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Combined sonodynamic therapy and X-ray radiation with methylene blue and gold nanoparticles coated with apigenin: Impact on MCF7 cell viability

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► ***Short report***

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ABSTRACT

Background: This study investigates the efficacy of sonodynamic and low-dose radiation with methylene blue and gold nanoparticles coated with apigenin sonosensitizer on breast cancer cells. ***Method and Materials:*** HR-TEM, UV-visible, zeta -potential, FTIR, and DLS were used to confirm the synthesized gold nanoparticles coated with apigenin. The concentration of gold nanoparticles coated with apigenin and methylene blue was calculated, and then ultrasound and 2 Gy x-rays were used to irradiate them. ***Results:*** The results showed that sonodynamic therapy in combination with radiation at a concentration of 8 µg/ml nanoparticles and 0.4 µmol methylene blue had a significant effect on cell death (0.26± 0.02). ***Conclusion:*** This study highlights the potential of sonodynamic therapy as a non-ionizing and non-invasive treatment, as well as the possibility of eliminating the side effects of cancer treatment by using green nanoparticles rather than chemical sensitizers.

INTRODUCTION

A growing number of cancer patients are treated with radiation therapy as their primary treatment option. New treatments are available, including gene therapy, immunotherapy, sonoimmunotherapy, photoimmunotherapy, sonodynamic therapy, and photodynamic therapy [1]. In sonodynamic therapy, sensitizers or drugs under ultrasound are employed [2]. Ultrasound waves have biological effects due to non-thermal and thermal interactions, acoustic radiation force, microstreaming, and acoustic cavitation. The collapse of these cavities results in high pressure and temperatures, which causes cell death. Treatment modeling of ultrasound parameters in the range of acoustic cavitation threshold with mechanical index (MI) parameter is essential for various medical applications [3]. Choosing non-toxic and effective acoustic sensitizers such as methylene blue (MB) is the first step in sonodynamic therapy [4].

Sensitizers are essential in enhancing drug effectiveness and reducing toxicity in tumor conditions [5]. Gold nanoparticles are widely used as radio-sensitizers due to their high energy absorption coefficient [6]. Flavonoid apigenin (Api), a green sensitizer, can stabilize nanoparticles and promote cancer cell death by preventing DNA damage repair [7]. Api also increases radiation in inhibiting proliferation, inducing apoptosis, and stopping cell growth in the G0/G1 phase [8].

This study aims to highlight the potential of combining complementary methods, specifically the use of gold nanoparticles coated with Api (Api@AuNPs) and MB, to enhance the efficiency of sonodynamic therapy and radiation for the treatment of MCf7 cells. Additionally, the study examined the incorporation of the parameter, MI (mechanical index), in sonodynamic therapy to enhance treatment outcomes.

METHODS AND MATERIALS

Synthesis of gold nanoparticles coated with apigenin (Api@AuNPs)

Api@AuNPs were synthesized through adding

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150 ml of 20 mM Api (Shaanxi Huike Botanical Development Co. China) solution to 10 ml of hot boiled water. ICP-OES (model: VISTA-PRO, Varian Co, Australia) was used to determine the concentration of nanoparticles. Zeta-potential (Zeta-check, Microtrac, Germany), UV-visible spectroscopy (model: NDNM96, NanoMabna Co, Iran), hydrodynamic diameter (DLS, NANO-flex Particle Sizer, Germany), and Fourier transform infrared spectroscopy (FT Nicolet Avatar 360 FTIR, Thermo Scientific, Courtaboeuf, France) were performed. Dimensions and morphology were checked using TEM (Zeiss EM 900, Germany). Nanoparticle diameter and size distributions were measured using DigitalMicrograph® software (Gatan Inc., California, US). MTT was used to assess the toxicity of Api@AuNPs nanoparticles after 24 hours.

Sonosensitizer (Methylene blue (MB))

Sigma–Aldrich Co. (St. Louis, MO, USA) provided MB as an acoustic sensitizer with Api@AuNPs. After 24 hours, toxicity was examined to determine the effective concentration.

Sonodynamic therapy protocol

An ultrasound device (Phyaction 190i, Germany) with a plane circular transducer extracted MI parameter [3] at 1 MHz and operated in continuous wave (CW) and pulse wave modes at 12%, 25%, and 50% duty factors. The ultrasound intensity was set at 2 W/cm^2, and radiation treatment time was selected to raise the temperature of the environment by one degree. Static wave propagation was avoided by a dampening layer.

Radiation therapy (6 MV X-ray)

The study utilized a 6 MV linear accelerator (Electa Co., Switzerland) for radiation therapy, seeding 10^5 cells into Petri dishes with a 30 × 30 cm^2 field size and 100 cm SSD. A single of radiation was administered with Perspex layers for complete scattering.

Protocol for treating MCF7 cell

The study involved MCF7 cells that were cultured in DMEM high glucose containing 10% FBS and 1% streptomycin. Until passage 3-4, the cells were incubated at 37°C with 5% carbon dioxide and 95% humidity. Different treatment protocols were applied to the cells, including radiation, ultrasound, MB, nanoparticles, etc. Following 24 hours of incubation with Api@AuNPs and 1 hour with MB, the cells were treated with ultrasound and radiation therapy.

MTT assay

The viability of cells was evaluated after 24 hours of incubation with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (DNAbiotech, Iran) assay. The viability was measured via an ELISA reader DANA, Model-DA3200, Iran.

Statistical analysis

The study compared various treatment protocols in MCF7 cells using PSS v.26 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism v.9 (GraphPad Software, Inc., San Diego, CA, USA), using one-way ANOVA at a 95% confidence level of 95%. Results are presented in terms of mean ± standard deviation.

RESULTS

The study found that radiation and sonodynamic therapy significantly reduced the death rate of cancer cells when MB and Api@AuNPs nanoparticles were present. Nanoparticles of Api@AuNPs were characterized using various techniques (figure 1), including UV-visible spectrum, FTIR spectrum, diameter quantification, and TEM image.

It was confirmed by UV-vis spectroscopy that Api@AuNPs' aqueous solution was stable, indicating that Api was present on gold nanoparticle surfaces. The FTIR evaluations showed several peaks in the Api spectrum, with 1632 cm^{-1} and 3432 cm^{-1} wavelengths in Api@AuNPs. Using an electron microscope, size was measured, with an average size of 19-30 nm. The prepared nanoparticles' average size was 30 nm, and the zeta potential of Api@AuNPs was -4 mV.

Mechanical index (MI) estimation

To evaluate the propagation of ultrasound, MI estimation was conducted (figure 2).

The highest MI was obtained at 2.00 W/cm^2, equal to 0.41 and 0.43 under propagating conditions. To examine acoustic cavitation interactions that are unstable, MI = 0.39 ≈ 0.40 was determined at a 1-MHz frequency, 2.00 W/cm^2, and a 2 cm distance from the transducer. It assumed MI = 0.39 so that 35 s, 45 s, 65 s, and 120 s were estimated to be the time of 2.00 W/cm^2 ultrasound in CW mode, and 50%, 25%, and 12% in pulse mode (figure 3).

Results showed a significant cell viability difference between CW and CTL modes. The optimal conditions for sonodynamic therapy were chosen, including 1 MHz frequency, 2 W/cm^2 intensity, and CW mode, with a distance of 2 cm between cells and the ultrasound source.

Sensitizers' cytotoxicity

The cytotoxic effect of MB (figure 4.A) on breast cancer cells and Api@AuNPs (figure 4.B) have been shown on MCF7 cells.

Figure 5 indicated a combined treatment of radiation and sonodynamic therapy with sensitizers significantly reduced the viability of cells with 0.26±0.02 in comparison with control ($P<0.05$). The study found that there is no notable difference between radiation and control, Api@AuNPs, MB, and

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control groups. Sonodynamic therapy combined with MB and Api@AuNPs significantly reduced cell viability, with a $P<0.05$ significance, indicating a much more significant decrease in the viability of cells than other treatment groups.

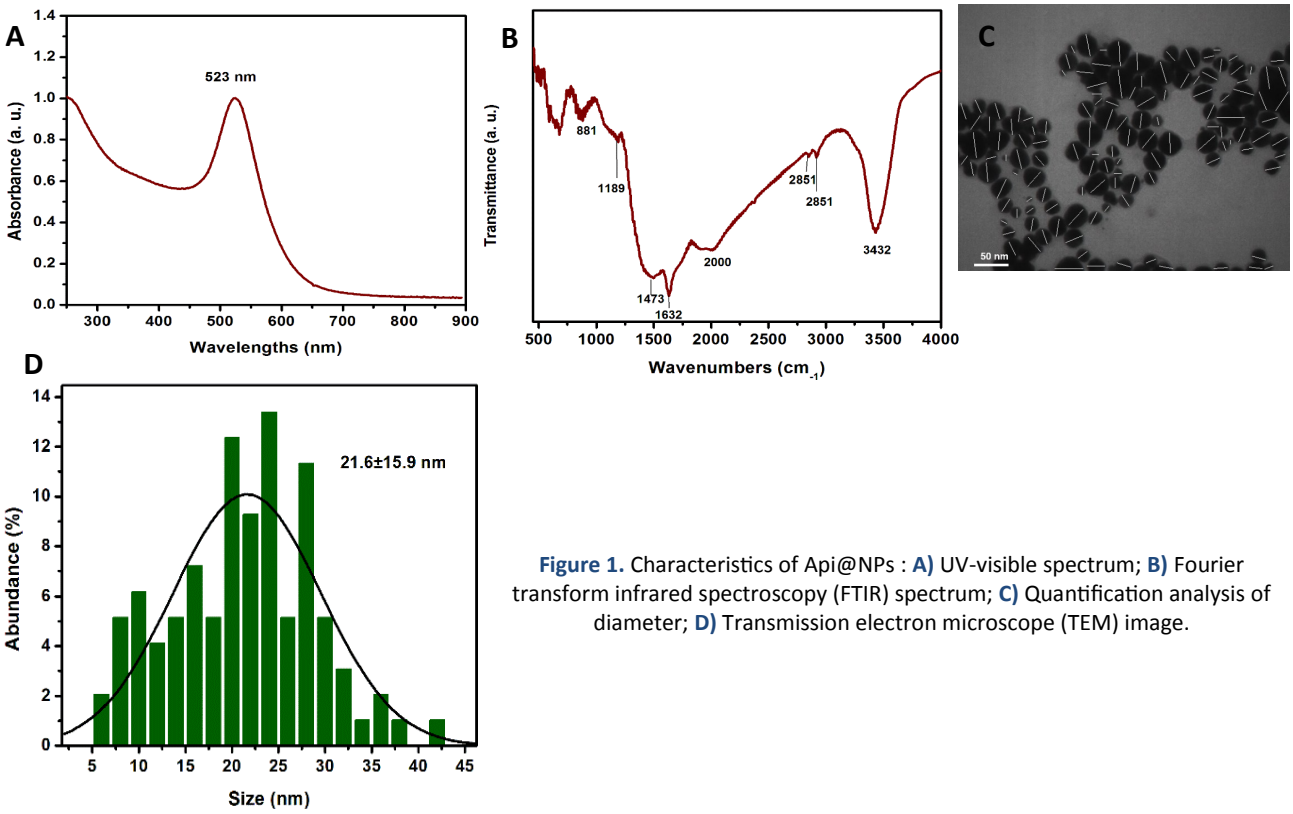

Figure 1. Characteristics of Api@NPs : **A)** UV-visible spectrum; **B)** Fourier transform infrared spectroscopy (FTIR) spectrum; **C)** Quantification analysis of diameter; **D)** Transmission electron microscope (TEM) image.

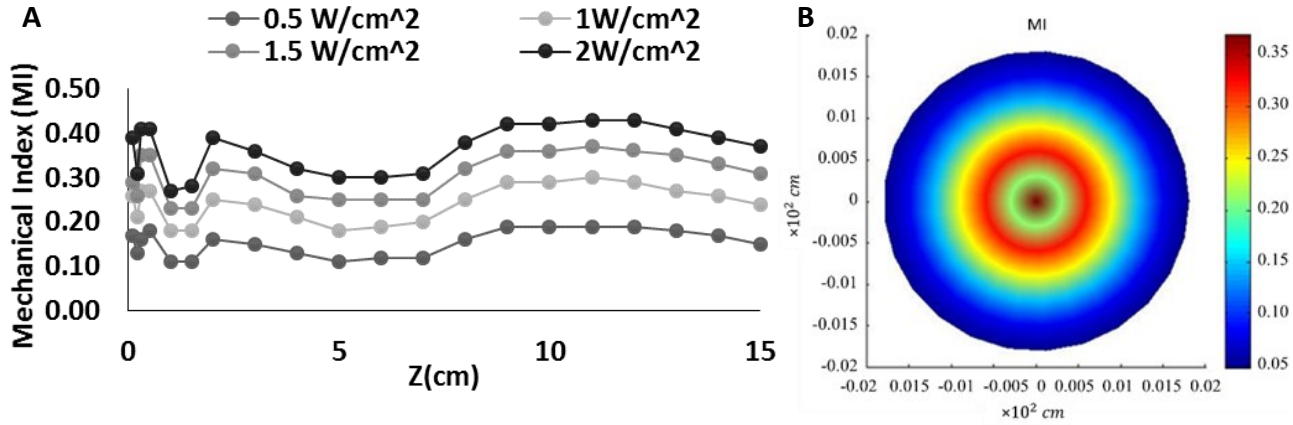

Figure 2. The estimation of mechanical index (MI): **A)** The axial mechanical index of a 1MHz frequency transducer in water at 37°C with intensities of 0.50, 1.00, 1.50, and 2.00 W/cm2 on the z axis in cm; **B)** Radial contour of MI with maximum of 0.39 at a 2 cm distance from the ultrasonic transducer. The color map from blue to red demonstrates an increasing rate of MI.

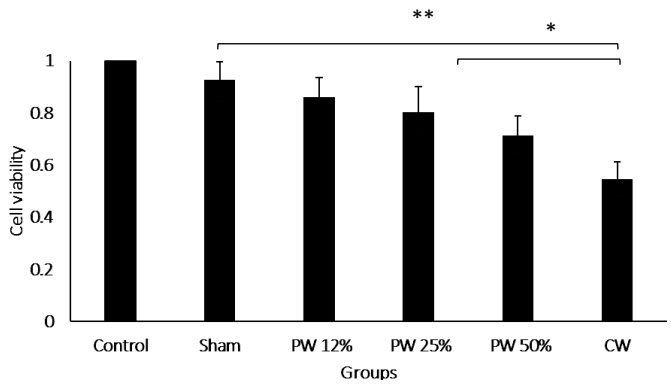

Figure 3. The cell viability of cultured cells after ultrasound treatment with MI= 0.39 in both continuous wave (CW) and pulse wave (PW) modes in various groups; Control, Sham, CW mode, and PW mode with duty factors of 12%, 25%, and 50%.

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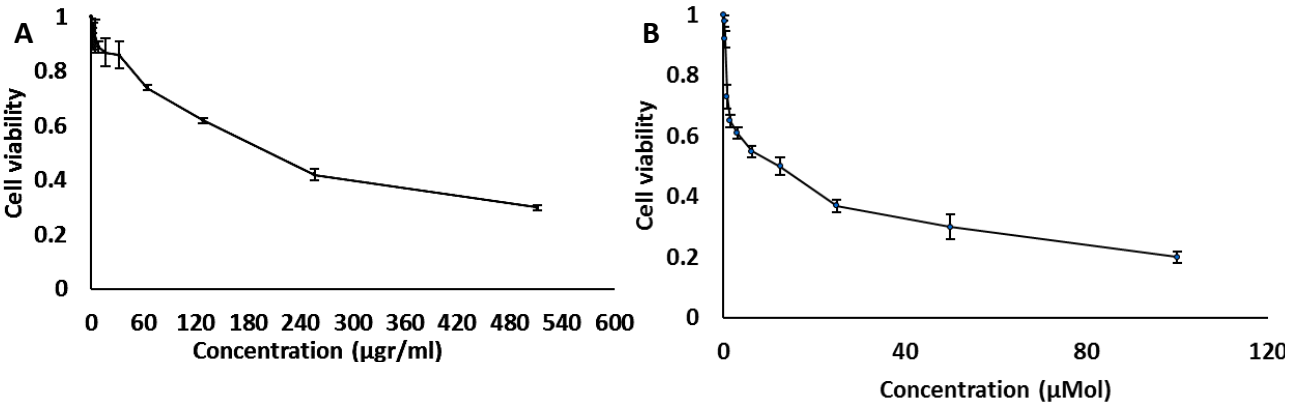

Figure 4. Sensitizer's cytotoxicity; **A)** Api@AuNPs (µgr/ml), **B)** MB (µMol). The study investigated the effect of radiation and sonodynamic therapy approaches on the death of 10% of cells when MB and Api@AuNPs were present (Figure 5), ultimately selecting a concentration of 8 µg/ml of nanoparticles and 0.39 µmol of MB.

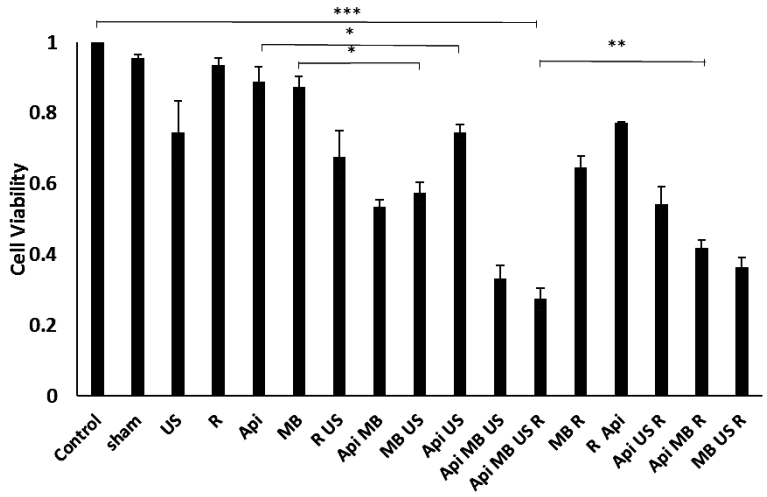

Figure 5. The cell viability of cultured cells after treatment with sonodynamic therapy with MI= 0.39 and radiation with 2 Gy in different groups: control, Sham, Ultrasound (US), Radiation (R) Gold nanoparticle coated with Apegnin (Api), methylene blue (MB), ultrasound and radiation (US R), nanoparticles with MB (Api MB), MB and ultrasound (MB US), nanoparticles and ultrasound (Api US), nanoparticles (Api MB US), ultrasound and radiation with MB and nanoparticles (Api MB US R), radiation with MB (MB R), radiation with nanoparticles (Api R), ultrasound and radiation with nanoparticles (Api US R), radiation with MB and nanoparticles (Api MB R), and ultrasound and radiation with MB (MB US R).

DISCUSSION

The study found that ultrasound waves above the acoustic cavitation threshold considerably enhanced the death of breast cancer cells when MB and Api@AuNPs were present, suggesting that non-ionizing radiation and sonodynamic therapy can synergize in apoptosis [5].

The study confirmed the characteristics of Api@AuNPs, confirming their stability and synthesis through UV-visible and FTIR measurements. Similar to Rajendran's study, small nanoparticles were obtained at boiling point temperature, and Api and Au were not detected in the UV-visible peak after nine centrifugation cycles (figure 1. A) [7]. Nanoparticle stability after six months was examined using UV-vis. Amini *et al.* found the spectrum peak at 523 nm [9]. Api on nanoparticle surface stabilizes them, with absorption bands 2919 and 2851 indicating CH stretching vibrations [10]. Figures 1. C and 1. D showed that nanoparticles correlate to smaller sizes provided, according to a study by Haiss *et al.* [11].

The study revealed that the MI threshold for water equivalent to the cell culture medium is higher than the selected threshold of 0.39, and axial propagated ultrasound intensity increases. Ahmadi *et al.* estimated mechanical and thermal indices, which are employed in high-frequency diagnostics to assess ultrasonic safety using numerical simulations [12]. This novel approach to therapeutic offers promising therapy improvements.

The CW mode, apart from MI, significantly outperforms the pulsed mode, as shown in figure 3. The results showed cell viability in the CW 62±3.1, 78±1.1 in 50%, 83±2.1 in 25 %, and 85%±3.1 in duty factor of 12.5% versus the control 100±3.2. The study reveals that ultrasound intensity, excitation duration, and duty cycle significantly impact cancer cell death, with T47D cell proliferation decreasing above 60% intensity and duty cycles [13]. Barati *et al.* discovered the antitumor effect of the ultrasound alone and in combination with the sonosensitizer in model of breast adenocarcinoma *in-vivo* [14].

The study likely selected concentrations of Api@AuNPs and MB according to a dose-response curve in order to achieve the desired therapeutic effect on cancer cells while minimizing side effects. MB, a photosensitizer [15], has been used in sonodynamic therapy to increase cell death through stable and inertial acoustic cavitation (figure 5). Studies have shown that MB increases the effectiveness of cell death in the presence of nanoparticles. Komori's study also confirmed the use

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of MB as a sonosynthesizer in the death of 180 sarcoma cells [16]. Moreover, Xiang's study on ovarian cancer cell line HO-8910 reported an increase in the effect of sonodynamic therapy in the presence of MB [17]. Sonodynamic therapy had 30% more deaths than ultrasound radiation alone and MB alone (13%). The rate of cell death in groups exposed to ultrasound waves in the presence of Api@AuNPs rose by 47% in comparison to the Api@AuNPs group. Gold nanoparticles absorb more radiation energy from photoelectric interaction, resulting in more secondary electrons and free radicals [18]. The presence of anti-cancer green elements on nanoparticle surfaces promotes biological tolerance and treatment effectiveness. Sonodynamic therapy enhanced the sensitivity of cells before radiotherapy. Liu *et al.* found a considerable improvement in the death of cancer cells with radiation (6 MV) in the presence of gold nanoparticles [19]. Api@AuNPs combined with laser have shown anti-cancer properties in photothermal therapy [20]. Radiation and sonodynamic therapy significantly affect MCF7 death.

CONCLUSION

Radiation and sonodynamic therapy, combined with green sensitizers, significantly impact breast cancer cell death. Radiation side effects can be reduced by combining a lower radiation therapy dose with sonodynamic therapy as a non-invasive and non -ionizing method. The design of treatment based on ultrasound wave interactions in sonodynamic therapy is hoped to improve treatment outcomes.

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***Ethical consideration*:** Ethical approval was obtained for this study of Iran University of Medical Sciences [IR.IUMS.REC.1400.647].

***Author contribution*:** Z.H-M, A.N, S.M.A, S.M.T and Z.A.K were responsible for conceiving and designing the study. S.M.T collected the data Z.H-M handled data processing and modeling, while S.M.A conducted data analysis. Z.H-M wrote the initial draft of the manuscript, and Z.A.K and A.N contributed to the manuscript revision.

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