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MOLECULAR IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI *ASPERGILLUS TAMARII* (TRICHOMACEAE)

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ABSTRACT

Microbial drug resistance threatens the effective prevention and treatment of infectious diseases, this has prompted a continuous search for new and effective antibacterial agents from diverse sources including endophytic fungi of plants and mushroom. *Aspergillus tamarii* is an endophytic fungus that has been isolated from different plants and mushrooms such as *Lycoperdon umbrinum*. This study was undertaken to identify the endophytic fungi isolated from *L. umbrinum* and to evaluate the antimicrobial activity of the extract from the endophytic fungi. The endophytic fungus was identified as *Aspergillus tamarii* (strain SRRC 108818S) based on sequence data analysis of the internal transcribed spacer region of rDNA. The extract from *Aspergillus tamarii* had significant antibacterial activities on *Salmonella typhi* (ATCC33458), *Staphylococcus aureus* (ATCC6538), *Bacillus subtilis* (ATCC6633), *Escherichia coli* (ATCC25922), with zone of inhibitions of 15.5 ± 0.707, 14.5 ± 0.707, 14 ± 0.00 and 23 ± 1.414 respectively at the highest concentration of 200 mg/mL. The extract however, does not have any effect on *Candida albicans* at all tested concentration. *Aspergillus tamarii* is an endophytic fungus that could be a potential source of antibacterial agent.

Keywords: Antibacterial activity, *Aspergillus tamarii*, Endophytic fungi, *Lycoperdon umbrinum*, Molecular identification

INTRODUCTION

Infectious diseases remain one of the major threats to human health. Although a number of natural and synthetic antimicrobial agents have been developed to kill pathogenic microorganisms, the discovery of new antimicrobial agent is essential to overcome the increasing levels of drug resistance by plant/human pathogens. More over there is the problem of insufficient number of effective antibiotics against diverse bacterial species (Song, 2008). Globally, infectious disease is one of the leading causes of death among children and adults. Nearly 15 million (>25%) of the 57 million annual deaths worldwide are caused by infectious disease (Anthony *et al*., 2005).

Antimicrobial drugs have been successfully used to treat target microbes causing infectious diseases for over 70 years. With the continued and irrational use of antimicrobial agents, many infectious agents have developed resistance by adapting to the drugs aimed at killing them through various resistance mechanisms, thereby making many antimicrobial agents less effective

against organisms they were previously active against. Thus, antimicrobial drug resistance has been recognized as a great threat to global public health and human civilization (Walker *et al*., 2009). In 2010, the World Health Organization advised all the countries to implement control procedures for the propagation of drug multi-resistant bacteria, highlighting the risks associated to the absence of alternative therapies against those microorganisms (WHO, 2010). The risks of antimicrobial drug resistance threaten not only the effective prevention of diseases but also prompt treatment of the ever-increasing range of infectious diseases, these combined has prompted a continuous search for new and effective antibacterial agents from diverse sources including endophytes of plants and mushroom (Gary *et al*., 2003).

Endophytic organisms are mostly microorganisms that do not necessarily cause any significant harm to its host while living in the intercellular spaces of the host tissues (Strobel and Daisy, 2003). They often spend their whole lifecycle colonizing inter and/or intracellular space of healthy tissues of the host plants, typically causing no symptoms or disease (Tan *et al*., 2001). There are over one million fungal endophytes that exist in nature (Petrini, 1991). Essentially many endophytic fungi are known to produce a large number of bioactive metabolites that exhibit antibacterial, antifungal, antiviral, antitumor, hypoglycemic, antiallergic, immunomodulating, antiinflammatory, hypolipidermic and hepatoprotective activities (Espenshade and Griffith, 1966). They have been acknowledged as an essential source of novel bioactive compound with potential applications in the field of agriculture, medicine and food industry; many endophytic fungi with potential health benefits have been isolated from several mushrooms (Zhao *et al*., 2010). It has been thought that due to the long period of coevolution between endophytes and the host plant/ mushroom, many endophytes are therefore capable of producing the similar bioactive compounds as those produced by the host plants(Strobel and Daisy, 2003). Endophytes provide an abundant source of novel bioactive secondary metabolites and several bioactive compounds that has been isolated and studied such as paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin (Zhao *et al*., 2010, Baby *et al.,* 2011). An increasing number of antimicrobial compounds are being isolated and characterized from endophytic fungi, the exploitation trend have become noticeable over recent years (Liang *et al*., 2012).

Aspergillus tamarii is an endophytic fungus and has been isolated from different plants and mushroom such as *Lycoperdon umbrinum* (Smith *et al*., 2001). *Aspergillus tamarii* has been widely used in the food industry for the production of soy sauce and in the fermentation industry for the production of different enzymes. Although known as allergenic and able to produce several toxic secondary metabolites, it is not considered dangerous and rarely encountered as a human pathogen. However, experts still advise that proper care should be taken when handling (Varga *et al*., 2011). It has been known to produce a wide range of secondary metabolite including malformin E, a cyclic pentapeptide (Ma *et al*., 2016).

MATERIALS AND METHODS

Collection and Identification

Lycoperdon umbrinum was collected from the botanical Garden, University of Ibadan in April, 2014.The endophytic fungus was isolated from the inner living tissues of the fruiting bodies the mushroom, samples were sliced off and maintained on Potato

Dextrose Agar (PDA), supplemented with 0.5% yeast extract in Petri dishes and incubated at 28°C for 7days and observed for development of spores (Kastner *et al*., 1994).After few weeks of incubation at room temperature, hyphal tips of the fungus were removed and transferred to fresh malt agar medium. Plates were prepared in duplicates to eliminate the possibility of contamination. Pure strains were isolated by repeated inoculation. All fungal isolates grown on isolation media were purified and preserved in a 2 mL Cryotube containing1 mL of 10% glycerol solution ($^{v}/_{v}$) and 5% lactose ($^{w}/_{v}$) at 80°C.

DNA isolation

Nuclear rDNA from the fungal isolates was extracted using a DNeasy® Plant Mini Kit (QIAgen). The lyophilized fungal mycelia were pulverized and disrupted with the help of glass beads. DNA was extracted from the pulverized mycelia according to the manufacturer's protocol.

DNA amplification

The isolated DNA was then amplified by Polymerase Chain Reaction (PCR using HotStarTaq Master Mix Kit (QIAgen). The ITS rDNA of the fungal isolate was amplified with primers ITS 1 (with base sequences TCCGTAGGTGAACCTGCGG) and ITS 4 (with base sequences TCCTCCGCTTATTGATATGC)
(Invitrogen), as primers, they were mixed with HotstarTaq Master Mix Kit and DNA template. Each PCR reaction mixture contained 5 10 ng of genomic DNA, 1 μM each of the primers ITS 1 and ITS 4, and 1 U of Hot start Taq Polymerase (Invitrogen) in a total volume of 50 μL. PCR thermal cycling was carried out in a PCR thermal cycler (BioRad) using the following programmed PCR cycle: Initial activation step in 95º C for 15 minutes to activate HotStarTaq®DNA Polymerase, followed by cycling steps which were repeated 35 times (Denaturing: 1 minute at 95º C, annealing: 1 minute at 56º C, extension: 1 minute at 72º C) with a final extension cycle at 72°C for 10 minutes.

Purification of PCR products and DNA sequencing

The PCR product was purified using 2% Agarose Gel Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 500 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Perfectprep® Gel Cleanup Kit (Eppendorf). Amplified fungal DNA (PCR product), was sequenced commercially and the base sequence was compared with publicly available databases as GenBank using the BLAST search tool.

The identification of the isolate was based on the sequence similarity cut off point for fungal species delimitation of at least ≥ 97% according to Brock *et al*. (2009) and with E-value cut-off 0.01.
The fungal identification was carried out at the Institute für Pharmazeutische Biologie und Biotechnologie, Heinrich Heine Universität Düsseldorf, Germany.

Culture of Isolate

Fresh fungal cultures (isolate) were transferred into flasks (500 mL) each containing 50 g sterilized rice media (solid cultures). The cultures were then incubated at room temperature without agitation for 30 days.

Extraction method

Ethyl acetate (250 mL) was added to the cultures and left overnight (12 h) and the rice media were then cut into small pieces to increase surface area and allow complete extraction for 72 h. Filtration was done followed by repeated extraction with ethyl acetate till exhaustion. After filtration, the filtrate was concentrated with a rotary evaporator, the dry residue obtained from fungal culture was partitioned between n-hexane and 90% methanol. The methanol fraction was concentrated and stored at low temperature for the antimicrobial assay.

Antimicrobial assay

Test Microorganisms

Typed culture bacteria strains; Gram positive bacteria: *Bacillus subtilis* ATCC6633 and *Staphylococcus aureus*– ATCC 6538; Gram negative: *Escherichia coli* ATCC 5922 and *Salmonella typhi* – ATCC 10231) and typed culture fungal strains *Candida albicans ATCC* 10231 obtained from Department of Microbiology University of Ibadan, Nigeria were used in this study.

Preparation of cultures

Overnight bacteria cultures were obtained by sub-culturing from the stored slopes. The stored bacteria slopes were then streaked with a sterile inoculating loop and inoculated into 5 mL nutrient broth. These were incubated appropriately for 18 hours at 37 ºC.

Preparation of crude extracts

Serial dilutions for the crude extract and was made (12.5 – 200mg/mL), and were used for the susceptibility test of the extracts.

Susceptibility studies

All the test microorganisms were tested for their susceptibilities by agar diffusion technique.

Preparation of bacterial seeded agar plates

A 1 in100 mL dilution of the overnight culture of each bacterium in appropriate medium was made by adding 0.1 mL into a test tube containing 9.9 mL of sterile distilled water. Using a sterile pipette, 0.2 mL of the 1 in 100 mL dilution of overnight culture of the test organism was seeded into 20 mL of melted and cooled agar medium and poured into sterile petri dishes after thorough mixing and allowed to set. Equidistant wells were then bored into the solidified medium using a sterilized cork borer of diameter 8 mm. 100 µL of the serially diluted extract was transferred into each of the wells in the inoculated agar and allowed to diffuse for 30 minutes. All plates were incubated at 37°C for 24 h, and the resulting inhibition zones were measured in millimeter (mm). The experiment was carried out in triplicate and antimicrobial activity was expressed as the mean of inhibition diameters (mm) produced by the extract. Gentamycin and ketoconazole were used as positive controls for bacterial and fungal strains respectively.

Determination of Minimum inhibitory concentration (MIC)

The MIC for the extract was determined by the agar dilution method. Serial dilutions of the extract were made to obtain a concentration range of 50 to 3.125 mg/mL. One mL of the extract from each dilution was mixed with 19 mL of molten agar and poured into sterile Petri dishes allowing the agar to set. The surface of the agar was allowed to dry before streaking with overnight culture of susceptible organisms. The plates were incubated appropriately for 24 hours and examined for the presence or absence of growth. The lowest concentration preventing visible growth of the organisms was taken as the minimum inhibitory concentration of the extract.

RESULT AND DISCUSSION

The result of the antimicrobial activity of the crude extract of *Aspergillus tamari* is presented in Tables 1 and 2. From Table 1, the extract had significant antibacterial activities on the bacteria species but does not have any effect on *Candida albicans*. However, the antibacterial activity of the extract varies in magnitude among the various selected bacterial species. The mean diameter of zone of inhibition ranges from 23 – 10 mm. Out of the four (4) bacterial culture tested, the extract had its highest mean diameter of zone of inhibition on *E. coli* (23 mm), followed by *S. typhii* (15.5 mm), *S. aureus* (14.5) and *B. subtilis* (14 mm) at 200 mg/mL. While, at the lowest concentration (2.5 mg/mL) the extract had zone of inhibition of 10 mm for all the bacterial species.

The MIC (Table 2) of the extracts varies from 6.25 mg/mL – 12.5 mg/mL. The MIC for *E. coli* and *S. aureus* was 6.25 mg/mL while, that of *S. typhii* and *B. subtilis* 12.5 mg/mL. The extract thus shows no obvious difference in susceptibility between both Gram positive and Gram negative antibacterial activity. However, there was no inhibition of growth on *C. albicans* by the extract.

Table 1: Mean ± Standard Deviation (S.D) of Zones of Inhibition (mm) of Extract of *Aspergillus tamari* on Typed Bacteria Cultures

	Mean ± S.D for Zones of Inhibition (mm) on test organisms				
Concentration of extract (mg/mL)	***Salmonella typhi (ATCC33458)***	***Staphylococcus aureus (ATCC6538)***	***Bacillus subtilis (ATCC6633)***	***Escherichia coli (ATCC25922)***	***Candida albicans (ATCC10231)***
200	15.5 ± 0.707	14.5 ± 0.707	14 ± 0.00	23 ± 1.414	_
100	13.5 ± 0.707	13 ± 0.00	13.5 ± 0.707	17 ± 0.00	_
50	13 ± 0.00	11 ± 0.707	13 ± 0.00	13.5 ± 1.41	_
25	10.5 ± 0.707	10 ± 0.00	11 ± 0.00	10 ± 0.00	_
12.5	10 ± 0.00	10 ± 0.00	10 ± 0.00	10 ± 0.00	_
Gentamycin (10 µg/ml)	16 ± 0.00	16 ± 0.00	18 ± 0.00	18 ± 0.00	_
Ketoconazole (20 µg/ml)					20 ± 0.00

There is a growing need for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. In fact, around 60% of the new drugs registered during the period 19812002 by the FDA as anticancer, antimigraine and antihypertensive agents were either natural products or based on them (Newman *et al*., 2003). The discovery of endophytic fungus has opened a new possibility in the search for bioactive secondary metabolite. Endophytic fungi can produce structurally and biologically diverse natural products and have become an important source of novel structures and strong biological compounds. A number of compounds antimicrobial activities have been isolated and reported in literature. But several endophytes that exit in nature are unknown (Strobel and Daisy, 2003; Kalyanasundaram *et al*., 2015).

Analysis of the antimicrobial inhibitory activity by agar dilution method showed that all the bacteria organisms were susceptible to the ethyl acetate extract of *A. tamari* as summarized in the Table 1. The extract displayed a concentration dependent antibacterial activity (Table 1). The extract however had no antifungal activity. At 12.5 and 25 mg/mL the extract had similar antibacterial activity on *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538, *S. typhii* ATCC 33458 with a zone of inhibition of 10 mm across. The extract displayed similar concentration dependent zones of inhibition on all bacteria at higher concentrations but had very significant activity on *E. coli* where the extract displayed a significantly higher degree of inhibition of 17.0 ± 0.00 and 23.0 ± 1.41 mm at 100 and 200 mg/mL respectively. However, the extract displayed no antifungal activity. The activity indices indicated that *E. coli* was the most susceptible while *Bacillus subtilis* is the least susceptible to the extract of *A. tamari*.

The extract had varying minimum inhibitory concentration (MIC) on the organisms as shown in Table 2. The MIC for *E. coli* and *S. aureus* was 6.25 mg/mL while, the MIC for *S. typhii* and *B. subtilis* was 12.5 mg/mL. The extract has more activity on *E. coli* and *S. aureus* than *S. typhii* and *B. subtilis*. The extract thus showed no obvious difference in susceptibility between both Gram positive and Gram negative antibacterial activity.

The result of this study has indicated that the antimicrobial activity of *A. tamari* (Isolated from *Lycoperdon umbrinum*) is similar to the report of the antimicrobial study of *Lycoperdon umbrinum* by Opige *et al*., (2006). The mushroom was observed to have moderate antibacterial activity against both Gram negative and Gram positive bacteria with similar zones of inhibition at the same concentration and bacterial culture. This therefore this suggested that *A. tamari* might be producing similar bioactive component as the host fungi (*L. umbrinum*).

Previous work had established that endophytic fungi isolated from *Taxus brevifolia* produced taxol similar to the host plant (*Taxus brevifolia*) which had potent anticancer activity (Wani *et al*., 1971). Other bioactive compounds that have been produce by endophytic fungi includes podophyllotoxin (Eyberger *et al*., 2006, Puri *et al*., 2016), deoxypodophyllotoxin (Kusari *et al*., 2009a), camptothecin, and structural analogs (Kusari *et al*., 2009b, Shweta *et al*., 2010), hypericin and emodin (Kusari *et al*., 2008, Kusari *et al*., 2009c), and azadirachtin (Kusari *et al*., 2012)

Table 2: Minimum Inhibitory Concentration (MIC) of Extracts of *Aspergillus tamari* on Test Organisms

Test organisms	Concentration of Extract (mg/mL)					
	50	25	12.5	6.25	3.13	1.56
Escherichia coli (ATCC25922)					+	+
Bacillus subtilis (ATCC6633)				+	+	+
Staphylococcus aureus (ATCC6538)					+	+
Salmonella typhi (ATCC33458)				+	+	+

Key: + = Growth; = No growth

CONCLUSION

It can be concluded that the *Aspergillus tamarii* extracts have potent antibacterial activity especially on *Escherichia coli*. The result of this study offers a scientific basis for the use of extracts of *Aspergillus tamarii* against bacteria associated diseases, and it could be an alternative source of potential antibiotics.

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