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Short communication

Isolation and identification of toxigenic fungi from infected peanuts and efficacy of silver nanoparticles against them

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ABSTRACT

Peanuts are vulnerable to fungal infections during long term storage. Fungi infecting peanuts are toxigenic and cause health hazards. The goal of this study was isolation and identification of fungi in peanuts and use of silver nanoparticles (AgNPs) to inhibit them. For this purpose firstly, we have isolated fungi from infected peanuts and identified on the basis of morphological and molecular study. Out of the total 54 fungal isolates, 47 were found to be *Aspergillus* spp. and other belongs to *Penicillium* spp. and *Macrophomina phaseolina.* Biochemical assay was performed to identify cultures of *Aspergillus flavus* from other species of this genus by inoculating it on Aspergillus Differentiation Medium (ADM). Thirty-one isolates were found to be *A. flavus*. Toxicity of *Aspergillus* spp. was evaluated on Yeast Extract Sucrose agar (YES) medium with an additive methylated β-cyclodextrin and nine isolates were found toxigenic. Secondly, AgNPs were synthesized from ten different plants and their characterization was performed using various analytical techniques such as UV–Visible spectrophotometer, Fourier Transform Infrared Spectroscopy, Zetasizer and Nanoparticle Tracking Analysis, etc. Further, antifungal potential of thus synthesized AgNPs was evaluated. All the synthesized AgNPs possess ability to inhibit fungal growth. *Cymbopogon citratus* leaf extract mediated AgNPs were found to have prominent antifungal potential against all test fungi and its minimum inhibitory concentration (MIC) was found to be 20 μg/ml. The biogenic approach proposed in the present study is eco-friendly, safe and economical viable. AgNPs also reported to have significant antifungal activity against toxigenic isolates of peanuts, hence such AgNPs can be effectively used for the management of toxigenic pathogens.

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1. Introduction

Peanut is the protein rich food hence used around the world as raw and in its various forms. Peanut sauce, peanut butter, peanut pickle and many other products are available in the market (Razzazi-Fazeli et al., 2004). People use these peanuts and its products but unfortunately unaware of the threat associated with it by the consumption of mycotoxin secreting fungi. Studies showed that almost all the regions of world are facing the problem of consumption of mycotoxins contaminated food by people ignorant of its consequences (Khiyami, El-Naggar, Almoammar, & Abd-Elsalam, 2015; Razzazi-Fazeli et al., 2004; Ruadrew, Craft, & Aido, 2013; Sultan & Magan, 2010). The major fungal flora like *Aspergillus, Penicillium* and *Fusarium* are commonly found associated with the contamination of peanuts. All these fungi were reported to be toxin producing with high impact on health under favorable conditions. Whereas, *Aspergillus* spp. are found to be more aflatoxigenic with carcinogenic effects (Kamika, Mngqawa, Rheeder, Teffo, & Katerere, 2014; Khiyami et al., 2015; Tran-Dinh, Kennedy, Bui, & Carter, 2009). Kamika et al. (2014) reported that 95% peanut samples were contaminated in the market of South Africa. In a survey by Tran-Dinh et al. (2009) in Vietnam reported that about 36% of peanut samples were positive for aflatoxins secreted by *Aspergillus flavus*. Prevalence of *A. flavus* and *A. niger* was reported on peanut kernels in Saudi Arabia by Khiyami et al. (2015). Risk of peanuts contamination by mycotoxigenic fungi increases during pre and post harvest, and highly susceptible at transport and storage during favourable conditions. Peanuts are mainly stored by conventional techniques, which are quite fruitful for short- term storage but not for long- term storage. Now, the question arises concerning preservation of peanuts for its long- term use, and this leads to find out promising approaches to the problem. Nanotechnology, is not new but came up with new ideas and has brought solution to this

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problem. Due to antimicrobial behaviour of nanoparticles, these have immense applications in agriculture, nutrition, medicine and health and some other sciences (Borase et al., 2015; Kathiravan et al., 2015; Santhosh, Yuvarajan, & Natarajan, 2015; Ulug, Haluk, Cicek, & Mete, 2015). Shinde, Lokhande, and Lokhande (2014) synthesized a thin film of silver by the treatment of *Psidium guajava* leaf extract and tested $AgNO_3$ for antimicrobial efficacy. In another study, Xia et al. (2016) demonstrated significant antifungal activity of AgNPs against *Trichosporon asahii*. AgNPs were also found effective against indoor mould (Ogar, Tylko, & Turnau, 2015). They reported that 30–200 mg/l concentration of AgNPs in building material is the best option than any other biocides. *Annona muricata* leaf extract- mediated AgNPs were synthesized by Santhosh et al. (2015) who reported larvicidal activity against vectors of malaria, filaria and dengue. Borase et al. (2015) studied *Ficus carica* leaf extract mediated AgNPs potential for inhibition of an enzyme urease, which plays a key role in pathogenicity of bacteria *Helicobacter pylori*. The results showed better inhibition of urease, thus help reduces the bacterial infection. Kathiravan et al. (2015) synthesized AgNPs from *Croton sparsiflorus* and studied its antimicrobial activity. Researchers are engaged in controlling fungal contaminations in various crops and much research is focused towards the control of aflatoxin producing *Aspergillus* (Pitt & Hocking, 2006; Reddy, Reddy, Abbas, Abel, & Murlidharan, 2008) but the problem regarding mycotoxigenic fungal contamination in stored peanuts still exist. In this study we have evaluated efficacy of AgNPs for the control of fungal contamination in peanuts.

2. Materials and methods

2.1. Solvents and chemicals

Potato dextrose agar (PDA), 70% ethanol, 1% sodium hypochlorite, potato dextrose broth (PDB), Roswell Park Memorial Institute (RPMI) 1640 medium (with L glutamine without sodium biocarbonate), Aspergillus differentiation medium (ADM), its chemical composition is yeast extract (20 gm), peptic digest of animal tissue (10 gm), ferric ammonium citrate (0.5 gm), dichloran (0.002 gm) and agar (15 gm) in 1000 ml distilled water and its pH was maintained at 6.3 $\pm$ 0.2 at 25 °C. Yeast extract sucrose agar (YES), methylated β cyclodextrin (Sigma-Aldrich), silver nitrate. All chemicals and media were purchased from HiMedia Pvt. Ltd., Mumbai.

2.2. Collection of samples

About 60 infected peanut samples were collected from local market of Amravati District (Maharashtra), Central India, in sterile sample bags and brought to laboratory for further use. The collected peanuts were of different varieties viz. Red natal, Parkash, Chandra, Chitra, Amber, Kaushal and mostly from Spanish bunch variety, which is commonly cultivated and available in this region.

2.3. Isolation of fungi

Collected peanuts were surface sterilized by washing three times with sterile distilled water for one minute each time, then washed by 1% sodium hypochlorite for 30 s two times and again rinsed in distilled water for 1 min two times. These peanuts were then cut into small pieces and kept for drying in laminar air flow. The pieces of surface disinfected peanut were then kept onto PDA medium in petri plates and incubated at 35 °C for 6 days. These fungi were then purified and stored on PDA slants for further experimental use.

2.4. Identification of isolates

2.4.1. Morphological study

Fungal morphology was studied by observing various phenotypic characters. Its macromorphological and micromorphological characters, viz., colour of colony shown on dorsal and ventral side, growth rate by measuring diameter of colony on seventh day of inoculation, texture of fungal colony and some microscopic characters like its fruiting body, conidial size and shape were studied (Samson et al., 2014). The morphology of all the isolated fungi was studied and compared with the identification key proposed by Barnett (1962), Nagamani, Kunwar, and Manoharachary (2006) and Mukerji and Manoharachary (2010). On the basis of the similarity and variations in morphological characters, all the fungi were categorized into six groups. Group I contains fungi with light- green to green colour on dorsal and white to pale-yellow on ventral side of the fungus colony, group II contains fungi with fawn to brown colour, whereas fungi showing cream-white to grey colour were placed into group III. The fungi showing black colour were split into two different groups by comparing the colour variations on its ventral side and fungi showing pale-yellow to white colour were put into group IV; however, fungi with black colour on its both sides were separated in another group V. Remaining fungi with white to grey in colour were grouped as VI.

2.4.2. Aspergillus Differentiation Medium (ADM)

ADM was used to differentiate culture of *Aspergillus flavus* from other species of this genus. All the isolates of *Aspergillus* spp. were inoculated on the petri plates containing ADM and incubated at 35 $\pm$ 2 °C for 3–5 days (Bothast & Fennel, 1974).

2.4.3. Yeast extract sucrose medium with additive methylated β cyclodextrin (YES βcyd)

Culture medium YES with an additive methylated β cyclodextrin was used for the detection of mycotoxic nature of isolated fungi. All the strains of *Aspergillus* isolated from infected peanuts were inoculated on petri plates containing YES βcyd and incubated at 35 $\pm$ 2 °C for 3–5 days in dark (Fente, Jaimezordaz, Vazquez, Franco, & Cepeda, 2001).

2.4.4. Molecular study

The isolates of fungi were grown on PDB for growth of fungal mat. These cultures were identified for purity by microscopic study then used for isolation of genomic DNA by the kit purchased from Chromous Biotech Pvt. Ltd., Bangalore, India according to manufacturer's instructions. Molecular identification was performed by amplifying internal transcribed spacer region (ITS) using ITS 1(5′TCC GTA GGT GAA CCT GCG G3′) as forward and ITS4 (5′TCC TCC GCT TAT TGA TAT GC3′) as reverse primer (White et al., 1990). Further, sequencing of amplicon was carried out at Xcelris Labs Ltd. Ahmedabad, Gujarat, India. These sequences were then submitted to the EMBL-EBI, European Nucleotide Archive, Cambridge, UK. Confirmed identification of these isolates was made by comparing these sequences with the sequences available in GeneBank database using online BLAST tool. This analysis provides results in the form of best matches with the available sequences in GeneBank. After BLAST analysis, the closest related species available at GenBank were selected for the construction of a phylogenetic tree.

2.5. Synthesis of AgNPs

Synthesis of AgNPs was carried out by the use of plant leaves. We have selected *Mangifera indica, Ziziphus mauritiana, Cymbopogon citratus, Tecoma stans, Delonix regia, Syzigium cumini, Chrysopogon zizanoides, Psidium guajava, Annona squamosa* and *Ficus*

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