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Abstract approved:

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Logging and forest thinning operations generate large amounts of residues in the form of small trees, branches and foliage that are usually left on the ground to decay or are burned. These resources are readily available, but it is uneconomical to remove them from the forest. The potential utilization of these resources was investigated for western juniper. This species is invading rangeland in the Interior West, altering the ecology of the land, and farmers are being encouraged to remove it. However, limited markets for the wood make removal costly. Developing uses for other parts of the stem could make removal more economically attractive. Western juniper foliage and twigs were steam distilled to obtain essential oils which were tested for their activity against subterranean termites and fungi. Residues from the steam distillation were pretreated with dilute sulfuric acid and digested with enzymes to determine their digestibility. The goal was to determine if an integrated operation involving steam distillation to remove

essential oils, followed by enzymatic digestion of extraction residues was feasible. Essential oil recovery from foliage averaged 0.77% wet weight, while yields from twigs were about 0.13% wet weight. Foliage essential oil showed high antifungal and termiticidal activities at the concentrations tested. Total sugar yields from foliage extraction residues (40-55%) were higher than those obtained from branchwood residues (15-25%). The highest yields were obtained at 121^{o}C and 1 hour pretreatment in 1% sulfuric acid. These results show that an integrated operation involving steam distillation of western juniper foliage to remove essential oil and enzymatic digestion of extraction residues is technically feasible. Additional work to determine the optimum distillation and pretreatment conditions for foliage would be needed.

POTENTIAL UTILIZATION OF WESTERN JUNIPER RESIDUES

by

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kennedy Sichamba, Author

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DEDICATION

To my family, especially mum and daddy for their support.

POTENTIAL UTILIZATION OF WESTERN JUNIPER RESIDUES

I.0 INTRODUCTION

Logging and forest thinning operations generate large amounts of residues in the form of small trees, branches, unwanted stem wood and foliage (a.k.a logging 'slash') that are left on the ground and often disposed of by burning. Up to 10 - 50% of the total above ground biomass is left behind as forest residues during harvesting activities (Oldenburger, 2006; Koopmans and Koppejan, 1997) depending on the species and the harvesting technologies. This figure may be higher in developing countries where most of the logging operations are done on a small scale. These resources can contribute to a forest's nutrient reservoir, reduce erosion and protect seedlings. However, excessive volumes of these resources result in management problems, including risk of fire - as residues become dry, they are a source of fuel to carry a ground fire into the crown - interfering with regeneration and requiring costly disposal treatments (Barger, 1979). Identifying uses for these resources could help solve these problems and ensure more efficient utilization of forest resources.

One potential area for product development from these residues is to explore the production of essential oils from various plant tissues. Plants that produce naturally durable heartwood contain extractives and essential oils which provide protection against bacteria, fungi, insects and other organisms (Schultz and Nicholas, 2001; Bakkali,

2008). Identifying the active oil components can contribute to development of environmentally benign wood protection systems (Wang et al. 2005). These residues are also potential raw materials for bio-fuels and other bio-chemical products. Lignocellulosic biomass consists of lignin and polysaccharides (cellulose and hemicelluloses). Polysaccharides can be broken down into simple sugars, which can then be converted to various products including bio-fuels, food additives or enzymes (Mussatto and Teixeira, 2010). Production of liquid bio-fuels can provide environmentally benign alternatives to fossil fuels.

Western juniper *(Juniperus occidentalis)* belongs to the *Cupressaceae* family (Bonner, 2000; Dealy 1990). It is a shrub or small tree growing to 20m tall (Adams, 2008). Until the late 1800s, western juniper was limited to rocky ridges or surfaces with scarce vegetation, (Miller et al. 2000). However, the species has expanded its range to more fertile and productive sites. Juniper expansion has been attributed to reduced forest fires, overgrazing and optimal climatic conditions (Miller and Rose, 1995; Miller et al. 2000). As western juniper woodlands expand, they tend to suppress the vegetation that previously occupied the site. Ranchers are particularly concerned about reduced foliage for livestock. It is therefore considered by many as a weed or pest (Adams 2008). Land owners are being encouraged to remove it to restore land productivity, but limited uses for juniper wood make removal costly. Historically, uses for western juniper wood

have been limited to low value products like fence posts and firewood. The poor form of the stem and internal defects have limited production of lumber products (Kurth and Ross, 1954). Efforts to control western juniper have included fire, herbicides and mechanical means such as chainsaws. These methods, however, have negative ecological consequences and can be expensive and time consuming. Recent efforts have focused on developing useful products from western juniper to support management activities.

Studies on western juniper utilization have focused mainly on steam distillation of oils from the heartwood and solvent extracts from heartwood and foliage. However, large amounts of foliage and twigs are left on site during western juniper harvesting. Developing uses for these residues could make removal more economically feasible.

Objectives

The objectives of this work were to:

1. Assess oil content of foliage and small branches of western juniper.

2. Explore bio-efficacy of the oils against fungi and termites.

3. Assess the fermentation potential of foliage and branches extraction residues.

2.0 LITERATURE REVIEW

Western juniper *(Juniperus occidentalis)* belongs to the *Cupressaceae* family (Bonner, 2000; Dealy 1990). It is a shrub or small evergreen tree growing to 20m tall (Adams, 2008). The trunk is typically short and heavily branched. Mature bark is thin, reddish brown, but weathers to grayish brown with broad, shallow furrows and flattened ridges. The leaves are scale-like, which conserves moisture in its dry, rocky environments (Adams, 2008, Miller et al. 2005). Mature female cones (often called 'berries' due to their shape) are small, and round, with smooth, leathery scales; green when young and bluish black when mature (Figure 2.1) (Miller et al. 2005).

Figure 2.1: Leaves, bark, cones and a juniper shrub (Factsheet, Virginia Tech).

Reproduction is by means of seeds. Female cones are fertilized from late April to late May (Bedell et al. 1993). Survival rates for the seedlings have been shown to be high

(Burckhardt and Tisdale, 1976). Western juniper may reproduce as early as 25 years of age if there is little competition from other vegetation, but trees become reproductive at about 50 to 75 years of age in most situations (Bedell et al. 1993; Miller and Rose 1995). Vegetative reproduction has also been observed in western juniper (Dealy 1990).

The range for western juniper extends to northeastern California, the northwest corner of Nevada, southwest Idaho, eastern and central Oregon and southeastern Washington (Figure 2.2) (Adams, 2008; Dealy 1990). The heaviest concentrations are found in rangelands of central Oregon, where western juniper grows in association with ponderosa pine (*Pinus ponderosa*) and big sagebrush (*Artemisia tridentate*). There are two geographically separated subspecies, *Juniperus occidentalis* var *occidentallis* in the northern part and *J. occidentallis* var *australis* in the southern part of its range (Dealy 1990).

2.1 Western juniper wood properties

2.1.1 Physical and mechanical properties

Western juniper wood varies from milky white to deep reddish-brown. The wood is richly colored, aromatic, surfaces well during milling, glues and finishes well and is more dimensionally stable than Douglas-fir or ponderosa pine (Miller et al. (2005).

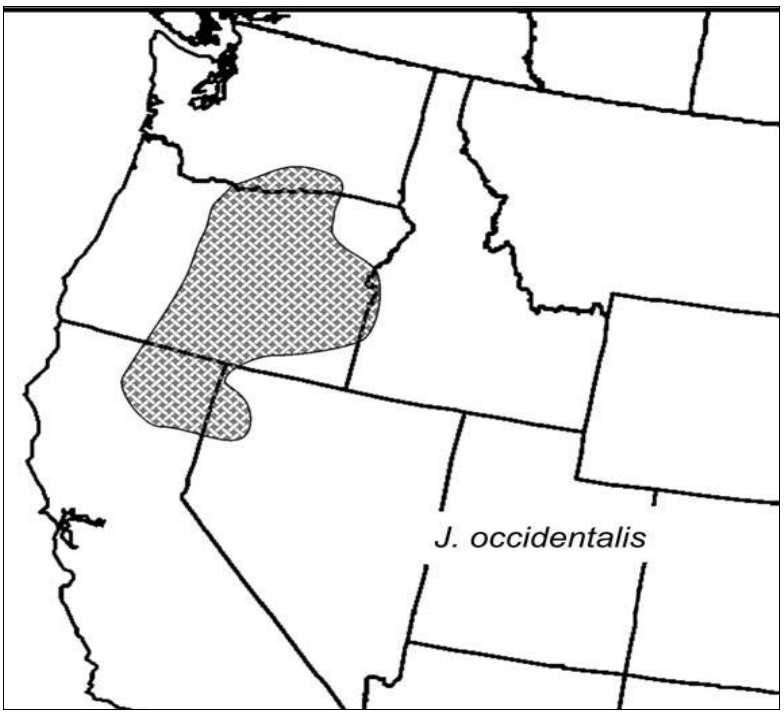

Figure 2.2 Native range of western juniper (Adams, 2008).

The heartwood is highly durable, and among the most decay resistant northwestern species for fence posts (Miller et al. 2005; Morrell et al. 1999). Physical and mechanical properties of western juniper wood are comparable to most softwoods (Table 2.1). However, the poor form of the bole and internal defects have limited the ability to produce lumber products from western juniper. Most older trees contain internal decay making recovery of lumber or pencil stocks difficult (Kurth and Ross, 1954).

Table 2.1. Mechanical and physical properties of western juniper and other commonly used woods measured at 12%. (Western Juniper factsheet, Oregon State).

Species	Specific Gravity	Density (kg/m^3)	Compression strength(kpa)	MOE ('000'kpa)	MOR (kpa)	Hardness(kg)
western juniper	0.44	496.57	43712.76	551.58	61639	283.94
Douglas-fir	0.48	544.62	50469.62	13444.77	85495	322.05
ponderosa pine	0.40	448.51	36680.10	8894.24	64811	208.65
eastern redcedar	0.47	528.60	41506.43	6067.39	60674	408.23
incense-cedar	0.37	600.46	35852.73	7170.55	55158	213.19
red oak	0.63	704.81	46608.58	12548.46	98595	585.13

2.1.2 Fiber and Chemical Properties

Western juniper fibers are shorter than those of most softwoods, but are longer than most hardwoods (Table 2.2). Fiber diameters are smaller than most softwoods, and about the same as hardwoods. Lignin content is much higher than most softwoods and hardwoods. Cellulose and hemicelluloses content are lower than most temperate softwoods and hardwoods. These properties make western juniper unsuitable for chemical pulping and paper production. High lignin may also make it a poor candidate

for production of bio-ethanol and other chemicals since lignin acts as physical barrier to the cellulose. It may, however, be suitable for reconstituted products like hardboard and MDF (Myers et al.1998).

Table 2.2. Fiber and chemical properties of western juniper vs. other common species at 12% mc. (Myers et al. 1998).

Species	Fiber/Vessel length (mm)	Fiber diameter. (mm)	Alpha cellulose (%)	Lignin (%)
Western juniper	1.60	0.012-0.031	38.6	35.5
Ponderosa pine	3.60	0.035-0.060	45.0	25.1
Lodgepole pine	3.50	0.035-0.055	47.3	25.9
Douglas-fir	4.50	0.035-0.055	52.6	28.0
White fir	3.50	0.035-0.050	49.1	27.8
W. hemlock	4.00	0.030-0.050	50.0	29.9
Red alder	1.20/ 0.85	-	44.0	24.1
Sugar maple	0.92/ 0.41	-	49.2	21.5
Red oak	1.32/ 0.42	-	46.0	23.9

2.2 Western Juniper Expansion

Until the late 1800s, western juniper was limited to rocky ridges or surfaces with scarce vegetation (Miller et al. 2000). The species range has, however, expanded to more fertile and productive sites. Juniper expansion has been attributed to reduced forest fire frequency, overgrazing and ideal climatic conditions for seed production and seedling development (Miller and Rose, 1995; Miller et al. 2000). Fire suppression and heavy livestock grazing removed herbaceous biomass that previously resulted in very hot fires (Miller et al. 2000). Without fire to control western juniper seedlings at their early stages of development, trees were able to survive and grow.

As western juniper woodlands expand, they tend to suppress the vegetation that previously occupied the site, and also alter the soil nutrient distribution. Western juniper encroachment suppresses the understory vegetation by drawing soil nutrients and intercepting precipitation. Miller et al. (2000) investigated the impacts of western juniper on plant composition and structure across several associations in southeastern Oregon and northeastern California. Their results showed that increases in juniper dominance led to declines of mountain big sagebrush (*Artemisia tridentate*) and aspen (*Populus tremuloides*) cover. They also found that herbaceous cover and species diversity declined and bare ground increased with increasing juniper dominance. Coultrap et al. (2008) also found a significant relationship between western juniper

canopy cover and understory species richness, shrub cover, forbs cover, total grass cover, cheatgrass cover, herbaceous productivity, and bare ground. Other studies also support these findings (Bates et al. 2005).

Western juniper invasion also has an impact on the distribution of soil nutrients. The roots of western juniper trees draw soil nutrients, depriving intercanopy spaces of soil nutrients. The pattern of soil nutrients under western juniper invaded soils is described as 'islands of fertility' because soil nutrients are concentrated under juniper trees (Klemmedson and Tiedmann, 2000; Doescher et al. 1987). Juniper roots also occupy the soil profile to the extent that the site is effectively closed for seedling establishment of desirable forage or browse species. (Miller et al. 2005). By suppressing understory vegetation, western juniper increases surface runoff and soil erosion.

The western juniper canopy also intercepts precipitation, thereby potentially reducing the amount of precipitation reaching the ground (Miller et al. (2005). Petersen and Stringham (2008) measured the infiltration, runoff and sediment yield in response to western juniper encroachment in south-east Oregon. They reported that western juniper-covered areas had very low depth of infiltration and high water runoff. Similar observations were reported by Pierson et al. (2007) who found that juniper-dominated hill slopes produced rapid runoff from low-intensity rainfall, and that large

interconnected patches of bare ground concentrated runoff into rills with much higher flow velocity resulting in high erosion. Less water and soil nutrients are available in the soils under juniper cover, thereby negatively affecting understory and intercanopy plant growth in areas with high juniper cover (figure 2.3).

Figure 2.3: Dense juniper stands with sparse understory vegetation (Gedney et al. 1999).

Landowners are being encouraged to remove western juniper to restore land productivity. However, limited markets for western juniper products make removal costly. Historically, the dominant uses for western juniper wood have been low-value products like fence posts and firewood (Kurth and Ross, 1954). Poor stem form and the presence of internal defects have limited the recovery of lumber products from western juniper. Other products including cement/wood fiber composites, particleboard, hardboard, decking, wall paneling, flooring, veneer, furniture, and novelty items, firewood, animal bedding, doors, cabinetry, rustic furniture and picture frame molding can also be successfully produced from western juniper wood. However, large branches,

a low volume per acre ratio, rocky terrain, and lack of a steady supply of raw, materials all limit the use of western juniper in these products (Miller et al. 2005).

Methods of eradicating western juniper have been extensively researched in an effort to improve land productivity. Some of the techniques used to eradicate western juniper include the use of herbicides and mechanical clearing using chain saws. (James et al. 1982). These methods have negative ecological consequences and can be expensive, labor intensive, and time consuming. Recently, researchers have concentrated on developing useful products from western juniper in order to support management activities. Research has mainly focused on exploring properties of natural oils of western juniper.

2.3. Extractive Composition and Properties

2.3.1 Extractive composition

The amounts of essential oil in western juniper heartwood vary between 1-3% depending on several factors like season, location, part of the tree from which the material was collected, and the operation conditions. Kurth and Ross (1954) investigated the effect of operating conditions on oil yield and quality of western juniper wood. Variables examined were particle size, distillation time, steam pressure and the effect of bark. About 12mm heartwood chips, hammer-milled chips or Wiley-milled

chips were steam distilled using different times and pressure. Oil yields increased with decreasing particle size. Highest yields (1.2%) were obtained from Wiley-milled chips, compared to 12mm chips (0.68 %). Oil yields increased with increasing pressure; however, the finest oil was obtained at low pressure. Maximum yields were obtained after 5-6 hours of steaming. The presence of bark slightly reduced oil yields.

An earlier study investigated the distribution of western juniper oil within the tree trunk (Bunert, 1954). Trees of about 400mm in diameter were felled and cross-sectional disks were cut from the butt and the top. Oil yields from the butt were about twice the yields from the top (2.23 vs. 1.09%). Higher yields were obtained by Burnet than by Kurth and Ross (2.23 vs 1.23 respectively) despite the fact that the material was collected from the same site; this discrepancy may be due to different seasons and operating conditions. Adams (1987) reported high essential oil yields (2.33%) from 50 - 100 mm diameter western juniper logs. Yields may have been slightly higher than for both Burnet (1954) and Kurth and Ross (1954) because Adams used only heartwood. Industrial scale steam distillation of western juniper wood was reported in a recent study by Yesenofski (1996). Low yields (0.17%) may have been due to due to the larger batch sizes (~100kg), and shorter distillation time (1-4hours).

The amounts and chemical composition of western juniper heartwood oil are comparable to the commercial sources of cedarwood (*Juniperus ashei* and *Juniperus virginiana*) (Table 2.3) (Adams, 1987). Western juniper contains higher amounts of cedrol, a commercial commodity, than *J. ashei* or *J virginiana*. However, western juniper oil has been reported to have a harsh odor and greasy taste (Kurth and Ross (1954), which limits its use for commercial application.

Table 2.3. Oil yield and percentage composition of commercially important components of cedarwood oil from *J. occidentalis*, *J.virginiana* and *J.ashei* (Adams 1987).

Compound	*J. occidentalis*	*J. virginiana*	*J. ashei*
α-cedrene	8.8	27.2	1.6
β-cedrene	2.6	7.7	1.6
Thujopsene	18.9	27.6	60.4
Cuparene	1.5	6.3	2.8
Cedrol	38.9	15.8	19.0
Widrol	1.6	1.0	1.1
Total oil yield (%)	2.33	3.2	4.0

Little work has been reported on essential oil yield from western juniper foliage. Yesenofski (1996) observed seasonal variations in foliage essential oils. Yields from

materials collected in fall were higher (0.34-0.46%) than those from materials collected in summer (0.17 -0.32%) from the same location. The oil is rich in monoterpenes and bornyl acetate. The main components are Sabinene (22%), p-cymene (11.4%), neryl formate (10.4%), α-pinene (10%), bonyl acetate (12.4%), sabinol (6.1%), α-terpinene (6.1%), γ-terpinene (4%) and d-limonene (2.6%) (Yesenofski, 1996; Tatrol, et al. 1973).

2.3.2 Biological properties of essential oils

Western juniper extracts have been shown to exhibit antimicrobial and termiticidal activities. Johnston et al. (2001) evaluated antimicrobial activity of essential oil and methanol extracts of western juniper wood against bacteria and yeasts (*Fusobacterium necrophorum, Clostridium perfringens, Actinomyces bovis* and *Candida albicans)* that cause infections in domestic animals. Western juniper heartwood essential oil was active against all the test microorganisms except *F. necrophorum*. It was also shown that α- and β-cedrene, among the main constituents of heartwood essential oil, were the active components. Methanol extracts were only active against *Actinomyces bovis,* with only a weak inhibitory effect against the other microbes. Strong antifungal activities of leaf extracts of western juniper have also been reported (Clark et al. 1990)

Adams et al. (1988) investigated the termiticidal activities of heartwood, bark/sapwood and leaves of western juniper. They found that subterranean termites (*Reticulitermes flavipes)* could not survive on heartwood sawdust nor could they survive on methanol or hexane heartwood extracts. Hexane extracts of the leaves also exhibited high termiticidal activities, but only minor activity was found in methanol extracts, which indicated that the non-polar components of leaf extractive were the active components. Craig et al. (2004) evaluated acute dermal toxicity of heartwood essential oil of western juniper on mice and rabbits. The oil only showed a positive stimulation on mice at 50% concentration, and at 100% concentration on rabbits.

2.4 Summary

Western juniper woodlands have expanded in the past century. As western juniper expands, it tends to displace plant communities that previously occupied the site. Removal costs are high as there are limited markets for western juniper products. Efforts have been made to develop markets for juniper products in order to offset management costs.

Like other junipers, western juniper wood contains appreciable amounts of essential oil. The oil properties, however, differ from commercial cedarwood oil, which limits its commercial application. Antimicrobial and termiticidal activities of essential

oils and solvent extracts of western juniper have been reported for various organisms. No work has been reported on oil content and biological properties of twigs. Enzymatic digestibility of juniper residues has also not been explored.

3.0 MATERIALS AND METHODS

Samples of foliage and twigs (5-10cm diameter) were collected from randomly selected healthy western juniper *(J. occidentalis*) trees located near Sisters, Oregon in January 2011. Foliage was separated from twigs and stored separately at 5^{o}C until use (Figure 3.1). Foliage was extracted within one week of collection to avoid loss of volatiles. Twigs were ground in a hammer mill to about 2-5mm diameter prior to extraction. Twigs were extracted within one month of collection.

Fig. 3.1 Western juniper twigs and foliage.

3.1. Steam distillation

Steam distillation of western juniper foliage and twigs was performed to obtain essential oils. Steam distillation was employed because of its simplicity and low cost compared to other methods. It also allows oils to be extracted at lower temperatures than the boiling points of these oils, which limits the potential for thermal

decomposition, while preserving their aroma. The principle behind steam distillation of plant materials is that steam passing through the material expands the intercellular pockets that hold the essential oil. Released essential oil is then entrained in the steam, until it reaches the condenser, where both steam and essential oils condense to a liquid.

Twigs milled to about 2 -5mm diameter and foliage tissues clipped to a maximum length of about 150mm were extracted in a steam distillation apparatus (Figure 3.2). A three-neck round bottomed boiling flask was ¾ filled with water and placed on a heating mantle. A handful of boiling chips were added to the flask to facilitate boiling. The juniper materials (2-3 kg) were weighed, and closely packed into a distillation column that was wrapped with an insulation material to prevent steam from condensing in the column. A condenser was securely clamped to the top end of the distillation column and connected to a cooling water line. It took about one hour for the water to start boiling. Vapors from the column (combination of steam and essential oils) were then cooled in the condenser, and collected in a flask as an oil-water mixture. The distillation system was operated until oil recovery was negligible (about 6-8 hours).

Oil was carefully decanted from the condensate mixture. The oil was weighed and the amount of oil recovered from a given mass of stems or foliage was used to determine percentage yield ((weight of oil/wet weight of the material)*100). The oil was

stored at 5^{o}C for biological tests, while foliage and twig residues were frozen for subsequent hydrolysis.

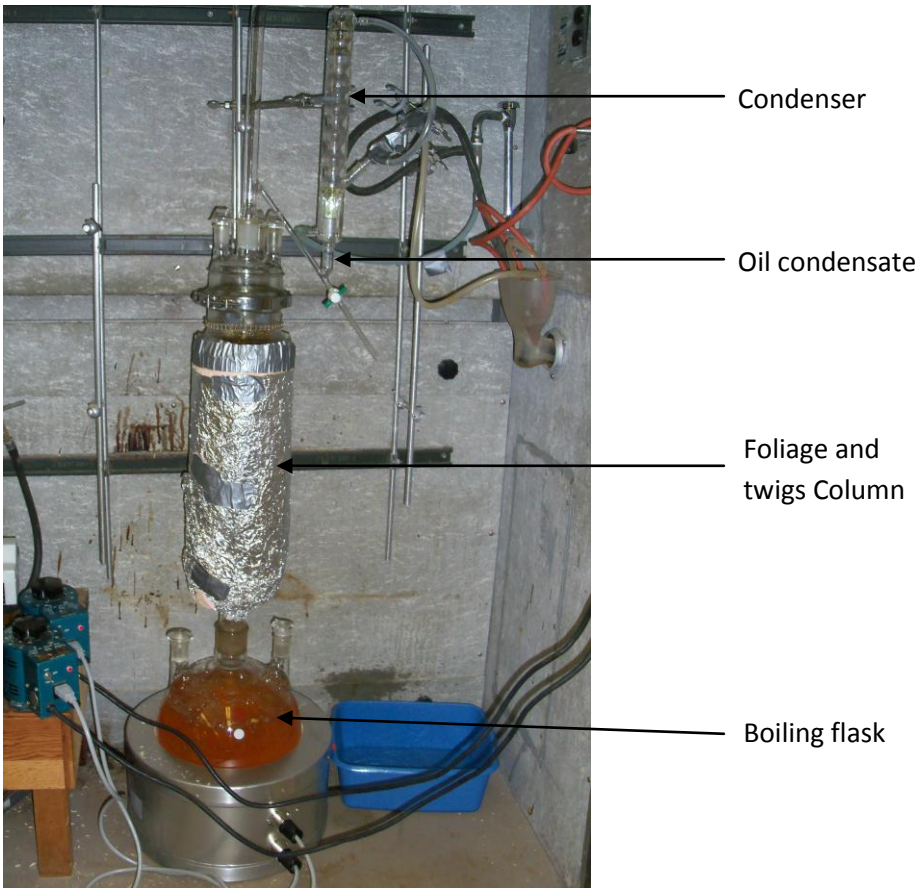

Figure 3.2. Steam distillation apparatus used to extract oils from western juniper foliage and stems.

3.2. Bio-efficacy tests

3.2.1 Antifungal activity

Essential oil from western juniper foliage was tested for its antifungal activity against *Postia placenta* (Fries) M. Larsen et Lombard (Isolate Madison 698) (brown rot fungus) and *Trametes versicolor* (L. ex Fr) Pilat (Isolate R-105) (white rot fungus).

Ponderosa pine (*Pinus ponderosa* L) wood blocks (20 mm x 15 mm x 5mm long) were oven dried at 103^{o}C for 24 hours, cooled in a desiccator and then weighed. The blocks were kept in the desiccator to limit moisture sorption until impregnation. Western juniper oil was diluted to 10, 20 or 30% (vol/vol) in 95% ethanol. The solution was impregnated in the blocks using a desiccator connected to a vacuum pump. The blocks were placed in a beaker, and a weight placed on top to prevent wood from floating. The oil solution, enough to cover the blocks, was then added to the beaker. The beaker was then placed in the desiccator, a vacuum was applied for 20 minutes, and then the vacuum was released. The beaker was removed from the desiccator, covered with aluminum foil and left to stand for 30 minutes. The blocks were then removed from the beaker, wiped with a paper towel to remove solution on the surface and weighed. Six replicates were prepared for each oil dilution. Control blocks were impregnated with 95% ethanol.

Retention of oil in wood blocks was determined using the formula:

$$\text{Retention (kg/m}^3\text{)} = (W_2\text{-}W_1) * C / V * 10$$

Where, W_1 was the dry weight of blocks, W_2 was the weight of blocks after impregnation, C was the concentration of oil in treating solution and V was the volume of the blocks (cm^3) (Table 3.1).

Table 3.1. Retentions of juniper oil at 10, 20 and 30% (vol/vol) dilutions delivered to ponderosa pine sapwood blocks.

Oil dilution (%)	Net retention (kg/m^3)*
10	48.29
20	99.41
30	140.56

*Values represent means of six blocks.

After impregnation, the blocks were exposed to the air for 48 hours, and then placed in a conditioning room for 21 days at 23^{o}C and 65% relative humidity. After conditioning, the blocks were sterilized by exposure to 2.5 mrads of ionizing radiation from a cobalt 60 source. The blocks were then placed on petri dishes (6 blocks per dish) containing 0.5% malt extract and 1% agar. Each plate contained three blocks treated with a given oil concentration and three untreated blocks. Fungal inoculum was cut

from the edge of the fungal culture, and aseptically placed between the blocks (Figure 3.3). Petri dishes were then sealed using wax film and incubated for 12 weeks at 28^{o}C.

After 12 weeks, mycelium was brushed off the blocks, which were weighed and reconditioned for 21 days at 23^{o}C and 65% RH. After conditioning, the blocks were weighed. Weights before and after fungal exposure were used to determine fungal associated weight loss.

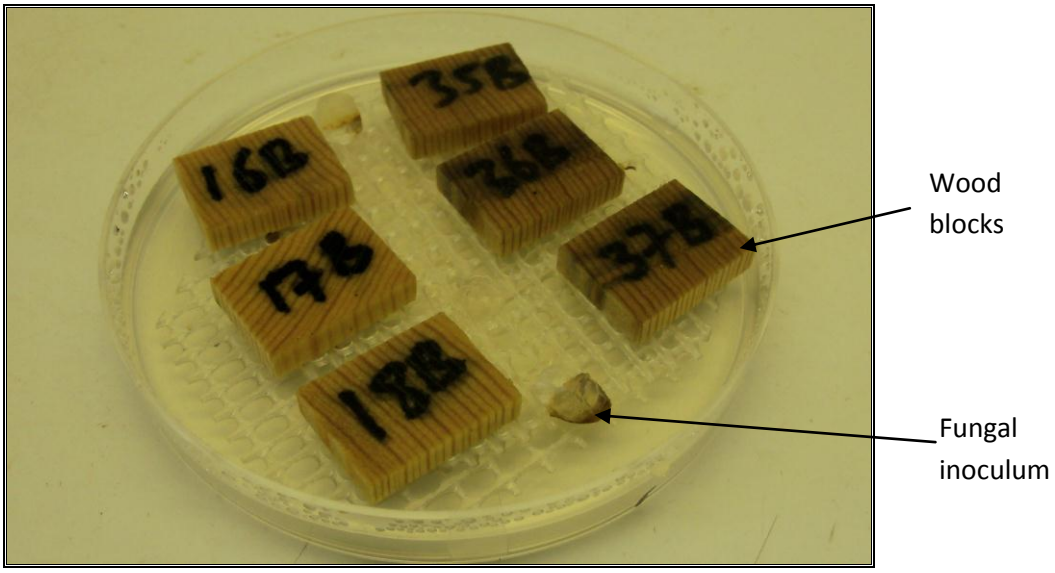

Figure 3.3. Petri dish arrangement for the decay tests.

3.2.2 Termite tests

Termiticidal activity of western juniper leaf oil against *Reticulitermes flavipes* was determined according to American Wood Protection Association Standard E1 (AWPA

2010) on southern yellow pine sapwood blocks impregnated with three concentrations (10%, 20%, or 30%) of the oil.

Southern pine sapwood blocks (19 x 19 x 6mm) were oven dried at 105^{o}C for 24 hours, and then cooled in a desiccator and weighed. The blocks were left in the desiccator until impregnation. Western juniper oil was diluted to the target concentration in 95% ethanol. Five blocks were prepared for each dilution. Two controls were prepared: southern yellow pine blocks treated with 95% ethanol and non-treated blocks. The blocks were impregnated using a vacuum desiccator as described above. The blocks were placed in a beaker to which the oil solution, enough to cover the blocks was added. The beaker was then placed in the desiccator and a vacuum was applied for 20 minutes. After 20 minutes, the vacuum was released and the beaker removed from the desiccator. The beaker was covered with aluminum foil and left to stand for 30 minutes. The blocks were then removed from the beaker, wiped with a paper towel to remove solution on the surface, and then weighed. Oil retentions in the blocks at each dilution are shown in Table 3.2. All the treated blocks were then exposed to open laboratory room conditions for 48 hours before conditioning them at 23^{o}C and 65% RH for about 21 days.

Table 3.2. Retentions of juniper oil at 10, 20 or 30% dilutions delivered to southern pine sapwood blocks.

Oil dilution (%)	Net retention (kg/m^3)*
10	71.62
20	148.75
30	208.63

*Values represent means of five blocks.

A no-choice (single test block per container) test procedure was used. Sand (150g) and water were added to a 450 ml glass jar, and the jar was allowed to stand for two hours. A test block was then placed on top of the sand in each jar, and about four hundred termite workers/soldiers (*Reticulotermes flavipes*) were added (Figure 3.4). Five replicate samples were prepared for each dilution. The jars were then kept at 25^{o}C and the bioassay terminated after four weeks. The sand was scraped from the blocks which were then oven dried to constant weight. The weight before and after exposure was used to determine percent weight loss. Live workers in each container were counted to determine insect mortality.

Figure 3.4. Glass jar arrangement for termite tests.

Termiticidal effects were evaluated by percent weight loss of the test blocks, termite mortality, and visual inspection of test blocks. Percent weight loss was determined using the formula:

$$\text{Weight loss (\%)} = (W_2\text{-}W_3)/W_2*100$$

Where, W_2 was the weight of conditioned test block before exposure to termites and W_3 was the weight of the conditioned test block after exposure. Termite mortality was calculated as number of dead termites after exposure per initial number of termites

added to the container. Visual rating of the test blocks was based on 0-10 grading scale, where 0 was complete destruction, and 10 was no evidence of termite attack.

3.3. Dilute acid pretreatments

Dilute sulfuric acid pretreatment combined with heat was performed on steam distillation residues of western juniper foliage and branchwood. Dilute sulfuric acid hydrolyses hemicelluloses and increases the surface area of biomass for subsequent enzymatic digestion. The objective of this phase of the experiment was to determine the ability of dilute sulfuric acid pretreatment to solubilize the carbohydrate fraction in western juniper foliage and twigs that had previously been steam distilled.

Foliage and twigs residues from steam distillation were dried at 50^{o}C for about 48 hours. A 1g sub-sample of this material was oven dried at 103^{o}C to constant weight. Weight before and after drying was used to determine moisture content at the time of treatment. All pretreatments were done using 10% solids. The material (25g) was weighed and transferred in a 500mL flask, to which 225 mL of 1% sulfuric acid was added. The mixtures were then exposed to one of the following time and temperature conditions: room temperature for 48 hours; steamed for one hour at 121^{o}C; or steamed for three hours at 121^{o}C. Heat treatments were performed in an autoclave. Each variable was tested on three replicates for both foliage and branchwood.

After pretreatment, the mixtures were filtered. The filtrates were retained for reducing sugar measurement. The pretreated residue was then repeatedly washed in cold water (about 12 wash waters) to neutralize the acid, then dried at 50^{o}C and retained for subsequent hydrolysis and fermentation. Reducing sugar content of the filtrate was measured by the Dinitrosalicylic Acid (DNS) reagent method (Adney and Baker, 1996). The DNS reagent was prepared by mixing 400mL DI water, 2.6g of dinitrosalicylic acid, 5g sodium hydroxide, 2.0ml phenol, 76g sodium potassium tartrate, and 2g of sodium metabisulfite. Glucose was used as a standard. Three milliliters of the filtrate was weighed and transferred into test tube to which 3mL of DNS reagent was added. The tubes were then boiled in a water bath for five minutes. Absorbance was measured using a spectrophotometer set at 540nm. The effect of pretreatment was determined by the concentration of reducing sugars in the filtrate. Higher reducing sugar concentration indicated more effective pretreatment. The reducing sugars should be readily accessible for the fermentation to ethanol.

3.4. Enzymatic Hydrolysis

Conversion of lignocelluloses to ethanol involves two major steps; (1) hydrolysis of polysaccharides to simple sugars, followed (2) by microbial fermentation of these simple sugars to alcohol. Acid hydrolysis and enzymatic hydrolysis are the most common techniques used to hydrolyze cellulose (Mussatto and Teixeira, 2010). Acid hydrolysis

uses concentrated mineral acids as a catalyst, and is conducted at higher temperatures than enzymatic hydrolysis (Mussatto and Teixeira, 2010). In addition, concentrated acids are toxic, and the acid has to be recovered for it to be economically feasible (Sun and Cheng. 2002). Enzymatic hydrolysis is more specific, and is conducted under milder conditions. In this study, enzymatic hydrolysis was evaluated using a mixed cellulase enzyme to determine the enzymatic digestibility of pretreated steamed western juniper branches and foliage.

Accellerase 1500 enzyme was obtained from Genencor (Rochester, New York). This is an enzyme complex produced with a genetically modified strain of *Trichoderma reesei*, and contains both hemi-cellulase and beta-glucosidases. Enzymes were refrigerated until use. Enzyme activity in filter paper units per milliliter (FPU/mL) was determined before use.

3.4.1. Determination of lignin and total carbohydrates in foliage

Lignin and total carbohydrates content in foliage was determined by first extracting foliage tissues with 2:1 ethanol-toluene mixture and hot water to remove the extractives. The extractive free foliage was treated with 72% sulfuric acid followed by hot water digestion to hydrolyze the carbohydrates, according to ASTM standards D1106 and D1105 (ASTM 2007). The insoluble residue remaining after this series of

experiments was the estimated acid insoluble lignin. Total carbohydrates were estimated by subtracting the weight of lignin and extractives from the original oven dry weight of the sample.

One gram of dried foliage tissues was weighed and sealed in dried cellulose bags. The bags were placed in a soxhlet extraction apparatus consisting of a soxhlet extractor equipped with a condenser (Figure 3.5). An oil bath was used as the heating source.

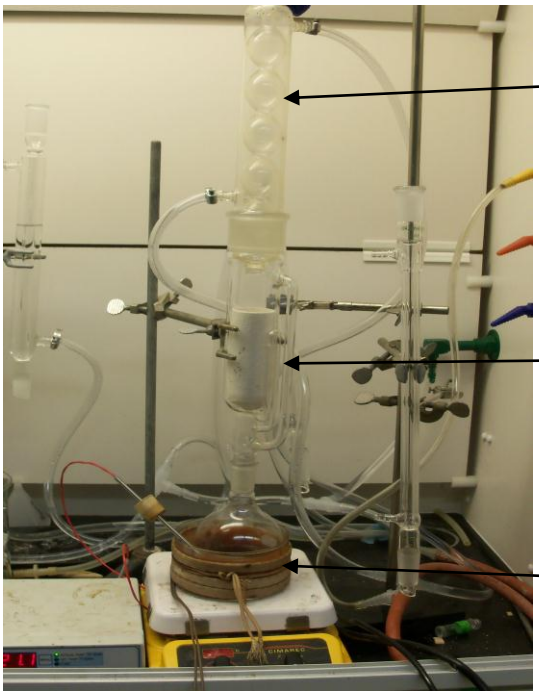

Figure 3.5. Soxhlet extraction set up to remove extractives from western juniper foliage.

The material was extracted for 6 hours in an excess of ethanol/toluene (sol/sol). The experiment was carried out in duplicate. After the extraction, the materials were

washed with 50mL of ethanol to remove residual toluene, and transferred to a 1000mL beaker. This material was digested with 400mL of hot water in a water bath for 3 hours. The material was then filter washed with 100mL of hot water, and dried at 105^{o}C to constant weight. Extractive content was determined by subtracting this weight from the original oven dry weight of the sample.

Additional samples subjected to extractive removal were dried at 50^{o}C for about 24 hours. The materials were then transferred into a 250ml beaker, to which 15mL of cold 72% sulfuric acid was added, and covered with aluminum foil. The beakers were allowed to stand for two hours at 20^{o}C in a waterbath, and then washed into a 1 liter beaker, to which 560mL of water was added to dilute the acid. This mixture was boiled for 4 hours in a water bath before being filtered into filtering crucibles, and oven dried at 105^{o}C to constant weight. The amount of lignin was estimated by subtracting the final weight from the initial oven dry weight of the sample.

3.4.2. Determination of enzyme activity

Enzyme activity was determined according to the National Renewable Energy Laboratory (NREL) standard procedure (Adney and J. Baker, 1996). This procedure used Whatman No. 1 filter paper as substrate to determine the dilution of original enzymes such that a 0.5mL aliquot of the dilution catalyzed 4% conversion of the substrate into

reducing sugars in 60 minutes. The original concentration of this dilution was used to calculate filter paper units (FPU) per milliliter of enzyme (FPU/mL = 0.37/concentration of enzymes). An enzyme filter paper unit is a micromole of reducing sugar produced by 1 ml of enzyme per minute.

Five enzyme dilutions (150, 200, 350, 500 and 750) were prepared in 0.05M sodium citrate buffer. Assay mixtures were prepared by placing a 1.0x6.0cm rolled filter paper strip in a test tube to which 1.0mL of sodium citrate buffer (pH 4.8) was added. The tubes were heated at 50^{o}C before the addition of 0.5mL of a given enzyme dilution (1.5mL total working volume). A reagent blank was prepared by adding 1.5mL of sodium citrate into a test tube. Enzyme controls were prepared by adding 1.0mL citrate buffer plus 0.5mL of enzyme dilution. One enzyme control was prepared for each dilution. A substrate control was prepared by adding 1.5mL sodium citrate buffer and a rolled filter paper to the tube.

Five concentrations (3.35mg/0.5mL, 2.5mg/0.5mL, 1.65mg/0.5mL,1mg/0.5mL) of glucose standard were prepared in DI water. Glucose standard tubes were prepared by adding 0.5mL of each enzyme concentrations and 1.0mL of citrate buffer in a test tube. Assay tubes, blank, controls and glucose standards were boiled in a water bath at 50^{o}C for 1 hour, then the reaction was stopped by adding 3mL of DNS reagent. All the tubes

were then boiled in a water bath for five minutes to react DNS with reducing sugars in solution, and then cooled in an ice water bath. About 0.2mL of this solution was then transferred into a cuvette and diluted with 2.5mL DI water. Absorbance was measured using a spectrophotometer set at 540nm. Glucose concentration was determined using a glucose standard curve.

3.4.3. Hydrolysis

Hydrolysis was done by slowly shaking scintillation vials containing assay mixtures, a substrate blank and an enzyme blank in an incubator set at 50^{o}C for 120 hours (Selig et al. 2008).

Assay mixtures were prepared by weighing about 0.27g of branchwood or 0.35g foliage in 20mL scintillation vials, to which 5mL 0.1M citrate buffer, 100µl 2% sodium azide solution and about 4.5mL DI water were added equating to a 10mL working volume. This corresponded to about 0.1g of cellulose per vial. The contents were heated at 50^{o}C before adding 0.12mL of Accellurase 1500 enzyme (60 FPU/g of cellulose loading). Substrate blanks were prepared by adding the same amount of biomass, 5mL buffer and 4.74 mL water in scintillation vials. The enzyme blank was prepared by adding 0.12mL cellulose enzymes, 5mL sodium citrate buffer and 4.88 mL DI water. Assay samples were run in triplicate. The vials were placed on a rotating shaker that was

incubated at 50^{o}C for 120 hours. The mixtures were then filtered, and the filtrate retained for glucose measurement.

Glucose concentration was measured using the DNS reagent method. The amount of cellulose digested (g), was calculated using the formula: glucose concentration * 0.9 * 10. Where, 0.9 was a collection factor for water added to cellulose upon hydrolysis and 10 was the total working volume in milliliters. Percent cellulose digestibility was then determined using the formula: Cellulose digestibility (%) = cellulose digested (g) /0.1 (g) *100

4.0 RESULTS

4.1 Steam Distillation

Oil yields from foliage were consistently higher than those from twigs (Table 4.1). Foliage yields ranged from 0.69% to 0.90%, while those from stems ranged from 0.09% to 0.18% on wet weight basis. Essential oil from foliage was greenish-yellow in color, with a strong burning odor. Essential oil from branches was pale yellow, with a mild taste.

Table 4.1. Essential oil yields from western juniper foliage steam distilled for 6-8 hrs.

Sample	% Yield	
	Foliage	Twigs
1	0.80	0.09
2	0.69	0.18
3	0.70	0.09
4	0.91	0.18
Mean	0.77	0.13

4.2 Bio-efficacy tests

4.2.1 Antifungal tests

Western juniper foliage oil was active against both the brown rot *(P. placenta*) and white rot (*T. versicolor*) fungi at all concentrations. Treated blocks exposed to the brown rot fungus lost about 1-3% weight, compared to 22-26% for the control blocks (Figure 4.1). Weight loss increased with increasing oil concentration. The highest weight losses (2.9%) were obtained from blocks treated with 30% oil, which was likely caused by chemical loss rather than biological attack. This was also evident from visual inspection of blocks after exposure. The 30% oil treatment completely inhibited mycelium growth, while mycelium grew on blocks treated with 10% oil (figure 4.2). These results suggest that the threshold for protection against *P.placenta* was below 10% (50 kg/m^3) oil.

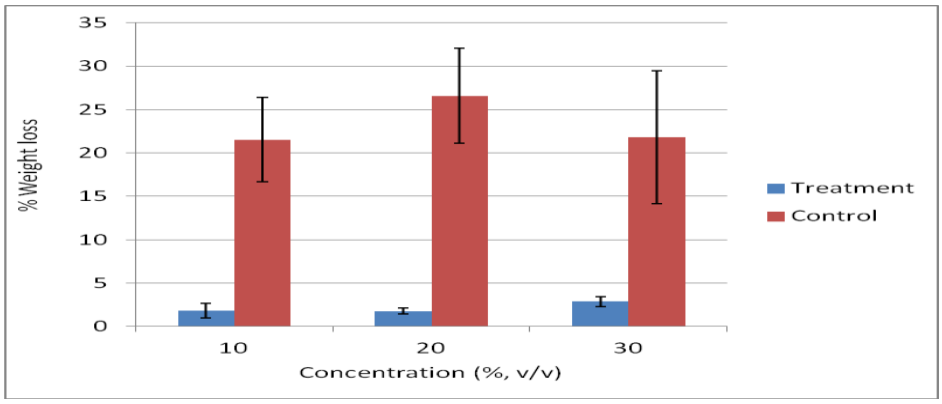

Figure 4.1. Weight losses (%) of oil -treated wood blocks exposed to *P. placenta* in an agar block decay test. Error bars represent one standard deviation.

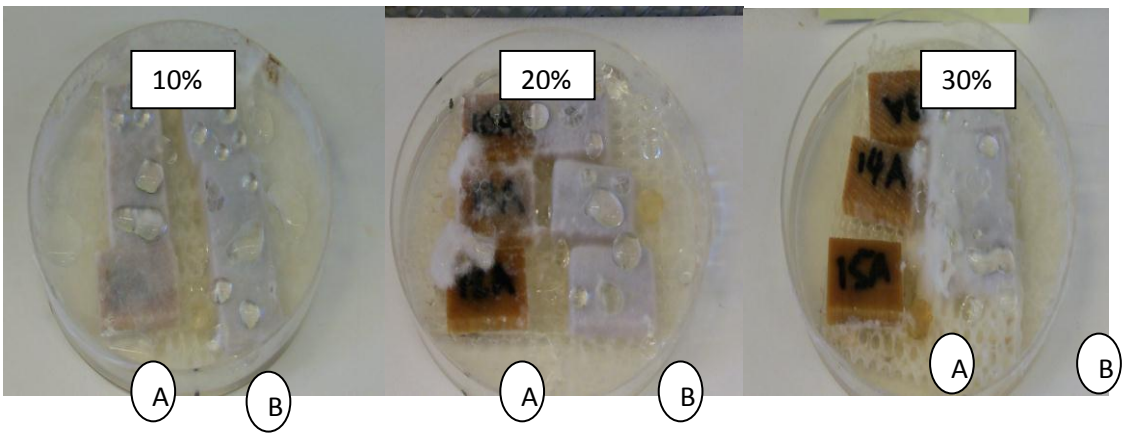

Figure 4.2. Test blocks treated with 10%, 20% or 30% (vol/vol) western juniper oil and exposed to *P. placenta*. A = treated blocks, B = controls.

Trametes versicolor was highly sensitive to western juniper leaf oil. No weight losses were observed in the treated or control blocks and mycelium growth was completely inhibited at all the concentrations (Figure 4.3).

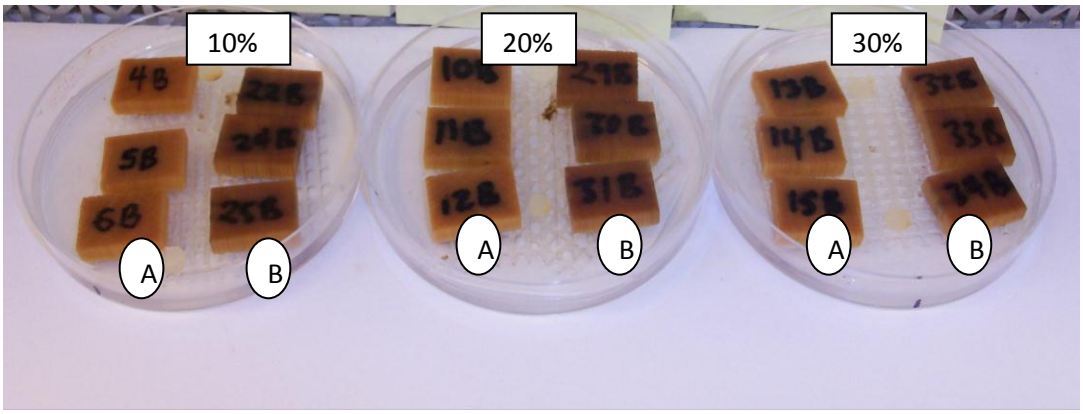

Figure 4.3. Test blocks treated with 10%, 20% or 30% (vol/vol) western juniper leaf oil and exposed to *T. versicolor*. A = treated blocks, and B = controls.

4.4.2 Termite tests

Western juniper foliage oil was active against termite attack at all concentrations tested. Ten percent juniper oil reduced weight loss of the blocks by 22% (Table 4.6) and about 79% of the termites were dead after four weeks, while only about 12% of the termites died in the control blocks (Table 4.6). The control blocks were heavily attacked, while light to moderate attack was observed in treated blocks (Figure 4.4).

Table 4.2. Wood weight loss, termite mortality and visual rating of blocks treated with 10, 20, or 30% juniper leaf oil and exposed to *R. flavipes* in an AWPA standard E1 test. Values are means of five replicates. Values in brackets are those of the controls for each dilution.

Juniper oil concentration (%)	Wood wt loss (%)		Mortality (%)		Visual rating*	
	Control	Treated	Control	Treated	Control	Treated
0	28.36	-	0	-	4	-
10	36.85	10.56	12	79	3.2	8.4
20	33.48	6.90	48	100	3.2	9.2
30	32.46	5.25	32	100	0	9.6

* Values range from 10 (no evidence of damage) to 0 (complete failure).

Termiticidal activity in blocks treated with 20% and 30% foliage oil was similar. In these higher concentrations, the oil reduced weight loss by 27%, and was 100% lethal, while about 90% of termites survived in the control blocks. Light attack was observed in treated blocks compared to heavy attack on the controls (Figure 4.4). The results suggest that the minimum amount of western juniper leaf oil that would prevent attack by *Reticulotermes flavipes* termites is between 10% and 20% (72kg/m3 – 148kg/m^3).

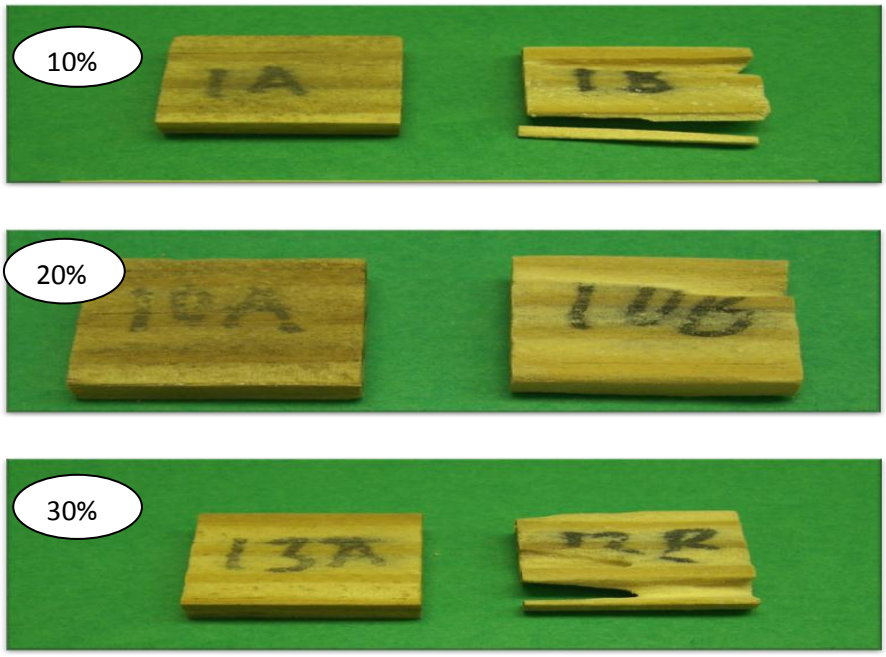

Figure 4.4. Wood blocks treated with 10, 20, or 30% (vol/vol) western juniper leaf oil (A) compared to non-treated controls (B) after exposure to *R.flavipes* termites in an AWPA standard E1 test.

4.3 Dilute Acid pretreatment

The highest sugar yield (0.26mg/mL) from foliage was obtained from samples treated at 121^{o}C for 1 hour (Figure 4.5). Longer pretreatment time (3 hours) at the same temperature produced lower sugar yields (0.23mg/mL), which suggested that the material had began to degrade. As expected, the lowest sugar yield (0.08%) was obtained at room temperature for 48 hours, which suggested that long reaction time produced no substantial improvement in on total sugar recovery. Sugar yields from branchwood were below the glucose standard range (0.1mg/mL – 6.7mg/mL), which suggested that none of the pretreatment conditions solubilized carbohydrates in branchwood.

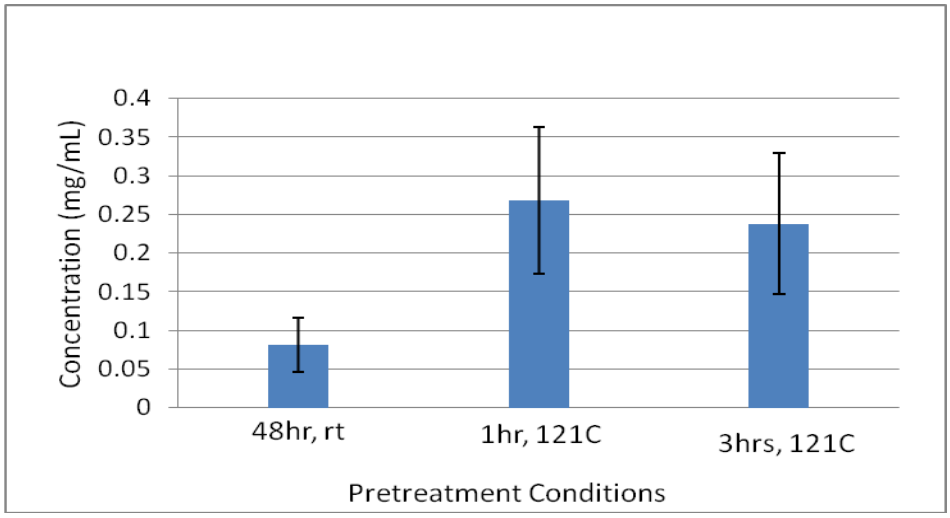

Figure 4.5. Effect of sulfuric acid pretreatment conditions on reducing sugar yield for foliage. Values represent means of three replicates, while error bars represent one standard deviation.

4.4 Enzymatic Hydrolysis

Dilute acid pretreated foliage and branchwood were digested with Accellerase enzyme (a complex preparation containing multiple enzymes) to determine their susceptibility to enzymatic digestibility. Percent cellulose digestion was calculated as the amount of glucose digested (g) per cellulose (g) added. The amount of glucose digested was an approximation based on total reducing sugars in the hydrolysate after hydrolysis, measured by DNS reagent. It was assumed that all the reducing sugars in the hydrolysate were glucose from cellulose hydrolysis. Activity of Accellerace enzymes was about 50 FPU/ml. Western juniper foliage consisted of about 35% carbohydrates and about 13% acid insoluble lignin. Extractives accounted for about 52% of the material. This is consistent with previously reported hexane and methanol extractive yields (45%) of ground western juniper foliage (Adams, 1987).

Total sugar yields from acid pretreated western juniper foliage ranged from 40% to 55%. These yields were higher than both the distilled control (30%) and non-distilled control (16%) (Figure 4.6). As expected, distilled material was more digestible than non-distilled material. The highest digestibility (55%) was obtained from the 1 hour acid-treated sample and was about 10% higher than yields obtained from 3-hour acid pretreated material. Sugar yields from materials heat pretreated for 3 hours (46%) were comparable to those from materials acid treated for 48hrs at room temperature (40%).

Room temperature pretreatment for 48 hours only improved the digestibility of foliage by about 5-10% (compared to the distilled control), suggesting that room temperature pretreatment did not markedly improve foliage digestibility.

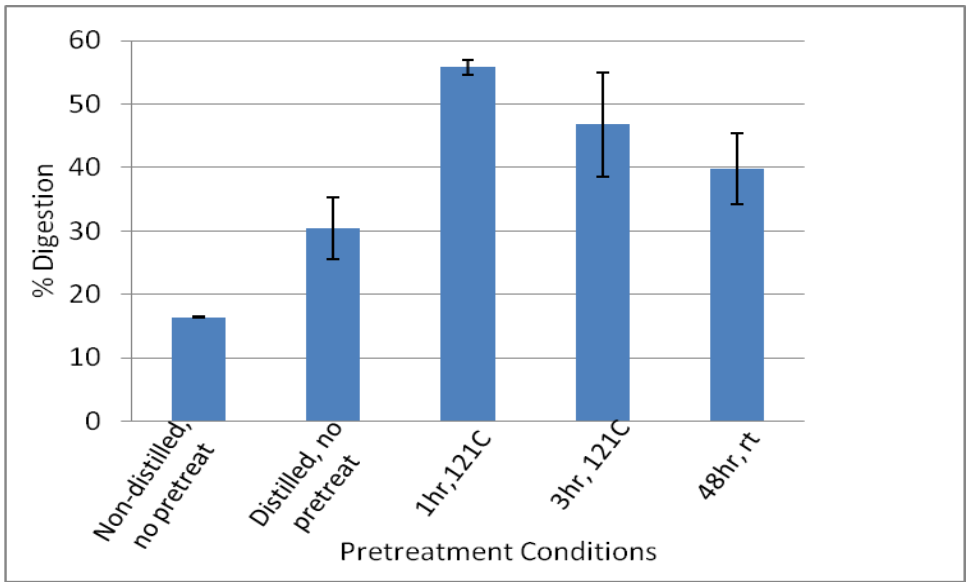

Figure 4.6. Total reducing sugar yields from western juniper foliage subjected to various pretreatment conditions as measured by DNS method. Values represent means of three replicates, while error bars represent one standard deviation.

Unexpectedly, the best branchwood digestion (24%) was obtained from the non-distilled, untreated control (figure 4.7). Yields from the distilled control and all the acid pretreated material were similar (12-13%) and were 11% lower than those obtained from non-distilled, non-pretreated control. This suggested that: (1) that steam distillation negatively affected branchwood digestibility and (2) 1% sulfuric acid

pretreatment of distilled branchwood, under the conditions tested had no effect on enzymatic digestibility. Three experiments were conducted to further explore these observations. In the first experiment, non-distilled branchwood was pretreated and digested with enzymes. The goal was to compare the digestibility of pretreated and non-pretreated material. The hypothesis tested was that non-pretreated material was more digestible than the pretreated material. The effect of drying at 50^{o}C (before and after acid pretreatment) on digestibility of branchwood was also investigated. Wet and dry materials were pretreated with 1% sulfuric acid at 121C for one hour at 15 psi, and then digested with enzymes. Non- pretreated material was used as the control. Experiments were performed in duplicate.

In contrast to distilled branchwood, pretreated material was more digestible (22%) than non-pretreated control (20%) (Figure 4.8). Although the differences were not large, this result reinforced the suggestion that steam distillation negatively affected branchwood digestibility. Sugar yields from dry and wet material were the same (22%), indicating that drying had no effect on material digestibility.

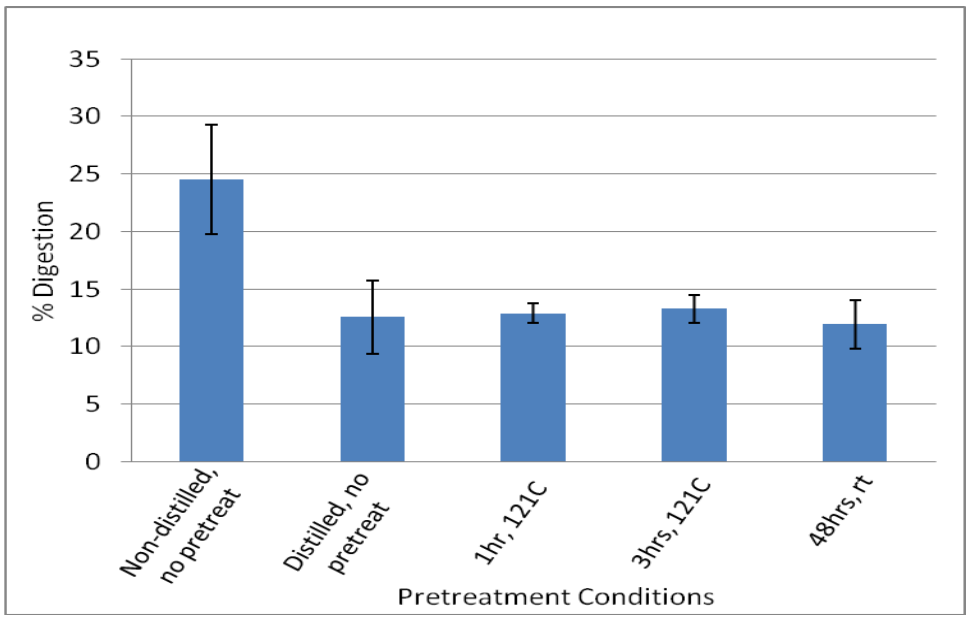

Figure 4.7 Total reducing sugar yields from western juniper branches subjected to various pretreatment conditions as measured by DNS method. Values represent means of three replicates, while error bars represent one standard deviation.

In the second experiment, the effectiveness of long term pretreatment (3 hours) and short term (1 hour) 1% sulfuric acid pretreatment on digestibility of undistilled branchwood were compared. Pretreated material was digested with enzymes for 48 hours. Sugar yields from the materials exposed to the two treatment conditions were comparable, but in contrast to distilled branchwood, long term pretreatment was about 3% more digestible than short term pretreatment (Figure 4.8). This also indicated that branchwood required harsher pretreatment conditions than foliage.

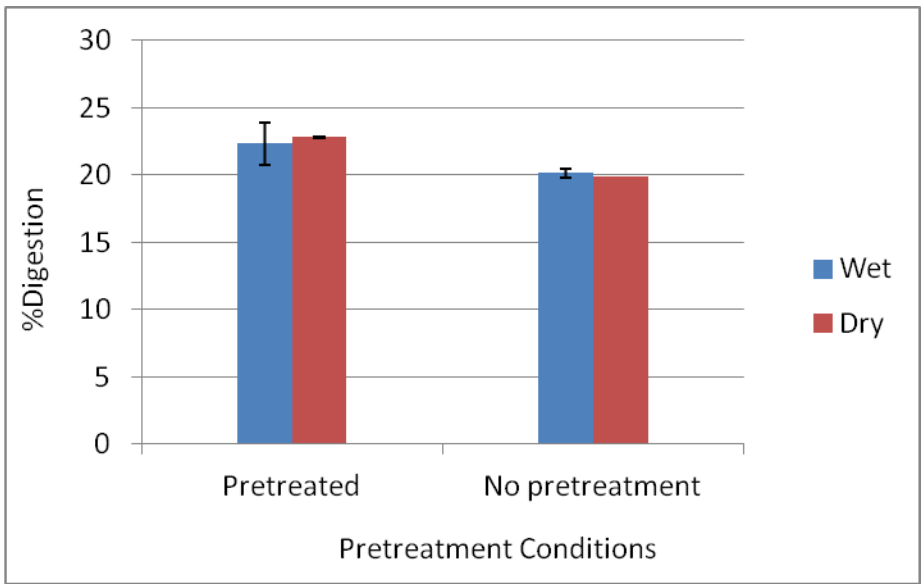

Figure 4.8. Total reducing sugar yields from enzymatic digestion western juniper branchwood pretreated with 1% H_2SO_4 acid at 121°C for 1 hour. Error bars indicate one standard deviation.

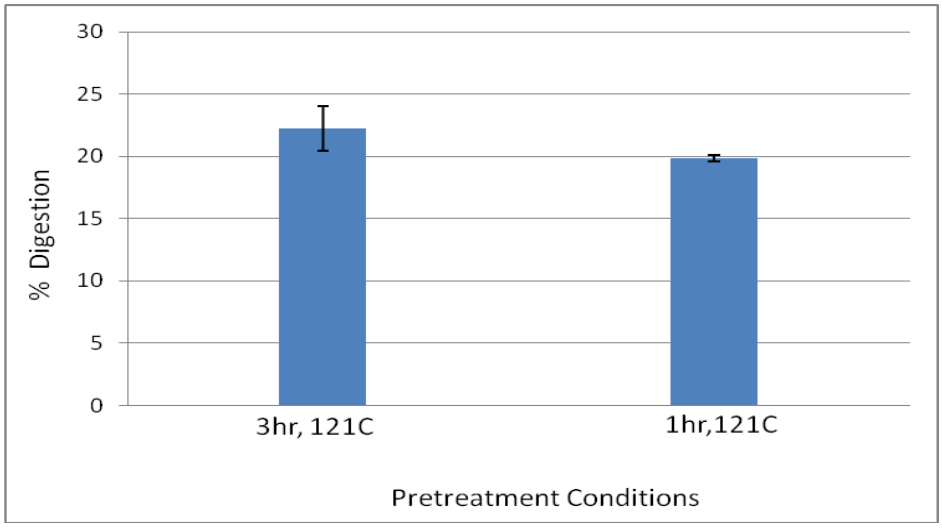

Figure 4.9.Total reducing sugar yield from enzymatic digestion of non-distilled western juniper branchwood pretreated with 1% H_2SO_4 at 121°C for 1 or 3 hours. Error bars represent one standard deviation.

The third experiment was performed to determine whether steam distillation had a similar (negative) effect on foliage digestibility. Non-distilled foliage was pretreated with 1% sulfuric acid at 121^{o}C for 1 or 3 hours and then digested with enzymes. The hypothesis tested was that non-distilled foliage was more digestible than steam distilled material. At the same pretreatment conditions, non-distilled foliage was less digestible (40-43%) than distilled material (46-55%), suggesting that steam distillation positively affected foliage digestibility (Figure 4.9). It was also interesting to note that like the distilled material, 1 hour pretreated material was slightly more digestible (45%) than material treated for 3 hours (41%).

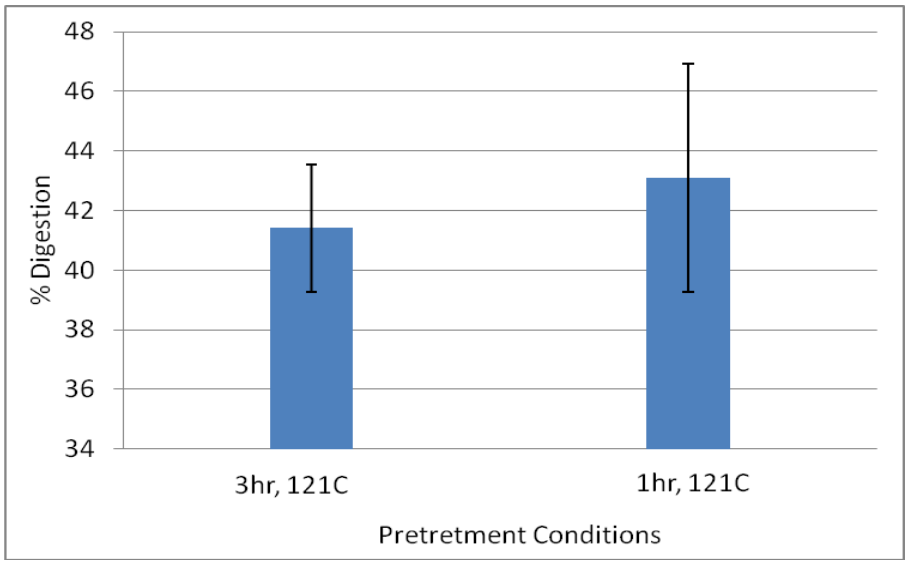

Figure 4.10. Total reducing sugar yield from foliage pretreated with 1% H_2SO_4 at 121^{o}C for 1 or 3 hours. Error bars represent one standard deviation.

Results of these tests indicated that: (1) steam distillation negatively affected enzymatic digestibility of western juniper branchwood, and positively affected foliage digestibility, and (2) branchwood required harsher pretreatment conditions than foliage.

5.0 DISCUSSION

5.1 Steam Distillation

Essential oils in plant materials are held in intercellular spaces and can be extracted by several methods including solvent extraction, hydro-distillation, and steam distillation Sjostrom 1981; Bakkali et al. 2008). High molecular weight extractives such as fats and phenolic compounds are extracted by organic solvents like ethanol, acetone and hexane, while tannins, inorganic salts and certain carbohydrates can be extracted by water (Sjostrom 1981). Volatile components of extractives, consisting mainly of monoterpenes and sesquiterpenes, can be extracted by steam, and are called essential oils because of their distinctive aroma. Yields and composition of essential oils varies among species, within different parts of the same tree, and among trees from different geographical locations (Rowell 2005; Sjostrom 1981; Adams, 1987). Seasonal variations in yields and composition of essential oils also have been reported in some species (Adams 1987; Vasek and Scora 1967). Yields also vary with the method of extraction used. A few studies have been conducted on yields of essential oils from western juniper from different parts of United States. Table 5 summarizes previous studies on yields of essential oils from western juniper from different parts of Oregon.

Table 5. Yields of essential oils from western juniper from Oregon

Source	Collection Site	Month Collected	Tree part	Distillation time (hrs)	% yields
Burnet (1954)	Prineville	December	Heartwood (top)	24	1.09
			Heartwood (butt)	24	2.26
Kurth and Ross (1954)	Prineville	May	Heartwood	4-6	1.4
Adams (1987)	Juntura	February	Heartwood	20	2.33
Yesenofski (1996)	Warm Oprings	November	Heartwood	1-4	0.4
			Foliage	1-4	0.4

Essential oil yields vary within different parts of the tree (Burnet, 1954). Seasonal variation in yields is evident from lower yields reported from material collected in May (Kurth and Ross, 1954), compared to yields from material collected from the same site in December (Burnet (1954). In addition, Yesenofski (1996) reported lower yields (0.04-0.32%) from materials collected in June, compared to the yields from materials collected in November (0.4%). Heartwood oil yields reported by Adams (2.33%) were comparable to those obtained by Burnet (2.26%) from material collected during the same season. Low yields reported by Yesenofski (1996) from shorter

distillation times (1-4hrs) and larger batches (~100kg) suggest that distillation time and batch size also affected yields. The purpose of this study was to determine essential oil yield from foliage and small branches (20-50mm) of western juniper from Sisters, Oregon.

Essential oil yield from foliage were relatively high (0.77% on wet weight basis). Yields could be higher if optimal loading was employed. Yields did not always correspond to the amount of biomass loaded in the column, that is, higher loading did not always result in higher essential oil yields. For example, more oil was obtained from a 2300 g batch than from a 2700 g batch (20 g vs. 18 g respectively). The same amounts of oil (19 g) were also obtained from 2400 g and 2700 g batches. This observation suggests that an optimal loading can maximize yield. Additional biomass did not result in corresponding higher oil yields. One possible explanation is that overpacking the distillation column with biomass while using the same steam pressure resulted in incomplete biomass distillation. Twigs associated with foliage also affected oil yield. These twigs did not contribute substantial amounts of oil, but contributed to the weight of extracted material. While yields could have been higher if the twigs were removed, commercial operations would not completely separate leaves from twigs.

Essential oil yields from foliage in this study were higher than those reported by Yesenofski (1996). Yesenofski used a specially designed apparatus to separate oil from the oil-water mixture. In this study, oil was purified by simply decanting oil from oil-water mixture, and it is likely that oil was not completely separated from water. The previous work also used larger batch sizes (90 to 100 kg) compared to those used in this study (2 to 4 kg). However, average distillation time in the previous study was 2 hours, while the average distillation time in this study was 6 hours. Lastly, the materials were collected from different sites.

Essential oil yields from foliage obtained in this study were lower than juniper foliage extractive yields (15.74% hexane extracts, 29.60% ethanol extract) (Adams 1987). One explanation for the huge difference in yield is that steam distillation extracts only the volatile components - mainly the monoterpenes, sesquiterpenes and sesquiterpenoids along with water soluble carbohydrates and tannins (Rowell, 2004). This leaves high molecular weight terpenes and aliphatic components that make up the majority of extractives removed using organic solvents.

Oil yield from branches was low (0.13% wet weight), corresponding to about 0.19% on dry weight basis (based on the 71% dry weight of branches). Extractive content in a tree decreases from the bottom of the tree upwards (Sjostrom, 1991).

Burnet (1954) also reported lower yields (1.09%) from 150mm diameter branches, compared to those from 400mm diameter logs (2.26) from the same trees. Branchwood is composed mainly of juvenile wood (Bowyer et al. 2007), which has a lower proportion of heartwood. In addition, branches used in this study were not debarked, and the higher proportion of bark in branchwood might have contributed to lower yields from branches (Bowyer et al. 2007). Kurth and Ross (1954) also observed reduced yields from western juniper chips steamed with bark compared to those distilled without bark. Any commercial distillation will likely use branchwood with bark because western juniper logs (and branches) are difficult to peel and the process would add costs. Therefore, these results show that western juniper foliage is a candidate for essential oil production because yields were relatively high and the material required little pre-processing. Further testing would be essential to optimize process conditions to maximize yields. Conversely, small branches were poor candidates for essential oil extraction. Oil yield was very low, and the material required size reduction before distillation, which added to the potential operation costs.

5.2 Bioefficacy tests

Essential oils play an important role in the protection of the plants against bacteria, fungi, insects and other organisms (Bakkali, 2008). Identifying the active oil components can contribute to the development of more environmentally benign wood

protection systems (Wang et al. 2005). Biological properties of the extractives and their abundance differ according to the method of extraction, species, plant organ, climate, soil composition and season (Bakkali, et al. 2008).

Previous studies on biological properties of western juniper focused on essential heartwood oils and solvent extracts of heartwood and leaves. Essential oil from western juniper heartwood has been shown to have antibacterial activity (Johnson et al. 2001). Hexane extracts of western juniper leaves have been shown to be active against *Cryptococcus neoformans* (Clark et al. 1990). Adams (1987), found that subterranean termites (*Reticulitermes flavipes)* could not survive on western juniper heartwood sawdust, nor could they survive on filter paper treated with hexane or methanol extracts of western juniper heartwood sawdust. He also reported high termiticidal activities in the hexane extracts of western juniper leaves. These studies did not test antifungal nor termiticidal activities of essential oil of western juniper foliage.

5.2.1 Antifungal Activities

Western juniper foliage oil was active against brown and white rot fungi (*P.placenta* and *T. versicolor* respectively) at all the concentrations tested. Weight losses in treated blocks exposed to *P.placenta* increased with increasing concentration, which reflected weight losses caused by chemical loss rather than fungal attack. It was

assumed that conditioned treated blocks reached the same equilibrium moisture content before and after exposure to fungi. It is possible that blocks did not reach the same equilibrium after exposure due to chemical losses, and this could cause weight losses in blocks.

T. versicolor was much more sensitive to western juniper leaf oil than was *P. placenta*. Microbial sensitivity to essential oils differs according to species (Andrews et al. 1980). Clark et al. (1990) found high antifungal activities in hexane extracts of western juniper foliage against *C. neoformans and S. cerevisiae,* while no activity was found in methanol extracts. On the other hand, high antifungal activity was observed against *T. mentagrophytes* in the methanol extracts. This suggested that non-polar components were active against *C. neoformans and S. cerevisiae*, while the more polar components were active against *T. mentagrophytes*. Differences in sensitivity to western juniper foliage oil between *T. versicolor* and *P. placenta* observed in this study may also be attributed to different active components. No antifungal activity against *T.versicolor* was observed with monoterpenes (a-pinene, b-pinene, p-myrcene and limonene) (Cheng et al. 2004), while α-cardinol and T-muurolol were both active. The antifungal activity of western juniper foliage oil against *T.versicolor* observed in this study may be attributed to monoterpenoids (such as sabinol) present in the oil. Monoterpenes (A-pinene, limone and p-cymene) are also present in western juniper

foliage essential oil and have been found to have antifungal activities (Filipowicz et al. 2003). These components could be active against *P. placenta*, but further tests would be required to ascertain the identity of the active components.

Terpenes inhibit fungal growth by disrupting the cytoplasmic membrane. Microbial sensitivity to terpenes varies according to cellular structure (Andrews et al. 1980). The difference in sensitivity to western juniper foliage oil between *P. placenta* and *T. versicolor* may reflect structural/physiological differences that render membranes less sensitive to specific oil components.

5.2.2 Termiticidal Activities

Western juniper foliage oil was active against *R. flavipes* at all concentrations tested. Oil retentions between 72kg/m^3 and 148kg/m^3 (10%-20% dilution) effectively prevented termite attack.

Termiticidal activities of western juniper foliage oil can be attributed to the monoterpernes which form the majority of foliage essential oil (Tatrol et al. 1973). Adams (1987) found high termiticidal activities in hexane extracts of western juniper foliage, while methanol extracts were less active. This indicated that the non-polar components were more active. Termiticidal activity by monoterpenes (β-myrcene, α-pinene, β-pinene and limonene) against termites (*Coptotermes formosanus)* has also

been reported (Cheng et al. 2004). Terpenes permeabilize the mitochondrial membranes, eventually leading to cell death (Bakkali et al. 2008). Further evaluation would be needed to ascertain the identity of the active compounds in western juniper foliage essential oils.

The results show that essential oils of western juniper foliage may be useful for controlling wood decay fungi and termites. Further research to determine the active components, modes of action, and environmental and human health issues would be necessary to determine if these oils have practical use for protecting wood. These tests were performed on unpurified oil, and isolation and purification of the active constituents may result in more lethal products. Western juniper foliage tissues are readily available from western juniper harvesting and are a potential source of bio-based fungicides or termiticides.

5.3 Dilute acid pretreatments

Sugars necessary for fermentation in lignocellulosic materials are trapped inside the lignocellulosic matrix structure of cellulose, hemicelluloses and lignin (Rowell, 2005). Pretreatment disrupts this structure and enhances enzyme access to cellulose. It converts lignocellulosic biomass from its native matrix form which is “recalcitrant to cellulase enzyme systems, into a form for which cellulose hydrolysis is more effective”

(Zheng et al. 2009). Pretreatment improves the digestibility of lignocellulosic material by breaking down the shield formed by lignin and hemicelluloses, disrupting the cellulose crystalline structures or reducing the degree of cellulose polymerization. Pretreatment methods can be grouped into three categories – physical, chemical and biological pretreatments (Zheng et al. 2009). Chemical pretreatment uses chemical agents like dilute mineral acids, alkaline and organic solvents. Dilute sulfuric acid is the most common acid based pretreatment method used, because it is relatively inexpensive and effective (Marzialetti et al.2008). Dilute acid breaks down the hemicelluloses and increases the porosity of the lignocellulosic material to improve enzyme access.

Essential oil yields from steam distillation of western juniper are low (1-2%) (Adams 1987; Kurth and Ross 1954), making steam distillation alone less competitive. Finding alternative uses for solid residues from steaming could improve process economics. One option is digestion of residues to produce fermentable sugars. This option is advantageous because steaming is a form of pretreatment, which could reduce other pretreatment costs. In this study, dilute sulfuric acid pretreatment of western juniper foliage and branchwood residues from steam distillation was performed using different times and temperatures. The goal was to improve the subsequent enzymatic digestibility of these residues.

Heating at 121^{o}C for 1 hour was the most effective treatment for hydrolyzing western juniper foliage. The yields decreased with increasing time at the same temperature. This can be attributed to time-dependent thermal degradation of sugars. Dilute sulfuric acid hydrolyses hemicelluloses to release sugars, and can continue to decompose those sugars to furfural and other degradation products (Hu et al., 2008). Marzialetti et al. (2008) also observed maximum yields of hemicellulose sugars from dilute sulfuric acid pretreatment of loblolly pine, with yields decreasing with time as the released sugars were subsequently degraded. Similar observations were reported from dilute sulfuric acid pretreatment of corn stover (Lloyd and Wayman, 2005), where hemicelluloses sugar yields initially increased with time and then decreased due to subsequent degradation of free sugars. As expected, the lowest sugar yield was obtained at room temperature for 48 hours, probably because of the low reaction temperature. Dilute sulfuric acid hydrolysis requires high temperatures (120C – 200C) to achieve acceptable rates of carbohydrate solubilization (Galbe and Zacchi 2002). Long reaction time had no substantial effect on carbohydrate solubilization. However, it was not possible to determine optimum pretreatment conditions for western juniper foliage from these data. Further evaluation would be needed to determine the maximum yield conditions. Short exposures (30-45 minutes) to high temperature (~160C) generally produce effective pretreatment conditions for dilute sulfuric acid pretreatment of lignocellulosic biomass (Marzialetti 2008; Lloyd and Wyman, 2005).

Sugar yields from branchwood were below the detection limit (0.1– 6.7mg/mL), which indicated that 1% dilute sulfuric acid treatment at either 121C or room temperature was not sufficient to solubilize carbohydrates. This may reflect the high lignin (35%, dry weight) content of western juniper wood which creates a physical barrier to the carbohydrates. The complex structure of softwoods makes them recalcitrant to enzyme attack after dilute acid hydrolysis (Galbe and Zacchi, 2002). Another possible cause for low yields was that the branches were not debarked. Bark acts as physical barrier to treatment, and this is especially true for branchwood which has relatively higher bark content (Bowyer et al. 2005). Generally, acid-catalyzed steam pretreatment has been shown to be an effective pretreatment method for softwoods (Nguyen et al. 2000; Soderstrom et al. 2002). Furthermore, steam distillation, which is also a form of pretreatment, should open up the wood structure and improve the digestibility of material. Further evaluations would be needed to determine the optimum pretreatment conditions for western juniper branchwood.

5.4 Enzymatic Hydrolysis

Lignocellulosic biomass consists mainly of cellulose, hemicelluloses and lignin (Sjostrom 1981). Polymer composition varies between softwoods and hardwoods and among species. Softwoods generally contain 40–45% cellulose, 7-14% hemicelluloses and 26-34% lignin (Rowell, 2005). Cellulose and hemicelluloses are sugar-based

polymers. Cellulose is a homopolymer containing only glucose sugar, while hemicelluloses are mixtures of heterogeneous polymers mainly composed of arabinose, galactose, glucose, mannose and xylose along with other minor components (Mussatto and Teixeira, 2010). Both cellulose and hemicelluloses can be broken down to their respective free sugars (hydrolysis), which can then be fermented by microorganisms to produce alcohol and other products. Hydrolysis can be catalyzed by either acids or enzymes. Acid hydrolysis is performed under harsh conditions (~160C, 20-30% acid), and can cause equipment corrosion problems. The process produces sugar degradation by-products, mainly furfural and hydroxylmethyl furfural, which can inhibit subsequent fermentation of sugars (Shahbazi and Zhang. 2010; Mussatto and Teixeira, 2010). Excess acid must also be recovered for the process to be economical (Sun and Cheng. 2002). Enzymatic hydrolysis has advantages over acid hydrolysis: it is more efficient, is performed under mild conditions (45-50^{o}C, pH 4.8-5.0), is highly specific, requires lower energy and gives high yields of glucose with low formation of by-products (Mussatto and Teixeira, 2010; Ballesteros, 2010). Enzymatic hydrolysis also has some drawbacks. It is a slow process and the enzymes are relatively expensive (Taherzadah and Karimu 2007). However, it is predicted that advances in enzyme production will improve the process economics (Ballesteros, 2010). The enzymatic approach is therefore a more promising method for commercial hydrolysis of lignocellulosic biomass.

Factors affecting enzymatic hydrolysis include structure of the substrate, lignin and hemicelluloses content, cellulose crystallinity, degree of cellulose polymerization, enzyme activity and operating conditions (Taherzadah and Karimu 2007; Ballesteros, 2010). Lignin and hemicelluloses act as physical barriers to cellulose, making enzyme access to cellulose difficult. Lignin also adsorbs the enzymes, reducing the amount of enzyme available to attack the cellulose. Cellulose crystallinity also limits enzyme access to cellulose (Pejo et al. 2008; Ballesteros, 2010). Pretreatment before enzymatic hydrolysis is essential to overcome these substrate-related challenges.

Enzymatic hydrolysis of softwoods has been studied on a number of species including balsam-fir (Jensen et al.2010), spruce (Bosch et al.2010; Soderstrom et al. 2002), pine (Wayman et al. 1986), Douglas-fir, (Nguyen et al. 1998) white fir, and ponderosa pine (Nguyen et al. 2000). Enzymatic hydrolysis of western juniper branchwood and foliage has not been studied. In our study, steam distilled, 1% sulfuric acid pretreated foliage and branchwood were digested with enzymes to determine enzymatic digestibility of these residues.

Steam distillation alone improved sugar recovery yield in foliage by about 10%. This was expected since steam hydrolyzes some hemicelluloses and opens up the lignocellulosic matrix, thereby increasing the surface area for enzyme access (Zheng et

al. 2009). The same mechanism is applied in steam pretreatment, except that steam pretreatment employs higher pressures. Hemicelluloses hydrolysis during steam pretreatment results from the combined action of acetic acid (and other acids) formed from acetyl groups in the hemicelluloses (autohydrolysis) and the acidic properties of water at high temperatures (Zheng et al. 2009). Sulfuric acid (1%) pretreatment for 1 hour was most effective, with more than 55% of the carbohydrates converted to free sugars. Sugar yield from the material pretreated for 3 hours was lower (40%) than that obtained from the 1 hour pretreatment. The harsh pretreatment conditions (121^{o}C, 3 hours) may have caused part of cellulose to be hydrolyzed during pretreatment, thus reducing the sugar potential of the remaining material. Weiqi et al. (2012) also reported a similar trend in enzymatic hydrolysis of dilute acid pretreated eucalyptus chips. They observed reductions in glucose yield with increasing pretreatment time. It is likely that hemicelluloses hydrolyzed during dilute acid pretreatment exposed the cellulose, which could also be partly hydrolyzed as pretreatment time increased.

Room temperature acid pretreatment for 48 hours only increased digestibility by about 5-10% (compared to the distilled control). This was not surprising because dilute acid hydrolysis requires high temperatures (120C – 200) to achieve acceptable rates of carbohydrate solubilization (Galbe and Zacchi 2002). Long reaction time had no noticeable effect on sugar recovery.

Overall, the highest sugar yield was obtained from foliage pretreated in acid for 1 hour at 121^{o}C. However, it was not possible to identify conditions for producing the maximum yield from this data. Further research would be required to determine the optimum pretreatment method for solubilizing western juniper foliage.

The results suggest that steam distillation negatively affected digestibility of branchwood. The non-distilled control material was about 10% more digestible than either acid pretreated or non-pretreated distilled material. Sulfuric acid pretreatment at all conditions tested did not appear to reverse the negative effects of steam distillation since total sugar yields from both acid-pretreated and non-pretreated distilled controls were about the same (11-13%). In a follow up experiment of enzymatic digestion of non-distilled, acid pretreated branchwood, sugar yields from the treated material (24%) was greater than that obtained from the non-pretreated control (20%). Although the difference was not large, these results also strengthened the suggestion that steam distillation negatively impacted western juniper branchwood digestibility. The results also indicated that, unlike branchwood, steam distillation positively affected foliage digestibility. Distilled foliage was more digestible (46-55%) than the non-distilled foliage (41-43%) under the same pretreatment conditions.

The negative effect of steam distillation on enzymatic digestibility of branchwood (which has high lignin content) may have been due to redeposition of lignin on cell wall surfaces during steam distillation. Donohoe et.al (2007) found that high temperature pretreatment of lignocelluloses under both acidic and neutral conditions, caused lignin to "coalesce into larger molten bodies that migrate within and out of the cell wall, and can redeposit on the surface of plant cell walls". They also reported that re-deposited lignin primarily accumulated in the pits and cell corners – zones that are important for the transport of pretreatment solution and enzymes through biomass structure – which can create physical barriers that inhibit access to carbohydrate molecules. Lignin re-deposition can negatively impact biomass saccharification (Selig et al. 2007). A specific melting point range for lignin imbedded in the matrix of lignocellulosic has not been found, but Selig et al.(2007) stated that it could be within the temperature range used during the pretreatment process (120C-200) or below. It is possible that the harsh steam distillation conditions (~100^{o}C, 6-8 hours) caused lignin in branchwood to undergo this transition and re-deposit on carbohydrate molecules, creating a physical barrier to both pretreatment solution and enzymes. This lignin transition could positively impact hydrolysis of lignocellulosic material because the coalescing exposed the carbohydrates to both pretreatment solution and enzymes (Jensen et al. 2010). This may have been the case with foliage, which has a much lower

lignin content (~13%) compared to wood material (35%). Further studies would be needed to confirm these observations.

Branchwood required harsher pretreatment conditions than foliage. In contrast to foliage, sugar yields from branchwood were higher in 3 hour pretreated material than material treated for 1 hour. Sugar yields from branchwood exposed to the same pretreatment conditions were about half that of foliage. This can also be explained by higher lignin content of western juniper wood compared to foliage. Similar observations were reported for enzymatic hydrolysis of balsam fir, a softwood with a high lignin content (35%) (Jensen et al. 2010). Balsam fir, aspen (a hardwood with a lignin content of 26.69%), and switchgrass (21.36% lignin) were pretreated with 0.25-0.75% sulfuric acid at 150-170C. Total sugar yield from balsam fir was only 21.1% compared to 88.38% and 97.6% from aspen and switchgrass, respectively. The extremely low sugar yield from balsam fir was attributed to the high lignin content. High lignin content along with a characteristic rigid structure cause softwoods to be more resistant to enzymatic attack after dilute acid hydrolysis since the “structure remains recalcitrant” (Galbe and Zacchi, 2002). Acid-catalyzed steam pretreatment has been shown to be an effective pretreatment method for softwoods (Nguyen et al. 2000; Soderstrom et al. 2002). It is also likely that the bark acted as physical barrier to enzymes since the branches were

not peeled. Additional studies would be needed to determine the optimum pretreatment conditions for western juniper branchwood with and without bark.

Previous studies have reported much higher sugar yields from enzymatic hydrolysis of softwood species than those found in this study. About 90% glucose was recovered from sulfuric acid-catalyzed steam pretreated Douglas - fir (Nguyen et al. 1998). Nguyen et al. (2000) reported about 82% sugar yield from enzymatic hydrolysis of acid-catalyzed, two-stage pretreated mixture of white fir and ponderosa pine; and 77% total sugar was obtained from two-stage, sulfuric acid-catalyzed steam pretreated spruce (S'oderstr'om et al. 2002). This difference is expected because of the different materials used; the inclusion of bark in this current study; and the different pretreatment methods and conditions.

6.0 CONCLUSIONS

Essential oil yields from foliage were relatively high (0.77% wet weight) and the material required little preprocessing. Yields obtained in this study were higher than those from previous studies (Yesenofski 1996), possibly because smaller batch sizes and longer extraction times were used. Yields from twigs were very low (0.13% wet weight); much lower than yields reported for western juniper stem (1.2-2.33%). (Adams 1987; Kurth and Ross 1954). This result agrees with previous findings that noted decreasing oil yields with increasing distance from the base of the stem (Burnet, 1954).

Leaf oil showed high antifungal activities against *P. placenta* and *T. versicolor*. *T. versicolor* was more sensitive to the oil than *P. placenta*. This could be due to their structural differences, as different organisms respond differently to essential oils. This study, however, could not determine the threshold for protecting against the two fungi. Additional tests would be required to determine the thresholds, as well as to determine the identity of active components. Leaf oil was also active against termites (*Reticulitermes flavipes*). Oil retentions of 72kg/m3 -148kg/m3 effectively prevented termite attack.

Steam distillation alone improved enzymatic hydrolysis of foliage residues by about 10%. Steam hydrolyses some hemicelluloses and opens up the lignocellulosic matrix, thereby increasing the surface area for enzyme access. Heating at 121^{o}C for 1 hour was the most effective pretreatment for enzymatic hydrolysis of foliage. Sugar yields decreased with increasing pretreatment time at the same temperature. The harsh pretreatment conditions (121^{o}C, 3 hours) may have caused part of the cellulose to be hydrolyzed during pretreatment, thus reducing the sugar potential of the remaining material. Similar observations have been reported on enzymatic hydrolysis of eucalyptus wood (Weiqi et al. 2012). These were preliminary tests; further tests on larger sample sizes would be necessary to determine the optimum pretreatment conditions for western juniper foliage hydrolysis. Since fermentation of the sugars produced was not performed; further tests would be required to determine the fermentability of these sugars. Sugar yields from enzymatic digestion of branchwood was low (10-25%), which reflected high lignin content (35%) in branches. Lignin acts as a physical barrier to both the enzymes and treatment solution. The results also suggest that steam distillation negatively affected enzymatic digestion of branchwood. This was probably due to redeposition of lignin on cell wall during steam distillation, which creates a physical barrier to both the treating solution and the enzymes. Similar observations were observed on enzymatic digestion of dilute sulfuric acid pretreated maize stems (Selig et al. 2007). Further tests would be needed to confirm these observations.

This study shows that there is potential for utilization of western juniper foliage. Essential oils from readily available western juniper foliage may be useful for controlling wood decay fungi and termites. Western juniper foliage may also be digested to simple sugars for subsequent fermentation to ethanol or other products.

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