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Antibacterial activity of *Rhizopus* specie isolated from rhizosphere of *Mentha Piperita*

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***Abstract*---**Soil is loaded by microbes which are capable of producing antibiotics. These compounds were used therapeutically and sometimes prophylactically in the control of infectious disease. Soil

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microbes can be grouped into Bacteria, Actinomycetes, Fungi, Algae, Protozoa and Nematodes. Fungi are unique eukaryotes which ingest food from outside and take nutrients through its cell walls and consists of tiny tubular mycelia known as hyphae. The fungi are well known in medical field due to the production of a wide variety of secondary metabolites. The current research study was focused on isolation, identification and antibacterial activity of fungi isolated from rhizosphere of Mentha Piperita (Mint), Abasyn University, Peshawar. The research study was conducted in the Microbiology Research Laboratory (MRL) of Abasyn University Peshawar. The soil sample was collected from rhizosphere by using sterile techniques. Serial dilution method was used to dilute the concentration of microbes present in soil sample. The Potato Dextrose Agar media was prepared and by pour plate method the sample was streaked on the plate. The plates were incubated at 280C for 72hours. The fungus was isolated by sub culturing technique and then identified by morphological and microscopic examination using Lactophenol cotton blue stain (LPCB). The fungus was identified as a member of genus Rhizopus. The metabolites production was done by using Potato Dextrose Broth (PDB) medium. The Rhizopus specie was grown in PDB at 28°C with continuous agitation of 100rpm for 10 days. The liquid culture was filtered through a Whatman filter paper. The filtrate was placed in a separating funnel and ethyl acetate was added in a ratio 1:1 of the filtrate. The ethyl acetate phase (lower phase) was recovered in a flask and evaporated through a rotary aspirator at 50 °C with a slight rotation (150rpm) and then the extract was collected in a glass vial. The antibacterial potential of extract was then determined against four identified bacterial samples collected from Microbiology Research Laboratory (MRL) of Abasyn University Peshawar. The bacteria were i.e., Klebsiella, Pseudomonas, Salmonella and Shigella isolated from diabetic foot ulcer patients. Nutrient agar medium was prepared and 24hour old bacterial inoculum was spread onto the surface of nutrient agar medium with the help of sterile cotton swab and wells were made for application of crude extract. The sample concentration 12 mg of the ethyl acetate extract per ml of DMSO, showed good activity in the form of inhibitory zones at the concentration rate of 1.2 mg/ml against Salmonella (17mm), S. epidermidis (17mm), Klebsiella (18mm) and Shigella (16mm). Whereas, at the concentration rate of 0.9 mg/ml the maximum zone of inhibition was observed against S. epidermidis (17mm), Shigella (15mm), Klebsiella (15mm) and Salmonella (14mm). Similarly, at the concentration rate of 0.6 mg/ml, the maximum zone of inhibition was observed against Klebsiella (15mm), S. epidermidis (15mm), Shigella (14mm) and Salmonella (13mm). Thus, it was concluded that soil sample of Mentha piperita consist of bioactive Rhizopus specie potent of inhibiting the bacterial growth.

***Keywords*---**Mentha piperita, antibacterial, antibiotics, bioactive.

Introduction

Soil microbes can be grouped into Bacteria, Actinomycetes, Fungi, Algae, Protozoa and Nematodes. Soil is loaded by microbes which are capable of producing antibiotics. Soil naturally contains 10^9 to 10^{10} microbes per gram (dry weight), which may present more than a million bacterial species. However, characterization of the small fraction of microbes that has been cultivated provides only a glimpse of their potential physiological capacity and influence on soil ecosystems. The absence of pure cultures or genome sequences makes it difficult to ascertain the roles of specific microbes in soil environments this is particularly true for bacteria in the Phylum bacteria which are broadly distributed in soils but poorly represented in culture (Batool *et al.,* 2017). Antibiotics are antimicrobial compounds produced by living microorganisms. These compounds were used therapeutically and sometimes prophylactically in the control of infectious disease. Over 4000 antibiotics have been isolated before but only 50 have achieved wide range usage. The other antibiotic compounds failed to achieve commercial importance for some reasons such as toxicity to human and animals, ineffectiveness or high production costs. The isolation of antibiotics from microorganisms is relatively easy as compared to chemical synthesis of antimicrobial agents. The isolation of antibiotics from microorganisms improved the discovery of novel antibiotics that could act as better chemotherapeutic agent (Helen *et al.,* 2012).Fungi are unique eukaryote which ingests food from outside and take nutrients through its cell walls. The reproduction of fungus takes place through spores. The body of fungi consists of tiny tubular cells known is hyphae. Like other animal fungi are also heterotrophs which cannot synthesize their own food (Ramezani *et al.*, 2019). The mode of nutrition of fungi are varied some are biotrophs mean feed on living organism some are saprophytes means feed on non- living organisms while some are necrotrophs means kill their host for food. Firstly, Fungus was thought to be prehistoric member of kingdom Plantae which is a little more develop than bacteria but now a days it is found that fungus are not ancient at all. The project tree of life in latest taxonomic classification explores that fungus and animal both fit in to one category Opisthokonta. The organisms which are studied as fungus are placed in a separate group known as kingdom fungi. The Eumycota known is true fungi, slime mold and Oomycetes (Hay *et al.*, 2019). *Rhizopus* is a genus of common saprophytic fungi on plants and specialized parasites on animals. They are found in a wide variety of organic substances, including "mature fruits and vegetables jellies, syrups, leather, bread, peanuts and tobacco. Some *Rhizopus* species are opportunistic human pathogens that often cause fatal disease called mucormycosis. This widespread genus includes at least eight species. *Rhizopus* species grow as filamentous, branching hyphae that generally lack cross-walls (i.e., they are coenocytic). They reproduce by forming asexual and sexual spores. In asexual reproduction, sporangiospores are produced inside a spherical structure, the sporangium. *Rhizopus oryzae* is used in the production of alcoholic beverages in parts of Asia and Africa. *Rhizopus stolonifer* (black bread mold) causes fruit rot on strawberry, tomato, and sweet potato are used in commercial production of fumaric acid and cortisone. Various species, including *R. stolonifer*, may cause soft rot in sweet potatoes and *Narcissus* (Menezes *et al.*, 2013). *Rhizopus* helps in soil nutrient enrichment since this species is grown in soil it ferments the fruits and vegetable in the soil inhibiting the growth and develops certain pathogens that

inhibits the growth of toxigenic fungus. In addition to that, there is even a type of Rhizopus (*Rhizopus microsporus*-fermented soybean tempe) that has proven to reduce colon carcinogenesis in rats by elevating factors of mucins, immunoglobulin A, and organic acids and give protection to piglets from *Escherichia coli*-infection by inhibiting adhesion to the intestinal membranes. The mating analysis has also been found which was comparative that this species structure is flexible in comparison with other species in the same genus. The topology length of the species genome is found to be three times bigger with the species (Silva *et al.*, 2012). *Rhizopus* species are able to produce different types of compounds of an enormous industrial importance i.e., enzymes and organic acids. Biosurfactants are products of the metabolism of living cells especially of bacteria, yeasts and filamentous fungi that may be produced extracellularly or as part of cell membranes. Their complex structural organization gives them important physico-chemical properties such as lowering surface and interfacial tensions between immiscible phase systems, promoting the formation of micelles through which hydrophobic compounds can be solubilized in water or vice-versa. In addition, these compounds are known to be efficient dispersing and emulsifying agents, exhibit high foaming and wetting abilities, and display low critical micelle concentration. These properties make biosurfactants molecules with a wide range of practical applications in the bioremediation of contaminated environments, enhanced oil recovery, as ingredients in the food processing industry, cosmetics and pharmaceutical industry (Pele *et al.*, 2018). Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome. *Rhizopus oligosporus* produces an antibiotic that limits Gram- positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. Natural sources can provide a huge range of complex and structurally diverse compounds. Recently, many researchers have focused on the investigation of fungi secondary metabolites as potential antimicrobial agents (Faisal *et al.,* 2016). The aim of our research work was evaluation of antibacterial activity of *Rhizpus* specieisolated from Rhizosphere of *Mentha Piperita.*

Materials and Method

The current research work was conducted in Microbiology Research Laboratory, Abasyn University, Peshawar. The methods that have been used in performing this research are describedin detail below:

Sample collection and processing

The samples of soil were collected from the rhizospheric region of *Mentha piperita* (mint) from Abasyn University, Peshawar. The soil sample was inoculated through serial dilution on fungal media i.e., PotatoDextrose Agar (PDA).

Isolation and Identification

The fungal specie was isolated by sub culturing technique and then identified by morphological characterization and microscopic examination. The morphology of the hyphae was observed by looking at the pigmentation (of spores and mycelium) and texture of the growth of fungi on PDA media.

Isolation of Secondary Metabolites from fungus

Potato Dextrose Broth (PDB) medium (250ml) was prepared and was autoclaved. The fungal culture was grown in PDB by inoculating the broth with fungal culture grown on Potato Dextrose Agar (PDA) and was incubated at 28°C with continuous agitation of 100rpm for 10 days. The liquid culture was filtered through a Whatman filter paper. The filtrate was placed in a separating funnel and ethyl acetate was added in a ratio 1:1 of the filtrate, to degrade the media components. Mix by inverting the bulb of funnel time to time. The mixture was left to settle down for several minutes (let the cap of the funnel open). The Ethyl Acetate phase (lower phase) was recovered in a flask and evaporated through a Rotary aspirator at 50 °C with a slight rotation 150rpm.The extract was collected in a glass vial (Manonmani *et al.*, 2005).

Collection of clinical samples

Four identified bacterial samples were collected from Microbiology Research Laboratory (MRL) of Abasyn University Peshawar. The identified bacteria were *Klebsiella, Pseudomonas, Salmonella* and *Shigella* isolated from diabetic foot ulcer patients. The ethyl acetate extract of fungus was screened against these bacterial strains. The agar well diffusion method was used for the screening of Antibacterial Activities.

Preparation of Bacterial Inoculum

Pure and isolated bacterial colonies (3-5 colonies) of the fresh bacterial culture are inoculated in test tubes containing equal amounts of nutrient broth and are incubated for 2-6 hours at 37°C.

Antibacterial activity procedure

Nutrient Agar Medium was prepared and was autoclaved at 121°C for 15 minutes at 5psi pressure. After autoclaving the molten nutrient agar was brought to Laminar Flow Hood and 20ml of molten medium was poured into sterilized petri plates and was allowed to become solid. After the solidification of agar medium, specific numbers of wells were dug in the media with the help of sterile metallic borer. The wells were evenly distributed in the petri plates and were at distance of 24mm from another where as one well in the center for control. Then 2–6-hour bacterial inoculum was spread onto the surface of nutrient agar medium with the help of sterile cotton swab. Entire surface of nutrient agar medium plate was streaked with the help of swab by turning the plate at 60° between the streaks. Test sample was prepared in concentration 12mg/ml of DMSO and was added into respective wells. Finally, the petri plates were kept in the laminar flow hood in a way that their faces were upward for 2-3 hours for the purpose of diffusion of the samples in the medium at room temperature and then they were transferred to the incubator. These petri plates were incubated for 24 hours at 37°C. After the incubation period, zones of inhibition were determined from the diameter of the wells.

Results

Identification of Fungi

The growth of isolate on PDA media after 7 days of incubation was examined and the growth characteristics were observed. The isolate showed black and white mycelium on PDA medium. The growth of the isolate is shown in the Figure. 4.1

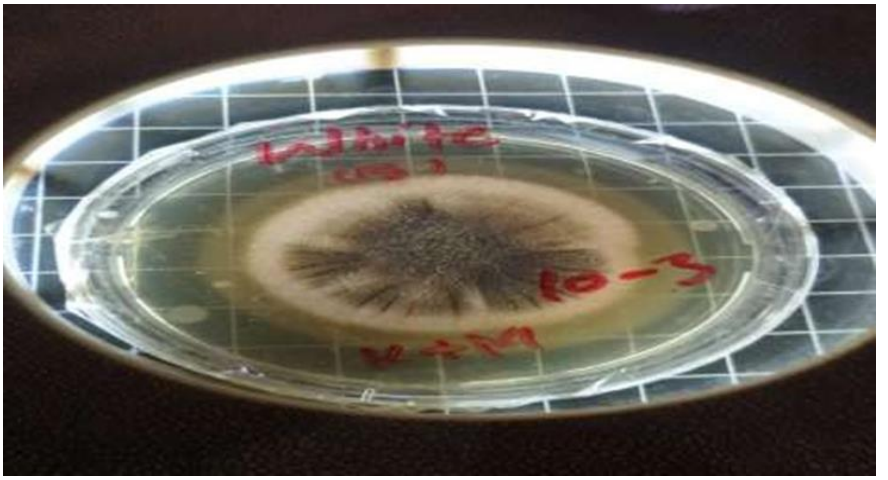

Figure 4.1 Rhizopus spp. growth on PDA media

The lactophenol cotton blue staining (LPCB) technique was performed and different structures of the isolate were observed. The isolate appeared to have a sporangium, a sac like structure containing spores with coenocytic hyphae. The structural characteristics are shown in Table 4.1 and Figure 4.2.

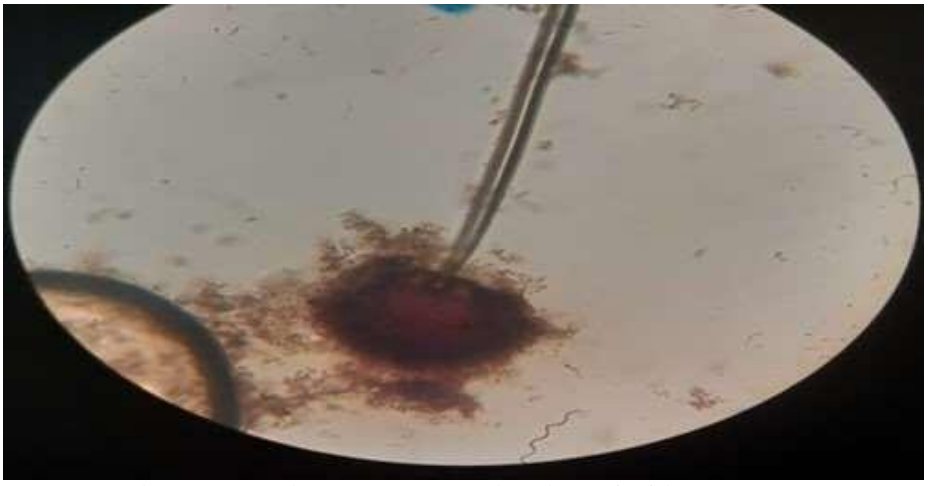

Figure 4.2 Microscopy Examination of Rhizopus spp

Table 4.1. Microscopic Examination of Isolated Specie

S. NO	Parameters	Isolate
1	Spore Producer	Sporangium
2	Hyphae	Coenocytic

Antibacterial Activity

The ethyl acetate extract of the *Rhizopus specie* was screened against four pathogenic bacteria i.e., *Salmonella, Shigella, Klebsiella* and *Staphylococcus epidermidis*. The results are shown in Table 4.2 and Figure 4.3. The sample concentration 12mg of the ethyl acetate extract per ml of DMSO, shows good activity in the form of inhibitory zones at the concentration rate of 1.2mg against *Salmonella* (17mm), *Shigella* (16mm), *Klebsiella* (18mm) and *S. epidermidis* (17mm). Whereas, at the concentration rate of 0.9mg the maximum zone of inhibition was observed against *salmonella*(14mm) and *shigella* (15mm), *Klebsiella* (15mm), *S. epidermidis* (17mm) and Similarly, at the concentration rate of 0.6mg, the maximum zone of inhibition was observed against *Salmonella* (13mm), *Shigella* (14mm) , *Klebsiella* (15mm) and *S. epidermidis* (15mm).

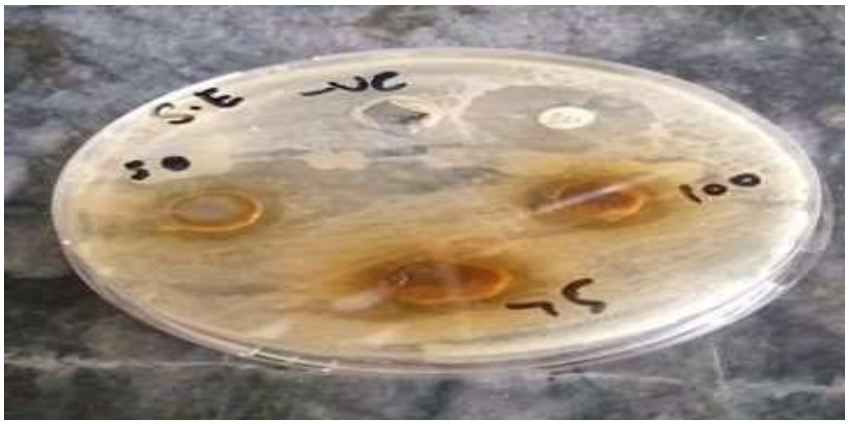

Figure 4.3 Antimicrobial activity of secondary metabolites of Rhizopus spp.

Table 4.2. Antibacterial activity of Crude Secondary Metabolite Extract of *Rhizopus* specie

S.no	BacterialStrains	ETHYL ACETATE EXTRACT (12mg/ml of DMSO)				
		ZONE OF INHIBITION (mm)				
	Salmonella	1.2mg	0.9mg	0.6mg	+ive Control Ceftriaxone	-ive Control DMSO
1.		17	14	13	25	0
2.	*Shigella*	16	15	14	23	0
3.	*S. epidermidis*	17	17	15	23	0
4.	*Klebsiella*	18	15	15	0	0

Discussion

Rhizopus is a genus of common saprophytic fungi on plants and specialized parasites on animals. They are found in a wide variety of organic substances, including "mature fruits and vegetables jellies, syrups, leather, bread, peanuts and tobacco. Some *Rhizopus* species are opportunistic human pathogens that often cause fatal disease called Mucormycosis. In the present study, *Rhizopus* specie was observed to grow as filamentous, branching hyphae of black center (matured hyphae) and white borders (immature hyphae). The microscopic examination revealed that the spores were contained in a sac like structure called sporangium with un-septate hyphae. The results are in agreement with Menezes *et al.*, (2013) as they concluded that *Rhizopus* species grow as filamentous, branching hyphae that generally lack cross-walls (i.e., they are coenocytic). They reproduce by forming asexual and sexual spores. In asexual reproduction, sporangiospores are produced inside a spherical structure, the sporangium. In current study, serial dilution of soil sample was done to obtain diluted concentration of soil samples i.e., 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. Then by pour plate method, the samples were streaked on Potato Dextrose Agar Plates (PDA). Chloromphenicol, a broad-spectrum antibiotic was poured into the media after autoclaving to avoid any bacterial growth in the medium. Similarly, in the previous study by Chandini and Rajeshwari. (2017) serial dilution method was used for dilution and same PDA media was used for isolation of fungi. In current study, Lactophenol Cotton Blue is a mixture of methyl blue a histological stain lactophenol. It is used in wet mount preparations for visualization of fungal structures especially in medical mycology, which is in line with the methodology followed by Parija *et al.,* (2001). In current study, the fungal culture was grown in Potato Dextrose Broth (PDB) byinoculating the broth with fungal culture grown on PDA and was incubated at 28 °C with continuous agitation of 100rpm for 10 days. The liquid culture was filtered through a Whatman filter paper. The filtrate was placed in a separating funnel and ethyl Acetate was added in a ratio 1:1 of the filtrate, to degrade the media components. Mix by inverting the bulb of funnel time to time. The mixture was left to settle down for several minutes (let the cap of the funnel open). The Ethyl Acetate phase (lower phase) was recovered in a flask and evaporated through a Rotary aspirator at 50 °C with a slight rotation 150rpm.The extract was collected in a glass vial. Same procedure was also performed by Manonmani *et al.*, (2005). In current research the ethyl acetate extract of the *Rhizopus specie* was used against four pathogenic bacteria i.e., *Salmonella, Shigella, Klebsiella* and *Staphylococcus epidermidis*. The extract showed good activity in the form of inhibitory zones at the concentration rate of 1.2 mg/ml against *Klebsiella* (18mm) followed by *S. epidermidis* (17mm). Whereas, at the concentration rate of 0.9 mg/ml the maximum zone of inhibition was observed against *S. epidermidis* (17mm) and at the concentration rate of 0.6mg/ml, the maximum zone of inhibition was observed against *Klebsiella* and *S. epidermidis* (15mm). While in the previous study by Takahashi *et al.* (2008) performed a study and isolated 200 fungal strains from soil samples of Serra do Cipó National Park in Brazil. The antibacterial activity of the fungal strains were determined against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Streptococcus pyogenes* and *Listeria monocyto genes*. About 67% of the extract showed activity against at least one of the tested

bacteria. Thirteen isolates were declared as the most common specie found in the studied soil samples (over 50% of all isolates).

Conclusions

From present study, it was concluded that the *Rhizopus* specie shows black and white mycelium on Potato Dextrose Agar medium. The crude metabolites extracted from *Rhizopus* specie showed good activity in the form of zones at the concentration rate of 1.2mg against *Klebsiella* (18mm) and *S. epidermidis* (17mm). Whereas, at the concentration of 0.9mg the maximum zone of inhibition was observed against *S. epidermidis* (17mm) and at the concentration rate of 0.6mg, the maximum zone of inhibition was observed against *Klebsiella* and *S. epidermidis* (15mm).

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