EXTRACTION AND PURIFICATION OF PODOPHYLLOTOXIN FROM EASTERN RED CEDAR (*JUNIPERUS VIRGINIANA* L.) IN OKLAHOMA

By

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Title of Study: EXTRACTION AND PURIFICATION OF PODOPHYLLOTOXIN FROM EASTERN RED CEDAR (*JUNIPERUS VIRGINIANA* L.) IN OKLAHOMA

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Abstract: Podophyllotoxin (ptox) is a pharmaceutical derived from plants used in a broad spectrum of medicinal treatments ranging from inflammatory diseases to cancer. Originally sourced from the rhizomes of the now endangered Indian mayapple (*Podophyllum emodi* Wall.), ptox can also be found in the foliage of eastern red cedar (ERC). Ptox has been extracted from the foliage of ERC for analytical purposes (Renouard et al., 2011) but no effort has been documented to extract at commercial scale, nor have any attempts to purify ptox from the ERC foliage extract been attempted. This work was intended to develop scalable processes and procedures to extract ptox from dried ERC foliage and then concentrate and partially purify it. Extraction and purification of ptox from ERC foliage required the determination of optimum extraction variables, combined with macroporous resin enrichment of ptox from the extract. The greatest ptox extraction yield was obtained using the combination of a 60ºC extraction temperature, 20% ethanol solvent, 1 h extraction duration and a 20:1 solvent:feedstock ratio. The ERC foliage extract was enriched from 0.5 to 0.6% in the field dried ERC foliage to 14% in a 70% methanol eluate from a PAD900 macroporous resin column with 70% recovery of ptox. Using the 70% methanol eluate, we determined that solvent precipitation with sodium carbonate, followed by extraction in ethyl acetate, and re-crystallization in a methanol:chloroform solvent can purify ptox from ERC foliage extract to 89% (dry weight), but with only 10% recovery of ptox throughout the process.

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CHAPTER I

INTRODUCTION

Podophyllotoxin (ptox; Fig. 1A), a natural product derived from plant extracts, is currently a pharmaceutical in high demand for antiviral (Ardalani et al., 2017) and anticancer drug therapies (Seegars et al., 2017). Ptox is a naturally occurring lignan, and acts as an antineoplastic and antiviral agent in the body by suppressing polymerization of tubulin, a component of microtubules. Ptox prevents the formation of mitotic spindles during cell division which causes cell cycle arrest (Ardalani et al., 2017). Ptox is not economically viable to synthesize, making synthetic sources unfeasible (Shah et al., 2021), leaving plant extracts to meet the demand for the ptox drug market (Izadifar and Baik., 2008). The high demand for the drug can be attributed to the many clinical uses that employ ptox or ptox derivatives (Liang et al., 2016).

Plants containing ptox in the rhizomes or leaves can be crushed or extracted and used as an antiviral topical solution (Fadeyi et al., 2013). Podofilox (0.5% ptox) is clinically prescribed as a topical solution to control symptoms from human papillomavirus (HPV) (Seegars et al., 2017). Ptox is cytotoxic to the cells that it comes in direct contact with, but not anticarcinogenic because it does not have a genetically targeted R-group compound (Figs. 1B-1D; Xiao et al., 2020). The antiviral activity of ptox can also be used to treat herpes simplex type I virus and measles (Yousefzadi et al., 2010). More recently, ptox derivatives in combination with other tested pharmaceuticals

have been known to treat autoimmune disorders and inflammatory diseases such as rheumatoid arthritis (drug CPH 82; Yousefzadi et al., 2010).

Ptox as a precursor compound can produce compounds genetically targeted against cancer cells and can be used to derive drugs used in cancer therapies (Ardalani et al., 2017; Canel et al., 2000; Seegars et al., 2017). Cancer is the second leading cause of mortality worldwide (Siegal et al., 2020), where anticancer chemotherapy drugs derived from taxanes and camptothectin dictated over $3 billion in market share (Medrado et al., 2014). Cancer cells duplicate out of control and cause tumorous tissue growths that can spread into the blood and throughout the body (Siegal et al., 2020). During mitosis, microtubules form mitotic spindles which are the necessary components for cell structural development, cell signaling, and cellular transport (Medrado et al., 2014). Inhibition of a cell from forming microtubules during mitosis leads to disruption of cell duplication and leads to subsequent cell death (Medrado et al., 2014). As a pharmaceutical agent, ptox derivatives slow the degradation and breaking of otherwise healthy DNA strands from topoisomerase II by stabilizing DNA binding sites that regulate transcription factors facilitating gene expression (Seegars et al., 2017). In 1983 the first ptox anticancer drug derivative, Etoposide (VP-16, VePesid®; Fig. 1C, Xiao et al., 2020), was approved by the Food and Drug Administration for use in lung and testicular cancer (Bedir et al., 2002). With some chemical alteration, additional ptox-based anticancer drugs have also entered the market as Epipodophyllotoxin (Fig. 1B; Xiao et al., 2020), Teniposide (VM-26; Uden et al., 1997 Fig. 1D; Xiao et al., 2020), and Etopophos (Bedir et al., 2002). Ptox derivatives have been and are currently being used in

clinical trials to form drugs targeted at malignancy (Ardalani et al., 2017; Xiao et al., 2020).

Ptox can be found in the foliage and/or roots of plant species in families Apiaceae, Berberidaceae, Cupressaceae, Lamiaceae, Linaceae, Polygalaceae, and some species of fungi (Ardalani et al., 2017). The cultivation and extraction of ptox from plants in these families has proved difficult, and the concentration of ptox on a dry weight basis (DWB) in the foliage and/or roots varies (Ardalani et al., 2017; Uden et al., 1991). Ptox was originally sourced commercially from the rhizomes of Indian (Himalayan) mayapple (*Podophyllum emodi* Wall., syn. *Podophyllum hexandrum*, syn. *Sinopodophyllum emodi* Wall.) which contains 4-6% ptox (DWB) in the rhizomes (Liu et al., 2015; Wang et al., 2017). Indian mayapple is native to the Himalayan mountains in China, Nepal, India, and Pakistan (Liang et al., 2016), and develops as a perennial rhizomatous shrub (34 cm height x 30 cm width) that is only found at higher elevations in the wild (Chaudhari et al., 2014). Indian mayapple must surpass low seed germination numbers, slow seed germination rates (1-5 months), and low seedling establishment in order to survive long enough to produce a harvest (Chaudhari et al., 2014). Without sustainable cultivation methods, Indian mayapple is now listed on The Convention on International Trading of Endangered Species due to overharvesting for ptox (Renouard et al., 2011; Wang et al., 2017).

Ptox has been reported in saprophytic fungi that grow on decaying plant matter and possess endophytic mycelia that produce secondary metabolites (Liang et al., 2016). Endophytic fungal species *Alternaria tenuissima* (Liang et al., 2016), and *Mucor fragilis* derived from cultures on decaying Indian mayapple rhizomes were found to contain the

pharmaceutical agents ptox and/or kaempferol (Huang et al., 2014). Surface tissue of the decaying Indian mayapple rhizomes were scraped into a petri dish in potato dextrose agar, sealed and incubated to allow for fungal growth, and the plant tissue was monitored over the course of 2 weeks, where successful endophytic fungi were analyzed with high-performance liquid chromatography (HPLC) and mass spectrophotometry for ptox (Huang et al., 2014; Liang et al., 2016). Fungi with the presence of ptox were isolated for reproduction and extracted for ptox, where the extracted fungi yielded ptox at 0.493% of mycelia dry weight (Huang et al., 2014). Fungal sources of ptox are low in concentration, but cultivation times are quicker because of the host-derived metabolites and transposons which duplicate genetic material from the host plant (Huang et al., 2014). Research still needs to be conducted on purification of ptox and other pharmaceuticals from fungal sources before cultivation can be considered.

Production of ptox from tissue cell cultures has been examined from *Callitris drummondii* (ptox 0.11% DWB), *Linum album* (ptox 0.11% DWB), *Linum nodiflorum* (ptox 0.20% DWB), *Podophyllum hexandrum* (ptox 0.09% DWB) *and Podophyllum peltatum* (ptox 0.34% DWB) plant species (Peterson and Alfermann, 2001; Uden et al., 1991). Sanitized plant parts were cultivated in vitro into cultures of callus tissues that cells could be separated from and frozen in liquid nitrogen so that the cells could be powdered and extracted for ptox (Yousefzadi et al., 2010). Optimized work on extraction methods were analyzed in *Linum album* seed tissue cell cultures (Yousefzadi et al., 2010), where the highest concentration of ptox was reported at a low 0.04% (DWB). Production of ptox from tissue cell cultures could be considered, but the plant species, plus conditions of the cultivation medium, lighting, and/or extraction technique need

further investigation to produce great enough yields to meet pharmaceutical demand (Peterson and Alfermann, 2001).

The American species of *Podophyllum*, American mayapple (*Podophyllum peltatum* L.), is a source for ptox when extracted from the leaves (Bedir et al., 2006). The concentration of ptox in American mayapple leaves is much lower (0.93%-2.52% DWB) (Bedir et al., 2006; Zheljazkov et al., 2011) than Indian mayapple rhizomes (4-6% DWB) (Liu et al., 2015; Wang et al., 2017). American mayapple can be found in eastern Canada and the United States as a native plant species in states such as Minnesota, Tennessee, Texas, and Florida (Bedir et al., 2006; Zheljazkov et al., 2011). American mayapple grows wild in mountainous regions, both moist and dry woodland areas as a perennial shrub (30 cm length x 23 cm width) and lays dormant in the summer (Zheljazkov et al., 2011). Due to low seed production, low fecundity, and poor vegetative propagation where one plant produces only one to two leaves for harvest per year, American mayapple has never been used as a ptox resource in the United States (Cantrell et al., 2012).

Ptox concentration in eight *Juniperus* species has also been reported for *J. chinensis* leaves (ptox 0.025% DWB), *J. communis* stem and leaves (ptox 0.02% DWB), *J.* x *media* leaves (ptox 0.097% DWB), *J. recurva* stem and leaves (ptox 0.06% DWB), *J. sabina* leaves (ptox 0.14% DWB), *J. scopulorum* leaves (ptox 0.17% DWB), *J. squamata* stem and leaves (ptox 0.005% DWB), and *J. virginiana* leaves (ptox 0.1% - 0.5% DWB) (Renouard et al., 2011). In this same study, ptox concentration was determined by extracting the plant parts into various solvents, where the highest amount of ptox was found in a 100% methanol extract of *Juniperus virginiana* (Renouard et al., 2011). Ptox

was present in the foliage (scales) of eastern red cedar (Renouard et al., 2011), with little to no presence in the bark, wood or seed (Gawde et al., 2009). Foliage from ERC trees do not vary in ptox concentration based on month of harvest, growing region, sex or stage of development (Renouard et al., 2011). Wood from ERC trees are used for essential oils, furniture, heartwood/cedar wood, and mulch, but in the timber industry the foliage from ERC is a byproduct often regarded as waste (Cantrell et al., 2012).

Native to North America, ERC can be found in Canada, the northeastern United States and is concentrated heavily across the U.S. great plains due to overplanting for windshields and erosion control in the 1940's (Donovan and Victoria, 2018; Starks et al., 2011; Wang et al., 2017). ERC is a dioecious woody perennial that will generate large dense populations quickly after introduction and establishment (Starks et al., 2011). ERC has a high growth rate, high fecundity, and can survive a wide range of edaphic and climatic conditions making it a competitive evergreen plant species that continues to grow and use resources year-round (Meneguzzo and Liknes, 2015). Since the 1800s ERC has been used to make furniture, fences, pencils, essential oil, gin and barrels for aging alcohol (Wang et al., 2017). Though production of ERC products has greatly declined over time for cheaper or synthetic alternatives, the United States government has supported ERC as a windbreak for buildings, to prevent erosion, and as shade trees for livestock for over 100 years (Donovan and Victoria, 2018). ERC has become a native invasive plant species that outcompetes other native species for sunlight (40 ft. tall at maturity; NC Extension 2021), water, and soil nutrients, ultimately reducing plant diversity in the invaded areas (Wang et al., 2017). ERC grows aggressively and encroaches into unwanted areas where management is laborious and expensive (Starks et

al., 2011). ERC foliage generates oils that are flammable, and has become a fire hazard in densely populated areas on the prairieland (Wang et al., 2017). ERC spread from plantings has led to rapid colonization which has profoundly changed species diversity, groundwater recharge, fire recurrences, nutrient cycling, and livestock forage availability (Donovan and Victoria, 2018).

In the early 1940's ERC was mass planted across eastern Oklahoma as erosion control (Starks et al., 2011), and again in 2001 over 1.8 million ERC trees were planted across the great plains as windbreaks (Donovan et al., 2018). In a study done in 2008, the 2001 ERC plantings are estimated to have caused over $218 million in economic loss to land value, groundwater recharge, and forage production across Oklahoma (Starks et al., 2011). Due to the encroaching and competitive nature of ERC, plantings have reduced land values while increasing the cost of land maintenance (NRCS, 2021). In Oklahoma, the USDA Natural Resources Conservation Service reported that one acre of ERC trees can absorb 55,000 gallons of water per year, and removal of ERC trees can cost up to $150 per acre (NRCS, 2021). The great plains provide a thriving grassland environment that is vulnerable to future ERC spread and invasions (Meneguzzo and Liknes, 2015). Though ptox is in much lower concentration in the foliage of ERC (0.6% dry weight basis; Liu et al., 2015) than in the rhizomes of Indian mayapple (4%-6% dry weight basis; Mulik and Laddha, 2015), the foliage of ERC is a fast-growing, relatively high foliage biomass and renewable resource found across most of North America.

Production of ptox from the foliage of ERC is possible, and tree utilization for this purpose could help reduce these problematic trees in Oklahoma. Focusing on ERC ptox production could reduce over-harvesting of Indian and American mayapple, while

contributing to removal of ERC trees growing in unwanted locations across the United States (Cantrell et al., 2012). Ptox production from ERC could help fund ERC removal in Oklahoma and other U.S. states, as well as generate a valuable and demanded pharmaceutical from an unwanted resource.

Locating ERC trees for removal in Oklahoma can be done through various sources and agencies; by contacting local farms, county extension offices, ODAFF (Oklahoma Food, Forestry and Wildlife) Agricultural Environmental Management Services division, or Oklahoma State Office Farm Service Agency to find unwanted ERC. Removal of ERC trees for foliage is a laborious task, and will require the trunk to be mechanically cut below the lowest limb, this will ensure the tree will not grow back or re-sprout (Hartzler, 2006). Felled ERC trees can be field dried for at least 3 months before being harvested. Field drying could help to reduce moisture and increase leaf brittleness which enhances foliage removal. After drying and removal of limbs, the limbs containing foliage can be bagged and held at ambient temperature before being processed. The trunk can be used for byproducts such as firewood, mulch, or left to decompose on site. Development of a mechanism specifically for the harvest of ERC foliage has never been done before, but some research has been conducted on generating a harvesting mechanism that could be used to defoliate ERC trees for removal by a Biosystems and Agricultural Engineering senior design team at OSU (2019-current).

Isolating ptox from the foliage of ERC requires extracting ptox into a solvent that is both compatible with later steps for concentration and purification, but that is also safe for use in scaled-up production. Ptox is soluble in organic solvents such as acetone, ethyl acetate, ethanol, propan-2-ol, butan-1-ol, and methanol (Gan et al., 2009). Ptox extraction

from the rhizomes of Indian mayapple has predominantly been done with methanol; large volumes of methanol were required to extract multiple kilograms of Indian mayapple (Bedir et al., 2006). When handled in larger volumes, methanol poses health risks from ingestion, inhalation, and/or skin exposure which may cause adverse health effects that include headache, dizziness, nausea, vomiting, partial to total loss of vision, kidney failure and death (CDC, 2011). American mayapple rhizomes have been extracted in ethanol concentrations that ranged from a very dilute 10% to a more concentrated 85%; a 30% aqueous-ethanol extract resulted in the highest yield of ptox (Izadifar and Baik., 2008). Ethanol is less dangerous than methanol, and both solvents could be recovered then purified for reuse (CDC, 2011). Ethanol solvent concentrations for extraction of ptox has been reported from 30% ethanol (Izadifar and Baik., 2008) to as high as 60% ethanol (Liu, 2015) for American mayapple. To speed the rate of extraction, and increase yield potential, heat has also been applied during a 1 h aqueous-ethanol extraction of American mayapple, with the maximum yield reported at 53 °C (Izadifar and Baik., 2008).

ERC extract contains unknown contaminants that require removal to reach pharmaceutical purity standard (Gawde et al., 2009). Extracting and isolating ptox requires a method to concentrate the ERC extract to reduce the volume of solvents required for further ptox purification steps. Macroporous resin enrichment is a method that has been used to concentrate and purify ptox from Indian mayapple rhizomes (Wang et al., 2018). Macroporous resins are highly adsorbent packing materials that provide binding sights for polar or nonpolar molecules which are compatible with the resins pore diameter, polarity, and/or specific surface area (Wang et al., 2018). When an adsorbent molecule and the polarity of a resin are compatible, the molecule will adsorb to the resin

binding sites and may be desorbed using a different, or more concentrated, solvent for collection (Wang et al., 2018). Ptox contains a lactone ring that is less polar when suspended in a solution, allowing for adsorption to weak polar or nonpolar resins. Resins with larger specific surface area offer higher adsorption capabilities (Wang et al., 2018), and can contribute to concentrating ptox from the extract.

Column chromatographic purification uses a cylindrical column packed with a macroporous resin (stationary phase) that is compatible with the extraction solvent (mobile phase). Extract can be loaded onto the column either with the use of gravity or with the assistance of a pump (Bajpai et al., 2016). Adsorption onto the resin purifies the target compound from other undesirable compounds which do not bind, and concentration of the target molecule which is present in the extract occurs due to elution into a volume which is less than that of the applied extract (Casas et al., 2016). Compounds separate from the extract and adsorb at different rates, some may not absorb at all and will flow off of the column, and the compounds that adsorb can be eluted with a compatible solvent for desorption (Bajpai et al., 2016). To test concentration and collection of a specific compound, adsorption and desorption properties of a few select resins were performed in a study to purify madecassoside and asiaticoside (anti-inflammatory plant extracts), where a dynamic desorption test was performed using ethanol with a step gradient starting at 10% and increasing to 90% to collect a purer and more concentrated compound (Jia and Xiuyang, 2008). Various macroporous resins have been tested on Indian mayapple extract to purify and concentrate ptox where D-101 (a nonpolar styrene resin from Cangzhou Bon; Hebei, China) was selected for optimal ptox adsorption and desorption efficiency (Wang et al., 2018). This project aimed to formulate

a protocol which can be used to extract, purify and concentrate ptox from ERC extract using macroporous resin. These parameters are defined on a laboratory scale in a manner where they can eventually be used to scale-up the entire process to meet the demand of ERC removal from Oklahoma lands as well as ptox production from the ERC plant extract. These works are broken into two chapters. Chapter II will define the parameters for ERC processing, extraction, and macroporous resin enrichment of extract. Chapter III will demonstrate further purification of ptox from ERC extract that was enriched with macroporous resins.

OBJECTIVES

The remainder of this thesis is divided into three chapters with chapter II addressing extraction and partial purification of ptox from ERC, chapter III addressing further purification of the ptox generated from work in chapter II, and chapter IV summarizing our success in addressing our research objectives. Our objectives are listed below for chapter II and III:

Chapter II objectives:

1. Identify variables required for extraction of ptox from ERC foliage.
2. Demonstrate use of extraction variables at a laboratory and a batch-laboratory scale.
3. Evaluate various resins for ptox concentration and purification from ERC extracts.
4. Demonstrate performance of the best resin in objective 3 in terms of concentrating and purifying ptox from ERC foliage extracts.

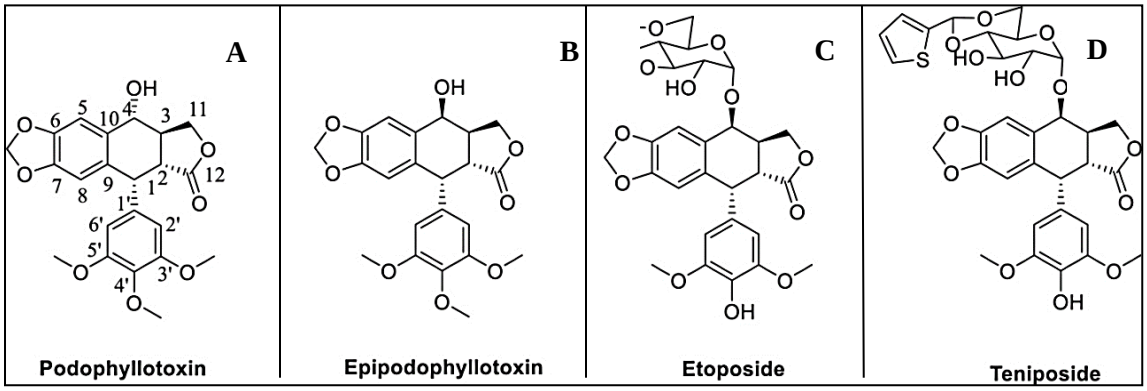
Chapter III objectives:

1. Further purify ptox from enriched ERC extract based on an adaptation to the ptox purification procedure for Indian mayapple extract by Mulik and Ladhha (2015).
    a. Demonstrate precipitation of ptox with Na_2CO_3.
    b. Demonstrate solubilization of ptox with ethyl acetate.
    c. Demonstrate crystallization of ptox using methanol:chloroform.
    d. Identify sources of loss of ptox throughout the procedure.

These objectives combined have an overall goal to create value for an invasive species in Oklahoma.

CHAPTER I FIGURES

Fig. 1. Podophyllotoxin (Fig. 1A) and podophyllotoxin derivatives (Figs. 1B-D) used as cancer therapies.[z]



[z] Xiao, J., M. Gao, Z. Sun, Q. Diao, P. Wang, and F. Gao. 2020. Recent advances of podophyllotoxin/epipodophyllotoxin hybrids in anticancer activity, mode of action, and structure-activity relationship: an update (2010–2020). European J. of Med. Chem. 208:112830-112830.

CHAPTER II

EXTRACTION AND MACROPOROUS RESIN ENRICHMENT OF PODOPHYLLOTOXIN FROM EASTERN RED CEDAR (*JUNIPERUS VIRGINIANA* L.) IN OKLAHOMA

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Abstract

Podophyllotoxin (ptox) is a pharmaceutical derived from plants used in a broad spectrum of medicinal treatments ranging from inflammatory diseases to cancer. Originally sourced from the rhizomes of the now endangered Indian mayapple (*Podophyllum emodi* Wall.), ptox can also be found in the foliage of eastern red cedar (ERC). Ptox has been extracted from the foliage of ERC for analytical purposes (Renouard et al., 2011) but no effort has been documented to extract at commercial scale, nor have any attempts to purify ptox from the ERC foliage extract been documented. This work was intended to develop scalable processes and procedures to extract ptox from dried ERC foliage and then concentrate and partially purify ptox. The greatest ptox extraction yield was obtained using the combination of a 60ºC extraction temperature, 20% ethanol solvent, 1 h extraction duration and a 20:1 solvent:feedstock ratio. This research demonstrates enrichment of ERC foliage extract from 0.5 - 0.6% in the field dried ERC foliage to 14%

in a 70% methanol eluate from a PAD900 macroporous resin column with 70% recovery of ptox based on the extraction and enrichment processes we defined.

Introduction

Podophyllotoxin (ptox), a lignan derived from plant extracts, has been shown to possess both cytotoxic and targeted anticancer activity by inhibiting and suppressing the formation of microtubules in malignant cells. Purified ptox is utilized as a scaffold compound to derive cancer therapy drugs (Ardalani et al., 2017; Canel et al., 2000; Seegars et al., 2017), but may be used without derivatization to treat various autoimmune, inflammatory or venereal diseases. Ptox-based anticancer drugs have entered the market as Epipodophyllotoxin, Etoposide, Teniposide, and Etopophos (Bedir et al., 2002; Uden et al., 1997; Xiao et al., 2020). Other ptox derivatives have been and are currently being used in clinical trials to form drugs targeted at malignancy (Ardalani et al., 2017; Xiao et al., 2020). Ptox was originally sourced from the rhizomes of Indian (Himalayan) mayapple (*Podophyllum emodi* Wall., syn. *Podophyllum hexandrum*, syn. *Sinopodophyllum emodi* Wall.) which contain 4-6% ptox (DWB) (Liu et al., 2015; Wang et al., 2017). Without sustainable cultivation methods, Indian mayapple is now listed on The Convention on International Trading of Endangered Species due to overharvesting for ptox (Renouard et al., 2011; Wang et al., 2017).

Ptox is also found in the foliage of various *Juniperus* species (Renouard et al., 2011; Ivanova et al., 2021), with the highest concentration found in eastern red cedar (ERC; *Juniperus virginiana* L.; 0.1-0.5% DWB) foliage. Native to North America, ERC can be found in Canada, the northeastern U. S. and is concentrated heavily across the

U.S. great plains due to overplanting for windshields and erosion control in the 1940's (Donovan et al., 2018; Starks et al., 2011; Wang et al., 2017).

In Oklahoma, ERC has become a native invasive plant species that outcompetes other native species for sunlight, water, and soil nutrients, ultimately reducing plant diversity in the invaded areas (Wang et al., 2017). Though ptox is in much lower concentration in the foliage of ERC than in the rhizomes of Indian mayapple, the foliage of ERC is a fast-growing, relatively high foliage biomass and renewable resource found across most of North America. Focusing on ERC foliage as a ptox resource for antiviral and anticancer drugs, rather than over-harvesting of Indian mayapple to extinction from its natural habitats, could help fund removal of ERC trees growing in unwanted locations across the United States (Cantrell et al., 2012).

Numerous studies have documented extraction of ptox from the foliage of ERC using buffered water/ethyl acetate (Gawde et al., 2009), aqueous methanol (80% methanol; Ivanova et al., 2021) and 100% methanol (Renouard et al., 2011). Water (Bedir et al., 2006), phosphate-buffered water (Zheljazkov et al., 2009) and ethanol-water (Izadifar and Baik, 2008) solvents have been used for ptox extraction from American mayapple (*Podophyllum peltatum* L.) leaves. The optimal temperature, duration of extraction and ratio of solvent:feedstock varies with each of these solvent systems.

Ptox purification after extraction has been reported for Indian mayapple by repeated precipitation (Mulik and Laddha, 2015) and partial purification has been accomplished using macroporous resins (Liu et al., 2015; Wang et al., 2018). In each case very high chromatographic purity was demonstrated. Macroporous resins have increased ptox purity from extracts by 7 to 27 fold, to 61 or 75% purity and at a recovery of 70 to

75%. It is unclear what these purities represented in terms of ptox purity in the dry matter of the purified resin eluates. Ptox purification from ERC foliage extracts has not been reported.

This project aimed to develop a protocol for extraction and partial purification of ptox from ERC foliage using macroporous resins, as a means to create value for this invasive species in Oklahoma.

MATERIALS AND METHODS

Plant materials:

ERC foliage was harvested from trees in the vicinity of Stillwater, Oklahoma. Branches were cut from trees and allowed at least three months to field dry before being transported to the Noble Research Center at Oklahoma State University for processing. The limbs containing foliage were cut into 40 cm sections and then dried to equilibrium weight in an oven at 40℃. Once equilibrium weight was reached (after approximately 7 days), the foliage was hand separated from the woody stems and weights were taken before the foliage was placed in 2-gallon freezer bags and held frozen (-20°C) prior to grinding.

Grinding was achieved (Cyclone Sample Mill; UDY Corp., Fort Collins, CO, U.S.) through a 1 mm steel screen to homogenize the sample and reduce the particle size. Ground foliage was collected into a 120 mL brown bottle (capacity is approximately 25 g dry foliage), and samples were labeled and stored for up to one month at room temperature to await extraction. Ground sample (500 mg) was weighed in triplicate and placed in an oven at 80℃ to achieve equilibrium weight (24 h). Moisture content (MC) was calculated by subtracting the initial weight (IW) from the dry weight (DW) and then dividing by the initial weight and multiplying by 100; MC = (((IW-DW)/(IW)) *100).

ERC foliage extraction:

Four variables were tested. Ethanol concentration (0 to 95 %, v/v), extraction temperature (25 – 90°C), extraction duration (1 – 2 h) and solvent: ERC feedstock ratio (5:1 to 80:1) were tested as described below to identify the best combination of extraction

parameters to achieve maximum ptox extraction from ERC foliage. Each combination was tested with a uniformly mixed batch of freshly ground ERC foliage.

a) Ethanol concentration: Ten aqueous ethanol solvents were tested (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 95% ethanol) against the 100% methanol solvent used by Renouard et al. (2011) for analytical ERC ptox determination. Samples (25 mg) were weighed using 12 replications into 2-dram vials and then extracted with 2 mL of the corresponding ethanol concentration with stirring for 1 h at room temperature. Vials were then centrifuged using a vacuum concentrator centrifuge (Speed Vac; Savant; Farmingdale, NY, U.S.) to spin down particulates at 3,000 g_n for 30 min. The supernatants were transferred to new vials and dried down completely in the Speed Vac for 1 h.

b) Extraction temperature: Extractions were carried out using a 20% ethanol (v/v) at room temperature (25 °C) and in a dry block heater at 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. Samples (25 mg) were weighed in sextuplicate into 2-dram vials and extracted with 2 mL of solvent for 1 h. Samples were centrifuged at 3,000 g_n for 30 min and supernatants were dried as described above.

c) Extraction duration: Extractions were carried out using 20% ethanol (v/v) at 60 °C for durations of 1 or 2 h. Samples (25 mg) were weighed in quadruplicate into 2-dram vials and extracted with 2 mL of solvent for 1 h. Samples were centrifuged at 3,000 g_n for 30 min and supernatants were dried as described above.

d) Solvent:feedstock ratio: Solvent:feedstock ratios (volume of extraction solvent: weight of foliage) were tested by extracting samples as dilute as 80:1 (2 mL solvent per 25 mg foliage) to as concentrated as 5:1 (2 mL solvent per 400 mg foliage). Samples (25 mg, 50 mg, 100 mg, 200 mg, and 400 mg) were weighed in quadruplicate into 2-dram vials and extracted with 2 mL of solvent (20% ethanol) for a 1 h duration at 60°C. Samples were centrifuged at 3,000 g_n for 30 min and supernatants were dried as described above.

Scale-up extractions were conducted to provide enough ERC extract for concentration and partial purification experiments. Extractions of up to 2 g of ERC foliage were performed in 50 mL centrifuge tubes with 40 mL of 20% ethanol solvent. In preliminary experiments we tested ptox yield using foliage that was crushed with a rolling pin versus foliage that was ground with a cyclone mill to pre-process ERC foliage prior to extraction. The crushed and ground 2 g of foliage was extracted in 40 mL 20% ethanol and extracted at 60 ℃ for 1 h with 1 min vortexing every 10 min. Samples were either centrifuged at 3,000 g_n for 3 h or vacuum filtered, where a 1 mL sample from each extracted foliage condition and was dried in a 2-dram. The extracts were transferred into 120 mL brown bottles which were stored in a refrigerator (3 ℃) to await purification trials.

Dried extract samples were reconstituted in 500 µl of methanol plus 500 µl of caffeine (0.1 mg caffeine mL methanol) added as an internal standard. Samples were vortexed and centrifuged at 3,000 g_n for 30 min in a Speed Vac to remove insoluble solids. The supernatants were carefully decanted into 8 mm auto sampler vials to await high-performance liquid chromatography (HPLC) analysis.

ERC extract column purification:

Four resins were chosen and provided from Purolite (Purolite; Glenville, MN, U.S.); Purosorb PAD900, PCG900C, PCG900M and PCG900F, ranging in particle diameter and price (Table 3). To activate the resins, two grams of each resin was mixed with 25 mL of 95% ethanol for 30 min and vacuum filtered using a sintered glass funnel. The resin was then re-equilibrated with 25 mL of 20% ethanol and vacuum filtered. After re-equilibration, the resins were dried in air prior to use.

To test resin adsorption capability, 25 mg of air-dried resin was weighed in quadruplicate into 2-dram vials and extract (1 mL) was added, vortexing at 10 min intervals for a duration of 1 h. The vials were centrifuged at 3,000 g_n for 30 min and the supernatants were decanted into a new vial and dried down completely in a Speed Vac for 2 hr. Dried supernatants were reconstituted for HPLC analysis as previously described and the resins were desorbed as described below.

To test resin desorption capability, resins were first rinsed for 1 h with 2 mL of 20% ethanol to rinse away unabsorbed compounds, centrifuged at 3,000 g_n for 30 min and the 20% ethanol supernatants were decanted into a new vial and dried completely. The washed resins were desorbed for 1 h with 2 mL of 40%, 60%, 70%, 80% and 95% ethanol and centrifuged at 3,000 g_n for 30 min. The supernatants were dried as described above and the resins were re-equilibrated with 20% ethanol for subsequent use.

Purolite PAD900 macroporous resin was utilized for the remainder of the study and was packed into a chromatographic column (0.8 cm x 4.7 cm) fitted with compression ends and fittings for attachment to a peristaltic pump

(Fisherbrand™ Variable-Flow Chemical Transfer Pump; Fisher Scientific, Waltham, MA, U.S.). Glass wool was packed at the outlet side (0.5 cm) prior to loading the column with 1.78 g of PAD900 resin. PAD900 resin was settled by initiating flow of 20% ethanol with the peristaltic pump at a flow rate of approximately 2 drops min (3.4 mL h). Final resin bed volume was 1.75 mL. Prior to ERC extract application, the column was activated with 10 mL (5.7 bed volumes) of 100% HPLC grade methanol (Fisher Scientific, Waltham, MA) and re-equilibrated with 10 mL of 20% ethanol.

The ptox holding capacity of PAD900 was tested by applying up to 300 mL of ERC extract (extract concentration 0.19 mg/ mL) and measuring ptox bleed from the column. The column was set at a flow rate of approximately 2 drops min and drops were collected with a fraction collector (Isco Retriever II; Teledyne Isco Inc., Lincoln, NE, U.S.) in 175 drop (approximately 5 mL) fractions. Each 5 mL fraction was collected into a 2-dram vial, and each vial (60 in total) was vortexed where an aliquot of 0.5 mL was taken and decanted into a new 2-dram vial to be dried down. Once dried, the samples were reconstituted in 500 µl of methanol and 500 µl of caffeine in methanol (0.1 mg/g) and transferred to an autosampler vial for HPLC analysis.

Subsequent ERC extract ptox column desorption was evaluated after application of 80 mL of extract and rinsing unbound substances with 10 mL of 20% ethanol as described above. Since subsequent ptox purification has typically been accomplished in methanol solvent, various concentrations of methanol were tested for ptox desorption from the column. A step gradient of diluted methanol (10 mL each), starting at 30% methanol and ending at 100% methanol in increasing increments of 10% were utilized to evaluate ptox desorption from the PAD900 packed column. Following 100% methanol

elution the column was re-equilibrated with 10 mL of 20% ethanol for subsequent reuse. During application, post-application column rinse, methanol elution and 20% ethanol re-equilibration steps flow rate was maintained at approximately 2 drops min, and column eluates were collected in 35 drop (approximately 1 mL) fractions. Volumes taken for HPLC analysis included 0.5 mL from each application and 20% ethanol fraction, and 100 µl of 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% methanol fractions. Samples were then dried in a Speed Vac for 1 h. Once dried, the samples were reconstituted in 500 µl of methanol and 500 µl of caffeine in methanol (0.1 mg caffeine mL) and transferred to an autosampler vial for HPLC analysis.

Subsequent 80 mL ERC extract application steps were collected in 20 mL fractions (approximately 700 drops) as opposed to 5 mL fractions, and any sources of loss of ptox during application was documented by sampling a 1 mL aliquot of each 20 mL application fraction. Application steps were dried down completely in a Speed Vac for 1 h. Once dried, the samples were reconstituted in 500 µl of methanol and 500 µl of caffeine in methanol (0.1 mg caffeine mL) and transferred to an autosampler vial for HPLC analysis.

We tested use of an optimized methanol step gradient in 10 mL increments for ptox elution starting at 40% methanol and proceeding to 70%, 80% and 100% methanol, then a final 20% ethanol rinse to re-equilibrate the column for subsequent extract application. Volumes taken for HPLC analysis of the optimized methanol step gradient procedure included 1 mL from each application and 20% ethanol fraction, 200 µl of 40%, 80% and 100%, and 50 µl 70% methanol fractions. Samples were then dried in a Speed Vac for 1 h. Once dried, the samples were reconstituted in 500 µl of methanol and 500 µl

of caffeine in methanol (0.1 mg caffeine mL) and transferred to an autosampler vial for HPLC analysis.

HPLC analysis:

Autosampler vials containing appropriately diluted samples plus caffeine (0.1 mg caffeine mL) as internal standard were placed into an automated sample injector (Thermo Scientific Dionex ASI-100; Thermo Scientific Dionex, Sunnyvale, CA, U.S.). Separation of compounds from a 10 µl injection was accomplished using a Kinetex 5 um XB C-18 100 Å column (250 x 4.6 mm; Phenomenex, Torrance, CA, U.S.) and a Dionex ICS 3000 liquid chromatograph at a flow rate of 0.8 mL min. The mobile phase consisted of 0.2% acetic acid (solvent A) and 100% methanol (solvent B). An elution gradient starting at 90% solvent A/10% solvent B was immediately linearly increased to 30% solvent A/70% solvent B over 40 min and then increased to 100% solvent B for the next 10 min. The column was rinsed with 100% solvent B for 10 min and then re-equilibrated to 90% solvent A/10% solvent B to prepare for the next run. Total run duration was 65 min. Peaks were detected at 290 nm using a Dionex ICS Series PDA detector. Fresh standards of ptox spiked with the internal standard (0.1 mg/ mL) were run with each sample run. Ptox from a sample was quantified relative to that of ptox in a standard sample run using the external standard. All samples were run in duplicate.

Data analysis:

Data was analyzed with SAS 9.4 (SAS Inc., Cary, NC, U.S.) using the GLIMMIX procedure. When appropriate differences among various treatment means were determined using Fisher's least significant difference at $p \leq 0.05$.

RESULTS

ERC foliage extraction:

Each solvent containing ethanol achieved greater ptox yield than the water control (Table 1). Interestingly, there were no significant differences in ptox yield among extractions containing between 10 and 95% ethanol (v/v). Subsequent extractions used a 20% ethanol (v/v) solvent as it was identified as a compromise between minimal cost and ensuring high extraction potential as compared to water alone. Yield was greatest when extraction temperature was greater than or equal to 60°C (Table 2). Yield at 40 °C was significantly less than that at 50 or 25 °C.

Using the selected 20% ethanol solvent and 60°C temperature, ptox yield declined with increasing feedstock amount in a curvilinear manner (Fig. 2). An inflection point was graphically determined at the 20:1 solvent:feedstock ratio, at which point the slope of the line decreased rapidly. Surprisingly, these results were unchanged at either 1 h or 2 h durations (Fig. 3).

Purolite resin evaluations:

All resins effectively absorbed ptox from the ERC extract and showed little ptox bleed during a subsequent 20% ethanol rinse (Fig. 4). Ptox elution profiles were different, with the large particle size, macroporous PAD900 resin exhibiting slightly lower ptox elution at the 40% and 60% ethanol elution steps compared to the smaller particle size resins, ultimately reaching a maximum ptox elution at 70% ethanol. Each of the smaller particle sized resins exhibited maximum ptox desorption at 60% ethanol concentration, with sharper elution of ptox from the smallest particle size resin (PCG900M) and almost

identical elution profiles from the other two intermediate particle size resins (PCG900C and PCG900F).

In preliminary trials we packed gravity-fed columns (10 mL capacity) with PAD900 and PCG900C resins (the two largest particle size resins). After loading the resins into a gravity-fed column, we noted substantially different flow rates with the 20% ethanol solvent. While PAD900 eluted at greater than 3 bed volumes per h (1.5 mL h) the PCG900C column quickly slowed to less than 1 bed volume per h (0.4 mL h; data not shown). Since one aim of our work was to develop a scalable and economical ptox purification process, we surmised that the smaller particle size of the PCG900C resin caused bed packing to an extent that would be difficult to achieve optimal column flow without the use of moderate pressure pumps. We decided to use the PAD900 resin in further tests.

ERC extract column purification:

Using PAD900, we packed a glass column with 1.78 g of resin (0.53 g resin DWB) and achieved column flow rate of 2 drops min with a peristaltic pump. To determine the ptox adsorption capacity, we applied up to 300 mL of ERC foliage extract (extract concentration 0.19 mg/ mL) to the column and collected 5 mL fractions of column eluate (Fig. 5). The resin appeared to be saturated with ptox at 245 mL (corresponding to 46.6 mg ptox applied), which was indicated when the concentration of ptox in the column eluate contained the same ptox concentration as the ERC foliage extract. Further evaluation of the extract ptox elution pattern from the column indicated that ptox was completely adsorbed to the PAD900 macroporous resin for up to 70 mL of extract application and only minimal ptox elution (<0.02 mg ptox mL column eluate) was

observed after 90 mL of extract application. To assure maximal extract application with minimal ptox loss we designated 80 mL of extract for column loading in subsequent experiments. Extract application and adsorption required approximately 23 h to complete when a consistent flow rate of 2 drops min was achieved.

After loading the column with 80 mL ERC extract (extract concentration 0.19 mg/ mL), a step gradient of diluted methanol (5 mL each), starting at 30% methanol and ending at 100% methanol in increasing increments of 10% was utilized to evaluate ptox desorption from the PAD900 packed column. Using the step gradient, we found that ptox eluted from the column in concentrations of methanol at 40% or higher with less than 3% loss to application and rinsing steps (Fig. 6). Between 50- 60% methanol there was a slight increase in ptox elution from the column, accounting for 10% of total ptox applied. The sharpest elution of ptox was seen in concentrations of 70% methanol (54% recovery) or higher. The remaining 33% of ptox eluted in 80- 100% methanol concentration range. The final column wash with 10 mL of 20% ethanol for re-equilibration prior to reuse resulted in no ptox elution.

Based on the results from the optimized methanol step gradient, we found the majority of ptox (70-75% of the quantity applied to the PAD900 macroporous resin column) eluted in the 70% methanol elution step, with only minor ptox elution during extract application and 20% ethanol column rinse (1-2% of total ptox applied), during the 40% methanol elution (4-6% of total ptox applied) and during the 20% ethanol re-equilibration (1% of total ptox applied) steps. Moderate ptox elution was noted in the 80% methanol (11-13% of total applied ptox) and the 100% methanol (6-9% of total applied ptox) elution steps.

HPLC chromatograms of appropriate dilutions from the ERC foliage extract (Fig. 8A) compared to the subsequent column elution steps (Figs. 8B-G) demonstrated variable apparent ptox purification during the elution steps. Within all chromatograms, the peak eluting at approximately 17.3 min was the caffeine internal standard (added prior to injection and not present in the samples themselves) and the peak eluting at approximately 37 min was ptox. In the ERC foliage extract, ptox was a prominent peak among numerous other unknown peaks eluting before and after ptox (Fig. 8A). During the 20% ethanol rinse ptox was a minor peak; some peaks which eluted within 10 to 11 min prior to ptox (25 to 26 min) were present but peaks eluting prior to 25 min or after the ptox peak were decreased in prevalence or absent (Fig. 8B). The 40% methanol elution step mostly mirrored the initial 20% ethanol column rinse with the exception that ptox increased slightly in prevalence (Fig. 8C). The 70% methanol elution step was substantially different, with ptox as the overwhelmingly prominent feature and only minor peaks eluting prior to and after the ptox peak (Fig. 8D). Within the 80% methanol elution step ptox was decidedly less prominent, there were very few peaks eluting prior to ptox and peaks eluting after the ptox peak became more prominent (Fig. 8E). The 100% methanol elution step mirrored the 80% elution step with the exception that ptox was notably less prominent (Fig. 8F). The 20% ethanol re-equilibration step exhibited very low ptox and very low peak intensity for other peaks, all eluting after the ptox peak (Fig. 8G). Purity of ptox at each step represented by the chromatograms in Figs. 8A-G was determined as a percent of podophyllotoxin in the dry matter and as a percent of podophyllotoxin verses all of the other detected chromatographic peaks (Table 4). Both dry matter and chromatographic peak ptox purity were higher during all column elution

steps compared to the foliage extract with the exception of the final 20 % ethanol re-equilibration step. Purity of ptox notably plateaued at the 70% methanol PAD900 macroporous resin elution step (14% purity as a component of dry matter and 89% purity as a component of detected chromatographic peaks).

DISCUSSION

Previously reported ptox extraction solvents have been 100% methanol for both ERC foliage (Renouard et al., 2011) and Indian mayapple rhizomes (Mulik and Laddha, 2015) or 80% methanol for *Juniperus* foliage (Ivanova et al., 2021), and water (Bedir et al., 2006), phosphate-buffered water (Zheljazkov et al., 2009) or ethanol-water (Izadifar and Baik, 2008) solvents for ptox extraction from American mayapple leaves. While we have found that 100% methanol works well as an analytical solvent, we were concerned about the potential hazards of methanol used in scaled up extractions due to significant inhalation and contact hazards (CDC, 2011). An ethanol-water solvent system was reported to be effective for ptox extraction from American mayapple leaves, showing a maximum yield of ptox using 30% ethanol (Izadifar and Baik, 2008). The present study tested various aqueous ethanol solutions and found that ethanol concentrations as low as 10% extracted the same amount of ptox as all higher ethanol concentrations (up to 95%) from ERC foliage (Table 1).

The optimum temperature for ptox extraction from American mayapple using an aqueous ethanol solution was reported to be 53°C (Izadifar and Baik, 2008) which is similar to the 60°C selected in the present study (Table 2). Ptox solubility was shown to be impacted by temperature with various solvents and the temperature relationship ultimately impacted duration needed for solubilization (Gan et al., 2009). Extraction duration has varied for ptox extraction, varying from as long as 48 h using 80% ethanol atroom temperature (Safarpoor et al., 2017), to 5 h using 100% methanol at room temperature (Renouard et al., 2011), to 10 min using 30% ethanol with sonication at 50 °C (Zhao and Baik, 2011). We did not find an increase in ptox yield by increasing the

extraction duration from 1 h to 2 h; in preliminary trials we did find that a 30 min extraction duration resulted in reduced ptox yield due to incomplete extraction. Extraction procedures in excess of 2 h were deemed impractical for scale up and therefore not tested.

Previously reported solvent to feedstock ratios for extraction of Indian mayapple ptox have ranged from 20:1 to 10:1 when using 70% ethanol at 70 °C for 1 h (Wang et al., 2013). This contrasts somewhat with the present study which found ptox yield (on a per gram DW basis) decreased rapidly at feedstock ratios less than 20:1. Although ptox yield was greatest for our highest solvent:feedstock ratio (80:1), the total ptox loss was less than 1 mg/ g foliage while the extraction throughput tripled for the 20:1 solvent:feedstock ratio. One aim for this study was to develop a cost effective, scalable system for ptox extraction from ERC foliage. We believe our choice of 20% ethanol as solvent, at the lowest effective temperature (60 °C), for the shortest time duration (1 h) and utilizing the greatest amount of feedstock within each extraction run (20:1 solvent:feedstock ratio) meets this goal.

One aim of our work was to develop a scalable process for ptox production from ERC foliage. Stirred reactors are an economical and scalable means to perform extractions – our extraction procedure would be best performed in such a system. Since ERC foliage contains substantially lower ptox than Indian or American mayapple, our extracts were substantially more dilute also. Substantial concentration of our more dilute extracts would be necessary to produce a comparable extract product for the downstream purification protocols designed around the mayapple products.

Macroporous resins have been used to purify ptox in Indian mayapple extracts containing in excess of 0.35 mg ptox mL (Liu et al., 2015). The choice for a compatible resin for ptox from an extract depended on each resin's ability to adsorb and then desorb ptox from the extract (Wang et al., 2018). We conducted adsorbance/desorbance tests on 4 macroporous resins differing in particle size (ranging from 300-1,200 µm to 20-50 µm), chemical structure (divinylbenzene versus polyvinylbenzene) and price ($285 kg to $3,700 kg). All resins adsorbed and desorbed ptox well. In previous work HPD300, a polystyrene macroporous resin, required 50% ethanol to desorb ptox (Liu et al., 2015) whereas D-101, a non-polar styrene macroporous resin required 60% ethanol to desorb ptox (Wang et al., 2018). In a similar manner, the maximal ptox desorption for polyvinylbenzene resins (PCG900C, PCG900F and PCG900M) was at a lower ethanol concentration (60% ethanol) than the divinylbenzene resin (70% ethanol). Using D-101 macroporous resin, Wang et al. (2018) found that the dynamic saturated adsorption capacity was 41.5 mg ptox g dry resin. We determined a higher adsorption capacity (the point at which column eluate ptox concentration equaled the extract ptox concentration) for our PAD900 macroporous resin of 88 mg/ g dry resin (46.6 mg ptox applied to 0.53 g dry resin). This total ptox load corresponded to 245 mL of applied ERC foliage extract. We chose to load the column with only 80 mL of ERC extract (containing 15 mg ptox), rather than the indicated 245 mL to achieve maximum ptox load capacity, to avoid ptox bleed from the column and subsequent loss from the column eluate. Although a small degree of ptox bleed was evident between 70 and 80 mL of ERC extract application to the column, the 0.01 mg mL concentration represented only 1% of total applied ptox, which was considered acceptable.

Although desorption of ptox from macroporous resins has typically been accomplished by increasing the concentration of ethanol, we noted that upstream purification processes typically utilize a methanol starting solvent (Mulik and Laddha, 2015). We surmised that once ptox was bound to the macroporous resin in the 20% ethanol solvent, we could exchange the column into aqueous methanol at a methanol concentration below that required for substantial ptox elution and achieve exchange of solvent. We packed a glass column with 1.78 g of PAD900 resin (0.59 g DB), occupying 1.75 mL, in order to conduct further tests. Using a step gradient to elute an 80 mL ERC foliage extract loading, we found that an initial column rinse with 10 mL of 20% ethanol (5.7 bed volumes), followed by another column rinse with 10 mL of 40% methanol, eluted only 5% of applied ptox but substantial peaks co-eluting prior to the ptox peak, and present in the ERC foliage extract, were also present in the column eluates. Since application of over 5 bed volumes of 40% methanol likely completely exchanged the 20% ethanol solvent with a methanol solvent we achieved solvent exchange and rinsed away a portion of the unknown contaminating peaks from the resin with these steps.

The 70% methanol column eluate chromatographic ptox peak was by far the major peak with minor unidentified apparent contaminating peaks co-eluting on either side of the ptox peak. Elution of a majority of ptox with 70% methanol coincided well with our ethanol ptox desorption data as seen in Fig. 4, but was slightly higher for our divinylbenzene-based PAD900 macroporous resin versus previously published data in which a D-101 styrene macroporous resin (60% ethanol) (Wang et al., 2018) and a second HPD-300 polystyrene macroporous resin showed maximum ptox desorption at 50% ethanol (Liu et al., 2015). All three resins above had similar particle sizes (300 to

1,200 µm) but different apparent matrices which contributed to ptox desorption at different ethanol concentrations. In contrast, this research showed the three resins having the smallest particle sizes (PCG900C, 100-200 µm; PCG900M, 50-100 µm; PCG900F, 20-50 µm) also exhibited maximum ptox desorption at 60% ethanol (Fig. 4). Desorption properties of the vinylbenzene macroporous resins may have changed due to slight differences in matrix (divinylbenzene for PAD900 eluting ptox maximally at 70% ethanol versus polyvinylbenzene for the other tested macroporous resins eluting ptox maximally at 60% ethanol). Since particle size of the three polyvinylbenzene resins decreased substantially (100-200 µm for the PCG900C macroporous resin to 20-50 µm for the PCG900F macroporous resin) while maximal ptox desorption concentration remained the same at 60% ethanol, we doubt that particle size contributed substantially to ptox desorption characteristics of these macroporous resins.

The substantial decrease in prevalence of unidentified apparent contaminating chromatographic peaks within the 70% methanol elution step, combined with predominance of apparent contaminating peaks in the 20% ethanol 40% methanol, 80% methanol and 100% methanol elution steps from the PAD900 macroporous resin, suggested that some purification of ptox was likely within the 70% methanol elution step.

Some purification of ptox was documented within the dry matter contained in the extraction and PAD900 macroporous resin elution steps. Whereas ptox from the ERC foliage was present at approximately 5-6 mg/ g foliage (0.5 to 0.6%), the dried extract contained 1.1% ptox, and the dry matter contained in PAD900 resin eluates contained increasing percentages of ptox up to a maximum of 14% in the 70% methanol eluate and then decreased in subsequent elution steps. Chromatographic peak purity of ptox (ratio of

the peak area units for ptox divided by the peak area of all detected peaks in the chromatogram) was by far greatest for the 70% methanol eluate at almost 90%. While chromatographic peak purity indicated a relatively high degree of ptox purity in some HPLC chromatograms, it was not a good quantitative measure of actual ptox purity in the dry matter of each fraction. HPLC chromatograms only measure peaks which can be separated on the column, using gradient conditions and detection parameters which are optimized for the compound(s) of interest. Since contaminating compounds may or may not have separated well using the chromatographic conditions optimum for ptox, and they may exhibit very different absorption maxima compared to the 290 nm used for ptox detection, we conclude that the dry matter content of ptox was the truest measure of ptox purity. The dry matter ptox purity in the 70% methanol eluate suggested that PAD900 macroporous resin enriched ptox from an ERC foliage extract by a little over one magnitude, from 1.1 % in the ERC foliage extract up to 14% in the 70% methanol eluate, and provided a means to capture the majority of ptox from the ERC foliage extraction (70%) into one concentrated fraction. The PAD900 macroporous resin provided a means to reduce original extract volume to ¼ of its original volume, to exchange the ptox from an ethanol to a methanol solvent system, and coincidently to increase ptox concentration in the dry matter by a little over one magnitude. The PAD900 macroporous resin also produced substantially less resistance to solvent flow versus the other, smaller particle size resins tested; this should require less expensive low pressure pumps to conduct scaled up column purification runs. The PAD900 macroporous resin appears to be quite suitable for continued re-use. We have adsorbed and desorbed ERC foliage extract at

least 40 times using the same packed column with little-to-no apparent degradation in column performance.

CONCLUSION

Field dried ERC foliage can be a source of ptox, and in doing so provide a reason for removing ERC trees invading land in rural and increasingly populated areas of Oklahoma and the US. Optimum conditions for ptox extraction were identified using 20% ethanol solvent at 60 °C for a duration of 1 h and at a solvent: ERC foliage weight ratio of 20:1. The PAD900 macroporous resin provided a means to concentrate ptox from 80 mL of ERC foliage extract into 20 mL of the 70% aqueous methanol eluate, capturing 70-75% of applied ptox into this fraction. Partial purification of ptox from 0.5 to 0.6% in the ERC foliage dry matter to 14% in the 70% methanol eluate dry matter was achieved using the combined extraction and resin purification procedures we developed. This degree of purification is encouraging, but further research is needed to achieve the higher purity which may be required for use of ptox as a pharmaceutical ingredient.

CHAPTER II TABLES

Table 1. Effect of ethanol solvent concentration on extraction yield of podophyllotoxin in mg podophyllotoxin/ g foliage from eastern red cedar foliage extract. [z]

Ethanol concentration (%, v/v)	Ptox yield (mg/ g)[y]
0	4.63 b
10	6.26 a
20	6.81 a
30	6.63 a
40	6.56 a
50	6.54 a
70	6.80 a
95	6.58 a

[z] Samples extracted in water, 10%, 20%, 30%, 40%, 50%, 70% and 95% ethanol for a 1 h duration at room temperature using an 80:1 (2 ml solvent/ 25 mg foliage) solvent:feedstock ratio.

[y] Means (n = 12) within a column and average yields followed by the same letter are not significantly different based on Fisher's LSD test at $P \leq 0.05$.

Table 2. Effect of extraction temperature on podophyllotoxin yield for eastern red cedar foliage extracts.[z]

Temperature[z] (°C)	Podophyllotoxin yield (mg/ g)
25[y]	6.49b[x]
40	4.87c
50	6.37b
60	7.14a
70	6.93a
80	7.17a
90	7.37 a

[z] Samples extracted in 20% ethanol for a 1 h duration at 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C at an 80:1 (2 ml solvent/ 25 mg foliage) solvent:feedstock ratio.

[y] Room temperature extractions were performed at 25°C and heated extractions (40°C - 90°C) were performed on a dry block heater.

[x] Means (n=6) within a column and average yields followed by the same letter are not significantly different based on LSD test at $P \leq 0.05$.

Table 3. Purolite macroporous resin characteristics of four tested resins, in order of largest to smallest particle size.

Resin name	Particle size (µm)	Resin Type	Price / kg (U.S. $)
PAD900	300-1,200	Macroporous divinylbenzene	$285
PCG900C	100-200	Macroporous polydivinylbenzene absorbent	$730
PCG900M	50-100	Macroporous polydivinylbenzene absorbent	$1,850
PCG900F	20-50	Macroporous polydivinylbenzene absorbent	$3,700

Table 4. Podophyllotoxin in eastern red cedar foliage extract and podophyllotoxin in eastern red cedar extract that was enriched using an optimized methanol step gradient on a PAD900 packed column. Podophyllotoxin by dry weight basis (%) and peak purity (%)[z] from a 20% ethanol rinse, followed by elution with methanol 40%, 70%, 80%, 100% and a 20% ethanol wash to re-equilibrate the column for subsequent runs.

Solvent	Ptox dry weight basis[y] (%)	Chromatographic peak purity (%)
Eastern red cedar foliage extract	1%	19%
20% ethanol rinse	4%	22%
40% methanol eluate	5%	16%
70% methanol eluate	14%	89%
80% methanol eluate	7%	47%
100% methanol eluate	3%	29%
20% ethanol wash/ column re-equilibration	2%	3%

[z] Samples extracted in 20% ethanol for a 1 h duration at 60℃ at an 80:1 solvent:feedstock ratio. 80 ml of eastern red cedar extract was then applied to a PAD900 packed column at a flow rate of 2 drops min. The column was rinsed with 10 ml of 20% ethanol, and eluted with 10 ml 40% methanol, 20ml 70% methanol, 10 ml 80% methanol, 10 ml 100% methanol and the column was re-equilibrated with 10 ml 20% ethanol.

[y] Values given represent the average of 4 PAD900 column purification procedures (80 mL eastern red cedar extract loads).

CHAPTER II FIGURES

Fig. 2. Effects of solvent:feedstock ratio on podophyllotoxin yield from eastern red cedar foliage extracted for 1 h at 60℃ and ranging from a dilute 80:1 (2 mL/ 25 mg), to a concentrated 5:1 (2 mL/ 400 mg). Values are expressed using the means of six replications.

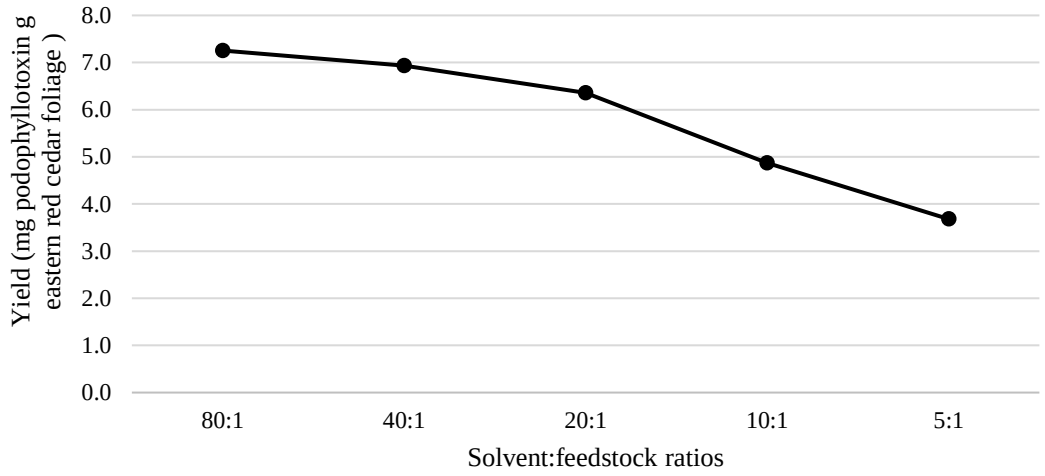


Fig. 3. Effects of extraction duration (1 h– 2 h) and solvent:feedstock ratio podophyllotoxin yield from eastern red cedar foliage extracts preformed at 60℃ and ranging from a dilute 80:1 (2 mL/ 25 mg), to a concentrated 5:1 (2 mL/ 400 mg). Values are expressed using the means of 4 replications.

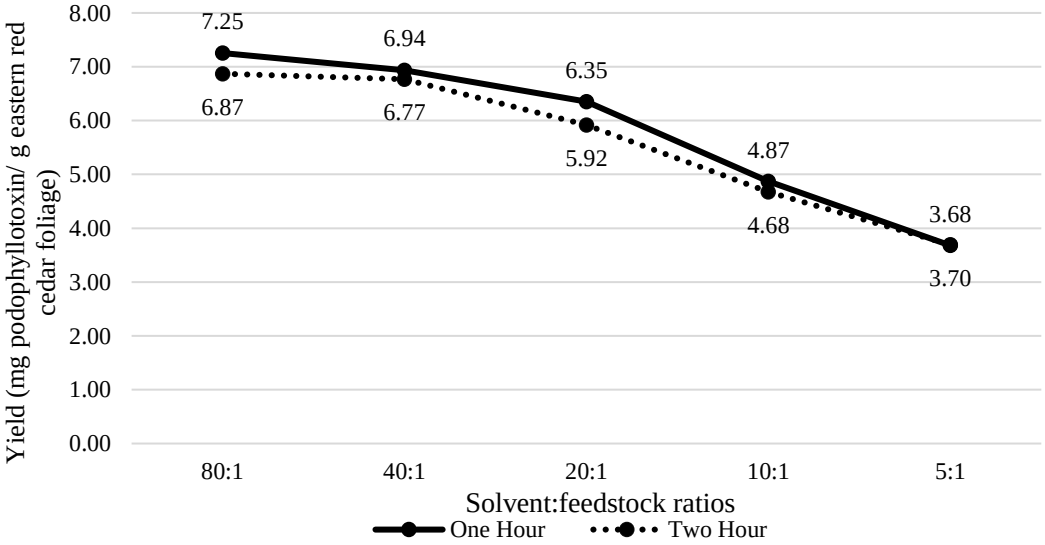


Fig. 4. Effects of using four different macroporous resins to concentrate and purify podophyllotoxin from 1 mL eastern red cedar foliage extract (extract concentration 0.15 mg/mL) using 500 mg resin sample in a 2-dram vial. Samples were allowed 1 h adsorption of 1 mL eastern red cedar extract. The 2-dram was then centrifuged at 3,000 g_n for 30 min and the supernatant was decanted from the resin, dried down completely in a Speed Vac for 1 h and reconstituted in 500 µl of methanol plus 500 µl of caffeine (0.1 mg caffeine mL methanol; added as an internal standard) for high-performance liquid chromatography analysis. This process was continued in the same 2-dram vial using 2 mL of a 20% ethanol rinse, 40%, 60%, 70%, 80%, and a 95% ethanol step gradient for a 1 h elution of podophyllotoxin per solvent.

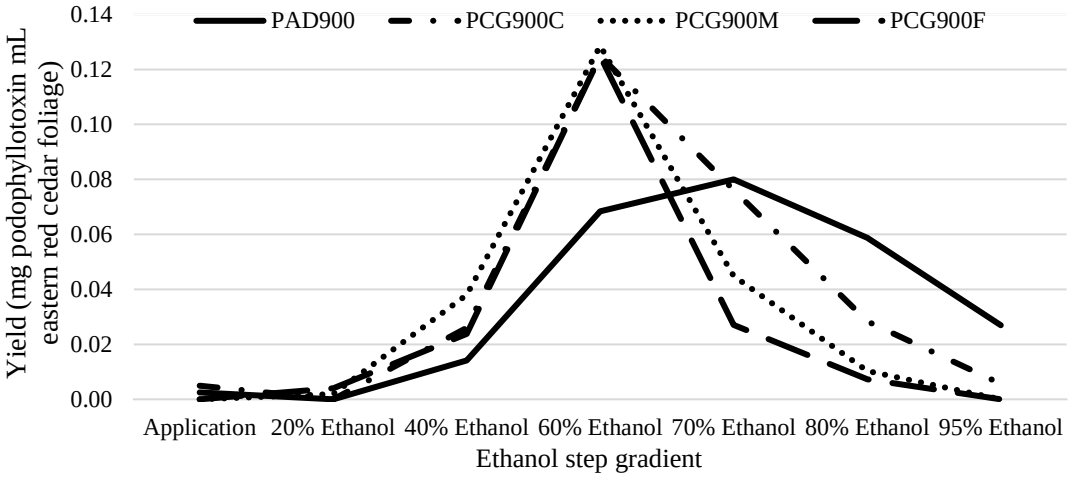


Fig. 5. Effects of attempting to overload peristaltic pump-fed column packed with 1.78 g PAD900 macroporous resin using 300 mL of eastern red cedar foliage extract (extract concentration 0.19 mg / mL). Saturation was reached at a 245 mL load of extract to the column, and lost 100% of the podophyllotoxin being applied.

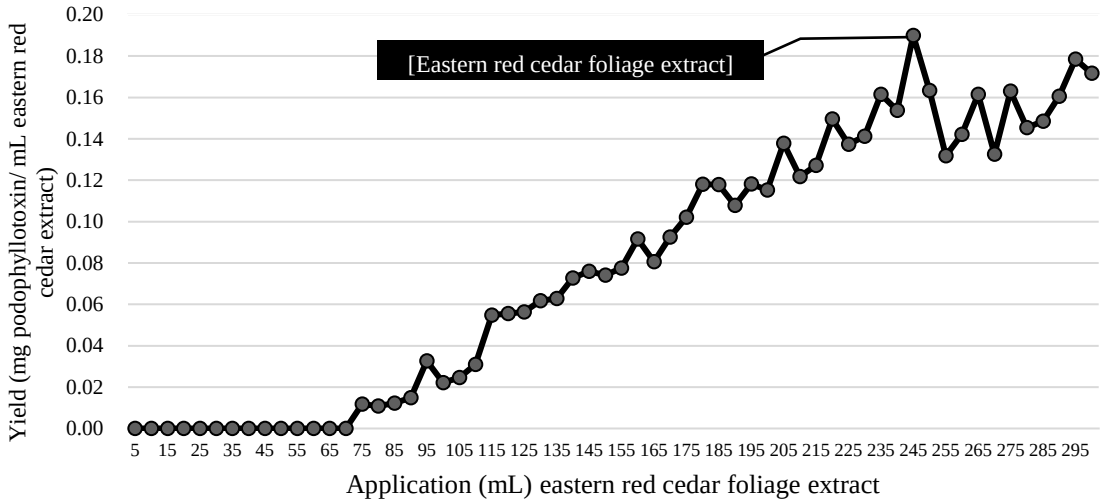


Fig. 6. Effects of attempting to elute podophyllotoxin from a peristaltic pump-fed column packed with 1.78 g PAD900 macroporous resin and exchange solvents from an ethanol solvent to a methanol solvent with use of a step gradient starting with a 20% ethanol rinse, exchanging for 30% methanol and increasing in increments of 10% to 100% methanol elutions, with subsequent washing of the column with 20% ethanol for re-equilibration using 10 ml rinses/elutions/washes.

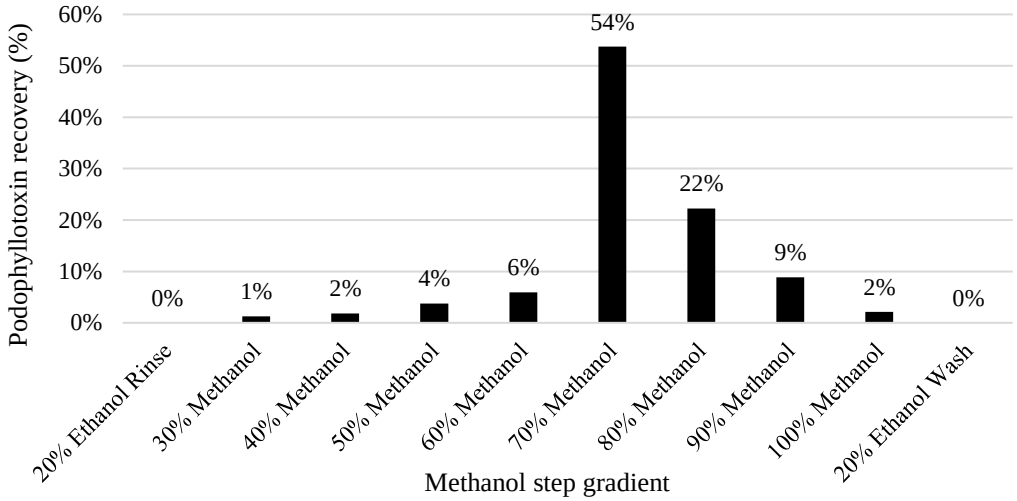


Fig. 7. Recovery (%) of podophyllotoxin from a PAD900 packed column loaded with 80 mL eastern red cedar extract (extract concentration 0.18 mg/ mL) using an optimized step gradient for elution and concentration of podophyllotoxin in a methanol solvent. Eastern red cedar extract was loaded onto the column and rinsed with 10 mL 20% ethanol, podophyllotoxin was eluted with 10 mL 40% methanol, 20 mL 70%, 10 mL of 80% and 100% methanol, with a 10 mL 20% ethanol wash for re-equilibration of the column for subsequent loads.

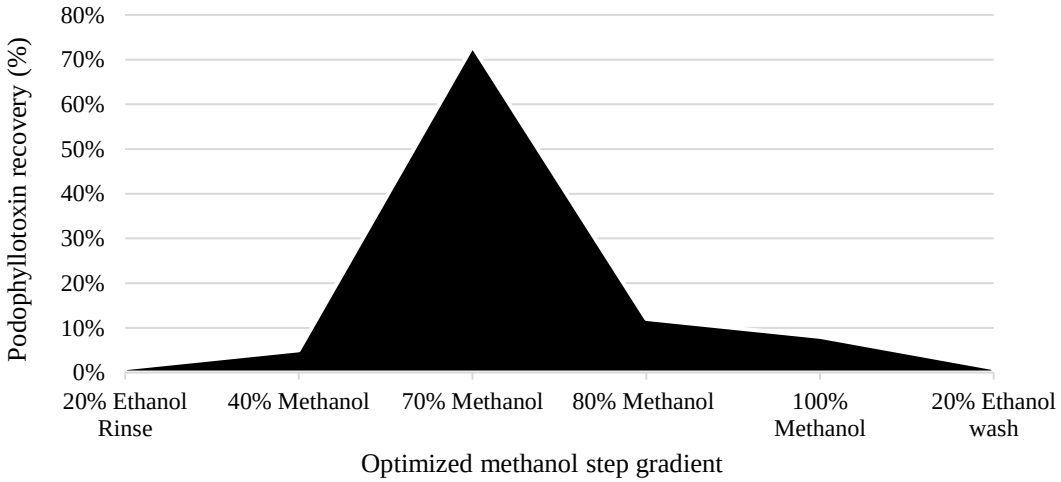
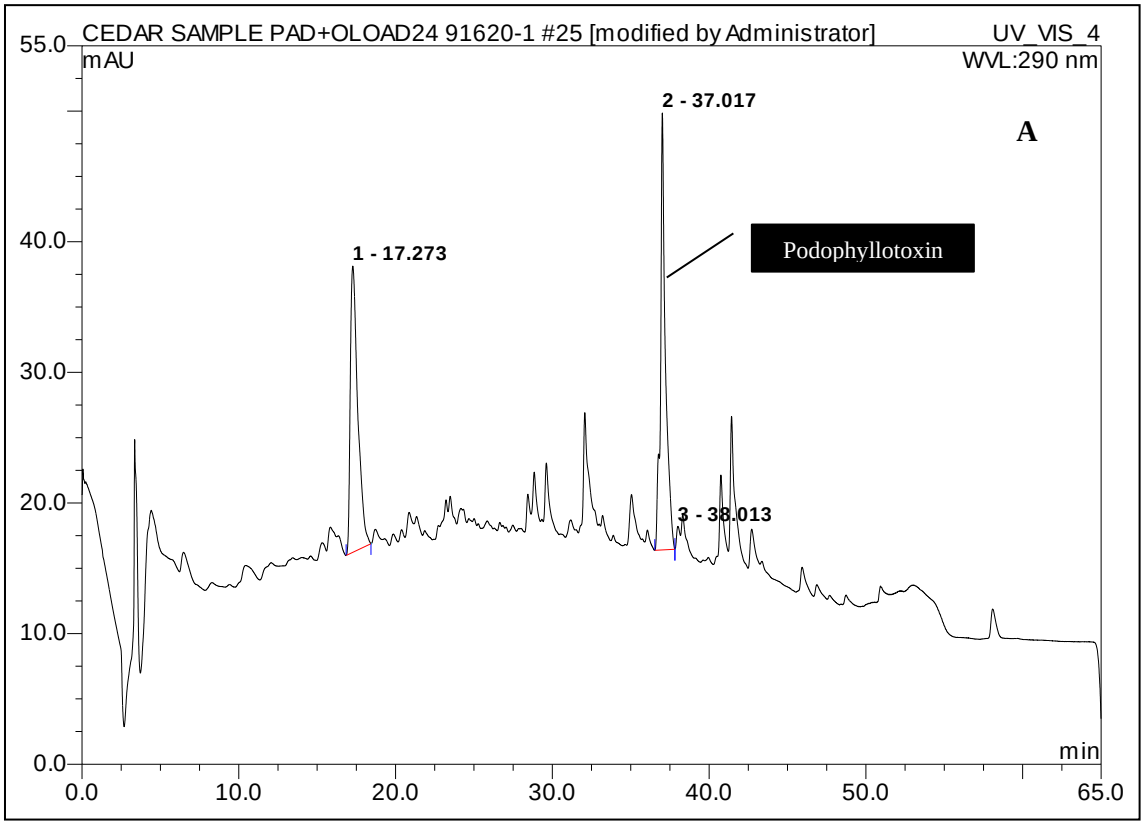
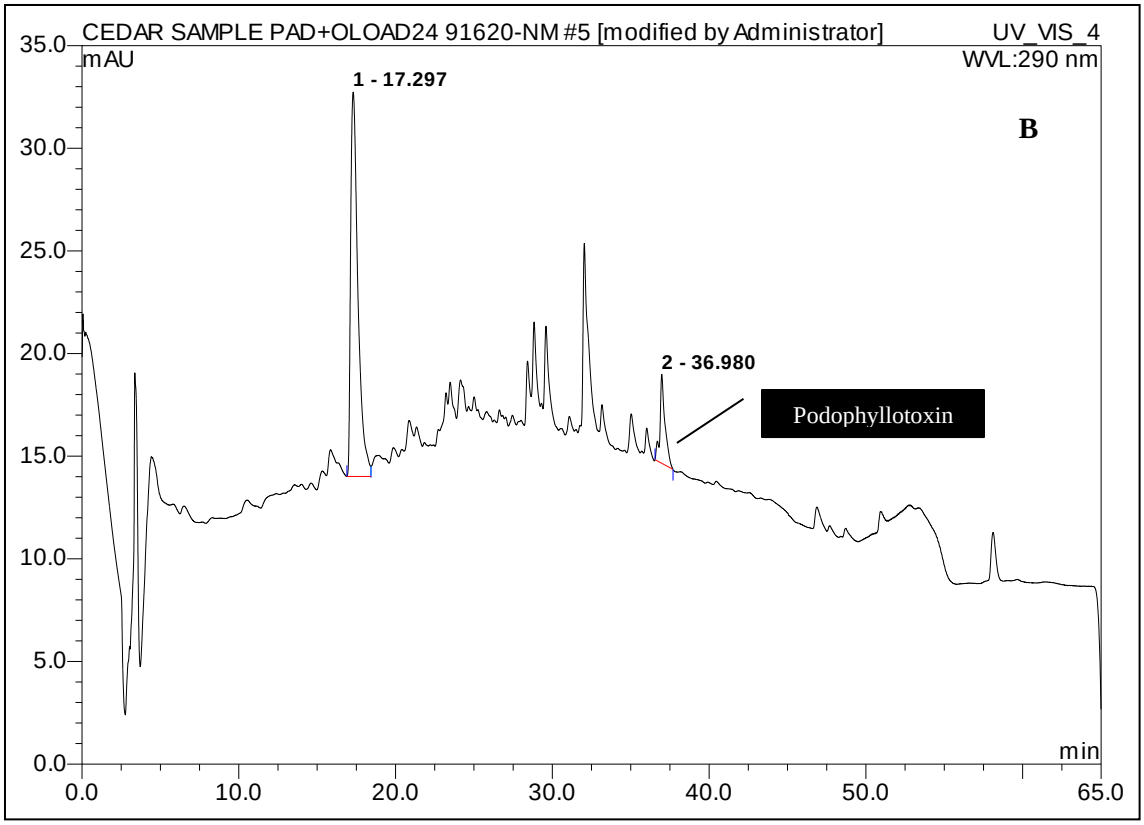


Fig. 8A-G. Chromatogram of eastern red cedar foliage extract (Fig. 8A). Chromatogram of compounds from eastern red cedar foliage extract that rinse from PAD900 packed column in 20% ethanol (Fig. 8B). Chromatogram of compounds from eastern red cedar extract which elute from PAD900 packed column in 40% methanol before podophyllotoxin (Fig. 8C). Chromatogram of concentrated podophyllotoxin in 70% methanol (Fig. 8D). Chromatogram of compounds from eastern red cedar extract which elute with and after podophyllotoxin in 80% methanol (Fig. 8E). Chromatogram of compounds from eastern red cedar extract which elute after podophyllotoxin in 100% methanol (Fig. 8F). Chromatogram of compounds from eastern red cedar extract which elute in 20% ethanol wash/re-equilibration of the column (Fig. 8G).

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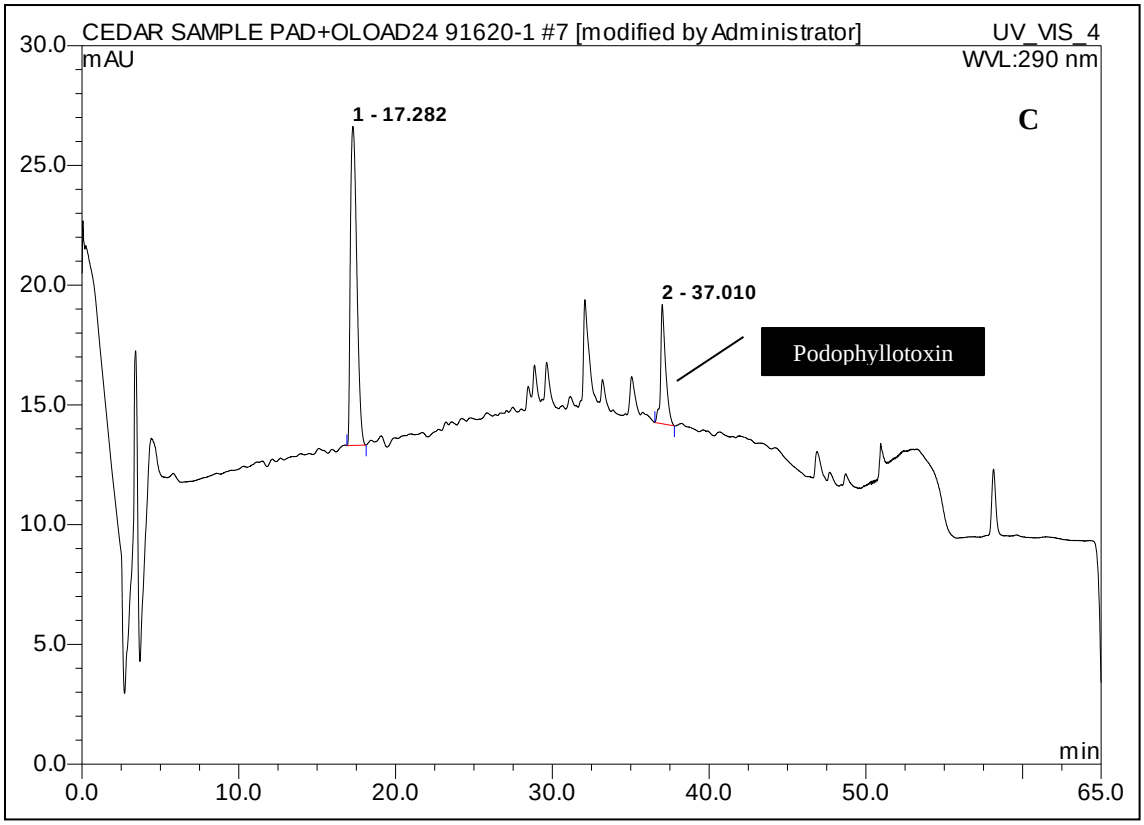
UV_VIS_4
mAU
WVL:290 nm
1 - 17.297
B
2 - 36.980
Podophyllotoxin
min
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
65.0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mAU
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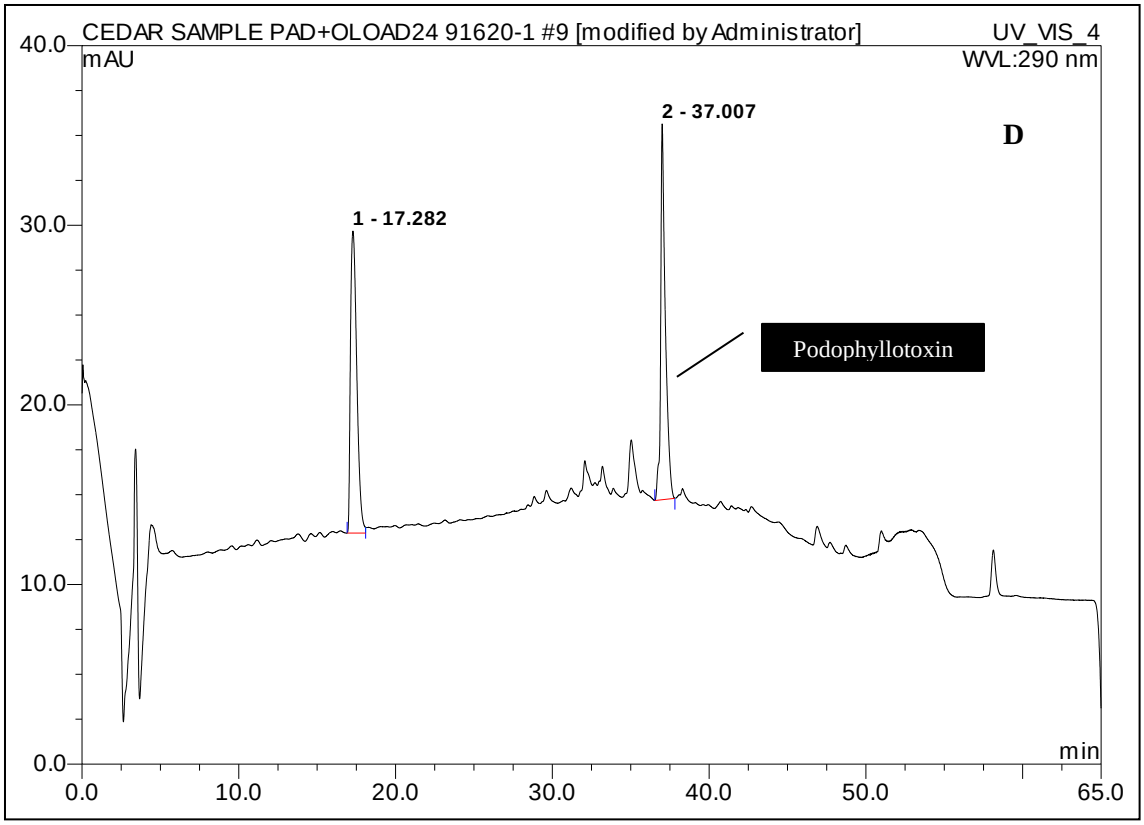
1 - 17.282
C
2 - 37.010
Podophyllotoxin
min
30.0
25.0
20.0
15.0
10.0
5.0
0.0
0.0
10.0
20.0
30.0
40.0
50.0
65.0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D
2 - 37.007
1 - 17.282
Podophyllotoxin
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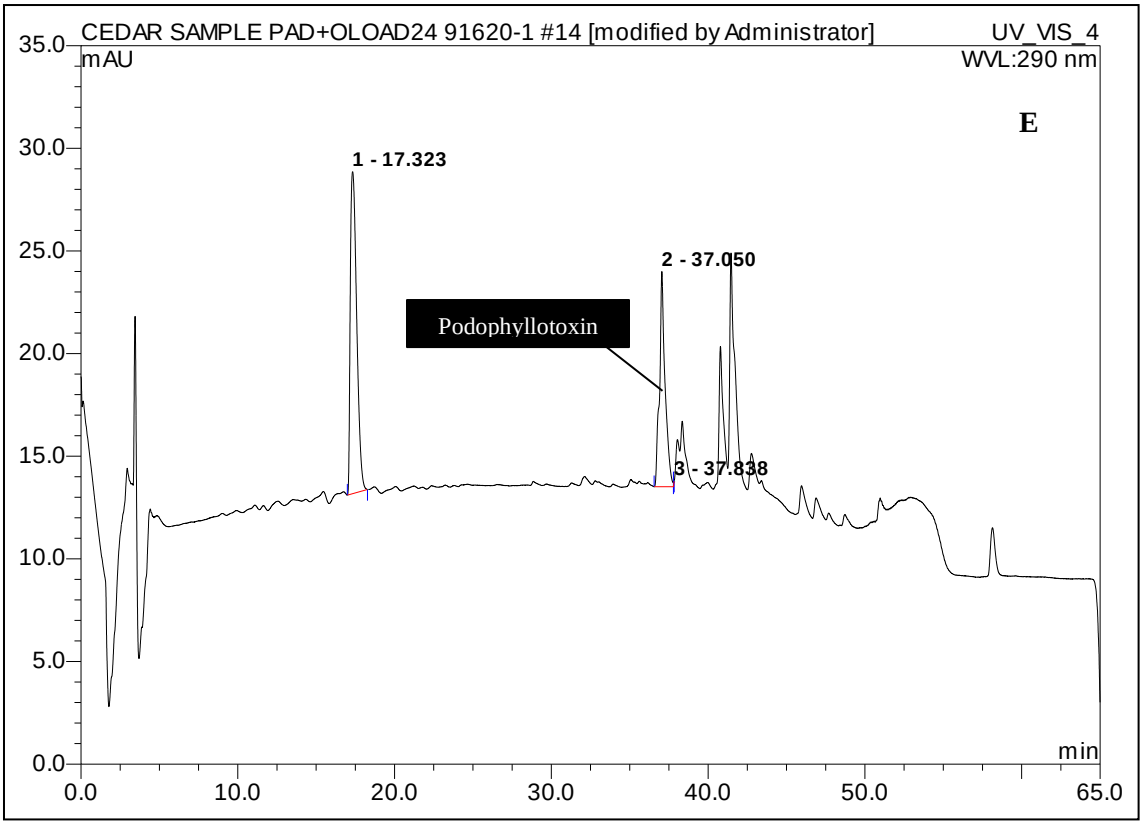
30.0
20.0
10.0
0.0
min
0.0
10.0
20.0
30.0
40.0
50.0
65.0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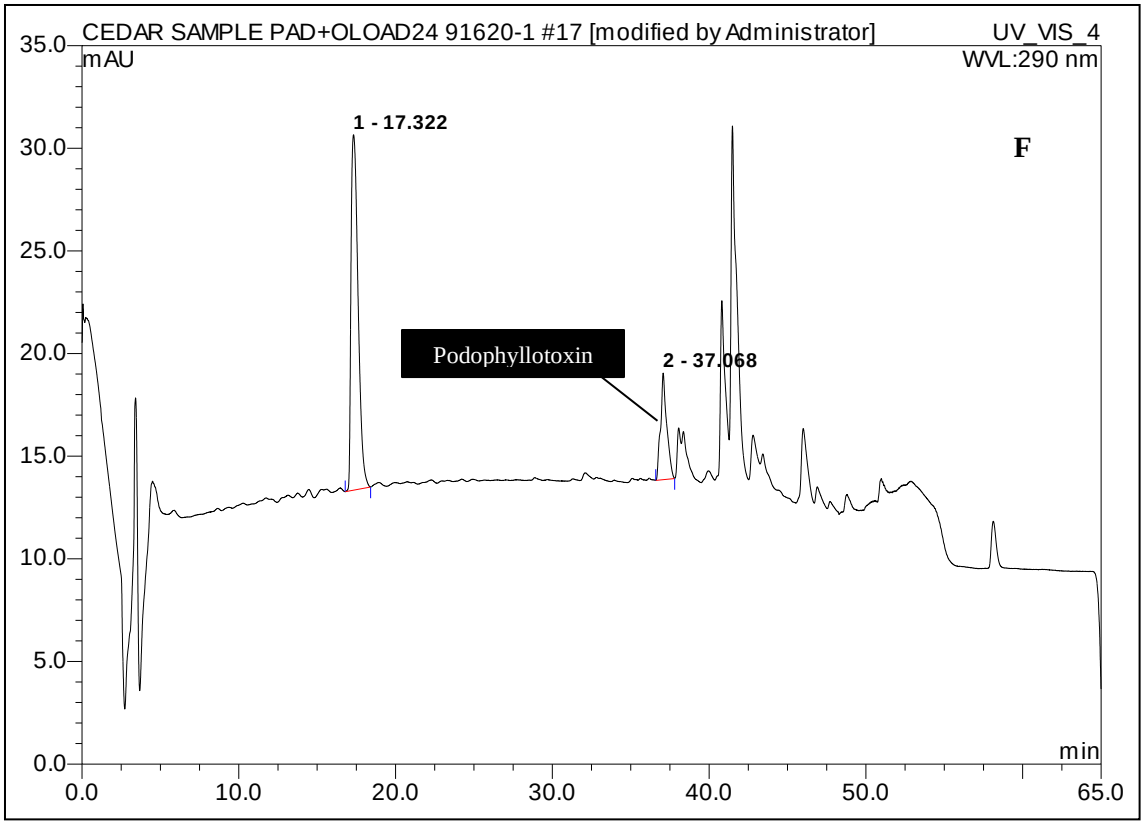
WVL:290 nm
E
1 - 17.323
2 - 37.050
Podophyllotoxin
3 - 37.838
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
min
0.0
10.0
20.0
30.0
40.0
50.0
65.0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2 - 37.068
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30.0
25.0
20.0
15.0
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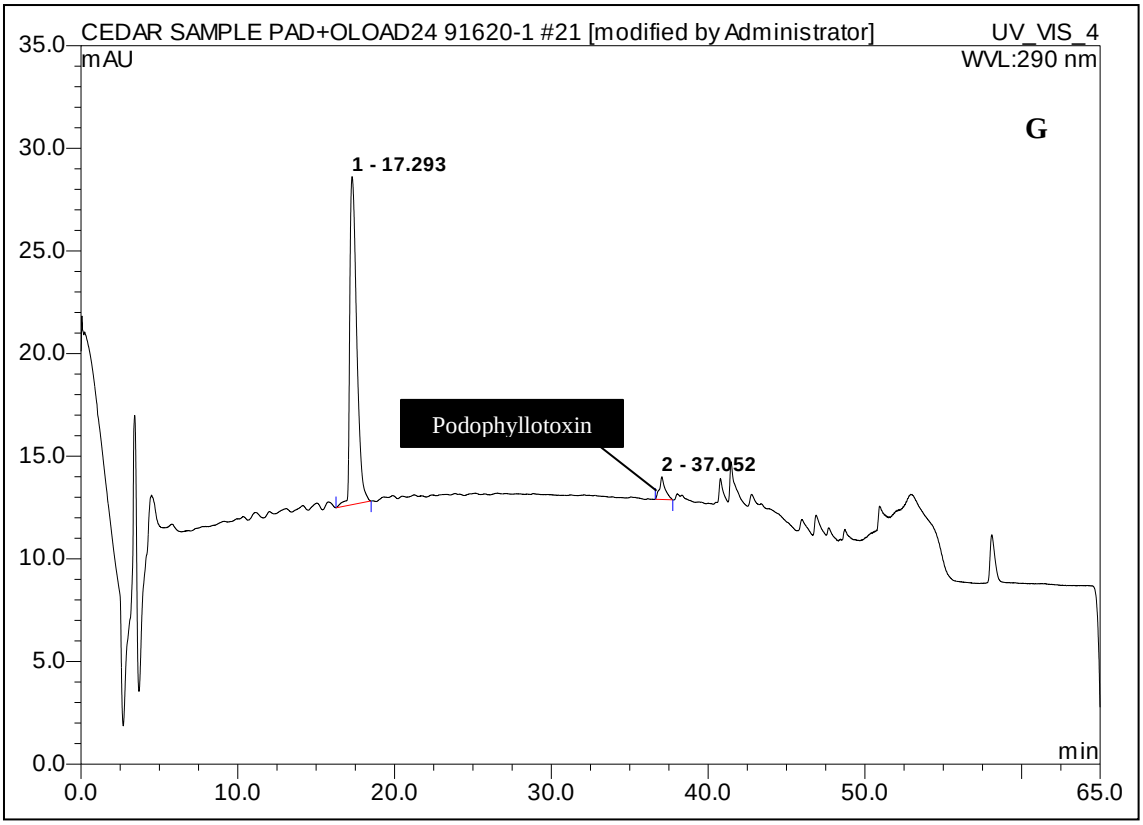
5.0
0.0
min
0.0
10.0
20.0
30.0
40.0
50.0
65.0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Podophyllotoxin
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30.0
25.0
20.0
15.0
10.0
5.0
0.0
min
0.0
10.0
20.0
30.0
40.0
50.0
65.0

CHAPTER III

FURTHER PURIFICATION OF PODOPHYLLOTOXIN EXTRACTED FROM EASTERN RED CEDAR SEPERATED BY STEP-GRADIENT FROM A PAD900 MACROPOROUS RESIN PACKED COLUMN

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Abstract

Eastern red cedar (ERC; *Juniperus virginiana* L.) is a source for podophyllotoxin (ptox); a pharmaceutical derived from plants used in a broad spectrum of medicinal treatments ranging from inflammatory diseases to cancer (Ardalani et al., 2017; Renouard et al., 2011; Seegars et al., 2017). Extraction and purification of ERC for ptox has been evaluated for the greatest ptox extraction yield which was obtained using the combination of a 60ºC extraction temperature, 20% ethanol solvent, 1 h extraction duration and a 20:1 solvent:feedstock ratio. The foliage extract was enriched from 0.5 to 0.6% in the field dried ERC foliage to 14% in a 70% methanol eluate from a PAD900 macroporous resin column with 70% recovery of ptox (Stenmark et al., 2021). This research aimed to further purify ptox from a PAD900 macroporous packed column from 14% ptox DW in the column eluate to 95.4% ptox DW in the re-solubilized crystalized ptox after repeated precipitation with neutralized 5% sodium carbonate and solubilization using ethyl acetate.

Introduction

Podophyllotoxin (ptox) is a target compound sought for use in antiviral topical solutions (Podofilox 0.5% ptox; Seegars et al., 2017), and genetically targeted anticancer and antitumor pharmaceuticals such as Epipodophyllotoxin, Etoposide (VP-16), and Teniposide (VM-26; Uden et al., 1997; Xiao et al., 2020; Yang et al., 2013). Chemical synthesis of ptox is an expensive and complex process, making pharmaceutical production reliant on ptox from plant extracts (Shah et al., 2021; Zhao and Baik, 2011). The original sources for ptox have either become endangered (rhizomes of Indian mayapple; *Podophyllum emodi* Wall.), or were never cultivated as a crop in the U.S. (leaves of American mayapple *Podophyllum peltatum* L.). Current research has been aimed at use of eastern red cedar (ERC; *Juniperus virginiana* L.) foliage for use in isolation of ptox.

ERC contains 0.1-0.5% ptox (DWB) (Renouard et al., 2011), and to extract 1 kg of foliage at our recommended solvent:feedstock ratio of 20:1 will require a cost effective and compatible solvent that is safe to use in large volumes. Ptox extraction from the rhizomes of Indian mayapple has predominantly been done with methanol; large volumes of methanol were required to extract multiple kilograms of Indian mayapple (Bedir et al., 2006). When handled in larger volumes, methanol poses health risks from ingestion, inhalation, and/or skin exposure which may cause adverse health effects (CDC, 2011). When handled in larger volumes, ethanol poses less health risks from ingestion, inhalation, and/or skin exposure than other solvents which have been reported to solubilize ptox (methanol, ethyl acetate, acetone; Gan et al., 2009). In order to safely generate enough ERC extract for ptox purification, large volume ethanol extractions

should be further explored.

Counter-current chromatography (CCC) (Yang et al., 2013) and high-speed counter-current chromatography (HSCCC) (Wang et al., 2013) has been reported for use in enriching ptox from Indian mayapple plant extracts. CCC utilizes a liquid-liquid chromatographic separation technique and does not require a solid sorbent for compounds to adsorb to (Wang et al, 2013; Yang et al., 2013). Selection of a compatible solvent for partitioning is crucial for CCC, where *n*-hexane, ethyl acetate, methanol, and water at 4:1:1:4 (v/v/v/v) (Wang et al., 2013), 4:6:3:7 (v/v/v/v) and 4:6:4:6 (v/v/v/v) (Yang et al., 2013) solvent systems have reported to separate and elute ptox from Indian mayapple extract with high resolution and purity (91.8% DW) (Wang et al., 2013). Determining a compatible solvent system for CCC requires careful attention the partition coefficient, so that high resolution samples can be produced. A low partition coefficient (below 0.2) can result in the extract application eluting close to the solvent front which reduces resolution of the target compound; a high partition coefficient (above 5.0) can result in broad peaks (Wang et al., 2013). Though CCC does not suffer from irreversible adsorption to a stationary phase, resolution of ptox in eluates can vary.

Column chromatographic purification using PAD900, HPD300, and D-101 macroporous resins has been reported to concentrate and partially purify large volumes of ptox from ERC (Stenmark et al., 2021) and Indian mayapple plant extracts (Liu et al., 2015; Wang et al., 2018). Macroporous resins are highly adsorbent packing materials that provide binding sights for polar or nonpolar molecules which are compatible with the resins pore diameter, polarity, and/or specific surface area (Wang et al., 2018). Adsorption of an extract onto a resin purifies the target compound from other undesirable

compounds which do not bind, and concentration of the target molecule which is present in the extract occurs due to elution into a volume which is less than that of the applied extract (Casas et al., 2016).

Compounds separate from the plant extract and absorb to the resin at different rates, some may not adsorb at all and will flow off of the column, and compounds that absorb along with the target compound can be eluted with a compatible solvent for desorption (Bajpai et al., 2016). Step and linear gradient elution has reported to provide clearer resolution of eluates when compared to isolation using isocratic elution techniques (Yang et al., 2013). Desorption of D-101 resin using an ethanol elution step gradient has been performed to provide resolution and further isolate ptox in column eluates of Indian mayapple (Wang et al., 2018). Using a step gradient for elution of ptox from a macroporous resin packed column also provides the flexibility to exchange solvents.

Based on the ptox purification procedure by Mulik and Laddha (2015) for Indian mayapple extracted in 100% methanol, further purification of ptox has been reported utilizing 5% sodium carbonate (Na_2CO_3) as a precipitation medium followed by extraction of the precipitate in ethyl acetate. Na_2CO_3 is reported to cause a precipitate of insoluble solids that is brown, thick and granular. The resulting precipitate requires resolubilization into ethyl acetate using a reflux apparatus for 3 h. Ptox is soluble in organic solvents such as acetone, ethyl acetate, ethanol, propan-2-ol, butan-1-ol, and methanol, and stable to high heat (Gan et al., 2009). Resolubilization in ethyl acetate helps to rid contaminants produced by the first sodium solution. Ethyl acetate extracts ptox from the precipitate back into solution for a second Na_2CO_3 precipitation of ptox which utilizes 1/10 the original Na_2CO_3 treatment volume using a reflux apparatus for 1 h

(Mulik and Ladhha. 2015). Precipitation and subsequent extraction in ethyl acetate of ptox from a concentrated extract provides a purer product for crystallization.

Crystallization can occur from a solution, by freezing, melting, or desublimation of a purified sample, and forms a solid of the target compound (Beckmann, 2013). Crystallization is a means to purify and recover the solid material of a target compound with the greatest distinguishing properties. Crystallization of ptox from a concentrated and purified Indian mayapple sample has been reported by boiling a methanol: chloroform (80:20 v/v) solution which produced 98% chromatographic peak purity (Mulik and Ladhha, 2015). No crystallization attempts have been made for ptox from ERC purified plant extracts.

This project aimed to create a process for further purifying ptox from ERC plant extracts that were purified with the use of macroporous resin enrichment. The goal of our research was to reach >90% ptox purity (DWB) using repeated precipitation, extraction, and recrystallization.

MATERIALS AND METHODS

Plant materials:

ERC foliage was harvested from trees in the vicinity of Stillwater, Oklahoma. Branches were cut from trees and allowed at least three months to field dry before being transported to the Noble Research Center at Oklahoma State University for processing.

Homogenization was achieved using a rolling pin to create a crushed material. ERC was crushed and stored at room temperature for up to one week to await an extraction.

ERC extraction:

Crushed ERC foliage was placed in an appropriately sized beaker, a magnetic stir bar was utilized, and the beaker was covered with parafilm. ERC foliage was extracted in 20% ethanol using stirring, at 60°C on a hot plate, for 1 h extraction duration at a 20:1 solvent:feedstock ratio. Samples could be either centrifuged at 3,000 g_n for 30 min or filtered through Miracloth (MillaporeSigma, Darmstadt, Germany) using vacuum filtration to remove particulates. Filtered extract was stored in 120 mL brown bottles in a refrigerator (3℃) for up to six months.

Macroporous resin enrichment:

Purolite PAD900 macroporous resin was utilized for the study and was packed into a chromatographic column (0.8 cm x 4.7 cm) fitted with compression ends and fittings for attachment to a peristaltic pump (Fisherbrand™ Variable-Flow Chemical Transfer Pump; Fisher Scientific, Waltham, MA, U.S.). Glass wool was packed at the

outlet side (0.5 cm) prior to loading the column with 1.78 g of PAD900 resin. PAD900 resin was settled by initiating flow of 20% ethanol with the peristaltic pump at a flow rate of approximately 2 drops min (3.4 mL h). Final resin bed volume was 1.75 mL. Prior to ERC extract application, the column was activated with 10 mL (5.7 bed volumes) of 100% HPLC grade methanol (Fisher Scientific; Waltham, MA, U.S.) and re-equilibrated with 10 mL of 20% ethanol.

The PAD900 macroporous resin column was loaded with 80 mL of ERC extract containing 0.15 to 0.19 mg ptox mL, rinsed with 10 ml 20% ethanol, and then eluted using a step gradient of 10 mL 40% methanol, followed by 20 mL of 70%, 10 mL of 80% and 100% methanol; a final 10 mL 20% ethanol wash was conducted to re-equilibrate the column for subsequent extract applications. The column was set at a flow rate of approximately 2 drops min and drops were collected with a fraction collector (Isco Retriever II; Teledyne Isco Inc., Lincoln, NE, U.S.) in 175 drop (approximately 5 mL) fractions. Volumes taken for HPLC analysis included 1 mL from each application and 20% ethanol fraction, 200 µl from 40%, 80% and 100% methanol eluate fractions, and 50 µl from the 70% methanol fraction. Samples were then dried in a vacuum concentrator centrifuge (Speed Vac; Savant; Farmingdale, NY, U.S.) for one h. Once dried, the samples were reconstituted in 500 µl of methanol and 500 µl of caffeine in methanol (0.1 mg caffeine mL) and transferred to an autosampler vial for HPLC analysis.

Precipitation of ptox from column eluates:

The remainder of the 70% methanol column eluate from the PAD900 packed column (containing approximately 10-13 mg ptox) was dried completely for 4 h in a 2-dram vial using a Speed Vac. Once completely dried, the 70% methanol column eluate

was solubilized in 1 mL 100% methanol and purified according to an adaptation of the procedure for Indian mayapple by Mulik and Laddha (2015) as described below:

Anhydrous Na_2CO_3 was mixed with deionized H_20 (5 g/ 100 mL) to form a 5% solution. The Na_2CO_3 solution was titrated with acetic acid (using a calibrated pH meter) from a basic (pH 12) to a neutral solution (pH 7-7.3). During the purification procedure 4 mL of neutralized 5% aqueous Na_2CO_3 was added to the 100% methanol (from the solubilized 70% methanol column eluate) and stirred on a magnetic stir plate for 24 h at room temperature to form a thick, dark, granular precipitate. The vial was centrifuged at 3,000 g_n for 30 min in a Speed Vac centrifuge and the Na_2CO_3 supernatant was decanted and saved for loss assessment.

The Na_2CO_3 pellet was dried in a Speed Vac completely before being reconstituted in 1 mL of ethyl acetate and extracted at 80 ℃ for 3 h. The vial was centrifuged for 15 min in a Speed Vac centrifuge and the ethyl acetate supernatant was decanted into a tared 2-dram vial and both the supernatant and the pellet were dried in a Speed Vac for 1 h. The ethyl acetate pellet was saved for loss assessment.

The dried ethyl acetate supernatant was precipitated with a second 0.4 mL treatment of neutralized 5% Na_2CO_3, and incubated for 3 h at 80°C with a stir bar on a heated stir plate; which formed a thick, tan precipitate in a dark yellow solution. The precipitate was then centrifuged at 3,000 g_n in a Speed Vac for 15 min. The supernatant was saved for loss assessment and the pellet was dried in a Speed Vac for 1 h.

The second Na_2CO_3 pellet was extracted a second time in 1 mL ethyl acetate at 80℃ for 1 h; which formed a clear yellow solution and a viscous precipitate. The vial

was centrifuged for 15 min in a Speed Vac centrifuge and the ethyl acetate supernatant was decanted into a tared 2-dram vial, and both the supernatant and pellet were dried in a Speed Vac for 1 h. The second ethyl acetate pellet was saved for loss assessment, and the second ethyl acetate supernatant was refrigerated (3 ℃) until further purification.

Ptox crystallization:

To crystallize ptox from the second ethyl acetate supernatant, the dried ethyl acetate supernatant was treated with methanol:chloroform. The second dried ethyl acetate supernatant was solubilized with 1 mL 100% methanol. After reconstitution into methanol, the sample was chilled to 4°C and 400 µl of chloroform was added. The samples were then transferred to a freezer (-20 °C) and incubated for 24 h for crystallization to occur.

After 24 h, any formed crystals were filtered through a chilled stainless steel Millipore filter holder (Millipore Corporation; Billerica, MA, U.S.) with a 0.45 µm Nylon 66 filter in the cold room at 4°C, and the filtrate was collected into a tared 2-dram vial. At room temperature ptox within the crystals was re-solubilized with methanol (200 µl, three times) from the filter, and eluted into a separate tared 2-dram vial. Both vials were dried down completely in a Speed Vac for 1 h and reconstituted in 1 mL 100% methanol where a diluted sample (50-100 µl) was taken for HPLC analysis. Purification and crystallization attempts were analyzed by HPLC for ptox by DWB% and on % chromatographic peak purity.

Loss assessment of ptox:

For loss assessment of ptox throughout the precipitation process, the 5% Na_2CO_3 supernatants were extracted by vortexing for 1 min with 1 mL of ethyl acetate (which generated a phase response), and centrifuging in a Speed Vac at 3,000 g_n for 15 min. The ethyl acetate phase was decanted into a tared 2-dram vial; the extraction was repeated three times on each Na_2CO_3 supernatant. The combined ethyl acetate extractions (3 mL) were dried in a Speed Vac for 1 h.

For loss assessment of the centrifugation pellets from the ethyl acetate extractions of the Na_2CO_3 precipitates, the ethyl acetate pellets were extracted with methanol (1 h/ 60 °C), and centrifuged at 3,000 g_n for 15 min in a Speed Vac. The supernatant was decanted into a tared 2- dram vial and dried for 1 h. After drying, samples were reconstituted into 1 mL 100% methanol where a diluted (500 µl) sample was taken and caffeine was added (500 µl, 0.1 mg/mL) for HPLC analysis.

HPLC analysis:

Autosampler vials containing appropriately diluted samples plus caffeine (0.1 mg caffeine mL) as internal standard were placed into a Thermo Scientific Dionex ASI-100 automated sample injector (Thermo Scientific Dionex; Sunnyvale, CA, U.S.). Separation of compounds from a 10 µl injection was accomplished using a Kinetex 5 um XB C-18 100 Å column (250 x 4.6 mm; Phenomenex, Torrance, CA, U.S.) and a Dionex ICS 3000 liquid chromatograph at a flow rate of 0.8 mL min. The mobile phase consisted of 0.2% acetic acid (solvent A) and 100% methanol (solvent B). An elution gradient starting at 90% solvent A/10% solvent B was immediately linearly increased to 30% solvent A/70% solvent B over 40 min and then increased to 100% solvent B for the next 10 min. The column was rinsed with 100% solvent B for 10 min and then re-equilibrated to 90%

solvent A/10% solvent B to prepare for the next run. Total run duration was 65 min. Peaks were detected at 290 nm using a Dionex ICS Series PDA detector. Fresh standards of ptox spiked with the caffeine internal standard (0.1 mg/ mL) were run with each sample run. Ptox from a sample was quantified relative to that of ptox in a standard sample run using the external standard. All samples were run in duplicate.

Data analysis:

Ptox in chromatograms from HPLC was identified and chromatographic peak purity (%) and ptox DWB (%) were analyzed. Sample responses ran separately from the standard were compared to that of the standard sample runs as an external standard.

RESULTS AND DISCUSSION

Scale-up extractions using up to 12 g of ERC foliage were done in order to produce a stock amount of ERC extract (up to 240 mL) which could be used for macroporous resin enrichment. Using our optimum conditions of crushed foliage extracted in 20% ethanol solvent, 60 °C extraction temperature, 1 h extraction duration and 20:1 solvent:feedstock ratio (Stenmark et al., 2021) we produced extracts from ERC crushed foliage containing 0.18 mg ptox mL. On a dry basis the extract contained on average 10 mg ptox/ g, compared to 5-6 mg ptox/ g foliage (Table 5). Crushed foliage extract yielded nearly the same amount of ptox as the finely ground foliage, but crushed foliage could be either vacuum filtered or centrifuged, where ground foliage had to be centrifuged. In scaled operation of our extraction procedure filtration could be a fast and efficient procedure for separating the spent foliage from the extract but centrifugation would be prohibitively expensive for the extract volumes to be processed. Crushed foliage was used for the remainder of the study in order to generate stock ERC extract. Stock ERC extract did not degrade in ptox over time and could be stored for up to 6 months when stored in 120 mL brown bottles and refrigerated at (3 °C).

Macroporous resin was one of our most costly ($285/kg) materials for purification and concentration of ptox from ERC foliage extract. Using PAD900, we observed that over 40 cycles of ERC foliage extract application, ptox desorption and column re-equilibration, the resin exhibited no change in column performance. Although we do not

yet know the life time of a packed column, the cost of the resin can be distributed across many extract purifications which reduces its effective cost.

Using a step gradient to elute an 80 mL ERC foliage extract application, we found that the majority (70-75%) of ptox eluted from the PAD900 macroporous resin column within the subsequent 70% methanol step (Stenmark et al., 2021). Less than 5% ptox eluted from the column in the steps prior. The 70% methanol column eluate was the purest fraction that eluted from the column by DW and peak purity (%), but we noted dark brown color compounds which eluted immediately from the column when the methanol solvent was increased from 40% to 70%. The 70% methanol fraction concentrated the ptox from 80 mL ERC foliage containing 5-6 mg/ g foliage to 20 mL 70% methanol, and produced a dry matter containing 10-13 mg ptox/ g. We were able to concentrate ptox in ERC extract to $1/4^{th}$ the original volume, while enriching the extract, and exchanging from an ethanol to a methanol solvent which could be used for further purification.

The 20 mL of ptox concentrated in 70% methanol was dried completely in a Speed Vac and reconstituted in 1 mL methanol for ptox precipitation. We found that when trying to precipitate ptox from 70% methanol without drying, the Na_2CO_3 step produced less precipitate. By resolubilizing the dried 70% methanol eluate into 1 mL 100% methanol we found that the Na_2CO_3 produced a greater precipitate. When analyzed with HPLC for ptox, the resolubulized 100% 1 mL of 100% methanol contained 9 mg ptox, 22% ptox DW and had 63% peak purity, compared to 13 mg ptox, 14% ptox DW and 50.7% peak purity for the original 70% methanol column eluate (Table 6). We suspect that some ptox was lost during the drying step or was not solubilized during the

reconstitution step, which indicated that a better drying procedure should be further investigated.

During initial testing with a non-neutralized Na_2CO_3 solution, we found that the ptox peak eluting at approximately 17.3 min had decreased in size by over 5 area units (AU), with a new prominent peak eluting 1-2 min prior to ptox (Fig. 9). Picropodophyllotoxin is produced when ptox is exposed to a basic solution (Medrado et al., 2014). This chemical change in the structure of ptox is an irreversible process that negates the anticarcinogenic activity of ptox. To correct this issue, we neutralized the Na_2CO_3 solution with a calibrated pH meter by titrating the basic solution from pH 12 to pH 7-7.3 using laboratory grade acetic acid. After we made this change to the precipitation solvent, the new peak did not occur upon HPLC and we assume that ptox did not irreversibly convert into picropodophyllotoxin.

Using the neutralized Na_2CO_3 we formed a precipitate (weighing on average 35 mg when dried); the Na_2CO_3 supernatant was saved for loss assessment and contained 29% ptox DW and had 7% peak purity. We extracted ptox using ethyl acetate from the dried Na_2CO_3 pellet. After the ethyl acetate extraction, the Na_2CO_3 pellet turned into a viscous brown material which could be spun down using centrifugation (3,000 g_n for 30 min). The ethyl acetate supernatant contained 5 mg ptox, 43% ptox DW and had 52% peak purity (Table 6). The ethyl acetate pellet was a small source of loss of ptox and contained 0.1 mg ptox, 0.7% ptox DW and had 6% peak purity (Table 6).

A second precipitation using neutralized 5% Na_2CO_3 produced a precipitate weighing on average 22 mg DW. The second Na_2CO_3 pellet when dried was used for a second ethyl acetate extraction. The second ethyl acetate solution was dried in a Speed

Vac and produced a solid weighing 18 mg. After a second ethyl acetate extraction, ptox in the ethyl acetate supernatant contained 3 mg ptox, 33% DW and had 48% peak purity (Table 6).

Crystallization of ptox from the dried ethyl acetate extraction step was first attempted using the 80:20 methanol:chloroform (v/v) solution reported by the procedure from Mulik and Ladhha (2015). This solution was attempted, but did not form a solid or crystallite substance on its own. The procedure from Mulik and Ladhha (2015) reported using methanol:chloroform with boiling to crystallize ptox from the solution. We did not witness ptox crystallizing in methanol:chloroform 80:20 (v/v) under boiling conditions. We decided that we should reconstitute the dried ethyl acetate into 100% methanol and chill it in a cold room before adding chloroform – which did not work. We then placed the sample into a freezer (-20°C) and incubated for 24 h for crystallization to occur. After 24 h we noticed that some crystals had formed within the solution, which dissolved back into solution upon warming to room temperature. In order to recover the crystals formed during incubation in the freezer, we took the samples from the freezer and filtered them through a pre-chilled 0.45 μm nylon 66 filter inside a cold room. We were then able to re-solubilize the crystals from the filter with room temperature 100% methanol, and when analyzed with HPLC the ptox purity for the filter retentate was assessed to contain 0.9 mg ptox, 89% DW and had 42% peak purity (Table 6).

Ptox recovery is documented in table 6. The first major source of loss is seen in the drying step where 13.12 mg of ptox in the 70% methanol eluate when dried completely in a Speed Vac and resolubilized in 1 mL of 100% methanol recovers only 9 mg ptox; 31.4% ptox loss. The second major source of loss of ptox occurs after the first

Na_2CO_3 precipitation. When extracted with ethyl acetate and analyzed for ptox with HPLC, the ethyl acetate supernatant that is decanted from the pellet contained 4.83 mg of the 9 mg ptox found in the 100% methanol starting solution; 46 % ptox loss. Minimal loss was seen in the ethyl acetate extracted pellet; 0.1 mg ptox, 0.7% ptox DW, and had 6% peak purity. The ethyl acetate extraction to the Na_2CO_3 precipitate was effective at recovering ptox from what precipitated from 100% methanol with minimal loss of ptox. After the second Na_2CO_3 precipitation we see loss of ptox from the purification process where 0.1 mg is lost to the Na_2CO_3 supernatant. The crystallization process recovers only 0.9 mg of ptox from the total 9 mg ptox in the starting 100% methanol starting solution; 10% recovery.

CONCLUSION

Using the methanol step-gradient on the PAD900 packed column, we were able to concentrate and partially purify ptox from an ERC foliage extraction. Throughout the purification procedures we noted the highest peak purity within the 70% column eluate fraction. Precipitation of ptox from the 70% column eluates proved difficult, where we noted chemical changes of ptox when the solution was basic (Fig. 9). Neutralizing the Na_2CO_3 prevented the chemical change in ptox to picropodophyllotoxin. Out of over 25 purification procedures, two successful purifications and crystallizations of ptox were observed where purity of ptox on a DW reached over 89%. Though the methanol:chloroform solution produced crystals upon freezing, the recovery of ptox, starting at 9 mg in 100% methanol starting solution was only 0.9 mg in the solubilized crystals for a 10% recovery. Throughout the purification procedure the major losses of ptox occurred during the precipitation with Na_2CO_3 and crystallization steps (sources of loss; Table 6). Even after a 24 h precipitation period and centrifugation of the precipitate, the Na_2CO_3 solution continued to precipitate, indicating that a longer precipitation period should be further investigated. In order to minimize losses of ptox during precipitation and crystallization, more research should be done to optimize a procedure for purification of ptox from ERC plant extracts that were enriched with macroporous resins.

CHAPTER III TABLES

Table 5. Podophyllotoxin in the foliage (mg/ g) verses podophyllotoxin in an extract (mg/ mL) from field dried, ground, and crushed eastern red cedar foliage extractions.[z]

Eastern red cedar foliage condition	Podophyllotoxin in foliage[y] (mg/ g)	Podophyllotoxin in extract (mg/ ml)
Field dried	5-6	---[x]
Ground	11	0.19
Crushed	10	0.18

[z] Samples extracted in 20% ethanol for a 1 h duration at 60℃ at a 20:1 solvent:feedstock ratio.

[y] Values given represent the average of 2 extractions with 4 replications.

[x] Extraction not preformed.

Table 6. Recovery of podophyllotoxin (mg/ mL) from an adaptation to the podophyllotoxin purification procedure from Mulik and Ludhha (2015).[z] Precipitation and crystallization was attempted to increase podophyllotoxin purity by dry weight basis (%); all samples were analyzed with high-performance liquid chromatography. Table 6 includes sources of loss of podophyllotoxin from the processes.

Purification step	Sample dry weight (mg)[x]	Podophyllotoxin dry weight basis (%)	Podophyllotoxin chromatographic peak purity (%)	Recovery (mg ptox/ mL)
Eastern red cedar foliage extract	13[y]	1	29	0.15
70% methanol eluate from column	5	14	51	13
100% methanol concentrated podophyllotoxin	5	22	63	9
Na_2CO_3 precipitation #1: loss	4	29	7	1
Ethyl acetate extraction #1	11	43	52	5
Ethyl acetate extraction #1: loss	15	0.7	6	0.1
Na_2CO_3 precipitation #2: loss	4	3	7	0.1
Ethyl acetate extraction #2	8	35	45	3
Ethyl acetate extraction #2: loss	7	0.8	7	0.1
Flow through from filter	3	12	46	0.3
Filter retentate	2	89	42	0.9

[z] Mulik, M.B. and K.S. Laddha. 2015. Isolation and characterization of aryltetralin type lignin roots of *Podophyllum emodi*. Int. J. Health Sci. Res. 5:537-540.

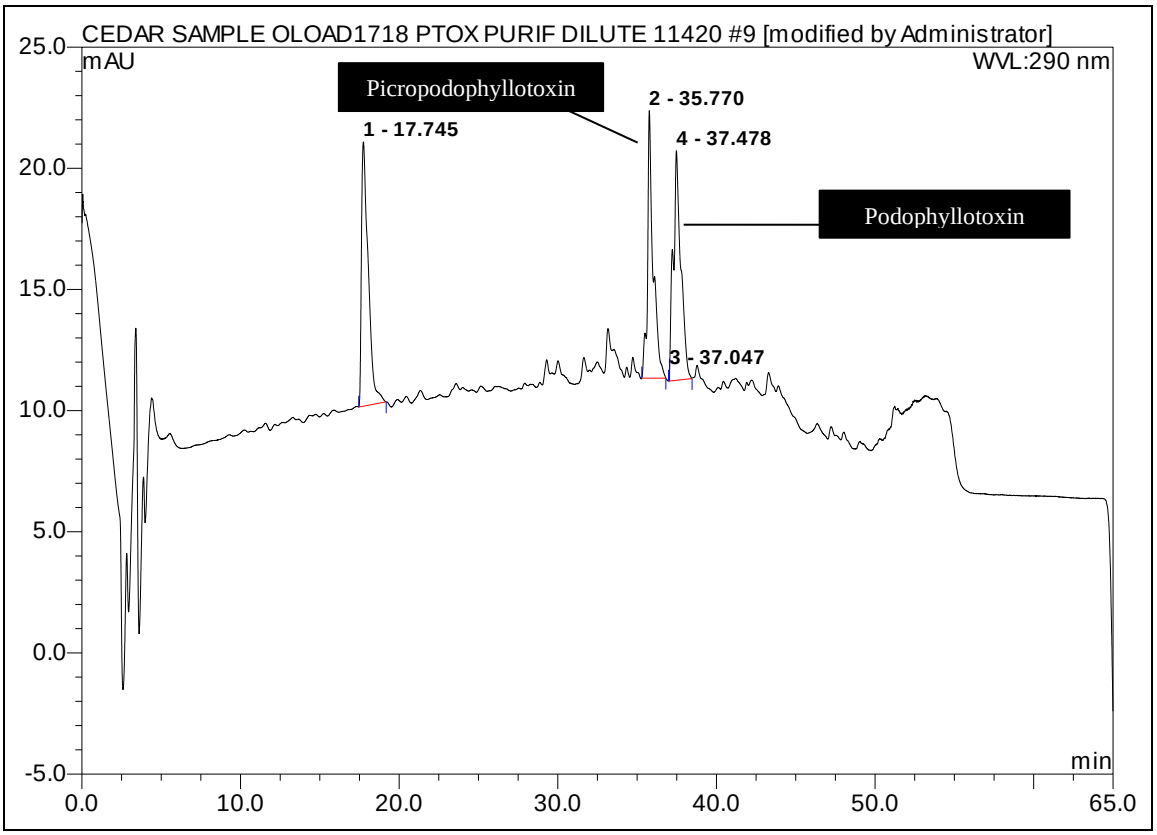
[y] Eastern red cedar foliage was extracted in 20% ethanol for a 1 h duration at 60℃ at a 20:1 solvent:feedstock ratio. Eastern red cedar extract (extract concentration 0.15 mg/ mL; 80 mL) was enriched with PAD900 resin using a methanol step gradient. Podophyllotoxin, concentrated in 20 mL 70 % methanol PAD900 column eluate (containing 13.1 mg podophyllotoxin), was dried completely in a Speed Vac and solubilized in 1 mL 100% methanol (containing 9.0 mg podophyllotoxin). The 1 mL of 100% methanol was precipitated with 4 mL Na_2CO_3. The precipitate was dried and extracted with 1 mL ethyl acetate, the supernatant was dried and precipitated with a second Na_2CO_3 (0.4 mL) treatment. The second Na_2CO_3 precipitate was dried and extracted with a second 1 mL of ethyl acetate (containing 3.01 mg podophyllotoxin). The second ethyl acetate extraction was crystallized a with methanol:chloroform solution that was

frozen (-20°C) for 24 h. The crystals were filtered (containing 0.34 mg podophyllotoxin) and solubilized from the filter with 100% methanol (containing 0.9 mg podophyllotoxin).

[x] values given represent the averages from 2 purification procedures; 2 replications per sample analyzed with high-performance liquid chromatography.

CHAPTER III FIGURES

Figure 9: Chromatogram of a non-neutralized sodium carbonate which was used to precipitate podophyllotoxin from a 100% methanol solution showing picropodophyllotoxin as the new dominant peak eluting at 35.7 minutes and podophyllotoxin eluting at 37.4 minutes.



CHAPTER IV

CONCLUSIONS

This chapter is presented to summarize the research conducted in chapters II and III including the results. The following objectives are listed for each chapter.

Chapter II conclusions:

1 Objective (1) was to identify variables required for extraction of ptox from ERC foliage. Variables tested for extraction were ethanol solvent concentration, temperature, extraction duration, and solvent:feedstock ratio.

   a. Ethanol concentration: Ethanol concentrations of 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 95% were tested using 25 mg foliage and 2 mL solvent for a 1 h extraction at room temperature. Aqueous ethanol at ≥10% concentration (v/v) showed no significant difference in yield and extracted nearly the same amount of ptox as concentrations as high as 95% ethanol (Table 1). Water alone was not as effective for ptox extraction. Ptox yield appeared to plateau using 20% or higher ethanol concentrations. We chose utilize 20% ethanol for continuation of the studies.
   b. Temperature: Temperatures ranging from 30-90°C were tested using 25 mg sample in 2 mL, 20% ethanol extraction solvent and a 1 h extraction duration. Temperatures above 60°C had no significant difference in yield of ptox when compared to extraction temperatures as high as 90°C. We chose to use 60°C as the lowest temperature that still produced high yields of ptox (Table 2).
   c. Solvent:feedstock ratio: We tested solvent:feedstock ratios using 25 mg, 50 mg, 100 mg, 200 mg, and 400 mg foliage in 2 mL 20% ethanol. Fig. 2 presents the yield of ptox from foliage at solvent:feedstock ratios varying from 2 mL extraction solvent:25 mg ERC foliage (80:1) to 2 mL extraction solvent:400 mg ERC foliage (5:1). Although ptox yield declined with increasing feedstock amount, there was an inflection point at 2 mL extraction solvent:100 mg ERC foliage (20:1 solvent:feedstock ratio) at which the decline was less than at lower solvent:feedstock ratios. We chose to utilize 20% ethanol, 60°C, and a feedstock ratio of 20:1 to increase feedstock throughput.
   d. Duration: Since extraction duration could impact ptox extraction yield at the various feedstock ratios, we tested the previously used 1 h extraction duration against a 2 h extraction duration (Fig. 3). We found little

difference in ptox extract yield by the 1 h increase in extraction duration. We continued experiments with 20% ethanol solvent, 60 °C incubation temperature using a solvent:feedstock ratio of 20:1 over an extraction duration of 1 h.

2 Objective (2) was to demonstrate use of extraction variables at a laboratory and a batch-laboratory scale. Using our optimum conditions described in objective 1, we tested UDY mill ground verses crushed foliage for batch-laboratory scale production of ERC extract. Using crushed foliage, we produced extracts from ERC foliage containing 0.18 mg ptox mL which was within range of the UDY mill ground foliage (0.15 to 0.19 mg ptox mL, Table 5). Crushed foliage extract yielded nearly the same amount of ptox as the finely ground foliage, but crushed foliage could be either vacuum filtered or centrifuged, where ground foliage had to be centrifuged. In scaled operation of our extraction procedure, filtration could be a fast and efficient procedure for separating the spent foliage from the extract but centrifugation would be prohibitively expensive for the extract volumes to be processed. Crushed foliage was used for the remainder of the study in order to generate stock ERC extract.

3 Objective (3) was to evaluate various resins for ptox concentration and purification from ERC extracts. We tested one divinylbenzene (PAD900) and three polyvinyldibenzene (PCG900C, PCG900F and PCG900M) resins differing in particle size and price (Table 3) for ERC extract ptox absorbance/desorbance properties.

a. We loaded 1 mL ERC foliage extract onto 500 mg of each of the four tested resins, allowing for a 1 h absorption, and rinsing with 1 mL of an ethanol step gradient from 20%, 40%, 60%, 70%, 80% and 95% to elute ptox (Fig. 4).
    i. All resins effectively absorbed ptox from the ERC extract and showed little ptox bleed during a subsequent 20% ethanol rinse.
    ii. Ptox elution profiles were different, with the large particle size, macroporous PAD900 resin exhibiting slightly lower ptox elution at the 40% ethanol elution step but then a wider ptox elution profile over higher ethanol concentration gradient steps and with maximum ptox elution at 70% ethanol.
    iii. Smaller particle size resins exhibited maximum ptox desorption at 60% ethanol concentration, with sharper elution of ptox from the smallest particle size resin (PCG900M) and almost identical elution profiles from the other two larger particle size resins (PCG900C and PCG900F).

b. We packed a column with PAD900 and PCG900C resins. During gravity-fed application of ERC extract the PAD900 resin exhibited 1.5 mL h flowrate, versus 0.4 mL h flow rate for the PCG900C column. Since an objective of this work was to develop a low cost, scalable ptox purification process from ERC, the higher flow rate for the PAD900 macroporous resin, combined with its lower cost, made it a resin of choice for continued experimentation.

4 Objective (4) was to demonstrate performance of the best resin in objective 3 in terms of concentrating and purifying ptox from ERC foliage extracts.

a. We tested desorption of ptox from a column containing PAD900 macroporous resin using a methanol step-gradient starting with an 80 mL load of ERC extract to the column. We rinsed the column with 10 mL

20% ethanol, switching to a 40% methanol eluate, followed by 20 mL elution of ptox in 70% methanol, and 10 mL of 80% and 100% methanol. The column was washed with 10 mL 20% ethanol to re-equilibrate the column for subsequent loads.

i. We achieved 80 mL loads of ERC extract with less than 1% loss of ptox from the column.
ii. We successfully exchanged ptox in 20% ethanol extract to a methanol solvent.
iii. We concentrated 15 mg ptox in 80 mL ERC extract to 13 mg ptox in 20 mL 70% methanol.
iv. We increased ptox concentration in the dry matter from 0.05% in ERC foliage to 14% in the 70% methanol column eluate.

Chapter III conclusions:

Chapter III had one objective- to further purify ptox from enriched ERC extract based on an adaptation to the ptox purification procedure for Indian mayapple extract by Mulik and Ladhha (2015). This objective was divided into four parts:

1. Demonstrate precipitation of ptox with Na_2CO_3.
2. Demonstrate solubilization of ptox with ethyl acetate.
3. Demonstrate crystallization of ptox using methanol:chloroform.
4. Identify sources of loss of ptox throughout the procedure.

1. Demonstrate precipitation of ptox with Na_2CO_3: We tested precipitation of ptox from the 70% column eluates that were dried completely and solubilized in 1 mL 100% methanol. During the purification procedure 4 mL of neutralized 5% aqueous Na_2CO_3 was added to the 100% methanol and stirred on a magnetic stir plate for 24 h at room temperature to form a thick, dark, granular precipitate.
    a. Neutralizing the Na_2CO_3 prevented the putative chemical change in ptox to picropodophyllotoxin (Fig. 9).
    b. Using the neutralized Na_2CO_3 we successfully formed a precipitate containing ptox. The Na_2CO_3 supernatant was saved for loss assessment and contained 1.18 mg ptox, 29.5% ptox DW and had 6.5% peak purity; 13% ptox loss.
    c. We conducted a second Na_2CO_3 treatment where we successfully formed a precipitate containing ptox. The Na_2CO_3 supernatant was saved for loss assessment and contained 0.12 mg ptox, 3% ptox DW and had 6.6% peak purity; 1.3% ptox loss.

2. Demonstrate solubilization of ptox with ethyl acetate: We extracted ptox using ethyl acetate from the dried Na_2CO_3 pellet on a stir plate for 3 h at 80°C.
    a. Ethyl acetate successfully solubilized ptox from the Na_2CO_3 pellet, and the ethyl acetate supernatant contained 4.83 mg ptox, with 43.1% ptox DW and had 51,7% peak purity (Table 6); 53.7% ptox recovery. The ethyl acetate pellet was a small source of loss of ptox and contained 0.1 mg ptox, 0.7% ptox DW and had 6.3% peak purity; 1% ptox loss.

b. When extracted a second time, the ethyl acetate successfully solubilized the second Na_2CO_3 pellet and we recovered 3.01 mg ptox with 35.8% ptox DW and 44.5% peak purity; 33.4% ptox recovery.

3. Demonstrate crystallization of ptox using methanol:chloroform: Crystallization of ptox from the dried ethyl acetate extraction of the second Na_2CO_3 pellet was first attempted using the 80:20 methanol:chloroform (v/v) solution. We reconstituted the dried ethyl acetate into 100% methanol and then placed the sample into a freezer (-20 °C) and incubated for 24 h for crystallization to occur. After 24 h we took the samples from the freezer and filtered them through a pre-chilled 0.45 μm nylon 66 filter inside a cold room. We re-solubilized the filter retentate with room temperature 100% methanol.
    a. The procedure from Mulik and Ladhha (2015) reported using methanol:chloroform with boiling to crystallize ptox from the solution. We did not witness ptox crystallizing in methanol:chloroform 80:20 (v/v) under boiling conditions.
    b. We decided that we should reconstitute the dried ethyl acetate into 100% methanol and chill it in a cold room before adding chloroform – which did not work.
    c. Placement of the solution in b) above into a freezer for 24 h did cause apparent crystals to form; if allowed to stand at room temperature the apparent crystals re-solubilized so separation of apparent crystals by filtration was conducted using the cold solvent.
    d. When analyzed with HPLC the ptox purity for the filter retentate contained 0.9 mg ptox, 89.3% DW with 41.5% peak purity (Table 6); 10% ptox recovery.

4. Identify sources of loss of ptox throughout the procedure: Recovery of ptox was assessed throughout the purification procedure by sampling from each step. Starting with the initial concentration step, the Na_2CO_3 precipitations, the ethyl acetate extractions, and ending with the crystallization attempts we examined the procedure for any major sources of loss of ptox as seen in table 6.
    a. The drying step where 13 mg of ptox in the 70% methanol eluate when dried completely in a Speed Vac and resolubilized in 1 mL of 100% methanol recovered only 9 mg ptox; 31.4% ptox loss.
    b. The first Na_2CO_3 precipitation lost 1.18 mg of ptox from the 9 mg starting amount; 13% ptox loss.
    c. The ethyl acetate extraction recovered 4.83 mg or 54% of ptox from the precipitate; 1% ptox loss in the ethyl acetate pellet.
    d. The second Na_2CO_3 precipitation lost 0.12 mg of ptox to the Na_2CO_3 supernatant; 1% ptox loss.
    e. The crystallization process recovered only 0.9 mg of ptox from the total 9 mg ptox for a 10% recovery; 90% ptox loss.
    f. With only 10% recovery of ptox from our adaptation to the ptox purification procedure for Indian mayapple extract by Mulik and Ladhha (2015), more research should be done to optimize a procedure for

purification of ptox from ERC plant extracts that were enriched with macroporous resins in order to minimize losses during ptox purification.

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