# Antibacterial activities of secondary metabolites of endophytic *Aspergillus fumigatus*, *Aspergillus* sp. and *Diaporthe* sp. isolated from medicinal plants

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**Key words:** antimicrobial, endophytes, flavonoids, saponins, terpenoids, thin layer chromatography.

**Zusammenfassung**: Heilpflanzen sind als gute Wirte von Pilzendophyten bekannt, die Verbindungen mit antimikrobiellen Aktivitäten produzieren. Es wurden *Aspergillus fumigatus* von *Cocos nucifera*, *Aspergillus* sp. von *Moringa oleifera* und *Diaporthe* sp. von *Origanum vulgare* auf ihre antimikrobiellen Aktivitäten untersucht. Ethylacetat-Rohextrakte von jedem Pilz wurden gegen *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* und *Bacillus subtilis* verwendet. Ferner wurde das Vorhandensein von Alkaloiden, Flavonoiden, Terpenoiden und Saponinen durch Dünnschichtchromatographie (DC) nachgewiesen. Die Ergebnisse zeigten eine teilweise Aktivität der Pilzextrakte gegenüber den meisten Testbakterien. Extrakte aus *Aspergillus* sp. zeigten die höchste antimikrobielle Aktivität gegen *P. aeruginosa*, *E. coli* und *S. aureus*. Der Extrakt aus *A. fumigatus* war der einzige, der eine antimikrobielle Aktivität gegen *B. subtilis* zeigte. Diese war jedoch nicht mit der Kontrolle Streptomycin vergleichbar. Flavonoid-, Terpenoid- und Saponinverbindungen wurden in allen Extrakten nachgewiesen, jedoch nicht quantifiziert. Es wird empfohlen, diese Verbindungen einzeln zu isolieren und hinsichtlich ihrer antimikrobiellen und anderen biologischen Aktivitäten zu charakterisieren.

**Abstract:** Medicinal plants are known to be good hosts of fungal endophytes that produce compounds with antimicrobial activities. In this study, *Aspergillus fumigatus* from *Cocos nucifera*, *Aspergillus* sp. from *Moringa oleifera* and *Diaporthe* sp. from *Origanum vulgare* were screened for their antimicrobial activities. Ethyl acetate crude extracts from each fungus were used against *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus* and *Bacillus subtilis*. Further, the presence of alkaloids, flavonoids, terpenoids and saponins were detected through thin layer chromatography (TLC). Results showed partial activity of fungal extracts to most test bacteria. Extracts from *Aspergillus* sp. showed highest antimicrobial activity against *P. aeruginosa*, *E. coli* and *S. aureus*. Extract from *A. fumigatus* was the only treatment which showed antimicrobial activity against *B. subtilis*. These were, however, not comparable with the control Streptomycin. Flavonoid, terpenoid and saponin compounds were detected in all extracts but was not quantified. It is recommended that these compounds be isolated and characterized individually for their antimicrobial and other biological activities.

Endophytes are microorganisms, bacteria and fungi, living within plant tissues without causing any apparent harm to the host (STONE & al. 2000). It is estimated that all plants possess at least one endophyte which then translates to not just the diversity of microorganisms living within tissues, but also the diversity of relationships with the host and abiotic factors. It is known that the plant-endophyte chemical communication and endophyte-to-endophyte interactions are key triggers to cryptic and novel natural products (KUSARI & al. 2012). This uniqueness can represent a large reservoir of biological resources from where to mine compounds useful in medicine, agriculture and various industries (MOUSA & RAIZADA 2013).

There had been a great stride in terms of research on secondary metabolites of endophytic fungi. However, because of the diversity of hosts, there are still many more that can be explored. Likewise, products of secondary metabolism, or in general natural products, are well adapted to nature and novel compounds can be expected from plants living in unique biotopes (SCHULZ & al. 2002). Thus, there is a need to routinely include fungal endophytes in the screening for novel compounds as candidates for drug manufacturing (SURYANARAYANAN & al. 2009).

Many fungal endophytes have been screened for their biological activities. *Phomopsis* sp. from leaves of *Aspidosperma tomentosum* (CORRADO & RODRIGUES 2004), *Verticillium* sp. from roots of wild *Rehmannia glutinosa* (YOU & al. 2009), and *Pichia guilliermondii* from *Paris polyphylla* var. *yunnanensis* (ZHAO & al. 2010) all exhibit antimicrobial properties against an array of test microorganisms. Many other fungal endophytes produce anticancer (KHARWAR & al. 2011, CHANDRA 2012), antimalarial (TANSUWAN & al. 2007), and nematicidal (LI & al. 2007) compounds.

Medicinal plants are valuable sources of fungal endophytes because they already have been used as alternative sources of medicine (KAUL & al. 2012). Plants known to be of ethnobotanical importance and produce notable drugs of medicinal value is one criterion in selecting plants for bioprospecting (TEJESVI & al. 2007). True enough, fungal endophytes from 81 Thai medicinal plants were screened against *Mycobacterium tuberculosis* and *Plasmodium falciparum* and tested against breast cancer cell lines (WIYAKRUTTA & al. 2004), and as many as 29 traditional Chinese medicinal plants revealed diversity in fungal endophytes (HUANG & al. 2008). Further, the medicinal plants *Lippia sidoides* CHAM. (DE SIQUIERA & al. 2011) and *Vitex negundo* L. (DESALE & al. 2013) have shown promising fungal endophytic activities to name a few.

It is on this note that fungal endophytes from commonly used medicinal plants in the province of Albay, Philippines, were screened. Fungal endophytes from common medicinal plants in the province of Albay, Eastern Philippines have already been reported (GUERRERO & al. 2019). Most of these medicinal plants are recommended by the Philippine Department of Health for their proven medicinal properties (BOY & al. 2018). However, the previous paper only enumerated fungal endophytes and did not investigate biological activities. Three species from among the isolates were selected based on literature search, growth in solid and liquid media, and screened for their production of secondary metabolites. Further, species were tested against microorganisms for their antimicrobial properties.

## Materials and methods

**Endophytic fungi:** Previously isolated endophytic fungi (GUERRERO & al. 2019) were used in this study as summarized in Tab. 1. Three of the most promising fungi were selected based on a set of criteria.

Tab. 1. Fungal endophytes isolated from medicinal plants in the province of Albay, Philippines

| Fungal code | Fungi ID | Collection site | Plant source | Culture |
|---|---|---|---|---|
| **REF-ISO-12** | *Aspergillus* sp. | Pio Duran, Albay | *Moringa oleifera* LAM. | |
| **REF-ISO-31** | *Aspergillus fumigatus* FRESENIUS | Pio Duran, Albay | *Cocos nucifera* L. | |
| **REF-ISO-23** | *Diaporthe* sp. | Ligao City | *Origanum vulgare* L. | |

These three isolates were selected after a preliminary assessment of their growth in both solid and liquid media and their ability to inhibit test bacteria through agar plug method. Each fungus was cultured and maintained in potato dextrose agar (PDA). Seven-day old cultures were used for each of the assays performed.

**Extraction and characterization of secondary metabolites:** An agar plug from each of the cultures was transferred to potato dextrose broth. All cultures were shaken every 3 days, for 5 min. at 150 rpm at room temperature for 21 days. Ethyl acetate (EtoAc) was added after 21 days to broth cultures (1:1 ratio) which were soaked overnight. Cultures with EtoAc were shaken for 2 h at 150 rpm. Mycelium was separated using Whatman filter paper No. 1. Polar and non-polar metabolites were separated with the use of a separatory funnel while the solvent was removed through rotary evaporation. Extracts were allowed to dry for 7–10 days.

Characterization through thin layer chromatography followed protocols of BIRADAR & RACHETTI (2013). Extracts were dissolved in either EtoAc or methanol (1 mg/ml). The prepared fungal extracts were applied in TLC plates with the use of capillary tubes. After drying, it was placed in TLC chambers accompanied by its respective solvent systems : methanol : ammonium hydroxide (17:3), chloroform: methanol (18:2), chloroform: glacial acetic acid : methanol : water (6:2:1:1), and benzene : ethyl acetate (1:1) for alkaloids, flavonoids, saponins, and terpenoids, respectively. The developed TLC plates were then air dried and were exposed to its accompanying spray reagents like MAYER's reagent for alkaloids, iodine vapor for saponins, 10 % $H_2SO_4$ + UV light for terpenoids and only UV light for flavonoids. After air drying, spots formed were noted and measured. Retention factor (Rf) was computed using the formula:

$$Rf = \frac{Distance\ traveled\ by\ solute}{Distance\ traveled\ by\ solvent}$$

**Antimicrobial activity assay:** Fungal extracts were tested for their antimicrobial activity using the disk diffusion method following the protocol of MARCELLANO & al. (2017). Bacterial suspensions were prepared from 24 h old culture of the test bacteria: *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus*, and *Bacillus subtilis*. All bacterial suspensions were standardized to contain approximately $1.5 \times 10^8$ CFU/ml based on 0.5 McFarland Standard. The bacteria were inoculated on dried

surface of a Mueller-Hinton Agar (MHA). Inoculum was incubated for 10–15 min to ensure any excess surface moisture to be absorbed and to ensure confluent lawn of bacteria growth before applying the test disks. The dried crude extracts were dissolved in 0.1 % dimethyl sulfoxide (DMSO) to a concentration of 100 mg/ml. Then, 30 µl of the dissolved extract was pipetted to 5-mm diameter sterile circular disks (Whatman Paper No. 1) and was allowed to dry for an hour at 35 °C. In each inoculated MHA plate, five paper disks with the extract were placed equidistantly. 30 µl of 0.1 % DMSO and 30 µl streptomycin (10 mg/ml) were used as negative and positive controls, respectively. All plates were incubated at room temperature for 24 h and 48 h and resulting zones of inhibition were measured.

**Statistical analysis:** All data were subjected to statistical analysis using Analysis of Variance (ANOVA) and Least Significant Difference (LSD) Test as post-hoc analysis. The software Statistical Tool for Agricultural Research (STAR) from the International Rice Research Institute (IRRI) was used.

## Results and discussion

**Characterization of secondary metabolites:** Three out of four secondary metabolites were found to be present in the fungal extracts (Tab. 2). Flavonoids, saponins and terpenoids showed distinct spots on the TLC plates. All fungal endophytes did not show presence of alkaloids. At least 2 flavonoid compounds, 2 saponins and 2 terpenoids were detected for *Aspergillus* sp. *Aspergillus fumigatus* had 3 detectable flavonoids, 1 saponin and 2 terpenoids. *Diaporthe* sp. had 4 flavonoids, 4 saponins and 3 terpenoids. These putative compounds were no longer identified in this study. Using methanol as the extracting solvent did not show significant difference in terms of number of compounds detected, except for *A. fumigatus* where at least two more flavonoids and one more terpenoid were detected compared with the ethyl acetate extract. It is recommended that the identification of these compounds be part of a follow up research.

Endophytic fungi are known producers of novel metabolites such as terpenoids, alkaloids, saponins and flavonoids (SOUZA & al. 2011, YU & al. 2010). The diversity of novel compounds which may be mined from fungal species, are not limited by the species themselves but likewise from where they were found or are associated with. This translates to a variety of compounds even when the same species are screened for as long as the species were found in different ecosystems or exist in different associations, such as endophytes as presented in this study. The mere association of fungi with plants provides them with unique compounds necessary for colonization of plant tissues (PETRINI & al. 1993).

**Antimicrobial activity:** Extracts from *Aspergillus* sp. showed the highest antimicrobial activity against *P. aeruginosa* both after 24 and 48 hours of incubation (Tab. 3). It was also partially active against *E. coli* and *S. aureus*. Its antibacterial activity was observed after 48 hours against *B. subtilis*. Extracts from *A. fumigatus* was partially active against *S. aureus* but none against the other test bacteria (Tab. 4). *Diaporthe* sp. showed partial activity against all bacteria except *B. subtilis* (Tab. 5). However, all isolates were not comparable to the zone of inhibition exhibited by streptomycin.

This study, however, recognizes the limitation of metabolite production. Because *in planta* association is important in the production of metabolites, the results may not fully reflect the overall capacity of the fungal endophytes to produce them. This limitation may tend to overlook some compounds not produced *in vitro* but are actively produced as means of chemical communication with their hosts (KUSARI & al. 2012). While the current study has optimized the conditions for secondary metabolite production, it did not take into consideration all parameters and has only focused on the use of synthetic

Tab. 2. Characterization of fungal crude extracts through thin layer chromatography (TLC).

| Metabolite | Solvent system | Fungal crude extract | Rf values | | Spray reagents |
|---|---|---|---|---|---|
| | | | **EtoAc** | **Methanol** | |
| Alkaloids | Methanol: conc. $NH_4OH$ (17:3) | *Aspergillus* sp. | No formation of distinct spots observed | No formation of distinct spots observed | Mayer's reagent |
| | | *Aspergillus fumigatus* | No formation of distinct spots observed | No formation of distinct spots observed | |
| | | *Diaporthe* sp. | No formation of distinct spots observed | No formation of distinct spots observed | |
| Flavonoids | Chloroform: methanol (18:2) | *Aspergillus* sp. | 0.62, 0.80 | 0.56, 0.78 | UV light |
| | | *Aspergillus fumigatus* | 0.74 | 0.60, 0.72, 0.8 | |
| | | *Diaporthe* sp. | 0.60, 0.80 | 0.56, 0.76 | |
| Saponins | Chloroform: glacial acetic acid: methanol: water (6:2:1:1) | *Aspergillus* sp. | 0.50, 0.92 | 0.50, 0.92 | Iodine vapors |
| | | *Aspergillus fumigatus* | 0.84 | 0.86 | |
| | | *Diaporthe* sp. | 0.58, 0.88 | 0.56, 0.92 | |
| Terpenoids | Benzene : ethyl acetate (1:1) | *Aspergillus* sp. | 0.62, 0.72 | 0.64, 0.74 | 10 % $H_2SO4$ & UV light |
| | | *Aspergillus fumigatus* | 0.68 | 0.60, 0.70 | |
| | | *Diaporthe* sp. | 0.58, 0.72 | 0.70 | |

Tab. 3. Zone of inhibition (mm ± SD) of EtoAc extracts of *Aspergillus* sp. against test bacteria.

| Treatment | Zone of inhibition (mm±SD) of EtoAc extracts of *Aspergillus* sp. against test bacteria | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram negative | | | | Gram positive | | | |
| | *Pseudomonas aeruginosa* | | *Escherichia coli* | | *Staphylococcus aureus* | | *Bacillus subtilis* | |
| | 24H | 48H | 24H | 48H | 24H | 48H | 24H | 48H |
| *Aspergillus* sp. | 20.67±1.25*b* | 21.00±2.83*b* | 12.67±1.70*b* | 12.33±2.36*b* | 14.67±2.87*b* | 14.00±2.94*b* | 0.00±0.00*b* | 3.00±4.24*b* |
| 0.1 % DMSO | 0.00±0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* | 0.00 ± 0.00*c* | 0.00 ± 0.00*c* | 0.00 ± 0.00*c* | 0.00±0.00*b* | 0.00±0.00*c* |
| Streptomycin (10 mg/ml) | 23.67±1.70*a* | 23.67±1.70*a* | 30.33±3.86*a* | 31.67±4.64*a* | 33.33±2.62*a* | 34.33±3.09*a* | 35.33±2.62*a* | 38.33±3.77*a* |

*Means followed by the same letters across column are not significantly different at 0.05 level of significance*

Tab. 4. Zone of Inhibition (mm±SD) of EtoAc extracts of *Aspergillus fumigatus* against test bacteria.

| Treatment | Zone of Inhibition (mm±SD) of EtoAc extracts of *Aspergillus fumigatus* against test bacteria | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram negative | | | | Gram positive | | | |
| | *Pseudomonas aeruginosa* | | *Escherichia coli* | | *Staphylococcus aureus* | | *Bacillus subtilis* | |
| | 24H | 48H | 24H | 48H | 24H | 48H | 24H | 48H |
| *Aspergillus fumigatus* | 2.67±3.77*b* | 2.67±3.77*b* | 0.00±0.00*b* | 0.00±0.00*b* | 13.00±1.63*b* | 12.67±2.87*b* | 12.00±1.73*b* | 6.33±4.64*b* |
| 0.1 % DMSO | 0.00±0.00*b* | 0.00±0.00*c* | 0.00±0.00*b* | 0.00±0.00*b* | 0.00 ± 0.00*c* | 0.00 ± 0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* |
| Streptomycin (10 mg/ml) | 21.00±2.94*a* | 21.67±3.40*a* | 30.67±0.47*a* | 33.00±2.83*a* | 32.33±2.62*a* | 32.67±2.36*a* | 38.67±3.30*a* | 37.67±2.49*a* |

*Means followed by the same letters across column are not significantly different at 0.05 level of significance*

Table 5. Zone of Inhibition (mm±SD) of EtoAc extracts of *Diaporthe* sp. against test bacteria.

| Treatment | Zone of Inhibition (mm±SD) of EtoAc extracts of *Diaporthe* sp. against test bacteria | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Gram negative | | | | Gram positive | | | |
| | *Pseudomonas aeruginosa* | | *Escherichia coli* | | *Staphylococcus aureus* | | *Bacillus subtilis* | |
| | 24H | 48H | 24H | 48H | 24H | 48H | 24H | 48H |
| *Diaporthe* sp. | 10.33±1.25*b* | 7.00±4.97*b* | 11.00±0.82*b* | 12.00±0.00*b* | 12.00±0.82*b* | 8.00±5.66*b* | 0.00±0.00*b* | 0.00±0.00*b* |
| 0.1 % DMSO | 0.00 ± 0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* | 0.00±0.00*c* | 0.00±0.00*b* | 0.00±0.00*b* |
| Streptomycin (10 mg/ml) | 23.00±3.56*a* | 21.67±1.70*a* | 27.00±1.41*a* | 27.67±2.36*a* | 28.33±0.94*a* | 27.33±2.36*a* | 35.67±1.70*a* | 36.67±1.70*a* |

*Means followed by the same letters across column are not significantly different at 0.05 level of significance*

culture media and temperature. New methods using plant tissues (ISAH & al. 2018) and use of epigenetic modifiers may overcome the inconsistent results from axenic culture procedures (VENUGOPALAN & SRIVASTAVA 2015). These, among others, are important in transforming endophyte studies into industrial bioprocesses (KUSARI & al. 2014).

The antibacterial activity of the fungal endophytes may mirror the activities of their host. While there were no parallel setups conducted in this study to test the antibacterial activities of the leaves, there are several studies available that point to the antibacterial activities of the plants. In a study by RAHMAN & al. (2009), *Moringa oleifera* extracts showed antibacterial activity against *P. aeruginosa* and *Staphylococcus aureus*, and *Aspergillus* sp. derived from *Moringa oleifera* in our study is partially active against the test bacteria. The same is true with *Origanum vulgare* having antimicrobial activity against *S. aureus* (SAEED & al. 2009) and *E. coli* (SAHIN & al. 2004), and *Diaporthe* sp. isolated from *Origanum vulgare* in our study is active against the same test organisms. This may not be observed in *Cocos nucifera*, however, since *Aspergillus fumigatus* in this study was isolated from the leaves. Most literature focusses on the coconut oil (NITBANI et al. 2016) and husk fibers (ESQUENAZI & al. 2002).

The pursuit of novel compounds from fungi is not new. In fact, it is in the kingdom fungi that the era of antimicrobials started with the discovery of penicillin from *Penicillium notatum* and allied species (RAPER & al. 1944). The anamorphic *Penicillium* and *Aspergillus* species, along with filamentous fungi, are among the widely studied species in relation to the production of novel metabolites (BLADT & al. 2013). The ubiquitous *Aspergillus* species have produced many novel compounds such as those derived from *A. niger* (VAROGU & CREWS 2000), *A. flavipes* (ROCHFORT & al. 2005), and *A. variecolor* (WANG & al. 2007), to name a few.

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