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BIOACTIVITIES OF DIVERSE ENDOPHYTES ISOLATED FROM THE STEM BARK OF *MORUS ALBA* L.

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ABSTRACT

Endophytes provide a plethora of potential substances that are used in modern medicine. There is an emergence of interest to discover potential compounds from endophytes due to their abundant availability from renewable sources, and low cost relative to chemically synthesized compounds. Studies have shown that *Morus alba* plant has potential antioxidant and antibacterial activities. Hence, in the present study we deals with the bioprospecting of endophytes of *Morus alba.* Stem bark of *M. alba* was collected from different regions of Gujarat. Total 13 bacteria and 14 fungal endophytes were isolated from the collected samples. Among 27 endophytic isolates, the extract of four isolates showed significant antibacterial activity with a diameter zone ranged from 8.0±0.0 to 22.33±1.262 mm with considerable minimum inhibitory concentration (MIC) values. These four extracts also showed significant inhibition of reactive oxygen species in 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Triterpenoids, flavonoids, and phenols were present in these extracts which might be responsible for these bioactivities. Results of this study concluded that these endophytes isolated from the stem bark of *Morus alba* L. have an important bioactivities *in vitro.* These extracts may prove good candidate for the antibacterial and antioxidant agents and can be exploited in pharmaceuticals after further development.

Keywords: Endophytes; *Morus alba*; Antibacterial; Antioxidant; Phenols; Terpenoids; Flavonoids

INTRODUCTION

Endophytes are the integrated and ubiquitous component of the plants, exist within almost all the plants in nature **(Sun & Guo, 2012)**. Exploration of endophytes offers a rich and diverse natural resource for the new drug scaffold. The resurgence of interest in these microorganisms for novel leads is due to their low toxicity, high biodegradability, abundant availability from renewable sources, and low cost compared to chemical synthesis **(Zhao, Shan, Mou, & Zhou, 2011)**. The importance of endophytes in providing bioactive compounds is evident by the discovery of epic anticancer drug "Taxol" from endophytic fungi *Taxomyces andreanae* of *Taxus brevifolia* plant **(Verma, Kharwar, & Strobel, 2009)**. Since then, a large number of bioactive metabolites as Cryptocandin **(Strobel *et al.*, 1999)**, Cryptocin **(Li, Strobel, Harper, Lobkovsky, & Clardy, 2000)**, Ambulic acid **(Li *et al.*, 2001)**, Pestaloside **(Lee, Yang, Schwartz, Strobel, & Clardy, 1995)**, Jesterone **(Li & Strobel, 2001)**, Torreyanic acid **(Ding *et al.*, 2009),** etc. have been extracted from endophytes and characterized for their potential activity. Several endophytes are yet to be discovered which can be exploited to develop novel drug leads. The diverse endophytes can offer an assorted and affluent repository of compounds having a broad spectrum of biological activities. In the present paper, we explored the possibility of finding biologically active diverse endophytes from the medicinal plant *Morus alba* L.

Morus alba L. has long been used commonly in ayurvedic and many traditional systems of medicine. This plant has a wide range of important pharmacological activities including antimicrobial, antioxidant, antidiabetic, anticancer, immunomodulation, etc. **(Devi, Sharma, Kumar, & Jeet, 2013)**. Hence, in the present study, we used the *Morus alba* plant. Harvesting plants for therapeutic purposes leads to their extinction and also affects the environment adversely. Alternatively, endophytes can offer an excellent source of remedial compounds that may be novel or similar to the compounds of the host plants (as in the case of taxol) **(Ryan, Germaine, Franks, Ryan, & Dowling, 2008)**. Besides, microorganisms can be grown in controlled conditions, easy to scale up, and genetic modulation can be feasible to get a higher yield of compounds. Reported study suggested that biologically active phytocompounds are present in the root and stem bark of *Morus alba* plant **(Ines Thabti et al, 2013)**. But, there are very few literature reports available for isolated endophytes from stem bark samples. Therefore, we isolated endophytes from the *Morus alba* stem bark and evaluated their antioxidant and antibacterial activities along with the recognition of a major group of bioactive compounds.

In the present study, we performed anti-bacterial, and antioxidant activities of bacterial and fungal endophytes isolated from the stem bark of *Morus alba*. In addition, qualitative assessments of the group of secondary metabolites present in potential extracts were also performed. Further, we identified the notable endophyte by 16S rRNA sequencing. Collectively, the present study facilitates the significance of *M. alba* endophytes in providing an untouched source of therapeutic agents.

MATERIALS AND METHODS

Plant sample collection

Total eight plant samples of *M. alba* were collected from the different regions of Gujarat between July 2017 to January 2018. For isolation of endophytes, the bark of each sample was placed into a sterile container and immediately processed.

Isolation of endophytes

The collected samples were washed by tap water and processed by surface sterilization procedure to eliminate epiphytic microorganisms by a slight modification of **Kjer *et al.* (2010)** method. All the samples were cut into small pieces followed by immersion in 70% ethanol for 1-2 min, in 4 % sodium hypochlorite (Himedia) for 3-4 min, in 0.01 % (v/v) Tween-20 (Himedia) for 1 min. Subsequently, rinse the samples with sterile water thrice. The efficiency of sterilization was confirmed by spreading water from the last washing into nutrient agar (NA) and potato dextrose agar (PDA) Petri plates. After surface sterilization, the samples were cut into small pieces (approximately 0.5 cm^2) and aseptically transferred to Petri plates containing NA and PDA media so that the freshly cut edges were in direct contact with the media surface. Next, the petriplates were incubated at 25°C for fungal and 37°C for bacterial growth **(Kjer, Debbab, Aly, & Proksch, 2010)**. All the plates were monitored at a regular time interval for growth of endophytes and pure endophytic isolates were preserved on Nutrient agar (for bacteria) and Potato dextrose agar (for fungi) media slants.

Coding of isolate

Each isolated culture was named using the first initials of source plant (Ma for *Morus alba*), subsequently "B" for bacteria and "F" for fungi. Afterward, isolate number and at last the region of sample collection site (C for central Gujarat, S for Saurashtra, N for north Gujarat, K for Kachchh, and SG for south Gujarat).

Mass cultivation/ fermentation of endophytes in a liquid medium

Each isolate was cultured in the respective medium (potato dextrose broth for fungi and nutrient broth for bacteria) and incubated on a rotary shaker at 120 rpm (bacteria at 37˚C for 48 hours and fungi at 25˚C temperature for 7 days). After that, the extractions of secondary metabolites produced by endophytes were carried out. Cultures of bacteria were centrifuged at 5000 rpm for 15 min and filtered with Whatman filter paper 1. Cultures of fungi were filtered with Cheesecloth. Bacterial pellets and fungal mycelia were discarded. Subsequently, filtrates were concentrated using a rotary evaporator under reduced pressure at 60°C.

Screening of antibacterial activity of extracts of endophytes

Test microorganisms

In the present study, Gram-positive bacteria i.e. *Bacillus subtilis* (MTCC 736) and *Staphylococcus aureus* (NCIM 2079) and Gram-negative bacteria i.e. *Escherichia coli* (NCIM 2065), *Pseudomonas aeruginosa* (NCIM 2200), and *Salmonella abony* (NCIM 2257) were selected from the microorganisms given in United States Pharmacopoeia (2000) and Indian Pharmacopoeia (2007) for antibacterial activity.

Antibacterial activity

Agar well diffusion method and Paper disk diffusion method were used for qualitative screenings of potential extract among all isolated endophytic extracts. In the agar well diffusion method, 100 µl extracts of endophytes (100 mg/ml in water) were inoculated in each well of Petri plates which were previously spread with test organisms. In the paper disk method, sterilized paper disks were placed on the Petri plates which were previously spread with test organisms. Approximately 20 µl extracts of endophytes (100 mg/ml in water) were injected on paper disk (injected 2 µl once and after it absorbed and dried, more 2 µl were injected and so on to achieved 20 µl in each disk). Gentamicin (1 mg/ml in water) was used as a positive control. All plates were incubated at 37°C for 24 hours. After the incubation period, antimicrobial activity was assessed by the measurement of inhibition of diameter zones (**Balouiri M *et al*, 2016**).

Minimum Inhibitory Concentration (MIC) analysis

The bioactive extracts that inhibited the growth of tested microorganisms at the lowest concentration, was further subjected to MIC assessment. MIC analysis was performed using the broth micro-dilution method. The endophytic extract samples were dissolved in sterile water. Serial dilutions were prepared in 96 well sterile microtiter plates. Subsequently, 50 µl nutrient broth and 50 µl test organisms were inoculated and incubated at 37°C for 24 hours. After incubation period, 40 µl of INT [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride] (0.2mg/ml) indicator dye was added in each wells. The red-pink color indicated the presence of bacterial growth and the growth inhibition was indicated by no color change. (**Andrews JM *et al*., 2001**).

Antioxidant activity by DPPH radical scavenging method

The antioxidant ability of endophytic extracts was evaluated by 1,1- diphenyl-2-picrylhydrazyl (DPPH) scavenging activity. In this method, 0.5 ml methanolic DPPH solutions (0.3 mM) was added to 0.5 ml of methanolic endophytic extracts of different concentrations (200, 400, 600, 800, 1000 µg/ml) in sterile 96 well microtiter plate. A diluted solution of 1:1 methanol and DPPH was used as control. Ascorbic acid was used as the positive control with similar concentrations as the samples. These solutions were gently mixed and incubated in the dark for 30 min at room temperature (25°C). Thereafter, the absorbance of the resulting solution was measured at 517 nm using a microplate reader (Thermo Scientific). The scavenging capabilities of samples were calculated using the following formula:

$$\text{DPPH Scavenging effect (\%)} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

The EC_{50} (effective concentration at which ROS radicals were scavenged by 50%) was calculated by interpolation from a linear regression analysis.

Qualitative phytochemical analysis

Qualitative analysis of various phytochemical groups such as alkaloids, triterpenoids, saponins, flavonoids, and phenols present in the four potential endophytic extracts was carried out using Dragendroff's test, Salkowski's test, Foam test, Shinoda test, and Ferric chloride test respectively.

Molecular identification of most potent endophyte

Genotypic identification of the potential endophytic bacteria (MaB03C) was carried out by 16S rRNA gene sequencing (at Xceleris Pvt. Ltd). The gene sequence was submitted to NCBI and also compared with already existing sequences in the NCBI database to recognize the similarities. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7.
The secondary metabolites produced by endophytic bacterial isolate MaB03C were compared with *Bacillus subtilis* (MTCC 736) using HPTLC. The mobile phase was Butanol: Propanol: Water in 4: 4: 2 ratio. The fingerprints of both extracts were observed and analyzed at 254 nm and 366 nm.

Statistical analysis

All the experiments were replicated three times. Data were analyzed using GraphPad Prism (GraphPad Software, SanDiego, CA) by one-way analysis of variance (ANOVA) with Dunnet's post-hoc test to determine the statistical significance. In each case $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Plant sample collection and isolated endophytes

Morus alba stem bark was collected from five different biogeographical zones in Gujarat viz. Kachchh, Saurashtra, South Gujarat, Central Gujarat, and North Gujarat to identify endophytic diversity (Table 1). All collected plant samples were authenticated by the research team of the department of phytochemistry and pharmacognosy, PERD Center. The voucher specimens of all the collected samples were deposited in the plant sample collection library of PERD Centre. Total 13 bacteria and 14 fungi were isolated from the collected bark samples and given experimental names. Later on, extracts of all the bacterial isolates and fungal isolates were prepared in broth media.

Table 1 Plant collection site of *Morus alba* and isolated endophytes

Collection site/Region	No. of isolated endophytes		Sample number / Voucher number
	Bacteria	Fungi	
Central Gujarat	5	7	Sample 1/ BVPPERD/PP/0717/07 Sample 2/ BVPPERD/PP/0717/08 Sample 3/ BVPPERD/PP/1117/10 Sample 4/ BVPPERD/PP/1117/11
North Gujarat	2	2	Sample 1/ BVPPERD/PP/1117/12
Saurashtra	2	2	Sample 1/ BVPPERD/PP/1217/21
Kachchh	2	1	Sample 1/ BVPPERD/PP/0118/02
South Gujarat	2	2	Sample 1/ BVPPERD/PP/0118/06

Antibacterial activity and MIC of endophytic extracts

In the present study, aqueous extracts of all bacterial and fungal endophytes were analyzed for their antibacterial properties. We obtained almost similar potential extracts in the results of both agar well diffusion and paper disk diffusion assay. However, the diameter of the zone of inhibitions was slightly different in both the methods because, in the Well diffusion assay, the extracts diffused throughout the medium while in the paper disk diffusion method, extract diffused only on the surface of the media. Based on screening assays, we noticed that out of 27 endophytes, 2 were found effective against *E. coli*, 5 have shown activity against *S. aureus*, 7 were effective against *B. subtilis*, 3 were effective against *P. aeruginosa* and 4 were found effective against S. *abony* (Table 2). The average zones of inhibition were found between 8.0 ± 0.0 to 22.33 ± 1.262 (Table 2). Among all, four endophytes namely MaB03C, MaB02N, MaB01SG, and MaF01C have shown significant antibacterial activity against multiple bacteria. The highest zone of inhibition was given by bacterial endophyte from the bark sample collected from North region of Gujarat (MaB02N) against *S. aureus* (Table 2).
Minimum Inhibitory Concentration (MIC) determination using the broth microdilution method uncovered, a bacterial endophyte from the central region of Gujarat (MaB03C) showed the most significant activity against all selected Gram-positive as well as Gram-negative bacteria (Table 3). The MIC values of MaB03C were 25, 50, 0.5, 50, and 50 mg/ml against *E. coli*, *S. aureus*, *B. Subtilis*, *P. aeruginosa*, and *S. abony* respectively.

Table 2 Results of agar well diffusion method for anti-bacterial activities of extracts of bacterial and fungal endophytes

Culture Code	Average zone of inhibition (in mm)				
	E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*S. abony*
MaB01K	-	-	-	-	-
MaB02K	-	-	-	-	-
MaB01S	-	-	-	-	-
MaB02S	-	-	-	-	-
MaB01C	-	-	9.66 ±0.385	-	-
MaB02C	-	13.66 ±0.385	12.66 ±0.192	-	-
MaB03C	12.66 ±0.385	15.0 ±0.577	16.33 ±0.509	14.0 ±0.333	14.0 ±0.577
MaB04C	-	-	-	-	-
MaB05C	-	-	-	-	-
MaB01N	-	-	-	-	-
MaB02N	-	22.33 ±1.262	-	-	8.67 ±0.192
MaB01SG	-	14.66 ±0.385	13.67 ±0.385	12.66 ±0.509	-
MaB02SG	-	-	-	-	-
MaF01K	-	-	-	-	-
MaF01S	9.0 ±0.0	-	12.0 ±0.0	-	8.67 ±0.192
MaF02S	-	-	-	-	-
MaF01C	-	-	11.33 ±0.385	11.33 ±0.192	10.33 ±0.192
MaF02C	-	-	-	-	-
MaF03C	-	-	-	-	-
MaF04C	-	-	-	-	-
MaF05C	-	-	-	-	-
MaF06C	-	-	-	-	-
MaF07C	-	-	-	-	-
MaF01N	-	-	-	-	-
MaF02N	-	-	-	-	-
MaF01SG	-	8.0 ±0.0	9.33 ±0.192	-	-
MaF02SG	-	-	-	-	-
PC (Gentamicin)	27.33 ±0.694	27.33 ±0.509	40.33 ±0.385	28.0 ±0.333	27.33 ±0.385

Table 3 Minimum inhibitory concentration (MIC) values of potential plant extracts against test organisms

Culture Code	Minimum inhibitory concentration (mg/ml)				
	E.coli	*S.aureus*	*B.subtilis*	*P.aeruginosa*	*S.abony*
MaB03C	25	50	0.5	50	50
MaB02N	100	25	100	100	100
MaB01SG	50	25	0.25	100	50
MaF01C	50	50	100	ND	25

ND: Not detected

Antioxidant activity of potential extracts

Many disorders including microbial diseases are associated with the generation of reactive oxygen species (ROS) (Liou & Storz, 2010). Nascent oxygen is a critical factor for the growth of bacteria **(Bahman, Rahnama, & Mazarei, 2017; Galadari, Rahman, Pallichankandy, & Thayyullathil, 2017; Prasad, Gupta, & Tyagi, 2017)**. Hence, we performed antioxidant activity using DPPH radical scavenging method (Hidayat, Fitri, & Kuswandi, 2017). The results of the present investigation indicated that the extracts of endophytes named MaB03C, MaB01SG, MaB02N, and MaF01C exhibited significant antioxidant activity (Fig.1) with the EC_{50} value of 6.64, 97.96, 72.11, and 226.33 µg/ml respectively.

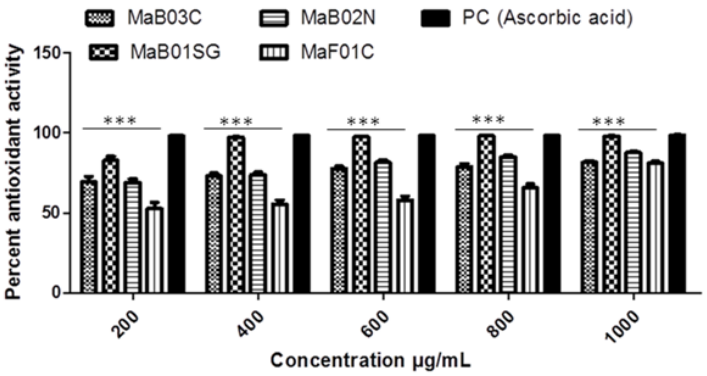

Figure 1 Percentage of antioxidant activity of selected endophytic extracts. These results are representative of the antioxidant experiment in triplicates with mean ± SEM. Statistical significance against Ascorbic acid as positive control; ***P < 0.001. (*** denotes significant value)

Qualitative phytochemical screening of endophytes

To evaluate a group of chemical compounds putatively responsible for the aforementioned biological activities, a preliminary phytochemical assessment of four potential endophyte extracts was carried out. Crude ethanolic extracts of these endophytes were tested for the presence of phenols, flavonoids, triterpenoids, alkaloids, and saponins. MaB03C has shown positive results in the triterpenoids test (Table 4). The bacterial extracts of MaB01SG have shown the presence of triterpenoids, flavonoids, and phenols (Table 4). The endophytic fungal extracts of the central region (MaF01C) have shown the presence of triterpenoid (Table 4). These chemical groups present in endophytic extract might be responsible for the biological activities.

Table 4 Phytochemical estimation of potential endophytic extracts

Endophyte's Extract	Alkaloids	Triterpenoid	Saponins	Flavonoids	Phenols
MaB03C	-	+	-	-	-
MaB01SG	-	+	-	+	+
MAB02N	-	-	-	-	-
MaF01C	-	+	-	-	-

+: present, -: absent

Molecular identification of most potent endophyte and HPTLC analysis

Combining results from all the bioactivities assessed in the present investigation, among all the endophytes, endophytic bacteria, MaB03C was found to deliver excellent antibacterial and antioxidant properties. The molecular identity of this endophyte has been established using the sequencing of the 16S rRNA gene. (NCBI accession number MH247236). BLAST analysis in NCBI revealed the sequence was more likely matched with *Bacillus* species. The first hundred hits were related to the *Bacillus* genus with higher similarity. Similar to the BLAST results, in phylogenetic analysis, MaB03C was grouped together *Bacillus subtilis* subsp spizizenii strain NBRC 101239 (accession no. NR 112686) (Fig S1).
Interestingly, this bacterial extract (MaB03C) also possesses antibacterial activity against *Bacillus subtilis* (MTCC 736) culture. Therefore, we extended our work to know the difference between both *Bacillus* species and their secondary metabolites by HPTLC fingerprinting. Best resolution and separation of compounds were obtained in Butanol: Propanol: Water in 4: 4: 2 ratio as mobile phase. The results of HPTLC revealed that all the bands and peaks were similar except one peak at 0.7 Rf as confirmed by comparative HPTLC spectral analysis (Fig 2). The results indicated that endophyte MaB03C was a different strain from *Bacillus subtilis* (MTCC 736).

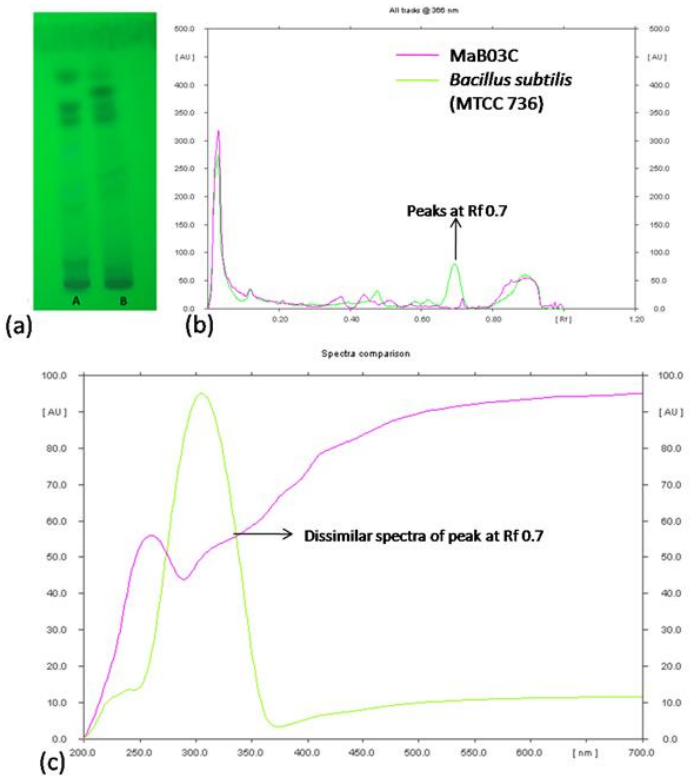

Figure 2 HPTLC of potential extracts of endophytes MaB03C and *Bacillus subtilis* MTCC culture (a) Screening and compounds of both extracts on TLC plate at 366 nm [in image A=MaB03C extract; B= *Bacillus subtilis* (MTCC 736) culture extract] (b) Scanning of each peaks (c) Spectrum of dissimilar peak (Rf=0.7) which depict that both bacillus cultures are different strain

CONCLUSION

Collectively, based on our findings we conclude that the extracts of endophytes isolated from the stem bark of *Morus alba* L. have an important antibacterial and antioxidant activity *in vitro*. The results of phytochemical analysis depicted that triterpenoids, flavonoids, and phenols were present in these extracts which might be responsible for biological activities. This study reinforced the assumption that endophytes of ethnomedicinal plants could be promising and an untouched resource of therapeutic compounds.

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