

Original article

Green synthesis of zinc oxide nanoparticles using *Azadirachta indica* A. Juss. leaves extract and its antibacterial activity against *Xanthomonas oryzae* pv. *oryzae*

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Received October 25, 2016; Revised November 10, 2016; Accepted November 15, 2016; Published online December 30, 2016

Abstract

A cost effective, simple and eco-friendly method has been developed to synthesize zinc oxide nanoparticles using *Azadirachta indica* A. Juss. leaf extract. Different combinations, viz., substrate concentration (0.1 and 0.2 M ZnSO₄), reducing agent (2.5 and 5.0 ml leaf extract) and sunlight irradiation durations (20, 40, 60 and 80 min) were assessed for their ability of precise stabilized zinc oxide nanoparticle synthesis. Leaf extract components serve as both reducing and capping agent, while sunlight acts as a catalyst in the synthesis process. The biosynthesized zinc oxide nanoparticles were characterized by surface plasmon response, dynamic light scattering (DLS) for size and fourier transform infrared spectroscopy (FTIR) for functional group identification. The intensity and number based smallest size of biosynthesized zinc oxide nanoparticles were 84.26 and 27.85 nm, respectively with FTIR spectral analysis, indicated that the plant extract acted as the reducing and capping agents on the surface of zinc oxide nanoparticles. Antimicrobial activity of synthesized zinc oxide nanoparticles were tested against rice pathogen, *Xanthomonas oryzae* pv. *oryzae* (Xoo) and showed a significant antibacterial activity compared to control.

Keywords: *Azadirachta indica* A. Juss., *Xanthomonas oryzae* pv. *oryzae*, zinc oxide nanoparticles, antibacterial

1. Introduction

Azadirachta indica A. Juss. popularly known as Neem, belongs to Meliaceae family, possess diverse medicinal properties. Leaf extract of *A. indica* contains phytochemicals and enzymes which take part in the conversion of metal compounds into nanoparticles (Gavhane *et al.*, 2012). The phytochemicals present in neem leaf extract acting as bioreductant are flavones, organic acids, ketones, amides and aldehydes; out of which flavones and organic acids are water-soluble phytochemicals that are responsible for the reduction of zinc ions into zinc nanoparticles (Sangeetha *et al.*, 2011).

Nanoscience and technology are among the most active research areas in modern material science which has emerged as a rapidly growing area with its immense applications in science and technology for the purpose of manufacturing new materials at the nanoscale level, possessing unique properties (Sosa *et al.*, 2003). Among different types of nanoparticles, zinc oxide nanoparticles have extensive applications in cosmetics and sunscreen lotions because of their efficient UV-A and UV-B absorption properties without scattering visible light (Schilling *et al.*, 2010). Zinc oxide nanoparticles possess excellent antimicrobial (Jin *et al.*, 2009; Gerloff *et al.*, 2012;), and anticancer properties (He *et al.*, 2011) and also used in agriculture (Rasmussen *et al.*, 2010).

During the past decade, synthesis of nanoparticles and their application has been one of the most important areas of research. Nanoparticle synthesis using physical and chemical processes are routinely utilized to synthesize metal nanoparticles, which allow one to obtain particles with the desired characteristics (Tsuji *et al.*, 2003). However, both these methods are usually expensive, labor-intensive, and are potentially hazardous to the environment and living organisms (Narayana and Sakthivel, 2010). To overcome these problems, green chemistry approach using different plant parts as a source of reducing agent has gained immense popularity (Makarov *et al.*, 2014).

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), is a severe disease in many rice-growing regions of the world including India (Babu *et al.*, 2003). Streptomycin is an aminoglycoside antibiotic which has been widely utilized in the treatment of bacterial diseases of humans and animals (Sundin and Bender, 1993), including the control bacterial blight of rice (Xu *et al.*, 2013). Development of resistance in Xoo against streptomycin was first reported by Shetty and Rangaswamy (1971), however, till date a very little work has been done on developing new antibacterial agents against Xoo. Looking to the current scenario and ever-changing climatic conditions, development of resistance in microbes will lead to unforeseen harmful effects for scientists and farmers. To overcome the antibiotic-mediated resistance, there is a need to develop new antimicrobial agents which would provide large-scale sustainable crop protection. Therefore, the present investigation was carried out using the antimicrobial property of medicinal plant neem coupled with the power of modern nanotechnology.

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DOI: 10.21276/ap.2016.5.2.9

2. Materials and Methods

2.1 Materials

The present study consists of synthesis, characterization and antimicrobial effect of biosynthesized zinc oxide nanoparticles. All the chemicals used in the present investigation were purchased from HiMedia Pvt. Ltd. India.

2.2 Preparation of neem leaf extract

A. indica leaf extract was used as a reducing agent for the synthesis of zinc oxide nanoparticles. About 5 g of finely cut dried leaves were added in 100 ml of distilled water and boiled for 15 min. The obtained aqueous extract was filtered through Whatman No. 1 filter paper. The filtrate was centrifuged at 6000 rpm for 10 min at 4°C to remove any suspended plant debris. The clear plant extract (PE) supernatant obtained was used for the synthesis of zinc oxide nanoparticles.

2.3 Synthesis of zinc oxide nanoparticles

For the synthesis of zinc oxide nanoparticles, 15.0 ml and 17.5 ml each of 0.1 M and 0.2 M $ZnSO_4$ solution was taken in a flask, followed by addition of 5 ml and 2.5 ml of plant extract, respectively to a final volume of 20 ml. This mixture was exposed to sunlight irradiation for 20, 40, 60 and 80 min (Moosa *et al.*, 2015). Samples were collected after each interval and physical characterization of yellowish-white precipitates after centrifugation at 6000 rpm for 10 min was carried out. The precipitates were then sonicated for 10 min at 40% amplitude in Q500 model, QSonica, USA.

2.4 Characterization using UV-visible spectrophotometer, DLS and FTIR

The absorption spectrum of synthesized zinc oxide nanoparticles was monitored using UV-Visible spectrophotometer (Beckman Coulter, DU730). Dynamic light scattering (Malvern Zetasizer, ZS 90) was used to check particle size (nm), polydispersive index (PDI) and count rate (Kcps) following the standard operating procedure at 25°C. Fourier transform infrared spectroscopy (FT-IR) (Perkin Elmer, Spectrum II) was used to check functional group present on the surface of zinc oxide nanoparticles. The oven dried zinc oxide nanoparticles powder was subjected to FTIR spectroscopy measurements. The FTIR spectra were generated using Perkin-Elmer Spectrum-II instrument in the diffuse reflectance mode at a resolution of 4cm^{-1} in KBr pellets.

2.5 Isolation of bacterial pathogen

Infected rice leaf samples were collected from the main Rice Research Station of Anand Agricultural University, Nawagam. Isolation of pathogenic bacteria was carried out as described by Mizukami and Wakimoto (1969). Briefly, leaf samples were cut into small sections of 3 mm size and surface sterilized with 0.1% $HgCl_2$ and washed thoroughly with sterile distilled water. These leaf sections were submerged in test tubes, containing 3 ml of sterile distilled water and incubated for 6 h. at room temperature. Loopful of this water was streaked on Peptone Sucrose Agar (PSA) (10 g peptone, 20 g sucrose and 15 g agar) and incubated at 28°C for 3-5 days. Single isolated colony obtained at the end of incubation period was used to assess antibacterial efficacy of biosynthesized zinc oxide nanoparticles.

2.6 Antibacterial assay

The antibacterial activity of the synthesized zinc oxide nanoparticles was checked against bacterial plant pathogen, *Xanthomonas oryzae* pv. *oryzae* by agar well diffusion method (Nanda and Saravanan, 2009). By using a sterile cork borer four wells of 6 mm diameter were made on a nutrient agar plate and 100 μl of each different dilutions of zinc oxide nanoparticles (5, 10 and 20 $\mu\text{g/ml}$) solution alongwith standard streptomycin (20 $\mu\text{g/ml}$) as a control, followed by incubation of plates at 37°C for 24 h. After incubation, the plates were observed for the zone of inhibition which was measured in terms of diameter (mm). Three test plates for each treatment were used, and results were expressed in Mean \pm S.E.

2.7 Statistical analysis

The simple statistical analysis was carried out for calculating the mean and the standard error of mean.

3. Results and Discussion

3.1 Synthesis of zinc oxide nanoparticles

Biosynthesis of zinc oxide nanoparticles occurred by exposing a mixture of aqueous $ZnSO_4$ and *A. indica* leaf extract under sunlight irradiation for 20, 40, 60 and 80 min. With increasing period of incubation, the color change occurs from transparent to yellowish brown in all treatments, indicating the bioreduction of zinc oxide particles. The color change is due to the excitation of surface plasmon resonance in solution (Noginov *et al.*, 2007; Isamilssa *et al.*, 2015). As shown in Figure 1, control with substrate alone without the addition of plant extract did not exhibit any change in color. The reduction of zinc ion, using *A. indica* plant leaf extracts is also evidenced from other studies (Oudhia *et al.*, 2015; Noorjahan *et al.*, 2015). Different parameters were optimized including the concentration of zinc sulphate, *A. indica* leaf extract and exposure to sunlight (20, 40, 60 and 80 min) which had been identified as factors affecting synthesis of zinc oxide nanoparticles (Moosa *et al.*, 2015).

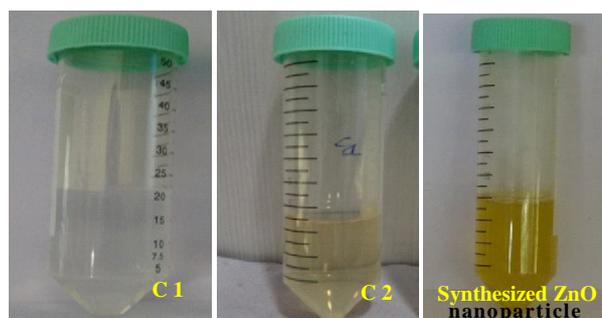


Figure 1: Color change due to surface plasmon resonance of zinc oxide nanoparticles exposed to sunlight. C1- $ZnSO_4$ +de-ionized water, C2-neem leaf extract +de-dionized water and synthesized ZnO nanoparticle - $ZnSO_4$ + neem leaf extract.

3.2 Characterization of synthesized zinc oxide nanoparticles

3.2.1 UV-visible spectroscopy

The absorption spectra obtained from UV-visible spectral analysis was used for characterizing zinc oxide nanoparticle. The biological synthesis of zinc oxide nanoparticles at different durations was confirmed using UV-visible spectral analysis. Characteristic broad peak in the UV-visible spectrum for zinc oxide nanoparticles ranges

from 230-330 nm (Revina *et al.*, 2007). In the present investigation, optical transitions have been observed for all the treatments which range from 332 - 346 nm (Table 1). Similar absorbance spectra was observed in fungus mediated synthesis of zinc oxide nanoparticles (Baskar *et al.*, 2013). It is clearly evident as depicted from the absorbance data that SPR absorbance tends to vary among different treatment combinations, owing to its sensitivity to nature, size and shape of the particles formed and also dependent upon particle to particle distance as well as its surrounding media (Kumar *et al.*, 2014).

3.2.2 Dynamic light scattering

(i) Size distribution by intensity

Characterization of zinc oxide nanoparticles solution was performed using Zeta Sizer Nano-ZS90. Dynamic light scattering (DLS) analyzed the velocity of particle movement by measuring dynamic fluctuations of light scattering intensity, caused by the random motion of the particle. This technique yields a hydrodynamic radius, or diameter, polydispersive index and counts rate of the particle present in the solution (Murdock *et al.*, 2008). The size distribution of DLS (Figure 2) indicates that the lowest size of zinc oxide nanoparticles is 84.26 nm under sunlight treatment exposed for 80 min, using 0.1 M ZnSO₄ and 2.5 ml of 5% neem leaf extract. The DLS results for the particle size in all the treatments are present in Table 1. The DLS histogram pattern of zinc oxide nanoparticles suspension synthesized using *A. indica* aqueous leaf extract suggest bioreduction of zinc oxide into nanoparticle (Figures 2 and 3). The PDI scale in all the treatments range from 0.124 to 0.383, indicates that the particle remains in disperse form in all the treatments.

(ii) Size distribution by number

For number weighted distribution, each particle in the solution is given equal weighting irrespective of its size which means proportional to a^3 . In the current study, different combinations effect like substrate concentration, reducing agent volume and exposure to sunlight are studied, hence, there is a need to observe distribution of size by number (Figures 4 and 5). The size distribution by number tends to change compared to intensity based distribution as the latter measures the intensity of scattered light proportional to sixth power of the particle diameter (Fissan *et al.*, 2014). Smallest size of 27.85 nm based on number distribution was recorded for zinc oxide nanoparticles, synthesized under 40 min sunlight exposed 0.1 M ZnSO₄ and 2.5 ml of plant extract (Figure 4 E). The best synthesis treatment combinations as obtained for size distribution by intensity, *i.e.*, 80 min sunlight reported the second lowest nanoparticle size by number. Similar decrease in size upon transformation from intensity to number based distribution was observed by Fissan *et al.* (2014). It is very well known that DLS based particle sizing could only be considered when normalization values for these nanoparticles are known. However, in most of the cases, these values are not available and hence, observed peaks is approximate values only. To overcome the issue, one can rely on number rather than intensity based distribution. The peak in particle-weighted description corresponds to larger particles accounting for 90% of population, however, upon conversion into number, the peak of the smaller particles corresponds >95% of the particle population (Elia *et al.*, 2014). Therefore, it is very much essential while interpreting the data to correlate intensity as well as number based distribution while using DLS system which has been very well carried out in the present study.

Table 1: Characterization of zinc oxide nanoparticles by dynamic light scattering analysis

Sunlight exposure (min)	Treatments		Size (nm)		PDI	Kcps	λ _{max} (nm)
	ZnSO ₄ (M)	Plant extract (ml)	Intensity based	Number based			
20 min	0.1	2.5	157.9	99.38	0.321	309.6	332
		5.0	167.6	102.0	0.379	314.5	332
	0.2	2.5	171.7	115.3	0.223	293.7	334
		5.0	168.0	94.57	0.156	297.1	334
40 min	0.1	2.5	151.7	27.85	0.246	500.2	346
		5.0	153.3	114.6	0.381	184.3	346
	0.2	2.5	172.1	55.93	0.367	335.05	332
		5.0	194.2	122.8	0.308	281.9	336
60 min	0.1	2.5	137.8	60.69	0.290	447.3	336
		5.0	302.7	172.0	0.383	379.6	334
	0.2	2.5	309.3	164.2	0.338	383.7	334
		5.0	315.8	221.2	0.336	195.9	336
80 min	0.1	2.5	84.26	33.25	0.337	252.96	336
		5.0	95.89	39.23	0.307	241.43	334
	0.2	2.5	96.34	70.19	0.124	169.1	332
		5.0	125.1	81.76	0.361	408.5	332

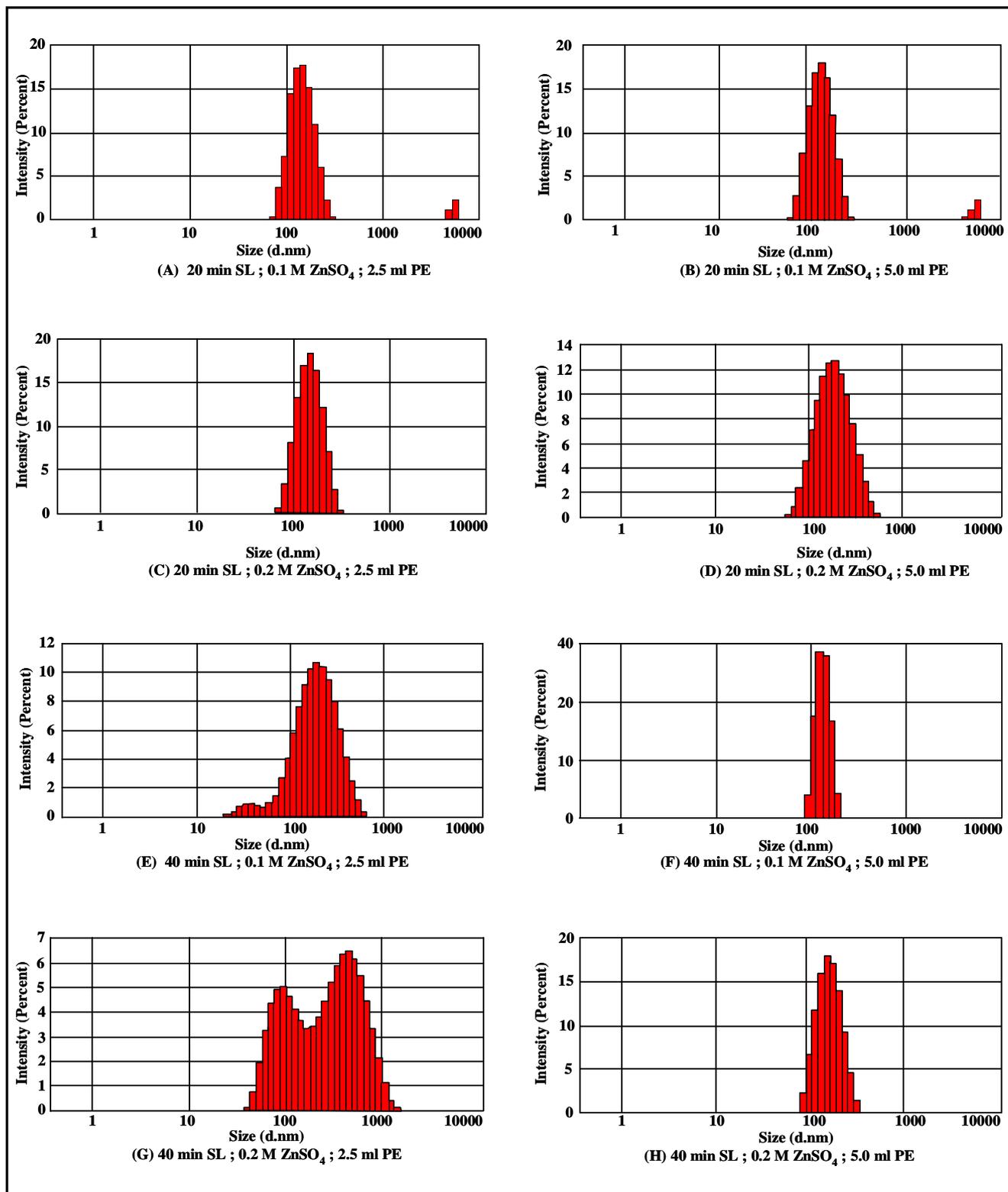


Figure 2: Size distribution by intensity of different biosynthesized zinc oxide nanoparticles at 20 and 40 min sunlight (SL) irradiation by dynamic light scattering.

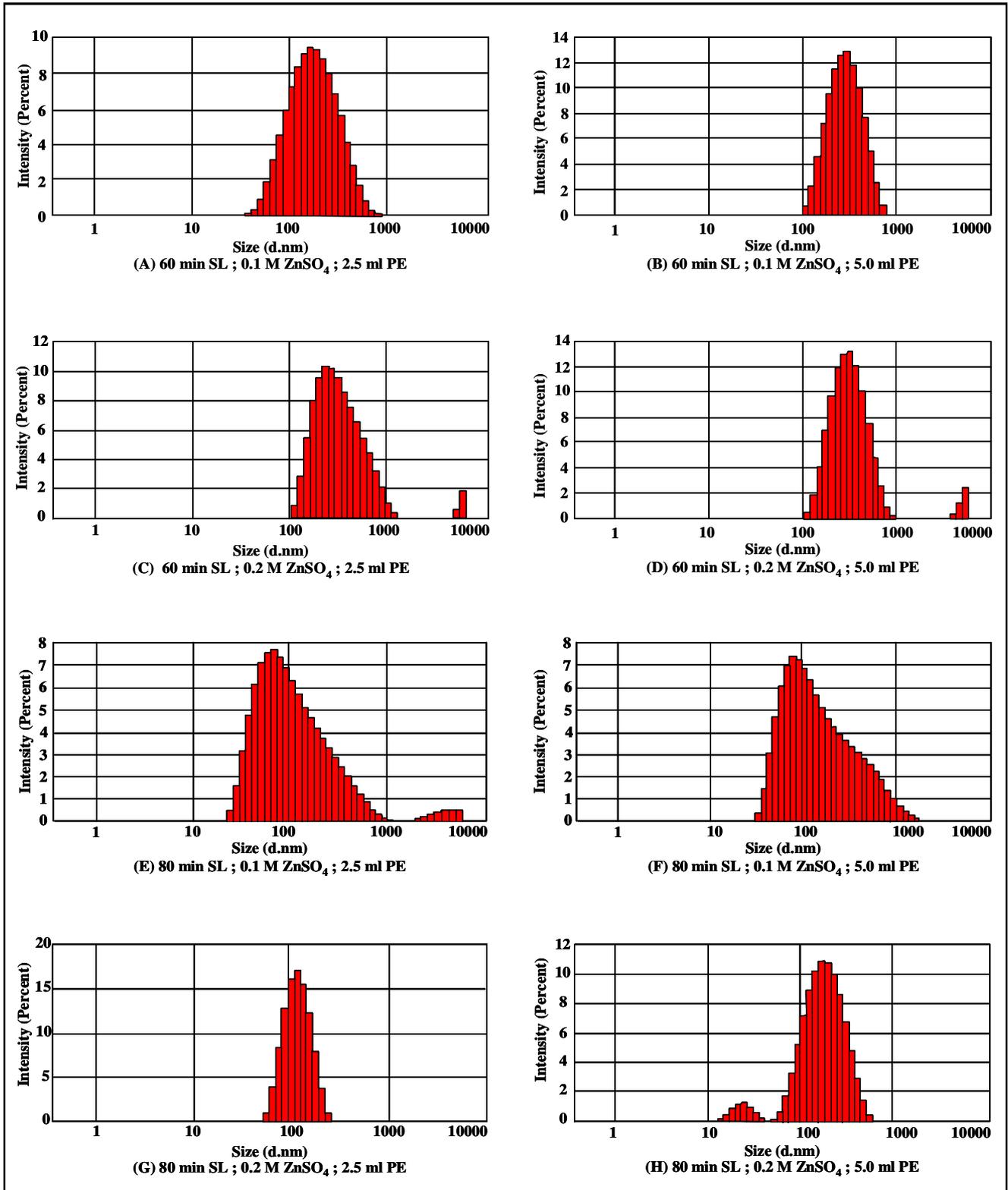


Figure 3: Size distribution by intensity of different biosynthesized zinc oxide nanoparticles at 60 and 80 min sunlight (SL) irradiation by dynamic light scattering.

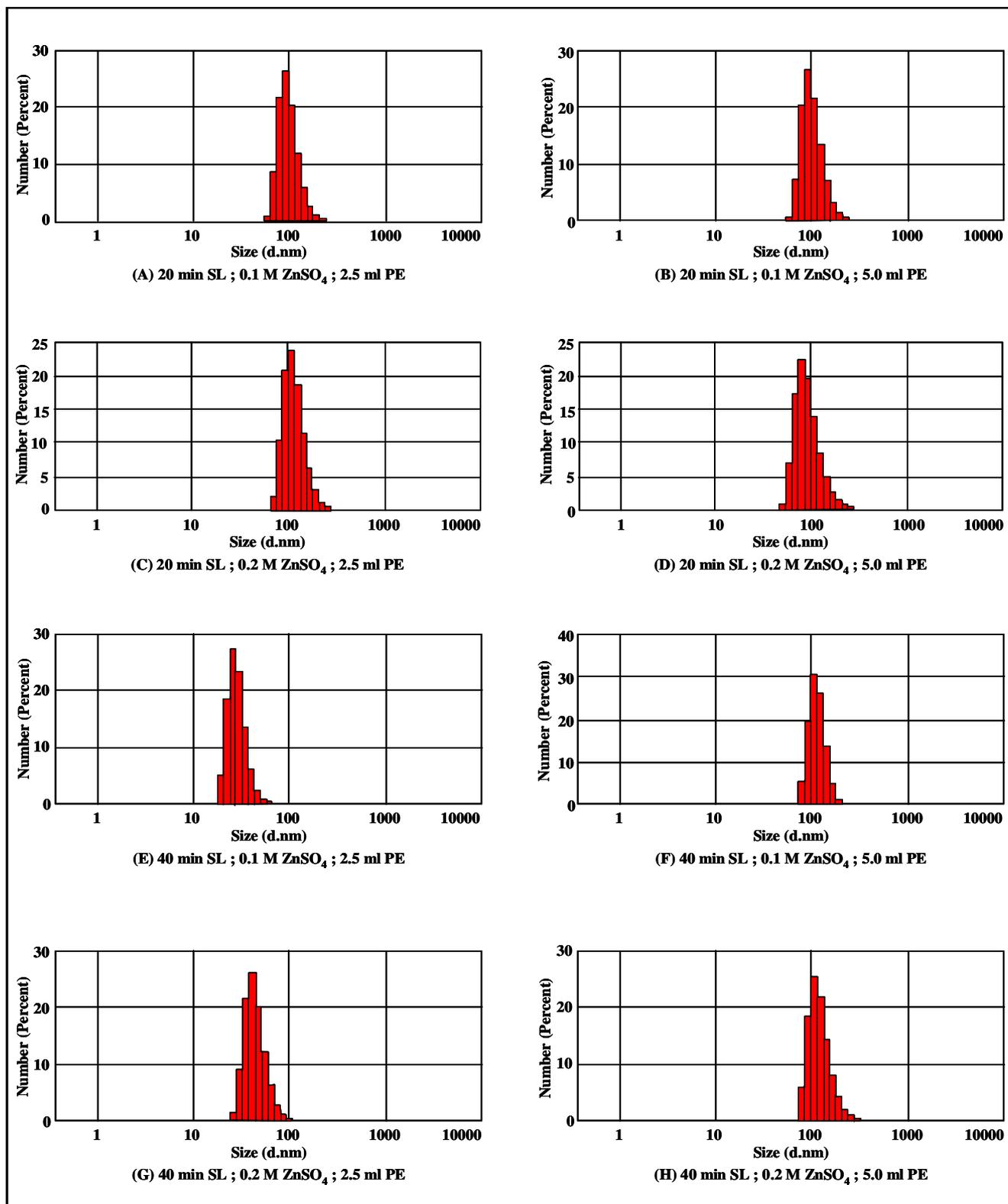


Figure 4: Size distribution by number of different biosynthesized zinc oxide nanoparticles at 20 and 40 min sunlight (SL) irradiation by dynamic light scattering.

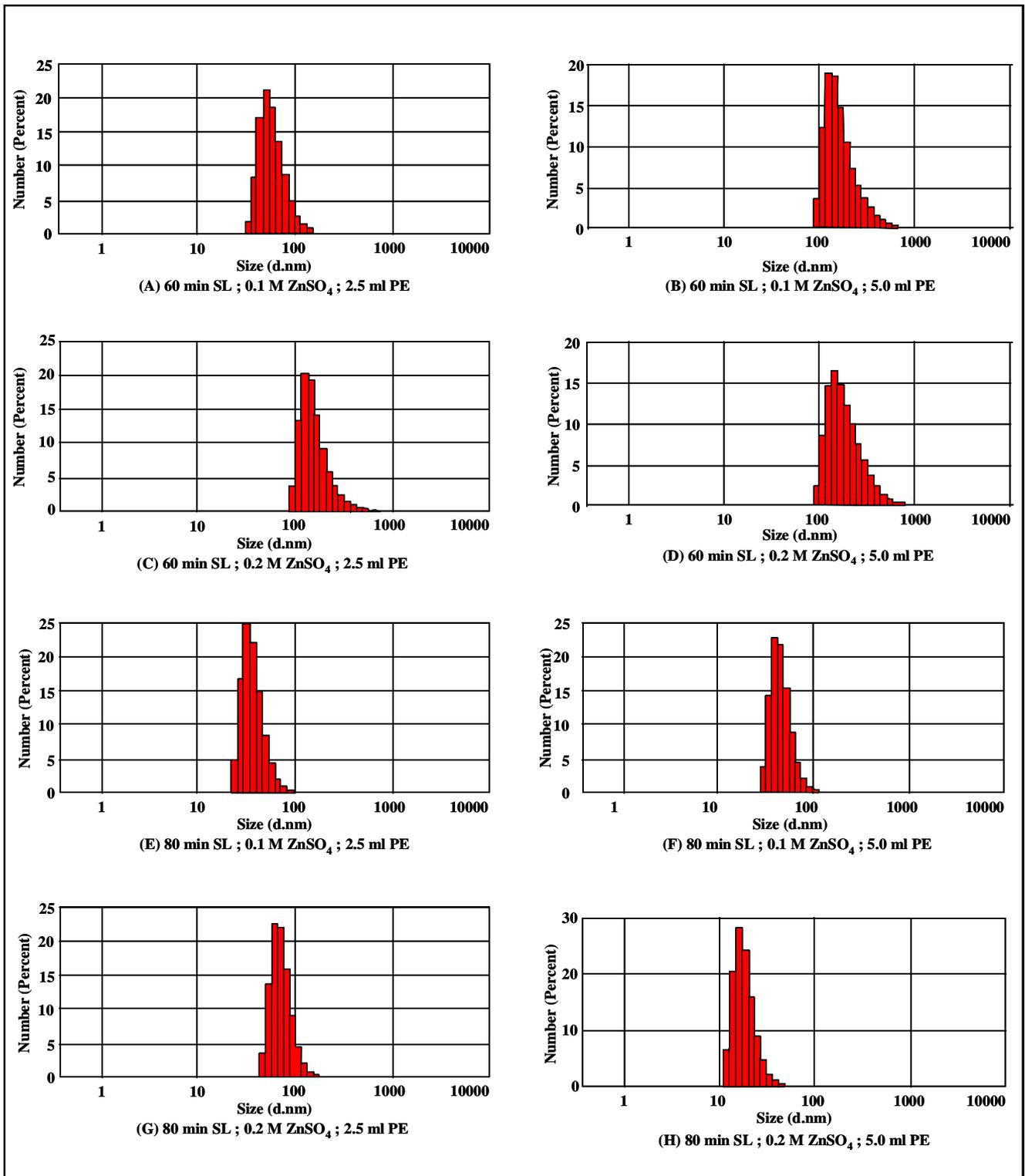


Figure 5: Size distribution by number of different biosynthesized zinc oxide nanoparticles at 60 and 80 min sunlight (SL) irradiation by dynamic light scattering.

(iii) Correlogram coefficient

As shown in the Figure 6, correlation coefficient exhibits two groups based upon the size of the nanoparticle synthesized at the end of incubation period under sunlight. The correlogram clearly suggest polydisperse nature of the biosynthesized nanoparticle. The correlogram of a DLS measurement can depict the nature of nanoparticle solution and can be extremely useful in estimating the size of nanoparticle (Barabadi *et al.*, 2014). Correlogram indicates mean size of the sample based upon time at which the correlation starts to significant decay. As the figure suggests, smaller the

particles, faster will be decaying and vice-versa. Similar results were reported by Barabadi *et al.* (2014) while optimizing process parameters for gold nanoparticles synthesis using microbes.

(iv) Cumulant fit

Similarly to correlogram, cumulant fit results obtained from DLS measurement corresponds to size dependent grouping of treatments. Larger particles have higher value compared to smaller particles (Figure 7). Similar trend of size dependent cumulant fit distribution was reported by Geißler *et al.* (2015) while characterizing polymeric nanoparticles using DLS and small angle X-ray scattering techniques.

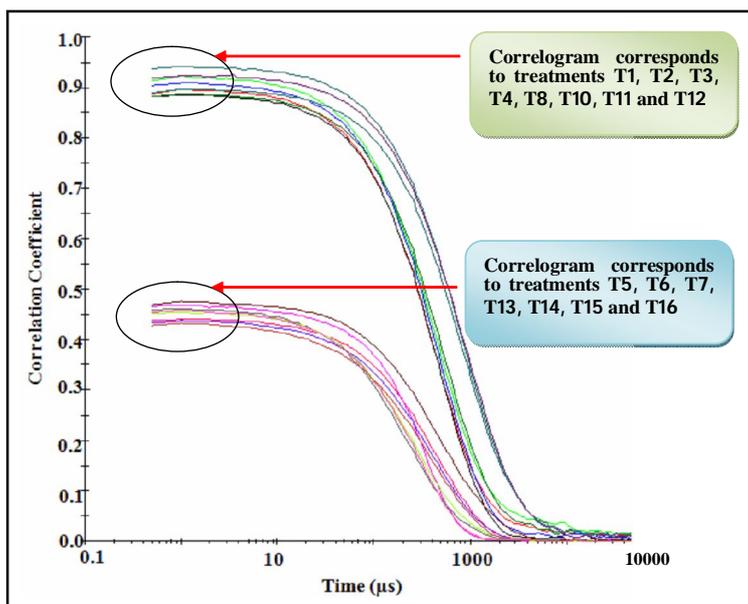


Figure 6: Correlation coefficient of different biosynthesized zinc oxide nanoparticles by dynamic light scattering.

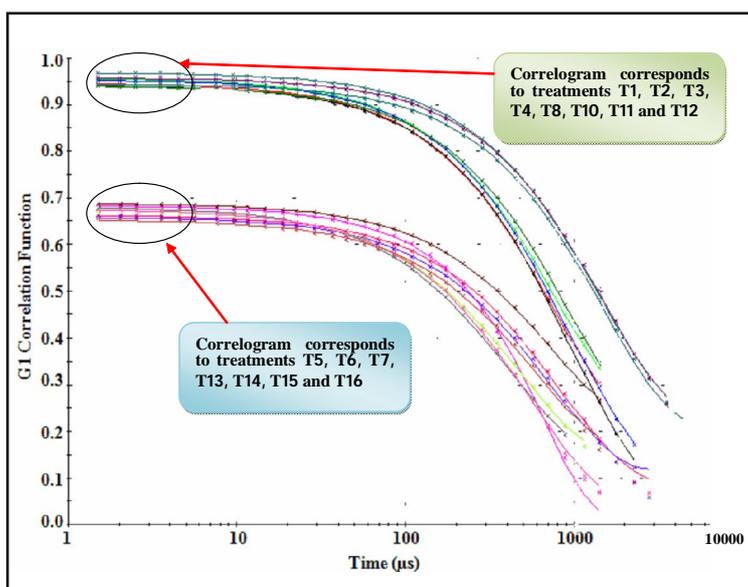


Figure 7: Cumulant fit error of different biosynthesized zinc oxide nanoparticles by dynamic light scattering.

3.2.3 FTIR spectroscopy

FTIR spectroscopy measures the absorption of infrared radiations by a sample and the results of such analysis are shown, using a wavelength. By this technique, it is possible to identify the biomolecules in plant extracts which play the crucial role in the reduction and stabilization of the synthesized nanoparticles (Senthilkumar and Sivakumar, 2014). The role of the neem leaf extract as a reducing and capping agent and presence of some functional groups was confirmed by FTIR analysis of zinc oxide nanoparticle. The representative spectrum of zinc oxide nanoparticles is shown in Figure 8 which suggest zinc oxide

absorption band in the region 400 and 600 cm^{-1} (Sangeetha *et al.*, 2011; Vani *et al.*, 2011; Rajiv *et al.*, 2013). The broad absorbance at 3412 cm^{-1} is attributed to the O–H stretching modes of vibration in hydroxyl functional group in alcohols and phenolic compounds. The band 1506 cm^{-1} corresponds to N–H stretching vibrations in amide-II; 1382 cm^{-1} represents monosubstituted alkynes (Vani *et al.*, 2011; Rajiv *et al.*, 2013). The observed peak at 1116.72 cm^{-1} denote -C-OC- linkages, or -C-O- bonds (Ahmed *et al.*, 2015). The observed peaks are mainly attributed to flavonoids and terpenoids present in plants extract. Functional groups exist in polyphenolic compounds of *A. indica* leaf extract which play an important role in bioreduction of zinc ions.

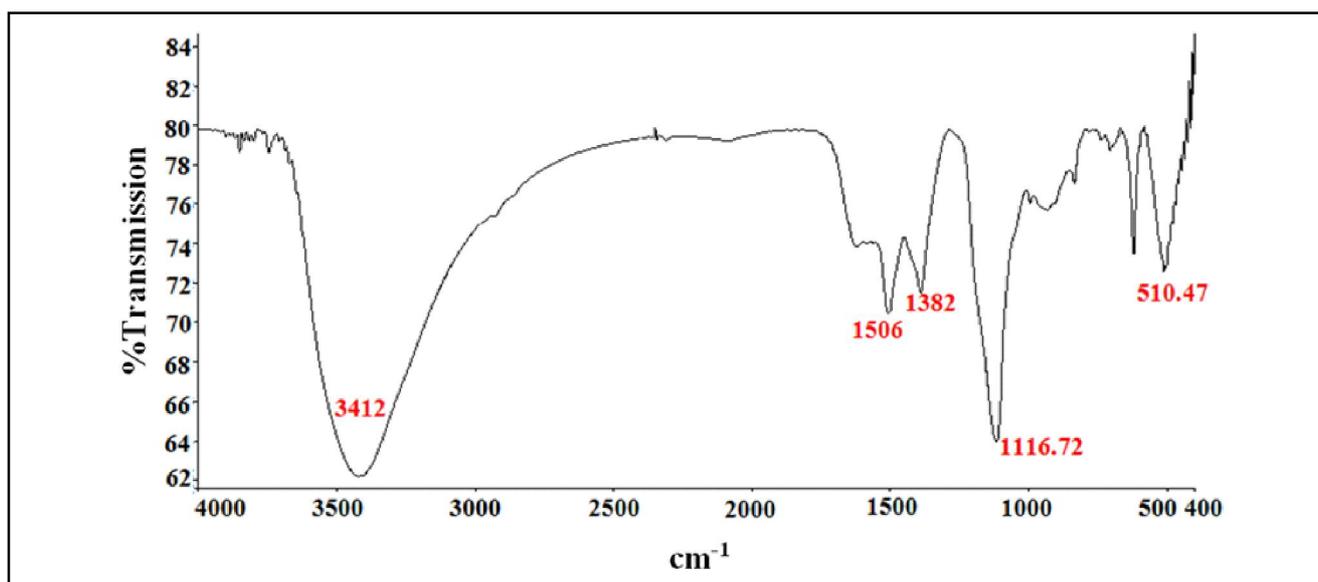


Figure 8: FTIR spectra of biosynthesized zinc oxide nanoparticles using 0.1 M ZnSO_4 + 2.5 ml PE (80 min).

3.2.4 Antibacterial activity of synthesized zinc oxide nanoparticles

Antimicrobial activity of green synthesized zinc oxide nanoparticles against bacterial plant pathogen *Xanthomonas oryzae* pv. *oryzae* was tested. All the treatment combinations yielded biosynthesized zinc oxide nanoparticles which showed antibacterial activity (Figure 9). The antibacterial activity was carried out by agar well diffusion method with antibiotic streptomycin as a control and green synthesized zinc oxide nanoparticles. The zone of inhibition (mm) was found at par for all the green synthesized zinc oxide nanoparticles as compared to antibiotic streptomycin, as shown in (Table 2). From the results, it can be confirmed that the maximum zone of inhibition found to be 35 mm in particles synthesized upon 80 min sunlight exposed using 0.1 M ZnSO_4 and 25 ml plant extract for 80 min. Concentration based zone of inhibition was observed at the end of incubation period which clearly suggest that the antibacterial activity of zinc oxide nanoparticles is concentration dependent and at lower concentration *i.e.*, 5 $\mu\text{g/ml}$, maximum zone of inhibition was observed for the smallest particle size and hence, it can be clearly seen that inhibition of bacterial growth is also nanoparticle size dependent. Further, as earlier reported by Divyapriya *et al.* (2014), the zinc oxide nanoparticles also have net positive charge on its surface which also plays an important role in enhancing its antibacterial activity. Zone of inhibition also indicates that the generation of surface oxygen species from zinc oxide

nanoparticles leads to damage of bacterial cell membrane, resulting in the death of the bacteria (Padmavathy and Vijayaraghavan, 2008; Sharma *et al.*, 2010).

4. Conclusion

The present study reports a green, eco-friendly and cost-effective approach for the synthesis of zinc oxide nanoparticles, using the *A. indica* leaf extract, which acts as a reducing and stabilizing agent. The intensity based size distribution of synthesized zinc oxide nanoparticles ranges from 84.25 to 315.80 nm while number based distribution ranges from 27.85 to 221.20 nm. The synthesized zinc oxide nanoparticles were found to be stable at room temperature as revealed by the polydispersive index due to the presence of polyphenol compounds in the plant extract. The correlogram and cumulant fit data also corresponds to size data and grouped the treatments based on the size data. The synthesized zinc oxide nanoparticles have shown good antibacterial activity against plant pathogen, *Xanthomonas oryzae* pv. *oryzae*. Thus, it is concluded from *in vitro* test that reported green synthesized zinc oxide nanoparticles can act as an effective antimicrobial agent in agriculture and detailed investigation on its field application should be carried out to assess the *ex vitro* antibacterial of zinc oxide nanoparticles.

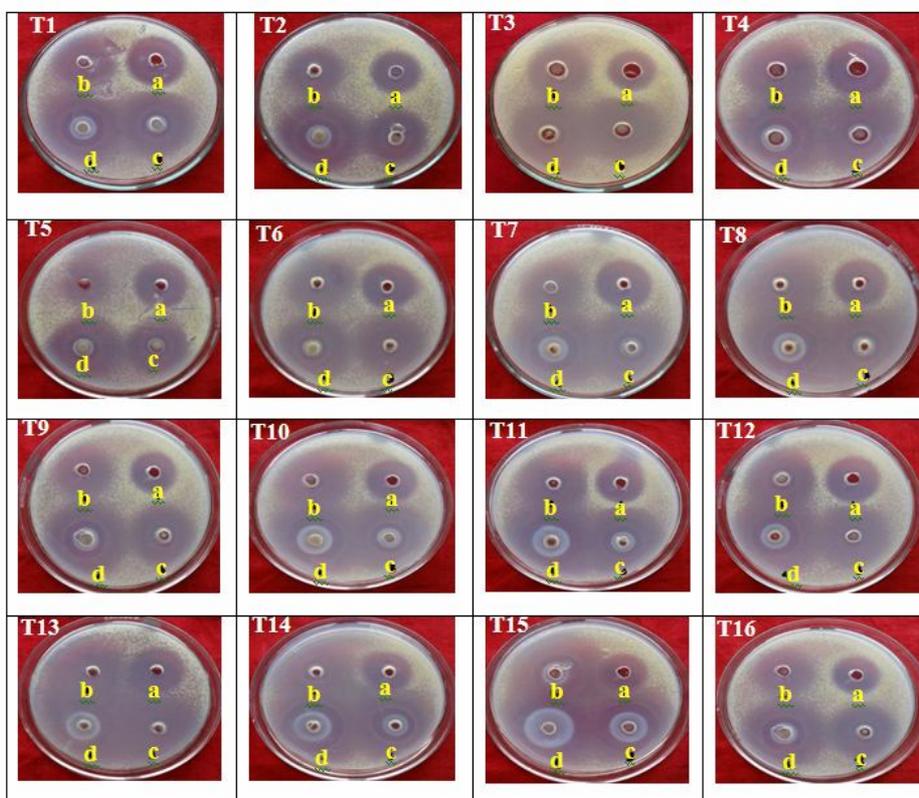
Conflict of interest

We declare that we have no conflict of interest.

Table 2: Determination of antibacterial activity of green synthesized zinc oxide nanoparticle against *Xanthomonas oryzae* pv. *oryzae*

Treatments				Green synthesized zinc oxide nanoparticles concentration			Strepto-cycline 20 µg/ml
Treatment code	Sunlight exposure duration (min)	ZnSO ₄ (M)	Plant extract (ml)	5 µg/ml	10 µg/ml	20 µg/ml	
T1	20 min	0.1	2.5	11.50±0.76	30.00±0.58	30.00 ±1.53	23.00 ± 0.58
T2			5.0	20.50±0.29	26.67±0.88	31.33 ±1.20	
T3		0.2	2.5	17.17±0.73	28.33±1.20	30.67 ±1.45	
T4			5.0	19.17±0.60	30.33±0.88	32.00 ±0.58	
T5	40 min	0.1	2.5	17.67±0.44	26.67±1.45	31.00 ±1.53	
T6			5.0	19.83±0.44	30.33±1.76	30.67 ±1.20	
T7		0.2	2.5	18.33±0.88	30.00±1.73	33.00 ±1.53	
T8			5.0	14.83±0.44	32.00±1.15	33.00 ±1.73	
T9	60 min	0.1	2.5	17.50±0.23	27.67±0.88	33.33 ±1.76	
T10			5.0	16.50±0.50	31.33±1.76	34.00 ±1.15	
T11		0.2	2.5	17.50±0.76	29.33±1.20	31.67 ±1.45	
T12			5.0	18.83±0.44	28.33±1.20	32.67 ±1.45	
T13	80 min	0.1	2.5	22.00±1.73	33.00±0.58	35.00 ±1.53	
T14			5.0	20.00±1.15	31.67±1.20	33.33 ±1.76	
T15		0.2	2.5	17.50±0.76	32.67±1.45	34.67 ±1.76	
T16			5.0	21.00±1.15	29.67±0.33	33.00 ±1.53	

Mean ± S.E.

**Figure 9:** Antibacterial activity of green synthesized zinc oxide nanoparticles against *Xanthomonas oryzae* pv. *oryzae*. (a) 20 µg/ml streptomycin, (b) 5 µg/ml zinc oxide nanoparticles, (c) 10 µg/ml zinc oxide nanoparticles and (d) 20 µg/ml zinc oxide nanoparticles. (For treatment number refer Table-2)

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