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RESEARCH ARTICLE

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# First Occurrence of Megastigmane Glucosides in a Plant of *Retama* Genus

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Chemical investigation of *Retama sphaerocarpa* collected in Algeria resulted in the isolation of two megastigmane glucosides, compounds **1** and **2**, along with a series of isoflavones and phenol derivatives. Compound **1**, named retamoside, was new and its structure was determined by extensive application of spectroscopic methods, including HRMS, 1D and 2D NMR and CD. The anti-inflammatory properties of co-occurring main megastigmane, saurobaccioside B (**2**) and structurally related vomifoliol (**3**) on LPS-stimulated murine macrophages RAW 274.7 have been evaluated.

**Keywords:** plant secondary metabolites, megastigmanes, Fabaceae, *Retama sphaerocarpa*, spectroscopic methods.

## Introduction

*Retama sphaerocarpa* (L.) Boiss is a leguminous shrubby species belonging to the tribe Genisteae of the family Fabaceae (subfamily Faboideae).[1] This perennial broom-like shrub, that is almost leafless and with evergreen photosynthetic stems, lives on a variety of soil types, under a large range of climatic conditions, tolerating extreme drought conditions.[1] *R. sphaerocarpa* is endemic to Mediterranean region and widely distributed in the Iberian Peninsula and North Africa including semi-desertic areas.[2] The plant is characterized by the ability to form symbiosis with nitrogen fixing bacteria and to use deep water resources under stressed environmental conditions.[3,4] Due to these characteristics, *R. sphaerocarpa* is widely used in revegetation and soil restoration projects playing an important ecological role in the dynamics of plant communities in arid and semi-arid regions.[5]

On the other side, *R. sphaerocarpa* is also employed as ethnobotanical remedy against diverse diseases in various Mediterranean countries.[6] Some examples include the traditional use as emetic in Morocco,[7] or against rheumatism and contusion pain in Spain,[8] or to cure rabies in animals and humans in Algeria.[9]

Given the environmental importance as well as the use in folk medicine, *R. sphaerocarpa* has been object of several chemical, ethnobotanical, and pharmacological studies.[10] The literature reveals that the chemistry of this plant is typically characterized by the presence of isoflavonoids[11–13] and quinolizidine alkaloids,[13–15] mainly, in accordance with the secondary metabolite profile of other *Retama* species. In addition, fatty acids, sterols, aliphatic alcohols, and terpenoids have been also reported in several phytochemical studies conducted on samples from distinct geographical areas. Nevertheless, due to the significance of *R. sphaerocarpa* in the Mediterranean ecosystem and

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in the economy of North African countries, it seems important to extend the knowledge about the chemical profile of this shrub with the aim at identifying minor compounds not yet described.

During our current chemical investigation of popular medicinal plants from Algerian desertic and semi-desertic regions [16], we have analyzed the metabolite content of aerial parts of *R. sphaerocarpa* collected at M'Sila, North Algeria, during May 2015. The AcOEt-soluble portion of the hydroalcoholic extract of dried aerial parts was chemically examined. As it was expected, the extract was found to be dominated by isoflavones, in agreement with already described studies on *R. sphaerocarpa*, with daidzein,[17–19] genistin[17,20,21] and isoprunetin[22–24] as main components. A series of phenol derivatives were also observed to co-occur in the extract including two megastigmane glucosides **1** and **2**, that were isolated together with related $C_{13}$-*nor*-isoprenoid vomifoliol (**3**) (*Figure 1*). Vomifoliol (=blumenol A) has been so far isolated from various plant sources, mainly as 9-O-glucosyl derivative, roseoside.[25–28] It may have been produced biogenetically by oxidative cleavage of carotenoid conjugated double bonds. Analogous with other degraded carotenoid *nor*-isoprenoids, such as β-ionone, vomifoliol is an important precursor in several natural fragrance and flavor substances.[29]

The spectroscopic analysis conducted on two purified megastigmane glucosides led us to characterize a new vomifoliol 9-*O*-glucoside, retamoside (**1**), along with saurobaccioside B (**2**), which was very recently reported from *Sauropus bacciformis*.[30] Here, the structure elucidation of **1** is described.

## Results and Discussion

A preliminary analysis of 1D and 2D NMR spectra of compound **1** immediately revealed strong structural similarities with co-occurring **2**.[30] The spectra contained signals attributable to a glycosyl moiety, a terpenyl portion and a phenolic unit. In addition, comparison of the part of NMR spectra accounting for terpenyl signals with those of co-occurring vomifoliol (**3**) clearly indicated that **1** was a glycosyl derivative of **3**, analogous with **2**.

Retamoside (**1**) had the molecular formula $C_{29}H_{38}O_{11}$ as deduced by the sodiated-molecular peak at *m/z* 585.2315 $[M+Na]^+$ in the HR-ESI-MS spectrum, that was consistent with the presence of an additional $-OCH_2$ unit with respect to compound **2**. The $^1$H-NMR spectrum of **1** (*Table 1*) showed resonances that were assigned, by analysis of $^1$H-$^1$H COSY, HSQC and HMBC experiments, to five methyl groups, two methylene groups, and fourteen methine groups. Additional eight quaternary carbons were detected by long-range HMBC spectra. The terpenyl moiety, the same as vomifoliol, exhibiting a cyclohexenone ring with three singlet methyl groups and a $C_4$ chain, was easily recognized by observation of the proton spectrum. In fact, this contained signals accounting for two geminal tertiary methyl groups (δ 0.99, 3H, s, $H_3$-11; δ 1.05, 3H, s, $H_3$-12), an α,β-unsaturated trisubstituted double bond bearing a methyl (δ 1.97, 3H, s, $H_3$-13; δ 5.89, 1H, s, H-4), an isolated methylene resonating as an AB system at δ 2.53 (1H, d, $J$=16.0, H-2a) and 2.17 (1H, d, $J$=16.0, H-2b), a disubstituted *trans*-double bond at δ 5.92 (1H, d, $J$=15.5, H-7) and 5.75 (1H, dd, $J$=15.5 and 7.3, H-8), and a carbinolic carbon linking a secondary methyl (δ 1.31, 3H, d, $J$=6.4, $H_3$-10; δ 4.53–4.45, 1H, m, H-9). In addition, a typical set of signals due to a β-glucosyl moiety was also observed in the $^1$H-NMR spectrum: $\delta_H$ 4.53 (1H, dd, $J$=12, 2.2, H-6′a), 4.34 (1H,

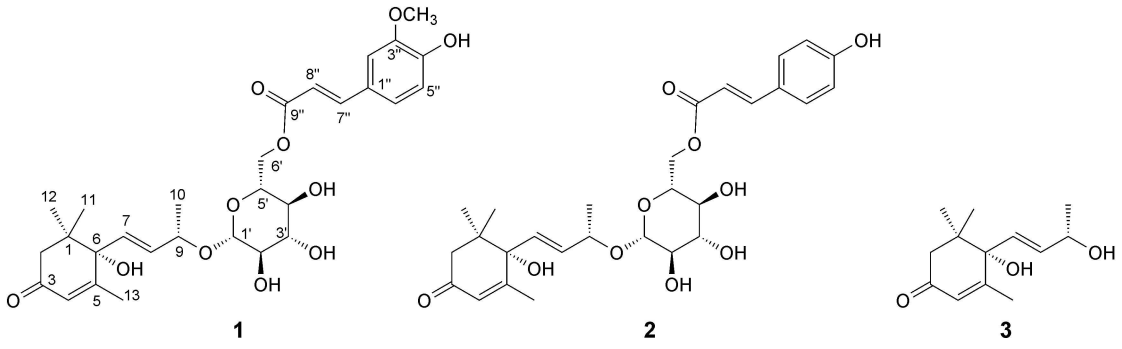


**Figure 1.** Structures of retamoside (**1**) and saurobaccioside B (**2**) isolated from *R. sphaerocarpa* along with $C_{13}$-*nor*-isoprenoid vomifoliol (**3**).

 

**Table 1.** $^{1}H$ and $^{13}C$-NMR spectroscopic data[a] for compound **1** in $CD_3OD$

| No. | $\delta_C$[b] | $\delta_H$ (*J* in Hz) |
|---|---|---|
| 1 | 41.8, C | – |
| 2 | 50.3, $CH_2$ | 2.53 (d, 16.0) |
| | | 2.17 (d, 16.0) |
| 3 | 200.0, C | – |
| 4 | 126.9, CH | 5.89 (s) |
| 5 | 166.2, C | – |
| 6 | 79.8, C | – |
| 7 | 133.5, CH | 5.92 (d 15.5) |
| 8 | 133.3, CH | 5.75 (dd, 15.5,7.3) |
| 9 | 74.4, CH | 4.53–4.45 (m) |
| 10 | 22.0, $CH_3$ | 1.31 (d, 6.4) |
| 11 | 24.3, $CH_3$ | 0.99 (s) |
| 12 | 23.0, $CH_3$ | 1.05 (s) |
| 13 | 19.2, $CH_3$ | 1.97 (s) |
| 1′ | 100.6, CH | 4.32 (d, 7.7) |
| 2′ | 74.5, CH | 3.29–3.23 (m) |
| 3′ | 77.9, CH | 3.36–3.32 (m) |
| 4′ | 71.3, CH | 3.36–3.32 (m) |
| 5′ | 75.4, CH | 3.44–3.40 (m) |
| 6′ | 64.5, $CH_2$ | 4.53 (dd, 12, 2.2) |
| | | 4.34 (dd, 12, 6.4) |
| 1″ | 127.0, C | – |
| 2″ | 111.3, CH | 7.22 (br. s) |
| 3″ | 149.0, C | – |
| 4″ | 150.3, C | – |
| 5″ | 114.6, CH | 6.84 (d, 8.0) |
| 6″ | 115.9, CH | 7.10 (dd, 8.0, 1.1) |
| 7″ | 146.7, CH | 7.66 (d, 15.8) |
| 8″ | 114.2, CH | 6.39 (d, 15.8) |
| 9″ | 168.3, C | – |
| OMe | 55.9, $CH_3$ | 3.94 (s) |

[a] Assignments aided by COSY, HSQC and HMBC ($J=7$) experiments. [b] By 2D NMR experiments.

dd, $J=12$, 6.4, H-6′b), 4.32 (1H, d, $J=7.7$, H-1′), 3.44–3.40 (1H, m, H-5′), 3.36–3.32 (2H, m, H-3′ and H-4 ′), and 3.29–3.23 (1H, m, H-2′). Further, the $^{1}H$-NMR spectrum of **1** contained signals due to the phenolic portion at $\delta_H$ 7.22 (1H, br. s, H-2″), 7.10 (1H, dd, $J=$ 8.0,1.1, H-6″), and 6.84 (1H, d, $J=8.0$, H-5″), and a methoxy singlet at $\delta_H$ 3.94, as well as olefinic resonances attributed to the conjugated *trans*-double bond at $\delta_H$ 7.66 (1H, d, $J=15.8$, H-7″), and 6.39 (1H, d, $J=15.8$, H-8″) (*Table 1*). These data suggested the presence in compound **1** of a *trans*-feruloyl (or 3-methoxy-4-hydroxycinnamoyl) moiety. The acyl unit esterified 6′-OH as it was suggested by the observed acylation shift on C-6′ methylene of the glucose residue (*Table 1*) and further supported by a diagnostic HMBC between glucose methylene $H_2$-6′ and C-9″ carboxy of *trans*-feruloyl moiety. On the other side, the β-glucosyl linkage was assessed to be at C-9 of the terpenyl unit by an indicative HMBC between H-9 and C-1′ anomeric carbon of glucose. Thus, the gross structure was determined as depicted in formula **1**. The assignment of all proton and carbon NMR values of compound **1** was aided by 2D NMR experiments ($^{1}H$-$^{1}H$ COSY, HSQC and HMBC) (*Table 1*) and agreed with the proposed planar structure.

The structure elucidation was completed by the assessment of the absolute configuration of C-6 and C-9 stereogenic centers. All (C-6, C-9)-stereoisomers of vomifoliol as well as the corresponding glucosyl derivatives, roseosides, have been found to occur in nature [e.g., 31, 32]. In the past, several conflicting data have been appeared in the literature with regards to the assignment of the absolute configuration at C-6 and C-9 due to the difficulty of discriminating diastereoisomers by spectroscopic methods. However, stereospecific syntheses of four possible (6*S*,9*S*)-, (6*S*,9*R*)-, (6*R*,9*S*)-, and (6*R*,9*R*)-diastereoisomers of both vomifoliol and roseoside have been also realized[33,34] and fully spectroscopic characterization has been reported allowing the secure assignment of the configuration of natural samples.

In retamoside (**1**) the configuration at C-6 was determined by comparing the circular dichroism profile of **1** with those reported for the pairs of synthetic C-6 epimers of vomifoliol and their glucosyl derivative roseosides.[34] As expected, the sign of Cotton effect in these compounds is depending only on the C-6 configuration whereas C-9 configuration does not affect the curve. Since CD spectra of **1** showed a positive Cotton effect at 241 nm, C-6 was assessed to have the *S* absolute configuration. On the other side, the 9*S* absolute configuration was assigned by comparing the $^{13}C$-NMR values of C-1′, C-9, and C-10 of **1** (*Table 1*) with those of 9*R* and 9*S* epimers of roseoside.[35] In fact, diagnostic differences of the $\delta_C$ values of this carbon set can be generally observed for 9*R* and 9*S* epimers of 9-hydroxy-megastigmane 9-*O*-glucopyranosides. Typically, C-1′, C-9, and C-10 resonate at 100.5–101.7, 74.7–76.3, and 22.3–22.6 ppm, respectively, in 9*R* *versus* 102.2–103.0, 77.3–79.1, and 21.2–21.8, respectively, in 9*S*.[35]

Thus, the structure of retamoside (**1**) was defined as (6*S*,9*S*)- 6′-*O*-*trans*-feruloyl-roseoside. Compound **1** differed from co-occurring saurobaccioside B (**2**) in the substitution pattern of phenol unit whereas the remaining part of the molecule including the configuration of the stereogenic centers was the same.

Interestingly, analysis of spectroscopic data of vomifoliol (**3**) co-occurring with **1** and **2** in *R.*

 

*sphaerocarpa* showed that the sample we isolated was not stereochemically pure revealing to be an epimeric mixture at C-6. In fact, a double set of signals were observed in the $^1$H-NMR spectrum of **3** for three methyl singlets linked to C-1 and C-5 as well as the vinylic protons of $\Delta^7$ double bond. However, the epimer with 6*S* configuration was prevalent as it was also supported by the positive CD curve of **3** even though $\Delta\varepsilon$ value at 243 nm resulted lower with respect to the literature.[34]

Megastigmane glycosides, of which roseosides are the most famous members, are an important group of natural products widely distributed in plants. Several types of glycosylation and functionalization including further esterification of glycosyl hydroxy groups have been found to occur. A close structural analog of compounds **1** and **2**, 6′-*O*-caffeoyl-(6*S*,9*R*)-roseoside, has been reported in the literature from Korean *Aster glehni*.[36] However, it is noteworthy that this class of metabolites has never been described from the genus *Retama*.

A series of interesting biological activities have been reported for megastigmane glycosides including inhibitory activity on leukotriene release,[33] insulin releasing properties,[37] antioxidant and XOD inhibitory activities,[38] and histamine release inhibitory properties[32,39] among others. Due to this, compound **2**, which was available in larger amount with respect to **1**, was investigated for its anti-inflammatory properties along with the co-occurring vomifoliol, which represents the apocarotenoid portion of these megastigmanes.

To provide the cytoprotective and anti-inflammatory activity of these two compounds, we used RAW264-7, a mouse macrophage-like cell lines treated with lipopolysaccharide (LPS from *E. coli*), known to induce inflammation and consequently cell death. As shown in *Figure 2*, both saurobaccioside B and vomifoliol (24 h before LPS) were able to reduce the LPS-induced cell death (0.5 μg/mL; 24 h), determined by the MTT assay. Saurobaccioside B exerted a protective action at a concentration of 25 μM, whereas vomifoliol is active only at 50 μM (*Figure 2*).

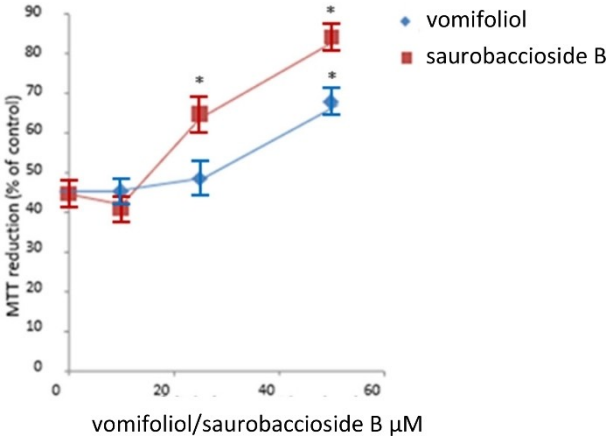


**Figure 2.** Effect of saurobaccioside B (**2**) and vomifoliol (**3**) on vitality of LPS-stimulated murine macrophages (RAW 264.7). Results represent the mean ± SEM of three to five separate experiments, each performed in duplicate. *$p < 0.001$ vs. control cells (one-way ANOVA followed by Bonferroni test).

## Conclusions

In this study, the metabolite content of the liposoluble extract of *R. sphaerocarpa* aerial parts from Algeria has been chemically investigated. Along with isoflavones and phenol derivatives that were already described from this species, two megastigmane glucosides, retamoside (**1**) and saurobaccioside B (**2**), have been isolated. Related $C_{13}$-*nor*-isoprenoid vomifoliol (**3**) has been also identified in the extract. New compound **1** has been fully characterized by spectroscopic methods. This is the first report of megastigmane compounds from plants of *Retama* genus. Compound **2** displayed anti-inflammatory activity on LPS-stimulated murine macrophages RAW 274.7 at 25 μM.

## Experimental Section

### *General Section*

Optical rotations were measured on a Jasco DIP 370 digital polarimeter. The UV spectra and CD curves were recorded on an Jasco V650 spectrophotometer and on a JASCO F815 spectropolarimeter, respectively. High-resolution mass spectra (HR-ESI-MS) were acquired on a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA). NMR experiments were recorded at the ICB-NMR Service Center. Chemical shifts values are reported in ppm and referenced to internal signals of residual protons ($CD_3OD$, δ 3.34 for H-atom, δ 49.0 for carbon). 1D and 2D NMR spectra were acquired on a Bruker Avance-400 spectrometer using an inverse probe fitted with a gradient along the Z-axis, and on a Bruker Avance III HD 400 MHz spectrometer equipped with a CryoProbe Prodigy. Analytical and preparative TLC were performed on precoated $SiO_2$ plates (Merck Kieselgel 60 F254, 0.25 and 0.5 mm), with detection provided by UV light (254 nm) and by spraying with

 

$Ce(SO_4)_2$ reagent followed by heating (120 °C). $SiO_2$ column chromatography was performed using Merck Kieselgel 60 powder (0.063–0.200 mm). HPLC separation was carried out using a Jasco PU-4180 liquid chromatograph equipped with a Jasco 4075 UV/Vis wavelength detector (210 and 254 nm) on a RP-Amide semipreparative column (Ascentis, 250×10 mm, 5 μm, Supelco).

### *Plant Material*

*Retama sphaerocarpa* was collected near Boussada (M'Sila region, northest, Algeria) in May 2015. The plant was identified by Dr. Khellaf Rebbas of the Mohammed Boudiaf-M-Sila University and a voucher specimen (code VRTM05015) was deposited at the Herbarium of the VARENBIOMOL Research Unit, University des Frères Mentouri, Constantine, Algeria.

### *Extraction and Purification*

Air-dried aerial parts of *R. sphaerocarpa* (4500 g) were macerated with a hydroalcoholic solution of MeOH/$H_2O$ (8:2, v/v) for 48 h at room temperature three times. After filtration, the organic solvent was evaporated to give a crude residue, which was suspended in water and successively extracted with $CHCl_3$, then AcOEt and finally butanol. The organic phases were concentrated to give the corresponding extracts: $CHCl_3$ (14.0 g), AcOEt (15.0 g) and butanol (116.0 g), respectively. A portion (7.7 g) of the AcOEt extract was fractionated by silica-gel column chromatography by eluting with a gradient (from 0 to 100%) of AcOEt in hexane to obtain 36 fractions. Selected fractions (C14, C17, C18, C19, C31, C32, C33) were taken into consideration after TLC chromatography analysis and preliminary $^1$H-NMR inspection. Isoprunetin (55.9 mg) was isolated as a precipitate from fraction C14 (348.0 mg), collected in hexane/ethyl acetate 6:4, and further purified by following precipitation in MeOH. Daizdein was the main compound present in fractions C17 (117.0 mg), C18 (80.0 mg) and C19 (44.0 mg) from which it was isolated by precipitation in hexane/ethyl acetate 1:1. Further crystallization in MeOH yielded 56.0 mg of pure daizdein. The remaining part of fraction C17 (60.0 mg) was purified by HPLC using as eluent gradient of $CH_3CN/H_2O$ (TFA 0.1%) starting from 60% of $CH_3CN$ to 100% of $CH_3CN$ in 30 min. Peak eluted at R*t* 13.4 has been identified as vomifoliol (7.0 mg). Fraction C31 (60.0 mg) was subjected to HPLC purification on a RP-Amide column using as eluent system a gradient of $CH_3CN$ in $H_2O$ starting from 30% to100% in 40 min and $CH_3CN$ 100% for further 5 min, with a flow rate of 2 mL/min. Peak eluted at R*t* 14.5 min was identified after NMR analysis and MS as saurobaccioside B (**2**, 1.7 mg). Fraction C32 (98.7 mg) was purified on $SiO_2$ column in a slow gradient of $CHCl_3/CH_3OH$ obtaining 14 sub-fractions (C32-1-C32-14). Subfraction C32-5 (37.5 mg) was further subjected to a $SiO_2$ column in the same eluent conditions to yield 14 subfractions (C32-5-1-C32-5-14). Subfraction C32-5-6 (10.7 mg) was loaded onto a semipreparative TLC, developed with $CHCl_3/CH_3OH$ 85:15, to give a UV compound at R*f* 0.75 that was identified as retamoside (**1**, 0.9 mg). Fraction C33 (196.3 mg) was purified by $SiO_2$ gel column chromatography with a gradient of MeOH in $CHCl_3$ to give 36.3 mg of pure genistin.

**Retamoside** (**1**): colorless oil; $[\alpha]_D = +1.3$ ($c = 0.04$, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 237 (4.90) nm; ECD ($c = 0.0032$, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 241 (+3.42), 323 (−0.3); $^1$H and $^{13}$C-NMR data see *Table 1*; HR-ESI-MS $[M+Na]^+$ *m/z* 585.2315 (calc. for $C_{29}H_{38}O_{11}$ Na 585.2312).

**Saurobaccioside B** (**2**): colorless oil; $[\alpha]_D = +6.8$ ($c = 0.06$, MeOH), lit.[30] $[\alpha]_D = +5.1$ ($c = 0.1$, MeOH); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 237 (4.61) nm; ECD ($c = 0.0032$, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 246 (+3.3), 322 (−0.7); $^1$H and $^{13}$C-NMR data in agreement with literature;[30] HR-ESI-MS $[M+Na]^+$ *m/z* 555.2207 (calc. for $C_{28}H_{36}O_{10}Na$ 555.2206).

**Vomifoliol** (**3**): white powder; $[\alpha]_D = +79.5$ ($c = 0.12$, $CHCl_3$), lit.[32] $[\alpha]_D = +184.3$ ($c = 0.1$, $CHCl_3$); UV (MeOH) $\lambda_{max}$ (log $\varepsilon$) 237 (4.90) nm; ECD ($c = 0.0019$, MeOH) $\lambda_{max}$ ($\Delta\varepsilon$) 243 (+8.2), 322 (−0.79); lit.[31] (c = 0.0021, MeOH) ($\Delta\varepsilon$) 241 (+16.5), 320 (−0.9).

### *Cell Culture*

The RAW 264.7 cell lines were purchased from Sigma–Aldrich (cat. No. 91062702). Cells were cultured in DMEM high glucose medium supplemented with 10% FBS and 1% penicillin/streptomycin, (HyClone Laboratories, Logan, UT, USA) in 100-mm dishes (Falcon, Becton Dickinson Labware, Francklin Lakes, NJ, USA) gassed with an atmosphere of 95% air- 5% $CO_2$. For experiments, the cells were plated into 35-mm culture dishes. At the treatment stage, total cell number was between 2.0 and $2.5\times10^5$ cell/dish.

 

*Cellular Viability Assay*

The decrease in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was assessed as a measure of cell viability.[40] Briefly, after treatments, the MTT solution (5 mg/mL) was added to the culture and incubated for an additional 4 h. The medium was then removed and replaced with one mL of dimethyl sulfoxide extracts using an ELISA 96% plate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm of the absorbance. Results are expressed as the percentage of MTT-reducing activity of treated versus untreated cells.

*Statistical Analysis*

Cellular viability data are expressed as mean ± SEM and were analyzed with GraphPad Prism 6 software, version 6.05 (GraphPad, Inc.). Statistical differences among groups were determined by either Student's t-test or two-way ANOVA followed by post hoc Bonferroni tests for comparison among means. A level of confidence of $P < 0.05$ was used for statistical significance.

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## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Conflict of Interest

The authors declare no conflict of interest.

## Author Contribution Statement

Conceptualization, M.L.C., and S.B.; methodology, O.S.B. L.P., and M.L.C.; investigation, O.S.B., A.B., and A.C.; writing – original draft preparation, M.G., and M.L.C.; writing – review and editing, M.L.C., M.C., and M.G.; All authors have read and agreed to the published version of the manuscript.

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