Comparison on Bactericidal and Cytotoxic Effect of Silver Nanoparticles Synthesized by Different Methods

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Abstract: Biologically synthesized silver nanoparticle are biocompatible for medical applications. The present work is aimed to synthesize silver nanoparticle using the fruit pulp of Tamarindus indica and to evaluate its antibacterial and anticancer activity against lung cancer cell lines. Antibacterial activity was assessed by well diffusion method. Cytotoxicity was evaluated using MTT assay. GC-MS of fruit pulp extract showed the presence of levoglucosenone, n-hexadecanoic acid, 9,12-octadecadienoic acid etc. Antioxidant activity of the fruit pulp was determined by DPPH assay, hydrogen peroxide scavenging assay and lipid peroxidation. The size of biologically synthesized silver nanoparticle varied from 50 nm to 76 nm. It was 59 nm to 98 nm for chemically synthesized silver nanoparticle. Biologically synthesized silver nanoparticle showed 26 mm inhibition zone against E.coli and chemically synthesized silver nanoparticle showed 20 mm. Antioxidant activity of fruit extract by DPPH showed 84 % reduction. The IC 50 of biologically synthesized silver nanoparticle against lung cancer cell lines was 48 µg/ml. It was 95 µg/ml for chemically synthesized silver nanoparticle. The increased activity of biologically synthesized silver nanoparticle was due to its smaller size, stability and the bioactive compounds capping the silver nanoparticle extracted from the fruit extract.

Keywords: Antioxidant activity, lung cancer cell lines, silver nanoparticles and Tamarindus indica.

1. INTRODUCTION
Lung cancer ranks second in the race causing mortality in Indians. According to the Indian Council of Medical Research cancer registry, “57,795 new cases were reported in 2010 and it is expected to rise up to 67,000 new cases annually by the year 2020” [1]. It is the common cause of death among other cancers. Increased incidences of lung cancer among young age reflect the changing life style with higher rate of smoking. It accounts for 9, 30, 000 deaths in 2010 [2]. It is a major type of cancer with low survival rate. Air pollution and passive smoking are responsible for lung cancer. Resistance of cancer cells to chemotherapeutic agents is an alarming signal for therapy. Currently successful treatment is given with targeted
therapy. But over time the cells will be recalcitrant to targeted therapy also and cancer recurs. Treatment of recurrent cancer with drugs will be worst. Resistance of lung cancers to drugs are correlated to the presence of two pumps named P-glycoprotein and Multidrug Resistance–associated Protein (MRP). So, there is an emerging need to develop innovative strategies to treat cancer. Recently more emphasize is lead on the effect of silver nanoparticles (Ag-NP) on cytotoxicity of cancer cells. Study by Kovács et.al.[3] demonstrated the anti proliferative effect of Ag-NP against drug resistant cancer cells and suggested the combination therapy with Ag-NP. Ag-NP have enhanced permeation effect that increases the efficiency of any drug. Generally antioxidants are prescribed along with chemotherapeutic agents. But the activity of antioxidant should not cancel the effect of Ag-NP which exert toxicity by the production of reactive oxygen species. When the Ag-NP are synthesized by biological route, they will be stabilized by capping agents from the biological source. If the biological source has active compounds with anticancer property, then the advantage of Ag-NP are more for cancer therapy.

2. MATERIALS AND METHODS

2.1 Chemicals
All chemicals were purchased from Hi media, Mumbai, India. Solvents used were of analytical grade.

2.2 Synthesis and Characterization of Ag-NP
Ag-NP was synthesized by the biological method using ethanol extract of T. indica fruit pulp (10%). To 100 ml of 1mM silver nitrate solution, 5ml of the extract was added. Ag-NP was synthesized by chemical reduction using sodium citrate. Ag-NP was prepared by slowly adding 2 % sodium citrate to 1 mM silver nitrate solution at 50 ºC. The solution was centrifuged at 10,000 rpm for 30 min. The dried residue was collected by drying in vacuum evaporator.

2.3 Extraction of Phytochemicals from T.indica
T. indica(PKM- 1) fruit pulp (5g/50ml) was extracted with 85% ethanol. The extract was centrifuged at 10,000 rpm for 15 min. The supernatant was evaporated, concentrated in the rotary evaporator and lyophilized.

2.4 GC-MS of T.indica fruit pulp
The lyophilized residue of T. indica fruit pulp extract was dissolved in 80 % ethanol and used for analysis in Clarus 500 Perkin Elmer. Elite-5 MS column was used. The flow rate of carrier gas was maintained at 1ml /min.

2.5 Antioxidant Activity of T.indica fruit pulp extract

2.5.1 DPPH Assay
The lyophilized T.indica powder (50µg/ml) was used for the DPPH assay [4]. Antioxidants in the fruit pulp reacts with DPPH, a purple coloured free radical and convert it to colourless 2, 2-diphenyl-1-picryl hydrazine. The magnitude of decolorization describes the scavenging activity. The reaction mixture consisted of 100 µl of 0.135 mM DPPH and 100 µl of fruit pulp
extract. A control was simultaneously maintained without DPPH. Antioxidant activity was determined using the equation 
\[
\frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}]}{\text{OD}_{\text{control}}} \times 100.
\]

2.5.2 Hydrogen Peroxide Scavenging Assay

Hydrogen peroxide scavenging efficiency was determined by the method of Ruch et al [5]. The reaction mixture contained 1mL (100 µg/mL) of fruit pulp extract and 0.6 mL of hydrogen peroxide (40 mM in phosphate buffer, 7.4). After 10 minutes of incubation at room temperature, the absorbance of hydrogen peroxide was measured at 230 nm. Control was maintained with extract alone without hydrogen peroxide. Hydrogen peroxide scavenging activity (%) was calculated using the equation 
\[
\frac{[\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}]}{\text{OD}_{\text{control}}} \times 100.
\]

2.5.3 Inhibition of Lipid Peroxidation

It measures the lipid peroxide formed using egg yolk emulsion [6]. The test reaction mixture (t) contained 5 µl egg yolk emulsion, 50 µl of fruit extract, 150 µl of trichloroacetic acid (20%) and 150 µl of thiobarbituric acid (0.67 % w/v). Egg yolk emulsion was prepared by diluting 10 ml of egg yolk with 90 ml of KCl (1.15 %). The mixture was incubated at 95°C for 1 h. It was cooled to room temperature and 400 µl of butanol was added and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was recorded at 532 nm. A control (c) was maintained without the fruit pulp extract. Inhibition (%) of lipid peroxidation was calculated using the equation \((1 - \frac{\text{OD}_t}{\text{OD}_c}) \times 100\).

2.6 Antibacterial Activity

Clinical isolate of *E. coli* was a gift from Inbiotics Clinical laboratory, Nagercoil, Tamilnadu, India. Antibacterial activity of Ag-NP and fruit pulp were assessed by agar well diffusion assay [7]. OD of fresh culture was adjusted to 0.08. 100 µl of culture was swabbed onto Mueller and Hinton agar medium. 100 µl of Ag-NP was loaded onto the well punctured in the plate. The plates were incubated at 37°C for 24 hours and the inhibition zone was measured.

2.7 Cytotoxicity Assay

Cytotoxic effect of Ag-NP was determined by MTT assay using lung cancer cell lines A549 [8]. MTT measures the reduction of yellow dye (3,4,5-dimethythiazol-2-y1)-2,5-diphenyl tetrazolium bromide) to an insoluble dark purple formazan product by mitochondrial succinate dehydrogenase. Formazan is solubilized by isopropanol and the color was measured in ELISA Reader at 570 nm. Color is proportional to the viable cells. Only live cells produce the enzyme. Cytotoxicity was calculated from the viable cells. Lung cancer cells were plated (10^4 – 10^6 cells) in 200 ml Phosphate Buffed Saline in 96-well plate. 20 µl of MTT solution was added and incubated at 37°C for 4 hours in darkness. To the supernatant, 200 µl of acidic isopropanol was added and mixed well. After one hour of incubation, the color was measured.

2.8 Statistical Analysis
All the experimental were replicated thrice. The results are expressed as of three replicates standard deviation.

3. RESULTS AND DISCUSSION

3.1 Synthesis and Characterization of Ag-NP

Ag-NP was synthesized using the ethanol extract of *T. indica* fruit pulp (biological method) and by sodium citrate (chemical method). Fig.1 represents the SEM images of Ag-NP. The size of biologically synthesized Ag-NP varied from 50 nm to 76 nm. It varied from 59 nm to 98 nm for chemically synthesized Ag-NP. Use of fruit extracts for the synthesis if Ag-NP was supported by Gnanajobitha et.al.[9]. Phytochemicals in fruits serve as reducing and capping agents.

![Figure 1. SEM of Ag-NP](image1.png)

(a) Biological method  
(b) Chemical method

**Figure 1. SEM of Ag-NP**

Fig. 2 shows the UV-Vis absorption spectrum of Ag-NP. Biologically and chemically synthesized Ag-NP showed absorption maximum at 410 nm and 425 nm respectively. Biologically synthesized Ag-NP showed absorption maximum at lower wavelength than chemically synthesized Ag-NP. Shift to lower wavelength is an indication of low particle size. Surface Plasmon resonance peak of Ag-NP can be fine tuned by changing the particle size.

![Figure 2. UV-VIS absorption spectrum of Ag-NP](image2.png)

(a) Biological method  
(b) Chemical method

**Figure 2. UV-VIS absorption spectrum of Ag-NP**
3.2 GC-MS of Tamarindus indica

Fig. 3 shows the GC-MS of the T. indica fruit pulp. Table 1 presents the compounds present in the fruit pulp along with their retention time and peak area. Fruits and seeds of exotic fruits are the richest sources of phenolic compounds, flavonoids, vitamins etc. They possess good antioxidant activity [10]

![Figure 3. GC-MS of T. indica fruit pulp](image)

| No. | RT  | Name of the compound | Molecular Formulae | MW  | Peak Area % |
|-----|-----|----------------------|--------------------|-----|-------------|
| 1.  | 2.92| Levoglucosenone      | C_{6}H_{6}O_{3}     | 126 | 26.53       |
| 2.  | 4.51| 2-Oxazolamine, 4,5-dihydro-5- (phenoxymethyl)-N-[(phenylamino)carbonyl]- | C_{7}H_{7}N_{3}O_{4} | 311 | 3.54       |
| 3.  | 6.35| á-D-Glucopyranoside, O-á-D-glucopyranosyl-(1.fwдарw.3)-á-D-fructofuranosyl | C_{16}H_{20}O_{9} | 504 | 9.05       |
| 4.  | 7.10| Phenol, 2,4-bis(1,1-dimethylethyl)- | C_{8}H_{14}O | 206 | 1.31       |
| 5.  | 8.16| Diethyl Phthalate     | C_{6}H_{12}O_{4}    | 222 | 6.22       |
| 6.  | 9.08| D-Glucose, 4-O-á-D-glucopyranosyl- | C_{8}H_{12}O_{11} | 342 | 6.29       |
| 7.  | 10.17| Tetradecanoic acid   | C_{14}H_{28}O_{2}  | 228 | 1.87       |
| 8.  | 10.85| 10-Methyl-E-11-tridecen-1-ol propionate | C_{14}H_{28}O_{2} | 268 | 1.03       |
| 9.  | 12.17| n-Hexadecanoic acid | C_{16}H_{32}O_{2}  | 256 | 16.30      |
| 10. | 14.14| 9,12-Octadecadienonic acid (Z,Z)- | C_{20}H_{34}O_{2} | 280 | 12.84      |
| 11. | 16.58| á-D-Mannofuranoside, farnesyl- | C_{20}H_{36}O_{6}  | 384 | 0.33       |
| 12. | 18.94| Benzene, 1-fluoro-4-nitro-2-(5-nitrofururylidinamino)- | C_{11}H_{8}F_{2}N_{3}O_{5} | 279 | 0.49       |
| 13. | 22.42| Squalene              | C_{30}H_{50}        | 410 | 0.41       |
| 14. | 24.27| Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester | C_{22}H_{34}O_{2} | 250 | 0.35       |
| 15. | 25.78| 1-Heptatriacotanol   | C_{20}H_{32}O_{2}  | 536 | 1.35       |
| 16. | 26.02| Vitamin A aldehyde   | C_{22}H_{34}O_{2}  | 284 | 1.86       |
| 17. | 26.66| 4-Isopropenyl-4,7-dimethyl-1-oxaspiro[2.5]octane | C_{20}H_{32}O_{2} | 180 | 0.47       |
| 18. | 28.06| Cholesterol-3-ol, 2-methylene-,(3â,5â)- | C_{36}H_{60}O_{2} | 400 | 3.74       |
| 19. | 30.05| 2H-Pyran, 2-(7-heptadecenyloxy)tetrahydro- | C_{22}H_{40}O_{2} | 336 | 2.38       |
| 20. | 31.17| (E,E)-7,11,15-Trimethyl-3-methylene- hexadeca-1,6,10,14-tetraene | C_{30}H_{52}       | 272 | 3.62       |
Levanoglucosenone. (preservative), n-Hexadecanoic acid (hypocholsteremic, lubricant, hemolytic, pesticide, anti androgenic, antioxidant, flavor, α reductase inhibitor), 9,12-Octadeadienoic acid (anticancer)-α-d-Glucopyranoside, O-α-D-glucopyranosyl 1-(fwdarw.3)-α-D-fructofuranosyl (preservative), 1-Heptatriacontanol (antimicrobial), squalene (antibacterial, antiageing, analgesic, antidiabetic, anti-inflammatory, hypocholesterolemic, antiulcerogenic, vasodilator, antispasmodic, antibronchitic, anticoronal antioxidant antitumor, immunostimulant, chemo preventive, lipoxygenase-inhibitor pesticide), vitamin A aldehyde, tetradecanoic acid (myristic acid) etc. are the major bioactive compounds in the fruit pulp. Cholestan-3-ol, 2-methylene-, (3α,5α)-(antimicrobial, anticancer antiasthma, anti-inflammatory antiarthritic).

The biological activity of phytochemicals are described in Dukes phytochemicals book [11].

3.3 Antioxidant Activity of T.indica

Fig. 4 represents the antioxidant activity of T.indica fruit pulp. Total antioxidant activity by DPPH assay was 84%, hydrogen peroxide scavenging activity was 88.35% and lipid peroxidation was 91.8%. Antioxidant activity of T. indica powder was extensively studied by Trila et.al.[12]. Luzia and Jorge [13] evaluated the antioxidant activity of T. indica seed coat. The present results corroborate with the observation of Ugwuona, and Onweluzo [14]. Fruits are a natural source of antioxidants. Antioxidants provide protection against oxidative stress [15] and prevents cancer [16]-[17]

Antioxidant activity is ascribed to the presence of polyphenols, flavonoids, diterpenes, fatty acid esters, alkaloids, glycosides etc. [18] Antioxidant activity of Tamarind fruit pulp was reported by Siddhuraju [19] and Razali et al. [20]

3.4 Antibacterial Activity of Ag-NP

Antibacterial activity of biologically synthesized Ag-NP was 28±3 mm. It was 22±4 mm for chemically synthesized Ag-NP. Increased antimicrobial activity of biologically synthesized Ag-NP is due to the small particle size and stability offered by bioactive compounds from the
fruit pulp extract. Polyphenolics, flavanoids and other secondary metabolites in fruits cause cell membrane damage leading to a leaky cell and death [21]. Heptatriacontanol, squalene and cholestan-3-ol, 2-methylene present in the T. indica fruit pulp possess antimicrobial properties.

3.5 Cytotoxicity of Ag-NP to Lung Cancer Cell Lines

Fig. 5a and 5b represents the Cytotoxic effect of Ag-NP against lung cancer cell lines. Cytotoxic effect of Ag-NP was dose dependent as evident from the fig 5b,5c and 5d. At 8 µg/ml of Ag-NP, 12.8 % of cytotoxicity and 5.8 % cytotoxicity were observed in biologically synthesized Ag-NP and chemically synthesized Ag-NP. As the concentration of Ag-NP was increased to 80 µg/ml, cytotoxicity in biologically synthesized Ag-NP was increased to 92.76 %. The IC50 of biologically synthesized Ag-NP against lung cancer cell lines was 48µg/ml. It was 95 µg/ml for chemically synthesized Ag-NP. Figure 5b shows the destruction of lung cancer cells by biologically synthesized Ag-NP is more than the other. Anticancer activity of Ag-NP against cancers were studied [22] –[23]. Ag-NP inhibits tumor cell by inhibiting RNA Polymerase [24]. Once Ag-NP’s are by internalization, they interfere with chromosome stability and mitosis [25]. They also mediate cytotoxic effect by increasing apoptosis by caspase 9 [26]. Still the complete molecular mechanism of cytotoxicity by Ag-NP is not well established.
Comparatively increased cytotoxic effect of biologically synthesized Ag-NP is due to the presence of compounds with anticancer property obtained from the fruit pulp of *T. indica*. Levanoglucosenone, octadecadienoic acid and cholestane-3-ol, 2-methylene present in *T. indica* has antitumor activity. n-Hexadecanoic acid and squalene present in *T. indica* are hypocholesteremic compounds that add to the cytotoxic effect by a different mechanism. Hypocholesteremic effect of *T. indica* fruit extract was experimentally proved in hamsters [27]. Hypocholesteremic compounds are recently focused for their antitumor effect. They exert their effect by functioning as an inhibitor of HMG-CoA reductase. Potential antitumor activity of HMG-CoA reductase inhibitors (statins) was described elaborately by Chan et.al.[28]. It decreases the expression of antiapoptotic protein Bcl-2 and increases the expression of the proapoptotic protein Bax [29].

Invasion and metastasis is caused by the translocation of Rho A from cytosol to membrane via prenylation. HMG-CoA reductase inhibitors block the production of geranylgeranyl pyrophosphate. Inhibiting its the synthesis automatically prevents the translocation of Rho A to membrane and hence terminates signal transduction [30]. Generally cancerous cells in S phase are resistant to radiation therapy. Studies by McKenna et.al.[31] and Miller et.al.[32], revealed that HMG-CoA reductase inhibitors can reverse the resistance of cancerous mass to radiation by arresting the cells in G1 phase. Therefore these inhibitors can be used along with radiation and chemotherapy. Anti proliferative effect of statins on lung tumor was reported earlier [33]. They also exhibit inhibitory effect on tumor induced angiogenesis. A major advantage of co administering doxycyclin with HMG-CoA reductase inhibitors is their ability to reverse the cardiotoxic effect of doxycyclin.

CONCLUSION

The study proved that Ag-NP synthesized using biological methods was more effective in its antibacterial and anticancer activity than chemical methods. *T. indica* fruit pulp contain many compounds with antimicrobial, anticancer and hypocholesteremic compounds. These bioactive compounds serves as capping agents of Ag-NP synthesized using biological methods. They augment the positive effects of Ag-NP. Therefore they can be used in conjunction with other chemotherapeutic agents. Further study is required to understand the molecular mechanism of action of bioactive compounds in improving the therapeutic value of Ag-NP.

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