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ANTIMICROBIAL AND CYTOTOXIC ACTIVITY OF *MACROPHOMINA PHASEOLINA* ISOLATED FROM GUMMOSIS INFECTED *CITRUS RETICULATA*.

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ABSTRACT

Macrophomina phaseolina is one of the pathogenic organisms of gummosis disease of orange tree (*Citrus reticulata).* The pathogen was identified from the observation of their colony size, shape, colour, mycelium, conidiophore, conidia, hyaline, spore, and appressoria in the PDA culture. The crude chloroform extracts from the organism showed antibacterial activity against a number of Gram positive and Gram-negative bacteria. The crude chloroform extract also showed promising antifungal activity against three species of the genus *Aspergillus.* The minimum inhibitory concentration (MIC) of the crude chloroform extract from *M. phaseolina* against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Shigella sonnie* were 128 µgm, 256 µgm, 128 µgm and 64 µgm/ml respectively. The LD_{50} (lethal dose) values of the cytotoxicity assay over brine shrimp of the crude chloroform extract from *M. phaseolina* was found to be 51.79 µgm/ml.

Key words: Gummosis disease, *Macrophomina phaseolina* , Antibiotic activity, MIC, Cytotoxicity.

INTRODUCTION

Orange (*Citrus reticulata*) is produced and consumed as fresh or processed. The popularity and demand for orange are providing a boast of the orange industry, but production is increasingly hampered by orange plant disease. Oranges were historically used for their high content of vitamin C, which prevents scurvy (Doris 1984) and also source of minerals (Gopalan *et al.* 2006, Coward 2007).

The gummosis has been well recognized as one of the most devastating diseases wherever orange are grown under dry and rainy season (Singh 1996). Gummosis is believed to be a condition of weak and injured trees

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and reported to be infectious. Gum exudes from blister containing gum pockets, usually located on the trunk. The wood beneath the blister shows a pink orange color (Wyllie 1988). This disease can affect the root, stem, leaves, blossoms and fruit (Singh 1996). Gummosis is caused by *Macrophomina phaseolina* (Dhingra and Sinclair 1974).

The resistance of the organisms increased due to indiscriminate use of commercial anti-microbial drugs commonly used for the treatment of infectious disease. This situation forced the researchers to search for new antimicrobial substance from various sources including fungi (Bauer *et al.* 1996). The study was designed to evaluate the antimicrobial activity and cytotoxicity of the chloroform extract of *Macrophomina phaseolinsa*. The underlying cause and mechanism of these types of properties could be helpful for understanding the nature of their antibiotic activities which also helps to find out the possibility of their utilization in treatment.

MATERIALS AND METHODS

The fungal materials were isolated from the 'gummosis' gum exudes of blister containing gum pockets, usually located on the trunk. The wood beneath the blister shows a pink-orange color collected from different orange fields of Agricultural Research Institute (BARI), Pahartali, Chittagong, Bangladesh. The specimens were collected in November-December. After collection, the samples were brought to the laboratory and detail observations on the symptoms of the gummosis disease infected orange fruits were made. After proper sterilization, 5-6 diseased orange fruits were incubated in humid chamber for 7-10 days at 28 ± 2^0C. The stock cultures were maintained on Potato Dextrose Agar (PDA). Sub-cultures were made at intervals of every 15 days. Examinations were made within 10-15 days under stereoscopic binocular microscope. Culture study was carried on with the culture obtained from the single spore.

Isolation of pathogen

Isolation of pathogen from the different host fruits was made following direct method, plating method and single spore were then isolated. A suitable portion of culture of pure organism from PDA plate was subjected to microscopic examination under a stereoscopic binocular microscope.

Isolation of the organism from artificially infected fruits with the organism

Previously surface sterilized and wounded by sterilized needle fruits were subjected to spraying by the conidial suspension of the purified organism

with automizer. The inoculated fruits were transferred into germ-free full humidly desiccators and incubated at 28±2^{0}C for 5-7 days. Observation was made after 5-7 days when fruits had developed characteristic lesions and compared with the naturally developed previously recorded symptoms. Re-isolation of the pathogen was made from the artificially infected fruits following the usual procedures described before. The morphological characters of the re-isolated organism were compared with the original isolates by which the fruits were inoculated.

Isolation of antibiotic from the culture medium

Potato Dextrose (PD) Broth was used for antibiotic production. PDA slants were inoculated with spores of the organism and were allowed to grow until sporulation occurred. Mature spores were transferred to conical flasks containing 25 ml of sterilized PD broth medium to make spore suspension and again aseptically transferred to large culture flasks (500 ml) each containing 300 ml of sterile PD broth medium. These were incubated at 28±2^{0}C for 10 days.

After 10 days of incubation, the medium in the flasks turned into yellow colour with thick, uniform mat on the surface. The liquid was then separated from its mycelial mat and filtered through a fresh piece of cotton, and then Whatman filter paper 2. The filtrate was preserved at +4^{0}C by adding 2/3 drops of toluene as preservative for the extraction of the antibiotics (metabolites).

100 ml of the culture filtrate was taken in a separating funnel. This was shaken for 30 minutes with 30 ml of chloroform for the first time. Then the lower layer having the chloroform extract was separated and kept in a suitable beaker. The remaining medium in the separating funnel was treated in the same manner and collected. The chloroform fraction thus obtained was evaporated under reduced pressure in a Rota-evaporator at 45^{0}C, until a yellowish solid mass was obtained and the weight of the solid extract was measured.

Determination of antibacterial activity

The *in vitro* sensitivity of the crude chloroform extract was determined by disc diffusion method against eight pathogenic bacteria (three Gram positive and five Gram negative) (Barry 1976 and Coyle 2005). The test organisms used were *Bacillus cereus* BTCC 19, *B. subtilis* BTCC 17, *B. megaterium* BTCC 18, *Staphylococcus aureus* ATCC 6539, *Escherichia coli* ATCC 25922 , *Salmonella typhi,* AE 14613, *Vibrio cholerae* and *Shigella sonnie* ATCC 25931.

Determination of antifungal activity

For fungal inoculums PDA pour plates were prepared. At the center of these 5 days old test fungi were transferred and incubated at (25±2) $^{\circ}$C. After 5

days of incubation they were ready for use. The poisoned food technique (Rahman 2008) was used to screen for anti-fungal activity.

Determination of Minimum Inhibitory Concentration (MIC) of crude chloroform extract

'Serial dilution technique' was followed using nutrient broth medium (Jones *et al.* 1985) to determine the MIC value of the compound against *Bacillus subtilis* , *Shigella sonnie*, *Staphylococcus aureus* and *E. coli.*

Cytotoxicity Test (Brine shrimp lethality assay)

Cytotoxicity was performed by using shrimp lethality assay (Meyer *et al*. 1982 and Persoone 1980). The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LD_{50} value was obtained from the best-fit line plotted concentration versus percentage of lethality.

RESULT AND DISCUSSION

The pathogen was identified from the observation that Mycelium was hyaline to pale brown color, septate, profusely branced Pycnidium are dark brown to black, round or globose or subglobose, ostiolate (mouth like opening at the upper part), embedded in the host tissue as solitary or gregarious, with 3-4 layers of heavily pigmented thick-walled cells on the outside (Fig.1). It also possesses conidiophores/phialides which are hyaline, short, obpyriform to cylindrical (Holliday And Punithalingam 1988). Conidia are pycnidiospore. Pycnidiospores are small, single celled, smooth, hyaline, elliptical (oblong) and thin-walled.

FIG. 1**:** GUMMOSIS DISEASE OF AN ORANGE TREE.

The antibacterial activity of the chloroform extract was tested against a series of Gram positive and Gram negative bacteria at a concentration of 500 µgm/disc and the results were compared with that of a standard antibiotic disc Kanamycin K- 30 (Table 1). From this study it is clear that the chloroform extracts of the pathogen showed significant antibacterial activities against all test organisms. However, the extract from *Macrophomina phaseolina* was found to be more sensitive against the Gram-negative bacteria than the Gram-positive bacteria. Kabir and Absar (2002) have reported almost similar antibacterial activity of the chloroform extract of *Colletotricum gloeosporioides*.

TABLE 1**:** ANTIBACTERIAL ACTIVITY OF THE CRUDE CHLOROFORM EXTRACT OF BROTH CULTURE FOR *MACROPHOMINA PHASEOLINA.*

Test bacteria	Diameter of zone of inhibition (mm)	
	500 µgm/disc	Kanamycin K- 30
Bacillus cereus	11	10
B. megaterium	28	25
B. subtilis	15	10
Escherichia coli	30	25
Salmonela typhae	32	27
Shigella sonnie	21	23
Staphylococcus aureus	26	24
Vibrio cholerae	16	12

Crude extract of *Macrophomina phaseolina* also showed a prominent significant degree of anti-fungal activity (Table 2). The maximum anti-mycotic activity was shown by crude extract (500µg) against *A. niger* which was 60.0%. Over all anti-mycotic activity of the *M. phaseolina* was very promising. Present research findings reveal that *Macrophomina phaseolinsa* can play a vital role in combating against antibiotic resistant fungi. Atienza *et al*. (1992) have reported

the isolation and identification of ethisolide as an antibiotic product from *Penicillin capsulatum*.

TABLE 2**:** ANTIFUNGAL ACTIVITY OF CRUDE EXTRACT OF *MACROPHOMINA PHASEOLINA*

Name of organism	Diameter of colony		% inhibition
	Control (mm)	Sample (500µg)	
Aspergillus niger Tiegh	47.5	19.0	60.0
A. ochraceus Wilhelm	29.0	21.0	27.6
A. ustus Bainier	25.0	17.0	32.0

The minimum inhibitory concentration (MIC) of the crude chloroform extract was determined against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Shegella sonnie* by serial dilution method. The concentrations at which first sign of inhibition observed in the experiment against respective test organisms are shown in the Table 3. Growth of the organism was observed in the test tube C_I (Medium + Inoculum) and no growth was observed in test tube C_M (Medium) & C_S (Medium + Sample). The minimum inhibitory concentrations (MIC) of the crude chloroform extract of the organisms are found at moderate concentration for both Gram positive and Gram-negative bacteria by serial dilution method.

TABLE 3**:** FIRST SIGN OF INHIBITION IN MIC DETERMINATION OF *MACROPHOMINA PHASEOLINA.*

Test organisms	MIC of the crude chloroform extract (µgm/ml)
Bacillus subtilis	128
Escherichia coli	128
Shigella sonnie	64
Staphylococcus aureus	256

The concentrations at which first sign of inhibition observed in the experiment against respective test organisms indicated that the crude chloroform extract has a moderate antimicrobial property. The MIC test of crude chloroform extract from *Macrophomina phaseolina* was found to be sensitive against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*, which might be due to the presence of phytochemicals in the crude chloroform extract. Kabir and Absar (2002) also reported similar sensitivity results of crude chloroform extract of *Colletotricum gloeosporioides* against *Escherichia coli* and *Staphylococcus aureus*.

The results of brine shrimp lethality assay clearly indicated (Fig. 2) the toxic effects of the extract. The results showed that the brine shrimp survival is inversely proportional to the amount of the extracts used with LD_{50} values. A test compound in order to be considered highly toxic, it needs to show shrimp death of 50% or more. In this assay each dose of crude extract over 500 µgm was found to be highly toxic. Whereas, doses below 500 µgm displayed little toxicity.

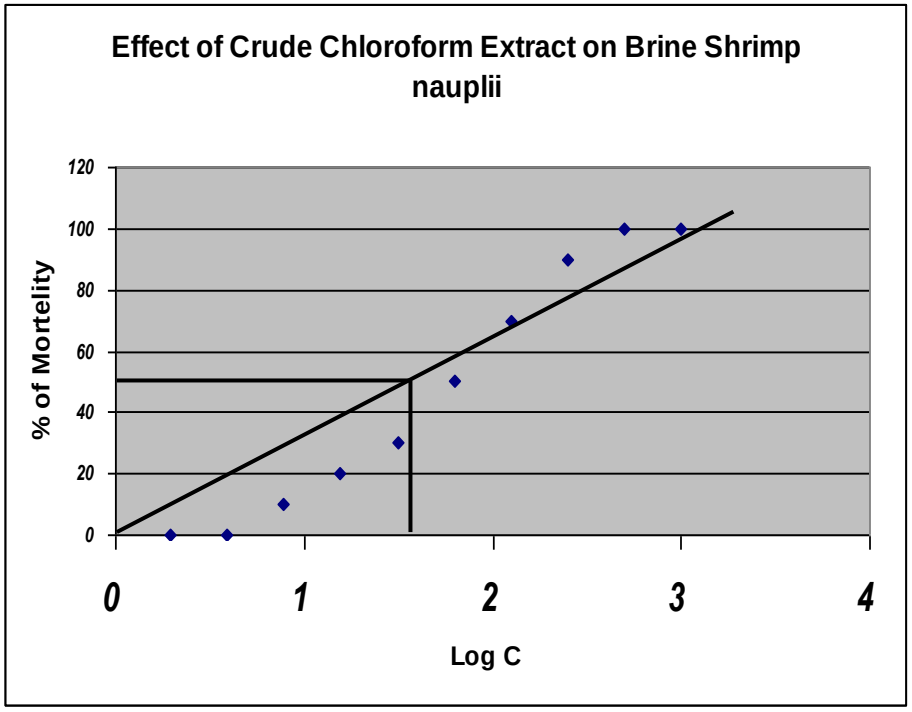

FIG. **2:** RESULTS OF BRINE SHRIMP LETHALITY BIOASSAY OF THE CHLOROFORM EXTRACT FROM *MACROPHOMINA PHASEOLINA.*

In the brine shrimp lethality bioassay, the chloroform extract of organism showed positive results, indicating that the extracts are biologically active. The mortality rate of brine shrimp nauplii was found to be increase with the increase of concentration of the sample and a plot of concentration versus percent mortality on graph paper gave an almost linear correlation between them. They had a notable antagonistic activity over both Gram positive and Gram-negative bacteria and the pathogen may play a vital role in combating against antibiotic resistant bacteria and fungi.

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