International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN: 0974-4290
Vol.7, No.5, pp 2460-2468, 2014-2015

Biosynthesis of silver nanoparticles using Impatiens balsamina leaf extracts and its characterization and cytotoxic studies using human cell lines.

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Abstract: Green syntheses of nanoparticles were abundantly used in nanoscience having greater application in as ecofriendly and non toxic. This study aims to analyse aqueous, ethanolic and methanolic extracts of *Impatiens balsamina (Ib)* flowers and Thin Layer Chromatography (TLC) studies for the presence of various phytochemicals. Synthesised silver nanoparticles using the aqueous extract of Ib flowers and also evaluate the anti cancer and antimicrobial activity. The presence of various phytochemicals viz., alkaloids, tannins carbohydrates, terpenes and glycosides were analysed by standard biochemical screening methods. The synthesised silver nanoparticles (SNPs) were characterised by using UV-Vis Spectroscopy, FTIR, XRD, SEM, TEM and also antimicrobial activity. The extracts contain various phytochemicals and also synthesised nanoparticles were found to be spherical in shape with average size in the range of 15 nm. We checked the cytotoxic properties of SNPs having greater inhibition of various cancer cellines. The results revealed that the aqueous extract of Impatiens *balsamina* flowers is a very good bioreductant for the synthesis of silver nanoparticles.
Keywords: Phytochemical analysis, silver Nanoparticles (SNPs), FTIR, XRD, SEM, TEM, antimicrobial studies, *Impatiens balsamina* flower extracts, cell lines.

1. Introduction:

Nanoparticles play an important role in all fields like biology and also medicine, etc. In medical field it is used to treat skin cancer and reduce bleeding in trauma patients [13]. There are different types of nanoparticles like those of metals, fibers, etc. among these silver nanoparticles have found many applications. Fabric containing bamboo-charcoal nanoparticles is used in masks owing to its antimicrobial properties [14]. Silver nanoparticles are used for deactivating HIV at low concentrations with less toxicity. It is also used in plasmonics, medicine, catalysis, photonics and optics .Silver nanoparticles are used as antiviral agents, as antimicrobials, anti-proliferative agents [15-17] and many more. Silver nanoparticles are not harmful to humans but act as effective agents against different bacteria [18-20], fungi [21-22] and yeast.

In India, since ancient times drugs used in traditional systems of medicines such as *Unani* and *Ayurveda*. The drugs are derived from the whole plant or from different organs, like leaves, stem, bark, root, flower, seed, etc. Some drugs are prepared from excretory plant product such as gum, resins and latex. Many secondary metabolites of plant are commercially used in a number of pharmaceutical compounds [1]. The isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. The scientific study of traditional medicines, derivation of drugs through bio prospecting and systematic conservation of the concerned medicinal plants are thus of great importance.

Impatiens balsamina L is an annual plant belongs to family *Balsaminaceae* commonly called as Balsam, Jewelweed. A major portion of pharmacological research is directed to anticancer drug designed to

made new molecular targets [2].Due to immense aptness of plants, variety of structurally diverse bioactive compounds, the potential chemical constituents in plants are capable of antitumor, anti-inflammatory, antidiabetic, antioxidant, BSA denaturation and also cytotoxic activities [3-12]. This plant juice used for against snakebites. [24].

Nowadays synthesis of nanoparticles using plant materials was greater application in the field of nanobiology. Plants having the number of alkaloids and terpenoids, flavonoids and other compounds are involved in the bioeduction of siver. Those are the ecofriendly and nontoxic to humans as well as animals. These are toxic to pathogenic microorganisms. *Impatiens balsamina* flower extract silver nanoparticles were checked against various cancer cell lines having greater diminishing properties.

This study explains novel approaches for green synthesis of solver nanoparticles using fresh flower extracts from the plant Impatience balsamina. As it plant had many medicinal applications. This flower mediated nanoparticles are greater medicinal application, we had gone through the characterization and applications in various human cell lines.

2. Material and Methods:

All the reagents used in the study were of analytical grade. Silver Nitrate ($AgNO_3$) was obtained from Sigma Aldrich.

2.1 Collection and processing of flower extract:

Fresh flowers of *Impatiens balsamina* were collected from, botanical garden, Osmania University Hyderabad, India. A 25 gms of fresh flowers were washed thrice thoroughly with distilled water to remove the dust particles, and used to prepare aqueous in 100 ml of deionised water, boiling for 15 min at 100 °C. Prepared ethanolic and methanolic extracts by prepared by orbital shaker incubation at 37 °C for 3 days.

Fig1: Impatiens balsamina flower

2.2 Preparation of Aqueous Extract:

5 gr of fresh flower material was taken in a clean 250 ml Erlenmeyer conical flask and 50 ml of sterile double distilled water was added and kept on a sand bath for 20 min. to prepare the aqueous flower extract. The extract was then filtered using Whattman No. 1 filter paper. The aqueous flower extract is used for further phytochemical analysis, synthesis of silver nanoparticles.

2.3 Preparation of Ethanolic Extract:

100 ml of 90% ethanol was added in 250 ml Erlenmeyer conical flask, followed by closing it with cotton plug and incubated on shaker incubator for 3 days to promote the formation of ethanolic flower extract by filtering the solution with Whattman No. 1 filter paper. The ethanolic flower extract is used for further phytochemical analysis and TLC by storing it in refrigerator by closing it with cotton plug in order to prevent the evaporation.

2.4 Preparation of Methanolic Extract:

100 ml of HPLC grade methanol was added in 250 ml Erlenmeyer conical flask and followed by closing it with cotton plug and incubated on shaker incubator for 3 days to promote the formation of methanolic flower extract by filtering the solution with Whatman No. 1 filter paper. The methanolic flower extract is used for further phytochemical analysis and TLC by storing it in refrigerator by closing it with cotton plug in order to evaporate the extract.

2.5 Preliminary investigation for the presence of phytochemicals:

The ethanolic, methanolic and aqueous extracts of *Impatiens balsamina* flowers were used to analysing phytochemical constituents such as alkaloids, tannins, glycosides, flavonoids, saponins, terpenes, carbohydrates by TLC and HPLC.

2.6 Biosynthesis of silver nanoparticles:

The aqueous extract obtained from flowers is used for the synthesis of silver nanoparticles. The extract was added to the 1mM silver nitrate solution in 1:10 ratio and then heated to 100^0C for 10mins.The colour change from pale yellow to reddish brown was observed due to the reduction of Ag^+ ions (Fig: 2).

Fig 2: Impatiens balsamina flower extract and Nanoparticles

3. Characterization of Silver Nanoparticles:

3.1 UV-Vis spectra analysis

The reduction of pure silver ions were monitored by the UV Visible Spectrum, using an Elico SL-159 UV spectrophotometer at 300-700 nm and flower extract as blank against silver nanoparticles solution for measuring the UV Visible spectrum shown in fig 3. This was the preliminary characterization for silver nanoparticles formed by the reduction of aqueous flower extracts of Ib.

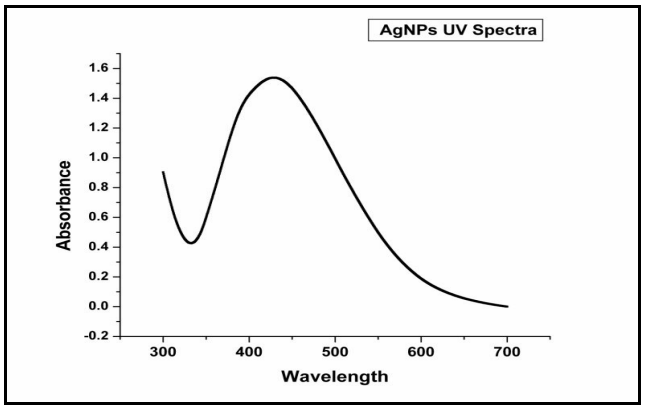

Fig. 3: UV-Vis spectra recorded reaction of 10^{-3} M aqueous solution of AgNO3 with *Impatiens balsamina flower* extract

3.2 FTIR analysis:

The residual solution was centrifuged at 10,000 rpm for 15 min. to remove any free biomass residue, and the resulting suspension was redispersed in 1 ml sterile distilled water. Thereafter the purified suspension was freeze dried to obtain dried powder. Finally, the dried nanoparticles were analyzed by FTIR using Paragon 500, Perkin Elmer-RX1 spectrophotometer to diffuse the reflectance mode for resolving 4cm^{-1} in KBr pellets.

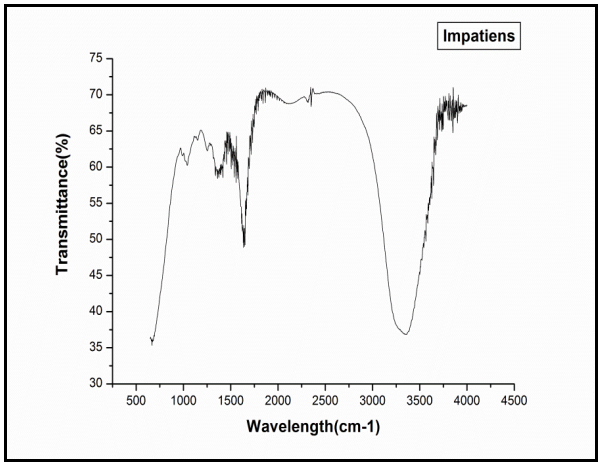

Fig.3 FTIR spectra of Ag Nanoparticles synthesized by reduction of Ag^+ ion by *Impatiens balsamina*

3.3 XRD analysis:

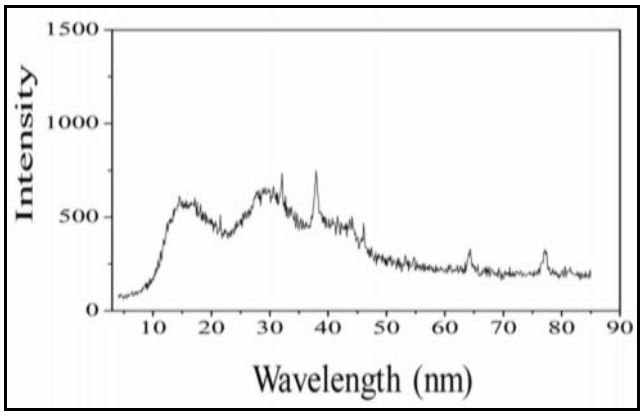

Fig. 4: XRD pattern of Ag nanoparticles synthesized by treating *Impateins balsamina flower* extract with AgNO3 solution:

Centrifuged and dried nanoparticles were used for X-ray diffraction (XRD). The XRD measurement was carried out by an X'Pert Pro X-Ray diffractometer generated at voltage 40 kV, a generator current of 30 mA with CuKα X-ray source (λ=1.54056A^o) at scanning rate of 2° min−1.

3.4 Scanning Electron Microscopy (SEM) Analysis

The morphology of the synthesized silver nanoparticles were identified by using Scanning Electron Microscope (SEM) with Zeiss 700 Scanning Electron Microscope (SEM).Thin films of the sample were prepared by dropping a very small amount of the sample on glass plates and then allowed to dry at room temperature.

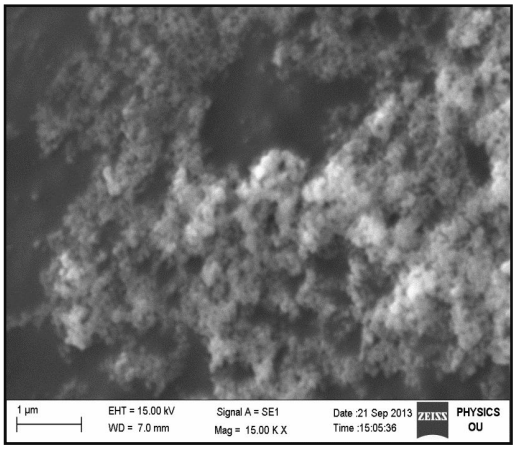

Fig. 5: SEM image of the silver nanoparticles synthesized by using Impateins balsamina flower extract

3.5 Transmission Electron Microscopy (TEM) Analysis:

The size of silver nanoparticles formed by the *Impateins balsamina* was done by the TEM image analysis. The TEM analysis was performed by using a TECHNAI FE 12 TEM instrument operating at 120 kV, which has a diffraction patterns recorded at selected areas to determine the particle structure and size of silver nanoparticles at a 660 mm camera length.

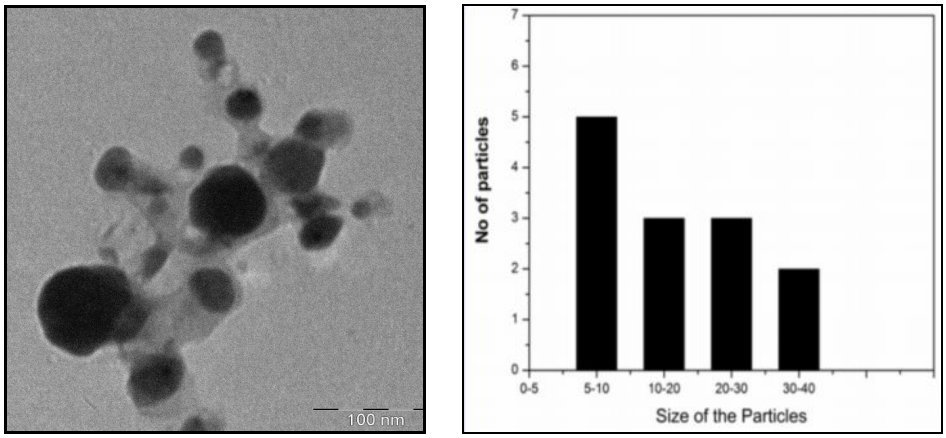

Fig. 6(A): TEM image of the silver Nanoparticles synthesized by using Impatiens balsamina flower extract. (5B) Histogram of particle size distribution of silver Nanoparticles.

3.6 Thin Layer Chromatography (TLC) Analysis:

The Aqueous, Ethanolic and methanolic extracts were definite for Thin Layer Chromatography (TLC). The solvent system used here is Ethyl acetate and Hexane (2:8) ratios were found to be the best for separation. After proliferating TLC of the methanolic extract, RF values were calculated for the spots were seen under UV illuminator for various compounds present in the extracts were shown in table 1.

Table 1: Phytochemical screening of Aqueous, Ethanolic and Methanol extracts of *Impatiens balsamina*

S. No	Primary and Secondary metabolites	Aqueous Extract	Ethanolic Extract	Methanolic Extract	Test Method
1.	Alkaloids	-	+	+	Siddiqui and Ali, 1997
2.	Tannins	-	+	+	Mukherjee PK; 2002
3.	Glycosides	-	++	++	Trease and Evans;1989
4.	Flavonoids	-	-	-	Siddiqui and Ali;1997
5.	Saponins	-	-	-	Siddiqui and Ali; 1997)

6.	Terpenes	+	++	++	Harborne; 973
7.	Carbohydrates	++	++	++	Krishnaveni et ai;1984

3.7 Antibacterial Activity studies:

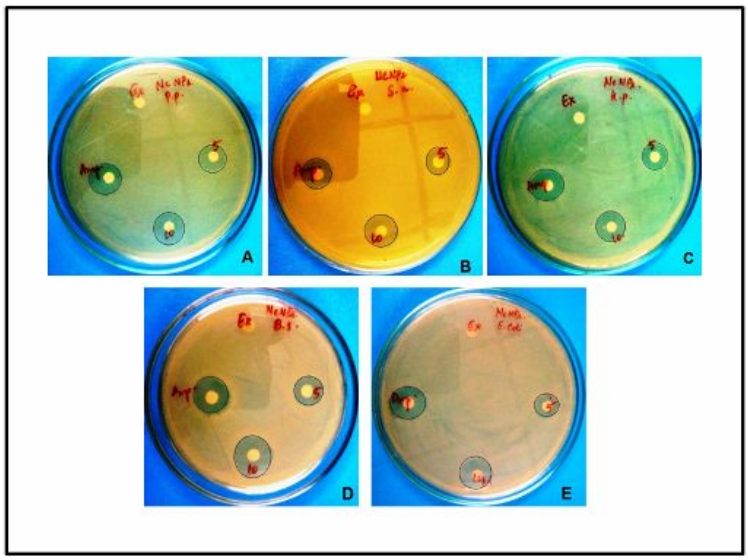

Fig.7 Antimicrobial activity of silver Nanoparticles synthesized by using Impatiens balsamina flower extract, A. *Pseudomonas putida*, B. *Staphylococcus aureus*, C. *Klebsheilla pnuemonea*, D. *Bacillus subtillis*, E. *Escherichia coli*.

The antibacterial activity of *Impatiens balsamina* flower extract and synthesized silver nanoparticles and crude extracts were evaluated by the agar disc diffusion method. For the determination of antibacterial activity, gram positive bacteria namely *Bacillus subtillis., Staphylococcus aureus* and *Pseudomonas putida*, and gram negative bacteria *Escherichia coli, Klebsheilla pnuemonea* were used.

3.8 Cell Culture and Nanoparticles Treatment:

The cell lines U937 (human histiocytic lymphoma), COLO205 (human Colon adenocarcinoma), B16F10 (mouse mealanocarcinoma) HepG2 (hepato cellular carcinoma) and HeLa (human cervix carcinoma) cell lines were obtained from the National Centre for Cellular Sciences (NCCS), Pune, India. Cells were used between passages 10 and 20 and cultured in RPMI -1640 (U937, COLO-205), DMEM (B16F10, HepG2) and MEM (HeLa) media, supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM NaHCO3, 2 mM -glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained in culture at 37° C in an atmosphere of 5% CO_2. At 85% confluence, cells were harvested using 0.25% trypsin and the cells (2 x 104) were seeded in each well containing 100µl of medium in 96 well plates. Cells were allowed to attach the surface for 24 h prior to nanoparticles exposure. IBNPs were suspended in complete cell culture medium (8mg/ml stock) and diluted to appropriate concentrations (25, 50, 75, 100, 150 and 200µg/ml). The dilutions of IBNPs were then sonicated using a sonicator bath at room temperature for 15 min at 40W to avoid nanoparticles agglomeration prior to administration to the cells. Selection of 25–200µg/ml concentration of IBNPs was based on a preliminary dose-response study (data not shown).

Results and Discussion:

The adding of *Impatiens balsamina* flower extract to 1mM silver nitrate solution lead to the appearance of a reddish brown colour solution after 15mins indicating the formation of silver nanoparticles. Further UV-Vis spectral analysis showed surface Plasmon resonance (SPR) band at 456 nm, a typical of silver nanoparticles. Fig.2 shows the UV-Vis spectra of silver nanoparticles synthesized by using *Impateins balsamina* flower extract.

The FTIR analysis was carried out to identify the possible biomolecules responsible for the reduction of Ag^+ ions and capping of the bioreduced nanoparticles synthesized by the aqueous flower extract of *Impatiens*

balsamina. FTIR spectra of Ag nanoparticles formed by reduction of Ag^+ ions (Fig. 3) using *Impateins balsamina flower* extract showed peak at 3248 cm-1(weak peak for O-H vibration), 1704 cm-1, 1682 cm-1, 1514 cm-1, 1381 cm-1, 1139 cm-1. The strong peak 3344 cm-1 is due to presence of phenolic group and for water present in the sticky part of the ethanol extract of the plant material. This result suggested the presence of flavonoids adsorbed on the surface of metal nanoparticles.

The biosynthesized silver nanoparticles by employing *Impateins balsamina flower* extract was further demonstrated and confirmed by the characteristic peaks observed in XRD analysis. The diffraction peak of silver nanoparticles at 2^{θ} values 38.10o, 44.16o, 64.52o, 77.45° are assigned to plane (111), (200), (220) and (311) of lattice plane of face centered cubic (fcc) for Ag nanoparticles respectively (Fig.4). The high intensity diffraction peak was observed at 38.18°, corresponding to the crystalline Ag. It is confirmed that the nanoparticles were composed of pure crystalline Ag and spherical in shape. It was further confirmed by SEM and TEM image analysis.

The SEM image of silver nanoparticles synthesized by using *Impateins balsamina flower* extract is shown in Fig.5, which shows distinct and clear image of synthesized silver nanoparticles having spherical shapes in the average size in the range between 5- 40 nm. This image further indicates that the silver nanoparticles are not aggregated i. e monodisperse in nature.

The TEM image of silver nanoparticles synthesised by using *Impateins balsamina* flower extract is shown in the Fig.6 which predominantly shows spherical shape with smooth surface morphology. The Histogram figure of the particles shows number of particles formed and also size of the particles were obtained on TEM grid. It is confirmed by histogram that silver nanoparticles size ranges within 5 - 40 nm.

The Aqueous, methanolic and ethanolic extracts were checked for Thin Layer Chromatography. Ethyl acetate and Hexane (2:8) ratios were found to be the best for separation of various compounds present. Terpenes and carbohydrates were present in the aqueous flower extracts of Ib and methanolic and ethanolic extracts were had Alkaloids, terpenoids, Tannins, glycosides, and terpenes and carbohydrates. These are involved in the reduction of silver nanoparticles.

Table 2: Antimicrobial activity of *Impatiens balsamina silver nanoparticles against following Gram positive and Gram negative bacteria.*

S. No	Name of the Organism	Inhibitory zones diameter in mm			
		Extract 5µl	**IbNps 5µl**	**IbNps 10µl**	**Ampicillin 5µl**
1.	*Pseudomonas putida*	-	19.31	22.76	22.49
2.	*Staphylococcus aureus*	-	20.9	27.7	25.4
3.	*Klebsialla pneumonia*	-	17.20	23.02	22.02
4.	*Bacillus subtillis*	-	17.1	21.9	26.2
5.	*Escherischia coli*	-	18.5	23.55	25.67

The antibacterial studies were studied with Gram positive strains like *Staphylococcus aureus, Bacillus subtilis, Klebsheilla pnuemonea, and Gram negative strains like Pseudomonas putida*, *Escherichia coli,* were used. Zone of inhibition was calculated shown in the Fig., 7A, B, C, D, E and Table.2. The plates are examined for the presence of growth inhibition, which is indicated by a clear zone surrounding each disc. The susceptibility of an organism to a plant extract is determined by the size of the zone. The gram negative bacteria had 14.5 mm, increased zone diameter than gram positive bacteria which is 13.2 mm. Because of gram negative bacteria having thick cell wall and polysaccharide layers as negative bacteria had thin cell wall. (Srivasthava S 2007)

Cytotoxicty was measured using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, according to the method of Mossman (1983). Briefly, the cells (2 x 104) were seeded in each well containing 100µl of medium in 96 well plates. After overnight incubation at 37 °C in 5% CO2, exactly 100µl of different test concentrations (25µg to 200µg/ml) of IBNPs were added to the cell suspension, which is equivalent to 5 to 40µg per 200µl of assay volume. The viability of cells was assessed after 24h, by adding 10µl of MTT (5 mg/ml) per well and incubated at 37°C for additional three hours. The medium was discarded and the formazan blue, which formed in the cells, was dissolved in 100 µl of DMSO. The intensity of colour

formation was measured at 570 nm in a spectrophotometer (Spectra MAX Plus; Molecular Devices; supported by SOFTmax PRO-5.4). The percent inhibition of cell viability was determined with reference to the control values (without test nanoparticles). The data were subjected to linear regression analysis and the regression lines were plotted for the best straight-line fit. The IC50 (inhibition of cell viability) concentrations were calculated using the respective regression equation, were shown in table 3.

Table 3: *In vitro* Cytotoxicity of biologically synthesized IBNPS against U937, Colo-205, B16F10, Hep G2 & HeLa cells by MTT assay.

S.NO	Cell line	$^{\S}IC_{50}$ values of IBNPs (µg/ml)	$^{\S}IC_{50}$ values of ^{a}Etoposide (µg/ml)
1	U937	84.17 ± 2.13	5.02 ± 0.62
2	Colo205	65.40 ± 2.41	4.07 ± 0.12
3	B16F10	196.5 ± 4.19	4.12 ± 0.42
4	HepG2	95.52 ± 4.08	3.12 ± 0.26
5	HeLa	93.27 ± 2.53	2.12 ± 0.32

The biological activities of IBNPs were prepared from flower extracts of ***Impatiens balsamina*** were evaluated to investigate their anti-proliferative/cytotoxic activities in five different types of human cancer cells including U937 (human histiocytic lymphoma), COLO205 (human Colon adenocarcinoma), B16F10 (mouse mealanocarcinoma) HepG2 (hepato cellular carcinoma) and HeLa (human cervix carcinoma) cells. Our results indicate that IBNPs were prepared from flower extracts of ***Impatiens balsamina*** are active against all cell lines including U937, Colo205, B16F10, HepG2 and HeLa at below 200µg/ml concentration (Table-3). COLO205 cells are more sensitive for NENPs (IC50: 65.40±2.41) followed by U937 (IC50: 84.17±2.13), HeLa (IC50: 93.27±2.53), HepG2 (IC50: 95.52±4.08) and B16F10 (IC50: 196.5±4.19). The order of sensitivity of human cancer cell lines towards the IBNPs is Colo205> U937 >HeLa >HepG2 >B16F10. The biologically synthesized IBNPs were exhibited cytotoxic properties against all the cell lines (<200 µg/ml) in a concentration-dependent manner. It is evident from the overall results, that the biologically synthesized IBNPs showed the potent anti-proliferative activity against Colo205 cell line, which is less cytotoxicity than the positive control, Etoposide (Table 3). Exponentially growing cells were treated with different concentrations of IBNPS for 24h and cell growth inhibition was analyzed through MTT assay. $^{\S}IC_{50}$ is defined as the concentration, which results in a 50% decrease in cell number as compared with that of the control cultures in the absence of an inhibitor and were calculated using the respective regression analysis. The values represent the mean ± SE of three individual observations. ^{a}Etoposide is a standard drug molecule employed as positive control.

Discussion:

The results of the qualitative screening of the phytochemical components such as alkaloids, tannins, glycosides, flavonoids, saponins, terpenes, carbohydrate in the aqueous, ethanolic and methanolic flower extracts of the plant species are shown in table 1. The presence of bioactive plant product visualises the separation of chemical constituents from the mixture of compounds through suitable chromatographic techniques. In the present study, in aqueous extract carbohydrates, terpenes show positive. In ethanolic extract the tests for the presence of alkaloids, carbohydrates, flavonoids, terpenes and glycosides shows positive. In methanolic extract the tests for the presence of alkaloids, carbohydrates, flavonoids, terpenes, tannins and glycosides shows positive These Phytochemical examinations focus on the distinct separation of the natural chemical constituents from plant extracts as they may be used for the clinical practices. Differential anticancer properties of the biologically synthesized IBNPS against cell lines (U937, COLO205, B16F10, HepG2 and HeLa) may be due to different mechanism of action. These preliminary results indicated that slight modification of methodology for biologically synthesis of IBNPS may yield as prospective anticancer drugs. Based on the present results, it is warranted that these IBNPS to be further evaluated on other cancer cell lines.

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