Characterization of non-polar compounds from Pinus ponderosa needles causing reproductive failure in mice during early gestation
by Yolanta Miroslawa Kubik

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Biochemistry
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Abstract:
Ingestion of hexane extracts of Pinus ponderosa needles causes reproductive failure in mice during early stages of gestation. Virgin mice of ICR strain were mated with a male of proven fertility. The day the copulatory plug was observed was designated day 1 of gestation. The hexane extracts were administered daily via stomach tube on day 1-5 of gestation. Implantation sites were stained by injection of pontamine sky blue dye on day 8 of gestation, 15 minutes before sacrifice. Interrupted implantation and embryonic resorption were observed in the uterus. The extract was found to be most effective around the time of implantation (day 5 of gestation). Through chromatographic analysis the active compounds were identified as non-conjugated diterpene resin acids: pimaric--0.8%, isopimaric--58.2%, and sandaracopimaric--3.3%. Ingestion of the above mixture of non-conjugated diterpene resin acids of ponder-osa pine needles during early gestation result in failure to maintain implantation and subsequent embryonic resorption in mice.

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ABSTRACT

Ingestion of hexane extracts of Pinus ponderosa needles causes reproductive failure in mice during early stages of gestation. Virgin mice of ICR strain were mated with a male of proven fertility. The day the copulatory plug was observed was designated day 1 of gestation. The hexane extracts were administered daily via stomach tube on day 1-5 of gestation. Implantation sites were stained by injection of pontamine sky blue dye on day 8 of gestation, 15 minutes before sacrifice. Interrupted implantation and embryonic resorption were observed in the uterus. The extract was found to be most effective around the time of implantation (day 5 of gestation). Through chromatographic analysis the active compounds were identified as non-conjugated diterpene resin acids: pimaric--0.8%, isopimaric--58.2%, and sandaracopimaric--3.3%. Ingestion of the above mixture of non-conjugated diterpene resin acids of ponderosa pine needles during early gestation result in failure to maintain implantation and subsequent embryonic resorption in mice.

INTRODUCTION

Pinus ponderosa (Western yellow pine) is a conifer that grows in the Western coastal states east through the Rocky Mountains, north to British Columbia, and south to Mexico. The needles are 5-11 inches long and grow in bundles of three or two and three.

Ponderosa pine needles have been reported to be poisonous to cattle, causing abortion during the late stages of gestation. "Pine needle abortion" as this phenomena is termed, has plagued ranchers for many years. In 1950, the Canadian Range Experimental Station in British Columbia was requested to determine if Pinus ponderosa needles were the causative agent in abortion in range beef cattle (MacDonald, 1950). Booth (1950) implies that non-infectious abortion was common in mountain areas abounding in ponderosa pine. This observation was substantiated by Halver (1953) when he observed that 31 out of 192 head of range heifers aborted when they had ingested ponderosa pine needles. Jacobsen (1952) reported that the "pine needle abortion" problem was responsible for numerous calf losses in nineteen, mostly mountainous, counties in Montana.

In 1959, Nicholson reported that consumption of ponderosa pine needles consistently caused abortion among range heifers. He also concluded that it was impossible to induce abortion with other species of conifers and confirmed that certain quantities of ponderosa pine needles would induce bovine abortion and/or the birth of stillborn or weak calves. Cows were found to be suffering from symptoms of starvation and were generally in poor physical condition.

It has been established experimentally that ponderosa pine needles do indeed cause bovine abortions, but more frequently the birth of weak calves that die shortly after birth (Gunn, 1949; Nicholson, 1957). At present, it is not known to what extent ponderosa pine needles are involved in the etiology of the"weak calf syndrome". Neither is it understood why cattle preferentially turn to ponderosa pine needles when other sources of forage are available.

In an attempt to elucidate the cause of pine needle induced abortion many investigators have turned to laboratory animals (rodents) as a biological assay system. Allen and Kitts (1961) examined several extracts (aqueous, ether and acetone) of ponderosa pine needles for estrogenic or anti-estrogenic effects on the uterus of the female

labortory mouse. They found the aqueous extract to contain anti-estrogenic compounds. The ether fraction had the greatest toxicity to adult mice and the acetone fraction resulted in the highest embryo mortality. In assessing the reproductive failure, it was observed that in all cases the mice showed signs of starvation. McClure (1959) reports that starvation alone can affect reproduction by inhibiting implantation.

Cook (1962) examined the antifertility aspects of "pine needle abortion", using acetone and ethyl acetate fractions of an aqueous extract of pine needles. The acetone fraction decreased uterine weight in immature females, but neither fraction caused reproductive failure.

In recent years, Chow, et al. (1972) have considered reproductive dysfunction in the female mouse due to ingestion of various extracts (volatile, acetone, and aqueous) of ponderosa pine needles. The acetone and volatile fractions have no effect on uterine weight or embryonic development. The aqueous extract, however, has a very dramatic effect (uterine wt./body wt. - aqueous .72 mg/g; control 1.00mg/g) on the uterine weight and also causes the greatest number of fetal deaths (11 when compared to two in the controls). This activity could be

destroyed by heating. The active compound(s) in ponderosa pine needles was concluded to be thermo-labile and water soluble.

Weideman (1973), in an attempt to further determine the active compound, extracted ponderosa pine needles by the method of Chow (1972) and administered the extract to immature rats supplemented with subcutaneous injections of estradiol in anticipation of uterine atrophy. Perplexing results were observed. Immature rats exhibited uterine and ovarian weights that were only slightly below that of controls. Some adult rats had functional corpora lutea while others did not. None of the necropsied animals exhibited signs of reproductive failure as described by other investigators. On the basis of this data Weideman concluded that he had not assayed the rats for a sufficient period of time. Weideman's inability to obtain similar results suggests to me that different types of compounds (ie. lipophilic) should be evaluated for their role in "pine needle abortion".

Cogswell (1974) evaluated the effects of ponderosa pine needles and extracts of pine needles on implantation, offspring viability and average litter size. Both aqueous and acetone extracts reduce average litter size. To

determine the critical period for disrupting embryonic development, Cogswell fed these same extracts on day 5 of gestation and observed that both extracts reduced the number of viable offspring and at a high concentration (2:1 ratio of extract to Purina Mouse Chow) both extracts disrupted embryonic development on day 6 or 7 of gestation. Mice lost weight, showed signs of gastric ulcerations and had blood filled implantation sites, suggesting embryonic resorption.

In addition to the reproductive failure aspects of "pine needle abortion", four other features have been considered: vitamin A deficiency, phosphorus deficiency, external parasites and toxic agents.

It was suggested that cattle turn to ponderosa pine neeldes as a source of vitamin A. After Muenscher (1945) concluded that range cattle did not find conifer needles palatable unless driven by starvation to eat them, Oh, et al. (1967) showed that monoterpene alcohols such as abinene, B-phellandrene, cis-acimene, and 1,8-cineole were unpalatable to browsing animals and had an inhibitory effect on the functioning of rumen microorganisms. Nicholson (1959) designed an experiment to address the hypothesis that pine needles are a source of vitamin A.

Using leptospirosis and brucellosis free heifers administered a ponderosa pine needle ration, he observed that one heifer aborted nine days after initiation of the feeding period. The other two animals gave birth to immature, weak calves which died shortly after birth. Blood vitamin A levels were analyzed to determine the effects of ponderosa pine needles on intestinal absorption. Results indicate a normal level of vitamin A in both experimental and control heifers.

During winter months range beef cattle maintained on near starvation rations frequently suffer from phosphorus deficiency which Rombouts and Guegen (1961) suggest may decrease fertility. The correlation between phosphorus deficiency and a pine needle craving was examined by Toner (1971). Results indicate that phosphorus deficient rats preferentially turn to pine needles as a source of phosphorus.

At this time it was suggested that external parasites (ie. fungi in the pine needles) could be responsible for the reproductive failure observed. Using an aqueous fungi, administered to mice on day 1 of gestation, Chow, et al. (1974) discovered that the extract of the fungi disrupted pregnancy, whereas the extract of the pine needles did not.

They concluded that the fungus in some manner converts the compounds in the needles to fetal-lethal materials. Utilizing "rejuvenated fungi", they demonstrated that fetal resorption occurred with ingestion of incubates of fungi, whereas the aqueous extract of the pine needles allowed for embryonic development. On the basis of this data, it was concluded that mycotoxins were the agents responsible for the reproductive dysfunction observed as opposed to compounds located in the needles.

In 1977, Anderson and Lozano investigated the toxicity characteristics associated with "pine needle abortion". Their results indicate that a mycotoxin is present in the aqueous incubates of pine needles. Necropsied mice showed no unusual organ cytology and therefore it was suggested that the greatest effect of ponderosa pine needle ingestion was on implantation. The extracted needles were thought to contain a water insoluble toxin that could be more toxic than the water soluble toxin.

In an attempt to return to the natural condition observed with range beef cattle, Neff, et al. (1979) administered a pine needle ration to mice from the ninth day of gestation until parturition. Results indicate a wasting syndrome, abortion and maternal death.

Adams, et al. (1979) in administering a pine needle ration to mice during late gestation (day 9-18) induced abortion and maternal death. In addition, a bacteria, Listeria monocytogenes, was isolated from the blood, uterus and cecum of the mice fed pine needle ration. In light of these results it was suggested that ponderosa pine needles in some manner enhance the growth of Listeria monocytogenes, a naturally occurring bacteria in the mouse.

Despite almost thirty years of investigations, the "pine needle abortion" problem is still not well understood. Although a number of aspects of this problem have been examined, the solution to this economic loss for the rancher remains unsolved. Some investigators feel that the compound responsible for the reproductive failure is located in the needles; others feel that it is a by-product of a pine needle fungus. Extensive experimentation utilizing an aqueous extract of pine needles suggests that an active compound is water soluble and thermo-labile.

Results have also suggested the possibility of lipophilic compounds being involved (Allen and Kitts, 1961 and Anderson and Lozano, 1977); however, until this study little work has been done in this area. A precedent has been established for lipophilic compounds being implicated in

reproductive problems (Farnsworth, et al. 1975), though not always in interruption of pregnancy during early gestation.

In lieu of this knowledge, the objectives of this thesis are to examine lipophilic molecules of ponderosa pine needles and evaluate their involvement in pine needle induced reproductive failure in laboratory mice during early gestation.

RESEARCH OBJECTIVES

This research study is divided into three areas of investigation:

1. Isolation of lipophilic material from ponderosa pine needles.

2. Demonstration of reproductive failure in mice due to ingestion of lipophilic compound(s) of ponderosa pine needles.

3. Purification and chemical identification of lipophilic compounds that are biologically active.

The purpose of this work is to isolate and identify the lipophilic compound(s) responsible for pine needle induced reproductive failure during early gestation in mice. With the knowledge of the identity of the active compound(s) responsible for pine needle induced reproductive failure during early gestation in mice, it might be possible in later studies to elucidate the mechanism by which this biological phenomena occurs. Once this was established, a method to counteract this affect could be developed thus solving the problem of economic loss for the rancher. Also, by understanding the mechanism involved, the active compound(s) could be utilized as a contra-

ceptive method during the early stages of gestation.

MATERIALS AND METHODS

Extraction of Pine Needles

Lipophilic compounds of the conifer Pinus ponderosa (collected from Grey Cliff, Montana) were obtained by cutting the needles into 1-2 cm lengths and extracting in hexane (3 ml/gm) for six hours on a mechanical shaker. This scheme (Figure 1) was repeated twice and the solvent was filtered and then cooled at 4°C for six hours. The resulting white precipitate that formed was filtered off utilizing a Buchner suction apparatus and the hexane was evaporated on a Brinkman/Buchi rotoevaporator (model Rotavapor-R) at 40°C. The extract, dissolved in hexane, was passed over a charcoal (Nuchar M.C.B.) column 2.2 cm in diameter and 5.1 cm in length. The solvent was evaporated and the extract stored in vials at 4°C until use.

Animals

Virgin female ICR mice (originally from Flow Laboratories, but bred and reared locally) weighing 28-32 grams, were placed two to a cage with a male of proven fertility and were examined daily for the presence of a copulatory plug (Parkes, 1926). The day the plug was observed was designated day 1 of gestation and treatments were initiated.

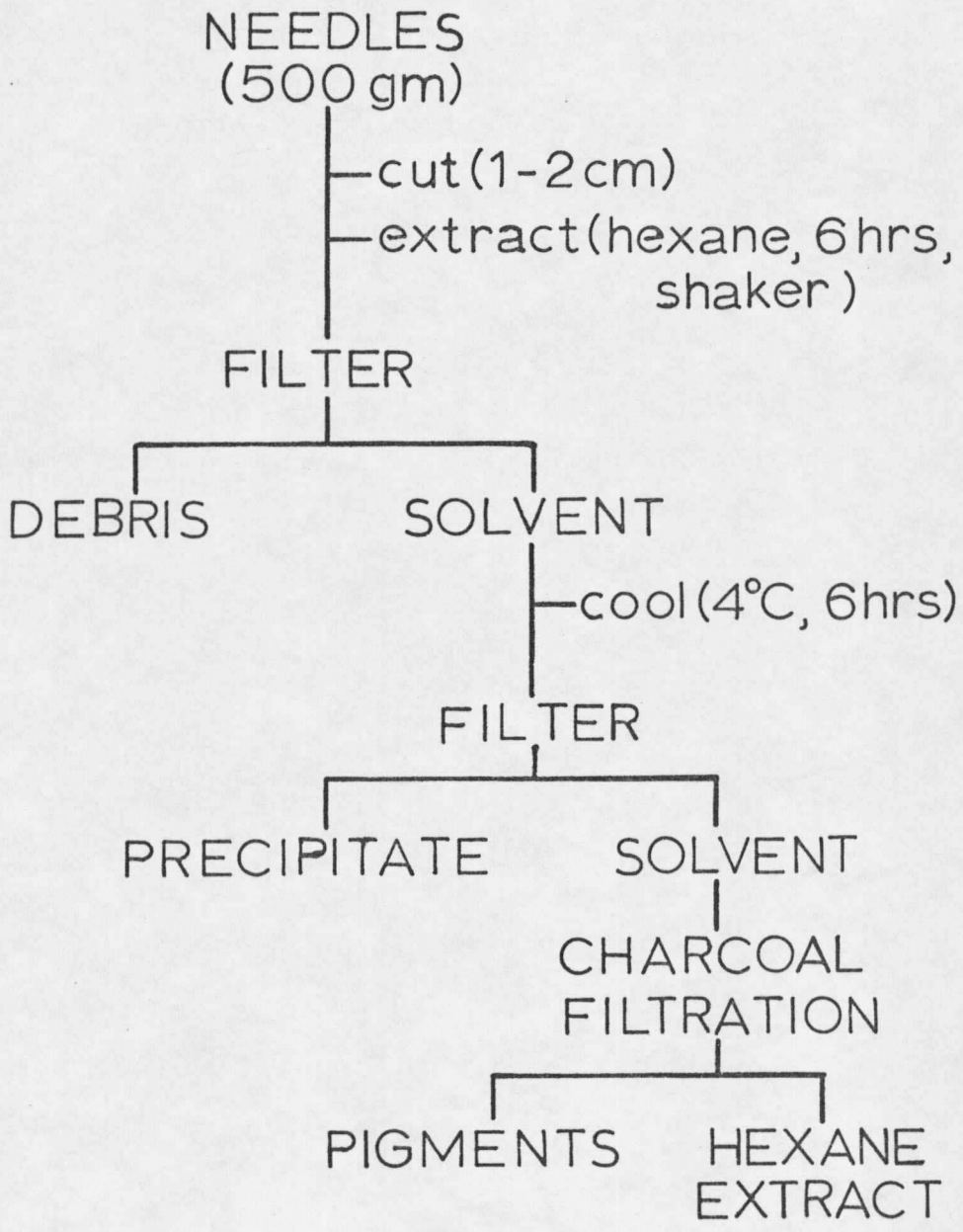

Fig.1 EXTRACTION SCHEME

On day 8 of gestation mice were sacrificed by cervical dislocation and uteri were excised and evaluated, by visual comparison to controls, for increased capillary permeability (positive pontamine blue reaction) and embryonic resorption. In this evaluation embryos smaller in size and weight and exhibiting no definition of form were considered to be in the process of resorption.

Pontamine Sky Blue Assay

Mice were injected with a 0.1 ml solution of 0.5% pontamine sky blue dye in 10% NaCl in the tail vein (Psychyos, 1961) to facilitate examination of the implantation site as displayed by increased capillary permeability. Fifteen minutes later mice were sacrificed and uteri were examined for the presence of implantation sites and resorptions as previously described.

Hexane Extract Ration

Hexane extract (500 grams of needles equivalent) was incorporated into lab chow at a 1:1 (weight equivalent of needles:weight of chow) ratio by dissolving the extract in 100 ml of diethyl ether and adding it to 300 grams of ground lab chow (Wayne Lab Blox). To this mixture was added 100 ml of distilled water and 100 ml of molasses

(unsulphured). The hexane extract ration was allowed to dry overnight, cut into pellets and administered ad lib on day 1-8 of gestation. Mice were allowed free access to water during the test period. Control mice had free access to lab chow and water.

Stomach Tube Administration

After establishing a bioassay system, the extract samples were dissolved in olive oil as a vehicle and administered via stomach tube at the concentrations and for the durations mentioned in results. Control mice received 0.2 ml per day of olive oil by stomach tube in addition to lab chow and water ad lib. The stomach tube apparatus consisted of a 1 cc Tuberculin syringe with a leur-lock tip, a B-D Yale 25G x 5/8" needle and a 4 cm length of 1 mm polyethylene tubing (Clay-Adams Intramedic).

Column Chromatography

Aliquots (3.0 grams) of hexane extract were eluted through an activated silica gel column having the following dimensions: total length 36.0 cm; with the top 9.0 cm having a diameter of 5.2 cm and the bottom 27.0 cm having a diameter of 3.0 cm. A slurry was prepared in hexane using 130 grams of Bio-Sil A (BioRad), 100-200 mesh.

Solvents utilized and fractions obtained are listed in results (Table 6).

Preparative thin layer chromatography was used to further purify the fractions obtained by column separation. The developing solvents were hexane:diethyl ether:acetic acid(75:25:1, v:v:v) or toluene:ethyl acetate (90:10, v:v).

Purified fractions were individually assayed for physiological activity.

Argentation Chromatography

The column fraction shown to interrupt implantation was further separated into two constituents by argentation thin layer chromatography. The 15% $AgNO_3$ plates were prepared by dissolving 5.6 gm of $AgNO_3$ crystals (J.T. Baker Chemical Company) in 85 ml of distilled water and adding this solution to 50 grams of silica (Adsorbosil-5, no binder, Applied Science Laboratories, Inc.). The slurry was applied to five glass plates(20 cm x 20 cm); when dry the silica was activated at 120°C for two hours.

The active column fraction (100-150 mg in hexane/plate) was applied to the silica and the plate was developed in hexane:diethyl ether:acetic acid (75:25:1, v:v:v). Bands were separated and eluted from the silica with toluene.