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Colletotrichum sp.- mediated synthesis of sulphur and aluminium oxide nanoparticles and its *in vitro* activity against selected food-borne pathogens

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ABSTRACT

Food spoilage is a major issue globally and million of people suffer from food-borne infections. Hence, there is a need to search for novel and effective antimicrobial agents. Nanoparticles act as antimicrobial agent, which can tackle the problem of food-borne pathogens. The present study includes mycosynthesis of sulphur nanoparticles (SNPs) and aluminium oxide nanoparticles (AlNPs) from *Colletotrichum* sp. and their characterization by UV-Vis spectroscopy, Nanoparticles Tracking and Analysis, Zeta potential measurement, X-ray diffraction and Transmission Electron Microscopy. Essential oils (EOs) were extracted from the leaves of *Eucalyptus globulus* and *Citrus medica*. Nanofunctionalized oils were formulated by combining nanoparticles with EOs. *In vitro* antimicrobial activity of SNPs, AlNPs, EOs and nanofunctionalized oils was evaluated against selected food-borne pathogens such as *Listeria monocytogenes, Salmonella typhi*, *Chromobacterium violaceum, Fusarium oxysporum* and *Aspergillus flavus*. The antimicrobial activity of SNPs was found to be maximum against *Salmonella typhi* (21 mm), whereas AlNPs were highly effective against *F. oxysporum* (22 mm). It was found that activity of antibiotics such as tetracycline, oxytetracycline, gentamicin, fluconazole, ketoconazole, amphoterecin B and nystatin. increase in combination with nanoparticles.Nanofunctionalized oil can be used as a novel antimicrobial agent the prevention of food spoilage caused by food-borne pathogens.

1. Introduction

The consumption of food contaminated with pathogenic bacteria and/or their toxins is a widespread health problem, being the major cause of food-borne diseases, such as campylobacteriosis, listeriosis, hemorrhagic colitis and salmonellosis (Käferstein, Mortarjemi, & Bettcher, 1997). Mead et al. (1999) estimated that food-borne agents annually affect 76- million people resulting into 5000 deaths in the United States. In fact, the identification and evaluation of new antimicrobial agents for the control of food-borne pathogens is a global challenge. Nanotechnology has potential for solving the problem of food-borne pathogens. Nanoparticles act as antimicrobial agent due to their small size and high surface/volume ratio, which allows them to interact with target bacteria (Le et al., 2011; Singh and Singh, 2011). Rai, Yadav, Bridge, and Gade (2009) coined the term “Myconanotechnology” [“Myco” meaning fungi and “nanotechnology” is the manipulation of materials with atleast one dimension in the range of 1–100 nm]. Filamentous fungi have the capacity to secrete proteins, produce large amount of extracellular enzymes, which help in better reduction of precursor material into nanosize material (Saxena, Sharma, Gupta, & Singh, 2014). Moghaddam, Namvar, Moniri, Paridah, and Mohamad (2015) reported that biologically synthesized nanoparticles possess high level of consistency. The knowledge about the mechanistic aspect has helped to control the morphology and size of the nanoparticles during the synthesis process (Yadav et al., 2015). Nanoparticles synthesized from *Colletotrichum* sp. were found to be very stable (ShivShankar, Ahmad, Pasricha, & Sastry, 2003; Rai, Yadav, & Gade, 2011).

Essential oils (EOs) are oily aromatic compound extracted from plants. They are considered as natural antimicrobial agent from ancient era (Mith et al., 2014). EOs are composed of terpenoids such

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as monoterpenes, sesquiterpenes, diterpenes and various molecules including acids, alcohols, aldehydes, aliphatic hydrocarbons, lactones, coumarins and homologs of phenyl propanoids (Nazzaro, Fratianni, Martino, Coppola, & Feo, 2013). EOs are hydrophobic and lipophilic in nature due to these attributes, EOs can pass through cell, cytoplasmic membrane, disrupts cell membrane mechanisms and can disturb different layers of polysaccharides, fatty acids, phospholipid content (Ramos et al., 2014). EOs can change the fluidity of cell membrane, which lead to membrane permeability and result into the leakage of ions and proteins. The permeabilization of outer and inner mitochondrial membrane leads to cell death (Faleiro, 2011; Ramos et al., 2014). EOs are volatile in nature and can decompose or evaporate at the time of food processing, preparation of antimicrobial film and formulation of drugs. To improve the stability of EOs at the time of processing and storage, nano-encapsulation of EOs has been applied in food and nutraceutical industries. Encapsulation of EOs can preserve and protect their functional properties, additionally, encapsulation increases antimicrobial activity of EOs (Hyldgaard, Mygind & Mygind, 2012; Hosseini, Zandi, Rezaei, & Farahmandghav, 2013). Encapsulation of EOs in biodegradable nanoparticles like chitosan can be used in food preservation (Hyldgarrd et al., 2012; Pecarsaki, Jugovic, Brankovic, Mihajilovski, & Jankovic, 2014).

In the present study, we report the mycosynthesis of SNPs and AlNPs from *Colletotrichum* sp. and evaluated antimicrobial activity of nanoparticles, EOs extracted from *Citrus medica* and *Eucalyptus globulus* and nanofunctionalized EOs (in combination with essential oils and nanoparticles) against selected food-borne *C. violaceum, L. monocytogenes, S. typhi*, *F. oxysporum* and *A. flavus*. The activity of SNPs and AlNPs was also evaluated in combination with tetracycline, oxytetracycline, gentamicin, fluconazole, ketoconazole, amphoterecin B and nystatin.

2. Materials and methods

2.1. Procurement of materials

Aluminium chloride, sodium thiosulphate, Muller-Hinton broth and agar, Brain Heart Infusion broth and agar, Potato Dextrose broth and agar, potassium bromide powder were purchased from Himedia PVT Ltd. Mumbai, India.

2.1.1. Preparation of fungal extract and synthesis of nanoparticles

Colletotrichum sp. (DBT 349) was procured from Department of Biotechnology, SGB Amravati University, Amravati, India. For the synthesis of nanoparticles, the fungus was inoculated in 100 mL Potato Dextrose Broth (PDB), and incubated for 7 d at 27 °C. Mycelia were harvested by filtering through Whatman's filter paper No 42 and rinsed thrice with sterile double distilled water. Later, mycelia were re-suspended in 100 mL sterile double distilled water and incubated at room temperature for 24 h. It was then passed through membrane filtration assembly. For synthesis of SNPs method described by Awwad, Salem, and Amany (2015) was modified and procedure of Ansari et al. (2015) was modified for the synthesis of AlNPs. For synthesis of SNPs, fungal filtrate was treated with 20 mM sodium thiosulphate ($Na_2S_2O_3$), and kept on magnetic stirrer for proper mixing, 1000 μl of concentrated hydrochloric acid was added in drop wise manner The suspended SNPs were centrifuged at 10,000 g for 20 min, washed thrice with distilled water, dried in oven and further used for characterization. For the synthesis of AlNPs, aluminium chloride was used as a precursor salt. The fungal filtrate was treated with 50 mM aluminium chloride ($AlCl_3$), heated in microwave oven at 640 W for 10 min. After nanoparticle synthesis precipitate was obtained at the bottom of the flask.

2.2. Detection and characterization of nanoparticles

2.2.1. Visual observation

The detection of synthesized nanoparticles was primarily carried out by visual observation. After the treatment of fungal filtrate with 20 mM $Na_2S_2O_3$ yellow turbid solution was formed, which indicates the formation of SNPs whereas, in case of AlNPs, brown precipitate was obtained at the bottom of the flask, which showed the formation of AlNPs.

2.2.2. UV-Vis spectrophotometer analysis

The preliminary detection of SNPs and AlNPs was made by using UV-Visible spectrophotometer (Shimadzu UV-1700, Japan). The aliquots of the synthesized nanoparticles were subjected to UV-Visible analysis by scanning the absorbance spectra from 200 to 800 nm.

2.2.3. Nanoparticle Tracking and Analysis (NTA)

Nanoparticle Tracking and Analysis is a laser-based light scattering system used for determining the size, particle size distribution and concentration of nanoparticles. Analysis was performed by NanoSight LM 20 using a beta version of the NTA 2.3 software (Nanosight Pvt. Ltd., UK). For sample preparation, 7 μl soultion of SNPs and AlNPs were diluted in 2 mL of nuclease free water.

2.2.4. Zeta potential analysis

Zeta potential is a measure of charge and stability of nanoparticles at pH 7, which was measured by using Zeta sizer (Malvern Zetasizer NanoZS90, UK). For sample preparation, 30 μl of synthesized nanoparticles solution was diluted in 2 mL of nuclease free water. For zeta potential measurement, 1000 μl of the sample was taken in zeta dip cell and placed in Zeta sizer for analysis .

2.2.5. X-ray diffraction (XRD)

X-ray diffraction (XRD) analysis was performed using Rigaku Miniflex II desktop X-ray diffractometer. For the sample analysis, powder samples of SNPs and AlNPs were mounted on sample holder ring. After analysis of the samples, Bragg's reflection pattern was obtained and compared with the standard reference file known as File Joint Committee on Powder Diffraction (JCPDS).

2.2.6. Transmission Electron Microscopy (TEM)

TEM (Philips model CM 12) analysis was carried out on carbon-coated copper grid. 5 μl of SNPs and AlNPs were placed on copper grids, and allowed to dry in infrared light for 30 min. Size and morphology of nanoparticles were determined by TEM analysis.

2.3. Extraction of essential oils (EOs)

EOs were extracted from the leaves of *Eucalyptus globulus* and *Citrus medica* by Clevenger apparatus (Souza et al., 2014).

2.4. Formulation of nanofunctionalized oils

Stock solution of (1mg/mL) nanoparticles was taken in deionized water as described by Ahmed, Hiremath, and Jacob (2016), and sonicated. Nanoparticles were mixed with 1 mL of EO. 10 μl of tween-80 was added to it; vortexed for 4 min, and sonicated for 30 min to prevent aggregation and deposition of nanoparticles.

2.5. Assessment of antimicrobial activity

2.5.1. Test bacteria

The pure cultures of the food-borne *Listeria monocytogenes* (MTCC 1143), and *Fusarium moniliforme* (MTCC 6636) were