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Screening of Antibiotic Producing Fungi from Soil

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Abstract: Fungi were isolated from the soil sample and it was characterized as *Aspergillus sp* by Lactophenol cotton blue staining. The antimicrobial activity of fungal extract was tested against pathogenic bacteria and fungi. The extract was tested against eight bacteria and five fungi. Then bioactive compounds were purified by silica gel column chromatography and it was identified by Gas Chromatography-Mass Spectrometry. Eight compounds such as Benzene, 1-fluoro-4-meth; 3-Benzyl sulfanyl-3-fluor; 1H-Indene,1,3-dimethyl-; 1H-Indole,1,2-dimethyl-; 3Beta-acetoxy-4,4,8,10,1; Benzene,1-fluoro-3-methyl-; 1H-Indole,1,2-dimethyl-; 1,2-Benzenedicarboxylic acid were identified. Cytotoxic activity of fungal extract on HepG2 were analysed by Trypan Blue dye exclusion method. Random amplified polymorphic DNA (RAPD) analysis was performed on Aspergillus sp isolated from soil sample. Nine primers such as OPA 01, OPA 02, OPA 03, OPA 04, OPA 05, OPA 06, OPA 07, OPA 08, OPA 09 were used for the RAPD analysis. DNA banding patterns generated by RAPD were scored for the presence (1) or for absence (0) of each amplified band. For genetic distance analysis, using NTSYS software Cluster analysis was based on similarity matrices using the unweighted pair group method analysis (UPGMA) program in the software package.

Keywords: Lactophenol, GC-MS, Cytotoxic, RAPD, NTSYS.

INTRODUCTION

Antibiotics are antimicrobial compounds produced by living microorganisms. These compounds were used therapeutically and sometimes prophylactically in the control of infectious diseases. Over 4000 antibiotics have been isolated before, but only 50 have achieved wide usage. The other antibiotic compounds failed to achieve commercial importance for some reasons such as toxicity to human and animals, ineffectiveness or high production costs[1].

Many antibiotics were produced by microorganisms as secondary metabolites. The isolation of antibiotics from microorganisms is relatively easy as compared to chemical synthesis of antimicrobial agents. The isolation of antibiotics from microorganisms improved the discovery of novel antibiotics that could act as better chemotherapeutic agents[2].

Fungi, which is separate from plants, animals, and bacteria. One major difference is that fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. Since the 1940s, fungi have been used for the production of antibiotics, and, more recently, various enzymes produced by fungi are used industrially and in detergents. Fungi are also used as biological pesticides to control weeds, plant diseases and insect pests. Many species produce bioactive compounds called mycotoxins, such as alkaloids and polyketides, that are toxic to animals including humans. In nature, antibiotics of fungal or bacterial origin appear to play a dual role: at high concentrations they act as chemical defense against competition with other microorganisms in species-rich environments, such as the rhizosphere, and at low concentrations as quorum-sensing molecules for intra- or interspecies signaling[3]. Other drugs produced by fungi include griseofulvin isolated from *Penicillium griseofulvum*, used to treat fungal infections[4] and statins (HMG-CoA reductase inhibitors), used to inhibit cholesterol synthesis. Examples of statins found in fungi include mevastatin from *Penicillium citrinum* and lovastatin from *Aspergillus terreus* and the oyster mushroom[5].

In this context, a study was conducted on the isolation, characterization, identification of fungi isolated from the soil sample collected from a market at Marthandam. The present study examined the antimicrobial activity against bacteria and fungi. The work also involves the purification and identification of antibiotic compound by silica gel column chromatography and GC MS analysis. The eluted fractions were tested for cytotoxic activity using cancer cell lines. DNA was isolated from these organisms and RAPD analysis was carried out in the study.

EXPERIMENTAL

Sample Collection: Fungi were isolated from soil collected from Marthandam, Kanyakumari district, Tamil Nadu, South India by serial dilution agar plating method. The soil samples were serially diluted up to 10^{-9} dilutions, in distilled water and 1ml sample from 10^{-8} to 10^{-9} were pour plated in Rose Bengal agar plates. The plates were kept for incubation at 28^0C to 37^0C for 24 to 72 hours in an inverted position. The fungal isolates were purified by pure culture techniques and refrigerated in agar slants for further studies.

Fungal Staining: Fungal staining was carried out by Lactophenol cotton blue staining technique. The preparation of fungal stain was viewed under low and high, dry magnification for the presence of characteristic mycelia and fruiting structures.

Antibiotic Sensitivity with Pathogens: The fungal isolates were tested against pathogens and their antibiotic sensitivity was determined by Disc Diffusion method**.** The bacterial lawn of each organism was prepared on the nutrient agar plates. One drop of fungal culture was added to sterile filter paper disc (size: 5mm) and allow to dry after each addition. The discs were then placed on air dried surface of the medium.

The plates were incubated at 37 ^{0}C for 24hours. After incubation the diameter of inhibition zones around the discs was measured.

Pathogenic Bacteria (Gram- positive and Gram -negative), and fungus were obtained from Microbial Type Culture Collection, Institute of Microbial Technology Chandigarh. The bacterial strains used *were Klebsiella pneumoniae, Escherichia coli, Bacillus megaterium, Staphylococcus epidermis, Streptococcus mutans, Enterococcus faecalis, Streptococcus pyogenes, Salmonella enteriticlis*. The fungal strains used were *Candida tropicalis, Aspergillus fumigatus, Aspergillus flavus, Candida albicans, Candida glabrata.* The bacterial and fungal strain cultures were maintained in nutrient and fungal broth as turbid growth and kept in refrigerator at 4 ^{0} C for further analysis.

Purification of Antibiotics: The purification of antimicrobial substance was carried out using silica gel (2.5x50) chromatography. Chloroform and Methanol 95:5(v/v) mixture was used as an eluting solvent. The column was left overnight until the silica gel (pro labo) was completely settled. 1 ml crude extract to be fractionated was added on the silica gel column surface and the extract was absorbed on top of silica gel. 10 fractions were collected (each of 5 ml) and tested for their anti microbial activity.

GC – MS Analysis: Gas chromatography-mass spectrometry analyses were performed using an Agilent GC-MS 5973 (Palo Alto, CA, USA) assembly equipped with a HP-5 cross-linked fused silica capillary column (25 m \ 0.32 mm \ 0.25 μm). Helium was used as carrier gas at 38 cm/s. The column total flow rate was 1 ml/min. General temperature conditions were: split/splitless injector at 280 °C, transfer line at 280 °C, source 230 °C, and column temperature program of 80°CD310 °C at 10 °C/min. Mass detection limits were 50D700 Da. Samples were reacted with BSTFA-pyridine (1:1, v/v) at room temperature for 30 min before analyses.

Viability Staining by Trypan Blue Dye Exclusion Method: Cytotoxic activity of fungal extract were analysed by Trypan Blue dye exclusion method. $HepG_2$ cancer cell line line was used for the determination of cytotoxic activity. Cells were maintained in DMEM (Dulbeccos modified eagles medium) supplemented with FBS (foetal bovine serum) and penicillin/streptomycin-L-glutamine and cultured in a humified atmosphere of 5% CO_2 and 95% air at 37°C in Thermo Hera Cell 150 incubator.

Cell lines in exponential growth phase were washed with phosphate buffer saline (PBS) solution and trypsinized and re-suspended in complete culture media. Cells were plated at 30,000 cells/well in 6 well plates and incubated for 24 hours during which a partial monolayer forms. After incubation the cells were exposed to various concentrations of the drugs, which is the plant extract (1000 g/ml, 500 g/ml, 250 g/ml, 150g/ml, 125 g/ml, 50g/ml and 25g/ml). The control well received only maintenance of medium. The plates were incubated at 37°C in a humidified incubator with 5% CO2 for a period of 24 hours. Morphological changes of drug treated cells were examined using an inverted microscope and compared with the cells serving as control. At the end of 24 hours incubation, cell viability was determined.

DNA Preparation: The genomic DNA isolation from fungi was described by the method[6,7]. CTAB method was the most commonly used protocol for the preparation of fungal genomic DNA.

RAPD Analyses: Nine primers designated OPA 01, OPA 02, OPA 03, OPA 04, OPA 05, OPA 06, OPA 07, OPA 08, and OPA 09 (Operon Biotechnology) were used. RAPD analysis was carried out essentially [8]. A 25µl PCR reaction mixture contained 10X PCR buffer- 1x , 25mM Mgcl- 2mM, 10mM dNTP mix- 600µM, 25Pico moles Primer- 5 Pico moles, Taq DNA polymerase- 0.75U, Template DNA- 50ng, Milli Q water- Variable. After the solutions were mixed, the tubes containing the mixtures were placed in a PTC-100 programmable thermal controller (MJ Research, Inc.). The thermal cycler was programmed for 1 cycle of initial denaturation for 4 minutes at 94°C. This was followed by 42 cycles programmed for

denaturation at 94°C for 50 seconds, annealing at 36°C for 1 minute, and extension at 72°C for 1.30 minutes. An additional cycle of at 7 minutes at 72°C was use for final extension followed by 40 cycles of 94°C for 1 min, 30°C for 2 min, and 72°C for 2 min and a final extension period of 72°C for 7 min. Randomly amplified products were analyzed by electrophoresis on a 1.2% agarose gel in Tris-borate-EDTA buffer (0.5MTris, 0.5Mboric acid, 10 mM EDTA [pH 8.0]) and visualized by ethidium bromide staining. The molecular size standards used were ranges from 100bp to 1kb.

Analysis of Data from RAPD: DNA banding patterns generated by RAPD were scored for the presence (1) or for absence (0) of each amplified band. All RAPD assays were repeated twice and only the reproducible bands were scored. For considering a marker as polymorphic, the absence of an amplified product in at least one species was used as a criterion.

RESULT AND DISCUSSION

Fungal Staining: On the basis of staining method (Lactophenol cotton blue) the bioactive compound producing fungi was identified as *Aspergillus* sp. The result was displayed in **Fig. 1.**

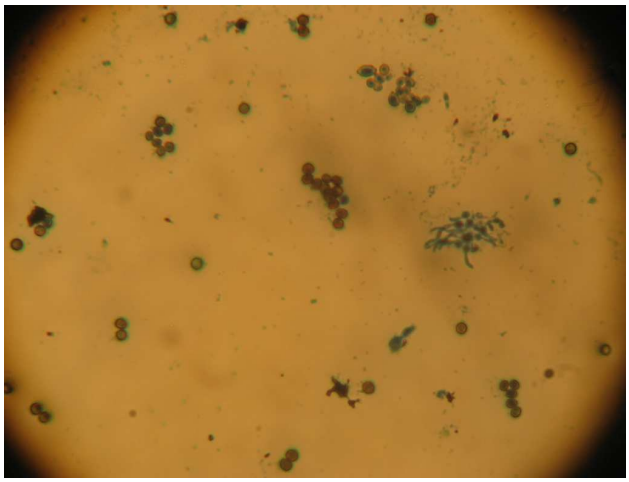


Fig-1: Fungal Staining

Antibiotic sensitivity with Pathogens: The bioactive compound producing fungi *Aspergillus* sp was selected and tested its antibiotic sensitivity with pathogens both bacteria and fungi. The organism showed both positive and negative interactions. In the negative interaction the organism produce inhibition zones which were measured and recorded. The values were listed in the **Table-1** and **Table-2**. The studies in isolated fungi involves the interaction of microorganisms includes bacteria and fungi. The microbial interactions were analyzed by the determination of the size of the inhibition zone[9].

Purification of Bioactive Compounds by Silica Gel Column Chromatography: Crude extract of *Aspergillus* sp was used for the purification of bioactive compound. Ten elutions were collected from fungi *Aspergillus* sp. The collected fractions were kept at 4^0C. The elutions were tested for their antimicrobial activities. The absorbances at 272nm of eluted fractions were represented in **Table-3.** The bioactive compounds were purified by silica gel column chromatography. Methodology of isolation and purification of antibiotics to a large extent depends on the properties of the antibiotic, its chemical nature and the environment in which it is accumulated[10]. Separation methods have been developed, and without doubt natural chemistry has greatly stimulated to the development of the refined techniques applied today.

Chromatographic methods including thin layer chromatography (TLC), column chromatography, gas chromatography (GC) and high performance liquid chromatography (HPLC), have made it possible to isolate compounds present in extremely small quantities. Spectral analysis including infrared (IR), ultraviolet (UV), NMR (nuclear magnetic resonance) spectroscopy and mass spectroscopy are used in the identification of antibiotics[11].

Table- 1: Antimicrobial Activity of Isolated Fungi using Bacterial (MTCC culture):

Sl.No	Bacteria (MTCC Culture)	Zone of Inhibition of isolated Fungi (cm)
1	*Klebsiella pneumoniae*	1
2	*Escherichia coli*	0.7
3	*Bacillus megaterium*	1.3
4	*Staphylococcus epidermis*	0.1
5	*Streptococcus mutans*	0.9
6	*Enterococcus faecalis*	1.1
7	*Streptococcus pyogenes*	1.4
8	*Salmonella enteriticlis*	0.3

Table- 2: Antimicrobial Activity of Isolated Fungi using Fungi (MTCC culture):

Sl.No	Fungi (MTCC Culture)	Zone of Inhibition of isolated Fungi (cm)
1	*Candida tropicalis*	Nil
2	*Aspergillus fumigatus*	Nil
3	*Aspergillus flavus*	Nil
4	*Candida albicans*	0.8
5	*Candida glabrata*	1.4

Table- 3: Absorbance of Eluted Fractions of *Aspergillus* sp:

Eluted Fraction	Absorbance at 272nm
1	0.3555
2	0.530
3	1.358
4	0.836
5	1.061
6	0.932
7	0.450
8	0.046
9	0.019
10	0.013

Antibacterial activity of *Aspergillus* sp against *Streptococcus pyogenes*: The eluted fractions of fungi were tested for their antibiotic sensitivity with pathogens. The elution produced inhibition zones which were measured and recorded. The values were listed in the **Table- 4.**

Table- 4: Antibacterial Activity of Eluted Fractions Using *Streptococcus pyogenes*:

Eluted Fraction	Zone Diameter (cm)
1	1
2	1.1
3	1.3
4	0.8
5	1.2
6	0.8
7	2
8	1.5
9	1
10	1.5

GC/MS Analysis of Fungal Extract: GC/MS Analysis indicated that the fungi *Aspergillus* sp extract showed totally 54 peaks. Eight compounds such as Benzene, 1-fluoro-4-meth; 3-Benzyl sulfanyl-3-fluor; 1H-Indene,1,3-dimethyl-; 1H-Indole,1,2-dimethyl-; 3Beta-acetoxy-4,4,8,10,1; Benzene,1-fluoro-3-methyl-; 1H-Indole,1,2-dimethyl-; 1,2-Benzenedicarboxylic acid were identified. The compounds present in peaks are given in the F**igure -2** and **Table– 5.** GCMS analysis reveals the production of 2-phenylethanol by one of the isolates JUBT 3M which was identified as *Aspergillus niger***.** This is the first report of production of 2-phenylethanol from endophytic **A. niger**[12].

Table-5: Compounds in Fungi *Aspergillus* sp extract:

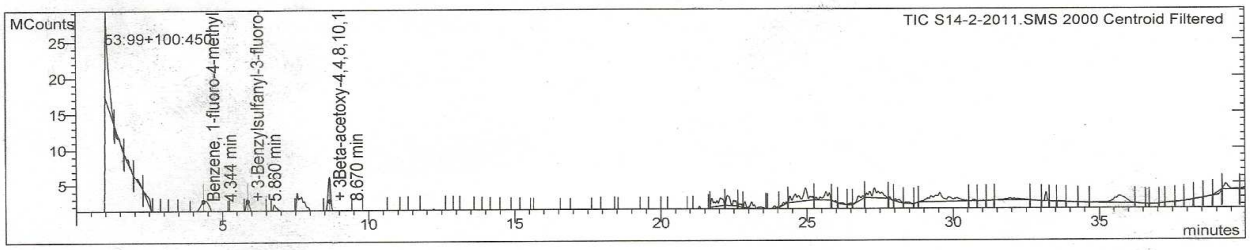
Number of peak	Retention Time (minutes)	Compounds
1.	4.344	Benzene, 1-fluoro-4-meth
2.	5.860	3-Benzyl sulfanyl-3-fluor
3.	6.282	1H-Indene,1,3-dimethyl-
4.	7.553	1H-Indole,1,2-dimethyl-
5.	7.634	3Beta-acetoxy-4,4,8,10,1
6.	4.425	Benzene,1-fluoro-3-methyl-
7.	6.769	1H-Indole,1,2-dimethyl-
8.	33.171	1,2-Benzenedicarboxylic acid

Print Date: 02 Apr 2011 15:14:46

Sample Report for s14-2-2011.sms

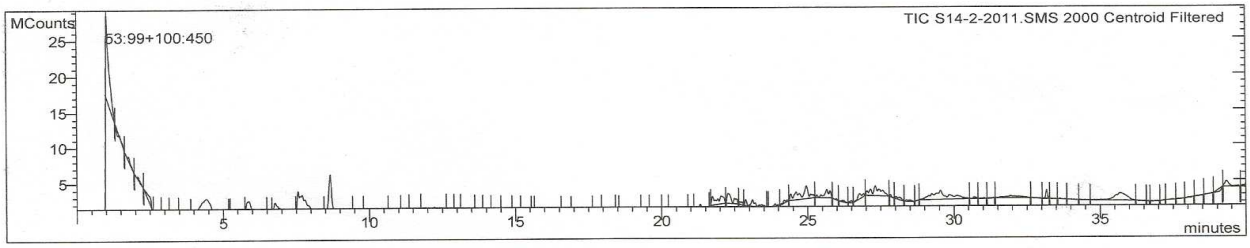
C.E.P.C. Laboratory & Technical Division

Sample ID:	S1	Operator:	
Instrument ID:	Varian GC/MS #1	Last Calibration:	10/5/2006 10:21 PM
Acquisition Date:	4/2/2011 12:38 PM	Data File:	...\4-11\s14-2-2011.sms
Calculation Date:	4/2/2011 1:42 PM	Method:	...ect metods\t.oil.mth
Inj. Sample Notes:	None		



Target Compounds

#	RT	Peak Name	Res Type	Quan Ions	Area	Amount/RF
1	4.344	Benzene, 1-fluoro-4-meth	Id.	108.9	1.506e+6	1505915 Counts
2	5.860	3-Benzylsulfanyl-3-fluor	Id.	91.0	3.018e+6	3017540 Counts
3	6.282	1H-Indene, 1,3-dimethyl-	Id.	127.9	348731	348731 Counts
4	7.553	1H-Indole, 1,2-dimethyl-	Id.	144.9	4.205e+6	4205455 Counts
5	7.634	3Beta-acetoxy-4,4,8,10,1	Id.	126.8	2.005e+6	2005387 Counts
6	7.794	3Beta-acetoxy-4,4,8,10,1	Id.	126.8	2.372e+6	2372152 Counts
7	8.670	3Beta-acetoxy-4,4,8,10,1	Id.	126.8	1.291e+7	12914277 Counts



Unidentified Peaks

Quan Ions: RIC
RF Used: 1.000

Spectrum Match Type: Normal-Forward
Match Thresh: 700

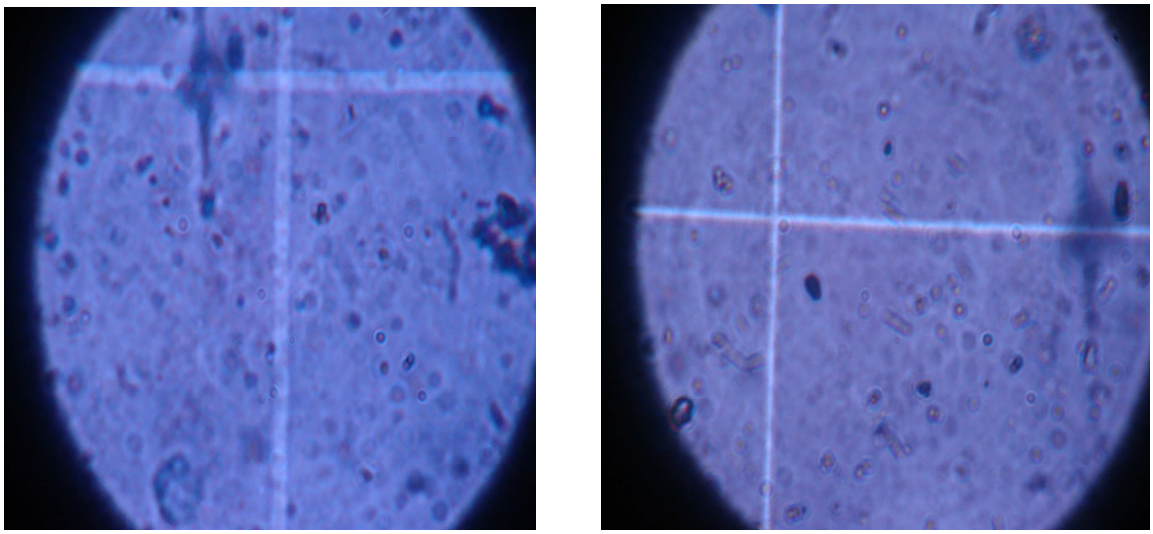
#	RT	Peak Name	Res Type	Area	Amount	R.Match
8	1.027	No Match	Unk.	3.732e+8	373213312	----
9	1.454	No Match	Unk.	5.149e+7	51488124	----
10	3.962	No Match	Unk.	2.753e+6	2753453	----
11	4.425	Benzene, 1-fluoro-3-methyl-	TIC	5.439e+7	54391812	764
12	4.827	No Match	Unk.	4.029e+6	4028559	----
13	4.924	No Match	Unk.	1.722e+6	1721759	----
14	5.307	No Match	Unk.	2.770e+6	2769544	----
15	5.564	No Match	Unk.	3.724e+6	3723550	----
16	6.118	No Match	Unk.	2.637e+6	2636580	----
17	6.769	1H-Indole, 1,2-dimethyl-	TIC	1.595e+7	15950529	732
18	7.698	No Match	Unk.	1.620e+7	16197537	----
19	21.742	No Match	Unk.	9.646e+6	9645563	----
20	22.036	No Match	Unk.	2.826e+7	28256650	----
21	22.270	No Match	Unk.	1.907e+7	19066436	----
22	22.494	No Match	Unk.	1.071e+7	10707921	----
23	22.875	No Match	Unk.	9.093e+6	9093127	----
24	23.885	No Match	Unk.	2.210e+6	2210312	----
25	24.200	No Match	Unk.	6.186e+6	6186369	----
26	24.295	No Match	Unk.	3.802e+6	3802324	----

Figure- 2: Chromatogram for Fungi *Aspergillus* sp extract

Cytotoxic Effect of *Aspergillus* sp Fungal Extract: The cytotoxic effects of *Aspergillus* sp fungal extract on HepG2 cell lines by Trypan Blue dye exclusion method. The Trypan Blue dye exclusion method of HepG2 cells after contact with 25µl, 50µl, 75µl, 100µl extract showed 16%, 28%, 34% and 47% metabolic activity respectively. The result was showed in **Table- 6** and figure- 3. Most of previous studies screened the potential of fungi extracts on antimicrobial activity, antifungal activities, antifouling, and cytotoxic activities on several cell lines[13,14].

Table- 6: Cytotoxic Effect of *Aspergillus* sp Fungal Extract against HepG2 cell line:

Concentration (µl)	Viable	Nonviable	Cytotoxic activity (%)
10	2028	185	8
25	1901	312	14
50	1800	413	19
75	1679	534	24
100	1452	701	32



Control **Test**

Fig- 3: Cytotoxic Effect of Fungal Extract on HepG2:

RAPDAnalysis: RAPD analysis in fungi, using 9 random primers for the genomic DNA. Among this nine primers, seven primers produced the reproducible bands. The amplicon size range from 100 – 500 base pairs. This data only for to screen the ambiguity of the primer amplification in this fungi. The result was recorded in **Table- 7** and **Figure- 4**. Using a molecular approach, arbitrary amplification of polymorphic DNA sequences, termed random

amplification of polymorphic DNA (RAPD) analysis on arbitrarily primed PCR (APPCR) typing [15,16] is one such new technique that is being used in many epidemiological studies. It is a fast, PCR based method of genetic typing based on genomic polymorphisms.

Table- 7: RAPD Analysis in the fungi using decamer primers:

S.No	Name of primer (Operon Tech)	Sequence of the primer	%GC	Amplicons size range (bp)	Total number of amplified bands
1	OPA 01	CAGGCCCTTC	70.00%	Nil	Nil
2	OPA 02	TGCCGAGCTG	70.00%	500-100	4
3	OPA 03	AGTCAGCCAC	60.00%	300-150	2
4	OPA 04	AATCGGGCTG	60.00%	500-200	3
5	OPA 05	AGGGGTCTTG	60.00%	500-400	2
6	OPA 06	GGTCCCTGAC	70.00%	Nil	Nil
7	OPA 07	GAAACGGGTG	60.00%	400	1
8	OPA 08	GTGACGTAGG	60.00%	600	1
9	OPA 09	GGGTAACGCC	70.00%	500-100	5

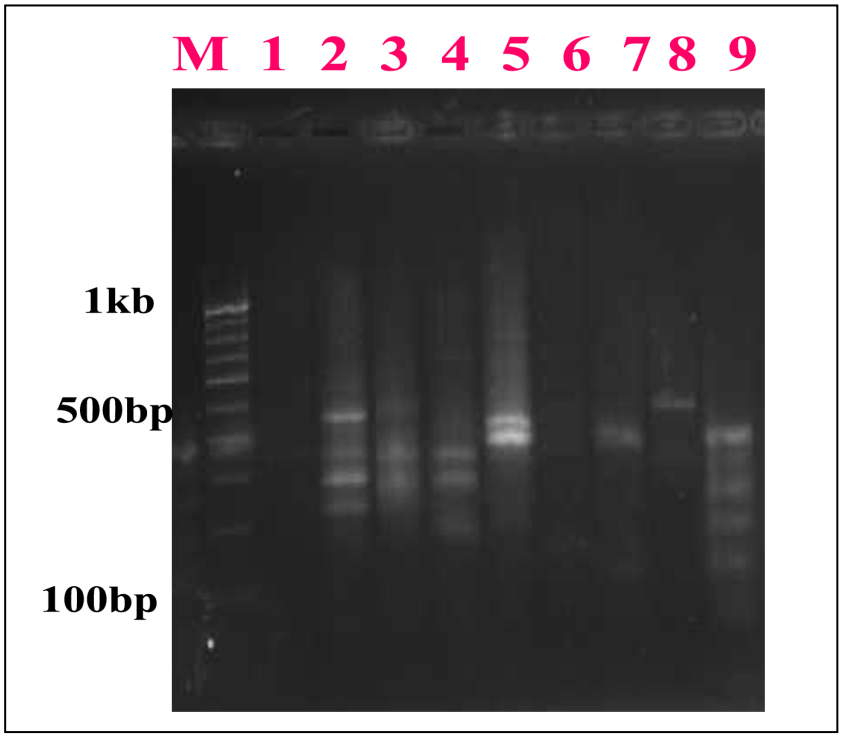


Fig- 4: RAPD Analysis of Fungi:

CONCLUSION

From this study, it can be concluded that the fungi *Aspergillus* sp showed potential interaction between the pathogenic microbes. The fungi were characterized only by fungal staining technique and it need further molecular level characterization. The bioactive compounds were characterized from the fungi and the fungal extracts showed antimicrobial, cytotoxic activities on HepG2 cell line. Data from the literature as well as our results reveal the great potential of fungal extracts showed various antimicrobial, cytotoxic activities and have not been completely investigated. Additional studies would be needed further to evaluate the potential of this fungal extracts for the isolation of novel antibiotics.

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