

# Screening Various Plant and Fungal Extracts Against *Clostridium perfringens* for Antimicrobial Activity

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## ABSTRACT

*Clostridium* is an anaerobic, endospore forming Gram-positive bacillus genus containing many important pathogenic species, including *Clostridium difficile* and *Clostridium perfringens*. Both of these pathogenic species have been implicated as the causative agents of serious gastrointestinal tract infections. Recently, there has been an emergence of antimicrobial drug resistant *Clostridium* species. Because of these concerns, a new drug is warranted. There is an ecological rationale that antimicrobial products could be synthesized in fungi and plants to ward off pathogens like *Clostridium*. To explore this further, plant and fungal specimens have been collected and a solvent extraction procedure has been performed. Crude extracts taken from various plants and fungal fruiting bodies have been screened against *C. perfringens* for evidence of antimicrobial activity. A total of 238 extracts from various fungi and plant species have been screened against *C. perfringens*. Of these, 25 showed antimicrobial activity against *C. perfringens*. Furthermore, a total of 17 fungal extracts demonstrated hemolytic activity and antimicrobial activity against *C. perfringens* while 8 extracts generated no hemolytic activity while showing antimicrobial activity. Further purification and analysis is currently being performed on promising crude extracts in order to unearth any potential novel antimicrobial agents.

## INTRODUCTION

*Clostridium perfringens* is one of the most common causes of foodborne illness in the U.S. (8). Drug resistant *C. perfringens* strains are becoming a major health concern. A study by Teuber indicated that copious use of antibiotics in agriculture is promoting a large antibiotic resistance problem in foodborne pathogens, including *C. perfringens* (7). According to the Public Health Agency of Canada, strains of *C. perfringens* have developed resistances of penicillin, tetracycline, erythromycin, chloramphenicol, metronidazole, and clindamycin (5).

In addition, *C. difficile*, the causative agent of *C. difficile* associated disease (CDAD), is the most commonly recognized cause of antimicrobial drug-associated diarrhea after antibiotic treatments lower natural flora levels in the intestinal tract. CDAD includes asymptomatic colonization, diarrhea and pseudomembranous colitis, toxic megacolon, sepsis, intestinal perforation, and death (6). Individuals infected with CDAD shed spores in their stool that can survive for up to 70 days in the environment and can be transported on the hands of healthcare personnel who have direct contact with infected patients or surfaces (9).

A recent study by the Centers for Disease Control and Prevention indicates that cases of CDAD in the United States are rapidly increasing in short stay hospitals, long term health care facilities, and the community (4). One study estimated the annual excess healthcare cost for CDAD in the U.S. to be greater than \$1.1 billion (2). Moreover, emerging strains of *C. difficile* are more readily capable of causing disease. For example, a fluoroquinolone-resistant strain of *C. difficile* responsible for hospital outbreaks in 11 U.S. states produces 16 to 23 fold more toxin than other strains (3).

Because of the rise of drug resistant strains of *C. perfringens* and *C. difficile*, new antimicrobials are needed. Naturally derived antimicrobials from plants and mushrooms could serve as sources for new antimicrobial drugs. There is an ecological rationale that antimicrobial products should be synthesized de novo in plants following microbial attack to protect the producer from pathogens (1). Mushrooms must also stave off degradation by various bacteria, thus this could be an untapped source of new antimicrobial drugs. Crude extracts taken from various plant and fungal extracts have been screened against *C. perfringens* for evidence of antimicrobial activity against this species. For this project, 238 fungal extracts and one plant extract were tested for activity against *C. perfringens*. Any extracts showing good activity against *C. perfringens* will be further purified and screened against other *Clostridium* including *C. difficile*.

## MATERIALS AND METHODS

### *Bacterial strains and growth conditions*

A wild type *Clostridium perfringens* strain was supplied by the UW-L culture collection. A clinical strain of *C. difficile* was supplied by the Marshfield Clinic. *C. perfringens* was grown overnight in thioglycolate broth at 37° C prior to any assays.

### *Disk diffusion assay*

A bank of organic solvent extracts had been prepared from 238 fungal extracts and one plant extract in dimethylsulfoxide (DMSO) at a concentration of 256 mg/ml. Disk diffusion assays were performed on the extracts against *C. perfringens*. Prior to inoculation, 5% sheep blood tryptic soy agar plates were reduced in a candle jar for at least 24 hours. The blood agar plates were inoculated with *C. perfringens* using a sterile cotton swab to create a lawn of confluent growth. Then, sterilized 6 mm diameter filter paper discs were placed on the sheep blood agar plates using a forceps. Eight µl of crude extract at a concentration of 256 mg/ml in DMSO were added to the paper discs. In the assay, DMSO was used as a negative control and tetracycline was used as a positive control for antimicrobial activity. The plates were then incubated at 37° C for 18-24 hours under anaerobic conditions using a Gas-Pak™ jar and H<sub>2</sub> gas generator with an anaerobic indicator. Plates were then examined to determine whether or not there was a zone of inhibition for *C. perfringens* growth and whether there was a zone of hemolysis. Data was recorded as diameters of zones of inhibition as well as the diameters of hemolysis zones, including type of hemolysis observed.

## RESULTS

Of the 238 extracts screened, 25 had anti-*C. perfringens* activity. However, only 8 extracts had both anti-*C. perfringens* activity and also a lack of hemolysis on the sheep blood agar. Seventeen extracts exhibited antimicrobial activity but were also hemolytic. Ninety extracts total had hemolytic activity. Fifty-two of these were α-hemolytic and 38 were β-hemolytic. The plant extract showed neither antimicrobial nor hemolytic activity

**Table 1.** Antimicrobial and hemolytic activity of fungal extracts against *Clostridium perfringens*

Crude extract type	Activity <sup>a</sup>
Hemolytic extracts	37.8%
Anti- <i>C. perfringens</i> extracts	10.5%
Hemolytic and anti- <i>C. perfringens</i> extracts	7.1%
Non-hemolytic and anti- <i>C. perfringens</i> extracts	3.4%

<sup>a</sup>Percent of total extracts

## DISCUSSION

Traditionally, molds have been the main source of antibiotics, and much effort has been placed into drug discovery from this source. The results of this study have demonstrated that fungal fruiting bodies are also a very good source for potential antimicrobial drugs. Altogether, 10.5% of all extracts tested had anti-*Clostridium* activity, and 3.4% of all extracts tested additionally demonstrated no hemolysis. The abundance of fungal fruiting bodies that have antimicrobial activity coupled with the relative ease of gathering, processing, and testing fungal fruiting bodies makes them an exceptional source for new drugs. It was interesting to note the high percentage of fungal extracts which are hemolytic. This characteristic may or may not be important as there may be multiple compounds within an extract that are separately causing either hemolysis or antimicrobial activity. Thus approximately one out of every ten fungal fruiting body extracts tested against *Clostridium* has potential to contain a novel anti-*Clostridium* drug.

This project has discovered several promising possibilities for finding an effective anti-*Clostridium* drug. Extracts which demonstrated good zones of inhibition and no hemolysis are being further fractionated and purified using thin layer chromatography as well as other purification methods. Once at sufficient purity, they will be screened against *C. difficile* and have a minimum inhibitory concentration defined. In addition, potent extracts will be tested against various other pathogenic microorganisms to ascertain a spectrum of activity. Finally, structural analysis will be performed on pure compounds. Hopefully, novel compounds will be identified with antimicrobial activity against *C. perfringens* and *C. difficile*.

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