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# Extremophiles as Biofactories of Novel Antimicrobials and Cytotoxics – An Assessment of Bioactive Properties of Six Fungal Species Inhabiting Rann of Kutch, India

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## Abstract

**Objective**: In this study we report the antibacterial and cytotoxic activity of the fungi isolated from Little Rann of Kutch, India. **Methods**: Antibacterial activity of crude extracts of 35 fungal isolates was assessed at an initial concentration of 40µg/ml against *Klebsiella pneumoniae* and *Escherichia coli*. Promising fungal isolates were identified by their morphology. Extracts of identified isolates were further tested at varying concentrations (10-40µg/ml) against *Staphylococcus aureus* and *Bacillus cereus* apart from *K. pneumoniae* and *E. coli*. Cytotoxic activity was assessed against cancer cell line MCF-7. Phenol, flavonoid and alkaloid estimation was done to understand the fungi's chemoprofile. **Findings**: In preliminary screening, six isolates showed significant activity against *K. pneumoniae* (14-17 mm) and *E. coli* (14-22 mm). These were identified as *Aspergillus flavus*, *A. cremeus*, *A. versicolor*, *A. terreus*, *Penicillium purpurogenum* and *Eurotium amstelodami*. On further evaluation of fungal extracts against four test pathogens, *A. versicolor* was found to be the most effective (8-11 mm), followed by *A. terreus* (8-10 mm) and *A. cremeus* (8-10 mm) at 10µg/ml each. *A. versicolor* and *P. purpurogenum* were found to be most cytotoxic, with $IC_{50}$ values of 6.26 and 6.30 respectively, followed by *A. flavus, E. amstelodami, A. cremeus* and *A. terreus*. Phenolic content was highest in *A. versicolor* (268.77±0.18 µg/mg GAE); flavonoid and alkaloid were maximum in *P. purpurogenum* - 91.17±0.44 µg/mg QE and 89.13±0.28 µg/mg PNE, respectively. **Improvements/Applications:** Six fungi isolated from Little Rann of Kutch, India, hold tremendous potential as a source of novel antimicrobials and cytotoxics. The results warrant more exhaustive bioassays and identification of active principles.

**Keywords:** Antibacterial, *Aspergillus*, Cytotoxicity, *Eurotium*, *Penicillium*, Rann of Kutch

## 1. Introduction

Ecological niches such as Antarctica[1], tropical regions[2], deep sea[3], habitats of extreme salinity, pH and temperature are being explored for microorganisms as such habitats are expected to harbour either commonly found microbes that produce complex and diverse natural products or previously unexplored fungal species. The metabolites produced by these fungi are expected to be source of novel drug lead compounds; these drugs are expected to be effective against new and evolved pathogens such as *Pseudomonas aeruginosa*[4], Ebola virus, Zika virus, West Nile virus[5] and *Acinetobacter baumanii*[6], that are posing fresh challenges to the medical community[7].

Little Rann of Kutch is located in the south-eastern region of the Great Rann of Kutch, a massive salt desert in the state of Gujarat, India. The region withstands scant and sporadic rainfalls in monsoon, extreme temperatures in summer, scarce vegetation, low soil organic and moisture content and very high surface salinity (281.6 ppm

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to 12,094 ppm) are characteristic features of this region[8]. This study reports the antimicrobial and cytotoxic potential of the crude extract of six fungi isolated from this extreme geographical region. Biochemical assays were done to study the fungi's chemoprofile and understand the underlying mechanism of their bioactivity.

## 2. Materials and Methods

### 2.1 Chemicals

All chemicals were of analytical grade, procured from Sigma-Aldrich (India) or Merck (India), unless otherwise stated, and used as received. Culture media were purchased from HiMedia (India). Double distilled water was used in all of the experiments.

### 2.2 Microorganisms

Thirty five soil fungi, previously isolated from the southern fringe zone of Little Rann of Kutch, India, were used for the study (our unpublished data). Briefly, fungi were isolated from the study region by serial dilution method and cultured on Czapek Yeast Autolysate agar (CYA), Malt Extract agar (MEA) and Potato Dextrose agar (PDA) plates[9]. The inoculated plates were incubated in dark at 25±1°C for 7 d. Subsequently, each morphologically distinct fungal colony was sub-cultured for obtaining pure isolates. The isolates were labelled as BBKF1 - BBKF35 in ascending order. No specific permissions were required for these locations/activities as the study region falls in a non-restricted area and prior permission from any authority is not required. The fungal isolates are being maintained on CYA agar at the institute's culture collection facility.

### 2.3 Cultivation and Extraction of Fungal Culture

Fungal extracts were prepared by the method reported earlier with slight modifications (VanderMolen et al. (2013)[10]. Agar plug from 7 days old culture of each fungus was inoculated in Czapek Dox broth (100ml) in Erlenmeyer flask and incubated in dark (10 d, 25±1°C). Mycelial mat was cut into fine pieces and suspended in 1:1 chloroform: methanol and kept overnight in a rotary shaker (120 rpm). The mixture was filtered and to the filtrate equal volumes of chloroform and distilled water was added and stirred vigorously in an orbital shaker (120rpm, 1h). The bottom organic layer was collected and evaporated to dryness in a Buchi Rotavac. The dried extract was suspended in 1:1 acetonitrile: methanol (100 ml) and hexane (100 ml) and stirred (120 rpm, 1h). The bottom organic layer was collected and evaporated to dryness in vacuo. Dimethyl sulfoxide (DMSO) extracts at a concentration of 1 mg/ml were prepared.

### 2.4 Antibacterial Assay

#### *2.4.1 Preliminary Screening for Antibacterial Activity*

Antibacterial activity of each of the thirty five fungal extracts was evaluated by agar well diffusion assay against the Gram negative bacteria *Klebsiella pneumoniae* (KJ938546) and *Escherichia coli* (MCC 2412). *K. pneumoniae* was obtained from the institute's culture collection facility and *E. coli* was acquired from the Microbial Culture Collection at NCCS, Pune, India. Bacteria were cultured and maintained on Mueller–Hinton agar (MHA). Bacterial inoculants were prepared in nutrient broth. The turbidity was adjusted to 0.5 McFarland standards. Each test organism (100 µl) was mixed with cooled MHA and poured into 80 mm Petridishes. Wells were cut and crude fungal extract (40 µg/ml, 100 µl) was poured. The plates were incubated (37°C, 24 h) and the zones of inhibition were measured. Tetracycline and rifampicin were used as positive controls. Six fungi exhibited significant antibacterial activity against the tested bacterial pathogens.

#### *2.4.2 Morphological Identification of Fungi with Significant Antibacterial Activity*

The six fungi showing antibacterial activity against Gram negative pathogens were selected for further analysis. Identification of the six fungal isolates was done by Agharkar Research Institute, Pune, India on the basis of their macro- and micro-morphology.

#### *2.4.3 In-depth Antibacterial Assay of Fungi with Significant Antibacterial Activity*

Crude extracts of the selected fungi were further tested at four different concentrations (10, 20, 30, 40 µg/ml) against two Gram negative bacteria *Bacillus cereus* and *Staphylococcus aureus*, in addition to *K. pneumoniae* and *E. coli*. *B. cereus* and *S. aureus* were acquired from the Microbial Culture Collection at NCCS, Pune, India.

### 2.5 Cytotoxicity Assay

The cytotoxicity of the fungal extracts against MCF-7 human breast cancer cell line was evaluated by MTT assay[11]. Cell line MCF-7 was acquired from National Center for Cell Science, Pune, India. Cells were cultured and maintained in Genome Biology Lab, Jamia Milia Islamia, New Delhi, India. Peripheral Blood Mononulcear Cell (PBMC) was used as control. PBMC was isolated from the blood sourced from blood bank at Jawaharlal Nehru Medical College, Aligarh, India. Blood sample was mixed with phosphate buffered saline (1:1 v/v) and centrifuged (400g, 30 min, 22 °C). The lymphocyte layer was harvested and washed thrice with PBS and centrifuged. The pellets were re-suspended in RPMI-1640 media. 10% antibiotic and antimycotic solution and fetal calf serum were added. Cells were seeded in 96-well tissue culture plate. Crude extracts at 5, 10, 15, 20 µg/ml were added and incubated for 48 hours at 37±2 °C. MTT dye was added and the resulting colour was read at 570 nm on ELISA plate reader. Cytotoxicity was calculated as:

$$\% \text{ of cytotoxicity} = (A_C - A_T)*100/ A_C$$

where, $A_C$ and $A_T$ are the mean value of absorbance of control and the treated cells respectively. The experiment was performed in triplicates.

### 2.6 Statistical Analysis

All the experiments were performed in triplicates. Data were expressed as mean values with standard deviation. Data were analysed by two-way analysis of variance (two-way ANOVA) for anti-bacterial assay. Data analysis was performed by linear regression for cytotoxicity assay. Tukey's procedure was used for significance of difference ($P < 0.05$). GraphPad Prism software (USA) was used for data analysis.

### 2.7 Determination of Total Phenol Content

Total phenolic content was determined according to the Folin-Ciocalteu (F-C) colorimetric method[12]. Gallic acid concentrations (25-300 µg/ml) were prepared and a calibration curve was obtained using a linear fit. The samples were analysed in triplicate.

### 2.8 Determination of Total Flavonoid Content

Total flavonoid content was estimated by the aluminium chloride method[13]. Quercetin was used as the standard and a calibration curve was prepared with concentrations ranging from 0-500 µg/ml. Samples were analysed in triplicate.

### 2.9 Determination of Total Alkaloid Content

Total alkaloid estimation of each of the crude extracts was done by spectrophotometric method of Dragendorff's reagent[14]. Standard curve was prepared using pilocarpine nitrate in HCl solution at different concentrations (750, 500, 400, 250, 200, 150 and 100 mg/l). Alkaloid contents were expressed as pilocarpine nitrate equivalents (µg/mg PNE).

## 3. Results

### 3.1 Preliminary Antibacterial Assay

Crude extracts of each of the thirty five morphologically distinct fungi were tested for their respective antibacterial activity. Six fungi showed significant broad spectrum antibacterial activity while the remaining had non-significant antibacterial activity.

### 3.2 Fungal identification

Based on colony characteristics and micromorphology, the six isolates were identified as *Aspergillus flavus*, *Aspergillus cremeus*, *Aspergillus versicolor*, *Aspergillus terreus*, *Penicillium purpurogenum* and *Eurotium amstelodami*.

### 3.3 In-depth Antibacterial Assay

Crude extracts of the six fungal isolates were tested at different concentrations against two Gram positive and two Gram negative bacterial pathogens. Clear zone of inhibition was observed to varying degrees, indicating the presence of antibacterial compounds in the extracts. Figure 1 gives a graphical representation of the antibacterial activity of fungal extracts at different extract concentrations against the test organisms - *K. pneumonia* (Kp), *B. cereus* (Bc), *E. coli* (Ec) and *S. aureus* (Sa). The bars represent zone of inhibition measured in mm including well diameter, expressed as Mean ± SD. On comparing the potency of the fungal crude extracts at 10 µg/ml concentration, *K. pneumonia* and *S. aureus* were found to be inhibited most by *A. versicolor*, *A. terreus* and *E. amstelodami*. *E. coli* was most susceptible to *A. versicolor* and *A.*

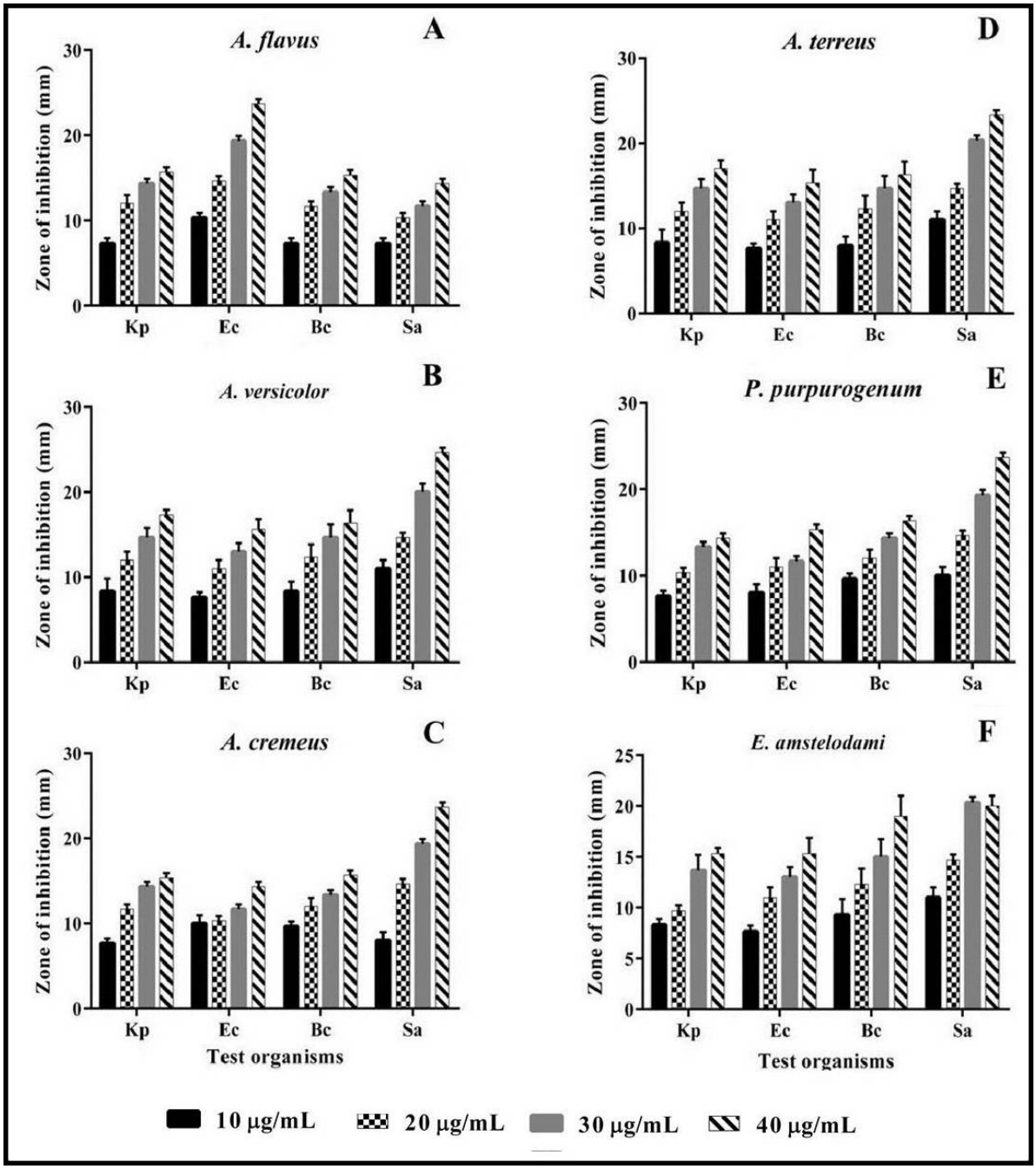


**Figure 1.** Antibacterial activity of fungal extracts against the test organisms measured as zone of inhibition. Data presented as mean ± SD.

*flavus* whereas *B. cereus* was inhibited most by *A. cremeus* and *P. purpurogenum*.

## 3.3 Cytotoxic Activity

MCF-7 cancer cell line and PBMC cell line were treated with different concentrations of the fungal extracts *in vitro*. Results obtained from MTT assay established that all six fungal extracts had significant cytotoxic activity against the cancer cells MCF-7, as compared to the healthy PBMC cells (Figure 2). Based on $IC_{50}$ values, *A. versicolor* (6.26) and *P. purpurogenum* (6.30) were found to be the most cytotoxic, followed by *A. flavus* (7.74), *E. amstelodami* (7.80), *A. cremeus* (7.93) and *A. terreus* (7.99), respectively (Figure 3).

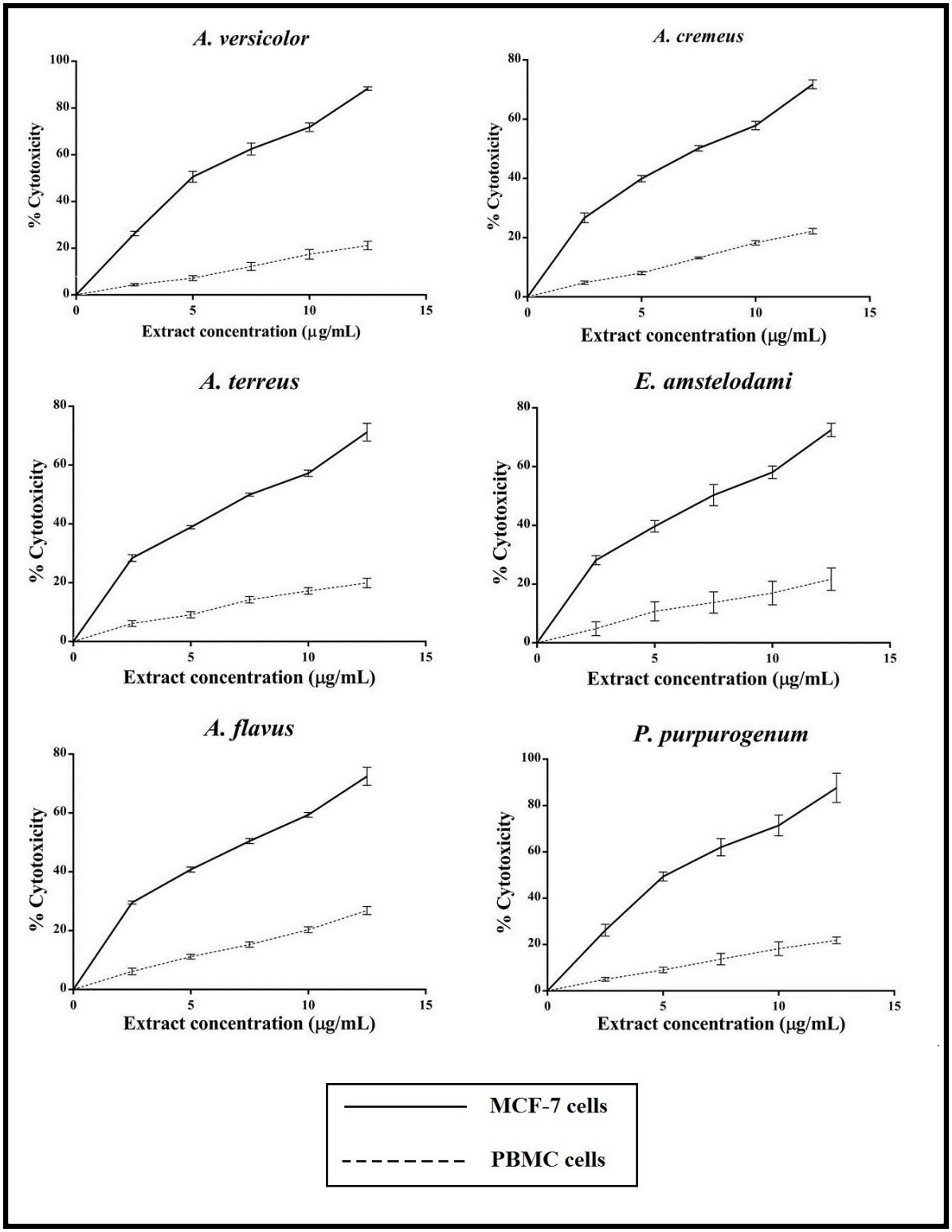


**Figure 2.** Cytotoxic activity of the fungal extracts against MCF-7 cancer cell line measured as % cells killed. The data are presented as mean ± SD.

**Table 1.** Phenol, flavonoid and alkaloid contents of the fungi. Data presented as mean± SD

| Fungus | Phenol (µg/mg GAE) | Flavonoid (µg/mg QE) | Alkaloid (µg/mg PNE) |
|---|---|---|---|
| *Aspergillus flavus* | 130.7±0.13 | 63±0.20 | 81.02±0.29 |
| *Aspergillus versicolor* | 268.77±0.18 | 73.537±0.20 | 71.23±0.41 |
| *Aspergillus cremeus* | 143.23±0.8 | 71.25±0.91 | 83.00±1.01 |
| *Aspergillus terreus* | 157.19±1.2 | 86.16±0.80 | 68.26±1.5 |
| *Penicillium purpurogenum* | 201.28±0.67 | 91.17±0.44 | 89.13±0.28 |
| *Eurotium amstelodami* | 182.36±0.35 | 56.45±0.66 | 76.87±0.41 |

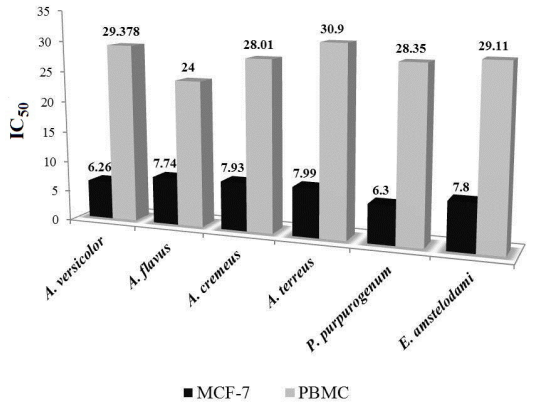


**Figure 3.** Comparative representation of the $IC_{50}$ values of the fungal extracts against MCF-7 and PBMC cell line.

### 3.4 Total Phenol, Flavonoid and Alkaloid Contents

The total phenol, flavonoid and alkaloid contents of the six fungal extracts have been summarized in Table 1. Total phenolic content was found to be highest in *A. versicolor* while flavonoid and alkaloid contents were highest in *P. purpurogenum*. The alkaloid, phenol and flavonoid contents in each of the six fungi were found to be significantly high.

## 4. Discussion

Microorganisms are naturally diverse and have been isolated from almost all known ecological habitats, even some extreme ones such as hydrothermal vents in the deep sea, soda and saline lakes and clouds[15]. Isolation of novel microorganisms is the crucial step in drug discovery programs. Although great strides have been made in drug formulation programs such as chemical synthesis of active principles, microbial natural products continue to be a promising and reliable source of drug lead compounds. Researchers are scanning unexplored ecological niches such as deep sea, rocks, insect guts, and regions of extreme salinity, pH and temperature for novel microbes. The rationale is that such habitats may harbor microbes having unique metabolic pathways. Such pathways may produce unusually complex and chemically diverse natural products. According to Hawksworth[16] there are an estimated 1.5 million different fungal species, of which approximately 0.1 million have been characterized. *Eurotiales* (*Aspergillus, Penicillium, Monascus*) within the phylum *Ascomycota* form the largest and most diverse producers of natural products, followed by *Xylariales*, *Hypocreales* and *Pleosporales*[17]. Nearly 4000 compounds comprising of antifungals, β-lactams, insecticides and toxins have been reportedly isolated from fungi alone. Members of the phylum Ascomycota, especially those belonging to the genera *Aspergillus* and *Penicillium*, produce metabolites possessing antibacterial, antifungal, insecticidal and nematicidal activities[18]. For example, *Aspergillus ochraceus* extract displays wide spectrum antibiotic activity against *Pseudomonas aeruginosa* and *E. coli*[19]. Intra- and extracellular metabolites of *Aspergillus terreus* Thom exhibit anti-fungal activity against *Aspergillus fumigatus*[20]. *A. niger* has been shown to have larvicidal and insecticidal activity[21].

Little Rann of Kutch is a barren saline wasteland that is low in organic carbon and nutrient contents. The intense summer heat evaporates the scarce moisture content, leaving behind a flat saline surface consisting of halite and gypsum crystals. The study was based on the premise that the fungi growing in hypersaline habitats possess complex metabolic pathways and secrete novel bioactive metabolites[22].

In the present study, thirty five fungi isolated from the salt desert of Little Rann of Kutch were investigated for

their antibacterial and cytotoxic activities. Six fungi - *A. versicolor*, *A. terreus*, *A. cremeus*, *A. flavus*, *P. purpurogenum* and *E. amstelodami* - were found to have potent broad spectrum antibacterial and cytotoxic activities. Each of the six fungal extracts displayed bactericidal activity against *S. aureus*, *B.cereus*, *E. coli* and *K. pneumonia*, with Gram positive bacteria having higher susceptibility as compared to Gram negative ones. Differences in cell architecture between the two bacterial types might be the cause of these variations[23]. An outer lipopolysaccharide membrane encapsulating Gram negative bacteria prevents the invasion of hydrophobic compounds[24].

For evaluating the cytotoxic potential, crude extracts of six fungi were interacted at different concentrations with MCF-7 cell line. The fungal extracts were strongly active with $IC_{50}$ values ranging from a minimum 6.26 µg/ml for *A. versicolor* to a maximum 7.99 µg/ml for *A. terreus*. The cytotoxicity of the fungal extract might be effected by one of the many mechanisms such as DNA cleavage, inhibition of key signalling enzymes, protein degradation and telomerase inhibition. Discovery of new compounds as anticancer agents is based on target-directed screening. The aforesaid enzymes are some of the most important and common targets for such screening- based drug discovery programs. Staurosporine[25] and balanol (PKC inhibitors)[26], clavaric acid (FTPase inhibitor)[27] and telemostatin (telomerase inhibitor)[28], are some of the cytotoxic compounds derived from microorganisms.

The phenol, flavonoid and alkaloid contents of each of the six fungal extracts were found to be significantly high. These compounds are known to exert antimicrobial, anti-tumoral, antioxidant, anti-allergic and anti-viral activities[29–31]. Phenols and flavonoids are reportedly involved in inhibition of nucleic acid biosynthesis and other metabolic processes[32,33]. Hydrolytic enzyme inhibition, perturbations of cell membrane and impediment of ATPase function, inactivation of microbial adhesins and non-specific interactions with carbohydrates are some of the reported mechanisms of action of phenolic compounds[34–38]. Alkaloid compounds have been used for centuries and continue to be an active component of modern day medicines. Quinine (antimalarial), quinidine and sparteine (antiarrhythmic), vincristine and vinblastine (anticancer agents) are few examples of alkaloid based drugs. Physostigmine (parasympathomimetic) and codeine (antitussive) serve as models for chemical synthesis of analogues[39]. High alkaloid content of the fungi might be one of the underlying factors for their bioactive properties.

A majority of antibacterial, antifungal and antitumor drugs trace their origin to microorganisms. Heavy dependence on microbial natural products as drug source for almost a century have given rise to speculations that the microbial resources have been exhausted and that the natural products have been amply studied. Combinatorial chemistry and combinatorial biosynthesis are believed to replace natural products as new drug source. Pharmaceutical companies are now substituting natural drug discovery programs with combinatorial chemistry based research[40]. However, microbial natural products still hold an edge over chemically synthesized compounds because of their higher steric complexity, structural and functional diversity and bioactivity[41]. As stated before, there are approximately 1.5 million fungal species of which only 0.1 million have been described to date. A large number of untouched habitats might be potential reserves of unusual microorganisms. Moreover, merely 0.1 to 5% of the known microorganisms can be cultured in vitro. The scientific community is not yet even fully aware of the true extent of microbial diversity. It would therefore be premature to rebut the potential of microorganisms as source of novel drug lead compounds.

This study is the first such attempt to explore the bioactive potential of the fungi isolated from the salty terrain of Little Rann of Kutch. The findings of the present study project the six fungal isolates of Little Rann of Kutch as a promising source of antimicrobials and cytotoxics. Since these fungi are adapted to survival in such extreme habitats, there is a high probability that they may synthesise novel metabolites of pharmaceutical importance. We are now in the process of purifying and identifying the bioactive compounds in these extracts. This maiden study warrants further exploration of the mycobiota of this unique ecological niche.

## 5. Conclusion

Crude extracts of six novel fungal strains isolated from the unexplored lands of Little Rann of Kutch, India exhibited significant antibacterial and cytotoxic activities. The bioactive properties of the extracts can be attributed to their significant levels of phenol, flavonoid and alkaloid compounds. Being an inhabitant of an extreme ecological niche, these fungi have the high possibility of acting as a fount of novel antimicrobials and cytotoxics.

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