

Article

Green Synthesis of Au-Pd Bimetallic Nanoparticles Using Aspalathin and Their Toxicity Study

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Abstract

Bimetallic nanoparticles have garnered significant attention in scientific literature due to their diverse applications and unique properties. Concurrently, green synthesis methodologies have emerged as environmentally friendly alternatives, reducing the ecological footprint of nanoparticle production. In this study, the efficient synthesis of Au-Pd bimetallic nanoparticles is presented, utilizing *Aspalathus linearis* (Burm.f.) R. Dahlgren, commonly known as green rooibos (GR), and its pure bioactive compound, Aspalathin (ASP). Integrating ASP as a pure compound into the green synthesis process offers precise control over nanoparticle characteristics, including size, morphology, and composition. Interestingly, the total extract forms an Au-Pd nanoparticle alloy, while aspalathin forms core-shell nanoparticles. Furthermore, cytotoxicity testing was carried out on selected cell lines to assess their impact on cell viability. The cytotoxicity test on cell lines and cellular uptake analysis demonstrated that none of the tested samples exhibited significant cytotoxic effects. ASP-conjugated bimetallic increased the uptake of the NPs by the cells more than the total extract. The results demonstrated that the Au-Pd bimetallic nanoparticles hold promise for biomedical applications, owing to their enhanced biocompatibility and tailored properties.

Keywords: *Aspalathus linearis*; aspalathin; green synthesis; bimetallic nanoparticles; core-shell nanoparticles; cytotoxicity; cellular uptake

1. Introduction

Nanotechnology is a popular area of science dealing with objects ranging from 1 to 100 nm in size, known as nanoparticles [1]. Nanoparticles can be synthesized by either chemical or biological methods. Furthermore, there is growing concern regarding the potential exposure and adverse effects of nanoparticles on both human health and the environment. Over recent years, numerous in vitro and in vivo studies have been conducted to explore the toxicological impacts and potential hazards associated with various nanoparticle exposures. However, there has been a significant gap in our understanding of the toxicity effects resulting from nanoparticle exposure. Consequently, depending on how various types of metal nanoparticles interact with organisms, the biosynthesized metallic nanoparticles with non-toxic surface functional molecules still demonstrate differing levels of toxicity. Research examining the toxicity of noble metals such as Ag, Au, and Pd has



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revealed that silver nanoparticles are highly toxic, primarily due to the gradual oxidation and release of highly reactive Ag^+ ions [2–4]. Due to this, biomedical applications for less harmful Au and PdNPs have shown popularity.

Nanoparticles formed from biological methods are most effective in controlling endemic diseases with fewer side effects [5,6]. Contrary to this, chemical methods involved in the synthesis of nanoparticles show adverse side effects because of the presence of toxic chemicals that get adsorbed on their surface [7–9]. Plant-based nanoparticle synthesis has gained more attention in several applications due to its eco-friendly nature in various fields. Plants are widely distributed, readily available, safer to handle and act as a source of several metabolites [8]. Furthermore, plant extracts are rich in secondary metabolites such as alkaloids, flavonoids, phenolic acids, and terpenoids. These secondary metabolites are capable of reducing ionic metals and assist in the formation of bulk metallic nanoparticles [10,11]. Nanoparticles are synthesized biologically from various parts of the plant, such as roots, stems, leaves, seeds, fruits, peels and flowers [12]. Therefore, synthesizing nanoparticles using plant extracts is a widely accepted method to produce green nanoparticles. Recently, many plant extracts [13–15] have shown capabilities to produce safe nanoparticles, including Au [16], Pd [17], and Au alloys [18].

The primary flavonoids in *Aspalathus linearis* (Burm.f.) R. Dahlgren leaves include aspalathin, nothofagin, orientin, vitexin, quercetin, and rutin [19,20]. The GR plant extract was reported in the synthesis of several metal nanoparticles, such as Au, Ag, and Pd [21,22]. Due to their ease of surface modification and application in a variety of fields, AuNPs have attracted serious attention across research communities [23–25]. On the other hand, PdNPs have been synthesized using biological plant extracts and microorganism derivatives, often environmentally and economically friendly materials [26,27]. Moreover, previous studies have reported the synthesis of PdNPs using *Syzygium aromaticum* extract [28,29]. Furthermore, Pd nanoparticle anticancer activity has been proven to induce apoptosis in cancer cells and inhibit their growth. The literature has shown that only a few reports have focused on green Pd synthesis to date; therefore, efforts to concentrate on Pd nanoparticle synthesis and anticancer potential are necessary [29].

Combining two metals with distinct properties in a single nano-system increases the number of distinct properties, allowing it to be used in a wide range of applications [30]. Akinfenwa and co-authors reported on the synthesis of nanoparticles using the pure compound, ASP, as a reducing and stabilizing agent [21]. Furthermore, to the best of our knowledge, no reports on the synthesis of Au-Pd alloy-structured bimetallic nanoparticles using GR extract and its compounds are available in the literature. As a result of the data on the use of monometallic Au and PdNPs in various applications, green-synthesized Au-Pd alloy-structured bimetallic nanoparticles can be used in various applications [31–33]. The scarcity of comprehensive data in toxic bioassay assessments of Au-Pd bimetallic nanoparticles against various biological agents, including microbes, cell lines, pathogens, parasites, and disease vectors, indicates the potential value of the novel insights provided by the current research on these nanoparticles [34].

In light of these considerations, this study directs its attention towards recent advancements in the green synthesis of Au-Pd bimetallic nanoparticles, offering a promising alternative to conventional methods reliant on environmentally unfriendly chemical agents.

2. Results

Through careful selection from a variety of plant extracts, *Aspalathus linearis*, commonly known as unfermented green rooibos (GR) plant extract, emerged as the preferred choice due to its rich polyphenol content. This study explores the formation of Au-Pd bimetallic nanoparticles, utilizing the unfermented GR extract as a primary plant source. The iso-

lation methodologies of ASP, together with its spectroscopic data, have been previously reported [21].

In this study, GR and ASP, with their intriguing chemical structure and potential reducing capabilities, serve as a vital component in the green synthesis of bimetallic nanoparticles.

2.1. Chemistry of Aspalathin

In the context of nanoparticle synthesis, ASP acts as a reducing agent, providing electrons to reduce metal ions into metallic nanoparticles. The reducing strength of ASP can be attributed to its chemical structure (Figure 1), which contains functional groups capable of electron donation. Flavonoids, including ASP, often have hydroxyl (-OH) groups and carbonyl (C=O) groups, which can participate in redox reactions. During the synthesis of nanoparticles, ASP can donate electrons to metal ions, leading to their reduction and subsequent formation of nanoparticles. The specific mechanism of reduction can vary depending on the metal ions and reaction conditions employed. The electron donation by ASP helps stabilize the metal ions in a reduced state, preventing their re-oxidation and allowing for the formation of stable nanoparticles.

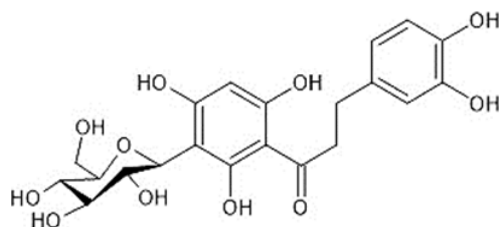


Figure 1. Chemical structure of Aspalathin (C-glucosyl dihydrochalcone).

2.2. Ultraviolet-Visible (UV-Vis) Spectroscopy

Figure 2A,B illustrates the spectral characteristics of GR and ASP-Au-Pd bimetallic nanoparticles, exhibiting an absorption peak in the visible range with a maximum at 525 and 540 nm, similar to the absorption spectrum of AuNPs.

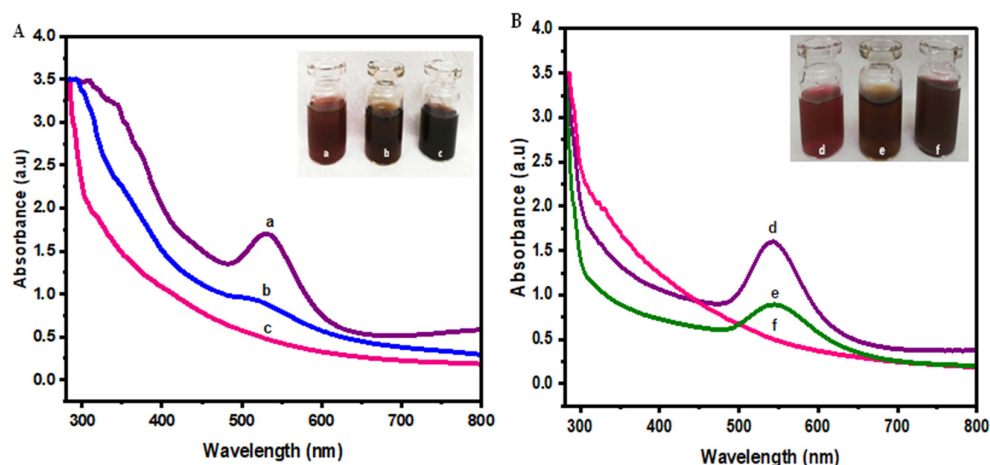


Figure 2. UV-Vis spectra of (A) GR-AuNPs (a), GR-Au-PdNPs (b), GR-PdNPs (c), as well as (B) ASP-AuNPs (d), ASP-Au-PdNPs (e), and PdNPs (f).

However, a blue shift in the SPR peak of Au-Pd bimetallic nanoparticles was observed in comparison to that of AuNPs. This shift is ascribed to the change in the composition and electronic structure of the nanoparticles due to the presence of Pd [35–37]. In addition, the observed shift in the SPR peak of the Au-Pd bimetallic nanoparticles formed from GR

extract was attributed to the alloying effect between Au and Pd, changes in nanoparticle size, and modifications in the local electromagnetic environment.

The ASP results were consistent with those reported by Chowdhury et al. [18]. This phenomenon agrees with previous studies that stated that the presence of a group 10 (d8s2) metal in bimetallic nanoparticles suppresses the surface plasma energies of group 11 (d10s1) metal [38]. This implies the formation of Au-Pd bimetallic nanoparticles in our samples. In addition, the absorbance of the Au-SPR band was slightly reduced. The decrease in the SPR band intensity provides evidence for the formation of alloyed bimetallic nanoparticles [36,37].

2.3. Dynamic Light Scattering (DLS)

The DLS analysis yielded valuable insights into the hydrodynamic diameters, size distribution, and stability of the synthesized bimetallic nanoparticles, as detailed in Table 1. The current reaction conditions produced average sizes of GR-Au-PNPs and ASP-Au-PdNPs ranging from 80.03 and 42.28, respectively. During DLS analysis, a thin electric dipole layer of the solvent adheres to the surface when a dispersed particle moves through a liquid medium. The particle movement in the medium is thereby influenced by the layer. As a result, as the particle moves under Brownian motion, the hydrodynamic diameter provides information about the inorganic core, any coating material, and the solvent layer attached to the particle [38,39]. Furthermore, the GR extract matrix, the polydispersity index (PDI) values of the nanoparticles were 0.362, while the ASP-formed nanoparticles had a PDI value of 0.381. Furthermore, the PDI information obtained from the DLS technique represented a degree of homogeneity of different particle sizes to the total number of particles [40,41].

Table 1. Hydrodynamic sizes, polydispersity index (PDI), and Zeta Potential (mV) of bimetallic nanoparticles obtained through the DLS technique.

Samples	Hydrodynamic Sizes (nm)	Polydispersity Index (PDI)	Zeta Potential (mV)
GR-Au-PdNPs	80.03	0.362	−18.2
ASP-Au-PdNPs	42.28	0.381	−21.7

Using this instrument, the Zeta Potential values (mV) and mobility of GR and ASP-NPs (cm/s V) were found to be −21.7 and −18.2, respectively, indicating the formation of an electrical double layer at the particle surface in the supporting matrix, as shown in Table 1 [42]. Nanoparticles with Zeta Potential values of −30 mV and lower are assumed to be mostly covered with negatively charged ions [43,44]. Positive Zeta Potential values have the opposite effect. As a result, the extent of Zeta Potential is a subject of repulsion and attraction that has been used to predict how long nanoparticle dispersions will remain stable. The AuNPs obtained negative Zeta Potential values that are typical of stable colloidal dispersions [43,44].

2.4. X-Ray Diffraction (XRD) Analysis

The XRD patterns of the monometallic and bimetallic nanoparticles were compared to confirm the formation of Au-Pd structures.

The XRD pattern of the Au-Pd nanoparticles (as shown in Figure 3) displays 2 θ peaks at 38.37, 44.49, 64.77, 77.83, and 81.97, which can be indexed to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) crystal planes, respectively. The diffraction peaks of Au-Pd were positioned between those of monometallic Au and PdNPs. Additionally, the alloyed sample slightly shifted to higher 2 θ values compared to those of the AuNPs, owing to the interactions

with Pd atoms following the formation of bimetallic Au-Pd alloyed structures [38,45]. This confirms that the Au-Pd alloy was formed rather than a mixture of mono-metals or core-shell nanoparticles. A similar phenomenon was reported by Song and co-workers as well as Wicaksono and co-workers [46,47].

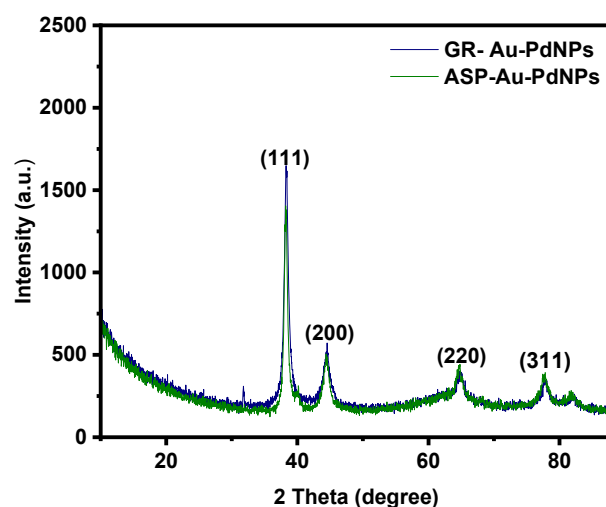


Figure 3. XRD pattern of Au-Pd bimetallic alloyed nanoparticles obtained using GR extract and its bioactive compound, ASP.

The Au-Pd bimetallic nanoparticles showed 2 θ peaks (Figure 3) at 38.28, 40.06, 44.42, 64.61, 77.67, and 81.65 corresponding to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) planes, respectively. Furthermore, as the Pd content increased, the Au-Pd bimetallic nanoparticles exhibited shifted Bragg peaks from pure Au to pure Pd. This suggests that the prepared nanoparticles were Au-Pd alloys. According to Vegard's law [48], the molar compositions of Au and Pd in the alloy nanoparticles can be obtained from the lattice parameter shifts calculated from the angular positions of the (1 1 1), (2 0 0), and (2 2 0) diffractions. All the bimetallic nanoparticles exhibited XRD peaks with fcc crystalline structures.

2.5. High-Resolution Transmission Electron Microscopy (HRTEM)

The HRTEM image, as presented in Figure 4A, illustrates that the bimetallic nanoparticles were predominantly widely scattered, featuring both isolated particles and nanoparticles with a mean core size of 34 nm.

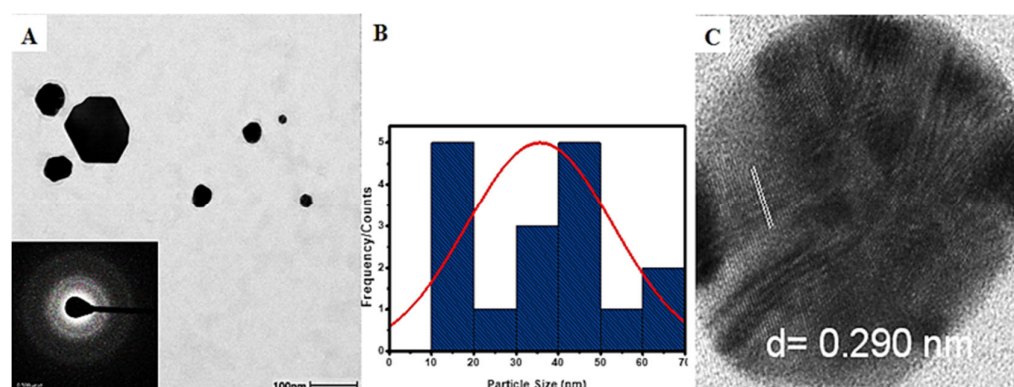


Figure 4. (A) HRTEM micrograph coupled with the Selected Area Electron diffraction (SAED) pattern insertion of GR plant extract synthesized Au-Pd alloyed bimetallic nanoparticles; (B) particle size distribution corresponding to micrograph A and (C) the d-lattice fringes of the nanoparticles.

Figure 4A presents the core size of the Au-Pd bimetallic nanoparticles, varied from 10 to 63 nm, with a particle size distribution of 33.78 ± 16.90 nm (Figure 4B). This suggests that the nanoparticles have a broad size range, with some particles being relatively small (10 nm) and others being relatively large (63 nm). HRTEM analysis revealed that GR plant extract improved the stability and dispersal of bimetallic nanoparticles, preventing agglomeration. Agglomeration of nanoparticles can have a negative impact on their performance and hinder their application. The stabilizing effect of GR extract suggests that this plant extract may function as a potential stabilizer for bimetallic nanoparticles, thus helping them to maintain their dispersed state and improving their functionality.

The SAED patterns are depicted in insertions of Figure 4A for the synthesized nanoparticles. The Au-Pd bimetallic nanoparticle SAED pattern in Figure 4A insertion revealed diffraction rings corresponding to crystal planes in the fcc structure [49]. The phase segregated metals may therefore be described as polycrystalline bimetallic nanoparticles [50].

Figure 4C shows a high-resolution image of the particles. Lattice fringes are visible, providing additional confirmation of the crystalline nature of the nanoparticles. The 0.290 nm d-spacing of the Au-Pd bimetallic nanoparticles reflects the distance between the crystallographic planes. Bimetallic nanoparticles have the properties of both metals, and their d-spacing can differ from that of single metals owing to alloying. This d-spacing corresponds to a crystallographic plane that displays the arrangement of the Au and Pd atoms [51]. These results corroborate the XRD patterns of the respective Au-Pd bimetallic nanoparticles.

Figure 5A shows that Au-Pd nanoparticles were well-dispersed owing to the stabilizing effect of the pure compound. The size of the Au-Pd bimetallic nanoparticles ranged from 8 to 47 nm, indicating that they had varying sizes within this range and a resulting particle size distribution of 21.31 ± 8.67 nm (Figure 5B). Nanoparticles of different sizes have distinct properties that can be beneficial in various environments. The irregular shapes of the alloyed nanoparticles may have been caused by the major compound used as the reducing agent and the uncritical synthesis conditions. HRTEM micrographs of Au-Pd bimetallic nanoparticles show dark Au-rich cores and lighter Pd-rich shells [47]. The Au cores and Pd shells of the Au-Pd bimetallic nanoparticles exhibited a core-shell structure, as reported by Han et al. [52]. Stabilization is necessary for the dispersibility and even distribution of nanoparticles. Aspalathin was likely adsorbed onto the surface of the nanoparticles, forming a protective layer that prevented agglomeration.

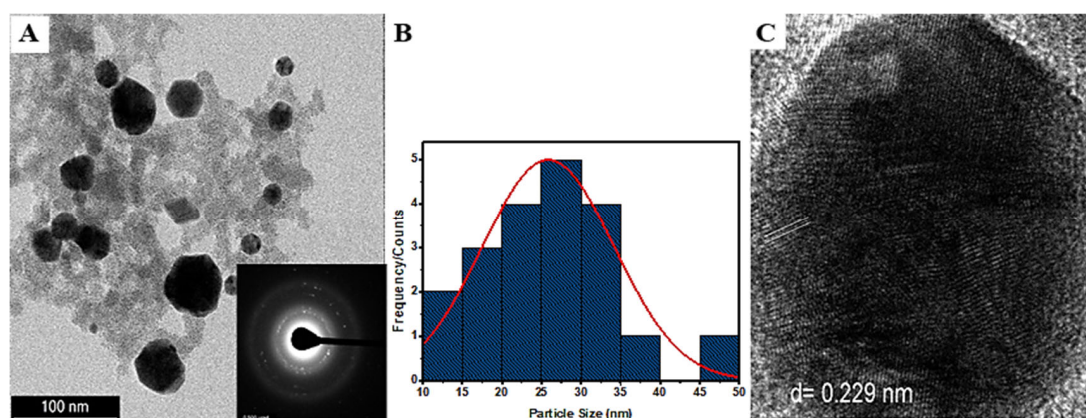


Figure 5. (A) HRTEM micrograph coupled with the SAED patterns of ASP-synthesized Au-Pd bimetallic nanoparticles, (B) histogram depicting the particle distribution of nanoparticles corresponding to micrograph (A,C), and the corresponding d-lattice spacing.

The HRTEM micrographs are coupled with the SAED pattern of the synthesized AuNPs (as shown in Figure 6A insertion), revealing the appearance of rings and bright spots in a circular pattern. The HRTEM micrograph in Figure 5A displays the SAED pattern

of the synthesized Au-Pd bimetallic nanoparticles. The bright spots that appear in a circular pattern correspond to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) reflections of the fcc Au-Pd bimetallic planes, which clearly indicates that the nanoparticles are poly-crystalline. The d-spacing of 0.229 nm (Figure 5C) for bimetallic nanoparticles indicates separation between their crystallographic planes. This specific d-spacing is related to the crystallographic plane that signifies the arrangement of both Au and Pd atoms [51].

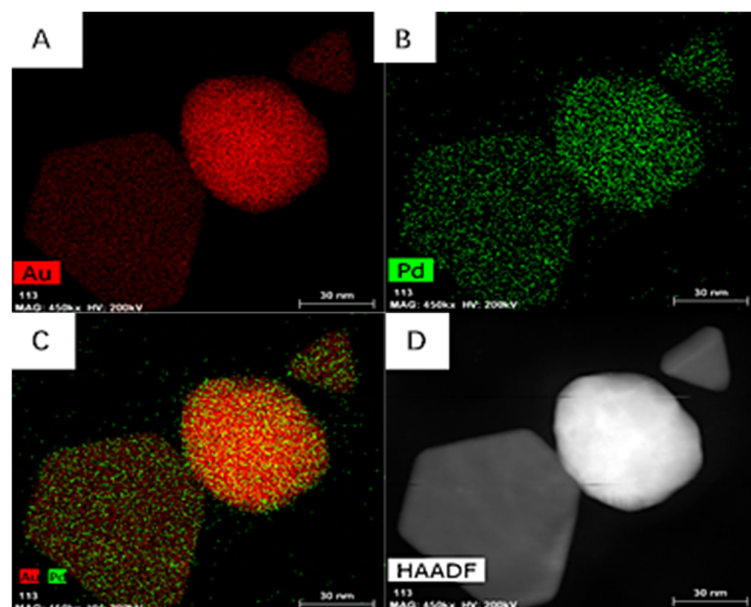


Figure 6. (A,B) Mapping images of individual Au (red) and Pd (green) atoms; (C) the overlaid image of Au and Pd atoms, and (D) STEM-HAADF Au-Pd alloyed bimetallic nanoparticles.

2.6. Scanning-Transmission Electron Microscopy-High Angle Annular Dark Field (STEM-HAADF)

In Figure 6, the STEM-EDX mapping method was employed to scrutinize the composition of the Au-Pd bimetallic nanoparticles [53]. The analysis aimed to examine the distribution of Au and Pd atoms. The coloured mapping images obtained exhibited an even dispersion of the Au (red) and Pd (green) atoms within the nanoparticle structure. The findings indicate an even distribution of Au and Pd atoms within the nanoparticles, implying a well-formed alloy structure rather than phase separation. Figure 6D shows a HAADF image of well-mixed Au-Pd alloy nanoparticles with uniformly dispersed bright spots that correspond to Au and Pd atoms, supporting the formation of an alloy with even dispersion of both elements [51].

The red and green images are associated with Au and Pd, respectively. Mapping images were acquired using the L-line spectra of the elements. An overlapping image is shown in Figure 7C. It can be observed that the elements have an inhomogeneous distribution in the nanoparticles, with zones with a high content of Pd (i.e., surface) and similar zones with a high content of Au (i.e., core) [52,54]. Furthermore, the overlaid image displays an Au centre coated or surrounded by Pd on the surface, as revealed by the STEM-HAADFX analyses. Moreover, Figure 7D displays the HAADF image, where bright areas correspond to regions with higher average atomic numbers, suggesting the presence of heavy or electron-dense elements (in this case, Au), while the dull areas reflect elements of lower atomic number (Pd) [52]. This result is consistent with the HRTEM (Figure 5A).

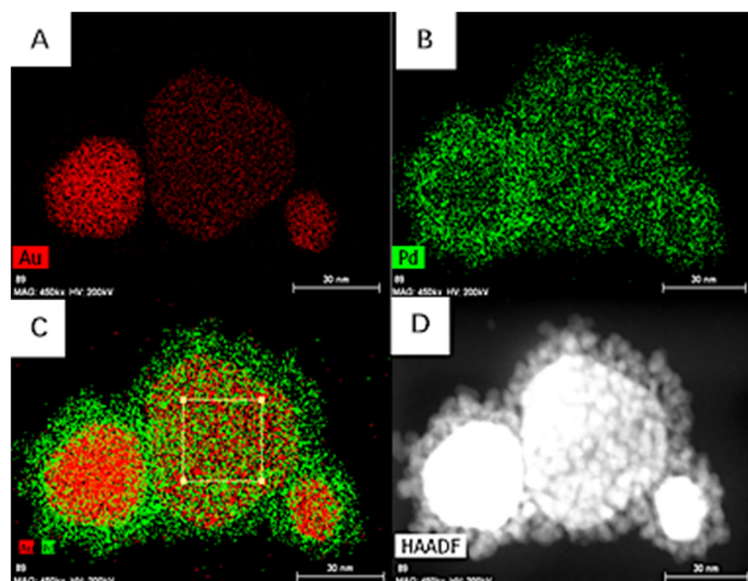


Figure 7. (A,B) Mapping images of individual Au (red) and Pd (green) atoms; (C) the overlaid image of Au and Pd atoms, and (D) STEM-HAADF Au-Pd core-shell bimetallic nanoparticles.

2.7. Attenuated Total Reflection-Fourier-Transform Infrared Spectroscopy (ATR-FTIR)

Ovais et al. [55] found that polyphenols are key reducing and stabilizing agents in green synthesis of metallic nanoparticles [55]. FTIR was used to identify the functional groups of the phytochemicals that reduce metal ions to a zero-valent form. FTIR analysis of GR extract (Figure 8) revealed peaks at 3360, 2929, 1638, 1385, 1251, 1061 cm^{-1} . The FTIR spectrum of the extract showed C=C vibration stretching at 1638 cm^{-1} and the O-H group of phenolic molecules at 3360 cm^{-1} [20,51]. The bands at 1385 and 1061 cm^{-1} indicated -C-O and -C-O-C stretching, whereas the 2929 cm^{-1} bands showed C-H asymmetric and symmetric stretching. These peaks suggest the presence of phenolic compounds in the extract. The FTIR vibrational bands of the GR plant extract were compared to those of its synthesized Au and PdNPs.

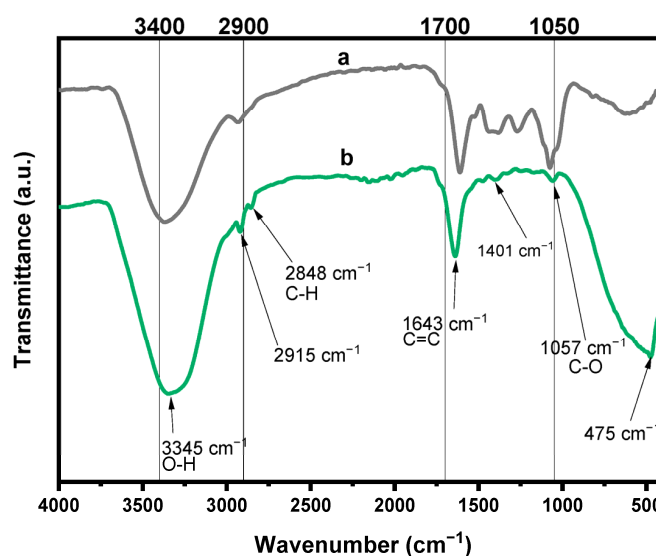


Figure 8. The FTIR spectra of the (a) GR extract, and (b) Au-Pd alloyed bimetallic nanoparticles.

The FTIR spectrum of Au-Pd alloyed bimetallic nanoparticles showed vibrational peaks at 3345, 2915, 2848, 1643, 1401, and 1057 cm^{-1} . The spectrum displayed a peak at 3345 cm^{-1} representing O-H stretching vibrations, while another at 2915 cm^{-1} indicated

the C-H stretching vibrations from the -CH₃ groups. The vibration of C=C in benzene was observed at 1643 cm⁻¹ [52,56]. Moreover, a small band at 1057 cm⁻¹ is associated with the C-H stretching vibrations of the aromatic rings [57].

The FTIR spectrum of ASP (Figure 9) showed peaks at 3378, 2974, 2918, 1746, 1624, 1256, 1077, and 820 cm⁻¹, representing free O-H, O-H hydrogen bonds, C-O carbonyl, C=C aromatic ring, and C-OH stretching vibrations. The peak at 3378 cm⁻¹ confirmed the O-H stretching of alcohols and the C=O stretching of carboxylic acid. The strong, intense 1624 cm⁻¹ band indicates C=C stretching of the alkene compound [58].

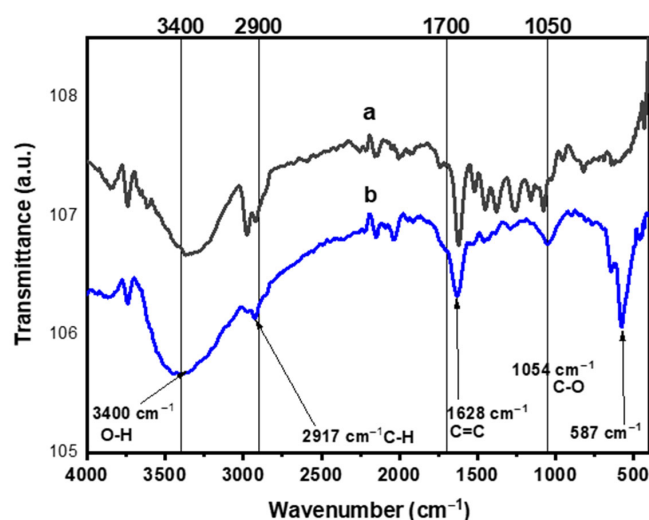


Figure 9. FTIR spectra of (a) ASP and (b) Au-Pd core-shell bimetallic nanoparticles.

The Au-Pd bimetallic nanoparticle spectrum showed vibrational frequencies of 3404 cm⁻¹ (O-H stretching of alcohols) and 1736 cm⁻¹ (C=O stretching of carboxylic acids). The alcohol bands were slightly stronger and weaker than those of ASP, Au, and PdNPs. Other frequencies included C-H stretching at 2853 cm⁻¹ and 2962 cm⁻¹, and O stretching at 1052 cm⁻¹. The peak at 1052 cm⁻¹ is due to the C-O-C and -C-OH stretching vibrations of secondary alcohols. The slight shifts may have been caused by Au³⁺ and Pd²⁺ interacting with the functional groups of ASP, confirming the nanoparticle coating. A comparison of the spectra of ASP and bimetallic nanoparticles showed that most of the absorption bands of ASP in the Au-Pd bimetallic nanoparticles had a slight peak shift. The O-H stretching mode shifted from 3312 cm⁻¹ in the extract to 3396 cm⁻¹ in the bimetallic nanoparticles, indicating that the ASP compound in the extract reduced the metal ions [52].

2.8. In Vitro Stability Study

The efficacy of nanoparticles in medical applications relies on their stability in biological solutions over a reasonable duration. As depicted in Figures 10 and 11, the assessment of stability was conducted at 0, 6, 12, 18, and 24 h of incubation in various biological media and buffers.

These included Dulbecco's Modified Eagle Medium (DMEM), glycine (GLY), sodium chloride (NaCl), cysteine (CYS), and phosphate buffers at pH 7 and 9, mixed with GR extract and ASP. Although the pH 9 solution exceeds the pH of human body fluids, investigating whether nanoparticles remain stable at such a high pH was deemed essential. These experiments were conducted both before and after incubation at 37 °C within the 0 to 24 h timeframe. The selected 24 h duration aims to accurately reflect the time required for complete medication bio-distribution within physiological systems. The expectation was that nanoparticles with stability would retain nearly identical wavelengths as the control samples over a defined time period before exhibiting any signs of instability.

2.9. Cytotoxicity Assays of Bimetallic Nanoparticles

Investigating the cytotoxicity of nanomaterials is crucial to predict their effects on cells. Nevertheless, there is a scarcity of research focusing on the synthesis of nanoparticles utilizing GR extract or its constituent ASP, along with the assessment of their cytotoxic effects. Various approaches have been employed for cytotoxicity analysis, with tetrazolium-based assays, such as the MTT assay, being the most widely used [59]. These assays measure the enzymatic activities associated with cellular metabolism in living cells, rather than counting the exact number of viable cells present [42,60]. Following 24 h of exposure to the tested samples, GR-Au-Pd, and ASP-Au-Pd bimetallic nanoparticles towards SKMEL-1, HaCAT, KMST-6 and HepG2 cells, the MTT assay was performed as shown in Figures 12 and 13.

Most of the tested samples yielded no significant ($p > 0.05$) reductions in cell viability at the tested concentrations as compared to the control. In this study, the tested samples showed no significant cytotoxic effect toward SKMEL-1 cells (as shown in Figure 12) at the tested concentrations (12.5, 25, 50, 100, and 200 $\mu\text{g/mL}$). Figures 12 and 13 display the assessment of cytotoxicity in HaCaT and KMST-6 cells, which had no significant cytotoxic effects when evaluated with all the samples.

2.10. Cellular Uptake of Green Synthesized Nanoparticles

The cellular uptake of metal nanoparticles used as carriers for therapeutic drugs is a crucial aspect being explored in the domain of nanomedicine [61]. These nanoparticles, often functionalized with drugs or therapeutic agents, are designed to be taken up by cells and deliver their cargo to specific targets within the body [62,63]. The uptake of Au-Pd bimetallic nanoparticles was confirmed through the ICP-MS analysis on each cell treated with the standard solutions of each metal to determine the concentration of each metal. Figure 14 illustrates the concentrations of Au-Pd bimetallic nanoparticles taken up by the respective cell lines.

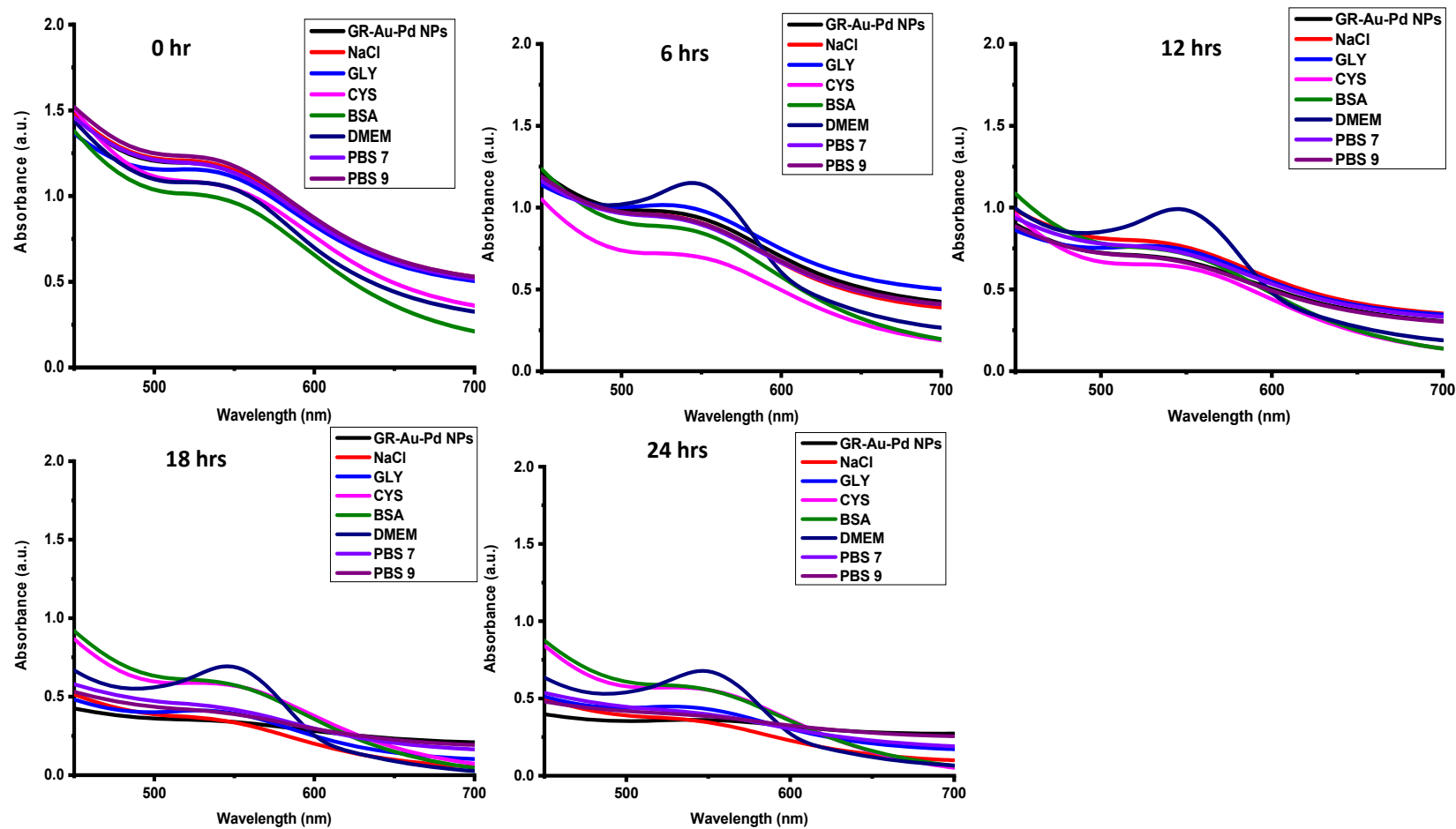


Figure 10. In vitro assessment of the stability of GR-Au-PdNPs at specified time intervals within biogenic media.

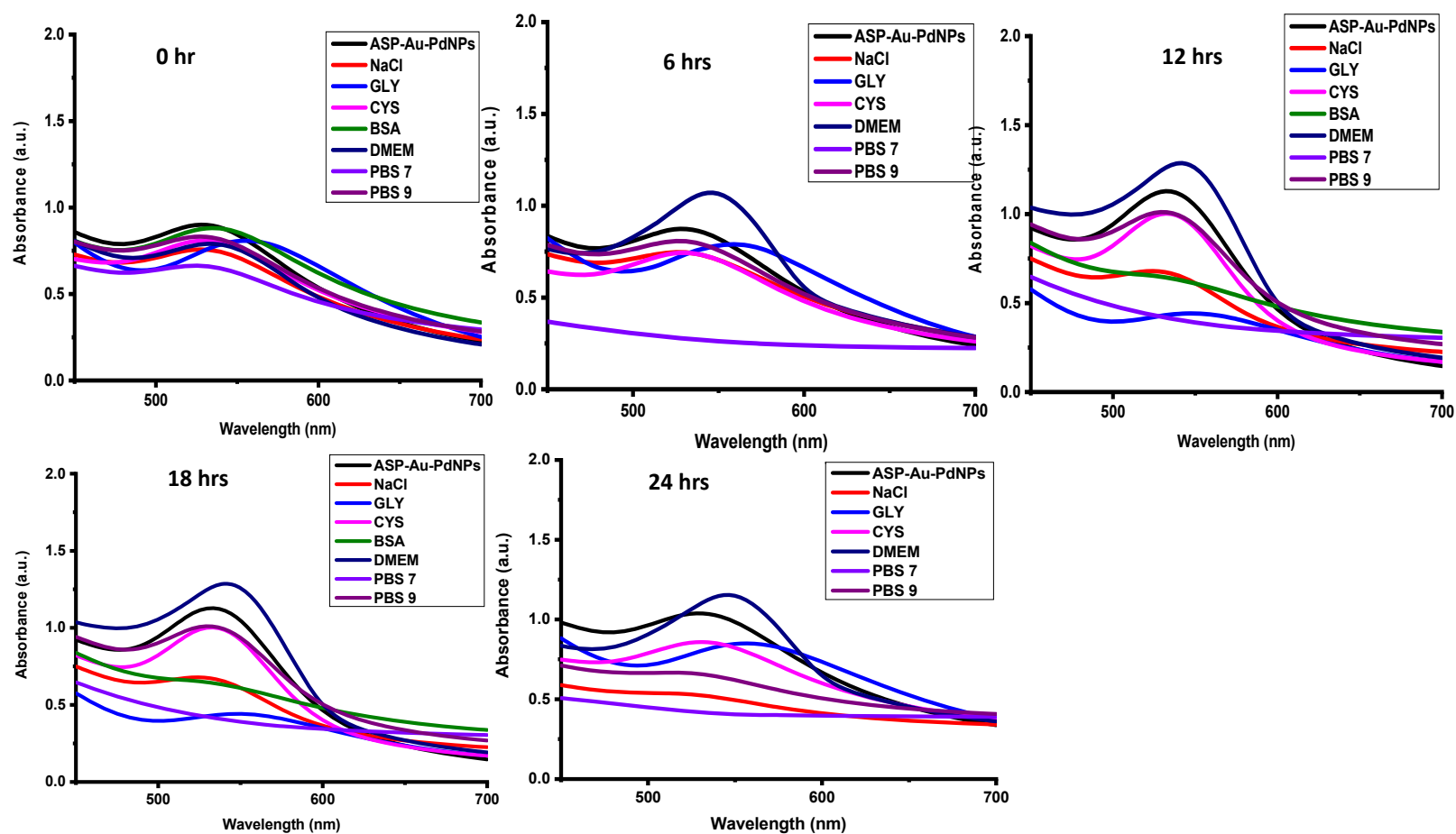


Figure 11. Assessing the in vitro stability of ASP-Au-Pd nanoparticles over given time intervals in biogenic media.

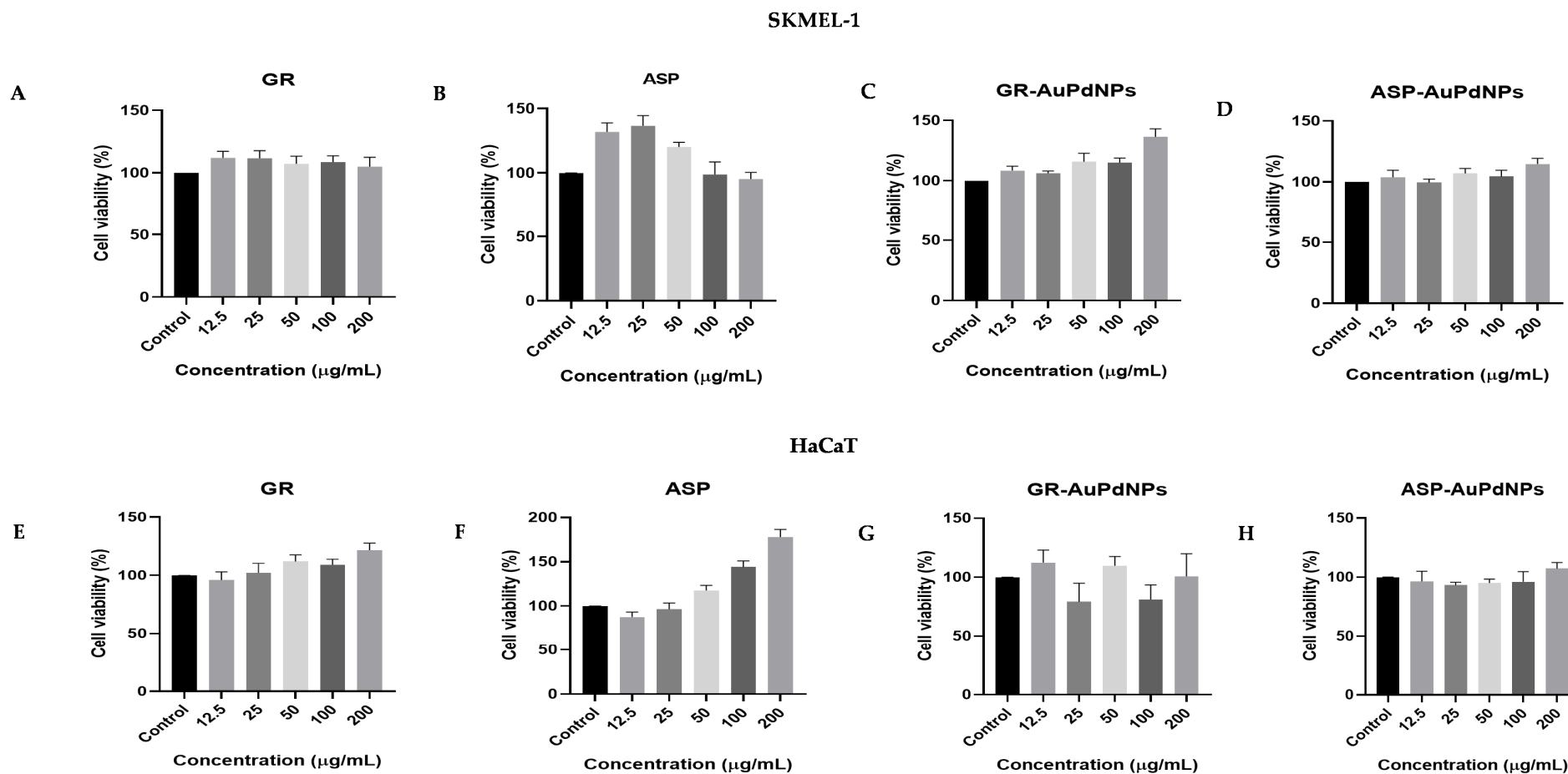


Figure 12. Cell viability of SKMEL-1 and HaCAT cells was assessed using the MTT assay during a 24 h exposure to different samples, including GR (A), ASP (B), GR-Au-PdNPs (C), ASP-AuPdNPs (D), GR (E), ASP (F), GR-Au-PdNPs (G), and ASP-AuPdNPs (H).

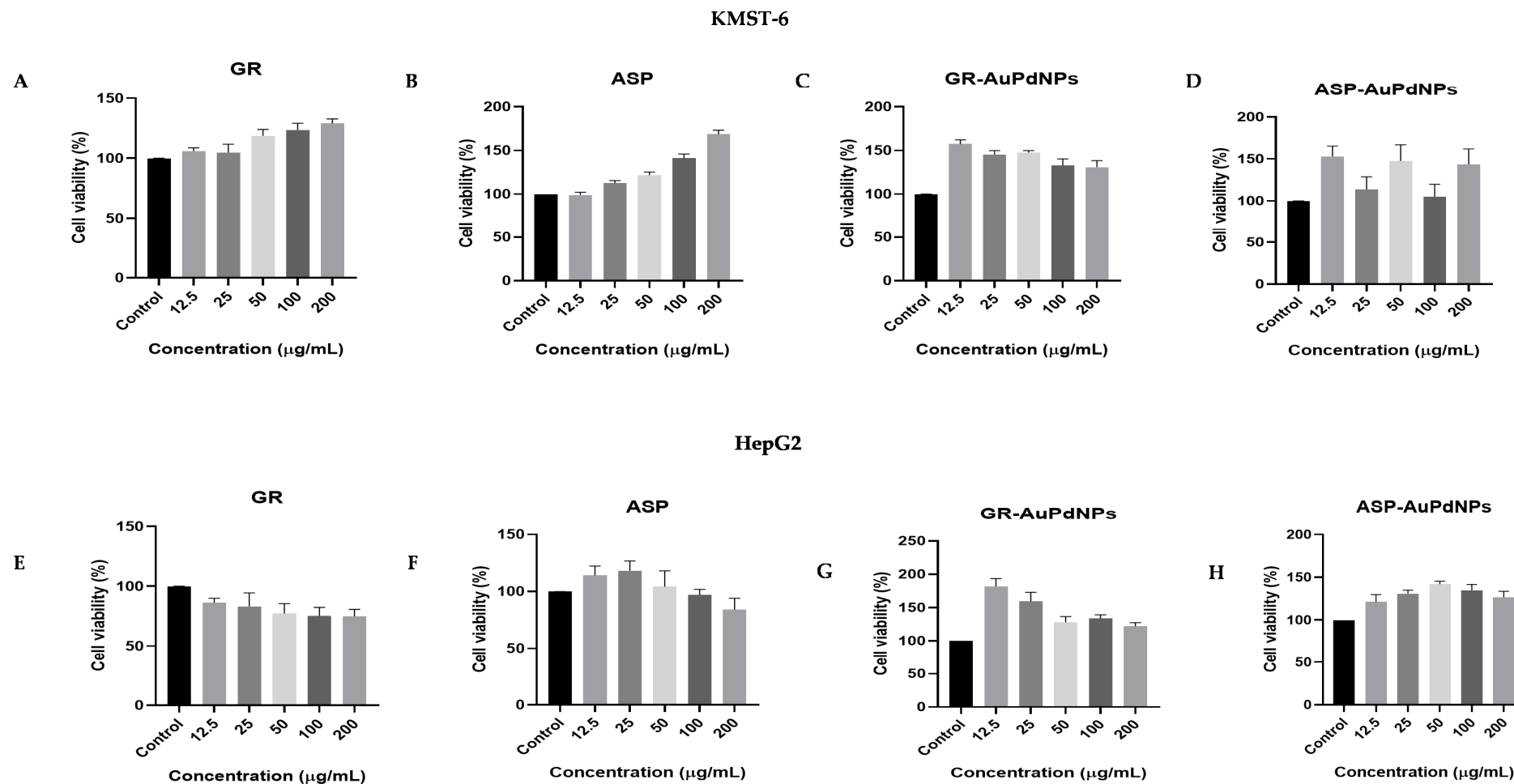


Figure 13. Cell viability of KMST-6 and HepG2 cells was assessed using the MTT assay during a 24 h exposure to different samples, including GR (A), ASP (B), GR-Au-PdNPs (C), ASP-AuPdNPs (D), GR (E), ASP (F), GR-Au-PdNPs (G), and ASP-AuPdNPs (H).

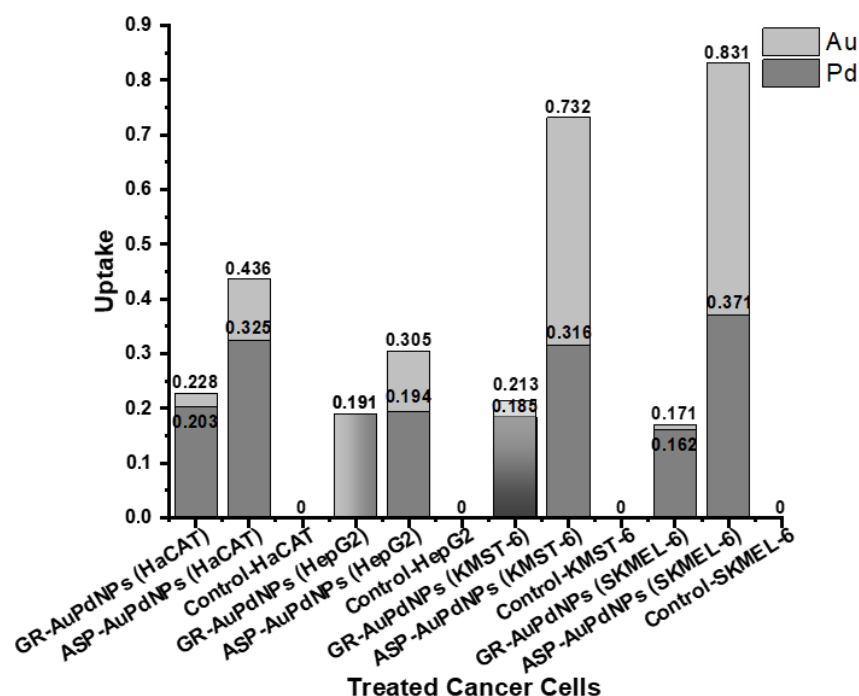


Figure 14. Cellular uptake results determined from ICP-OES measurements of Au-Pd bimetallic nanoparticles. The y-axis represents normalized fractional values (unitless), not percentages.

3. Discussion

The article introduces the green synthesis of Au-Pd bimetallic nanoparticles utilizing the total extract of GR, enriched with its bioactive compound ASP. For the synthesis of Au-Pd bimetallic alloyed nanoparticles, a visible absorption band with a maximum at 525 nm and 540 nm was observed in the UV-Vis spectrum when GR and ASP were used, respectively, which were shifted compared to the absorption spectra obtained previously for the AuNPs without the Pd [64]. Similar to previous studies, the spectrum of the Au₅Pd_{2.5} sample showed a comparable absorption band when prepared with an Au/Pd ratio of 2:1 [18]. Based on the primary analysis of the UV-Vis data, the GR-Au-Pd bimetallic nanoparticles were formed in an alloyed configuration. In contrast, there was minimal alloying between Au and Pd in the ASP-Au-Pd bimetallic nanoparticles because the SPR peak position of the nanoparticles was close to the weighted average of the SPR peak positions of the pure Au and PdNPs. This suggests that the two metals remained separate within the nanoparticles, forming a core-shell structure [47].

Through DLS, information pertaining to the physicochemical properties of metal nanoparticles is obtained, particularly their hydrodynamic diameter and size distribution. The results obtained are then compared with those obtained using HRTEM [65]. While there are notable differences between the visual results obtained from DLS and HRTEM, the latter provides the actual size and configuration of the electron-dense metal nanoparticles. Conversely, the former indicates a larger size, accounting for the hydrodynamics of the particles within the liquid medium. DLS measures the hydrodynamic radius of a nanoparticle, encompassing the coating on its surface, whereas HRTEM measures the nanoparticle core size [16,66]. Hence, the hydrodynamic diameter of the GR-Au-Pd bimetallic nanoparticles was larger than the core size determined by HRTEM.

HRTEM analysis showed that GR and ASP-Au-Pd bimetallic alloyed and core-shell nanoparticles were polydispersed in morphology consisting of predominantly spherical and diamond shapes within the range of 3 to 47 nm, as well as 8 to 47 nm. The hydrodynamic diameters obtained were 80 nm and 42 nm for GR and ASP-Au-Pd bimetallic nanoparticles,

respectively. The use of a pure compound provided particles that occurred at a smaller size, suggesting that using a pure material or compound as a reducing agent was essential for the formation of Pd and Au-Pd bimetallic nanoparticles. This means that the purity of the material used as a reducing agent was crucial for the successful synthesis of Au-Pd bimetallic nanoparticles. The obtained Polydispersity Index (PDI) values of 0.362 and 0.381 for GR and ASP Au-PdNPs, respectively, suggest a nearly uniform and monodisperse size distribution, as corroborated by HRTEM results [67].

Zeta Potential values were measured at -18.2 mV for GR and -22 mV for ASP-Au-Pd bimetallic alloyed nanoparticles. Consequently, the Au-Pd bimetallic alloyed nanoparticles demonstrated a stable, non-agglomerated nanomaterial within the acceptable range [21,43,63]. The most stable ASP-Au-Pd bimetallic nanoparticles are essential for maintaining their physicochemical properties and functionality during storage and administration. Crystallinity of the bimetallic nanoparticles was validated through XRD and SAED analyses, supporting the feasibility of green synthesis of bimetallic nanoparticles from bioactive compounds [43,67]. STEM-EDX mapping was employed to scrutinize the composition of the Au-Pd bimetallic nanoparticles, revealing an even dispersion of Au (red) and Pd (green) atoms throughout the nanoparticle structure in the obtained coloured maps. These results supported the formation of an alloy with even dispersion of both elements. This is due to the complex interactions among multiple compounds in the GR extract that favoured the formation of alloyed bimetallic nanoparticles rather than distinct core-shell structures. In contrast, Au-Pd bimetallic nanoparticles synthesized using ASP exhibit a core-shell configuration due to the specific interactions and functionalities of the isolated compound, highlighting a plausible outcome yet to be attained in green nanotechnology research. At present, we cannot propose a definitive mechanistic pathway (Figure 15) for the formation of the core-shell structure when using ASP, nor for the alloy formation observed with the total extract [21]. However, a reasonable interpretation can be based on the difference in standard reduction potentials between Au^{3+}/Au ($E^\circ \approx +1.50$ V; $\text{AuCl}_4^-/\text{Au} \approx +1.00$ V) and Pd^{2+}/Pd ($E^\circ \approx +0.92$ V; $\text{PdCl}_4^{2-}/\text{Pd} \approx +0.62$ V). The higher redox potential of Au^{3+} favours its preferential and faster reduction, leading to initial Au nucleation, followed by Pd deposition onto the preformed Au surface. Such sequential reduction is consistent with the formation of a core-shell architecture.

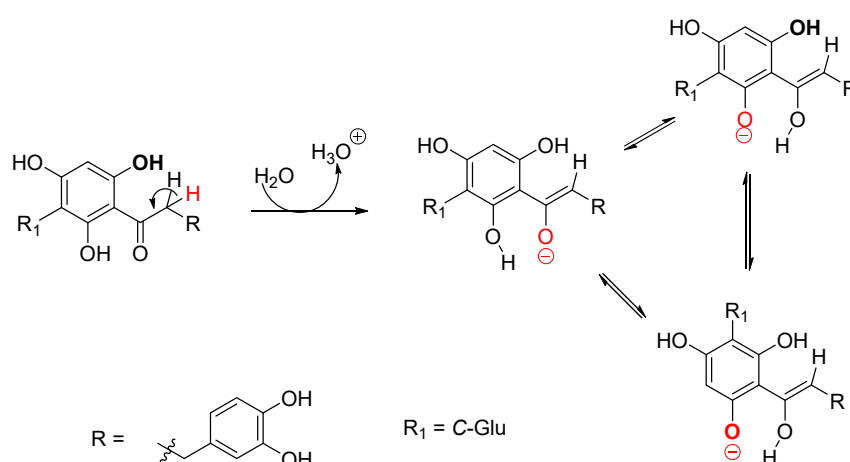


Figure 15. The proposed reduction mechanism of aspalathin.

In contrast, the total extract represents a complex multicomponent phytochemical system containing diverse reducing and coordinating agents. These components may exhibit different affinities and reduction tendencies toward Au^{3+} and Pd^{2+} , thereby modifying their effective reduction kinetics. In addition, differential complex formation between the metal

ions and multiple phytochemicals, especially phenolics, may alter their redox behaviour and nucleation rates. As a result, the reduction processes may occur more simultaneously, promoting co-nucleation and atomic interdiffusion, ultimately leading to alloy formation rather than a distinct core-shell structure. These characteristics are similar to previous reports [51,63]. Understanding these differences is crucial for tailoring nanoparticle synthesis approaches to achieve desired characteristics and functionalities.

The FTIR GR and ASP spectra were studied against their bimetallic nanoparticle spectra. The FTIR profile suggested that the formation of Au-Pd bimetallic alloy nanoparticles corresponded to phenolic compounds. It may be assumed that the polyphenols in GR extract may function as a reducing agent as well as a capping agent. Compounds bearing hydroxyl (-OH) groups were abundant in the extract; notably, chalcones may contribute to the reduction of the metal salts to their corresponding metallic nanoparticles.

Figures 10 and 11 show a decline in the absorption band intensity of ASP-Au-Pd and GR-Au-Pd bimetallic nanoparticles, which suggests it may be linked to elevated ionic levels present in NaCl, PBS 7, and PBS 9 solutions. A high concentration of ions can potentially screen electrostatic repulsion between nanoparticles, rendering them more prone to aggregation. Additionally, higher pH values, particularly in PBS 7 and 9, might impact the stability and surface charge of the nanoparticles. Glycine as an amino acid is known to interact with nanoparticles, capping phenolic molecules and may contribute to aggregation in this specific system, as indicated by the observed reduction in intensity. It is plausible that the interaction between glycine and nanoparticles in this scenario does not provide adequate steric or electrostatic stabilization [64,68]. Additionally, the presence of amine groups may have an impact on how the particles aggregate. Proteins have strong binding sites (-COOH and -NH₂), especially phenolic compounds that might make this kind of coordination with Au-Pd bimetallic nanoparticles possible [41,66].

Contrary to the Zeta Potential results, the findings revealed GR-Au-PdNPs to be the most stable. This is due to the numerous phytochemicals in the GR extract that contribute to the formation of nanoparticles and function as capping agents. This means that how the phytochemicals interacted positively impacted how they interacted with the biogenic media. Additionally, the tetrazolium-based MTT assay demonstrated that GR, ASP, and Au-Pd bimetallic nanoparticles did not significantly diminish cell viability in SKMEL-1, HaCAT, KMST-6, and HepG2 cells at varying concentrations, indicating a lack of notable cytotoxic effects and suggesting proliferation-like or metabolic stimulation effects across diverse cell lines. The observed cell viability values exceeding 100% in some cases reflect increased cellular metabolic activity relative to the untreated control, which is a known feature of tetrazolium-based assays and does not represent a true increase in cell number. Moreover, none of the investigated samples induced 50% cell death at any of the tested concentrations. This observation aligns with the findings of Akinfenwa et al. [21], who reported similar results for the cytotoxicity studies of GR-AuNPs and ASP-AuNPs against SH-SY5Y and HepG2 cell lines [21]. Moreover, the scarcity of literature concerning the biomedical assessment of cancer cells using green-synthesized Au-Pd bimetallic nanoparticles warrants attention. It is noteworthy that researchers in China have recently introduced a novel platinum (IV) conjugate termed ketoplatin, which combines ketoprofen and cisplatin. This innovative prodrug exhibits substantially reduced systemic toxicity compared to cisplatin and demonstrates a remarkable increase in anticancer efficacy, approximately 50-fold greater [69].

Furthermore, it is noteworthy that none of the biosynthesized Au-Pd bimetallic nanoparticle samples exhibited significant cytotoxicity when compared to their corresponding extract or compound. This consistent lack of cytotoxicity suggests the biocompatibility and potential safety of these green-synthesized bimetallic nanoparticles for biomedical

applications. The coexistence of Au and Pd in bimetallic nanoparticles exhibited a synergistic effect, enhancing cellular uptake. Thus, it is worth mentioning that the Au-Pd bimetallic nanoparticles evaluated with HepG2 cells featured a well-balanced composition, comprising equal proportions of Au and Pd, precisely set at a concentration of 0.191 for both elements.

The size of nanoparticles significantly affects their behaviour in biological systems. Smaller nanoparticles (<100 nm) tend to exhibit enhanced cellular uptake and tissue penetration, making them ideal for targeted drug delivery and imaging of cancer cells. Thus, the uptake of ASP-Au-PdNPs by KMST-2 (0.316 µg/mL-Pd and 0.732 µg/mL-Au) and SKMEL-1 (0.371 µg/mL-Pd and 0.831 µg/mL-Au) cells demonstrated significantly higher levels compared to the uptake of the metallic nanoparticles. This suggests a potential synergistic effect or enhanced cellular uptake efficiency of the bimetallic nanoparticles compared to their monometallic counterparts. These findings also imply that an increased dosage of nanoparticles on cell lines may obstruct the interface between the cells and the reactive sites, leading to reduced permeation into the cells [68]. Furthermore, cancer cells absorbed higher concentrations of Au-Pd bimetallic nanoparticles, attributed to their optical properties, inert nature, biocompatibility, bio-conjugation potential, and low toxicity.

4. Materials and Methods

Chemicals, such as sodium tetrachloroaurate (III) dihydrate $\text{NaCl}_4 \cdot 2\text{H}_2\text{O}$, palladium (II) chloride (PdCl_2), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), and glycine (GLY), were procured from Sigma-Aldrich (St. Louis, MO, USA). L-cysteine (CYS) and Folin–Ciocalteu’s (FC) phenol reagent originated from Boehringer Mannheim GmbH in Mannheim, Baden–Wurttemberg, Germany. Dulbecco’s Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), and Trypsin/Ethyl Diamine Tetra Acetic Acid (EDTA) were obtained from Gibco Invitrogen in Karlsruhe, Germany. Phosphate-Buffered Saline (PBS) was purchased from Lonza (Cape Town, South Africa). Bovine Serum Albumin (BSA) was sourced from Miles Laboratories in Pittsburgh, PA, USA. Additionally, Dimethylsulphoxide (DMSO), Penicillin, Streptomycin, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromine (MTT), and Trypan Blue (TB) were acquired from Sigma-Aldrich in Steinheim, Germany.

Cell lines, namely SKMEL-1 (human melanoma cell line), HaCAT (human epidermal keratinocyte cell line), KMST-6 (human fibroblast cell line), and HepG2 (liver hepatocellular carcinoma cell line), were procured from the American Type Cell Culture (ATCC) in Manassas (MAX), VA, USA.

4.1. Synthesis of Nanoparticles

Synthesis of Au-Pd Bimetallic Nanoparticles

The bimetallic nanoparticles (Au-Pd nanoparticles) were formed (as shown in Figure 16) by maintaining a molar ratio of 2:1 by first forming Au nanoparticles. An aliquot of the prepared AuNPs was transferred into a beaker, and a solution of 10 mM PdCl_2 was added at a volume ratio of 1:1 under constant and vigorous stirring at 70 °C for 2 h. A brown solution observed indicated that the Au-Pd bimetallic nanoparticles were successfully synthesized. The colloidal solutions were allowed to cool to room temperature before going through several centrifugal washing steps. This is done to remove unreacted substances that may still be present in the solution.

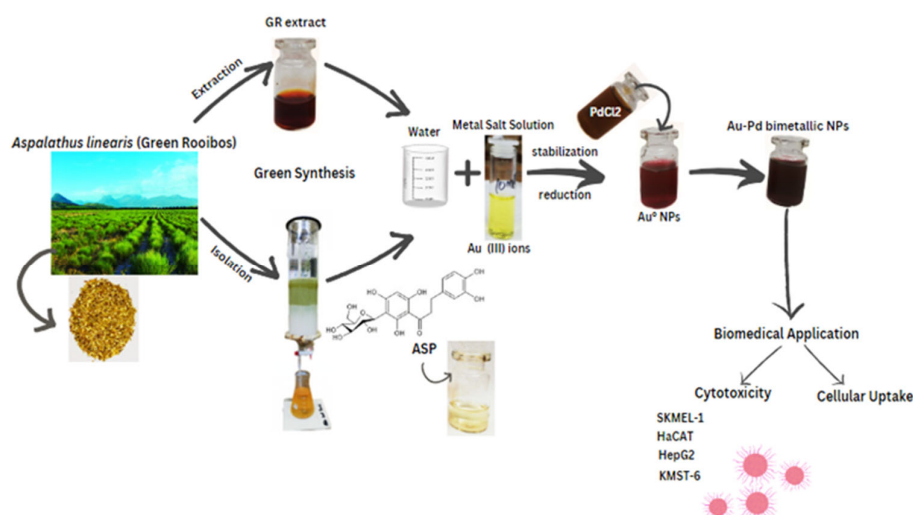


Figure 16. Schematic diagram summarizing the steps involved in the green synthesis of Au-Pd bimetallic nanoparticles.

4.2. Characterization: Type of Instrumentation

4.2.1. X-Ray Diffraction (XRD)

The XRD technique was used for the determination of the crystal structures of crystalline materials. The XRD patterns for the lyophilised samples of the powdered Au-Pd bimetallic nanoparticles supported on the plant extract were acquired on a Bruker AXS GmbH (Karlsruhe, Germany) D8 Advance diffractometer (voltage 40 KV; current 40 mA). The XRD spectra were recorded in the range 30–90° (2 θ) using a CuK α (λ = 0.154 nm) monochromatic radiation X-ray source and in a step-scan of $\Delta 2\theta$ = 0.05°.

4.2.2. Ultraviolet-Visible (UV-Vis) Spectroscopy

The formation of Au-Pd bimetallic nanoparticles was analyzed by BMG LABTECH-SPECTROstar-Nano ultraviolet-visible (UV-Vis) spectroscopy (Ortenberg, Germany) to confirm the peak surface plasmon resonance of the Au-Pd NPs at ~530 nm. Spectra were recorded at room temperature as a function of wavelength at a resolution of 1 nm between wavelengths 220 nm and 650 nm using a double-beam spectrophotometer. Synthesized Au-Pd bimetallic nanoparticles samples were diluted at a 1:1 ratio of base solvent and the nanoparticles.

4.2.3. Dynamic Light Scattering (DLS)

The Malvern Zeta-Sizer Nano-Series Nano-zs90 (Worcestershire, UK), equipped with both Zetasizer and Zeta Potential functionalities, was utilized to analyze Au-Pd bimetallic nanoparticles. This instrument facilitates the measurement of approximate average sizes for colloids, nanoparticles, and molecules without requiring sample agitation. The analysis involved studying a 1 mL (1000 μ L) diluted sample in quartz glass cuvettes (similar to those used for UV-Vis analysis). Key parameters, such as temperature (set at 25 °C), the base solvent (water), and cuvette settings (disposable cell for Zeta Sizer and disposable folded capillary cell for Zeta Potential), were carefully configured, aligning with a greener methodological approach.

4.2.4. High-Resolution Transmission Electron Microscope (HRTEM) Imaging

The size, shape, crystallinity and elemental composition of the Au and Pd nanoparticles produced in the solution were determined using HRTEM coupled with EDX. In this research project, the TEM micrographs will be obtained using the FEI TECNAI F20 HRTEM

(Eindhoven, The Netherlands) operated at 200 kV and equipped with a detector for X-ray analysis. The Au-Pd bimetallic nanoparticle samples were prepared by drying a single drop of undiluted solution on a carbon-coated copper grid and drying under an electric bulb.

4.2.5. Fourier-Transform Infrared (FTIR) Spectroscopic Analysis

The ATR-FTIR analysis was used to identify the biomolecules responsible for synthesizing and capping Au-Pd bimetallic nanoparticles using *Aspalathus linearis* plant extract. The PerkinElmer, Inc., Spectrum (Version 10.4.2) ATR-FTIR spectrometer (Waltham, MA, USA) was used to analyze samples. A drop of distilled water was used as a blank, and the background was removed from the sample scan. A drop of the sample was placed on the instrument, and a scan measuring percentage over the range of wavenumbers from 400 to 4000 cm^{-1} was taken.

4.3. Biological Studies

4.3.1. Stability Study of Nanoparticles

The in vitro stability study involved combining 200 μL of solutions containing Au-Pd NPs with 100 μL of biogenic amino acids such as 0.5% glycine, Phosphate-Buffered Saline (PBS at pH 7 and pH 9), and a 0.5% NaCl solution. This procedure was conducted in a 96-well plate. Subsequently, the mixture underwent incubation at a temperature of 37 $^{\circ}\text{C}$ for various durations. UV-Vis spectroscopy was employed to evaluate the stability of AuNPs, PdNPs, and Au-Pd bimetallic nanoparticles in these media, focusing on observing the preservation of their absorbance maxima at 6 h intervals over a 24 h period.

4.3.2. Cell Culture

This research involved the use of SKMEL-1, HaCAT, KMST-6 and HepG2 cell lines. These cells were grown in a controlled environment at 37 $^{\circ}\text{C}$ with a mixture of 95% air and 5% CO_2 following sterile procedures. HaCAT, KMST 6 and HepG2 cells were cultured separately in DMEM growth medium. This medium was supplemented with 10% Fetal Bovine Serum (FBS) along with penicillin (100 $\mu\text{g}/\text{mL}$). Streptomycin (100 $\mu\text{g}/\text{mL}$) at a concentration of 1%. The SKMEL-1 cells, on the other hand, were cultured in complete RPMI growth medium supplemented with 10% FBS and penicillin/streptomycin at the same concentration as mentioned before.

4.3.3. Cytotoxicity Assay

The cytotoxicity was evaluated using MTT [70], which involved 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. Briefly, SKMEL-1, HaCAT, KMST-6 and HepG2 cell lines were individually placed in 96-well cell culture plates with a density of 5000 cells per well. They were left to attach. Subsequently, the medium was replaced with the fresh medium containing increasing concentrations (12.5, 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$) of the samples tested: GR and ASP-Au-Pd bimetallic nanoparticles. This was maintained for a duration of 24 h. As for the control samples used, cells were incubated in the medium. After the incubation period of 24 h, MTT solution (5 mg/mL) was added to each well (10 μL) and was incubated for an additional 4 h at a temperature of 37 $^{\circ}\text{C}$. Therefore, the resulting MTT crystals were solubilized in DMSO, and the absorbance was measured at a wavelength of 570 nm for each well. The calculation of percentage cell viability was determined using the following formula:

$$\% \text{Cell Viability} = (\text{Absorbance of treated well}) / (\text{Absorbance of untreated well}) \times 100$$

4.3.4. Statistical Analysis

Information from the present study was accurately documented and underwent statistical analysis using GraphPad Prism for Windows, version 8.4.3, developed by GraphPad Software in San Diego, SD, USA. To ensure reliability, the experiments were carried out in triplicate. After analyzing the summary statistics, which included conducting the Kolmogorov–Smirnov test for a normal distribution, the data underwent analysis using the independent *t*-test in instances where a normal distribution was observed. Statistical significance was established at $p < 0.05$.

4.3.5. Cellular Uptake of Biosynthesized Nanoparticles

In 6-well plates, 1×10^5 SKMEL-1, HaCAT, KMST-6, and HepG2 cells treated with biosynthesized GR and ASP-Au-Pd bimetallic nanoparticles were individually seeded and incubated for a 24-hour period. After the incubation period, the medium was aspirated, and PBS was used to rinse the cells. In 15 mL tubes, the cells were trypsinized, centrifuged at 3000 rpm, re-washed with PBS, and centrifuged again. After discarding the PBS, aqua regia (14 mL) was used to digest the nanoparticles at 80 °C briefly, resulting in the release of metal ions. The released metal ions from the cells were quantified using ICP-OES (Inductively Coupled Plasma-Optical Emission Spectroscopy) analysis. For ICP-OES analysis, Au and Pd standard solutions were each prepared at a concentration of 100 ppm, followed by subsequent serial dilutions yielding concentrations of 1 ppm, 2.5 ppm, 5 ppm, 10 ppm, 15 ppm, and 20 ppm for standard calibrations. The cellular uptake of nanoparticles was correlated with the Au and Pd ion concentrations in the cells.

5. Conclusions

This research illustrated the capability of the water extract from GR and its compound in the production of bioactive Au-Pd bimetallic nanoparticles. Both the GR plant extract and ASP proved effective in synthesizing Au-Pd bimetallic nanoparticles. Moreover, the MTT assay indicated that these nanoparticles, derived from the GR extract and ASP, did not significantly reduce cell viability in various cell lines. This suggests minimal cytotoxic effects and supports cell proliferation across diverse cell lines. The synergistic effect of coexisting Au and Pd in bimetallic nanoparticles enhanced cellular uptake, particularly in cancer cells, owing to their optical properties, inert nature, biocompatibility, bio-conjugation potential, and low toxicity. The outcomes also highlighted the ASP compound's role in reducing and stabilizing the synthesis of bimetallic nanoparticles, suggesting its potential application in various biomedical fields.

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