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ANTINEOPLASTIC ACTIVITY OF SYNTHESIZED GOLD NANOPARTICLES AND GOLD NANOCONJUGATE AGAINST CERVICAL CANCER HELA CELL LINE

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ABSTRACT The present study focused on a pharmacognostic approach over the usage of plant materials for nanoparticles synthesis. 3 medicinal plants were selected for the study based on their medicinal properties. Nanoparticles were synthesized with each of the plant extract and screened for anticancer effects. All the three nanoparticles showed good anti proliferative effects against cervical cancer. In order to achieve a synergistic effect, a nanoconjugate was synthesized using all the three bioactive extracts. The gold nanoconjugate was biosynthesized using green extracts of 3 medicinal plants namely Guazuma ulmifolia, Aegles marmelos and Justicia gendarussa. The in vitro cytotoxicity was checked in Vero cell line and in vitro anticancer activity was checked in cervical cancer HeLa cell line. The result signified non-toxicity of the nanoparticles with minimum viability of 80% in all the tested concentrations. The anticancer activity was checked in cervical cancer HeLa cell line. The inhibitory concentration was achieved at much less concentration for the nanoconjugate than the individual nanoparticles.

KEYWORDS : Nanoparticles, Nanoconjugate, Trio extract, Pharmacognosy, Vero cell line, HeLa cell line, cervical cancer.

INTRODUCTION

The biosynthesis of silver nanoaparticles using microorganism *Bacillus subtilis* was reported by Saifuddin and co-workers (1). The synthesis of silver particles through *Aspergillus niger* was investigated by Sadowski. (2). In another study, *Fusarium oxysporum* has been exploited for the extracellular synthesis of silver nanoparticles (3). Absar and coworkers has reported on the extra and intracellular biosynthesis of gold nanoparticles by *Trichothecium* species (4). Gold nanoparticles were synthesized using sundried biomass of *Cinnammum canphora* leaf. (6). Gold nanoparticles can be used to enhance the biorecognition of cancer drugs like DTIC (13). Doxorubicin, an anticancer drug was conjugated with gold nanoparticles (14)

METHODS:

Table 1: Plants Used In The Study

S.No. Scientific name	Common name	Referred as	Part used
1. Guazuma ulmifolia	Bay cedar	GU	Bark
2. Aegles marmelos	Indian bael	AM	Leaves
3. Justicia gendarussa	Willow-leaved justicia	JG	Leaves
4. Combination (1+2+3)	Nanoconjugate	GAJ	1+2+3

PREPARATION OF PLANT EXTRACT

All the 3 specimens used in this study were collected from our college campus, Women's Christian College, Chennai. The formal identification of the plant material used in the study was identified by Dr. G. Jeya Jothi, Loyola College, Chennai. Five grams each of *Aegles marmelos* (AM), *Justicia gendarussa* (JG) leaves and *Guazuma ulmifolia* bark (GU) were washed well and cut into small pieces. The plant materials were taken in 250 milliliter Erlenmeyer flasks, 50 milliliter of double distilled water was added and boiled for 10 minutes. All these extracts were allowed to cool and filtered separately through a Whatman filter paper. The filtrates were stored at -18^{0}C in the refrigerator.

SYNTHESIS OF GOLD NANOCONJUGATE:

The pH of chloroauric acid was checked and adjusted to alkaline. The synthesis was carried out between chloroauric acid and the trio plant extract (18)(19). The three extracts were mixed in equal proportions to achieve the trio plant extract. The trio extract was added to chloroauric acid solution at room temperature. The trio extract was used as the reducing and stabilizing agent for the synthesis. The light yellow color of chloroauric acid was transformed into dark wine red color indicating the formation of gold nanomaterial.

CHARACTERIZATION OF NANOPARTICLES:

UV-VIS SPECTRASCOPY ANAYLSIS

1 milliliter of sample was diluted with 30 milliliter of double distilled water and analyzed for the synthesized nanoparticles. The UV-vis spectra of the AuNPs and AuNCs were obtained using a Shimadzu UV 1800 - UV Visible spectrophotometer operating in the wavelength between 200 - 800 nm.

HRSEM ANALYSIS (HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY)

The Nanoparticles and Nanoconjugate were further subjected to electron microscopic imaging to understand the morphology and dimensions of the nano formulations. 10 microliter of sample was placed on the glass cover slip and air dried for one day. Then the HRSEM was carried out at SAIF, IIT Madras, Adyar.

TEM & SAED ANALYSIS

A tiny drop of the analyte was placed on the TEM grid and air dried. Followed by, the Transmission Electron Micrographs of the samples were recorded through Philips CM 200 camera, operating at voltages between 20-200 kv, with a resolution of 2.4 A^o at the Centralized Instrumentation facility (SAIF), at IIT Bombay, Mumbai. SAED pattern analysis was carried out to interpret the nature of the synthesized nanomaterials. Generally a SAED pattern was recorded along with the TEM characterization.

ZETA POTENTIAL

The strength of the biosynthesized nanomaterials was interpreted by Zeta potential analysis. It was determined by measuring the positive or negative charges associated with the NPs surface. This charge would prevent the nanoparticles from aggregation and will ensure highly stable nanoformulations. Generally the Zeta potential measured towards either positive range or negative range. At both of these ranges, the material has the strongest stability.

PHYTOCHEMICAL ANALYSIS THE PLANT EXTRACTS

The green extracts were analyzed for the various phytocompounds such as terpenoids, flavonoids, saponins, tannins, phenols, Coumarins, Triterpenoids and the results were tabulated. These phytocompounds and secondary metabolites acted as the reducing and stabilizing agents aiding biosynthesis of nanoparticles.

IN VITRO CYTOTOXICITY OF THE SYNTHESIZED NANOCOMPOUNDS

In vitro cytotoxicity of the synthesized nanoparticles and nanoconjugate was checked in VERO cell line through MTT assay for 3 days. The cell line used in the study was procured from TRPVB, TANUVAS, Chennai. In day one, 1x10^4 cells were added into each well of 96 well plates and the plates were incubated for 24 hours. In day 2, after the overnight adherence of cells, various concentrations of AuNPs and AuNCs were added. The treated cells were incubated for 24 hours at 37^{o}C in CO_2 incubator. In day 3, 10 µl (5 mg/ml) of MTT was added to each well. The cells were incubated for 3 – 4 hours in MTT dye facilitating mitochondrial enzyme activity. After the

incubation, 100 µl of DMSO was added and incubated for an hour. Finally the absorbance was read at 540 nm in the ELISA plate reader. Percentage of cell viability was calculated using the formula, cell inhibition = (O.D of treated cells/O.D of control) x 100

ANTICANCER ACTIVITY OF THE SYNTHESIZED NANOCOMPOUNDS

In vitro cytotoxicity of the synthesized nanoparticles and nanoconjugate was checked in HeLa cervical cancer cell line through MTT assay for 3 days. The cell line used in the study was procured from TRPVB, TANUVAS, Chennai. In day one, $1x10^4$ cells were added into each well of 96 well plates and the plates were incubated for 24 hours. In day 2, after the overnight adherence of cells, various concentrations of AuNPs and AuNCs were added. Then the cells were incubated for 24 hours at 37°C in CO_2 incubator. In day 3, 10 µl (5 mg/ml) of MTT was added to each well. The cells were incubated for 3 – 4 hours in MTT dye facilitating mitochondrial enzyme activity. After the incubation, 100 µl of DMSO was added and incubated for an hour. Finally the absorbance was read at 540 nm in the ELISA plate reader. Percentage of cell viability was calculated using the formula, cell inhibition = (O.D of treated cells/O.D of control) x 100.

RESULTS AND DISCUSSION

BIOSYNTHESIS OF GOLD NANOPARTICLES

The plants used for the synthesis of the gold nanoparticles (AuNPs) and nanoconjugate (AuNC).

Fig 1 A, B, C: *Justicia Gendarussa, Aegles Marmelos Leaves And Guazuma Ulmifolia* Bark

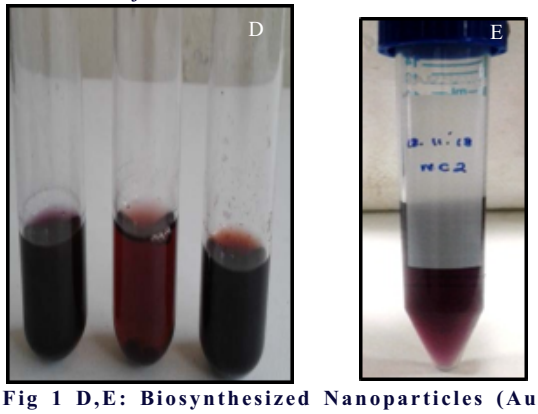


Fig 1 D,E: Biosynthesized Nanoparticles (AuNPs) Nanoconjugate (AuNC)

The gold nanoparticles and nanoconjugate were successfully biosynthesized using the three plant extracts individually and as trio extracts. The complete synthesis of nanomaterials took place in less than 30 minutes at ambient room temperature. The synthesized AuNPs and AuNC were dark wine red in appearance.

UV VISIBLE SPECTROPHOTOMETER ANALYSIS

The preliminary confirmation of synthesized nanoparticles was analyzed in UV visible absorption spectrum, which showed the specific peak for nanoparticles between 500-600 nm. The peak confirmed the formation of AuNPs and AuNC.

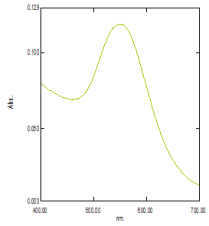


Fig 2a: UV spectrum of AuNPs synthesized

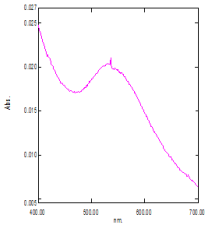


Fig 2b: UV spectrum of AuNPs synthesized

by *J. gendarussa* (λ_{max}-551 nm)

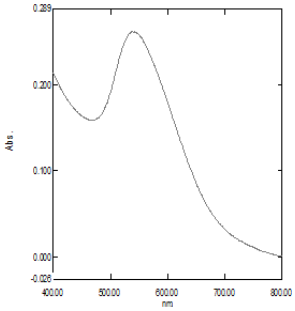


Fig 2c: UV spectrum of AuNPs synthesized By *G. ulmifolia* (λ_{max} -539 nm)

by *A. marmelos* (λ_{max}-536 nm)

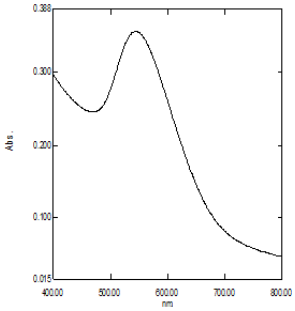
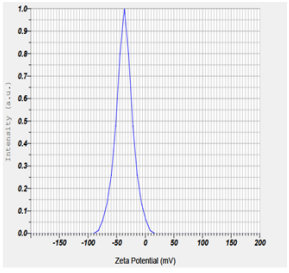


Fig 2d: UV spectrum of AuNC synthesized by *Trio Extracts* (λ_{max}-543 nm)

ZETA POTENTIAL

The synthesized nanomaterials were analyzed through Zeta potential. When the Zeta potential measured was towards more positivity or negativity, highly stable nanoparticles system was achieved (7).



- Zeta potential (Mean) AuNPs : -31.9mV
- Zeta potential (Mean) AuNPs : -32.2 mV
- Zeta potential (Mean) AuNPs: -30.5 mV
- Zeta Potential (Mean) AuNC: -36.7 mV

Fig 3: Zeta Potential Analysis

If the zeta potentials recorded was greater than +30 mV or less than −30 mV, the particles were stable and strongly cationic or anionic (8). The zeta potential observed showed that the nanoparticles were highly stable.

HRSEM ANALYSIS

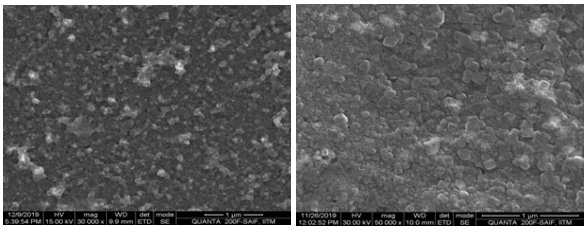


Fig 4a, b: HRSEM Images Of The Nanoparticles

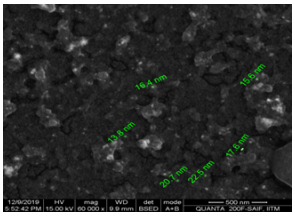


Fig 4c: Size Of Nanoparticles In HRSEM

The dimension of the nanoconjugate was 13 nm onwards with an average of 20nm. This was indeed an appreciable nanoparticle size attained through biosynthesis approach and particularly a novel attempt using the Trio extract. The particles were mostly spherical in formation, well dispersed.

TEM & SAED ANALYSIS

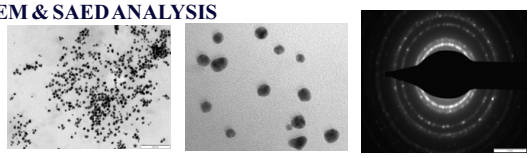


Fig 5a: TEM image of AuNC at 200nm b) TEM image of AuNC at

20nm c) SAED pattern of AuNC

The transmission electron microscopic images recorded were in line with the SEM imaging micrographs. The biosynthesized AuNC were spherical in morphology, mono dispersed and the dimension ranged between 13nm and 20nm. From the above image ©, it could be observed that the SAED pattern presented bright spots. Hence the biosynthesized nanoconjugate was absolutely crystalline in nature.

PHYTOCHEMICAL ANALYSIS OF THE PLANT EXTRACTS:

The green extracts of the plant was analyzed for different phytochemicals and secondary metabolites. The observed results were listed in Table 2.

Table 2: Phyto Chemicals Present In The Plant Extract

S.No.	Compounds	G.ulmifolia Bark	J.gendarussa leaves	A.marmelos leaves
1.	Coumarins	+	+	+
2.	Saponins	+	+	+
3.	Flavanoids	+	+	+
4.	Terpenoids	+	+	+
5.	Triterpenoids	+	+	+
6.	Carbohydrates	+	+	-
7.	Phenols	Brown color	Blackish Green color	Green color
8.	Tannins	+	+	Brownish green color
9.	Alkaloids	+	-	+
10.	Proteins	+	+	+

*+ = indicated presence of the compound, *_= indicated absence of the compound

IN VITRO CYTOTOXITY STUDY

The *in vitro* cytotoxicity of the AuNC was determined in VERO cell line. Cytotoxicity was checked for series of concentrations of the nanoconjugate. The results showed that the biosynthesized AuNC had very minimal toxicity at all the tested concentrations. It was observed that the minimum percentage of viability was observed to be 71% and maximum was 100%.

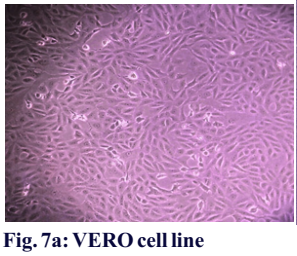


Fig. 7a: VERO cell line

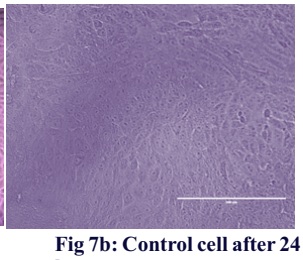


Fig 7b: Control cell after 24 hrs

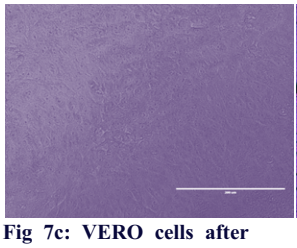


Fig 7c: VERO cells after treated with AuNC

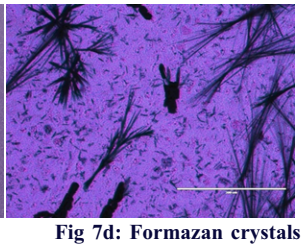


Fig 7d: Formazan crystals formed followed by MTT dye incubation

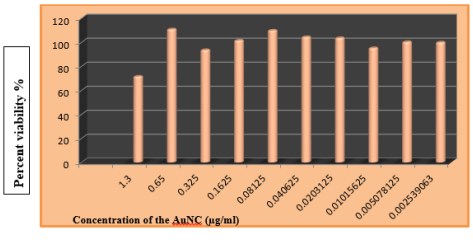


Fig 8: Percent of viability in VERO cell line

ENHANCED ANTICANCER ACTIVITY OF NANOCONJUGATE

Individually, the biosynthesized AuNPs were checked for their potential anticancer activities. Each nanoparticle showed different levels of anticancer efficiency. On an effort to bring about an improved synergistic effect, the conjugate was formulated. Finally, the AuNC was checked for its antineoplastic effect.

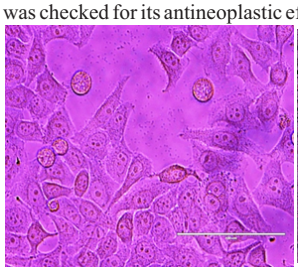


Fig 8a: untreated HeLa cell line

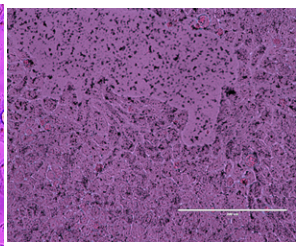


Fig 8b: HeLa cell line after treated with AuNC

From the above images, it could be observed that AuNC treated HeLa cell line showed distinctive degeneration of cells and cell death compared to the untreated control cell line. The results implied that the AuNC showed a cumulative activity than the individual compounds. There was a collective bioactivity from the three extracts in the trio extract. Thus the pharmacognistic properties of the biological sources could be exploited in the therapeutic control of diseases.

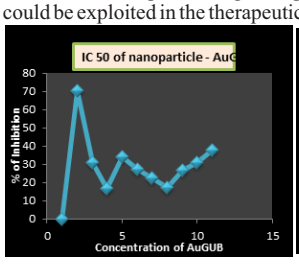


Fig 9a: IC 50 of nanoparticle – AuGUB

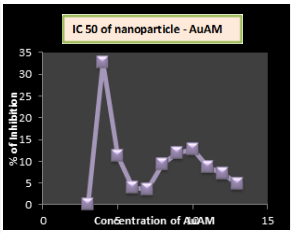


Fig 9b: IC 50 of nanoparticle - AuAM

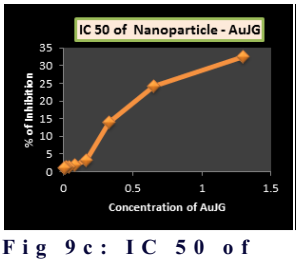


Fig 9c: IC 50 of nanoparticle – AuJG

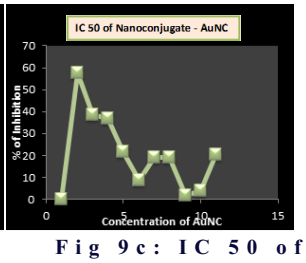


Fig 9c: IC 50 of nanoconjugate - AuNC

Individual nanoparticle was synthesized using single plant extract. Upon screening of nanoparticles for the anticancer efficiency, all three plants were used for the synthesis of a nanoconjugate to achieve synergistic effect. The IC_{50} was 28.12ng/ml for AuGUB, 66.91 ng/ml for AuAM, 22.609ng/ml for AuJG and 6.374ng/ml for the nanoconjugate. It could be noted that the inhibitory concentration was achieved at much less concentration for the AuNC than the individual nanoparticles. When there were collective bioactive compounds involved in the biosynthesis from more than one plant extract, a better cumulative effect was achieved. This nanoconjugate approach can be further applied for the synthesis of improved nanomaterials.

CONCLUSION

A nanoconjugate (NC) comprising of the 3 extracts (Trio extract) was synthesized based on the results of anticancer activity. The nanoconjugate was biosynthesized using 3 medicinal plants namely *Guazuma ulmifolia, Aegles marmelos* and *Justicia gendarussa.* The nanoconjugate showed a combined synergistic effect and increased anticancer activity than the nanoparticle. The *in vitro* cytotoxicity was checked in Vero cell lines by MTT assay. The anticancer activity was screened in HeLa cell line. These results implied that the nanoconjugate exhibited a synergistic effect combining the bioactivity of all the three plants.

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