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Isolation and Antibacterial Activity of Endophytic Fungi from *Melochia umbellata* (Houtt)

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A study observing endophytic fungi from medicinal plant *Melochia umbellata* and their antibacterial potencies has been done. Twelve species of endophytic fungi were successfully isolated from *Melochia umbellata* leaves and twigs. Four of them showed an activity in the antagonist test against *Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae and Shigella dysentriae.* The active isolates were fermented in potato dextrose yeast (PDY) medium at 25°C for 21 days with agitation of 150 rpm. The secondary metabolites were extracted from the fermentation medium using ethyl acetate and the mycelia were extracted using methanol. The ethyl acetate and methanol extracts were assessed for their antibacterial activity against the pathogenic bacteria and some of the isolates extract showed antibacterial activity with inhibition zone obtained were between 0 and 10.51 mm. From ethyl acetate extracts, We were obtained MUD5 94 mg with inhibition zone 8.08 mm against *E. coli*, 10.51 against *S. dysentriae*, 7.81 mm against *P. aeruginosa*, 9.00 mm against *V. cholerae,* MUR4 116 mg with inhibition zone 8.61 mm against *E. Coli*, MUR5 was obtained 93 mg with inhibition zone 7.54 mm against *E. coli*, and MUR6 96 mg with inhibition zone 7.44 mg against *P. aeruginosa*. While from methanol extracts were obtained MUR6 516 mg with inhibition zone 9.3 mm against *P. aeruginosa*. Phytochemical identification revealed that the active extracts contain alkaloids, flavonoids and steroids. From this present work, it can be concluded that these endophytic fungi could be promising source of bioactive compounds and can be used for further study.

Keywords: Antibacterial Activity; Endophytic fungi; *Melochia umbellata; Mycelia*.

The discovery of natural products and novel bioactive molecules have played major role in the search for new drugs.[2] The existence of microorganisms in various plant tissues (such as root, fruit, stem, seed, leaf, etc.) provide a mutual relationship without causing any symptom of diseases are called endophytes.[1] Generally, the plant tissues are protected from infectious agents by the endophytic fungi by producing various bioactive compounds. The endophytic fungi reside asymptomatically in the internal tissues beneath epidermal cell layers and lives within the intercellular spaces of the tissues. The endophytic fungi also may penetrate to the living cells of all higher plants[2]. They are good and interesting source of antibiotics. Natural products from endophytic microbes have been observed to be able to inhibit or kill a wide variety of harmful disease-causing agents but not limited to Phytopathogens, as well as bacteria, fungi, viruses and protozoan that affect humans and animals[1].

Isolation of Endophytes is a critical step which requires sensitivity to recover a maximum number of colonized Endophytes and should remove the epiphytic microbes which are present on the plant surface. The collected plants for studying endophytic communities should look apparently healthy and disease-free plant to decrease the possibility of pathogenic and saprobe species contaminant, and to avoid the isolation of pathogenic endophytic microorganisms around it[2].

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Studies about the endophytic fungi have been widely conducted in some countries. Recent study showed that *Melochia umbellata* leave extract triggered antibacterial activity against *Staphylococcus aureus,* and *Shigella dysentriae* with inhibition zone of 10.5 mm (2500 ppm) and 9.36 mm (2500 ppm), respectively[3]. In this study, we focus on the isolation and identification of endophytic fungi of *Melochia umbellata* and the screening of their antibacterial activity. We are also determining for the phytochemical compounds in the extracts of endophytic fungi. We hope this study will contribute to the invention of new antibiotic candidate which can provide benefits to the health problems.

MATERIALS AND METHODS

Sources for Endophytic Fungi

Leaves and twigs were thoroughly washed with mild detergent and running tap water and then air–dried. After which they were surface sterilized by using 3 step surface sterilization start with submerging them in 75%ethanol for 3 min. Further sterilization was performed by using 5.3% sodium hypochlorite solution for 5 min, and 75% ethanol for 0.5 min, sequentially. After sterilization, samples were washed with sterile water to remove ethanol residues. Each leaf was cut into 1 cm in size and twigs were cut into two pieces. Samples were placed at the surface of sterile potato dextrose agar (PDA) medium and incubated for 3-5 days, at 25°C. The fungal isolates were identified based on their morphological characters[6, 7, 8, 9].

Mass cultivation and extraction of metabolites of endophytic fungi

The fungal endophytes were mass cultivated on potato dextrose yeast (PDY) by placing agar blocks of actively growing pure culture (3mm in diameter) in 250 ml Erlenmeyer flasks that contain 100 ml medium. The flasks were

Table 1. Zone of Inhibition produced by endophytic fungi on human bacterial pathogens

Extract	Endophytic Fungi	Diameters of Inhibition zone (mm) *Escherichia coli*	*Shigella dysentriae*	*Pseudomonas aeruginosa*	*Vibrio cholerae*	Amount of extract (mg)
Ethyl acetate	MUD1	-	-	-	-	-
	MUD2	-	-	-	-	-
	MUD3	-	-	-	-	-
	MUD4	-	-	-	-	-
	MUD5	8.08±0.053	10.51±0.153	7.81±0.0781	9.00±0.097	94
	MUD6	-	-	-	-	-
	MUR1	-	-	-	-	-
	MUR2	-	-	-	-	-
	MUR3	-	-	-	-	-
	MUR4	8.61±0.215	-	-	-	116
	MUR5	7.54±0.262	-	-	-	93
	MUR6	-	-	7.44±0.301	-	96
Methanol	MUD1	-	-	-	-	-
	MUD2	-	-	-	-	-
	MUD3	-	-	-	-	-
	MUD4	-	-	-	-	-
	MUD5	-	-	-	-	257
	MUD6	-	-	-	-	-
	MUR1	-	-	-	-	-
	MUR2	-	-	-	-	-
	MUR3	-	-	-	-	-
	MUR4	-	-	-	-	97
	MUR5	-	-	-	-	614
	MUR6	-	-	9.30±0.106	-	516

incubated at room temperature for 21 days with periodical shaking at 150 rpm. After incubation period, culture media and mycelia were separated by filtration and extracted with the method described by Dasale *et al* (2013).The Fungi mycelia was sonicated in 100 ml methanol for 30 minutes. The mycelia extract was filtered and evaporated. The culture media was extracted by liquid-liquid extraction using ethyl acetate in the same volume with the media. Extraction was repeated three times and the organic solvent of collected extract was evaporated under reduced pressure. The crude extract was then dissolved in Dimethyl sulphoxide (DMSO) for antibacterial bioassay.

Antibacterial Activity

Test Organisms

There were 4 strains of pathogenic bacteria which were used for the antibacterial activity of fungi. They were *E. coli, S. dysentriae, P. aeruginosa,* and *V. cholerae.*

Antagonist test

Endophytic fungi cultures were transferred to petri dish which consisted of nutrient agar medium and pathogenic bacteria. The dishes were incubated for 24 hrs, at 37°C.

Antibacterial activity test

The agar well diffusion assay method was used to examine the antibacterial activity of

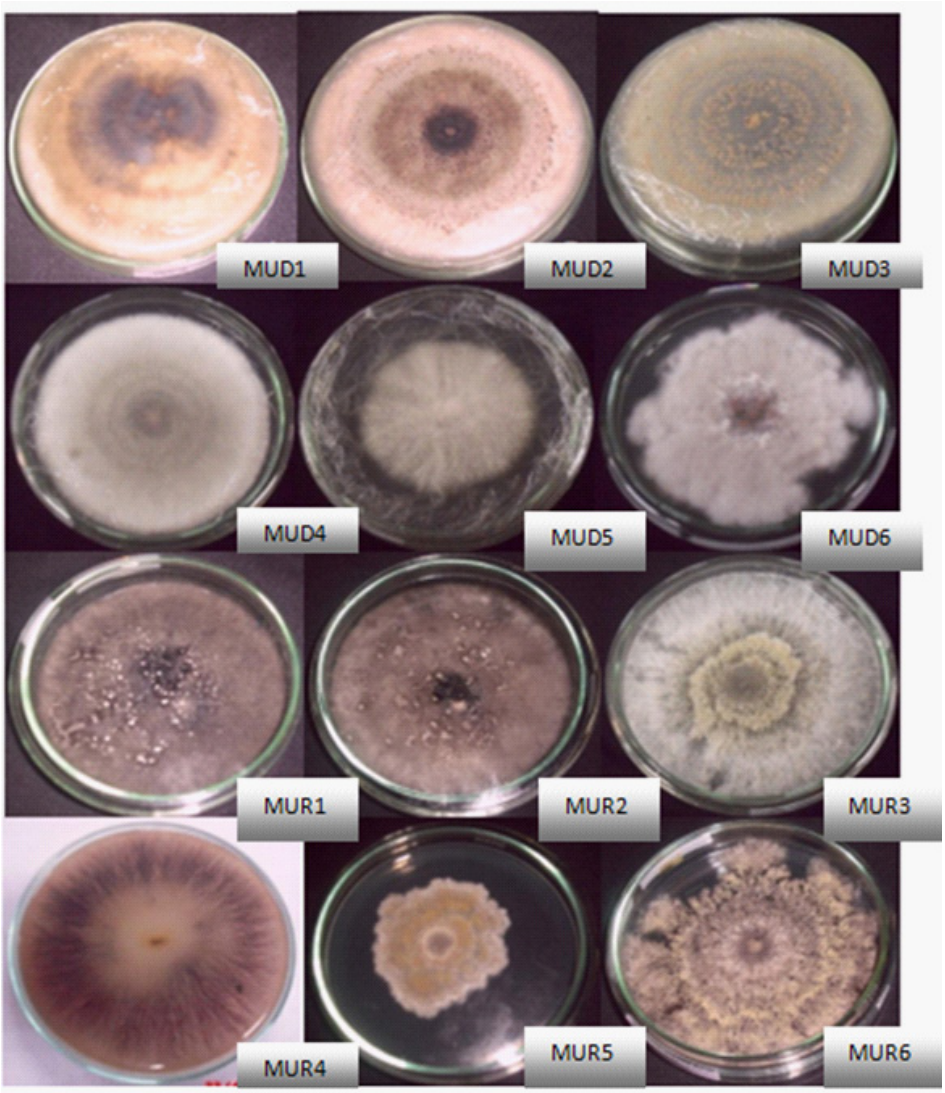
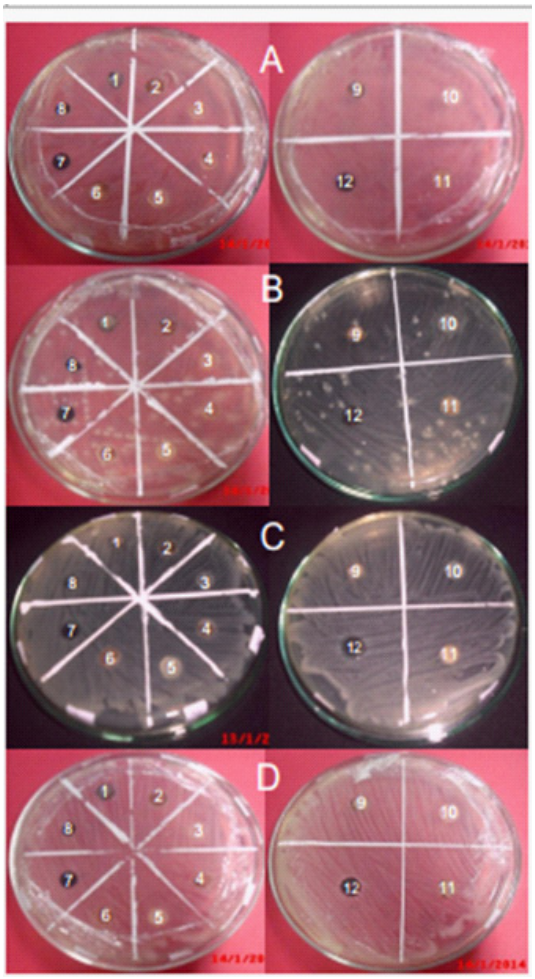


Fig. 1. Endophytic Fungi Isolates

fungi. In this method, wells were aseptically made in seeded media using sterile cork borer and an amount of 20 µl bioactive metabolite was dropped in the previously prepared wells and incubated at 37°C in bacteriological incubator for 24 hrs. Finally, plates were observed for zones of inhibition and their diameter was measured with Antibiotic zone scale.



A. Antagonist test against *Escherichia coli*
B. Antagonist test against *Pseudomonas aeruginosa*
C. Antagonist test against *Shigella dysentriae*
D. Antagonist test against *Vibrio cholera*

1-12 are the 12 endophytic fungi isolate examined in the antagonist test
1 : MUD1 7 : MUR1
2 : MUD2 8 : MUR2
3 : MUD3 9 : MUR3
4 : MUD4 10 : MUR4
5 : MUD5 11 : MUR5
6 : MUD6 12 : MUR6

Fig. 2. Antagonist test result

Phytochemistry identification

The active antibacterial isolate extracts were identified for their phytochemistry compounds. These were done by thin layer chromatography method. The extracts were eluted using hexane: ethyl acetate (2:1) and the results were observed under UV 254, 366 nm and visible (after sprayed with 10% H_2SO_4). For Alkaloid identification, the spots were sprayed with dragendorf reagent and for flavonoids identification by using sitroboric reagents[11, 12].

RESULTS

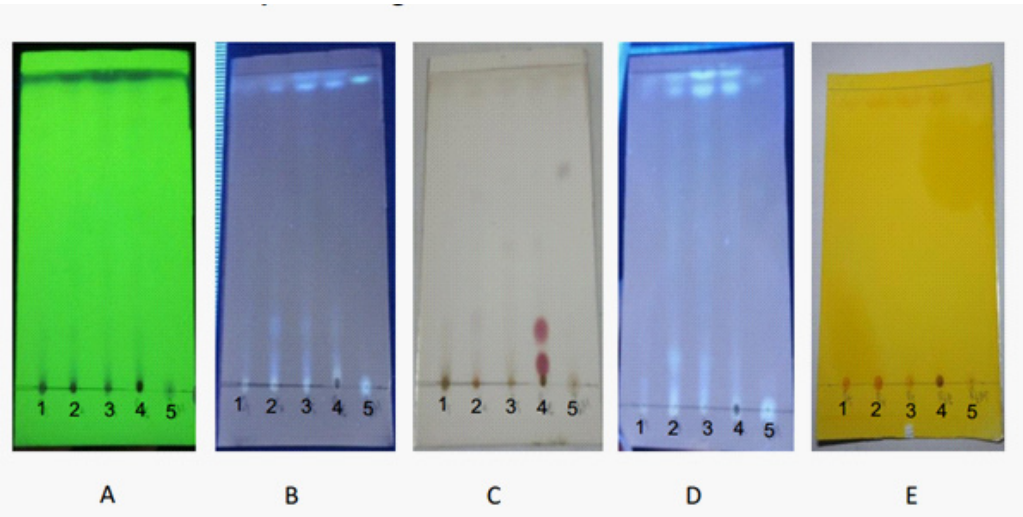
Isolation of Endophytic fungi

In the present study, fungal strains were isolated from leaves and twigs of *Melochia umbellata*. A total of 12 fungi was isolated. Four most active isolates from antagonist test which showed the biggest inhibition zone were fermented. The active endophytic fungi were fermented for 21 days using PDY to obtain the secondary metabolites. The fermentation broth or supernatant was extracted with ethyl acetate and the biomass was extracted with methanol. Screening of endophytic fungi to determine antibacterial activity was executed by agar well diffusion method against 4 pathogenic human bacteria (*Escherichia coli, Shigella dysentriae, Pseudomonas aeruginosa,* and *Vibrio cholerae*).

Extraction of isolates in 200 ml PDY medium resulted in 94 mg ethyl acetate extract from MUD5 isolates, 116 mg extract MUR4, 93 mg extract MUR5 and 96 mg extract MUR6. The methanol extract obtained were 257 mg from MUD5 isolates, 97 mg extract MUR4, 614 mg extract MUR5 and 516 mg extract MUR6.

In antibacterial activity, MUD5 showed high inhibition zone, 8,08 mm against *E. coli*, 10,51 against *S. dysentriae*, 7,81 mm against *P. aeruginosa*, 9,00 mm against *V. Cholerae.* Diameter of inhibition zone of MUR4 was 8,61mm against *E. Coli*, MUR5 was 7,54 mm against *E. Coli*, and MUR6 was 7,44 mg against *P. Aeruginosa*. While from methanol extracts were obtained MUR6 with inhibition zone 9,3 mm against *P. Aeruginosa.*

Phytochemical identification were carried out for all the active extracts and showed that ethyl



A. UV 254 1. Ethyl acetate extract of MUD5
B. UV 366 2. Ethyl acetate extract of MUR4
C. H_2SO_4 10% 3. Ethyl acetate extract of MUR5
D. Sitroboric 4. Ethyl acetate extract of MUR6
E. Dragendorf 5. Methanol extract of MUR6

Fig. 3. Phytochemical identification

acetate extract of MUD5, MUR4, MUR5, MUR6 contained alkaloid compound based on red spot formation on thin layer chromatography after spraying of Potassium Bismuth Iodide solution. Flavonoids content of all active extracts showed by the light green spot on uv 366 after being sprayed with sitroboric. From the preliminary phytochemical screening also revealed the presence of steroids in ethyl acetate extract of MUR6 based on the formation of red spot after the spot being sprayed with H_2SO_4 10%. (Figure 3).

DISCUSSION

Various species of endophytic fungi made an ecological niche in the inner space of plants. The fungi interact with their environment in a positive manner in the role of improving plant defense and disease control. Endophytic fungi isolation from medicinal plant results in the production of bioactive metabolites which has a great activity against microbes. Hence, scaling up the production of bioactive metabolites is necessary to fulfill the demand of agriculture and pharmaceutical industries. In this study, we have isolated 12 endophytic fungi from *Melochia umbellata*. Ethyl acetate extract from fungi isolate (MUD5) shows activities against *E. coli, S. dysentriae, P. aeruginosa, and V. cholerae.* MUR6 produce active secondary metabolites against *P. aeruginosa,* both in the supernatant and the biomass. Phytochemical identification showed that all active extract consist of alkaloids, steroids and flavonoids compound.

CONCLUSION

The present investigation was an attempt to search for potential endophytic fungi from *Melochia umbellata*. The study revealed the active antibacterial extracts which were extracted with ethyl acetate and methanol. Four active ethyl acetates and 1 active methanol extract were examined for their antibacterial activities based on their inhibition zone diameters. Therefore, further determination of active metabolites content in the extract needs to be investigated quantatively and completely. Besides that, TLC bioautography need to be developed in case to investigate the active antibacterial fraction.

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