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EVALUATION OF CYTOTOXIC POTENTIAL OF TERMINALIA BELLERICA ROXB SILVER NANOPARTICLES AGAINST PANCREATIC CANCER ASPC-1 CELL LINE

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ABSTRACT

Silver is a second most valuable metal in the world which might be used for ornamental as well for medical treatment purposes. After the arrival of nanotechnology in science field,the treatment of diseases become more advanced than compared to previous era, Nanoparticles green synthesis from plants also possess excellency in the pharmacological area. However, the silver nanoparticles from *Terminalia bellerica* roxb in the treatment of cancer is taken for the present study, the UV-Vis, FT-IR, SEM analysis revealed the potency of silver nanoparticles of *Terminalia bellerica* Roxb in pancreatic cancer cell line AsPC-1. The MTT study shows the maximum anticancer activity against AsPC-1 of *Terminalia bellerica* roxb. In the present study, We critically assess the role of silver nanoparticles in biomedical application.

KEYWORDS: FT-IR, MTT assay, SEM, *Terminalia bellerica* roxb, UV- Vis.

INTRODUCTION

Nanotechnology in Medicine

Nanotechnology and especially nanomaterials have received much consideration because their structure and properties differ appreciably from those of molecules, atoms, and bulk materials.[1] The synthesis of metal nanoparticles has been widely discussed in the literature

due to their distinctive chemical and physical properties, which have many potential purposes.[2] The utilization of non-toxic solvents, biodegradable materials and low-cost green chemicals are central to resources synthesis and processing, considering the green reaction method of these strategies. The stabilizer, reaction medium, and green reducing agent are three key factors in the synthesis and stabilization of metallic nanoparticles.[2]

Silver nanoparticles production

The use of plant and plant extract in nanoparticle synthesis is considered advantageous over microbial based system because it reduces the elaborate process of maintaining cell cultures. The particle size growth can also be controlled by altering synthesis conditions like P^H, reductant concentration, temperature, mixing ratio of the reactants etc., The plant based synthesis can be carried out either extracellular or intracellular. Intracellular synthesis takes place inside the plant whereas the extracellular synthesis occurs *in vitro*.

Fig 1: Green Synthesis of Silver Nanoparticles.

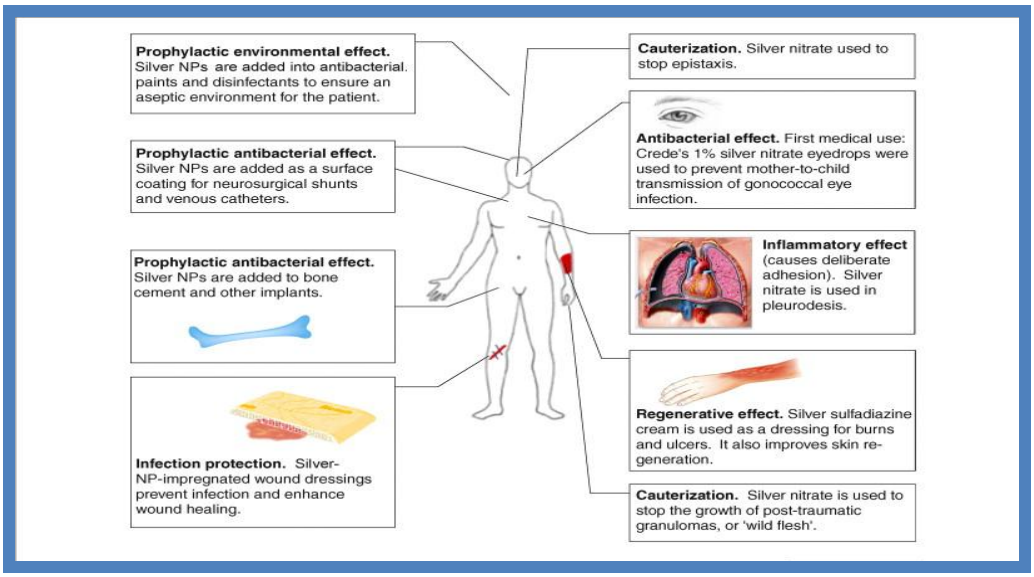


Fig 2: Silver Nanoparticles-Role in medicine.

Cancer

Pancreatic cancer is one of the most aggressive human malignancies. It represents the fourth most frequent cause of cancer-related death and the second most frequent cause, after colorectal cancer, when considering digestive tract cancers alone.

Pancreas is an important retroperitoneal organ with exocrine and endocrine functions. Tumors of pancreas are divided into two groups.[3]

1) Non-endocrine pancreas tumors
2) Endocrine pancreas tumors.

Non-endocrine pancreas tumors are categorized as benignant and malignant. Benignant non-endocrine tumors of the pancreas: adenoma, cystadenoma, lipoma, fibroma, lymphangioma and neuroma. Malignant tumors of the pancreases have different histological features i.e.

1) Ductal adenocarcinoma
2) Cystadenocarcinoma and
3) Other (sarcomas, metastatic etc) malignant tumors.

Diabetes Mellitus (DM) with an onset after 45 years of age may sometimes foresee pancreatic cancer. Pancreatic cancer may sometimes manifest as acute pancreatitis. Today, there are opinions that pancreatic cancer originates from a genetic disposition (stem cell disease).[4,5,6,7] Presence of some colonic polyp types of cancer in the family or personal history increases the likelihood of pancreas cancer.

Risk factors for pancreatic cancer

1. Diabetes mellitus
2. Cigarette Smoking
3. Pancreatitis
4. Alcohol Consumption
5. Diet
6. Excess Weight (Overweight and obesity)

Medicinal plants with anticancer activity

Plants are the chief source of natural products that are used in medicine. Generally, populations that consume a high level of natural herbal products have a reduced incidence of

cancer.[8] For this reason, extracts from different plants have been extensively studied. The present study is focused to screen traditionally used medicinal plants for anticancer effect. The present study was carried out for the potency of *Terminalia bellerica* in anticancer activity.

MATERIALS AND METHODS

Plant collection

The dried fruits of *Terminalia bellerica* were collected from local market in Mannargudi, Tiruvarur district in Tamilnadu.

Collection, Identification and Authentication of plant materials

The plant species namely *Terminalia bellerica* plant was collected from in and around Mannargudi, Thiruvarur District, Tamil Nadu, India. The plant was identified with the help of the Flora of Presidency of Madras and authenticated by Dr.S. John Britto, RAPINAT Herbarium and Center for Molecular Systematics, St. Joseph's college, Tiruchirappalli (Voucher number of the specimen, DS 008).

Preparation of Extract

The dried fruits were pulverized in the blender and sieved for use. The coarse free sample is soaked in ethanol for 72hrs.The coarse free filtrate was filtered by means of Whatmann No.1 filter paper. The extract was kept in a boiling water bath to evaporate ethanol. The ethanol free extract was used for preliminary analysis.

Synthesis of Ag nanoparticles using dried fruits extract

Development of AgNPs-Fruit extract (10ml) was added to 100 ml of 5mM and 10mM aqueous silver nitrate solution (1:9) at room temperature. The formation of AgNPs was indicated by the development of yellowish-dark brown color in the variation of P^H, first the P^H of the extract was adjusted before adding silver nitrate solution. The incubation was done for 5hrs. The observations were made after each hour, to encompass a change in color from light greenish to dark brownish. After each hour small amount of the reaction mixture was centrifuged at 18,000 rpm for 25 min and the supernatant was collected and pellet was stored at 4^0C.

Fig 3: Green synthesis of AgNPs of *Terminalia bellerica.*

Preliminary Phytochemical screening

Pytochemical analysis of the extract was conducted following the procedure of Indian pharmacopeia (1985).By this analysis, the presence of several Phytochemicals like alkaloids, flavonoids, tannins, saponins, esters, resins, sugars and glycosides, were tested.

The UV and FT-IR spectroscopic analysis were carried out under standard procedures.

Scanning electron microscope (SEM)

In this research work, Jeol JSM-6480 LV SEM machine was used to characterize mean particle size and morphology of nanoparticles. The freeze dried sample of Ag NPs solution was sonicated with distilled water and small drop of this sample was placed on glass slide and allowed to dry. A thin layer of platinum was coated to make the samples conductive. Jeol JSM -6480 LV SEM machine was operated at a vacuum of the order of 10-5 torn. The accelerating voltage of the microscope was kept in the range 10-20 KV. Compositional analysis on the sample was carried out by the energy dispersive X-ray spectroscopy (EDS) attached with the SEM.

***In-Vitro* CYTOTOXIC ACTIVITY OF MTT ASSAY**

Purification of biosynthesized silver nanoparticles

To remove the excess silver ions, the silver colloids were centrifuged at 10,000 rpm for 15 minutes and washed three times with millipore water. A dried powder of silver nanoparticles was obtained by freeze drying in lyophiliser for further characterization.

Cell lines

Pancreatic cancer cell lines (AsPC-1 cell line) were used in this study and procured from National Centre for Cell Sciences (NCCS), Pune, India.

Culture medium

Human pancreatic cell lines AsPC-1 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 25 µg/mL amphotericin B. The cells were cultured in a 37°C incubator in a humidified atmosphere containing 5% co_2.

Preparation of Stock solution

Stock solutions (20 mg/mL) of the silver nanoparticles synthesized from *Terminalia bellerica* were dissolved in DMSO and serially diluted with complete growth medium containing 50 µg/mL of gentamycin to the desired concentrations (10, 20, 40, 80 and 100 µg/mL). Untreated control cultures received only the vehicle (DMSO<1%).

Cell Viability assay

MTT Assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. Cancer AsPC-1 cells were seeded at the density of 2×105 cells/well into 6 well plates and treated with the naoparticles for 24 h. The cells were permitted to adhere for 24 hours, and the growth medium (MEM) removed using micropipette and the monolayer of cells washed twice with MEM without FBS to remove dead cells and excess FBS. 1ml of medium (without FBS) containing different dilution of drugs were added in respective wells; 200 µl MTT (5 mg/ml in PBS) were added to each well, and the cells incubated for a further 6-7 hrs in 5% CO_2 incubator. After removal of the medium, 1ml of DMSO was added to each well. The effect of nanoparticles on cell growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as 100% viable. The cells were then exposed to with the medium alone (as positive control). Concentrations of the AgNPs ranging 10-100 µg/ml and gemcitabine (10µg), the standards were used for the study. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product(Horiuchi *et al.*,1988).

The cells are then solubilised with an organic solvent (eg. isopropanol) and released the solubilised formazan product. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. The cells were incubated with the nanoparticles for 24h and 48 h and the cell mortality were checked. The plates were placed on a shaker for 15 min and the absorbance was read on an enzyme-linked

immunosorbent assay (ELISA) reader at 570 nm. Each experiment was carried out in triplicate and the half maximal inhibitory concentration (IC_{50}) of the nanoparticles as the percentage survival of the cells was calculated according to the formula provided below:

Percentage of viable cell concentration was calculated thus:

$$\text{Viability (\%)} = [(\text{Mean sample OD/Untreated Control OD})] \times 100$$

RESULTS AND DISCUSSION

Terminalia bellerica is a rich medicinal value herb which is widely employed for many diseases. The ethanolic extract of *T. bellerica* reduced the silver nanoparticle biologically and eco-friendly. The biosynthesized silver nanoparticle was explored against pancreatic cancer cells. The silver nanoparticles from *T.bellerica* ethanolic extract exhibit significant anticancer activity. This study insights the *T. bellerica* synthesized AgNPs could be an effective applicability in drug preparation for pancreatic cancer. Table 1 shows the Qualitative analysis of phytoconstituents of fruits of *terminlia bellerica* the result revealed that plant extract showed presence of alkaloids, flavonoids, Terpenoids, Tannins, Phenol, Glycosides, saponins.

Table 1:Preliminary Phytochemicals Analysis.

S.No.	Name of the compound	Result
1.	Alkaloids	+
2.	Flavonoids	+
3.	Terpenoids	+
4.	Tannins	+
5.	Phenol	+
6.	Glycosides	+
7.	Saponins	+

+ = Presence

The phytochemical compounds of *Terminalia bellerica* were qualitatively analyzed. The 70% ethanolic extract showed the presence of alkaloids, flavonoids, Terpenoids, Tannins, Phenol, Glycosides saponins. From this analysis, it reveals that *Terminalia bellerica* possess good extract of phenolic and tannin as secondary metabolites suggest that *Terminalia bellerica* may possess antioxidant and anticancer activities.

Table 2: UV-Vis analysis of ethanolic extract of *Terminalia bellerica.*

S.No.	Chromophore	Transition	λmax (nm)
1.	• Poly-unsaturated and aromatic • N=N	n→π*	190-380 nm
2.	N=O	σ →π*	630-700
3.	NO_2	σ → σ *	>1000

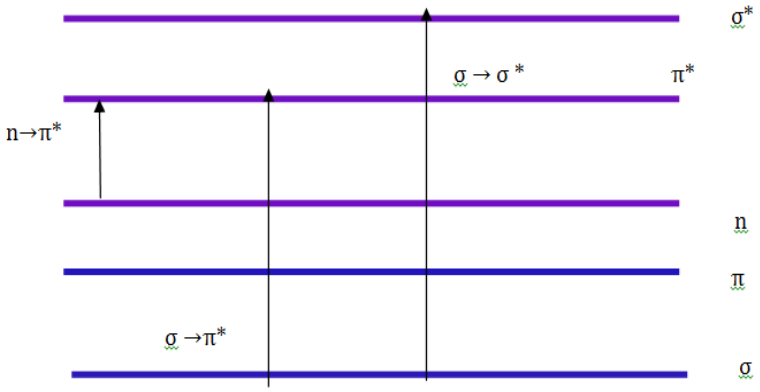


Fig 4: Energy changes of electronic transitions.

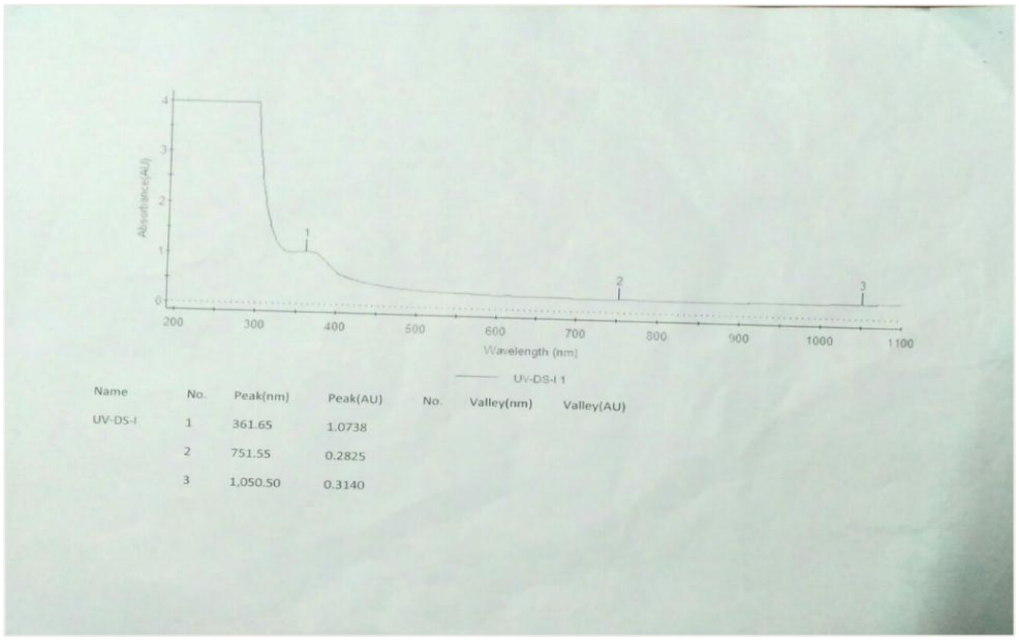


Fig 5: UV-Vis Analysis of Ethanolic Extract of *Terminalia bellerica.*

The UV-VIS spectroscopy offers a simple, technique to identify the main phytochemicals, discriminating between the lipophilic and hydrophilic molecules in relation to the polarity.

The UV-VIS Spectrum of the *Terminalia bellerica* indicates the presence of phytochemicals containing the functional groups poly-unsaturated and aromatic compounds, N=O, NO_2 Which indicates the secondary metabolites presence such as flavone, alkaloids, piperidine,

isoquinoline flavone, alkaloids, piperidine, isoquinoline, which exhibits the transition energy from of orbital n to π. The absorbance of colour is due to the transition of n→π*, σ →π*, σ → σ * orbital from ground state to excited state.

Table 3: FT-IR analysis of ethanolic extract of *Terminlia bellerica.*

S.No.	Wavelength(Nm)	Functional Groups	Compound Identified
1.	3432.74	Amine N-H Stretch	Primary amines produce two N-H stretch absorptions, secondary amides only one, and tetriary none.
		Amide N-H Stretch	As with amines, an amide produces zero to two N-H absorptions depending on its type.
2.	2083.21(m or s)	Alkyl C-H Stretch	Alkane C-H bonds are fairly ubiquitous and therefore usually less useful in determining structure.
3.	1637.72(v)	Alkenyl C-H Stretch Alkenyl C=C Stretch	Absorption peaks above 3000 cm^{-1} are frequently diagnostic of unsaturation
4.	1384.31	symmetric (medium)	**Nitro compounds** O **N+** O -
5.	1244.89	(aromatic) **ethers**	Alkoxy C O
6.	1059.06	alkoxy C-O	C-O
7.	668.21	cis disubstituted alkene	R R C C H H

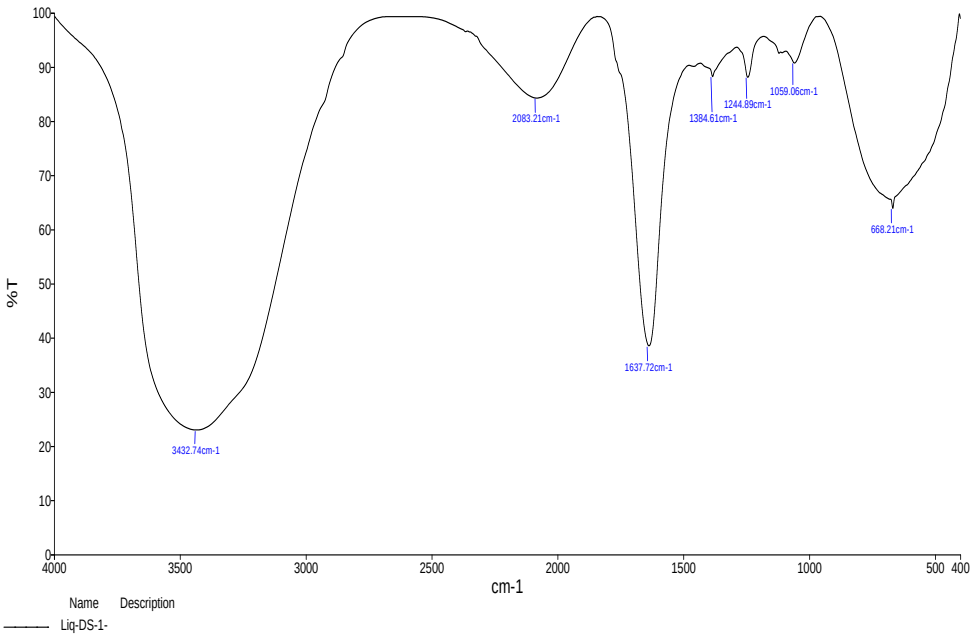
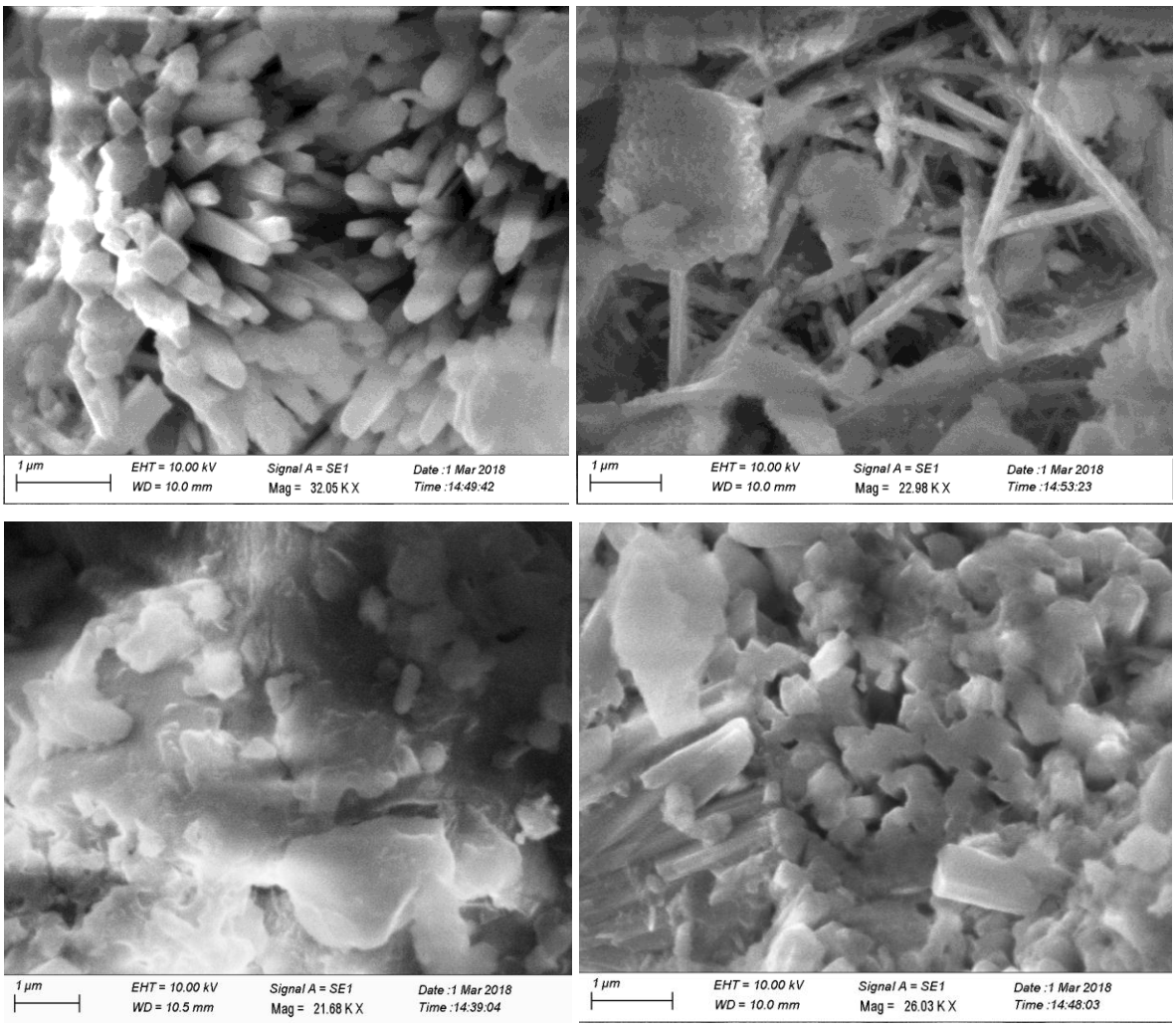


Fig 6: FT-IR analysis of *Terminalia bellerica.*

All these compounds belong to secondary plant metabolites as per researcher explanations.[10] The presence of glycosides and alkaloids in Terminalia bellerica may be associated with their use by traditional medicine practitioners in healthcare systems in the treatment of cough, fever and cold.[10] These were confirmed by FT-IR spectrophotometer study that predicted the presence of the groups: O-H, C-H, C=C, C=O, C≡N, N-H, C-H, carbonates and nitrates stretching. The presence of characteristic functional groups of carboxylic acids, anhydrides, alcohols, phenols, amines, amides,esters, ethers, sulphur derivatives, glycosides, nitrates, nitriles and carbohydrate could be responsible for the various medicinal properties of *Terminalia bellerica*.[11] Based on the functional group analysis, Terminalia bellerica doesn't contain any toxic compounds.

Table 4: SEM analysis in ethanolic extract of *Terminalia bellerica.*



The SEM images of the AgNPs are shown in Table:4. It is seen that AgNPs of different shapes were obtained in case of *Terminalia bellerica* fruit extracts being used as reducing and

capping agents. *Terminalia bellerica* fruit extracts formed approximately spherical, cuboidal cylindrical AgNPs, respectively. This may be due to availability of different quantity and nature of capping agents present in the *Terminalia bellerica* fruit extracts. This is also supported by the shifts and difference in areas of the peaks obtained in the FT-IR analysis.

Table 5: *Invitro* MTT Assay of ethanolic extract of *Terminalia bellerica.*

S.No	Treatment	Conc (µg/ml)	Absorbance 570nm At 24 hrs	IC_{50}	% Cell viability
1.	AsPC-1 cells Untreated		0.267		100.0 ± 7.3
2.	AgNPs treated	10	0.228	15.77	85.3 ± 5.2
3.		20	0.169		63.4 ± 5.9
4.		40	0.107		40.1 ± 3.3
5.		80	0.084		31.6 ± 1.9
6.		100	0.055		20.9 ± 1.2
7.	Gemcitabine	10	0.044		16.4 ± 1.1

Values are mean ± SEM expressed as (n=3); *P<0.001, as compared with AsPC-1 incubated cells.

IC50 value of the AgNP synthesized from *Terminalia Bellerica* – 15.77µg

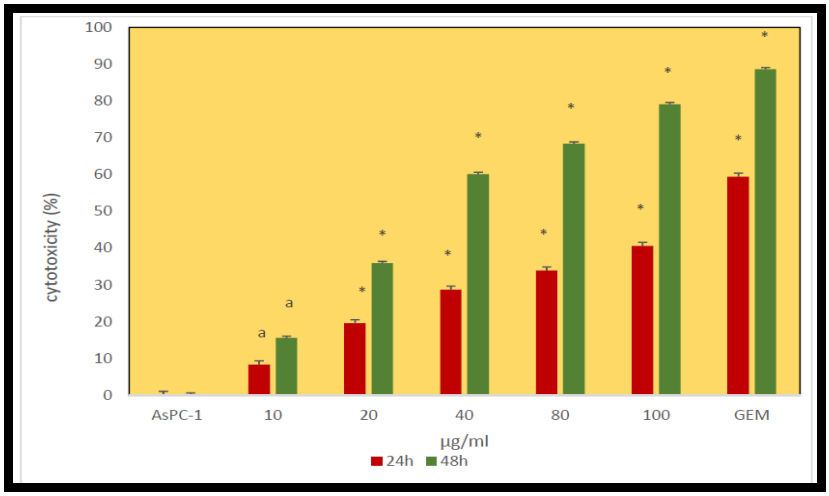


Fig 7: The AgNPs showed cytotoxicity on the AsPC-1 cell line. The potent cell Mortality was at the concentration 100µg/ml then decreased significantly with the decreased concentration of extract.

Values are mean ± SEM expressed as (n=3); aP<0.05 and *P<0.001

The ability of the cells to survive at toxic levels has been the basis of most cytotoxicity assays. It depends both on the number of viable cells and on the mitochondrial activity of cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay is based

on the assumption that dead cells or their products do not reduce tetrazolium. Tetrazolium salts are reduced only by metabolically active cells. Thus MTT can be reduced to a blue coloured formazan by mitochondrial enzyme *succinate dehydrogenase*. The amount of formazan produced is directly proportional to the number of active cells.[12]

In the present study, the ethanolic extract of the fruits of *Terminalia bellerica* was screened using MTT for its cytotoxicity against one cell line AsPC-1 at the intervals of 24 & 48 hrs. Among these 2 intervals the AsPC-1 shows no mortality and shows maximum cell viability which indicates that the metabolically active constituents present in the cell responsible for TCA cycle key enzymes *succinate dehydrogenase*. The concentration of 10µg/ml of plant extract shows maximum % of cell viability (85%) than compared to the standard drug Gemcitabine with % of cell viability of about 16%.

SUMMARY AND CONCLUSION

The reaction mechanism of AgNPs and factors affecting particle size are also clarified. Significant advantages of these methods over previous ones include: it has a short reaction time; relatively uniform particles with small diameter are produced; the reaction proceeds rapidly at room temperature; organic solvents are not used, and used chemical reagents are water soluble, cheap, easy to deal with, not producing hazardous by-products and environmentally friendly; and the resulting particles are easily separated from the reaction mixture.

Pancreatic cancer has a lower incidence compared to other forms of cancer but with an increased mortality remains a major public health problem. Pancreatic cancer is closely associated with the risk factors such as diabetes, obesity, smoking, pancreatitis and diet, pancreatic cancer etiology remains unknown. Pancreatic cancer and diabetes are in a close correlation and is evidenced in many studies. An explanation may be an increased mitogenic function of insulin in patients treated with insulin or having an oral treatment with sulphonylureas or other drugs stimulating insulin secretion. Finding a therapeutic solution, able to decrease the high rate mortality of pancreatic cancers. However, this is a preliminary study and hence there is a detailed progressive work should be carried out. More efforts are needed to explore potent anticancer plants from the mother earth and save humans around the world from cancer which will be carried out in future.

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