Zinc oxide nanoparticles (ZnO-NPs) *via* green approach for enhanced antimicrobial activity against food-borne pathogen (F-BP) bacteria

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Abstract:

A simple, eco-friendly, and biomimetic approach using *Turbinaria ornata* (TO) aqueous extract was developed for the formation of zinc oxide nanoparticles (ZnO-NPs). We have synthesized eco-friendly TOZnO-NPs which has economic benefits without employing any stabilizer and a chemical surfactant. Green TOZnO-NPs structural and textural features were characterized by various analytical techniques such as UV-visible spectrophotometer, XRD, SEM, EDX, DLS, Fe-SEM and FTIR. *In-vitro* antimicrobial activity of TOZnO-NPs was studied on selective Gram-negative and positive food-borne pathogen (F-BP) bacteria. Antimicrobial activity portrayed higher inhibition against Gram-negative then Gram-positive. Higher inhibition 16 mm against *Escherichia coli* (NCIM 2931) and minimal 12 mm in *Staphylococcus aureus* (NCIM 5021) was observed. Anti-microbial mechanism of TOZnO-NPs employed a biphasic phenomenon persuading by osmotic shock that could attack the cell wall directly leads to death. These findings clearly implicate that green TOZnO-NPs is a new paradigm to mitigate the F-BP bacteria.

Keywords: Green synthesis; *T. ornata*; Zinc oxide nanoparticles; food-borne pathogen (F-BP) bacteria ; Anti-bacterial activity

1. Introduction:

Metal nanomaterials (NPs) and nanocomposites (NCs) are attracting researchers across globe due to their superior magnetic, chemical, optical and electrical properties. These properties of metal nanomaterials are remarkable in various application sections containing nonlinear optical machinery, nano-electronical devices, catalysis, bio-medical, etc (Khan et al., 2017). Green nanotechnology is recently, emerging as a rapidly growing field in science and technology with its application for the purpose of manufacturing eco-friendly nanoscale materials. There is a growing necessity to develop eco-friendly synthesis of NPs/NCs considered which does not use toxic materials in the procedures. Green synthetic method employing green extracts have drawn attention as a simple non-toxic, bio-safe, bio-compatible and viable alternative to chemical and physical methods (Krishnan et al., 2015). Green synthesis of zinc oxide nanoparticles (ZnO-NPs) used commercially produced synthetically which have some advantages, compared to physical and chemical methods, such as lower cost, white appearance (Vigneshwaran et al., 2006). Among various NPs, ZnO-NPs considered to be most promising semiconductor act as a green promising technology provides alternative ways for anti-bacterial activity are effective at killing pathogenic and non-pathogenic bacteria. ZnO-NPs are believed to be non-toxic, bio-safe, and bio-compatible have been also used as drug carriers, cosmetics and fillings in medical applications.

Widespread bacterial resistance induced by the abuse of antibiotics eagerly needs the exploitation of novel antimicrobial agents and strategies. World Health Organization (WHO) has estimated contamination of water by various types of microorganism has long been a great concern for human health (WHO, 2011). Since the last decade food-borne pathogen (F-BP) bacteria emerged as a global prophylactic hindrance for the community and nosocomially acquired infections. Severe threat community health due to a progressive rise in anti-biotic resistance (ABR) and are emerging pathogens whose resistance profiles provide a major challenge for public health (Zheng et al., 2018). Countless articles reported improved therapies of antibacterial and that a single effective antibiotic can resist over 70 % of infections caused by pathogen (Ashbolt, 2015; Li and Webster, 2018).

Currently, over 70% of bacterial nosocomial infections in the United States (USA) are resistant to one or more antibiotics traditionally used to eliminate those (Cabello et al., 2006). The last few years have seen an enormous increase of a host of ABR. In recent years, much emphasis has been put on the safety aspect of foods and water owing to cross contamination caused by spoilage or pathogenicmicroorganisms. The USA, Center for Disease Control and

Prevention (CDC) estimated that at least 90,000 deaths to bacterial infection, more than half caused by ABR (Rawashdeh and Haik, 2009). People who become infected with DRS pathogens usually spend more time in the hospital and require a form of treatment that uses two or three different antibiotics which are less effective, more toxic and expensive (Dolliver et al., 2008). Partially metabolized antibiotics along with their excreta are commonly discharged either to sewage treatment plants or untreated to environmental waters or soils (Singer et al., 2016). The particular concern are effects of antibiotics used for treating infections or for farming purposes in a selection of F-BP bacteria, with an impact on human health.

In the present study it can be used as a bio-factory for the green synthesis of ZnO-NPs a quite novel leading a green viable and facile methodology are playing a major role in the field of applications (Saravanan et al., 2013; Zareet al., 2019). Literatures have the witnesses for the green synthesis of NPs is a promising and environmental favourable material with some exciting properties widely investigated and owing to their broad range of applications. The opportunity deals with a simple, eco-friendly synthesis in the current research and to evaluate the use of aqueous extracts from brown marine macroalgae *T. ornata* (TO) is to get the bio-reducing agent for the ZnO-NPs synthesis as a natural product inspired method (Aziziet al., 2014). The green synthesized TOZnO-NPs showed exciting antibacterial activity againstgram-negative and positivehuman pathogenic bacteria (HPB) as model organisms. These findings could expand our knowledge in the biosynthesis of TOZnO-NPs and future environmental and bio-applications of TOZnO-NPs.

2. Experimental Methods:

2.1. Collection and extraction of seaweed

Marine macroalgae *Turbinaria ornata* (TO) species were collected from the intertidal zone at low tide, in November 2018, from the coastal area of Mandapam region (latitude 78° 8’ East and longitude 9° 17’ North) at the Gulf of Mannar area coast of Tamil Nadu, South India inthe Indian Ocean. Seaweed species were collected by hand using sterile plastic packets and polyethylene bags washed with seawater to remove debris, shells, sand and associated epifauna/ epiphytes (Krishnan et al., 2015).

Seaweed samples were transported to the laboratoryand then cleaned thoroughly with tap water followed by distilled water to remove surface salt. After cleaning, they were dried in the shade at room temperature (28 $\pm$ 2 ^{0}C) for a weekand then ground in an electric mill (Preethi Zodiac MG-218 750 W Juicer Mixer Grinder) to less than 0.50 mm. From that 20 g of milled seaweed powder were mixed with 200 mL of double distilled water and kept in a boiling water bath at 80 ^{0}C for 30 mints. After cooling, the crude seaweed extract (SE) was filtered through a Whatman No.1 filter and stored in a refrigerator at 4 ^{0}C until further analysis in due course.

2.2. Green synthesis of zinc oxide nanoparticles (ZnO-NPs)

An aqueous solution of 0.5 M zinc nitrate hexahydrate [$Zn(NO_3)_2 \cdot 6H_2O$] and seaweed extract (SE) was used for the green synthesis of ZnO-NPs. Each SE the reaction mixture was prepared by adding 5 mL of SE to 95 mL of 0.5 M $Zn(NO_3)_2$ solution in a 250 mL Erlenmeyer flask and kept in a boiling water bath at 70 ^{0}C. The color of mixture reaction was changed from dark brown to a pale white color during the reaction, indicating the synthesis of TOZnO-NPs. Among the ten seaweeds, *Turbinaria ornata* (TO) extract produced a remarkable color changed dark brown to a pale white color within 12 hrs indicates the biosynthesis of TOZnO-NPs by surface plasmon resonance (SPR), which were confirmed by UV-vis spectroscopy (Rajaboopathi and Thambidurai, 2017).

2.3. Characterization of zinc oxide nanoparticles (ZnO-NPs)

For further characterization, synthesized TOZnO-NPs were purified by repeated centrifugation at 10,000 rpm for 15 mints. The resultant pellet was re-suspended in Milli-Q water and freeze drying lyophilizer (FD-10-MR) for 12 hrs. From this TOZnO-NPs phase, purity, particle size/ morphology and composition were determined by X-ray diffraction (XRD) analysis further recorded by a diffract meter (Nicolet Model: 6700). Scanning electron microscopy (SEM) equipped with energy dispersive spectroscopy (EDS) (SEM; HITACHI, S-3000H). Size distribution and theaverage size of the nanoparticles were estimated on the basis of field-emission scanning electron microscope (Fe-SEM; JSM-6360LA). The chemical structure and functional group of the TOZnO-NPs was examined by using Fourier transformed infrared spectroscopy (FTIR- Spectrum RX 1, Male Perkin Elmer) analysis by KBr pellet technique in a range 4000 cm^{-1} to 400 cm^{-1}.

2.4. Gram-negative and positive food-borne pathogen (F-BP) bacteria for testing

As a model organisms Gram-negative and positive F-BP bacteria were procured such as *Escherichia coli* (NCIM 2931); *Klebsiella pneumonia* (NCIM 2883); *Proteus mirabilis* (NCIM 2241); *Pseudomonas aeruginosa* (NCIM 5029); *Salmonella typhimurium* (NCIM 2501); *Vibrio cholera* (MTCC 2501) and positive *Bacillus subtilis* (NCIM 2920);

Micrococcus luteus (NCIM 2871); *Staphylococcus aureus* (NCIM 5021) and *Staphylococcus epidermis* (NCIM 2493). These cultures were obtained from the Council of Scientific and Industrial Research - National Chemical Industrial Microorganisms (CSIR-NCIM), Pune, India, and the Council of Scientific and Industrial Research - Microbial Type of Culture Collection and Gene Bank (CSIR-MTCC), Chandigarh, India.

2.5. In-vitro antimicrobial screening against F-BP bacteria

Microbial culture selective medium, standard disks, sterile swabs and HiAntibioticZone Scale-C were purchased from Hi-Media Mumbai, India. F-BP bacteria were sub-culturd into fresh nutrient broth (NA) medium for 24 hrs at 28 ± 2^0C. All isolates were analysed by pure culture spread plating method with 0.1 mL suitable dilutions. Each strain was swabbed uniformly into the individual Muller Hinton Agar (MHA) plates using sterile cotton swabs were used to inoculate suspensions prepared in sterile 0.85% saline matching an optical density of 0.5 McFarland standards corresponding to 10^8 CFU mL^{-1}on the surface of agar plates for homogeneous growth and allowed to dry (Krishnan et al., 2017).

Well diffusion assay/method was used to evaluate the*in-vitro* antibacterial activity of SE and green TOZnO-NPs against certain F-BP bacteria on MHA plates. Lyophilized TOZnO-NPs powder were dissolved in Milli-Q water and sonicated for 20 mints in order to prevent the agglomeration of particles. Four wells each of 6 mm diameters were made on F-BP bacteria coated each plate. Then SE and dissolved TOZnO-NPs solutions were loaded into each well at different concentrations such as 20, 40, 60, and 80 µg mL^{-1}, respectively. Theplates were incubated at 37 ± 2 ^{0}C for 24 - 48 hrs, after incubation the growth inhibition zones were measured by a ruler/ Hi Antibiotic Zone Scale-C (Harinee et al., 2019).

3. Result and Discussion:

Synthesis of TOZnO-NPs reliable, nontoxic and eco-friendly methods has become a matter of great interest in recent times due to their various advantageous properties and applications in a variety of biomedical applications fields. ZnO-NPs preparation by green synthesis approaches has many advantages over conventional methods involving chemical agents associated with environmental toxicity. Green synthesized TOZnO-NPs have recently emerged as promising materials in the biomedical sciences because of their antimicrobial activities towards a wide variety of F-BP bacteria and virus including type 1and hepatitis Bvirus. It'swidely appliedintechnological application that has been established at the molecular levelwhichrepresents an economic alternative to chemical and physical methods (Hosseini et al., 2008).

All seaweeds were able to synthesized TOZnO-NPs with variations in the color intensity with respect to time which is attributed to the excitation of surface plasmon (SPR) vibrations in metal nanoparticles. Among the ten seaweeds extract *T. ornata* (TO) produce a remarkable color changed within 12 hrs dark brown to a pale white color during the reaction. Green technology is emerging as rapidly growing fields in nanotechnology intend for the development of sustainable eco-friendly materials for biological applications. TO seaweed extract ZnO-NPs were confirmed through visual assessmentis shown in Figure 1. For synthesis of TOZnO-NPs, desired amount of zinc nitrate and seaweed extract were appropriately mixed and reacted. The reaction color of mixture reaction was changed from dark brown to a pale white color indicating the formation of TOZnO-NPs. The changes in color formation may be attributing to the interaction of functional groups present in the seaweed extract with zinc nitrate to reduce into Zn^0 ions and stabilize the TOZnO-NPs. This green approach appears to be a cost effective, require extensive labour and time alternative to conventional physical and chemical methods of synthesis.

The UV–vis absorption spectrum of the green TOZnO-NPs and *Turbinaria ornata* (TO) broth is shown in Figure. 2. Sharp absorption in the wavelength of 350 nm elucidating the characteristic peak of ZnO-NPs which arises due to the phenomenon of SPR. The characteristic color change was due to the excitation of SPR and a temperature dependent reduction of $Zn(NO_3)_2$ that is used to confirm the green nanoparticles (TOZnO-NPs). Figure. 3 shows the XRD pattern of the green TOZnO-NPs shows strong diffraction peaks at 31.73°, 34.39°, 36.20°, 47.56°, 56.60°, 63.02°, 66.48°, 69.1°, 72.6°, and 76.86° corresponds to (100), (002), (101), (102), (110), (103), (112), (201) and (004) respectively. All the recorded diffraction peak intensities exhibited heaxogonal wurtzite crystalline structure of ZnO coincided well with JCPDS No 36 -1451. Average crystallite size of the nanoparticles was determined through Scherer's formula:

$$D = (k\,\lambda / \beta\cos\theta) \times 100 \qquad (1)$$

Where, K is the shape factor (k=0.9); β is the full width at half maximum (FWHM); θ is the Bragg's angle and λ is the x-ray wavelength (λ=1.5406). It indicates the particles had acceptable crystalinity with face centred cubic structure in the form of the green TOZnO-NPs aggregates.

Through SEM analysis shows the particle size, shape and morphological structure of green TOZnO-NPs formed were agglomerated with a hexagonal structure. Figure. 4 showed a spherical particle size ranging from 15 nm to 50 nm. This agglomeration is due to polarity and electrostatic attraction of ZnO-NPs agglomeration due to the presence of organics in *T. ornate* as stabilizing agents. The EDS analysis implied reduction elemental Zn+ signals that peaked at 92 % higher percentages agglomeration of ZnO-NPs and other carbon elements comprised a portion of 5 % present in Figure. 5. Fe-SEM study further confirmed which were clearly indicated spherical, crystalline, and poly-dispersed TOZnO-NPs sizes of 20 nm, which is well matched with the measured crystal diameter obtained from XRD pattern. The magnified image showed rectangular morphology of ZnO-NPs as indicated in Figure. 6 also illustrated that synthesized nanoparticles self-assembled to form agglomerates due to the polarity and electrostatic attraction.

The FTIR spectra of biosynthesized TOZnO-NPs and the control spectrum of seaweed extract recorded in the range of 400 - 4000 cm^{-1} shows in Figure. 7. FTIR absorption peak at 3432, 1609 cm^{-1} ascribed to the presence of O-H Stretching mode (Yuvakkumar et al., 2014). Two strong sharp peaks appears around 1402 cm^{-1} and 1609 cm^{-1} corresponds to C-H hydroxyl group and C=O carbonyl group (Jeyabharathi et al., 2017). Significant FTIR band observed at lower wavenumber i.e., below 500 cm^{-1} represents Zn-O stretching vibration mode (Matinise et al., 2017).

The selected HPB stains were evaluated against green TOZnO-NPs. Anti-microbial effect was based on the configuration, shape, size, species-specific characteristics and concentrations of nano-compounds which play an important role in pertaining antagonistic activity (Wang et al., 2017). The maximum-inhibition zone of 16 mm was obtained against Gram-negative *Escherichia coli* (NCIM 2931) whereas a minimum-inhibition zone 12 mm was obtained against Gram-positive *Staphylococcus aureus* (NCIM 5021) probably due to their EPS secretion in Table. 1. Gram-positive bacteria have thick wall composed of multilayers of peptidoglycan compare to negative bacteria complex cell wall structure with a layer of peptidoglycan between outer membrane and cytoplasmic membrane (Shanmugam et al., 2014; Rocaet al., 2015). In both cases, anti-microbial mechanism of TOZnO-NPs employed a biphasic phenomenon persuading by osmotic shock which damage the cell membranes thereby internalization of TOZnO-NPs inside the cell eventually induce ROS followed by oxidative stress and cell death (Costaet al., 2018).

4. Conclusion

Green process has been developed by using marine macroalgae brown *Turbinaria ornata* (TO) mediate zinc oxide nanoparticles through a complete green synthetic method. Structural and textural features of green synthesized TOZnO-NPs were characterized by various analytical techniques. Anti-microbial screening higher inhibition activity occurred 16 mm was observed against *Escherichia coli* (NCIM 2931) and minimal 12 mm in *Staphylococcus aureus* (NCIM 5021) due to the secretion of extracellular polymeric substance (EPS) from Gram-positive bacteria. The results have suggested that modification of TOZnO-NPs can efficiently target and kill both Gram-positive and negative bacteria. It will be a suitable paves the way for environmental the biosynthesized ZnO-NPs prepared from *T. ornata* is expected to have notable applications in pharmaceutical industries and biomedical fields.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Legends to table and figure captions:

Table 1: *In-vitro* antibacterial activity of green synthesized TOZnO-NPs against food-borne pathogen (F-BP) bacteria.

Figures:

Figure 1: Visible observation color change of synthesized green TOZnO-NPs
Figure 2: UV–vis spectroscopy
Figure 3: XRD pattern mixed phase of face-centred cubic (fcc) structures
Figure 4: SEM observation particle size and distribution of synthesized green TOZnO-NPs
Figure 6: Fe-SEM observation of synthesized green TOZnO-NPs.
Figure 7: FTIR spectra of seaweed extract (alone) and green synthesized TOZnO-NPs from *Turbinaria ornata* (TO) seaweed extract.

Figure 1: Visible observation color change of synthesized green TOZnO-NPs

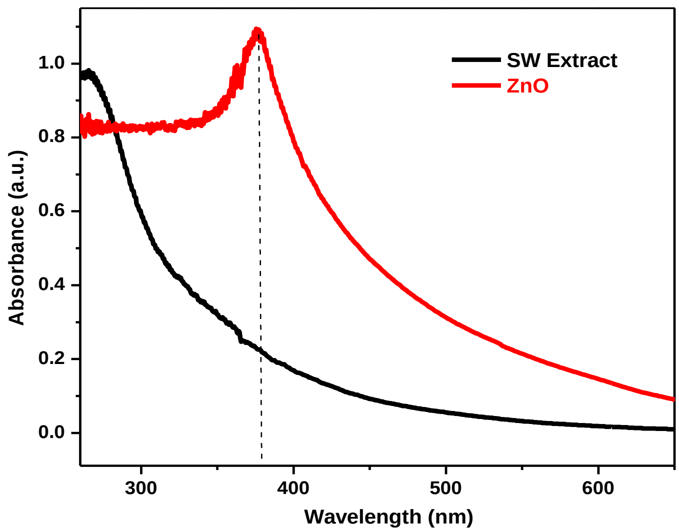

Figure 2: UV–vis spectroscopy

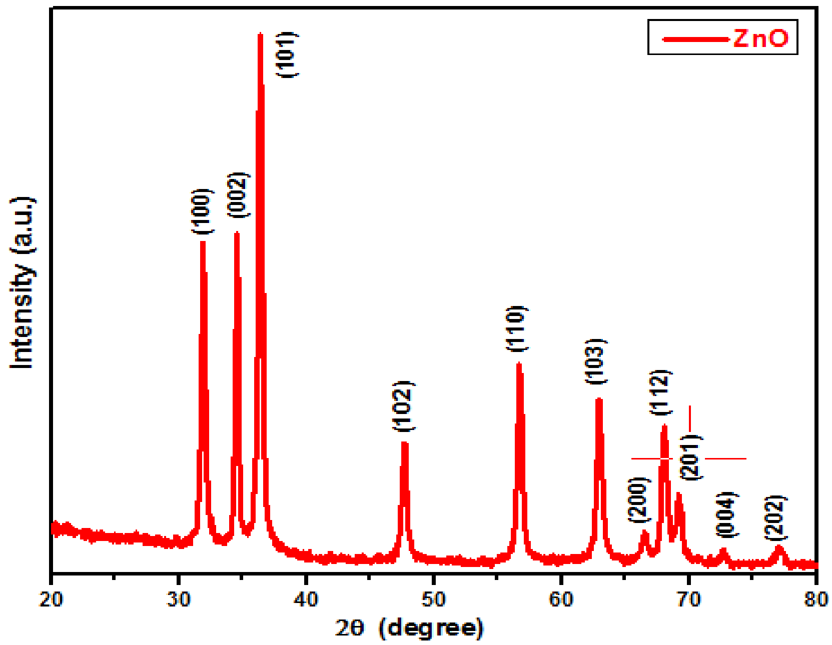

Figure 3: XRD pattern mixed phase of face-centred cubic (fcc) structures

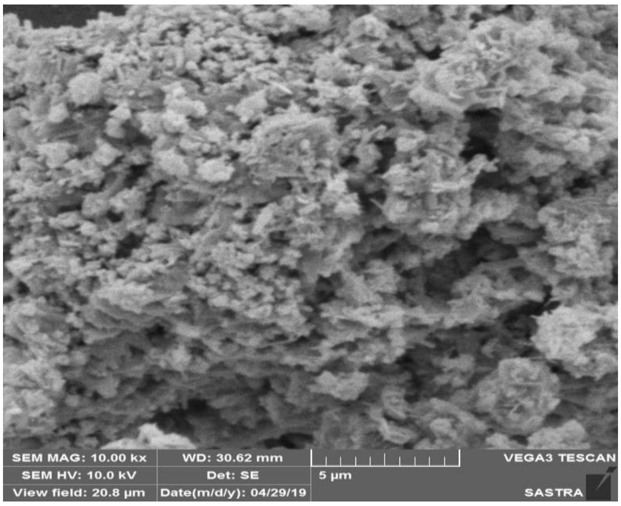

Figure 4: SEM observation particle size and distribution of synthesized green TOZnO-NPs

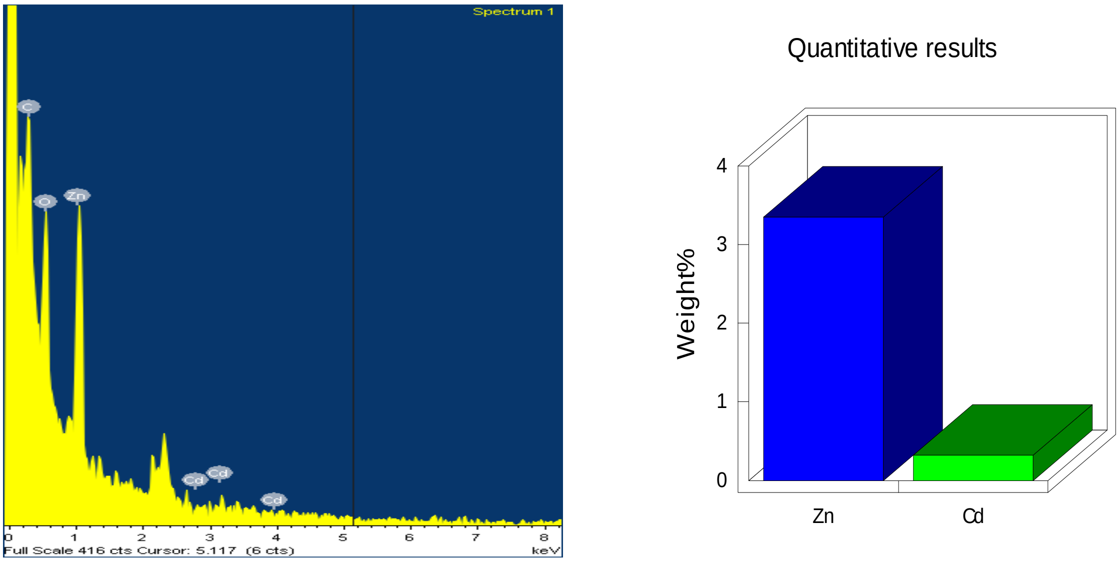

Figure 5: EDS Spectrum image of green synthesized TOZnO-NPs

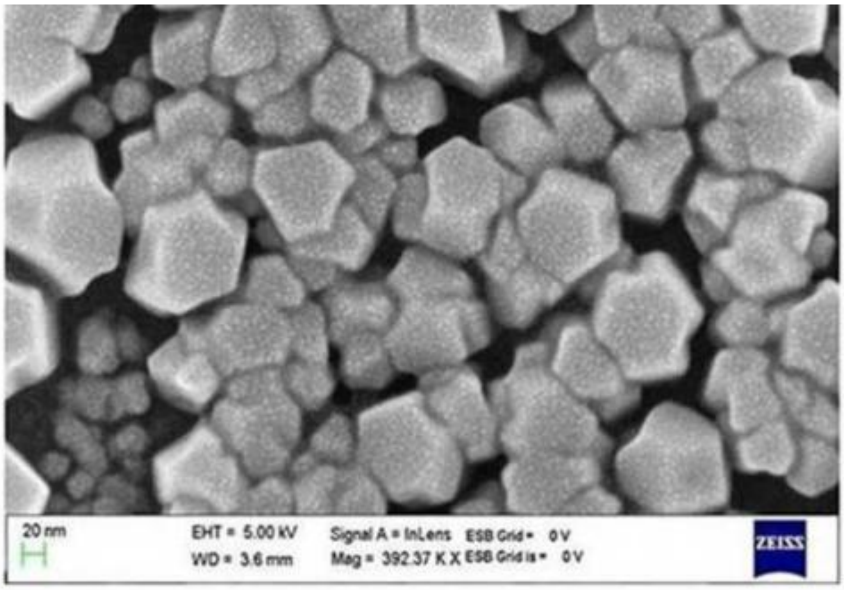

Figure 6: Fe-SEM observation of synthesized green TOZnO-NPs.

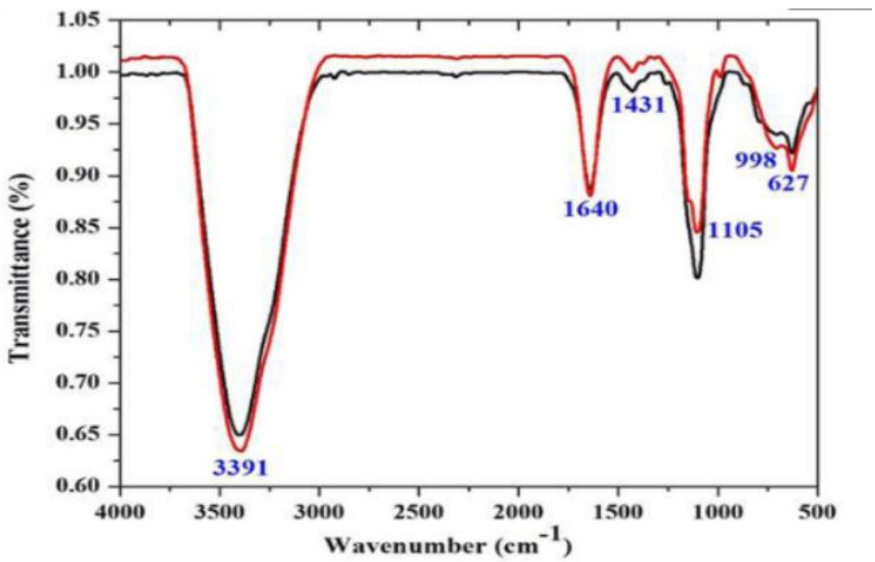

Figure 7: FTIR spectra of seaweed extract (alone) and green synthesized TOZnO-NPs from *Turbinaria ornata* (TO) seaweed extract.

Table 1: *In-vitro* antibacterial activity of green synthesized TOZnO-NPs against food-borne pathogen (F-BP) bacteria.

S. No	food-borne pathogen (F-BP) bacteria	Inhibition zones (mm) of green synthesized TOZnO-NPs			
		20 µg mL^{-1}	40 µg mL^{-1}	60 µg mL^{-1}	80 µg mL^{-1}
Gram Negative					
	Escherichia coli	10	10	14	16
	Klebsiella pneumonia	08	08	12	14
	Proteus mirabilis	06	10	13	14
	Pseudomonas aeruginosa	08	10	12	13
	Salmonella typhimurium	08	08	12	14
	Vibrio cholera	06	08	12	13
Gram Positive					
	Bacillus subtilis	00	06	08	10
	Micrococcus luteus	00	06	06	08
	Staphylococcus aureus	06	08	10	12
	Staphylococcus epidermis	06	08	10	10