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Antimicrobial Activities of Secondary Metabolites from Warm Spring Fungi.

Akinyemi A Fajilade T. O
Department of Science Technology, Federal Polytechnic Ado-Ekiti, Nigeria

Abstract
Water from warm spring was screened for the presence of some fungi using different nutrient media. Antimicrobial activity of secondary metabolites of the isolates were evaluated against *Escherichia coli, Staphylococcus aureus*, *Bacillus subtilis* and *Klebsiella pneumoniae.* Agar well diffusion method was utilised in the evaluation of the antimicrobial activity and it was discovered that each of the secondary metabolites showed antibacterial activities against the test bacteria. The result showed good prospects in the search for new antibacterial drugs.
Keywords: Warm spring fungi, metabolites, bioactive compounds

1. Introduction

Fungi has been reported to be the most prolific natural but under exploited source of novel compounds after plants because of its enormous biodiversity and it is estimated that a conservative 1.5 million species are recorded worldwide (Hawksworth, 1991; Savage, 1995; Hawksworth, 2001). Dreyfuss and Chapella (1994) estimated that 4000 secondary metabolites of fungi origin are known to produce biological activities and the vast majority comes from the soil environment. Screening such fungi for new bioactive compounds usually leads to re-isolation of already known metabolites (Lee and Oh,2006). However, exploring other ecological niches than soil when selecting for target organisms for bioactive investigation has been emphasized (Gloer, 1997). Some of the relatively unexplored fungal groups are the fresh water fungi, marine fungi and the fungal endophytes (Dreyfuss and Chapella, 1994). Scientist have been able to collect and identify fungi that flourish in extreme environments for decades. They include cold, hot, alkaline pools, acidic, salty water, high pressure regions and from niches with above normal level of radiation (Onifade, 2007) . However, the search for new secondary metabolites from microorganism in general, has long been confounded by the observation that different strains belonging to the same species can produce different secondary metabolites while taxonomically diverse strains can produce identical metabolites (Jensen *et al*., 2007). The discovery and identification of new safe drugs without severe side effects has become an important goal of research in the biomedical science (Lull *et al*., 2005). In view of the serious problems of chemotheraphy, resistance of bacteria and fungi, the newly emerging old and new pathogens, it has been predicted that the fungal products will gain eminent importance mostly in the pharmacology and perhaps agriculture. (Berdy, 2005). The aim of this study was to examine fungi isolated from warm spring and investigate their secondary metabolites for antimicrobial activities.

2. Materials and methods

2.1 Sampling

Samples were taken from Ikogosi warm spring, in Ekiti, Nigeria. Aseptically, the cap and cover of a sterile sample bottle was removed and the neck of the bottle was then plunged downwards about 30 cm below the surface and tilted slightly upwards to let it fill completely before carefully replacing the cap and cover. Where there was no current, the bottle was pushed forward horizontally until it was filled. The water sample collected was taken to the laboratory and processed within 24 hours.

2.2 Isolation and identification of fungal isolates

Standard dilution was carried out on water samples collected from various part of the spring after which it was placed in Yeast malt agar and potato dextrose agar plates as described by Vicente *et al* (2001). All the fungal stock cultures was maintained on Sabouraud Dextrose Agar. Identification was based on their sexual and asexual structures produced in culture. The fungal isolates were identified based on their morphological and reproductive characters. Colonies were analysed, slides were prepared from cultures using lactophenol cotton blue stain and examined with a microscope (Barnett and Hunter, 1998).

2.3 Inoculation and fermentation

Ten fungal inoculum's discs was transferred into each of fifteen 1 L Erlenmeyer flasks containing 250 ml of fermentation medium. The medium consisted of yeast extract, 0.4 %; malt extract, 1.0 % and glucose, 0.4 %; pH 7.0. All the flasks were incubated at 28^{o}C ± 2^{o}C in a rotary shaker (130rpm) for 14 days. During incubation, a flask was removed at random every 48hour and fermented medium processed as described by Onifade (2007).

2.4 Extraction procedure

The broth cultures were passed through 0.45mm filter paper under vacuum to separate the mycelia from the culture broth. The wet mycelia were weighed, blended and extracted with ethyl acetate (1:1w/v). The pH of the broth was noted after which it was extracted with equal volume of ethyl acetate (1:1).The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuum to dryness in pre-weighed vials after which the metabolites was weighed. This was later diluted with dimethyl sulfoxide (DMSO) and used for the antimicrobial assay.

2.5 Pathogens used for the antimicrobial activity

A total of four bacterial pathogens were used for the antimicrobial activity. These were *Bacillus subtilis* (NCIB 3610), *Klebsiella pneumonia* (NCIB 4183), *Staphylococcus aureus and Escherichia coli.* They were obtained from the Department of Microbiology, Obafemi Awolowo University (OAU), Ile-Ife, Nigeria and OAU teaching hospital.

2.6 Antibiotic assay

The antimicrobial activity of the extracts was determined by the agar well diffusion method. Agar plates were inoculated with broth cultures of the test organisms. Five millimeter diameter wells were punched into the agar and filled with 30µL of each extract dilution. The plates were then incubated at appropriate temperatures for 24h after which zones of inhibition was measured.

3. Results and discussion

A total of twenty colonies comprising of four different fungal species were isolated from the warm spring. The four species were *Aspergillus fumigatus, Penicillium italicum, Aspergillus flavus and Aspergillus niger.* A representative of each of the species were selected for fermentation and subsequent screening of their metabolites for antimicrobial activities. The final pH of the fermented culture medium for the various fungal isolates is shown in Fig. 1. It was observed that the culture of *A. niger* had the lowest pH of 5.54 at the end of 14 days of fermentation.

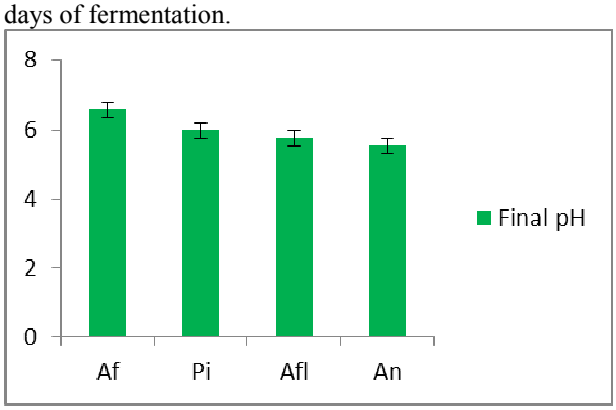

Fig. 1 Final pH of the fermented media

Af= *A fumigatus*, Pi=*P italicum*, Afl=*A flavus* and An=*A niger*

The pH of the growth medium is important because it affect cell membrane function, cell morphology and structure, uptake of various nutrients and product biosynthesis (Fang and Zhong, 2002). Fungi generally metabolise acids to decrease pH and the media becomes acidic, but growth is retarded when it becomes too acidic (Lin *et al*., 1973). Digrak and Ozcelik (2000) reported that growth and metabolites production by fungi were influenced by the interacting effects of pH, simple sugars and temperature. Although, *A niger* had the lowest pH in this study, the highest mycelium mass was with the *A flavus* (Table 1). Strobel et al. (2004) stated that incubation temperature, medium composition, and degree of aeration affect the amount and kinds of compounds that are produced by fungi. Also, Santos et al. (2015) stated that the culture medium, agitation, and temperature can increase or reduce the production of the bioactive compounds by fungi. In this study, the highest dry weight of filtrate extract was in *A fumigatus* while the lowest weight was with *A niger***.**

Table 1. Effects of fermentation on the final mycelial weight, volume of filtrate and dry weight of culture filtrate extract at 14 days.

Species	Wet weight of Mycelia (g)	Final volume of filtrate (ml)	Dry weight of filtrate extract (mg)
A fumigatus	12.00	156	68
P italicum	10.15	236	13
A flavus	19.00	170	72
A niger	16.23	230	7

values show mean of three replicates

The antimicrobial activity of the secondary metabolites extract from the four fungi screened showed that two of the metabolites extract showed antimicrobial activity against all the test pathogens (Fig. 2).

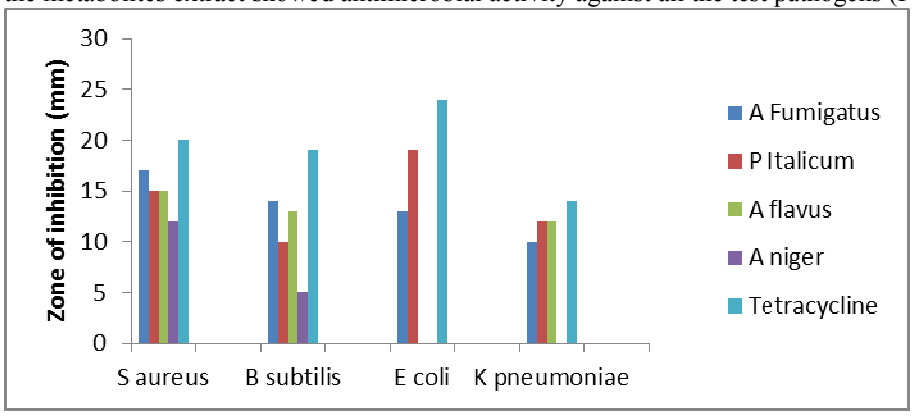

Fig. 2 Antimicrobial activity of the extracted secondary metabolites from warm spring fungi, (n=3).

Syahn et al., (2012) reported the production of secondary metabolites with broad and strong bactericidal effects by *A fumigatus, A flavus* and *Aspergillus Spp* strains from a polluted river. In this study, metabolites from *A fumigatus, A flavus* and *P italicum* generally showed zone of inhibtion against the pathogens though the activity against *K pneumoniae* was very low compared to other pathogens. However, it was discovered that metabolites from A niger showed zone of inhibition against *S aureus* and *B subtilis* but had no activity against *E coli* and *K pneumoniae*. This contradicts the findings of Kalyanasundaram et al. (2015) which reported that crude extract from *A niger* showed a zone of inhibition against *K pneumoniae.* Although, the standard antiboitic used in this study showed higher zones of inhibition than the secondary metabolites tested, however the results are comparable because crude extracts were used and future work will attempt to identify the active molecules from these extracts.

4. Conclusion

There is still a general call for the production of new antibiotics and chemotherapeutic agents that are highly effective and possess low toxicity. Therefore, increasing the quality and quantity of bioactive compounds by exploring fungal groups from other ecological niches like the warm spring as it has been demonstrated in this study would increase the chances of finding new leads for such therapeutic agents.

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