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IN-VITRO STUDY OF ANTI-FUNGAL ACTIVITY USING WHOLE *ARGEMONA MEXICANA* MEDICINAL PLANT

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ABSTRACT

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This study was undertaken to identify the antifungal activities of *A. mexicana* medicinal plant. Three types of solvents extract (water, methanol, and acetone) were collected through a series of process like drying, grinding and extraction from the total of this medicinal plant material. The antifungal activity was determined by disc diffusion method with the visual observation of the clearing zone of inhibition against one type of fungus. Water, methanol and acetone extract were screened for antifungal activity against *Aspergillus terreus* fungus. Only methanol extract of *A. mexicana* medicinal plant prominently shows the antifungal activity against *A. terreus*. Therefore, it might be said that *A. mexicana* could be used as a potential specific antifungal agent as it is being prescribed in an ancient medicinal practice name 'Ayurveda'.

Key words: *antifungal activity, Argemona mexicana, in vitro activity, medicinal plant etc.*

INTRODUCTION

Human race was started its journey millions of years ago from the caves of hills and maintained their life in adverse conditions. Since they had been suffering from diseases, so they identified the leaf sap from plant as medicine for prevention or to cure of disease. Generally, medicine or drugs is made from microbial or plant's bioactive chemical sources as well as synthesized through chemical engineering. Plant using as medicine was started from the time of immemorial and it is also continuing until now. In all of the early civilization, there was much interest on medicinal plants. All about 5,000 years ago, the earliest written evidence of the use of medicinal plants for the preparation of medicines was found in the Sumerian clay slabs of Nagpur, India. It consists of 12 recipes for making medicines which mention more than 250 different plants, some of which are alkaloids like Poppy, Hyos and Mandrake. Emperor Ginseng wrote "Pent'sao" the Chinese book on roots and grass, discusses 365 medicines (dried parts of medicinal plants), many of which are still used today: raisins, camphor, theae folium, podophyllum, great yellow gentiana, ginseng, jimson weeds, cinnamon bark, and ephedra around 2500 BC. However, in 77 BC, Dioscorides wrote his great treatise, "De Materia Medica", which dealt with the nature and properties of all medicinal substances known at that time (Biljana Bauer *et al*. 2012).

As it is known that 'antimicrobial' is a drug that kills or inhibits the multiplication of microbes like bacteria, fungi, viruses and protozoa. The history of anti-microbial agent has been dynamic, characteristic by the constant emerge of new challenges which is followed by investigation, discovery and production of new medicine. For example, 'liamycin' is an antifungal drug (polyenes antibiotics) that isolated from *S. pimprina* and developed by Hindustan antibiotics at pitnpri. It is similar to nystatin antifungal drug but more water-soluble (Edward 2019).

It is also identified that more than 100000 fungus species are strictly saprophytic, they live on dead organic matter which they help decompose. Some about 50 species, cause diseases in human and about as many cause diseases in animals, most of them superficial diseases of the skin or its appendages. More than 10000 species of fungi however can cause diseases in plants as well (Matthew *et al*. 2012).

In this study, an antifungal activity was determined against *A. terreus* fungus. The most harmful fungus is *Aspergillus* and it is also called *Eurotiurn*. The genus *Aspergillus* is known since long before and its economic as well as academic importance has resulted in an extensive study of its taxonomy, physiology and genetics. *A. terreus* represents the asexual stages of genera belonging to the family *Eyrotiacea*. The genus, through worldwide in distribution, is more prevalent in tropical countries. Conidia of *Aspergillus* are always present in the air and cause contamination in bacterial and cell culture in laboratory. It is commonly found on rotting oranges and *phyllanthus* fruits (Yung and Kenneth, 1967).

A. terreus is an emerging microbial agent of invasive aspergillosis in immuno compromised patients in several medical centers in the world. So, it attains apprehension attention in the worldwide because of its resistance to antifungal drug like amphotericin B, *in vivo* and *in vitro*, resulting in poor response to antifungal therapy and high mortality (Shallu *et al.* 2015). Although, *A. terreus* is frequently found in the environment, *A. fumigatus* is by far the main cause of 'IBPA' as 'Invasive bronchopulmonary aspergillosis' is a life-threatening disease in immunocompromised patients. However, once *A. terreus* establishes infection in the host, disease is as fatal as *A. fumigatus* infections (Silvia *et al.* 2012).

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So far, it is also known that *in vitro* studies have been done using *A. mexicana's* plant against anti-diabetic activity, anti-cancer activity, CNS related activities, wound recovering action, anti-microbial activity, antioxidant activity, anti-inflammatory, analgesic, antipyretic activity, hepatoprotective activity, anti-fertility activity, antiallergic activity, nematicidal activity, allelopathic effect, antihelmintic activity, larvicidal activity, antifeedant action (Nancy and Praveena, 2017). Therefore, our present study is aimed at screening for antifungal activities from whole *A. mexicana's* plant's extract against medically very concern *A. terreus* fungus or specifically to screen out more sensitivity and higher specific anti-fungal activity which will likely to be utilized as an alternative and promising anti-fungal drug candidate.

MATERIALS AND METHODS

Collection and preparation of plant material:

A. mexicana medicinal plants were collected from Digholia, Khulna, Bangladesh as this plant's local name is 'Shiyal Kata' as well. The collected plant materials were placed on fresh paper for drying using sunlight. After a week, moisture free plant material was collected and subjected to dry machine for making absolute dried plant material. Then, collected plant material was finely powdered using a grinding machine and stored in air-tight container with an aseptic condition.

Extraction of plant material with different solvents:

In this investigation, screening of different solvents extract of *A. mexicana* plants was made to study their antifungal activity. Powdered material was extracted successively using three different solvents like distilled water, methanol and acetone, using the concept of the nature of solubility and distribution of these active ingredients. The precise mode of extraction naturally depends on the texture and water content of the plant material to be extracted and on the type of substances to be isolated. The classical chemical procedure for obtaining organic constituent from the dried plant tissues is extracted continuously the powdered material in Soxhlet extractor with a range of solvents. Extraction of this plant material was stored in the 4°C refrigerator to avoid loss. Powdered material which weight is 200 grams was extracted with 400 ml of distilled water at 70°C in a Soxhlet extraction apparatus. For this reason, the powdered plant material was taken at first in a round filter paper which lowered portion was pinched off and after placement of the upper portion was filled with cotton. Then, it was placed in a Soxhlet extraction apparatus. Distilled water was poured into the upper portion of the apparatus so that the filter paper with powder was soaked. Once 3 cycles were finished, and then the filter paper with powdered plant material was kept in an untouched condition for 24 hours. After 24 hours, the temperature was maintained at 65°C. The extraction was carried out until the process was completed. The same procedure was followed when methanol and acetone were used but temperature was maintained at 40°C. When the extraction method was completed, the end product was taken in a glass beaker. Then extraction using water was concentrated at 65°C temperature, methanol and acetone extracts were concentrated at 40°C temperature.

Determination or the weight or the plant extract: The weight of the extracted plant product was taken by an ultra-sensitive modern electric balance. For this reason, the individual product was taken out completely on a tracing paper and weights were recorded.

Test microorganism: To determine the antifungal activity, *Aspergillus terreus* was collected from microbial biotechnology laboratory, Khulna University. *A. terreus* was inoculated in Saboy root liquid media into 500 ml flask with aseptic condition and was kept at room temperature in a few days for fungal growth. After a few days, fungal culture was observed in the flak as mycelium is grown. These growth cultures were kept at 4°C temperature in refrigerator for storing.

Preparation of culture media: Two types of media were prepared for the screening test. One was Saboy root solid media (peptone 1 gram, Dextrose 4m gram, Agar 1.6 gram) and another was Saboy root liquid media (peptone 1 gram, Dextrose 4 gram). Both of the medium were dissolved in distilled water. Medium were sterilized with autoclave 121°C for 15 minutes. Liquid medium was placed at 4°C refrigerator for storing. In case of solid media, media is poured in the petri dishes (20 ml/petri dishes) in a laminar airflow cabinet and was allowed to solidify for 30 minutes at room temperature.

Preparation of fungal inoculum: A smooth (not over growth) monolayer is necessary for the test. For producing smooth fungal monolayer on the solid agar medium, small glass beads were taken in one drum vial with 2 ml distilled water and sterilized. A small portion of fungal mycelium from stock culture was transferred to 1 drum vial by forceps in a laminar airflow cabinet. Then, this drum vial was vortex for pulverization of mycelium.

Preparation of the disc: Whatman filter papers were used to prepare the disc. By using punching machine, the Whatman papers were punched and discs were made in circular 5 mm diameter. Then, these discs were autoclaved and dried in incubator to prevent contamination. Sterilized paper discs were placed in a sterilized petri dish. The discs were impregnated separately with the measured volume of target extracts using a micropipette and left for a period in an aseptic condition for the complete evaporation of solvent. Negative control discs were also prepared using methanol or acetone in order to determine whether this solvent does have

any antifungal activity or not. Also, standard antifungal discs were prepared like using paper discs putting into anti-fungal nystatin suspension drug (The ACME Laboratories Ltd, Bangladesh). Nystatin is an anti-fungal drug has anti-fungal activity (Semis *et al.* 2015).

Diffusion of discs for screening antifungal activity: Inoculum of *A. terreus* was transferred separately to solidify media plate using micropipette and spread gently for making smooth monolayer. The excess inoculum was removed from the plate using micropipette. Diffusion assay is based on the activity of antibiotic of antifungal compound to diffuse from a confined source through the nutrient agar gel and create a concentration gradient. If the agar media is seeded with a sensitive organism, a zone of inhibition will result where the concentration exceeds the Minimum inhibitory concentrations (MIC) for those particular organisms. The discs were impregnated with different solvents were placed on these plates at a reasonable distance from each other using a sterile needle. A number of simultaneous events was occurred during this time. Initially, the dried disc was absorbed water from the surrounding test medium and drug dissolves in it. Then, the drug migrates through the adjacent test medium due to concentration gradient. This result was turn into a gradual change of drug concentration in the agar surrounding each disc. The plates were inverted and incubated in 48 hours at 27.5°C and checked for clearing zone. The activity of antifungal is evidenced by the presence of a clear zone of inhibition surrounding the disc where the drug presents in inhibitor concentration. Disc diffusion method is highly effective for rapidly growing microorganisms and the activities of the test drugs are expressed by measuring the diameter of the zone of inhibition. Generally, the more susceptible of the test organism, the larger is the zone of inhibition. The principle factors, which determine the size of the zone of inhibition, are: 1. intrinsic antimicrobial sensitivity of the drug. 2. Growth rate of the test organisms. 3. Diffusion rate of the drug which is related to its water solubility. 4. Concentration of test organisms inoculated in the medium. 5. Concentration of drug per disc. 6. Thickness of the test medium in the petri dishes.

Determination of zone of inhibition using medicinal plant extract:
After 48 hours, incubation the antifungal activity of the drug and plant extracts was determined for measuring the zone of inhibition (in mm) at the outside of the bottom petri dishes by a transparent scale. Inhibitory zones were obtained by the samples and were compared with that of the standard positive control nystatin anti-fungal drug disc.

RESULT AND DISCUSSION

Determination of the weight of the plant extraction: Methanol, acetone and water were used as solvents for extraction. From total plant material, 10.5 grams was obtained from water extract, 8.24 grams was obtained from methanol extract and 7.62 grams was obtained from acetone extracts. To determine the zone of inhibition of antifungal products, initially crude extracts of methanol, and water were tested for antifungal activity against *A. terreus*.

Table 1. Extraction yield from the plant *A. Mexicana*

Weight of plant material used for each extraction procedure (gram)	Weight of water extraction (gram)	Weight of methanol extraction (gram)	Weight of acetone extraction (gram)	Extraction Yield%		
				Water (%)	Methanol (%)	Acetone (%)
200	10.4	8.24	7.62	5.2	4.12	3.81

a) **b)** **c)**

Fig. 1. a) Nystatin is an antifungal drug's chemical structure **b)** *Argemona mexicana* medicinal plant **c)** *Aspergillus terreus* grows in petri dishes.

Also, to determine the concentration required using methanol extraction from plant for observing antifungal potency, discs were made with a series of concentration like 400 µg/disc, 800 µg/disc, 1600 µg/disc 6000 µg/disc, 12000 µg/disc, 18000 µg/disc and 24000 µg/disc.

a) **b)**

Fig. 2. a) Photograph shows the zone of inhibition using water, methanol, and acetone along with nystatin drug as a positive control. b) Zone of inhibition is shown by methanol extract against *A. terreus*.

In this experiment, methanol extract of 18000 µg/disc and 24000 µg/disc were showed the zones of inhibition as 18 mm and 19 mm size respectively.

Table 2. To determine the zone of inhibition using *A. mexicana* medicinal plant extract against *A. terreus*.

Concentration (µg/disc)	Diameter of zone of inhibition (mm)								
	Drug			Drug			Methanol Control		
	H	V	A	H	V	A	Methanol	Acetone	Water
6000	8	8	8	0	0	0	0	0	0
1200	9	9	9	0	0	0	0	0	0
1800	10	10	10	**18**	**18**	**18**	0	0	0
2400	12	12	12	19	19	19	0	0	0

Note: H= Horizontal, V=Vertical, A=Average.

So, finally it can be said that methanol extract of 18000 µg/disc is the lowest concentration that can produce zone of inhibition against *A. terreus.*

Tropical counties like Bangladesh has high incidence of infectious diseases due to its favorable climatic condition for multiplication of microorganisms specially fungi like onchomycosis, tinea pedis, crytococcosis, moniliasis, blastomycosis, candidasis, dermal candida infections, dermatophytoses as fungal diseases. It is also known that *Aspergillus* is a very harmful fungus, which causes in all animals a form of pneumonia with many of the symptoms of tuberculosis (Chris and David, 2015). Different plants and plant materials have been suggested by different treatise for the treatment of various diseases like *A. mexicana* has been considered for much usefulness in different treatise in the Indian sub-continent prescribed in various skin diseases in and Ayurveda, Unani. This medicinal plant is a spiny herbaceous annual plant with yellow flowers and latex grow in wasteland and roadside in all parts of the country like Bangladesh (Saurabh *et al.* 2012). Therefore, it deserves to be an excellent topic for study its antifungal activities because of its much used in traditional medical treatment and availability. In this study, it was found that the lowest concentration of the plant extract in methanol was 18000 /disc. In fact, this is very high concentration for the plant to show any antifungal activity, especially against *A. terreus*. It can be anticipated that this plant extracts contain also other non-related compound so finally using this medicinal plant extracts become highly concentrated one to show anti-fungal activity. It is highly essential to determine specific bioactive compound using chromatography method like high-performance liquid chromatography (HPLC) and that will reduce its concentration from crude extracts which will be paved the way to increase specificity and sensitivity against *A. terreus*, also specific bioactive compound's structure can be determined by using UV, Mass spectrometry, NMR etc. experiments for further study. (Sasidharan *et al.* 2011) & (Abdullahi and Haque, 2020). As it is known, acetone extraction was not shown any antifungal activity probably due to its polar difference for extraction active anti-fungal compound (Abdullahi and Haque, 2020). It is highly desirable to identify an alternative specific and sensitive anti-fungal compound due to this fungus shows resistance against common anti-fungal drug as it is showed. Moreover, it is very likely that methanolic extract of *Argemone mexicana* has some antifungal activity against some other fungus as it was studied (Joel *et al.* 2020). Now-a-days, *Aspergillus* co-infection in patients with severe coronavirus disease 2019 (COVID-19) pneumonia leading to fatal. (Yuto *et al*. 2021). Nevertheless, this medicinal plant also has some promising anti-viral activity as well (Yuh-Chwen *et al.* 2003). It might be worthy also for screening of anti-viral study against present novel SARS-CoV-2 virus disease Covid-19 as a future study.

CONCLUSION

In this study, methanol extract of *A. mexicana* medicinal plant specifically shows the antifungal activity against *A. terreus*. Furthermore, studies like anti-fungal screening, very specific chemical identification and testifying as

a medicine *in-vitro* and *in-vivo* will be evaluated in near future using this ancient *A. mexicana* medicinal for the betterment of mankind as it has immense importance and potentiality.

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