

Ponderosa Pine Needle-Induced Abortion in Beef Cattle: Identification of Isocupressic Acid as the Principal Active Compound

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A crude acid fraction isolated from the needles of ponderosa pine (*Pinus ponderosa* Laws) induced abortions when fed to late-term pregnant beef cows. The major components identified in this abortifacient fraction were four long-chain fatty acids (palmitic, linoleic, oleic, and stearic acids), four abietane diterpene acids (isopimaric, dehydroabietic, abietic, and neoabietic acids), and two labdane diterpene acids (imbricataloic and isocupressic acids). From this crude acid fraction isocupressic acid was isolated and dosed by gavage to five pregnant cows starting on day 250 of pregnancy. Four of the five cows aborted calves when administered isocupressic acid at dosages between 99 and 152 mg/kg (twice daily). At a lower dosage of 66 mg/kg no abortion occurred. No abortifacient activity was observed after a mixture of the abietane diterpene acids was gavaged at dosages of 77 and 120 mg/kg (twice daily); but at higher dosages (238 and 255 mg/kg) these compounds were toxic, and two cows died. It was concluded that the primary abortifacient constituent in ponderosa pine is isocupressic acid and that a separate toxic syndrome may occur from ingestion of high levels of some abietane diterpene acids in pine needles.

INTRODUCTION

Consumption of needles from ponderosa pine (*Pinus ponderosa* Laws) by cattle in late stages of pregnancy is known to cause abortions (MacDonald, 1952; Stevenson et al., 1972; James et al., 1977). Abortions are characterized by weak uterine contractions, occasional incomplete cervical dilation, excessive mucus discharge, birth of a small weak calf, and retained fetal membranes (Stevenson et al., 1972; James et al., 1989). Calves born during the last trimester of pregnancy as a result of cows ingesting pine needles are generally born alive and their survival depends on gestational age and intensity of postnatal care they receive. Complications subsequent to the abortion are frequent and include septic metritis, agalactia, rumen stasis, and death if treatment is not prompt. Occasionally, cows die from an apparent toxicosis induced by the pine needles (James et al., 1977).

Numerous investigators have attempted to isolate and identify the chemical constituents in pine needles that induce abortions in cows. From the available literature on this subject, potential abortion-producing components from ponderosa pine needles have been reported as soluble in water, methanol, ethanol, 1-butanol, acetone, ether, chloroform, and hexane; described as both heat stable and thermolabile; and reported to have antiestrogenic activity (Allen and Kitts, 1961; Allison and Kitts, 1964; Chow et al., 1972; Anderson and Lozano, 1979). Implicated compound classes have included prostaglandins (Shue, 1983) and abietane diterpene acids (Kubik and Jackson, 1981). Water-soluble lignols (Manners et al., 1982), labdane diterpene acids (Zinkel and Magee, 1991), and teratogenic piperidine alkaloids (Tawara et al., 1993) have also been isolated from ponderosa pine needles; however, no data are available implicating these compounds as possible abortifacients.

Many of these reported attempts to isolate the abortifacient compound of ponderosa pine needles have focused on the use of small animal (mice and rats) bioassay

procedures. These studies found that the ponderosa pine needles, and some fractions extracted from those needles, cause embryo resorption and fetal and maternal toxicity in rodents (Allen and Kitts, 1961; Chow et al., 1972; Anderson and Lozano, 1979; Kubik and Jackson, 1981). None of these studies have demonstrated abortions similar to those when pregnant cows ingest ponderosa pine needles. Therefore, we have continued to use pregnant cattle as our primary assay. Recently, James et al. (1994) described the results of feeding trials using ponderosa pine needles and various solvent extracts and residues therefrom, demonstrating that the abortifacient components could be removed from the pine needles by solvent extraction. In a continuation of that effort, we report here on the extensive fractionation of the ponderosa pine needle extract, guided by assay testing in pregnant cows, and the isolation and identification of the major compounds of the abortifacient active materials.

MATERIALS AND METHODS

Plant Material. Pine needles were collected from *P. ponderosa* trees in January 1992 in the John Day area of central Oregon. This particular site has a history of pine needle abortion cases (Stevenson et al., 1972). Collections were made by clipping the last 15–25 cm of the branches from recently harvested trees. Pine bark material was collected in the same area from harvested trees in 1987. Plant material was allowed to dry at ambient temperatures and then stored in burlap bags. Before feeding or extraction, the dry plant material, including the stems, tips, and needles, or bark was ground to pass a 2-mm screen.

Solvent Extraction and Partition. Before extraction, the collected pine materials were initially tested in feeding trials with pregnant cows to determine the amount (dosage) required to consistently induce abortions (James et al., 1994). A single dose was established at 2.7 kg of dry pine needles. Since animals were dosed twice daily until the induced parturition, or up to 10 days, a total of 54.5 kg of pine needles was required for each cow. Extracts and fractions from a complete 10-day requirement of needles (54.5 kg) were prepared as follows. Freshly ground pine needles (54.5 kg), in six batches (9.1 kg each), were extracted by steeping with methylene chloride (CH₂Cl₂, 30 L/batch). The extracting needles were stirred three times daily, and the CH₂Cl₂ solution was drained after 48 h. Fresh CH₂Cl₂ (25 L) was added,

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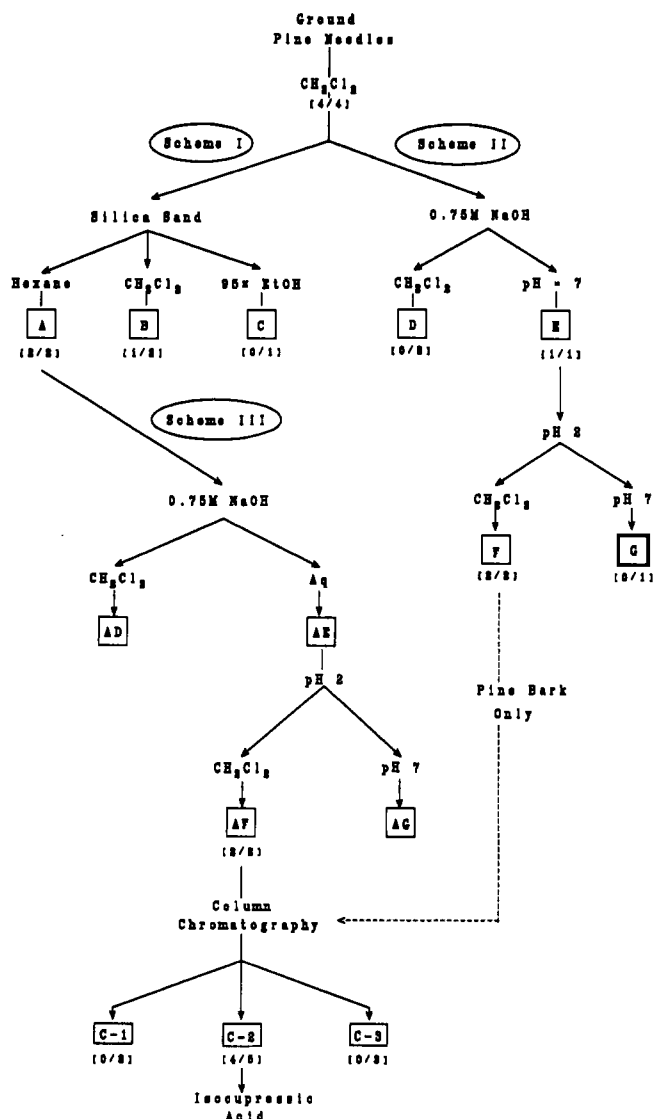


Figure 1. Procedure for extraction and fractionation of pine needles and results of feeding trials with assay results noted (no. of abortions/no. of animals tested).

and the needles were extracted for 48 h and then drained. The resulting CH_2Cl_2 solutions were then concentrated (*in vacuo*, 55 °C) and stored (5 °C) as a 30–50% (w/v) solution in CH_2Cl_2 . The CH_2Cl_2 extracts were further partitioned as outlined in Figure 1 and detailed below.

Silica Sand/Solvent Extraction—Scheme I. Each dose equivalent (5.0% of the total pine needle CH_2Cl_2 extract obtained from the original 54.5 kg of pine needles) was mixed with 5.7 kg of silica sand (70 mesh) and air-dried in a fume hood. The sand/extract mixture was then placed in an 8-L glass bottle fitted with a bottom drain valve. Hexane (2 L) was added and the mixture stirred. The solvent was then drained under reduced pressure. The sand was washed with additional hexane (2 L), and the two extracts were combined and concentrated to yield test fraction A. The sand was then extracted in a similar manner using CH_2Cl_2 (4 L) to yield test fraction B, and then 95% ethanol (4 L) to yield test fraction C.

Base Extraction—Scheme II. A second batch of pine needles (54.5 kg) was extracted, and then each dose equivalent (5.0% of the total CH_2Cl_2 extract) was mixed with CH_2Cl_2 (1.2 L) and 0.75 M NaOH (1.5 L) and allowed to stand for 24 h. The CH_2Cl_2 solution was drained and concentrated to give fraction D. The aqueous base fraction was neutralized (pH 7) with concentrated HCl and stored (–15 °C) to give test fraction E. After fraction E was found to cause an abortion, additional material was adjusted to pH 2 with HCl and extracted with CH_2Cl_2 (2 L). The CH_2Cl_2 was drained and concentrated to give test fraction F. The acidic aqueous layer was adjusted back to pH 7 with NaOH to yield fraction G.

Scheme III. Fraction A was prepared as described above using Scheme I, and then two-dose equivalents of fraction A were base extracted as described in Scheme II to yield fractions AD, AF, and AG. On the basis of the results from the feeding trials of fractions D and G isolated during Scheme II, the corresponding fractions AD and AG were not tested, and only fraction AF was tested for abortifacient activity. One dose of fraction AF weighed approximately 120 g (4% dry weight of the plant material).

Chemical Analyses of Fraction AF. A small aliquot (100 mg) of fraction AF was methylated with methyl iodide similar to the procedure previously described by Garner and Park (1991). The resulting methyl esters were analyzed by gas chromatography using a Hewlett-Packard HP 5890 gas chromatograph equipped with a split/splitless injector and a FID detector. The column was a DB-1 (J&W Scientific) capillary column (30 m × 0.32 mm i.d.). Injector temperature was 250 °C, and detector temperature was 325 °C. Oven temperature was programmed at 200 °C for 1.0 min, 200–300 °C at 10 °C/min, with a final temperature of 300 °C for 5 min. Gas chromatography/mass spectrometry analyses were obtained on a Shimadzu QP-1000 GC/MS system using splitless injection onto a SE-30 equivalent methyl silicone (Quadrex) capillary column (50 m × 0.2 mm i.d.). Oven program was 200 °C for 1.0 min, 200–300 °C at 5 °C/min, and 300 °C for 5.0 min. Electron impact spectra were recorded at 70 eV with an ion source temperature of 250 °C, and chemical ionization spectra were recorded using NH_3 reagent gas. Mass spectra of the free acids were recorded using a heated solids probe. GC/MS was also performed on a Hewlett-Packard 5890 Series II instrument equipped with a 5971 mass-selective detector operating at 70 eV, an on-column injector, and a 60-m × 0.32-mm i.d. SE-30 capillary column. The column was temperature programmed from 120 to 300 °C at 10 °C/min. Peaks were tentatively identified by probability-based matching with the Benchtop/PBM mass spectral library (Palisade Corp., New York) and subsequently identified by comparison of relative retention times (GC) and matching of mass spectra to previously reported data (Zinkel et al., 1971; Cambie et al., 1981; Foster and Zinkel, 1982; Zinkel et al., 1985; Fang et al., 1989; Han and Zinkel, 1990; Zinkel and Magee, 1991) and available standard samples.

IR spectra were recorded on a Perkin-Elmer Model 281 scanning infrared spectrometer as a thin film between two NaCl plates. Thin-layer chromatography (TLC) analyses were made using silica gel 60 coated glass plates (10 cm × 20 cm, 0.25 mm thick) developed with hexane/ethyl acetate (60/40). Developed plates were dried and then placed in iodine vapor for visualization. HPLC analyses were made using a Hewlett-Packard 1090 liquid chromatograph, reversed-phase column (250 mm × 4.6 mm, C_{18} nest group), and HP diode array UV detector monitored at 210 nm. Separation was achieved using a linear gradient of methanol/water (70–100% methanol, 25 min) at a flow rate of 1.0 mL/min. NMR spectra were recorded using a Varian 300XL spectrometer.

Isolation of Isocupressic Acid (6). Initially, isocupressic acid (6) was isolated from pine needle fraction AF. After the first feeding trial, a simpler procedure was found for the isolation of isocupressic acid from pine bark. Pine bark (72.7 kg) was extracted with CH_2Cl_2 and processed using Scheme II to yield bark fraction F. Column chromatography was then used to isolate isocupressic acid from pine needle fraction AF and pine bark fraction F.

The chromatography column was prepared by packing a 15-cm × 100-cm glass column with 2.8 kg of silica gel (70–230 mesh) in a hexane (8 L) slurry. One dose equivalent of pine needle fraction AF (120 g) or bark fraction F (110 g) was added to the top of the column as a 30% solution in hexane/ethyl acetate (50/50). The column was eluted using a stepwise gradient of hexane/ethyl acetate in 4-L amounts starting at 90/10 and increasing the ethyl acetate, in 5% increments, up to 50/50. The column was then flushed with methanol (10 L). Starting with the elution of hexane/ethyl acetate (70/30), smaller fractions (1 L) were collected and analyzed for isocupressic acid (6) by TLC. All fractions eluting before isocupressic acid were combined and concentrated to give test fraction C-1. Fractions found to contain isocupressic acid were combined to yield fraction C-2, and the remaining fractions and methanol wash were combined and concentrated to give fraction C-3. Fraction C-2 was assessed to be greater than 80% isocupressic acid (6) by GC, HPLC, NMR, and TLC. Identification of isocupressic acid (6) was confirmed

Table 1. Results of Feeding Trials with Pine Needle Extract and Extract Fractions

treatment	no. of abortions/no. of animals tested	interval to parturition ^a
CH ₂ Cl ₂ extract	4/4	6, 3, 4, 9
fraction A	2/2	3, 6
fraction B	1/2	19, 23
fraction C	0/1	32
fraction D	0/2	37, 34
fraction E	1/1	5
fraction F	2/2	2, 7
fraction G	0/1	23
fraction AF	2/2	5, 6

^a Interval (days) from start of treatment (250 days) to parturition. Normal term pregnancy = 35 days (285 days gestation). Abortion = interval less than 20 days (270 days gestation) and retained fetal membranes.

Table 2. Feeding Trials with Mixture of Abietane Diterpene Acids (Rosin Gum) and Isolated Isocupressic Acid

treatment	dose (mg/kg/2× daily)	no. of abortions/no. of animals tested	interval to parturition
rosin gum			
33g/dose	77	0/1	32
50	120	0/1	29
100	255, 238	0/2	N/A ^c
column fractions			
C-1	68, 82, 131 ^b	0/3	35, 46, 29
C-2 ^c	66, 99, ^b 109, 147, 152	4/5	33, 8, 6, 3, 2
C-3	35, 43, 54 ^b	0/3	46, 24, 31

^a N/A, cattle died before calving. ^b Fractions isolated from ponderosa pine needles. All others were isolated from pine bark. ^c >80% isocupressic acid.

by comparison of spectroscopic data with that previously reported (Cambie et al., 1981; Shimizu et al., 1988; Fang et al., 1989). NMR and MS data collected on the isolated isocupressic acid are summarized below.

Isocupressic Acid (6): ¹H NMR (300 MHz, CDCl₃) δ 0.62 (s, 3H), 1.25 (s, 3H), 1.67 (s, 3H), 4.17 (d, *J* = 7.0 Hz, 2H), 4.54 (s, 1H), 4.87 (s, 1H), 5.40 (t, *J* = 7.0 Hz, 1H); ¹³C NMR (75.5 MHz, CDCl₃) δ 183.6 (C=O, C-19), 147.9 (C-8), 140.5 (C-13), 122.9 (C-14), 106.5 (C-17), 59.3 (C-15), 56.3 (C-5), 55.5 (C-9), 44.2 (C-4), 40.4 (C-10), 39.1 (C-1), 38.7 (C-12), 38.4 (C-7), 37.9 (C-3), 29.0 (C-18), 26.0 (C-6), 21.9 (C-11), 19.9 (C-2), 16.3 (C-16), 12.8 (C-20); EI MS (70 eV) *m/z* (relative intensity) 320 M⁺ (0.4), 305 (6), 302 (6), 287 (14), 275 (5), 259 (11), 257 (8), 241 (12), 189 (22), 175 (10), 167 (19), 161 (16), 149 (26), 147 (24), 133 (33), 121 (95), 107 (53), 93 (65), 81 (89), 67 (62), 55 (100); CI MS (NH₃) *m/z* 338 [M + NH₄]⁺ (3), 320 [M + NH₄]⁺ - H₂O (25), 303 MH⁺ - H₂O (100), 257 (65), 247 (8).

Animal Feeding Trials. The protocol for animal use in this research was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Utah State University, Logan. Thirty-two pregnant beef cows (average weight of 430 kg) with known breeding dates were divided into groups and gavaged test fractions A–G, AF, rosin gum (a mixture of abietane diterpene acids), and column fractions C-1, C-2, and C-3 (Tables 1 and 2). Test materials were prepared for feeding trials by dividing the total extract (from 54.5 kg of pine needles), or test fractions, into 20 equal doses. Each dose equivalent was then mixed with 0.45 kg of ground alfalfa hay. Any residual solvent was removed from the alfalfa by air-drying in a fume hood for at least 2 h. The alfalfa/pine needle extract mixture was then administered by gavage (hand pumped with stomach tube) to pregnant cows, starting on day 250–252 of pregnancy, in two doses per day (morning and afternoon) for up to 10 days as previously described (James et al., 1994). All dosages in the text and Table 2 are listed as milligrams of (test material) per kilogram of animal body weight (twice daily).

Rosin gum (Sigma Chemical) was dissolved in CH₂Cl₂ (0.67 g/mL) and then dispersed on alfalfa hay and gavaged to pregnant cows. The initial dosages of rosin gum were 238 and 255 mg/kg (twice daily), which was approximately equal to the total diterpene acid content of one dose of fraction AF. The rosin gum dosage

was reduced to 120 mg/kg and subsequently 77 mg/kg (twice daily), the latter of which was equal to the amount of only abietane diterpene acids present in fraction AF.

An average gestation period for Hereford beef cattle is 285 days, and fetal membranes are normally expelled in less than 6 h after calving (Morrow, 1986; Werven et al., 1992). The induced parturition was considered to be premature or an abortion if it occurred before 270 days of gestation and fetal membranes were retained for more than 12 h. Because of the expense and size of the cattle and the copious amounts of test material needed, a positive response (abortion) in two cows was considered sufficient to justify testing subsequent fractions. Only the most active fractions were pursued further.

RESULTS

Earlier feeding trials determined that methylene chloride (CH₂Cl₂) was an effective solvent in removing the abortifacient activity from pine needles (James et al., 1994). When the resulting crude organic extract was tested, all four cows aborted calves within 3–9 days and retained fetal membranes (Table 1). This crude organic extract justified further fractionation and we proceeded using the procedures as outlined in Figure 1.

Fractionation of Pine Needle Extract. Scheme I. The concentrated CH₂Cl₂ extract was mixed with silica sand and extracted with three solvents encompassing a range of polarity from low (hexanes), medium (methylene chloride), and high polarity (95% ethanol), yielding test fractions A, B, and C, respectively. Fraction A was active, causing abortions in two cows tested (Table 1). The activity of fraction B was weak, causing one abortion that occurred 19 days after the initial dosage (day 269 of gestation). This animal retained the fetal membranes for several days, and therefore the event was considered an abortion. Fraction C did not cause an abortion in a single cow that was dosed.

Scheme II. The pine needle extract was partitioned between CH₂Cl₂ and 0.75 M aqueous NaOH solution. The CH₂Cl₂ layer was concentrated to yield test fraction D, which was not active. The remaining aqueous base was neutralized to pH 7 with HCl to yield test fraction E. Fraction E caused an abortion in the single cow dosed (Table 1). Additional fraction E material was prepared and separated further by adjusting to pH 2 with HCl and extracting with CH₂Cl₂ to yield test fractions F and G. Fraction F induced abortions in two cows tested, after 2 and 7 days, respectively. Fraction G was not active.

Scheme III. Fraction A was again obtained from a separate pine needle extraction and Scheme I procedure and then partitioned further using the base extraction procedure of Scheme II. Only fraction AF was tested since prior results from fractions D and G showed no activity. Two cows aborted calves after 5 and 6 days (Table 1). The abortions induced by fraction AF were clinically identical (retained fetal membranes and weak calves) to those observed when pine needles are gavaged.

Characterization of Fraction AF. The acidic nature of fraction AF was established from the isolation procedure. IR spectroscopy of the crude mixture confirmed the presence of a carboxylic acid group with a strong C=O stretch at 1680 cm⁻¹. The mixture of crude acids was converted to the corresponding methyl esters using methyl iodide (MeI) and analyzed by gas chromatography (Figure 2A). Components of fraction AF were identified after analysis by gas chromatography/mass spectrometry and included four long-chain fatty acids (palmitic, linoleic, oleic, and stearic acids), four abietane diterpene acids (isopimaric, dehydroabietic, abietic, and neoabietic acid), and two labdane diterpene acids (isocupressic and imbricatolonic acid) (Figure 3). Other smaller peaks in the GC chromatogram of fraction AF remain unidentified but

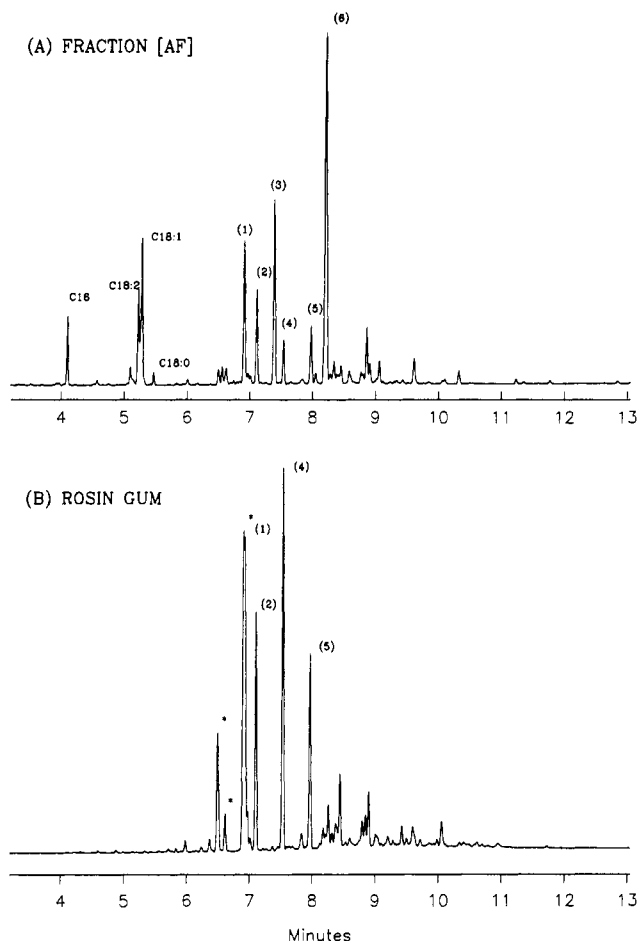


Figure 2. (A) GC chromatogram of the methylated fraction AF. Peaks identified were palmitic acid (C16), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), isopimaric acid (1), dehydroabietic acid (2), imbricataloic acid (3), abietic acid (4), neoabietic acid (5), and isocupressic acid (6) as methyl esters. (B) GC chromatogram of the methylated commercially available rosin gum material. (*) Other isomers of abietic acid.

appear from the GC/MS data to be other diterpene acids and phthalate esters. On the basis of additional data described below, their identifications have not been pursued further.

Assay Testing of Diterpene Acid Mixture (Rosin Gum). Rosin gum was dosed by gavage into four cows as a method of testing the abortifacient activity of the abietane diterpene acids found in pine needle fraction AF. Rosin gum is the common name for a commercially available naval stores product and is a mixture of diterpene resin acids (mostly abietic acid and related isomers) obtained from the stumps or the exudate of pine trees. The GC chromatogram obtained upon methylation of the rosin gum material is shown in Figure 2B. The four abietane diterpene acids (isopimaric, abietic, dehydroabietic, and neoabietic acid) identified in fraction AF were present in the rosin gum material as major components (peaks 1, 2, 4, and 5, Figure 2B). Isocupressic and imbricataloic acids (peaks 6 and 3, Figure 2A), the labdanes of fraction AF, were not found in the rosin gum.

At dosage levels of 77 and 120 mg/kg (twice daily) the rosin gum showed no abortifacient activity and both cows calved at full term (Table 2). This dosage was equivalent to or slightly greater than the amounts of those same components as found in fraction AF. At higher dosages (238 and 255 mg/kg, twice daily), the rosin gum was toxic and the animals became depressed and stopped eating, followed by rumen stasis, tympany, respiratory distress, and peripheral neuropathy, a result similar to that

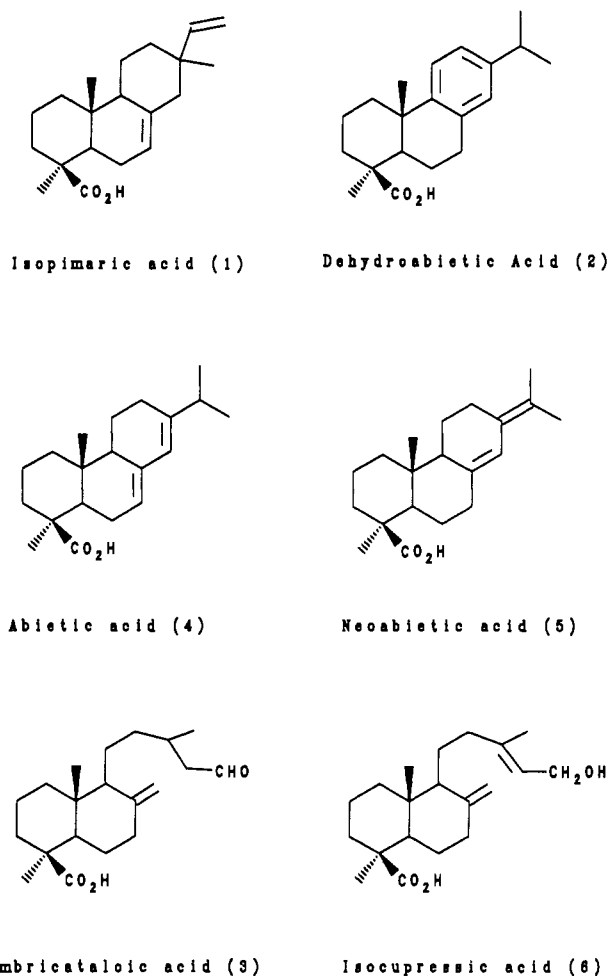


Figure 3. Chemical structures of the major diterpene resin acids identified in fraction AF. Numbers in parentheses correspond to GC peaks in Figure 2A.

previously observed in feeding trials of ponderosa pine branch tips (Panter et al., 1990). Both animals had to be euthanized and were subsequently necropsied. However, no abortions occurred in any of the feeding trials of the rosin gum.

Isolation and Testing of Isocupressic Acid (6). Isocupressic acid (6) was isolated from pine needle fraction AF and later from pine bark fraction F by large-scale column chromatography. To ensure that the abortifacient activity was not lost, all material eluting from the column was tested. Compounds eluting from the column before isocupressic acid were combined to make a mixture of fatty acids and the other diterpene resin acids (fraction C-1). Fraction C-2 contained isocupressic acid (>80%) and a small amount of neoabietic acid (5), while fraction C-3 contained mostly unknown polar material (Figure 4). Fractions C-1, C-2, and C-3 isolated initially from pine needles and then from pine bark were individually tested for abortifacient activity (Table 2). No abortions occurred when fraction C-1 or C-3 was administered to six pregnant cows; however, two of three cows dosed fraction C-2 (isocupressic acid) aborted after 6 and 8 days. The resulting dosage levels for the isocupressic acid (calculated after the feeding trials and figured from the weight of the cow and the amount of isolated isocupressic acid) were 109 and 99 mg/kg (twice daily) for the two cows that aborted and 66 mg/kg for the cow that calved normally. As final confirmation, more isocupressic acid (6) was isolated from pine bark and tested in two additional cows. The dosages were increased to 147 and 152 mg/kg (twice daily). Both cows aborted calves after 3 and 2 days,

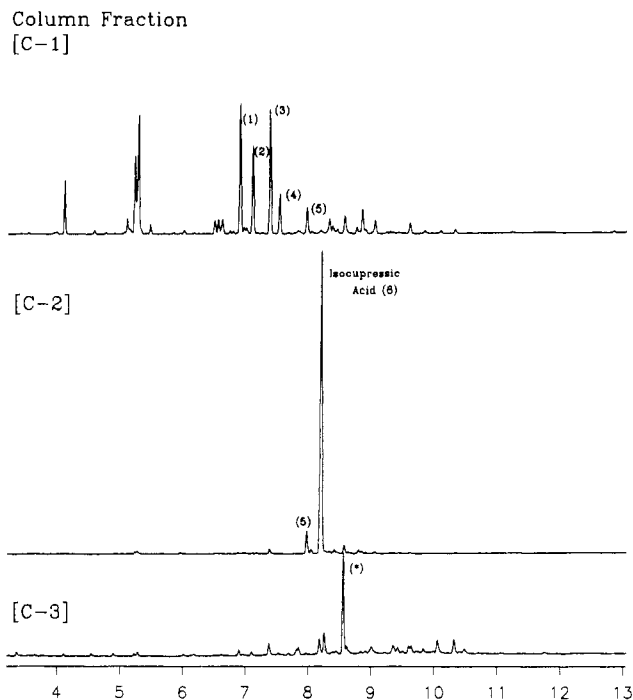


Figure 4. GC chromatograms of column fractions from isolation of isocupressic acid from pine needle fraction AF. (*) Bis(2-ethylhexyl) phthalate.

respectively. In total, four of five cows dosed fraction C-2 (isocupressic acid) aborted calves.

DISCUSSION

Fractionation of the ponderosa pine needle extract, and subsequent testing of the various fractions for abortifacient activity, led to the isolation of a crude acid fraction (identified as fraction AF) that was found to induce abortions in the late-term pregnant cows. The major components of fraction AF were identified as a mixture of long-chain fatty acids and diterpene resin acids. The occurrence of long-chain fatty acids was not unexpected, and diterpene resin acids are common constituents found in many pine species. In fact, the individual diterpene acids identified in fraction AF (Figure 3) have been previously isolated from needles of *P. ponderosa* and fully characterized (Fujii and Zinkel, 1984; Zinkel and Magee, 1991).

It seemed unlikely that the long-chain fatty acids were the abortifacient compounds in pine needles because of their ubiquitous occurrence in other plants that are not abortifacient; however, the activity of fraction AF could potentially be attributed to its diterpene acid content. Bioactivity has been reported for certain diterpene acids. Kaurenoic, trachylobanoic, and several other tricyclic diterpene acids reportedly inhibit larval development in several *Lepidoptera* species (Elliger et al., 1976). Two diterpenes, kaurenoic and grandiflorenic acids, were reported to have potent uterotonic activity in a guinea pig uterine strip assay (Page et al., 1992), and abietic acid and related isomers have exhibited antimicrobial activity (Himejima et al., 1992). A mixture of eight abietane diterpene acids and several unsaturated fatty acids isolated from ponderosa pine needles has been reported to cause reproductive failure in mice (Kubik and Jackson, 1981). Thus, we examined the diterpene acids identified in fraction AF further for abortifacient activity in cows.

Large quantities of individual purified diterpene acids were unavailable for testing. The four abietanes, isopimaric (1), abietic (4), dehydroabietic (2), and neoabietic (5) acids, were available as the major components in a

commercially available mixture known as "rosin gum". When the rosin gum was fed to four pregnant cows, no abortifacient activity was found. However, this material was nephro- and neurotoxic at the higher dosage levels and may account for the toxicity observed in the feeding of pine branch tips (Panter et al., 1990). A complete toxicological and histopathological investigation of the observed intoxications will be reported elsewhere. On the basis of the feeding trials with the rosin gum, we concluded that the four abietanes (isopimaric, abietic, neoabietic, and dehydroabietic acids) identified in fraction AF were not the abortifacient components of the pine needles, eliminating all but isocupressic and imbricataloic acids as possible active compounds (peaks 6 and 3, respectively, in Figure 2A).

Isocupressic acid (6) was originally isolated from the pine needles by column chromatography of fraction AF and was then later isolated from ponderosa pine bark fraction F. Pine bark was shown to induce abortions in cattle in earlier experiments (Panter et al., 1990; James et al., 1994) and was used here to replace a limited supply of abortifacient active needles in order that further feeding trials might be completed with isocupressic acid. In testing the three column fractions isolated from pine needles and pine bark, fraction C-2 was the only material found to induce any abortions. Fraction C-2 was assessed to be >80% isocupressic acid by GC, HPLC, TLC, and NMR. Neoabietic acid (5) was present in fraction C-2 isolated from the pine needles at a concentration of 5.7%. However, this compound cannot be an abortifacient component because it was fed at even higher levels as part of the rosin gum material (see Figure 2B, peak 5) without causing an abortion. Neoabietic acid was also not found in fraction C-2 isolated from pine bark (data not shown). It is unlikely that the other low-level contaminants account for any activity because of their very low concentrations and their occurrence in fractions C-1 and C-3 which did not induce abortions.

The isolated isocupressic acid (6) was tested in five cows (Table 2) and at dosage levels ranging from 66 to 152 mg/kg (twice daily). Four of the cows aborted calves. A single cow, which received the lowest dosage at 66 mg/kg (a result of using a large cow, 482 kg, and a smaller amount of isolated isocupressic acid), did not abort. Although the number of test animals was small, an explanation for the one cow that did not abort is offered by a preliminary dose/response relationship. On the basis of the data from Table 2, an oral dosage of approximately 100 mg/kg (twice daily) is required to induce the abortion. This dosage appears reasonable when compared to the value calculated from the original dosage of ponderosa pine needles. For example, our original pine needle dose was 2.7 kg/dose (twice daily), and current assay of the needles reports the isocupressic acid concentration to be 1.7% (dry weight). Assuming an average weight of a pregnant cow as 430 kg, the effective dose of isocupressic acid received from the 2.7 kg of pine needles would be 107 mg/kg (twice daily).

In addition to isocupressic acid (6), there may be other diterpene acids of ponderosa pine that have abortifacient activity. Imbricataloic acid (3) is another labdane acid and was isolated as part of the pine needle fraction AF. Its structure is very similar to that of isocupressic acid. Imbricataloic acid was part of column fraction C-1, which did not cause any abortions, yet as a single component it may not have been at a high enough dosage to be active. Isocupressic acid derivatives, acetylisocupressic and succinylisocupressic acids, have also been isolated from ponderosa pine (Zinkel and Magee, 1991). In fact, these two labdane acid esters were reported to be the major diterpene acids in many of the ponderosa pine samples

examined by Zinkel and Magee (1991) and accounted for over 50% of the acid content in a central Oregon collection. Zinkel and Magee (1991) also suggested that the occurrence of isocupressic acid may be partially an artifact resulting from hydrolysis of acetyl- and succinylisocupressic acids during analytical procedures. Therefore, ester hydrolysis during base/acid extractions (Scheme II of our procedure) of the pine needle extract may account for the absence of the acetyl and succinyl derivatives in fraction AF. The fact remains that fraction AF and column fraction C-2 (isocupressic acid) caused abortions when administered to pregnant cows.

We might also suggest that the abietane-type acids may have some function in the abortifacient event. We demonstrated with feeding trials of the rosin gum and column fraction C-1 that these compounds will not induce the abortion by themselves; however, they are toxic, causing depression, decreased appetite, and even death if administered at a high enough dosage. A pregnant cow receiving a sublethal dose (as might happen from consumption of ponderosa pine needles) may become ill (depressed and stop eating). The condition of the pregnant cow combined with the effect of isocupressic acid (the abortifacient) might expedite the abortion process. In addition, it may at this time be difficult to distinguish between postabortion complications and intoxication from abietane diterpene acids.

In summary, we have identified isocupressic acid (6) as a major abortifacient component in ponderosa pine needles based on the extraction and isolation from ponderosa pine needles and bark and subsequent assay in pregnant cows. Imbricatolonic acid (3) may also be an active component based on a labdane structure very similar to that of isocupressic acid. We intend to examine further isocupressic acid, imbricatolonic acid, and related labdane diterpene acids for abortifacient activity and a comprehensive study of structure/activity relationships. The identification of the active compound responsible for inducing abortions in cattle is an important first step in the overall management of the pine needle abortion problem.

ACKNOWLEDGMENT

We thank Joyce Johnson, Terrie Wierenga, and Kermit Price for their help with sample and dose preparations and plant extractions; Lew Dell Balls, Rex Probst, and Mike Semrad for the care and handling of the animals and preparation of plant material; and Carl Elliger for standard samples of diterpene resin acids.

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Received for review August 10, 1993. Revised manuscript received December 8, 1993. Accepted December 20, 1993.*

* Abstract published in *Advance ACS Abstracts*, February 1, 1994.