Archives of Razi Institute, Vol. 76, No. 5 (2021) 1333-1341

DOI: 10.22092/ari.2021.356366.1832

Original Article

Effect of the Crude Extract of Coprophilous Fungi on Some Bacterial Species Isolated from Cases of Mastitis

Jasim, A. S[1], Abass, B. A[1 *], Al-Rubayae, I. M[2]

1. Department of Microbiology and Parasitology, College of Veterinary Medicine, University of Basra, Basrah, Iraq
2. Department of Biology, College of Science, University of Basra, Basrah, Iraq

Received 4 October 2021; Accepted 29 October 2021
Corresponding Author: basil.abbas@uobasrah.edu.iq

Abstract

Coprophilous fungi are a large group of fungi mostly found in herbivore dung and have an exclusive life cycle. This group of fungi produces many important metabolites which can be consumed in medicine or agriculture. The present study aimed to investigate the antibacterial effects of these fungi on bacterial mastitis. A total of 50 dung samples were collected from four herbivores (cows, buffalos, sheep, and camels) from different areas of Basra. The moist chamber method was used for each sample to establish a fungal fruiting body and detect the type of the fungi. The coprophilous fungi included *Aspergillus sp. (A. niger, A. fumigatus, A. flavus, A. terrus), Chaetomium sp., Sordaria sp., and Podospora sp.* which belong to the Ascomycetes class. PCR test was performed using the ITS region for confirmatory detection of species. The highest and the lowest number of isolated species was associated with cow dung and camel dung, respectively. The antimicrobial property of three different partitioned extracts (petroleum ether [F1], ethanol [F2], and water [F3]) prepared from some fungal mycelia was evaluated in vitro. All fractions were tested to detect antimicrobial activity using the disc diffusion assay against five pathogenic bacteria *Staphylococcus aureus*, *Streptococcus Enterobacter*, *Proteus mirabilis*, and *E. coli*. which is isolated from bovine mastitis. Data revealed that all fractions could inhibit the tested bacteria. However, inhibitory activity was found to be dependent on (6i) the used fungal strains; (ii) the extracted solvent; and (iii) the tested bacteria. In general, the petroleum ether extracts (F1) derived from all fungi displayed the highest inhibitory activity against the testing bacteria. In conclusion, the present study concluded that the extracts prepared from the fungal mycelia contain bioactive compounds with antibacterial properties. This study was first conducted in Iraq and further studies are required to develop new treatments.

Keywords: Antibiotic activity test, Coprophilous fungi, Herbivore dung, Iraq

1. Introduction

Coprophilous (dung-loving) fungi are a distinctive large group of saprophytic fungi modified to grow in herbivore dung (1). Coprophilous fungi are dung-inhibiting fungi and are more commonly found in domesticated farm mammals such as cow, sheep, buffalo, goat, and sheep; while, they are less prevalent in birds and wild mammals (2). Fungi that can develop and survive on herbivore dung form a limited group of microorganisms commonly known as coprophilous fungi (3). These fungi adapted to these rich and distinct conditions and display exceptional morphological features as having a life cycle that begins during the grazing of herbivores on vegetation and foodstuffs. Although many fungi spores are ingested during feeding, most are destroyed under the high temperature and gastric juices in the gastrointestinal tract (4). The spores of coprophilous fungi protect themselves in various ways. Once they are excreted from the droppings, they are transferred to the plants and re-ingested by the herbivores to complete their life cycle. This ecological group includes highly specific species

that can survive in the gastrointestinal tract of animals (5). However, these fungi identify an important component of ecosystems that is responsible for reutilizing nutrients in the animal dung and provide important storage for ecological, paleontological, and palynological studies (6, 7). This group is an important source of antibiotics, enzymes, and biological control agents (8, 9). Therefore, the secretion potential of coprophilous fungi containing enzymes to destroy the plant cell wall is significant (10). Many secondary or specific metabolites produced in the life cycle of coprophilous fungi have been discovered and confirmed to have antimicrobial, antioxidant, and anti-inflammatory activities (11, 12). Natural products derived from microbes are potentially useful in numerous fields, including agriculture, industry, and medicine. Microbial metabolites have been the main source of most antibiotics currently available on the market from a medical point of view (13). Since the revolution in antibiotic discovery during the last century, the trend of resistance of pathogenic bacteria to antibiotics has dramatically enhanced, especially in the last few years (14). Therefore, there is an urgent need to screen new antimicrobial agents that can play a vital role in controlling bacterial infections caused by multi-resistant pathogens (15). However, to the best of our knowledge, no reports available on herbivores dung samples. The present study aimed to isolate and identify saprophytic and coprophilous fungi from different samples of herbivore dung in Basrah, and to investigate the effect of antibacterial activity of their crude extract on some bacterial mastitis. Part of a research program focusing on fungal diversity examines some fungal strains that are capable of producing active metabolites against bacterial pathogens.

2. Materials and Methods

2.1. Samples Collection

A total of 50 dung samples were collected during the study from four herbivores, including caws, buffalos, sheep, and camels which normally lived in Basra. The stool sample containers were transferred to the veterinary laboratory unit using a plastic icebox. Dung samples were dried in the shade and stored at room temperature (28°C) in paper envelopes.

2.2. Moist Chamber Method

The moist chamber method was used to isolate the saprophytic and coprophilous fungi from dung samples. For this purpose, an appropriate amount of dung is moistened using sterile distilled water and incubated at room temperature. After three days, the samples were examined for fruiting bodies with a dissecting microscope. Long incubation periods of up to 60 days were performed to develop fungi and their fruiting bodies. A small amount of sterile water with chloramphenicol antibiotic was occasionally added to the dishes to keep the dung constantly moist (16, 17). No culture medium from the dung samples was used for growing saprophytic and coprophilic fungi, since the fungi grew on its host, and received the moister produced from the wet condition prevailing inside the blotting paper covering Petri dishes. Periodically, the plates were microscopically examined to detect fungal growth, sporulation, and fruit body formation. Growing fungi were directly isolated using a fine sterile needle to collect fungal spores from the dung samples, and transferred to the PDA (Potato Dextrose Agar, Merck, Germany) and PCA (Potato Carrot Agar) media (18, 19).

2.3. Observation of Macrofungi

In cases that fruit bodies were formed, fungal structures were separately removed, mounted in pure potassium hydroxide or sterile distilled water, and tested under a dissecting microscope with higher magnification (3).

2.4. Observation of Microfungi

The fungal growth was identified based on the morphological features (colony growth form, color, texture on different agar media, and spore formation) using light microscopy following the taxonomic guidelines. Slides were prepared, stained with lactophenol cotton blue, and observed using a light microscope at lower (×10) and higher (×40)

magnification (20-22).

2.5. Molecular Detection

2.5.1. Polymerase Chain Reaction

DNAs were extracted from the harvested fungal isolates grown on PDA for 8 days in dark and fresh mycelia according to the manufacturer instructions of Presto™ Stool DNA Extraction Kit (*Geneaid, South Korea*). The forward (ITS1: 5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse (ITS4: 5'-TCCTCCG CTTATTGATATGC-3') primers were used to target the ITS region of 18S rRNA gene (23). According to the manufacturer instruction of Maxime PCR Premix (*Intron, Korea*), the tube of the master mix was prepared at a final volume of 20µl. The protocol of the Thermal cycler (BIO-RAD, USA) system was designed as follows (Table 1):

Table 1. The protocol of the thermal cycler (BIO-RAD, USA) system

No. of Cycle	Step	Temperature	Time
1	Initial denatuartion	95^0 C	4 minutes
35	Denatuartion	95^0 C	30 seconds
	Annealing	56^0 C	30 seconds
	Extension	72^0 C	1 minute
1	Final Extension	72^0 C	6 minutes
-	Hold	4^0 C	-

A total of 10µl of each PCR product in addition to 5µl of DNA ladder were electrophoresed into agarose-gel (1%) stained with ethidium bromide. Visualization was performed using the UV Transilluminator (Clinx Science, China).

2.5.2. Sequence Analysis

Positive DNAs were sequenced, and the data were analyzed based on Megablast search analysis in the NCBI GenBank nucleotide database (www.ncbi.nlm.nih.gov).

2.6. Extraction and Fractionation of Fungal Crude Metabolites

Seven fungal strains were used in the present study; *Aspergillus niger, A. terrus, A. Fumigates, A. flavus, Chaetomium, Sordaria,* and *Podospora*. According to a design recently presented by Synytsya, Monkai (24), the fungal crude metabolites were extracted and fractionated (Figure 1).

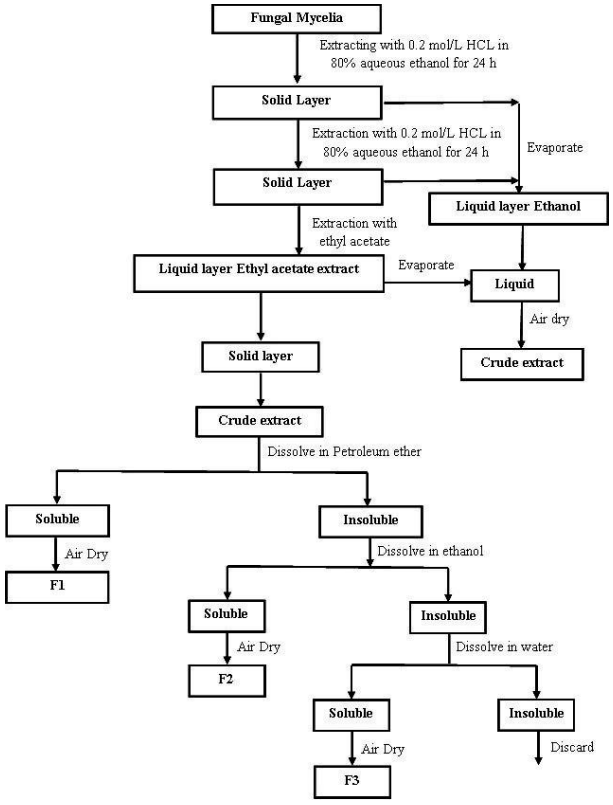


Figure 1. Extraction and fractionation of fungal crude metabolites (24)

2.7. Antibacterial Screening Test

Crude fungal extracts were screened for antibacterial activity against five pathogenic bacteria *Staphylococcus aureus, Streptococcus enterobacter, Proteus mirabilis,* and *E. coli* which were previously isolated from bovine mastitis samples in Basra. After overnight incubation at 37°C on the brain-heart infusion broth culture, antimicrobial activity assay was measured using the crude extract on Mueller Hinton agar medium plates. Then, antimicrobial activity assay was performed by the modified paper disc assay (25).Each experiment was performed in two replicates. The values of the growth of the inhibition zone around the discs were measured by millimeter (mm).

2.8. Statistical Analysis

All the values were expressed as mean ± standard deviation (M±SD), and variations between the means were analyzed by one-way ANOVA using SPSS software (version 20). A p-value less than 0.05 is statistically significant.

3. Results

A total of 4 genera and 4 species of coprophilous fungi belonging to class *Ascomycota sp*. were obtained to form the fruiting bodies for all collected dung samples in the moist chamber (Table 2).

Table 2. Frequency and distribution of coprophilous fungal species (*Ascomycota sp*.)

Fungal species	Frequency %	No. of isolated fungal species				Total
		Cow	Buffalo	Sheep	Camel	
Aspergillus niger	17.71	15	15	8	10	48
Aspergillus fumigatus	17.34	20	10	7	10	47
Aspergillus flavus	16.6	7	18	10	10	45
Aspergillus terreus	15.13	15	8	10	8	41
Sordaria	12.91	10	10	15	0	35
Podospora filiformis	11.8	10	12	10	0	32
Chaetomium	8.49	10	2	8	3	23
Total	-	87	75	68	41	271

During isolation and identification of coprophilous fungi from herbivore dung samples, the populations of *Aspergillus sp.* were dominant in comparison to *Chaetomium sp.*, *Sordaria sp*., and *Podospora sp*. Statistically, the highest value was reported for *Aspergillus niger* (17.71%) followed by *A. fumigatus* (17.34%), *A.flavus* (16.6%), and *A. terreus* (15.13%). Other coprophilous fungi; *Sordaria sp*. (12.91%), *Podospora filiformis* (11.8%), and *Chaetomium sp.* (8.49%) species were reported with a significant decrease in concentration (Figure 2).

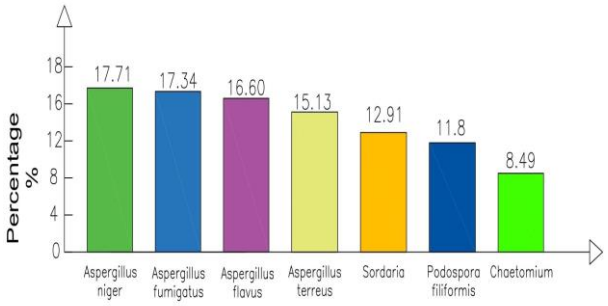


Figure 2. Results for occurrence coprophilous *Ascomycota sp.* fungal isolates

The relative presence of the fungi isolated from the dung samples showed that there were significant differences in the frequency of each fungus in the dung samples as well as their distribution in the herbivore dung (Figure 3)

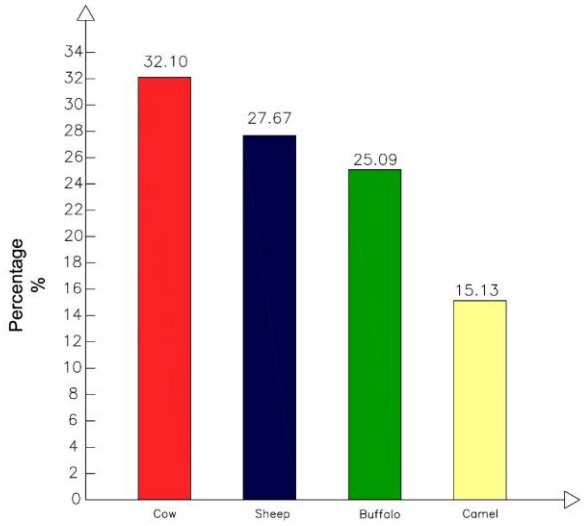


Figure 3. Prevalence of fungal isolates among herbivores

The results of the DNA amplification revealed the positive groups of fungi at 500bp for the gene of ITS ITS4 (Figure 4). The highest levels for isolated fungi were obtained from cows, sheep, and buffalos. While the lowest level of coprophilous fungi was isolated from camel dung. Differences in these results may be due to the nature of camels feeding on desert halophytes, which limits the growth of these fungi during the study period, or due to the physicochemical structure of this type of dung (9, 20).

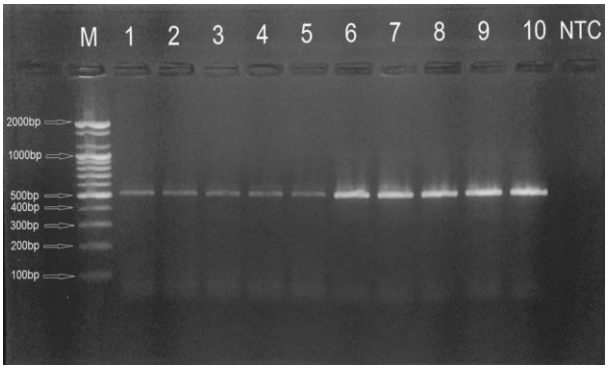


Figure 4. Result of conventional PCR to detect the 18SrRNA ITS gene of coprophilous *Ascomycota sp.* fungal isolates
M: Ladder marker (100-2000bp); Lanes 1-10: Positive samples at 500bp;
Lane NTC: Negative control

The difference in number and type of fungi isolated is probably a reflection of physiochemical and type of plant species consumed by these animals (Figure 5). Factors affecting the diversity of coprophilous mycoflora are nutritional and ecological factors, pH, aeration, humidity, temperature, light periodicity, competition, and predation (26). The result indicated that all the fungal extracts tested were active and were able to inhibit at least one of the testing bacteria with different a spectrum which was significant at $P<0.05$. Different solvents used to prepare mycelial extracts also have different degrees of inhibitory activity.

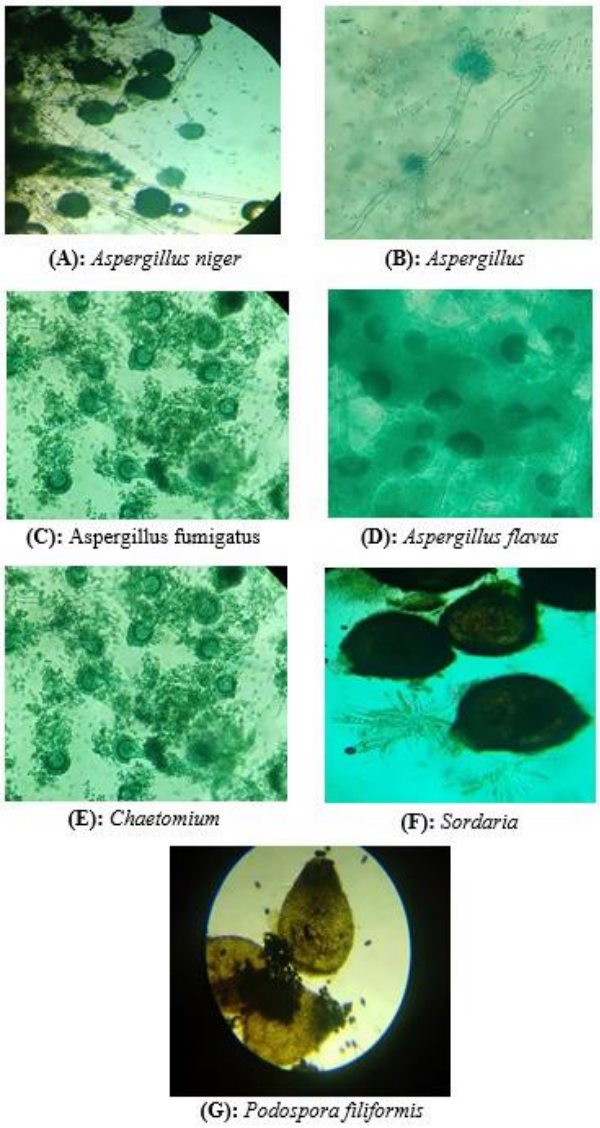
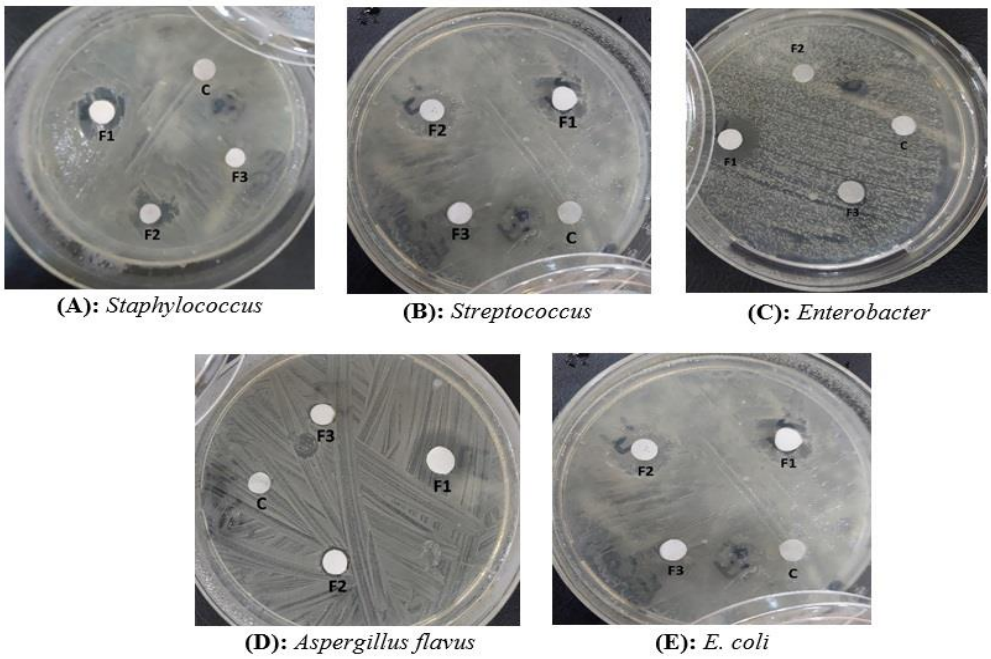


Figure 5. Coprophilous fungi Ascomycetes using light microscope

The petroleum ether (F1) extracts prepared from most fungal mycelia were more effective in inhibiting a wide range of the testing bacteria, including both gram-positive and gram-negative bacteria (Table 3, Figure 6). The only exception was the F3 extract derived from *Chaetomium* sp., which showed inhibitory activity against the gram-negative bacteria.

Table 3. Antibacterial activity of fungal extracts (F1: Petroleum ether; F2: Ethanol; F3: Water)

Fungus	Extract	*Staphylococcus aureus*	*Streptococcus*	*Enterobacter*	*Proteus mirabilis*	*E. coli*
Sordaria spp.	F1	15±1.41	10±2.82	8±1.41	0±0.0	12.50±2.12
	F2	11±1.41	7±1.41	0±0.0	5±2.82	12.5±2.12
	F3	0±0.0	4±1.82	4±1.65	7±1.41	1.5±0.12
Chaetomium spp.	F1	14.5±0.7	7±2.82	10±1.41	8.5±0.7	0±0.0
	F2	10.5±2.12	9±1.41	7±1.41	0± 0.0	1.5±0.12
	F3	2±0.41	6±1.41	5±0.01	2.5±0.53	0± 0.0
Podospora	F1	15±1.41	0±0.0	12±1.41	7.5±0.7	8±1.41
	F2	7±1.41	2±1.41	0±0.0	1.5± 2.12	8±5.65
	F3	10.5±2.12	3±0.41	2±0.41	0±0.0	1.5±0.12
Aspergillus. niger	F1	13.5±2.12	14±2.82	8±1.41	12.5±3	7±0.0
	F2	8.5± 0.7	7±1.41	0±0.0	0±0.0	3.5± 0.7
	F3	4± 1	2±1.41	10±2.82	0± 0.0	3±0.82
Aspergillus fumigatus	F1	20±1.41	14.5±2.12	10±4.24	0± 0.0	7.5± 0.7
	F2	0±0.0	0±0.0	3.5±0.7	12±1.41	7.5±2.12
	F3	3±1.41	4±4.24	11±1.41	4±2.82	0±0.0
Aspergillus flavus	F1	0±0.0	12±1.41	13.5±2.12	0±0.0	0±0.0
	F2	4±1.41	7.5±2.12	8±1.41	1±0.41	6.5±0.7
	F3	0±0.0	5±2.82	0±0.0	8±1.41	6.5±2.12
Aspergillus terreus	F1	17±8.48	14±1.41	9.5±0.7	6±1.41	12±1.41
	F2	0±0.0	12±1.41	3±2.82	0±0.0	4±1.41
	F3	9±1.41	3±0.41	4±0.24	0± 0.0	0±0.0



(A): *Staphylococcus* **(B):** *Streptococcus* **(C):** *Enterobacter*

(D): *Aspergillus flavus* **(E):** *E. coli*

Figure 6. Result of antibacterial activity test of crude extract of fungal isolates (F1: Petrolleum ether; F2: Ethanol; F3: Water; C: Negative control)

There is a significant increase in multi-resistant bacteria to commonly used antimicrobial agents that are emerging as a serious medical problem worldwide. Therefore, fundamental requirements are needed to manage and control this challenge through progressive alternative approaches that led to discovering a new source of antibacterial compounds. Fungi are a major source of beneficial bioactive compounds such as antibiotics and plant-growth-promoting compounds used in medicine and agriculture (24, 27).For this reason, the present study focuses on screening a new active antibacterial agent from coprophilous fungi.

Fungal metabolites are diverse and show various biological activities (28). The results obtained from the present study further confirm this by highlighting antibacterial activity. Based on the available findings, fungi are one of the important biological sources for the discovery of antibiotics. The in vitro experiments have revealed that the coprophilous fungal strains produced metabolites exhibit antibacterial activity to varying degrees which depends on the producing strain, extracting solvents, and testing microbes. The fungi selected in this study are common saprobes coprophilous some of which are widely known to produce active extracellular metabolites with antimicrobial activity. For example, *Aspergillus* species secrete a plethora of metabolites and are industrially used as biocontrol agents (29). *Chaetomium* species are also known to produce various bioactive compounds including mycotoxins. In addition, some new compounds with potential use in medicine have been isolated and described from these fungi; these include chaetochromones from *Chaetomium* (30-32).

It should be noted that this is one of the few things that deal with fungal mycelial extracts. Generally, many researchers have focused their interest on extracellular metabolites for many reasons. Some advantages include: (i) the fungal cells remain viable; (ii) the extraction of metabolites is simple; and (iii) large-scale production is convenient. However, the extract obtained from the fungal cells (i.e., intracellular substances) also exhibited biological activity. In some cases, extracts obtained from fungal mycelia displayed stronger activity (24, 33). Our results further confirm the potential use of fungal metabolites in the medical and pharmaceutical industries. The data obtained are expected to open up a new perspective in medical research to focus on the importance of a preliminary screening study, which is a key step in pharmaceutical channel research. *Aspergillus* species have high efficiency in producing secondary metabolites. The efficacy is due to the existence of massive genomic and proteome. Interestingly, all crude extracts of *Aspergillus* sp. sufficiently inhibited the growth of gram-positive and gram-negative pathogenic bacteria. Meanwhile, not all *Aspergillus* sp. inhibits the growth of gram-negative bacteria. The best explanation for this result could be the possibility of a low molecular chemical effect on gene cluster of antibiotic production that could lead to producing a new active secondary metabolite that has antibacterial activity (27). The results of this study revealed that fungal mycelial extracts of four coprophilous fungi differed in their antimicrobial activity against tested bacteria strains. This change is based on the ability of fungal isolates in producing inhibitory metabolites against pathogenic bacteria.

Authors' Contribution

Study concept and design: A. S. J.
Acquisition of data: B. A. A.
Analysis and interpretation of data: B. A. A.
Drafting of the manuscript: I. M. A.
Critical revision of the manuscript for important intellectual content: B. A. A.
Statistical analysis: A. S. J.
Administrative, technical, and material support: B. A. A.

Ethics

All the procedures during the current study were approved by the University of Basra, Basrah, Iraq animal care and ethics committee.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Thilagam L, Nayak B, Nanda A. Studies on the diversity of coprophilous microfungi from hybrid cow dung samples. Int J PharmTech Res. 2015;8:135-8.
2. Mueller RC, Scudder CM, Whitham TG, Gehring CA. Legacy effects of tree mortality mediated by ectomycorrhizal fungal communities. New Phytol. 2019;224(1):155-65.
3. Ghosta Y, Poursafar A, Fathi Qarachal J. Study on coprophilous fungi: new records for Iran mycobiota. Rostaniha. 2017;17(2):115-26.
4. Vašutová M, Mleczko P, López-García A, Maček I, Boros G, Ševčík J, et al. Taxi drivers: the role of animals in transporting mycorrhizal fungi. Mycorrhiza. 2019;29(5):413-34.
5. Miranda V, Sede S, Aranda-Rickert A, Rothen C, Scervino JM, Barros J, et al. Taxonomy, life cycle and endophytism of coprophilous fungi from an underground desert rodent. Fungal Ecol. 2020;43:100872.
6. Florenzano A. The history of pastoral activities in S Italy inferred from palynology: a long-term perspective to support biodiversity awareness. Sustainability. 2019;11(2):404.
7. Richards TA, Jones MD, Leonard G, Bass D. Marine fungi: their ecology and molecular diversity. Ann Rev Mar Sci. 2012;4:495-522.
8. Karwehl S, Stadler M. Exploitation of fungal biodiversity for discovery of novel antibiotics. How to Overcome the Antibiotic Crisis. 2016:303-38.
9. Richardson MJ. Diversity and occurrence of coprophilous fungi. Mycol Res. 2001;105(4):387-402.
10. Peterson R, Grinyer J, Nevalainen H. Secretome of the coprophilous fungus Doratomyces stemonitis C8, isolated from koala feces. Appl Environ Microbiol. 2011;77(11):3793-801.
11. El-Shahat H. Endophytic fungal research in Egypt: Present status. Microbial Biosystems. 2020;5(1):122-7.
12. Rangarajan S, Verekar S, Deshmukh SK, Bellare JR, Balakrishnan A, Sharma S, et al. Evaluation of anti-bacterial activity of silver nanoparticles synthesised by coprophilous fungus PM0651419. IET Nanobiotechnol. 2018;12(2):106-15.
13. Sarrocco S. Dung-inhabiting fungi: a potential reservoir of novel secondary metabolites for the control of plant pathogens. Pest Manag Sci 2016;72(4):643-52.
14. Fair RJ, Tor Y. Antibiotics and bacterial resistance in the 21st century. Perspect Medicinal Chem. 2014;6:PMC. S14459.
15. Nikaido H. Multidrug resistance in bacteria. Annu Rev Biochem.2009;78:119-46.
16. Carris L, Little C, Stiles C. Introduction to fungi. The plant health instructor. Washington State University, Kansas State University, and Georgia Military College. 2012.
17. Sánchez C. Cultivation of Pleurotus ostreatus and other edible mushrooms. Appl Microbiol Biotechnol. 2010;85(5):1321-37.
18. Kakde UK, Kakde HU. Incidence of post-harvest disease and airborne fungal spores in a vegetable market. Acta Botanica Croatica. 2012;71(1):147-57.
19. Sharma G. Influence of culture media on growth, colony character and sporulation of fungi isolated from decaying vegetable wastes. J Yeast Fungal. 2010;1(8):157-64.
20. Altayyar IA, Ismail AS, Abdullah SK. A preliminary study of Coprophilous Fungi in North of Jordan.Development. 2017;4:6.
21. Moghalles M, Al-Bader S. Isolation and identification of Fungi from the Animal Dung in Thamar-Yemen and Estimation of the Antagonistic Activity of Papulospora sp. against Fusarium oxysporum. Yemeni J Agric Vet Sci. 2014;1(2):22-26.
22. Piasai O, Manoch L. Coprophilous ascomycetes from Phu Luang wildlife sanctuary and Khao Yai national park in Thailand. Agriculture and Natural Resources. 2009;43(5):34-40.
23. Ding J, Jiang X, Guan D, Zhao B, Ma M, Zhou B, et al. Influence of inorganic fertilizer and organic manure application on fungal communities in a long-term field experiment of Chinese Mollisols. Applied Soil Ecology. 2017;111:114-22.
24. Synytsya A, Monkai J, Bleha R, Macurkova A, Ruml T, Ahn J, et al. Antimicrobial activity of crude extracts prepared from fungal mycelia. Asian Pacific Journal of Tropical Biomedicine. 2017;7(3):257-61.
25. Ellis M. More dematiaceous hyphomycetes. Ed. Commonwealth Mycological Institute. Kew, Surrey, England. 608 pp. 1971.

26. Richardson M. Records of coprophilous fungi from the Lesser Antilles and Puerto Rico. Caribbean Journal of Science. 2008;44(2):206-14.
27. Zutz C, Bandian D, Neumayer B, Speringer F, Gorfer M, Wagner M, et al. Fungi treated with small chemicals exhibit increased antimicrobial activity against facultative bacterial and yeast pathogens. Biomed Res Int. 2014;2014.
28. Ellis MB. Dematiaceous hyphomycetes. Dematiaceous hyphomycetes. 1971.
29. Ellis MB, Ellis JP. Microfungi on miscellaneous substrates: an identification handbook: Croom Helm; 1988.
30. Al-Ani LKT. The Importance of Endophytic Fungi from the Medicinal Plant: Diversity, Natural Bioactive Compounds, and Control of Plant Pathogens. Medically Important Plant Biomes: Source of Secondary Metabolites: Springer; 2019. p. 189-238.
31. Nielsen KF. Mycotoxin production by indoor molds. Fungal Genet Biol. 2003;39(2):103-17.
32. Pimentel MR, Molina G, Dionísio AP, Maróstica Junior MR, Pastore GM. The use of endophytes to obtain bioactive compounds and their application in biotransformation process. Biotechnol Res Int. 2011;2011.
33. Majhenič L, Škerget M, Knez Ž. Antioxidant and antimicrobial activity of guarana seed extracts. Food Chem. 2007;104(3):1258-68.