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Canadian boreal pulp and paper feedstocks contain neuroactive substances that interact in vitro with GABA and dopaminergic systems in the brain

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HIGHLIGHTS

- Conifer pulp feedstocks were tested in vitro for dopaminergic/GABAergic activity.
- Conifer phytochemicals were tested in the same assays.
- Interaction with neuroendocrine endpoints could lead to reproductive disruption.
- Neuroendocrine effects of pulp mill effluents could be of phytochemical origin.
- Potential exists for natural product development from Canadian conifer species.

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ABSTRACT

Pulp and paper wood feedstocks have been previously implicated as a source of chemicals with the ability to interact with or disrupt key neuroendocrine endpoints important in the control of reproduction. We tested nine Canadian conifers commonly used in pulp and paper production as well as 16 phytochemicals that have been observed in various pulp and paper mill effluent streams for their ability to interact in vitro with the enzymes monoamine oxidase (MAO), glutamic acid decarboxylase (GAD), and GABA-transaminase (GABA-T), and bind to the benzodiazepine-binding site of the GABA(A) receptor (GABA(A)-BZD). These neuroendocrine endpoints are also important targets for treatment of neurological disorders such as anxiety, epilepsy, or depression. MAO and GAD were inhibited by various conifer extracts of different polarities, including major feedstocks such as balsam fir, black spruce, and white spruce. MAO was selectively stimulated or inhibited by many of the tested phytochemicals, with inhibition observed by a group of phenylpropenes (e.g. isoeugenol and vanillin). Selective GAD inhibition was also observed, with all of the resin acids tested being inhibitory. GABA(A)-BZD ligand displacement was also observed. We compiled a table identifying which of these phytochemicals have been described in each of the species tested here. Given the diversity of conifer species and plant chemicals with these specific neuroactivities, it is reasonable to propose that MAO and GAD inhibition reported in effluents is phytochemical in origin. We propose disruption of these neuroendocrine endpoints as a possible mechanism of reproductive inhibition, and also identify an avenue for potential research and sourcing of conifer-derived neuroactive natural products.

1. Introduction

Conifer forests have long been used in the pulp and paper mill industry as a source of cellulose fiber. Following cellulose extraction, effluents generated from this industry are rich in plant secondary metabolites which, after treatment, are released to aquatic ecosystems. These by-products from wood processing have demonstrated the potential to interfere with normal neuroendocrine processes in exposed fish populations

Abbreviations: DA, dopamine; GABA, gamma-aminobutyric acid; MAO, monoamine oxidase; GAD, glutamic acid decarboxylase; GABA-T, GABA-transaminase; GABA(A)-BZD, GABA(A) receptor benzodiazepine-binding site; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

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(Basu et al., 2009; Waye and Trudeau, 2011). Reproductive and metabolic disruptions of wild fish downstream of pulp and paper mills (resulting in smaller gonads, larger livers, later age to maturity, and increased condition factor) have been an important research focus for environmental toxicologists and endocrinologists for the last decade and a half (for reviews, see Hewitt et al., 2006; MacMaster et al., 2006; Parrot et al., 2006) with concerted efforts towards solutions (Hewitt et al., 2008). Furthermore, fathead minnows exposed to effluents in laboratory studies exhibit a rapid and reversible inhibition of spawning (Kovacs et al., 2007). Disruption at the level of the brain has been an overlooked mechanism involved in these perturbations, whereby effluent chemicals interfere with hypothalamic neurotransmitter and neuropeptide pathways critical for the regulation of pituitary and gonadal functions (Basu et al., 2009; Popesku et al., 2008). This has led to the neuroendocrine disruption hypothesis for the actions of pulp and paper mill effluents to inhibit reproductive processes in fish (Basu et al., 2009; Waye and Trudeau, 2011).

In vitro screening studies (Basu et al., 2009, 2012; Milestone et al., 2012) showed that effluents and wood feedstock extracts are highly active at enzyme and receptor sites in the neuroendocrine GABAergic and dopaminergic systems which tightly regulate the reproductive axis (Popesku et al., 2008). Prior to this work, very few studies had been undertaken to assess the impact of pulp mill effluents on the fish brain. One study by Van Der Kraak et al. (1992) demonstrated the effects of effluents on pituitary function, where luteinizing hormone (LH) levels were lower in populations of white sucker (*Catostomus commersonii*) downstream of mill discharge. When these exposed fish were injected with a gonadotropin-releasing hormone (GnRH) agonist, the LH surge and subsequent ovulation seen in control fish were not observed.

We have previously reported on the different neuroendocrine bioactivities of extracts from Canadian hardwood feedstocks (Basu et al., 2012) and mixed feedstocks used in Brazil, New Zealand, and Canada (Milestone et al., 2012), however there is a lack of information on the most commonly pulped and phytochemically distinct boreal conifer species. Therefore the objective of the present study was to screen the neuroendocrine activities of extracts from nine boreal conifer species and sixteen conifer-derived phytochemicals against a battery of in vitro assays focussing on components in the dopamine (DA) and gamma-aminobutyric acid (GABA) neurotransmitter systems. It is important to note that a number of the phytochemicals in the present report have been identified in final mill effluents (for example, resin acids) or detected in a Canadian softwood kraft mill chemical recovery condensates associated with hormonal disruptions (Belknap et al., 2006). Furthermore, the neurotransmitter systems we explore have well-documented and critical roles in neuroendocrine control mechanisms (Basu et al., 2009; Popesku et al., 2008; Trudeau, 1997) and numerous neurological ailments from anxiety or epilepsy (Awad et al., 2007, 2009; Mullally et al., 2011) to Parkinson's and its associated disorders (Weintraub and Burn, 2011).

We measured the enzymatic activities of monoamine oxidase (MAO), glutamic acid decarboxylase (GAD), and GABA-transaminase (GABA-T), and binding to the GABA(A) receptor benzodiazepine binding site (GABA(A)-BZD). We chose these assays owing to the fact that in vertebrates, GABA and dopamine are among the most abundant neurotransmitters and play important roles in diverse behavioral processes while controlling the endocrine axes.

MAO is one of the enzymes responsible for metabolizing the neurotransmitters dopamine, serotonin, and norepinephrine, thereby inactivating them. There are two sub-types of MAO in mammals (MAO-A and MAO-B) which have differing substrate selectivity and inhibitor sensitivity. In goldfish (*Carassius auratus*), there is only one form of MAO, corresponding more closely to the mammalian MAO-A; that is, it is more sensitive to MAO-A type inhibitors (Figueroa et al., 1981). Dopamine is the most important inhibitor of the reproductive axis in fish, inhibiting release of GnRH in the hypothalamus and LH from the pituitary. GAD is the enzyme responsible for synthesizing the neurotransmitter GABA, GABA-T is responsible for its metabolism. There are three receptors for GABA. The GABA(A) receptor is important for stimulating GnRH in the hypothalamus. The GnRH in turn stimulates LH release from the pituitary which causes ovulation in females, sperm release in males, and sex steroid production in both sexes (Popesku et al., 2008; Trudeau, 1997). When an agonist binds to the benzodiazepine-binding site of the GABA(A) receptor (GABA(A)-BZD), the activity of GABA is potentiated (Smith and Olsen, 1995).

We tested the inhibition of MAO and GAD enzyme activities in goldfish brain extracts with the following objectives: 1) to determine the potential of these chemicals to disrupt neuroendocrine functions related to reproduction in fish exposed to pulp mill effluents and 2) to determine the pharmacological potential of conifer extracts and their secondary metabolites in vertebrates. To further explore the pharmacological potential of conifers and their phytochemicals, we included the GABA-T enzyme activity and GABA(A)-BZD receptor-binding assays that are currently established in our lab and optimized with brain preparations from rats (*Rattus norvegicus*). These two assays, as well as the GAD assay, have previously been used to examine the effects on the GABAergic system of plants traditionally used by the Q'eqchi Maya in the treatment of epilepsy and anxiety (Awad et al., 2007, 2009). The GABAergic and dopaminergic systems are important targets for the pharmaceutical treatment of many neurological disorders ranging from Parkinson's disease (such as the MAO inhibitor rasagiline), to anxiety (GABA(A)-BZD agonists such as diazepam) and epilepsy (GABA-T inhibitors such as vigabatrin). Unfortunately the pharmaceutical treatments for these disorders, such as diazepam or vigabatrin, can have serious side effects with prolonged usage; therefore the exploration for safer alternatives was an important avenue to consider during our work on these neuroendocrine targets.

2. Methods

2.1. Plant collection

Common juniper (*Juniperus communis* L.), white spruce (*Picea glauca* (Moench) Voss), black spruce (*Picea mariana* (Mill.) BSP.), balsam fir (*Abies balsamea* (L.) Mill.), and jack pine (*Pinus banksiana* Lamb.) were collected in September 2007 near Mistissini, QC, Canada. White cedar (*Thuja occidentalis* L.), eastern hemlock (*Tsuga canadensis* (L.) Carr.), tamarack larch (*Larix laricina* (Du Roi) Koch), and white pine (*Pinus strobus* L.) were collected near Denholm, QC, Canada, in 2008. Plants were classified according to Scoggan (1978). Voucher numbers are shown in Table 1 and are deposited at the herbarium of the University of Ottawa, Ottawa, ON, Canada.

2.2. Phytochemical standards

Standards for the resin acids neoabietic acid, abietic acid, levopimaric acid, palustric acid, sandaracopimaric acid and isopimaric acid were provided by FPInnovations (Pointe-Claire, QC, Canada), while dehydroabietic acid, geranyl linalool, 4-ethylguaiacol, vanillin, veratraldehyde, manool, isoeugenol, 3-hydroxy-5-methoxystilbene, and pinosylven, described in Belknap et al. (2006) and Parrott et al. (2011), were provided by

Table 1
List of Eastern Canadian conifer species tested, with Latin and common names and voucher numbers for the herbarium at the University of Ottawa. Specimens were classified according to Scoggan (1978).

Plant	Voucher no.
Abies balsamea L. (balsam fir)	19980
Picea mariana (Mill.) BSP (black spruce)	19981
Juniperus communis L. (common juniper)	19982
Picea glauca (Moench) Voss (white spruce)	19983
Larix laricina (Du Roi) Koch (tamarack larch)	19984
Pinus banksiana Lamb. (jack pine)	19985
Tsuga canadensis (L.) Carr. (eastern hemlock)	19986
Thuja occidentalis L. (white cedar)	19987
Pinus strobus L. (white pine)	19988