# Research Report

*Evaluation of antifungal activity of CHCx formulation against Saprolegnia parasitica*

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## Background and Context

Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. The contribution of aquaculture to global supplies of fisheries products continues to grow, increasing from 3.9% of total production by weight in 1970 to 32.4% in 2004. Worldwide, the sector has grown at an average rate of 8.8% per year since 1970, compared with only 1.2% for capture fisheries and 2.8% for terrestrial farmed meat production systems over the same period. Between 1986 and 2001, Canadian aquaculture production grew at an average annual rate in excess of 19%. In 2004, Canadian aquaculture output reached 152,523 tonnes, valued at $597 million, representing 13% of total fisheries production in Canada and 24% of its value (Statistics Canada 2008). While the development of this industry has been impressive, its growth, has not kept pace with other leading fish-farming nations; Canada ranks only twenty-second among aquaculture producing nations and accounts for only 0.3% of global aquaculture production (FAO 2008). The present size of our aquaculture industry, however, does not reflect its potential. With abundant natural resources, established technical and management expertise, and favourable access to international markets, Canada has the ability to become a global leader in aquaculture. Nevertheless, projected industrial growth will not be realized without investment in research and development leading to tangible technological development to overcome current constraints.

Fungal diseases are the second largest cause of mortality in aquaculture, particularly in the cultivation of shellfish and fish species. One of most destructive pathogens and that having the greatest economic impact in freshwater aquaculture sectors is the fungus *Saprolegnia parasitica* (Ramaiah, 2006). It is endemic to all freshwater habitats around the world and is partly responsible for the decline in natural populations of salmon and other freshwater fish. The common fungus *S. parasitica* is a ‘water mold’ that forms cotton-like tufts composed of branched filaments (mycelium; Morin 2006)

*Saprolegnia* is part of the phylum comycetes, a group of filamentous protists with about 500 species; they are non- photosynthetic aquatic organisms that resemble fungus but are not of this group. *Saprolegnia* is considered an opportunistic pathogen that is saprotrophique and necrotrophique (Bruno and Wood, 1999). Some groups *Saprolegnia* possess filaments that are considered necessary for attachment to the host (Willoughby, 1994) including, the zoospores of *S. parasitica* (Pickering and Willoughby, 1982). *Saprolegnia* has a complex life cycle that includes sexual and asexual reproduction (Meyer, 1991). Different species of *Saprolegnia* are able to germinate under different environmental conditions and nutrients (Bruno and Wood, 1999; Willoughby, 1985) and can tolerate a fairly wide range of temperatures ranging from 3 ° C to 33 ° C, which reflects the thermal preferences of the host (Pickering and Willoughby, 1982).

Fish, become sensitive to *Saprolegnia* when exposed to stressful conditions (handling, high temperature, low oxygen concentration). Stress increases the level of corticosteroids in blood plasma, which suppresses the inflammatory reaction and increases the protein catabolism, governed by the steroid (Pickering, 1994). In the final phase of the disease, protein deficiency leads to atrophy of skeletal muscles and suppression of the synthesis of collagen. The lack of collagen leads to poor regeneration of lesions on the skin (Willoughby and Pickering, 1977). *Saprolegnia* spp can infect the dermal layer of fish and eggs, resulting in a condition referred to as saprolegniosis (Stoskopf, 1993). When the aquatic fungus is present in sufficient quantities on dead eggs, it may subsequently spread to other eggs, causing massive egg loss. In fry, it can infect the digestive tract at start feeding as well as skin, gills and fins of the animal. Among the adult fish, the fungus can infect the skin, including injuries caused when handling fish during their transfer. The infection can spread through the skin of the animal (dermis) and the underlying muscles, causing significant tissues necrosis. Saprolegniosis creates significant physiological perturbations (eg disruption of the osmoregulatory system) as well as being a gateway for microbial infections (Pickering and Willoughby, 1982).

Evidence suggests widespread infection in most freshwater fish species (Bruno and Wood, 1999) left untreated, saprolegniosis can quickly escalate, resulting in up to 50% mortalities of a given population. (Mayer, 2000). Current losses to the global salmon farming industry are estimated at several tens of millions of kilograms per year (Torto-Alalibo and al., 2005).

Since 1936, infections caused by *Saprolegnia* were treated with malachite green, an organic dye and effective biocide. Malachite green is inexpensive and is characterized as being very effective regarding its antifungal and antimicrobial properties however its use has become a very controversial at the international level; since 2002, its use has been banned in many regions of the world including the European Union, USA, numerous Asian countries, and Canada. Malachite green has an affinity for DNA, altering its functions and structure, owing to its mutagenic and carcinogenic properties. The use of malachite green is now in question because of the risks posed to consumers (eg effects on immune and reproductive systems human teratogenic and carcinogenic properties) from potential residues in treated fish (Morin and Boucher, 2005). Indeed, residues of malachite green continue to be detected in different products from aquaculture. This speaks loudly to the effectiveness of the compound and more importantly to the need for effective alternatives.

The banning of malachite green led to the resurgence of *Saprolegnia* infections. Alternative antifungal products permitted for use are sodium chloride, formalin (Parasite-S™, or Formalin-R™ solution), hydrogen peroxide (Perox-Aid™) and bronopol (Pyceze™) for eggs and fish (MAPAQ, 2006); affected fish or eggs are treated daily for 3-14 days (depending on product) using a static bath with a duration generally between 30-60 minutes. Apart from salt, substitutes for green malachite can be potentially carcinogenic (eg formalin), toxic to humans (eg formalin vapours, reactivity of hydrogen peroxide) or be costly to use (ex. Bronopol). Moreover, bronopol is not currently registered in Canada and as such requires a veterinarian prescription to be applied. Hence, there is an urgent need of new alternative products to control saprolegniosis.

Over the past 2 decades, there have been significant efforts to replace synthetic pesticides and therapeutics in all agricultural sectors. In recent years, a large number of synthetic pesticides have been banned in the western world because of their undesirable attributes such as high and acute toxicity, long degradation periods, accumulation in the food chain and an extension of their power to destroy both useful and harmful pests (Barnard et al.

1997). Natural products offer a vast, virtually untapped reservoir of bioactive compounds with many potential uses.
One of these uses is in agriculture to manage pests and as therapeutics, with less risk than with synthetic compounds that are toxicologically and environmentally undesirable.

Green plants represent a reservoir of effective chemotherapeutants and can provide valuable sources of natural pesticides (Rice et al 1998). Reports are available on the use of active agents from higher plants, in place of chemical fungicides, that are non-phytotoxic, systemically active, and easily biodegradable (Hostettmann & Wolfender, 1997). Studies have demonstrated the use of several plant by-products, which possess antimicrobial properties, on several pathogenic bacteria and fungi (Lis-Balchin & Deans 1996).

## Progress Related to Current Proposal

### i) Hypothesis:

Cinnamaldehyde, a major constituent of essential oil from cinnamon and selected phytochemicals (tannic acid and a neem oil-based product) demonstrate anti-fungal activity against a variety of fungal species. These compounds can be used to treat and/or prevent *Saprolegnia parasitica* infections in trout.

### ii) Objectives:

Prior to in vivo antifungal efficacy studies, initial candidate screening assays will:

a) Validate the anti-fungal activity of the selected compounds using a rapid in vitro assay.
b) Evaluate the toxicity of the compound via a standardized homologous in vivo assay.

### iii) Experimental Methods:

Cinnamaldehyde and tannic acid were purchased from Sigma. A patented product Formula CHCx registered by the CFIA and UBA authorized for use in the food processing industry was obtained from Eckhard Canada (Delhi ON).
An anti-fungal screening assay was developed by inoculating *S. parasitica* (clinical isolate from local fish facility shown to be highly virulent) at the center of a glucose-yeast agar plate and by adding 70μl of the anti-fungal product in a plug made in the agar. After transferring the mycelium, the testing dishes were incubated at 26 ± 2 C, 70% relative humidity. When the mycelium of fungi reached the edges of control dishes (without compounds added) after approximately 5 days, the antifungal indices were calculated. Each test was repeated four times, and the data averaged. If an inhibition zone was detected further analysis were performed to quantify inhibition effectiveness. The micro-plate hemp seed method described by Stueland et al. (2005) was used to determine the minimum inhibitory concentration (MIC) of the compounds. Once the MIC was obtained, this concentration was tested in vivo using rainbow trout to determine toxicity. Toxicity was evaluated for cinnamaldehyde and tannic acid using a standard method recommended by Environment Canada (EPS 1/RM/9). The time required to obtain 50% mortality (LT50) was performed by adding ten fry (0.3g) in a 30-l aquarium with static water maintained at 15°C; the test were in triplicate.

## IV) Results:

As reported with other fungal species, cinnamaldehyde demonstrated antifungal activity in vitro against a highly-virulent *S parasitica* field isolate at 250 ppm. However the compound demonstrates aesthetic properties in vivo at concentrations above 50 ppm. At the MIC, onset of fish mortality is rapid, with an $LT_{50}$ of 25 minutes (Table 1). As a result, the evaluation of trans-cinnamaldehyde in in vivo efficacy studies (bath treatment) will not be pursued. Tannic acid and CHCx appear to be a more appropriate candidate, as it demonstrates effective antifungal activity and less acute toxicity. Further efficacy testing is currently on-going. Toxicity testing will reveal their potential for in vivo efficacy testing.

**Table 1.** In vitro antifungal activity (MIC) and in vivo toxicity ($LT_{50}$) values for trans-cinnamaldehyde and tannic acid, and a neem-oil based compound

| | MIC (ppm) | $LT_{50}$ (hours) |
|---|---|---|
| Trans-cinnamaldehyde | 250 | 0.3 |
| Tannic acid | 175 | 5.6 |
| CHCx | 300 | TBD |

[a]TBD: In vivo toxicity to be determined

## V) Conclusions/Perspectives:

Although the application of cinnamaldehyde for bath treatment of fish appears to be limited due to its anaesthetic properties, its application for an oral treatment to prevent intestinal fungal infections of first feeding fry needs to be verified. Additional compounds show promise for fungus control and treatment, and merit further evaluation in efficacy studies and toxicity studies.

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