

An In-vitro Biosynthesis of Zinc Oxide Nanoparticles Using Rich Flavonoid Extract from the Petals of *Delonix regia* and Evaluation of their Antioxidant and Anticancer Properties

Sathyabama S¹, Sankaranarayanan S^{2*}

¹Assistant Professor, Department of Botany, Stella Maris College (Autonomous), Chennai-600 086, Tamil Nadu, India

²Assistant Professor Department of Botany, Government Arts college, Chengam Road, Tiruvannamalai-606 603 Tamil Nadu, India

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ABSTRACT

Objective: To determine the influence of antioxidant and cytotoxicity activities of flavonoid rich fraction from the petals of *Delonix regia* mediated biosynthesis of Zinc oxide nanoparticles (ZnO NPs).

Methods: Synthesized ZnO NPs were estimated total phenolic content and flavonoid from flavonoid rich fraction were determined. The scavenging radicals were estimated by ABTS, lipid peroxide, nitric oxide inhibition, metal chelating method. The synthesized ZnO NPs were characterized by FTIR and SEM.

Results: FTIR spectra of synthesized ZnO NPs exhibited prominent peaks at 3752.5 67.1 cm⁻¹ (alkynes), 1638.2 cm⁻¹, 1400.3 cm⁻¹ (alkanes), and 1052.0 cm⁻¹ (C-O absorption). The morphological characterization of synthesized ZnO NPs was analysed by SEM which showed spherical shape and clusters with an average size of 32.58 nm. The synthesized ZnO NPs showed more antioxidant and anticancer activity than the standard. The plant aqueous extract and synthesized ZnO NPs were found to possess maximum antioxidant activity when compared with ascorbic acid. Total content of phenolic compounds and flavonoid (mg/g) in petal flavonoid rich fraction and synthesized ZnO NPs were found to be 85.4 and 18.3 mg/g, respectively. The flavonoid rich fraction synthesis ZnO NPs showed a dose- and time-dependent inhibitory effect on the growth of A549 (p53 wt) lung cancer cells ($P < 0.05$). IC₅₀ was 12.33 µg/ml, and the maximal inhibition of cell growth (>80%) was obtained at 20 µg/ml. **Conclusions:** Green synthesized ZnO NPs provides a promising approach can satisfy the requirement of large-scale industrial production bearing the advantage of low-cost, eco-friendly and reproducible.

Keywords: ZnO nanoparticles, *Delonix regia*, FTIR, electron microscopy, antioxidant, anticancer.

INTRODUCTION

The field of nanotechnology is one of the most active areas of research in modern materials science. Green-mediated synthesis and characterization of nanoparticles have emerged as a significant division of nanotechnology from the last decade, chiefly from precious metals such as gold, silver, platinum, copper and zinc (Ankamwar et al., 2005). A great deal of effort has been put into the biosynthesis of inorganic material, especially metal nanoparticle using microorganisms and plants (Farooqui et al., 2010). As biomolecules are very sensitive to the solution pH and temperature, there is a general need to synthesize metal oxide semiconducting nanoparticles for possible applications in biological sensing, biological labeling, drug and gene delivery, and nanomedicines (Jain et al., 2005; Visaria et al., 2006). In particular, due to their easy fabrication, environmentally friendly nature, and non-toxic synthesis route, ZnO nanoparticles can provide a better option for various biological applications. However, water solubility and biocompatibility of ZnO nanoparticles are the main requisites for biological applications. In this regard, Bauermann et al. (2006) reported the bio-friendly

synthesis of ZnO nanoparticles in aqueous solution at near neutral pH and low temperature (37 °C). Another approach regarding the use of ZnO nanoparticles in biological applications was recently reported by Hanley et al. (2008). The authors reported the preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. Meeting point on the assorted and valuable biological activities of medicinal plants has worldwide grown in recent years. Medicinal plants not only supply somewhat inexpensive drugs with insignificant side effects, but they are also sources of other beneficial substances including phytochemicals. Phytochemicals are phenolics, polyphenolics and flavonoids, which are known as bioactive compounds responsible for the antioxidant and anticancer properties. Flavonoid extract synthesis nanoparticle system provides a viable option to overcome the problem associated with solubility, bioavailability and biocompatibility with various entrapping systems (Mohan S and Nandhakumar, 2014). *Delonix regia* is an ornamental medium-sized tree, widely planted in avenues and gardens in all warmer and damper parts of India. It is consistently voted among the top five most beautiful

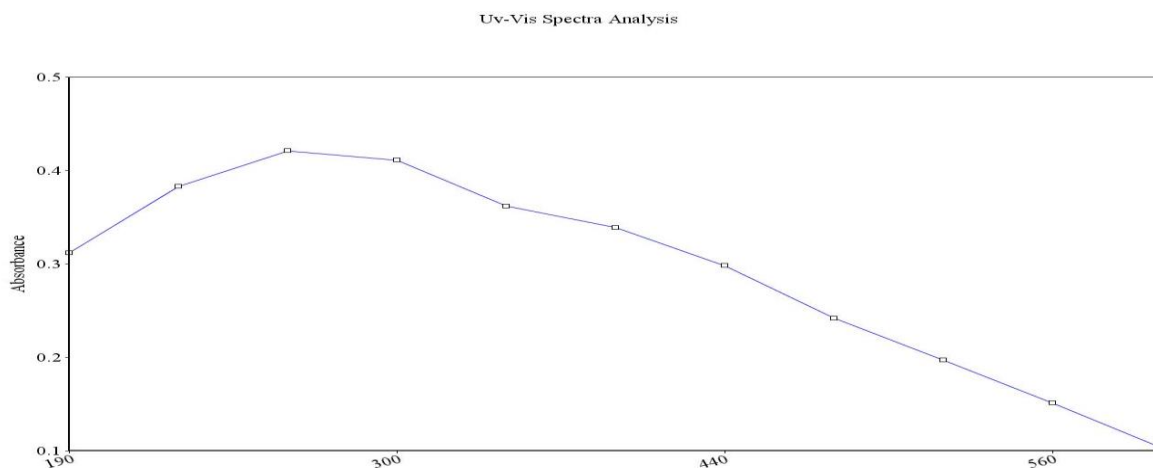


Figure 1: Characterization of ZnO NPs from rich flavonoid extracts of petals *Delonix regia* by UV-VIS absorption spectroscopy

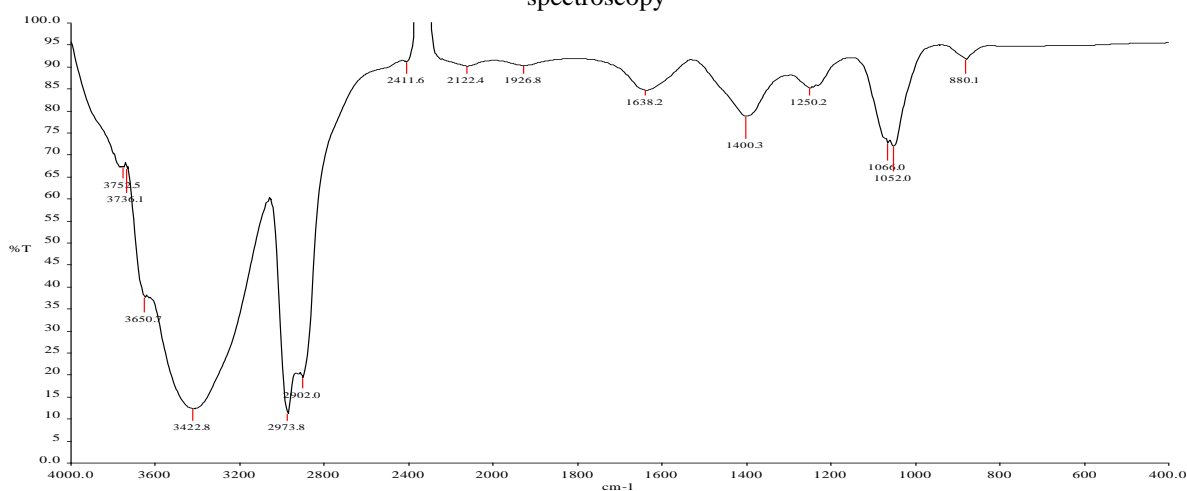


Figure 2: Characterization of ZnO NPs by FTIR

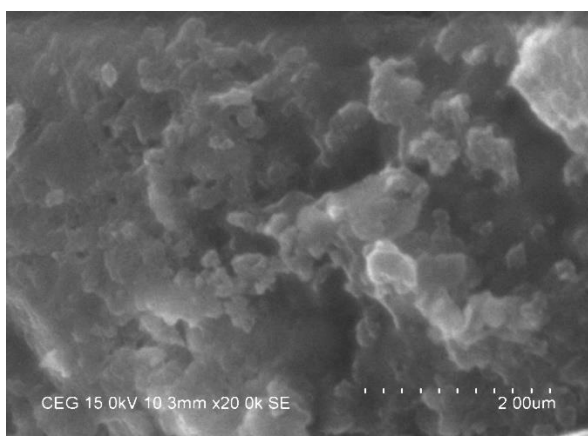


Figure 3: Surface morphology and size of the ZnO NPs

flowering trees in the world. Large red-orange in color having five petals, one petal contains also white color streaks and little bit big as compare to other petals, four spreading scarlet or orange-red petals up to 8 cm long having same size and colour, a fifth upright petal called the standard, which is slightly larger and spotted with yellow and white. It is an impressive range of medicinal and biological properties, has been used in the folk medicine

systems of several civilizations like for the treatment of constipation, inflammation, arthritis, hemiplegia, leucorrhoea and rheumatism (Shabir et al., 2011). The knowledge obtained from this investigation could also assist the design of flavonoid extract synthesis ZnO nanoparticles effective delivery systems for the protection and release of bioactive flavonoids that have potential to improve human health or increase the shelf life of pharmaceutical or food products. This nanoparticulated system may be suitable for their use in pharmaceutical applications and for the development of medical foods in the near future.

MATERIALS AND METHODS

Plant Materials

Petals of *Delonix regia* were collected from Sri Sairam Siddha Medical College and Research Centre, Chennai, Tamil Nadu, India. Plants were authenticated by Dr. S. Sankaranarayanan, Head Department of Medicinal Botany Sri Sairam Siddha Medical College and Research Centre, Chennai, Tamilnadu, India.

Preparation of extract

The shade dried petals was ground to fine powder and sieved. Exactly 100g of the finely grounded petals were soaked in sterile distilled water at room temperature for 24

hrs. The extract was filtered using Whatman filter paper No.1 and centrifuge the solution at 5000 rpm for 10 minutes for removable of residue. The clear water extracts partition with Petroleum ether, and chloroform for elimination other than flavonoid metabolites. Clear water extracts were subjected to quantification of flavanoid.

Determination of total Phenolics

The concentration of total phenolics in the methanol extract of *Delonix regia* were determined by using Folin-Ciocalteu reagent and calibrated externally with gallic acid. Briefly, about 0.2 ml methanol extract and 0.2 ml of Folin-Ciocalteu reagent were added and mixed vigorously. After shaking for 4 min, 1 ml of 15% Na₂CO₃ was added, and finally the mixture was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm using Deep Vision 1371 spectrophotometer. The concentration of the total phenolics was estimated as mg of gallic acid equivalent by using an equation obtained from gallic acid calibration curve. The quantification of phenolic compounds in all the fractions was carried out in triplicate and the results were averaged [Singleton et al., 1999].

Biosynthesis of ZnO nanoparticles

Delonix regia mediated biosynthesis of Zinc oxide nanoparticles and stabilized by following the method of Daneshvar et al. [2008]. Typically, 50 mL of rich flavonoid extract (50 mg/L) at 1.1-fold critical micelle concentration was mixed with 50 mL of 1 mM ZnNO₃. This solution was incubated at 80 °C for 30 min under vigorous stirring to form rich flavonoid extract and Zn complex solution. To obtain the colloidal rich flavonoid extract and ZnO nanoparticles, few drops of 1 M NaOH solution were added under vigorous stirring. The color change was monitored by visual inspection and by measuring absorbance by UV-Vis spectrophotometer (Deep vision, (1371) UV-Vis Spectrophotometer, India). The resulting white precipitate was obtained by centrifugation at 5000 rpm for 10 min, washed with sterilized RO water and dried at 70 °C under vacuum oven. The dried rich flavonoid extract synthesis ZnO nanoparticles were grounded and stored in amber colour container until further use.

Characterization of Synthesized ZnO nanoparticles

The UV absorbance of the synthesized ZnO nanoparticles was measured in UV-100, Spectrophotometer operated at a resolution of 1 nm. Characterization involves FTIR analysis of the synthesized ZnO nanoparticles by scanning it in the range 400-4000 cm⁻¹ at are solution of 4cm⁻¹. These measurements were carried out on a Perkin Elmer spectrum in the diffuse reflectance. The surface morphology of ZnO nanoparticles was examined with the JEOL JEM 2100 high-resolution transmission electron microscope.

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay

ABTS radical scavenging activity of rich flavonoid extract synthesis ZnO nanoparticles were determined according to Re et al [1999]. ABTS radical was freshly prepared by adding 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to yield an

absorbance of 0.70 at 734 nm and the same was used for the antioxidant assay. The final reaction mixture of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Vitamin-C. Similarly, in the test group, 1 ml reaction mixture comprised 950 µl of ABTS solution and 50 µl of the rich flavonoid extract synthesis ZnO nanoparticles solution. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV-Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound.

Inhibition of Lipid Peroxidation activity

Lipid peroxidation induced by Fe₂₊-ascorbate system in egg yolk by the method of Bishayee and Balasubramaniyam [1971], was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al [1979]. The reaction mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20mM, pH 7.0); KCl (30mM); FeSO₄ (NH₄)₂SO₄.7H₂O (0.06mM); and various concentrations of rich flavonoid extract synthesis ZnO nanoparticles in a final volume of 0.5ml. The reaction mixture was incubated at 37°C for 1 h. After the incubation period, 0.4ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The butanol-pyridine layer was removed and its absorbance at 532 nm (Deep Vision (1371) UV-Vis Spectrophotometer) was measured to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard. Inhibition of lipid peroxidation (%) by the rich flavonoid extract synthesis ZnO nanoparticles was calculated according to 1 - (E/C) X 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample ($Abs_{532+TBA} - Abs_{532-TBA}$).

Superoxide radical scavenging assay

This assay was based on the capacity of the rich flavonoid extract synthesis ZnO nanoparticles to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich, 1971) in the presence of the riboflavin-light-NBT system, as described earlier Tripathi and Pandey et al., [1999]; Tripathi and Sharma [1999]. In brief, each 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, NBT (75 µM) and different concentration of test sample solution. It was kept in front of fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV-Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark and served as blanks. The percentage inhibition of superoxide generation was measured by comparing the absorbance of the control and those of the reaction mixture containing test sample solution.

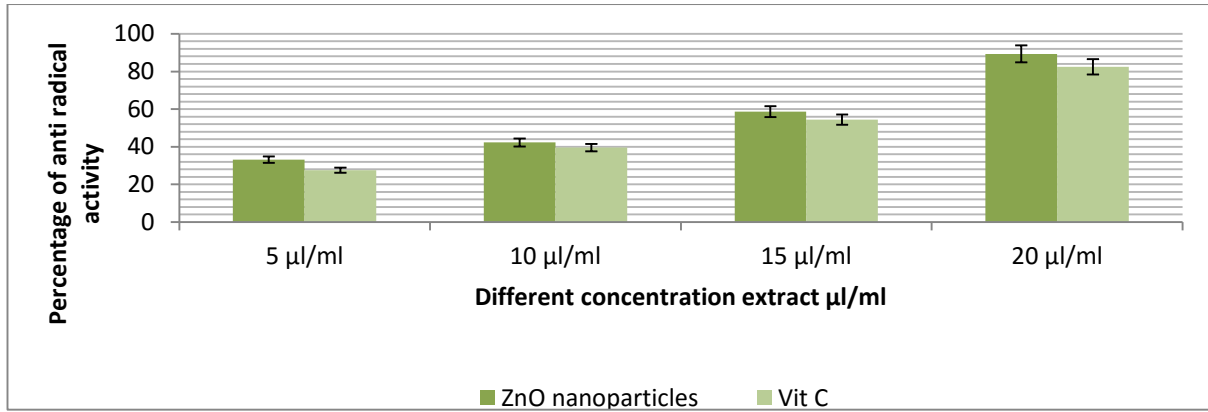


Figure 4: ABTS radical scavenging activity of rich flavonoid extract synthesis ZnO nanoparticles

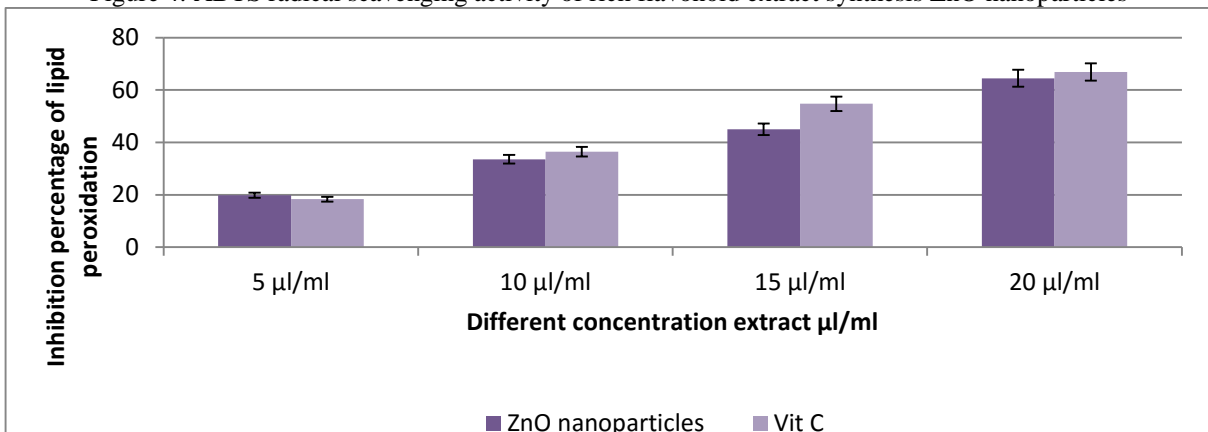


Figure 5: Inhibition of Lipid Peroxidation activity by rich flavonoid extract synthesis ZnO nanoparticles

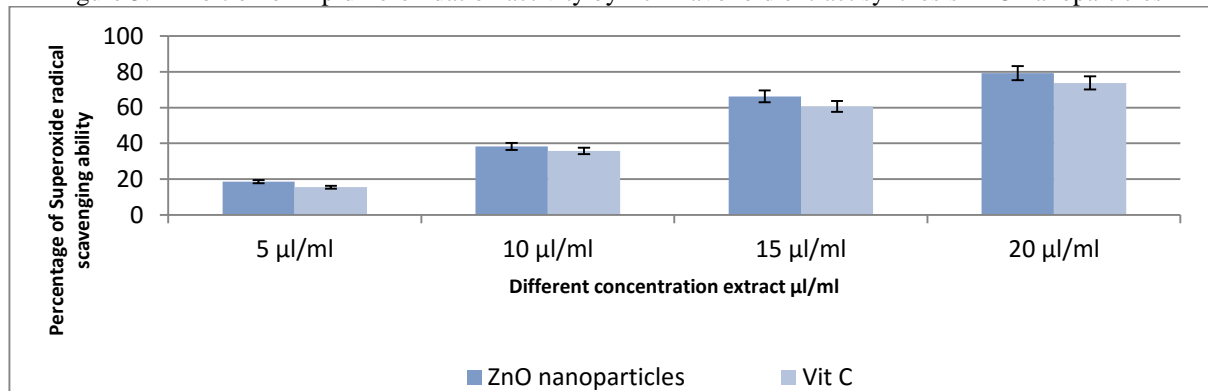


Figure 6: Superoxide radical scavenging activity of rich flavonoid extract synthesis ZnO nanoparticles

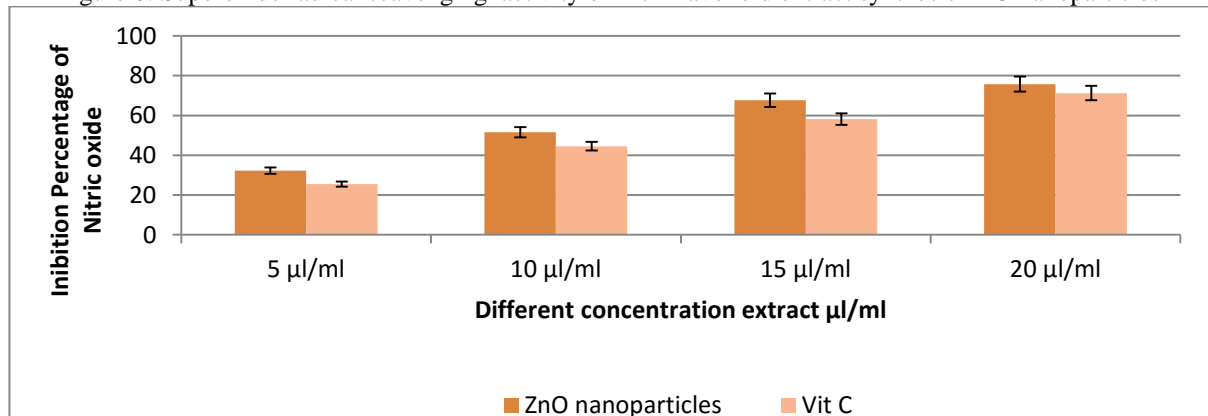


Figure 7: Nitric oxide radical scavenging capacity of rich flavonoid extracts synthesis ZnO nanoparticles

% Super oxide radical scavenging capacity = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid extract or standard.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging capacity of rich flavonoid extracts synthesis ZnO nanoparticles were measured according to the method described by Olabinri et al [2010]. 0.1ml of sodium nitroprusside (10mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of three different solvent extract and incubated at room temperature for 150min. After incubation period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N- (1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the reaction mixture was read at 546nm against blank. All readings were taken in triplicate and Vitamin C was used as the standard. The % inhibition was calculated by following equation.

% Nitric oxide radical scavenging capacity = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 was the absorbance of control and A_1 was the absorbance of different solvent extract.

Cell line and culture

Human lung cancer cells A549 (p53 wt), were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured following their instructions. Human lung cancer cell lines used were: A549 (p53 wt), the cells were grown in RPMI 1640 media.

Cytotoxicity assay Methyl Thiazolyl Tetrazolium (MTT assay)

The cytotoxicity of rich flavonoid extracts synthesis ZnO nanoparticles determined by tetrazolium (MTT) assay (Selvakumaran et al., 2003). Cells (2×10^3 /well) were plated in 100 μ l of medium/well in 96-well plates. After overnight incubation, rich flavonoid extracts synthesis ZnO nanoparticles was added at various concentrations (5,10, 15 and 20 μ g/ml), 5 wells for each concentration. After treatment with rich flavonoid extracts synthesis ZnO nanoparticles for 1, 2, 3, 4, and 5 days, 20 μ l of 5 mg/ml MTT (pH 4.7) was added to each well and cultivated for another 4 h. The supernatant was removed and 100 μ l DMSO was added per well. Samples were then shaken for 15 min. The absorbance at 570 nm was measured with microplate reader (Bio-Rad), using wells without cells as blanks. All experiments were performed in triplicate. The effect of rich flavonoid extracts synthesis ZnO nanoparticles on the proliferation of cancer and normal cells was expressed as relatively cell viability, using the following formula (Kim et al., 2006).

OD of drug treated sample

Percent viability = $\frac{\text{OD of drug treated sample}}{\text{OD of none treated sample}} \times 100$

OD of none treated sample

Statistical analysis

The antioxidant assays were ascertained using one way analysis of variance (ANOVA). Furthermore, Duncan's post hoc test was applied, so as to determine the statistically significant different values. All statistical handling was performed using SPSS software, v. 14.0 (SPSS, Chicago, Ill., U.S.A).

RESULT AND DISCUSSION

Synthesis and characterization of ZnO NPs from rich flavonoid extracts of petals *Delonix regia* by UV-VIS absorption spectroscopy

The formation of ZnO NPs was initially confirmed visually. The change in color of the reaction mixture due to surface Plasmon resonance singularity provides a convenient signature to indicate the formation of ZnO NPs. The pH was determined by using Digital pH meter. The pH of the reduced solution with Nanoparticle synthesized was found to be 6.7 and 5.3. The absorption spectra of rich flavonoid extracts obtained from the petals *Delonix regia* were compared with the absorption spectra of ZnO NPs prepared using these extracts in order to reveal the formation of ZnO phyto-nanoparticles. The absorption spectra of ZnO phyto-nanoparticles were recorded every four hours once and up to 24 hours (Fig-1). Formation of zinc nanoparticles in aqueous colloidal solution were confirmed using Ultraviolet-visible spectral analysis. Zinc oxide nanoparticles normally show a broad peak in the UV-vis spectrum in the range of 230–330 nm (Revina et al. 2007).

Characterization of ZnO NPs by FTIR

FTIR measurements of rich flavonoid extracts synthesized ZnO NPs was carried out to identify the possible biomolecules responsible for the reduction capping of and efficient stabilization of the bio-reduced ZnO NPs. The FTIR spectra of the rich flavonoid extracts synthesized ZnO NPs was shown in Fig-2. The rich flavonoid extracts displayed a number of adsorption peaks, reflecting its complex nature. The spectrum showed the bands for the functional groups located at 3752.5, 3736.1, 2973.8, 2902.0, 2411.6, 1638.2, 1400.3, 1,275, 1,155 and 880.1 cm^{-1} . The strong band of $\text{C}=\text{O}$ stretch (carbonyls) was recorded at 1,672.48 cm^{-1} . The medium bands of $\text{C}-\text{C}$ (in ring) stretch (aromatics) and $\text{C}-\text{H}$ stretch (alkyl halides) were recorded at 1066 and 1052 cm^{-1} .

Morphology of rich flavonoid extracts synthesis ZnO NPs by SEM

The surface morphology and size of the nanoparticles were obtained by Scanning Electron Microscopy (SEM) analysis. The Fig-3 shows the ZnO NPs synthesized by the petals rich flavonoid extract of *Delonix regia*. The electrostatic interactions and hydrogen bond between the bio-organic capping molecules bond are responsible for the synthesis of copper nanoparticles using plant extract. It was shown that spherical and relatively uniform shape of the copper nanoparticles was confirmed in the range of 65–184nm. The quantitative and qualitative analysis of elements may be concerned in the formation of ZnO NPs (Fig-3).

Free radical-scavenging ability of ABTS assay

The radical scavenging ability measured by ABTS assay is given in Fig-4 and expressed as IC_{50} 163.82 value which donates the amount in μ g of vitamin C equivalent per gram of a dried sample. In this assay lower amount required to give IC_{50} indicates higher free radical scavenging activity. ZnO NPs synthesized by the petals rich flavonoid extract exhibited significantly higher free radical activities than Vitamin C. All of the amounts of ZnO NPs synthesized

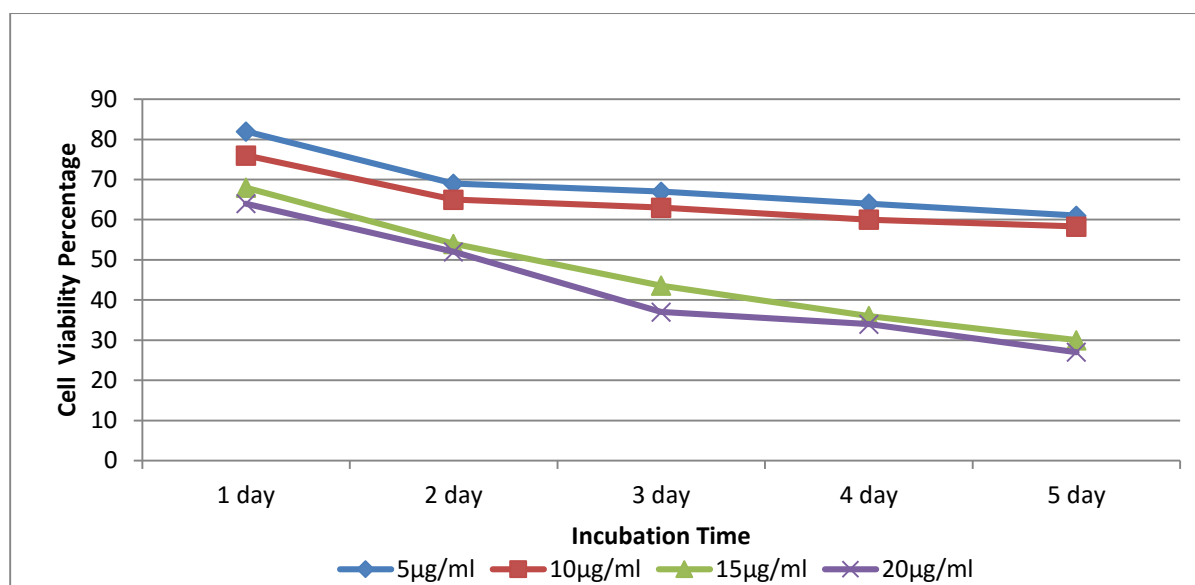


Figure 8: Cytotoxic activity of the flavonoid extract synthesis ZnO NPs against human lung cancer cells

showed higher activities than control and these differences were statistically significant ($P < 0.01$). ABTS, exhibited a dose-response relationship, with maximum attainable inhibition of the free radicals in ABTS assays ranging between 70% and 80%. Despite the fact that these methods have similar reaction mechanisms and do not necessarily measure the same activity (Prior et al., 2005), the ABTS methods clearly indicated that the studied ZnO NPs possess variable but considerable antiradical activities. It is also well known that the antioxidant effect of ZnO NPs including rich flavonoid extract of *Delonix regia* can be attributed mainly to radical scavenging activity.

Inhibition of Lipid Peroxidation

In this assay, the peroxidation induced by ferrous sulfate in egg yolk homogenates and ascorbic acid as reducing agents. Hydroxyl radicals are generated by mixing Fe^{3+} and ascorbate which attack the biological material. This leads to the formation of MDA (malondialdehyde) and other aldehydes, which form a pink chromogen with TBA (2-thiobarbituric acid), absorbing at 532 nm. As shown in Figure 5, the ZnO NPs demonstrated considerable amount of lipid peroxidation inhibitory effect by 64.48%, while Vitamin C significantly inhibited lipid peroxidation by 66.89%. The results were concentration-dependent and considered statistically significant ($P < 0.05$). The flavonoid and other phenolic chemicals mechanism may restrain lipid peroxidation through diverse chemical device, including free radical quenching, electron transfer, radical addition or radical recombination. In addition, lipid peroxidation of cell membrane is associated with various physiological events such as atherosclerosis, inflammation and liver injury (Kabouche et al., 2007).

Superoxide radical scavenging assay

The scavenging of H_2O_2 by flavonoid extract synthesis ZnO NPs may be attributed to their nanoparticle, which could donate electrons to H_2O_2 , thus neutralizing it to water. The H_2O_2 scavenging capacity of ZnO NPs may be credited to the structural features of their active materials, which decide their electron contribute capability

(Wettasinghe and Shahidi, 2000). The capability of ZnO NPs to scavenge H_2O_2 is shown in Fig. 6 and compared with vitamin C as standards. ZnO NPs was proficient of scavenging H_2O_2 in a dose-dependent manner. Twenty-micrograms of ZnO NPs exhibited 79% scavenging activity on H_2O_2 . On the other hand, at the same concentration vitamin-C showed 73% activity. These results indicated that ZnO NPs possess effective H_2O_2 scavenging activity IC_{50} value 12.21 but higher than Vitamin-C IC_{50} value 12.36. However, there was statistically a very significant correlation between those values and control ($P < 0.05$). The H_2O_2 scavenging effect of same dose (20 µg) of flavonoid extract synthesis ZnO NPs and standards decreased in the order of $>$ Vitamin-C. *Nitric oxide radical scavenging activity*

In the present study, the nitric oxide radical quenching activity of the flavonoid extract synthesis ZnO NPs was detected and compared with the standard ascorbic acid. The exhibited the maximum percent inhibition of 75% at a concentration of 20 µg/mL, with an IC_{50} value of 30.51 µg/mL, in a concentration-dependent manner (Fig-7). However, ascorbic acid exhibited maximum percent inhibition of 72%, with an IC_{50} value of 42 µg/mL (Fig-7). There was statically significant correlation between those values and control ($P < 0.05$). The scavenging activity of the extract against nitric oxide was detected by its ability to inhibit the formation of nitrite through direct competition with oxygen and oxides of nitrogen in the reaction mixture. Nitric oxide is a potent pleiotropic mediator of physiological processes, such as smooth muscle relaxation, neuronal signalling, inhibition of platelet aggregation and regulation of cell-mediated toxicity. Moreover, in pathological conditions, nitric oxide reacts with superoxide anion and forms potentially cytotoxic molecules, such as peroxynitrite (Alasalvar et al., 2006). *Cytotoxic activity of the flavonoid extract synthesis ZnO NPs against human lung cancer cells*

The flavonoid rich fraction synthesis ZnO NPs showed a dose- and time-dependent inhibitory effect on the growth

of A549 (p53 wt) lung cancer cells ($P < 0.05$). IC_{50} was 12.33 $\mu\text{g/ml}$, and the maximal inhibition of cell growth (>80%) was obtained at 20 $\mu\text{g/ml}$. The result of cytotoxic activity of the flavonoid extracts synthesis ZnO NPs against human lung cancer cells. The anticancer activities might be associated with their anti-oxidant properties (Son et al., 2003), and therefore, it might be assumed that flavonoid rich fraction synthesis ZnO NPs has anticancer activity against Human lung cancer cells.

CONCLUSIONS

The results revealed that antioxidant and cytotoxicity exhibited dose- and time-dependent effects for different kinds of ZnO NPs. ZnO encourage oxidative stress, decreases viability, and increases cell death in Human lung cancer cells A549. Finally, there is still little knowledge about the feature of ZnO toxicity associated with the nanoparticle sizes, as well as how they are transported in cells and how nanoparticles interact with the cell membrane and organelles.

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