Assessment of Antifungal Efficacy of Green Synthesized Sulphur Nanoparticles Using *Ocimum Basilicum* Leaves Extract

Himanshi[1], Deepika P. Joshi[1], Hemant Dasila[2] and Manvika Sahgal[2]

[1]*Department of Physics, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, 263145 Uttrakhand, India*
[2]*Department of Microbiology,Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, 263145 Uttrakhand, India*
Corresponding author Email –himsept08@gmail.com

Abstract- A large number of plant pathogens belongs to the kingdom fungi, which are responsible for some catastrophic diseases in crops. The diseases that are common in fruits and vegetables are mostly fungi originated. In the present work, an attempt has been made to evaluate the antifungal activity of biosynthesized sulphur nanoparticles (SNPs). Sulphur is an essential component in both agriculture and technology. *Ocimum basilicum* (Basil) leaves extract has been used as a surfactant in an acidic (oxalic) medium to synthesize Sulphur nanoparticles. Oxalic acid is a weak acid that meets the requirement of green synthesis fairly. Different characterization techniques such as XRD, UV-Vis, SEM and FTIR have been employed to assess the nanoparticles. Uv-Vis spectrum shows a peak at 298nm, which confirms the formation of Sulphur nanoparticles. A highly crystalline nature with crystalline size 58nm has been revealed by the XRD analysis. The linkage of biomolecules on the surface of Sulphur nanoparticles has been analyzed by the FTIR spectrum. The adsorption of biomolecules on the surface is responsible for ceasing the agglomeration process and increasing their adaptability with nature. SEM data shows the quasi-spherical shape of nanoparticles. Well diffusion assay has been performed to study the antifungal activity of SNPs against *Alternaria triticina*, *Aspergillus niger* and *Fusarium oxysporum*, and significant growth inhibition has been observed in the case of *Aspergillus niger*.

Keywords: Green synthesis; Oxalic acid; Sulphur nanoparticles; Antifungal activity.

1. Introduction

Over the decades, sulphur has proven its worth in both agriculture and industrial field due to its astonishing attributes and functionality. Sulphur is one of the macro nutrients needed by crops to ensure food sustainability. It is an essential component for the production of some metabolites such as enzymes, proteins, amino acids and vitamins in plants as well as in living beings. It also helps the plants to fight against abiotic stress and diseases [1-3]. For a long time, sulphur has been used as a fungicide and antimicrobial against different pathogens. It is used to control apple scab diseases in cold weather and also used in various crops such as grapes, strawberries and many other vegetables as an antifungal agent [4].

Nowadays, we are dealing with an era of nanoparticles and nanotechnology. Nanoparticles have always surprised human beings due to their magnificent properties and applications. Sulphur nanoparticles have gained popularity in the past few years due to their vast use in almost every field like agriculture, high specific capacity sulphur-lithium batteries, industries, for carbon nanotubes modification, pharmaceuticals, antibacterial efficacy and catalytic properties [5-7]. Lots of chemical methods have been employed by the researchers to synthesize sulphur nanoparticles such water/oil microemulsion method with the use of hazardous H_2S accompanied by novel biodegradable iron chelates [8], reverse microemulsion method [9] and electrochemical method in both non-solvent and solvent mediums [10]. All these methods possess a number of demerits as they use perilous chemicals, harsh environmental conditions and high expenses. Extensive use of these methods has threatened the ecological safety and security. A balance between the modern technologies and Mother Nature is needed for the sustainable use of all the resources available and for the betterment of mankind. In order to achieve this goal, researchers have introduced 'the green synthesis of nanoparticles'. Use of biological entities such as plants and microorganisms for the production of nanoparticles is a revolutionary step to coexist with nature [11-12]. Lots of literature is available for the green synthesis of metal nanoparticles such as gold, silver and copper etc. [13-

14]. But, only a few works has been done for the synthesis of sulphur nanoparticles despite their magnificent applications and essentiality. Paralikar and Rai used *Catharanthus roseus* (Vinca), *Azadirachta indica* (Neem), *Polyalthia longifolia* (Ashoka) and *Mangifera indica* (Mango) with precursor sodium polysulphide to synthesize sulphur nanoparticles [15]. Khairan *et al.* reported spherical sulphur nanoparticles of size 55.613nm using aqueous garlic extract as a capping and stabilizing agent [16]. Salem *et al*. used *Punica granatum* peel extract along with sodium thiosulphate as precursor in acidic medium for the production of sulphur nanoparticles [17]. And even fewer works has been done for the assessment of antifungal activity of green prepared sulphur nanoparticles.

The present work focuses on the antifungal activity of green synthesized sulphur nanoparticles using *Ocimum basilicum* (Basil) leaves extract as a surfactant. Basil is a year-long herb belonging to the family *Lamiaceae* and genus *Ocimum L.* It is considered a holy plant in India. Oxalic acid (organic acid) has been used throughout the process to provide acidic medium. *Alternaria triticina*, *Aspergillus niger* and *Fusarium oxysporum* plant pathogens have been taken to study the antifungal efficacy of SNPs.

2. Materials and Methods

2.1 *Materials*

Oxalic acid dihydrate ($C_2H_2O_4.2H_2O$), Sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$, 99.5%) and potato dextrose agar have been purchased from Himedia. Fresh *Ocimum basilicum* leaves have been taken from the surroundings of the university campus. Deionized double distilled water has been used as a solvent throughout the process.

2.2. Culture collection

The isolated fungi strains of plant pathogens *Alternaria triticina*, *Aspergillus niger* and *Fusarium oxysporum* have been collected from department of microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar.

2.3 Preparation of aqueous extract of Ocimum basilicum leaves

Firstly, the collected *Ocimum basilicum* leaves have been washed using tap water about a couple of times and thereafter, they have been cleaned using deionized double distilled water 5-6 times. All the dust particles and physical impurities have been washed off. After drying them for about an hour, they are finely chopped into small pieces. 50g of these has been dissolved in 500mL of distilled water and boiled for 25 minutes. The cooled-down mixture has filtered using filter paper Whatman No-1 to pull out the undesired part. Thereafter, the extract has been centrifuged at 5000 rpm for 10 minutes in order to remove any biological impurities present. The extract has been stored in freeze for further use.

2.4 Synthesis of sulphur nanoparticles

The present work deals with a safe and environment-friendly method to synthesize the SNPs as given by Khairan *et al.* with some modest changes [16]. 24.8g of precursor $Na_2S_2O_3.5H_2O$, 99.5% has been mixed up with 250mL of *Ocimum basilicum* leaves extract and sonicated for 40 minutes at 30^0C. The uniform sonicated solution has been further diluted using 250mL of deionized double distilled water with mild stirring. Thereafter, 10% oxalic acid has been added to the solution drop-wise under continuous stirring at 30^0C and a yellow precipitate is observed. The schematic representation of synthesis is given in figure 1. The mixture has been centrifuged at 6000 rpm for 5 minutes. The precipitate has been collected and washed repeatedly using double distilled water to remove any impurity. It has been placed in a hot air oven to dry at 50^oC for 8 hours and collected for further use. The possible disproportion reactions involved in this instance is as follows:

$$\mathbf{Na_2S_2O_3.5H_2O + C_2H_2O_4 \rightarrow Na_2C_2O_4 + SO_2 + S\,(\downarrow) + H_2}$$

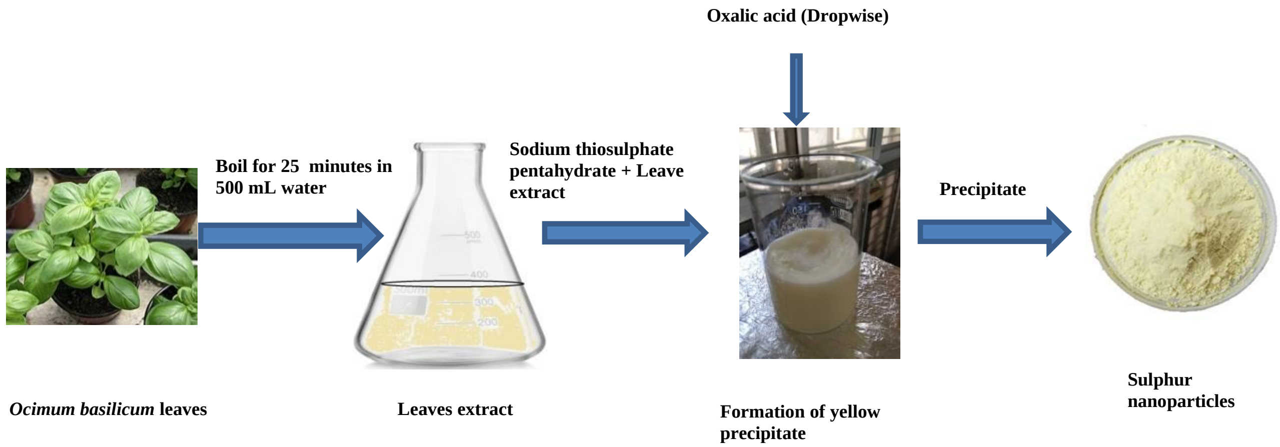

Figure 1: Schematic representation of synthesis of Sulphur nanoparticles

2.5 Well diffusion assay

To study the antifungal effect of sulphur nanoparticles, a well diffusion assay is performed. Inoculum carrying the fungus strains to be tested is spread on nutrient plate filled with potato dextrose agar (PDA) using a sterile spreader. Thereafter, the two wells each of 8mm are punched into the agar medium. The wells are filled with 0.4mM solution of sulphur nanoparticles and left to diffuse thoroughly at room temperature for two hours. Then, the plates are incubated for a week in upright position at 37^0 C. The incubated plates without any sulphur nanoparticles in the wells are taken as control in the each case of fungi strains

2.6 Characterization techniques

Different characterization techniques have been used to study the optical, structural, morphological and compositional properties of sulphur nanoparticles. UV-Vis spectroscopy has been carried out using Perkin Elmer UV/VIS Lambda 365 instrument with a range of 190nm to 800nm. The crystalline nature of SNPs is explored by Bruker D-8 Advanced Eco XRD furnished with Cu-K_α radiation source along with Ni as a filter in 2θ range 10^o-90^o. Bruker Alpha FTIR spectrometer is used to study the composition of *Ocimum basilicum* leaves extract and adsorption of biomolecules on the surface of sulphur nanoparticles. Scanning Electron Microscopic morphological analysis has been performed to examine the morphology of nanoparticles. The purity of nanoparticles is examined using EDX technique with FEI Quanta-200 MK-2.

3. Results and Discussion

For the sustainable development of science and technology, harmony between nature and science/technology is a must. Green synthesis of nanoparticles has emerged as a stepping stone to accomplish this goal. Green synthesis of sulphur nanoparticles is a simple and one-step process. *Ocimum basilicum* leaves extract has been used as a green surfactant throughout the process. Since the green surfactant is rich in a variety of secondary metabolites such as ketones, carboxylic acids, proteins, alcohols, phenols and alkyl group etc., which also exhibit reducing properties according to the literature [18-19], the precursor has been added to the extract and sonicated for 40 minutes, but no chemical change has been observed. This indicates that it does not show any reducing property with sulphur precursor. Therefore, Oxalic acid has been used as a reducing agent to synthesize SNPs.

3.1 Uv-Vis analysis:

UV-Vis spectrum for the synthesized nanoparticles shows a peak at 298nm as shown in figure 2. It confirms the availability of sulphur nanoparticles as the optical spectrum for sulphur nanoparticles lies between 245-300nm [20]. The sharp peak indicates that the SNPs have a small range for particle size distribution.

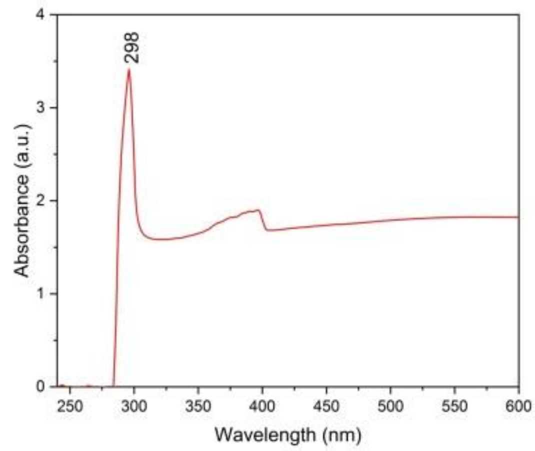

Figure 2: UV-Vis spectrum of SNPs

3.2 FTIR analysis:

The presence of biomolecules in the extract and their adsorption on the surface of sulphur nanoparticles have been studied using Fourier-transform infrared spectroscopy. FTIR spectrum for the extract and the SNPs has been shown in figure 3. The spectrum of the extract shows a broad sharp peak at 3286 cm^{-1} corresponding to O-H stretching of alcohols due to intra-molecular bonding and N-H stretching. The weak peak at 2141 cm^{-1} and sharp strong peaks at 1634 cm^{-1} and 580 cm^{-1} are due to the presence of C≡C stretching of monosubstituted alkyne, C=C stretching of monosubstituted alkene and halo compounds respectively. The FTIR spectrum for synthesized SNPs shows peaks at 3746 cm^{-1}, 1610 cm^{-1}, 1516 cm^{-1}, 1304 cm^{-1} and 785 cm^{-1} corresponding to O-H stretching of alcohols, C=C stretching of alkene in α,β-unsaturated ketone, N-O stretching of the nitro group, S=O stretching of sulphone and halo compound respectively. Some biomolecules such as alcohols, alkenes and halo compounds which are predominantly observed in both the extract and the sulphur nanoparticles are responsible for capping and even distribution of SNPs. The presence of the nitro group and sulphone at the surface of sulphur nanoparticle is due to the linkage of alcoholic and amide group of protein with the nanoparticles resulting in stabilizing and casing of sulphur nanoparticles. The small changes in the peak stipulate the biomolecules are perfectly adsorbed at the surface of SNPs and responsible for capping and stabilization. No other significant new peaks are observed for sulphur nanoparticles indicating only the adsorption of phytochemical on the surface of SNPs, no chemical reaction.

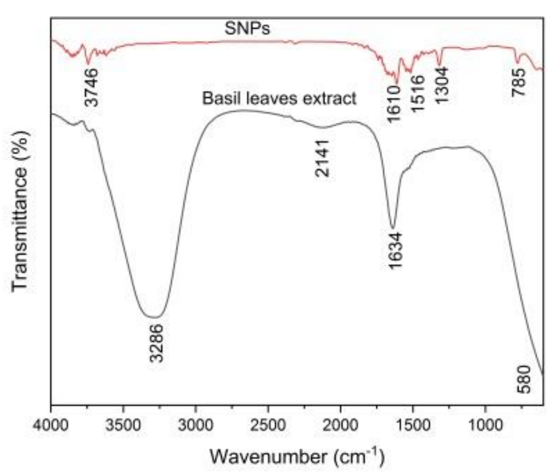

Figure 3: FTIR spectrum of extract and SNPs

3.3 X-ray Diffraction analysis:

XRD technique has been employed to study the crystal nature of SNPs. The diffraction pattern shows a strong peak at 23.14^0 corresponds to the crystallographic plane 222 as shown in figure 4. This data is consistent with the data given by pdf no 00-008-0247, International Centre for Diffraction Data (ICDD) for the standard orthorhombic Sulphur pattern. The average crystalline size 58nm is calculated using the Debye-Scherrer formula given as:

$$D=K.\lambda/\beta.\cos\theta$$

Where D stands for the diameter of nanoparticles, λ represents the wavelength of X-ray radiation source given as 0.1546, β is FWHM (full width at half-maximum value), θ is the half diffraction angle in radian and K is the Scherrer constant with a value of 0.9. The small crystalline size specifies that the *Ocimum basilicum* leaves extract works exquisitely as a surfactant and oxalic acid is responsible for the fast nucleation process.

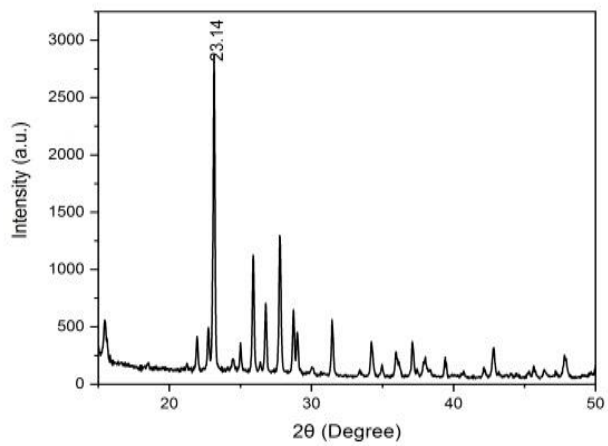

Figure 4: XRD graph of SNPs

3.4 FESEM analysis:

The morphology and uniformity of sulphur nanoparticles has been studied using field emission scanning electron microscope (FESEM). Finely grounded sulphur nanoparticles powder is subjected to the scanning. It shows that the nanoparticles are of irregular shape (quasi-spherical) with a size distribution from 60-90nm range as shown in figure 5. The process of agglomeration is slowed down due to the presence of green surfactant resulting in small size SNPs. It indicates that green surfactant *Ocimum basilicum* extract along with reducing agent oxalic acid binds perfectly with the nanoparticle and which is in agreement with above discussed FTIR results.

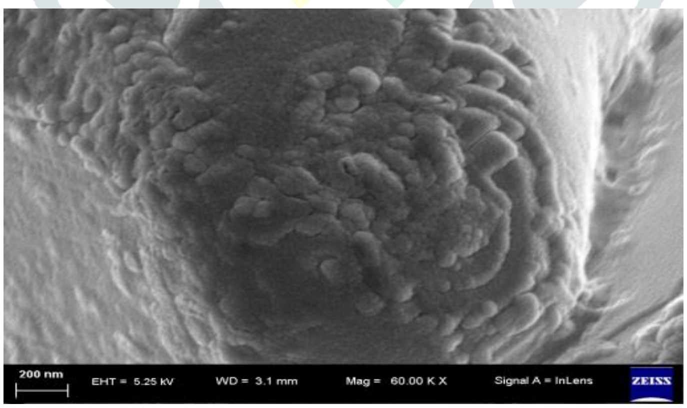

Figure 5: SEM image of SNPs

3.6 Assessment of antifungal activity of sulphur nanoparticles:

The antifungal effect of synthesized sulphur nanoparticles has been studied against three plant pathogens namely *Alternaria triticina*, *Aspergillus niger* and *Fusarium oxysporum.* All these three exhibit devastating effects on some particular crops such as *Alternaria* is responsible for blight disease which reduces the yield tremendously [21] and *Fusarium oxysporum* effects the banana crop [22-23]. A concentration of 0.4mM has been used against these plant pathogens. A significant inhibitory effect on the growth of spore has been observed in the case of *Aspergillus niger*, while no notable inhibitory effect is observed with the *Alternaria triticina* and *Fusarium oxysporum* as shown in figure 6. It may be because of the small concentration of sulphur nanoparticles as previous studies revealed that with increasing the concentration of nanoparticles, the spore inhibition increases [24]. Different species of plant pathogens need different amounts of fungicides and different compositions. At this particular concentration, the zone inhibition occurs only with *Aspergillus niger*.

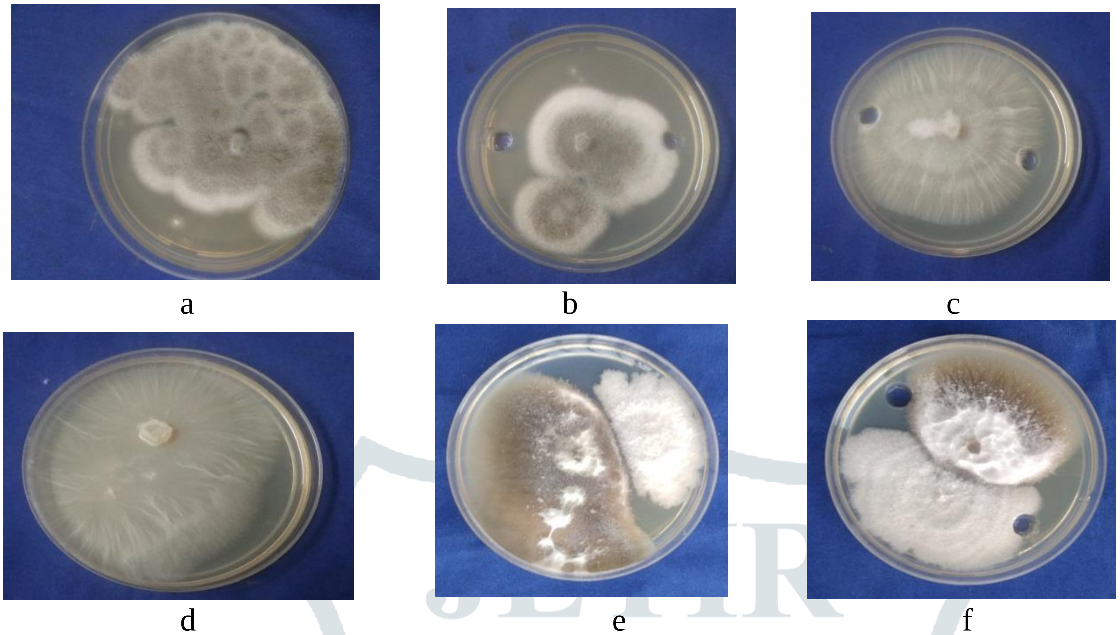

Figure 6: a) Aspergillus niger control; b) Aspergillus niger treated with SNPs; c) Fusarium oxysporum control; d) Fusarium oxysporum treated with SNPs; e) Alternaria triticina control; f) Alternaria triticina treated with SNPs.

4. Conclusions

Sulphur nanoparticles play an inconceivable part in agriculture and industrial development. Green synthesis of sulphur nanoparticles is considered as a boon to the agriculture as they do not possess any toxic effect. Highly crystalline orthorhombic SNPs have been synthesized in the present work using basil leaves extract as surfactant with crystalline size 58nm and nanoparticles size 60-90 nm. There is no additional layer of any harmful chemical at the surface of nanoparticles is observed, only the biomolecules are available as revealed by FTIR analysis. Uniformly distributed quasi-spherical SNPs are synthesized. Treatment of different plant pathogens with sulphur nanoparticles reveals the antifungal nature of the nanoparticles, which is highly dependent on the concentration of the nanoparticles.

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