Assessment of the Antimicrobial Activities of the Secondary Metabolites Produced by Pure Cultured \textit{Trichoderma koningii}, \textit{Rhizopus stolonifer} and \textit{Fusarium oxysporum}

John Kenneth Mensah\textsuperscript{1,*}, Charles Kwoseh\textsuperscript{2}, Nicholas Banahene\textsuperscript{1}, Samuel Aweinatey Atuilik\textsuperscript{1}, Daniel Oppong\textsuperscript{1} and Malik Borigu\textsuperscript{2}

\textsuperscript{1}Department of Chemistry, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana
\textsuperscript{2}Department of Crop and Soil Sciences, College of Agriculture and Natural Resources, KNUST, Kumasi, Ghana
*Corresponding author’s E-mail: jkmensah75@yahoo.com

\textbf{A R T I C L E  I N F O}

\textbf{Article type:} Research article
\textbf{Article history:} Received April 2015
\textbf{Keywords:} Trichoderma koningii, Rhizopus stolonifer, Fusarium oxysporum, Antimicrobial, Secondary metabolites, Broth dilution assay

\textbf{A B S T R A C T}

Fungi have been source organisms for drug discovery, but remain unexplored source materials for natural product in Ghana. This study provides preliminary antimicrobial susceptibility assessment of crude extracts of secondary metabolites derived from select soil-habitat fungi. Pure cultures of \textit{Trichoderma koningii}, \textit{Rhizopus stolonifer} and \textit{Fusarium oxysporum}, isolated from soil, were fermented for secondary metabolites in Sabouraud Dextrose Broth (SDB) for 21 days. Bioactive compounds were isolated with ethylacetate and crude extracts screened for antimicrobial activities against a panel of microorganisms including \textit{Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans} (fungus) in broth dilution assay. Extracts displayed variable antimicrobial activities that were microorganism-specific and comparatively low in relation to the strength of the activities displayed by the clinically used standard agents Ciprofloxacin (antibacterial) and Ketoconazole (antifungal). This study provides access to previously unexplored extract source materials for anti-infectives.

\textsc{Capsule Summary:} Ethylacetate extracts of the secondary metabolites of pure cultured \textit{Trichoderma koningii}, \textit{Rhizopus stolonifer} and \textit{Fusarium oxysporum} displayed variable antimicrobial activities against a panel of four microbes.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\hline
\end{tabular}
\end{table}

\textbf{INTRODUCTION}

Fungi synthesize chemically diverse and often biologically active compounds as defense weapons (Yi et al., 2009), as chemical signals (Dufuor and Rao, 2011), as competition weapons (Cueto et al., 2001) and as adaptive response chemical signals (Hay, 2009). Cultured soil fungi have, therefore, been productive sources of drugs worldwide that includes cyclosporin, penicillins, and some statins (Butler, 2004). But fungi remain unexplored source materials for natural product leads in Ghana. In the continuing effort to identify fungal secondary metabolites with medicinally-relevant anti-infective properties, an evaluation of crude extracts of select fungal cultures for potential antimicrobial therapeutic leads has been undertaken.

Crude fungal secondary metabolites selected for antimicrobial evaluation were derived from a broad fungal bio-diversity spectrum that include genetically-diverse strains as \textit{Trichoderma koningii}, \textit{Rhizopus stolonifer} and \textit{Fusarium oxysporum}. All selected fungi are soil-habitants and are amenable to standard methods of culture in readily available media that support secondary metabolism in vitro. Chosen strains are also proven rich sources of media-specific biologically active secondary metabolites including Beauvericin (Logriece et al., 1999). Besides \textit{Fusarium oxysporum} and \textit{Trichoderma koningii} the
literature is silent about *Rhizopus stolonifer* ability to produce anti-infective secondary metabolites. The scientific rationale that undergirds this study is that the pool of secondary metabolites that emerge from these diverse strains will exhibit significant structural and substantial chemical diversity and might even encompass novel chemotypes that are potentially capable of exhibiting potent antimicrobial activities towards the clinically problematic multi-drug resistant microbial pathogens. And that success in this endeavor will certainly provide clinicians with a powerful new treatment option against problematic multi-drug resistant pathogens.

This study provides preliminary antimicrobial susceptibility assessment of crude extracts of secondary metabolites derived from three select soil-habitat fungi. Antimicrobial activity of the extracts assayed by broth dilution methods against a panel of microorganisms that include *Staphylococcus aureus* (gram positive bacterium), *Escherichia coli* (gram negative bacterium), *Candida albicans* (fungus) and *Pseudomonas aeruginosa* (gram negative bacterium) were variable. Although extracts displayed differential levels of activity that were substantially lower than clinically used pure standard agents Ciprofloxacin (antibacterial) and Ketocanazole (antifungal), this study provided access to previously unexplored source of natural product extract materials for anti-infectives.

**MATERIALS AND METHODS**

**Chemicals**

Nutrient broth, nutrient agar and Sabouraud broth were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol and Methanol were obtained from Merck Chemical Supplies (Damstadt, Germany). All reagents were of analytical grade.

**METHODS**

Sterilization of all flasks and media was accomplished by autoclaving at 120 °C for 30 min. All fermentation studies were conducted in 500-mL Erlenmeyer flasks containing 100 mL of Sabouraud media within the flasks closed with cotton plugs.

**Culture and maintenance of microorganisms**

The panel of microbes used for the assessment of antimicrobial activity were obtained from ATCC (USA) and were stored in liquid nitrogen in 20% glycerol. Microbes were maintained by regular sub-culturing on nutrient agar (bacterial) and on potato dextrose agar (PDA) (fungi) before storage at 4° C prior to experimental use.

**Isolation of fungi from soil**

Soil samples were obtained from farms located in KNUST campus. About 1 g soil sample was suspended in 9 mL sterile distilled water and the mixture vortexed vigorously. The soil allowed to settle from the suspension and the supernatant was filtered and then diluted by transferring 1 mL to a second test tube containing 9 mL of sterile water. An aliquot (100 µL) of the diluted filtrate was spread evenly on potato dextrose agar (PDA) medium supplemented with 50 µg/mL chloramphenicol in a petri dish and incubated at 37°C for 7 to 10 days. Individual fungal colonies were removed and repeatedly sub-cultured until pure fungal cultures were obtained.

**Identification of fungi**

Spores of the pure fungal culture were mounted on a microscope slide and examined under a light microscope (Leica, Germany). Fungi were identified via comparison of observed spore morphological features and culture characteristics with published notes available in the standard reference manual (Barnett and Hunter, 1986).

**Inoculation of media with fungal isolate**

Sabouraud broth was sterilized by autoclaving at 120 °C for 30 min. Sterilized media was inoculated under aseptic conditions with a single isolated fungal colony selected from the pure cultured fungi on PDA. Inoculated media was kept at room temperature (27-28 °C) with intermittent shaking for 21 days.

**Extraction of secondary metabolites**

Culture supernatant was filtered to separate mycelia from broth. Filtrates were partitioned three times with equal volumes of ethylacetate. The organic fractions were combined, dried over anhydrous sodium sulphate, filtered and reduced to dryness on a rotavap. Dried residues were stored at -20 °C until needed for bioactivity studies. Yield of extracts ranged from 250 mg to 350 mg.

**Quality control**

To assure that extracts were derived solely from the cultured fungi of interest, an aliquot of the fermentation culture was pipetted and its contents cultured on PDA. Fungal strain were authenticated after microscopic visualization. All cultures were found to contain only the fungi of interest and harbored no additional microbe.

**Diagnostic TLC**

To determine the number of possible compounds in each extract, a 20 µL of each ethylacetate extract was dissolved in 100 µL chloroform. An aliquot 10 µL of the resulting solution was spotted at the origin of a 10- by 5-cm TLC glass plate coated with silica gel (Fisons Scientific silica gel S/0790/53, Loughborough England) that was approximately 500 µm in thickness. The spots were allowed to dry and eluted with chloroform for 60 min. Developed plates were air-dried and product spots were visualized with iodine vapor. Rf values of visible spots were estimated.

**Basic phytochemical screening**
The ethylacetate extract was assayed for its phytochemical constituents using protocols previously described (Trelease and Evans, 1984).

**Alkaloids:** Acidified solution of alkaloid produce a white-yellowish precipitate upon the addition of a few drops of Mayer’s reagents. Extracts were initially heated on a boiling water bath with 2% hydrochloric acid. The mixture was allowed to cool and few drops of Mayer’s reagent added. A yellow precipitate indicated the presence of alkaloids.

**Terpenoids and steroids:** To a small amount of the extract (2 mg) was added (250 µL of acetic anhydride and 250 µL of chloroform). Concentrated sulphuric acid (1 mL) was added slowly and a red-violet color indicated the presence of terpenes while a green-bluish color was indicative of the presence of steroids.

**Flavonoids:** To a small amount of the extract was added 1.5 mL of 50% methanol and the solution warmed. Metal magnesium was then added followed by 5-6 drops of concentrated hydrochloric acid. The formation of a red color indicated the presence of flavonoids while an orange color indicated the presence of flavones.

**Tannins:** To 0.5 mL of an aqueous solution of the extract was added 1-2 drops of ferric chloride solution. A blue color indicated the presence of gallic tannins while a greenish-black color indicated the presence of catecholic tannins.

**Coumarins:** To 500 µL of an aqueous solution of the extract was added 750 µL of 10 % NaOH. The formation of a yellow colour is indicative of the presence of coumarins.

**Saponins:** To 500 µL of an aqueous solution of the extract was added 1 mL of 1% sodium bicarbonate and the solution shaken. A persistent froth is indicative of the presence of saponins.

**Saponins**

**Phytochemical Screening**

**RESULTS AND DISCUSSIONS**

**Phytochemical Screening**

Fungi constitute outstanding source organisms for antimicrobial, antioxidants, anticancer, antidiabetic and immunosuppressive drug leads. This study assessed the antimicrobial activities of the ethylacetate extracts of the fermentation culture of three fungal species: *Trichoderma koningii*, *Rhizopus stolonifer* and *Fusarium oxysporum*.

All three fungi are uniformly characterized by saprophytic feeding within soil-habitat. *Trichoderma koningii* is a saprophyte that produces diverse bioactive compounds used for competitive inhibition against other fungi and pathogenic microorganisms within its milieu (Song et al., 2006). *Rhizopus stolonifer* is a cosmopolitan filamentous fungi with mycelia that persist in soil and in other moist environments and that enables the biosynthesis of diverse bioactive compounds within its resident environment (Hernandez-Lauzardo et al., 2006). *Fusarium oxysporum* is a common saprophyte and a pervaasive plant endophyte that is a proven rich source of biologically active secondary metabolites (Liu, 2011).

While the contemporary literature reveals that the examined species are rich sources of bioactive secondary metabolites, none of the reported metabolites were obtained via fermentation in Sabouraud media and none has been examined for antimicrobial activities against the specific tested panel of microbial strains (Lu and Shen, 2004; Hsu and Koh, 2011).

TLC analysis revealed extracts that are mixtures of at least two compounds with distinct Rf values (Table 1). Since the TLC spots were specific for the stationary and mobile phase used, the possibility exists that more spots representative of other bioactive compounds would be revealed under better resolution provided by a different mobile phase or by another stationary phase.
Trichoderma koningii showed four evenly spaced spots. Rhizopus stolonifer extract revealed two spots representative of compounds that were highly polar and that congregated close to the baseline of the TLC plate. Fusarium oxysporum revealed a pattern of TLC separation that was similar to that of Rhizopus stolonifer with minimal movement of the two spots from the baseline (Table 1). The presence of the observed phytochemicals is often predictive of bioactivity of the constituent compound.

Inhibitory effect of the extracts on microbial growth was assessed via broth dilution assay and means minimal inhibitory concentrations (MIC), in the mg/mL range, are provided in Table 2. Each extract showed inhibitory activity against more than one test microorganism. Test microbes were differentially susceptible to the inhibitory effect of the extract and showed variations in broth dilution MICs that appear to be test microorganism-specific. Trichoderma koningii extract was only weakly inhibitory on Staphylococcus aureus. Inhibitory bioactivity of Rhizopus stolonifer on Staphylococcus aureus was on a level that was

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Number of spots from TLC and Rf values</th>
<th>Phytochemicals present in extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichoderma koningii</td>
<td>Four Rf: 0.27, 0.40, 0.51 and 0.92</td>
<td>Alkaloids, Reducing Sugars, Terpenoids, Saponins and Tannins and Steroids.</td>
</tr>
<tr>
<td>Rhizopus stolonifer</td>
<td>Two Rf: 0.115 and 0.29</td>
<td>Alkaloids, Reducing Sugars, flavonoids, Anthraquinones, Terpenoids and Saponins.</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>Two Rf: 0.04 and 0.13</td>
<td>Flavonoids, Terpenoids, Saponins and Tannins.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Test organism</th>
<th>Agar diffusion assay MIC (mg/mL) OF EXTRACT</th>
<th>Agar diffusion assay MIC (mg/mL) OF ciprofloxacin</th>
<th>Agar diffusion assay MIC (mg/mL) OF fluconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichoderma koningii</td>
<td>Staphylococcus aureus</td>
<td>3.0</td>
<td>0.031</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td>2.4</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>1.2</td>
<td>0.062</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Candida albicans</td>
<td>2.4</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Rhizopus stolonifer</td>
<td>Staphylococcus aureus</td>
<td>2.4</td>
<td>0.031</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td>No effect</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>2.4</td>
<td>0.062</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Candida albicans</td>
<td>2.0</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>Staphylococcus aureus</td>
<td>0.31</td>
<td>0.031</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td>No effect</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>0.16</td>
<td>0.062</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Candida albicans</td>
<td>2.50</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>
similar to that of Trichoderma koningii. In contrast, Fusarium oxysporum extract was more bioactive on Staphylococcus aureus than on the other microbes. Extracts from Rhizopus stolonifer and Fusarium oxysporum showed no inhibitory activity on Pseudomonas aeruginosa at the assayed concentration and extracts from Trichoderma koningii were inhibitory on Pseudomonas aeruginosa only at a higher concentration (2.4 mg/mL). Extracts from Fusarium oxysporum had a strongest antibacterial activity against E. Coli. Similarly, Trichoderma koningii extracts exhibited maximum growth inhibition against E. Coli in contrast to extracts of Rhizopus stolonifer that displayed the least inhibitory activity against E. Coli.

Extracts showed antifungal activities on Candida albicans as well. The most potent antifungal activity against Candida albicans was shown by Rhizopus stolonifer extract. Trichoderma koningii extract had better fungicidal effect on Candida albicans than Fusarium oxysporum extract although both inhibitions were observed at higher MICs values. In all cases, bioactivity of the standard drugs (antibacterial Ciprofloxacin and antifungal Fluconazole) used as positive controls were higher with MICs at lower concentrations (at least 2-fold to at most a 100-fold) than that of the extracts.

Reports abound about antimicrobial compounds produced by specific strains of Fusarium, the only endophytic fungi among the list (Liu, 2011) and by several strains of Trichoderma (Song et al., 2006). Antimicrobial extracts from Rhizopus stolonifer have not been reported in the literature.

Although bioactivity was minimal with MICs above the range of clinically useful concentrations, the possibility that one or more synthetic analogs of the constituent compounds might exhibit potent antimicrobial activity should spur further research designed to derive useful anti-infective medicinal agents from the extracts of these cultured fungi. Consequently, the next phase of this research study would be to purify each fungal extract to single pure bioactive constituents. Using activity-guided fractionation of the active constituents with agar diffusion assay as the primary screens, the fraction(s) that contain the antibiotic activity can be isolated. Structure determination using NMR, IR and mass spectroscopy can be conducted on the isolated active fractions. Analog synthesis of the most promising active pure compounds can then be accomplished and analogs can then be screened for their ability to inhibit specific microorganisms in vitro and in vivo. The possibility for the development of potent antimicrobial compounds using the bioactive extract constituents as templates should stimulate further research into the bioactivities of fungal extracts.

CONCLUSION

Ethyl acetate extracts of the secondary metabolites of pure cultures of Trichoderma koningii, Rhizopus stolonifer and Fusarium oxysporum displayed variable antimicrobial activities against Staphylococcus aureus (gram positive bacterium), Escherichia coli (gram negative bacterium), Pseudomonas aeruginosa (gram negative bacterium) and Candida albicans (fungus). Although the three fungal source organisms are extensively studied, their secondary metabolites have not been thoroughly examined for antimicrobial activities against the panel of tested microbes. The levels of antimicrobial activities displayed were substantially lower than clinically used pure standard agents Ciprofloxacin (antibacterial) and Ketoconazole (antifungal). This study increased the repertoire of natural product sources available for investigation in Ghana and provided access to previously unexplored source of extract materials for anti-infectives.

REFERENCES


Butler, M.S., 2004 Review: The Role of Natural Product Chemistry in Drug Discovery. Journal of Natural Products; 67(12): 2141-2153


www.bosaljournals/csp/
Trichoderma koningii SMF2. Federation of European Microbiological Societies: 121-124.

