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ORIGINAL ARTICLE

Evaluation of Wound Healing Potential of (5-formylfuran-2-yl)Methyl Benzoates by *In Vitro* Cell Culture Studies

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ABSTRACT

Objectives: 5-hydroxymethylfurfural (5-HMF) derivatives, found in many natural products, exhibit various biological activities. Benzoate derivatives enhance cellular re-epithelialization without inducing cytotoxicity. This study aimed to evaluate the wound-healing potential of six benzoate derivatives of 5-HMF by usingan *in vitro* cell culture method.

Materials and Methods: Six ester derivatives of 5-HMF were synthesized, and the molecular structures were elucidated by proton-1 nuclear magnetic resonance, carbon-13 nuclear magnetic resonance, and Quadrupole time-of-flight mass spectrometry. The effects of the compounds on neuronal and fibroblast cell survival were determined *in vitro* using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The total oxidation state (TOS) and total antioxidant capacity (TAC) were measured spectrophotometrically. The effects of the lead compounds (M3 and M5) on fibroblast migratory potential were evaluated using a wound-healing scratch assay.

Results: The MTT test showed that M2, M3, M5, and M6 did not damage fibroblasts at any tested concentration (10^{-1} µM–10^{-2} µM). M3 and M5 increased fibroblast cell numbers at all tested concentrations ($p \leq 0.05$ to $p \leq 0.001$). It was observed that M2, M3, M5, and M6 at concentrations of 10^{-1} µM and 1 µM did not damage the neuronal cells. Also, M3 and M5 did not damage neuronal cells at 10 µM. M3 and M5 exhibited lower oxidant activity ($p \leq 0.001$) than other compounds in TOS tests, and showed higher antioxidant activity than other compounds in TAC tests. In the scratch test, the wound area in the M3 group was 72% on the first day and decreased to 32% on the second day ($p \leq 0.001$). On the other hand, in the M5 group, the wound area was 81% on the first day and decreased to 25% on the second day ($p \leq 0.001$).

Conclusion: M3 and M5 promote cell migration and have the highest potential for wound healing among the compounds tested.

Keywords: Ester derivatives of 5-hydroxymethylfurfural, cell viability assay, total antioxidant capacity, cell migration

INTRODUCTION

The skin acts as a barrier, protecting the body against assaults by external agents, such as chemicals, microorganisms, and physical insults. Therefore, a successful and short wound healing process is critical in practice.[1] Fibroblasts are critical for promoting normal wound healing, including the breakdown of the fibrin clot and the formation of collagen structures. Fibroblasts migrate to the skin wound and form granulation tissue.[2]

To understand the biological behavior of a substance, it is necessary to determine its toxic or non-toxic effects on cells. *In vitro* cytotoxicity assays are performed in cell culture to evaluate substances with drug-like properties or to investigate their toxic profiles.[3]

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Oxidative stress causes oxidative damage, which leads to tissue damage or delayed healing of damaged tissues.[4] Therefore, the total oxidation state (TOS) test is used to predict the oxidation potential of a compound in cells. Similarly, the total antioxidant capacity (TAC) test is used to assess the antioxidant potential of a compound within cells.[5]

5-hydroxymethylfurfural (5-HMF) (Figure 1) is an organic compound consisting of a furan ring with aldehyde and alcohol functional groups, and is formed from the dehydration of carbohydrates such as fructose, glucose, sucrose, cellulose, and inulin.[6] 5-HMF can be formed by the Maillard reaction when sugar-containing foods, such as honey, are heated under acidic conditions.[7] In addition, 5-HMF and its derivatives are found in many plant extracts.[8]

Some preclinical studies suggested that 5-HMF might be carcinogenic.[9] On the other hand, studies reveal aspects of 5-HMF and its derivatives that may benefit human health.[10] 5-HMF acts as an antioxidant,[11] protective against hypoxic injury,[12] anti-inflammatory,[13] tyrosinase inhibitor.[14] Antidiabetic, antiproliferative, antibacterial, antisickling, and analgesic properties of 5-HMF derivatives (ester, alkyl ether, and aryl ether) have been reported in the literature.[15-18]

Antioxidant, antifungal, antipsoriatic, and antithrombotic effects of salicylic acid and benzoic acid derivatives have been reported.[19,20] Benzoate derivatives have also been reported to improve cell re-epithelialization without cytotoxic effects.[21]

Wound healing is a complex biological process that involves hemostasis, inflammation, re-epithelialization, and tissue maturation, which are interconnected and occur sequentially. One of the most important factors affecting wound healing is oxidative stress.

This study aimed to evaluate the wound healing potential of 6 benzoate derivatives of 5-HMF by using the *in vitro* cell culture method. For this purpose, 5-HMF ester derivatives of nonsubstituted, 4-OCH3, 4-F, 4-F3C, 2-OH, and 4-CN benzoic acids were synthesized. Then, the wound-healing potential of these synthesized compounds was evaluated *in vitro* using MTT cytotoxicity tests in primary neurons and fibroblasts, and by TAC and TOS assays. In addition, the wound-healing capacities of the compounds on injured L929 fibroblasts were evaluated using an *in vitro* fibroblast migration assay.

MATERIALS AND METHODS

Chemistry

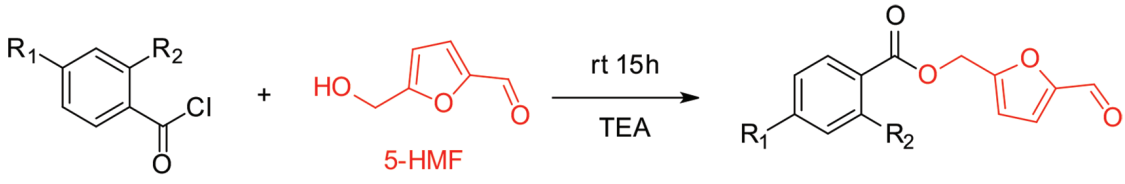
^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker FT-400 spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz), using $CDCl_3$ as the solvent and tetramethyl silane as the internal standard. Coupling constants are given in hertz (Hz). Q-TOF LC-MS mass spectra were recorded on an Agilent 6530 Accurate-Mass instrument. The Melting points of the compounds were determined using an Electrothermal 9100 melting-point apparatus. All chemicals were obtained from Merck or Sigma-Aldrich.

General procedure for the synthesis of 5-HMF

One equivalent of D-fructose (3.6 g) was dissolved in dimethyl sulfoxide (DMSO) (40 mL) in a 100-mL glass bottle. After that, 0.1 equivalent of $FeCl_3{\cdot}H_2O$/activated charcoal (135 mg and 800 mg, respectively) was added to the mixture as the catalyst. The reaction mixture was heated at 90 °C in an oil bath with magnetic stirring for 5 h. Afterward, 80 mL of water was added, and the mixture was extracted three times with 30 mL portions of ethyl acetate. The organic layer was separated, and the solvent was evaporated. The product could be directly employed in the next step. Additionally, the residue was purified by column chromatography (4:1 petroleum ether/EtOAc) to obtain pure 5-HMF, completely separated from DMSO, as a brownish-yellow syrup (77% yield).[22]

General procedure for the synthesis of (5-formylfuran-2-yl) methyl benzoate derivatives

5-HMF (366 mg, 1 mmol) and benzoyl chloride derivatives (1.5 mmol) were dissolved in 8 mL of CH_2Cl_2. Afterward, the mixture was cooled to 0 °C, and trimethylamine (606 g, 2 mmol) was added slowly (Figure 1). The resultant reaction mixture was stirred at 0 °C for 30 min, then at room temperature for 15 h. Then the mixture was concentrated, extracted with CH_2Cl_2/H_2O, and the organic layer was dried over Na_2SO_4. The resultant crude oil was purified by silica gel column chromatography and eluted EtOAc/hexanes (6:4), yielding 80-93% (Figure 1).



M1 (R_1:-H R_2:-H)
M2 (R_1:-OCH_3 R_2:-H)
M3 (R_1:-F R_2:-H)
M4 (R_1:-F_3C R_2:-H)
M5 (R_1:-H R_2:-OH)
M6 (R_1:-CN R_2:-H)
M7 (R_1:-H R_2:-$O(CO)CH_3$)

Figure 1. Synthesis of the target compounds
5-HMF: 5-hydroxymethylfurfural

(5-Formylfuran-2-yl)methyl benzoate (M1)

Yield: 80%, mp: 64-66 ˚C, White crystal.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.38 (s, 2H, CH_2), 6.68 (d, 1H, J:3.5 Hz, CH_{furan}), 7.24 (d, 1H, J:3.5 Hz, CH_{furan}), 7.45 (d, 2H, J:7.7 Hz, Ph-H), 7.58 (d, 1H, J:7.4 Hz, Ph-H), 8.34-7.85 (m, 2H, Ph-H), 9.65 (s, 1H, CHO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 58.2, 112.84, 121.94, 128.50, 129.23, 129.84, 133.48, 152.85, 155.52, 165.94, 177.90. HRMS (Q-TOF) *m/z* Calcd for [M+H]+ 231.0579, found. 231.0651.

(5-Formylfuran-2-yl)methyl 4-methoxybenzoate (M2)

Yield: 78%, mp: 40-42 ˚C, Yellow crystal.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 3.86 (s, 3H, Ph-OCH_3), 5.35 (s, 2H, CH_2), 6.67 (d, 1H, J:3.5 Hz, CH_{furan}), 6.92 (d, 2H, J:8.9 Hz, Ph-H), 7.25 (d, 1H, J:3.5 Hz, CH_{furan}), 8.01 (d, 2H, J:8.9 Hz, Ph-H), 9.64 (s, 1H, CHO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 58.48, 58.01, 112.70, 113.75, 121.54, 131.94, 152.78, 155.15, 155.92, 163.76, 165.73 177.98. HRMS (Q-TOF) *m/z* Calcd for [M+H]+ 261.0685, found. 261.0756.

(5-Formylfuran-2-yl)methyl 4-fluorobenzoate (M3)

Yield: 83%, Yellow liquid.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.36 (s, 2H, CH_2), 6.67 (d, 1H, J:3.3 Hz, CH_{furan}), 7.10 (d, 2H, J:8.7 Hz, Ph-H), 7.23 (d, 1H, J:3.6 Hz, CH_{furan}), 8.0 (d, 2H, J:5.4 Hz, Ph-H), 9.64 (s, 1H, CHO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 58.31, 112.91, 115.55, 115.8, 122.04, 125.51, 132.38, 132.48, 132.74, 132.84 152.87, 155.41, 164.98, 177.99. HRMS (Q-TOF) *m/z* for $[M+H]^+$, calculated: 249.0485, found: 249.0483.

(5-Formylfuran-2-yl)methyl 4-(trifluoromethyl)benzoate (M4)

Yield: 92%, mp: 79-81 ˚C, Yellow crystal.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.41 (s, 2H, CH_2), 6.71 (d, 1H, J:3.5 Hz, CH_{furan}), 7.25 (d, 1H, J:3.5 Hz, CH_{furan}), 7.71 (d, 2H, J:8.2 Hz, Ph-H), 8.17 (d, 2H, J: 8.1 Hz, Ph-H), 9.66 (s, 1H, CHO). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 58.61, 113.17, 121.83, 122.16, 124.87, 125.54, 125.51, 129.98, 130.26, 132.47, 154.86, 164.77, 177.88. HRMS (Q-TOF) *m/z* Calcd for [M+NH_4]+ 316.0791, found. 316.0791.

(5-Formylfuran-2-yl)methyl 2-hydroxybenzoate (M5)

Yield: 78%, mp: 67-69 ˚C, Yellow crystal.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.40 (s, 2H, CH_2), 6.71 (d, 1H, J:3.2 Hz, CH_{furan}), 6.88 (d, 1H, J:7.5 Hz, Ph-H), 6.98 (d, 1H, J:8.3 Hz, Ph-H), 7.25 (d, 2H, J: 3.3 Hz, Ph-H), 7.47 (d, 1H, J:7.6 Hz, Ph-H), 7.85 (d, 2H, J: 7.8 Hz, Ph-H), 9.66 (s, 1H, CHO), 10.53 (s, 1H, Ph-OH). ^{13}C-NMR (100 MHz, $CDCl_3$) δ (ppm): 58.31, 111.68, 113.29, 117.67, 119.39, 121.79, 130.09, 136.29, 152.99, 154.64, 161.78, 169.44, 177.92. HRMS (Q-TOF) *m/z* Calcd for [M+H]+ 247.0528, found. 247.0601.

(5-Formylfuran-2-yl)methyl 4-cyanobenzoate (M6)

Yield: 90%, mp: 91-93 ˚C, White crystal.

^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm): 5.43 (s, 2H, CH_2), 6.68 (d, 1H, J:3.6 Hz, CH_{furan}), 7.23 (d, 1H, J:3.2 Hz, CH_{furan}), 7.73 (d, 2H, J:8.7 Hz, Ph-H), 8.13 (d, 2H, J: 8.4 Hz, Ph-H), 9.63 (s, 1H, CHO). ^{13}C-NMR (100 M*Hz*, $CDCl_3$) δ (ppm): 58.79, 113.29, 116.85, 117.82, 121.78, 130.34, 132.31, 133.08, 153.04, 154.58, 164.34, 177.84. HRMS (Q-TOF) *m/z* Calcd for [M+Na]+ 278.0430, found. 278.0425.

Cell cultures

Since the compounds were dissolved in DMSO and applied in cell culture studies, DMSO was used as the control. The effects of doses of M1-M6 (0.1, 1, 10, and 100 µg/mL), which were identified as non-toxic in MTT tests on neuronal cell lines, were evaluated in other *in vitro* experiments (TAC, TOS, Cell Migration Test).

Primary neuron culture

Sprague-Dawley rat pups younger than 24 hours were used to obtain cortical and brain neurons. After the rats were swiftly decapitated, the excised cortices were transferred to 5 mL of Hanks' Balanced Salt solution; macro-fragmentation was performed with a scalpel, and microlysis was performed using trypsin-ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin-0.02% EDTA). The cells were then centrifuged at 1200 rpm for 5 min. Fresh medium [88% Neurobasal medium, Gibco, USA, 10% fetal bovine serum (FBS, Gibco, USA), 2% B-27 supplement (ThermoFisher, Germany), 0.1% antibiotic (penicillin-streptomycin), and amphotericin B (Thermo Fisher, Germany)] was added to the cells that had settled to the bottom.[23]

L929 fibroblast culture

L929 mouse fibroblasts were obtained from the Medical Pharmacology Department, Atatürk University (Erzurum, Türkiye). Then, the cells were centrifuged at 1200 rpm for 5 min. The cells were suspended in fresh medium [89% Roswell Park Memorial Institute, 10% FBS, and 1% antibiotic (penicillin-streptomycin) and amphotericin B (ThermoFisher, Germany)]. They were grown in T-25 culture flasks until they reached 70-80% confluency. The prepared flasks were stored in an incubator with 5% CO_2 at 37 ˚C. When 80% of the flasks were covered by cells, the cells were removed from the flasks with trypsin-EDTA (0.25% trypsin-0.02% EDTA) and centrifuged. The supernatant was discarded, and the cell suspension was distributed homogeneously to 24-well tissue culture plates at 400 µL/well (40,000 cells/well). It was incubated at 5% CO_2 and 37 ˚C until the cells reached confluence.

Cell cultures MTT assay

The direct contact test was applied to determine the cytotoxicity of the materials. The evaluation was performed using the MTT reagent (Sigma Aldrich Inc., St. Louis, USA) containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide. To determine cytotoxicity by the MTT test, a mixture was prepared consisting of 1 mL of 5 mg/mL MTT solution in PBS. The outer surface was covered with aluminum foil and was kept in the refrigerator. After the culture media from the incubated cells were removed, the previously prepared samples were placed into each well and incubated again for 24 hours at 37 ˚C in a 5% CO_2 atmosphere. Thus, the cytotoxic effects of the materials were evaluated at the end of 24 hours. A total of 99.4 µL of the mixture containing DMSO, 0.6 mL HCl, and 10 g sodium lauryl sulphate was added to each well at 100 µL/well to dissolve

the formazan crystals formed following MTT application, and the plates were incubated again for 4 hours. After this process, the absorbance (optical density) was measured using a spectrophotometer (μQuant, Bad Friedrichshall, BioTek) at a wavelength of 570 nm. Data are presented graphically following SPSS analysis.[24]

TAC-TOS assay

The TOS and TAC were evaluated by spectrophotometric measurement. The cell culture medium was harvested when the experiment was complete. According to the manufacturing process, the TAC absorbance at 660 nm (Trolox equiv/mmol L-1) and the TOS absorbance at 530 nm (H_2O_2 equiv/mmol L-1) were evaluated.

At the end of the experiment, morphological changes were assessed using an inverted microscope (Leica, USA). Images of all treatment groups, exposed to the doses for the desired period, were taken at 20× magnification.

Inspections with an inverted microscope

In cell culture, the distance between wound edges in six randomly selected areas for each dose was measured in μm.

Statistical analysis

The results are presented as the mean ± standard error. Statistical comparisons between groups were calculated using one-way analysis of variance and Tukey's honestly significant difference method. All calculations were performed using SPSS version 20. $p<0.05$ and $p<0.001$ were considered statistically significant in all tests. To determine normality, the Shapiro-Wilk tests were used.

L929 cell line migration test

L929 fibroblast cells cultured in tissue culture plates had their medium renewed every three days until the cells in the wells reached 100% growth. When the desired density was achieved, wounds were created in the cell monolayers on tissue culture plates using a 100 μL pipette tip. The synthesized substances (M1-M6) were administered at concentrations of 0.1, 1, 10, and 100 μM. After the doses were administered, the experiment was terminated upon establishment of the first bridge in the wound.

Ethics

Ethics committee approval of the study was received from the Atatürk University Animal Experiments Local Ethics Committee (approval number: 49, dated: 26.02.2024) and Atatürk University Non-Interventional Research Ethics Committee (approval number: 120, dated: 29.03.2024).

RESULTS

According to the *in vitro* fibroblast MTT assay results (Figure 2), compound-treated groups exhibited higher viability rates (95-123%) than the control group ($p<0.001$). In particular, the group treated with compound M3 at 10 μM exhibited the highest viability compared with the control group ($p<0.001$).

The *in vitro* fibroblast TAC assay data for all compounds are summarized in Figure 3. The TAC levels of the compounds in fibroblast cell culture were 1.41-1.87 Trolox equivalents (mmol L-1). In particular, the groups treated with compounds M3 and M5 at 10 μM exhibited the highest antioxidant capacity compared with the control group ($p<0.001$).

The *in vitro* fibroblast TOS assay data for all compounds are summarized in Figure 4. The TOS level in the M3-treated group at 100 μM was significantly lower than that in the control group ($p<0.001$).

The *in vitro* neuronal MTT assay data for all compounds are summarized in Figure 5. In MTT tests, when the compounds were applied to neuronal cells at 0.1 μM, cell survival exceeded 90%. In MTT tests, when M1, M2, M4, and M6 were administered to neuronal cells at 10 μM, statistically significant toxic effects were observed (for M1, M2, M6 $p<0.05$ and for M4 $p<0.001$). Significant toxicity was observed only at 100 μM in the groups administered M3 and M5 ($p<0.001$).

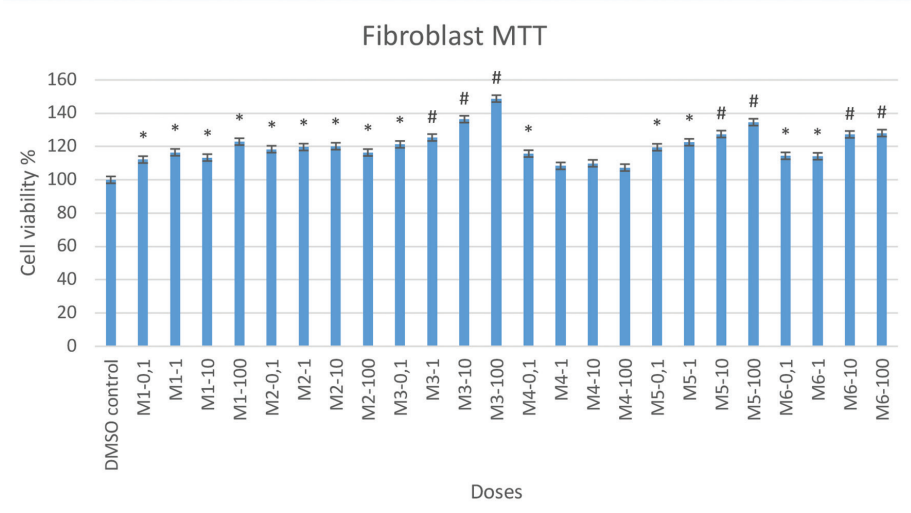


Figure 2. *In vitro* fibroblast MTT assay viability ratio of the groups (*$p<0.05$ and #$p<0.001$ compared to the control group)
DMSO: Dimethyl sulfoxide, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

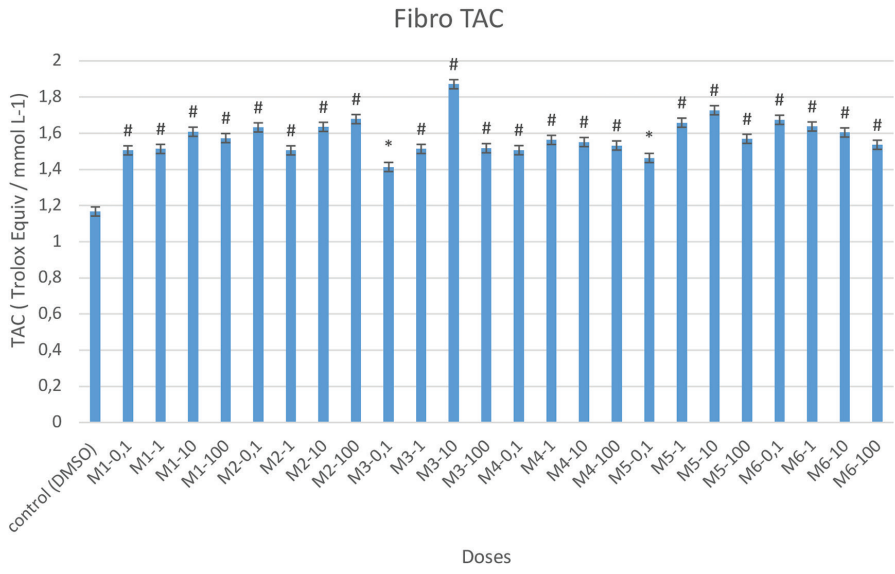


Figure 3. *In vitro* fibroblast TAC assay data (*p<0.05 and #p<0.001 compared to the control group)
DMSO: Dimethyl sulfoxide, TAC: Total antioxidant capacity

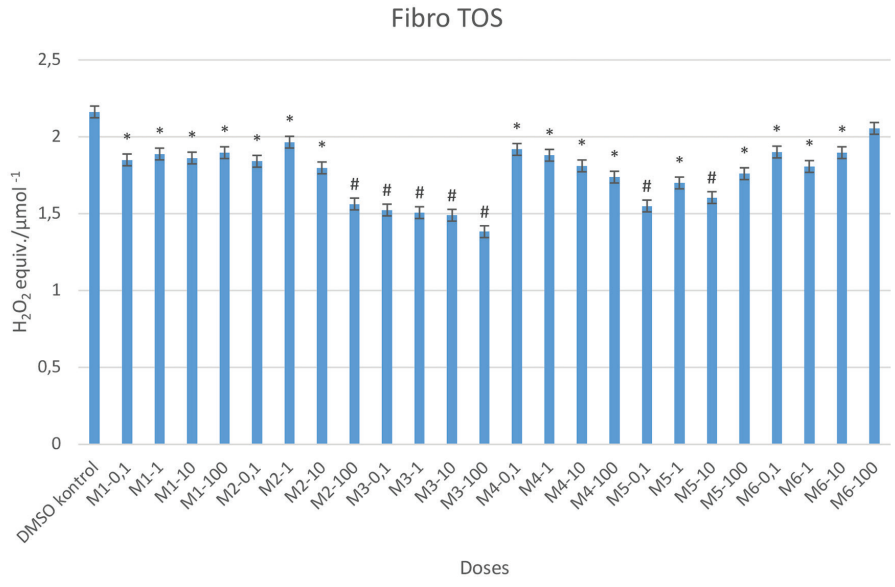


Figure 4. *In vitro* fibroblast TOS assay data (*p<0.05 and #p<0.001 compared to the control group)
DMSO: Dimethyl sulfoxide, TOS: Total oxidation state

The *in vitro* neuron TAC assay data for all compounds are summarized in Figure 6. TAC levels of compounds in neuronal cell cultures ranged from 0.66 to 2.44 Trolox equivalents (mmol L-1). In particular, the groups treated with compound M5 at concentrations of 0.1, 1, and 10 µM exhibited the highest antioxidant capacity compared to the control group (p<0.05).

The *in vitro* neuronal TOS assay data for all compounds are summarized in Figure 7. TOS levels of the compounds in neuronal cell culture were determined at a concentration of 0.1 µM and ranged between 0.79 and 1.45 H_2O_2 equivalent mmol L^{-1}. In particular, the group treated with compound M5 at 0.1 µM exhibited the lowest oxidant capacity compared with the control groups (p<0.001).

Based on TAC and TOS tests, among the compounds tested, the 4-F derivative (M3) and the 2-OH derivative (M5) were found to be the safest with respect to oxidative stress in neuronal cells in this study (Figures 3, 4, 5, and 7).

In this study, the effects of 5-HMF benzoates on wound healing were investigated at concentrations ranging from 0.1 to 100 µM. The cell migration test results for the control and the least cytotoxic compounds (M3 and M5) are summarized in Figures 8. Findings from inverted microscopy are summarized in Table 1. Statistically significant differences in wound closure were found between the groups.

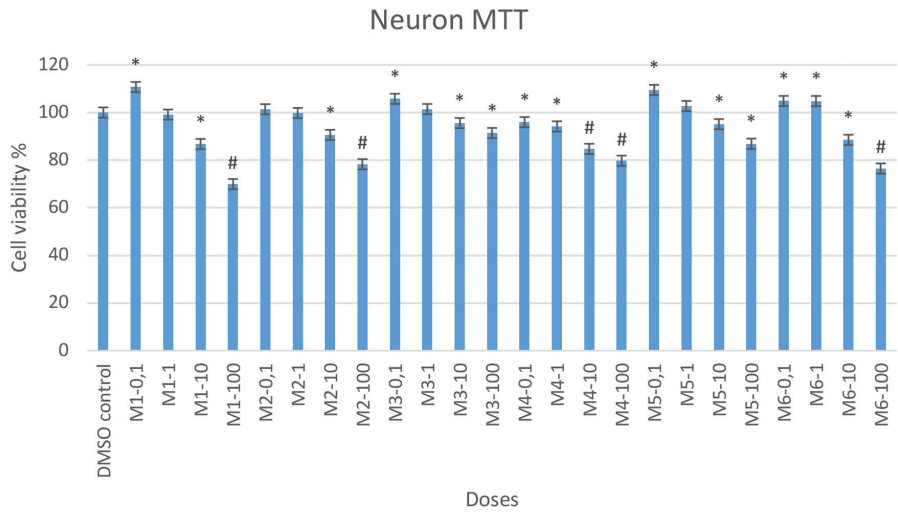


Figure 5. *In vitro* Neuron MTT assay viability ratio of the groups (*$p<0.05$ and #$p<0.001$ compared to the control group)
DMSO: Dimethyl sulfoxide, MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

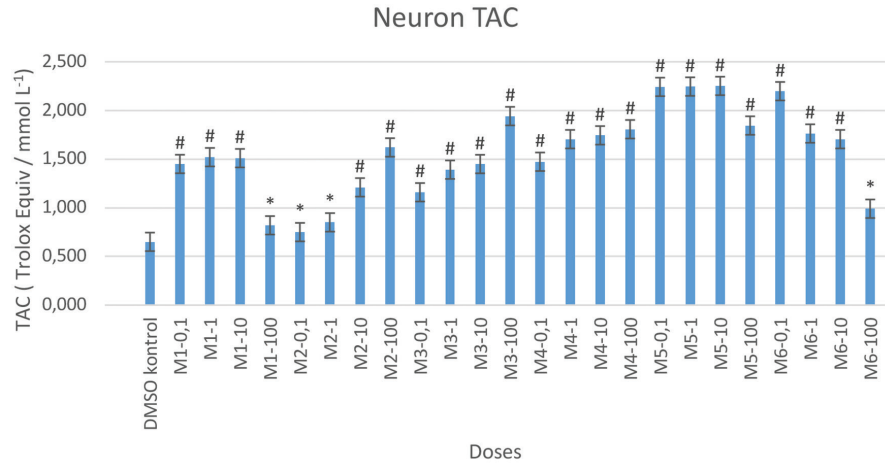


Figure 6. *In vitro* neuron TAC assay data (*$p<0.05$ and #$p<0.001$ compared to the control group)
DMSO: Dimethyl sulfoxide, TAC: Total antioxidant capacity

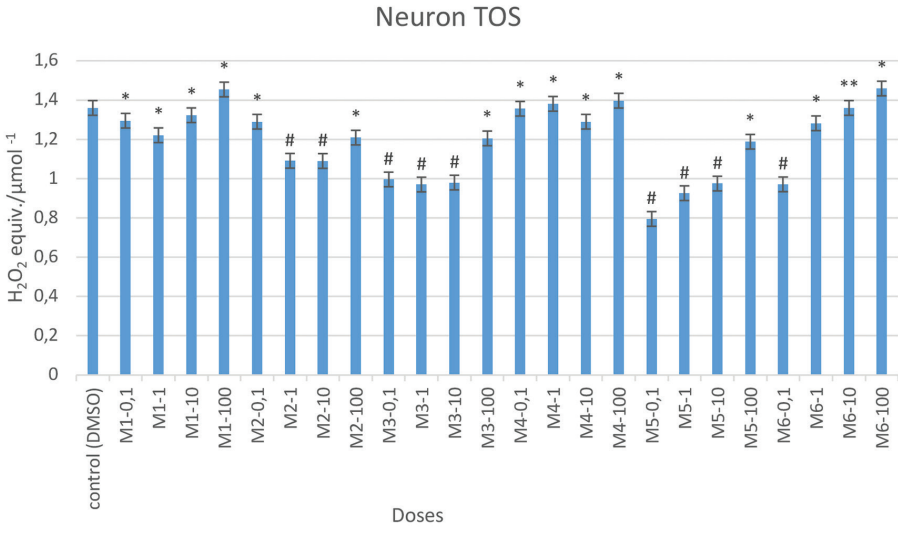


Figure 7. *In vitro* Neuron TOS assay data (*$p<0.05$ and #$p<0.001$ compared to the control group)
DMSO: Dimethyl sulfoxide, TOS: Total oxidation state

The best wound closure was achieved at a concentration of 10 μM, compared with applications of M3 and M5 across the 0.1-100 μM concentration range (Figure 8).

Table 1. Inverted microscopic findings

Groups	1st day	2nd day
Control	71.12±3.16^{a,A}	72.24±0.16^{a,A}
M3-0.1 μM	69.44±2.04^{a,A}	51.64±3.12^{b,B}
M3-1 μM	50.78±1.26^{b,A}	53.16±4.06^{b,A}
M3-10 μM	72.09±2.14^{a,A}	32.11±5.02^{c,B}
M3-100 μM	51.24±0.98^{b,A}	52.78±3.08^{b,A}
M5-0.1 μM	71.64±0.28^{a,A}	34.04±4.18^{b,B}
M5-1 μM	33.24±2.78^{b,A}	42.34±2.88^{c,b}
M5-10 μM	81.02±2.06^{c,A}	25.18±3.84^{d,B}
M5-100 μM	25.02±2.06^{d,A}	26.56±1.16^{d,A}

^{a,b,c}Shows the difference between groups in the same column (p<0.001), ^{A,B}Shows the difference between groups in the same row (p<0.001)

DISCUSSION

The first step of the reaction, the synthesis of 5-HMF, occurs at a moderate temperature in DMSO. DMSO is less toxic than other polar aprotic solvents such as dimethylformamide and dimethylacetamide.[25] The second step of the reaction (synthesis of the benzoate derivatives) occurs at room temperature in CH_2Cl_2. In summary, energy consumption in the reaction processes is low. In both cases, the products can be isolated with high yields and selectivity.

One strategy in drug development is to replace a hydrogen atom with a fluorine atom.[18,26] In drug modification, fluorine substitution changes the chemical and physical properties of the drug molecule, such as stability, solubility, molecular polarity, intramolecular hydrogen bonding, and charge-transfer capacity.[27] Thus, fluorine substitution in a drug molecule can influence pharmacokinetic, pharmacodynamic, and toxicological properties.[28] In this study, fibroblast viability was high in the group treated with the 4-fluoro derivative (M3). According to Figure 2, fibroblast viability increased as the M3 dose rose from 0.1 to 10 μM. Fibroblasts play an important role in wound healing, aggregating in the dermis at the wound margin to produce collagen.[29]

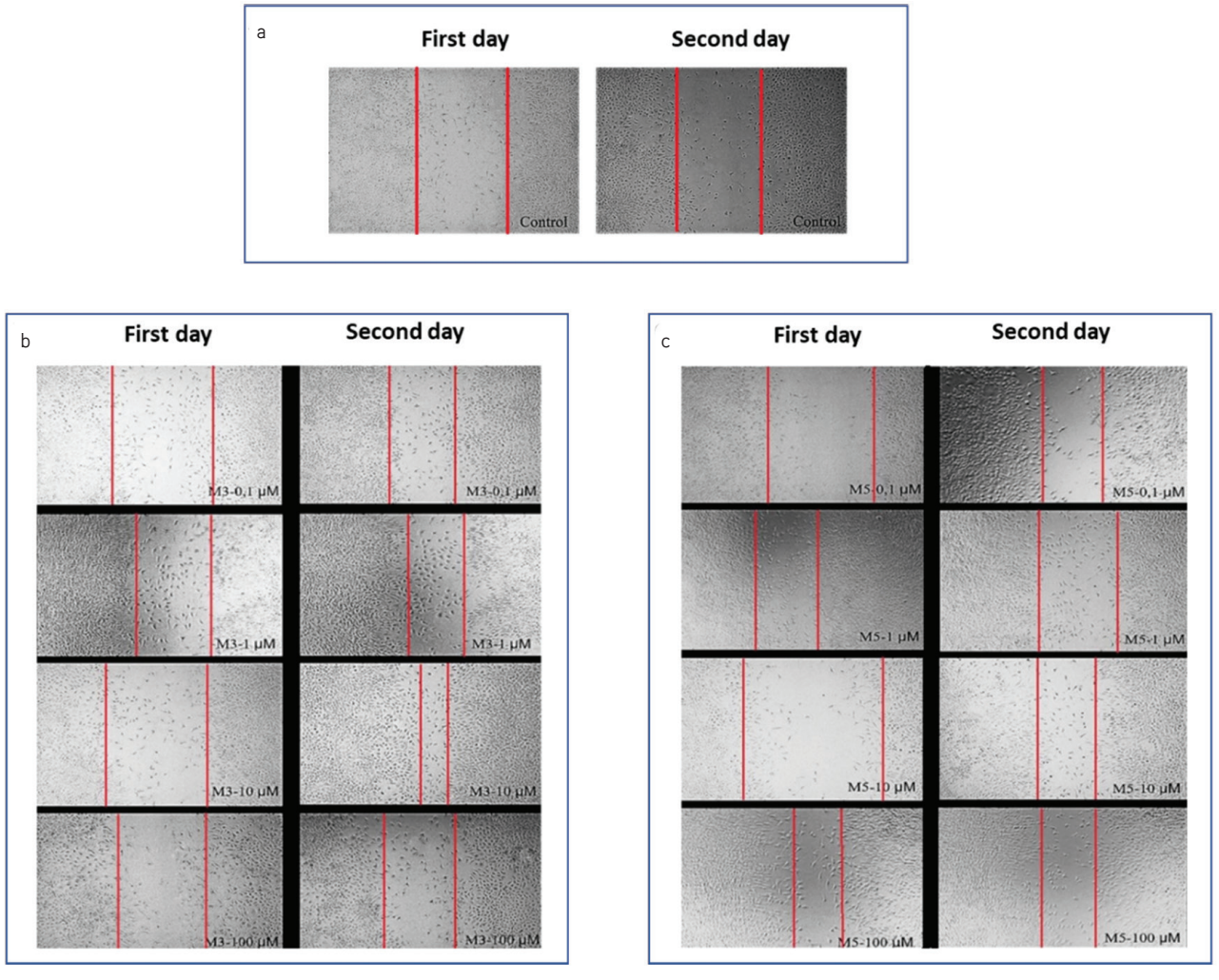


Figure 8. Cell migration test images of the groups (a) control, (b) M3 (0.1-100 μM)-treated, and (c) M5 (0.1-100 μM)-treated

A high TAC assay level and a low TOS assay level in cells indicate a safe oxidative-stress profile for the compound.[30] Among the benzoic acid derivatives (2-hydroxy, 3-hydroxy, 4-hydroxy, 2,6-dihydroxy, and 2,3-dihydroxy methyl benzoate) in the literature, the monohydroxybenzoic derivative with the hydroxyl group in the ortho position relative to the carboxylate group was found to exhibit the strongest antioxidant properties.[31] In this study, M5, a 2-hydroxybenzoate derivative, demonstrated notable antioxidant activity, as indicated by high TAC and low TOS levels.

One way to assess compound toxicity is to conduct *in vitro* studies using cells or cell lines.[32] The importance and popularity of *in vitro* experiments have increased because they are more frequently used prior to *in vivo* studies and because *in vitro* technology saves both time and money. Reproducible and reliable analyses from *in vitro* experiments can be obtained more easily and inexpensively than from *in vivo* experiments.[33] The central nervous system innervates all body regions, including cutaneous structures. Therefore, dermatological studies need to evaluate, during the early stages of drug development, whether a drug candidate damages neurons.[34] Damage to neuronal cells reduces fibroblast proliferation; therefore, synthesized molecules should both increase fibroblast numbers to promote wound healing and avoid damaging neuronal cells. Neuropathic ulcers are often observed in diabetic patients. In diabetic patients, Insulin resistance and hyperglycemia are associated with increased production of reactive oxygen species and elevated oxidative stress.[35] Increased oxidative stress in cells leads to inflammation in tissues. Inflammation in tissues also leads to dysfunction in nerve cells. Treatment of both the underlying cause of neuropathy and the ulceration is important in the management of neuropathic ulcers.[36] In this study, the groups treated with the compound exhibited higher viability of neuronal cell cultures than the control groups ($p<0.001$). According to the *in vitro* neuronal MTT test results in Figure 5, the compounds did not show cytotoxicity in neuronal cell cultures at concentrations of 0.1 and 1 µM. A reduction in cell viability was observed in neurons treated with M1 (non-substituted), M2 (4-CH_3), M4 (4-Cl), and M6 (4-CN) at 10 µM concentration, whereas no significant toxic effect was observed in neurons treated with M3 and M5 at 10 µM concentration.

In the literature, the wound-healing potential of benzyl benzoate derivatives has been evaluated using anti-inflammatory assays *in vitro* and *in vivo*, as well as with *in vivo* excision wound models. In the related study, it was reported that benzyl benzoate derivatives inhibited albumin denaturation and COX-2, and promoted wound healing more rapidly than the standard compound (nitrofurazone).[37]

Cell migration tests help assess the wound healing potential of compounds.[38] The wound healing assay is easily performed for small molecule screening[39] and drug discovery.[40] In this study, the cell migration results for the M3- and M5-treated groups were consistent with the MTT, TAC, and TOS assay results. *In vitro* cell migration assays show that the wound-healing process is accelerated as the migration rate and number of fibroblasts migrating into the scratched area increase.[41] In this study, M3 and M5 had a strong effect on both viability and wound healing at a concentration of 10 µM.

The findings obtained in this study demonstrate that compounds M3 and M5 have the potential to promote wound healing. However, to more comprehensively assess the efficacy of these compounds, it is crucial to conduct comparative studies against reference substances with well-established wound-healing effects reported in the literature. Agents such as allantoin, dexpanthenol, and silver sulfadiazine are widely used as reference compounds whose wound-healing effects have been demonstrated in both clinical and experimental studies. Comparison of compounds M3 and M5 with such references will enable further studies, particularly *in vivo* animal models, to demonstrate their therapeutic value more clearly. In this respect, our study lays the foundation for future comprehensive preclinical evaluations.

CONCLUSION

According to the MTT results, compounds M3 (4-fluoro derivative) and M5 (2-hydroxy derivative) stand out for their ability to increase cell viability. The study focused on M3 and M5 compounds because fibroblast viability was higher in the M3-treated group, whereas neuronal viability was higher in the M5-treated group. M3 and M5 did not increase oxidative stress in the TAC and TOS tests. As a result, M3 and M5 promote cell migration and have the highest potential for wound healing among the compounds tested. In further studies, the albumin-denaturation and COX-2-inhibition potentials of 5-HMF benzoates can be investigated. Secondly, the wound-healing potential of these derivatives can be investigated using *in vivo* diabetic wound models. Furthermore, nanotechnology-oriented strategies facilitate the delivery of drugs to the target site with accurate concentrations and release patterns, and often serve as bioactive carriers and therapeutic agents for the treatment of severe wounds. Therefore, in further studies, nano-formulations of M3 and M5 compounds can be prepared, and their wound-healing properties can be studied in diabetic wound models.

Ethics

Ethics Committee Approval: Ethics committee approval of the study was received from the Atatürk University Animal Experiments Local Ethics Committee (approval number: 49, dated: 26.02.2024) and Atatürk University Non-Interventional Research Ethics Committee (approval number: 120, dated: 29.03.2024).

Informed Consent: Not required.

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Footnotes

Authorship Contributions

Surgical and Medical Practices: F.Y., H.Ş.D., Concept: M.K., A.H., Design: M.K., A.H., Data Collection or Processing: M.K., F.Y., H.Ş.D., Analysis or Interpretation: M.K., F.Y., H.Ş.D., A.H., N.Ç., Literature Search: M.K., Writing: M.K.

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