BIOSYNTHESIS OF ZINC OXIDE NANOPARTICLESUSING *CALOTROPIS GIGANTEA*L. (MA-YO-GYI),CHARACTERIZATION AND ANTIMICROBIAL ACTIVITIES

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ABSTRACT

This research deals with biosynthesis of zinc oxide nanoparticles using *Calotropis gigantea*L. (ma-yo-gyi), characterization and antimicrobial activities. The aim of this research is to synthesize, characterize and study the antimicrobial activities of biosynthesized zinc oxide nanoparticles. Leaves of *Calotropis gigantea* L. (Ma-yo-gyi) were collected from Kathitkan village in Aung Lan Township, Magway Region and identified in Department of Botany, Pyay University. The phytochemical screening of aqueous leaves extract of *Calotropis gigantea* L. was carried out by test tube method and aqueous leaves extract was also characterized by FT IR technique. Zinc oxide nanoparticles were biosynthesized fromzinc nitrate by method Iand method II using aqueous leaves extract of *Calotropis gigantea* L. as a reducing agent. Thermal stability of green synthesized zinc oxide nanoparticles by method I was studied by Thermogravimetric-Differential Thermal Analyzer (TG-DTA). The effects of calcination temperature on crystallize sizes of biosynthesized ZnO nanoparticles by method I was also studied. The crystallinity and purity of biosynthesized zinc oxide nanoparticles by methods I and II werestudied by X-ray Diffraction (XRD) technique. The biosynthesized ZnO nanoparticles by both methods were found to be crystalline in nature and which has hexagonal structure. The average crystallize sizes were calculated by using Debye-Scherrer equation and found to be in the range of (25-33) nm. The antimicrobial activities of biosynthesized ZnO nanoparticles on six microorganisms such as *B. subtilis, B. pumilus, C. albicans, S. aureus, E. coli* and *P. aeruginosa* were studied by ager well diffusion method. It was found that the high antimicrobial activities of biosynthesized ZnO NPs (23-26 mm) on gram positive and negative bacteria strain except *P. Aeruginosa* and also high activity on fungus like *Candida albicans.* For the investigation of the storage time of guava and tomato fruits at room temperature, biosynthesized ZnO nanoparticles coated on surface of these fruits and found to be fresh without any moltuntilone month.

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Keywords ZnO nanoparticles, biosynthesis, *Calotropis gigantea*L., antimicrobial activity

Introduction

Nowadays, nanotechnology has grown to be an important research field in all areas. For several years, scientists have constantly explored different synthetic methods to synthesize nanoparticles (Sridhara *et al*., 2013).Because of their unique physicochemical and optoelectronic properties, nanoparticles are of particular interest for a number of applications as catalysts, chemical sensors, electronic components, medical diagnostic imaging, pharmaceutical products, and medical treatment protocols. Conventional synthesis of nanoparticles can involve expensive chemical and physical processes that often use toxic materials with potential hazards such as environmental toxicity, cytotoxicity and carcinogenicity(Alagumuthu and Kirubha, 2012). Use of biological organisms such as microorganisms, plant extract or plant biomass could be an alternative to chemical and physical methods for the production of nanoparticles (Sridhara *et al*., 2013). In comparison with microorganisms, the plant approach is more advantageous since it does not need any special, complex and multi-step structures. Furthermore, synthesis in plants tends to be faster than microorganisms, is more cost-effective and is relatively easy to scale up for the production of large quantities of nanoparticles. Plant extracts containing bioactive alkaloids, phenolic acids, polyphenols, proteins, sugars and terpenoids are believed to have an important role in first reducing the metallic ions and then stabilizing them (Shah *et al*., 2015). Zinc oxide nanoparticles stand out as one of the most versatile materials, due to their diverse properties, functionalities and applications. They also possess antimicrobial actions against some bacteria and fungi. As far as synthesis of zinc oxide nanoparticles is concerned they can be synthesized by chemical methods but in recent times due to evolution of green chemistry, biogenic synthesis of ZnO nanoparticles is also possible by using different plant extracts. The biosynthesis of ZnO nanoparticles is much safer and environment friendly compared to chemical synthesis because it does not lead to formation of toxic byproduct (Sabir *et al.*, 2014). The aim of this research is to

synthesize, characterize and study the antimicrobial activities ofbiosynthesized zinc oxide nanoparticles using plants extracts.

Scientific Classificationof *Calotropisgigantea L.* (Chandrabhan *et al.*, 2011)

Kingdom	:	Plantae
Order	:	Gentianales
Family	:	Apocynaceae
Subfamily	:	Asclepiadoideae
Genus	:	*Calotropis*
Species	:	*C. gigantean*
Botanical name	:	*Calotropisgigantea*L.
Myanmar name	:	Ma-yo-gyi

Materials And Methods

Preparation and Characterization of Aqueous Leaves Extract of *C.gigantea*

The extract of *C.gigantea* leaves was prepared by placing 20 g of clean and driedcrushed leaves in 250 mL glass beaker along with 200 mL of distilled water. The mixture was then boiled for 20 min until the color of aqueous solution changed from watery to brown-yellow. Then, the mixture was cooled to room temperature and filtered with Whatman No. 1 filter paper. The presence of functional groups in the aqueous leaves extract sample was investigated by test tube method and characterized by FT IR technique.

Preparation and Characterization of Zinc Oxide Nanoparticles

For the synthesis of zinc oxide nanoparticles by (Method I) 50 mL of *C.gigantea* leaves extract was taken and heated to 60-80ºC using a magnetic stirrer heater. 5 g of zinc nitrate was added to the above solution and maintained the temperatures at about 80ºC. This mixture was continue heated until it reduced to a deep yellow colored paste and then collected in a ceramic crucible and a light yellow colored powder was obtained by calcination at

200ºC, 300ºC, 400ºC, 500ºC and 600ºC for 2 h in muffle furnace (Sabir *et al*., 2014).

Method II was also used for biosynthesis of zinc oxide nanoparticles. A 50 mL of *C.gigantea* leaves extract was heated to 60-80ºC using a magnetic stirrer heater. 5 g of zinc nitrate was added and this mixture is then stirred and heated to achieve good homogeneity. The mixture was heated without stirring to obtain gel and then heated to obtain finally the desired product ZnO in the form of ash powder. A pale yellow colored powder was calcined in muffle furnace at 500ºC for 2 h and finally white powder ZnO was obtained. The biosynthesized zinc oxide nanoparticles were characterized by TG-DTA and XRD techniques.

Study on Antimicrobial Activities of Zinc Oxide Nanoparticles

One mL each of the bacterial suspension of 24 h of nutrient agar was added in each of the petri-dishes. Immediately after hardening of the agar well were made with a 10 mm sterile cork borer from each seeded agar. After removing the agar disks, the wells were incubated at 37°C for 18-24 h. The diameters of the inhibition zone were measured and recorded in mm.The well plate diffusion method was used to test the antibacterial action of the zinc oxide nanoparticles on 24 h broth culture of the organisms used.

Determination of Storage Time of Fruits at Room Temperature by Coating withZnO Nanoparticles

Storage time of guava and tomato fruits at room temperature were investigated by coating the solution of biosynthesized ZnO nanoparticles (0.01 g in 100 mL distilled water) on surface of fruits by layer coating method. The test samples were marked as control for non-coating and S and S_1 for coating with nanoparticles synthesized by methods I and II, respectively. All samples were kept at normal room temperature for observation of results.

Results and Discussion

PhytochemicalsPresent in Aqueous Extracts of *C. gigantea*Leaves and Characterization

The aqueous extract of *C.gigantea* leaves was screened for the presence of various bioactive phytochemicals. The analysis revealed the presence of glycosides, alkaloids, polyphenols, flavonoids, tannins, saponins and α-amino acids in these aqueous leaves extract (Table 1). The functional groups present in aqueous extract of *C.gigantea* leaves were determined by FT IR techniqueas shown in Table 2 and Figure 1. The broaden peaks at 3342 cm^{-1} corresponds to the stretching vibration of OH group. The band at 2941 cm^{-1}corresponds to stretching vibrations of CH group. The bands at 1599 cm^{-1} can be attributed to C=C stretching vibrations of aromatic compounds. The spectrum showed bands at 1394 cm^{-1} corresponding to the sp^3 C–H bending of alkanes. The bands at 1192 and 1109cm^{-1} can be attributed to C-O stretching vibration. The spectrum showed bands at 655 and 617cm^{-1} corresponding to C-H bending for H-bond to sp^2 carbon (Coates, 2000). These are tabulated in Table 2.

Table 1 Phytochemicals Screening in Aqueous Leaves Extracts of *C. gigantea*

No.	Tests	Test Reagents	Observations	Remark
1	Glycosides	10% Lead acetate solution	White ppt.	+
2	Carbohydrates	10% α-naphthol, conc: H_2SO_4	Red ring not form	ND
3	Alkaloids	Mayer's reagent	Orange colour	+
4	Polyphenols	10%$FeCl_3$, 5%$[K_3Fe(CN)_6]$	Blue black colour	+
5	Flavonoids	Conc:HCl, Conc:H_2SO_4, Mg ribbon	Yellow colour	+
6	Tannins	Gelatin, 1%$FeCl_3$	White ppt.	+
7	Saponins	Distilled water	Frothing	+
8	Steroids	Acetic anhydride, Conc: H_2SO_4	No greenish blue colour	ND
9	α-Amino acids	Ninhydrin	Purple spot	+

10	Reducing sugars	Benedict's solution	No brick-red ppt.	ND

(+) present (ND) Not Detected

Table 2 FT IR Data of Leaves Extract of *C.gigantea*

No.	Wavenumber (cm^{-1})		Band Assignment
	Observed Values	Literature Values*	
1	3342	3650-3200	-OH stretching
2	2941	2960-2850	-CH stretching
3	1599	1650-1550	C=Cstretching
4	1394	1400-1393	C-H bending
5	1192, 1109	1250-1050	C-O stretching
6	655, 617	1000-600	C-H bending

*Coates, 2000

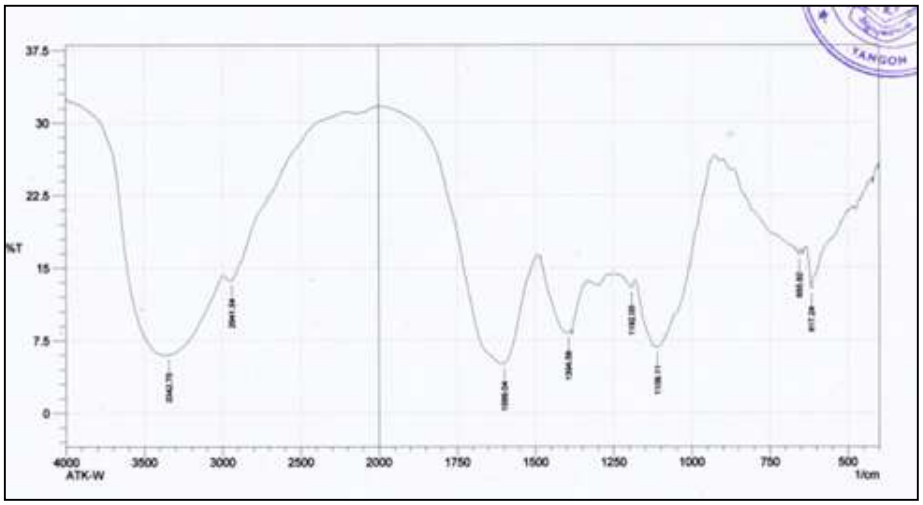

Figure1 FT IR spectrum of aqueous leaves extract of *C.gigantea*

Characterization of Biosynthesized Zinc Oxide Nanoparticles before

Calcination TG-DTA analysis

Thermal stability of biosynthesizedzinc oxide by method I was investigated by TG-DTA technique and the resultant TG-DTA thermogram is shown in Figure 2. TG-DTA data of the prepared zinc oxide nanoparticles before calcinations showed the total weight loss 52.98% in the temperature range of 38.59-601.76°C. This is attributed to the evaporation of trapped water in the crystal, decomposition of organic residue and zinc nitrate to zinc oxide in the preparation of biosynthesized zinc oxide. The TG-DTA thermogram indicated the two endothermic peaks at 139.56°C and 198.98°C corresponding to the loss of trapped water anddecomposition of organic matter in sample, respectively. The exothermic peak at 369.97°C was related with complete transition of zinc nitrate to crystalline zinc oxide nanoparticles.TG-DTA data indicated that the biosynthesized zinc oxide was found to be thermally stable in the temperature range of 460-500°C.

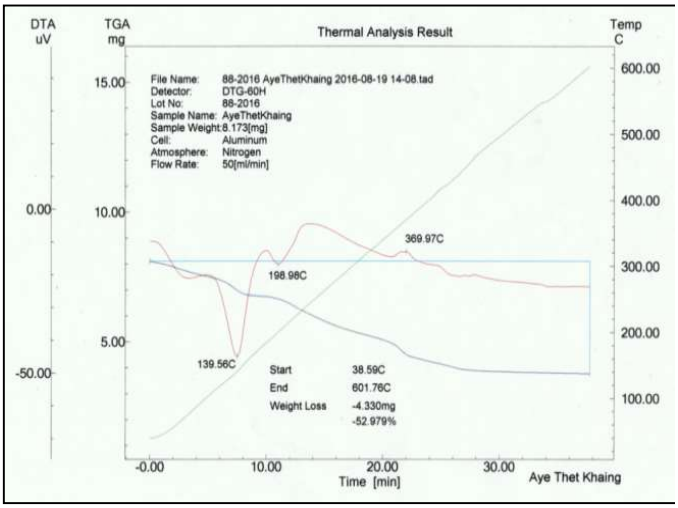

Figure 2 TG-DTA thermogram of biosynthesized ZnO nanoparticles by method I before calcination

XRD analysis

The structures of the zinc oxide nanoparticles biosynthesized by methods I and II before calcination were studied by XRD technique (Figures 3 and 4). In Figure 3, the XRD pattern of zinc oxide nanoparticles is matched with PDF file of 89-0138 of zinc hydroxide, $Zn(OH)_2$. It can be seen that some noise peaks, other peaks and broadness of the peak which indicated the amorphous nature and the presence of phytochemical and impurities in the prepared ZnO nanoparticles. Figure 4 shows the XRD pattern of zinc oxide nanoparticles biosynthesized by method II. It can be seen that in addition to characteristic peaks of ZnO, the presence of impurity peaks were observed because of the presence of phytochemicals and impurities in the prepared ZnO nanoparticles.

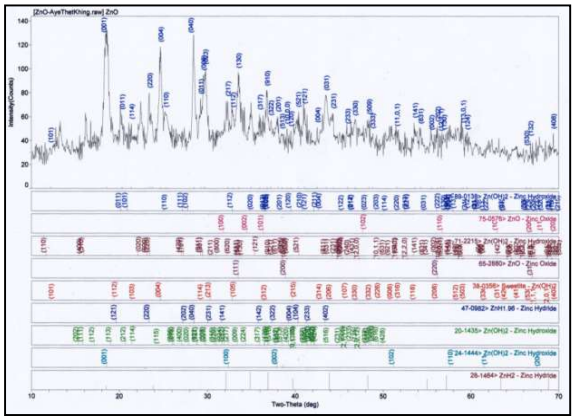

Figure 3 XRD diffractogram of biosynthesized ZnO particles by method I before calcination

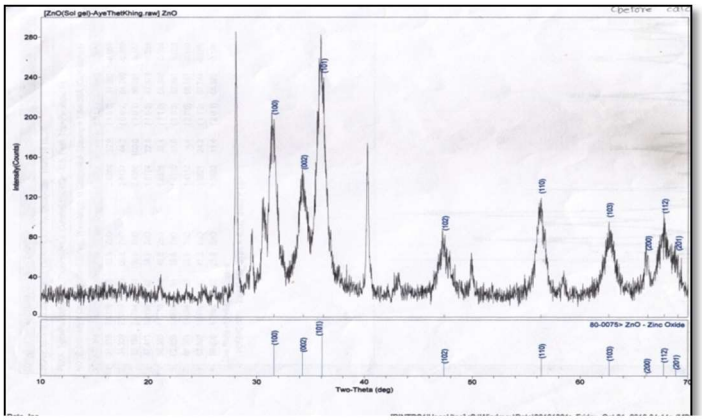

Figure 4 XRD diffractogram of biosynthesized ZnOparticles by method II before calcination

Characterization of Biosynthesized Zinc Oxide Nanoparticles after Calcination XRD analysis

The Powder XRD patterns of the ZnO samples calcined at different temperatures are shown in Figure 5. When the temperature was increased, decomposition began and hexagonal ZnO started to appear. The hexagonal ZnO crystal was initially detected at 300°C and broad diffraction peaks became nice and sharp at both 500 and 600°C. The average lattice constant of ZnO nanoparticles calcined at 500 °C and 600 °C were found to be a, b=3.2534Å, c=5.2118Å and a, b=3.2736Å, c=5.2255Å, respectively. The average lattice constants of ZnO nanoparticles calcined at 500 °C are close to literature value of a, b=3.2475 to 3.2501 Å, c=5.2042 to 5.2075 Å. This result is consistent with DTA data. In DTA curve, the phase transition temperature was found to be 369.97°C. Therefore, it can be seen that ZnO nanoparticles with well-developed crystal structures appeared at the calcination temperature between 400 and 500°C. From these studies, in order to get pure crystal of hexagonal ZnO, the calcination temperature of about 500°C was chosen.

The effect of calcination on crystalized structure of biosynthesized zinc oxide nanoparticles by method II was also studied at the calcination temperature of 500°C. crystalized structure of the zinc oxide nanoparticles biosynthesized by method II at 500°C were analysed by X-ray diffractometer (Figure 6). There are nine typical diffraction peaks, (100) at 31.557°, (002) at 34.227°, (101) at 36.051°, (102) at 47.344°, (110) at 56.365°,(103) at 62.662°, (200) at 66.272°,(112) at 67.680° and (201) at 68.933° which may be assigned to the characteristic peaks of hexagonal ZnO crystal and are matched with library card number89-1397 ZnO. High purity and crystallinity of ZnO nanoparticles was obtained at that temperature.

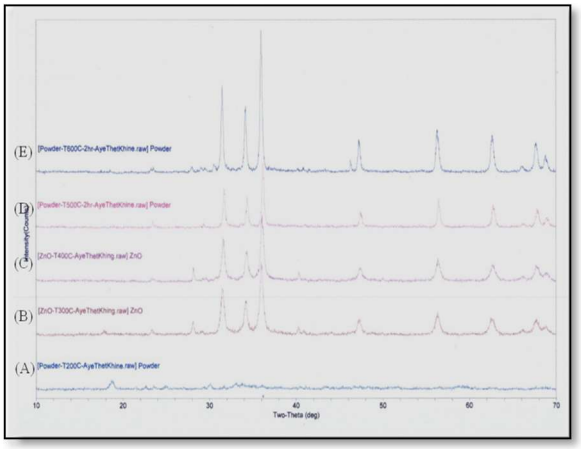

Figure 5 XRD diffractograms of biosynthesized ZnO nanoparticles by methodI calcined at (A) 200°C (B) at 300°C (C) at 400°C (D) at 500°C (E) at 600°C for 2 h

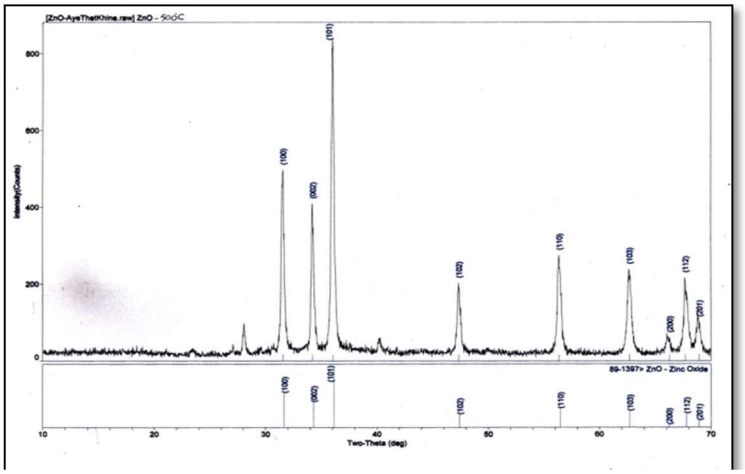

Figure 6 XRD diffractogram of biosynthesized ZnO nanoparticlesprepared by method II calcined at 500 °C for 2 h

Average Crystallite Sizes of Biosynthesized Zinc Oxide Nanoparticles

The average crystallite sizes of biosynthesized zinc oxide particles were calculated by using Debye-Scherrer equation, $t=0{\cdot}9\lambda/\beta\cos\theta$, where t is the crystallite size, λ the wavelength of the X-ray used, β is the full width at half maximum (FWHM) of the peak inradians and θ is the diffraction angle or the Bragg angle of the peak (Jenkins and Snyder, 1996). After calcined at 400°C, amorphous nature disappeared and crystalline nature of ZnO appeared but its crystal structure has some impurities peaks. When the calcination temperature was higher than 500°C, the XRD patterns showed the strong diffraction peaks of ZnO. Moreover, the characteristic peaks of ZnO became sharper and stronger when the calcination temperatures change from 300 °C to 400°C, 500°C and 600°C, indicating that ZnO are getting better nanocrystalline size. The data revealed that the crystallite size increased with the increase in the final calcination temperature. Their difference in crystallization and crystallite size could be mainly attributed to the relative calcination temperature. The average crystallite sizes of biosynthesized zinc oxide nanoparticles by method I at different calcination temperatures were calculated to be 24.82 nm at 300°C, 25.99 nm at 400°C, 31.26 nm at 500°C and 30.31 nm at 600°C. It was found that average crystallite size increased with increase in calcination temperature up to 500°C but decreased in calcination temperature of 600°C which may be due to agglomeration in the calcinations process. However, the average crystallite size of biosynthesized zinc oxide nanoparticles by method II after calcination at 500°C found to be 27.00 nm. Similarly, the lattice parameters of ZnO nanoparticles were much closed to literature value at calcination temperature of 500°C. The data are shown in Table 3. In order to obtain smaller ZnO particles with well-developed crystal structures, calcination

temperature of 500°C was chosen for the study of the application of ZnO nanoparticles.

Table 3 Average Crystallite Size and Lattice Parameters of Biosynthesized Zinc Oxide Nanoparticles at Different Calcination Temperatures

Method	Calcination temperature (°C)	Average crystallite size (nm)	Lattice Parameters(Å)	
			a=b	c
Method I	300	24.82	3.2709	5.2215
	400	25.99	3.2636	5.2116
	500	31.26	3.2534	5.2118
	600	30.31	3.2736	5.2255
Method II	500	27.00	3.2692	5.2200

Antimicrobial Activities of Zinc Oxide Nanoparticles

Antimicrobial activities of biosynthesized zinc oxide nanoparticles using aqueous leaves extract of *C. gigantean* prepared by methods I and II against bacteria like *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *E. coli* and fungi like *Candida albicans* were quantitatively evaluated in culture media by using Agar well diffusion method. It was observed that the growth of inhibition zones of all bacteria and fungi were solely high in both biologically synthesized zinc oxide nanoparticles except *Pseudomonas aeruginosa*. High antimicrobial activities was observed against *Bacillus subtilis* (25 mm), *Staphylococcus aureus* (24 mm), *Bacillus*

pumilus (23 mm), *E. coli* (25 mm), *Candida albicans* (23 mm) and low activity was shown against *Pseudomonas aeruginosa* (16 mm) by using zinc oxide nanoparticles biosynthesized by method I. Similarly, high antimicrobial activity was observed against *Bacillus subtilis* (26 mm), *Staphylococcus aureus* (24 mm), *Bacillus pumilus* (25 mm), *E.coli* (24 mm), *Candida albicans* (26 mm) and low activity was shown against *Pseudomonas aeruginosa* (14 mm) by using zinc oxide nanoparticles biosynthesized by method II. The results are shown in Table 4 and Figure 7. Antimicrobial effects of zinc oxide nanoparticles obeyed a dual action mechanism of antimicrobial activity i.e, the bacterial and fungicidal effect of zinc ions membrane disrupting effect of the polymer subunits. The microbial activity of particles depends on the stability in the cultured medium too. The result confirmed that the treated microbial cells were damaged showing leakage of proteins and nucleic acid into nutrient agar media. These particles which can be prepared in a simple, rapid and cost effective manner are suitable for the formulation of new types of microbial materials.

Basically the detected active oxygen species generated by these metal oxide particles could be the main mechanism of their antibacterial activity. The antibacterial mechanism of ZnONPs involves the direct interaction between ZnO nanoparticles and cell surfaces affecting cell membrane permeability; afterwards these nanoparticles enter and induce oxidative stress in bacterial cells, which results in thein hibition of cell growth and eventually cell death; the demonstrated antibacterial activity of ZnONP recommends its possible application in the food preservation field. It can be applied as a potent sanitizing agent for disinfecting and sterilizing food industry equipment and containers against the attack and contamination with foodborne pathogenic bacteria. The NPs of ZnO showed both toxicity on pathogenic bacteria and beneficial effects on microbes, which has bioremediation potential and is a strong root colonizer (Molina *et al*., 2006).

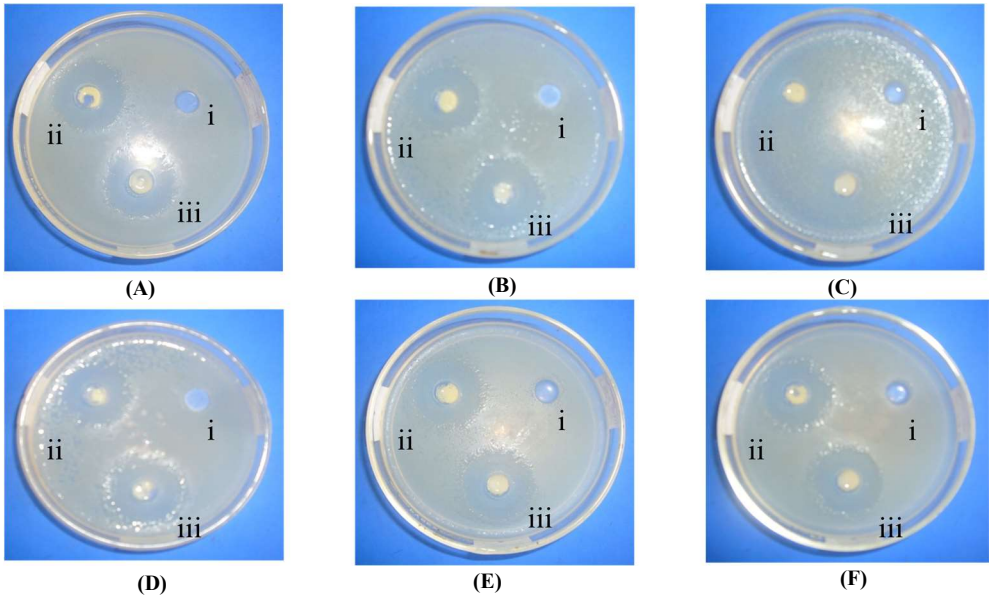

Figure7 Antimicrobial activitivies of biosynthesized zinc oxide nanoparticles on six microorganisms (A) *Bacillus subtilis* (B) *Staphylococcus aureus* (C) *Pseudomonas aeruginosa* (D) *Bacillus pumilus* (E) *Candida albicans* (F) *E. coli*
(i) Control, (ii) ZnO NPs (method I), (iii) ZnO NPs (method II)

Table 4 Antimicrobial Activity of Biosynthesized Zinc Oxide Nanoparticles on Different Species of Microorganism (Agar Well Diffusion Method)

Microorganisms	**Diameter of inhibition zone (mm)**	
	Method I	**Method II**
Bacillus subtilis	25(+++)	26(+++)
Staphylococcus aureus	24(+++)	24(+++)

Pseudomonas aeruginosa	16(++)	14(+)
Bacillus pumilus	23(+++)	25(+++)
Candida albicans	23(+++)	24(+++)

Agar well - 10 mm, 10 mm ~ 14 mm (+), 15 mm ~ 19 mm (++), 20 mm above (+++)

Coating of Biosynthesized Zinc Oxide Nanoparticles on Fruits

Storage time of guava and tomato fruits at room temperature were investigated by coating ZnO nanoparticles on surface of fruits by layer coating method. The test samples were marked as control for non-coating and S and S_1 for coating with zinc oxide nanoparticles biosynthesized by methods I and II as shown in Figure 8. All samples were kept at normal room temperature for observation of results. Although the control sample keeping after tomato for 15 days and guava for 20 days at room temperature start to rot, the tested samples without any molt were found to be fresher than the controlled until one monthstorage. It can be deduced that coating the fruits with biosynthesized ZnO nanoparticles have highly resistant to oxidation (Jeong *et al.,* 2005).

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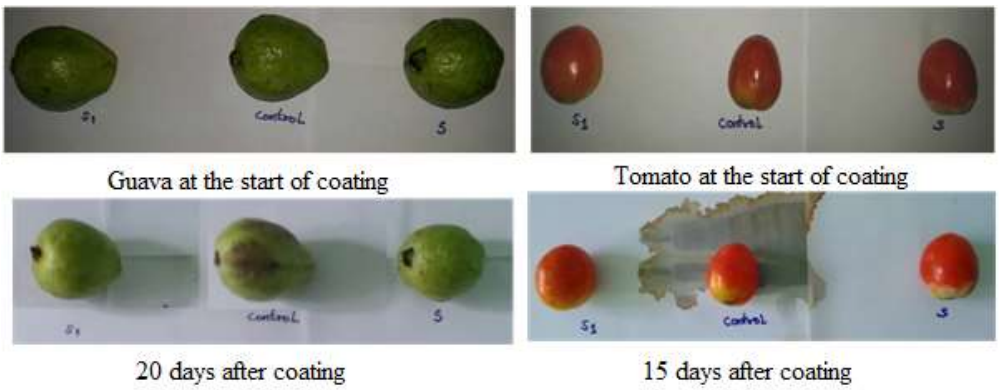

Figure 8 Coating of biosynthesized ZnO nanoparticles on guava and tomato S and S_1 = fruits coating of ZnO NPs biosynthesized by methodsI and II respectively

Conclusion

ZnO nanoparticles were successfully biosynthesized from zinc nitrate by methods I and II. Aqueous leaves extract of *C. gigantean* (Ma-yo-gyi) was used as reducing and stabilizing agents. Phytochemical screening of aqueous leaves extracts of *C.gigantea* (Ma-yo-gyi) indicated the presence of phytochemicals such as α-amino acids, glycoides, polyphenols, saponins, alkaloids and flavonoids. FT IR confirms the presence of above phytochemicals in aqueous leaves extracts of *C.gigantea* which can stabilized the nanoparticles. From TG-DTA data, decomposition of $Zn(NO)_3$ to ZnO start at 369.9ºC andcomplete formation of ZnO was temperature between 400-500ºC. XRD data confirm complete formation of pure ZnO at 500ºC with hexagonal phase and consistent with literature value of lattice parameters. From study on antimicrobial activities of biosynthesized ZnO nanoparticles, high antimicrobial activities of ZnO nanoparticles on six microorganisms proved to a pronounced influence on inhibiting the growth of both gram positive, gram negative bacteria strain except *P. aeruginosa* and also high activity on fungus like *Candida albicans.* Thus, it is concluded that the reported biosynthesized ZnO nanoparticles can act as an effective antimicrobial agent. Coating of the guava and tomato fruits with biosynthesized zinc oxide nanoparticles (Methods I and II) by layer coating method at room temperature had benefits for the ability to retard fruit pathogens and highly resistant to oxidation, increase the storage time of guava

and tomato at room temperature. From these results, the biosynthesis of zinc oxide nanoparticles by methods I and II are much safer and environment friendly compared to chemical synthesis because it does not lead to formation of toxic by-product chemicals.

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