Endophytic fungi associated with the black mangrove *Avicennia germinans* in Cabo Rojo, Puerto Rico: their antimicrobial potential.

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

BIOLOGY

UNIVERSITY OF PUERTO RICO

MAYAGÜEZ CAMPUS

2018

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Endophytic fungi associated with the black mangrove, *Avicennia germinans,* in Cabo Rojo, Puerto Rico: their antimicrobial potential.

ABSTRACT

The search for new antimicrobial compounds has been of great interest in recent years. These compounds are naturally produced by microorganisms including endophytic fungi isolated from mangroves. This study focuses on endophytic fungi isolated from leaves and seeds of the black mangrove (*Avicennia germinans*) from Bahía Salinas, Cabo Rojo, Puerto Rico. We isolated 20 genera of fungal endophytes from which nine identified strains were tested for antimicrobial secondary metabolite production. Fungal strains from *Penicillium* (BSI-HV-1(2)) sp., *Aspergillus flavus* (BSI-HV-2(1)), *Aspergillus clavatus* (BSI(MH)-HJ-2(1)), *Stereum* (BSI-R-2(1)) sp., *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *Purpureocillium* (BSII(3.5)-R-3(2)) sp., *Simplicillium* (BSI(1.5)-R-1(1)) sp., *Hortaea* (BSII(3.5)-HC-1(2)) sp. and *Bionectria* (BSI(MH)-HC-3(1)) sp. were selected for analysis. Four bacterial strains: *Escherichia coli, Pseudomonas* sp., *Serratia* sp. and *Staphylococcus* sp. and two yeast strains of *Candida albicans* and *C. tropicalis* were used to perform bioassays (ATCC certified strains). Growth curves were prepared for yeasts and bacteria. Fungal extracts were added after lag, exponential and stationary growth phases of each bacteria and yeast culture. Our results showed that *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)) extracts strongly decreased the growth of *C. albicans, P. aeruginosa,* and *E. coli*. Only *A. flavus* (BSI-HV-2(1)) decreased the growth of *C. tropicalis*. *Stereum* (BSI-R-2(1)) sp. enhanced the growth of *E. coli* and *A. flavus* (BSI-HV-2(1)) enhanced the growth of *S. aureus*. These results could open a way for future discoveries of new antimicrobial or antifungal compounds from the black mangrove endophytes in Puerto Rico.

Hongos endófitos asociados al mangle negro, *Avicennia germinans*, en Cabo Rojo, Puerto Rico: su potencial antimicrobial

RESUMEN

La búsqueda de nuevos compuestos antimicrobiales ha sido de gran interés durante los últimos años. Estos compuestos pueden ser producidos naturalmente por muchos organismos, entre ellos, hongos aislados de mangles. Este estudio se enfoca en los hongos endófitos aislados de las hojas y semillas del mangle negro (*Avicennia germinans*) en Bahía Salinas, Cabo Rojo, Puerto Rico. Aislamos 20 géneros de hongos endófitos de los cuales se escogieron 9 para determinar si los metabolitos secundarios producidos tenían alguna capacidad antimicrobial. Las especies de hongos utilizadas fueron *Penicillium* (BSI-HV-1(2)) sp., *A. flavus* (BSI-HV-2(1)), *Aspergillus clavatus* (BSI(MH)-HJ-2(1)), *Stereum* (BSI-R-2(1)) sp., *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *Purpureocillium* (BSII(3.5)-R-3(2)) sp., *Simplicillium* (BSI(1.5)-R-1(1)) sp., *Hortaea* (BSII(3.5)-HC-1(2)) sp. y *Bionectria* (BSI(MH)-HC-3(1)) sp. Utilizamos cuatro cepas de bacterias para realizar los bioensayos: *Escherichia coli*, *Pseudomonas* sp., *Serratia* sp. y *Staphylococcus* sp., así como dos cepas de levaduras, *Candida albicans* y *C. tropicalis* (cepas certificadas ATCC). Se realizaron curvas de crecimiento para determinar las fases de crecimiento de cada bacteria y levadura. Luego se añadió el sobrenadante del hongo (extractos) para ver cómo los metabolitos secundarios excretados por el mismo afectaban la curva normal de crecimiento en las fases lag, exponencial y estacionaria de cada espécimen. Se encontró que los extractos de *A. flavus* (BSI-HV-2(1)) y *A. clavatus* (BSI(MH)-HJ-2(1)) disminuyeron el crecimiento de *C. albicans, P. aeruginosa* y *E. coli*. Sólo *A. flavus* (BSI-HV-2(1)) disminuyó el crecimiento de *C. tropicalis*, mientras que los extractos de *Stereum* (BSI-R-2(1)) sp. y *A. flavus* (BSI-HV-2(1)) aumentaron el crecimiento de *E. coli* y *S. aureus*, respectivamente. Estos resultados abren nuevas puertas a

posibles descubrimientos de nuevos productos antimicrobiales provenientes de hongos endófitos de mangle negro en Puerto Rico.

ACKNOWLEDGEMENTS

I would like to thank all the people that make this work possible. To my chairman, Matias J. Cafaro, for his guidance, patience and dedication on every step of my journey since I started to work in his laboratory.

To all the members of the Symbiosis Laboratory; my mentor as an undergraduate student, Angélica Olmo, and the ones who took part in this research, specially Zuleida Valentin, Edaris Rodríguez, and Victoria Cabán.

To the Department of Biology faculty members, specially: Sandra Maldonado, Carlos Ríos Velázquez, Luis Ríos, Magaly Zapata, Carol Rivera among others; for being supportive and providing their knowledge and help.

To the BRIDGES to Doctorate Program and their coordinators, for funding and guideline throughout these years (Grant number R25GM058389).

Without you all I would not have become the scientist that I am today. Thanks.

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Chapter 1. General Overview

1.1 Introduction

Antibiotics have been widely used for 80 years since their first discovery in 1928 by Alexander Fleming (ACS 2016). Since then, microorganisms have developed resistance against many drugs. Infections by multi drug-resistant microorganisms (MDRO) have limited treatments, increasing morbidity in patients. Thus, the discovery and development of new compounds is critical, but where could we find them? There are many ecological niches that can harbor potential drugs for the treatment of MDROs such as marine environments (Hughes and Fenical 2010; Rahman et al. 2010), and also being produced by different organisms like plants, bacteria and fungi (Aminov 2010).

Marine environments, such as mangroves, provide a unique ecological system with a variety of adaptations, increased productivity and irreplaceable value. Mangroves are woody plants found in coastal areas around the tropics, characterized by different tree species, which can withstand high salt concentrations (Alongi 2008; Kathiresan and Bingham 2001). They also serve as natural barriers for coastal erosion and hurricanes (Lugo and Snedaker 1974), and are habitats for many organisms, such as bacteria, archaea, algae, insects, marine invertebrates, fungi among others (Alongi 2008; Polidoro et al. 2010; Costa et al. 2012; Castro et al. 2014; Hong et al. 2015). On this remarkable habitat, we can find microorganisms associated with the inner tissues of plants known as endophytes. Many of these microorganisms are capable of producing secondary metabolites, which inhibit growth of other organisms including pathogenic bacteria and yeasts (Cheng et al. 2009; Silva et al. 2011).

An endophyte is an organism that lives in between plant cells (mostly bacteria or fungi). They appear to be present in all plants (Rodriguez et al. 2008) and several studies suggest that

fungal endophytes have a symbiotic relationship with several plants (Petrini 1986). These organisms are very important for the plant in different ways: they can protect the host plant from herbivores and from plant pathogens (Mousa and Raizada 2013), boost host fitness (Spiering et al. 2006), promote drought tolerance or avoidance (Malinowski and Belesky 2000), and increase salt tolerance (Baltruschat et al. 2008). In some cases, endophytes can also invade plant tissues acting as pathogens (Rodriguez et al. 2008). They can be found in a myriad of plant species, sometimes associated with a specific plant in mutualistic, parasitic, or commensal symbiotic relationships (Arnold and Lutzoni 2007).

Endophytic fungi, especially associated with mangroves, have been reported many times in Southeast Asia (Table 1.1), but in the Caribbean, especially in Puerto Rico, there are no known reports of mangrove fungal endophytes. These fungi, besides being an important component of the plants microbiome (Porras-Alfaro and Bayman 2011), also produce important secondary metabolites that have antimicrobial, antifungal and anticarcinogenic properties (Bhimba and Joel 2012). In addition, fungi (not only fungal endophytes), are essential in multiple life processes (Wessels 1999; Harms et al. 2011). For example, they are the principal decomposers of organic compounds (Boer et al. 2005). Thus, research in countries of limited studies like Puerto Rico is very important to explore new areas. An estimated of the total number of fungal species indicates that there are about 5.1 million fungi, based on a fungus to plant ratio of 6:1 (Blackwell 2011). With the current technology, it would take about 1,170 years to only describe 1.4 million fungi (Hibbett et al. 2007), representing a substantial window of opportunities for new discoveries of new fungal species. Until 2016, there were 11,268 species isolated from the Caribbean, 30% of those represented by Puerto Rico's fungal species in where 3,315 species have been described (24% endemic to the island) (Cantrell-Rodriguez 2016).

In addition to contribute to the identification of new fungal species and, given the necessity for new fungal studies as a means to discover new compounds with medical applications, we decided to study the antibiosis potential of fungal endophytes from the black mangrove *Avicennia germinans* in Puerto Rico. Since fungal endophytes play a role in producing important bioactive compounds for the benefit of the plant and it has been showed that they can produce medicinal compounds, it is worth study them from mangrove forests in the Caribbean.

1.2 Literature Review

1.2.1 Mangroves in Puerto Rico

Puerto Rican mangroves are classified in two groups according to climate regions: the northern-coast and the southern-coast mangroves. The northern-coast is characterized by high precipitation, wave energy and river runoff (since the majority of Puerto Rican rivers flow into the Atlantic Ocean), and is also dominated by basin (interior) and riverine (commonly flooded by river water) mangroves. On the other hand, the southern-coast shows less precipitation and peripheral (tide-dominated) mangroves. Both regions are considered subtropical with an equal temperature regime (Lugo and Cintrón 1995; Martinuzzi et al. 2009). Both are distributed along most of Puerto Rico's coasts (Figure 1.1) (Martinuzzi et al. 2009).

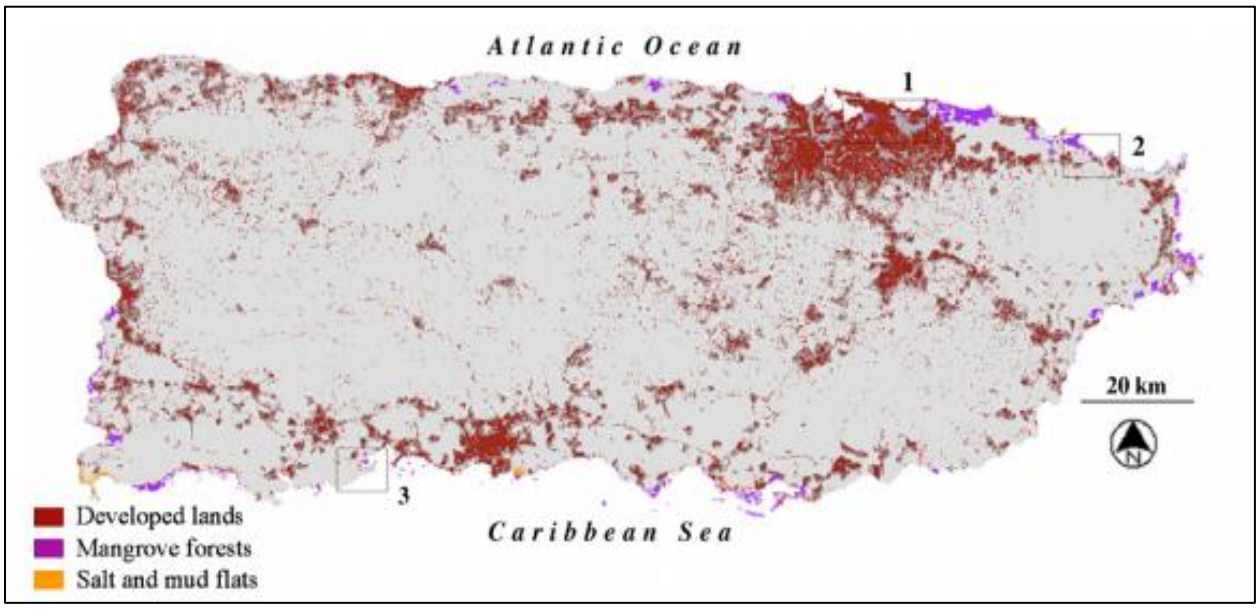

Figure 1.1: Distribution of mangrove forest in Puerto Rico (purple areas) (Martinuzzi et al. 2009).

Mangroves are formed by a variety of plant species around the globe. There are four types of mangroves in Puerto Rico: (i) red mangrove (*Rhizopora mangle*), (ii) white mangrove (*Laguncularia racemosa*), (iii) black mangrove (*Avicennia germinans*), and (iv) buttonwood mangrove (*Conocarpus erectus*) (not considered a mangrove because it cannot grow in salty soils) (Lugo and Snedaker 1974; Chinea and Agosto 2007). The black mangrove, *Avicennia germinans*

is abundant in Puerto Rico's mangroves and is one the most tolerant species to environments with high salinity (Holdridge 1940; Lugo and Cintron 1975; Sobrado 1999).

Avicennia germinans possess a peculiar characteristic that attracts the attention for our study. The plant possesses specialized glands to excrete the excess absorbed salt through its leaves (Scholander et al. 1962) (Figure 1.2). Thus, it represents a unique environment for microorganisms associated with this plant (Lugo and Snedaker 1974). Black mangrove can be found in other continental tropical countries around the world like North America (Florida and Texas coasts), Bahamas, Bermuda, Mexico, Africa, Australia, India, and Thailand (Kathiresan and Bingham 2001; Little and Wadsworth 1964; Nieves et al. 2002).

Figure 1.2: Salt crystals observed on a leaf from *Avicennia germinans*. (http://www.westafricanplants.senckenberg.de/root/index.php?page_id=14&id=2263).

1.2.2 Mangrove-associated fungal endophytes and their medical properties

Mangrove endophytes have been studied in the past years due to their production of secondary metabolites. These compounds possess an important role in the medical field due to their antimicrobial, antifungal, anticancer, and antimalarial properties (Bhimba et al. 2011; Strobel and Daisy 2003). Secondary metabolites are not only produced by fungi, but also, by many organisms including plants and bacteria (Croteau et al. 2000; Vining 1990). Plant secondary metabolite production has been studied for many years placing them as the major producers of compounds with medical applications (Rodríguez et al. 2008). Several studies have shown that secondary metabolites from fungal endophytes can improve the survival of the host plant and its capacity to produce its own compounds (Ludwig-Müller 2015). In fact, studies have shown that some compounds originally believed to be plant-produced are actually produced by their fungal endophytes (Kaul et al. 2012; Alvin et al 2014).

Compounds with antibiotic, antifungal and anticancer activity have been isolated from fungal species. The most well-known example of a fungal antibiotic is the discovery of penicillin by Alexander Fleming in 1928 (Fleming 1929), the first compound showing antibacterial properties. Since then, and with the emergence of a large number of pathogens developing resistance to existing drugs, the search for new compounds with antibiotic properties of fungal etiology has been uninterrupted through decades (Vassal et al. 2013; Alanis 2005).

In addition to identify compounds for disease treatments, investigations are also conducted to study the roles of endophytes in agriculture and biofuel production (Strobel et al. 2008). Crop plants inoculated with certain endophytes may provide disease or parasite resistance by producing compounds like cryptocandin (useful in the control of the wine grape pathogen *Botrytis cinerea*), produced by *Cryptosporiopsis quercina* (Li et al. 2000), and jesterone (an oomycete antibiotic for

plant pathogens) from *Pestalotiopsis jesteri* (Strobel and Daisy 2003). The latter, has been successfully synthetized maintaining its biological properties (Mehta and Pan 2004). Also, other endophytes may convert cellulose and other carbon sources into "myco-diesel" hydrocarbons and other hydrocarbon derivatives (Strobel et al. 2008). Subsequently, secondary metabolites from endophytes have become of primordial interest for the scientific community in many areas.

A vast number of studies about the diversity and properties of mangrove fungal endophytes have been reported around the world (Tables 1.1 and 1.2). The majority of these reports are from eastern countries (Gilbert et al. 2002; Liu et al. 2007). Apart from Brazil and Panama, investigations in western countries are more limited. More detailed and comprehensive information about compounds produced by mangrove-associated fungi has been reported in many reviews over the years by specific dates [May 2012 to April 2014 (Chagas et al. 2015)], different biotopes (Schulz et al. 2002), and specific properties like biofungicides (Kumar and Kaushik 2012), among others (Bhimba et al. 2011; Bhimba and Joel 2012: Blunt et al. 2014; Pandi et al. 2011; Thatoi et al. 2013). Some compounds produced by mangrove endophytes showing antibacterial or antifungal activities are shown on Table 1.3.

Table 1.1: Previous reports of fungal endophytes isolated from mangrove plants.

Type of mangrove	Fungal Isolates				Country	Reference
Avicennia schaueriana *Laguncularia racemosa* *Rhizophora mangle*	*Guignardia* sp. *Colletotrichum gloeosporioides* *Glomerella cingulata* *Sphaerosporium sp.* *Chloridium virescens* *Microsphaeropsis arundinis*	*Penicillium pinophilum* *Periconia cambrensis* *Phoma herbarum* *P. diachenii* *P. obscurans* *Sordaria prolífica* *Torula ellisii*	*Nodulisporium* sp. *Periconia cambrensis* *Phomopsis archeri* *Phyllosticta* sp. *Preussia minima* *Fusarium lateritium*	*Scopulariopsis sphaerospora* *Sordaria prolifica* *Sphaerosporium equinum* *Torula ellisii* *Trichoderma pseudokoningii* *Hormonema* sp.	Brazil	Costa et al. 2012
	Diaporthe sp. *Colletotrichum* sp. *Fusarium* sp. *Trichoderma* sp. *Xylaria* sp. *Arthothelium* sp. *Chrysoporthe* sp. *Coniothyrium* sp. *Coprinellus* sp.	*Curvularia* sp. *Epicoccum* sp. *Eutypa* sp. *Gelasinospora* sp. *Lasiodiplodia* sp. *Neosartorya* sp. *Neurospora* sp. *Nigrospora* sp. *Phaeoramularia* sp.	*Phaeoseptoria* sp. *Phanerochaete* sp. *Pseudallescheria* sp. *Scolecobasidium* sp. *Valsa* sp. *Guignardia* sp. *Penicillium* sp. *Aspergillus* sp. *Alternaria* sp.	*Botryosphaeria* sp. *Endothia* sp. *Neofusicoccum* sp. *Pestalotiopsis* sp. *Pichia* sp. *Periconia* sp. *Cylindrocladium sp.*		De Souza et al. 2013
Laguncularia racemosa	*Aspergillus niger*	*Curvularia pallescens*	*Guignardia bidwelii*	*Paecilomyces variotii*		Silva et al. 2011
Avicennia officinalis *Kandelia candel* *Excoecaria agallocha* *Rhizophoramucronata*	*Aspergillus flavus*				India	Ravindran et al 2012
Acanthus ilicifolius	*Alternaria* sp.	*Aspergillus* sp.	*Cumulospora* sp.	*Pestalotiopsis* sp.		Maria et al. 2005
Rhizophora apiculate	*Acremonium sp.* *Diaporthe sp.*	*Hypoxylon sp.*	*Pestalotiopsis sp.*	*Phomopsis sp.* *Xylaria* sp.		Buatong 2010
Rhizophora apiculate	*Glomerella* sp. *Sporormiella minima* *Acremonium* sp. *Alternaria alternata*	*Aureobasidium* sp. *Cladosporium cladosporioides* *Curvularia lunata* *Curvularia pallescens*	*Drechslera* sp. *Nodulisporium* sp. *Pestalotiopsis* sp. *Phialophora* sp. *Fusarium* sp.	*Phoma* sp. *Phomopsis* sp. *Phyllosticta* sp. *Pithomyces* sp. *Sporothrix* sp.		Kumaresan and Suryanarayanan 2002
Acanthus ilicifolius	*Colletotrichum* sp. *Acremonium* sp. *Cumulospora marina*	*Alternaria* sp. *Aspergillus* sp. *Cladosporium* sp.	*Cytospora* sp. *Dycima* sp. *Fusarium* sp.	*Paecilomyces* sp. *Phoma* sp. *Pestalotiopsis* sp.		Maria and Sridhar 2003
Aegiceras corniculatum *Avicennia marina* *Avicennia officinalis* *Bruguiera cylindrica* *Ceriops decandra* *Excoecaria agallocha* *Lumnitzera racemosa*	*Acremonium* sp. *Alternaria* sp. *A. alternata* *Aspergillus* sp. *A. niger* *Camarosporium* sp. *Chaetomium* sp.	*Cladosporium* sp. *Colletotrichum* sp. *Drechslera* sp. *Curvularia* sp. *Glomerella* sp. *Mammaria* sp.	*Memnoniella* sp. *Paecilomyces* sp. *Penicillium sp.* *Phialophora* sp. *Phoma* sp. *Phomopsis* sp.	*Pithomyces* sp. *Phyllosticta* sp. *Selenophoma* sp. *Sporormiella minima* *Sporothrix* sp. *Trimmatostroma* sp.		Kumaresan and Suryanarayanan 2001
Bruguiera gymnorrhiza	*Phyllosticta* sp.	*Pestalotiopsis* sp.		*Colletotricum* sp.	Japan	Izumi et al. 2001
Sonneratia caseolaris *Sonneratia hainanensis* *Sonneratia ovata* *Paracaseolaris* *Sonneratia apetala*	*Cytospora* sp. *Diaporthe* sp.	*Fusarium* sp. *Glomerella* sp.	*Mycosphaerella* sp. *Phoma* sp.	*Phomopsis* sp. *Stemphylium* sp.	China	Xing et al. 2010
Kandelia candel	*Phomopsis* sp.	*Pestalotiopsis* sp.	*Guignardia* sp.	*Xylaria* sp.		Pang et al. 2008
Sonneratia alba	*Alternaria sp.*					Kjer et al. 2009
Avicennia sp.	*Penicillium* sp. *Aspergillus* sp.	*Guignardia* sp. *Curvularia* sp.	*Diaporthe* sp. *Eupenicillium* sp.	*Neosartorya* sp. *Cladosporium* sp.	Malaysia	Ling 2013
Avicennia germinans	*Aspergillus ustus*				Panama	Facey et al. 2016

Table 1.2: Reports of fungal endophytes producing active compounds from mangrove plants.

Type of mangrove	Fungal Isolates	Pathogen	Activity/ Compounds	Country/ Reference
Laguncularia racemosa	*Aspergillus niger* *Curvularia pallescens* *Guignardia bidwelii* *Paecilomyces variotii*	*Staphylococcus aureus* *Bacillus subtilis* *Enterococcus faecalis* *Micrococcus luteus* *Escherichia coli* *Pseudomonas aeruginosa*	Antibiotic	Brazil/ Silva et al. 2011
Sonneratia alba	*Alternaria* sp.	*Staphylococcus aureus*	Xanalteric Acids I and II	China/ Kjer et al. 2009
Acanthus ilicifolius	*Alternaria* sp. *Aspergillus* sp. *Cumulospora* sp. *Pestalotiopsis* sp.	*Bacillus subtilis* *Enterococcus* sp. *Klebsiella pneumoniae* *Pseudomonas aerugionosa* *Salmonella typhi* *Staphylococcus aureus* *Candida albicans* *Trichophyton metagrophytes*	Antibiotic and Antifungal	India/ Maria et al. 2005
Rhizophora apiculate	*Acremonium sp.* *Diaporthe sp.* *Hypoxylon sp.* *Pestalotiopsis sp.* *Phomopsis sp.* *Xylaria* sp.	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Microsporum gypseum* *Candida albicans* *Escherichia coli* *Cryptococcus neoformans*	Antibiotic and Antifungal	India/ Buatong et al. 2011
Avicennia officinalis	*Aspergillus* sp. *Penicillium* sp. *Acremonium* sp. *Curvularia* sp. *Cladosporium* sp. *Phoma* sp. *Fusarium* sp.	*Pseudomonas aeruginosa* *Escherichia coli* *Aeromonas hydrophila* *Bacillus cereus* *Staphylococcus aureus* *Edwardsiella tarda* *Vibrio harveyi* *Vibrio fluvialis* *Vibrio cholera* *Vibrio parahaemolyticus* *Vibrio proteolyticus* *Vibrio vulnificus* *Candida albicans* *Aspergillus flavus* *Aspergillus fumigatus*	Antibiotic and Antifungal	India/ Neema et al. 2015
Rhizophora mucronata	*Pestalotiopsis* sp.	N/A	Chromones production Asthma treatment	China/ Xu et al. 2009; Netzer et al. 2012
Excoecaria agallocha	*Phomopsis* sp.	*Candida albicans* *Fusarium oxysporum*	Phomopsins	China/ Huang et al. 2008
Kandelia candel	*Sporothrix* sp.	N/A	Sporothrins	China/ Wen et al. 2009
Avicennia sp.	*Penicillium* sp. *Curvularia* sp. *Diaporthe* sp. *Aspergillus* sp. *Guignardia* sp. *Neosartorya* sp. *Cladosporium* sp. *Eupenicillium* sp.	*Bacillus cereus* *Bacillus subtilis* *Staphylococcus aureus* *Micrococcus luteus* *Escherichia coli* *Pseudomonas aeruginosa* *Vibrio anguillarum* *Saccharomyces cerevisiae* *Candida albicans* *Aspergillus niger*	N/A	Malaysia/ Ling 2013
Not specified	*Aspergillus* sp. *Colletotrichum* sp. *Fusarium* sp. *Guignardia* sp. *Penicillium* sp. *Pestalotiopsis* sp. *Phomopsis* sp. *Talaromyces* sp. *Trichoderma* sp.	*Fusarium oxysporum*	Antagonism	Indonesia/ Rahmansyah and Rahmansyah 2013
Rhizophora apiculate *R. mucronata* *Ceriops decandra* *Sonneratia alba* *Lumnitzera littorea* *Avicennia alba* *Acanthus ilicifolius* *Xylocarpus granatum* *Xylocarpus moluccensis* *Thespesia populneoides*	*Phyllosticta* sp. *Cladosporium* sp. *Colletotrichum* sp. *Phomopsis* sp. *Xylaria* sp.	*Bacillus subtilis* *Pseudomonas aeruginosa* *Escherichia coli* *Staphylococcus aureus* A375 (human malignant melanoma), SW620 (human colorectal adenocarcinoma), Kato III (human gastric carcinoma), HepG2 (human liver hepatoblastoma) and Jurkat (human acute T cell leukemia)	Antibacterial and anticancer	Thailand/ Chaeprasert et al. 2010

Table 1.3: Compounds from mangrove endophytic fungi with antimicrobial or antifungal activity.

Type of mangrove	Fungal Isolates	Compound	Activity	Reference
Rhizopora mucronata	*Pestalotiopsis* sp.	Pestalotiopen A	Antibacterial: *Enterococcus faecalis*	Hemberger et al. 2013
Laguncularia racemosa	*Diaporthe phaseolorum*	3-Hydroxypropionic acid (3-HPA)	Antibacterial: *S. aureus* and *Salmonella typhi*	Sebastianes et al. 2012
Avicennia sp.	*Penicillium*	Quinolactacin	Quinolone antobiotic	Ling 2013
	Diaporthe	Cyclo(tyrosylprolyl)	Antibiotic	
Acanthus ilicifolius	*Talaromyces* sp.	Talaromyone A	Antibacterial: *B. subtilis*	Cai et al. 2017
Kandelia candel	*Talaromyces* sp.	Norlichexanthone	Antibacterial: *P. aeruginosa* Antifungal: *A. niger*, *C. albicans* and *F. oxysporum*	Lui et al. 2010
		Secalonic Acid A	Antibacterial: *E. coli*, *S. aureus*, *S. ventriculi* and *P. aeruginosa* Antifungal: *A. niger*, *C. albicans* and *F. oxysporum*	
		Stemphyperylenol	Antibacterial: *S. aureus*, *S. ventriculi* and *P. aeruginosa* Antifungal: *A. niger*, *C. albicans*	
Kandelia obovata	*Talaromyces amestolkiae*	5-Hydroxy-7-methoxy-2-methyl-benzofuran-3-carboxylic acid	Antibacterial: *E. coli*, *S. aureus*, *B. subtilis* and *S. epidermidis*	Chen et al. 2016
Ceriops tagal	*Penicillium* sp.	Deoxytalaroflavone	Antibacterial: *S. aureus*	Jin et al. 2013
		7-Hydroxy-Deoxytalaroflavone		
Kandelia candel	*Penicillium aculeatum*	Chromone derivative	Antibacterial: *Salmonella* 2.0	Huang et al. 2017
Sonneratia alba	*Alternaria* sp.	Xanalteric acids I and II	Antibacterial: *S. aureus*	Kjer et al. 2009
Porteresia coarctata	*Penicillium chrysogenum MTCC 5108*	(3, 1′-didehydro-3[2″ (3‴, 3‴;-dimethyl-prop-2-enyl)-3″-indolylmethylene]-6-Mepipera-zine-2, 5-dione)	Antibacterial: *Vibrio cholera*	Devi et al. 2012
Pongamia pinnata	*Nigrospora sp. MA75*	Griseophenone C	Antibacterial: MRSA, *E. coli*, *P. aeruginosa*, and *P. fluorescens*	Shang et al. 2012
Not specified	*Nigrospora* sp.	4-Deoxybostrycin and its derivative Nigrosporin	Antibacterial: (MDR) *Mycobacterium tuberculosis*	Wang et al. 2013

In summary, all these studies show that fungal endophytes isolated from mangroves possess high potential for the discovery of new drugs that may serve as antifungals, antimicrobials, and anticarcinogens. In Puerto Rico, reports about mangrove-associated fungi are few; although they include information about foliar taxa they present no evidence of secondary metabolite

production (Nieves-Rivera et al. 2002; Nieves-Rivera 2005). Thus, continue to develop research in this area is warranted to explore endophytes taxa and their properties. The research presented here is focused on selected endophytic fungi isolated from the black mangrove (*Avicennia germinans*) and their effect in the growth of selected bacterial and yeasts pathogens.

1.3 Goals

1.3.1 Hypothesis

1. If fungal endophytes showing antimicrobial activity have been reported for a myriad of plants (including mangroves), then fungal endophytes from the black mangrove *Avicennia germinans* in Puerto Rico should also produce extracellular secondary metabolites with antimicrobial potential.

1.3.2 Objectives

1. Isolate and identify fungal endophytes from the black mangrove *Avicennia germinans.*

2. Determine if the secondary metabolites produced by selected endophytes from *A. germinans* possess antibacterial or antifungal activity.

Chapter 2. Identification of cultivable fungi associated with the black mangrove *Avicennia germinans*.

2.1 Introduction

Avicennia germinans, commonly known as black mangrove for the color of the trunk and heartwood, is a tree that grows in tropical and subtropical countries around the world. These shrubs are founded in coastal zones and swamps, where the seawater reaches, providing the necessary high salinity concentrations for its growth (Andreu et al. 2010; Nieves-Rivera et al. 2002). *A. germinans* growth fluctuates between three and twelve meters according to environmental conditions (Nieves-Rivera et al. 2002). Its seeds are enclosed in a fruit where the cotyledon and root are already germinating. When the seeds fall, they produce vertical roots called pneumatophores. This specialized root allows oxygen acquisition necessary in this environment due to water inundations. Leaves are pointed and oval shaped with a green topside and greyish underneath. They can grow from 2 to 3 inches long and possess specialize glandules for salt excretion (Andreu et al. 2010; Lugo and Snedaker 1974; Nieves-Rivera et al. 2002).

In Cabo Rojo, Puerto Rico, this species can be found in various territories of the National Wildlife Refuge between red mangroves (*Rhizophora mangle*), found in standing waters, and the white mangrove (*Laguncularia racemosa*), a more highland tree (FWS 2010; Lugo and Snedaker 1974). This mangrove forms part of an ecosystem that harbors an endless number of organisms including plants, birds, fishes, bacteria, archaea and fungi, among others (Kathiresan and Bingham 2001). Fungal studies associated with this mangrove are very limited.

In Puerto Rico, there is one study of fungi associated with *A. germinans* leaves. Nieves and colleagues (2002) found a planthopper (*Petrusa marginata*) on *A. germinans*, which excreted sugary honeydew on which the fungus *Asteridiella sepulta* grew, being considered a foliar fungus of the plant. Other studies have reported fungi associated with *A. germinans* but, to our knowledge,

only two studies are from endophytes including one in Panama (Gilbert et al. 2002) (no identification of isolates) and the other in Jamaica (Facey et al. 2016) reporting the fungus *Aspergillus ustus*. Some of the other reports of non-endophytes associated with *A. germ*inans were in different western countries. In Panama, *Phellinus swieteniae, Trichaptum biforme,* and *Ceriporia alachuana* were isolated from *A. germinans* (Gilbert and Sousa 2002). In Mexico, six species where isolated from the rhizosphere of the mangrove: *Aspergillus* sp., *Blastomyces* sp., *Fusarium* sp., *Peniciliium* sp., *Acremonium* sp. (Martin-Rodríguez et al. 2014), and *Aspergillus niger* (Vazquez et al. 2000). Moreover, in Florida, Olexa and Freeman (1975) described *Phyllosticta hibiscina* as a fungal pathogen causing necrosis in *A. germinans*, while *Nigrospora sphaerica* was found causing chlorosis in the plant leaves (Osorio et al. 2014). In Texas and Bermudas, *Acremonium* sp., *Aspergillus flavus, A. niger, Cladosporium cladosporiodes,* and *Nigrospora sphaerica* were reported in leaves and stems (Koehn and Garrison 1981) and *Halosarpheia fibrosa* was found associated with mangroves (Kohlmeyer and Kohlmeyer 1977). Thus, the relatively few studies on *A. germinans* fungal endophytes communities makes this study exceptional.

2.2 Materials and Methods

2.2.1 Sampling area

Mangrove leaves and seeds were collected randomly from Bahía Salinas at Cabo Rojo, Puerto Rico (17°56'27.8"N and 67°11'32.86"W) [Appendix A]. This area is part of the Boquerón National Wildlife Refuge making it a unique and irreplaceable environment (Nieves-Rivera et al. 2002) [Appendix B]. Bahia Salinas possess soils of high salinity, poor water availability, and a sandy composition derive from volcanic material. Its annual average precipitation is 36 inches (last 17 years), with a minimum of 15 inches in 1997 and a maximum of 58 inches in 1998. The climate is tempered during the year, with a highest average temperature of 31.6 ºC and a lowest average temperature of 21.6 ºC (NWS 2010).

2.2.2 Leaves and seeds samplings

Samples were collected randomly from healthy *A. germinans* trees found in the road that led to the Cabo Rojo Lighthouse. Seeds and young, mature and almost falling leaves were placed separately in plastic bags and transported to the laboratory for processing during the day. We performed three samplings during the following dates: (i) June 2013 (Bahia Salinas I: BSI), (ii) January 2014 (Bahia Salinas II: BSII), and (iii) August 2015 (Bahia Salinas III: BSIII). In the second and third sampling, precipitation volumes were 25% and 50% below the normal annual average reported for each month respectively (NOAA 2016). Also, Puerto Rico was suffering from a drought crisis during the third sampling where only 2 to 3 inches of precipitation were reported in Cabo Rojo for August 2015 (USDA 2015) [Appendix C].

2.2.3 Leaves and seeds processing

Mangrove leaves and seeds were surface-sterilized with hypochlorite (50%) for one minute and ethanol 70% for 3 minutes to prevent contamination from epiphytic species (Costa et al. 2012; Morell-Rodríguez 2008). We used a sterile hole-puncher to obtain circular segments of the leaves

and seeds (we used the cotyledons as well as the roots inside the seeds). Then, we placed three circular pieces per Petri dish of 4 different media: (i) Potato Dextrose Agar (PDA), (ii) PDA 1.5% NaCl, (iii) PDA 3.5% NaCl, and (iv) an enriched medium prepared with mangrove leaf extract (inoculation in each media were made in triplicates). All media were autoclaved and served into petri dishes inside the hood to avoid contamination. The medium containing the leaf extract was prepared using a blender and its salinity was measured with a portable refractometer (salinity of leaves medium: 4%). We added 10 grams of black mangrove leaves in 1L of distilled water. The mixture was filtered with Whatman paper grade 1. Finally, 6.5 grams of agar were added and the medium was autoclaved. All media were supplemented with antibiotics (penicillin and streptomycin; 5g per liter). After inoculation, the plates were incubated at 25.0 °C until visible fungal growth was observed emerging from the borders of the segments. For one month, each plate was screened daily for endophytes using a dissection microscope to assure that the isolate was not from the external tissue of the leaves, cotyledons or roots.

2.2.4 Macroscopic and microscopic culture-dependent identification method

Macroscopic and microscopic observations were recorded for the identification of fungal isolates. Wet mount slides and moist chambers were prepared for observation under a compound microscope. Taxonomic keys were used for the identification of the specimens where different culture media were used according to each key [*Nigrospora* (Ellis 1971), *Aspergillus* (Klich 2002), and *Penicillium* (Pitt 1988)]. Isolates highly difficult to identify were subjected to molecular analysis.

2.2.5 Molecular identification using the ITS gene region

DNA extraction was performed using grinding and Cetyl Trimethyl-Ammonium Bromide (CTAB) chemical method (Doyle and Doyle 1987). The mycelium was transferred to a sterile mortar (pre-cooled at -80°C) and grounded to a fine powder (this is necessary to break the strong chitin fungal cell walls). Then, the sample was transferred to a microtube to continue with the CTAB extraction protocol. Approximately 500µL of CTAB were added to each sample to promote cell lysis. Then, we use 500µL of chloroform to extract the DNA and performed the precipitation with one volume of 100% isopropanol and washed with 100µL of 70% ethanol. Finally, each sample was resuspended in 50µL of TE buffer (1/10; 10mM Tris-HCl and 0.1mM EDTA) and stored at -20°C.

PCR amplification of the internal transcribed spacer (ITS) region was carried out using ITS4 [5'-TCCTCCGCTTATTGATATGC-3'] and ITS5 [5'GGAAGTAAAAGTCGTAACAAGG-3'] primers (White et al. 1990). The ITS is a portion of DNA localized between the small and large subunits of ribosomal RNA (rRNA) genes. Eukaryotes possess two ITS regions: (i) ITS1, located between 18S and 5.8S rRNA genes and (ii) ITS2, located between 5.8S and 26S rRNA genes (in plants) (Bellemain et al. 2010). This region has been considered as a universal barcode for fungal specimens (Schoch et al. 2012). Genomic DNA of 1:10 and 1:20 dilutions were used as template with 0.6mM of ITS primers, 0.8x PCR green buffer, 3nM $MgCl_2$, 0.16 mM dNTPs and 5U Taq polymerase for a final volume of 25 µL per reaction. The following thermal parameters were used: 95°C: 5min (1x); 94.0 °C: 1:30min, 52.5 °C: 30sec, 72.0 °C: 1min (35x), and 72.0 °C: 5min (1x). Sanger sequencing was conducted to identify the fungal genera from each specimen at the High-Throughput Genomics Unit of the University of Washington, Seattle, WA (UW-htSEQ). We compared our sequences with the ones in the National

Center for Biotechnology Information (NCBI) data base, using the program BLASTn®. This led to a more accurate fungal identification.

2.2.6 Identification of fungi associated with the black mangrove *Avicennia germinans*: Sequence Analysis

Once the sequence analysis was ready, we downloaded our sequences from the UW-htSEQ webpage. Then, we edited them using the program BioEdit Sequence Analysis Editor (Hall 1999) to eliminated unreliable nucleotides (noise and nucleotides with a query coverage below 20%). To perform the identification, the program BLASTn® (Altschul et al. 1990), which align and compare our sequences with the ones in the GenBank® (Benson et al. 2013) of the National Center for Biotechnology Information (NCBI) was used. For this analysis, a comparison using reference sequences from the GenBank was made. This led us to a list of similar sequences to ours, from which we selected the one with the higher percent of query coverage and identification. Having our sequences identified we proceeded to perform a phylogenetic analysis using the program CLC Main Workbench 7.6.4. The specific steps followed were: (i) create alignment, (ii) select the sequences to be analyzed, (iii) select an alignment (very accurate), and (iv) create a phylogenetic tree (Neighbor-Joining method; nucleotide distance measure: Jukes-Cantor; Boostrap: 1000). Other parameters included: (i) gap open cost: 10.0, (ii) gap extension cost: 1.0, and (iii) gap cost: as any other (Quiagen 2016). This program performed multiple sequence alignments between our sequences and the ones retrieved from the data base, creating a phylogenetic tree that shows the evolutionary relationships among them. Differences between taxa can be evaluated with the boostrap percent showed in the tree (Figure 2.12).

2.3 Results

2.3.1 Fungal endophytes from *A. germinans*.

A total of 104 specimens within 20 genera associated with *A. germinans* leaves and seeds were identified from culture-dependent methods using molecular, macroscopic and microscopic characteristics [Appendix D]. All isolates were classified by samplings (BSI, BSII, and BSIII; where BS: Bahia Salinas), by culture media [Potato Dextrose Agar (PDA) 0% NaCl, PDA 1.5% NaCl, PDA 3.5% NaCl, and MH 4% salinity (measured)], and isolation source (leaves and seeds) (Table 2.1). In the first sampling (BSI), 54 specimens were isolated within 13 different genera (*Hortaea, Penicillium, Bionectria, Teratosphaeria, Purpureocillium, Acremonium, Cladosporium, Bipolaris, Nigrospora, Aspergillus flavus, Aspergillus clavatus, Aspergillus niger, Wrightoporia, Stereum, Pochonia, Simplicillium,* and *Nemania*), but the final number was reduced to 41 due to contamination by mites. Nevertheless, from samplings BSII and BSIII only 30 endophytes were isolated within 8 genera (*Hortaea, Penicillium, Bionectria, Teratosphaeria, Purpureocillium, Acremonium, Amorosia littoralis,* and *Physalospora*), and 20 isolates also within 8 genera (*Hortaea, Penicillium, Bionectria, Teratosphaeria, Purpureocillium, Acremonium, Cladosporium, Aspergillus,* and *Bipolaris*), respectively. The most abundant genera of our isolates were *Hortaea* (76.3%), *Penicillium* (50.5%), and *Cladosporium* (35%). These three genera were the only ones present in the three samplings (BSI, BSII and BSIII), while 3 genera were present in two of the samplings and the other 14 were only on one of the samplings (Figure 2.1). Similar to the results on the different samplings, *Hortaea, Penicillium,* and *Cladosporium* were the only genera present in all culture media (Figure 2.2 to 2.5). Nevertheless, the most number of isolates was reported for PDA 1.5% NaCl and PDA 3.5% NaCl followed by PDA (no salt added) and the leaves medium in descending order. Differences among genera were also found; *Stereum* and *Simplicillum* were only present in seeds and *Nemania, Wrightoporia, Aspergillus niger, A. flavus, A. clavatus, Pochonia,*

Bipolaris, *Amorosia* and *Physalospora* were only present in the leaves. The remaining genera were found in both leaves and seeds (Figure 2.6).

In summary, a total of 91 fungi were isolated during three samplings from the leaves and seeds of the black mangrove *Avicennia germinans* in four different culture media. The majority of the genera were isolated in the first sampling, from the leaves and on 1.5% salinity PDA medium.

Table 2.1: Fungal isolates from *A. germinans*. Isolates were retrieved from three samplings (BSI, BSII and BSIII), on four different culture media [PDA, PDA (1.5% NaCl), PDA (3.5% NaCl) and leaves medium] from seeds and leaves.

	Leaves			
	PDA	**PDA (1.5% NaCl)**	**PDA (3.5 % NaCl)**	**Leaves Medium**
BSI	*Nigrospora* sp. *Aspergillus niger* *Aspergillus flavus* *Penicillium* sp. *Wrightoporia* sp. *Hortaea* sp.	*Nigrospora* sp. *Nemania* sp. *Pochonia* sp.	*Hortaea* sp. *Penicillium* sp.	*Aspergillus clavatus* *Bionectria* sp. *Hortaea* sp.
BSII	*Penicillium* sp.	*Teratosphaeria* sp. *Purpureocillium* sp. *Physalospora* sp. *Penicillium* sp. *Acremonium* sp. *Amorosia* sp.	*Teratosphaeria* sp. *Penicillium* sp. *Hortaea* sp. *Amorosia* sp.	*Bionectria* sp. *Hortaea* sp. *Penicillium* sp.
BSIII	*Bipolaris* sp. *Penicillium* sp. *Teratosphaeria* sp.	*Cladosporium* sp. *Teratosphaeria* sp. *Penicillium* sp.	*Cladosporium* sp. *Hortaea* sp.	N/A*
	Seeds			
BSI	*Nigrospora* sp. *Stereum* sp. *Hortaea* sp.	*Hortaea* sp. *Simplicillium* sp. *Acremonium* sp. *Bionectria* sp.	*Penicillium* sp. *Hortaea* sp.	N/A*
BSII	*Purpureocillium* sp.	N/A*	*Purpureocillium* sp. *Penicillium* sp.	N/A*
BSIII	N/A*	*Cladosporium* sp. *Penicillium* sp. *Bionectria* sp.	*Cladosporium* sp.	*Aspergillus* sp. *Purpureocillium* sp. *Cladosporium* sp.

*N/A= there was no visible growth of endophytes.

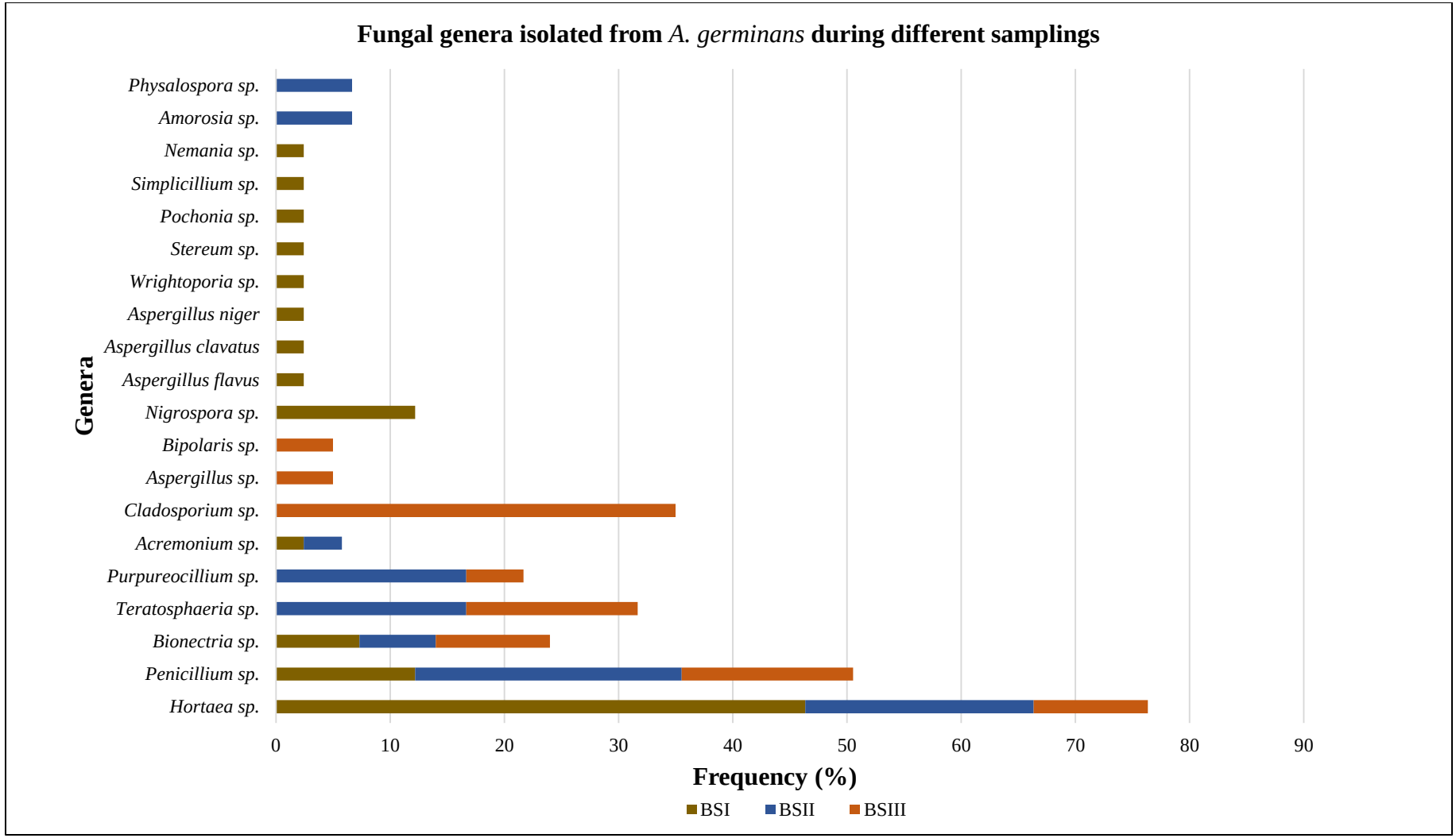

Figure 2.1: Frequency of fungal genera isolated from the black mangrove *Avicennia germinans* from samplings: BSI, BSII, and BSIII.

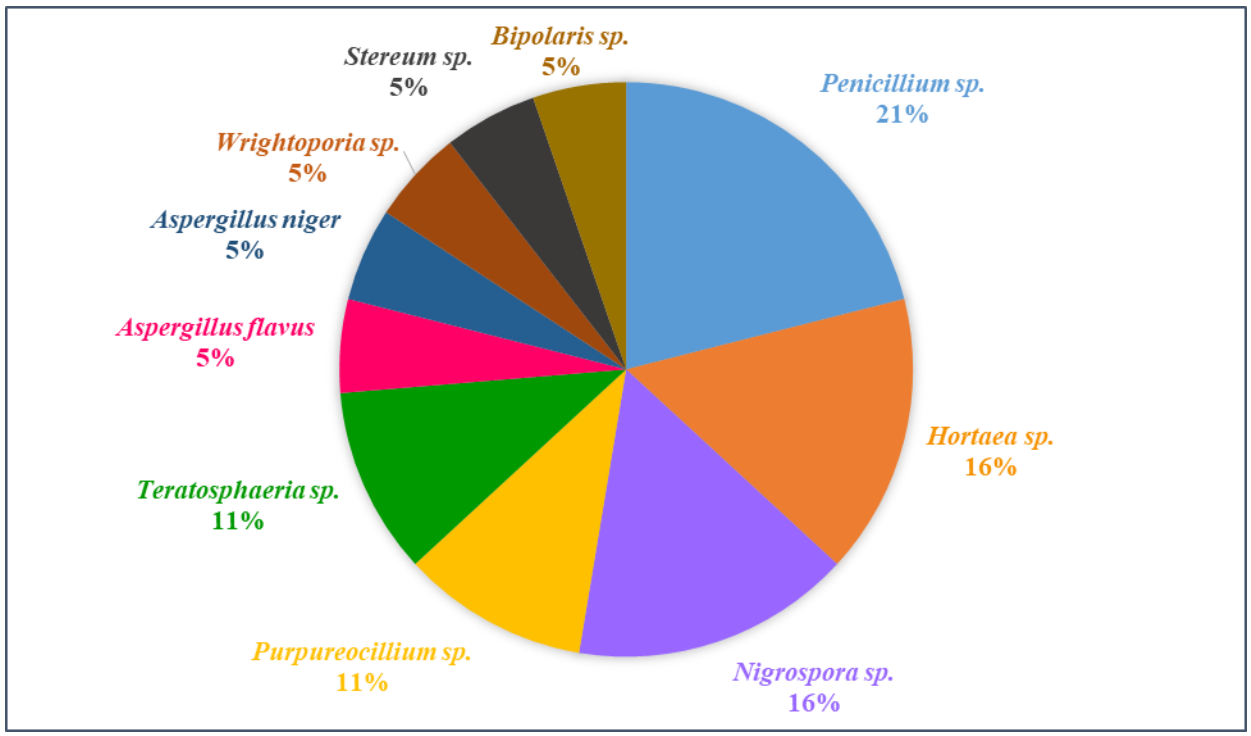

Figure 2.2: Fungal genera isolated from *A. germinans* in potato dextrose agar (PDA) (n=19).

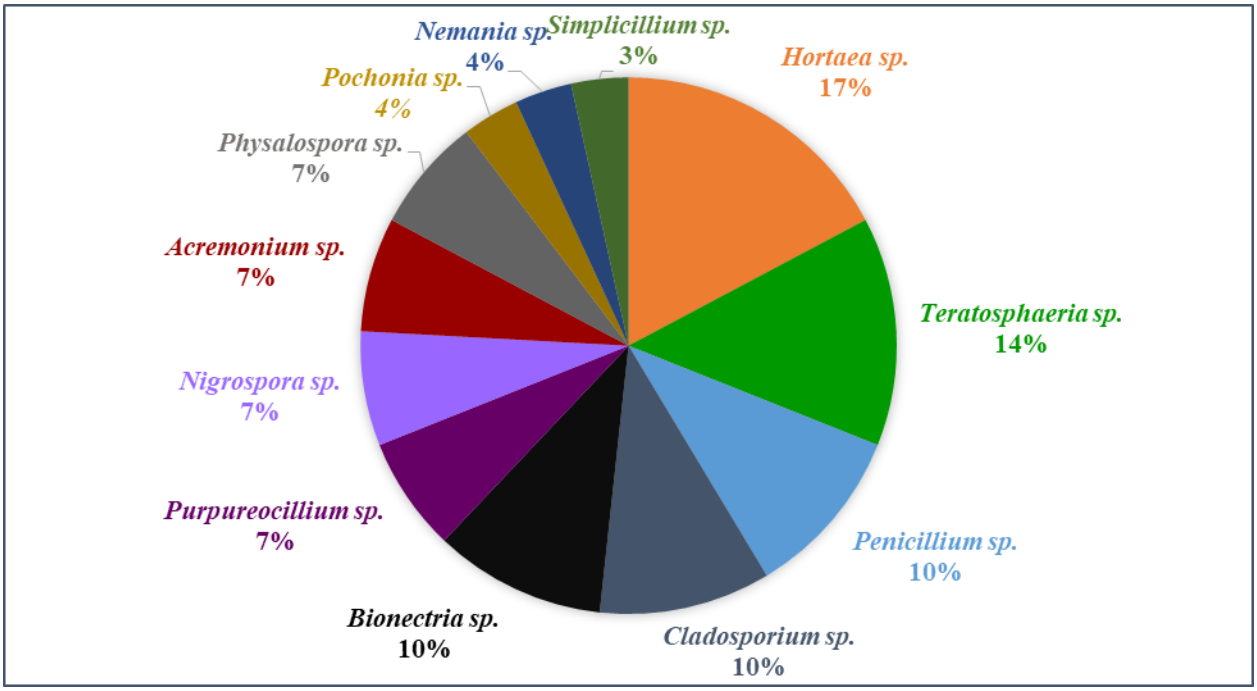

Figure 2.3: Fungal genera isolated from *A. germinans* in PDA 1.5% salinity (n=29).

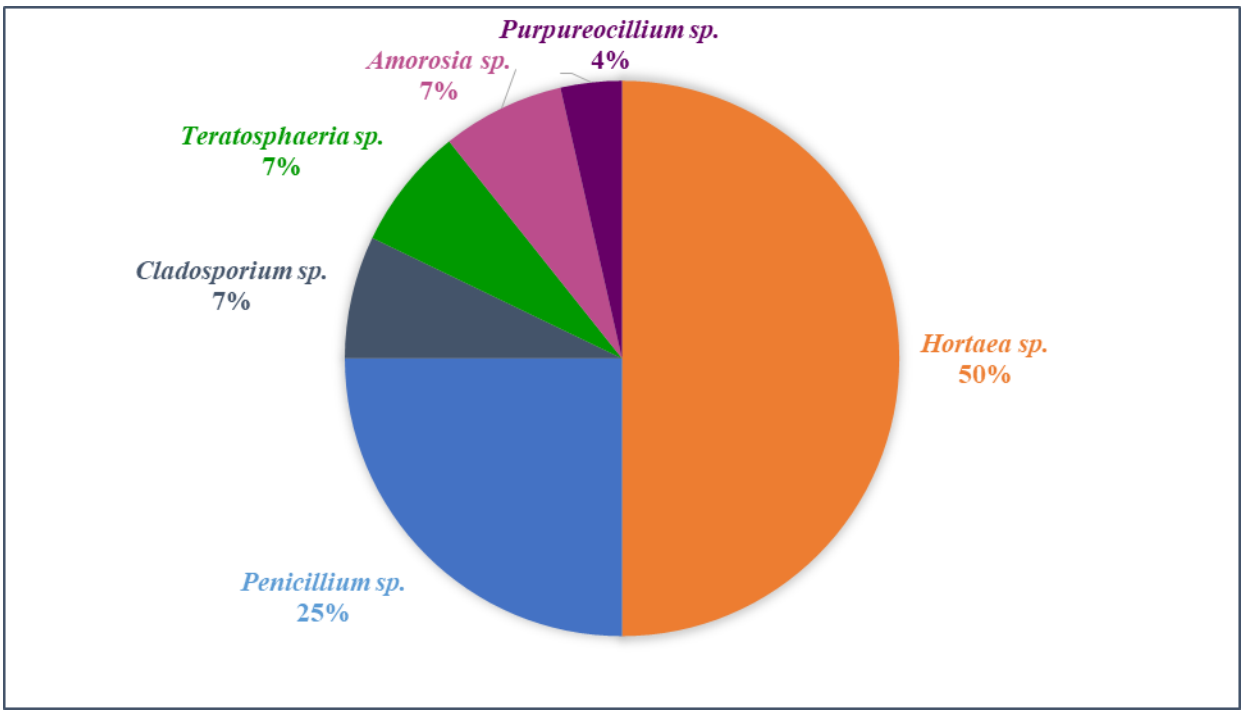

Figure 2.4: Fungal genera isolated from *A. germinans* in PDA 3.5% salinity (n=28).

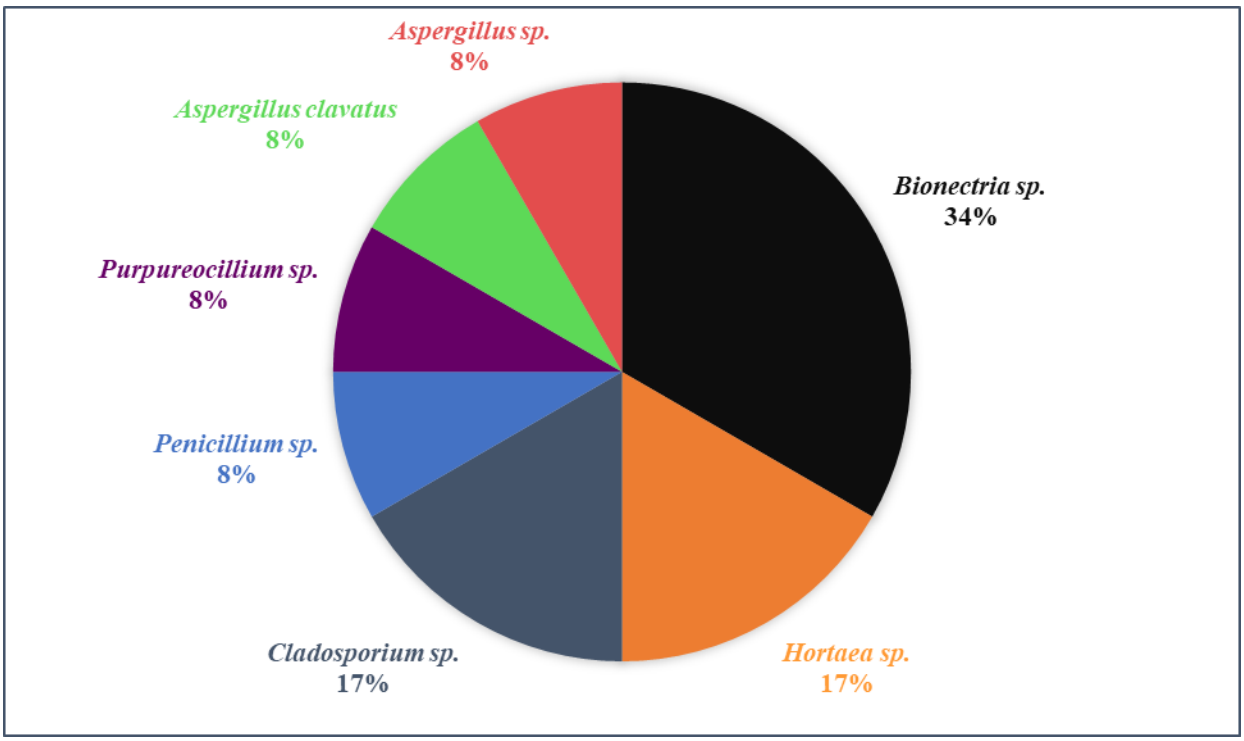

Figure 2.5: Fungal genera isolated from *A. germinans* in Leaves Media (4% salinity) (n=12).

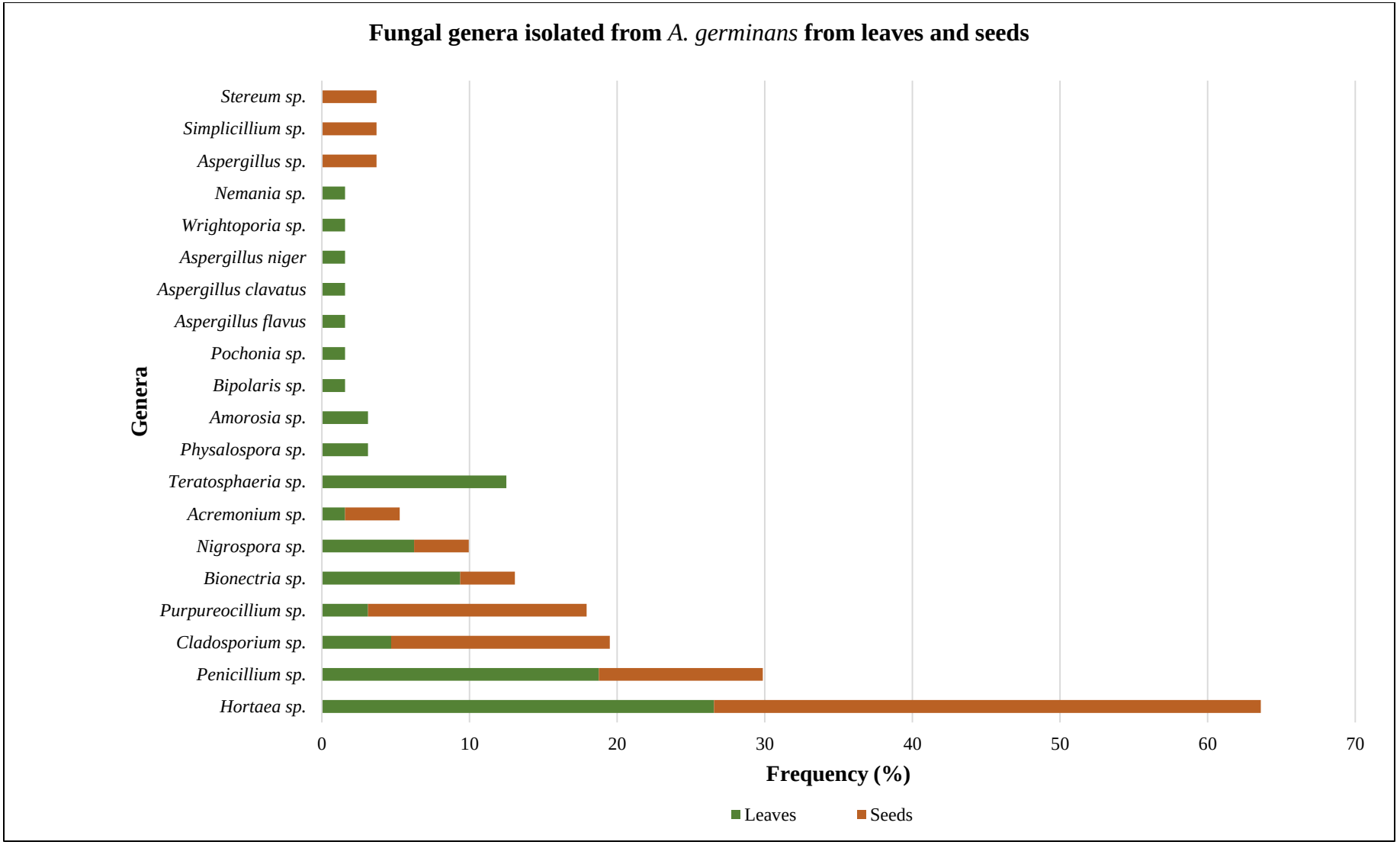

Figure 2.6: Frequency of fungal genera isolates from the black mangrove *Avicennia germinans* from leaves (n=64) and seeds (n=27).

2.3.2 Macroscopic and microscopic identification of *A. germinans* endophytes.

From all the isolates, only three genera, including five species, were identified at the species level using microscopic and macroscopic characteristics: (i) *Aspergillus flavus,* (ii) *Aspergillus clavatus,* (iii) *Aspergillus niger,* (iv) *Penicillium waksmanii,* and (v) *Nigrospora (Khuskia oryzae).* Macroscopic and microscopic descriptions for each specimen are listed below. For these methods, different culture media were used according to each taxonomic key (Ellis 1971; Klich 2002; Pitt 1991).

2.3.2.1 *Aspergillus clavatus* (BSI(MH)-HJ-2(1))*:*

Macroscopic description: CYA25 medium: colony diameter: ~ 46 mm; adverse: greyish blue; reverse: incrusted in the agar; mycelium: umbonated in the middle and greyish blue; exudate: a little; soluble pigment: present. CYA37 medium: colony diameter: ~18.1 mm; adverse: blue with white borders; reverse: full of folds, dark brown center with white borders; mycelium: elevated in the center, turquoise blue; exudate: a little; soluble pigment: present. CY20S medium: colony diameter: ~48.5 mm; adverse: greyish blue; reverse: pale yellow with folds; mycelium: folds in the center, light greyish blue halo near the center; exudate: no present; soluble pigment: present. MEA medium: colony diameter: ~31.5 mm; adverse: whitish and blue; reverse: whitish without folds; mycelium: white and blue; exudate: no present; soluble pigment: no present. CZ medium: colony diameter: ~25 mm; adverse: greyish blue; reverse: pale yellow without folds; mycelium: greyish blue and incrusted in agar (beige-whitish); exudate: no present; soluble pigment: no present. Microscopic description: uniseriate. STIPE: length: could not be clearly seen; width: ~ 21 µm; surface texture: smooth. VESICLE: diameter: 177x55 µm; shape: piriform. CONIDIA: length: 3.33 µm; shape: elliptic; surface texture: smooth (Figure 2.7).

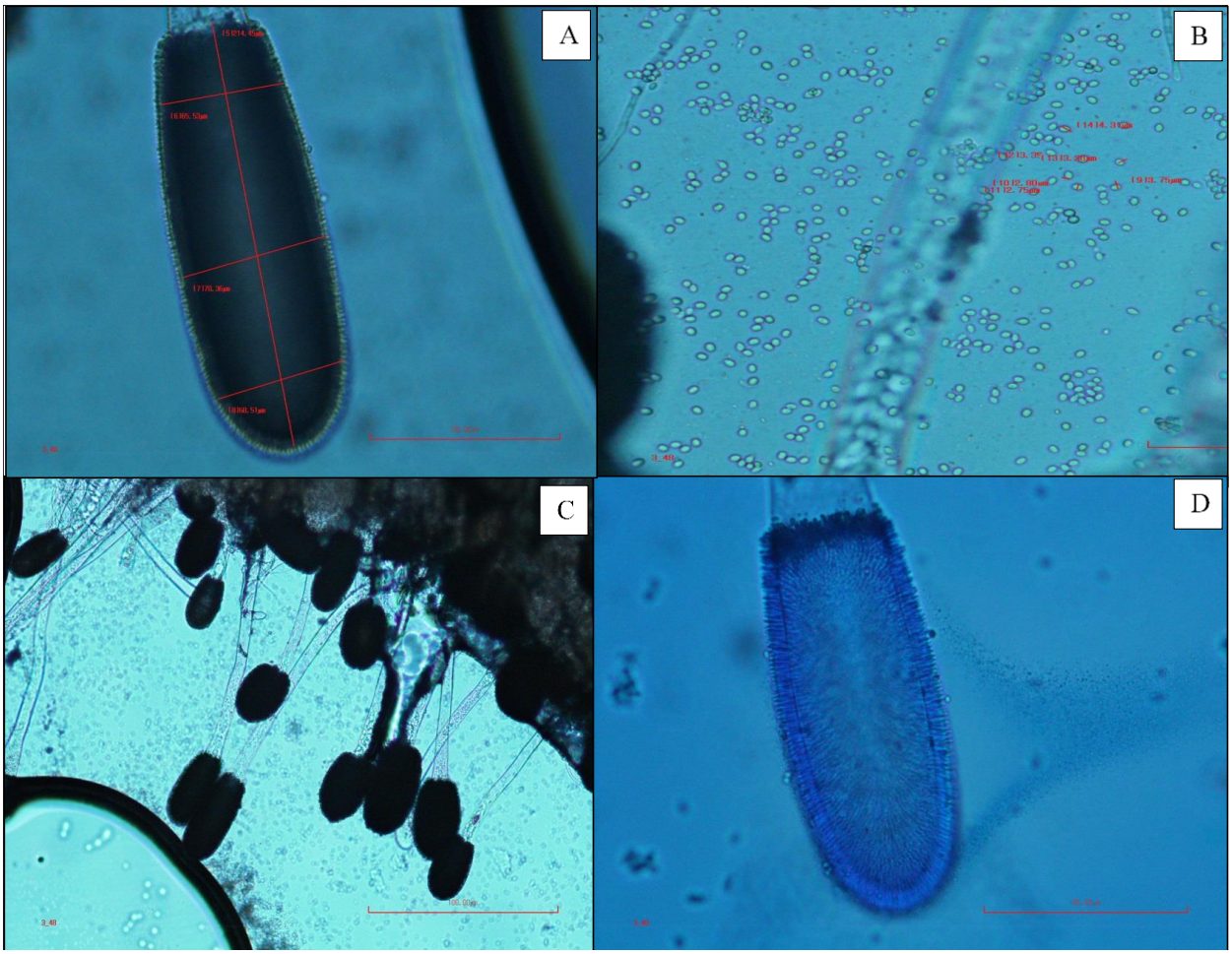

Figure 2.7: *Aspergillus clavatus* (BSI(MH)-HJ-2(1)) structures. Measurements from vesicles, conidia and conidiophore were obtained for identification. Vesicles (A and D), conidia (B) and conidiophore (C). Measures of the vesicle (A) and conidia (B) are shown. Scale: 100µm.

2.3.2.2 *Aspergillus flavus* (BSI-HV-2(1))

Macroscopic description: CYA25 medium: colony diameter: ~47 mm; adverse: green; reverse: pale yellow with irregular folds in the center that became radial in the edges; mycelium: green; exudate: no present; soluble pigment: no present. CYA37 medium: colony diameter: ~46 mm; adverse: greenish; reverse: brown in the center with folds, lighter color in the edges; mycelium: olive-green; exudate: no present; soluble pigment: no present. CY20S medium: colony diameter: ~50.25 mm; adverse: green; reverse: pale yellow with folds; mycelium: green; exudate:

no present; soluble pigment: no present. MEA medium: colony diameter: ~44 mm; adverse: greenish; reverse: no folds, green and whitish; mycelium: green and elevated; exudate: no present; soluble pigment: no present. CZ medium: colony diameter: ~34 mm; adverse: green; reverse: dark brown with folds; mycelium: dark green; exudate: present; soluble pigment: no present. Microscopic description: biseriate. STIPE: length: ~486.5 µm; width: ~6.82 µm; surface texture: a little rough. VESICLE: diameter: ~22 µm; shape: circular-globose. CONIDIA: length: ~4 µm; shape: circular-globose; surface texture: smooth to lightly rough (Figure 2.8).

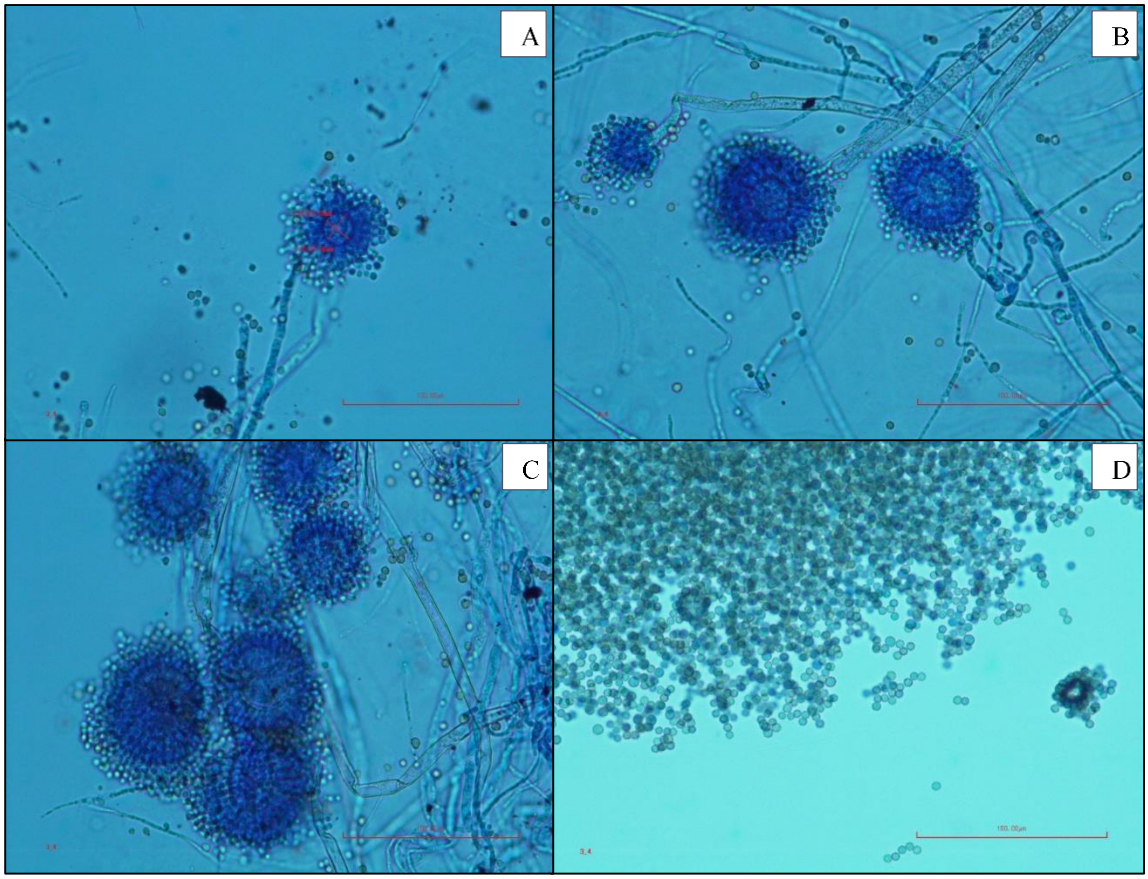

Figure 2.8: *Aspergillus flavus* (BSI-HV-2(1)) structures. Measurements from conidiophores and conidia were obtained for identification. Conidiophores (A-C) and conidia (D). Scale: 100µm.

2.3.2.3 *Aspergillus niger (BSI- HV-1(1)):*

Macroscopic description: CYA25 medium: colony diameter: ~ 42 mm; adverse: dark brown; reverse: pale yellow with folds; mycelium: dark brown; exudate: no present; soluble pigment: no present. CYA37 medium: colony diameter: ~ 37.25 mm; adverse: brown; reverse: pale yellow with folds; mycelium: brown; exudate: no present; soluble pigment: no present. CY20S medium: colony diameter: ~ 50.25 mm; adverse: brown; reverse: pale yellow with folds; mycelium: brown; exudate: no present; soluble pigment: no present. MEA medium: colony diameter: ~ 25.17 mm; adverse: brown; reverse: various folds; mycelium: dark brown almost black; exudate: no present; soluble pigment: no present. CZ medium: colony diameter: ~ 36 mm; adverse: dark brown; reverse: various folds; mycelium: dark brown; exudate: no present; soluble pigment: no present. Microscopic description: biseriate. STIPE: length: ~ 373 µm; width: ~ 19.2 µm; surface texture: smooth. VESICLE: diameter: 41 µm; shape: circular. CONIDIA: length: ~4.24 µm; shape: circular-globose; surface texture: rough (Figure 2.9).

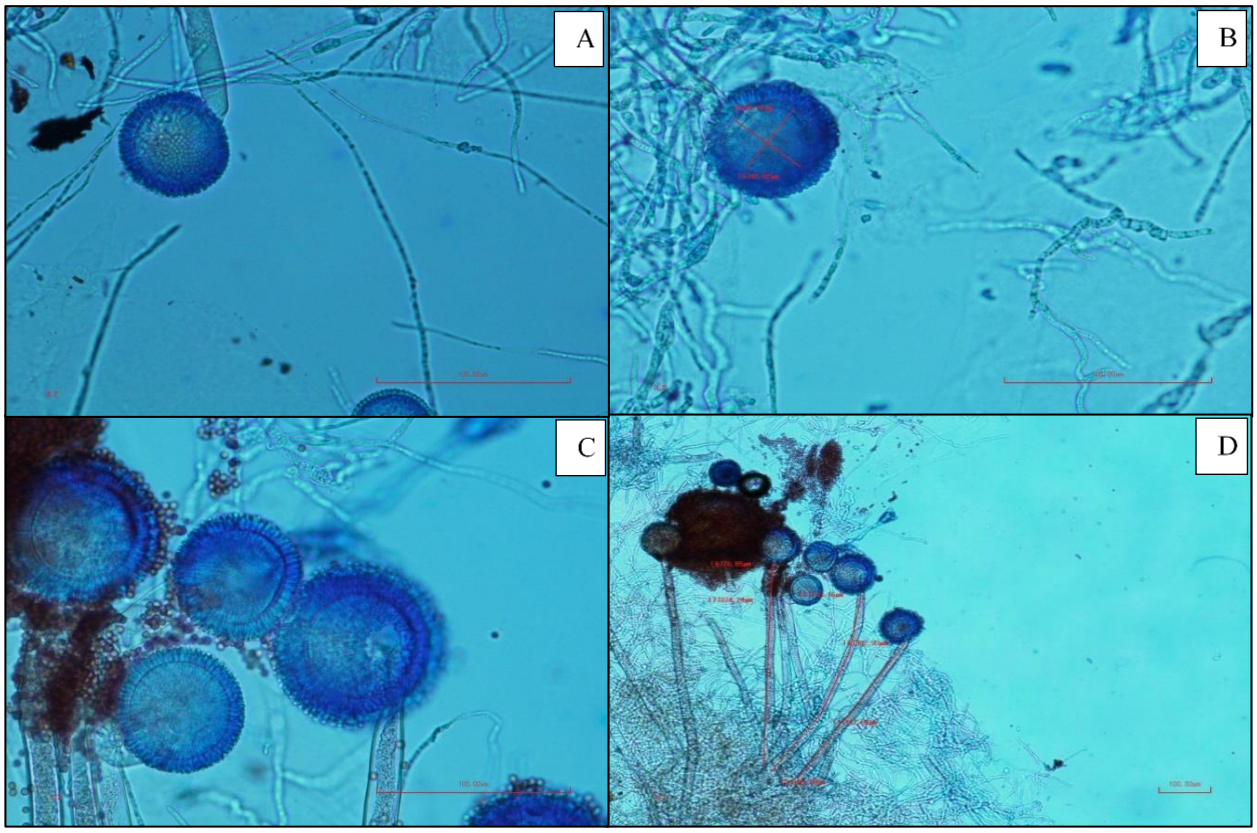

Figure 2.9: *Aspergillus niger* structures. Measurements from vesicles and conidiophores were obtained for identification. Vesicles (A-C) and conidiophores (D). Measures of the vesicle (B) and conidiophores (D) are shown. Scale: 100µm.

2.3.2.4 *Penicillium waksmanii* (BSI-HV-1(2))*:*

Macroscopic description: CYA25 medium: colony diameter: ~28.25 mm; adverse: greenish-blue; reverse: pale yellow in borders and orange in center with folds; mycelium: umbonate in center with folds, velvety; exudate: present in large amount, amber color; soluble pigment: present, yellowish. CYA37 medium: colony diameter: ~16 mm; adverse: greenish-blue with white borders; reverse: pale yellow in borders and orange in center with folds; mycelium: umbonate in center with folds; exudate: present in large amount, amber color; soluble pigment: present, yellowish. CYA5 medium: colony diameter: ~3-4 mm; adverse: white; reverse: pale yellow. MEA medium: colony diameter: ~20.6 mm; adverse: greenish-blue with grayish zones,

beige under the agar; reverse: pale yellow; mycelium: fasciculate; exudate: no present; soluble pigment: no present. G25N medium: colony diameter: ~20.5 mm; adverse: greenish-blue with grayish zones; reverse: pale yellow in borders and orange in center with folds; mycelium: velvety, umbonate in center; exudate: no present; soluble pigment: present, yellow-orange. Microscopic description: STIPE: length: ~60.4 µm. PENICILLI: biverticilated, irregular terminal; RAMI: no present. METULA: ~2-4; length: ~12 µm, smooth, no vesiculated. PHIALIDES: ~3-5; length: ~7.1 µm. CONIDIA: 5 surface texture: a little rough. VESICLE: diameter: ~22 µm; shape: circular-globose. CONIDIA: length: ~5 µm, spherical (Figure 2.10).

2.3.2.5 *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1))*:*

Macroscopic description: adverse: grayish with black and white areas; reverse: black with white areas, black dots; mycelium: covered the entire petri dish; exudate: no present; soluble pigment: could not be seen. Microscopic description: CONIDIA: length: ~11.1 to 17.0 µm (Figure 2.11).

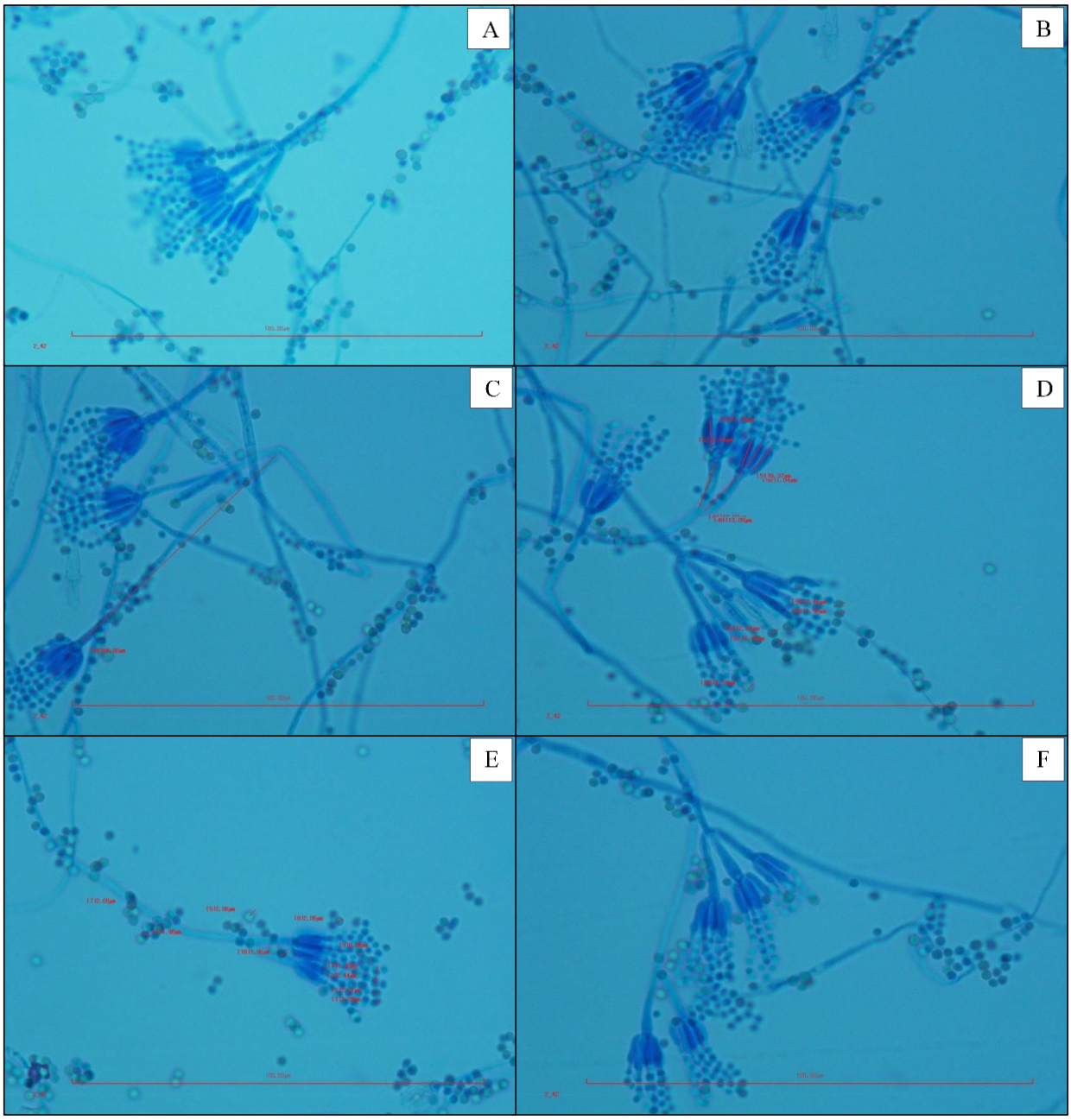

Figure 2.10: *Penicillium waksmanii* (BSI-HV-1(2)) structures. Measurements from conidiophores, metulae and phialides were obtained for identification. Conidiophores (A, B, C and F), metulae and phialides (D), and conidia (E). Measures of the conidiophore (C), methula and phialides (D) and conidias (E) are shown. Scale: 100µm.

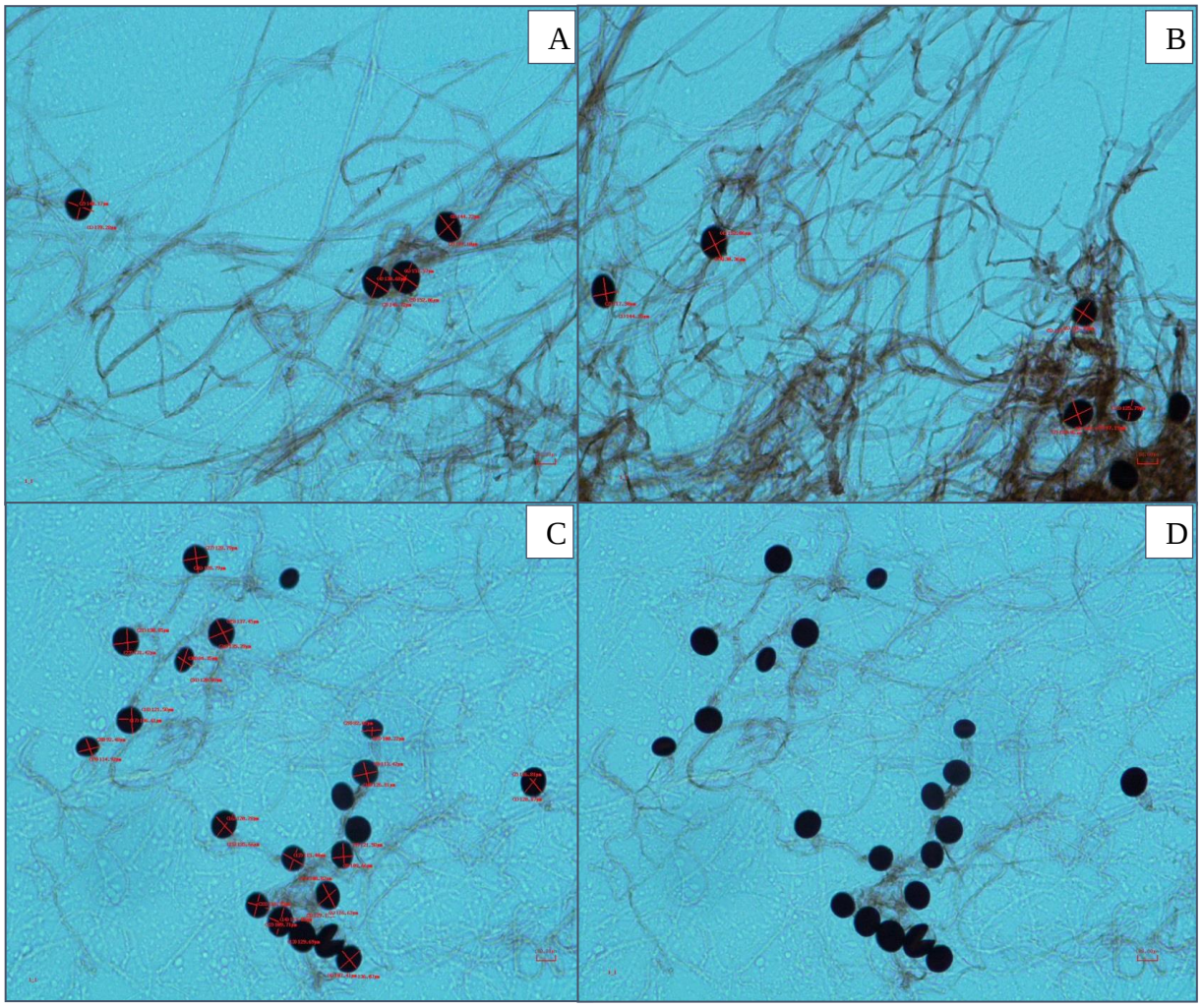

Figure 2.11: *Nigrospora* (*Khuskia oryzae*) (BSI(1.5)-HJ-2(1)). Measurements from conidia were obtained for identification. Conidia (A-D). Measures of conidias are shown (A-C). Scale: 100µm.

2.3.2 Phylogenetic analysis of black mangrove fungi

We performed this analysis to have a more consistent view of the different groups of fungal endophytes associated with *A. germinans*. The results showed 4 different classes between the isolates: (i) Agaromycetes (2 specimens), (ii) Dothidiomycetes (43 specimens), (iii) Eurotiomycetes (19 specimens) and (iv) Sordariomycetes (27 specimens). In our data, only two basidiomycetes, *Stereum* and *Wrightoporia*, were associated with *A. germinans* (Figure 2.12).

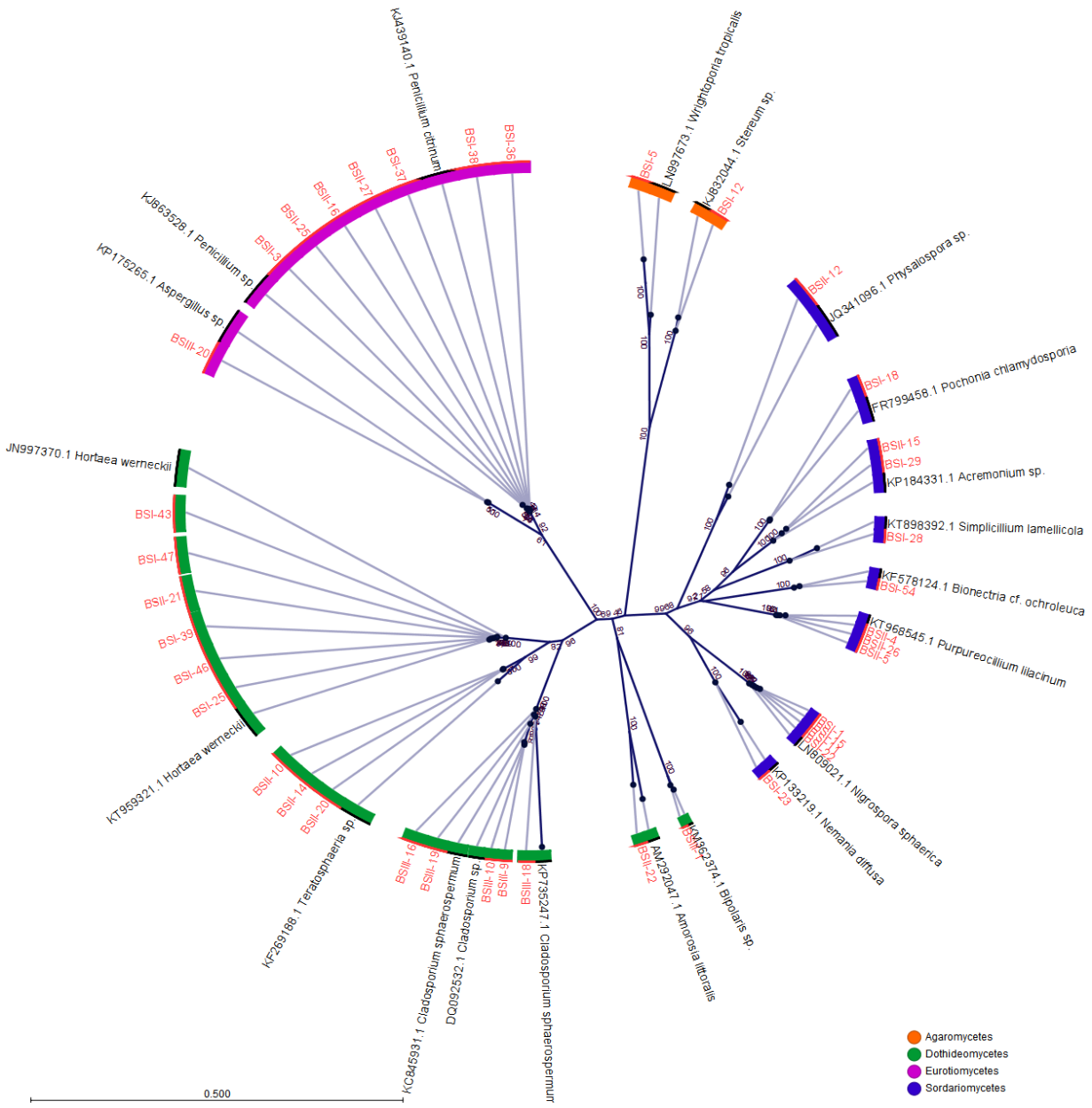

Figure 2.12: Phylogenetic Neighbor-joining tree based on ITS region gene sequences isolated from the black mangrove *A. germinans*. Isolates belonging to the same class are colored: Agaromycetes (orange), Dothidiomycetes (green), Sordariomycetes (blue), and Eurotiomycetes (purple). The ITS region is composed for approximately 500-600 base pairs for fungi. The numbers at the nodes indicate bootstrap support values (>50%).

2.4 Discussion and Conclusion

Fungal endophytes represent an important community in many environments. They can help their host plant by providing resistance to disease and herbivores, and also reducing stress to abiotic factors. Endophytes, especially associated with marine environments, have been studied due to their capacity to produce medicinal compounds (Rodríguez et al. 2008). Also, new species have been found in strict symbiosis relationships with a host plant, rising awareness about the myriad of fungal species that are waiting to be discovered. Given this, our first objective was to isolate and identify the fungal endophytic community associated with seeds and leaves of the black mangrove *Avicennia germinans*.

Since our goal was not to compare the fungal diversity between seasons, we isolated *A. germinans* specimens in three random samplings. To our surprise, we found different amounts of fungal isolates during each sampling. This could have been caused by precipitation and drought changes during those times; given that for the second and third sampling the precipitation was 25% and 50% below the normal average for this zone, respectively. Also, during the summer and fall of 2015, Puerto Rico was under a severe drought (USDA 2015). This correlates with studies showing that the amount of precipitation affects the abundance of endophytes (U'Ren et al. 2012). Although other studies have shown that endophytes confer abiotic stress resistance like high salinity, drought and extreme temperatures (Kandalepas et al. 2015), this does not specifically means that endophyte diversity and abundance remain unaffected as shown in our data.

Fungal endophytes were isolated in different culture media at different salinity concentrations. We expected to retrieve more specimens in the leaf medium since it contains the principal compounds of their natural habitat, but more endophytes were isolated in PDA medium with 1.5% salinity and the lowest from the leaf medium. However, there was almost no difference

between that medium and the one containing 3.5% salinity (29 and 28 isolates, respectively). A possible explanation for these results is that the leaf medium did not possess the nutrients that are available in PDA medium, thus reducing the amount of specimens able to grow in it. Although a study in Panama demonstrated that the high salinity of *A. germinans* leaves, compared with other mangroves, *Rhizophora mangle* and *Laguncularia racemosa*, contributed to a low recover of specimens (Gilbert et al. 2002), in our study, more isolates were obtained from higher salinity media. Among the three more common genera associated with *A. germinans*, *Aspergillus* and *Cladosporium* have been reported as two of the most common endophytes in mangroves (Liu et al. 2007). Even though we were able to identify to species 5 of the 20 genera by macro and microscopic characteristics, there was some degree of uncertainty with *Penicillium waksmanii* since we found some discrepancies with the measurements described in the literature. To address this singularity, multiple measurements should be performed from different wet mounts of the organism.

Endophytes were not only isolated from the leaves but also from the seeds of the black mangrove. The purpose of this was to see if there was any species associated specifically with the seed that could be vertically transferred in this plant. Only *Stereum* and *Simplicillium* were found solely in *A. germinans* seeds. No conclusions about a specific association of these two species with a vertical transmission in this mangrove can be reached at this point; more studies must be performed since only one isolate was recovered from some species in a sole sample.

Molecular and phylogenetic studies showed only four phylogenetic classes present in our study, 89 ascomycetes and 2 basidiomycetes: *Stereum* and *Wrightoporia*, but no relation were found between certain taxa and a specific sampling, media or isolation source. We also need to consider that most of the species isolated appeared only once in all our samples. To have a better

understanding about the diversity and the conditions that may affect the abundance of *A. germinans* endophytes, a more systematic sampling must be made under different conditions such as comparisons between dry and wet seasons and extensive studies on specific parts of the plant. In general, all fungal isolates have been reported as endophytes in other mangroves and non-marine plants [Appendix E]. However, to the best of our knowledge, some genera are reported as mangrove endophytes for the first time in this study: *Physalospora, Pochonia, Simplicillium, Stereum, Teratosphaeria and Wrightoporia.* Some of these genera have been isolated from mangroves or marine environments but not as endophytes. *Physalospora* (Olexa 1976) and *Stereum* (Cavalcanti et al. 2016) were found associated to the red mangrove *Rhizophora mangle* in Brazil, and *Pochonia* was found on mangrove sediments in China (Li, Wang, 2016). Moreover, there are no known reports of fungal endophytes associated to the black mangrove in Puerto Rico, thus this represents the first report of fungal endophytes associated to *A. germinans* in Puerto Rico.

Chapter 3. Antibiotic and Antifungal Assays

3.1 Introduction

Antibiotics were first described by Selman Waksman in 1941 as "small molecules made by a microbe that antagonize the growth of other microbes" (Clardy et al. 2009). These compounds are important in medicine due to their potential as antimicrobials, antifungals and anticancer, among others (Bhimba and Joel 2012; Strobel et al. 1999). They are byproducts of the secondary metabolism of certain organisms like bacteria, fungi and plants (Croteau et al. 2000; Vining 1990). They have served to overcome numerous illnesses; thus, giving us a better quality of life. One of the most relevant challenges for the scientific and medical community today is the problem with antibiotic resistant organisms. There are many reports where pathogens that were thought to be neutralized have become resistant to the existent drugs; more pernicious in nosocomial infections. For example, *E. coli*, although a microorganism associated with the normal human gastrointestinal tract, can cause urinary tract and blood streams infections and some strains are resistant to cephalosporins and fluoriquinoles antibiotics (Tanwar et al. 2014). Similarly, *Candida* strains, responsible for candidiasis, have been reported to be resistant to fluconazole and echinocandins (Tanwar et al. 2014). Other nosocomial pathogens of medical relevance that have developed antibiotic resistance include: (i) *Staphylococcus aureus* causing fatal infective endocarditis and necrotizing pneumonia, has developed resistance to methicillin and its alternative treatments: vancomycin, distamycin and linezolid (Haaber et al. 2017); some strains are also resistant to penicillin, erythromycin, rifampicin, gentamicin and clindamycin (Rağbetli et al. 2016); (ii) *Pseudomonas aeruginosa* causing severe respiratory infections especially in patients suffering from cystic fibrosis and immunocompromised patients at the intense care unit and some strains are resistant to cephalosporins, fluoroquinolones, and carbapenems (Cabot et al. 2016); (iii) *Serratia marcescens* causing urinary tract infections, septicemia, meningitis, wound infections and

affecting neonatal intensive care units being resistant to ampicillin, cephalosporins, β-lactams, aminoglycosides and fluoroquinolones (Haifei et al. 2012).

Different approaches have been studied to overcome these problems. Among them there are antibiotic-antibiotic combinations, where two antibiotics are combined to produce stronger effect (Worthington and Melander 2013), but to find compatible drugs that will actually present synergism could be difficult (Richardson 2017). Sequential regimens are also studied, where two or more drugs are alternated during the treatment period (Fuentes-Hernández et al. 2015). Also, combinations of an antibiotic with a non-antibiotic compound that increases the activity of the antibiotic or blocks the resistant mechanism of the pathogen have been explored (Worthington and Melander 2013). Another different approach given by Ayhan and coworkers (2016) is to re-sensitize the resistant bacteria with a treatment with specifically designed anti-sense oligonucleotides.

Despite all these great efforts to fight antibiotic resistance, as Tawar et al. (2014) expresses, the discovery of novel drugs is one of the best chances to overcome this problem in combination with the actual knowledge of drug resistance. Given this, several researches have been made searching in different ecological niches for possible new drugs. Some of the most successful work has been done on marine environments, since they harbor a myriad of species capable of producing compounds with the desired effects (Hughes and Fenical 2010; Rahman et al. 2010). Among these, fungal compounds have caught the attention of recent investigations (Cheng et al. 2009; Silva et al. 2011).

The first approach to see if there are any active compounds with potential antibiotic activity is to determine the effect on microbial growth of specific pathogens. Bacterial and yeast pathogens

are ideal to test these effects on growth because rapid and easy bioassays can be developed. Optical density measures determined by spectrophotometry reflect the proportional number of cells in a liquid culture medium (Monod 1949). Using this method, different curves can be prepared and analyzed (Gompertz growth) (Zwietering et al 1990). In this work, we focused on the analysis of *A. germinans* endophyte fungal extracts on bacterial and yeast pathogens using growth curves to detect any effect that produces growth decrease of pathogens.

3.2 Materials and Methods

3.2.1 Fungal Extracts

Fungal isolates were incubated in 250mL Erlenmeyer flasks with Malt Extract medium (ME) on a shaker at 120 rpm for 2 weeks prior extractions (Morell-Rodríguez 2008). The term "fungal extracts" is referring to the supernatant collected from each specimen after 2 weeks of incubation giving enough time for the fungal isolates to produce extracellular secondary metabolites. The supernatant was collected in falcon tubes and subjected to two different treatments: (i) a 0.22µm filter was used to filter the extract and (ii) the extract was autoclaved. Also, the supernatant (as initially collected), was centrifuged at 15,871 rcf for 5 minutes and used for the assays as the "untreated" treatment. Thus, three different fungal extract treatments were evaluated: (i) untreated, (ii) filtered and (iii) autoclaved. Only the supernatant (containing extracellular secondary metabolites) was used to determine a possible antibacterial and antifungal activity. Nine fungal isolates showing potential to produce secondary metabolites to be used as antimicrobials on other studies were used for this analysis: *Penicillium* (BSI-HV-1(2)) sp., *Aspergillus flavus* (BSI-HV-2(1)), *Aspergillus clavatus* (BSI(MH)-HJ-2(1)), *Stereum* (BSI-R-2(1)) sp., *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *Purpureocillium* (BSII(3.5)-R-3(2))

sp., *Simplicillium* (BSI(1.5)-R-1(1)) sp., *Hortaea* (BSII(3.5)-HC-1(2)) sp. and *Bionectria* (BSI(MH)-HC-3(1)) sp. All extracts were collected in triplicate from each fungus.

3.2.2 Antibacterial and Antifungal Assays

Yeast and bacteria growth curves were used for the analyses; where the standard growth curves of each microorganism were compared with growth curves of each specimen treated with different fungal extracts. Tryptic Soy Broth (TSB) and Yeast Mold (YM) media were used for the assays with bacteria and yeasts, respectively. The bacteria used included: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 10145), *Serratia marcescens* (ATCC 13880) and *Staphylococcus aureus* (ATCC 6538), provided by Mrs. Magaly Zapata from the Microbiology Department and the yeasts: *Candida albicans* (ATCC 42266) and *Candida tropicalis* (ATCC 4563), provided by Mrs. Carol Rivera from the Mycology Laboratory; both from the University of Puerto Rico- Mayaguez Campus. Previous to the experiments, standard growth curves for each microorganism were performed to determine the different growth phases. A biophotometer (Eppendorf, Biophotometer Plus) was used to measure the absorbance and plot a graph of absorbance vs. time to obtain the curves. Bacteria and yeast were inoculated in 250mL Erlenmeyer flasks and placed in a shaker at 150 rpm for 10 hours. Samples were taken every 30 minutes before the fourth hour and every hour after that same time. Before each absorbance measurement the samples were vortexed vigorously to obtain a homogeneous mixture, especially the yeasts which flocculate. All measures were performed in triplicate.

We divided the experiments in three assays, in the first assay we used extracts from: *Penicillium* (BSI-HV-1(2)), *Simplicillium* (BSI(1.5)-R-1(1)), *Purpureocillium* (BSII(3.5)-R-3(2)), *Bionectria* (BSI(MH)-HC-3(1)) and *Aspergillus flavus* (BSI-HV-2(1)) against: *Escherichia coli*, *Pseudomonas aeruginosa*, *Serratia marcescens* and *Staphylococcus aureus*, and the yeasts:

Candida albicans and *Candida tropicalis*. The extracts (2.5mL) were added after 2 to 3 hours of inoculation since the logarithmic phase started at that time for bacteria and 3 hours for *C. tropicalis* and *C. albicans*. Then, a second assay was performed given the results from the first assay, where only two bacteria and one yeast strain were used: *E. coli*, *P. aeruginosa* and *C. albicans.* Four different fungal extracts were used for these analyses: *Stereum* (BSI-R-2(1)), *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *Hortaea* (BSII(3.5)-HC-1(2)) and *Aspergillus clavatus* (BSI(MH)-HJ-2(1)). Since *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)) presented relevant results (a significant decrease in the growth of the tested organisms was observed), a third assay was performed where the fungal extracts were not only added at the beginning of the log phase but also in the lag phase and at the beginning of the stationary phase. Positive controls using nystatin/cyclohexamide and penicillin/streptomycin were used for the antifungal and antibacterial assays, respectively. Also, controls adding water and ME were performed. All assays were performed in triplicate.

3.2.3 Statistical Analysis

The analysis of the data was performed using the GraphPad Prism version 6.00 for Windows. Using this program, we were able to compare the growth curves of all specimens adjusting the data to a model designed for growth curves (Gompertz Model) (Winsor, 1932; Zwietering et al. 1990). This model uses an equation that analyses some features of a growth curve: (i) an asymptote in the curve (representative of the stationary phase), (ii) the growth rate and (iii) the displacement along the x-axis. The equation used was: $y = (YM)e^{-(\ln\left(\frac{Y0}{YM}\right))e^{-kx}}$; where YM represents the upper asymptote (stationary phase); ln(Y0/YM) sets the displacement along the x axis (translates the graph to the left or right); "k" sets the growth rate [y scaling], Y0 represents the lower asymptote and "e" is Euler's Number (e = 2.71828...) (Zwietering et al. 1990; Manterola

2016). This equation is available on the Graph Pad program and once you insert your data it runs an ANOVA (F-test) comparing those specific parameters from each set (YM, Y0 and K). An output window shows all the values from the analysis including the p-value and if the compared curves are considered the same or different. To perform all the procedure on the program: (i) introduce the data on the tables, (ii) go to "analyze"; on the "X and Y axis" select "Nonlinear regression (curve fit)" and also select the data set you want to compare, (iii) then, under "fit", select "Gompertz growth" and under "Fitting method" select "Least squares (ordinary) fit", (iv) once those parameters are set, under "Compare", select "Does one curve adequately fit all the data sets?", the comparison method "Extra sum-of-squares F-test" and the p-value: 0.05.

3.3 Results

3.3.1 First Assay

Among the selected bacteria and yeasts, only *Aspergillus flavus* (BSI-HV-2(1)) extracts significantly affected the growth of *E. coli* (all extracts), *Candida albicans* (all extracts) and *Candida tropicalis* (untreated and filtered extracts) (Table 3.1) (Figures 3.1, 3.2 and 3.3). The rest of the extracts did not have a significant negative effect on the growth curves of the microorganisms (p-value > 0.05) (Table 3.1) [Appendix G and H]. Unexpectedly, untreated extracts from *A. flavus* (BSI-HV-2(1)) enhanced growth of *S. aureus* (Figure 3.4). Statistical analysis showed a p-value less than 0.05 for the ones that were reported to have a significant effect on the bacterial and yeast growth [Appendix R].

Table 3.1: Results showing fungal extracts that significantly (p-value <0.05) affected (+) and unaffected (-) the growth of bacteria and yeasts.

	Did the fungal extracts affect the bacteria and yeasts standard growth curve?					
	BACTERIA				**YEASTS**	
EXTRACTS	***E. coli***	***P. aeruginosa***	***S. marcescens***	***S. aureus***	***C. albicans***	***C. tropicalis***
Positive Control	+	+	+	+	+	+
Distilled Water (control)	-	-	-	-	-	-
ME medium (control)	-	-	-	-	-	-
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	-	-	-	-	-	-
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	-	-	-	-	-	-
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	-	-	-	-	-	-
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	-	-	-	-	-	-
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	-	-	-	-	-	-
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	-	-	-	-	-	-
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	-	-	-	-	-	-
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	-	-	-	-	-	-
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	-	-	-	-	-	-
Penicillium (BSI-HV-1(2)) *(untreated)*	-	-	-	-	-	-
Penicillium (BSI-HV-1(2)) *(autoclaved)*	-	-	-	-	-	-
Penicillium (BSI-HV-1(2)) *(filtered)*	-	-	-	-	-	-
A. flavus (BSI-HV-2(1)) *(untreated)*	+	-	-	+	+	+
A. flavus (BSI-HV-2(1)) *(autoclaved)*	+	-	-	-	+	-
A. flavus (BSI-HV-2(1)) *(filtered)*	+	-	-	-	+	+

*(+): there was a significant difference (p-value <0.05) when compared to the standard curve including decreased and enhanced growth; (-) no significant difference (p-value>0.05) was observed.

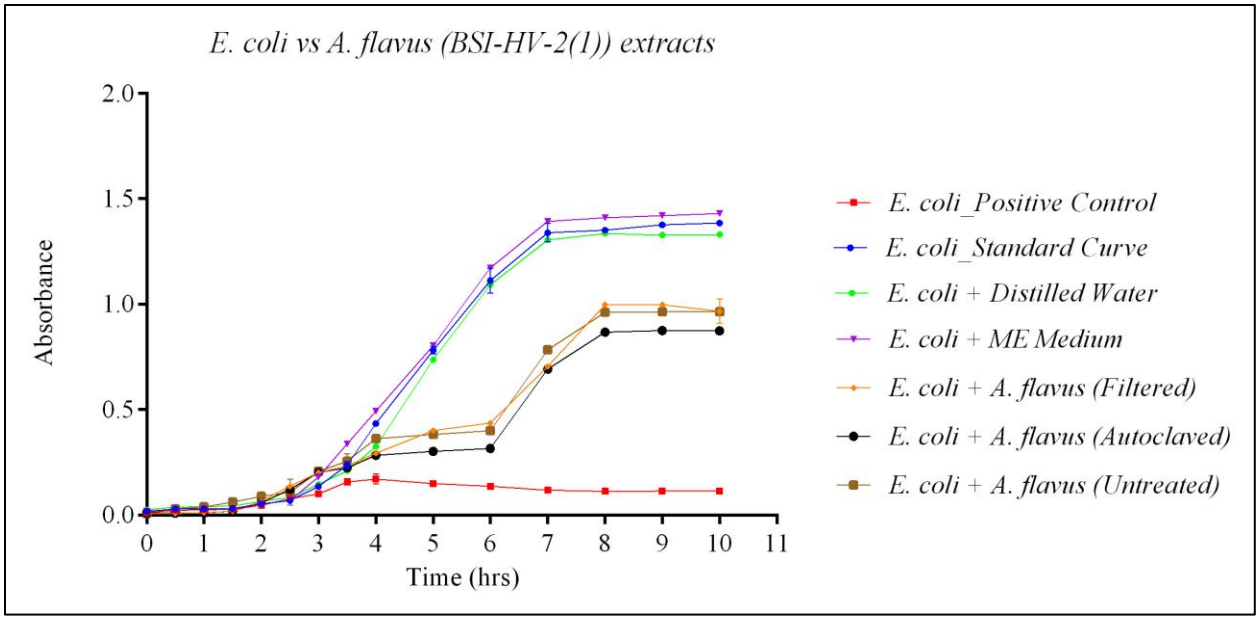

Figure 3.1: *E. coli* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added after 2.5 hours of incubation. All measures were performed in triplicate.

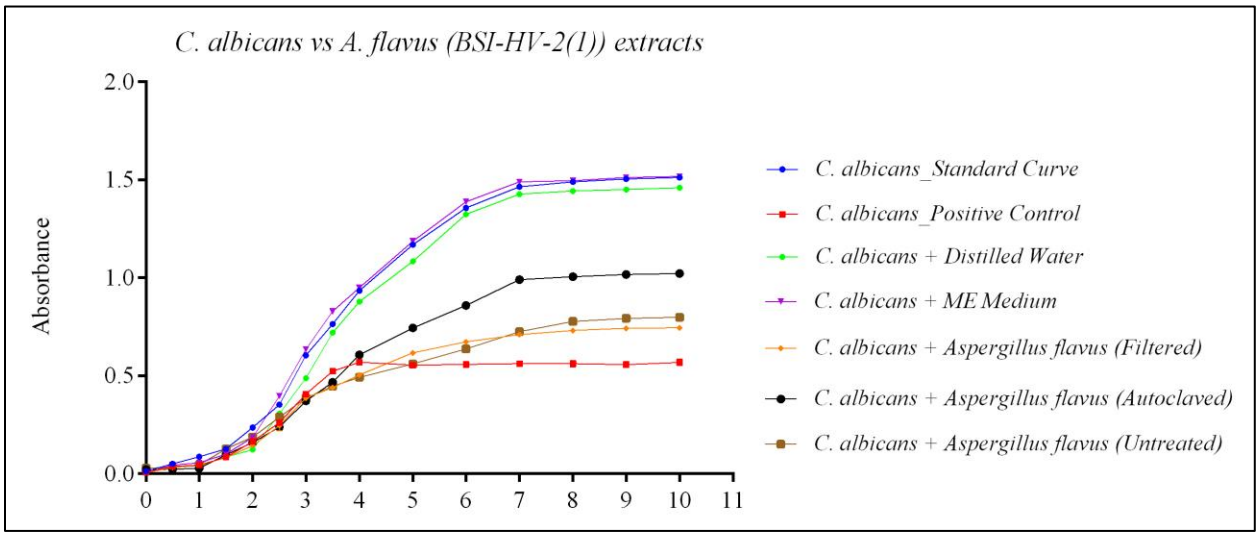

Figure 3.2: *C. albicans* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

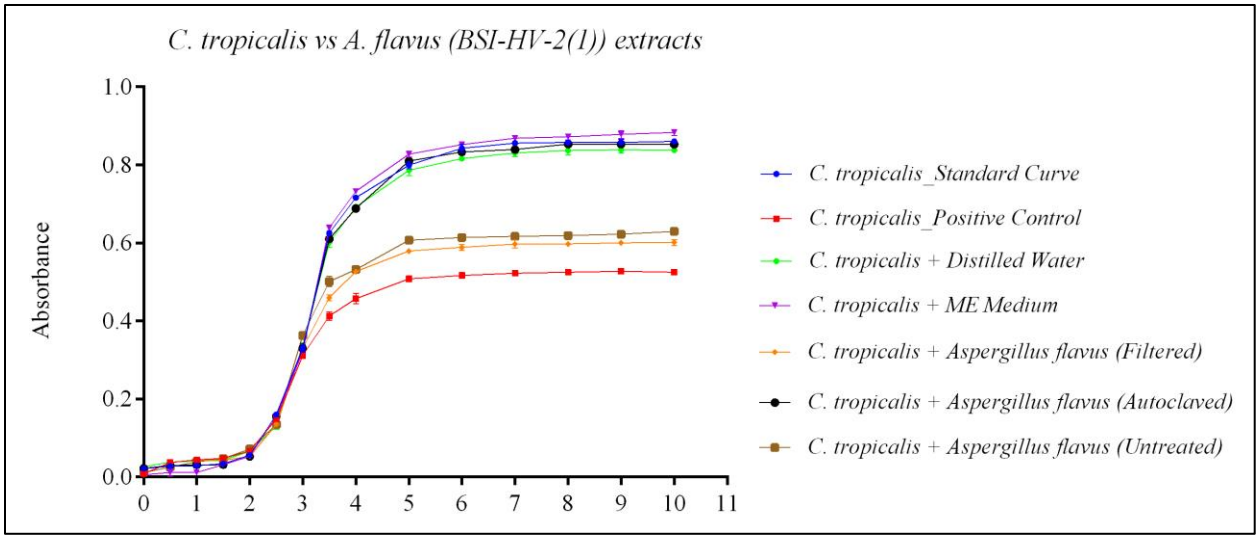

Figure 3.3: *C. tropicalis* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

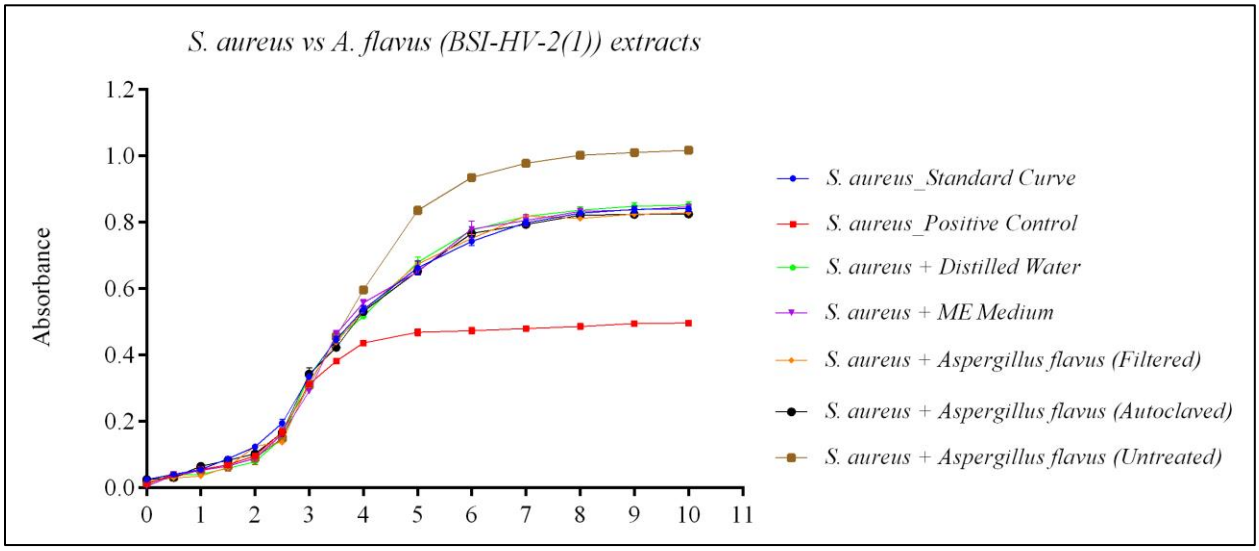

Figure 3.4: *S. aureus* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added after 2 hours of incubation. All measures were performed in triplicate.

3.3.2 Second Assay

Candida albicans and *E. coli* were most affected in the first assay; hence, they were selected to be tested with other fungal extracts. As another control, non-affected *P. aeruginosa* was selected over *S. marcescens* due to its clinical relevance as a pathogen. Then, four new fungal extracts were selected to perform assays: *Stereum* (BSI-R-2(1)), *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *Hortaea* (BSII(3.5)-HC-1(2)) and *Aspergillus clavatus* (BSI(MH)-HJ-2(1)). Surprisingly, extracts from *Stereum* (BSI-R-2(1)) (untreated and autoclaved) increased the growth of *E. coli* (Figure 3.5), but extracts from *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)) (all treatments) (Figure 3.6) and *A. clavatus* (BSI(MH)-HJ-2(1)) (all treatments) (Figure 3.7) decreased its growth. On the other hand, extracts from *Hortaea* (BSII(3.5)-HC-1(2)) [Appendix L] and *Stereum* (BSI-R-2(1)) (filtered) (Figure 3.5) have no effect on the growth curve of *E. coli*. Only *A. clavatus* (BSI(MH)-HJ-2(1)) extracts (all treatments) decreased growth of *P. aeruginosa* and *C. albicans* (Figures 3.8 and 3.9), while the other extracts had no significant effects on the growth curves of these organisms [Appendix M and N]. Once again, statistical analysis showed a p-value less than 0.05 for the ones that were reported to have a significant effect on the bacterial and yeast growth [Appendix R]. All results are showed on Table 3.2.

Table 3.2: Results showing fungal extracts that significantly (p-value <0.05) affected (+) and unaffected (-) the growth of *E. coli*, *P. aeruginosa* and *C. albicans*.

	Did the fungal extracts affect the bacteria and yeast standard growth curve?		
	BACTERIA		**YEAST**
EXTRACTS	***E. coli***	***P. aeruginosa***	***C. albicans***
Positive Control	+	+	+
Distilled Water (control)	-	-	-
ME medium (control)	-	-	-
Stereum (BSI-R-2(1)) (untreated)	+	-	-
Stereum (BSI-R-2(1)) (autoclaved)	+	-	-
Stereum (BSI-R-2(1)) (filtrated)	-	-	-
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (untreated)	+	-	-
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (autoclaved)	+	-	-
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (filtrated)	+	-	-
Hortaea (BSII(3.5)-HC-1(2)) (untreated)	-	-	-
Hortaea (BSII(3.5)-HC-1(2)) (autoclaved)	-	-	-
Hortaea (BSII(3.5)-HC-1(2)) (filtered)	-	-	-
A. clavatus (BSI(MH)-HJ-2(1)) (untreated)	+	+	+
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved)	+	+	+
A. clavatus (BSI(MH)-HJ-2(1)) (filtered)	+	+	+

*(+): there was a significant difference (p-value <0.05) when compared to the standard curve including decreased and enhanced growth; (-) no significant difference (p-value>0.05) was observed.

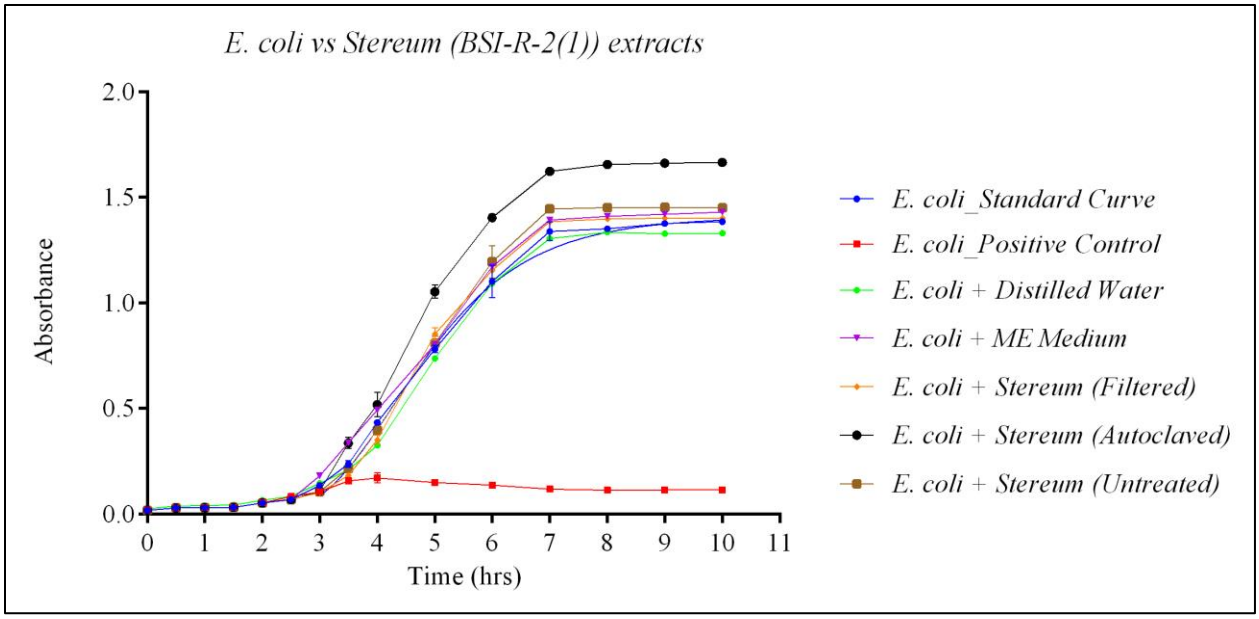

Figure 3.5: *E. coli* growth curves under different treatment from extracts of *Stereum* (BSI-R-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

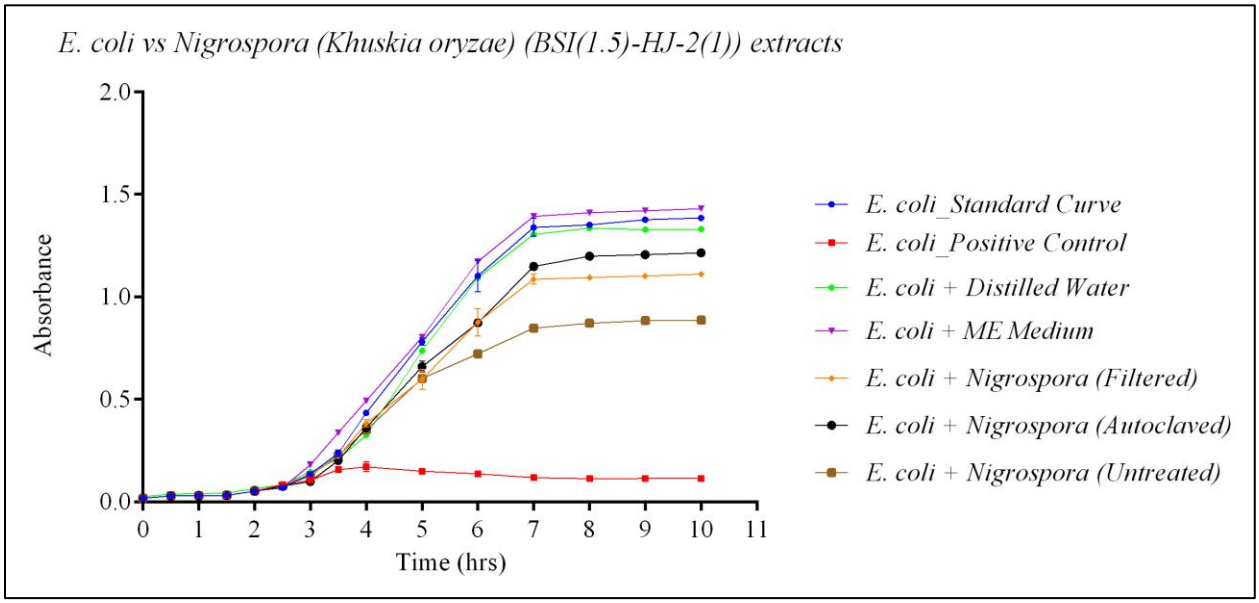

Figure 3.6: *E. coli* growth curves under different treatment from extracts of *Nigrospora* (*Khuskia oryzae*) (BSI(1.5)-HJ-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

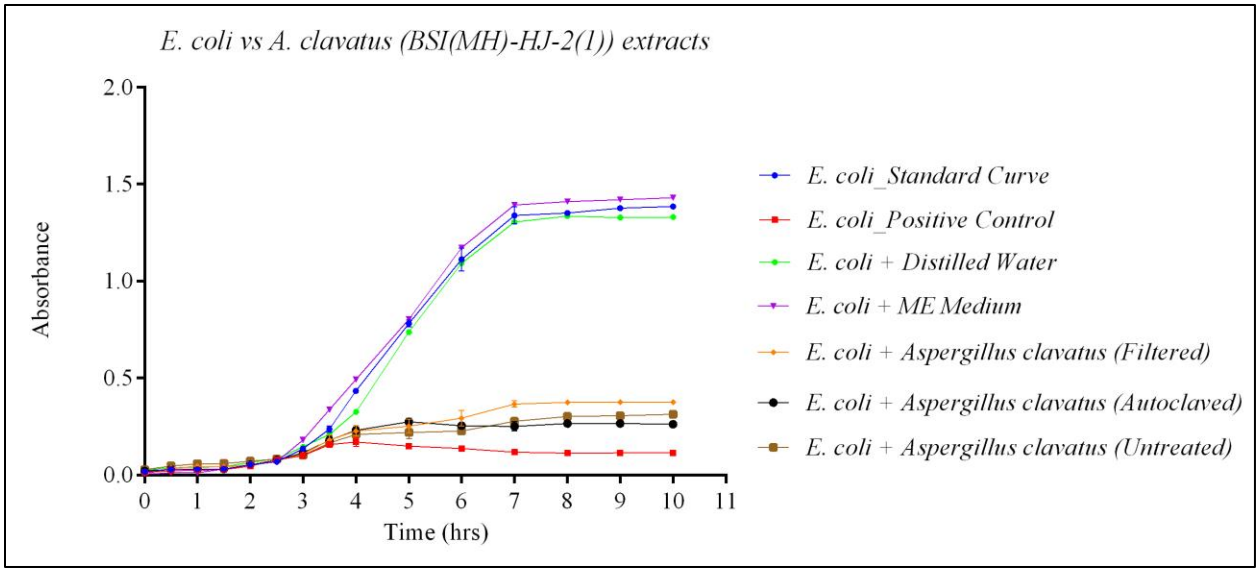

Figure 3.7: *E. coli* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

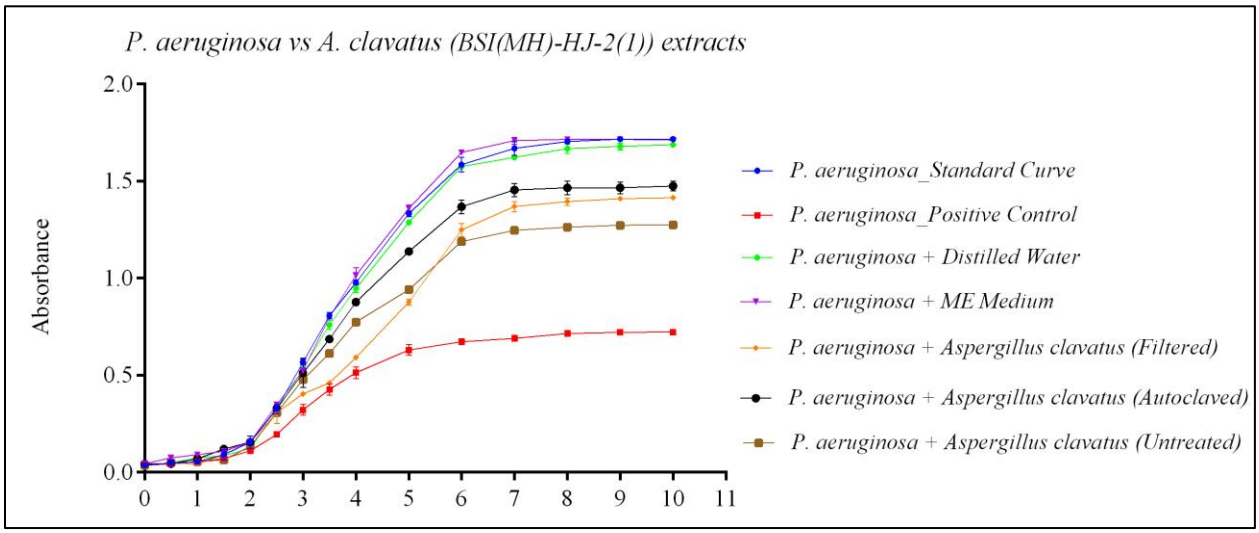

Figure 3.8: *P. aeruginosa* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added after 2 hours of incubation. All measures were performed in triplicate.

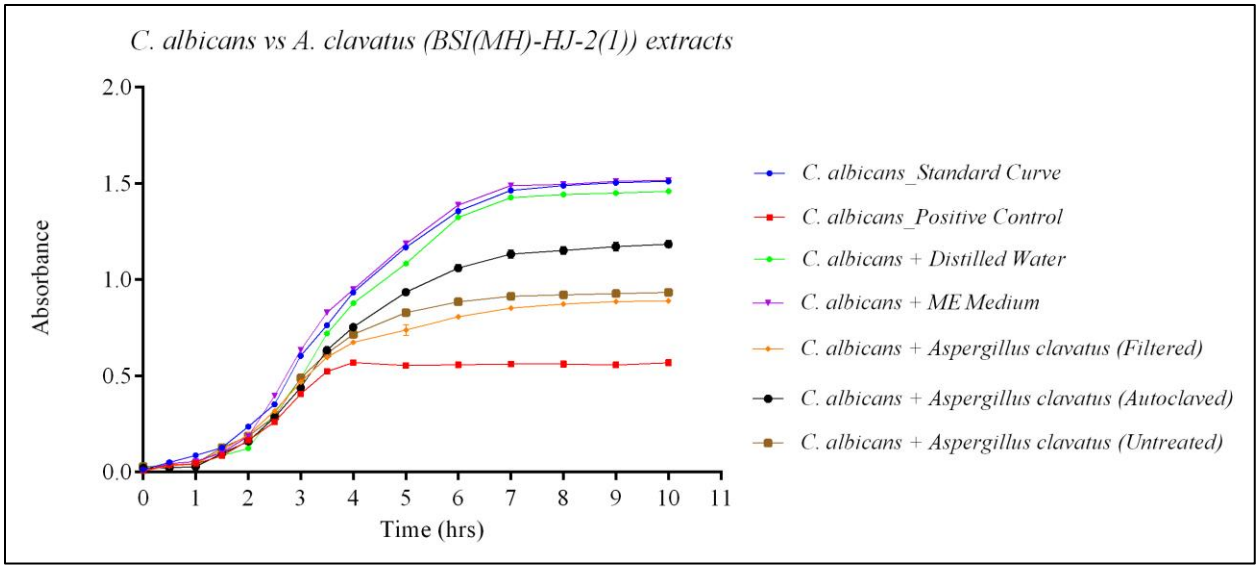

Figure 3.9: *C. albicans* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added after 3 hours of incubation. All measures were performed in triplicate.

3.3.3 Third Assay

Since *Aspergillus flavus* (BSI-HV-2(1)) and *Aspergillus clavatus* (BSI(MH)-HJ-2(1)) extracts were the ones that showed most growth inhibition within the first two assays, we proceeded to see their effects on each grow phase on the model strains selected. For the lag phase results, we observed a stronger decrease of growth for *E. coli*, *P. aeruginosa* and *C. albicans* (Figures 3.10 to 3.15), resulting in an increased lag phase and overall less growth. On the contrary, when the extracts were added at the beginning of the stationary phase the results did not show any significant differences on the growth curves when compared with the standard [Appendix P and Q]. Statistical analysis showed a p-value less than 0.05 for the ones that were reported to have a significant effect on the bacterial and yeast growth [Appendix R]. All results are showed in triplicate.

Table 3.3: Results showing fungal extracts that significantly affected (+) and unaffected (-) the growth of *E. coli*, *P. aeruginosa* and *C. albicans*. Extracts were added at the beginning of the curve (lag phase) and in the stationary phase.

	Did the fungal extracts affect the bacteria standard growth curve?		
	BACTERIA		**YEAST**
EXTRACTS	***E. coli***	***P. aeruginosa***	***C. albicans***
Positive Control	+	+	+
Distilled Water (control)	-	-	-
ME medium(control)	-	-	-
Lag Phase			
A. flavus (BSI-HV-2(1)) *(untreated)*	+	+	+
A. flavus (BSI-HV-2(1)) *(autoclaved)*	+	+	+
A. flavus (BSI-HV-2(1)) *(filtered)*	+	+	+
A. clavatus (BSI(MH)-HJ-2(1)) *(untreated)*	+	+	+
A. clavatus (BSI(MH)-HJ-2(1)) *(autoclaved)*	+	+	+
A. clavatus (BSI(MH)-HJ-2(1)) *(filtered)*	+	+	+
Stationary Phase			
A. flavus (BSI-HV-2(1)) *(untreated)*	-	-	-
A. flavus (BSI-HV-2(1)) *(autoclaved)*	-	-	-
A. flavus (BSI-HV-2(1)) *(filtered)*	-	-	-
A. clavatus (BSI(MH)-HJ-2(1)) *(untreated)*	-	-	-
A. clavatus (BSI(MH)-HJ-2(1)) *(autoclaved)*	-	-	-
A. clavatus (BSI(MH)-HJ-2(1)) *(filtered)*	-	-	-

*(+): there was a significant difference (p-value <0.05) when compared to the standard curve including decreased and enhanced growth; (-) no significant difference (p-value>0.05) was observed.

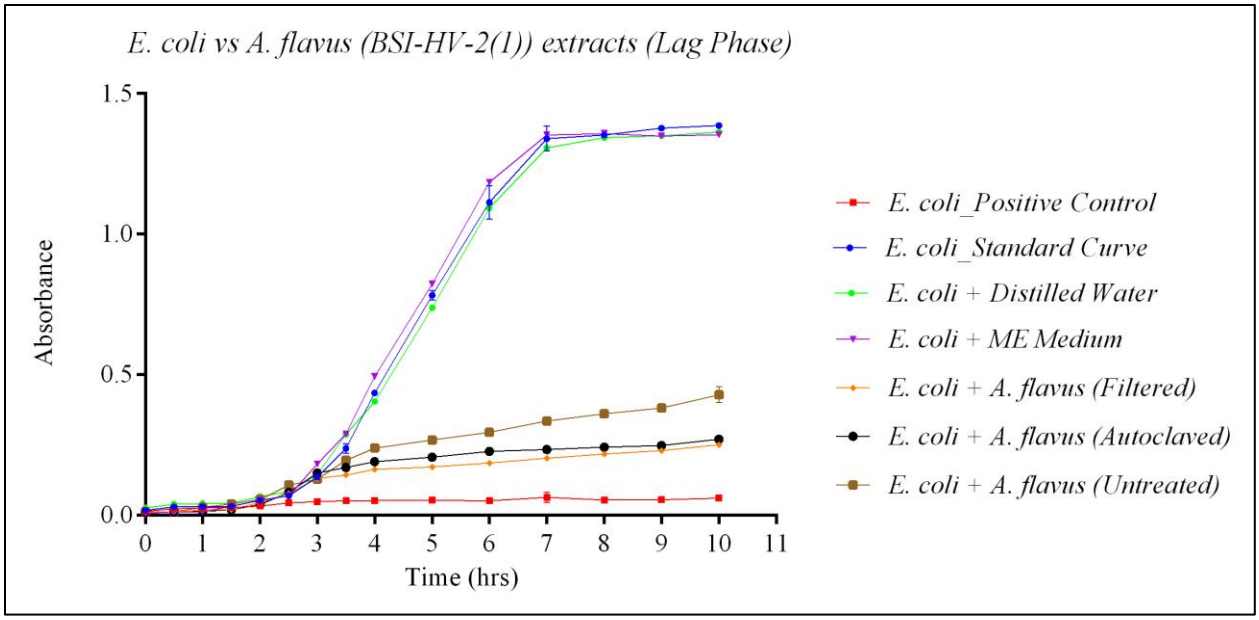

Figure 3.10: *E. coli* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

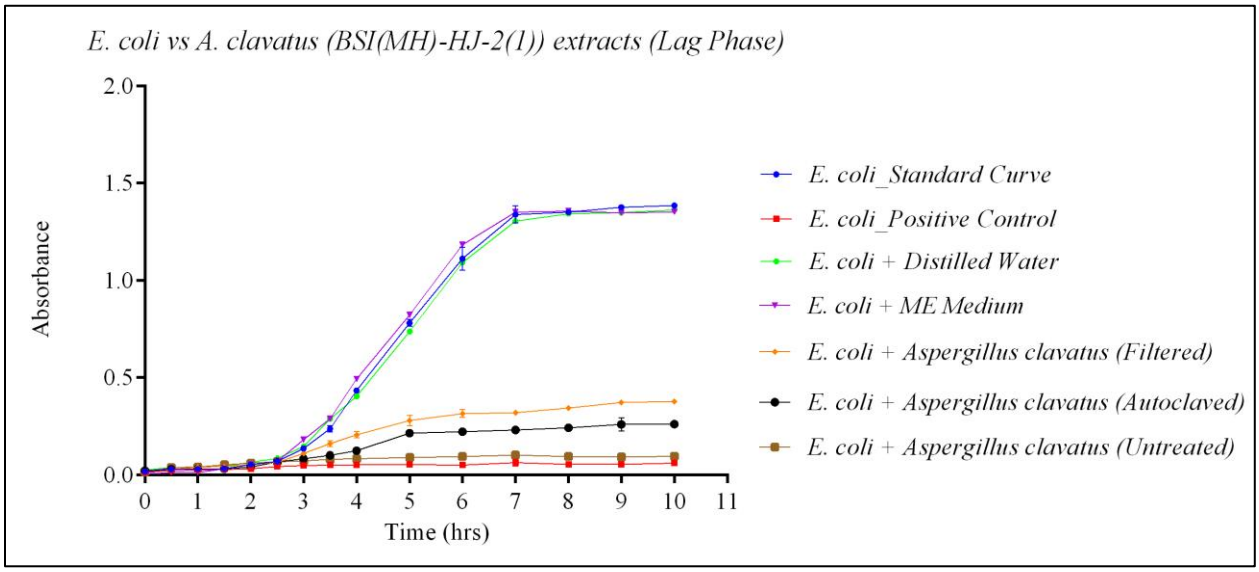

Figure 3.11: *E. coli* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

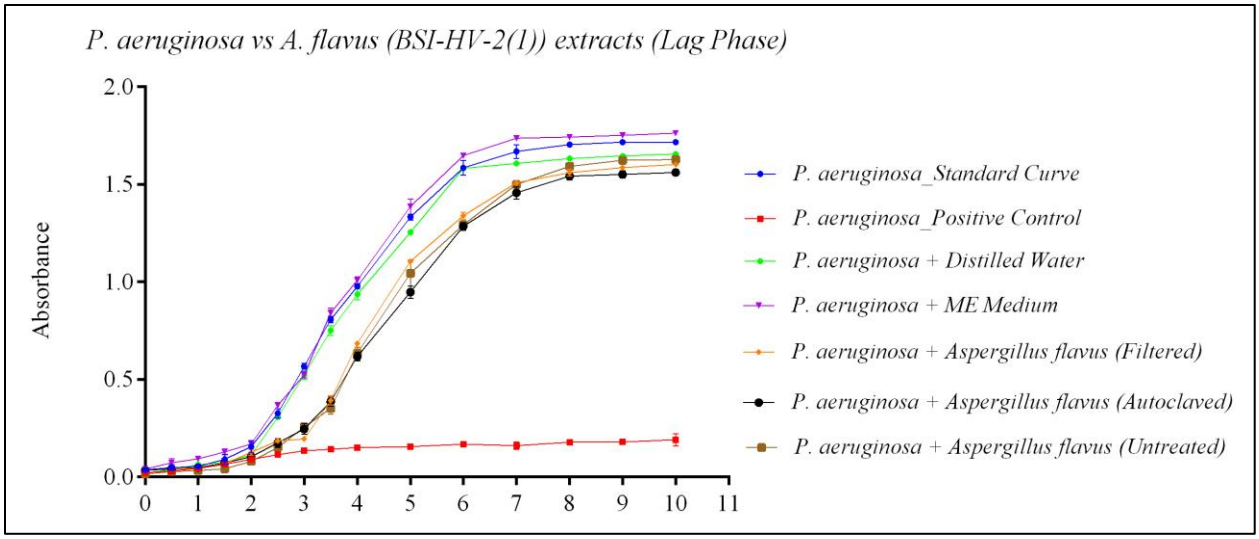

Figure 3.12: *P. aeruginosa* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

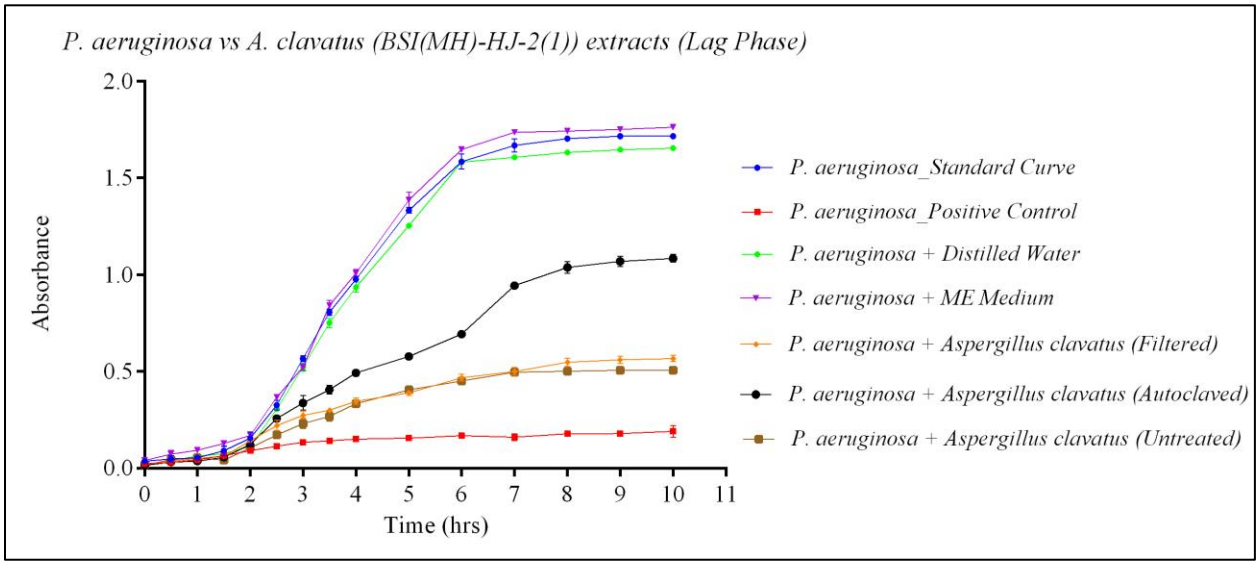

Figure 3.13: *P. aeruginosa* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

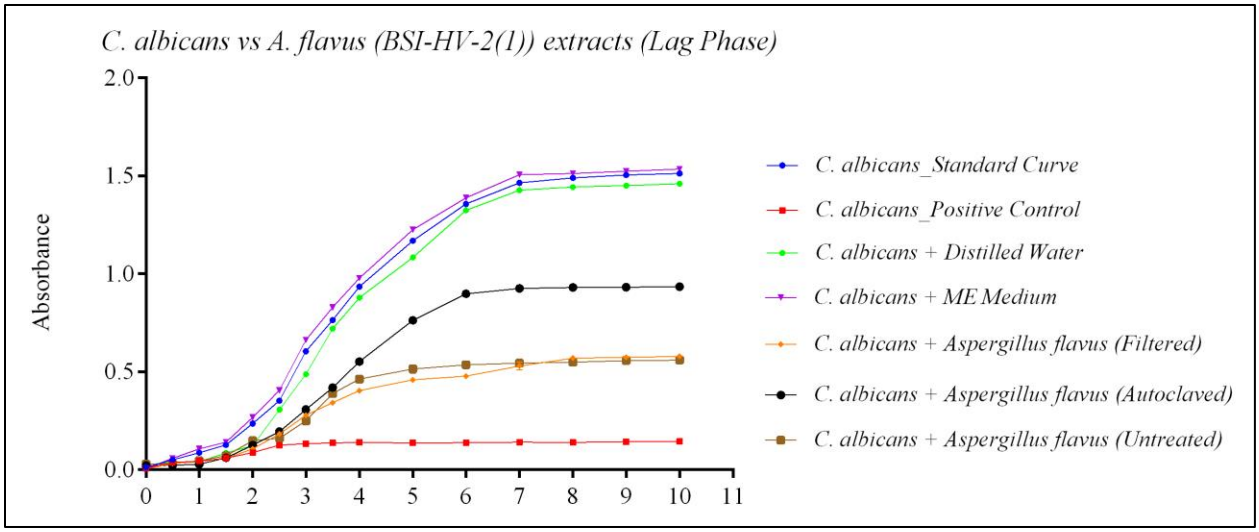

Figure 3.14: *C. albicans* growth curves under different treatment from extracts of *A. flavus* (BSI-HV-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

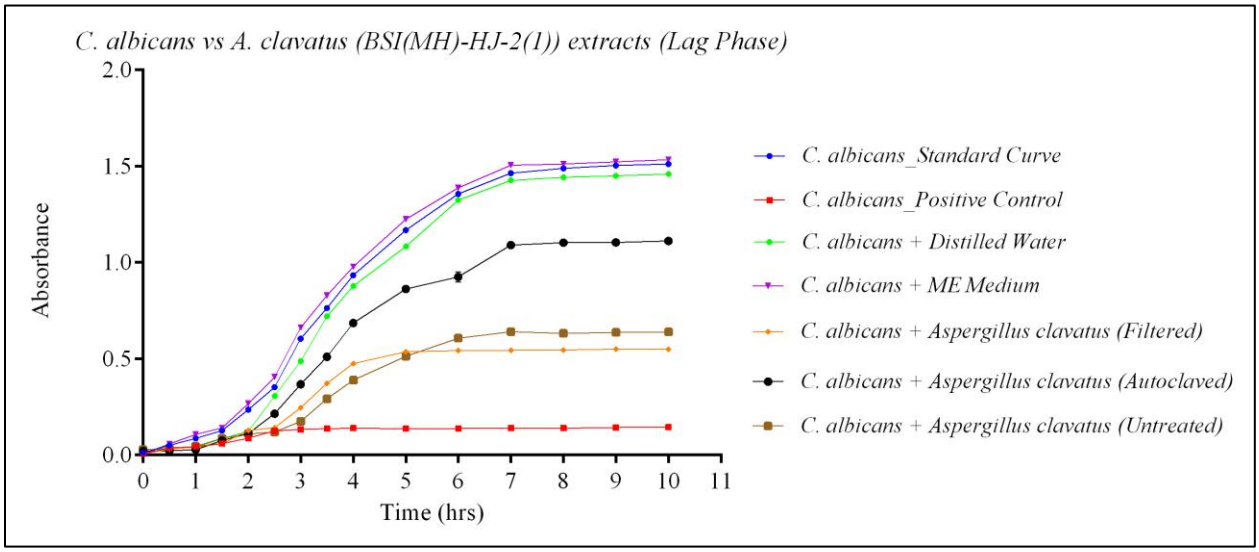

Figure 3.15: *C albicans* growth curves under different treatment from extracts of *A. clavatus* (BSI(MH)-HJ-2(1)). Extracts were added when incubated. All measures were performed in triplicate.

3.4 Discussion and Conclusion

This study was expected to find fungal extracts with the capability to decrease growth of certain microorganisms considered pathogens. On the contrary, unexpected results were obtained where the extracts of *A. flavus* (BSI-HV-2(1)) (untreated) enhanced growth of *S. aureus* (Figure 3.4) and extracts from *Stereum* (BSI-R-2(1)) (autoclaved and untreated) enhanced growth of *E. coli* (Figure 3.5). Bacteria or fungi can benefit from specific compounds that are produced by the other partner when interacting in a synergist relationship (Frey-Klett et al. 2011). Nutritional interactions have been seen in other systems, but they are usually about competition.

In the case of *A. flavus* (BSI-HV-2(1)) extracts, (i) compounds released by the fungus (added as extract from the supernatant) could possess hydrolytic enzymes that help the bacteria obtain nutrients or used them directly as source of energy (Boer et al. 2005); (ii) fungal spores could be promoting the survival of the bacteria as seen on some bacterial communities that feed on *Glomus geosporum* outer spore layer (Frey-Klett et al. 2011; Roesti et al. 2005); (iii) or *S. aureus* could also be producing a compound that help *A. flavus* (BSI-HV-2(1)) to grow since there was no treatment for this extract, however this is less probable since fungi growth rate is much more slower than bacterial growth (Frey-Klett et al. 2011; Sitharashmi et al. 2015). In addition, *A. flavus* (BSI-HV-2(1)) could be producing a compound that protects *S. aureus* from other agents as seen in *Candida albicans-S. aureus* interactions (Kong et al. 2016).

For the enhanced growth of *E. coli* by the autoclaved extract of *Stereum* (BSI-R-2(1)) the explanation could be a little more of a challenge. Perhaps there could have been a compound(s) that was/were decomposed by the high temperature and pressure of the autoclave process and *E. coli* was able to use it as source of energy. To have some insight about this singularity, further

experiments could be prepared in where a reducing sugar analysis (Miller 1959) can be made before and after autoclaving the extract.

On the other hand, extracts from *Nigrospora (Khuskia oryzae)* (BSI(1.5)-HJ-2(1)), *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)) decreased growth of *E. coli*, but with *A. flavus* (BSI-HV-2(1)) extracts, it recovered and started to grow again. Despite this, it did not grow to the same extent as the standard curve (Figure 3.1). Since the extracts from that result were added during the beginning of the logarithmic phase, *E. coli* may have had the chance to recover from the negative effect, a behavior not seen when the extract was added in the lag phase (Figure 3.10). Among the 3 fungal extracts that affected *E. coli* growth, the ones from *A. clavatus* (BSI(MH)-HJ-2(1)) were the more effective, given to the point that when the analysis was performed in the lag phase, they worked very similarly to the positive control, showing no significant difference between them ($p<0.05$) (Figure 3.7). All extracts from *A. clavatus* (BSI(MH)-HJ-2(1)) also decreased the growth of *P. aeruginosa* (Figure 3.8) and *C. albicans* (Figure 3.9), but not to the same extent.

A. clavatus (BSI(MH)-HJ-2(1)) extracts presented a major decrease in the growth of the selected bacteria and yeast maybe due to its production of various toxic secondary metabolites. Among them, the fungus produces clavacin (best known as patulin), cytochalasin E and tryptoquivalines (López-Díaz and Fannigan 1997; Varga et al. 2007). Given that the majority of the extracts showed lower effect after the autoclaved treatment, it is probable that these compounds are sensitive to high temperature and pressure, such as patulins, which possess a melting point of 111ºC (O'Neil 2006). Also, Gomes and coworkers (2014), found no effect from tryptoquivalines on the growth of *E. coli* and *P. aeruginosa* and cytochalasin E, which possess a melting point of 206-208 ºC (Cole and Cox 1981). These are only a few examples of potential compounds produced

by *A. clavatus* (BSI(MH)-HJ-2(1)), but we cannot discard the possibility that the observed effects could be caused by other compounds with antimicrobial activity.

The other microorganisms affected by the extracts of *A. flavus* (BSI-HV-2(1)) were *C. albicans* and *C. tropicalis. C. albicans,* was affected by all the extracts (Figure 3.2) but *C. tropicalis* was not affected by the autoclaved extracts (Figure 3.3). In this case, it is probable that the compound(s) that performed the activity is affected by the high temperature (121ºC) and pressure (15 psi) of the autoclaving process.

A. flavus (BSI-HV-2(1)) extracts was the other treatment that presented significant effects in decreasing the growth of bacteria and yeasts [Appendix R]. It is relevant that this organism produces substances known as aflatoxins (Klich 2007). These toxins are considered major carcinogens (lung and liver mostly) (Wong and Hsieh 1976) and bacterial mutagens (Stark et al. 1979). Thus, they could be directly responsible for growth decrease. Nevertheless, latest studies have found lactic acid producers (Ahlberg et al. 2015) and probiotic bacteria that mitigate the effects of aflatoxins (Gacem and Ould El Hadj-Khelil 2016).

When we examined the effect of the extracts of *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)) added at the lag and the stationary phase, results were as expected. If there was a decrease in growth when the extracts were added at the start of the log phase in the first assay, for the lag phase it was expected to see a major decrease in growth since the extracts were added earlier (Figures 3.10 to 3.15). Interestingly, in addition to that major growth decrease for the case of *E. coli* with the extracts of *A. clavatus* (BSI(MH)-HJ-2(1)) (Figure 3.11), the extracts of the untreated treatment acted very similar to the positive control (penicillin/streptomycin mix). When we performed an analysis comparing the slopes of the two “curves”, there were no

significant differences among them (p-value<0.05). For the treatment at the stationary phase no significant effect was seen since the bacteria was already at its maximum growth.

These results show a potential for extracts from *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)), which can be producing a compound or compounds that decrease the growth of pathogens besides the ones known until today since they are recovered from a different environment, thus affecting the compounds they produce.

Future Work

To have further insight on the fungal endophytic community associated to the black mangrove *Avicennia germinans* several experiments can be addressed. For example, experiments comparing fungal diversity between seasons (e.g. dry and wet seasons) since we observed a decrease in the amount of isolates. Also, perform several samplings to determine the distribution of endophytes on different parts of the plant to observe if there is any specific association with a specie or a vertical transmission, by the endophytes associated to the seeds, in this mangrove.

To address some of the uncertainties with the antimicrobial and antifungal assays, various experiments could be performed in the future. For example, analyzing the fungal supernatants (extracts) by performing extractions in fractions using a column chromatography and identifying possible compounds of interest. Also, instead of incubating only the fungus to produce secondary metabolites by its own, an experiment adding bacteria strains to it could produce new insights given that the bacterial-fungus interaction can activate gene clusters that produce secondary metabolites, which are not expressed under normal growth conditions (Brakhage and Schroeckh 2011). This was seen when a marine isolate of *Pestalotia* produced pestalone (a chlorinated benzophenone antibiotic) when it was co-cultivated with marine alpha proteobacterium (CNJ-328) (Frey-Klett et al. 2011).

For the results of the enhanced growth of *S. aureus* by *A. flavus* (BSI-HV-2(1)) (untreated) extracts, an experiment to address this situation could be as follows: take four samples (one of the extract, a second one of the inoculated medium with the bacteria, another one when the extract is added, and a final sample at the end of the experiment) and prepare a wet mount of each sample and compare the amount of fungus (if there is any) and bacteria. In addition, an antibiotic such as penicillin/streptomycin (which we know for this results, affect the growth of *S. aureus* significantly) could be added to the extract of *A. flavus* (BSI-HV-2(1)) and then perform the assay once again with *S. aureus* to see if the is a protection by *A. flavus* (BSI-HV-2(1)) to *S. aureus* as seen in *Candida albicans* (Kong et al. 2016).

Similarly, for the results of the enhanced growth of *E. coli* by *Stereum* (BSI-R-2(1)) (autoclaved) extracts, to see if there are really compounds degrading due to the autoclaving process that could serve as source of energy (like sugars) to *E. coli* from extracts of *Stereum* (BSI-R-2(1)), a reducing sugar analysis by the 3,5-dinitrosalicylic acid (DNS) method (Miller 1959) could be performed to the extract before and after autoclaving to see if the amount of reducing sugars increase.

General Conclusions

In this research, we were able to study fungal endophyte community in the black mangrove *A. germinans* and determined their potential as producers of secondary metabolites with antimicrobial properties. To our knowledge, this is the first report of fungal endophytes associated to *A. germinans* in Puerto Rico in where the fungi *Physalospora*, *Pochonia*, *Simplicillium*, *Teratosphaeria*, *Wrightoporia* and *Stereum* were identified for the first time as mangrove endophytes. Finally, *A. flavus* (BSI-HV-2(1)) and *A. clavatus* (BSI(MH)-HJ-2(1)) showed a potential to produce compounds with antimicrobial and antifungal activity. This findings opens a window to the discovery of new compounds with antimicrobial activity that could be used to treat diseases.

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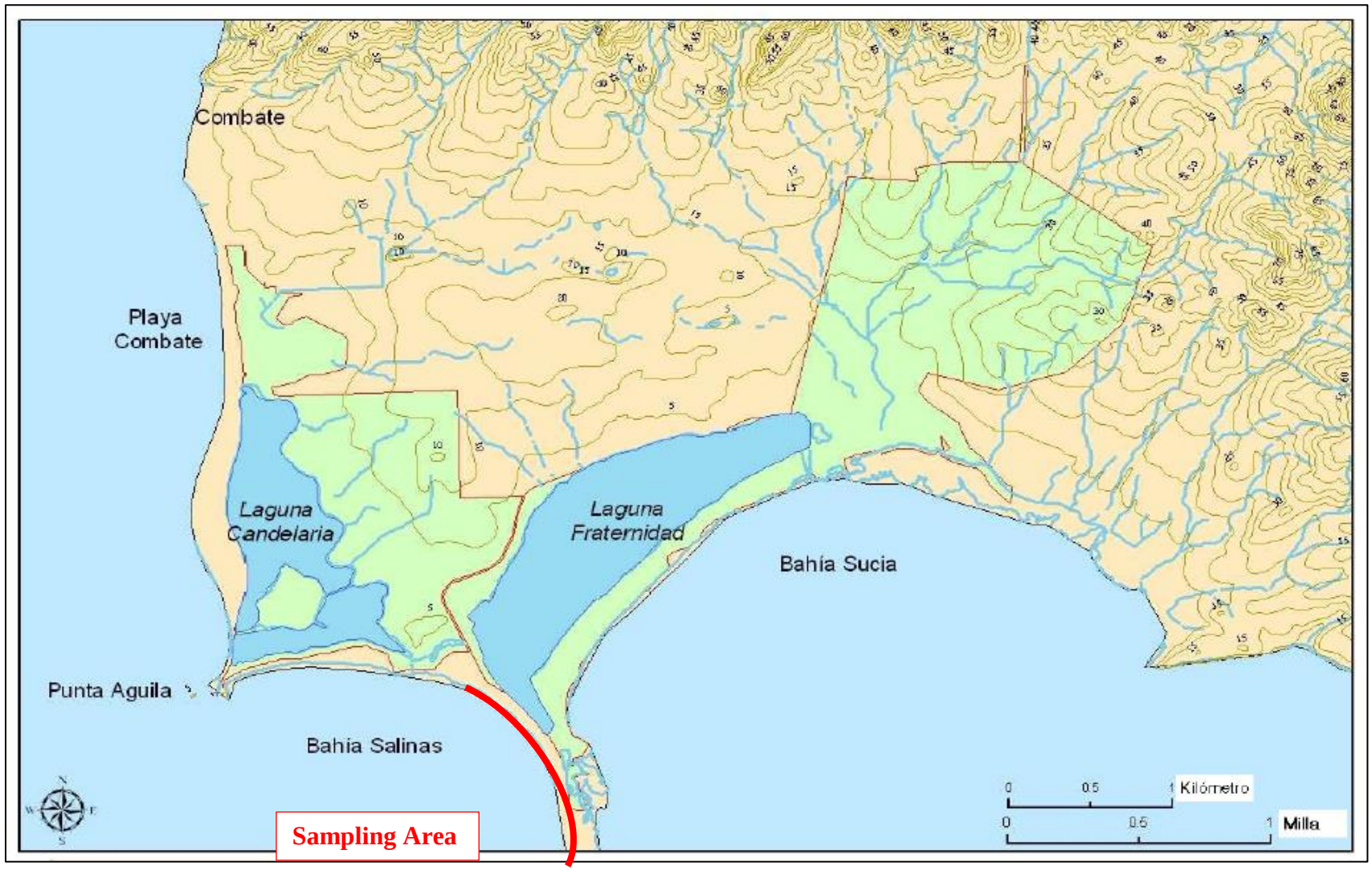
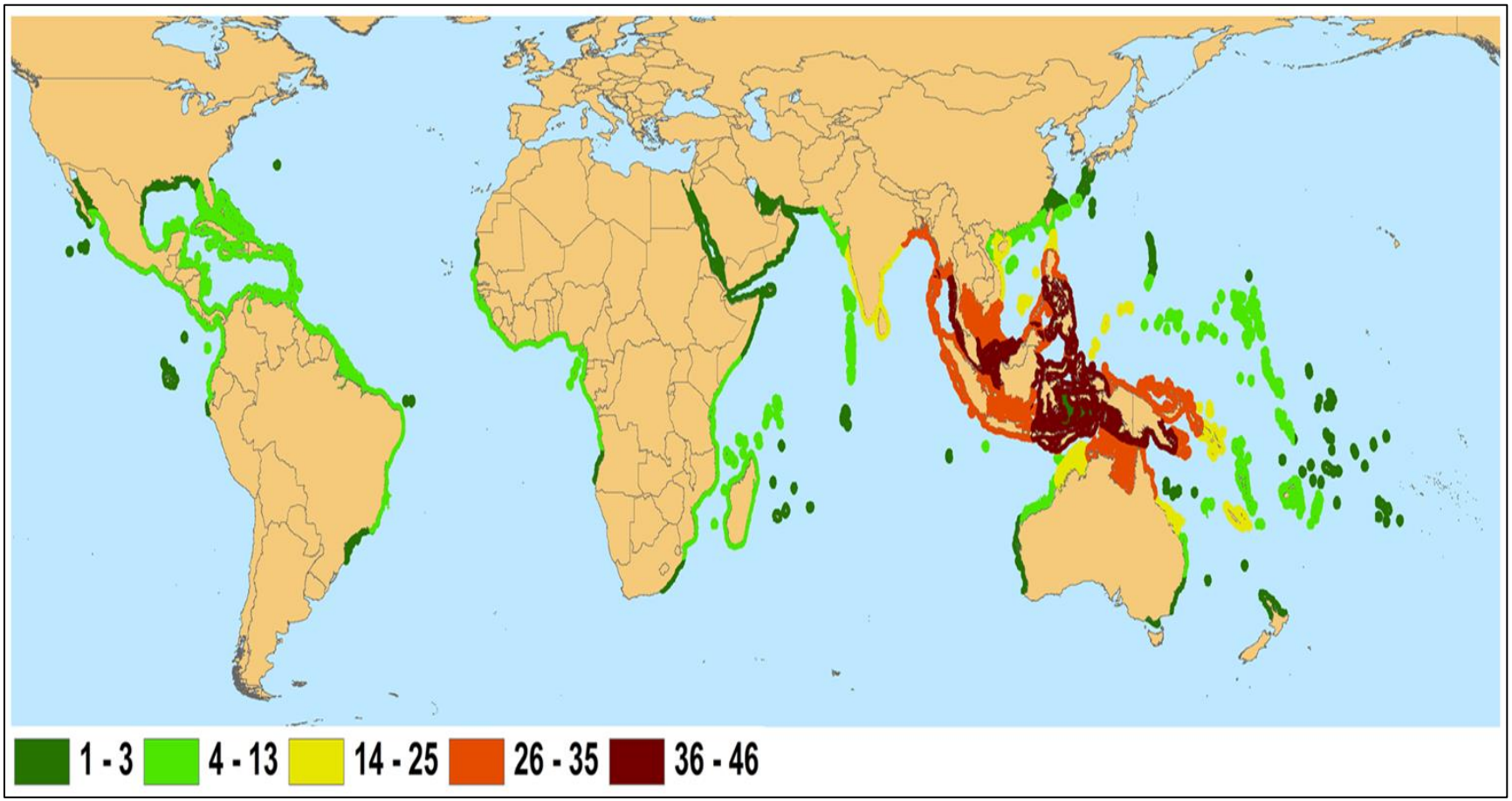
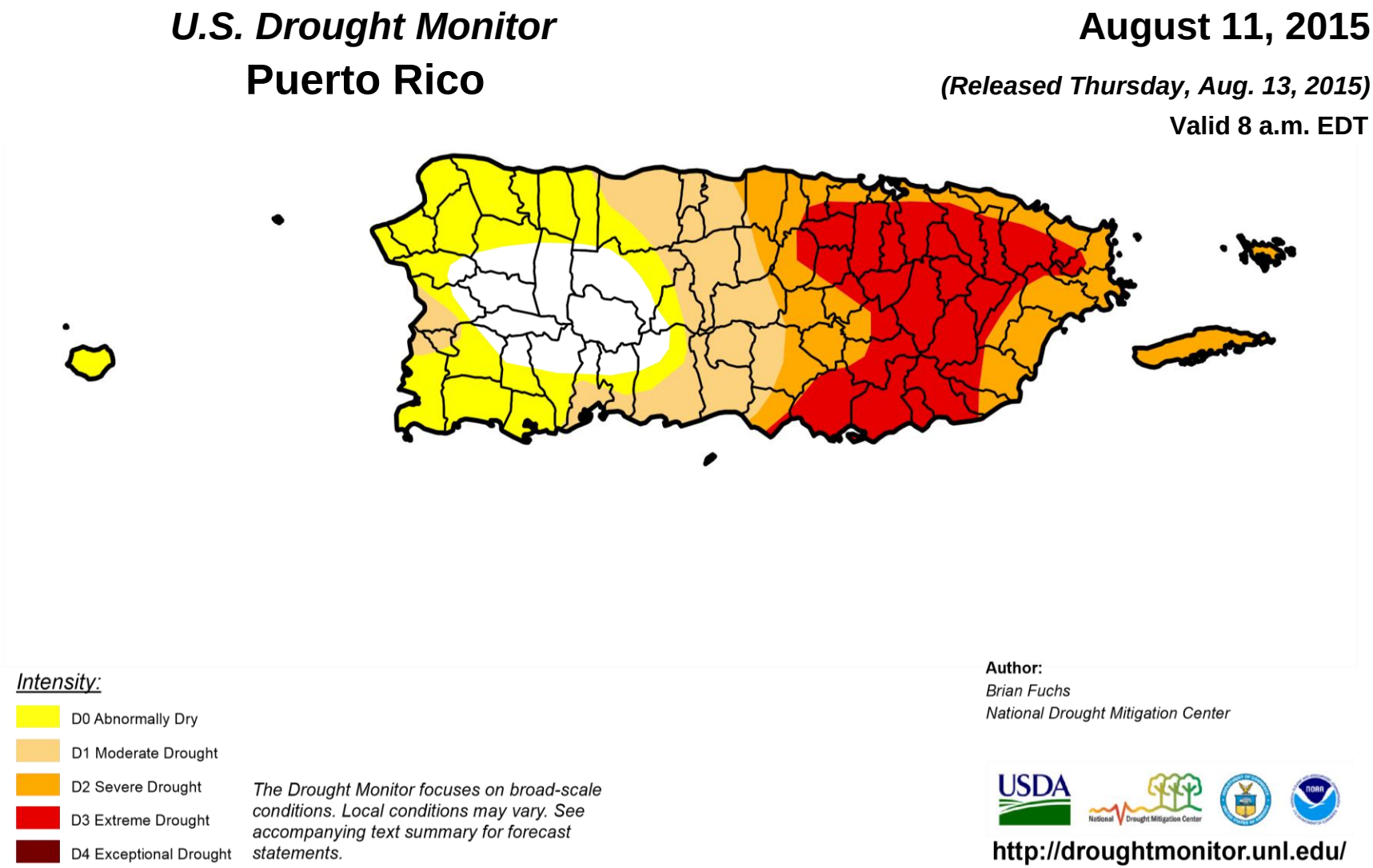
Appendix A: Topographic map of the Cabo Rojo National Wildlife Refuge (FWS 2010).



Appendix B: Mangrove Species Richness: Native distributions of mangrove species (Polidoro et al. 2010).



Appendix C: Map of drought areas in Puerto Rico in August 2015 (USDA 2015).



Appendix D: Fungi isolated from the back mangrove *Avicennia germinans* in Bahia Salinas, Cabo Rojo, Puerto Rico.

BSI							
DNA/PCR Codes	**Plates Codes**	**Accesion Number**	**Identification**	**Phylum**	**Class**	**Query Covarage / Max Identity**	**Source**
1	BSI-HJ-3(1)	LN809021.1	*Nigrospora sphaerica*	Ascomycota	Sordariomycetes	99/100	Cave air sample/Spain
2	BSI-HV-1(1)	N/A	*Aspergillus niger*	Ascomycota	Eurotiomycetes	N/A	N/A
3	BSI-HV-1(2)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
4	BSI-HV-2(1)	N/A	*Aspergillus flavus*	Ascomycota	Eurotiomycetes	N/A	N/A
5	BSI-HV-3(1)	LN997673.1	*Wrightoporia tropicalis*	Basidiomycota	Agaromycetes	84/91	Leaves endophyte/Phillipines
6	BSI-HV-3(2)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
7	BSI-HC-1(1)	N/A	*Nigrospora sphaerica*	Ascomycota	Sordariomycetes	N/A	N/A
8	BSI-HC-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
9	BSI-HC-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
10	BSI-HC-3(2)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
11	BSI-C-2(1)	LN809021.1	*Nigrospora sphaerica*	Ascomycota	Sordariomycetes	98/100	Cave air sample/Spain
12	BSI-R-2(1)	KJ832044.1	*Stereum* sp.	Basidiomycota	Agaromycetes	90/100	Rubber tree leaf/ Peru
13	BSI-R-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
14	BSI-R-3(2)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
15	BSI(1.5)-HJ-2(1)	KC492457.1	*Nigrospora* sp.	Ascomycota	Sordariomycetes	98/100	Mango endophyte/China
18	BSI(1.5)-HV-3(1)	LN808894.1	*Pochonia chlamydosporia*	Ascomycota	Sordariomycetes	100/99	Cave/Spain
22	BSI(1.5)-HC-2(1)	LN809021.1	*Nigrospora sphaerica*	Ascomycota	Sordariomycetes	100/97	Cave air sample/Spain
23	BSI(1.5)-HC-3(1)	KP133219.1	*Nemania diffusa*	Ascomycota	Sordariomycetes	99/99	Plant endophyte/Ecuador
24	BSI(1.5)-C-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
25	BSI(1.5)-C-2(2)	JN997370.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	99/99	Silicone scuba diving equipment/Spain
26	BSI(1.5)-C-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
28	BSI(1.5)-R-1(1)	KT898392.1	*Simplicillium lamellicota*	Ascomycota	Sordariomycetes	99/98	Plant/Iran
29	BSI(1.5)-R-1(2)	KP184331.1	*Acremonium* sp.	Ascomycota	Sordariomycetes	95	Arthropods/Portugal
30	BSI(1.5)-R-3(1)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A
31	BSI(3.5)-HJ-1(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
35	BSI(3.5)-HV-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
36	BSI(3.5)-HV-3(1)	KJ439140.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	99/99	Root vegetable/Brazil
37	BSI(3.5)-HC-1(1)	KJ439140.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	100/99	Root vegetable/Brazil
38	BSI(3.5)-HC-2(1)	KJ862538.1	*Penicillium sp.*	Ascomycota	Eurotiomycetes	98/100	Plant endophyte/China
39	BSI(3.5)-HC-3(1)	KT959321.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	98/99	Coral fungi/China
40	BSI(3.5)-HC-3(2)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
42	BSI(3.5)-C-1(1)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
43	BSI(3.5)-C-1(2)	KT959321.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	99/99	Coral fungi/China
44	BSI(3.5)-C-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
45	BSI(3.5)-C-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
46	BSI(3.5)-C-3(2)	KT959321.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	98/99	Coral fungi/China
47	BSI(3.5)-R-3(1)	KT959321.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	99/99	Coral fungi/China
48	BSI(MH)-HJ-2(1)	N/A	*Aspergillus clavatus*	Ascomycota	Eurotiomycetes	N/A	N/A
51	BSI(MH)-HV-3(1)	N/A	*Hortaea werneckii*	Ascomycota	Dothideomycetes	N/A	N/A
53	BSI(MH)-HC-2(1)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A
54	BSI(MH)-HC-3(1)	KF578124.1	*Bionectria ochroleuca*	Ascomycota	Sordariomycetes	98/99	Inner root/Blazil

Appendix D**:** Fungi isolated from the back mangrove *Avicennia germinans* in Bahia Salinas, Cabo Rojo, Puerto Rico.

BSII							
DNA/PCR Codes	**Plates Codes**	**Accesion Number**	**Identification**	**Phylum**	**Class**	**Query Covarage / Max Identity**	**Source**
1	BSII-HV-2(1)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
2	BSII-HC-2(1)	KT385760.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	100/100	Airborne fungi/Thailand
3	BSII-R-1(1)	KT968545.1	*Purpureocillium lilacinum*	Ascomycota	Sordariomycetes	100/99	Mitochondrion/China
4	BSII-R-2(1)	KT968545.1	*Purpureocillium lilacinum*	Ascomycota	Sordariomycetes	99/100	Mitochondrion/China
5	BSII(1.5)-HJ-3(1)	N/A	*Purpureocillium* sp.	Ascomycota	Sordariomycetes	N/A	N/A
6	BSII(1.5)-HV-1(1)	N/A	*Purpureocillium* sp.	Ascomycota	Sordariomycetes	N/A	N/A
7	BSII(1.5)-HV-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
8	BSII(1.5)-HV-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
9	BSII(1.5)-HC-1(1)	KF269188.1	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	100/92	Leaf endophyte/Australia
10	BSII(1.5)-HC-1(2)	N/A	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	N/A	N/A
11	BSII(1.5)-HC-2(1)	N/A	*Physalospora* sp.	Ascomycota	Sordariomycetes	N/A	N/A
12	BSII(1.5)-HC-2(2)	JQ341096.2	*Physalospora* sp.	Ascomycota	Sordariomycetes	97/94	Ebony endophyte/Cameroon
13	BSII(1.5)-HC-2(3)	KF269188.1	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	100/92	Leaf endophyte/Australia
14	BSII(1.5)-HC-3(1)	KP184331.1	*Acremonium* sp.	Ascomycota	Sordariomycetes	98/99	Rubber tree/Thailand
15	BSII(1.5)-HC-3(2)	KJ863528.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	100/99	Plant endophyte/China
16	BSII(3.5)-HJ-3(1)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
17	BSII(3.5)-HV-1(1)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
18	BSII(3.5)-HV-3(1)	N/A	*Amorosia* sp.	Ascomycota	Sordariomycetes	N/A	N/A
19	BSII(3.5)-HC-1(1)	KF269188.1	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	99/92	Leaf endophyte/Australia
20	BSII(3.5)-HC-1(2)	JN997374.1	*Hortaea werneckii*	Ascomycota	Dothideomycetes	98/99	Coral fungi/China
21	BSII(3.5)-HC-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
22	BSII(3.5)-HC-2(2)	AM292047.1	*Amorosia littoralis*	Ascomycota	Dothideomycetes	87/91	Marine intertidal sediment/Bahamas
23	BSII(3.5)-HC-2(3)	N/A	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	N/A	N/A
24	BSII(3.5)-HC-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
25	BSII(3.5)-R-3(1)	KJ439140.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	99/99	Root vegetables/Brazil
26	BSII(3.5)-R-3(2)	KT968545.1	*Purpureocillium lilacinum*	Ascomycota	Sordariomycetes	100/99	Mitochondrion/China
27	BSII(MH)-HV-2(1)	LC105679.1	*Penicillium citrinum*	Ascomycota	Eurotiomycetes	98/100	Manuscript/Indonesia
28	BSII(MH)-HV-3(1)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A
29	BSII(MH)-HC-2(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
30	BSII(MH)-HC-3(1)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A

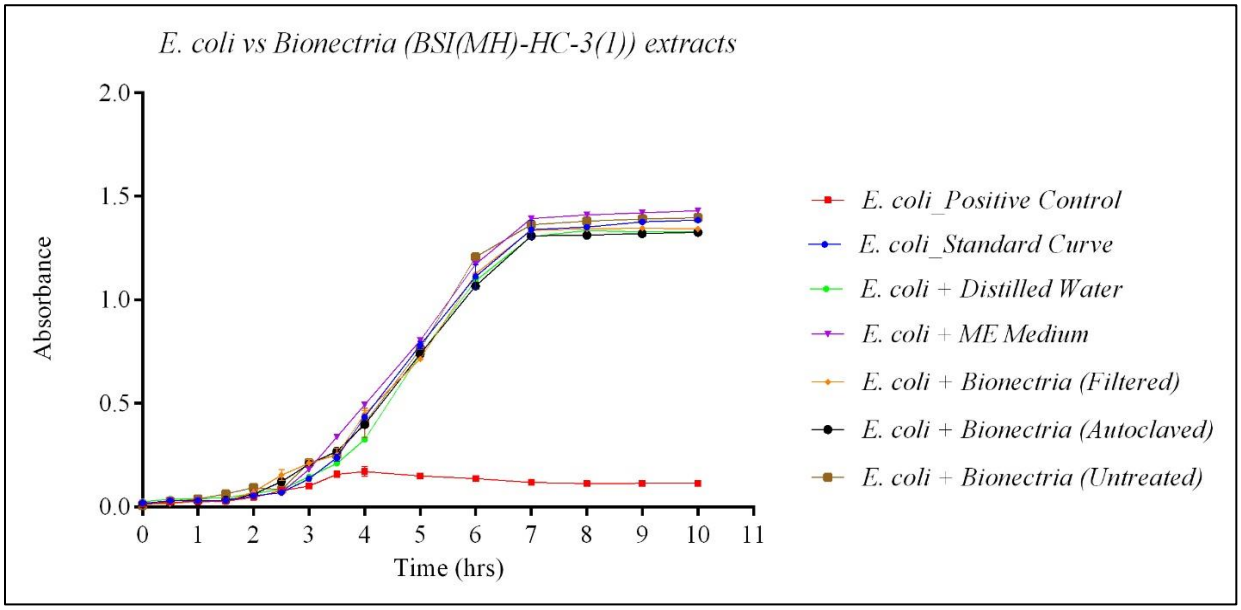
Appendix D**:** Fungi isolated from the back mangrove Avicennia germinans in Bahia Salinas, Cabo Rojo, Puerto Rico.

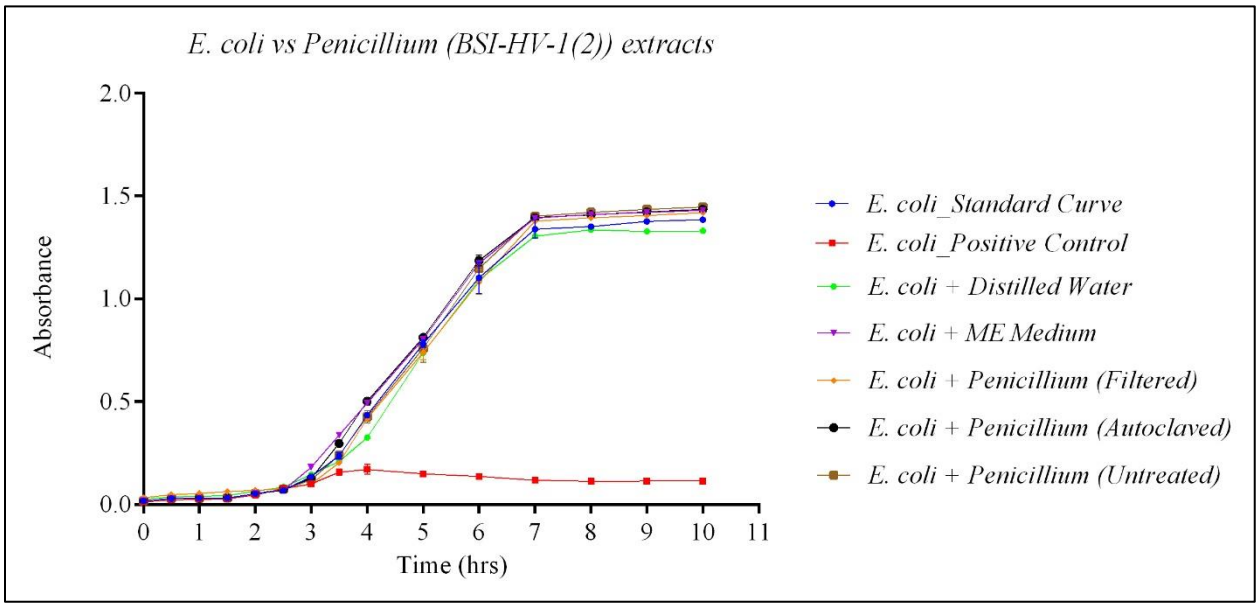
BSIII							
DNA/PCR Codes	**Plates Codes**	**Accesion Number**	**Identification**	**Phylum**	**Class**	**Query Covarage / Max Identity**	**Source**
1	BSIII-HC-1(2)	KM362374.1	*Bipolaris* sp.	Ascomycota	Dothideomycetes	98/100	Grapevine endophyte/Brazil
2	BSIII-HC-1(2)(1)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
3	BSIII-HC-1(2)(2)	N/A	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	N/A	N/A
4	BSIII-HC-2(1)	N/A	*Teratosphaeria* sp.	Ascomycota	Dothideomycetes	N/A	N/A
5	BSIII(1.5)-HC-1(1)	N/A	*Cladosporium* sp.	Ascomycota	Dothideomycetes	N/A	N/A
6	BSIII(1.5)-HC-2(1)	N/A	*Teratosphaeria sp.*	Ascomycota	Dothideomycetes	N/A	N/A
7	BSIII(1.5)-HC-2(2)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A
8	BSIII(1.5)-HC-2(3)	N/A	*Penicillium sp.*	Ascomycota	Eurotiomycetes	N/A	N/A
9	BSIII(1.5)-HC-3(1)	JN851005.1	*Cladosporium sphaerospermum*	Ascomycota	Dothideomycetes	99/99	Coral reef/China
10	BSIII(1.5)-R-3(1)(1)	KC845931.1	*Cladosporium sphaerospermum*	Ascomycota	Dothideomycetes	98/99	Noni tree fungi/China
11	BSIII(1.5)-R-3(1)(2)	N/A	*Penicillium* sp.	Ascomycota	Eurotiomycetes	N/A	N/A
12	BSIII(1.5)-HC-3(2)	N/A	*Bionectria* sp.	Ascomycota	Sordariomycetes	N/A	N/A
13	BSIII(3.5)-HC-2(1)	N/A	*Cladosporium* sp.	Ascomycota	Dothideomycetes	N/A	N/A
14	BSIII(3.5)-HC-2(2)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
15	BSIII(3.5)-HC-3(1)	N/A	*Hortaea* sp.	Ascomycota	Dothideomycetes	N/A	N/A
16	BSIII(3.5)-R-3(2)	KP735247.1	*Cladosporium sphaerospermum*	Ascomycota	Dothideomycetes	97/100	Chicken feces/Philippines
17	BSIII(MH)-R-1(1)	N/A	*Purpureocillium* sp.	Ascomycota	Sordariomycetes	N/A	N/A
18	BSIII(MH)-R-2(1)	KP735247.1	*Cladosporium sphaerospermum*	Ascomycota	Dothideomycetes	97/100	Chicken feces/Philippines
19	BSIII(MH)-R-2(2)	DQ092532.1	*Cladosporium* sp.	Ascomycota	Dothideomycetes	98/99	Marine sponge/Hawaii
20	BSIII(MH)-R-3(1)	KT803070.1	*Aspergillus terreus*	Ascomycota	Eurotiomycetes	99/99	Cotton root/China

Appendix E: Isolates from this experiment founded as endophytes or mangrove endophytes.

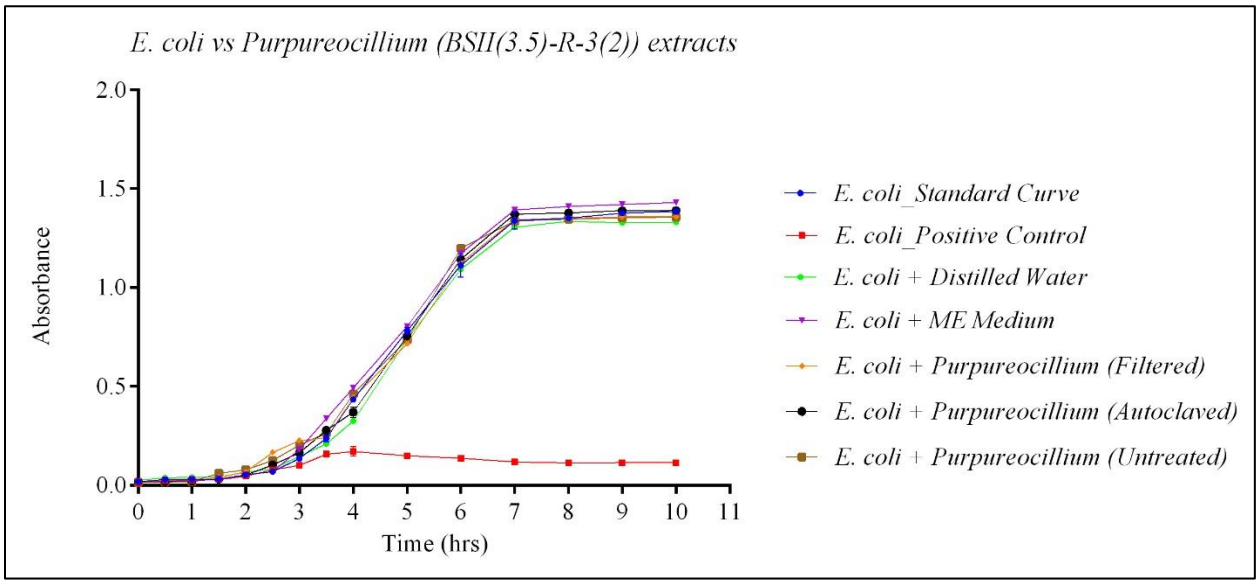
Fungus	Plant	Country/Reference	Mangrove	Country/Reference
Acremonium	*Coffea arabica*	Colombia/ Vega et al. 2008	*Rhizophora mucronata*	India/ Ananda and Sridhar 2002
Amorosia	Not found	Not found	*Avicennia marina*	China/Li, Xiong, et al. 2016
Aspergillus sp.	*Casuarina equisetifolia*	Puerto Rico/ Bayman et al. 1998	*Rhizophora mucronata*	India/Ananda and Sridhar 2002
Aspergillus clavatus	*Myoporum bontioides*	China/Li et al. 2017	*Avicennia marina*	India/Bharathidasan and Panneerselvam 2015
Aspergillus flavus	*Hibiscus tiliaceus* *Boswellia sacra*	China/Wang et al. 2012 Saudi Arabia/El-nagerabi et al. 2014	*Avicennia marina*	India/Bharathidasan and Panneerselvam 2015
Aspergillus niger	*Sesbania bispinosa*	India/Anita et al. 2009	*Avicennia alba*, *Sonneratia* and *Laguncularia racemosa*	Indonesia/ Rahmansyah and Rahmansyah 2013 Brazil/Silva et al. 2011
Bionectria	*Nothapodytes foetida*	India/ Samaga et al. 2013	*Sonneratia caseolaris*	China/ Ebrahim et al. 2012
Bipolaris	*Ocimum sanctum* *Enhalus acoroides*	India/ Chowdhary and Kaushik 2015 Thailand/ Supaphon et al. 2012	Not specified	Thailand/ Doilom et al. 2017
Cladosporium	*Coffea arabica*	Puerto Rico/ Vega et al. 2008	*Kandelia candel*, *Aegiceras corniculatum* *Avicennia marina* and *Rhizopora apiculata*	China/Li, Xiong, et al. 2016 India/Kumaresan and Suryanarayanan 2002
Hortaea	*Bletilla ochracea*	China/ Tao et al. 2008	*Aegiceras corniculatum*, *Avicennia marina and Bruguiera gymnorrhiza*	China/Chen et al. 2012; Li, Xiong, et al. 2016
Nemania	*Pinus tabulaeformis*	China/Guo et al. 2003	*Rhizophora apiculata*	Thailand/Doilom et al. 2017
Nigrospora	*Manilkara bidentata*	Puerto Rico/ Lodge et al. 1996	*Rhizophora mucronata*	India/Ananda and Sridhar 2002
Penicillium	*Manilkara bidentate* *Casuarina equisetifolia*	Puerto Rico/ Lodge et al. 1996 Puerto Rico/ Bayman et al. 1998	*Rhizophora mangle*	Brazil/Costa et al. 2012
Physalospora	*Diospyros crassiflora*	Cameroon/ (Douanla-Meli and Langer 2012)	Not found	Not found
Pochonia	*Hordeum vulgare*	Spain/Larriba et al. 2015; Maciá-Vicente et al. 2009	Not found	Not found
Purpureocillium	*Gossypium hirsutum*	EEUU/Castillo-López et al. 2014	*Kandelia candel*	China/ Gong et al. 2017
Simplicillium	*Enhalus acoroides* *Pinus thunbergii*	Thailand/ Supaphon et al. 2012 Korea/ Min et al. 2014	Not found	Not found
Stereum	*Hevea* *Pinus thunbergii*	Peru, Mexico, Brazil and Cameroon/ Martin et al. 2015 Korea/ Min et al. 2014	Not found	Not found
Teratosphaeria	*Pinus clausa*	EEUU/ Padumadasa et al. 2018	Not found	Not found
Wrightoporia	*Theobroma gileri*	Ecuador/ Thomas et al. 2008	Not found	Not found

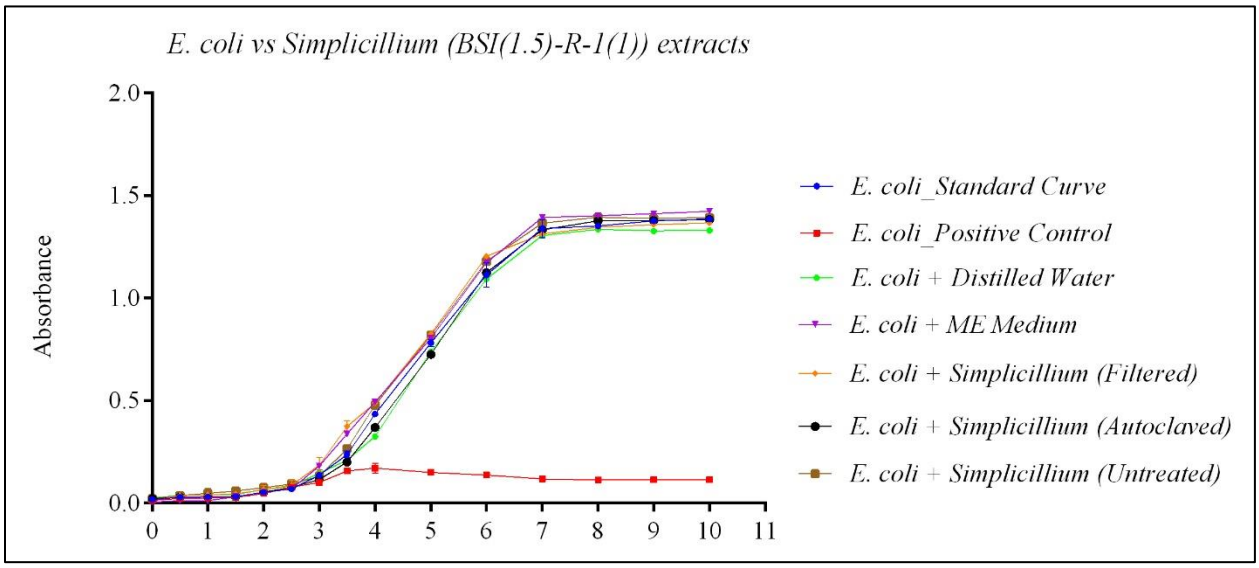
Appendix F: First Assay: *E. coli* growth curves showing not significant differences between treatments (p-value > 0.05).





Appendix F**:** First Assay: *E. coli* growth curves showing not significant differences between treatments (p-value > 0.05).





Appendix G: First Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).

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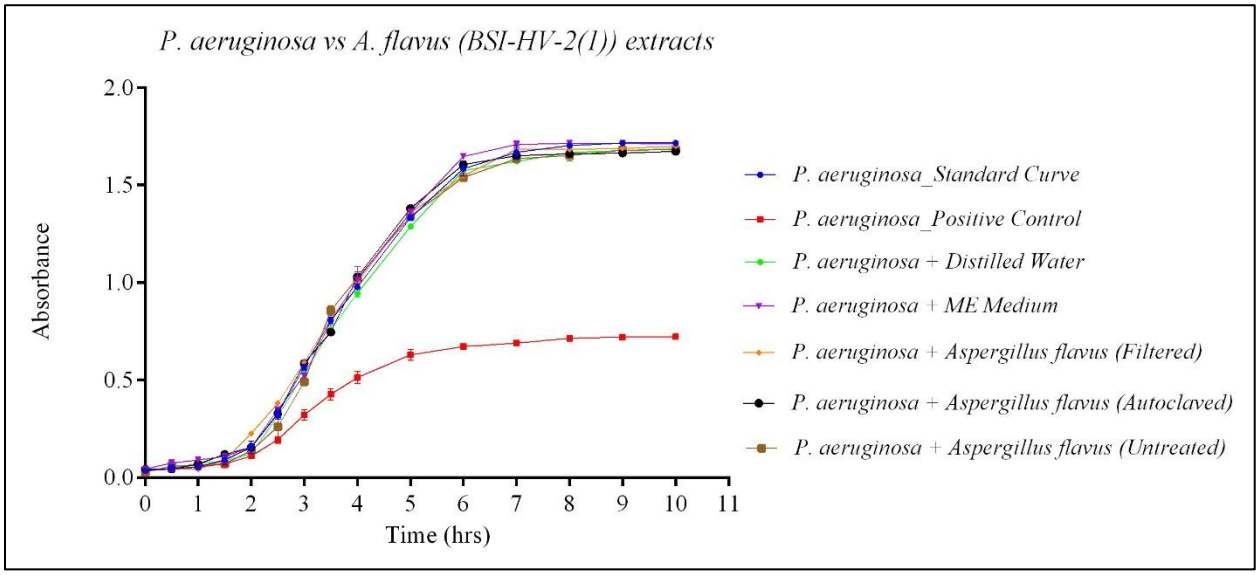
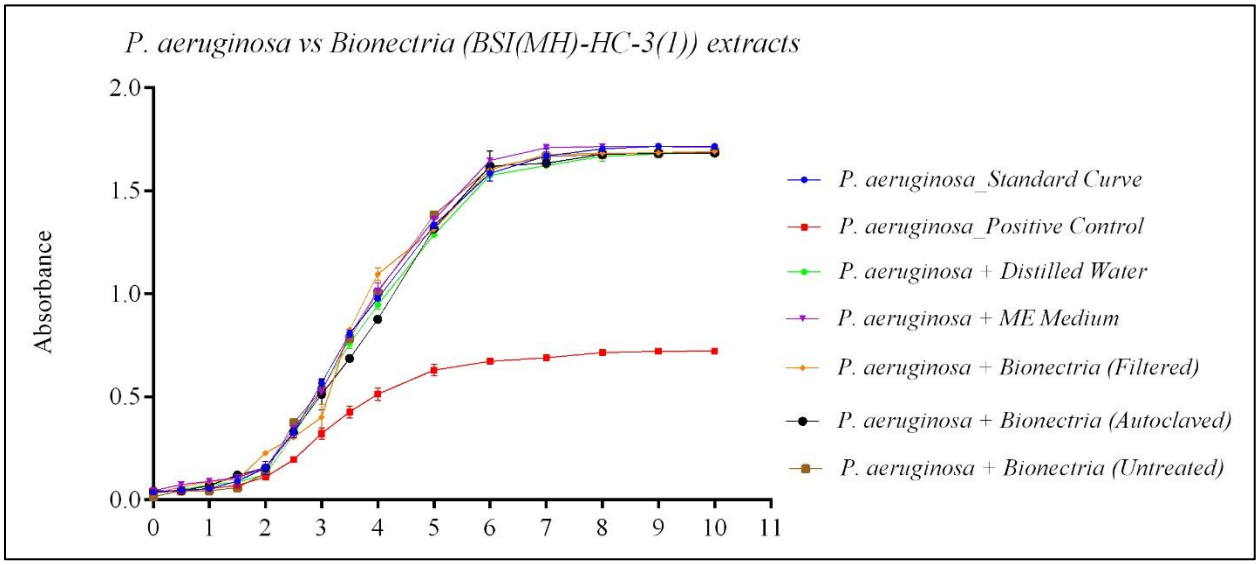
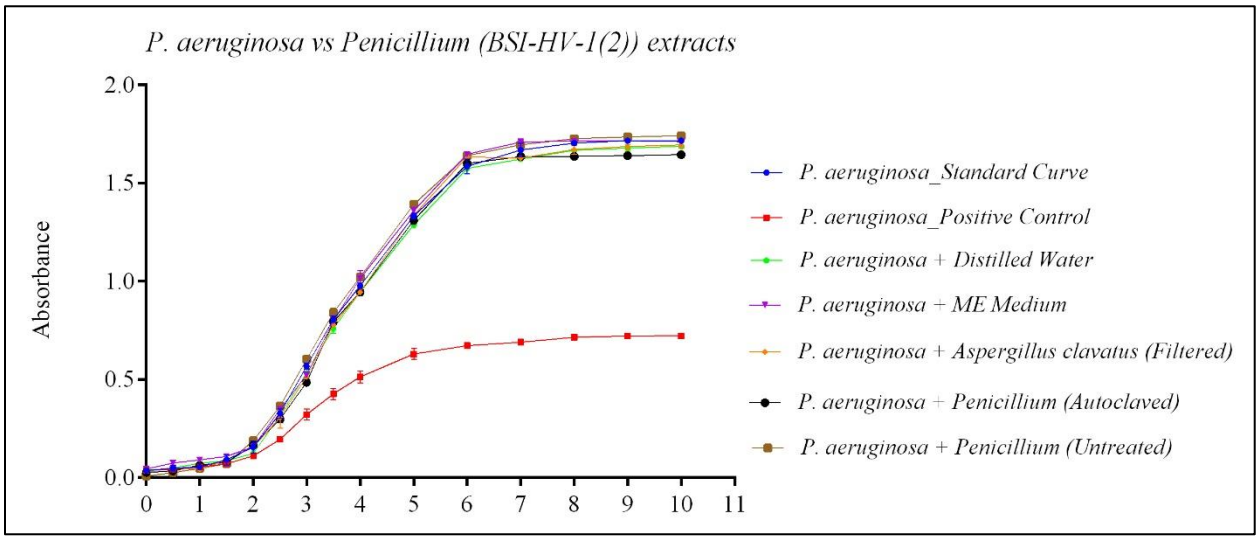
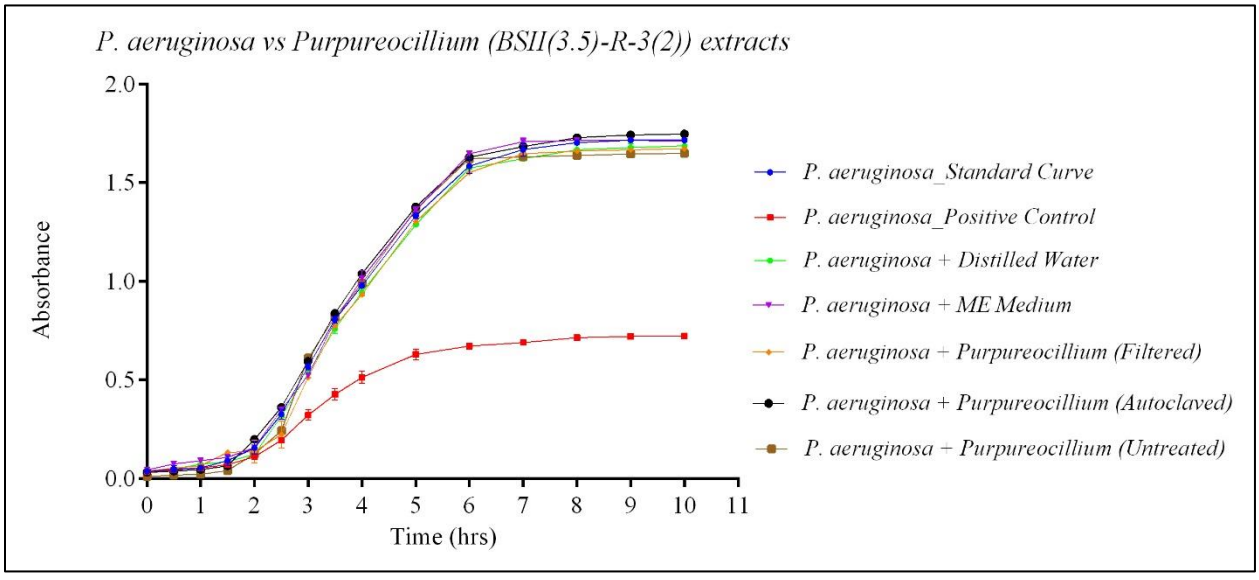


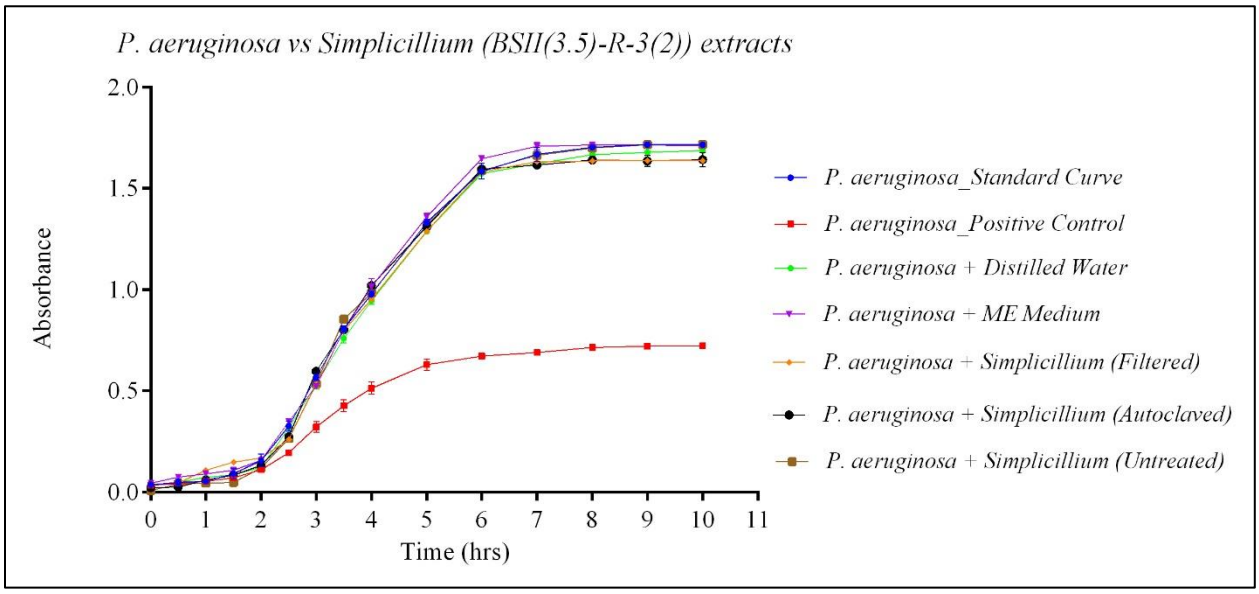


Appendix G**:** First Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).

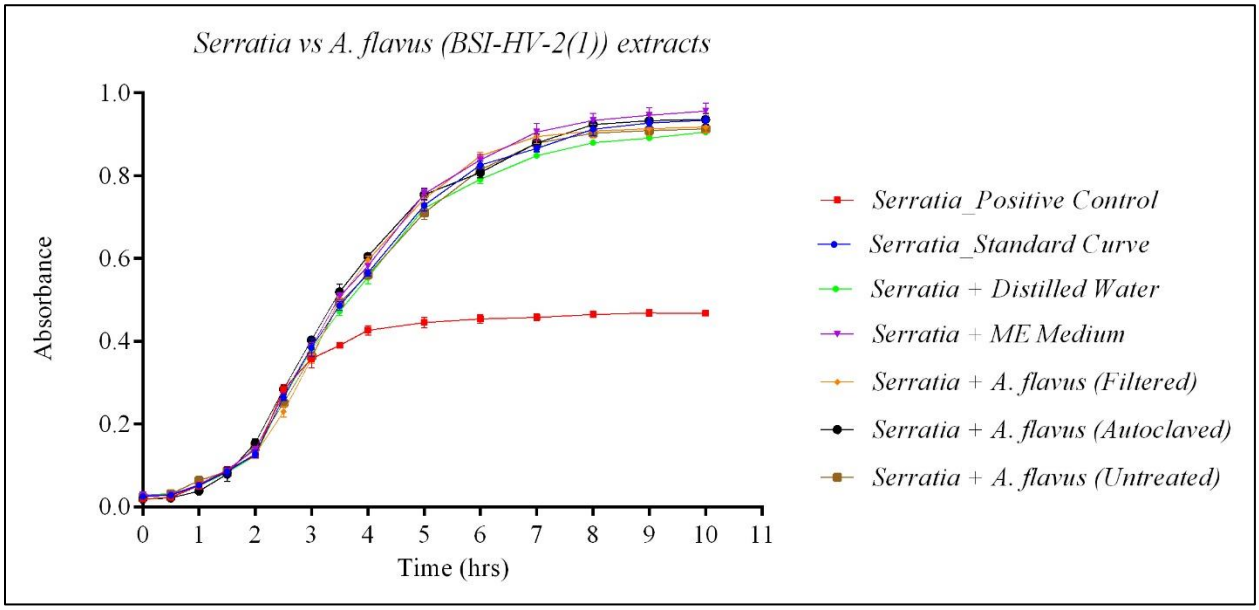


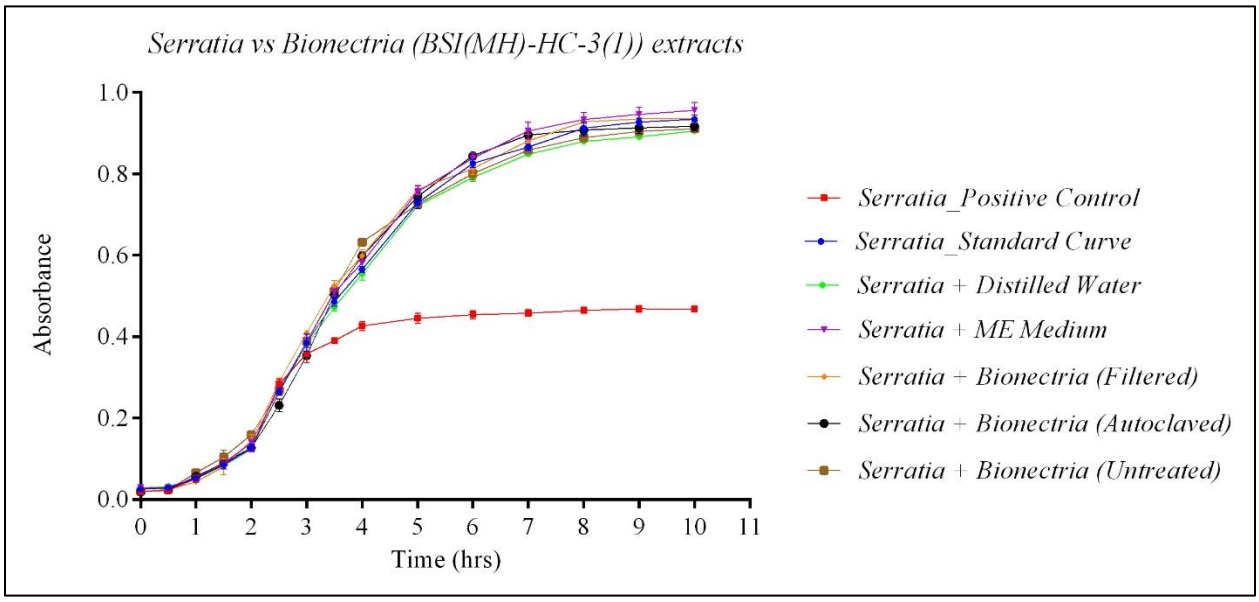


Appendix G**:** First Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).

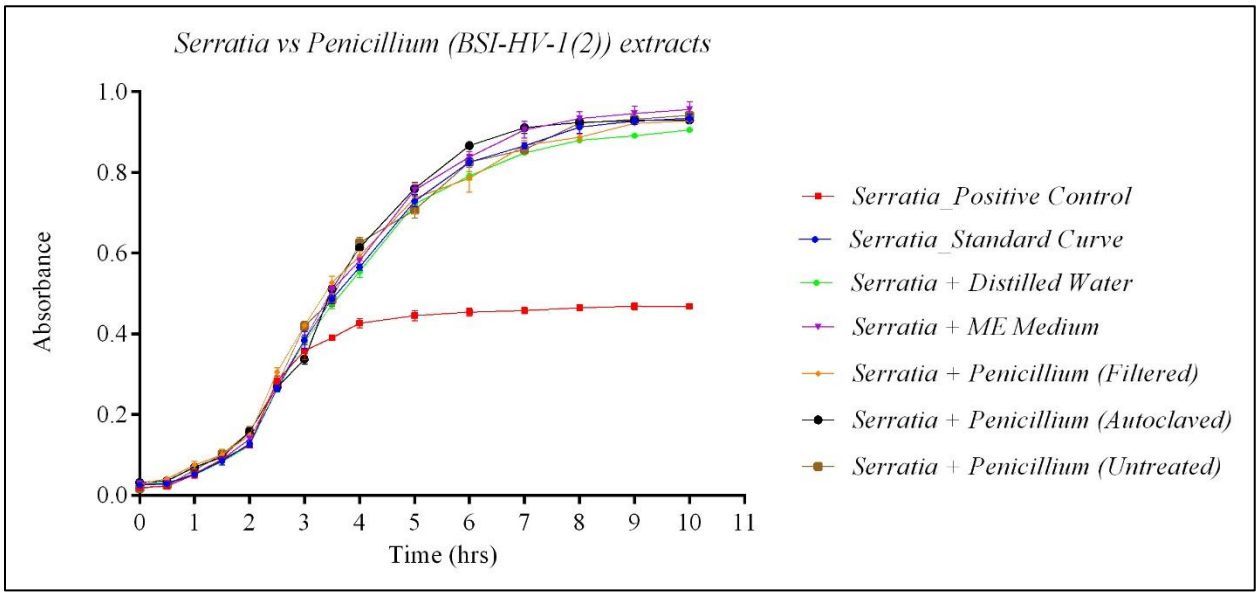


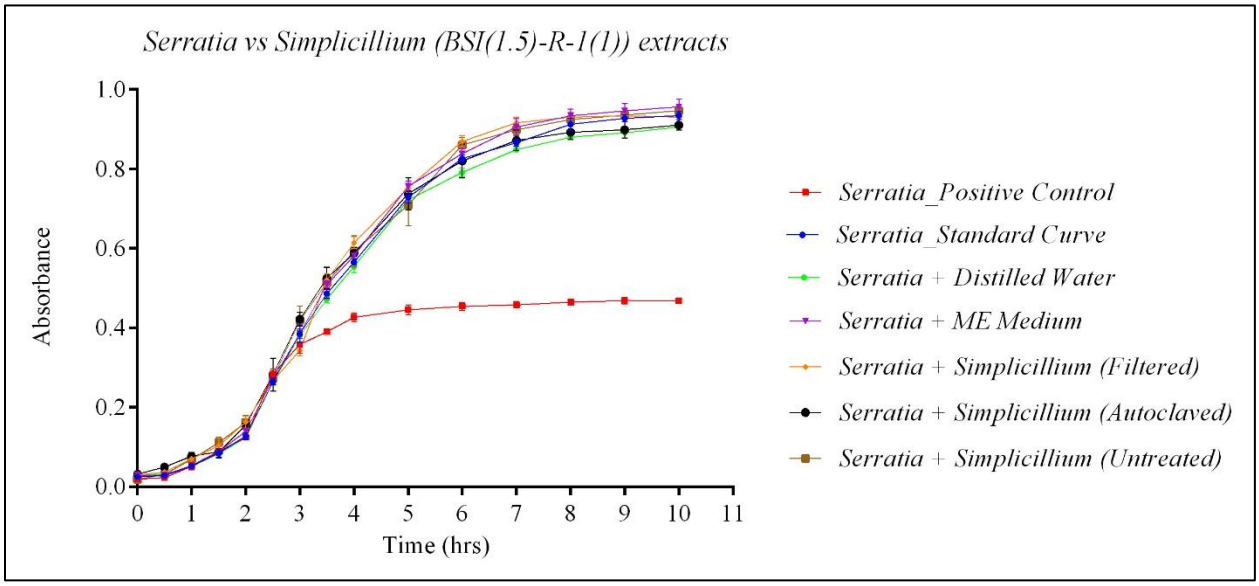
Appendix H: First Assay: *S. marcescens* growth curves showing not significant differences between treatments (p-value > 0.05).



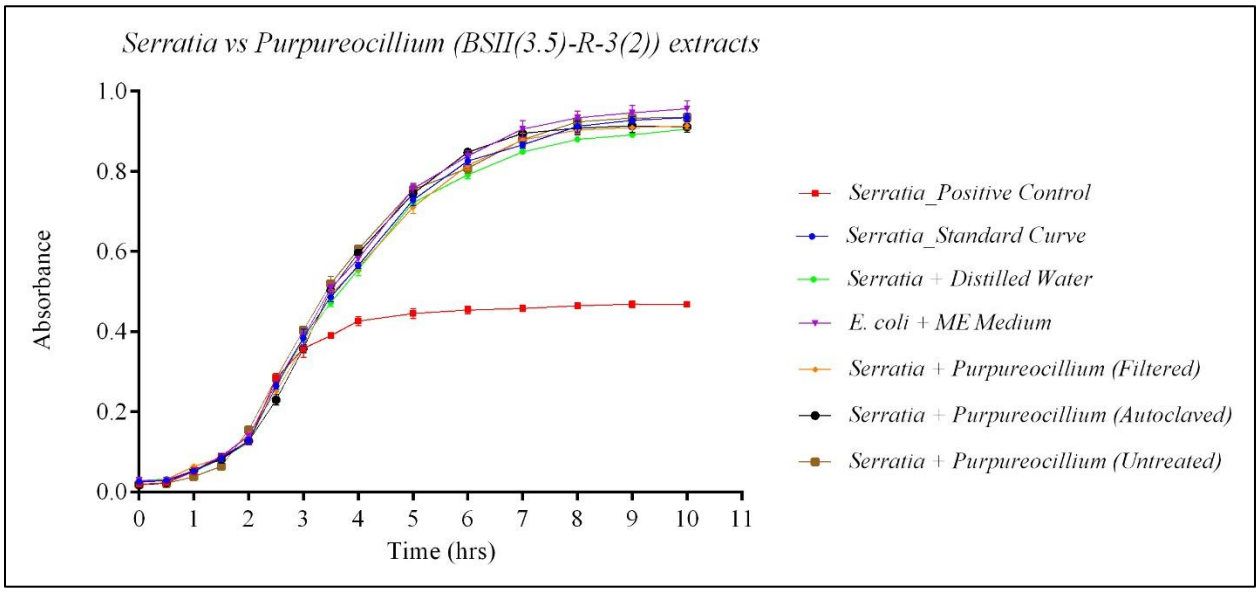


Appendix H**:** First Assay: *S. marcescens* growth curves showing not significant differences between treatments (p-value > 0.05).

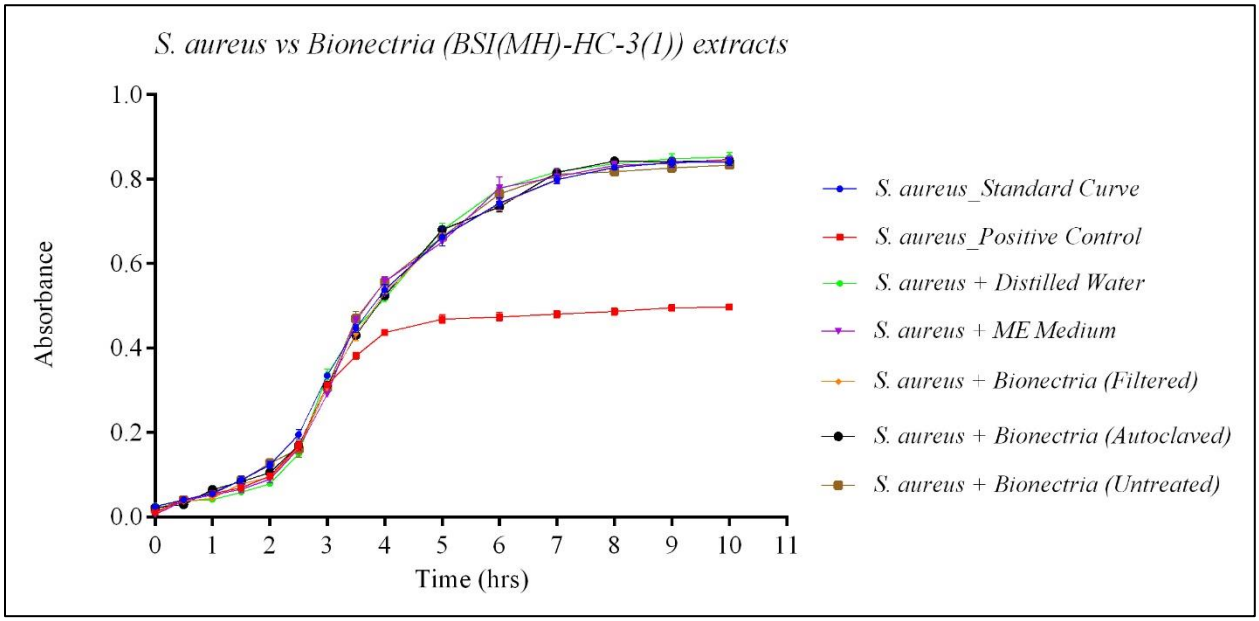


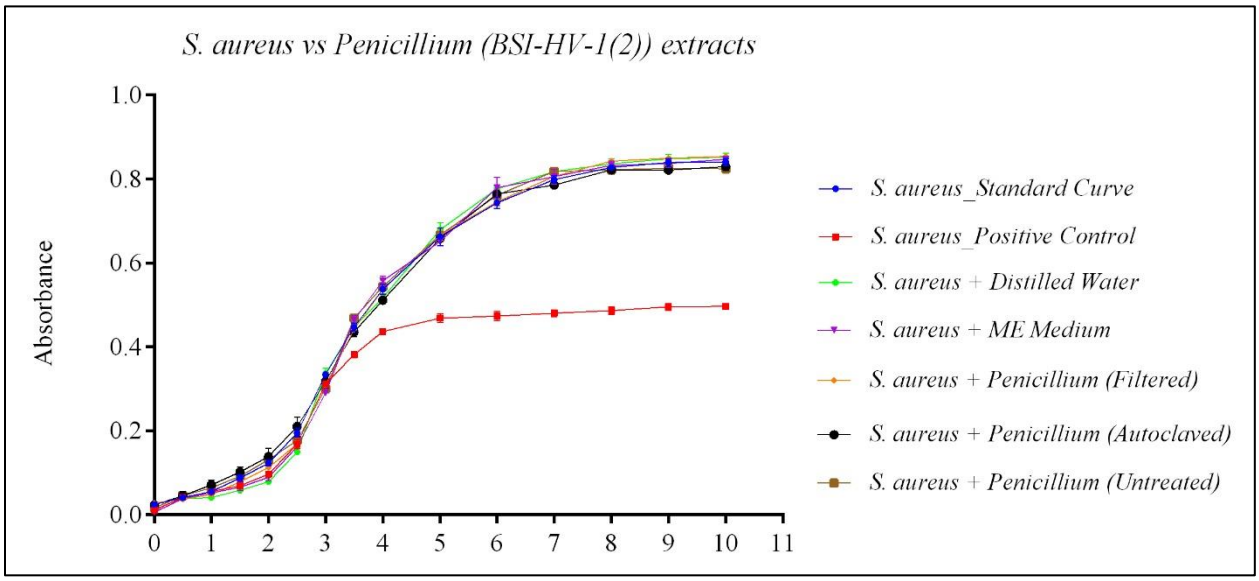


Appendix H**:** First Assay: *S. marcescens* growth curves showing not significant differences between treatments (p-value > 0.05).

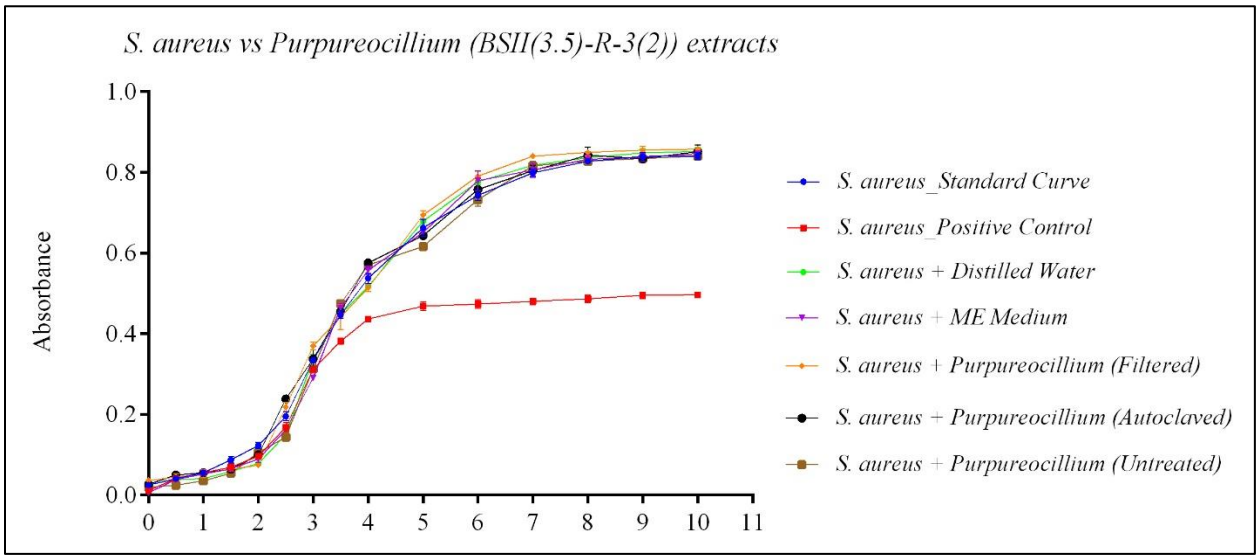


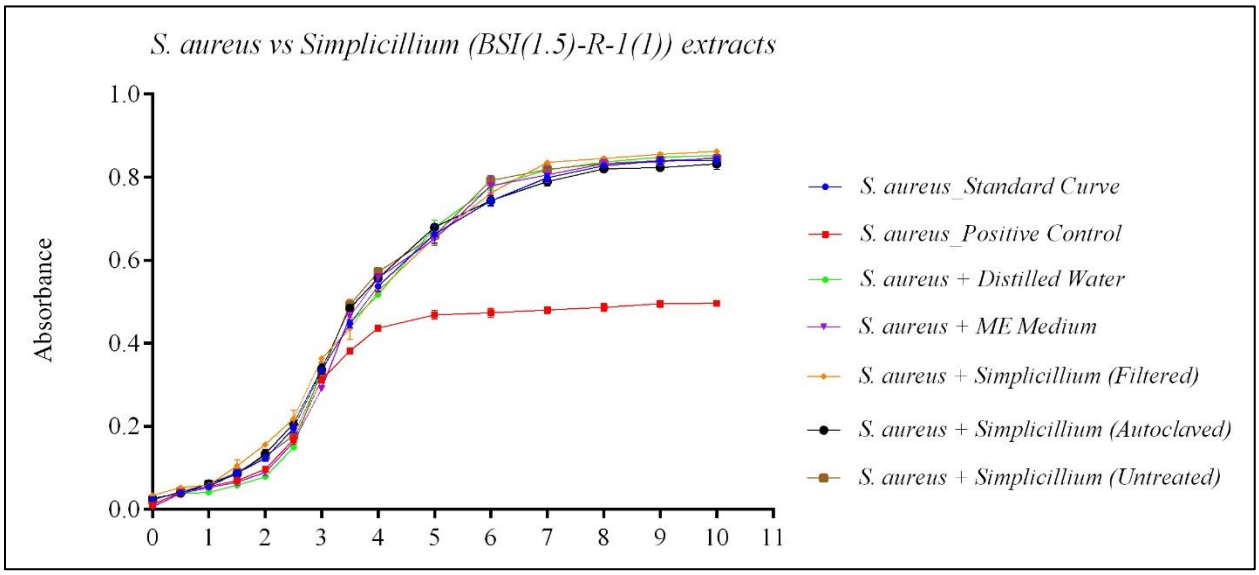
Appendix I: First Assay: *S. aureus* growth curves showing not significant differences between treatments (p-value > 0.05).



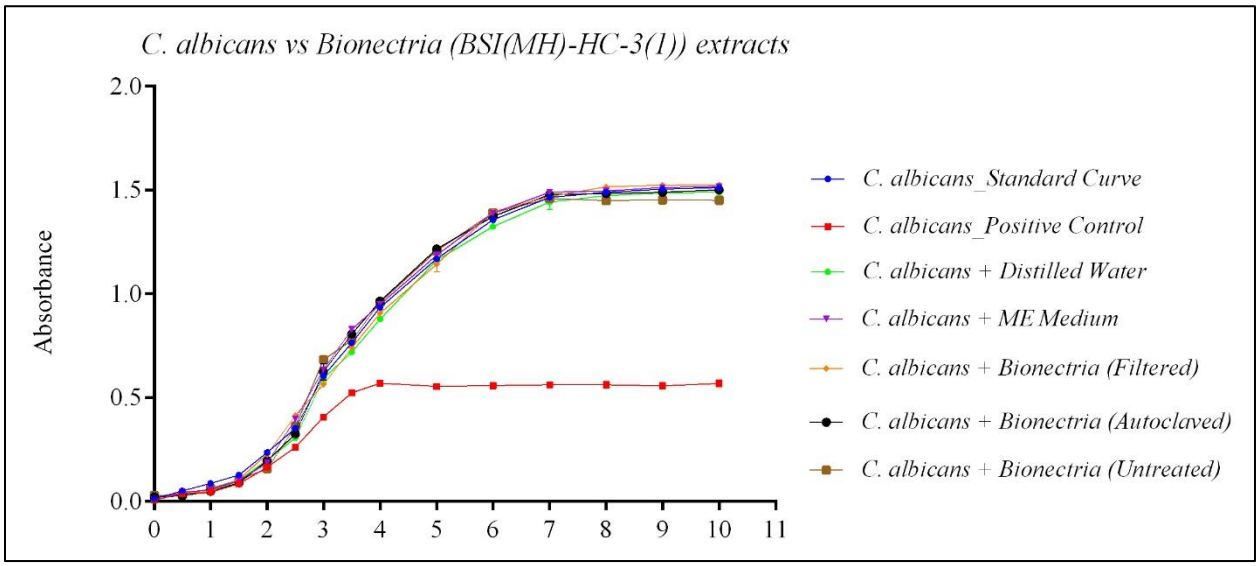


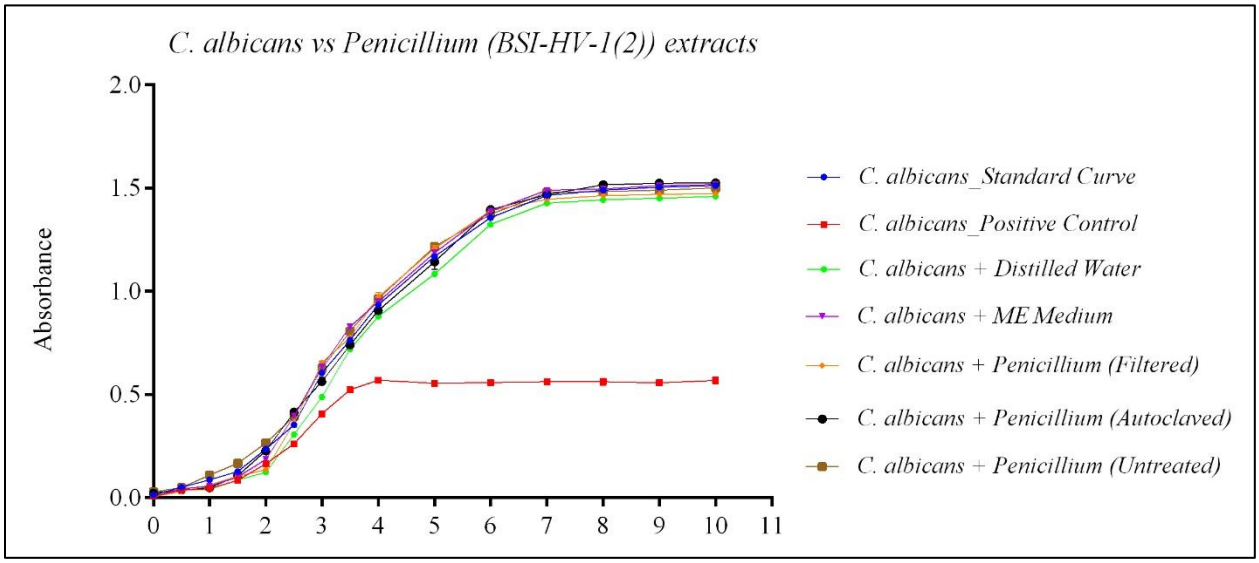
Appendix I**:** First Assay: *S. aureus* growth curves showing not significant differences between treatments (p-value > 0.05).



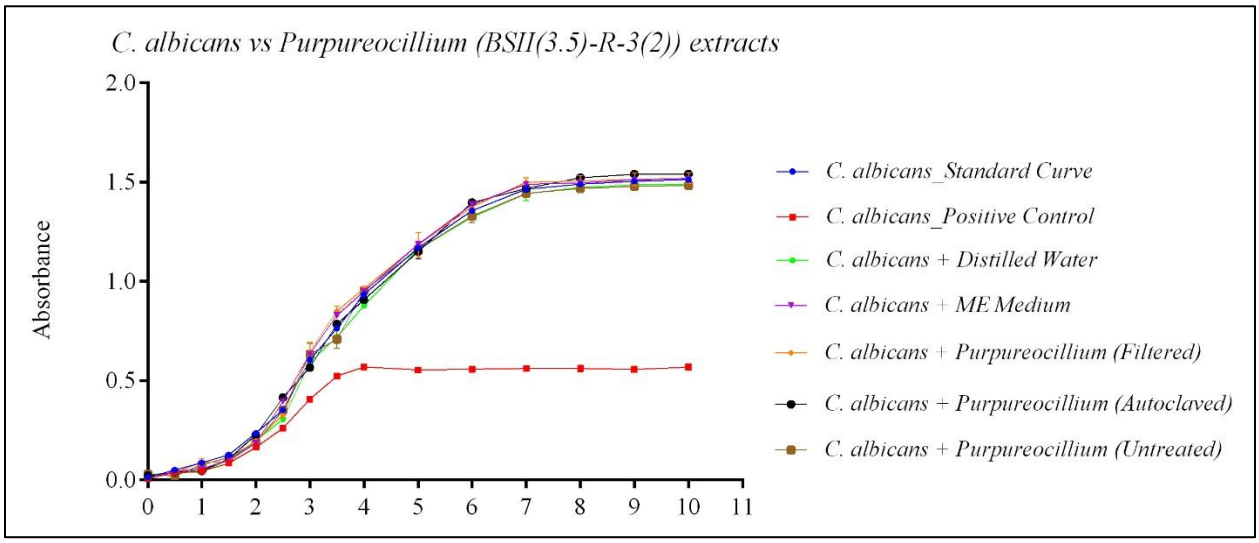


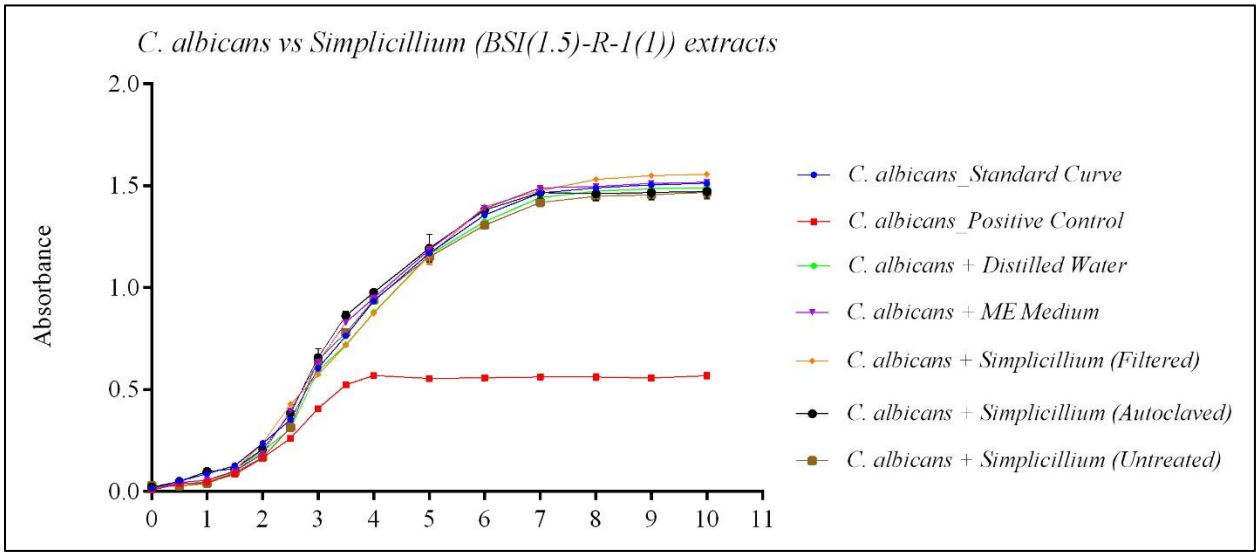
Appendix J: First Assay: *C. albicans* growth curves showing not significant differences between treatments (p-value > 0.05).



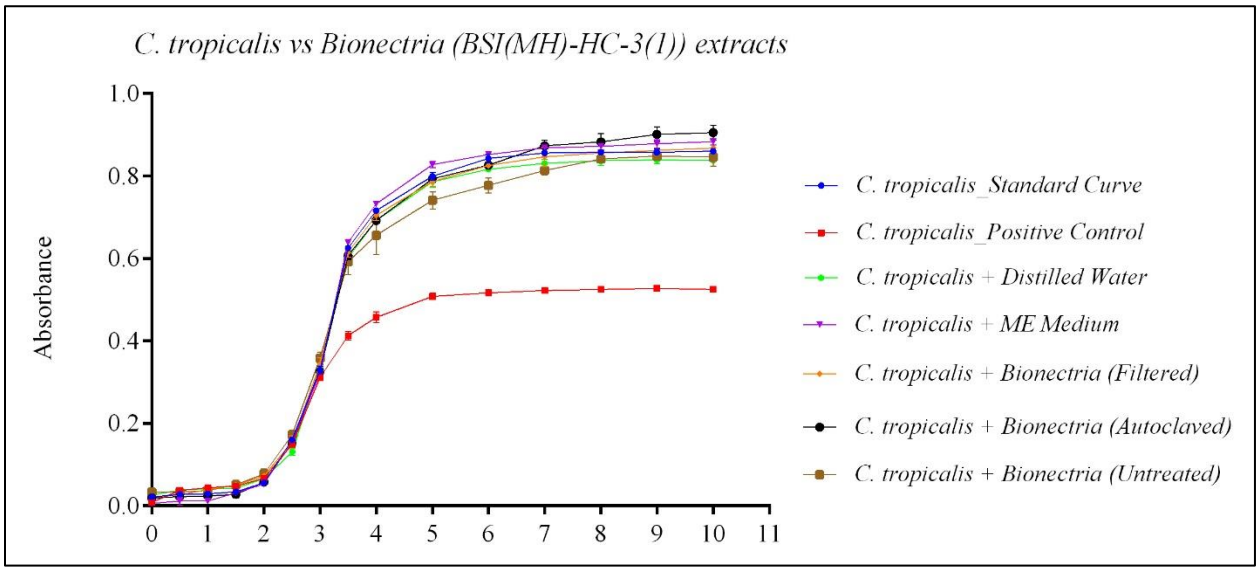


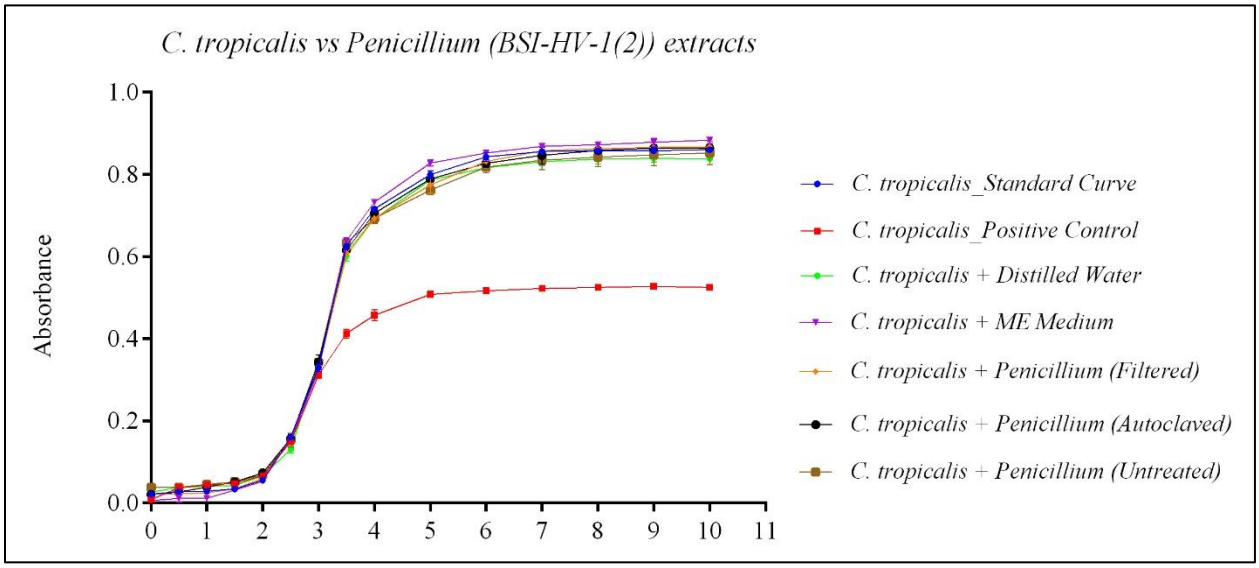
Appendix J**:** First Assay: *C. albicans* growth curves showing not significant differences between treatments (p-value > 0.05).



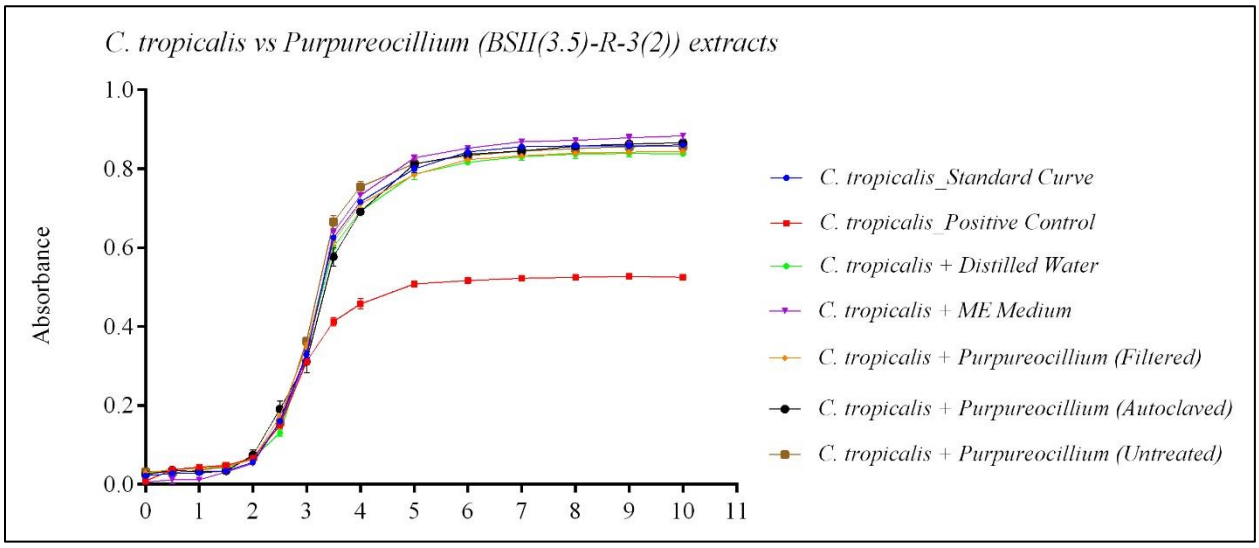


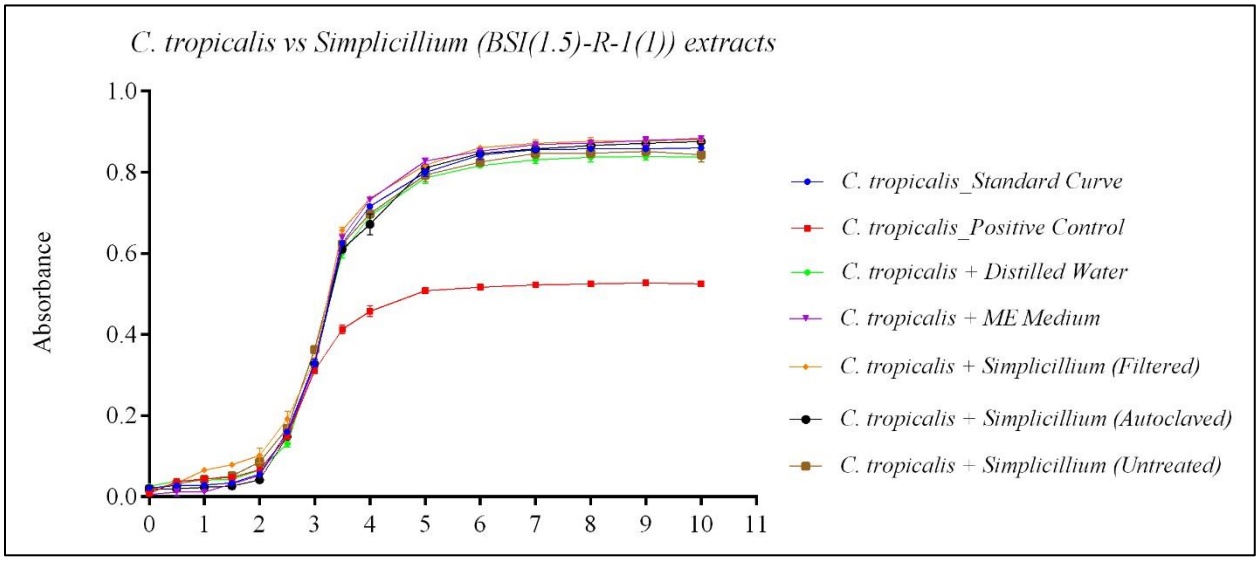
Appendix K: First Assay: *C. tropicalis* growth curves showing not significant differences between treatments (p-value > 0.05).



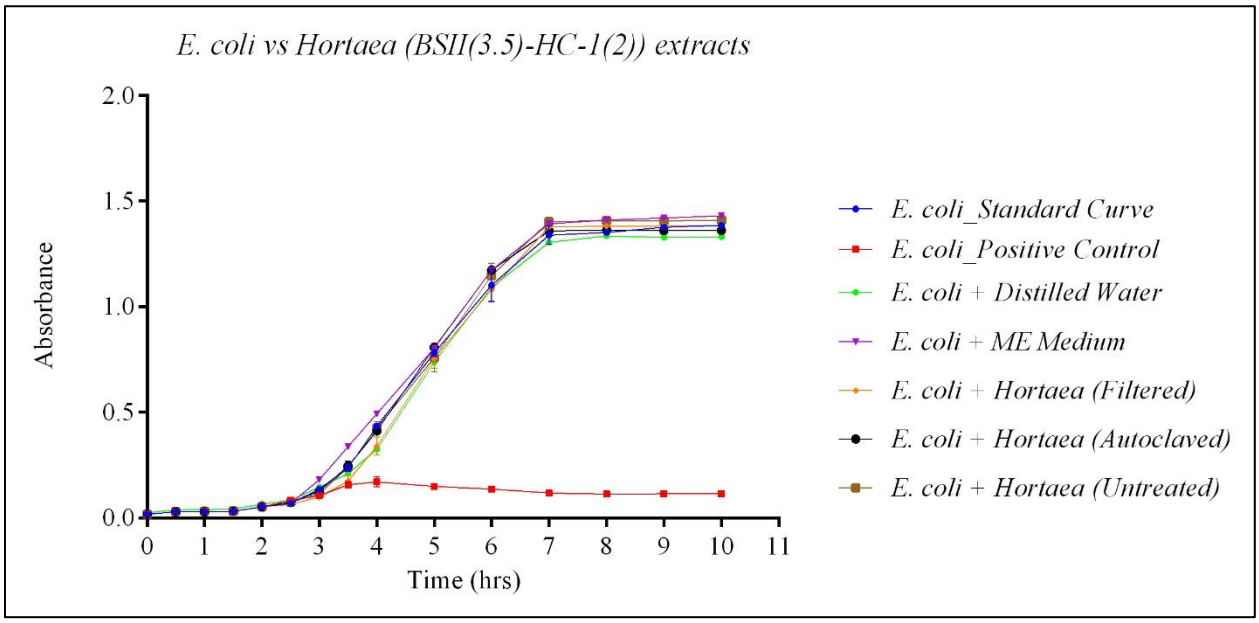


Appendix K**:** First Assay: *C. tropicalis* growth curves showing not significant differences between treatments (p-value > 0.05).

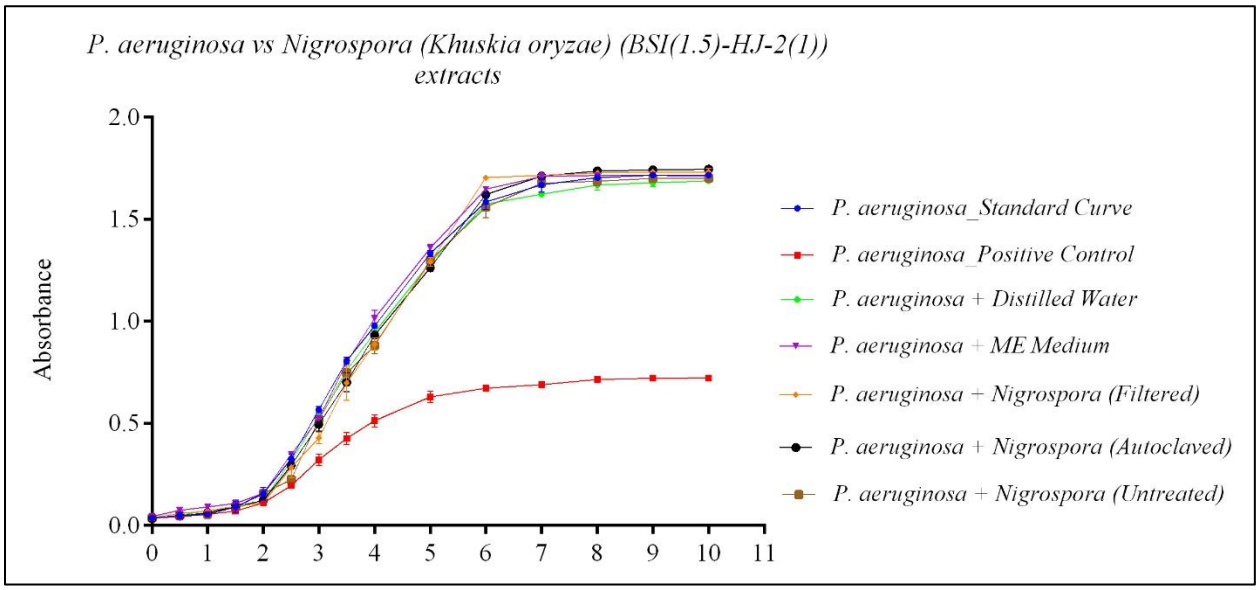


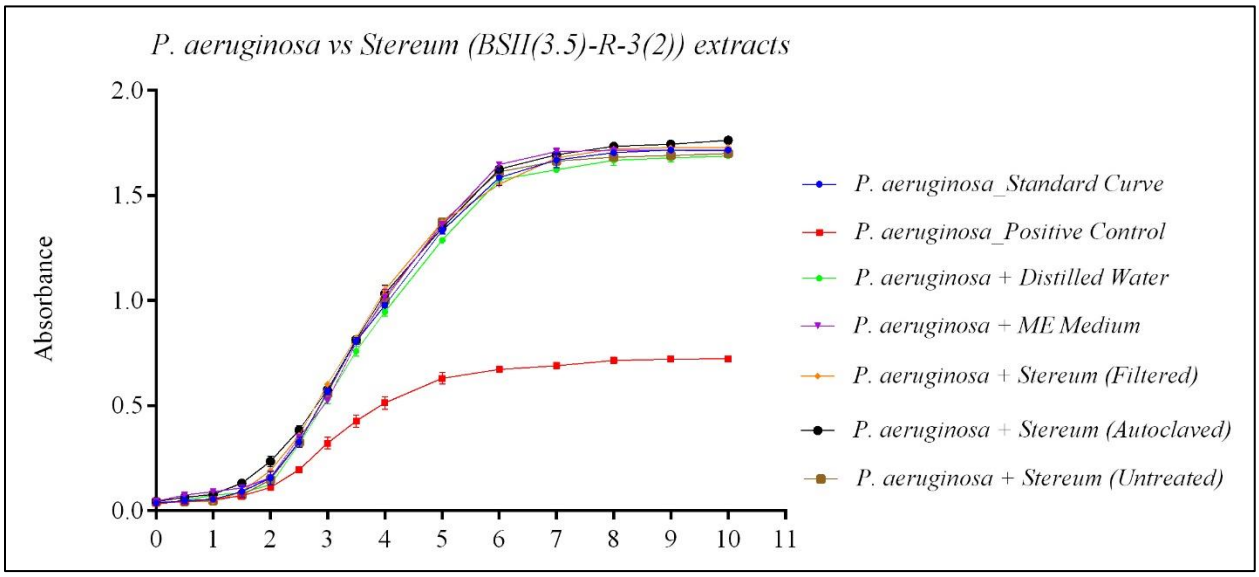


Appendix L: Second Assay: *E. coli* growth curves showing not significant differences between treatments (p-value > 0.05).

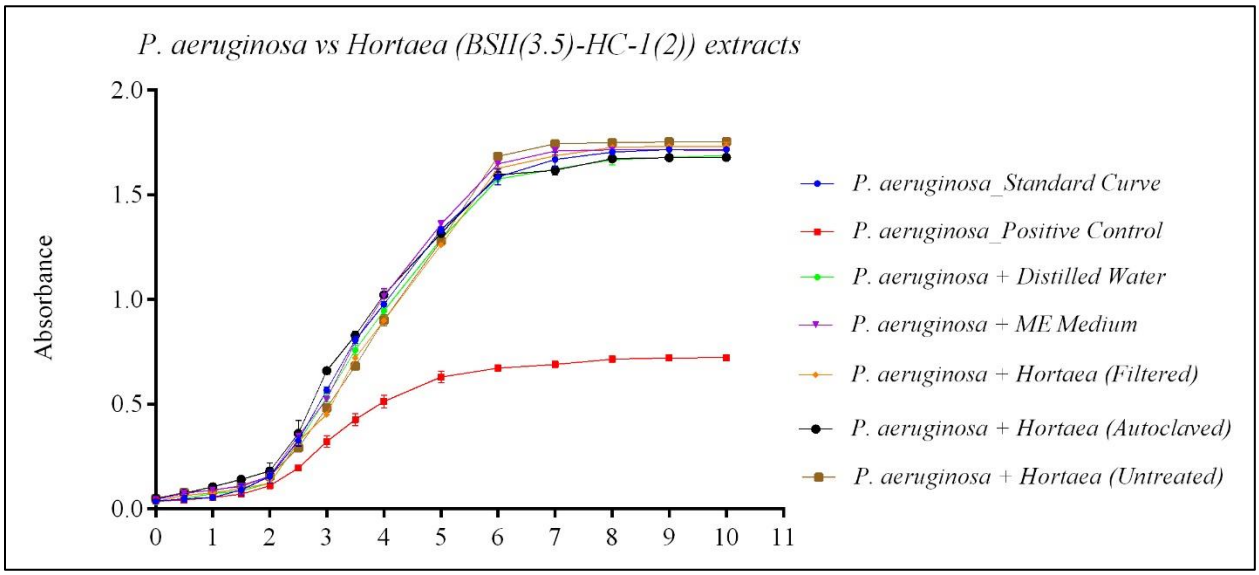


Appendix M: Second Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).

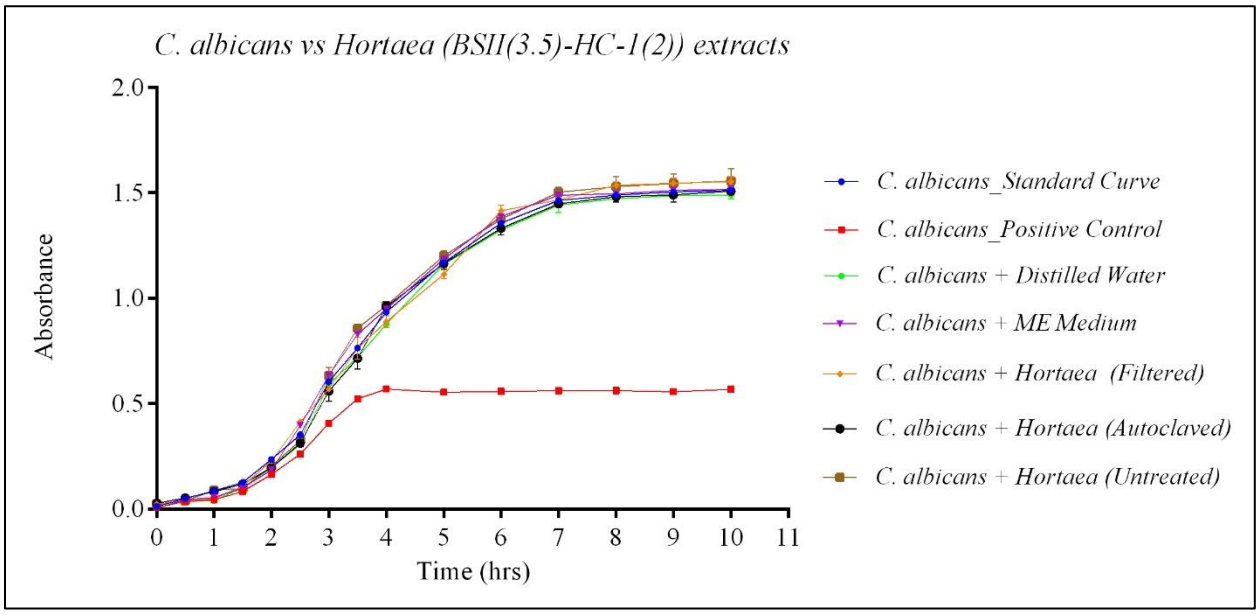


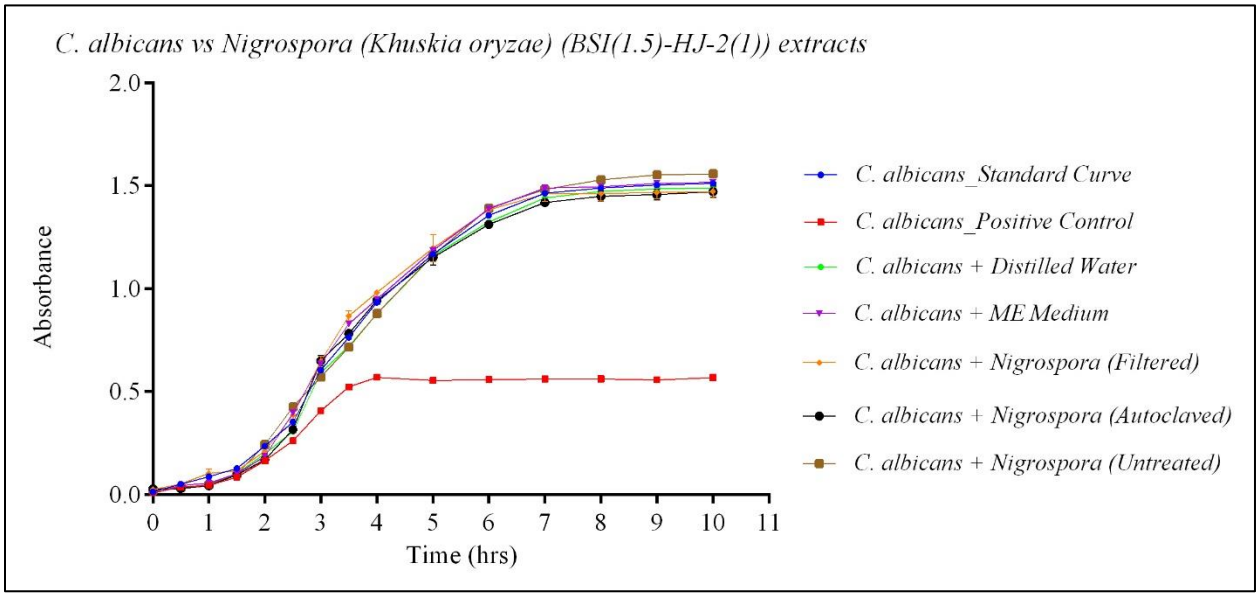


Appendix M**:** Second Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).

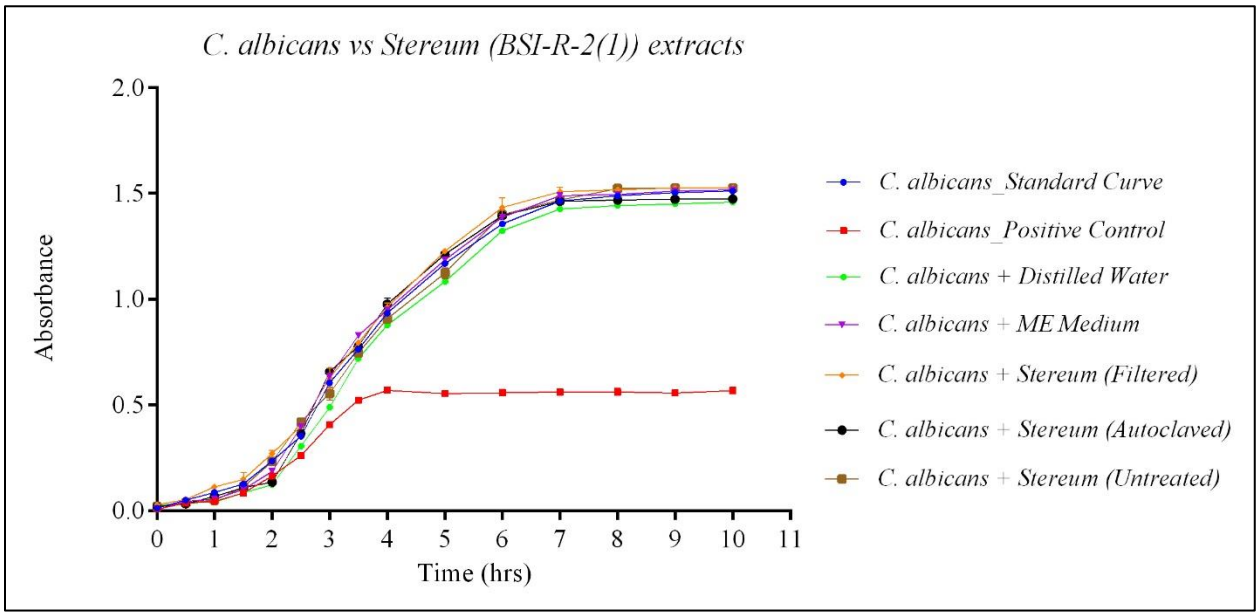


Appendix N: Second Assay: *C. albicans* growth curves showing not significant differences between treatments (p-value > 0.05).

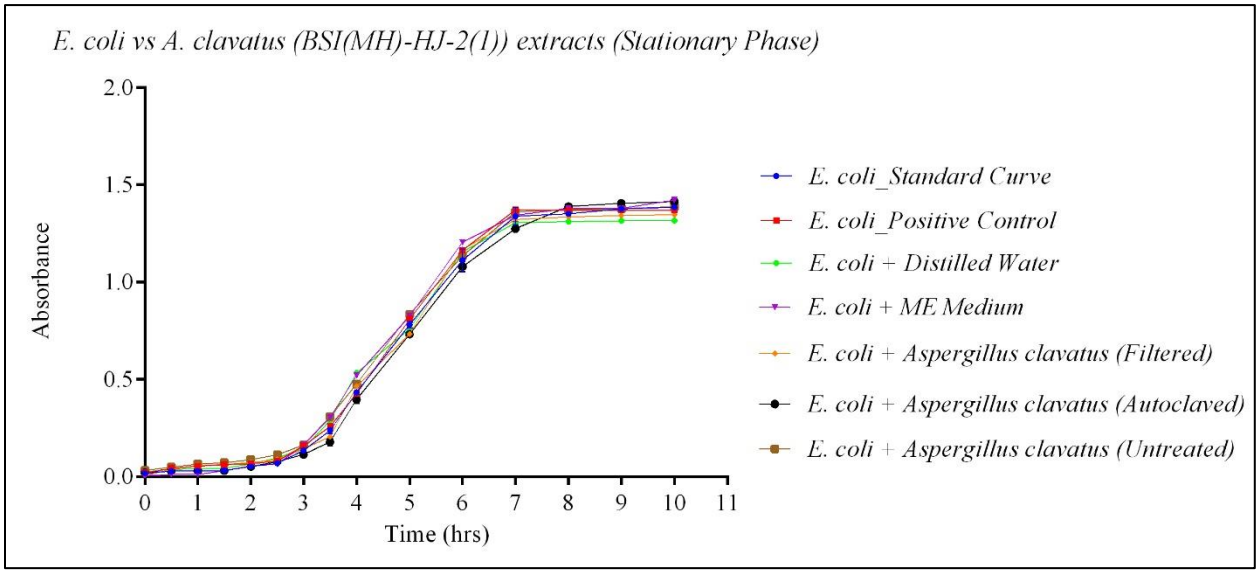


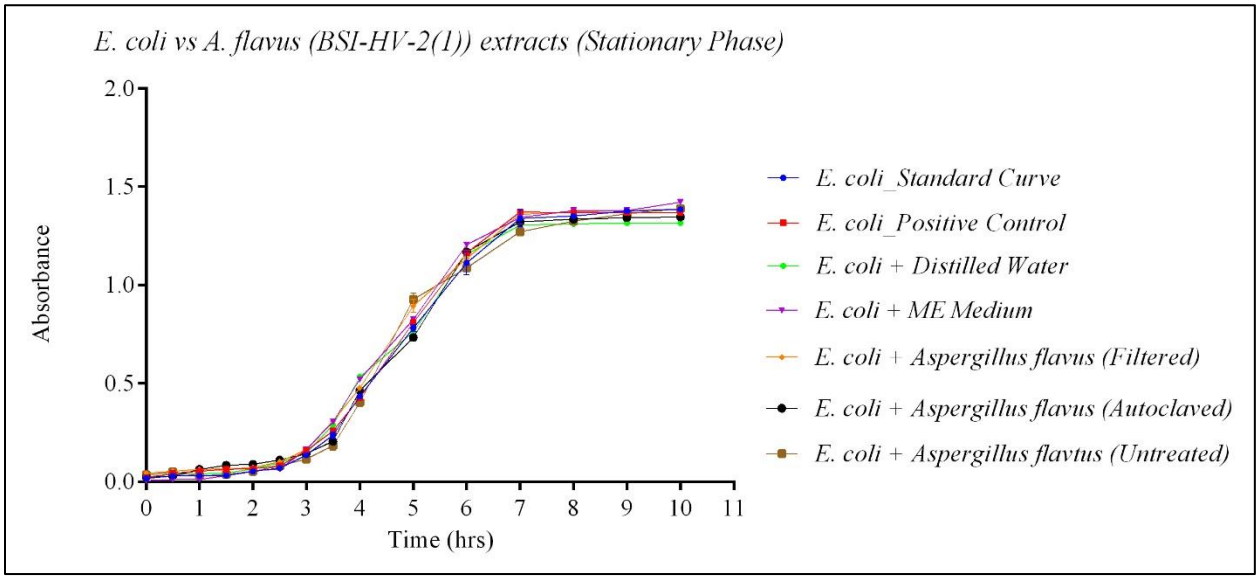


Appendix N**:** Second Assay: *C. albicans* growth curves showing not significant differences between treatments (p-value > 0.05).

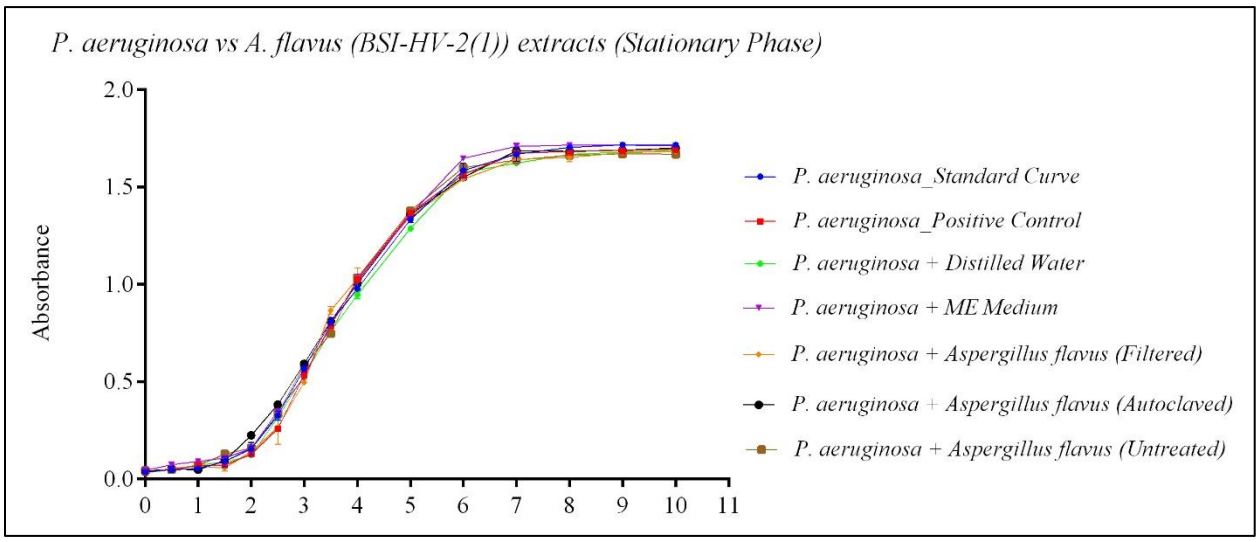


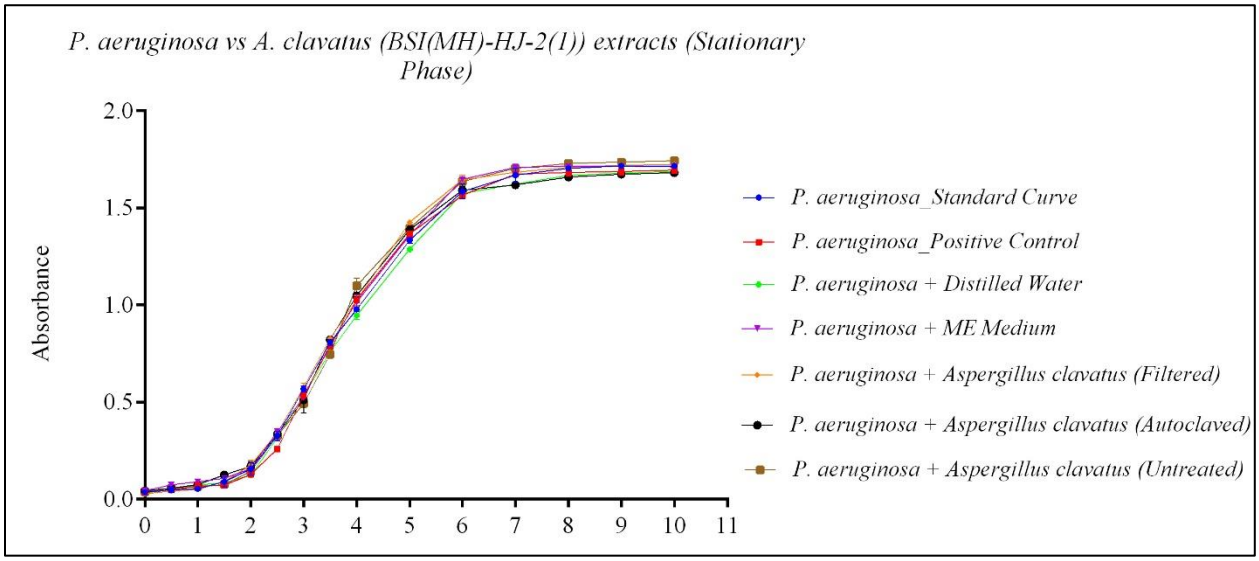
Appendix O: Third Assay: *E. coli* growth curves showing not significant differences between treatments (p-value > 0.05).



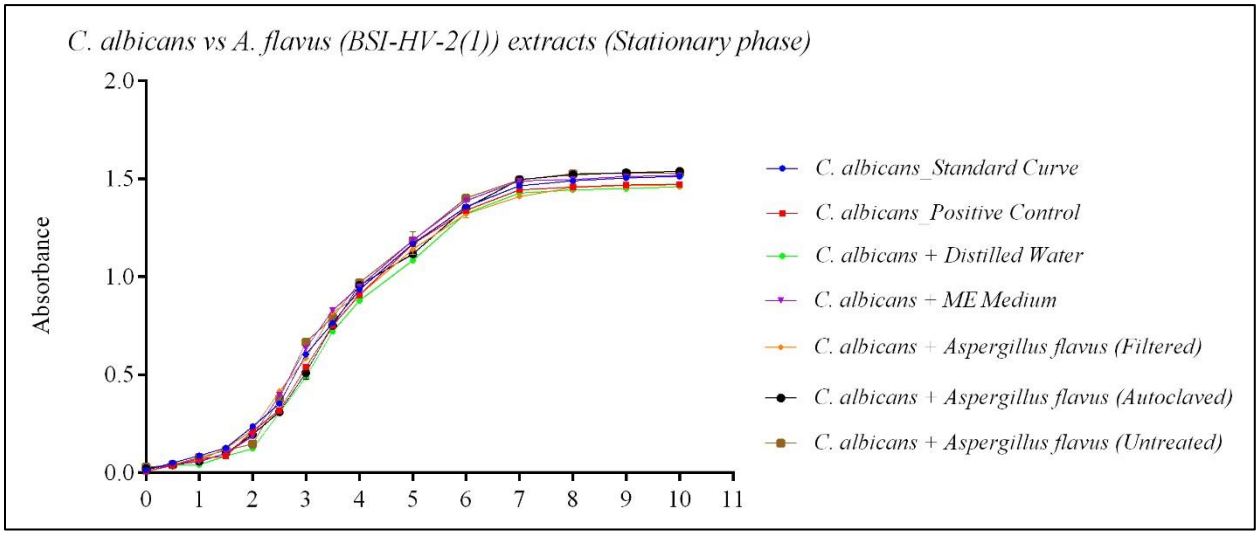


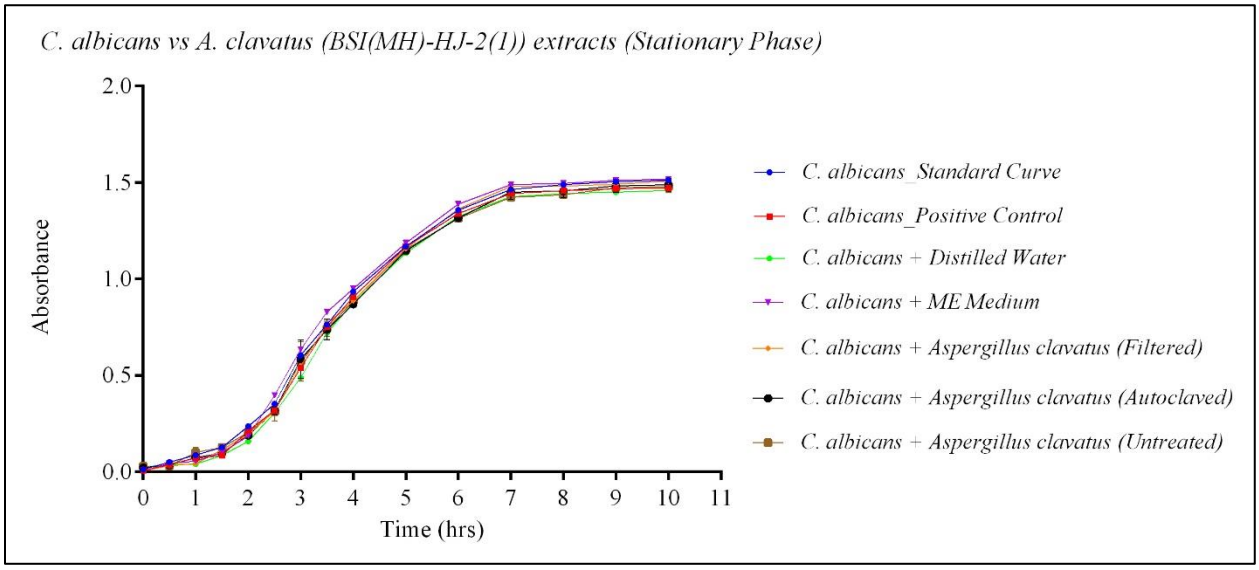
Appendix P: Third Assay: *P. aeruginosa* growth curves showing not significant differences between treatments (p-value > 0.05).





Appendix Q: Third Assay: *C. albicans* growth curves showing not significant differences between treatments (p-value > 0.05).





Appendix R: Statistical Analysis Data

	E. coli						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	1.434	4.823e-011	.7517				
Positive Control	.1336	2.068e-008	1.533	12	1432 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	1.391	4.613e-014	.7133	12	1.326 (3,24)	.2891	One curve for all data sets
ME medium (control)	1.484	1.356e-008	.7113	12	2.908 (3,24)	.0553	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	1.446	8.811e-012	.7864	12	0.9505 (3,24)	.4320	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	1.439	4.574e-014	.7974	12	0.8906 (3,24)	.4602	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	1.415	1.141e-008	.7361	12	2.951 (3,24)	.0529	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	1.430	1.050e-008	.7111	12	0.4186 (3,24)	.7413	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	1.464	3.316e-010	.7267	12	0.1572 (3,24)	.9240	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	1.454	4.732e-007	.6452	12	0.6289 (3,24)	.6035	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	1.465	3.660e-010	.7359	12	0.2840 (3,24)	.8364	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	1.407	1.174e-007	.6661	12	1.577 (3,24)	.2210	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	1.436	2.020e-007	.6616	12	0.6709 (3,24)	.5782	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	1.506	1.809e-011	.7466	12	1.858 (3,24)	.1638	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	1.484	1.356e-010	.7561	12	2.949 (3,24)	.0531	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	1.477	8.729e-012	.7444	12	0.6875 (3,24)	.5685	One curve for all data sets
Stereum (BSI-R-2(1)) (untreated)	1.508	6.209e-017	.8411	12	4.524 (3,24)	.0119	Different curve for each data set
Stereum (BSI-R-2(1)) (autoclaved)	1.706	3.643e-016	.8676	12	69.79 (3,24)	< 0.0001	Different curve for each data set
Stereum (BSI-R-2(1)) (filtrated)	1.439	3.589e-023	.9195	12	2.563 (3,24)	.0784	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (untreated)	.9121	4.474e-008	.7263	12	214.1 (3,24)	< 0.0001	Different curve for each data set
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (autoclaved)	1.276	8.399e-009	.6737	12	24.79 (3,24)	< 0.0001	Different curve for each data set
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (filtrated)	1.169	5.495e-008	.6739	12	45.32 (3,24)	< 0.0001	Different curve for each data set
Hortaea (BSII(3.5)-HC-1(2)) (untreated)	1.475	1.453e-012	.7748	12	0.8579 (3,24)	.4763	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (autoclaved)	1.413	2.020e-015	.8437	12	0.4983 (3,24)	.6870	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (filtered)	1.439	1.442e-017	.8324	12	1.764 (3,24)	.1808	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Log Phase)	.3459	0.01891	.3607	12	1203 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Log Phase)	.2685	1.849e-007	1.021	12	1183 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Log Phase)	.4050	0.003168	.4856	12	1006 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Lag Phase)	.09711	0.02131	.5878	12	2140 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Lag Phase)	.2775	0.003090	.4683	12	1376 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Lag Phase)	.3860	0.001617	.5260	12	1105 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Stationary Phase)	1.448	1.427e-008	.7144	12	1.248 (3,24)	.3144	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Stationary Phase)	1.469	7.477e-011	.7178	12	1.531 (3,24)	.2321	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Stationary Phase)	1.401	1.623e-012	.7901	12	0.1545 (3,24)	.9257	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated) (Log Phase)	1.367	0.01139	.2852	12	70.47 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved) (Log Phase)	1.312	0.008473	.2736	12	93.64 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (filtered) (Log Phase)	1.364	0.006592	.3021	12	80.60 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (untreated) (Lag Phase)	.4163	0.005651	.4563	12	1050 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved) (Lag Phase)	.2466	1.186e-005	.9140	12	1447 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (filtered) (Lag Phase)	.2295	0.001808	.6216	12	1492 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (untreated) (Stationary Phase)	1.381	3.258e-018	.8882	12	0.6773 (3,24)	.5745	One curve for all data sets
A. flavus (BSI-HV-2(1)) (autoclaved) (Stationary Phase)	1.404	8.201e-012	.7763	12	0.1225 (3,24)	.9459	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered) (Stationary Phase)	1.434	5.819e-010	.7617	12	1.903 (3,24)	.1561	One curve for all data sets

Appendix R**:** Statistical Analysis Data

	P. aeruginosa						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	1.754	7.259e-005	.7263				
Positive Control	.7267	0.0001401	.7868	12	1323 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	1.721	4.294e-005	.7307	12	1.770 (3,24)	.1799	One curve for all data sets
ME medium (control)	1.766	2.850e-005	.7552	12	0.3358 (3,24)	.7996	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	1.736	4.788e-006	.7943	12	0.8526 (3,24)	.4789	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	.670	3.262e-006	.8271	12	2.764 (3,24)	.0639	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	1.688	3.398e-005	.7501	12	1.499 (3,24)	.2402	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	1.674	2.872e-007	.8770	12	3.007 (3,24)	.0501	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	1.777	8.020e-005	.7395	12	2.879 (3,24)	.0569	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	1.709	9.240e-006	.7653	12	2.065 (3,24)	.1316	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	1.723	8.263e-006	.7942	12	0.6728 (3,24)	.5771	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	1.746	7.154e-005	.7015	12	1.448 (3,24)	.2536	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	1.727	8.533e-006	.7873	12	0.1860 (3,24)	.9049	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	1.755	6.360e-005	.7474	12	2.987 (3,24)	.0511	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	1.688	7.869e-006	.7837	12	2.190 (3,24)	.1154	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	1.728	1.553e-005	.7619	12	0.6456 (3,24)	.5933	One curve for all data sets
Stereum (BSI-R-2(1)) (untreated)	1.728	9.939e-006	.7901	12	0.6523 (3,24)	.5893	One curve for all data sets
Stereum (BSI-R-2(1)) (autoclaved)	1.807	0.0006384	.6630	12	2.461 (3,24)	.0871	One curve for all data sets
Stereum (BSI-R-2(1)) (filtrated)	1.759	0.0001281	.7267	12	1.760 (3,24)	.1817	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (untreated)	1.748	2.281e-005	.7269	12	2.534 (3,24)	.0808	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (autoclaved)	1.803	3.959e-005	.7028	12	2.934 (3,24)	.0539	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (filtrated)	1.793	2.934 (3,24)	.7635	12	2.988 (3,24)	.0510	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (untreated)	.1.830	3.878e-005	.6980	12	2.244 (3,24)	.1090	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (autoclaved)	1.719	0.0003852	.7026	12	1.915 (3,24)	.1541	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (filtered)	1.793	4.758e-005	.6966	12	2.467 (3,24)	.0865	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Log Phase)	1.306	0.0003915	.6813	12	194.8 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Log Phase)	1.518	0.0003382	.6837	12	53.58 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Log Phase)	1.529	0.001763	.5248	12	70.04 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Lag Phase)	.5239	0.003832	.5872	12	2537 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Lag Phase)	1.230	0.01501	.3773	12	422.5 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Lag Phase)	.5785	0.01485	.4874	12	2180 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Stationary Phase)	1.769	1.953e-006	.8150	12	1.904 (3,24)	.1559	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Stationary Phase)	1.703	1.191e-005	.7965	12	0.9445 (3,24)	.4347	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Stationary Phase)	1.757	1.647e-005	.7849	12	1.934 (3,24)	.1510	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated) (Log Phase)	1.690	1.212e-006	.8363	12	1.924 (3,24)	.1527	One curve for all data sets
A. flavus (BSI-HV-2(1)) (autoclaved) (Log Phase)	1.711	1.766e-005	.7811	12	0.6199 (3,24)	.6089	One curve for all data sets
A. flavus (BSI-HV-2(1)) *(*filtered) (Log Phase)	1.741	0.0002763	.7024	12	1.470 (3,24)	.2477	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated) (Lag Phase)	1.683	7.481e-007	.6747	12	106.8 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved) (Lag Phase)	1.637	7.196e-006	.6354	12	94.24 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (filtered) (Lag Phase)	1.632	3.436e-008	.7486	12	52.17 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (untreated) (Stationary Phase)	1.710	2.634e-005	.7720	12	0.6601 (3,24)	.5846	One curve for all data sets
A. flavus (BSI-HV-2(1)) (autoclaved) (Stationary Phase)	1.742	0.0002929	.7005	12	1.446 (3,24)	.2542	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered) (Stationary Phase)	.684	4.196e-007	.8630	12	1.880 (3,12)	.1868	One curve for all data sets

Appendix R**:** Statistical Analysis Data

	C. albicans						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	1.546	0.001725	.4672				
Positive Control	.5712	9.290e-009	1.341	12	1038 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	1.520	0.0006131	.6734	12	2.793 (3,24)	.0621	One curve for all data sets
ME medium (control)	1.536	0.0005334	.7137	12	1.682 (3,24)	.1974	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	1.470	0.0001988	.7470	12	2.900 (3,24)	.0557	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	1.493	0.0004041	.7501	12	2.925 (3,24)	.0543	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	1.605	0.002878	.6001	12	2.132 (3,24)	.1226	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	1.509	0.0007597	.6852	12	0.8465 (3,24)	.4820	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	1.579	0.001934	.6324	12	0.8681 (3,24)	.4712	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	1.537	0.0003361	.7299	12	1.437 (3,24)	.2566	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	1.478	9.975e-005	.7885	12	2.590 (3,24)	.0763	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	1.514	0.0001231	.7649	12	2.846 (3,24)	.0588	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	1.571	0.001863	.6302	12	0.6026 (3,24)	.6196	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	1.539	0.002608	.6530	12	1.869 (3,24)	.1618	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	1.572	0.001883	.6297	12	0.5883 (3,24)	.6286	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	1.488	7.244e-005	.7911	12	2.874 (3,24)	.0572	One curve for all data sets
Stereum (BSI-R-2(1)) (untreated)	1.578	0.002322	.6185	12	0.6324 (3,24)	.6013	One curve for all data sets
Stereum (BSI-R-2(1)) (autoclaved)	1.496	8.017e-005	.7883	12	2.532 (3,24)	.0810	One curve for all data sets
Stereum (BSI-R-2(1)) (filtrated)	1.576	0.002428	.6488	12	2.967 (3,24)	.0521	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (untreated)	1.607	0.002762	.6016	12	2.429 (3,24)	.0900	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (autoclaved)	1.473	0.0002160	.7455	12	2.563 (3,24)	.0784	One curve for all data sets
Nigrospora (Khuskia oryzae) (BSI(1.5)-HJ-2(1)) (filtrated)	1.495	0.0004290	.7486	12	2.972 (3,24)	.0519	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (untreated)	1.567	0.0005225	.7056	12	2.192 (3,24)	.1151	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (autoclaved)	1.528	0.0005749	.6809	12	1.137 (3,24)	.3540	One curve for all data sets
Hortaea (BSII(3.5)-HC-1(2)) (filtered)	1.600	0.002810	.6040	12	1.413 (3,24)	.2635	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Log Phase)	.9365	0.0002429	.8372	12	469.3 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Log Phase)	1.189	0.0003267	.7159	12	236.7 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Log Phase)	.8807	0.0008811	.7890	12	681.3 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Lag Phase)	.6685	0.0002468	.6588	12	913.9 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Lag Phase)	1.129	0.0001451	.6969	12	258.0 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Lag Phase)	.5602	1.546e-006	.9787	12	1090 (3,24)	< 0.0001	Different curve for each data set
A. clavatus (BSI(MH)-HJ-2(1)) (untreated) (Stationary Phase)	.5606	0.001115	.6561	12	2.768 (3,24)	.0637	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (autoclaved) (Stationary Phase)	1.518	0.0008014	.6632	12	2.775 (3,24)	.0632	One curve for all data sets
A. clavatus (BSI(MH)-HJ-2(1)) (filtered) (Stationary Phase)	1.536	0.0004643	.66846	12	1.942 (3,24)	.1497	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated) (Log Phase)	.8112	0.01704	.5083	12	859.5 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved) (Log Phase)	1.057	0.002386	.5826	12	530.3 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (filtered) (Log Phase)	.7466	0.001640	.6985	12	1298 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (untreated) (Lag Phase)	.5649	0.0001673	.8678	12	1158 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved) (Lag Phase)	.9642	5.074e-005	.7292	12	512.7 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (filtered) (Lag Phase)	.5725	0.001884	.6664	12	1745 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (untreated) (Stationary Phase)	1.555	0.0004760	.7108	12	1.545 (3,24)	.2287	One curve for all data sets
A. flavus (BSI-HV-2(1)) (autoclaved) (Stationary Phase)	1.570	0.0005764	.6642	12	2.219 (3,24)	.1120	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered) (Stationary Phase)	1.494	0.001844	.6595	12	2.656 (3,24)	.0714	One curve for all data sets

Appendix R**:** Statistical Analysis Data

	S. aureus						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	.8543	0.001021	.6552				
Positive Control	.4931	6.421e-007	1.099	12	179.4 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	.8570	3.040e-005	.7678	12	2.192 (3,24)	.1151	One curve for all data sets
ME medium (control)	.8475	3.021e-005	.7746	12	1.503 (3,24)	.2391	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	.8557	0.0002852	.7240	12	0.7354 (3,24)	.5412	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	.8365	0.0006319	.7046	12	0.7579 (3,24)	.5287	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	.8914	0.004558	.5728	12	1.750 (3,24)	.1836	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	.8387	6.963e-005	.7535	12	0.7299 (3,24)	.5442	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	.8535	0.0006119	.6895	12	0.2958 (3,24)	.8280	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	.8732	0.0002395	.7118	12	1.437 (3,24)	.2565	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	.8429	0.0001871	.7294	12	0.3338 (3,24)	.8010	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	.8612	0.0003802	.6781	12	0.6670 (3,24)	.5805	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	.8497	0.0001751	.7078	12	1.244 (3,24)	.3159	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	.8465	0.0004189	.6974	12	0.1123 (3,24)	.9520	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	.8542	0.002325	.6125	12	0.3687 (3,24)	.7763	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	.8638	0.0003886	.6789	12	0.7006 (3,24)	.5609	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated)	1.027	1.322e-006	.8108	12	61.65 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved)	.8413	0.0002988	.7014	12	0.4182 (3,24)	.7416	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered)	.8375	5.227e-005	.7608	12	1.294 (3,24)	.2992	One curve for all data sets

Appendix R**:** Statistical Analysis Data

	S. marcescens						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	.9441	0.001758	.6350				
Positive Control	.4650	1.807e-005	.162	12	521.3 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	.9106	0.001530	.6479	12	2.879 (3,24)	.0570	One curve for all data sets
ME medium (control)	.9674	0.001717	.6404	12	2.809 (3,24)	.0611	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	.9641	0.004075	.6054	12	2.832 (3,24)	.0597	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	.9187	0.002411	.6584	12	2.787 (3,24)	.0625	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	.9606	0.001499	.6568	12	1.965 (3,24)	.1462	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	.9368	0.001096	.6851	12	2.632 (3,24)	.0731	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	.9299	0.0002942	.7219	12	1.484 (3,24)	.2442	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	.9343	0.001982	.6282	12	0.5138 (3,24)	.6767	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	.9139	0.001905	.6674	12	2.852 (3,24)	.0585	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	.9327	0.0003799	.7105	12	1.107 (3,24)	.3656	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	.9409	0.001312	.6776	12	2.950 (3,24)	.0530	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	.9470	0.003471	.6174	12	1.385 (3,24)	.2713	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	.9549	0.001077	.6715	12	1.565 (3,24)	.2237	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	.9279	0.004204	.6237	12	2.983 (3,24)	.0513	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated)	.9340	0.001973	.6286	12	0.5217 (3,24)	.6714	One curve for all data sets
A. flavus (BSI-HV-2(1)) (autoclaved)	.9391	0.001402	.6731	12	2.868 (3,24)	.0576	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered)	.9328	0.0003551	.7134	12	1.266 (3,24)	.3084	One curve for all data sets

Appendix R**:** Statistical Analysis Data

	C. tropicalis						
	Parameters			**Degrees of Freedom**	**F (DFn, DFd)**	**p-value**	**Preferred Model/ Conclusion**
EXTRACTS	**YM**	**Y0**	**K**				
Standard Growth Curve	.8555	3.961e-032	1.493				
Positive Control	.5254	1.049e-016	1.395	12	192.0 (3,24)	< 0.0001	Different curve for each data set
Distilled Water (control)	.8326	1.919e-036	1.531	12	0.7728 (3,24)	.5206	One curve for all data sets
ME medium (control)	.8726	0.000e+000	1.553	12	0.5602 (3,24)	.6464	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(untreated)*	.8458	2.981e-020	1.360	12	0.8494 (3,12)	.4932	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(autoclaved)*	.8653	5.518e-025	1.384	12	0.3640 (3,24)	.7795	One curve for all data sets
Simplicillium (BSI(1.5)-R-1(1)) *(filtered)*	.8784	1.567e-018	1.328	12	0.7007 (3,24)	.5609	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(untreated)*	.8507	0.0000000	1.670	12	0.7739 (3,24)	.5200	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(autoclaved)*	.8617	3.380e-016	1.245	12	0.7738 (3,24)	.5201	One curve for all data sets
Purpureocillium (BSII(3.5)-R-3(2)) *(filtered)*	.8677	6.406e-027	1.455	12	0.5213 (3,24)	.6717	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(untreated)*	.8282	2.348e-013	1.203	12	2.516 (3,24)	.0823	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(autoclaved)*	.8801	1.613e-019	1.295	12	0.7567 (3,24)	.5294	One curve for all data sets
Bionectria (BSI(MH)-HC-3(1)) *(filtered)*	.8527	1.956e-024	1.403	12	0.1105 (3,24)	.9531	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(untreated)*	.8369	3.576e-027	1.450	12	0.3975 (3,24)	.7560	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(autoclaved)*	.8539	1.865e-023	1.389	12	0.1055 (3,24)	.9561	One curve for all data sets
Penicillium (BSI-HV-1(2)) *(filtered)*	.8565	9.207e-024	1.376	12	0.2479 (3,24)	.8620	One curve for all data sets
A. flavus (BSI-HV-2(1)) (untreated)	.6210	3.703e-030	1.585	12	80.97 (3,24)	< 0.0001	Different curve for each data set
A. flavus (BSI-HV-2(1)) (autoclaved)	.8496	3.413e-028	1.442	12	0.1762 (3,24)	.9115	One curve for all data sets
A. flavus (BSI-HV-2(1)) (filtered)	.5979	5.243e-031	1.576	12	113.1 (3,24)	< 0.0001	Different curve for each data set