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ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM MORINGA OLEIFERA FOR THE ANTI MICROBIAL ACTIVITIES

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ABSTRACT

Being poorly investigated, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. In the present study we aimed the purification of endophytic fungal strains from *Moringa oleifera* plant, the isolation, identification, characterization and the preliminary evaluation of its pharmaceutical potential. The herbal powder was subjected anti-microbial activity by disk diffusion technique. The activity results revealed that compounds showed promising activity against gram postive bacteria *Staphylococcus aureus, Bacillus,* and fungi *Candida,* It was found to be ineffective aganist gram negative bacteria such as *Pseudomonas, E.coli and A.niger*. Further work is proposed in optimising the plant for better pharmacological activity.

KEYWORDS: *Moringa oleifera, Staphylococcus aureus,* Anti-microbial, *Pseudomonas* and *E.coli.*

INTRODUCTION

Endophytic fungi are a kind of fungi that colonize and reside in living and internal tissues of plants without causing any disease to the plant and reproduce by vegetative and sexual methods**.** The endophytic microorganisms penetrate plants tissue mainly by the root. However aerial parts such as stomata, flowers cotyledons also can serve as entrance

(Kobayashi DY et al., 2000). Endophytes from medicinal plants are a potential source of a diverse array of bioactive metabolites which can be used for the development of some potent drugs. Many authors have isolated endophytic microbes from various medicinal plants with antioxidant (Huang WY et al., 2007), antibacterial (Gangadevi V et al., 2008), antimicrobial (Sette LD et al., 2006; Souwalak P et al., 2006). Further many more examples are there in which endophytes producing various secondary metabolites such as taxol (Li JYet al., 1996), asperagenase (Theantann T et al., 2007), campothecin (Touseef A et al., 2006), as anticancer compounds and artimisinin (Tiwari R et al., 2008) as antimalarial etc.

Moringa oleifera is the most widely cultivated species belongs to the plant family Moringaceae. Among the array of domestic medicinal plants which are used in day today life. It is one of the best known and highly distributed medicinal plants throughout the Asian continent. It has traditionally been used in the treatment of malaria, parasitic diseases, skin diseases, hypertension and diabetes. It has been demonstrated that *Moringa oleifera* exhibits pharmacological properties such as antioxidant, anti-inflammatory, anti-cancer, anti-hyperglycemic and anti-hyperlipidemic properties (Dhanalakshmi R et al., 2013).

Antimicrobial activity has been extensively studied in *Moringa oleifera* by cup plate method. Crude extracts of *Moringa oleifera* demonstrate different antimicrobial activities. In this appraisal, we sum up the research progress in understanding and characterizing the anti-microbial activity of *Moringa oleifera* tissues including anti-bacterial and anti-fungal activity, and discuss the potential use of *Moringa oleifera* in the control of pathogenic microbes (Oxford AE et al., 1939). In the present study we establish the variety of endophytic mycoflora in *Moringa oleifera*.

MATERIAL AND METHODS

Nutritive properties

Every part of *M. oleifera* is a storehouse of important nutrients and antinutrients. The leaves of *M. oleifera* are rich in minerals like calcium, potassium, zinc, magnesium, iron and copper. Vitamins like beta-carotene of vitamin A, vitaminB such as folic acid, pyridoxine and nicotinic acid, vitaminC, D and E also present in *M. oleifera*. Phytochemicals such as tannins, sterols, terpenoids, flavonoids, saponins,anthraquinones, alkaloids and reducing sugar present along withanti-cancerous agents like glucosinolates, isothiocyanates, glycoside compounds and glycerol-1-9-octadecanoate (Kandasamy P et al., 2015).

Fig 1: ***Moringa Oleifera***.

Collection of sample

M. oleifera leaves were collected in the botanical garden. Healthy and mature trees were carefully chosen for sampling. The leaves were collected from a plant packed in sterile zip-lock bags transported in to the laboratory. The samples were then processed for isolation of endophytes immediately to reduce the chance of contamination (Rajeswari S et al., 2014).

Isolation of fungal endophytes

Samples will be cleaned under running water to remove soil adhering to roots and then air dried. Leaves stem and roots will be partitioned and before sterilization, cleaned roots will be cut in to pieces of 5 cm long. Roots will be surface sterilized by 4% sodium hypochlorite for 5 min, 70% ethanol for 1 min and sterile distilled water for 1 min 2–3 times. The surface sterilized roots pieces are transferred to an alcohol sterilized mortar and macerated separately in to suspension using distilled water and serial dilutions were made. . Highly sterile conditions were maintained for the isolation of endophytes and the entire process was carried out inside the laminar air flow. The diluted aliquots will be transferred on sterile potato-dextrose-agar (PDA) plate. After incubation at 23-25°C for 7–14 days, predominant isolates of fungi will be picked up and purified. Culture purity will be determined from colony morphology (Pawle G et al., 2014; Amin N et al., 2014).

Fermentation and Extraction

Endophytic fungal isolates will be grown on PDA plates at 23-25°C for 7–14 days depending on growth rate. Purified isolates of each fungus will be inoculated and fermented separately into a 3000 ml Erlenmeyer flask containing 600 ml of potato-dextrose broth (potato infusion from 200 g potatoes+20 g of dextrose, pH 5.1± 0.2, 24 g/L). After incubation at 23-25°C for 21 days under stationary condition, each fungal culture is filtered through four layer of cheese cloth and homogenized at 4000 rpm to separate the mycelia from broth. The filtrate will be extracted with 300 ml of ethyl acetate/chloroform three times. The organic phase will be

separated to dryness under reduced pressure using rotary evaporator (Super fit Rota vap, PBU-6) and weighed to constitute crude extract. The fungal crude fractions will be tested for anti-microbial activity against gram positive bacteria, gram negative bacteria and fungi.

***In Vitro* Screening for Anti-Microbial Activity**

Antimicrobial Activity Procedure

1) Media Used

Brain Heart Infusion agar.

2) Temperature

Bring agar plates to room temperature before use.

3) Inoculums preparation

a. Using a loop or swab, transfer the colonies to the plates.
b. Visually adjust turbidity with broth to equal that of a 0.5 McFarland turbidity standard that has been vortexed. Alternatively, standardize the suspension with a photometric device.

4) Inoculation of Agar plate

a. Within 15 min of adjusting the inoculums to a McFarland 0.5 turbidity standard, dip a sterile cotton swab into the inoculums and rotate it against the wall of the tube above the liquid to remove excess inoculums.
b. Swab entire surface of agar plate three times, rotating plates approximately 60º between streaking to ensure even distribution. Avoid hitting sides of petriplate and creating aerosols.
c. Allow inoculated plate to stand for at-least 3 minutes but no longer than 15 min before making wells.

5) Stock solution preparation

Prepare the stock solution weighing 10mg of compound and dissolve it in 1ml of DMSO

6) Addition of compound into plate

a. Take hollow tube of 5mm diameter, heat it. Press it on above inoculated Agar plate and remove it immediately by making a well in the plate. Likewise, make five well on each plate.
b. With the help of micropipette add 75µl, 50µl, 25µl, 10µl and 5µl in each well.

7) Incubation

a. Incubate plates within 15 min of compound application.
b. Invert plates, and stack them no more than five high.
c. Incubate for 18-24 hrs at 37 ºC in incubator.

8) Reading plates

a. Read plates only if the lawn of growth is confluent or nearly confluent.
b. Measure diameter of inhibition zone to nearest whole millimeter by holding the measuring device.

Note

a. In anti-fungal disc diffusion method, Sabouraud agar medium is used instead of Brain heart infusion agar.
b. For Facultative anaerobes, incubate plates in the CO_2 Jar and keep the jar in the incubator at 37 ºC.
c. For Anaerobic organisms, incubate plates in the An-aerobic jar and keep the jar in the incubator at 37 ºC.

Antimicrobial activity of the synthesized compounds was determined, using a slightly modified cup plate method. Brain Heart Infusion agar was used for the growth of bacterial strains Bacterial strains such as gram-positive (*S. aureus* and *Bacillus*) and gram-negative (*E.coli*, *A. niger & Pseudomonas)* and Fungi *(Candida).* All the test compounds were dissolved in DMSO at a concentration of 1 mg/ml. Each plate was inoculated 75µl, 50µl, 25µl, 10µl and 5µl in each well. The plates containing bacteria were incubated at 37^0C for 24 hrs, the positive antimicrobial activity were read based on the growth inhibition zone and compared with amoxicillin, as shown in **Table-1.**

RESULTS AND DISCUSSION

Endophytic microorganisms have established ever-increasing attention due to their potential in produce toxins, pharmaceuticals, enzymes, and growth factors. The revision of endophytic micro biota may expose about the contact between the plant and environmental conditions. In the present work, we isolated endophytes from leaves of *M. oleifera* plants. The leaf segments showed a maximum repository for endophytic fungi than the stem segments. The large amount of the endophytes isolated were bacteria, which is was interesting because the works till now only reported the isolation of endophytic fungi from tissues of this plant. It

was observed that the highest density of bacteria was found in the leaves of plants from botanical garden

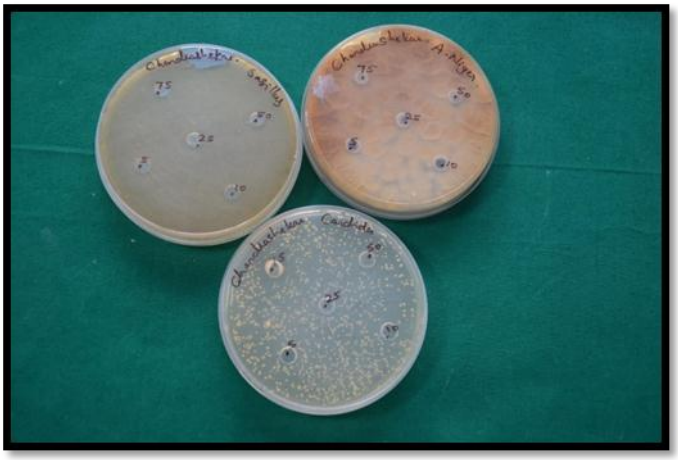
The herbal extract was screened for anti bacterial activity using amoxicillin as standard reference (1µg/mL) as shown in **Table 1.** The macroscopic observation of isolated bacteria allowed distinguishing different colorations of the colonies, such as white, gray, cream, yellow, orange and pink. The colonies presented mucoid to smooth textures. Some showed a drier aspect with regular and irregular borders and without producing pigments, indicating that they are actinobacteria. In regard to the fungi, the isolates were grouped in morphotypes .The textures were cotton wool, sandy, dry, serous, granular, powdery and warty while the borders were regular, irregular or radiated.

In general, the *moringa* leaves showed significant antibacterial activity but they are more specific to particular strains of bacteria.

From the biological data, it was evident that leaves showed promising activity against gram postive bacteria *Staphylococcus aureus*, *Bacillus*, and fungi *Candida* as shown in **Fig 2**. It was found to be in-effective aganist gram negative bacteria such as *Pseudomonas and E.coli* as shown in **Fig 3**. However the antimicrobial activity of the extract compounds against the tested organisms was found to be promising with that of respective standard drug at tested dose level.

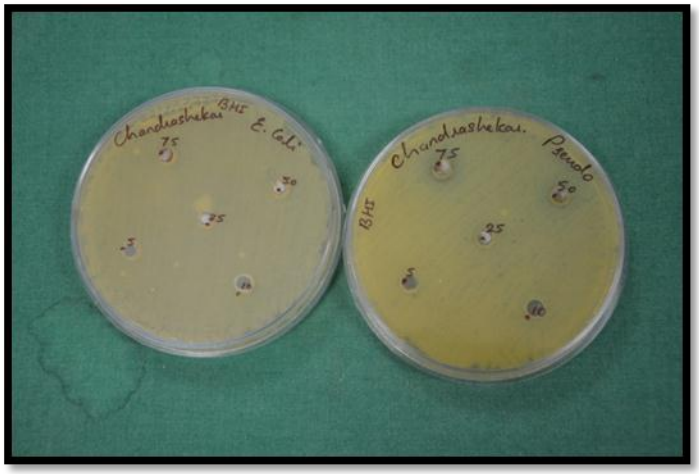
Table 1: Antimicrobial activity of *Moringa oleifera* by Disc Diffusion Method.

	Zone of Inhibition (mm)				
Herbal Powder Extract	**75 µl/ml**	**50 µl/ml**	**25 µl/ml**	**10 µl/ml**	**05 µl/ml**
Staphyllococcus	23	18	15	13	12
Bacillus	16	13	12	10	R
Pseudomonas	R	R	R	R	R
E.coli	R	R	R	R	R
Candida	16	14	12	10	R
A. niger	R	R	R	R	R
Amoxycillin	24	18	16	14	12



Disc Diffusion Method

Figure 2: Anti-microbial activity of *Moringa oleifera* on *Staphylococcus aureus, Bacillus, and fungi Candida.*



Disc Diffusion Method.

Figure 3: Anti-microbial activity of *Moringa oleifera* on *Pseudomonas and E.coli.*

CONCLUSION

Endophytic fungi were successfully isolated from *Moringa oleifera*; further studies have to be conducted to screen the novel bioactive compounds which were used in various fields like medicine, agriculture, environment etc. The above results stimulate works on the bio-prospection of the endophytes obtained. The tree as a native to India can become a great source of income for the nation if this potential for highly nutritional food is exploited by the industries and researchers.

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