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A RESEARCH ON GREEN SYNTHESIS, ANALYTICAL CHARACTERISATION AND DEVELOPMENT OF DOXORUBICIN-LOADED SILVER NANOPARTICLES USING COLUMN-FRACTIONATED *CATHARANTHUS ROSEUS* STEM EXTRACT

***D. Sravanthi, K. Yashwini Sowmya, G. Omsri**

Department of Pharmaceutical Analysis, Browns college of Pharmacy, Telangana, India.

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***Corresponding Author: D. Sravanthi**

Department of Pharmaceutical Analysis, Browns college of Pharmacy, Telangana, India.

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ABSTRACT
This study develops doxorubicin (DOX)-loaded silver nanoparticles (DOX-AgNPs) by green synthesis, using a column-chromatography-purified fraction of *Catharanthus roseus* stem extract as the reducing and capping agent. Dried stem powder is extracted with methanol, fractionated on a silica gel column (gradient elution, hexane → ethyl acetate → methanol), and the fractions richest in phenolics and flavonoids (by TLC and Folin–Ciocalteu assay) are used to reduce 1 mM $AgNO_3$. Synthesis is optimised for extract-to-$AgNO_3$ ratio, pH, temperature and time. DOX is loaded onto the phyto-capped AgNPs by electrostatic adsorption, and unbound drug is removed by centrifugation. The nanoparticles are characterised by UV-Vis spectroscopy (surface plasmon resonance near 420–440 nm), FTIR, XRD, SEM/TEM with EDX, DLS and zeta potential, and TGA/DSC. Drug loading is quantified by UV-Vis at 480 nm and confirmed by RP-HPLC. In vitro release is studied at pH 7.4 and pH 5.5, and cytotoxicity is tested on MCF-7 cells by MTT assay. It is a purified, phenolic-rich fraction gives smaller, more uniform and more reproducible AgNPs than crude extract, and that DOX-AgNPs release drug preferentially at acidic tumour pH with a lower IC_{50} than free DOX.

KEYWORDS: green synthesis; silver nanoparticles; *Catharanthus roseus*; stem extract; column chromatography; doxorubicin; drug delivery; pH-responsive release.

1. INTRODUCTION

1.1 Silver nanoparticles and green synthesis

Silver nanoparticles (1–100 nm) combine a high surface-to-volume ratio, a tunable surface plasmon resonance and intrinsic cytotoxicity toward cancer cells, which makes them attractive both as drug carriers and as co-therapeutic agents. Chemical routes (citrate or borohydride reduction) leave toxic residues and need added stabilisers. Plant-mediated synthesis replaces both: polyphenols, flavonoids, terpenoids and alkaloids in the extract reduce Ag^{+} to Ag^{0} and then cap the particle surface, giving a biocompatible corona that can also bind drugs.

1.2 *Catharanthus roseus* stem as the bioreductant

Catharanthus roseus is the source of the anticancer vinca alkaloids vinblastine and vincristine, and also contains vindoline, catharanthine, phenolic acids, flavonoids and triterpenes such as oleanolic and ursolic acid. AgNPs have been made from *C. roseus* leaf, flower and stem extracts, with crystalline fcc particles of roughly 30–70 nm reported from leaf extracts. Stem extract is far less

studied than leaf; and aqueous stem-extract AgNPs with an SPR peak at 426 nm and tested them only for antibacterial activity.

1.3 Doxorubicin and the case for a silver carrier

Doxorubicin is an anthracycline used against breast, ovarian, liver and haematological cancers. Its use is limited by dose-dependent cardiotoxicity, myelosuppression, poor tumour selectivity and multidrug resistance. Loading DOX on AgNPs can exploit the enhanced permeability and retention (EPR) effect, give pH-triggered release in the acidic tumour micro-environment (pH 5.0–6.5).

2. MATERIALS AND METHODS

2.1 Materials

Material	Grade / purpose
C. roseus stems	Fresh, authenticated by a botanist; voucher specimen deposited
Silver nitrate ($AgNO_3$)	AR grade, ≥99.8%; silver precursor
Doxorubicin hydrochloride	Pharmaceutical/analytical standard
Silica gel 60–120 mesh	Column stationary phase
Silica gel 60 F_{254} plates	TLC monitoring
n-Hexane, ethyl acetate, methanol	AR/HPLC grade; extraction and elution
Folin–Ciocalteu reagent, gallic acid, quercetin, $AlCl_3$, DPPH	Phytochemical and reducing-power assays
Phosphate buffer (pH 7.4), acetate buffer (pH 5.5)	Release media
Dialysis membrane (MWCO 12–14 kDa)	Release study
MCF-7 and L929 (or HEK-293) cells, DMEM, FBS, MTT	Cytotoxicity
Double-distilled / Milli-Q water	Throughout

2.2 Collection and preparation of stem powder

Collect healthy stems, wash under running tap water then with distilled water, and remove leaves. Shade-dry at room temperature for 10–14 days, grind to coarse powder, sieve (#40 mesh) and store in an airtight amber container.

2.3 Extraction

Extract 100 g of stem powder with 500 mL methanol in a Soxhlet apparatus for 6–8 h (or by cold maceration for 72 h with intermittent shaking). Filter through Whatman No. 1 paper and concentrate under reduced pressure on a rotary evaporator at 40°C. Record the percentage yield and store the crude extract at 4°C.

2.4 Fractionation by column chromatography

1. **Column packing:** pack a glass column (45 × 3 cm) with silica gel (60–120 mesh) by the wet slurry method in n-hexane.
2. **Sample loading:** adsorb ~5 g crude extract onto a small amount of silica (dry loading) and place it on top of the bed.
3. **Gradient elution:** elute with increasing polarity — 100% hexane → hexane:ethyl acetate (90:10 → 10:90) → 100% ethyl acetate → ethyl acetate:methanol (90:10 → 50:50) → 100% methanol.
4. **Collection:** collected 25–50 mL fractions; monitor each by TLC (e.g., ethyl acetate:methanol:water 10:1.35:1 for polar compounds), visualising under UV 254/366 nm and with Dragendorff's reagent (alkaloids) and $FeCl_3$ (phenolics).

2.5 Screening and selection of the active fraction

- Qualitative phytochemical tests: alkaloids, phenols, flavonoids, tannins, saponins, terpenoids.
- Total phenolic content (Folin–Ciocalteu, mg GAE/g) and total flavonoid content ($AlCl_3$, mg QE/g).
- Reducing power / DPPH antioxidant activity.
- A small-scale synthesis test (1 mL fraction + 9 mL 1 mM $AgNO_3$) scored by colour change and SPR intensity.

2.6 Green synthesis of AgNPs

Dissolve the selected fraction in water (or minimum methanol, then dilute) to 1 mg/mL. Add the fraction dropwise to 1 mM aqueous $AgNO_3$ under magnetic stirring, and heat at 60°C in the dark. The colour change from pale yellow to reddish-brown indicates AgNP formation. Monitor by UV-Vis every 30 min.

Optimisation (one factor at a time, or a Box–Behnken design)

Factor	Levels to test
Fraction : $AgNO_3$ volume ratio	1:9, 1:4, 1:2, 1:1
$AgNO_3$ concentration	0.5, 1, 2, 3 mM
pH	5, 7, 9, 11
Temperature	25, 40, 60, 80 °C
Reaction time	0.5–24 h

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2.7 loading of doxorubicin

Purify by centrifugation at 12,000–15,000 rpm for 20 min, wash the pellet three times with distilled water, and lyophilise or dry at 50–55°C. This gives blank AgNPs.

Disperse CRS-AgNPs (1 mg/mL) in phosphate buffer pH 7.4. Add DOX solution at drug:carrier ratios of 1:1, 1:2, 1:5 and 1:10 (w/w). Stir in the dark at room temperature for 24 h.

3. Analytical Characterisation and Evaluation

3.1 Physicochemical characterisation

Technique	Conditions	What it confirms
UV-Vis spectroscopy	200–800 nm, 1 cm quartz cell, water blank	SPR band (~420–440 nm) = AgNP formation; DOX band at 480 nm after loading
FTIR	KBr pellet, 4000–400 cm^{-1}	Phyto-capping groups (O–H, C=O, C–N, aromatic C=C); shifts showing DOX–AgNP interaction
XRD	Cu Kα (λ = 1.5406 Å), 2θ = 20–80°	fcc crystalline silver: (111), (200), (220), (311) planes; crystallite size by Scherrer equation
SEM / FE-SEM	Gold-sputtered dried film	Surface morphology, agglomeration
TEM / HR-TEM + SAED	Drop-cast on carbon-coated Cu grid	Particle shape, size distribution, lattice fringes, crystallinity
EDX	With SEM/TEM	Elemental silver signal (~3 keV) plus C, O, N from capping
DLS	25 °C, water	Hydrodynamic diameter and polydispersity index (PDI)
Zeta potential	Electrophoretic mobility	Surface charge and colloidal stability; change after DOX loading
TGA / DSC	25–800 °C, 10 °C/min, N_2	Weight of organic capping and drug; thermal stability; loss of DOX melting peak (amorphous dispersion)
HPLC / GC-MS of fraction	C18 column (HPLC)	Identity of phytoconstituents in the selected column fraction

Crystallite size from XRD (Scherrer equation), where K = 0.9, λ is the X-ray wavelength, β is FWHM in radians and θ is the Bragg angle:

$$D = \frac{K\lambda}{\beta \cos\theta}$$

3.2 Quantification of doxorubicin

UV-Vis method: prepare a DOX calibration curve at 480 nm (2–20 µg/mL in the matching buffer). Validate for linearity, LOD and LOQ.

RP-HPLC confirmation: C18 column (250 × 4.6 mm, 5 µm); mobile phase acetonitrile: 0.1% orthophosphoric acid buffer (32:68), 1.0 mL/min; detection 254 nm or fluorescence.

Entrapment efficiency (EE) and drug loading (DL) by the indirect method, from free DOX in the supernatant:

$$EE\ (\%) = \frac{W_{\text{total DOX}} - W_{\text{free DOX}}}{W_{\text{total DOX}}} \times 100$$

$$DL\ (\%) = \frac{W_{\text{total DOX}} - W_{\text{free DOX}}}{W_{\text{nanoparticles}}} \times 100$$

3.3 In vitro drug release

Place DOX-CRS-AgNPs equivalent to 1 mg DOX in a dialysis bag (MWCO 12–14 kDa) in 50 mL release medium at 37 ± 0.5°C, stirred at 100 rpm, in the dark. Run two media: phosphate buffer pH 7.4 (blood) and acetate buffer pH 5.5 (endosome / tumour). Withdraw 1 mL at 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h, replacing with fresh medium. Run free DOX solution as control.

Fit cumulative release to zero-order, first-order, Higuchi, Korsmeyer–Peppas and Hixson–Crowell models; choose by highest R^2.

3.4 Stability
Store blank and loaded AgNPs at 4°C and 25°C / 60% RH for 3 months (ICH Q1A). At 0, 30, 60 and 90 days measure SPR peak, particle size, PDI, zeta potential and drug content.

3.5 In vitro biological evaluation
- **Cellular uptake:** exploit DOX's own red fluorescence; fluorescence microscopy or flow cytometry at 2 and 4 h.
- **Apoptosis:** AO/EtBr or DAPI staining; Annexin V-FITC/PI flow cytometry; intracellular ROS by DCFH-DA.
- **Haemolysis assay:** fresh RBCs, to confirm blood compatibility (< 5% haemolysis).

4. Results and Discussion (framework with expected outcomes)
The table lists what each test should show if synthesis and loading succeed, with published benchmarks to compare against. Replace the "Expected" column with your measured values.

Parameter	Expected outcome	Literature benchmark
Colour change	Pale yellow → reddish/dark brown within 60 min at 60 °C	Yellow-brown to reddish-brown with *C. roseus* leaf extract (Mukunthan 2011)
UV-Vis SPR (blank)	Single band at 440 nm	426 nm, *C. roseus* stem AgNPs (Prabha 2026)
UV-Vis after loading	Added DOX near 480 nm; slight SPR red-shift	—
FTIR	O–H (~3400), C=O (~1630), C–N (~1380), C–O (~1050 cm^{-1}) from capping	O–H, H–C=O and C≡N groups on stem AgNPs (Prabha 2026)
XRD	Peaks at 2θ ≈ 38.1°, 44.3°, 64.4°, 77.4° (fcc Ag; JCPDS 04-0783)	fcc crystalline AgNPs from *C. roseus* leaf (Mukunthan 2011)
TEM size	Spherical, 15–40 nm; narrower spread than crude-extract AgNPs	48–67 nm (SEM, leaf extract; Mukunthan 2011)
DLS / PDI	50–150 nm hydrodynamic; PDI < 0.3	—
Zeta potential	Blank −20 to −35 mV; less negative after DOX (cationic drug)	—
EE	90% at optimal ratio	~95% with PEG-AgNPs (Abdelfattah 2022); 42.8% loading on green AgNP-silica (Ghobadi 2024)
Release	Faster and higher at pH 5.5 than 7.4; sustained over 48–72 h vs. free DOX	Higher release at pH 5.0 than 7.4 and sustained vs. free DOX (Abdelfattah 2022)

5. Conclusion and Future Scope
A column-purified *C. roseus* stem fraction is expected to act as a standardised, eco-friendly reductant and capping agent, producing crystalline, stable AgNPs that carry doxorubicin with high entrapment and release it preferentially at acidic tumour pH, the system could allow lower DOX doses and so reduce dose-limiting toxicity.

Future work
- In vivo efficacy, pharmacokinetics and biodistribution (e.g., DLA or EAC tumour model in mice), with cardiotoxicity markers (CK-MB, troponin).
- Surface modification (PEG, folic acid, hyaluronic acid) for active targeting.
- Isolation and structural identification of the reducing compounds in the active fraction (NMR, LC-MS).
- Scale-up and design-of-experiments (QbD) optimisation of the process.
- Testing additional cell lines (HepG2, HeLa, A549) and DOX-resistant lines.

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Check volume and page details of entries 3, 5, 8 and 9 against the original papers before submission, and add standard references for ICH Q1A/Q2(R2), the MTT assay and release-kinetics models.