IJPSR (2017), Volume 8, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 11 April, 2017; received in revised form, 14 June, 2017; accepted, 29 June, 2017; published 01 December, 2017

GREEN SYNTHESIS OF SILVER NANOPARTICLES USING *TABERNAEMONTANA DIVARICATA* AND *IN-VITRO* CYTOTOXICITY INVESTIGATION AGAINST HUMAN LUNG ADENOCARCINOMA

Navinchandra G. Shimpi* and Minakshi Jha

Department of Chemistry, University of Mumbai, Santacruz (East), Mumbai - 400098, Maharashtra, India.

Keywords:

Green synthesis, A549
Silver nanoparticles (AgNPs),
TEM, AFM, Anticancer activity,

Correspondence to Author:
Navinchandra G. Shimpi

Associate Professor,
Faculty of Physical Chemistry,
University of Mumbai, Santacruz
(East), Mumbai - 400098,
Maharashtra, India.

E-mail: navin_shimpi@rediffmail.com

ABSTRACT: The present study aims to synthesize highly stable and dispersed cytotoxic silver nanoparticles (AgNPs) from *Tabernaemontana divaricata* (*T. divaricata*) leaves extract, by using noble green approach at room temperature. Phytocomponents were investigated using general methods and found good agreement with reported literature. Green synthesized AgNPs were characterized using UV-Vis, FT-IR, XRD, TGA, FEG-SEM, EDX, TEM, SAED and AFM spectroscopy techniques. XRD and SEAD confirmed polycrystalline nature. TEM study shows spherical morphology with size range 6-20 nm. Suforhodamine B (SRB) assay revealed potent cytotoxicity of AgNPs on A549 cells with IC_{50} value 32.3µg/ml. As per SRB assay in case of AgNPs, GI_{50} = 37.1µg/ml, TGI = 68.9µg/ml, LC_{50} = > 80µg/ml, while *T. divaricata* exhibits GI_{50}, TGI, LC_{50} values = > 80µg/ml, indicating greater biocompatibility of AgNPs in comparison to *T. divaricata* leaves extract. This investigation reveals that *T. divaricata* mediated AgNPs has a great potential for the development of an efficient anticancer nano drug with minimal side effects to combat against life-threatening lung cancer.

INTRODUCTION: Now-a-days the world is facing an alarming situation because of lung cancer, which is the major cause of cancer death in man and women with the increasing mortality rate day by day [1]. According to data of 2012, 1.8 million People diagnosed with lung cancer and so far 1.6 million deaths reported due to this severe disease [2]. World health organization (WHO) report shows lung cancer five years survival rate (17.8%) is lower than another cancerous site such as Prostate (99%), breast (90.5%), colon (65.47%).

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.8(12).5100-10

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (12).5100-10

As per report more than half of people with lung cancer died within one year of being diagnosed, which clearly indicates about its high incidence and low survival rate. Lung carcinoma is abnormal and uncontrolled cell growth in tissues of lungs, which can be spread beyond lung by metastasis process into other parts of the body.

Some common observed symptoms from this disease are weight loss, blood in cough, shortage of breathing, wheezing and chest pain. The existing cytotoxic agents used for treatment (surgery, radiotherapy, chemotherapy, broncho-scopy and targeted cell therapy) are expensive and inefficient due to poor solubility, poor targeting efficacy and drug resistance with severe side effects [3]. Due to these disadvantages, the discovery and identification of new anticancer agents has now become the demand of society, which should be

biocompatible, cost effective with low side effects on the human immune system. Modern nanotechnology can provide the solution by incepting green chemistry approaches with good control over particles size facilitates an important platform for development of suitable and efficient nanomedicine with biomedical applications. To design efficient nanomedicine and nanocarrier, plant mediated green synthetic approach is most promising that generates free noble silver nano-particle with high therapeutic potential for various biomedical applications such as biological tagging, cell labelling, cancer therapy, bio sensing, molecular imaging, pharmaceutical applications [4], drug delivery [5] and wound healing [6]. Green approach towards synthesis of nanomedicine is one of the best choice due to fast reduction rate of metal salt, one pot room temperature synthesis, eliminates the use of toxic and expensive chemicals, eco-friendly, requires less processing time and the procedure requires no specific condition unlike chemical and physical methods.

Green syntheses of silver nanoparticle (AgNPs) have been studied previously using *Glycyrrhiza glabra* [7], *Sapindus mukorrosai* [8], *Leea indica* (Burm. f.) Merr [9], *Rhizopus stolonifera* [10], *Saraca indica* [11], *Parkia speciosa* Hassk [12], *Syzygium jambos* (l) [13], *Adenium obesum* [14], *Annona muricata* [15], *Ipomoea aquatica* forsk [16], *Clerodendrum phlomidis* [17]. These results have shown that AgNPs can be synthesized in environment friendly method using plant extract. Still, the potential of the plants as a green precursor for development of nanomedicine and nanocarrier is yet to be fully explored by using medicinal plant. *Tabernaemontana divaricata* (*T. divaricata*) belongs to the family Apocynaceae and subfamily Rauvolfioideae, is a glabrous, evergreen plant having medicinal importance which is selected for AgNPs synthesis. In the present study phytomediated biosynthesis of stable AgNPs from *T. divaricata* at room temperature was carried out, characterizations and *in-vitro* analysis to investigate the anticancer efficiency of AgNPs against A549 human lung cancer cell was investigated.

MATERIALS AND METHODS:

Chemicals Used: Silver nitrate ($AgNO_3$ > 99.9%) was purchased from s. d. fine chemicals limited, Mumbai (India) and used without further purification. DMSO supplied by Hi-media, India. Source of cell line was NCCS, Pune. RPMI 1640 and Adriamycin were procured from sigma Aldrich. Bovine serum, L-glutamine and all other chemicals were procured from Merck Ltd. Mumbai (India).10mg/ml bovine insulin in 25mM HEPES, pH 8.2 was procured from sigma cat. No I0516. 10 mM unbuffered Tris base solution was used during SRB assay. The fresh and healthy leaves of *T. divaricata* were collected from Kalina campus, University of Mumbai. Deionized (DI) water and ethanol was used throughout the nano drug AgNPs synthesis. Solvents were double distilled before the experiment.

Preparation of Fresh Leaves Extract: Fresh and healthy leaves of *T. divaricata* (**Fig. 1A**) were washed thoroughly in tap and double distilled water. Finally, the leaves ware rinsed with de-ionized water to make them free from dust and other contaminants. Further leaves were dried at 50 °C for 20 minutes to remove the moisture in hot air oven. The plant leaves extract was prepared using 25gm of finely chopped leaves in ethanol (200 ml) and 50ml of deionized water (80:20) which was refluxed at 35 °C for 60 minutes. Further this refluxed solution was filtered through whatman 1 filter paper and stored at 4 °C for screening of phyto components and AgNPs synthesis.

Synthesis of AgNPs: $AgNO_3$ (1mM) stock solution was prepared in deionized water. The plant leaves extract (20ml) was added to silver nitrate solution (80ml) drop wise with constant stirring and incubated for 24 hours. The reaction was carried out under dark condition to avoid photo activation and reaction mixture was kept undisturbed during incubation. During this process, the colour of the solution changes from light green to dark brown after incubation. The formation of AgNPs was monitored through UV-Vis spectra. Further to isolate the particles, the solution was centrifuged, and particles were collected after discarding supernatant. The collected AgNPs were allowed to dry in hot air oven at 50 °C for one hour.

Phytocomponents Screening: The Phyto-components screening of leaves extract (LE) was performed as per available method and reported as follows (**Table 1**).

TABLE 1: PRELIMINARY SCREENING TESTS OF PHYTOCHEMICALS PRESENT IN LEAF EXTRACT OF *T. DIVARICATA*

Phytocomponents test	Procedure and results
Alkaloids (Wagner test)	1% HCl was added to 2ml LE and steamed, then 1ml of this solution was reacted with 6 drops of wagner reagent, brownish red ppt. confirmed the presence of alkaloid.
Flavonoid (NaOH test)	Extract was treated with dilute NaOH followed by addition of dilute HCl. Yellow solution obtained, turned colorless with dilute HCl, and indicates the presence of flavonoid
Phlobatanin (HCl test)	2ml of 1% HCl was added to 2 ml leaf extract and boiled. Red precipitate was not obtained showed absence of Phlobatanin.
Saponin (Foam test)	5ml of distilled water was added to 0.5ml of filtrate and shacked well, Persistence of froth indicated the presence of saponin
Tannin (Bramer's test)	10% alcoholic $FeCl_3$ was added to 2-3ml of metabolic extract in the ratio of 1:1, Greenish grey color of the solution was observed indicated towards presence of tannin
Terpenoid (Salkowski test)	5ml LE + 2 ml $CHCl_3$ + 3 ml concentrated H_2SO_4. Color of interface was reddish
Triterpenoid	brown 1.5 ml leaf extract + 1ml Libermann-Buchard reagent (acetic anhydride + conc. H_2SO_4) formation of green color indicated presence of triterpenoid. Squalene we obtained in GC-MS also.

Characterization: The AgNPs were analyzed by UV-Vis spectroscopy (Shimadzu UV 2450) to investigate optical properties based on unique surface Plasmon resonance. The bio reduction of AgNPs using *T. divaricata* leaves extract was monitored by sampling the reaction mixture scanned at room temperature at a wavelength of 300-600 nm range with a resolution of 1nm. Phase identification and crystalline nature of AgNPs was confirmed using X- ray diffraction (Shimadzu XRD 7000). The analysis was performed with an operating voltage 40.0 KV, current 30.0 mA with CuKα radiation (λ =1.54060Ao), scan range 20-80 degree, step size 0.0200 degree, count time 0.60 sec, slit DS 0.50 degree, SS 0.50 degree, RS 0.15 mm. Thermal stability and weight loss of AgNPs was studied on thermal gravimetric analyzer at 30-1000 °C.

Sample was kept in aluminium pan sample holder and exposed to heating under continuous nitrogen purging (20ml/min). Fourier transform infrared spectra (FTIR) was carried out to identify different types of bio-molecules in leaves extract of *T. divaricata* and those associated with AgNPs using Perkin Elmer Frontier (model no 91579) in the range of 4000-400 cm^{-1} at a resolution of $4cm^{-1}$. Field emission gun -scanning electron microscope (FEG-SEM) analysis was done by JEOL JSM-7600F combined with EDX to study the size, shape and elemental composition of AgNPs. The topographic information was obtained by transmission electron microscope (PHILIPS TEM CM 200) at an accelerating voltage of 200KV and resolution 0.24nm.

The sample was prepared by coating AgNPs solution onto carbon coated copper grid. After two minutes film is get deposited and extra solution was removed by using a blotting paper and TEM grid was allowed to dry completely before loading. At last of the image recording one selected area was inserted into the beam path by blocking the entire electron beam except for the small fraction passing through one of the hole, and only this section contributed to SAED on the screen. To study surface texture of biosynthesized AgNPs, atomic force microscopy (Bruker's Dimension Icon, US) was used in tapping mode with f (frequency) 50-90 KHz and K (cantilever's force constant) 0.4 N/m.

***In vitro* Cytotoxicity of Synthesized AgNPs:** The cytotoxicity of synthesized AgNPs against A549 cells as measured by sulforhodamine B (SRB) assay [18, 19]. Briefly A549 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2ml L-glutamine. The cells were inoculated into 96 well microtiter plates in 100µL medium. After cell inoculation, the micro plates were incubated at 37 °C in a 5 % CO_2, 95% air and 100 % relative humidity for next 24 hours. To evaluate anticancer activity of the *T. divaricata* capped AgNPs, the cultured cells were treated in separate plates with different concentration of silver nanoparticles and plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 minutes at 4 °C.

The supernatant was discarded; the plates were washed and dried. Sulforhodamine B (SRB) solution (50µl) at 0.4 % (w/v) in 1 % CH_3COOH was added to each of the wells and incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing. Bound stain was eluted with 10 mM tris base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength. % of control cell growth = [mean OD_{sample} – mean $OD_{day\ 0}$ / mean $OD_{neg\ control}$ – mean $OD_{day\ 0}$] x 100

% growth inhibition = 100 - % control cell growth
% cells killed = 100 – [mean OD_{sample} / mean OD_{day0}] x100

Each treatment condition was replicated thrice and Statistical analysis was done, morphology of the cells was captured as images.

Statistical Analysis: Data was reported as mean ± SD of three replicates of each experiment. Results were analyzed using one-way variance analysis (ANOVA). For comparison of multiple groups one-way ANOVA with Tukey's HSD (honest significant difference) test was used. Anova analysis of *T. divaricata* mediated AgNPs and *T. divaricata* leaves extract are provided in tabular form in electronic supplementary information (ESI). The results with $p < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSIONS: The potential of *Tabernaemontana divaricata* plant leaves extract to reduce silver nitrate and synthesis of high quality AgNPs was initially detected by colour change from green to brown. The formation and stability of incubated AgNPs was confirmed by optical UV-Vis spectra in the wavelength range 300-600 nm. Silver nanoparticles possess free electrons, which gives surface plasmon resonance band because of the combined vibration of electrons of silver nanoparticle in resonance with light wave. In this study, maximum absorbance was obtained at 414 nm (**Fig. 1B**). According to previous report spherical AgNPs contributes to absorption band at around 400-420nm in UV-Vis spectra. The formation of surface plasmon resonance peak depends upon formed particles and their size and shape.

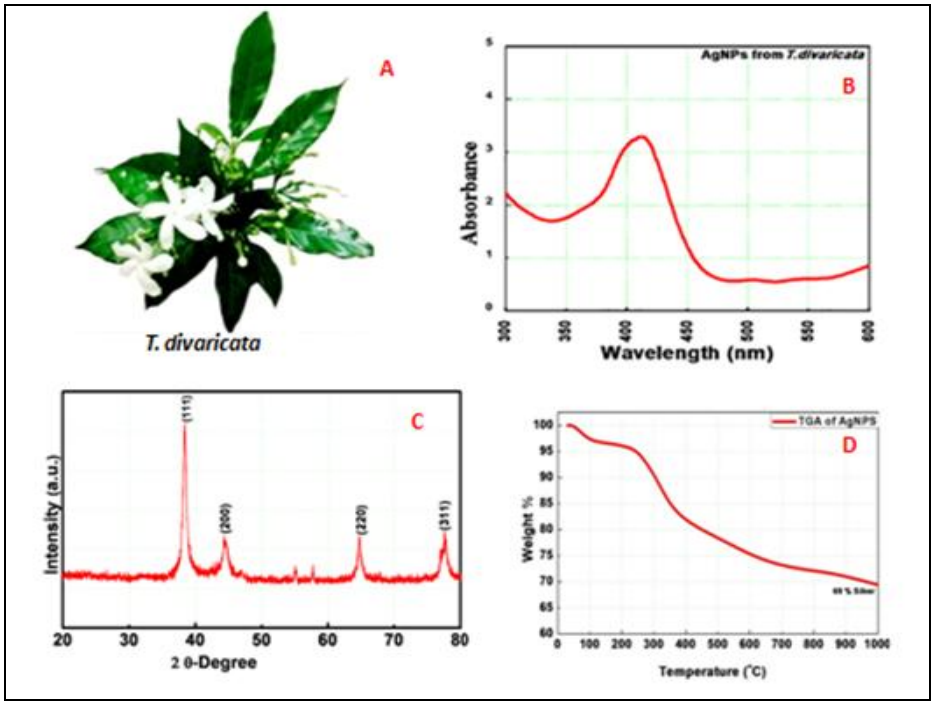

FIG. 1: (A) FRESH AND HEALTHY LEAVES OF *T. DIVARICATA* FOR EXTRACTION PROCESS. (B). THE ULTRAVIOLET-VISIBLE SPECTRA OF *T. DIVARICATA* SILVER NANOPARTICLE (AgNPs). THE ABSORPTION SPECTRA EXHIBITED A STRONG BROAD PEAK AT 414 nm. (C). X-RAY DIFFRACTOGRAM PATTERN CORRESPONDING TO THE DIFFRACTION FROM (111), (200), (220), (311) PLANES OF FCC LATTICE OF *T. DIVARICATA* MEDIATED SILVER NANOPARTICLES, COMPLETELY MATCHED WITH JCPDS CARD NO. 04-0783, REVEALS POLYCRYSTALLINE NATURE OF AgNPs (D) TGA OF CAPPED AgNPs PREPARED USING *T. DIVARICATA* LEAVES EXTRACT

The nature of crystalline AgNPs was confirmed by XRD pattern (**Fig. 1C**). The Bragg reflection was observed at 2θ = 38.11°, 44.27°, 64.42°, 77.46° represents the (111), (200), (220) and (311) planes of centrosymmetric face centered cubic structure of silver. (See ESI JCPDS card 00-004-0783). Average crystalline size was calculated by applying Scherrer's equation in which FWHM value was taken from highly intense (111) plane.

$$D = K\lambda / \beta\cos\theta, \text{ or } D = 0.94\lambda / \beta\cos\theta$$

Where K = Dimensionless space factor or Scherrer's constant (0.94), λ = wavelength of Cu Kα radiation with value 0.1546 nm, β = Full width at half maximum (FWHM) in radian and θ = Bragg angle. The average size of AgNPs was 14.9 nm.

In TGA plot (**Fig. 1D**) we got three successive weight losses. First step weight loss of 3.39% at 70 °C was observed due to loss of moisture from sample. AgNPs began to degrade at around 220 °C. Another dominant weight loss was 17.9% was observed this behaviour is most likely as a consequence of the surface desorption of the bio organic compounds present in AgNPs powdered sample. Total loss of 30.45% was observed in complete thermal degradation. The result indicates that phyto-components of *T. divaricata* are responsible for the reduction of Ag^{+} to Ag^{0} nanoparticle may be a thermally less stable compound, which gets extracted in solvent extract.

FT-IR spectra of *T. divaricata* leaves extract showed the absorption bands at 3308, 2978, 1642, 1043, 1453, 1084, 877 and 612cm^{-1} (**Fig. 2a**). The strong intense band observed at 3308cm^{-1} assigned to intermolecular bonded O-H stretching of alcohol. The peak at 2978cm^{-1} indicated ν(C-H) symmetric vibration of saturated hydrocarbon, The presence of C=C stretch observed at 2133cm^{-1}, -CH_2, -CH_3 bending was appeared at 1453cm^{-1}, C-N bonding was observed at 1084cm^{-1}, band at 1043 cm^{-1} attributed to ν(C-OH) stretching of ester, -C-H out of plane bending was assigned at 877 cm^{-1} and band at 612 cm^{-1} was characteristic of C-Cl. Band at 1642cm^{-1} implies the appearance of amide I group which confirms the existence of protein molecule in plant extract. The change in stretching vibrations observed with AgNPs at 3283cm^{-1} indicated the intermolecular H bonded –OH band with a shift from 3308 cm^{-1} of *T. divaricata* leaves extract. Similarly, bending of carbonyl that was observed at 1622cm^{-1} with AgNPs, was shifted from 1642cm^{-1}. Some more shifting in band around 518 cm^{-1} of AgNPs which was out of plane ring bending shifted from 612cm^{-1} of *T. divaricata* leaves extract.

Other shifted vibration was observed at 729cm^{-1} which indicates –NH_2 group. Out of plane bending (832 cm^{-1}) indicates -C-Cl, -C-C stretches. Vibration band at 2847cm^{-1} indicates N-H stretching of amide II, alkane C-H stretching observed at 2915cm^{-1}. The strong absorption at 1453cm^{-1} (**Fig. 2a**) of *T. divaricata* for COO– symmetric stretch of carboxylic acid is shifted towards lower frequency 1376 cm^{-1} (**Fig. 2b**). The band at 1462cm^{-1} attributed to methylene scissoring vibration from protein. This study suggests that peptides of protein through carboxylate group of amino acid have strong affinity to bind free Ag^{0} so that protein forms a bio coating to prevent agglomeration (**Fig. 6**). Band at 1376cm^{-1} observed due to residual $AgNO_3$ present. The new absorption band at 463cm^{-1} corresponds to characteristic peak of silver nanoparticle –O=C bond formation. The comparison of FTIR spectra of *T. divaricata* leaves extract and AgNPs revealed the involvement of aromatic amine which is alkaloid, Protein, alcohol as well as carbonyl group which mediated the reduction, capping and stabilization of AgNPs formed.

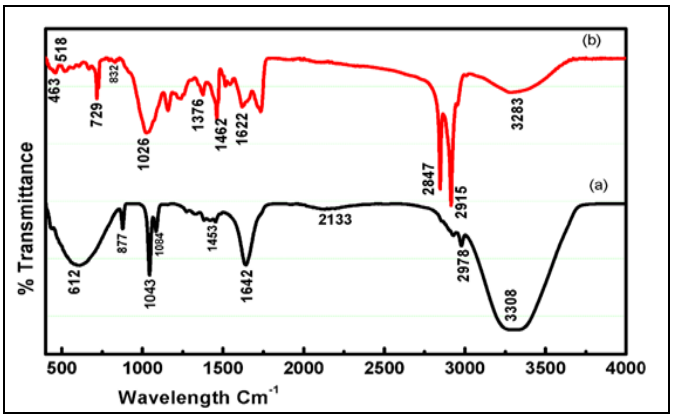

FIG. 2: FTIR SPECTRUM OF (A) *T. DIVARICATA* LEAVES EXTRACT AND (B) BIO SYNTHESIZED AgNPs

The morphology of synthesized nanoparticles was characterized by scanning electron microscope (**Fig. 3**).

SEM image exposed that nanoparticles were well distributed without aggregation; mostly spherical shape particles were observed. EDX studies of AgNPs (**Fig. 3C**) exhibited the strong signal of Ag at 3 Kev due to excitation of surface Plasmon resonance.

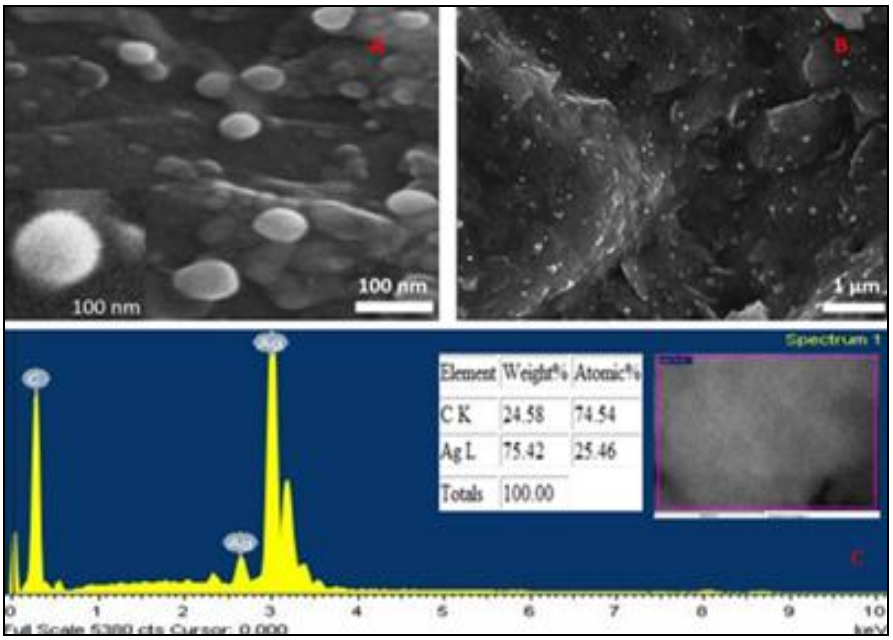

FIG. 3: FEG - SEM MICROGRAPH (A-B) UNDER DIFFERENT MAGNIFICATIONS SHOWING MORPHOLOGY AND SPHERICAL SHAPE OF AgNPs SYNTHESIZED USING *T. DIVARICATALE* (C) REPRESENTS EDS SPECTRA CONFIRMING THE PRESENCE OF AgNPs

TEM study indicates about spherical morphology without agglomeration with size range 6-20nm (**Fig. 4**). TEM image completely confirmed about the nano size of synthesized AgNPs.

The highly crystalline nature of these nanoparticles was also confirmed by selected area diffraction pattern. The four bright circular rings represent (111), (200), (220) and (311) planes.

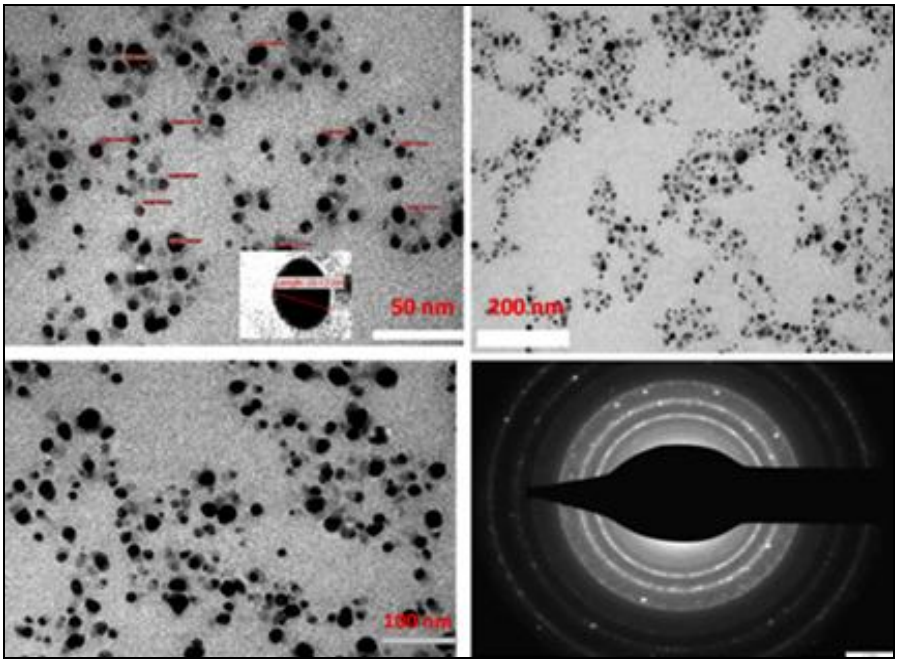

FIG. 4: TEM IMAGES OF *T. DIVARICATA* MEDIATED SILVER NANOPARTICLES (AgNPs) WITH SPHERICAL MORPHOLOGY AND SIZE RANGE 6-20nm, IMAGES SHOWS THE MORPHOLOGY OF NANOPARTICLES ON 50nm SCALE, 200nm SCALE, 100nm SCALES AND SELECTED AREA ELECTRON DIFFRACTION PATTERN. SAED PATTERNS SHOWS CONCENTRIC RINGS WITH INTERMITTENT BRIGHT DOTS INDICATING HIGHLY POLY-CRYSTALLINE NATURE OF SILVER NANOPARTICLES

3D topographic image of typical tapping mode AFM indicated about well arranged, regular and well segregated uniform distribution of AgNPs. Roughness R_{max} was 37.8nm. Image Rq was 4.98 nm and image Ra was 3.95nm. The histogram of height distribution, horizontal line profile (plotted using Gwyddion 2.46 software) and 3D structure are shown below (**Fig. 5**). The film of AgNPs on silicate glass microscope slide displayed dense and uniform packed structure which could provide suitable and biocompatible surface for specific anticancer application.

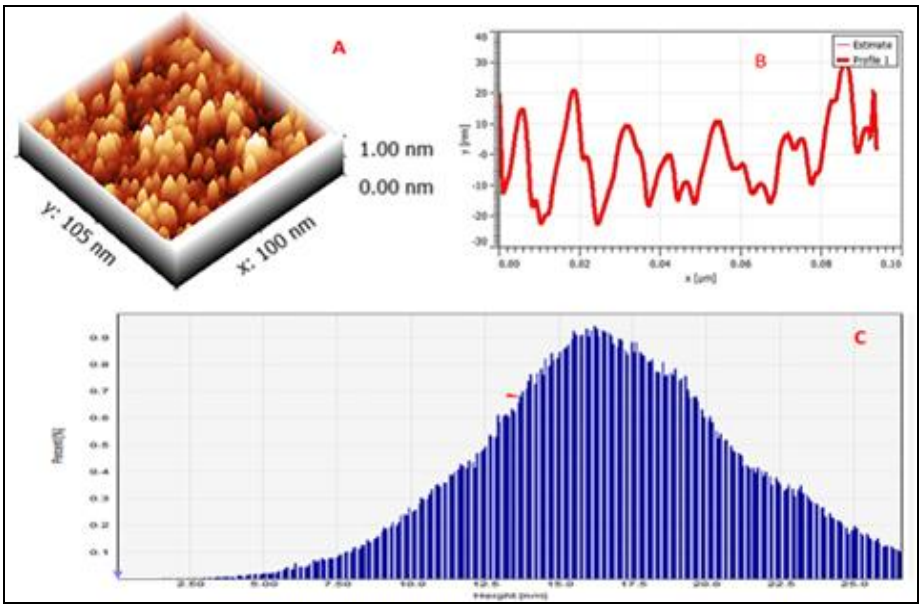

FIG. 5: AFM STUDIES OF SILVER NANOPARTICLES THIN FILM SYNTHESIZED FROM LEAVES EXTRACT OF *T. DIVARICATA* (A) 3D IMAGE (B) HORIZONTAL LINE PROFILE OF AgNPs THIN FILM (C) HISTOGRAM OF HEIGHT DISTRIBUTION

FIG. 6: THE PLAUSIBLE PROPOSED GROWTH MECHANISM FOR FORMATION AND STABILIZATION OF AgNPs

Possible Growth Mechanism of AgNPs Formation: The *T. divaricata* leaves extract contains primary metabolites along with alkaloid, phenol, protein, flavonoid, saponin *etc*. FT-IR study also revealed about wide range of reducing agents, which are responsible for reduction (Ag^+ to Ag^0), capping and stabilization of AgNPs without agglomeration. The constituents of leaves extract not only successfully reduced silver salt but also exhibited excellent tenacity against agglomeration. The capping of *T. divaricata* leaves extract bio-molecule on the surface of AgNPs is evidenced

from FTIR curve (**Fig. 2a**), where absorption bands of AgNPs is shifted with respect to pure *T. divaricata* leaves extract.

Carbonyl group from amino acid residue and peptide of protein have greater tendency to bind silver nanoparticle [20]. Carboxylate group (obtained in FTIR) present in amino acid has greater tendency to act as surfactant by developing a protein layer on silver nanoparticles. Currently E. Vierling *et al.*, has reported that heat shock proteins (10-25 KDa) are found in many leaves extract [21]. Michal Ruerk reported about presence of various dehydrin proteins (12-17 KDa) in plant leaves extract [22], hence presence of protein may not be denied and further FTIR also support the presence of protein which became responsible for nanoparticle stabilization. With the protection of protein, AgNPs aggregation process is prevented and simultaneously growth of AgNPs is controlled resulting in encapsulated smaller and stabilized silver nanoparticles. Step by step schematic representation of chemistry behind AgNPs formation and stabilization is demonstrated as follows (**Fig. 6**).

***In vitro* Testing for Anticancer Reactivity of AgNPs:** Current literature suggests that AgNPs may induce reactive oxygen species and contribute towards cellular damage and cell death process [23]. Presence of many kinds of alkaloids from *T. divaricata* is already reported by T.S. Kam [24]. Antibacterial, antioxidant and antimicrobial studies of the leaves of *T. divaricata* are also reported. To the best of our knowledge no any report available on anticancer activity of *T. divaricata* mediated AgNPs against A549 cell line.

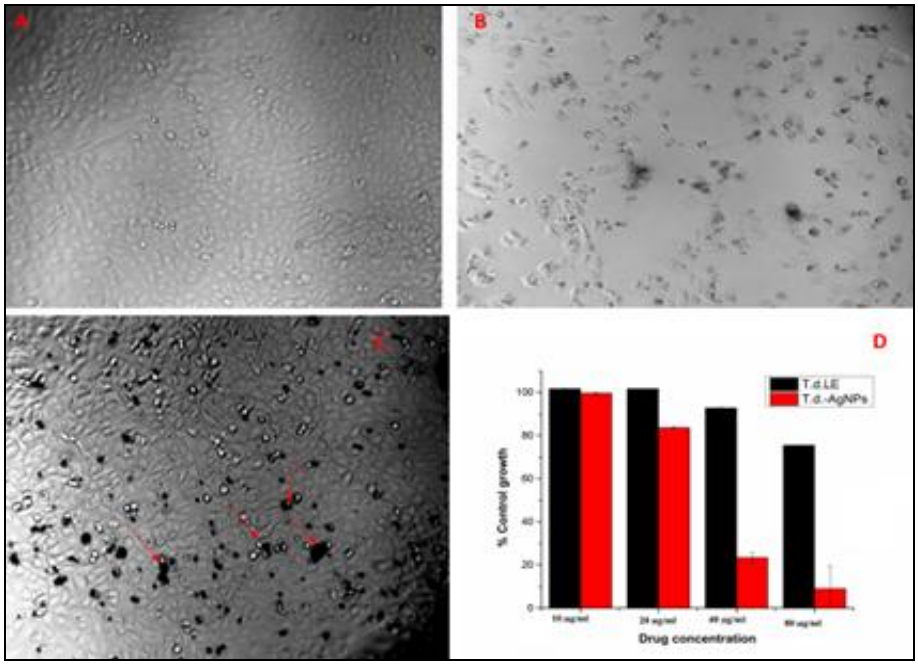

FIG. 7: CYTOMORPHOLOGICAL FEATURES OF (A) CONTROL, (B) POSITIVE CONTROL (ADRIAMYCIN), (C) A549 HUMAN LUNG CANCER CELL LINE TREATED WITH AgNPs COLLOIDAL NANO DRUG, WHICH GOING THROUGH INTRACELLULAR SUICIDE PROGRAM INVOLVING MORPHOLOGICAL CHANGES LIKE CELL SHRINKAGE, OXIDATIVE STRESS, LOSS OF MEMBRANE STABILITY, CELL BURST LEADING TO APOPTOSIS (D) *IN VITRO* CYTOTOXIC EFFICACY OF *T. DIVARICATA* MEDIATED AgNPs ON A549 HUMAN LUNG CANCER CELL LINE. IN PLOT, INCREASED CONCENTRATION OF AgNPs (10-80mg/ml) (X-AXIS) INHIBITS THE VIABILITY OF CELLS (Y-AXIS). STATISTICAL ANALYSIS WAS DONE BY A ONE-WAY ANOVA

All the data were expressed in mean ± SD of three experiments and presented in **Table 2**.

The enhanced cytotoxic effect is due to presence of bioactive compounds (summarized in GC-MS results table shown in ESI) present in *T. divaricata* leaves extract. The *in-vitro* cytotoxic activity of AgNPs synthesized by using *T. divaricata* leaves extract was tested on A549 human lung cancer cell lines by Sulforhodamine B (SRB) assay. Different concentrations of colloidal silver nanoparticles were able to reduce cell viability of A549 human lung cancer cell line, in a dose dependent manner

(**Fig. 7**). A dose dependent decrease in the viability of A549 human lung cancer cells were observed on treatment with AgNPs. Morphological variations such as retardation in cell growth, loss of membrane stability, cell shrinkage and Cell death phenomenon were observed in A549 human lung cancer cell line treated with colloidal nanosilver (**Fig. 7**). Statistical analysis is summarized as follows (**Table 2**).

Dose dependent effect of *T. divaricata* leaves extract mediated AgNPs on A549 human lung cancer cell line. Statistical analysis is based on One-way ANOVA at 5% level of significance.

TABLE 2A: ANOVA ANALYSIS OF *T. DIVARICATA* MEDIATED AgNPs

AgNPs Concentrations	Mean 99.65	SD	95% CI
10	83.66	6.91	99.65± 13.54
20	23.42	3.56	83.66±6.97
40	9.01	11.83	52±22
80	33.8	31.64	49.01±62.01

*Coeff Var. = 0.34889, Root MSE = 17.25949, Data mean = 49.44.

TABLE 2B: ANOVA ANALYSIS OF *T. DIVARICATA* LEAVES EXTRACT

T. divaricata LE Concentrations	Mean 101.73	SD	95% CI
10	102.1	3.95	3.95±7.742
20	92.7	2.10	2.10±4.131
40	75.5	2.33	2.33±4.58
80	2.57	1.05	1.05±2.065

*Coeff var. = 0.02781, Root MSE = 2.582, Data mean = 92.87

As per SRB assay in case of AgNPs, GI_{50} =37.1µg/ml, TGI =68.9µg/ml, LC_{50}=>80µg/ml was obtained, while *T. divaricata* leaves exhibits GI_{50}, TGI, LC_{50} values >80µg/ml, which indicates greater biocompatibility of AgNPs in comparison to *T. divaricata* leaves extract. The IC_{50} was obtained at 32.3µg/ml concentration indicating greater biocompatibility as determined in previous studies by Sankar *et al*., [25], Panniappan *et al*., [26], Venkatesan *et al*., [27], Reddy *et al*., [28], Govender *et al*., 29., Xia *et al*., [30] and Kanipandian *et al*., [31]. Anticancer activity of previous reported AgNPs along with IC_{50} details are summarized in **Table 3**.

TABLE 3: COMPARISON OF GREEN SYNTHESIZED AgNPs FOR ANTICANCER ACTIVITY AGAINST A549 HUMAN LUNG CANCER WITH PREVIOUS REPORTS

S. no	Anti-cancer activity of AgNPs	Plant material use for AgNPs synthesis	IC_{50} (µg/ml)	Reference
1	A549 Lung cancer cells	*Origanum vulgare*	100	Renu sankar *et al*., (2013) [25]
2	A549 Lung cancer cells	*Cymodocea serrulata*	100	P. Paaniappan *et al*., (2014) [26]
3	A549 Lung cancer cells	*Rosa damascena*	80	B. Venkatasen *et al*., (2014) [27]
4	A549 Lung cancer cells	*Accoruscalmus rhizome*	53.2	J. N. Reddy *et al*., (2014) [28]
5	A549 Lung cancer cells	*Albizia adianthifolia*	43	R. Govender *et al*., (2013) [29]
6	A549 Lung cancer cells	*Taxus yunnanensis* Callus	40.3	Q. H. Xia *et al*., (216) [30]
7	A549 Lung cancer cells	*Gossypium hirsutum*	40	Kanipandian *et al*., (2014) [31]
8	A549 Lung cancer cells	*Tabernaemontana divaricata*	32.3	Present work

CONCLUSION: Green Synthetic approach provides an opportunity to design and develop nanomedicine and it has a bright future in new generation of cancer therapeutics. Data from this study revealed the advantage of green method as *T. divaricata* leaves extract was successfully utilized as bio reductant and played a triple role of reducing, capping and stabilizing agent, in fabrication of highly efficient and stable AgNPs at room temperature. The crystalline nature was verified with XRD and SAED pattern. Major bio reductant functional group was analyzed by FTIR. SEM and TEM indicated towards spherical morphology with particle size range 6-20nm. Furthermore, *T. divaricata* derived AgNPs exhibited strong *in vitro* cytotoxic activity against A549 human lung cancer cell line by SRB assay and results indicates that by increasing the concentration of AgNPs, cell death of A549 human lung cancer cells increases.

This is the first report on anticancer activity of green synthesized AgNPs using *T. divaricata* leaves extract against human lung cancer A549 cell. We believe that our finding extends the significant application of AgNPs in biomedical research and cytotoxic potential of AgNPs may lead to discover efficient chemotherapeutic agent against A549 and many other injurious cancer cells, simultaneously inherent cytotoxic potential opens a door towards futuristic effective nanocarrier in biomedicine for targeted drug delivery and effective treatment.

ACKNOWLEDGEMENT: One of the authors (M. Jha) is thankful to the university grants commission, UGC, New Delhi, India, for financial support. We would like to gratefully acknowledge The Head, SAIF, IIT Mumbai for TEM and FEG-SEM studies and The Head, ACDSF, ACTREC for anticancer studies.

CONFLICT OF INTEREST: The authors report no conflict of interest in this work.

REFERENCES:

1. Key statics for lung cancer 2016 (updated 16/05/2016) Available from http://www.cancer.org/cancer/lungcancer-nonsmallcell/detailedguide/non-small-cell-lung-cancer-key-statics.
2. Falk S and Williams C: Lung Cancer-the facts. Oxford University Press, Third Edition 2010.
3. Desantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS and Jemal A: Cancer treatment and survivorship statistics. CA-Cancer Journal of Clinicians 2014; 64: 252-271.
4. Hedaginal BR and Taranath TC: Characterization and antimicrobial activity of biogenic silver nanoparticles using leaf extract of *Thunbergia alata* bojer ex sims. International Journal of Pharmaceutical Sciences and Research 2017; 8(5): 2070-2081.
5. Gulsonbi M, Parthasarathy S, Bharatraj K and Jaysankar V: Green synthesis, characterization and drug delivery applications of a novel silver/carboxymethylcellulose – poly (acrylamide) hydrogel nanocomposite. Ecotoxicology and environmental safety 2016; 134(2): 421-426.
6. Jerley AA and Margret AA: Mycosynthesis of silver nanoparticles and antibacterial effects in wound dressing. International Journal of Pharmaceutical Sciences and Research 2012; 3(6): 1698-1704.
7. Pandian N and Chidambaram S: Antimicrobial, cytotoxicity and anticancer activity of silver nanoparticles from *Glycyrrhiza glabra*. International Journal of Pharmaceutical Sciences and Research 2017; 8(4): 1633-1641.
8. Mishra S and Thakur M: Role of microwave assisted extraction for isolation of saponins from *Sapindus mukorrosai* and synthesis of its stable bio functionalized silver nanoparticles and its hypolipidaemic activity. International Journal of Pharmaceutical Sciences and Research 2016; 7(7): 2959-2965.
9. Rokhade VK and Taranath TC: Phytosynthesis of silver nanoparticles using fruit extract of *Leea indica* (burm. F.) Merr. and their antimicrobial activity. International Journal of Pharmaceutical Sciences and Research 2017; 8(3): 1319-1325.
10. Rahim KA, Mahmoud SY, Ali AM, Almaary KS, Mustafa MA and Husseiny SM: Extracellular biosynthesis of silver nanoparticles using *Rhizopus stolonifera.* Saudi Journal of Biological Sciences 2017; 24: 208-216.
11. Garg S: Microwave-assisted rapid green synthesis of silver nanoparticles using *saraca indica* leaf extract and their antibacterial potential. International Journal of pharmaceutical science and research 2013; 4(9): 3615-3619.
12. Fatimah IS: Green Synthesis of Silver Nanoparticles Using Extract of *Parkia speciosa* Hassk Pods assisted by microwave Irradiation. Journal of advanced research 2016; 7(6): 961-969.
13. Dutta PP, Bordoloi M, Gogoi K, Roy S, Narzary B, Bhattacharya DR, Mohapatra PK and Mazumder B: Antimalarial silver and gold nanoparticles: Green synthesis, characterization and *in-vitro* study. Biomedicine and pharmacotherapy 2017; 91: 567-580.
14. Faraha MA, Ali MA, Chen SM, Li Y, Hemid FM, Tarbous FM, Anazi KM and Lee J: Silver nanoparticles synthesized from *Adenium obesum* leaf extract induced DNA damage, apoptosis and autophagy via generation of reactive oxygen species. Colloids and Surfaces B: Bio-interfaces 2016; 141: 158-169.
15. Shaniba VS, Aziz AA and Kumar PRM: Phyto-mediated synthesis of silver nanoparticles from *annona muricata* fruit extract, assessment of their biomedical and photo-catalytic potential. International Journal of Pharmaceutical Sciences and Research 2017; 8(1): 170-181.
16. Sivaraman D, Panneerselvam P, Muralidharan P, Prabhu TP and Kumar RV: Green synthesis, characterization and anti-microbial activity of silver nanoparticles produced using *Ipomoea aquatica* forsk leaf extract. International Journal of Pharmaceutical Sciences and Research 2013; 4(6): 2280-2285.
17. Sriranjani R, Srinithya B, Vellingiri V, Brindha P, Anthony SP, Sivasubramanian A and Muthuraman MS: Silver nanoparticle synthesis using *Clerodendrum phlomidis* leaf extract and preliminary investigation of its antioxidant and anticancer activities. Journal of Molecular Liquids 2016; 220: 926–930.
18. Vichai V and Kirtikara K: Sulforhodamine B Colorimetric assay for cytotoxicity screening. Protocol 2006; 1(3): 1112 - 1116.
19. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the national cancer institute 1990; 82(13): 1107-12.
20. Annamalai J and Nallamuthu T: Green synthesis of silver nanoparticles: characterization and determination of antibacterial potency. Applied nano science 2016; 6: 259–265.
21. Vierling E, Harish LM and Chen Q: The Major Low-Molecular-Weight Heat Shock Protein in Chloroplasts Shows Antigenic Conservation among Diverse Higher Plant Species. Molecular and cellular biology 1989; 9(2): 461-468.
22. Rurek M: Diverse accumulation of several dehydrin-like proteins in cauliflower (*brassica oleracea* var. botrytis), *arabidopsis thaliana* and yellow lupin (*lupinus luteus*) mitochondria under cold and heat stress. BMC plant biology 2010; 10: 181.
23. Zhu B, Li Y, Lin Z, Zhao M, Xu T, Wang C and Deng N: Silver nanoparticles induce hepg-2 Cells apoptosis through ROS-mediated signalling pathways. Nanoscale Research Letters 2016; 11: 198
24. Kam TS, Pang HS and Lim TM: Biologically active indole and bisindole alkaloids from *Tabernaemontana divaricata*. Organic and Biomolecular Chemistry 2003; 1: 1292-1297.
25. Sankar R, Karthik A, Prabhu A, Karthik S, Shivashangari KS, Ravikumar V: *Origanum vulgare* mediated biosynthesis of silver nanoparticles for its antibacterial and anticancer activity. Colloids and Surfaces B: Biointerfaces 2013; 108: 80–84.
26. Paaniappan P, Satishkumar G, Sankar R: Fabrication of nano-silver particles using *Cymodocea serrulata* and its cytotoxicity effect against human lung cancer A549 cells

line. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 2015; 138: 885-890.
27. Venkatesan B, Subramanian V, Tumla A, Vellaichamy E: Rapid synthesis of biocompatible silver nanoparticles using aqueous extract of *Rosa damascena* petals and evaluation of their anticancer activity. Asian Pacific Journal of Tropical Medicine 2014; 7(1): S294-S300.
28. Nakkala JR, Mata R, Gupta AK, Sadras SR: Biological activities of green silver nanoparticles synthesized with *Acorous calamus* rhizome extract. European Journal of Medicinal Chemistry 2014; 85: 784–794.
29. Govender R, Phulukdaree A, Gengan RM, Anand K, chuturgoon AA: Silver nanoparticles of *Albizia adianthifolia*: sthe induction of apoptosis in human lung carcinoma cell line. Journal of Nanobiotechnology 2013; 11:5.
30. Xia QH, Ma YJ, Wang JW: Biosynthesis of silver nanoparticles using *Taxus yunnanensis Callus* and their antibacterial activity and cytotoxicity in human cancer cells. Nanomaterials 2016; 6 (9): 160.
31. Kanipandian N, Thirumurugan R, A feasible approach to phyto-mediated synthesis of silver nanoparticles using industrial crop *Gossypium hirsutum* (cotton) extract as stabilizing agent and assessment of its *in vitro* biomedical potential. Industrial Crops and Products 2014; 55: 1–10.

How to cite this article:
Shimpi NG and Jha M: Green synthesis of silver nanoparticles using *Tabernaemontana divaricata* and *in-vitro* cytotoxicity investigation against human lung adenocarcinoma. Int J Pharm Sci Res 2017; 8(12): 5100-10.doi: 10.13040/IJPSR.0975-8232.8(12).5100-10.