

# Evaluating bionanoparticle infused fungal metabolites as a novel antimicrobial agent

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## ABSTRACT

Therapeutic properties of fungal metabolites and silver nanoparticles have been well documented. While fungal metabolites have been used for centuries as medicinal drugs, potential of biogenic silver nanoparticles has recently received attention. We have evaluated the antimicrobial potential of *Aspergillus terreus* crude extract, silver nanoparticles and an amalgamation of both against four pathogenic bacterial strains. Antimicrobial activity of the following was evaluated – *A. terreus* extract, biogenic silver nanoparticles, and a mixture containing extract and nanoparticles. Four pathogenic bacteria - *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* were used as test organisms. Phenol, flavonoid, and alkaloid content of extract were determined to understand the chemical profile of the fungus. The extract contained significantly high amounts of phenols, flavonoids, and alkaloids. The extract and biogenic silver nanoparticle exhibited significant antibacterial activity at concentrations of 10 µg/ml and 1 µg/ml, respectively. When used in combination, the extract-nanoparticle mixture showed equally potent antibacterial activity at a much lower concentration of 2.5 µg/ml extract + 0.5 µg/ml nanoparticle. Given its high antibacterial potential, the fungal extract can be a promising source of novel drug lead compounds. The extract – silver nanoparticle mixture exhibited synergism in their antibacterial efficacy. This property can be further used to formulate new age drugs.

**Key words:** Antibacterial, *Aspergillus terreus*, biogenic silver nanoparticle, Rann of Kutch

## INTRODUCTION

Microorganism-based medicinal drugs have been around for a century. Today, a plethora of medications is available against a wide range of conventional diseases. Most of these trace their origin to a microbial source. Metabolites sourced from microorganisms, particularly fungi and actinomycetes, have been utilized as antibiotics (cephalosporins),<sup>[1]</sup> immune-suppressants (cyclosporin),<sup>[2]</sup> antibacterial

and antifungal agents (flindersine),<sup>[3]</sup> and anticancer compounds (carnosol and isouvaretin).<sup>[4]</sup> Newly emerging diseases such as Zika and Ebola virus, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and β-lactam-resistant *Streptococcus pneumoniae*, demand a concerted effort toward formulating novel drugs for a postantibiotic era.<sup>[5]</sup> Nanotechnology-based therapeutics have provided a breakthrough in the field of medical science with wide ranging applications such as drug and gene delivery, detection of proteins and pathogens, and targeted elimination of tumor cells and as antimicrobials, to name a few. Bionanotechnology employs nanoparticles synthesized on biological platforms such as algae, fungi, yeast, bacteria,

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actinomycetes, and plants.<sup>[6-8]</sup> Biologically synthesized nanoparticles, or bionanoparticles, are as efficient as their chemical counterparts and are more economical to produce.

## MATERIALS AND METHODS

### Chemicals and reagents

All chemicals were of analytical grade, obtained from Sigma-Aldrich (India) or Merck (India). Culture media were purchased from HiMedia (India). Rifampicin was procured from Sigma-Aldrich (India).

### Isolation and identification of *Aspergillus terreus*

*Aspergillus terreus* isolated from the salt desert of Little Rann of Kutch, India, was used for the study. Fungal isolation was performed by serial dilution method. Briefly, 1 g soil was dissolved in sterile phosphate-buffered saline and  $10^{-1}$ ,  $10^{-3}$ , and  $10^{-5}$  dilutions were prepared. From each dilution, 100  $\mu$ l was pipetted out and dispensed onto Czapek Yeast Autolysate agar (CYA) plates.<sup>[8]</sup> Plates were incubated upside down in dark at  $25 \pm 1^\circ\text{C}$  for 4 days. Fungal colonies were identified on the basis of macro- and micro-morphology. Colonies bearing resemblance to *A. terreus* were further subcultured on CYA and observed for colony characteristics such as growth rate, texture, extent of sporulation, mycelial color, and reverse colony color. Culture identification services of Gujarat Genomics Initiative, Gujarat, were utilized for molecular identification based on 18S rDNA sequencing.

### Bionanoparticle

Silver bionanoparticles (AgNPs) were synthesized from *Chlorella pyrenoidosa* (NCIM 2738) procured from National Center for Industrial Microorganism, Pune, India. Batch cultures of *C. pyrenoidosa* were grown in Bold's basal medium. The culture was used in the mid-exponential growth phase. Nanoparticles were synthesized using cell extract by the methodology outlined by Aziz *et al.*, 2015.<sup>[9]</sup> Culture was collected in falcon tubes and centrifuged. The pellet was washed thrice with distilled water followed by boiling for 5 min. The suspension was cooled and centrifuged at 10,000 rpm for 10 min. The supernatant was harvested and used as a cell extract. About 10 ml cell extract was mixed with 90 ml 1 mM  $\text{AgNO}_3$  solution for 24 h. Change in the color indicated the formation of silver nanoparticles. Transmission Electron Microscope (Philips, EM-410 LS) was used to observe the morphology of the biosynthesized Ag NPs. The size of the AgNPs was determined to be  $8 \pm 2$  nm. Energy dispersive X-ray analysis was performed to confirm the presence of elemental silver in the AgNPs.

### Fungal metabolite extraction

Metabolites were extracted from the mycelium.<sup>[10]</sup> Agar plug from a 7-day-old *A. terreus* culture was inoculated in Czapek Dox Broth (100 ml) in Erlenmeyer flask and incubated in dark (10 d,  $25 \pm 1^\circ\text{C}$ ). Mycelial mat was harvested,

shredded, and suspended in 1:1 chloroform:methanol and kept overnight in a rotary shaker (120 rpm). The mixture was filtered, and equal volumes of chloroform and distilled water were added to the filtrate and shaken in an orbital shaker (120 rpm, 1 h). Bottom organic layer was collected and evaporated to dryness in a Buchi Rotavapor. The dried extract was suspended in 1:1 acetonitrile:methanol (100 ml) and hexane (100 ml) and stirred (120 rpm, 1 h). The bottom organic layer was collected and evaporated to dryness. The extract was suspended in dimethyl sulfoxide at a concentration of 1 mg/ml and stored at  $-20^\circ\text{C}$ .

### Antibacterial assays

#### Antibacterial assay using fungal extract

Antibacterial activity of *A. terreus* extract was evaluated against *Klebsiella pneumoniae* (KJ938546), *Escherichia coli* (MCC 2412), *S. aureus* (MCC 2408), and *Bacillus cereus* (MCC 2243) by agar well diffusion method. *K. pneumoniae* was obtained from the institute's culture collection facility. *E. coli*, *B. cereus*, and *S. aureus* were acquired from the Microbial Culture Collection at National Centre for Cell Science, Pune, India. Bacteria were cultured on Mueller–Hinton agar. Bacterial inoculants were prepared in nutrient broth at 0.5 McFarland standards. Each test organism (100  $\mu$ l) was mixed with cooled Mueller–Hinton agar and poured into 80 mm petri dishes. Wells were cut out, and 100  $\mu$ l crude extract at 5, 10, 15, and 20  $\mu$ g/ml concentrations were poured. The plates were incubated ( $37^\circ\text{C}$ , 24 h) and the zones of inhibition were measured. Rifampicin was used as positive control. All experiments were performed in triplicates.

#### Antibacterial assay using silver bionanoparticles

Antibacterial efficacy of AgNPs was assessed against the bacterial pathogens at four different concentrations (0.5, 1, 1.5, and 2  $\mu$ g/ml) using the methodology adopted in the previous section.

#### Antibacterial assay using extract + silver bionanoparticle formulation

Based on the individual antibacterial potency, suspensions containing varying proportions of fungal extract and AgNPs were prepared sequentially and were analyzed for their antibacterial activity.

### Determination of phenol, flavonoid, and alkaloid content of fungal extract

Total phenolic content was determined according to the Folin–Ciocalteu colorimetric method.<sup>[11]</sup> Gallic acid was used as a standard, and a calibration curve was prepared for concentrations ranging from 25 to 300  $\mu$ g/ml and a calibration curve was obtained using a linear fit.

Total flavonoid content was estimated by the aluminum chloride method.<sup>[12]</sup> Quercetin concentrations (0–500  $\mu$ g/ml) were prepared, and a calibration curve was obtained using a linear fit.

Total alkaloid estimation was done by spectrophotometric method of Dragendorff's reagent.<sup>[13]</sup> Standard curve was prepared using pilocarpine nitrate in HCl solution at different concentrations (750, 500, 400, 250, 200, 150, and 100 µg/l). Alkaloid contents were expressed as pilocarpine nitrate equivalents (µg/mg PNEs). All the samples were analyzed in triplicates.

## RESULTS

### Isolation and identification

On the basis of colony morphology and microscopic observations, *A. terreus* was tentatively identified. Sequencing of internal transcribed spacer (ITS) 1-5.8S-ITS 2 region of rDNA confirmed the identity of the fungus as *A. terreus*.

### Antibacterial activity of fungal extract

Zone of inhibition was observed against all the test organisms, indicating the broad spectral nature and presence of antimicrobial compounds in the extracts. Figure 1a gives a graphical representation of the zone of inhibition observed against the bacteria at different extract concentrations. Except for *S. aureus* which was inhibited at 5 µg/ml, the remaining showed significant inhibition only at concentration  $\geq 10$  µg/ml. Gram-positive bacteria were more susceptible as compared to the Gram-negative strains.

### Antibacterial activity of silver bionanoparticles

Figure 1b represents the antibacterial activity of nanoparticles against the test pathogens. The nanoparticles were found to be extremely potent against all the bacterial strains, with clear zone of inhibition at 1 µg/ml.

### Antibacterial activity of extract + silver bionanoparticle formulation

Synergistic effect of AgNPs – metabolite mixture was assessed. Based on the individual antibacterial potency of the extract and AgNPs, concoctions containing different proportions of each were prepared and tested against the bacteria [Figure 2]. Initially, the AgNPs concentration was kept constant at 1 µg/ml, the minimum concentration at which it exhibited bactericidal effect, whereas the concentration of extract was varied [Figure 2a]. About 2.5 µg/ml extract + 1 µg/ml AgNPs showed bactericidal activity higher than either 10 µg/ml extract or 1 µg/ml AgNPs. Further, keeping the extract concentration constant at 2.5 µg/ml, the AgNPs concentration was varied and tested on bacterial pathogens [Figure 2b]. Established on the minimum concentration at which significant clear zones were observed against all four bacteria, 2.5 µg/ml extract + 0.5 µg/ml AgNPs formulation was found to have maximum potency at the lowest concentration.

### Phenol, flavonoid, and alkaloid content

The extract was found to contain remarkably high amounts of phenol ( $157.19 \pm 1.2$  µg/mg gallic acid equivalent [GAE]),

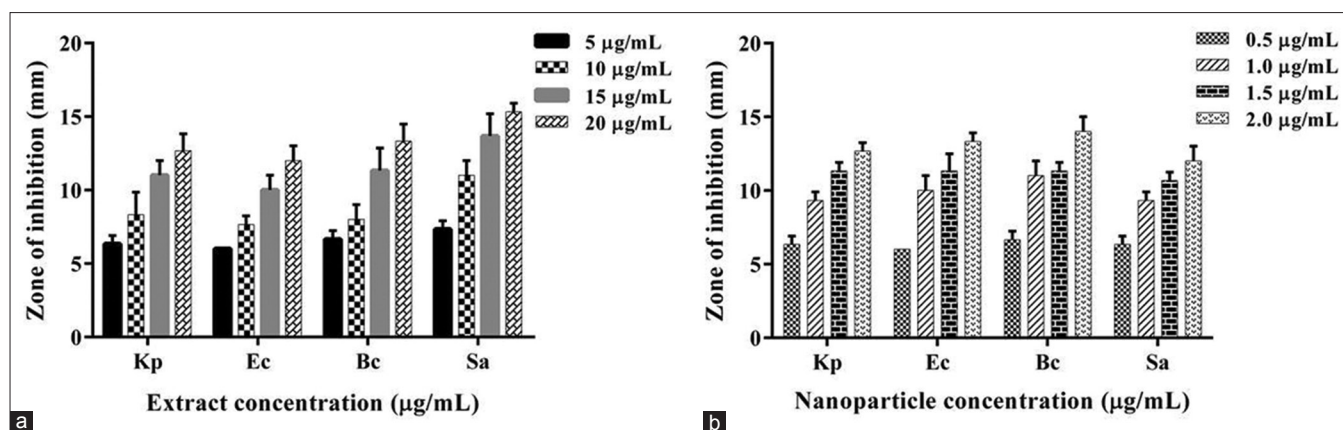
flavonoid ( $86.16 \pm 0.80$  µg/mg quercetin equivalent [QE]), and alkaloid ( $68.26 \pm 1.5$  µg/mg PNEs).

## DISCUSSION

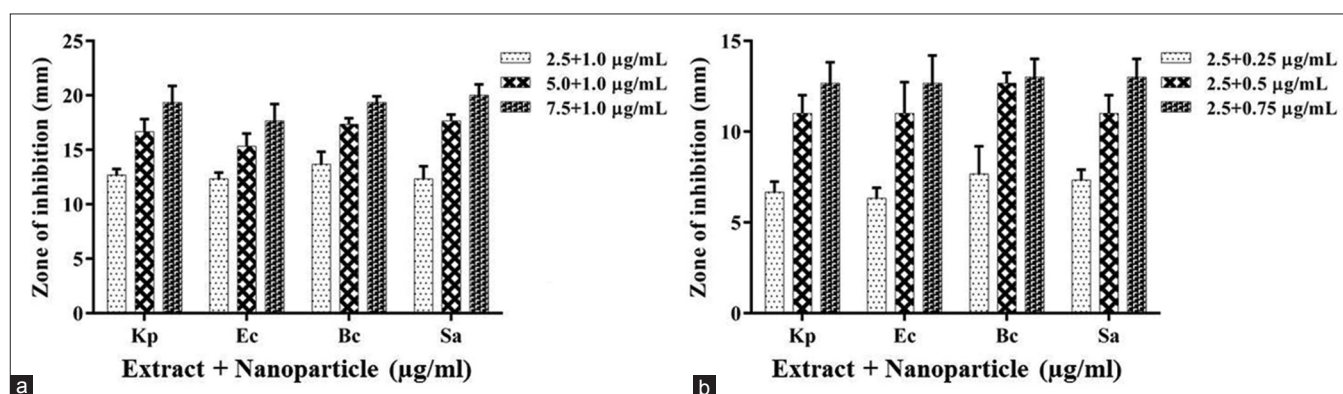
Members of the phylum Ascomycota, especially *Aspergillus*, produce metabolites that have antibacterial, antifungal, insecticidal, and nematocidal activities.<sup>[14]</sup> *A. terreus*, a halotolerant soil fungi isolated from the salt desert of Little Rann of Kutch, India, was tested for its antibacterial potential. The fungal extract showed statistically significant bioactivity at a minimum concentration of 10 µg/ml against all four bacterial strains, indicative of its broad spectrum nature. Antibacterial potential of biologically synthesized silver nanoparticles was assessed. AgNPs were found to be extremely potent with inhibition observed at 1 µg/ml concentration against all test organisms. In addition, to investigate if any synergism existed between the metabolites and AgNPs in their antimicrobial action, mixtures containing varying proportions of each were tested. Interestingly, extract + AgNP mixture at 2.5 + 0.5 µg/ml concentration was found to exhibit higher bactericidal activity than either component and at statistically significant lower concentrations, indicating a synergism between the two [Figure 3]. Gram-positive strains were found to be more susceptible as compared to the Gram-negative strains in all three cases. Differences in cell architecture between Gram-negative and Gram-positive bacteria might be the cause of these variations. The Gram-negative bacteria possess an outer membrane of lipopolysaccharide that prevents the invasion of hydrophobic compounds and are therefore more tolerant to antibacterial agents.<sup>[15]</sup>

Silver has been traditionally used in various topical antimicrobial formulations. Silver is preferred over other metals as it shows higher toxicity to microorganisms and lower toxicity to mammalian tissue. Nanoparticles are more effective in their toxicity because of their large surface area to volume ratio.<sup>[16]</sup> Although the exact underlying mechanisms are yet to be fully elucidated. Some researchers have proposed that AgNPs (1–10 nm size) act by binding to the cell membrane and altering its permeability. AgNPs disrupt the bacterial respiratory chain leading to generation of reactive oxygen species (e.g., hydrogen peroxide) and resultant oxidative stress and cell damage.<sup>[17,18]</sup> In addition, the NPs have a high affinity for phosphorus and nitrogen and therefore, post their entry into the cell, these bind with proteins implicated in inhibition of cellular metabolism.<sup>[16,19-22]</sup> Biogenic AgNPs have shown to have still higher microbicidal effect than their chemical counterparts.

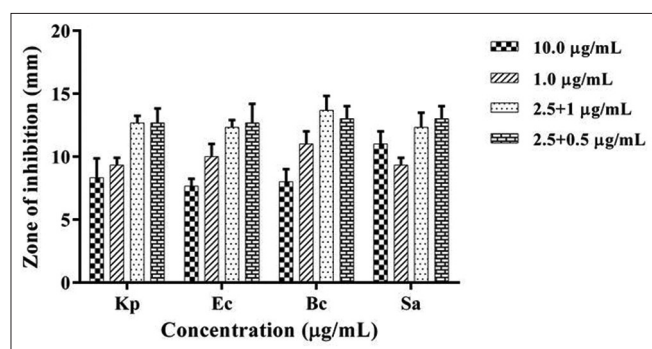
Phenols and flavonoids exert antimicrobial, antitumoral, antioxidant, antiallergic, and antiviral activities.<sup>[23,24]</sup> At  $157.19 \pm 1.2$  µg/mg GAE,  $86.16 \pm 0.80$  µg/mg QE, and  $68.26 \pm 1.5$  µg/mg PNE, respectively, the phenol,



**Figure 1:** Antibacterial activity of fungal extract (a) and silver nanoparticles (b) at different concentrations measured as a zone of inhibition against pathogenic bacteria, *Klebsiella pneumoniae*, *Escherichia coli*, *Bacillus cereus*, and *Staphylococcus aureus*. Experiment was performed in triplicates, the error bars represents mean  $\pm$  standard deviation



**Figure 2:** Antibacterial activity of extract + silver nanoparticles. Silver nanoparticles concentration kept constant at 1 µg/ml, extract concentration was varied (a). Taking the minimum concentration of extract showing significant activity, 2.5 µg/ml, concentration of silver nanoparticles was varied (b). Experiment was performed in triplicates, the error bars represents mean  $\pm$  standard deviation



**Figure 3:** Comparative analysis of the antibacterial efficacy of fungal extract and silver nanoparticles alone and in combination against pathogenic bacteria. Antibacterial activity at (2.5 + 1) µg/ml extract + silver nanoparticles was higher than 10 µg/ml extract and 1 µg/ml silver nanoparticle, respectively (2.5 + 0.5) µg/ml extract + silver nanoparticle found to be as effective as (2.5 + 1) at  $P$  value -  $0.0001 < 0.01 < 0.05$ . Experiment performed in triplicates. Error bars represents mean  $\pm$  standard deviation

flavonoid, and alkaloid contents of the fungal extract were found to be significantly high. Antibacterial activity of the extract may be ascribed to the high content of these

compounds which have been reported to be involved in inhibition of nucleic acid biosynthesis and other metabolic processes.<sup>[25,26]</sup> Growth inhibition of bacteria might be brought about by inhibition of hydrolytic enzymes, inactivation of microbial adhesins, and cell envelope transport proteins and nonspecific interactions with carbohydrates.<sup>[27]</sup>

The findings of the present study have yet again highlighted the significance of the fungal metabolites and AgNPs as potential antimicrobial agents.<sup>[28-30]</sup> The extract needs to be analyzed further for isolation and identification of active principles. Biogenic silver nanoparticles are not only extremely effective antimicrobial agents, as deduced from this study, but also economical. Although the AgNPs and fungal extract displayed significant antibacterial activity, a mixture containing both in much lower concentration was found to be equally potent, thereby displaying a synergistic effect. It would be interesting and vital to understand the underlying mechanism. Further characterization studies are being done on the extract-AgNPs mixture to get clues to this synergism.

## CONCLUSION

The fungal extract of *A. terreus* and biogenic silver nanoparticles are potent antimicrobial agents. However, their potency is even higher and at much lower concentration, when used in combination. Further characterization studies need to be done to understand the underlying mechanisms.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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