Evaluation secondary metabolite extract produced by *Aspergillus terreus* isolated from poultry droppings as antimicrobial agent

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Abstract
The purpose of this study was to separate fungi that produce antibiotics from bird droppings.

Place and Time of Study: Between October 2022 and March 2023, poultry droppings were gathered from farms and marketplaces in Poultry fields in Basra / Karma Ali.

Approach: A total of 150 samples of bird droppings were gathered. Subsequently, 1 gram of freshly deposited chicken droppings were measured and diluted in a sequential manner. The resulting dilutions were then cultivated for a duration of five days on malt extract agar and potato dextrose agar. Microscopical and morphological description of *Aspergillus terreus*. The mushroom extract's minimum inhibitory concentration (MIC) against pathogenic bacteria was determined using the agar well diffusion technique. The extract's aggressive behavior was evaluated against a total of four bacterial isolates, encompassing both Gram-positive and Gram-negative strains.

Results: The results showed that the fungal extract isolated from *Aspergillus terreus* showed aggressive behavior against pathogenic bacteria, as extracts of *Aspergillus terreus* secondary metabolites were effective against *Klebsiella pneumonia*, *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli* in a range of doses where the inhibitory concentration was the lowest for *Escherichia coli* and *Staphylococcus aureus* is 15 mg/ml, while the minimum inhibitory concentration for *Bacillus cereus* and *Klebsiella pneumonia* was 33.5. When the extracts are refined, they can be used to create new medicinal molecules from natural sources.

Keywords: Metabolite, *Aspergillus terreus*, poultry droppings, antimicrobial agent

1. Introduction
The pharmaceutical and industrial sectors have long been interested in the eukaryotic organisms that make up the fungal kingdom because they provide important natural sources of biologically active secondary metabolites compared to other elements made from plants, and the biochemicals extracted from fungi are active agents with unexplored potential. (Hung and Lin 2017) [18]. According to a recent Food and Drug Administration (FDA) assessment, microorganisms were responsible for around 25% of the 38% of medications derived from natural materials. The importance of microbes as a long-term pipeline for the development of novel drugs has been highlighted by these findings (Newman and Cragg 2016) [14]. Chemicals derived from natural products provide substantial and uninterrupted resources for medicinal chemistry applications. As a result, most leading pharmaceutical firms have focused on evaluating microbial natural extracts to develop high throughput libraries. (Hung and Lin 2017) [8]. Secondary metabolites, which are organic molecules, are synthesized by organisms through a limited number of fundamental biosynthetic pathways that deviate from primary metabolic pathways. These compounds are often synthesized by certain families and genera of animals. (Pazouki & Panda, 2000) [18].

Since ancient times, people have been drawn to fungi because they provide a rich source of secondary metabolites. Secondary metabolism is typically employed for conflict, animosity, or self-preservation against other species. The functions of these secondary metabolites, which include defense against pathogens and pests, etc., are known to be important in helping an organism adapt to its environment. Moreover, they serve as a crucial source for high-quality pharmaceuticals (Devi, et al 2020) [5]. The filamentous fungi found in animal manure, which belong to the phylum Ascomycota, and the most important of which is the...
2. Materials and Methods

2.1 Sample Collection

At the University of Basra/Karma Ali, samples of poultry waste were taken from domestic chickens and commercial poultry. We extracted fresh droppings from the chicken houses using disinfected plastic forks. Poultry litter that could not be collected with a plastic spoon was swabbed by passing a sterile swab over each sample until it darkened. Within an hour of collection, the samples were serially numbered and brought to the laboratory in plastic containers for processing.

2.2 Sample Processing

A 1 gram sample of poultry manure was subjected to homogenization in 10 milliliters of sterile water. The mixes were subjected to repeated dilution at a 10-fold ratio. The bacterial strains known as 10, 10-6, 10-7, and 10-8 were subsequently cultured on malt extract agar (MEA) and potato dextrose agar supplemented with chloramphenicol. The culture plates were incubated for a duration of five (5) days, during which daily monitoring was conducted till observation was made. Growth of fungal colonies. Maghraby et al., 1991) [10].

2.3 Isolation of Fungi

The pure culture was established by growing the mixed fungal culture individually in fresh PDA media. The dishes were then subjected to another five (5) days of incubation at room temperature and after growth of the pure fungal colony they were kept tightly in the refrigerator. Until I use it in the second step of the study (Norhafizah, 2012) [16].

2.4 Identification and Classification of Fungal Isolate

2.4.1 Microscopic Examination

From each isolated fungal culture, a minute quantity of cell and agar was extracted and subsequently transferred onto a microscope slide. The slides were dyed with lactic phenol blue and subsequently covered with a coverslip. The slides were then viewed using a light microscope at a low magnification of X40. The study observed several microscopic characteristics, such as the mycelial end, branching, hyphae structure, and the existence of spores.

The physical and cultural properties of the fungal isolates were compared to those of other fungal isolates in order to identify them and classify them according to the established categorization in the fungal atlas. (Norhafizah, 2012 & Adegunloye and Adejumuo, 2014) [16, 1].

2.6 Characterization of molecular genetics

The Prestomini gDNA yeast kit (Geneaid) was used to isolate fungal genomic DNA. For further study, genomic DNA was stored at -80°C; the conserved ITS region was amplified using the universal primer pair technique. The quantity, purity, and titer of genomic DNA products were evaluated using NanoDrop, electrophoresis, and polymerase chain reaction (PCR) (Mirhendi et al., 2006) [11]. The PCR products were submitted to Macrogen, Korea, for sequencing (http://dna.macrogen.com). Using the Basic Local Alignment Search Tool (BLAST), the sequence was aligned with the public database to compare the homology in GenBank Figure (2).

2.7 Bacterial Organisms Used for the Screening

The effectiveness of the fungal extract was tested against four isolates of pathogenic bacteria, which are Klebsiella pneumonia, bacteria isolated from Wound swab, Staphylococcus haemolyticus, isolated from cesarean section infections, Bacillus cereus isolated from food, Escherichia coli isolated from urinary tract infections, Staphylococcus aureus. Isolated from Nasial swab, they were obtained from the microbiology laboratory and the applied microbiology laboratory in the College of Science / University of Basra.

2.8 Preliminary Screening of Fungi Isolates for Antagonism

The disk diffusion method was followed to test the effectiveness of the active substances of the crude extract, as the weight of 0.2 ml of the crude extract of each fungus was dissolved in 1ml of the organic solvent DMSO. The bacteria were activated on nutrient agar medium and after 24 hours of incubation at 36 degrees, a suspension of each strain was made. Petri shower dishes containing 0.1 of the bacterial suspension were injected for each strain of bacteria and the number of cells (10^6) was spread by sterile swabs on MHA medium and left for one hour, A cork drill was used to create a 6 mm diameter hole in the center of the dish. Subsequently, 100 ml of the extract was injected into the dish. The dishes were then placed in an incubator set at a temperature of 37 °C for a duration of 24 hours. The examination of the plates involved the evaluation of inhibitory activity by the measurement of the width of the inhibition zone in millimeters. (Balouiri et al., 2016) [3].

2.9 Extraction of Secondary Metabolite

Fungal cultures represented by fermentation media were filtered separately if the fermentation media was decanted, the media were placed in centrifuge tubes at 6000 rpm for 10 minutes, filtered using Whatman No1 filter papers, and then the pH was adjusted to 3. By adding drops of hydrochloric acid HCL at a concentration of 0.1, then the active ingredients were extracted from the filtrate by adding a volume of the filtrate to an equal volume of ethyl acetate. Then the extracts were collected and placed in sealed
containers for preservation in the refrigerator at a temperature of 4 °C.

2.10 Minimum Inhibitory Concentration (MIC) Determination
The MIC was estimated using the agar diffusion method, as outlined by Ikegbunam et al. in 2018. The extract was prepared at different quantities (500, 250, 125, 65, 33.5, 15) and put to agar wells (6 mm in diameter) on culture plates of the test organisms. A positive control (Chromophenicol disks) and a negative control (DMSO) were also utilized. The wells were subsequently incubated at a temperature of 37 °C for a duration of 18 hours. The zone of inhibition was measured and recorded.

2.11. Statistical Analysis
The means ± standard deviation (SD) for all data were determined using SPSS Statistical Packages of Social Sciences (Version 26; USA) by the use of static analysis. The researchers employed the least significant difference test (LSD) to examine the disparity between averages. A statistical significance level of \( p < 0.05 \) was deemed significant.

3. Results
3.1 Identification of Fungal Isolates from Poultry Droppings
The phenotypic diagnosis of the filamentous fungi isolated in the current study was based on the color of the colony, its height, and its ability to form reproductive spores. The differential dye ectophenol for fungi was used. It was based on the shape and type of conidia in diagnosing various fungi Figure (1).

3.2 Molecular characterisation method
A. terreus isolate was identified using the advanced molecular characterisation method. The ITS region was amplified as a conserved region and sequenced. The ITS region sequences with a size of about 500-600 bp were compared with the sequences of the public database, which were deposited in the GeneBank (Fig 2). The homology was investigated using the BLAST. The identified A. terreus isolate was deposited at the GenBank with the accession number MT558939.1.

3.4 Production and extraction of bioactive metabolites from A. terreus: The secondary metabolites of the fungi produced in the fermentation medium were extracted after 30 days of incubation, and the organic acid ethyl acetate proved highly efficient in extraction, as shown in the following figure (3).

Fig 1: The phenotypic diagnosis of A. terreus

Fig 2: Identification of A. terreus species by molecular methods

Fig 3: Growth of the A. terreus strain and preparation of bioactive metabolites extract. A: Dried extract of organic solvent at room temperature. B: Extraction of bioactive metabolite by ethyl acetate. C: Growth of A. terreus strain on PDB medium for 30 days at 27 °C.)
3.5. Biological Activity
The secondary metabolite extract of *A. terreus* exhibited significant antibacterial activity against the four unique bacterial isolates examined, yielding highly favorable outcomes. A notable disparity in inhibition rates was seen between *Staphylococcus aureus II*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Bacillus cereus*, including both Gram-positive and Gram-negative bacteria. (Figure 4).

![Fig 4: Showing the inhibitory activity of the fungal extract, (Aspergillus terreus) against pathogenic bacteria at a concentration of (1000 μg/ml), A: Escherichia coli (Gram-), B: Staphylococcus aureus (Gram+).](image)

3.4. Measurement of the minimum inhibitory concentration of the fungal extract against pathogenic bacteria
Different concentrations of the fungal extract were used to determine the minimum inhibitory concentration for the growth of pathogenic bacteria. We used 6 concentrations of the extract, which are (500, 250, 125, 65, 3, 3.5, and μg /ml), the inhibitory concentration was measured as the inhibitory concentration was the lowest for *Escherichia coli* and *Staphylococcus aureus* is 15 mg. /ml, while the minimum inhibitory concentration for *Bacillus cereus* and *Klebsiella pneumonia* was 33.5 Table (1), Figure (5). Significant differences were also calculated for the inhibition rates of pathogenic bacteria Table (2).

Table 1: Minimum Inhibitory Concentration rate of *A. terreus* secondary metabolite extract against bacterial test organism mg/ml (Green color refers to MIC values)

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>500 μg/ml</th>
<th>250 μg/ml</th>
<th>125 μg/ml</th>
<th>15 μg/ml</th>
<th>33.5 μg/ml</th>
<th>15 μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

![Fig 5: Varying zones of inhibition based on varying A. terreus extract doses. MIC values for Bacillus cereus](image)

Table 2: Showing comparison between different pathogenic Bacteria using different Concentrations of the crude. (S: Significant difference between groups (p value <0.05), *groups with different letters are significantly different based on the p-values)

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Types of Bacteria</th>
<th>Inhibition zone mean</th>
<th>Mean Labels</th>
<th>LSD p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td>18.5 9.5 8 5.5 3 1 18.5 9.75 A</td>
<td>3.292 0.044&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>E. coli</em></td>
<td>19.5 10 7.5 4.5 2.5 1 22 9.57 A</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>B. cereus</em></td>
<td>13 8 5 2.5 1.5 0 18.5 6.93 B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>K. pneumoniae</em></td>
<td>12.5 9.5 5 1.5 0 17 6.64 B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean Labels</td>
<td>15.875 b 9.25 c 6.375 d 3.5 E 2 e 0.5 f 19 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td></td>
<td>0.03&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
4. Discussion
The most significant bioactive substances for treating infectious disorders are antibiotics. However, the rise of microorganisms resistant to several drugs has created fundamental obstacles to the effective treatment of infectious diseases. Thus, there has been a growing interest in finding effective antibiotics due to the burden of highly frequent multidrug resistant infections worldwide. In a prior investigation, secondary metabolites and fungi that produce antibiotics were isolated using samples of chicken litter from several poultry farms in Ihiala that were chosen at random. According to earlier research, one of the most crucial steps in isolating several species of strong antibiotic-producing fungi was to choose various prospective locations, such as soil roots and chicken litter (Abo-Shadi et al., 2010) [2]. Because biotic and abiotic processes interact in poultry farms, they offer a favorable environment for microbial activities (Okoli 2006) [17].

The Aspergillus genus possesses a robust ability to synthesize most of the essential pharmaceutical compounds, including butenolide, aspergivones, aurasesperone, and Asperchondols H. (Wang et al., 2015, and C.H. Liu, 2015 & Li et al., 2018) [20, 19].

An significant species of Aspergillus, Aspergillus terreus is used in the commercial manufacturing of lovastatin, a medication that lowers cholesterol, and itaconic acid, a biobased chemical. These industrial applications have proved the superior fermentation capability. The genome data showed that more research is needed to fully understand A. terreus’ exceptional ability to synthesize natural products. Recent developments in genome mining approach have facilitated the identification of compounds derived from diverse cryptic biosynthetic gene clusters. Several metabolic engineering research have been conducted on commercial strains of itaconic acid and lovastatin in order to improve the production techniques (Huang et al., 2021) [7].

A. terreus has the capability to create an extra metabolite. This chemical demonstrated significant efficacy against clinical bacterial isolates in laboratory environments. The analysis of the extract revealed the presence of the phenolic compound 2-(4-hydroxyphenyl) tetrahydro-3, 4, which has significant antibacterial, antifungal, and antivirulence properties. The compound furandiol was first identified in the species A. terreus. (Naser, et al., 2023) [13].

In the current study, the ability of the isolated fungi to inhibit the growth of pathogenic bacteria has been revealed initially, and we have used two types of bacteria, gram-positive bacteria, which are Staphylococcus aureus bacteria. And bacteria negative for the gram stain, which are Escherichia coli bacteria, and both pathogenic bacteria isolated from patients with urinary tracts were obtained from the laboratory of applied microbiology in the Department of Life Sciences of the College of Science, University of Basrah. Very encouraging outcomes were obtained when the secondary metabolite extract of A. terreus was tested against the four different bacterial isolates that were the subject of the investigation. On the other hand, a substantial difference was observed in the rates of spike inhibition for both Gram-positive and Gram-negative bacteria in Staphylococcus aureus II, Klebsiella pneumoniae, Escherichia coli, and Bacillus cereus.

The creation of new drugs requires new sources. Given the importance of secondary fungal metabolites that have proven therapeutic efficacy, and given the rapid expansion of pathogenic bacteria and the emergence of drug-resistant strains, which represent serious clinical problems in the treatment of infectious diseases, the search for new antibiotics is required. (Cadelis, et al., 2022 & Ng et al., 2015) [4, 15].

Conclusion
In conclusion, the comprehensive study undertaken in this research elucidated the identification, molecular characterization, production, and biological activity of bioactive metabolites extracted from Aspergillus terreus. Through phenotypic diagnosis and advanced molecular methods, the fungal isolate was accurately identified and deposited in the GenBank. The efficacy of ethyl acetate in metabolite extraction was demonstrated, leading to the production of bioactive compounds exhibiting significant antibacterial activity against various pathogenic strains. The
determination of minimum inhibitory concentrations further underscored the potency of the fungal extract against both Gram-positive and Gram-negative bacteria. Importantly, this study highlights the potential of *A. terreus* as a valuable source of secondary metabolites with therapeutic applications, particularly in combating multidrug-resistant bacterial infections. These findings underscore the importance of exploring fungal resources for novel antibiotic discovery, especially amidst the growing challenge of antibiotic resistance in clinical settings. Moving forward, further research into *A. terreus* and similar fungal species holds promise for addressing the pressing need for effective antimicrobial agents in the face of evolving infectious diseases.

**References**


