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Research Article

EVALUATION OF ANTIBACTERIAL ACTIVITY OF ENDOPHYTIC FUNGI *ASPERGILLUS JAPONICUS ISOLATED FROM TRIDAX PROCUMBENS* L.

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ABSTRACT

Objective: The main purpose of the present study was to isolate the endophytic fungi from *Tridax procumbens* L. and evaluate their antibacterial activity at different concentration of compatible solvents extracted metabolites against the test pathogenic bacterial strain, as well as the molecular characterization of potent endophytic fungal isolate that showed the maximum antibacterial activity.

Methods: The endophytic fungi were isolated from the different parts of the collected *T. procumbens* plant. Screening of endophytic fungi for the antibacterial activity was scrutinized against five pathogenic bacteria such as *Bacillus subtilis*, *Streptococcus pyogenes, Escherichia coli*, *Klebsiella pneumoniae,* and *Salmonella typhimurium* using agar well diffusion method. After screening, the metabolite of the potent fungal isolate was extracted using different solvents by solvent-solvent extraction procedure and observed their antibacterial activity. For molecular identification of the fungi, the DNA was extracted, quantified, and amplified using two oligonucleotide primers ITS4 and ITS6 in polymerase chain reaction.

Results: In the present study, five endophytic fungi were isolated from medicinal plant *T. procumbens* and screened for their antibacterial activity against *E. coli* (22.60±0.32 mm), *S. typhimurium* (19.26±0.23 mm), *S. pyogenes* (16.36±0.18 mm), *K. pneumoniae* (14.26±0.54 mm), and *B. subtilis* (14.43±0.27 mm) bacterial strain. The endophytic fungal strain *A. japonicus* isolated from *T. procumbens* was showed the significant antibacterial activity against the pathogenic bacteria. The morphological identification of all the isolated endophytic fungi was observed on the basis of their macroscopic and microscopic characteristics, and the molecular identification of the potent fungal strain was observed through 18s rRNA sequence analysis. Using solvent-solvent extraction technique, different solvent residues of the potent fungal metabolite were extracted in benzene, n-,butanol and toluene. The n-butanol solvent extract exhibited a maximum zone of inhibition against the test bacterial strains.

Conclusion: The present study reveals that the endophytic fungi serve as a potential source for the production of effective bioactive compounds.

Keywords: Antibacterial activity, n-Butanol extract, *Tridax procumbens* L., Bioactive compounds.

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INTRODUCTION

As the population of the world are increased, it is suffering from various health problems caused by certain drug resistance bacteria, parasite protozoans, and fungi. Therefore, an intensive search for the development and invention of new effective agents to deal with these problems is now underway [1]. Since ancient times, people had exploded the nature, particularly, plants in search of new drugs. This has resulted in use of a large number of medicinal plants with curative properties which help in the treatment of various diseases caused by microorganisms [2]. A number of research observed that there are a number of endophytic fungi present inside the plants and protect their host from insects and other herbivores. The term endophyte refers to the group of microorganisms which is part of its life cycle, invades the tissue of living plants, and causes asymptomatic infection [3]. Endophytic fungi have been reported to produce similar compounds to that of the host, and it was confirmed when taxol was obtained from an endophytic fungi isolated from *Taxus brevifolia* [4]. Endophytic fungi are source of bioactive compounds which have wide range of application in pharmaceutical and agrochemical [5-7]. Bioactive compounds isolated from endophytic fungi belong to several classes such as alkaloids, peptides, steroids, and phenol [6]. These fungi produce novel antibacterial, anticancer, antiviral, antioxidant, insecticidal, and antimalarial compounds that can be utilized by pharmaceutical industries [8,9]. Sandhu *et al*. [10] isolated the endophytic fungi from *Calotropis procera* (Linn.) R. Br. of Jabalpur region and tested their antibacterial activity against *Klebsiella pneumoniae, Bacillus subtilis, Escherichia coli, Staphylococcus epidermidis,* and *Pseudomonas aeruginosa.* Therefore, in the present investigation, the endophytic fungi were isolated from the medicinal plant *Tridax procumbens* and observed their antibacterial activity against the pathogenic bacteria.

MATERIALS AND METHODS

Collection of plant sample

In the present investigation, the mature and healthy plant leaves were collected from the medicinal plant *T. procumbens* from Majholi region, Jabalpur (M.P.), India. Plant leaves were collected in sterilized polythene bags and processed for the isolation of endophytic fungi within 12 h after collection. Plant specimen has been identified/authenticated by respective floras [11-13].

Isolation of endophytic fungi

The collected fresh leaves were rinsed in running tap water to remove dust and debris. Rinsed leaves were selected for surface disinfection under aseptic condition. The leaves were cut into 3–4 mm diameter segments and surface disinfected in sterilized distilled water for 1–2 min, then treated with 4% sodium hypochlorite (NaOCl) for 2 min and immersed in 70% ethanol for 2 min, and again washed in sterilized distilled water for 2 min in laminar airflow. After that, the segments were placed onto the sterilized filter paper for dry and then transferred into Potato Dextrose Agar (PDA) medium Petri plates and incubated

in fungal incubator at 26±1°C for 5–7 days. The pure colonies of the endophytic fungi were further transferred on the PDA plates at 26±1°C for 5–7 days. The pure cultures of the isolated fungi were preserved on PDA slant without antibiotic at 4°C.

Phenotypic identification of isolated endophytic fungi

The morphological identification of the isolated fungi was done by slide culture technique given by Aggarwal and Hasija, 1980 [14], and Domsch *et al.* 2007 [15]. The endophytic fungi were characterized on the basis of their color, size and shape of spores, hyphae characters, and reproductive structures [16].

Source of test bacterial strain

The pathogenic bacteria used in the study such as *Salmonella typhimurium* microbial type culture collection (MTCC) 733, *E. coli* MTCC 1679, *Staphylococcus aureus* MTCC 96, *B. subtilis* MTCC 441, and *K. pneumoniae* MTCC 4032 cultures were obtained from MTCC and GenBank, Institute of Microbial Technology (IMTECH), Chandigarh, India. The organisms were preserved at 4°C in the presence of glycerol (15% v/v) for longer periods.

Screening of isolated endophytic fungi

The isolated endophytic fungi were subcultured in Petri plates containing PDA and incubated in a fungal incubator for 6–7 days at 26±1°C. Sterile cork borer of 8 mm was used to cut the portion of mycelia mat and transferred to 100 mL pre-sterile PDB and incubated at 26±1°C in a fungal incubator for 7th, 14th, and 21st day [17]. Under aseptic conditions, the metabolized broths were filtered by Whatman filter paper no.1 and centrifuged at 8000 rpm for 8 min. The pellet was discarded and the supernatant refiltered to get cell-free culture filtrate (CFCF) for the estimation of antibacterial activity.

Molecular identification of potent fungi

After screening, the molecular identification of potent fungal strain was performed by 18S rRNA sequence analysis. For the molecular identification, the DNA of potent fungal was isolated by LETS method [18]. In this method, loop full conidia were inoculated to the 10 mL media and that was incubated for 16-30 h. After that, the mycelia was harvest, washed with distilled water, and lyophilized by liquid nitrogen and crushed in a motor-pastel by adding 0.7 mL extraction LETS buffer (0.1 M LiCl, 10 mm EDTA, 10 mm HCL [pH 8], and 0.5% SDS). After that, crushed mycelia was poured in the centrifuge tubes and vortex for few minutes. After that, 1 mL phenol:chloroform:isoamyl alcohol (25:24:1) were added and vortexed for 1 min at medium speed and centrifuged at 3000 rpm for 5 min. There were two layers formed in the tube as aqueous layer and pellet. The pellet was discarded and the aqueous layers was transferred in the other sterilized tube and add 1 mL absolute chilled ethanol in the aqueous layer and placed the tube on dry ice for 15 min. After that spinned for 15 min in a micro-centrifuge at 10,000 rpm for 10 min. The supernatant was removed and the pellet dissolved in TE buffer for further use.

Quantification and amplification of fungal DNA

The quantity of the isolated DNA was checked in ultraviolet (UV)-visible spectrophotometer. From the stock, 1 µL DNA was mixed with 49 µL sterile distilled water to get 50 times dilution. The A260/280 was recorded to check the purity of DNA. Polymerase chain reaction (PCR) amplification of ITS region was done in 20 µL of reaction mixture containing PCR buffer, ×1 (Kappa, SA); $MgCl_2$, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; primer, 1 picomol and template DNA, 50 ng. Sterile nuclease-free water is used as negative control. The oligonucleotide primers used for PCR amplification are ITS4 and ITS6. The 5'-3' sequence for ITS4 is TCC TCC GCT TAT TGA TAT G, with 50 GC%, 51.0°C Tm value, and 19 bp length and 700 bp product size. Similarly, the 5'-3' sequence for ITS6 is GAA GGT GAA GTC GTA ACA AGG, with 60 GC%, 56.0°C Tm value, and 21 bp length. Approximately 50 ng genomic DNA was used as the template for amplification. The temperature conditions were as follows: Initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 48°C for 30 s, and extension at 72°C for 1 min 30 s. The reactions were optimized for each individual sample; the range of temperatures found to be useful was 48°C with 1°C increments in annealing temperature until satisfactory amplification was observed. The presence of a single product between 700bp was verified by agarose gel electrophoresis. The remaining PCR product (90 µL) was purified for subsequent manipulations using a QIA-quick-spin PCR purification column (Qiagen), following the manufacturer's protocol. Phylogenetic tree analysis was performed using the MEGA software version 4.0.

Fermentation and extraction

The PDB medium was inoculated with conidial suspension of the potent endophytic fungal prepared from the 7-day-old culture in sterilized distilled water and incubated the flask at 26±1°C in fungal incubator for 14 days under stationary condition. After fermentation, broth was filtered with the help of Whatman filter paper no. 1 and the CFCF was centrifuged in cooling centrifuge (REMI) at 10,000 rpm for 10 min (4°C) to remove the cell debris. The separated supernatant was further subjected to solvent extraction using different solvents in the order of their increasing polarity such as toluene, benzene, and n-butanol by solvent-solvent extraction procedure. In this method, the metabolites was dissolved in the solvent, and then, it was taken in separating funnel, shaken vigorously for 15 min, and kept stationary 15–20 min to separate organic phase from aqueous phase. The upper layer was separated and collected in another flask, and the solvent extracts were evaporated in Rotary Vacuum Evaporator (Buchi R-300 Rota-vapor, Buchi Co. Germany). The extract residue was dissolved in double-distilled water and stored at 4°C to be used as stock solution for antibacterial activity.

***In vitro* antibacterial activity by agar well diffusion method**

For antibacterial activity, agar well diffusion method was performed given by Newyork, 1972 [19]. Nutrient agar medium (NAM) plates were prepared and 25 µL of bacterial suspension was spread out with the help of glass spreader on the NAM plates to make bacterial lawn and allowed to dry for 10 min. The agar wells were prepared by scooping out the medium with a sterile cork borer which had 8 mm diameter. The wells were then filled with 75 µL of the fungal crude extract and incubated at 37°C for 24 h. After the incubation period, the zone of inhibition was measured using HiMedia antibiotic zone scale and compared with the control. All experiments were performed in triplicate.

Statistical analysis

In the present study, antibacterial activity was conducted in triplicate and the results were calculated as mean (±) standard deviation. Data were analyzed with one-way ANOVA and significant differences between mean values at $p<0.05$ were determined using SPSS program (16.0 versions).

RESULTS

Isolation and phenotypic identification of isolated endophytic fungi

In the present investigation, five endophytic fungi were isolated from leaves of *T. procumbens* collected from Majholi, Jabalpur (M.P.), India, as shown in Table 1. Identification of fungal strains was done using standard protocol of Aggarwal and Hasija, 1980 [14], and Domsch *et al.* 2007 [15]. On the basis of their cultural and microscopic properties, these fungi show different characteristics and successfully identified as *Aspergillus japonicus* (SG1), *Aspergillus niger* (SG2), *Alternaria* sp. (SG3), *Fusarium* sp. (SG4), and *Penicillium* sp. (SG5) as shown in Fig. 1.

Screening of isolated endophytic fungi from the plant

For screening the antibacterial activity of isolated endophytic fungi from the leaves of plant, all fungal isolates were inoculated in PDB for 7th, 14th, and 21st day and incubated at 26±1°C in a fungal incubator. During the screening of endophytic fungi, CFCF of 7th, 14th, and 21st day were examined for their antibacterial activity against the test bacterial strain as shown in Fig. 2 and depicted in Table 2.

Table 1: Phenotypic characteristics of isolated endophytic fungi from leaves of *T. procumbens*

Fungal isolate	Macroscopic characteristic	Microscopic characteristic	Probable genera/species
SG1	Colony wrinkled, near mummy brown, mycelium white, reverse uncolored	Conidial heads radiate, hyaline to pale brown, smooth, vesicles subglobose to globose, conidia spherical or ellipsoidal	*Aspergillus japonicus*
SG2	Colonies were powdery in texture and black in color with conidial production, reversed plate showed pale yellow colored due to pigmentation	Hyphae septate hyaline dichotomously branched vesicle, round, radiate head, black conidia, spores erect, simple and thick walled	*Aspergillus niger*
SG3	Woolly colonies greenish-black or olive brown with a light border	Single conidia, smooth-wall, dark in color, septate hyphae	*Alternaria* sp.
SG4	White pink color, smooth swarming and raised colony	Multi-celled spores, conidia are oval shaped and attached to conidiophores arising from a septate mycelium	*Fusarium* sp.
SG5	White and cottony colonies	Single-celled spores, conidia in chains develop at the end of the sterigma from the medulla of the conidiophores arise from a septate mycelium	*Penicillium* sp.

Table 2: Screening of isolated endophytic fungi against five pathogenic bacteria

Name of fungi	Day of incubation period	Zone of inhibition (in mm)				
		B. subtilis	***S. pyogenes***	***E. coli***	***S. typhimurium***	***K. pneumoniae***
A. japonicus	07	00.00±0.00	00.00±0.00	06.11±0.54	00.00±0.00	00.00±0.00
	14	14.43±0.27	16.36±0.18	22.60±0.32	19.26±0.23	14.26±0.54
	21	08.31±0.54	00.00±0.00	08.16±0.47	00.00±0.00	00.00±0.00
Fusarium sp.	07	05.05±0.39	00.00±0.00	07.23±0.58	00.00±0.00	00.00±0.00
	14	14.76±0.55	00.00±0.00	16.33±0.35	13.56±0.20	16.53±0.33
	21	09.10±0.47	00.00±0.00	11.23±0.52	00.0±0.00	00.00±0.00
Alternaria sp.	07	00.00±0.00	12.24±0.26	10.00±0.17	00.00±0.00	07.15±0.32
	14	09.56±0.45	09.40±0.26	10.53±0.25	00.00±0.00	09.56±0.36
	21	11.04±0.19	00.00±0.00	13.23±0.36	08.04±0.72	06.16±0.14
A. niger	07	13.71±0.28	09.22±0.42	17.32±0.25	00.00±0.00	12.24±0.58
	14	08.50±0.22	06.05±0.47	12.23±0.54	10.06±0.46	00.00±0.00
	21	08.32±0.54	00.00±0.00	10.20±0.31	00.00±0.00	00.00±0.00
Penicillium sp.	07	00.00±0.00	12.04±0.56	07.08±0.38	00.00±0.00	00.00±0.00
	14	11.30±0.09	08.40±0.23	16.36±0.47	10.02±0.80	00.00±0.00
	21	00.00±0.00	14.16±0.32	12.20±0.17	00.00±0.00	09.50±0.41

A. japonicus: Aspergillus japonicus, A. niger: Aspergillus niger, B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, E. coli: Escherichia coli, K. pneumoniae: Klebsiella pneumoniae, S. typhimurium: Salmonella typhimurium

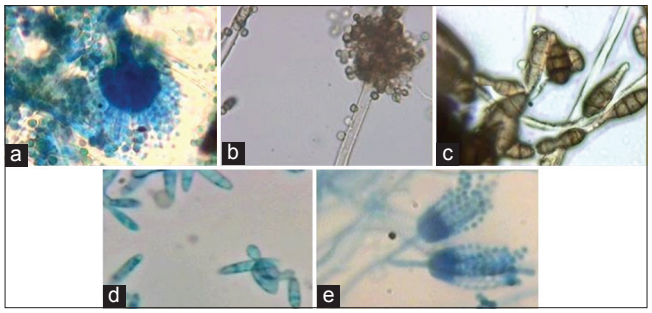

Fig. 1: Light microscopic (×40) observation of endophytic fungi (a): SG1 - *Aspergillus japonicus*, (b) SG2 - *Aspergillus niger*, (c) SG3 - *Alternaria* sp., (d) SG4 - *Fusarium* sp., (e) SG5 - *Penicillium* sp.

Molecular identification of potent fungus

After screening the antibacterial activity of the endophytic fungi, the potent fungal strain *A. japonicus* was showed and the maximum antibacterial activity against the test bacteria was further identified by molecular sequencing using 18S rRNA sequence analysis. In the molecular study, two primers, namely, ITS 4 (TCC TCC GCT TAT TGA TAT G) and ITS6 (GAA GGT GAA GTC GTA ACA AGG) were used for molecular identification. Based on BLAST search of rRNA gene sequence, the endophytic fungi were found to be closest homology to *A. japonicus* and submitted to the NCBI GenBank with accession no. KY218732 (Fig. 3). Phylogenetic tree was constructed based on the closest relationship with consensus sequences and shown in Fig. 4.

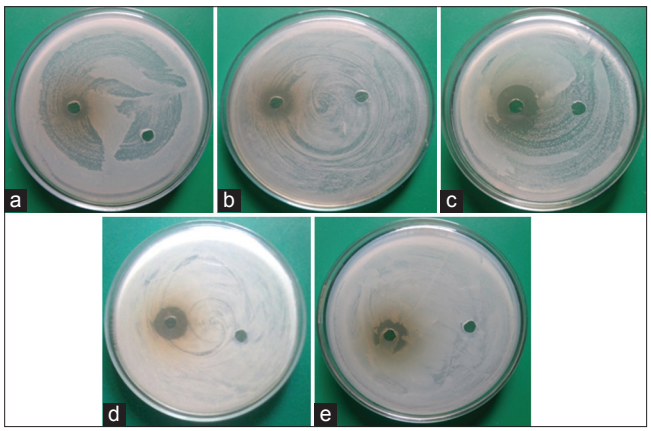

Fig. 2: Antibacterial activity of 14 days old cell-free culture filtrate of *Aspergillus japonicus*, (a) *Bacillus subtilis*, (b) *Streptococcus pyogenes*, (c) *Escherichia coli*, (d) *Salmonella typhimurium*, (e) *Klebsiella pneumoniae*

***In vitro* antibacterial activity of potent fungi**

In the present investigation, metabolites of endophytic fungi *A. japonicus* that showed the maximum zone of inhibition against the test bacterial strain were extracted with a number of organic solvent such as benzene, toluene, and n-butanol (1:1 v/v) and concentrated in rotary vacuum evaporator. After evaporation of solvent, the metabolites were mixed

with sterilized distilled water and observed their antibacterial activity by agar well diffusion method, and the fraction of n-butanol extract was showed maximum antibacterial activity against *B. subtilis, E. coli*, MTCC1679, *K. pneumoniae* MTCC4032, *S. typhimurium* MTCC733, and *Streptococcus pyogenes* MTCC96 as shown in Table 3. The optimum inhibitory concentration of n-butanol fungal extract was also observed at different concentrations from a range of 25–800 µL. The antibacterial activity of the n-butanol extract was increased as the concentration was augmented, and after 800 µL concentration, the antibacterial activity of the solvent extracted metabolites was fairly stable as depicted in Table 4.

Table 3: Zone of inhibition of potent fungal filtrate in different solvents (in mm)

Pathogenic bacteria	Zone of inhibition (in mm)											
	Toluene (µL)				Benzene (µL)				n-Butanol (µL)			
	25	50	75	100	25	50	75	100	25	50	75	100
B. subtilis	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	06.1±0.00	07.6±0.00	09.2±0.00	10.4±0.00	07.3±0.00	12.8±0.00	15.1±0.00	15.8±0.00
S. pyogenes	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	09.0±0.00	14.6±0.00	17.2±0.00	19.1±0.00
E. coli	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	10.4±0.00	12.0±0.00	14.3±0.00	16.2±0.00	11.3±0.00	17.5±0.00	25.2±0.00	27.6±0.00
S. typhimurium	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	11.2±0.00	11.7±0.00	13.2±0.00	14.5±0.00	05.3±0.00	13.1±0.00	21.4±0.00	24.2±0.00
K. pneumoniae	00.0±0.00	00.0±0.00	00.0±0.00	00.0±0.00	12.0±0.00	13.2±0.00	14.1±0.00	15.7±0.00	00.0±0.00	08.2±0.00	14.3±0.00	16.8±0.00

B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, E. coli: Escherichia coli, K. pneumoniae: Klebsiella pneumoniae, S. typhimurium: Salmonella typhimurium

DISCUSSION

Endophytic fungi are the most promising bioagent that has a huge source of various bioactive compounds. Mainly, these compounds could be classified as alkaloids, terpenoids, quinones, steroids, isocoumarins, saponins, lactones, phenylpropanoids, and phenols [9,20]. Many of these compounds are being used for the treatment of a number of diseases [7]. Thus, in the present work, endophytic fungi were isolated from the medicinal plant *T. procumbens* L. and observed their antibacterial against the bacterial strain. Sandhu *et al.* 2016 [21] reported seven endophytic fungi such as *Aspergillus niger*, *Penicillium citrinum*, *Cladosporium* sp., *Curvularia lunata*, *Aspergillus* sp., *Alternaria* sp., and *Aspergillus fumigates* isolated from *Rauwolfia serpentina* and the morphological identification was completed based on mycelia type, aerial color, shape and kind of spores, presence of foot cells, conidiophores, and the characteristics of spores. In another study, Tran *et al.* 2010 [22] isolated six endophytic fungi form phyllodes of *Acacia* species and molecular identification was done through 18S rRNA gene sequencing. In the present study, five endophytic fungal strains were isolated from *T. procumbens* and identified as *Aspergillus japonicus, Fusarium* sp., *Aspergillus niger, Alternaria* sp., and *Penicillium* sp. using phenotypic characteristics, and the potent fungal strain was identified on the basis of 18S rRNA sequencing using ITS 4 and ITS6 primers. Parenicova *et al.* 2001 [23] used DNA sequence of the ITS1 and ITS2 region to make sure the close relationship between *A. japonicus* and *A. aculeatus.* In the present study, phylogenetic tree was constructed and that showed, 99% similarity with *A. Japonicus*. During the screening of endophytic fungi, CFCF of 7th, 14th, and 21st day was examined for their antibacterial activity. *A. japonicus* showed the maximum inhibitory effect against selected pathogenic bacteria. The optimum inhibitory concentration of n-butanol fungal extract was also observed at different concentrations that the antibacterial activity was elevated as the concentration of n-butanol extracted metabolites was increased. Similarly, Meenupriya and Thangaraj, in 2011 [24], also observed the inhibitory concentration of ethyl acetate extract of the *Aspergillus ocharaceus* against the four pathogenic bacteria from a range of 25–1000 µL and the maximum antibacterial activity was examined as the concentration of the solvent extract was increased.

CONCLUSION

Endophytic fungi are those microorganisms that survive interior of plants an especially leaf, stems, and roots without any apparent harm to host. Endophytes are rich sources of bioactive metabolites, which can be potentially used in the field of medicine, agriculture, and industries. In the present investigation, fungal endophyte *A. japonicus* isolated from *T. procumbens* showed the maximum antibacterial activity against the test bacterial strain. Further study can be performed to identify the bioactive compounds present in the extract. *A. japonicus* exhibited the most significant inhibitory activity against five pathogenic bacteria. The isolation of these antibacterial compounds from the endophytic fungi and identification of bioactive compounds can be a crucial approach to search of novel natural products.

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Table 4: Well-diffusion method standardization (n-butanol fungal extract low and high concentration)

Concentration (µL)	Zone of inhibition (in mm)				
	B. subtilis	*S. pyogenes*	*E. coli*	*S. typhimurium*	*K. pneumoniae*
Low concentration					
25	07.40±0.21	09.10±0.26	11.37±0.52	05.20±0.12	00.00±0.00
50	12.20±0.34	14.12±0.53	17.34±0.36	13.23±0.74	08.16±0.52
75	15.13±0.71	17.30±0.45	25.31±0.78	21.42±1.05	14.20±0.94
100	15.52±0.45	19.11±0.34	27.26±0.57	24.13±0.65	16.04±0.32
High concentration					
200	17.30±0.62	22.42±0.57	29.05±1.12	26.18±0.38	19.21±0.57
400	19.50±0.44	23.14±0.49	30.23±0.84	27.42±0.05	21.14±0.25
600	20.33±0.37	24.46±0.87	31.12±0.92	28.13±0.76	22.40±0.54
800	20.30±0.85	25.19±0.68	31.60±0.30	29.06±0.43	23.27±0.35

B. subtilis: Bacillus subtilis, S. pyogenes: Streptococcus pyogenes, E. coli: Escherichia coli, K. pneumoniae: Klebsiella pneumoniae, S. typhimurium: Salmonella typhimurium

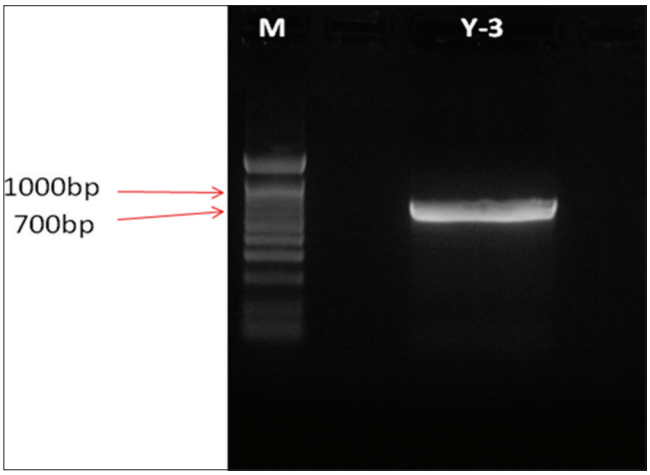

Fig. 3: 1% Agarose Gel data showing the band of Amplified DNA (700bp),

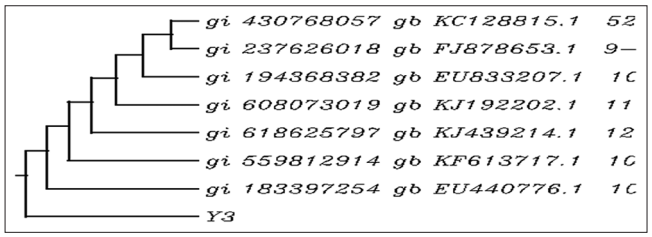

Fig. 4: Phylogenetic analysis of the obtained sequence of ITS Region with Aspergillus *japonicas* sequences from Genbank

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