same as described above for the mice. All rabbits had access to Certified Rabbit Diet (Harlan Teklad, Indianapolis, IN) ad libitum. Water was provided to the animals in all studies ad libitum. Periodic analyses of the water are performed and the results are archived at Calvert Preclinical Services Inc. There are no known contaminants in the diet or water which at the levels detected would be expected to interfere with the purpose, conduct or outcome of the study.

2.3. Local lymph node assay

The mice were weighed on days 1 and 6. Groups of five mice were treated with 25 μl on the dorsal surface of each of the ears once per day for 3 days with either the vehicle, olive oil or the test article, western juniper or Port-Orford-cedar extracts, at concentrations of 0.5%, 5% and 50% or the positive control, 0.1% dinitrochlorobenzene (DNCB) in dimethyl sulfoxide (DMSO). On day 6, the mice were injected with 20 μCi of ^{3}H-thymidine. Five hours later, the mice were euthanized with CO_2 and the draining auricular lymph nodes were removed. The lymph node cells were precipitated with 5% trichloroacetic acid (TCA) and the pellets counted in a ß-scintillation counter to determine incorporation of the ^{3}H-thymidine. The mean decays per min (DPM) for each group was determined. Increases in ^{3}H-thymidine incorporation relative to vehicle-treated control were derived for each group and recorded as stimulation indices (SI). The criterion for a positive response is that one or more concentrations of a test article elicit a three-fold or greater increase in isotope incorporation relative to the vehicle control.

2.4. Acute dermal irritation assay

Within 24 h before the test, the fur was removed from the dorsal area of the trunk of each rabbit, being careful to avoid abrading the skin. In the first set of dermal irritation experiments utilizing the 20-week-old rabbits, an undiluted Port-Orford-cedar extract or western juniper extract was administered once (0.5 ml/site) on the clipped skin of two rabbits. The extract was applied to a small area of skin (~5 cm × 5 cm) and covered with a gauze patch. The patch was held in contact with the skin with a sheet of rubber dam. The trunk of the animal was wrapped with an elastic bandage dressing which was held in place with non-irritating tape

for the duration of the exposure period. Access by the animal to the patch and resultant ingestion/inhalation of the test article was prevented.

At the end of the 4 h exposure period, residual extract was removed using gauze and water without altering the existing response or the integrity of the epidermis. Each site was unwrapped and scored according to a technique described by Draize (1959). The scoring system examined the skin for the presence of erythema and edema. The former was graded as 0 for no erythema, with erythema scores of 1 for very slight, 2 for well defined, 3 for moderate to severe and 4 for severe to eschar formation. Edema was scored in a similar manner with 0 indicating none, 1 very slight, 2 slight, 3 moderate and 4 severe. A score for each animal was determined using the immediate, 24, 48 and 72 h observations for calculations and dividing by four. The primary irritation index (PII) is the sum of the scores for all of the animal scores that is divided by 6. The PII is considered slight if less than 2, moderate if between 2 and 5 or severe if greater than 5. Due to moderate to severe erythema and slight edema recorded in the first two rabbits administered the Port-Orford-cedar extract, the extract was diluted with olive oil (1:1) and applied in a similar manner to the remaining four rabbits while the western juniper concentration remained undiluted. In a second set of experiments, using three female and three male 24-week-old rabbits, four intact skin sites per animal received either 5.0% or 0.5% concentrations of western juniper extract or Port-Orford-cedar extract in olive oil. The application and observation times were identical to those described above. Body weights were recorded at the beginning and termination of the study. All animals were euthanized by an overdose of Euthasol (Virbac, Fort Worth, TX) following experiment termination.

2.5. Statistical analysis

Evaluation of equality of means of the data from the local lymph node assay was made by a one way analysis of variance using the *F* distribution to assess statistical significance using Systat 9.01 (SPSS Inc., Chicago, IL). If statistically significant differences between the means were found, a Dunnett's test was used to determine the degree of significance from control means. The design of the acute dermal irritation study is such that statistical analysis was not necessary using the Draize Evaluation (Draize, 1959).

3. Results and discussion

Fig. 1 and Table 1 illustrate the major components of western juniper oil and Port-Orford-cedar oil extracts. The analyses of the components indicated that they were identical to those that had been isolated previously (Adams, 1987; Tucker et al., 2000). The concentration of extracted oil on a dry weight basis from the western juniper shavings was 1.68% (Adams, 1987) while the Port-Orford-cedar oil was 1.88% (Dr. D. Walker, Essex Laboratory, Personal Communication, 29 December 2003).

The results of the local lymph node assay in the mice are seen in Table 2. Based on data from this study, Port-Orford-cedar oil at concentrations of 0.5%, 5% and 50% did not induce a hypersensitivity response and therefore is not considered to be a sensitizer. Only western juniper oil extract at 50% concentration showed a positive response of 3.33 SI with 3.0 or greater representing a positive response and indicating a potential sensitizer. Lesser concentrations of 0.5% and 5% did not show a positive stimulation response.

The most severe dermal response of the acute dermal irritation study occurred in the initial two rabbits tested with undiluted Port-Orford-cedar extract (Table 3). The PII score in these rabbits was 3.3. However, when this extract was diluted (1:1) with olive oil, the PII score dropped to 0.625. In the second set of dilution experiments, extracts of Port-Orford-cedar oil at 5% and 0.5% had PII scores of 1.1 and 0.3, respectively. At the 0.5% concentration of Port-Orford-cedar oil extract, the 0.3 score represented only two rabbits which showed a score of 1 in erythema (barely perceptible) in the initial 24 h; all the other four rabbits scored 0. With the 5.0% concentration, one animal out of the six total showed a defined edema and erythema to day 8 and then recovered. Another rabbit showed no edema and very slight erythema only at 24 h. The four remaining rabbits had 0 for a score in both categories throughout the study. By the end of the experiment, all six rabbits scored 0. Undiluted western juniper oil had a PII score of 2.7 indicating moderate irritation. However, at 5% concentration, western juniper oil extract had only very slight erythema (barely perceptible) and no

Table 2
(A and B). local lymph node assay results for western juniper oil (*J. occidentalis*) and Port-Orford-cedar oil (*Chamaecyparis lawsoniana*) extract toxicity study

Group	Treatment	Dose (%)	DPM[a] (mean ± S.E.M.)	SI[b]	Results
(A) Results from western juniper oil					
1	Vehicle	–	1,131 ± 166	–	–
2	Western juniper oil	0.5	1,555 ± 181	1.37	–
3	Western juniper oil	5	935 ± 238	0.83	–
4	Western juniper oil	50	3,767 ± 519	3.33	+
5	DNCB[c]	0.1	18,310 ± 2,068***	16.19	+
(B) Results from Port-Orford-cedar oil					
1	Vehicle	–	1,469 ± 148	–	–
2	Port-Orford-cedar oil	0.5	803 ± 255	0.55	–
3	Port-Orford-cedar oil	5	1,379 ± 447	0.94	–
4	Port-Orford-cedar oil	50	2,579 ± 584	1.76	–
5	DNCB[c]	0.1	19,286 ± 3,134***	13.13	+

Test/control ratio of 3.0 or greater represents a positive result.
[a] DPM: decays per min.
[b] SI: stimulation index.
[c] DNCB: dinitrochlorobenzene.
*** Statistically significant difference compared to the vehicle control group ($P < 0.001$).

edema in two rabbits at 24 h that was resolved by 48 h. No signs of skin irritation were seen with the 0.5% dilution. Thus, at 0.5% concentration, western juniper oil extract was found to be a non-irritant. Finally, no changes in weight were noted nor were there any toxic clinical effects from any of the substances tested.

A recent review examined the use of relevant skin sensitization test methods (Kimber et al., 2001). Three primary objectives of this review were to evaluate which methods best determined (a) relative potency, (b) the threshold dose necessary for induction of skin sensitization and (c) risk assessment. It was determined that for de novo investigations, the local lymph node assay is the recommended method for assessment of the influence of a new formulation on skin sensitizing potency. Utilizing the local lymph node assay to test both western juniper and Port-Orford-cedar oil extracts, it was found that neither assay showed a positive response at levels to which animals would be commonly exposed on bedding made from shavings of these species. This conclusion is based on the less than 2% by dry weight of western juniper or Port-Orford-cedar oil that is extracted by steam distillation.

Two pilot studies appear to support the interpretation that exposure to low concentrations of oil, such as in animal bedding made of western juniper or Port-Orford-cedar shavings, will not elicit a hypersensitivity response (Blythe et al., 2001). These studies were conducted at Oregon State University with animals housed for greater than 6 months on western juniper shavings. Twelve healthy adult horses of mixed breeds were bedded on western juniper shavings for a minimum of 12 h per day and for 24 h per day during inclement weather. Baseline photographs were taken of the legs and ventral abdomen immediately prior to and at the end of the study. Blood samples were taken at the same times and analyzed for complete blood counts and for the following chemical concentrations: blood urea nitrogen, creatinine, creatine kinase, asparatate amino transferase, gamma glutamyl transferase, total bile acids, total protein, albumin and bilirubin. The horses were examined daily for any possible foot, limb or abdominal lesions. During and at the end of the study, there was no evidence of any skin lesions or any other clinical or biochemical abnormalities. In a parallel study, eight dogs, primarily Labrador Retrievers, were housed for 198 days on similar western juniper shavings. Physical examinations and blood analyses were identical to those evaluated in the horses. None of the parameters from any of the dogs had a statistically significant change and there were no signs of dermal hypersensitivity or abnormalities.

In summary, this study shows that low concentrations of oil extracts from either western juniper or

Table 3
Dermal observation post treatment scores in rabbits with western juniper oil and Port-Orford-cedar oil at 0.5% and 5% concentrations

Rabbit number	1 h		24 h		48 h*	
	Erythema	Edema	Erythema	Edema	Erythema	Edema
(A) Dermal observations for 5.0% western juniper						
1	1	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	1	0	0	0
4	0	0	1	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0

(B) Dermal observations for 0.5% western juniper had all values equal to 0

Rabbit number	1 h		24 h		48 h		72 h		day 5		day 6		day 7		day 8		day 9	
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
(C) Dermal observations for 5.0% Port-Orford-cedar																		
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1	0	2	1	2	2	2	2	2	2	2fl*	2	1fl*	1	1fl*	0	0	0
6	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Rabbit Number	1 h		24 h		48 h*	
	Erythema	Edema	Erythema	Edema	Erythema	Edema
(D) Dermal observations for 0.5% Port-Orford-cedar						
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	1	0	0	0	0	0
4	0	0	1	0	0	0
5	1	0	1	0	0	0
6	0	0	0	0	0	0

Erythema was scored as follows: no erythema = 0, very slight erythema (barely perceptible) = 1, well-defined erythema = 2, moderate to severe erythema = 3, severe erythema (beet redness) to slight eschar formation (injuries in depth = 4. Edema formation was scored as follows: no edema = 1, very slight edema (barely perceptible) = 1, slight edema (edges of area well-defined by definite raising) = 2, moderate edema (raised approximately 1 mm) = 3, severe edema (raised more than 1 mm and extending beyond area of exposure) = 4, fl*: flaking of skin indicating recovery.

* At 48 h to day 9, all values equaled 0 in all rabbits.

Port-Orford-cedar had no toxic effects. Further, they did not elicit a hypersensitivity reaction nor an acute skin irritation at the low concentrations to which animals bedded on these materials would be exposed.

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