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The Antimicrobial Effects of Secondary Metabolites of Anguillan Fungi

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SD and AS designed and supervised the study. Author MB performed the experiment and statistical analysis, wrote the protocol and managed the literature searches. Authors MB and AS managed analyses of the study and wrote the first and final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Many drugs have been isolated from fungal species. This study aims at identifying fungal species isolated in Anguilla in order to determine the antimicrobial effect of their secondary metabolites from pure culture against *Staphylococcus aureus* by demonstrating the presence of a zone of inhibition in the culture plate.
Methods: Samples were cultured and sub-cultured to isolate pure culture on potato dextrose yeast agar (PDYA) and chosen for further studies by the presence of surface exudates. Those cultures that produced copious amounts of surface exudates were examined for antimicrobial effects by further testing.
Results: Antimicrobial testing of MB0725C *(P. digitatum)* samples did not result in any evidence of antimicrobial properties. However, MB0725A (*P. chrysogenum)* punch biopsy-agar overlay produced a 11 mm ZOI, whereas crude exudates testing resulted in a 27 mm ZOI. Crude culture filtrate of potato dextrose broth (PDB) did not result in any ZOI for either MB0725A or C. Sensitivity testing on samples collected from Yeast Extract Lactose Broth (YELB) on Day 3, Day 6, Day 9 and

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Day 12 resulted in ZOI of 11 mm, 13, 15 and 17 mm respectively. The change in pH for MB0725A liquid culture in PDB versus YELB was significantly different (N=12, P<0.0001).
Conclusion: MB0725A was an excellent producer of surface exudates and further experiments showed that its secondary metabolites had antimicrobial effects against *Staphylococcus aureus* using Kirby-Bauer sensitivity testing.

Keywords: Fungal species; antimicrobial testing; penicilium; zone of inhibition.

1. INTRODUCTION

Since the discovery of penicillin in 1928 by Alexander Fleming and its mass production by Sir Howard Florey and Ernst Chain from Oxford University, many fungal species have been investigated and used to produce an array of modern day medicines [1-4]. The antibiotics penicillin and cephalosporin; for example, are isolated from *Penicillium* sp. and *Acromonium* sp. respectively, whereas the immunosuppressant drug Cyclosporin-A is isolated from *Tricoderma sp*. and *Cylindrocarpon* sp. [1-4]. The statin drugs, used for lowering cholesterol are isolated from *Aspergillus* sp. of fungi [5]. Currently, the secondary metabolites produced by higher order fungi, such as the triterpenoids from the reishi mushroom; *Ganoderma lucidum,* are being investigated for their cytotoxic effects on cancer cells [6,7].

Many of the drugs isolated and used in medicine today have been discovered from soil sample, from fruit and from mushroom biopsy directly [1-8]. Once cultured, these organisms grow rapidly and once their growing medium has been depleted of nutrients and become stressed they produce secondary metabolites in the form of liquid exudates which are of interest here. Currently, the literature is lacking on the species of fungi growing in Anguilla and their biological uses in modern medicine.

The aim of this study is to identify fungal species isolated in Anguilla in order to determine the antimicrobial effect of their secondary metabolites from pure culture against *Staphylococcus aureus* by demonstrating the presence of a zone of inhibition in the culture plate. In the first experiment, Agar biopsy of pure fungal culture will be used in agar overlay technique on Mullen-Hinton Agar with *Staphylococcus aureus* to determine the antimicrobial effects of metabolites produced within the agar by measuring zone of inhibition. For the second experiment, surface exudates produced by fungal colonies excreted above agar level on top of colony will be examined using Kirby-Bauer disk diffusion technique and assessed for antimicrobial activity using Muller-Hinton Agar with *Staphylococcus aureus* by measuring zone of inhibition. For the last experiment, surface culture of fungi will be started using spores on Potato Dextrose Broth (PDB) and Yeast Extract Lactose Broth (YELB). The pH of cultures will be measured daily and the antimicrobial effects of broth filtrates examined for day 3, 6, 9, and 12. Antimicrobial effects will be examined using Kirby-Bauer disk diffusion technique on Muller-Hinton Agar with *Staphylococcus aureus* by measuring zone of inhibition.

2. MATERIALS AND METHODS

2.1 Sample Collection

The soil samples were collected from 6 different locations around Anguilla, British West Indies. Soil samples were collected from farmer's fields, wet salt-pond areas and dry sandy areas. Samples were stored in plastic zipper bags and kept at 4℃ until further use. Spoiled sour orange samples were also collected and stored similarly. White bread was moistened with sterile distilled water and left exposed to outdoor air for 30 minutes and then sealed in a jar to allow spores to germinate.

2.2 Growing Media

2.2.1 Isolation and sporulation media and solid media for metabolite production

Potato dextrose yeast agar (PDYA) was modified from the recipe according to [9]. Briefly, a 150 g of washed and diced potatoes are boiled in 500 ml of water. After straining, 1 gram of brewer's yeast is added and boiled for another 10 minutes. After removing from the heat source, the yeast is allowed to settle to the bottom and the liquid is decanted, discarding the solids. A stir rod is added, heated again to boiling and the stirrer set to 200 rpm. 10 cc's of dextrose syrup is slowly added. The volume is returned to 500 ml with

distilled water. For initial growth media using soil samples, 0.5 cc of 100 mg/ml of gentamycin is added. All additional subculture media and sporulation media was the same however; they did not contain antibiotics. After 2 minutes of boiling, 9 g of agar-agar powder is added and boiled to dissolve. Media is autoclaved for 45 min at 121℃ at 15 psi.

2.2.2 Liquid metabolite production media

Two media types were tested including minimal media and specialized penicillin production media. Potato dextrose broth (PDB) was modified according to [9] by boiling 150 g potatoes in 500 ml of water with the addition of 10 g dextrose syrup. Yeast extract lactose broth (YELB) was made according to [10] but modified by boiling 10 g of yeast extract, 20 g of lactose, 4 gram of dextrose syrup, 0.5 g L-cysteine and 2 g of calcium carbonate in 500 ml distilled water. Media pH is adjusted to 5.5 using hydrochloric acid before autoclaving and dispensed into 125 ml aliquots in 500 ml flasks and autoclaved for 45 min at 121℃ at 15psi before inoculation. 0.5 ml of spore suspension containing 5 x 10^7 spores/ml is added to each flask after cooling. Cultures are incubated in the dark at 25℃ for 12 days. Upon collection of daily samples for pH measurement, 1 ml of 0.1 g/ml of L-cysteine is added to each broth [11].

2.3 Isolation and Subculture for Pure Fungal Culture

2.3.1 Isolation from soil, fruit and bread samples

Soil was diluted 1:10 (%w/v) in distilled autoclaved water and vigorously shaken for 60 seconds. The solutions were allowed to settle for 5 minutes and an aliquot of 500 µl of each was plated and spread on sterile potato dextrose yeast agar (PDYA) containing gentamycin to prevent bacterial contamination. Similarly, spores from fruit were rubbed with a sterile loop, mixed with 2 ml of distilled autoclaved water and 500 µl of this spore suspension was plated and spread on PDYA medium. Bread mold was harvested with a loop and streaked on to PDYA medium. All cultures were labelled and sealed and incubated at 25℃ in the dark for 4 days.

2.3.2 Selection of species and subculture for pure culture

Fungal cultures are selected based on their ability to produce metabolites. Visually, those cultures which produced copious amounts of surface exudates on the surface of the culture are selected for further experiments. Those species later identified to be pathogenic or harmful were discarded. Briefly, small portions of each specimen were obtained with a sterile loop and plated to PDYA to produce a pure culture. Similarly, slide culture on PDYA of selected organisms was done in order to aid in microscopic examination and identification [12].

2.3.3 Spore production and spore suspension

For spore production 5 mm x 5 mm area of sporulating mold was removed from culture using a sterile scalpel blade. This was placed into 2ml of sterile distilled water and aspirated back and forth in order to disperse spores. One-half ml is aspirated and, leaving the agar behind, is placed in the centre of PDYA and spread evenly around the plate to make a “lawn culture” for spore propagation. These cultures were incubated at 25℃ in the dark for 5-10 days. When cultures were heavily sporulating as noted by homogeneous coverage of green-blue spores on top of the culture, a spore suspension was made by flooding mold lawn cultures with 10 cc’s autoclaved distilled water. The culture is agitated by hand for 2 min resulting in a dark green or blue suspension of spores. Spore density was calculated using a hemocytometer. Spore suspension is diluted to approximately 5 x 10^7 spores/ml as described by [13].

2.4 Antimicrobial Testing Using Kirby-Bauer and Muller-Hinton II Agar

2.4.1 *Staphylococcus aureus*

Antimicrobial testing is conducted in the department of Pathology at Princess Alexandra Hospital according to their standard operating procedures. Hospital standard; gram positive, hemolytic positive, catalase positive, coagulase positive *Staphylococcus aureus,* is used for sensitivity testing. Briefly, 5 colonies of the specimen are isolated from blood agar with a sterile loop and placed in normal saline solution and Vortexed briefly to mix. The turbidity of the solution is compared to standards and adjusted as needed. A lawn culture is made and sensitivity testing is conducted using Kirby-Bauer method on Muller-Hinton II agar.

2.4.2 Agar overlay sensitivity testing

Biopsy of pure fungal cultures on PDYA are taken from 24 day old cultures using a sterile

metal punch producing a 6 mm (same diameter as sensitivity paper disk) diameter biopsy. The fungal biopsy is placed fungal side up in the centre of a Muller-Hinton II agar Petri dish seeded with *Staphylococcus aureus* and incubated at 35℃ for 24 hours. Zone of inhibition is measured in millimeters.

2.4.3 Solid agar exudates-metabolite sensitivity testing

Exudates produced on top of pure fungal cultures are extracted using pipette and 35 µl of the exudates is pipette onto sterile standard sensitivity discs. The disc is then placed in the center of a Muller-Hinton II agar Petri dish seeded with *Staphylococcus aureus* and incubated at 35℃ for 24 hours. Zone of inhibition is measured in millimeters.

2.4.4 Broth filtrate sensitivity testing

Five ml of broth filtrate produced from liquid surface culture is taken daily using sterile technique. The pH is measured daily. Samples from PDB and YELB on day 3, 6, 9 and 12 were stored in the fridge for further analysis on day 13. Briefly, 35 µl of broth filtrate is pipetted onto paper sensitivity discs and placed onto Muller-Hinton II agar seeded with *Staphylococcus aureus* and incubated at 35℃ for 24 hours. Zone of inhibition is measured in millimeters.

3. RESULTS

3.1 Species Identification and Selection of Trial Organisms

The majority of fungal species identified from soil included aspergillus (*A. niger and A. flavus), mucor and penicillium* sp. Four penicillium sp. were isolated from soil and identified using slide culture technique and microscopy. These included *P. chrysogenum* (MB0725A), *P. purpurogenum* (MB0725B), and *P. verrucosum* (MB0725E). Citrus fruit samples included *P. citrinum* (MB0725D) and bread samples yielded 2 species including *P. digitatum* (MB0725C) and one unidentifiable grey specimen (MB0725F). Of these, only 2 specimens; MB0725A and MB0725C showed copious surface exudates metabolite production in initial culture and in pure culture at 24 days when grown on solid PDYA (Fig. 1). These two specimens; MB0725A and MB0725C are further used in surface liquid culture in PDB and YELB.

3.2 Agar Overlay

The agar overlay technique showed promising results in MB0725A with a zone of inhibition at 24 hours of 11 mm using the Kirby-Bauer sensitivity testing method on Muller-Hinton Agar and *Staphylococcus aureus* as the test organism (Fig. 2). MB0725C did not show any zone of inhibition.

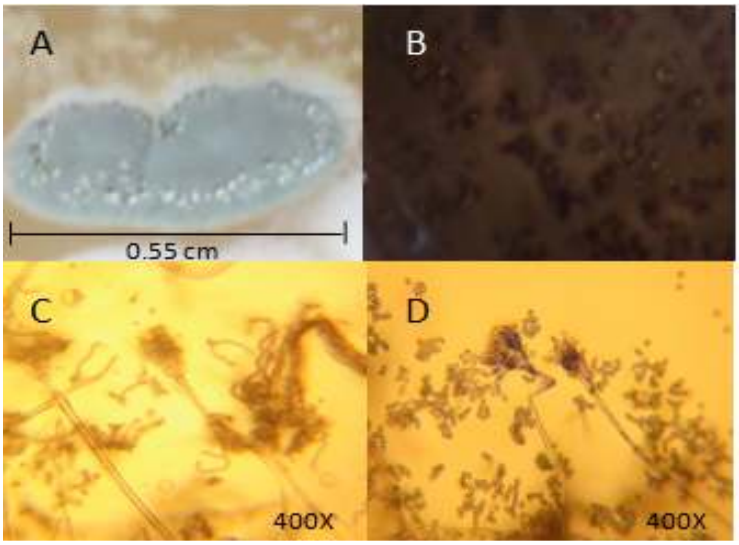

Fig.1. Selection of fungi based on the production of surface exudates. (A) MB0725A (*P. chrysogenum)* with surface exudates (B) MB0725C (*P. digitatum)* with surface exudates. (C) Microscopic examination of slide culture of MB0725A identified as *P. Chrysogenum* (D) MB0725C identified as *P. digitatum* using 1:20 methylene blue staining at 40X

Fig. 2. Agar overlay of fungal biopsy reverse side view. A) MB0725 A showing a zone of inhibition of 11mm on Muller-Hinton Agar with *Staphylococcus aureus* whereas B) MB0725C did not show any zone of inhibition

3.3 Surface Metabolic Exudates Sensitivity Testing

The metabolic exudates of MB0725A on standard paper disc showed promising results with a zone of inhibition 27 mm using the Kirby-Bauer sensitivity testing method on Muller-Hinton Agar with *Staphylococcus aureus* as the test organism (Fig. 3). MB0725C did not show any zone of inhibition.

3.4 Surface Liquid Culture pH Change Over 12 Days of Culture

The pH did not change drastically over the 12 day period of surface culture of MB0725A in PD broth. The mean pH is 5.59 (N=12, variance=0.007, SD=0.0793). However, the pH of the YELB broth changed significantly over the 12 day period with a mean pH of 7.73 (N=12, variance= 1.075, SD=1.04) (Fig. 4). T-test of the values comparing these pH results showed significantly different change in pH between these two media types ($P< 0.0001$, 95% CI= -2.764 to -1.516, SE= 0.301, DF=22 and t=7.1074). However, the pH did not change drastically over the 12 day period of surface culture of MB0725C in PD broth or YELB liquid media. The mean pH for PD broth was 5.94 (N=12, variance=0.048, SD=0.219) and the mean pH for YELB was 6.05 (N=12, variance=0.055, SD=0.235). T-test of these values showed no significant difference between the pH means for this organism ($P=0.2582$, 95% CI= -0.302 to 0.0823, SE= 0.093, DF=22, and t=1.1862) in either liquid media.

3.5 Broth Filtrate Sensitivity Testing

The broth filtrate for MB0725A sensitivity for PDA did not show any inhibition at Day 3, Day 6, Day 9, or Day 12 of collection. However, in YELB there was a zone of inhibition of *Staphylococcus aureus* for all time points. Specifically, Day 3 filtrate resulted in a 11 mm zone of inhibition, Day 6 resulted in a 13 mm zone of inhibition, Day 9 resulted in a 16 mm zone of inhibition and Day 12 resulted in a 17 mm zone of inhibition (Figs. 6 A, B). There was no zone of inhibition for MB0725C over all time points for both PD and YEL broth.

4. DISCUSSION

Media composition, including the sugar carbon source, micronutrient salts and pH, has been previously shown to regulate fungal colony morphology and metabolite production [10,13,14]. The yeast added to the potato dextrose yeast agar supplied the required nutrients necessary to produce these secondary metabolites on the solid media as nutrients have depleted. However, other commercially available ingredients, including corn steep liquor, contain the optimal carbon sugars and nitrogen sources, employed in high-yield production of penicillin. It is not surprising to learn that PD broth (without yeast extract) is a very minimal media and it did not support any detectable metabolite production. This is the case since precursor molecules (including adequate carbon and nitrogen source and essential amino acids such as L-cysteine are missing) [11,13]. The advanced production

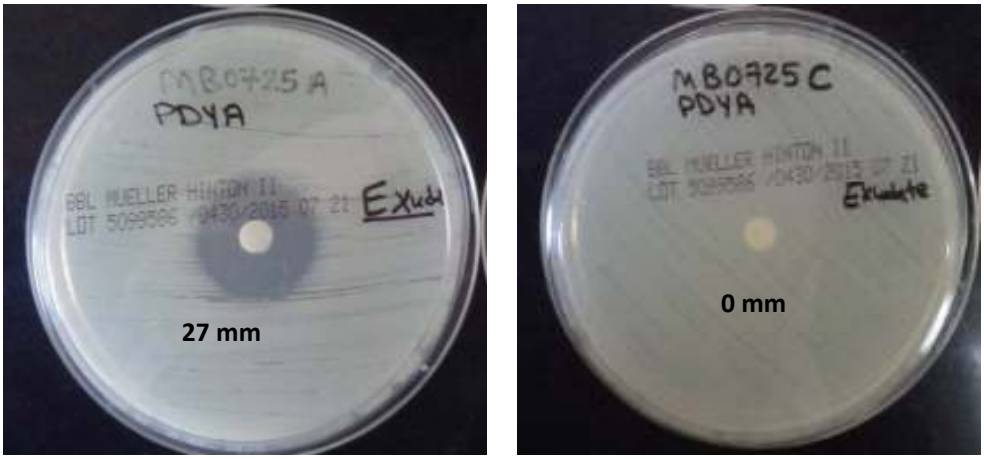

Fig. 3. Surface exudates sensitivity testing. (A) Surface exudates of secondary metabolites of MB0725A showed a zone of inhibition of 27 mm with the Kirby-Bauer sensitivity testing method on Muller-Hinton II agar using *Staphylococcus aureus* as the test organism. (B) Surface exudates of MB0725C did not show any zone of inhibition

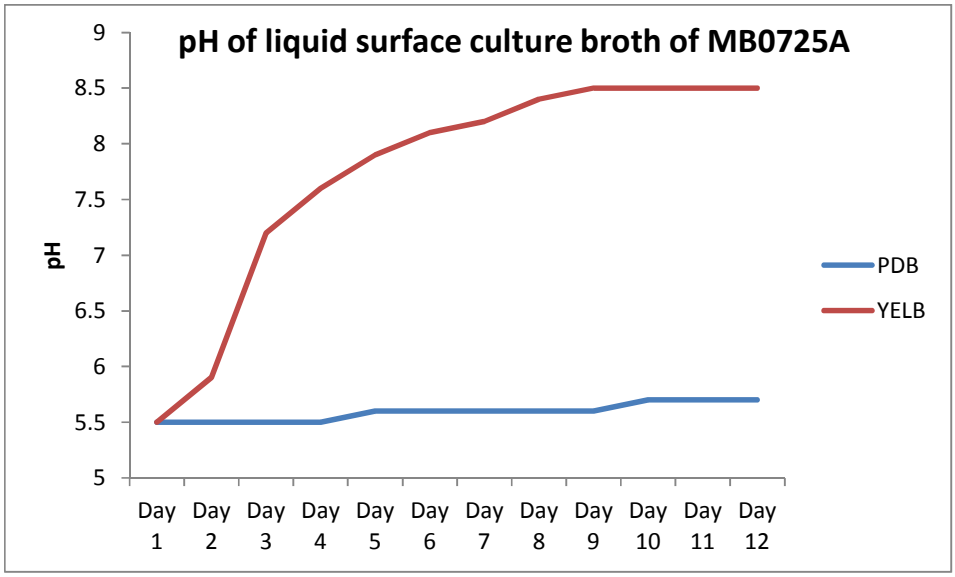

Fig. 4. Change in pH over 12 days of liquid culture broth of MB0725A in potato dextrose broth (PDB) and yeast extract lactose broth (YELB)

media including lactose as the carbon sugar source and L-cysteine as a precursor amino acid to penicillin formation greatly enhanced the production of secondary metabolites leading to the antimicrobial effects seen in Kirby-Bauer sensitivity testing. In particular, the lactose as a carbon source allows for slow degradation to its decomposition components of glucose and galactose, whereby glucose can be metabolized by the fungi for growth [10,13,15]. Since lactose has a slow rate of decomposition to glucose, the fungi are continuously in a stressed environment but supported as well required for continued metabolite production. The use of more advanced media, such as corn steep liquor Medias with lactose would greatly enhance our production of secondary metabolites [10,13,15]. Also, early on in the developmental stages of penicillin production, it was found that agitated liquid culture techniques greatly enhanced metabolite production and to date this is the method used (with engineered and mutated strains of *P. chrysogenum)* [16,17].

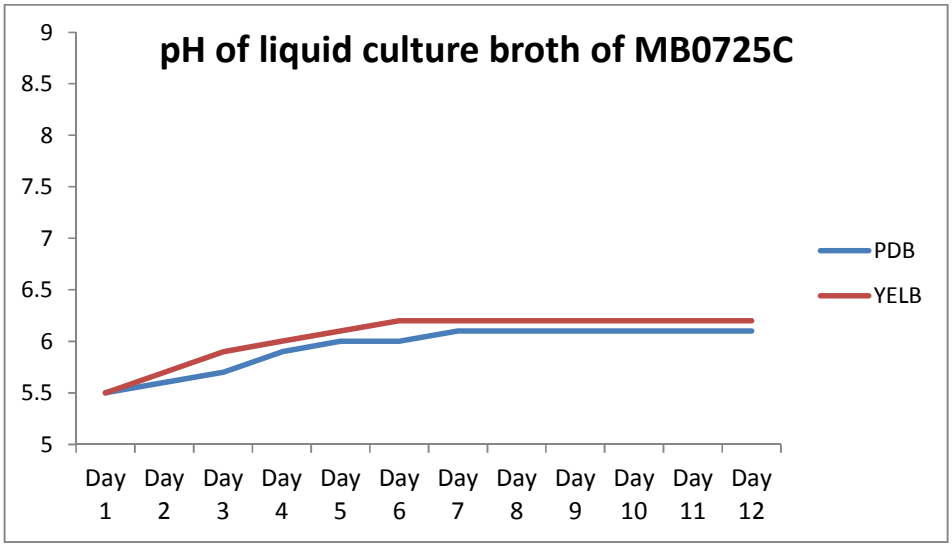

Fig. 5. Change in pH over 12 days of liquid culture broth of MB0725C in potato dextrose broth (PDB) and yeast extract lactose broth (YELB)

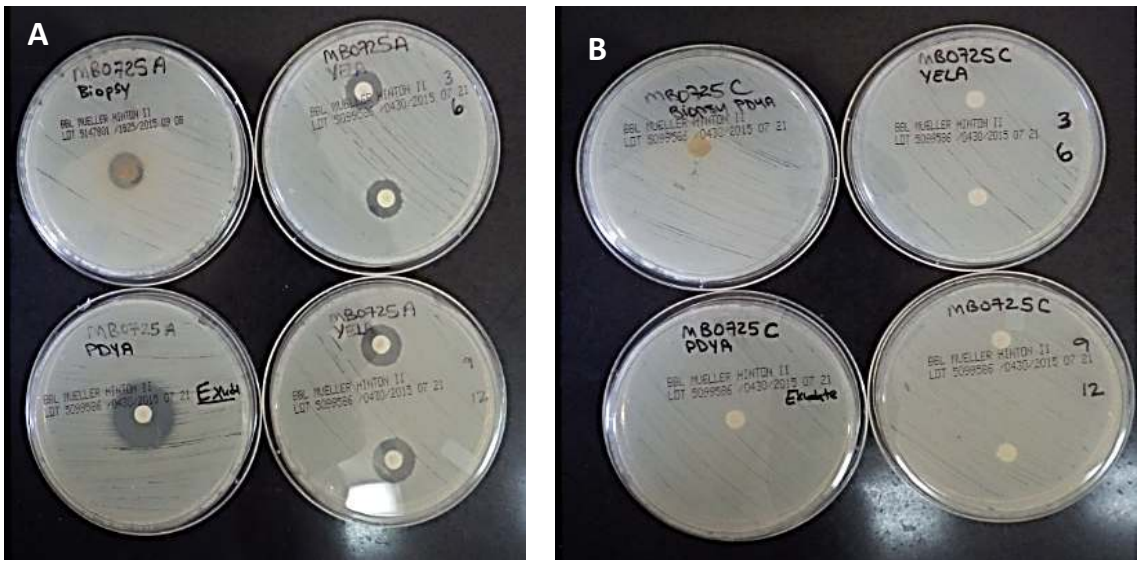

Fig. 6. Kirby-Bauer sensitivity testing of metabolites produced by fungi in Anguilla British West Indies. A) MB0725A sensitivity testing on Muller-Hinton agar with *Staphylococcus aureus. Top left*- potato dextrose biopsy of 24 day old culture*, bottom left*- surface exudates, *vertical right top and bottom* Broth filtrate in YELB broth on Day 3 of culture (11mm), and Day 6 (13mm), Day 9 (15 mm) and Day 12 (17mm). B) MB0725C showed no inhibition for all experiments

The color of the agar beneath MB0725A on potato dextrose yeast agar became darker in colour gradually over the first 2 weeks of culture. This is due to secondary metabolite excretion into the media as previously reported by Fleming during early studies on *Penicillium* culture [18]. Since MB0725A was able to produce metabolites in the PDYA agar, it was assumed that MB0725C would be able to as well. The color change noted in the agar of MB0725C was not nearly as drastic as that in MB0725A. Even though MB0725C did produce copious amounts of exudates above the fungal culture, no zone of inhibition was noted when agar biopsy was plated in the Kirby-Bauer

method for sensitivity testing. This is consistent with previous research suggesting that *P. digitatum* does not produce penicillin [19]. Although MB0725C was noted as *P. digitatum*, identifying *Penicillium* sp. with microscopy alone is not an effective way to identify this large group of fungi [20]. Basically, multiple media types, microscopy and genomic studies are required to specifically identify the species isolated [20]. Unfortunately, the identity of our isolates is based strictly on color and microscopy and so further studies are needed to verify the species of fungus isolated.

The Kirby-Bauer method for sensitivity testing failed to produce any inhibition of *Staphylococcus aureus* for all culture filtrate samples when the discs containing the liquid samples were dried at 35℃ previous to plating. This may be due to the fact that the crude filtrate metabolites produced are not heat stable at this temperature. However, penicillin G produced through extraction methods are stable for a short period of time (10-14 days) at room temperature (20℃) and stable for short periods of time (minutes) up to 100℃ [18].

The study was then conducted using 35 µl of crude culture filtrate (35 µl was measured to be the amount absorbed without leakage) and immediately put in 5℃ until use within the hour. This method was able to produce results and show antimicrobial effects (Fig. 6).

Although it is an educated assumption that the organism identified is *P. chrysogenum* using microscopy and the metabolite produced is penicillin-G, this may not be the case at all. Further studies using HPLC and mass spectroscopy on extracts of the metabolite need to be conducted in order to determine the metabolite produced. It is possible that the organism MB0725A is not *P. chrysogenum* and the antimicrobial metabolite produced by it is not penicillin-G. It is possible that the organism is a new species not previously identified and the metabolite produced a novel possible therapeutic molecule not previously described either. A specialist in identifying *Penicillium* species using microscopy and genomics must be used.

The pH change or the lack there of, of the culture broths over the 12 day culture period was a good predictor of secondary metabolite production. The minimal surface culture media of potato dextrose broth (PDB) did not have any drastic pH change over the culture period of 12 days. However, the specialized production media, yeast extract lactose broth (YELB) did show a drastic pH change over the culture period becoming markedly alkaline (8.5-9) within the first 7-10 days. This is consistent with previous studies on surface culture of *Penicillium* [10].

The increased size of the zones of inhibition by MB0725A filtrate as culture days progress is intuitive; however the large zone of inhibition of 11 mm at Day 3 (Fig. 6) was unexpected. However, a large increase in pH was noted between Day 2 and Day 3 with a pH difference of 1.3 which is the greatest pH difference recorder in this study in one day.

5. LIMITATIONS

There were a number of limitations in this study. Most specifically it was the inability to find resources on the small island of Anguilla. In particular, the media types used were engineered using commonly available materials. For instance, lactose was isolated from reconstituted fat-free milk powder, removal of fats and album proteins and re-crystallization of the lactose crystals over 3 day period. Similarly, the common stain used for fungal staining in current literature favored to be Lacto-phenyl Cotton Blue (LPCB). Our lab was unable to attain this stain and so over 50 staining experiments were conducted to optimize the staining using various collection methods, dilutions of methylene blue and various incubation times.

Since there was no availability of pure penicillin-G on the island at the time of this study, it was impossible to make standard disc to compare different concentrations with our filtrate zones of inhibition using the Kirby-Bauer method to determine the units/ml of production. However, 10 international unit standards (10IU) were tested and produced a 10 mm zone of inhibition in multiple trials using the *Staphylococcus aureus*. Further experiments should be conducted using penicillin-G standardized discs of various concentrations and comparison of zones of inhibition using Kirby-Bauer sensitivity testing to determine production concentration. Similarly, the hospital standard organism used in these experiments is not classified as ATCC certified *Staphylococcus aureus.* Further experiments should include the use of standard ATCC strains of *Staphylococcus aureus.*

6. CONCLUSION

Although some species of fungi may produce copious amounts of metabolites in the form of surface exudates on potato dextrose yeast agar (PDYA), it does not necessarily mean that these liquids have antimicrobial properties. For example, two isolates MB0725A and MB0725C both produced large quantities of surface exudates, however only MB0725A had antimicrobial properties against our test organism. Furthermore, media composition is directly related to the amount of metabolite produced and is based on basal salts, carbon sources and nutrients. Agar overlay technique proved an easy and acceptable technique for screening fungal cultures for antimicrobial metabolite production. Yeast extract lactose broth (YELB) was able to support the production of secondary metabolites when all other culture media were unavailable. As previously reported, a rise in pH is associated with production of metabolites in culture. Although surface culture of *Penicillium* sp. has not been employed for decades in large scale manufacture of antibiotics, it was a sufficient technique to simply screen organisms for antimicrobial properties. Slide-culture method made microscopy easier for identification compared to other tested methods. Further studies using MB0725A should be conducted as described.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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