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ORIGINAL PAPERS

SMART NANOSTRUCTURED DRUG CARRIERS FUNCTIONALIZED WITH POLYPHENOLS AND DOXORUBICIN FOR ENHANCED GLIOBLASTOMA THERAPY

Cristina-Teodora DOBROTĂ[1,2,3] Gheorghe TOMOAIA[3,4], Olga SORITAU[5], Cristina-Lavinia ROŞOIU[6], Aurora MOCANU[7], Ossi HOROVITZ[8], Maria TOMOAIA-COTISEL[3,9 *]

[1] Research Center of Excellence in Physical Chemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11 Arany Janos St., 400028 Cluj-Napoca, Romania; (cristina.dobrota@ubbcluj.ro)
[2] Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeş-Bolyai University, 44 Republicii St., 400015 Cluj-Napoca, Romania,
[3] Academy of Romanian Scientists, 3 Ilfov St. 050044 Bucharest, Romania
[4] Department of Orthopedics and Traumatology, Iuliu Hatieganu University of Medicine and Pharmacy, 47 General Traian Moşoiu St., 400132 Cluj-Napoca, Romania; Academy of Romanian Scientists, 3 Ilfov St., 050044 Bucharest, Romania, (tomoaia2000@yahoo.com).
[5] The Oncology Institute "Prof. Dr. Ion Chiricuţă", 34-36 Republicii St., 400015 Cluj-Napoca, Romania, (olgasoritau@yahoo.com)
[6] Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeş-Bolyai University, 44 Republicii St., 400015 Cluj-Napoca, Romania, (cristina.rosoiu@stud.ubbcluj.ro)
[7] Research Center of Excellence in Physical Chemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11 Arany Janos St., 400028 Cluj-Napoca, Romania, (mocanu.aurora@gmail.com)
[8] Research Center of Excellence in Physical Chemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11 Arany Janos St., 400028 Cluj-Napoca, Romania, (ossihor@yahoo.com)
[9] Research Center of Excellence in Physical Chemistry, Faculty of Chemistry and Chemical Engineering, Babeş-Bolyai University, 11 Arany Janos St., 400028 Cluj-Napoca, Romania

[*]Corresponding author e-mail: Maria Tomoaia-Cotisel mcotisel@gmail.com

Abstract. This study investigates gold nanoparticles multi-functionalized with resveratrol, piperine, icariin, and doxorubicin for the treatment of glioblastoma multiforme (GBM) stem cells. MTT assays were used to evaluate cancer cell viability as a function of formulations (compositions) and exposure time. These results show that the combination of doxorubicin, which is essential for strong cytotoxic effects, with polyphenols, that are responsible primarily for enhancing the doxorubicin targeting to the cancer cells, leads to an improved anticancer effect through synergistic interactions. Smart gold nanoparticles functionalized with doxorubicin and polyphenols improved the delivery and the efficacy of the low doxorubicin doses, suggesting reduced toxicity to healthy cells and enhanced drug targeting to cancer cells. Overall, the developed multifunctional nanostructures demonstrated a promising enhanced anticancer potential and may represent an effective strategy for future glioblastoma therapy.

Keywords: cancer therapy, gold nanoparticles, doxorubicin, polyphenols, glioblastoma, synergistic effect

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1. Introduction

Cancer continues to represent a major global health burden, driving the continuous search for more efficient diagnostic tools and therapeutic approaches. Although substantial progress has been achieved in oncology over recent decades, current treatments still face important limitations, particularly regarding toxicity toward healthy tissues and the development of therapy resistance [1]. Consequently, modern cancer research is increasingly focused on innovative strategies, as smart drug carriers, capable of synergistic effects on selectively eliminating tumor cells while minimizing adverse effects on normal cells [2,3].

Conventional chemotherapy and immunotherapy are commonly associated with considerable toxicity. Patients frequently experience myelosuppression, gastrointestinal disturbances, alopecia, and anemia during treatment, while long-term complications such as cardiotoxicity and neurotoxicity can significantly reduce quality of life [4]. Furthermore, advanced targeted therapies and immunotherapeutic agents are often financially inaccessible in many low- and middle-income countries. These limitations have stimulated growing interest in natural bioactive compounds as safer complementary or alternative anticancer agents.

Glioblastoma multiforme (GBM), the most aggressive primary tumor of the central nervous system, presents an additional therapeutic challenge due to its highly invasive character and resistance to standard therapies [5,6]. Despite multimodal treatment approaches combining surgery, radiotherapy, chemotherapy, targeted therapies, and supportive care, patient prognosis remains extremely poor [7,8]. GBM progression is associated with marked tumor heterogeneity, genetic instability, activation of oncogenic signaling pathways such as PI3K/AKT, and overexpression of drug efflux transporters including P-glycoprotein (P-gp), which contributes significantly to multidrug resistance [9,10]. In addition, glioblastoma stem cells play a crucial role in tumor recurrence, angiogenesis, and resistance to both chemotherapy and radiotherapy [11].

Given the increasing incidence of chemo- and radioresistance together with the severe side effects induced by conventional therapies, attention has shifted toward phytochemicals with chemo preventive and therapeutic potential [12-14]. Numerous natural compounds isolated from fruits, vegetables, and

medicinal plants — including curcumin, berberine, quercetin, piperine, resveratrol, epigallocatechin gallate, coumarins, silymarin, and lycopene — have demonstrated promising antitumor activities [2,15]. These compounds may interfere with abnormal intracellular signaling pathways, suppress inflammation, inhibit angiogenesis, and induce different forms of programmed cell death such as apoptosis, ferroptosis, and necroptosis [16]. Moreover, many phytochemicals possess strong antioxidant properties, protecting healthy tissues from oxidative damage generated during anticancer therapies through scavenging reactive oxygen and nitrogen species and reducing lipid peroxidation [17].

Despite their therapeutic promise, the clinical application of phytochemicals is frequently hindered by poor solubility, low bioavailability, limited chemical stability, and rapid systemic elimination [18]. Nanotechnology-based delivery systems have emerged as an effective solution to overcome these drawbacks by improving drug stability, enhancing targeted delivery, and increasing therapeutic efficacy [19].

Gold nanoparticles (GNPs) have attracted considerable attention in nanomedicine due to their versatile biological and physicochemical properties [20]. Their synthesis relies on the reduction of Au^{3+} ions to metallic Au^{0}, using either chemical or biological reducing agents. Conventional chemical methods commonly involve trisodium citrate or sodium borohydride, while green synthesis approaches employ biomolecules such as resveratrol, polysaccharides, enzymes, peptides, or natural pigments [21-24]. Green-synthesized GNPs are particularly attractive because they offer improved biocompatibility and reduced environmental impact [25].

In oncology, GNPs have demonstrated potential applications in imaging, drug delivery, and cancer therapy [26]. Functionalized or centrifuged GNPs, although less extensively investigated, have shown the ability to induce cytotoxicity and apoptosis in several cancer cell types. To improve nanoparticle stability and minimize aggregation or toxicity, various capping agents may be employed, including resveratrol, piperine, and icariin [2].

Piperine, the major alkaloid found in *Piper nigrum* and *Piper longum*, possesses both antioxidant and anti-inflammatory properties, with reported protective effects on normal cells and cytotoxic activity toward cancer cells [27]. Resveratrol, a polyphenol abundant in grape skin, is known for its anti-inflammatory, antioxidant, and moderate anticancer properties [28], whereas icariin also exhibits significant anti-inflammatory and protective biological activities [29]. Together, these biomolecules represent promising candidates for improving the therapeutic performance of GNPs and conventional anticancer drugs such as doxorubicin [30].

Doxorubicin remains one of the most effective chemotherapeutic agents due to its capacity to induce apoptosis in malignant cells; however, its clinical

use is limited by severe dose-dependent toxicity. Combining doxorubicin with biofunctionalized GNPs may enhance antitumor activity while reducing the required therapeutic dose and protecting healthy tissues. In the present approach, gold nanoparticles were synthesized using resveratrol-mediated reduction, followed by centrifugation and purification steps designed to remove residual reaction compounds and obtain nanoparticles suitable for subsequent functionalization [2].

The biological activity of the resulting nanocomposites was evaluated using MTT viability assays. The objective was to assess their ability to induce apoptosis in glioblastoma cells while simultaneously reducing toxicity toward normal cells. By combining centrifuged GNPs with selected phytochemicals and doxorubicin, this study aimed to develop multifunctional nanostructures capable of enhancing anticancer efficacy, overcoming resistance mechanisms, and improving the safety profile of conventional chemotherapy.

Materials and Methods

Gold nanoparticles (GNPs) were synthesized using aqueous solutions prepared with bi-distilled deionized water. Tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.5%) and sodium hydroxide (NaOH, reagent grade, ≥98%) were obtained from Merck (Darmstadt, Germany), while trans-resveratrol (≥99%, HPLC assay) was supplied by Sigma-Aldrich (Buchs, Switzerland). For the functionalization of the synthesized GNPs, doxorubicin hydrochloride (~98%) purchased from Sigma-Aldrich (Munich, Germany), piperine (≥98%, HPLC assay) obtained from Alfa Aesar (Karlsruhe, Germany), and icariin analytical standard (≥94%) from Sigma-Aldrich (Steinheim, Germany) were employed. Dimethyl sulfoxide (DMSO) was acquired from Sigma-Aldrich (Schnelldorf, Germany). Dulbecco's phosphate-buffered saline (PBS), free of $CaCl_2$ and $MgCl_2$ and adjusted to pH 7.4, was purchased from Alfa Aesar (Karlsruhe, Germany). The MTT tetrazolium reagent, namely 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (≥98%), was also obtained from Sigma-Aldrich (Buchs, Switzerland).

The **synthesis** of gold nanoparticles, GNPs, is revealed in Fig. 1. Their **functionalization** with polyphenols and doxorubicin is shown in Fig. 2, as following.

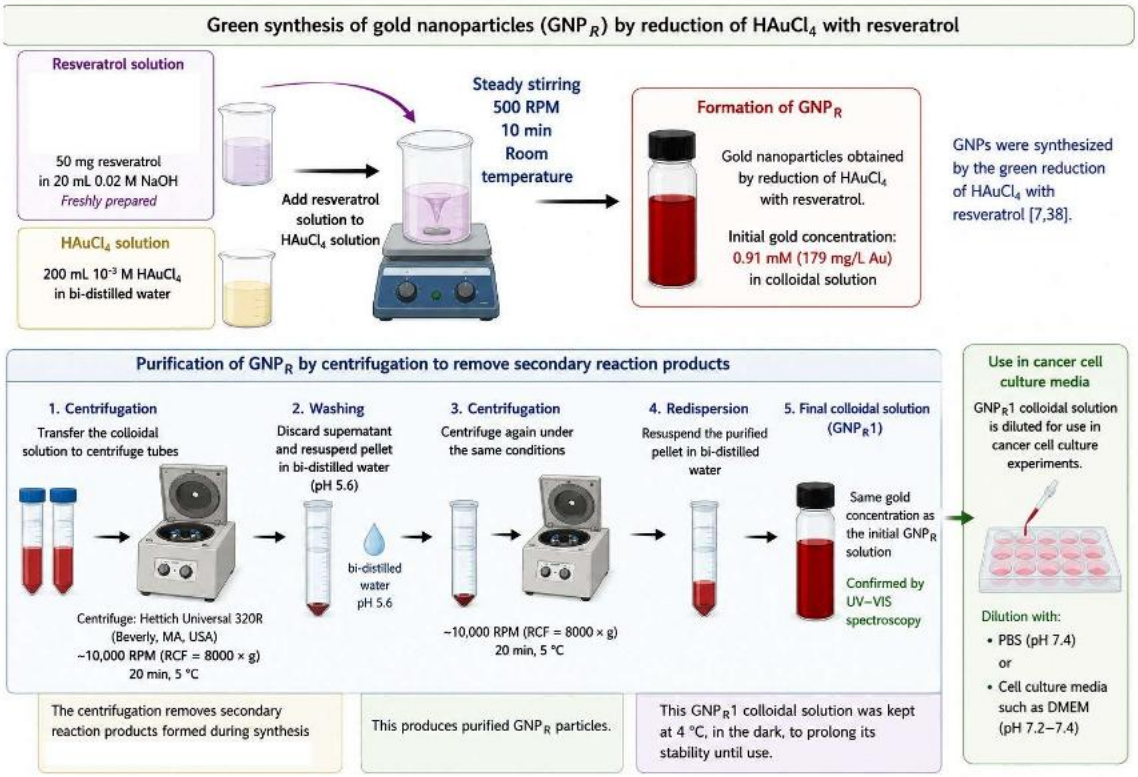

Figure 1. Schematic illustration of green synthesis of gold nanoparticles (GNPs) by reduction of $HAuCl_4$ with resveratrol.

The GNPs were synthesized by the green reduction of $HAuCl_4$ with resveratrol [2,3, 31] as can be seen in Fig. 1. The GNP_R1 (also noted $GNP_{R}1$ or GNP_{R1} in Fig. 1) colloidal solution was further used in functionalization of GNP_R1 particles with selected natural polyphenols, resveratrol, R, piperine, P, and icariin, Ic, as can be seen in Fig. 2. Polyphenols are recognized as targeting molecules to cancer cells. The functionalized GNP_R1 particles with these polyphenols give GNP_R1@R/P/Ic different nanocarriers. They can be further functionalized with doxorubicin, D, leading to multi-functionalized GNP_R1@R/P/D/Ic nanocarriers (also noted GNP_{R1}@R/P/D/Ic in Fig. 2).

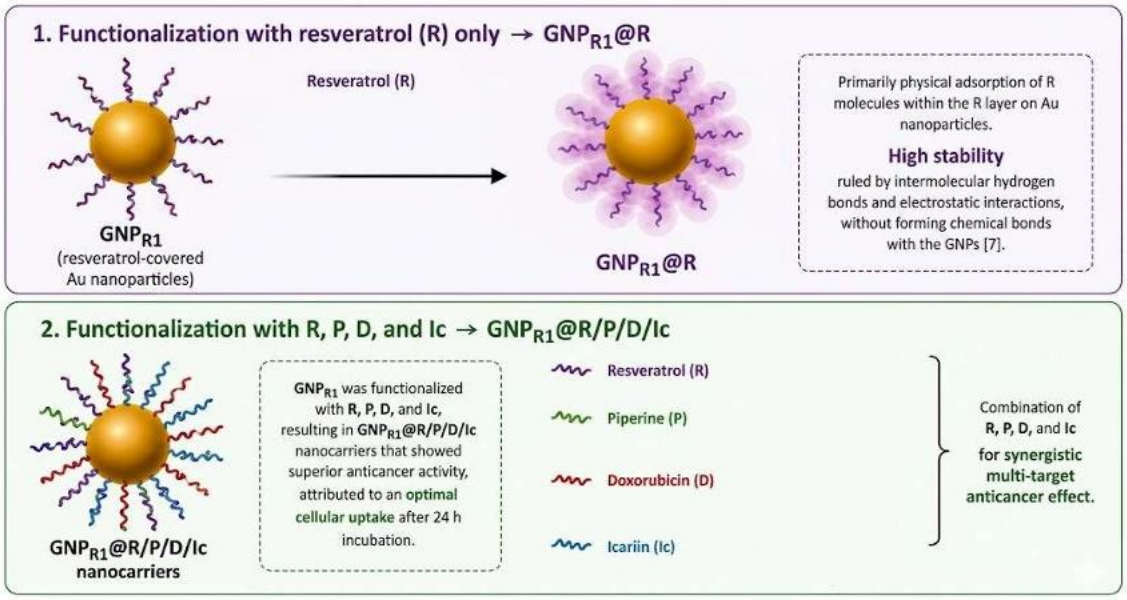

Figure 2. Schematic presentation of functionalization of gold nanoparticles with resveratrol, piperine, icariin and doxorubicin.

In this study a **tumor stem cell** line was used derived from a primary **glioblastoma culture** (GBM). Tumor stem cells isolated from a patient with high-grade glioblastoma were characterized by immunocytochemistry and reverse transcription polymerase chain reaction (RT-PCR). Glioma cells expressed stem cell markers such as CD133, CD105, CD90, Nanog, Oct 3/4, CXCR4, as well as glial cell markers: nestin, glial fibrillary acidic protein (GFAP) and neurofilament protein (NF). These cells presented a high proliferative capacity and were radio-chemo-resistant [32].

Comparison among the effects induced by gold nanoparticles, GNP_R1, synthesized by green method, and functionalized with polyphenols, resveratrol, piperine and icariin, as well doxorubicin, on glioblastoma tumor stem cells, is investigated by **MTT assay**.

For the MTT assay, GBM stem cells were counted using a hemocytometer, and seeded into 96-well plates at a density of 1×10^4 cells/well in 200 µL complete culture medium. Cells were cultured in vitro in stem cell medium supplemented with growth factors and then, the capacity of the surviving stem-like precursors to form tumor spheres and to continue to proliferate were tested. After 24 h of incubation, the cells were treated with functionalized gold nanoparticles, polyphenols, and/or doxorubicin at a 1/20 dilution. Doxorubicin was tested at concentrations of 2.1, 6.25, and 12.5 µg/mL in order to compare nanoparticle effects with clinically relevant treatments.

Cell viability was assessed after 24 h using the MTT assay, based on the reduction of tetrazolium salt to formazan crystals by viable cells. Following incubation with MTT solution (1 mg/mL) for 1 h at 37 °C in the dark, the formed crystals were dissolved in DMSO, and absorbance was measured at 492 nm using a BioTek Synergy 2 microplate reader. Cell viability was expressed as relative viability (%) compared to untreated control cells, considered as 100% MTT assay.

The data obtained from the MTT viability assay were analyzed using GraphPad Prism 5 software. **Statistical evaluation** was performed using two different methods. Repeated-measures ANOVA followed by Dunnett's multiple comparison test was applied to compare treated groups with untreated control samples. In addition, comparisons between two selected groups were carried out using the Mann–Whitney test. For all analyses, differences were considered statistically significant at $p < 0.05$ (*).

Results and Discussion

Glioblastoma (GBM) is considered one of the most malignant and difficult-to-treat primary brain tumors due to its highly invasive behavior, intense vascularization, marked cellular heterogeneity, and strong resistance to standard therapeutic approaches. One of the major limitations in brain cancer treatment is the restricted ability of therapeutic agents to penetrate the blood–brain barrier (BBB) [33]. Because of the BBB's highly selective permeability, only a very small fraction of administered drugs (1%) can reach brain tissue, resulting in poor therapeutic efficiency against glioblastoma [34,35]. Consequently, the development of alternative therapeutic strategies capable of overcoming these barriers has become essential. In this context, various therapeutic molecules and drugs covalent attachment onto the surface of gold nanoparticles, have shown considerable potential as efficient carriers for biomolecule delivery [36-38].

After conjugation and functionalization, these nanocomplexes are able to cross the cellular membrane as a single system, facilitating the intracellular delivery of therapeutic agents. Among the natural biomolecules most frequently employed for nanoparticle functionalization are piperine, resveratrol, and icariin, due to their recognized anticancer properties [2].

Doxorubicin is a broadly used chemotherapeutic drug with proven efficacy against numerous cancer types. However, several studies have reported important adverse effects associated with its administration, including hematological, cardiotoxic, and testicular toxicity [39-41]. Recent evidence suggests that nano formulations of doxorubicin may not only enhance its therapeutic activity and broaden its anticancer spectrum but also improve its safety profile by reducing systemic toxicity [42].

In the first part of the experiment, the individual and combined effects of polyphenols and doxorubicin on cell cultures were evaluated. The chemical composition and concentration of the tested solutions are presented in the legend of Figure 3. In this stage, the relatively high concentrations of doxorubicin used are notable: 10 μg/mL and 6.65 μg/mL. The effects of the applied treatments are shown in Figure 3 (as bars graph).

From the analysis of the data in Fig. 3, it can be observed that the study began with high concentrations of individual and combined polyphenols, as well as a very high concentration of doxorubicin (10 μg/mL). Treatment variants (compositions, noted Comp, or samples) 5–11 all contain a lower concentration of doxorubicin (6.65 μg/mL) and progressively decreasing concentrations of polyphenols.

The analysis of the data (% viable cells) presented in Figure 3 shows that experimental variant 1, represented by doxorubicin at a concentration of 10 μg/mL, exhibits the strongest reduced viability effect, of approximately 76% compared to the control sample. This situation can indicate either cell death (cytotoxicity) or an inhibition of cell proliferation/metabolism (cytostatic effect), or both.

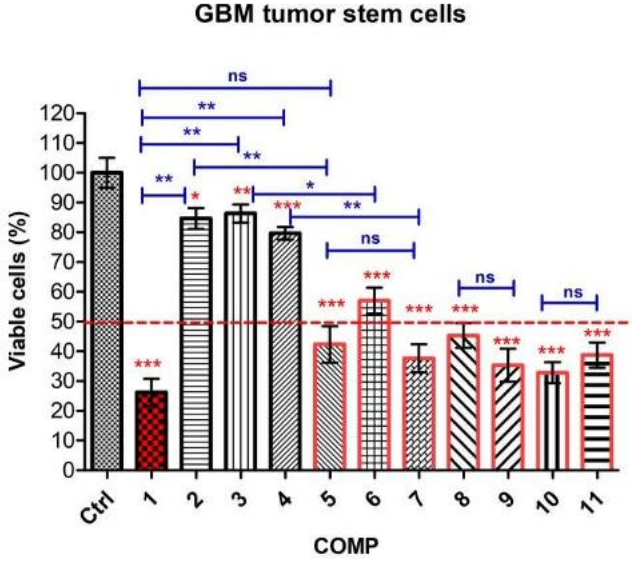

Figure 3. Viable cells in % of control (Ctrl), from MTT assay, for the glioblastoma stem cells, after 24 hours of exposure to free polyphenols: resveratrol, R, piperine, P; R and P mixed solution (for molar ratio R/P = 1), and icariin, Ic, with or without doxorubicin, D. Control, 1-D 10 μg/mL, 2-R 1.5 μg/mL, 3-P 2 μg/mL, 4-R 1.11 μg/mL; P 1.39 μg/mL (R/P = 1); 5-R 0.5 μg/mL, D 6.65 μg/mL, 6-P 0.665 μg/mL, D 6.65 μg/mL, 7-R 0.37 μg/mL, P 0.46 μg/mL (R/P = 1); D 6.65 μg/mL, 8-Ic 1.665 μg/mL; D 6.65 μg/mL, 9-R 0.25 μg/mL; Ic 0.83 μg/mL; D 6.65 μg/mL (Ic/R =1.12), 10-P 0.335 μg/mL; Ic 0.83 μg/mL; D 6.65 μg/mL, 11-R 0.19 μg/mL; P 0.25 μg/mL; Ic 0.625 μg/mL; D 6.65 μg/mL (R/P=0.94; R/Ic=0.90; P/Ic =0.96).

In Fig. 3 statistical analysis performed with GraphPad Prism 5, is given by repeated measures ANOVA followed by Dunnett multiple comparison test results, which are illustrated with red stars, and paired ***t-test*** results with blue brackets and blue stars. Statistical significance was set at $p \leq 0.05$ and was noted as *p<0.05, **p<0.01 and ***p<0.001.

However, considering the very high toxicity of doxorubicin in clinical practice, it would be desirable for its level to be as low as possible. Regarding the individual effects of resveratrol (sample 2) and piperine (sample 3), administered at moderate doses, a slight reduction in cell viability was observed, namely 18% and 15%, respectively, compared to the control sample. Sample 4 indicates the combined effect of resveratrol and piperine at medium doses.

Samples 5 to 11 contain doxorubicin at a concentration of 6.65%, therefore at a lower dose, together with individual or combined polyphenols in different formulations. It can be observed that the presence of doxorubicin is essential for reducing cell viability below 50% compared to the control. It is noteworthy that individual polyphenols added to the composition at low doses exhibit a distinct cytotoxicity action.

Thus, resveratrol (sample 5) induces 54% cytotoxicity, icariin (sample 8) 51%, and piperine (sample 6) 44%. The best results were recorded in the case of combined variants containing doxorubicin together with two polyphenols, where they may exert a synergistic effect. Thus, variants 7 and 9, containing resveratrol and piperine, and resveratrol and icariin, respectively, showed very comparable (similar) reduced viability values of approximately 63%. The most pronounced inhibition, 65%, was observed for variant 10, which contains piperine and icariin alongside doxorubicin.

The complex treatment containing all three polyphenols exhibited a cytotoxicity of approximately 60% compared to the control sample. Therefore, the formulation with a potential reduced toxicity on human healthy cells and maximum cellular cytotoxicity coupled with a possible inhibitory effect, as demonstrated on cell viability (%) is the one (composition 10) containing piperine and icariin together with doxorubicin**.**

In the next stage of the experiment, the administered formulations contained gold nanoparticles functionalized with resveratrol, piperine, and icariin, together with very low doses of doxorubicin: 0.70 μg/mL, 0.52 μg/mL, and 0.42 μg/mL. The chemical composition and the concentration used can be observed in Fig. 4.

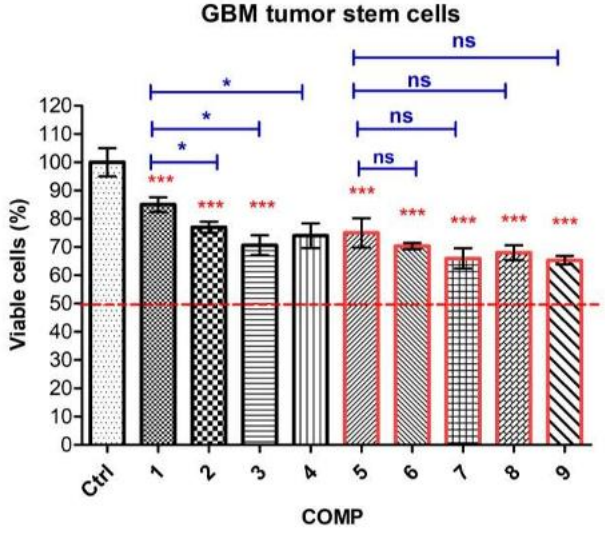

Figure 4. Viable cells in % of control (from MTT assay) of tumor stem cells, derived from glioblastoma (GBM), after 24 hours of exposure to GNP-R1 functionalized with biocompounds (polyphenols) and doxorubicin. Control (Ctrl); 1-GNP_R1 5.9 µg/mL; 2-GNP_R1 5.9 µg/mL; P 0.67 µg/mL; 3-GNP_R1 5.9 µg/mL; P 0.46 µg/mL and R 0.37 µg/mL (P/R = 1.0); 4-GNP_R1 5.9 µg/mL; Ic 1.67 µg/mL; 5-GNP_R1 5.9 µg/mL; D 0.70 µg/mL; 6-GNP_R1 4.5 µg/mL; R 0.38 µg/mL; D 0.52 µg/mL; 7-GNP_R1 4.5 µg/mL; P 0.34 µg/mL; R 0.28 µg/mL (P/R = 1.0); D 0.52 µg/mL; 8-GNP_R1 4.7 µg/mL; Ic 1.33 µg/mL; D 0.42 µg/mL; 9-GNP_R1 4.7 µg/mL; R 0.2 µg/mL; Ic 0.67 µg/mL (Ic/R = 1.1); D 0.42 µg/mL.

Statistical analysis representation: repeated measures ANOVA followed by Dunnett multiple comparison test results are illustrated with red stars and ***t-test*** results with blue brackets and blue stars. Statistical significance was set at $p \leq 0.05$ and was noted as *p<0.05, **p<0.01 and ***p<0.001.

The analysis of the data presented in Figure 4 shows that the percentage of reduced cell viability ranges between 17% and 33%, depending on the administered formulation. A treatment variant can indeed be used as a treatment composition, properly formulated. Consequently, variant 1, represented by gold nanoparticles reduced with resveratrol, recorded the lowest cytotoxicity and inhibition, namely 17%.

By introducing piperine into the formulation (noted variant 2 or composition 2), an improvement in the effect was observed, with the cytotoxicity increasing to 24%. Variant 3 (GNP_R1 5.9 µg/mL; P 0.46 µg/mL and R0.37 µg/mL; P/R = 1.0) represents the formulation, refering to the physical and chemical process used to combine them in the GNP_R1 functionalized with resveratrol and piperine, GNP_R1@R/P, exhibiting a cytotoxicity of 27%.

Variant 4 contains icariin and shows a cytotoxicity percentage of 25%. Starting with variant 5, doxorubicin was introduced into the formulation.

Interestingly, variants 4 and 5 displayed similar values of reduced cell viability effect, although a greater variability of the results was observed in the case of doxorubicin administration, indicating a more uniform effect induced by the icariin treatment.

Resveratrol from composition 6 (GNP_R1 4.5 µg/mL; R 0.38 µg/mL; D 0.52 µg/mL) specifically affected GBM tumor stem cells, with a cytotoxicity value of 30 %. The effect of variant 8 (GNP_R1 4.7 µg/mL; Ic 1.33 µg/mL; D 0.42 µg/mL), on GBM tumor stem cells, is comparable with the effect of variant 6, showing almost the same cytotoxicity.

Variants 7 and 9, containing piperine, resveratrol, and doxorubicin, and respectively resveratrol, icariin, and doxorubicin, exhibited a reduced cell viability at level of 33%. Therefore, the optimal formulation is obtained by the composition number 9, containing resveratrol and icariin (Ic/R = 1.1), and the lowest dose of doxorubicin (D 0.42 µg/mL).

Doxorubicin exerts its anticancer activity (cytotoxicity) through several complementary mechanisms, including intercalation between DNA base pairs, which disrupts DNA replication and transcription [43]; inhibition of topoisomerase II leading to irreversible double-stranded DNA breaks [44], and generation of reactive oxygen species (ROS) that induce oxidative stress and damage cellular components such as DNA, proteins, and lipids [45].

In addition, doxorubicin activates apoptotic signaling pathways involving p53, BAX, and caspases [46], promotes cell cycle arrest particularly in the G1 and S phases [47], and preferentially accumulates in tumor tissues due to the increased permeability and irregular structure of tumor blood vessels, thereby enhancing its cytotoxic effects against cancer cells [48].

Doxorubicin is commonly administered intravenously, frequently in combination with other chemotherapeutic agents in order to enhance its antitumor efficacy. Despite its effectiveness against cancer cells, doxorubicin also affects normal cells, particularly those from the bone marrow, gastrointestinal tract, and cardiac tissue. One of the most severe adverse effects associated with this drug is cardiotoxicity, which may progress to heart failure, especially following high cumulative doses [49].

To reduce systemic toxicity and improve tumor selectivity, various advanced delivery strategies have been developed. These include nanoparticle- and liposome-based systems designed to increase drug accumulation at the tumor site, as well as targeted delivery approaches in which antibodies or specific ligands are conjugated to the doxorubicin molecule to selectively bind receptors overexpressed on cancer cells, thereby enhancing therapeutic efficiency while minimizing damage to healthy tissues [50].

The functionalization of gold nanoparticles (GNPs) with resveratrol and doxorubicin, as proposed in this study, represents a promising approach in cancer therapy by integrating the unique physicochemical properties of gold nanoparticles with the therapeutic potential of polyphenols [51,52]. This strategy is designed to improve drug delivery efficiency and bioavailability, enhance anticancer efficacy, and minimize systemic toxicity by enabling more selective targeting of cancer cells [53, 54].

Conclusions

This study demonstrates that doxorubicin is the principal contributor to cytotoxic activity, as its presence is essential for achieving significant reductions in cell viability, particularly below the 50% threshold. However, its use is limited by dose-dependent toxicity, which supports the need for dose minimization strategies.

At the same time, the tested polyphenols (resveratrol, piperine, and icariin) show moderate but consistent reduced cell viability, probably based on their primarily intrinsic antiproliferative effects. When used individually, they produce only slight to moderate effects on cell viability, but their biological impact becomes more relevant in combination with gold nanoparticles and doxorubicin.

A key finding of this study is the clear evidence of synergistic effects when doxorubicin is combined with polyphenols. Formulations containing two polyphenols alongside doxorubicin consistently show higher anticancer activity than single-component or simpler combinations, indicating that polyphenols can potentiate chemotherapeutic efficacy of doxorubicin.

The introduction of functionalized gold nanoparticles further improves the therapeutic profile by enabling effective anticancer activity even at very low doxorubicin concentrations. This suggests that nanoparticle-based delivery can enhance drug efficiency, while potentially reducing systemic toxicity.

The most favorable balance between anticancer efficacy and reduced drug toxicity is achieved in formulations of GNPs functionalized combining resveratrol and piperine (P/R =1) or resveratrol and icariin (Ic/R=1.1) with low-dose doxorubicin, which provide strong and stable cytotoxicity. This discovery highlights the importance of smart multifunctional nanostructured carriers designed for targeting drug delivery to glioblastoma tumor stem cells, to optimize chemotherapy by enhancing anticancer activity while lowering drug-associated side effects.

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