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Original Article

Biofabrication of Ruthenium Oxide Nanoparticles Using Leaf Extracts of *Causonis trifolia* (L.) and Evaluation of their Biocompatibility, Antioxidant, and Anti-Skin Cancer Potentials

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Abstract

Green synthesis of ruthenium nanoparticles (RuNPs) is gaining significant attention due to its environmentally friendly, cost-effective, and readily accessible nature. However, few studies have reported on green synthesis and characterization of RuNPs compared to other metal NPs. This study describes how to make RuNPs using water extracts from *Causonis trifolia (L.)* leaves as a natural reducing agent. The characterization of RuNPs proved that the particles exhibit a prominent characteristic absorption peak at 491 nm and show functional groups corresponding to various bioactive phytochemical constituents, such as flavonoids, alkaloids, phenolics, and terpenoids that are involved in the capping of NPs. The particles had a granular appearance with an irregular surface, ranging in size from 37 to 63 nm and an average size of 48 nm. These particles were confirmed to be tetragonal structures with 73 % elemental Ru and possess a high degree of purity. The MTT assay assessed the cytotoxicity of RuNPs on A-431 skin cancer cell lines and Vero non-cancerous cells. The results indicated that the NPs exhibit significantly high activity against cancer cells and less activity against non-cancer cells. The NPs exhibit the IC_{50} concentration of 267.10 µg/mL, whereas standard doxorubicin shows 130.34 µg/mL. These NPs effectively reduce DPPH radicals with an IC50 concentration of 115.58±0.232 µg/mL, demonstrating their ability to act as antioxidants. Hence, it can be concluded that the RuNPs synthesized are nontoxic, safe for further exploration in biomedical applications, and showcased as eco-friendly and effective agents in cancer treatment and antioxidant therapy.

Keywords: Anti-Skin Cancer Activity, A-*431 Cell Lines, Causonis trifolia (L.); Green Synthesis, Ruthenium Nanoparticles*

Introduction

People around the world have used plants as an essential source of medicine since ancient times. The World Health Organization (WHO) reported that globally more than 80% of the population relies on herbal and traditional medicines as part of their routine healthcare (Selvaraj *et al*., 2019). Medicinal plants create special compounds called secondary metabolites, like alkaloids, flavonoids, terpenoids, and phenolics, which are important for the plants and also provide significant health benefits for people. Green synthesis of NPs utilizing plant bioactive compounds is an innovative approach that combines nanotechnology with sustainable chemistry that produces nanoparticles in an eco-friendly manner (Becker, Manske & Randl, 2022). Unlike conventional nanoparticle production methods that often rely on toxic chemicals and high-energy processes, green synthesis utilizes plant-derived bioactive constituents that work as natural reducing and stabilizing agents. This method of production was treated

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as more environmentally friendly and cost-effective and yields nanoparticles with enhanced biocompatibility that makes them significantly applicable for various medical applications. As a sustainable and scalable alternative, green synthesis has gained significant attention for producing nanoparticles with potential uses in medicine, agriculture, and environmental remediation (Mahmoud, 2020).

The size and shape of NPs are crucial because they directly affect the physical, chemical, electrical, and optical characteristics (Kusada *et al*., 2013). Ru is a transition metal within the platinum group, but it is more affordable than palladium and platinum. RuNPs have various applications, including producing diesel fuels (Kang *et al*., 2009), powering methanol fuel cells (Liu *et al*., 2006), dehydrogenation reactions (Su *et al*., 2007), breaking down azo dyes (Gupta *et al*., 2013), and removing organic contaminants from water (Perkas *et al*., 2005). The traditional RuNPs fabrication methods include sonochemistry (Kumar *et al.,* 2010), microwave irradiation (Gupta *et al*., 2013), electrochemistry (Rahman *et al*., 2011), and hydrothermal processes (Dikhtiarenko *et al*., 2012). However, these techniques involve complex procedures that consume more time and cost and involve toxic chemicals, which makes them less ideal for the fabrication of RuNPs. Thus, simple, eco-friendly, and cost-effective alternatives need to be developed for the fabrication of RuNPs, and green chemistry offers a sustainable way to produce these NPs.

The herbal plant *Causonis trifolia* (L.) is commonly known as three-leaf cayratia or bush grape, which is found in tropical and subtropical regions of Asia and Africa. It contains a wide range of bioactive compounds that contribute to its pharmacological properties and can be utilized to treat fever, pain, respiratory disorders, and gastrointestinal issues (Hazra *et al*., 2023). Various studies demonstrated that *Causonis trifolia* possesses significant antioxidant activity that helps neutralize harmful free radicals and potentially lowers the risk of chronic diseases. Additionally, the plant exhibits anti-inflammatory activity, antimicrobial efficiency, anticancer potential, blood sugar regulation, and hepatoprotective benefits (Kumar *et al*., 2011).

Recently, a few studies reported on the production of RuNPs utilizing extracts of plants such as *Gunnera perpensa (*Mfengwana & Sone, 2023), *Gloriosa superba (*Gopinath *et al*., 2014), *Anacyclus pyrethrum (*Nisha, Vidyalakshmi & Razack, 2020), *Aspalathus linearis* (Ismail *et al*., 2016), fishtail fern, holy basil, rosy periwinkle, and sago palm (Gupta *et al*., 2019). These reported studies inadequately explore the pharmacological significance of RuNPs. In the literature, one study reported the utilization of the leaf extract of *Causonis trifolia* (L.) for fabricating zinc oxide NPs (Usha Rani *et al*., 2023). No research has shown how to make RuNPs using Causonis trifolia, so this study aimed to find a new way to create RuNPs using leaf extracts from *Causonis trifolia (L.).* Further, the pharmacological significance of the synthesized RuNPs was evaluated. This approach is not only cost-effective but also offers potential applications in biomedicine and pharmaceutical fields.

Materials and Methods

Reagents and Chemicals

The reagent-grade chemicals utilized in this study, including Ruthenium(III) chloride hydrate ($RuCl_3.xH_2O$), Methanol, DPPH (*2,2-Diphenyl-1-picrylhydrazyl*) and Dimethyl Sulfoxide (DMSO) were purchased from Merck chemicals, Mumbai. RPMI (Roswell Park Memorial Institute) Medium, MTT (Methylthiazolyl diphenyl-tetrazolium bromide) and Phosphate-Buffered Saline (PBS), and Triton X-100 were purchased from Fisher Scientific, Mumbai. The skin cancer (A-431) cell lines and Vero cells (non-cancerous) were procured from the National Centre for Cell Sciences, Pune, India, and were cultured on RPMI media. The standard doxorubicin drug was procured from Zydus Healthcare Limited, Hyderabad, India.

Collection and Processing of Plant Material

In this study, fresh *Causonis trifolia* plants were collected from the coastal region of Machilipatnam, Krishna district, Andhra Pradesh, India. The taxonomical identification of plant material was performed

by Prof. Ch. Srinivasa Reddy, Department of Botany, SRR & CVR Government Degree College, Vijayawada. The department stored the plant specimen under the voucher number SRR-CVR/2024/P05. The healthy leaves that were free from visible damage or signs of disease were carefully collected and were washed thoroughly with distilled water to eliminate any surface impurities, dust, and contaminants. They were then shade-dried at room temperature for several days, and completely dried leaves were finely powdered through a mechanical grinder.

Five (5) grams of leaf powder was weighed and was placed into an airtight jar containing 100 mL of freshly boiled deionized water. This mixture was kept undisturbed for 5 hours to ensure that the phytochemical compounds from the leaf powder were fully extracted in the water. After extracting phytochemical compounds, the resultant mixture was filtered carefully to remove any larger particles. It was then centrifuged for 10 minutes at 4000 rpm to separate any remaining particles. The clear upper layer of extract was collected and stored at 4°C.

Synthesis of RuNPs

The RuNPs were synthesized by following a modified version of the method reported in literature (Mfengwana & Sone 2023). The procedure briefly: 5 grams of ruthenium chloride hydrate was combined with 30 mL of *Causonis trifolia* leaf extract. The solution color quickly turned dark grey, confirming the visible sign of reduction of ruthenium ions (Ru^{3+}) to elemental ruthenium (Ru^{0}). Then the extract comprising chelated ruthenium was continuously stirred at 60°C for 24 hours, followed by drying at 80°C over two days. This drying process yields a black powder of RuNPs, which was collected and utilized for further study.

Characterization of RuNPs

The optical characteristics of both RuNPs and leaf extract were examined with a double-beam UV-visible spectrophotometer (JASCO, V-560, Japan) within the 200–800 nm wavelength range. The optical band gap of NPs was further measured through UV-diffuse reflectance spectrophotometer (DRS; HO-SP-DRS100, India). The TEM analysis of synthesized NPs was performed on a Jeol JEM 2100 microscope (Japan) within an operating voltage of 120 keV. NPs were thoroughly washed with methanol followed by distilled water and were then mounted onto carbon-coated copper grids for TEM studies. The elemental composition and particle size of NPs were assessed through Scanning Electron Microscopy (SEM) combined with Energy Dispersive X-ray Spectroscopy (EDX) and were performed on a Jeol 6390LA (Japan) instrument equipped with an OXFORD XMX-N detector. The crystal structure and size of the NPs were studied using X-ray Diffraction (XRD) on a Bruker D8 Advance (USA) machine that uses Cu Kα radiation. The XRD analysis of NPs was performed within a 2θ range of 20° to 100° for precise evaluation of crystal characteristics. A DR-525 particle size analyzer (Brookhaven, USA) was employed to evaluate particle size and zeta potential of NPs.

Biocompatibility Potentials

The compatibility and safety of the synthesized RuNPs were checked by doing a hemolytic test with human red blood cells, using a changed method from Iqbal et al., 2021. The procedure Briefly, 1 mL of fresh blood was collected in an EDTA tube, and erythrocytes in the blood sample were separated by centrifugation at 12,000 rpm for 10 min. Then the cell pellet was separated and washed with PBS solution. Erythrocyte suspension solution was prepared by mixing 200 µL of red blood cells with 9.8 mL of PBS. A 100 µL portion of this suspension was then treated with different concentrations of RuNPs and incubated at 36°C for 1 hour. This was again centrifuged, and supernatant was taken in a 96-well plate, and release of hemoglobin was determined through spectrophotometer at 540 nm using positive (Triton X-100) and negative (DMSO) controls. The hemolysis percentage was evaluated by the following equation given by Sæbø *et al.,* 2023.

$$\%\ \text{Hemolysis} = \frac{absorbance\ of\ sample - absorbance\ of\ negative\ control}{absorbance\ of\ positive\ control - \ absorbance\ of\ negative\ control} \times 100$$

Antioxidant Activity of RuNPs:

The antioxidant activity was evaluated using a slightly modified DPPH assay protocol described by El-Borady, Fawzy & Hosny, 2023. In this method, 1 mL of DPPH (0.1 mM) solution was combined with 3 mL of different concentrations of RuNPs and leaf extract, ranging from 1 µg/mL to 250 µg/mL. This study utilizes ascorbic acid as a reference or standard. A control DPPH solution that doesn't contain any sample was also prepared and was treated as a control. This was thoroughly shaken and allowed to stand for 30 min at room temperature. Then the absorbance of all prepared test solutions was measured at 571 nm using a spectrophotometer. The accuracy of test results was ensured by performing the analysis in triplicate. Prasanna, Sunil, and Arun Kumar (2018) provided the formula for determining the % DPPH scavenging activity.

$$\%\ \text{DPPH Assay} = \frac{A0 - A1}{A0} \times 100$$

In the equation, A0 is control sample absorbance and A1 is test absorbance. Based on results obtained in various test concentration studies, dose-response curves were plotted for both aqueous extract and NPs. These dose-response curves were utilized to calculate its corresponding IC_{50} concentration which represents the concentration that requires 50% inhibition of DPPH radicals.

Anti-Skin Cancer Activity of RuNPs:

The cell viability of RuNPs was tested using the MTT assay utilizing skin cancer (A-431) cell lines as described by Naiel *et al.,* 2022. In brief, 1 × 10^5 cells/mL of A-431 cell lines were seeded in a 96-well plate (100 µL/well) containing different (25 to 500 µg/mL) concentrations of RuNPs and *Causonis trifolia* extract. These plates are incubated for 24 hours at 37°C and after removing growth medium, two-fold dilutions of samples were prepared in RPMI medium with 2% serum. An MTT solution (5 mg/mL in PBS) was added (8-20 µL/well), shaken, and incubated at 37°C with 5% CO_2 for 4 hours until formazan crystals formed. The crystals were dissolved in DMSO (200 µL) and absorbance was measured at 560 nm. The accuracy of test results was ensured by performing the analysis in triplicates. The cell viability was determined with the formula:

$$\%\ \text{Cell Viability} = \frac{A0 - A1}{A0} \times 100$$

In the equation, A0 is the absorbance of the control sample, and A1 is the absorbance of the test sample. Based on results obtained in various test concentration studies, dose-response curves were plotted for both aqueous extract and NPs. These dose-response curves were used to find the IC_{50} concentrations, which are the amounts needed to reduce the A-431 cell lines by 50%. The doxorubicin standard drug at a concentration equivalent to the concentration of RuNPs was utilized as a positive control in this study.

After 24 hours, all test samples were examined under an inverted phase-contrast tissue culture microscope (Olympus CKX41) with a camera (Optika Pro5 CCD). The obtained microscopic images were observed for any visible changes in cell shape, such as cell shrinkage, rounding, or the presence of granules and vacuoles in cell cytoplasm. The correlation of these results proved the anti-cancer potential of synthesized RuNPs.

Results

A noticeable reduction of ruthenium metal and formation of RuNPs was observed when mixing *Causonis trifolia* leaf extract to ruthenium metal solution. The reduction of ruthenium metal was confirmed visually by change in reaction mixture color from pale color to dark blackish-brown color. The change in reaction mixture and formation of RuNPs was further confirmed by UV-visible spectral analysis. The absorption spectrum reveals a prominent absorption peak at 491 nm (Figure 1). Simultaneously, the plant extract was heated under reflux conditions and its absorbance was monitored. The absorption spectrum of plant extract doesn't show any absorption peak at this wavelength indicates that the complete reduction of Ru^{3+} ions to their metallic Ru^0 form.

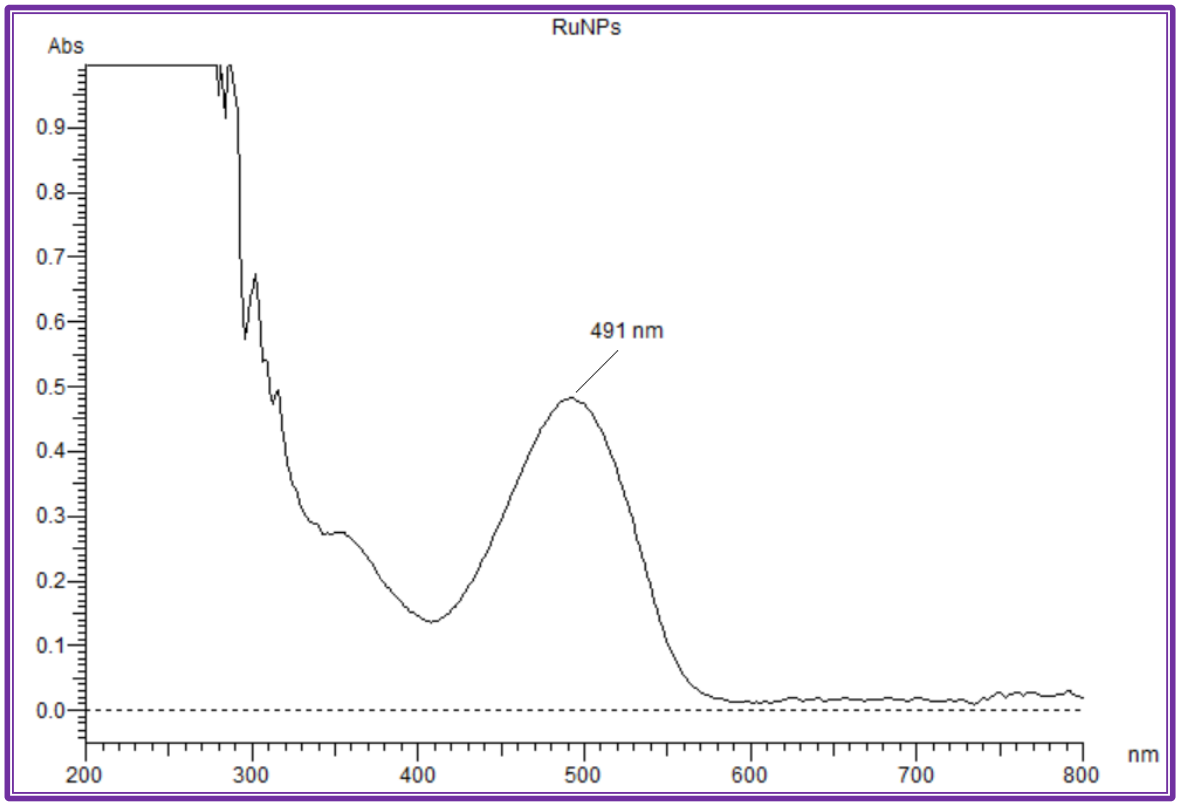


Figure 1: *UV-visible absorption spectrum of RuNPs synthesized using aqueous leaf extract of Causonis trifolia*

FTIR analysis was conducted to evaluate the biomolecules in *Causonis trifolia* leaf extract that facilitated the reduction of Ru^{+} ions and the stabilization of the synthesized RuNPs (Figure 2). A strong IR band at 3360 cm^{-1} indicates the N-H stretching vibration associated with primary amines whereas the band at 2924 cm^{-1} corresponds to the stretching of aliphatic C-H bonds. The bands located at 1355 cm^{-1} and 1602 cm^{-1} are due to the presence of NO_2 stretching and C=C stretching respectively. The observed IR bands at 1093 cm^{-1} and 1301 cm^{-1} are associated with –C–O–C stretching and C–O stretching bands respectively. This analysis results suggests the presence of various phytochemical compounds in *Causonis trifolia* leaf extract that contribute to reduction and stabilization of RuNPs during synthesis.

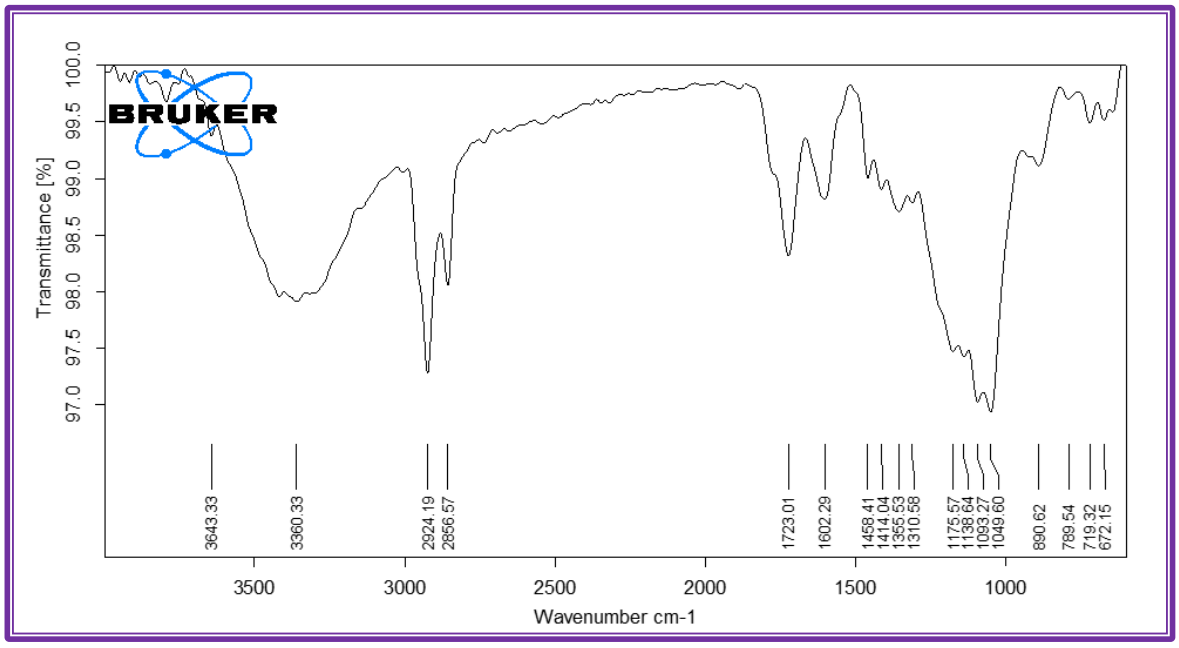
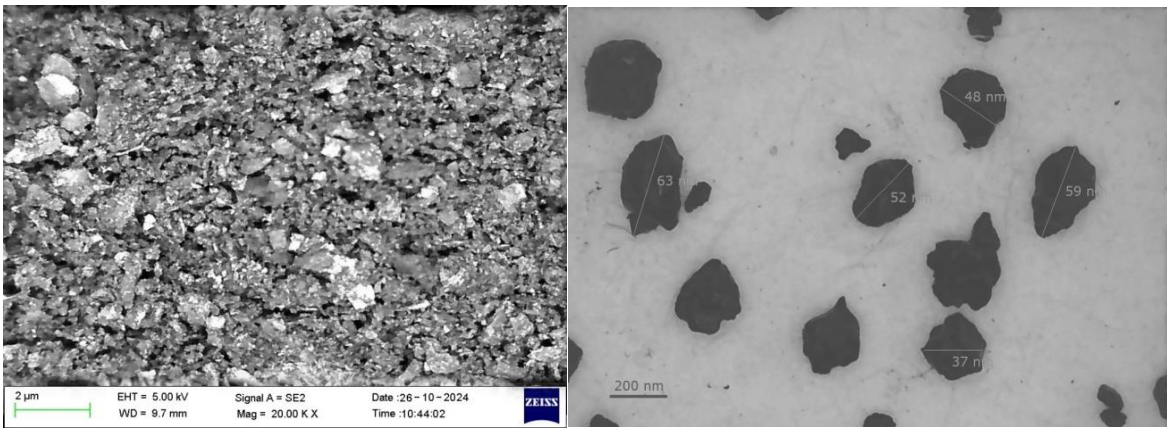


Figure 2: *FTIR spectrum of RuNPs synthesized in this study*

The SEM and TEM analysis provide evidence of the morphology and particle size distribution of the synthesized RuNPs. Figure 3A shows that the texture as viewed in the SEM is quite rough and uneven with high surface irregularities. The RuNPs have a thick, grainy structure, with the particles sticking together in clumps because the high surface energy of the nanoparticles encourages them to cluster. The irregular surface and granular appearance of the RuNPs provide evidence of their non-uniformity, likely due to the use of a plant-based extract as a capping agent, which causes deviations in their shape and size. In the HRTEM study (Figure 3B), the nanoparticles were found to vary in size from 37 to 63 nm, with an average size of 48 nm, indicating a moderate range of sizes. TEM analysis indicates that the NPs have uneven surfaces and lack a clear crystal structure, which is common for nanoparticles made using plant extracts. Additionally, RuNPs are spread out evenly on the grid without clumping together, showing that the plant extract has successfully coated and stabilized the particles.



a b

Figure 3: SEM (A) and TEM (B) analysis results of RuNPs synthesized using aqueous leaf extract of Causonis trifolia

The EDX spectrum (Figure 4) shows that RuNPs were successfully made, highlighted by a strong peak at about 2.6 keV, which represents ruthenium. This result verifies that ruthenium is the major element in the sample, with a 73% composition in the formed NPs. Additionally, the spectrum visualizes the presence of smaller peaks corresponding to carbon (C) at 0.3 keV, oxygen (O) at 0.5 keV, potassium (K) at 3.3 keV, and magnesium (Mg) at 1.25 keV. The presence of carbon and oxygen was maybe due to organic compounds derived from *Causonis trifolia* leaf extract that was used in the green synthesis process. The signals corresponding to Mg and K were observed, which may originate from the plant's natural extract or the coastal environment where the plant leaves were collected. These extra elements indicate that the RuNPs might be covered or supported by natural materials from the plant extract, which could improve the stability and effectiveness of the NPs.

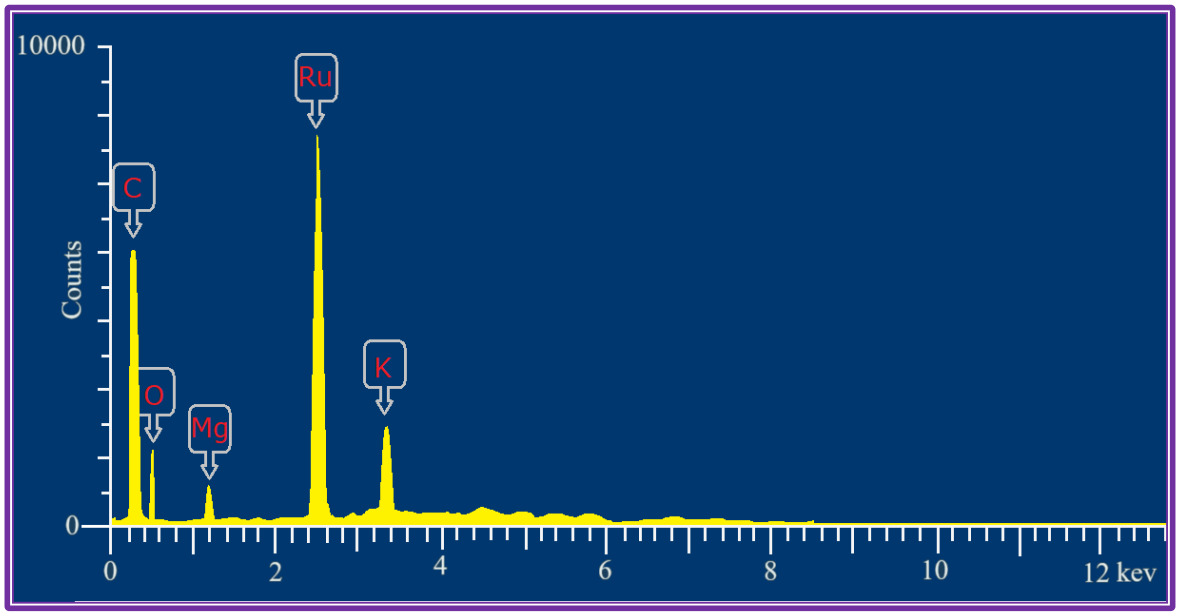


Figure 4: *EDX analysis results of RuNPs synthesized using aqueous leaf extract of Causonis trifolia*

The X-ray Diffraction (XRD) analysis of RuNPs made with the water extract from *Causonis trifolia* leaves shows clear peaks that confirm the particles are crystalline. The XRD pattern (Figure 5) shows peaks at 2θ values at 27.8, 38.1, 45.0, 57.8, 68.7 and 74.2 corresponding to the 110, 020, 210, 220, 301 and 311 crystal planes. The most intense peak at the (220) plane indicates a high degree of crystallinity and suggests that this is the preferred orientation in the synthesized RuNPs. The Bragg reflections at its corresponding Miller indices can be attributed to the tetragonal structure of synthesized RuNPs with JCPDS# 88-0322. No minor or unassigned peaks observed in the XRD spectrum suggest that there are no significant impurities detected and prove that the NPs have a high degree of purity.

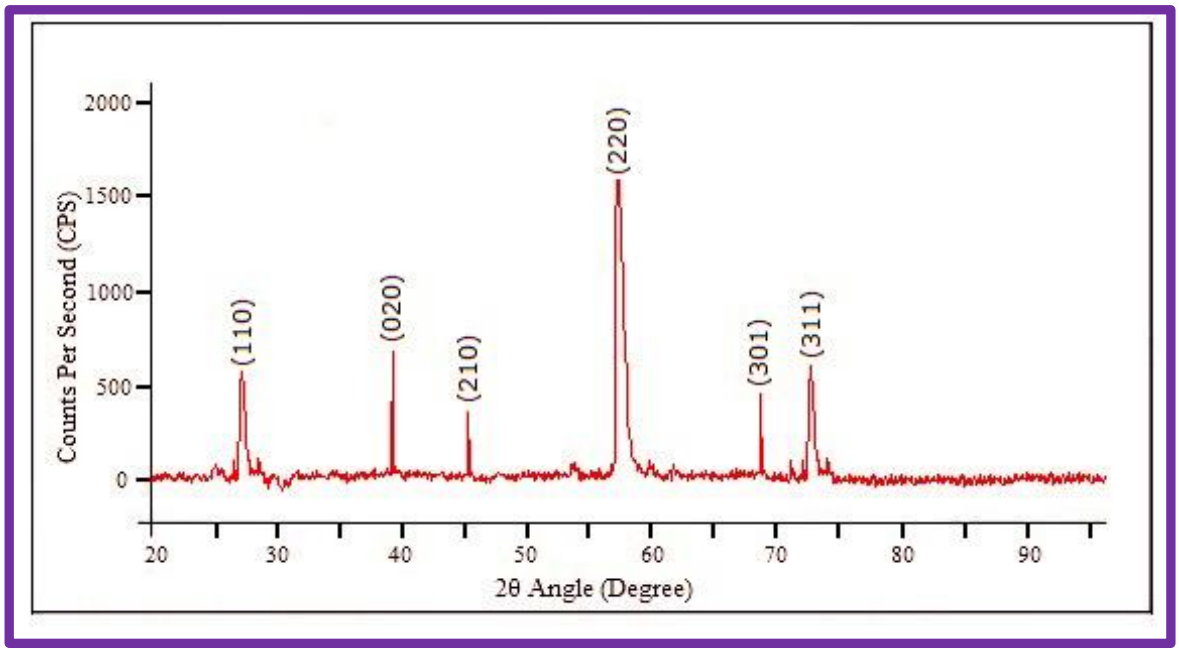


Figure 5: XRD analysis results of RuNPs synthesized using aqueous leaf extract of *Causonis trifolia*

Biocompatibility Potentials

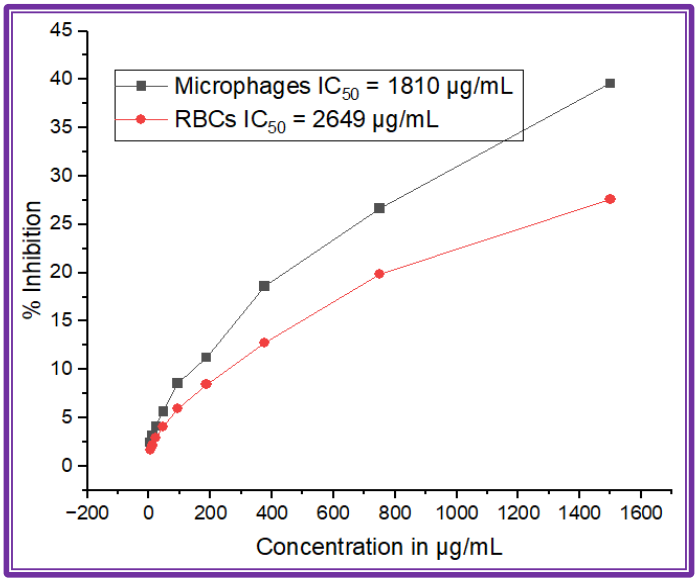
The safety and compatibility of the synthesized RuNPs with biological systems were thoroughly examined by following biosafety guidelines. As per guidelines, biochemical substances are classified based on their hemolytic effects, in which substances that cause more than 5% hemolysis are treated as hemolytic; those with 2–5% are slightly hemolytic; and those with less than 2% hemolysis are treated

as non-hemolytic substances. The erythrocytes were exposed to various concentrations of RuNPs, from 5.859 to 1500 µg/mL, to evaluate the hemolytic properties of synthesized RuNPs (Table 1). The reaction of RuNPs with the erythrocytes was noticed using a UV-visible spectrophotometer. The results showed a dose-dependent effect, where the highest concentration (1200 µg/mL) caused a 27.59±0.153 % release of hemoglobin from the red blood cells, indicating a significant hemolytic effect at this level. However, at lower concentrations, the RuNPs display very nominal hemolytic activity. Similarly, macrophages were exposed to the same concentration of RuNPs, and dose-dependent results were noticed for the synthesized RuNPs. The IC50 concentration was noticed to be 2649±1.281 µg/mL for RBCs and 1810±1.693 µg/mL for the inhibition of macrophages, confirming the non-toxic properties of RuNPs when used in low amounts. Hence it can be confirmed that the NPs synthesized in this study were safe and biocompatible. Figure 6 displays the results noticed in the biocompatible study of synthesized RuNPs.

Table 1: Biocompatibility potentials result of RuNPs synthesized in this study

S.No	Concentration in µg/mL	% Inhibition noticed	
		Microphages mean±SD	RBCs mean±SD
1	5.859	2.46±0.036	1.71±0.073
2	11.719	3.13±0.020	2.15±0.087
3	23.438	4.15±0.040	2.91±0.095
4	46.875	5.66±0.068	4.10±0.113
5	93.75	8.58±0.079	5.92±0.106
6	187.50	11.25±0.043	8.51±0.152
7	375	18.59±0.085	12.75±0.121
8	750	26.67±0.120	19.82±0.257
9	1500	39.58±0.155	27.59±0.369

Results presented in table are mean ± standard deviation (n = 3), $p < 0.05$



Results presented in graph are mean ± standard deviation (n = 3), $p < 0.05$

Figure 6: *Biocompatibility assay results of RuNPs synthesized using aqueous leaf extract of Causonis trifolia*

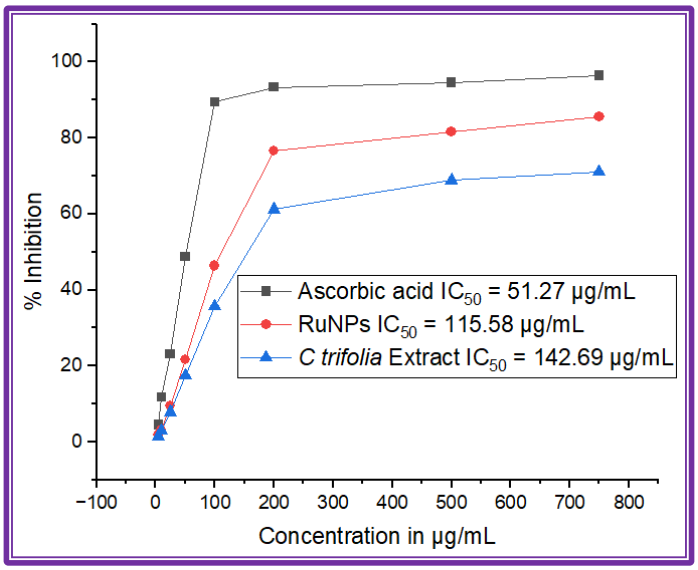
Antioxidant activity of RuNPs

Figure 7 shows that RuNPs display significant antioxidant activity by neutralizing free radicals and radical inhibition potential was increased with increase in the dose of NPs. At the highest (750 µg/mL) concentration tested, the DPPH radical scavenging activity was 85.58±0.093 % for RuNPs, 71.14±0.103% for *C trifolia* extract and 96.36 % for ascorbic acid (Table 2). The IC_{50} concentration was calculated to be 142.69±0.245 µg/mL for plant extract and 115.58±0.232 µg/mL for plant extract mediated RuNPs whereas 51.27±0.119 µg/mL for standard ascorbic acid.

Table 2: Antioxidant activity results achieved in the study

S. No	Concentration in µg/mL	% DPPH inhibition noticed for		
		Ascorbic	RuNPs	*C. trifolia* Extract
1	5	4.59±0.035	1.85±0.029	1.34±0.006
2	10	11.73±0.067	3.26±0.034	2.91±0.012
3	25	23.18±0.066	9.58±0.039	7.84±0.024
4	50	48.76±0.062	21.63±0.026	17.52±0.032
5	100	89.43±0.122	46.39±0.049	35.69±0.051
6	200	93.36±0.151	76.68±0.077	61.25±0.108
7	500	94.58±0.079	81.58±0.126	68.91±0.112
8	750	96.36±0.157	85.58±0.99	71.14±0.057

$n = 3$, $p < 0.05$



Results presented in graph are mean ± standard deviation ($n = 3$), $p < 0.05$

Figure 7: DPPH inhibition assay results of RuNPs synthesized using aqueous leaf extract of *Causonis trifolia*

Anti-Skin Cancer Activity of RuNPs

The utilization of plant-based materials to synthesize nanoparticles for anticancer treatment has emerged as a promising approach in cancer treatment. The combination of plant compounds with nanotechnology can improve therapeutic effectiveness, reduce side effects, and support patient-centered treatments. The NPs formulations can make Ru complexes work better at targeting cancer cells while causing little to no harm or side effects. Hence the RuNPs synthesized in this study were tested against skin cancer (A-431) cell lines for cancer-fighting activity. This study utilizes doxorubicin as a standard drug and Vero cells as non-cancerous cells.

Figure 8 demonstrates the comparative results achieved for the extract from *Causonis trifolia* leaves, extract-mediated RuNPs, and standard drugs against the inhibition of growth of skin cancer (A-431) cell lines and non-cancerous Vero cells. The results demonstrated that, at a low concentration of 5 µg/mL, the A-431 A-431cell growth inhibition of 3.58±0.058 %, 1.88±0.011%, and 0.90±0.063 % was noticed for the standard drug, RuNPs, and aqueous leaf extract, respectively, suggesting that very nominal growth inhibition occurred at the lowest concentration studied. As the concentration of the test sample increases, the growth inhibition also increases by following dose-dependent activity and reaches 38.36±0.085 %, 18.72±0.051%, and 11.63±0.082 % inhibition at 100 µg/mL concentration, respectively. At a very high test concentration of 1000 µg/mL, the inhibition of A-431 cell growth was 91.23±0.269, 69.24±0.306, and 39.53±0.299 % for the standard drug, RuNPs, and aqueous leaf extract, respectively. The IC_{50} concentration was calculated using standard graphs, and the results show that the NPs have an IC_{50} concentration of 267.10±0.369 µg/mL, while the standard drug and leaf extract have IC_{50}

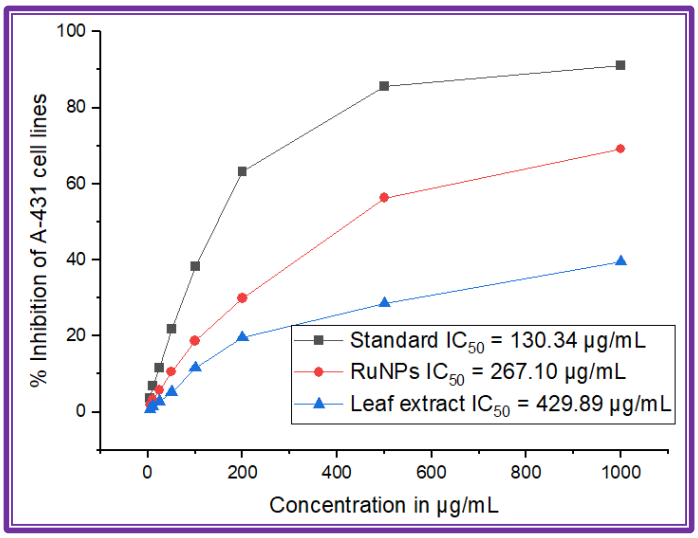
concentrations of 130.34±0.308 µg/mL and 429.89±0.528 µg/mL, respectively, indicating that the synthesized RuNPs are very effective.

Table 3: Anti-skin cancer activity results for standard, extract and RuNPs

S. No	Concentration in µg/mL	% Inhibition noticed for		
		Ascorbic acid	RuNPs	*C. trifolia* Extract
1	5	3.58±0.009	1.88±0.005	0.90±0.013
2	10	6.91±0.017	3.39±0.011	1.54±0.015
3	25	11.53±0.023	5.72±0.019	2.87±0.038
4	50	21.85±0.36	10.60±0.024	5.22±0.021
5	100	38.36±0.039	18.72±0.029	11.63±0.036
6	200	63.22±0.041	29.90±0.034	19.63±0.042
7	500	85.58±0.052	56.31±0.016	28.62±0.049
8	1000	91.23±0.021	69.24±0.078	39.53±0.055

$n = 3$, $p < 0.05$

A.



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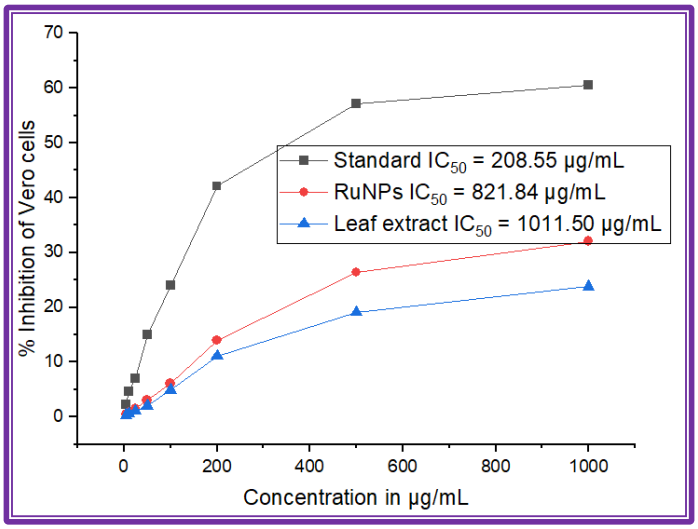
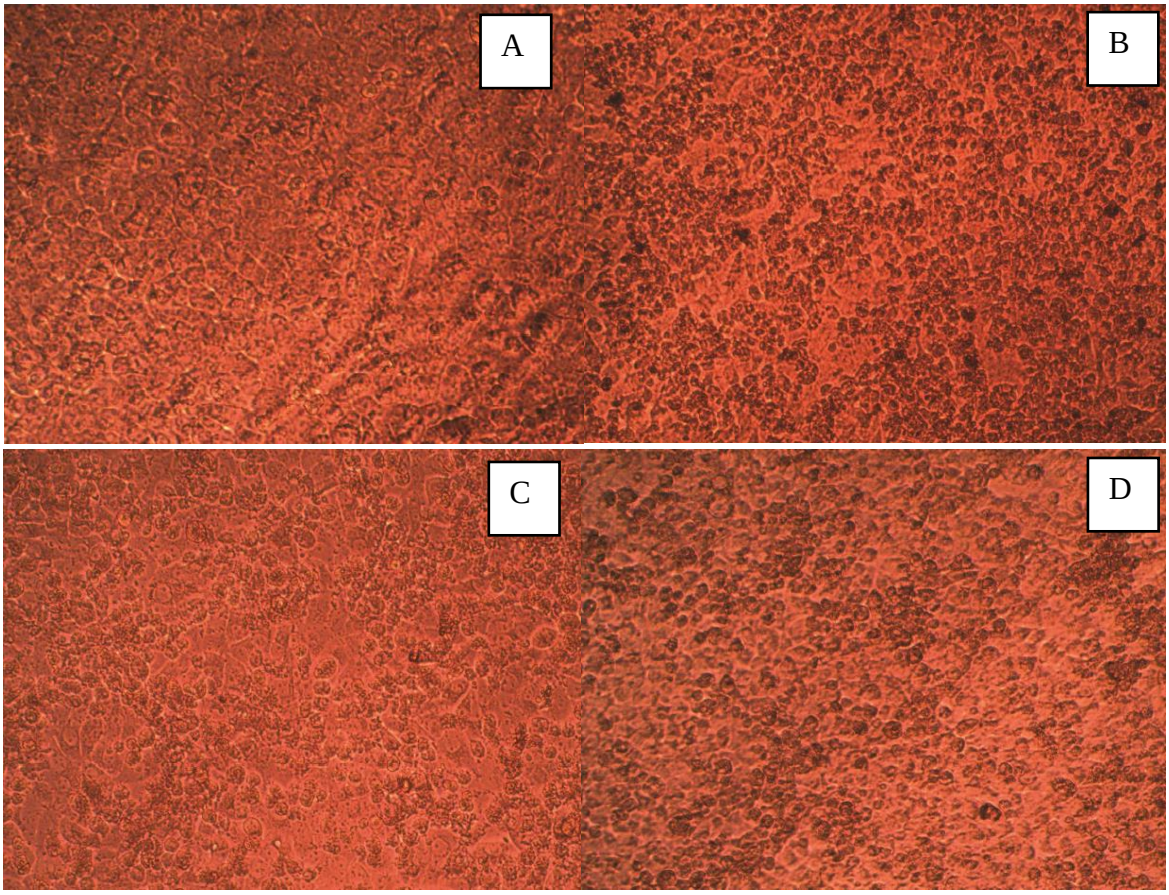


Results presented in graph are mean ± standard deviation ($n = 3$), $p < 0.05$

Figure 8: *The comparison of anti-skin cancer ability of Causonis trifolia leaf extract, extract mediated RuNPs and standard drug against Skin cancer (A-431) cell lines (A) and non-cancerous Vero cells (B)*

Similarly, the toxic nature of studied samples was tested against non-cancerous Vero cells to evaluate the selective inhibition ability of synthesized NPs. The results indicate that the standard doxorubicin exhibits an IC50 concentration of 208.55±0.628 µg/mL, whereas synthesized RuNPs show 821.84±1.251 µg/mL, and leaf extract shows 1011.50±1.528 µg/mL, suggesting that the plant extract and the synthesized RuNPs show very nominal inhibition activity against Vero cells compared to the standard drug.

The efficiency of inhibiting cancer cell growth by synthesized RuNPs was further confirmed by performing microscopic observation of cancer cells treated with standard drug, leaf extract, and RuNPs synthesized in this study. All test samples after 24 hours of treatment display notable changes in cell shape and structure, as shown in Figure 9. The cells treated with the standard drug as well as synthesized RuNPs show significant variations, including blebbing (formation of small bubbles on the cell surface), granulation, and shrinkage, which suggest that the activity is due to apoptosis. These types of morphological changes were not noticed for non-cancerous cell lines treated with RuNPs, suggesting that the NPs were non-toxic to non-cancerous cell lines, which further confirms the targeted anti-cancer efficiency of RuNPs.



A=Control, B=Drug treated, C=Extract treated, D=Nano treated

Figure 9: *Morphological observation of A-431 cell lines treated with Causonis trifolia leaf extract, extract mediated RuNPs and standard drug*

Discussion

The green synthesis has emerged as a sustainable, cost-effective, and environmentally friendly approach to produce nanomaterial. Among metal NPs, RuNPs are less explored, particularly through green synthesis methods using plant extracts as reducing and capping agents. Hence, this study aimed to synthesize and characterize RuNPs using aqueous extracts of *Causonis trifolia* (L.) leaves and

evaluate their antioxidant activity, biocompatibility, and cytotoxicity against A-431 skin cancer cell line applications.

The UV-Vis spectroscopy analysis showed a strong absorption peak at 491 nm, which indicates that NPs were successfully formed, and these results matched what has been reported in previous studies (Gopinath et al., 2014). The FTIR results showed that there were important chemical groups linked to natural compounds like flavonoids, alkaloids, phenolics, and terpenoids, which are key for keeping the nanoparticles stable. The FTIR frequencies noticed in the synthesized RuNPs were aligned with findings reported in the literature (Gopinath *et al*., 2014). The synthesized RuNPs exhibited granular morphology with irregular surfaces and a nanoscale size range of 37–63 nm, averaging 48 nm. The four-sided crystal shape and high purity of 73% confirm that the made nanoparticles are of good quality, making them appropriate for use in medicine.

The NPs exhibits exceptional DPPH radical scavenging activity in RuNPs, with an IC50 concentration of 115.58 ± 0.232 µg/mL, highlighting their antioxidant potential, which is crucial in mitigating oxidative stress-related diseases. The NPs have greater antioxidant activity compared to plant extracts because they have a greater surface area, which improves radical scavenging activity. Redox reactions are enhanced by the presence of metal ions, whereas phytochemicals derived from plants stabilize and extend antioxidant activity. The combined effect of metal ions and bioactive compounds also improves the transfer of electrons, making nanoparticles more effective at neutralizing reactive oxygen species. Both the water extract and the RuNPs made with the extract show better antioxidant activity because of their secondary metabolites. The compounds, like phenolics and terpenoids, in plant extracts are known for their strong antioxidant properties, which act as stabilizing agents for the RuNPs and hence exhibit high DPPH radical inhibition abilities. The ability of the synthesized RuNPs to inhibit the DPPH radical, which is a measure of antioxidant strength, was found to be much better than what has been reported in previous studies (El-Amier et al., 2024), showing that the nanoparticles made in this study are more effective.

The cytotoxicity results from the MTT assay show that the RuNPs had significant anticancer activity against A-431 cells from skin cancer with an IC50 concentration of 267.10 µg/mL and exhibit less cytotoxicity toward Vero non-cancerous cells. This selective cytotoxicity demonstrates their potential as a safe and effective therapeutic agent for cancer treatment. An ideal new anticancer drug should specifically target and inhibit cancer cell growth with little or no influence on the healthy/normal cells (Anjum *et al*., 2021). In this study, the results indicate that the synthesized RuNPs showed a stronger inhibitory effect on cancer cells, with very little inhibitory activity against non-cancerous healthy cells. This selectivity suggests that RuNPs synthesized may be suitable as potential anticancer drug candidates to treat cancer. The NPs are proved to have biocompatibility, and irregular-shaped NPs have greater active sites exposed, which affect cell uptake and biomolecule interaction. Although the shape improves bioavailability, it may cause cytotoxicity through excessive oxidative stress. Surface charge, hydrophilicity, and aggregation behavior also influence NP stability and biological response. These findings provide a significant opportunity for further exploration of RuNPs in biomedical and therapeutic applications.

This study produces promising results but has certain limitations in terms of cytotoxicity evaluation because this study was conducted using only A-431 cell lines for skin cancer, which does not provide a comprehensive understanding of the RuNPs' effects against cancer treatment. Therefore, future research should look at how RuNPs affect a wider variety of cancer cell lines and perform in vivo studies to check their effectiveness, how they spread in the body, how they are processed, and any possible long-term harmful effects.

Conclusion

A straightforward and inexpensive way was used to make RuNPs by using the beneficial chemicals from *Causonis trifolia* (L.) leaves, which change Ru^{3+} into Ru^{0} to create RuNPs. The extract from Causonis trifolia leaves is rich in various bioactive compounds that act as an electron donor to aid in this reduction process. The formation of RuNPs was confirmed with several methods that showed that

the NPs were circular to irregular in shape with a size range of 37 to 63 nm. The XRD results further confirm the metallic nanostructure of particles with 73% elemental Ru. The synthesized nanoparticles demonstrated notable cytotoxic and antioxidant activities with significant biocompatible potential. This biosynthesis of NPs has several advantages, including affordable prices and significant applicability in biomedical, pharmaceutical, and food fields. It can be scaled up for commercial production. These RuNPs can be used in targeted drug delivery systems because their tiny size helps them hold onto drugs well and release them in a controlled way at tumor sites. Surface modification with antibodies or ligands would allow better selectivity for cancer cells and reduce toxicity for healthy tissues. Their antimicrobial activity also renders them good candidates for wound healing, antimicrobial coatings, and pharmaceuticals. Future research could look into using new technology to create NPs that have specific sizes, shapes, and even distribution, which could improve how plant-based nanomaterials are used in medicine.

Conflict of Interest

The authors declare that they have no competing interests.

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