In vitro primerjava svežih in liofiliziranih 3D natisnjenih materialov za oskrbo ran z vgrajenim ekstraktom *P. major*

Comparative Evaluation of Fresh and Freeze-dried 3D-printed Wound Dressings with *Plantago Major* Extract

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Izvleček

Namen: *Namen študije je bil pripraviti ekstrakt iz zeli širokolistnega trpotca ter oceniti njegovo varnost in vpliv na metabolno aktivnost (MA) fibroblastov v širokem razponu koncentracij. Poleg tega smo preučili učinek suhega in svežega 3D-tiskanega materiala za oskrbo ran (obloge) na fibroblastih, da bi bolje razumeli njegov potencial pri celjenju ran.*

Metode: *Pripravili smo metanolni ekstrakt trpotca in testirali njegov vpliv na MA fibroblastov v širokem razponu koncentracij. S 3D tiskom smo izdelali obloge (sestavljene iz metilceluloze, alginata in nanofibrilirane celuloze) z vgrajenim ekstraktom ter preučili vpliv suhe in sveže obloge na MA fibroblastov in celjenje ran.*

Rezultati: *Ekstrakt je bil varen pri koncentracijah 1–1.000 µg/mL,*

Abstract

Aim: *In this study, we prepared an extract of* Plantago major L. *(PM) herb and assessed its safety and impact on fibroblast metabolic activity (MA) across a broad concentration range. Additionally, we examined the effects of dry and fresh three dimensionally (3D)-printed wound dressings (abbreviated: dressing) on fibroblasts and evaluated their influence on cell migration and proliferation by a scratch assay.*

Methods: *We prepared a methanolic PM extract and investigated its effect on fibroblast MA across various concentrations. Using 3D printing, we fabricated dressings composed of methylcellulose, alginate, and nanofibrillated cellulose, incorporating the extract. Subsequently, we assessed the effects of dry and fresh dressings on fibroblast MA as well as on migration/proliferation by a scratch assay.*

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medtem ko je bil pri 10.000 µg/mL citotoksičen. MA fibroblastov se je povečevala do 1.000 µg/mL, nato je začela padati, kar nakazuje na hormetični učinek. Suha obloga je povečala MA fibroblastov na 1,2 v primerjavi s kontrolo (1) in svežo oblogo (0,99). Test zapiranja raze je pokazal, da obe oblogi podobno učinkovito podpirata migracijo/proliferacijo fibroblastov.

Zaključki: *Naši* in vitro *testi podpirajo tradicionalno uporabo širokolistnega trpotca pri celjenju ran in lahko predstavljajo osnovo za nadaljnje raziskave te rastline v kliničnih študijah.*

Results: *The extract had no cytotoxic effects at concentrations of 1–1,000 µg/mL, but became cytotoxic at 10,000 µg/mL. Fibroblast MA increased up to a concentration of 1,000 µg/mL, after which it began to decline, indicating a hormetic effect. The dry dressing enhanced fibroblast MA 1.2-fold more than the control (normalized to 1.0) and the fresh dressing (0.99-fold). The scratch assay confirmed that both dressings supported fibroblast migration/proliferation equally.*

Conclusions: *Our* in vitro *results support the traditional use of PM in wound healing and can serve as a basis for future investigation of this plant in clinical trials.*

INTRODUCTION

PM has been widely used in traditional medicine for wound healing. Studies have confirmed that it contains bioactive compounds, including plantamajoside, verbascoside, and aucubin, which may contribute to its therapeutic properties (1). However, its cellular effects, including potential hormetic effect (the biphasic response to different concentrations), have not been well documented (2). This study aimed to evaluate the safety and bioactivity of a methanolic extract of PM on fibroblast MA and its potential hormetic effect. Hormesis is described as a biphasic effect, with low dose stimulation and a high dose cytotoxic effect. At low levels, biological, physical, chemical, or physiologic stressors can activate adaptive cellular responses that enhance repair and restoration processes in damaged tissues or organs. These responses frequently result in a performance or recovery level that surpasses that of unstimulated controls—by as much as 30–60% (3). By contrast, higher doses of the same stimuli can become detrimental, leading to cytotoxic effects and impaired function (3).

Additionally, we investigated the integration of PM extract into three-dimensionally (3D)-printed dressings made from methylcellulose (MC), alginate, and nanofibrillated cellulose (NFC). Three-dimensional printing is an emerging technology in wound care that facilitates the fabrication of personalized dressings tailored to the wound's specific shape and size (4). It also enables the precise incorporation of bioactive compounds, making it a promising drug-delivery system (5). The use of multicomponent hydrogels allows for the customization of diverse microstructures and interconnected pore networks, which facilitate efficient oxygen, nutrient, and metabolic waste transport (6). Both natural and synthetic materials can be utilized in 3D printing. Among natural materials, alginate, cellulose derivatives, collagen, and fibrin are widely used because of their biocompatibility, biodegradability, and non-toxicity (6).

The impact of dry and fresh dressings on fibroblast MA and migration was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) and scratch assays, respectively. Understanding the role of PM extract and its incorporation into advanced biomaterials is essential for developing innovative wound care solutions. By investigating the effects of PM extract on fibroblast function, our study aimed to bridge the gap between traditional medicine and modern wound healing.

MATERIALS AND METHODS

Materials

Dry, crushed herb of PM was purchased from Caelo (LOT 558-1 kg, Germany). Alginic acid sodium salt (ALG, Mw: 80 kDa) and MC (Mw: 658.7 kDa, 25 cP) were obtained from Sigma-Aldrich, Germany. Cellulose nanofibril suspension (NFC, 3% (w/v) at 1.0 g/cm³ aqueous gel, with a nominal fiber width of 50 nm and lengths of several hundred microns) was obtained from The Process Development Center, University of Maine (Orono, USA). Ultra-pure water (Resistivity18.2 mΩ·cm at 25°C) from the ELGA Purelab water purification system (Veolia Water Technologies, UK) was used to prepare all solutions. All other chemicals were of analytical grade.

Methods

Plant material extraction

The dry PM herb was ground into powder with an electric grinder. The plant material was extracted with 70% (v/v) MeOH with a 1:20 plant to solvent extraction medium ratio at 60°C under reflux, with moderate stirring for 2 h. The plant material was removed by vacuum filtration and the filtrate was concentrated under reduced pressure at 40°C (Rotavapor® R-100, BUCHI, Labortechnik AG, Switzerland). The remaining liquid was frozen at –80°C and lyophilized (VirTis BenchTop 6K, SP Industries Inc., PA, USA) overnight to obtain a viscous extract (*extractum spisum*) with a drug extract ratio 5.6:1.

Preparation of 3D printed dressing with added 0.1 wt.% PM viscous extract

A dressing was prepared, consisting of 10 wt.% MC, 5 wt.% ALG), and 1.5 wt.% NFC, a composition shown in our earlier study to be suitable for fibroblast viability and growth (7). For dressing with added extract, 0.1 wt.% PM extract was dissolved in water, which was first mixed with NFC, followed by the addition of ALG and MC. The gel was 3D printed using a pneumatic 3D printer (Vitaprint IRNAS, Slovenia) with extrusion nozzles (Nordson EFD, USA) with a diameter of 0.25 mm. The pressure was set to 4 bars. The dressings were of cylindrical shape with a 20 mm diameter, 1.8 mm height, and layers composed of 18 filaments. The 3D printed dressings were cross-linked with 5 wt.% CaCl2 for 1 min and wiped with a low-lint towel. Some of the dressings were immediately soaked in cell medium (see section: Cell-based testing), while others were frozen at –80°C, and lyophilized overnight (VirTis BenchTop 6K).

Cell-based testing

MTT assay with PM extracts

Human skin fibroblasts (abbreviated as fibroblasts) (ATCC CCL-110™, LGC Standard, United Kingdom) were cultured in Advanced Dulbecco's Modified Eagle's Medium (ADMEM; ThermoFisher, Germany) supplemented with 5% (v/v) fetal bovine serum (FBS), together with penicillin, streptomycin, and glutamine (each at 10% v/v), at 37°C in a humidified atmosphere containing 5% CO2 (7). Fibroblasts were seeded in 96-well microtiter plates at a density of 10,000 cells/well and incubated at 37°C under a 5% CO2 atmosphere for 24 h (8).

The viscous PM extract was prepared as a stock solution of 10 mg/mL (in cell medium), filtered through a 0.22 µm filter, and diluted with the cell medium to obtain final concentrations of 1, 10, 100, 1,000, and 10,000 µg/mL, which were added into respective wells with a final volume of 100 µL. The control well contained fresh medium alone. Cells were incubated for 24 h at 37°C under an atmosphere of 5.0 wt.% CO2.

The MTT assay is one of the most widely applied methods which determines MA as an indicator of cell viability (9, 10). The water-soluble yellow dye MTT is converted to insoluble purple formazan in living cells by mitochondrial reductases. The insoluble formazan is subsequently dissolved in dimethyl sulfoxide (DMSO) and quantified at 570 nm (11). Fibroblasts were used in our study because they are the most abundant dermal cell type and are involved in wound healing. They produce collagen, fibronectin, and proteoglycans, which are major components of the extracellular matrix (12). MTT reagent (10% (v/v) in medium with 5% (v/v) FBS) was added to each

well. After incubation for 3 h and the formation of purple formazan crystals, the medium was carefully discarded, and DMSO (100 µL) was added to dissolve the crystals. Absorbance was determined at 570 nm using a Varioskan Multiple Reader (ThermoFisher Scientific, USA).

All experiments were performed in quadruplicate, with a blank control (sample/control without MTT) for each set. Results are expressed as the mean value with standard deviation (SD). Cell MA was calculated using the following equation:

$$\text{Cell viability} = \frac{(A_s - A_{sb})}{(A_c - A_{cb})} \qquad \textit{Equation 1}$$

where As is the absorbance of the sample, Asb is the absorbance of the sample blank, Ac is the absorbance of the control, and Acb is the blank absorbance of the control.

Dressing biocompatibility by the MTT assay

Cell MA was evaluated according to Mosmann (13), and meeting the ISO 10993-5 and ISO 10993-12 regulations, by the extract method. The assay was performed as described above with the following changes:

Dry dressing containing 0.1 wt.% PM extract and fresh dressing with 0.1 wt.% PM extract were incubated in the cell culture media (ADMEM, supplemented with 5% (v/v) FBS) for 24 h at 37°C under a 5% CO2 atmosphere. The following day, the conditioned medium from the dressing was transferred onto the cells, either undiluted or at a 1:2 dilution, with a final medium volume in each well of 100 µL. Control cells were incubated in fresh cell culture medium supplemented with 5% (v/v) FBS. The remainder of the protocol was unchanged.

Scratch assay with the dressings

The scratch assay is a widely used *in vitro* method for evaluating the wound closure rate by assessing cell migration and proliferation. A scratch is manually created on a confluent cell monolayer using a pipette tip, and cell movement into the wound area is monitored under a microscope (19).

The scratch assay was performed according to (14) and (15). Fibroblasts were seeded in 24-well plates (50,000 cells/well) and incubated overnight at 37°C in ADMEM supplemented with 5% (v/v) FBS under a 5% CO2 atmosphere. Fresh and dry dressings were incubated overnight in 3 mL ADMEM supplemented with 5% (v/v) FBS under identical conditions as in the MTT assay.

The following day, a scratch was created using a 200 µL sterile pipette tip. The cells were washed with fresh medium, which was subsequently discarded. Control wells received fresh medium containing 5% (v/v) FBS, while the medium from the dressing incubation was transferred onto cells in the experimental wells (100 µL). The cells were incubated overnight at 37°C under a 5% CO2 atmosphere.

Images were obtained using an optical microscope (Axiovert 40, Zeiss, Germany) immediately after the scratch was made and at 4 and 24 h post-scratch. All experiments were conducted in triplicate. The area of the wound was quantified using ImageJ software (National Institutes of Health, USA) with the wound healing plugin (16) and the percentage of wound closure was calculated from the following equation:

$$\%\ \text{Wound closure} = \left(\frac{(A_0 - A_n)}{A_0}\right) \times 100 \qquad \textit{Equation 2}$$

where A0 is the initial wound area, and An is the wound area after 4 or 24 h.

Statistical analysis

All numerical values are reported as the mean ± SD. The Shapiro–Wilk test confirmed the normal distribution of experimental data. Levene's test was applied to assess the equality of variances. Because all datasets were well modeled by a normal distribution and homoscedastic, one-way analysis of variance (ANOVA) followed by Dunnetti's post-hoc test was performed accordingly. P-values < 0.05 were considered to be statistically significant. Statistical analysis was performed using SPPS Statistics 27 (IBM Corp., USA).

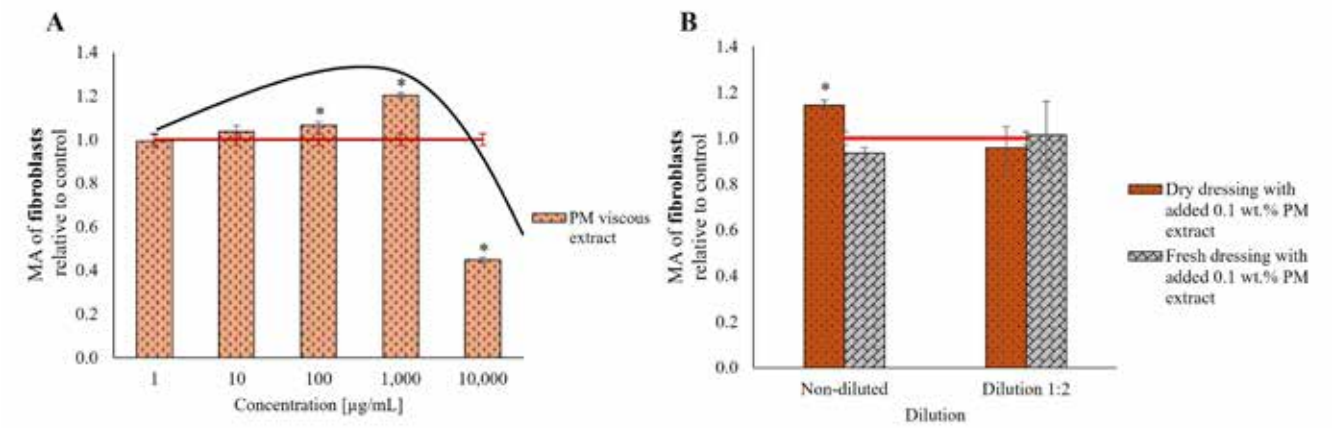

Figure 1. *A: MTT assay with PM viscous extract in a wide range of concentrations. Black curve represents the so-called hormetic effect. B: MTT assay with dry or fresh dressing with added 0.1 wt.% PM viscous extract.-*

RESULTS

MTT assay with PM extract and dressings

The MTT assay results are presented in Figure 1. Part of the results were obtained in the scope of our previous study (17).

Figure 1A illustrates fibroblast MA following treatment with different PM viscous extract concentrations. There was a steady increase in fibroblast MA from 1 to 1,000 µg/mL, followed by major decrease at 10,000 µg/mL, indicating cytotoxicity. This biphasic response suggested the presence of a hormetic effect, characterized by low-dose stimulation and high-dose inhibition. The black curve in the graph represents this hormetic trend, typically showing a 30%–60% greater response compared to the control (18).

Figure 1B illustrates fibroblast MA when treated with conditioned medium from the fresh and dry dressings. The dry dressing increased MA by 1.2-fold compared to the control, indicating enhanced fibroblast activity. However, this effect diminished with 1:2 dilution, reaching MA levels comparable to the control. By contrast, the fresh dressing exhibited a slightly lower relative MA than the control, though this difference was not statistically significant. Upon dilution, MA aligned with that of the control.

Scratch assay with dressing with incorporated PM extract

The migration/proliferation of untreated fibroblasts (control) or in the presence of conditioned medium from the dressings were observed at 0, 4 and 24 h after the scratch was applied. The results are shown in Figure 2. The extent of wound closure was quantified by measuring the scratched area over time. The percentage of wound closure was calculated to compare the effects of different conditions. The results are summarized in Table 1.

Table 1: Percentage wound closure after 4 and 24 h.

	% Wound closure after 4h	% Wound closure after 24h
Control	18.9±13.3	89.4±11.1
Dry dressing with added 0.1 wt.% PM extract	16.9±5.3	86.9±13.2
Fresh dressing with added 0.1 wt.% PM extract	11.5±11.2	85.0±10.0

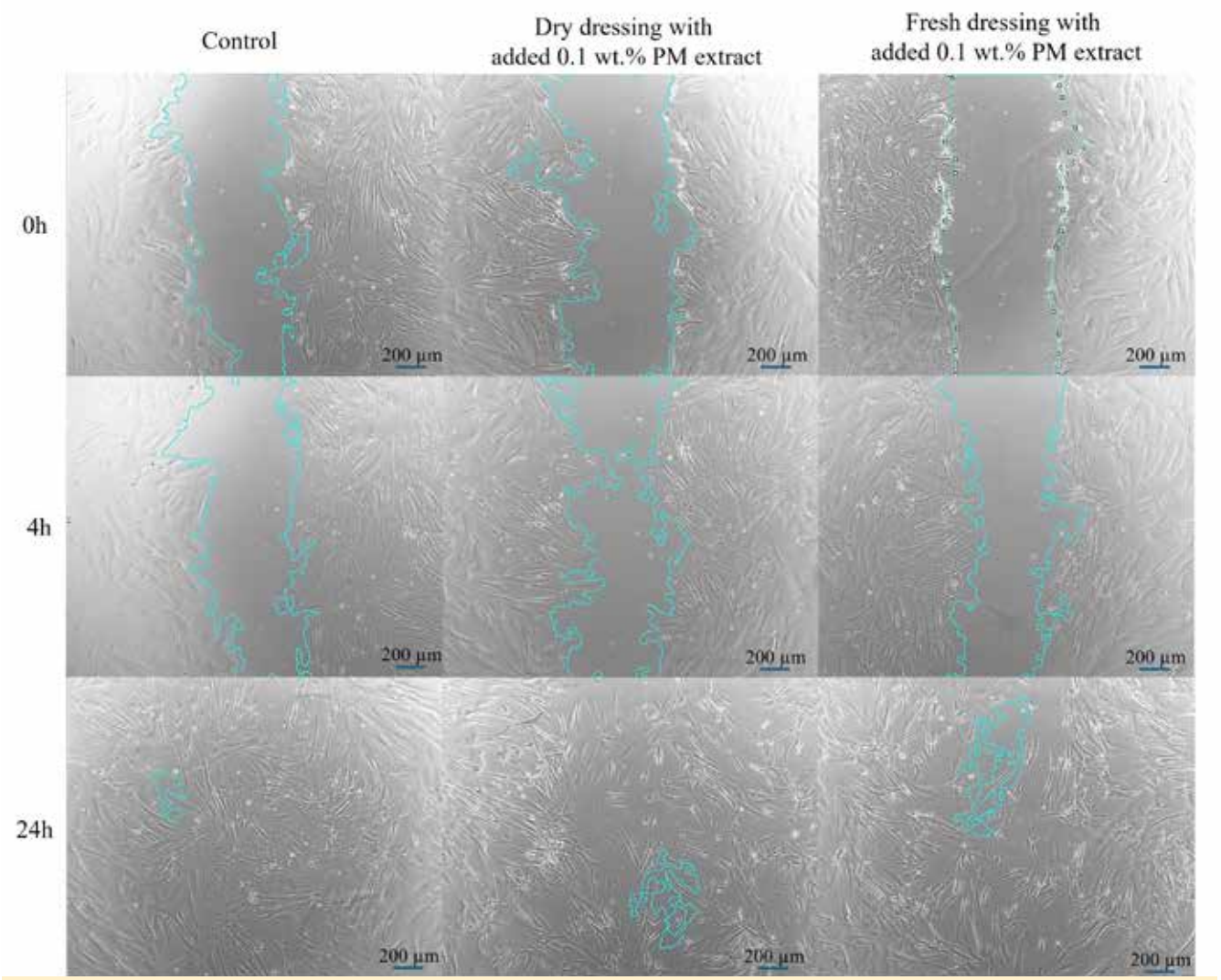

Figure 2. *Scratch assay performed with the conditioned medium from the fresh and dry dressings with added 0.1 wt.% PM extract, compared to control. The micrographs were obtained at 0, 4, and 24 h after scratching, and the percentage wound closure was calculated using ImageJ with the wound healing plugin. The experiments were conducted in three parallel groups. Blue-defined regions represent the area without cells and were used to calculate the wound closure.*

After 24 h, the control wound "healed" with 89.4% ± 11.1% closure, while wounds treated with fresh and dry dressings healed with 86.9% ± 13.2% and 85.0% ± 10.0% closure, respectively. These results indicated no significant difference in wound closure among the groups. While at 4 h the fresh dressing did exhibit a slightly lower average wound closure percentage compared to the control and dry dressing, this difference was not statistically significant. On the basis of the MTT results, the slight reduction in fibroblast MA with the fresh dressing appeared to correlate with slower fibroblast migration in the scratch assay.

DISCUSSION

Hormesis is characterized by a biphasic dose–response, where low concentrations stimulate biological activity,

whereas high concentrations exhibit inhibitory or cytotoxic effects. The observed hormetic effect (Figure 1A) reflected previous findings in various biological systems. Hormesis has been widely reported not only in cellular models, but also in plant growth studies, however, reports specifically addressing this phenomenon in PM remain lacking. For example, Perven et al. demonstrated that an aqueous extract of *Moringa oleifera* promoted shoot growth in *Lepidium sativum* at lower concentrations, whereas higher inhibited shoot growth and reduced root length (20). Hormetic responses to *M. oleifera* have been reported in many other studies, including animal reproduction and sperm preservation (21, 22), neuronal systems (23, 24), bone formation (25), and immune cell responsiveness (26).

Erdal Altıntaş and Aytar Çelik also observed a biphasic effect when testing PM water and ethanol extracts on fibroblast MA (27). Although the authors did not describe it as hormesis, this effect could be clearly observed (Figure 3).

The findings in the present study provided novel evidence of hormetic behavior of PM extracts, which, to our knowledge, has not been previously reported. These insights highlight PM's potential for dose-dependent therapeutic applications, particularly in wound healing, where controlled stimulation of fibroblast activity is beneficial.

The impact of dressing freezing and lyophilization on fibroblast MA was evaluated by testing dry and fresh dressings on fibroblasts cells (Figure 1B). The dry dressing significantly increased MA compared to both the control and the fresh dressing. Upon dilution, the stimulatory effect of the dry dressing diminished. Lyophilization modifies the dressing's structure, enhancing porosity and thus increasing the surface area, which may accelerate and improve the release of bioactive compounds from the PM extract, thereby stimulating fibroblast MA (28). By contrast, the fresh dressing retains more residual moisture, potentially affecting swelling, diffusion, and the gradual release of active compounds into the medium (29). Moreover, in the fresh dressing, stronger interactions between the active PM compounds and the polymer matrix may form, potentially slowing extract release (30).

The scratch assay results demonstrated that the dressings effectively promote wound closure, with almost 90% closure observed after 24 h, comparable to the control (Figure 2, Table 1). This suggested that the dressing provides a favorable environment for cell migration and proliferation, which are crucial for the initial stages of wound healing (31). Zubair et al. conducted a scratch assay using aqueous and ethanolic extracts of fresh and dry PM leaves on oral epithelial cells (32). The authors found that ethanol-based extracts promoted cell proliferation and migration at concentrations of 0.1 and 1.0 mg/mL, but exhibited cytotoxic effects at 10 mg/mL. Notably, the hydroethanolic extract of dry leaves was more effective at 1.0 mg/mL than at 0.1 mg/mL,

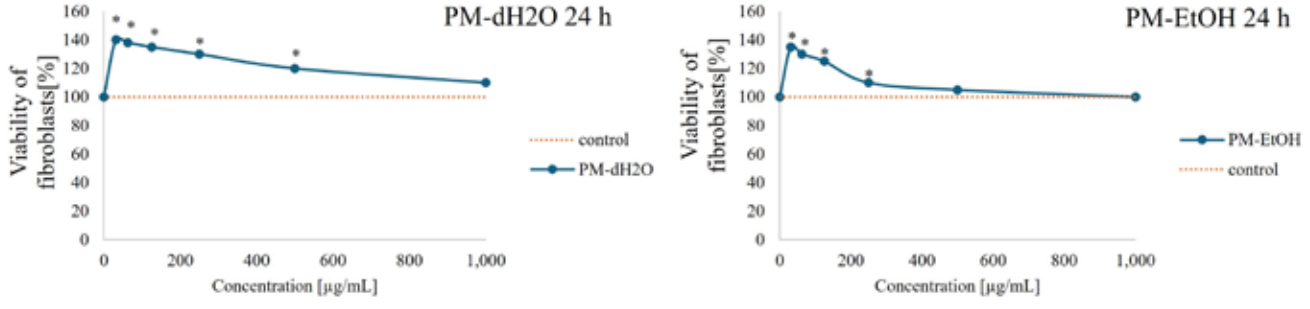

Figure 3. *Hormetic effect of PM water and ethanol extracts. The fibroblast MA was tested with a wide range of concentrations (31.25–1000.0 µg/mL). (The graphs were modified from Erdal Altintas and Aytar Celik with permission. Open access journal, license CC-BY 4.0 (27)).*

achieving 100% wound closure after 24 h compared to 90% at the lower concentration. This suggested a potential hormetic effect of PM, where moderate concentrations enhance cell activity, whereas higher concentrations may be detrimental. The limitation of this study is the use of *in vitro* models and a single cell type, which may not fully represent the complexity of *in vivo* wound healing. Future studies should include *in vivo* validation and broader dose–response analyses to clarify the therapeutic potential and mechanistic basis of PM dressings in wound healing.

CONCLUSION

This study demonstrated that PM extract is safe for fibroblasts at concentrations up to 1,000 µg/mL, with cytotoxic effects observed at 10,000 µg/mL. Notably, to our knowledge, this is the first study to provide evidence of a hormetic effect of PM extracts. The successful incorporation of PM extract into a 3D-printed dressing enabled further evaluation on fibroblasts, revealing that the dry dressing had a more pronounced stimulatory impact on MA compared to the fresh dressing. Dressing lyophilization prolongs its shelf life and enhances the stability of the incorporated extract, allowing the product to be stored at room temperature and making it more suitable for clinical use. Additionally, the scratch assay confirmed that both dressings were equally effective at promoting wound closure, reinforcing the traditional use of PM in wound healing applications. These findings highlight the potential of PM-based biomaterials in advanced wound care and encourage further research into their mechanisms of action and *in vivo* efficacy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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