

Diosgenin, a Plant-Derived Sapogenin, Exhibits Antiviral Activity *in Vitro* against Hepatitis C Virus

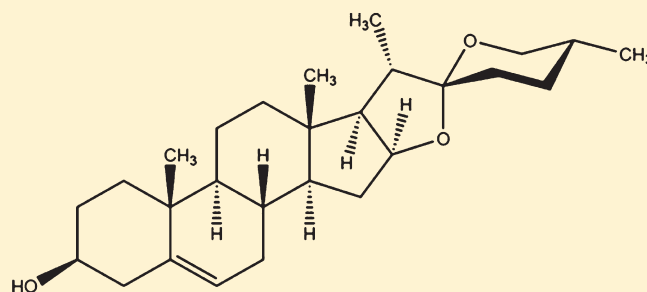
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ABSTRACT: Diosgenin (3β -hydroxy-5-spirostene, **1**), a plant-derived sapogenin, is used as a dietary supplement. However, the biological effects of **1** related to viral replication remain unexplored. In this study, the effects of **1** on hepatitis C virus (HCV) replication were evaluated. Based on a reporter-based HCV subgenomic replicon system, **1** was found to inhibit HCV replication at low micromolar concentrations. The EC_{50} (concentration at which 50% of HCV replication is inhibited) of **1** was $3.8 \mu\text{M}$. No cellular toxicity was observed at this concentration. Diosgenin (**1**) also significantly reduced the levels of viral RNA and viral proteins as evaluated by quantitative real-time reverse transcriptase PCR and Western blot analysis, respectively. In addition, in an alternative HCV antiviral system more closely aligned to all steps involved in the HCV infection and life cycle, **1** totally abolished HCV replication at $20 \mu\text{M}$. Moreover, **1** reduced the phosphorylation of signal transducer and activator of transcription 3. A combination of **1** and interferon- α exerted an additive effect on the resultant anti-HCV activity.

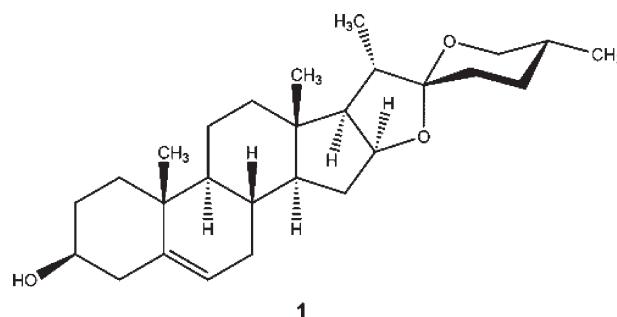


Diosgenin (**1**)

Hepatitis C virus (HCV) infection is a significant health problem that causes chronic hepatitis and liver cirrhosis. It is estimated that worldwide approximately 170 million individuals are afflicted by chronic HCV infection.¹ HCV, a member of the Flaviviridae, is a positive-sensed RNA virus with a genome encoding a single polyprotein of approximately 3000 amino acids. Inside the infected host cells, this polyprotein is cleaved into at least 10 structural and nonstructural proteins through the action of both host and viral proteases.^{2,3}

Interferon- α (IFN- α) or its pegylated form, in combination with ribavirin, is the only recommended treatment for HCV infection at present. This combination therapy is limited by its only partial efficacy.⁴ Furthermore, IFN- α -based therapy is poorly tolerated, expensive, and suitable only for certain patient populations.⁵ Consequently, it is important to discover new therapeutics that are more effective and economical than the current agents. Complementary and alternative herbal supplements for HCV therapy have been addressed and discussed.⁶ Diosgenin (3β -hydroxy-5-spirostene, **1**), a plant-derived sapogenin, is derived from the tubers of *Dioscorea* species (yams).⁷ Earlier studies have indicated that **1** has a wide range of biological properties,^{8–12} such as inhibition of intracellular reactive oxygen species (ROS) levels¹³ and vasoconstriction.¹⁴ It has been demonstrated that **1** inhibits the proliferation of cancer cells through a variety of mechanisms.^{10,15–19} Altogether, these preclinical and mechanistic studies suggest the potential of **1** as a multi-target-based therapeutic or chemopreventive agent against several types of tumors. Interestingly, it has also been demonstrated that **1**

can attenuate plasma cholesterol.^{20–23} A previous study has shown that HCV requires elements of the cholesterol biosynthetic pathways for efficient replication.²⁴ Moreover, several reports have indicated that statins, cholesterol-lowering drugs, can attenuate the replication of subgenomic HCV-1b replicons and inhibit RNA replication of Japanese patient with fulminant hepatitis (JFH-1) HCV.^{25–27} A recent report has proposed that statins may replace ribavirin in combination therapy with interferon.²⁸ However, the biological actions of **1** involved in HCV replication remain unexplored. The objective of the present study was to investigate whether diosgenin (**1**) can inhibit the replication of HCV.



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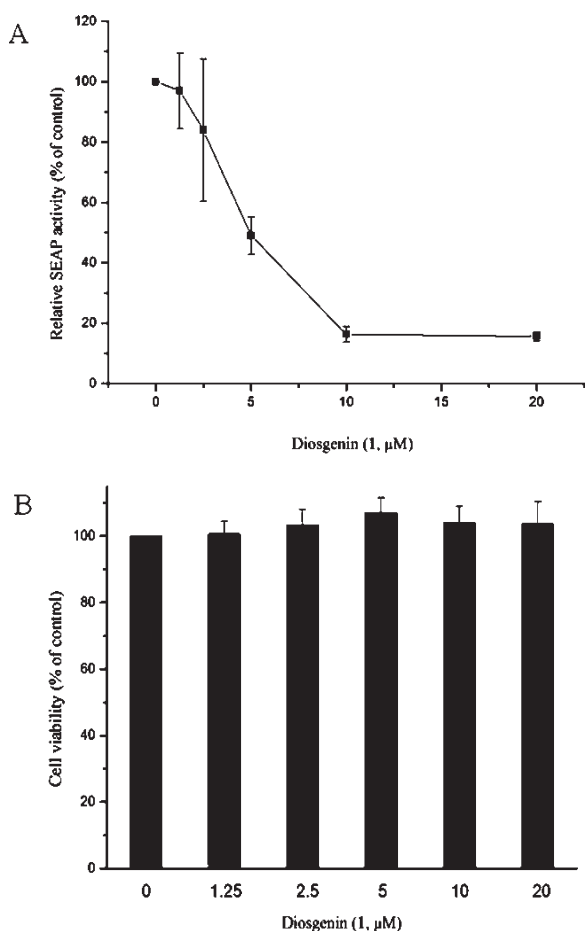


Figure 1. (A) Ava5-EG(Δ 4AB)SEAP cells were engineered by stable integration of the EGFP(Δ 4AB)SEAP reporter gene into Ava5 cells. Ava5-EG(Δ 4AB)SEAP cells were treated with the indicated doses of diosgenin (**1**). SEAP activities in the culture medium were measured to reflect the extent of HCV replication inside cells. (B) Cellular toxicity was evaluated using MTS assay. Results are expressed as means \pm standard deviations for three replicate wells.

RESULTS AND DISCUSSION

Effect of Diosgenin (1**) on HCV Replication in the SEAP-Based Reporter System Based on Ava5-EG(Δ 4AB)SEAP Cells.** Studies on HCV have been hampered in the past by the lack of an efficient cell culture system, until the advent of a subgenomic replicon system, such as the use of Ava5 cells.^{29,30} Our group has demonstrated previously the feasibility of using engineered Ava5 cells expressing a reporter gene, EG(Δ 4AB)SEAP, to reflect accurately the level of HCV replication by measuring levels of SEAP enzyme activity in the cell culture medium.³¹ Using the SEAP reporter assay, Ava5-EG(Δ 4AB)SEAP cells were treated with **1** at various doses. After 48 h, the culture medium was exchanged with fresh medium containing the same concentration of **1**, with the cells incubated for a further 24 h. Subsequently, the culture medium was harvested to determine SEAP activities. As shown in Figure 1A, **1** decreased SEAP activity in a dose-dependent manner, with the EC_{50} value determined as 3.8 μM . Additionally, no apparent toxicity was observed for cells treated with **1** over this concentration range, as revealed by the MTS assay (Figure 1B). As such, the effect of **1** on HCV replication was clearly revealed. For some time, *Dioscorea* species have been used to supplement the

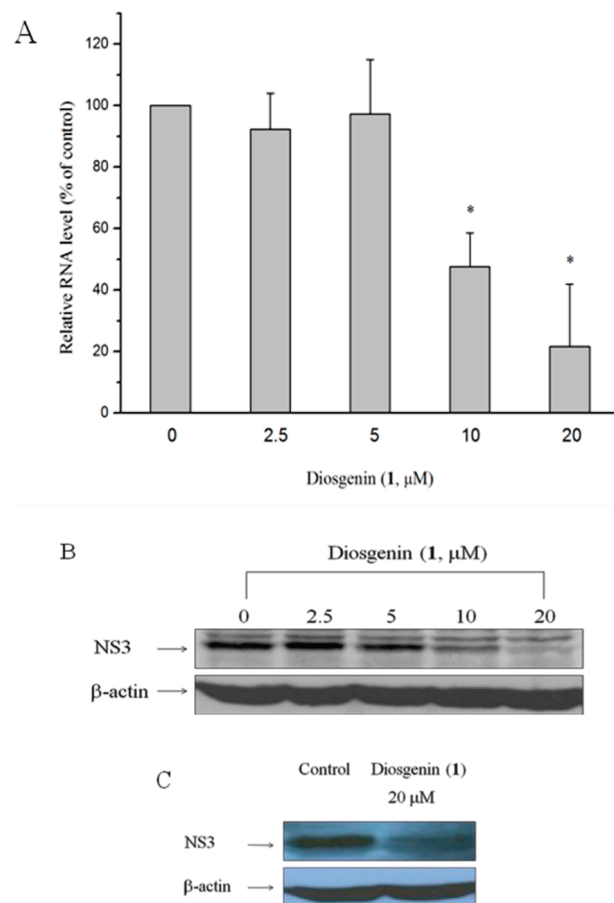


Figure 2. (A) The HCV RNA level was quantified by RTqPCR. The cells were treated with diosgenin (**1**) for 72 h. Expression of HCV RNA levels were reduced by **1** in Ava5 cells. HCV RNA levels obtained for samples not treated with test compound were designated as 100%. Each experiment was performed in triplicate, and error bars reflecting standard deviations are shown ($*p < 0.05$ compared to the control group). (B) The intracellular HCV NS3 protein levels were analyzed using a Western blotting assay. HCV NS3 proteins were inhibited by diosgenin (**1**) in a dose-dependent manner. (C) Diosgenin (**1**) significantly reduced HCV NS3 protein levels in an alternative HCV antiviral system constituting all steps involved in HCV infection and replication.

diet.^{15,32,33} Diosgenin is produced on acid hydrolysis of the saponins present in *Dioscorea* species. Furthermore, derivatives of *Dioscorea* species have been employed to treat a variety of ailments, such as hypercholesterolemia,^{34,35} diabetes,^{36,37} and gastrointestinal ailments.³⁸ Many of the observed benefits of *Dioscorea* species may be attributed to diosgenin (**1**). In the present study, it was demonstrated that **1** possesses anti-HCV activity, which constitutes a newly identified activity of this compound.

Effect of Diosgenin (1**) on Levels of HCV RNA and Viral Protein NS3.** Detection of HCV RNA levels in Ava5 cells upon treatment with **1** confirmed the anti-HCV effect of this compound. In this experiment, Ava5 cells were treated with **1**, and cells were harvested at 72 h after compound treatment. The cellular HCV RNA levels were analyzed by RTqPCR. As shown in Figure 2A, a dose-dependent decline occurred in HCV subgenomic RNA levels upon treatment with **1**. These results clearly indicated that **1** inhibits the replication of HCV. Next, whether **1** can alter the level of HCV NS3 protein was examined. Ava5 cells

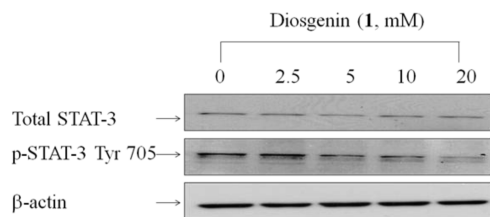


Figure 3. Diosgenin (**1**) suppresses phosphorylation levels of STAT3 in a dose-dependent manner. Ava5 cells were treated with the indicated concentrations of **1** for 72 h. The phosphorylation of STAT3 and STAT3 proteins was detected by Western blotting analysis. Total levels of the STAT3 protein were not affected by **1**.

were treated with **1** at various concentrations for 72 h, with cell lysates harvested and evaluated by Western blotting analysis. As shown in Figure 2B, the levels of viral NS3 proteins declined upon treatment with **1**. The levels of loading control β -actin remained unchanged. After treatment with **1** for 72 h, viral NS3 proteins in the HCV subgenomic replicon cells were clearly reduced in a dose-dependent manner. Additionally, because Ava5 and Ava5-EG(Δ 4AB)SEAP cells containing the subgenomic replicon systems do not recapitulate all steps of the HCV replication cycle, whether **1** can alter HCV NS3 protein production in an alternative HCV antiviral system constituting all steps involved in the viral life cycle was also examined.^{39–41} The stably transfected Huh7 cell line expressing EG(Δ 4B5A)SEAP protein, designated Huh7-EG(Δ 4B5A)SEAP cells, and the Japanese patient with fulminant hepatitis (JFH-1) strain of HCV were employed to examine the anti-HCV effect of **1**. After treatment of cells with **1** for eight days, the levels of HCV NS3 protein in Huh7-EG(Δ 4B5A)SEAP cells infected with the JFH-1 strain virus were evaluated. As shown in Figure 2C, **1** significantly reduced the levels of HCV NS3 protein at 20 μ M in this alternative system for evaluation of anti-HCV effects. No apparent toxicity was observed for cells treated with **1** over the course of the experiment (data not shown). Despite the development and clinical evaluations of numerous synthetic small-molecule inhibitors, it remains important to also discover novel anti-HCV agents that originate from natural products as potential new drugs, as has been addressed by Newman and Cragg.⁴²

Inhibition of Constitutive Signal Transducer and Activator of Transcription 3 (STAT3) Phosphorylation by Diosgenin (1) in Ava5 Cells. Although the anti-HCV activity of **1** was revealed clearly, the exact mode of action is as yet unknown. Since STAT3 plays a critical role in HCV replication,⁴³ it was investigated as to whether or not **1** can mediate phosphorylation of STAT3 in Ava5 cells. Ava5 cells were incubated with different concentrations of **1** for 72 h, and whole-cell extracts were prepared and the phosphorylation of STAT3 was examined by Western blotting analysis using antibody recognizing STAT3 phosphorylation at tyrosine 705. As shown in Figure 3, it was found that **1** suppresses phosphorylation levels of STAT3 in a dose-dependent manner, with no effect on the production of STAT3 protein. These results demonstrated clearly that **1** inhibits the phosphorylation of STAT3 in Ava5 cells. Thus, besides an anti-HCV effect, **1** has also been demonstrated to exhibit an inhibitory effect on phosphorylation of STAT3. Interestingly, it has been demonstrated that **1** inhibits constitutive activation of STAT3 without affecting the levels of total STAT3 protein.⁴⁴ On the basis of these findings, it appears reasonable to infer that one of the mechanisms by which **1**

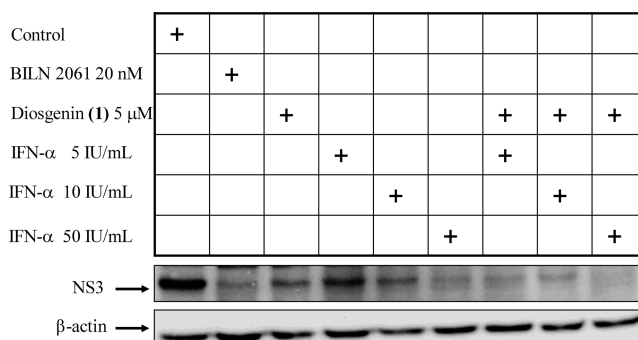


Figure 4. (A) Additive effect of diosgenin (**1**) on the production of HCV NS3 proteins in Ava5 cells. The additive effect refers to the presence of a co-regulatory effect by both **1** and IFN- α that was greater than the effects induced by either compound alone. The cells were treated with **1** (5 μ M) or IFN- α (5, 10, 50 IU/mL) alone and in combination for 72 h. NS3 proteins were detected by Western blot analysis, and the abundance of β -actin protein was also analyzed as an internal standard.

mediates its anti-HCV effect may be through suppression of STAT3 phosphorylation.

Diosgenin (1) Potentiates the Anti-HCV Effect of IFN- α . Since a significant proportion of HCV patients do not respond satisfactorily to IFN- α -based therapy, whether or not diosgenin (**1**) can potentiate the anti-HCV activity of IFN- α is of prime concern. This study evaluated the effect of **1** in combination with IFN- α on HCV replication for 72 h by Western blotting analysis. A potent inhibitor of NS3/4A, BILN 2061 (BILN)⁴⁵ was used as a positive control in this experiment. As shown in Figure 4, minimal levels of NS3 were left in the BILN-treated cells, while **1** alone at 5 μ M partially inhibited HCV replication, as evidenced by the level of remaining viral NS3. IFN- α at 5 or 10 IU/mL exerted partial anti-HCV effects, whereas at 50 IU/mL, nearly complete inhibition of HCV was observed. When 5 μ M of **1** was combined with IFN- α at different doses, all treatments revealed stronger anti-HCV efficacy in this cell-based assay than in counterparts performed without **1**. No cellular cytotoxicity was observed at the concentrations of the different study subjects used, either alone or in combination (data not shown). These results indicate that nearly complete inhibition of HCV replication could be achieved when a low concentration of **1** was combined with low doses of IFN- α .

Recent studies have provided evidence that intake of diosgenin (**1**) regulates certain aspects of acquired immunity, including the enhancement of IFN- γ expression.⁴⁶ This is important because IFN- α /IFN- γ combinations displayed highly synergistic anti-HCV effects.⁴⁷ Very recently, Huang et al. described the use of **1** in enhancing the modulatory T-cell immunity in the intestine of mice with food allergy.⁴⁸ Moreover, the Th1/Th2 cytokine balance has been implicated in the successful HCV treatment by IFN- α and ribavirin combination therapy.⁴⁹ Therefore, it will be important to evaluate whether **1** can benefit the treatment of HCV infection through immunomodulatory effects on T-cell immunity.

To the best of our knowledge, the present study demonstrates for the first time the anti-HCV effect of diosgenin (**1**). Our findings warrant further investigation to examine whether **1** can become an adjuvant to current IFN- α -base therapy. Future research is needed to establish whether this compound is safe and efficacious enough to be incorporated into evolving HCV therapeutic regimens.

EXPERIMENTAL SECTION

Test Compounds and Reagents. Diosgenin (**1**, purity $\geq 95\%$) and IFN- α were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture medium, fetal bovine serum (FBS), antibiotic-antimycotic mixture, and other culture reagents were purchased from Invitrogen Life Technologies Corporation. (Carlsbad, CA). The MTS assay reagents were purchased from Promega Corp. (Madison, WI). Start DNA Master SYBR Green I kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany). BILN 2061 (BILN) was obtained from Acme Bioscience, Inc. (Belmont, CA).

Cell Culture. Ava5 cells are Huh7-derived cell lines.²⁹ The Ava5 cells contain the 5' end of the HCV genome, including the internal ribosomal entry site of the encephalomyocarditis virus and 12 amino acid codons of the core protein, the neomycin phosphotransferase (*neo*) gene, the nonstructural genes NS3 to NSSB of HCV genotype 1b, and the 3'UTR. The Ava5 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, penicillin (50 U/mL), streptomycin (50 mg/mL), and 1% nonessential amino acids as a basal medium at 37 °C with 5% CO₂. Huh7-GL cells contain a chromosomally integrated genotype 2a HCV cDNA and generate infectious virus (JFH-1) continuously.⁴⁰ Huh7-GL cells were kindly provided by Dr. Guangxiang Luo and maintained in basal medium plus 1% nonessential amino acids, 1% sodium pyruvate, and 5 μ g/mL blasticidin (Invitrogen).

Secreted Alkaline Phosphatase (SEAP) Reporter Protein Assay. Our previous study demonstrated the feasibility of using Ava5 cells that stably express the EG(Δ 4AB)SEAP reporter gene to reflect anti-HCV activity by measuring SEAP activity.⁵⁰ Ava5-EG(Δ 4AB)SEAP cells were seeded in 96-well plates at a density of 6×10^3 cells per well. After incubation for 24 h, the cells were treated with **1** at various concentrations. Two days after treatment with **1**, the culture medium was removed and replaced with fresh medium containing **1** at the same concentrations used in the initial incubation; the cells were incubated for one more day. The culture medium was then assayed for SEAP activity by using a Phospha-Light assay kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

Quantitative Real-Time Reverse Transcriptase PCR (RTqPCR). Whether diosgenin (**1**) inhibits HCV replication was examined by collecting cells and then using RTqPCR to detect viral RNA. Ava5 cells were seeded in a six-well plate at a density of 1.5×10^5 cells/well. Following incubation overnight, cells were treated with **1** at various concentrations for 72 h. Upon completion of treatment, total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and resuspended in 10 μ L of 0.1% diethyl pyrocarbonate water. The RT-PCR was performed as described elsewhere. Primers for HCV RNA (genotype 1b) were as follows: forward primer 5'-CTCGACGTTGT-CACTGAA-3' and reverse primer 5'-CCATCCGAGTACGTGC-3'.

Western Blotting. To determine the effect of diosgenin (**1**) on production of HCV NS3 proteins in cells, a Western blotting assay was used. Cells were washed three times in PBS and then lysed with a lysis buffer (50 mM Tris, 150 mM sodium chloride, 1% Triton X-100). The cell lysates were collected and clarified by centrifugation at 15000g for 15 min at 4 °C. Equivalent amounts of all lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein in the gel was transferred to a polyvinylidene difluoride membrane (Millipore, NYSE:MIL). Subsequently, the membrane was blocked with 5% nonfat milk overnight at 4 °C and then incubated for 1 h at room temperature with anti-NS3 antibody, antiphosphorylated STAT3 (Tyr705), or anti-STAT3 antibody. The membrane was also probed with a mouse monoclonal anti-human β -actin antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The amount of β -actin served as a loading control.

Analysis of Test Compound Combinations. To examine the effects of test compound combinations, Western blotting assays were

applied. The interaction effects between diosgenin (**1**) and IFN- α were used in a test compound combination analysis. Test compounds were added to cell culture medium for 72 h. At the end of treatment, the attached cells were washed three times with PBS and stored at -80 °C for further analysis of Western blotting experiments.

Detection of the Effect of Diosgenin (1**) on Intercellular HCV Activity.** The constructions of pEG(Δ 4B5A)SEAP plasmid were performed as described elsewhere. Huh7-EG(Δ 4B5A)SEAP cells were employed to test the effect of antiviral drug treatment. The expression levels of intercellular NS3 protease in HCV-JFH-1-infected Huh7-EG(Δ 4B5A)SEAP cells after administration with **1** were then estimated by seeding cells at a density of 5×10^4 /well in a 24-well plate. Following overnight incubation, cells were infected with HCV-JFH-1 at a multiplicity of infection (MOI = 0.02) for 6 h. Subsequently, unbound viruses were removed by medium replacement, and cells were treated with 20 μ M **1**, which did not cause cytotoxicity, in basal medium for 7 days. Upon completion of test compound treatment, cell lysates were collected and prepared to detect the expression levels of intercellular NS3 protease using Western blotting analysis, as described previously.³⁹

Cytotoxicity Testing. Cells were seeded in 96-well plates and incubated overnight at 37 °C under a 5% CO₂ atmosphere. The medium was then replaced with fresh medium containing various concentrations of diosgenin (**1**), with the incubation continued for five days. The effects of **1** on cell viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay. Briefly, 100 μ L of MTS reagents were added to each well according to the manufacturer's instructions and the plates were incubated for 2 h. Cell viability was measured at 490 nm with a plate reader, and ethanol was used as a control solvent.

Statistical Analysis. All the means obtained are presented with their standard deviations. The statistical significance of the difference in the parameters was determined by a Student's *t* test. A *p* value of <0.05 was considered statistically significant.

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