https://doi.org/10.31925/farmacia.2024.1.22 **ORIGINAL ARTICLE**

DESIGN AND EVALUATION OF PROPOLIS-HPC/PVP ELECTROSPUN NANOFIBERS FOR POSSIBLE CUTANEOUS APPLICATIONS

IMREN ESENTÜRK-GÜZEL [1,2#], EVREN ALGIN YAPAR [3#], SIMONA CAVALU [4]*, İBRAHIM TUĞKAN ŞEKER [5], EBRAR İNAL [6,7], MURAT KARTAL [6,8], RABIA ÇAKIR [2,9], RABIA YILMAZ ÖZTÜRK [2.9], FERIHA GÖKSU [10], SIBEL BÖLEK [10], AYYÜCE TÜRKMEN [1]

[1]*Department of Pharmaceutical Technology, Hamidiye Faculty of Pharmacy, University of Health Sciences, Istanbul, Türkiye*
[2]*Türkiye Biotechnology Institute, Health Institutes of Türkiye (TUSEB), Istanbul, Türkiye*
[3]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Sivas Cumhuriyet University, Sivas, Türkiye*
[4]*Department of Preclinical Sciences, Faculty of Medicine and Pharmacy, University of Oradea, Bihor, Romania*
[5]*Department of Gastronomy and Culinary Arts, School of Tourism and Hotel Management, Sivas Cumhuriyet University, Sivas, Türkiye*
[6]*Department of Pharmacognosy, Faculty of Pharmacy, Bezmialem Vakıf University, Istanbul, Türkiye*
[7]*Institute of Health Sciences, Bezmialem Vakıf University, Department of Pharmacognosy and Natural Product Chemistry, Istanbul, Türkiye.*
[8]*Phytotheraphy Research Center, Bezmialem Vakıf University, Istanbul, Türkiye*
[9]*Department of Bioengineering, Faculty of Chemical and Metallurgical Engineering, Yıldız Technical University, Istanbul, Türkiye*
[10]*Department of Food Technology, Experimental Medicine Research and Application Centre, University of Health Sciences, Istanbul, Türkiye*

**corresponding author: simona.cavalu@gmail.com*
[#]Authors with equal contribution.

Manuscript received: November 2023

Abstract

The aim of the study was to prepare propolis loaded hydroxypropyl cellulose and polyvinyl pyrrolidone (HPC/PVP) nanofibers, suitable for cutaneous applications. LC-HRMS analyses were applied for the identification and quantification of the bio-compounds in the propolis extract. Electrospinning was applied in order to prepare propolis HPC/PVP nanofibers with increasing concentration of propolis 10%, 20% and 30 % (v/v) and subsequently characterized in terms of morphology, wettability, thermal degradation and biological properties. SEM images showed homogenous features of the nanofibers and average diameter between 197.2 - 268.4 nm, high antioxidant activity, very good biocompatibility and no cytotoxicity. Particularly, with the highest propolis concentration, 30-PE/HPC/PVP nanofiber formulation is a promising alternative to conventional treatments for cutaneous applications.

Rezumat

Scopul studiului a fost de prepararea unor nanofibre pe bază de hidroxipropilceluloză și polivinil pirolidonă (HPC/PVP) încărcate cu propolis, pentru aplicații cutanate. Analizele LC-HRMS au fost aplicate pentru identificarea și cuantificarea biocompușilor din extractul de propolis. Tehnica *electrospinning* a fost aplicată pentru prepararea nanofibrelor de propolis-HPC/PVP cu o concentrație de propolis de 10%, 20% și 30% (v/v) și ulterior caracterizate: din punct de vedere morfologic, umectabilitate, degradare termică și proprietăți biologice. Imaginile SEM au arătat caracteristici omogene ale nanofibrelor și un diametru mediu între 197,2 - 268,4 nm, activitate antioxidantă ridicată, biocompatibilitate foarte bună și lipsă de citotoxicitate. Formularea 30-PE/HPC/PVP, cu cea mai mare concentrație de propolis, este o alternativă promițătoare la tratamentele convenționale pentru aplicații cutanate.

Keywords: electrospinning, nanofibers, propolis, polyvinyl pyrollidone, hdroxypropylcellulose, biocompatibility

Introduction

Although the chemical composition of propolis depends on several factors such as plant sources, geographical location, month of collection, species of the bee, it is generally accepted that flavonoids are the major constituents in propolis, being responsible for its biological activities including antioxidant, antibacterial, antiviral, antifungal, anti-inflammatory and antitumor activities [1-5]. Particularly, the rich phenolic content and high antioxidant activity of propolis originated from Anatolia were proved by previous studies [1, 2].
Being a large reservoir of bio-compounds, it was already demonstrated to be able of stimulating dermal cells proliferation, in addition to bear antimicrobial or anti-inflammatory properties [6]. Wound healing management is always challenging, and acute or

chronic wounds remains a major clinical problem. The treatment strategy in the case of severe skin damage is to ensure rapid wound closure and re-epithelialization, providing a moist environment along with topical nutrition to promote the new tissue growth. Due to its complex composition with antioxidant and antimicrobial activity, propolis was used in different pharmaceutical and cosmetic formulations, proving an effective protective mechanism in management of burns wounds, by accelerating the healing process, decreasing the generation of reactive oxygen species and reducing inflammation [6-9].

Propolis-based polyvinyl alcohol hydrogel was previously developed and the wound-healing activity was proved *in vivo*, in a study conducted by Kapare *et al*. [7], while nano emulsion formulations based on propolis and tea tree oil were demonstrated to be efficient in wound healing process, both *in vitro* and *in vivo*, by Abdellatif *et al*. [8]. The topical application of propolis on diabetic wounds was also effective, as demonstrated *in vivo* by Hozzein *et al*. [9], showing that propolis significantly improves the closure of diabetic wounds and reduces the levels of inflammatory cytokines (IL-1β, IL-6, TNF-α and MMP9) to normal levels [9]. On the other hand, electrospun nanofibers are attracting attention especially due to success in modulating delivery of hydrophilic or lipophilic active pharmaceutical ingredients and high patient compliance with good biocompatibility. Both natural and synthetic polymers and their blends can be used in preparing nanofibers, with high surface areas to volume ratio which provides efficient delivery of drugs. Various polymers or their blends such as polylactic acid, poly(lactide-co-glycolide), polyurethane, cellulose acetate, polycaprolactone, etc. containing nanofibers have been researched for biomedical applications in terms of drug delivery and wound healing [10-13].

Nowadays, environmental, infectious and biological factors inducing skin damage are accompanied by sun burns, acne wounds and scars, age spots and wrinkles, which are worldwide problems. For these complex situations, there is no single product that can be used effectively without side effects, suitable for long-term application. To provide a solution to these special needs, in this study, we propose the development of a nanofiber mat containing propolis from Sivas-Türkiye, well known for its quality. The preparation of the nanofibers was achieved by using a combination of two polymers: hydroxy propyl cellulose (HPC) and polyvinylpyrrolidone (PVP). HPC is a non-ionic derivative of natural cellulose, widely used in pharmaceutical and cosmetic applications as emulsifier, stabilizer, suspending, thickener, tablet binder, film forming agent, and extended release-matrix former, but also in biomedical applications such as wound dressings [14, 15]. In this study, HPC was chosen due to its nontoxicity, biocompatibility, and biodegradability properties, being both water and organic soluble, allowing to obtain homogeneous dispersion with propolis ethanolic extract suitable for electrospinning [14, 16]. PVP is one of the most widespread polymers used in pharmaceutical and biomedical applications being biocompatibility, non-toxic, with amphiphilic properties. It can be included in polymer blends to enhance spinnability of the solutions during nanofiber production by electrospinning [17, 18].

The aim of this study was to prepare propolis containing nanofiber formulations and to determine the optimum propolis concentration suitable for wound healing process, by physico-chemical characterization *via* SEM, FTIR spectroscopy, contact angle and TGA measurements along with *in vitro* antioxidant activity, cytotoxicity and biocompatibility assays for potential cutaneous applications.

Materials and Methods

Materials

The starting materials and reagents employed for nanofibers preparation were: organic certificated crude propolis purchased from Engür A.Ş. (Sivas, Türkiye), hydroxy propyl cellulose (HPC, Mw = 100.000 g/mol) purchased from Sigma (Germany), polyvinyl-pyrrolidone (PVP, Mw = 1.300.000 g/mol) from Acros Organics (USA), ethanol and methanol (analytical grade) purchased from Merck (Darmstadt, Germany). *In vitro* assay was performed using mouse fibroblast NIH 3T3 cells (ATCC®, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), Pen-Strep, Triton X-100 (100 µl/mL penicillin and 100 µg/mL streptomycin), 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT), phenazine methosulfate (PMS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Foetal bovine serum (FBS) was purchased from Gibco, Scotland. RPMI-1640 medium and morpholinepropanesulfonic acid (MOPS) were obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was provided from Dojindo Laboratories (Kumamoto, Japan). Penicillin, streptomycin and glutaraldehyde solution (25% aqueous solution) were purchased from Sigma Chem Co. (St. Louis, MO, USA) and deionized water was supplied from Merck Millipore Milli-Q ultrapure water system (Merck Millipore, Molsheim, France).

Methods

Preparation and characterization of ethanolic propolis extract

In order to facilitate grinding due to the extremely sticky nature of propolis, raw propolis samples were frozen at -18°C for 24 hours, and then ground in a laboratory ball mill (MSE Technology, Türkiye) into a fine powder. For wax removal, the powder was transferred to trays and kept in an oven at 60°C for

120 minutes, then solidified again, and wax separated by mechanical treatment. The ethanolic extract was obtained by a method described in literature [19, 20] with some modifications. For this purpose, 100 g of ground propolis was subjected to extraction, while shaking (145 rpm/15 min *per* hour) at 50°C for 5 days in 1000 mL (70 w/w) ethyl alcohol. At the end of the 5th day, the extract was first subjected to a coarse filtration (non-woven fabric 50 µm) and then filtered through filter paper with a pore diameter of 20 µm to obtain ethanolic propolis extract (PE). The condensation of the extract was obtained with 1:10 ethanol/propolis ratio by evaporating the alcohol to 1:5 ratio.

LC-HRMS analyses were performed for identification and quantification of the bio-compounds in propolis extract. The experimental parameters are listed in Table I.

Table I

Laboratory and equipment conditions

<table>
<tr><td colspan="4">Laboratory Environmental Conditions
Temperature: (22. 0 ± 5.0°C)
Relative Humidity: (50 ± 15%) RH</td></tr>
<tr><td colspan="3">HPLC equipment
Thermo Dionex Ultimate 3000</td><td>Mass Spectrometry equipment
Thermo ORBITRAP Q-EXACTIVE</td></tr>
<tr><td colspan="3">Mobil phase A: 1% Formic acid – H_2O
Mobil phase B: 1% Formic acid – MeOH
Column: Troyasil C18 HS-150 x 3 mm, 5 µm
Flow (mL/min): 0.35</td><td rowspan="8">Ion Source: ESI
Mass Scanning Range (m/z): 100 - 900
Sheath gas flow rate: 45
Aux gas flow rate: 10
Spray voltage (kV): 3.80
Capillary temp. (°C): 320
Aux gas heater temp (°C): 320
S-lens RF level: 50.0
Definitions: ILMER Library</td></tr>
<tr><td rowspan="7">Gradient</td><td>Time</td><td>B%</td></tr>
<tr><td>0.00</td><td>50</td></tr>
<tr><td>1.00</td><td>50</td></tr>
<tr><td>3.00</td><td>100</td></tr>
<tr><td>6.00</td><td>100</td></tr>
<tr><td>7.00</td><td>50</td></tr>
<tr><td>15.00</td><td>50</td></tr>
</table>

Preparation of nanofibers by electrospinning

0.6% (w/v) HPC and 8% (w/v) PVP were dissolved in ethanol by stirring at 400 rpm overnight at room temperature to obtain empty HPC/PVP solution. In order to prepare propolis-containing HPC/PVP solution, ethanolic propolis extract (PE) was added to the polymer blend at increasing 10%, 20% and 30% (v/v) concentrations, the mixtures being stirred at 400 rpm overnight, at room temperature, to get homogeneous solutions (PE/HPC/PVP). The viscosity of HPC/PVP and PE/HPC/PVP solutions was assessed using a viscometer (Brookfield Engineering, Middleboro, MA, USA) fitted with Spindle 52 at 50 rpm, 25°C, while the pH and conductivity were measured using a multimeter (Eutech Instruments PC2700, Landsmeer, The Netherlands) at room temperature.

In order to perform the electrospinning procedure, the homogeneous solutions were separately placed into 2 mL syringes with a flat-end metal needle (inner diameter 0.7 mm) and the syringe was settled horizontally on the syringe pump of electrospinning unit (NE-100, Inovenso, Istanbul, Türkiye). The feeding rate of the polymer solution was controlled by syringe pump adjusted to a volume flow rate of 1 mL/h, while 18 kV and 21 kV voltage were supplied for empty and propolis-containing nanofibers, respectively. Electrospinning was carried out at room temperature and the obtained nanofiber formulations are summarized in Table II and Table III.

Table II

Electrospinning solutions

Solutions	% HPC (w/v)	% PVP (w/v)	% PE (v/v)
HPC/PVP	0.6	8	0
10-PE/HPC/PVP	0.6	8	10
20-PE/HPC/PVP	0.6	8	20
30-PE/HPC/PVP	0.6	8	30

Table III

Nanofiber electrospinning conditions

Nanofibers	Voltage (kV)	Distance (cm)	Flow rate (mL/h)
HPC/PVP	18	15	1
10-PE/HPC/PVP	21	15	1
20-PE/HPC/PVP	21	15	1
30-PE/HPC/PVP	21	15	1

Physico-chemical characterization of nanofibers mats

Electrospun nanofiber mats were investigated by field emission scanning electron microscopy (FE-SEM; SU7000, Hitachi, Japan) in terms of morphology of the surface and fibre diameters. The samples were coated with ultra-thin gold film. FTIR (Fourier Transform Infrared) spectra were recorded using the instrument Thermo Scientific, Nicolet iS50 FT-IR (Madison, WI, USA) in a range of 500 - 4000 cm^{-1} to assess the incorporation of propolis in HPC/PVP polymeric mixture, and OMNIC 8.0 software was used for data analysis.

The surface wettability and hydrophilicity of the nanofibers were evaluated by measuring the contact angle (Attension Theta Flex, Biolin Scientific, Oy, Finland). The nanofiber mats were cut in 1 cm × 1 cm size, placed on the contact angle analysing plate, and 5 µL deionized water was dropped through the computer-controlled process on 0 - 10 s intervals, while the photographic image was recorded immediately. The contact angle was defined by fitting the Young-Laplace equation around the droplet, the data being analysed with the software OneAttension Version 3.1.

Thermal gravimetric analysis (TGA) was employed to evaluate the decomposition pattern and decrease in sample mass as a function of temperature using TGA device (TGA 8000, PerkinElmer, Walthman, MA, USA), operating in the temperature interval from 25 to 600°C with a rate of 10°C min/min under N_2 atmosphere [21].

In vitro assays

Antioxidant activity and radical scavenging capacity of the nanofibers were evaluated using the stable DPPH radical according to the method described by Singh *et al*. [22]. The reduction of DPPH was recorded spectrophotometrically at 517 nm using a UV-spectrophotometer (Multiskan-Sky UV-Vis, Thermo Fisher Scientific Inc., Massachusetts, USA). Briefly, the nanomaterials were allowed to interact with freshly prepared methanolic DPPH solution (80 µM), and after 30 minutes incubation in darkness, at room temperature, the absorbance was measured at 517 nm against blank (methanol). The antioxidant capacity was calculated using the equation (1):

$$\% \text{ Radical Scavenging Activity} = [(A_0 - A_1)/A_0] \times 100, \quad (1)$$

where, A_0 was the absorbance of DPPH free radical solution in methanol, A_1 the absorbance of the sample.

The cytotoxicity of the nanofibers was evaluated by XTT assay using mouse fibroblast cell line (NIH/3T3) according to the cytotoxicity test reference ISO10993–5 standards. The NIH/3T3 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM F-12) with 10% foetal bovine serum (FBS) and 1% Pen-Strep (100 µl/mL penicillin and 100 µg/mL streptomycin) and incubated at 37°C and 5% CO_2 (NUVE, EC160, Türkiye). The confluent culture was trypsinized and centrifuged at 1000 rpm for 5 min, and the viable cells were counted with a haemocytometer. The cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C for 24 h. Prior to treatment, nanofibers were filtered through 0.45 µm (pore size) filter membranes for size-occlusion sterilization. Then, experimental cell groups were incubated with nanofibers samples in a concentration range between 0.1 mg/mL and 1 mg/mL for 24 h. Positive control groups were treated with Triton X-100 and control groups were supplemented with normal media. After 24 h of incubation, 100 µL fresh medium containing 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) with phenazine methosulfate (PMS) was added to each well, and placed in the incubator for 4 h. Absorbance was measured at 450 nm using a Lab Line MultiPlate Reader. The percentage of cell viability was calculated according to the equation (2) [23]:

$$Cell\ viability\ (\%) = \frac{Optical\ density\ of\ sample}{Optical\ density\ of\ control}\ x\ 100, \quad (2).$$

In order to examine the biocompatibility of the nanofibers, the indirect XTT assay was employed based on the extraction method [24]. The extraction of samples was performed according to the ISO 10993–5 standards. To prepare the nanofiber extracts, 1 mL of the culture medium without FBS was added to each 5 mg nanofiber mat and kept in the incubator at 37°C for 7 days. NIH/3T3 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and incubated at 37°C for 24 h. Then, the culture medium was removed, and 90 µL of sample extracts from 1st, 3rd and 7th day along with 10 µL FBS were added to each well. The cells were allowed to interact with these nanofiber extracts for 24 h in the incubator at 37°C in 5% CO_2. After 24 h, 100 µL XTT with PMS was added to each well and incubated for another 4 h. Absorbance was measured at 450 nm using a Lab Line MultiPlate Reader (Lab Line, Cairo, Egypt). The cell viability was calculated according to the equation (2).

Statistical analysis

All the results are presented as the mean ± standard deviation of at least three to six experiments. The statistical analysis was performed using the one-way ANOVA with $P < 0.05$ as the level of significance (GraphPad Software, Inc., version 7.0a, La Jolla, CA, USA).

Results and Discussion

Identification and quantification of the bio-active compounds in propolis extract

The qualitative and quantitative determination of bio-active compounds in propolis extract, performed with LC-HRMS, are presented in Table IV (LC-HRMS chromatograms presented in supplementary material S1).

Table IV

Compound amounts determined in propolis sample LC-HRMS (mg/L)[a]

Compound	RT[b]	Quantification (mg/L)[c]	m/z
Pyrogallol	2.28	82.496 ± 3.600	125.024
(+)-Catechin	2.39	10.408 ± 0.648	289.071
Chlorogenic acid	2.49	39.960 ± 1.864	353.087

Compound	RT[b]	Quantification (mg/L)[c]	m/z
3,4-Dihydroxybenzaldehyde	3.01	50.144 ± 3.032	137.024
Verbascoside	3.03	0.136 ± 0.034	623.198
Caffeic acid	3.22	6300.400 ± 2.992	179.035
Luteolin-7-rutinoside	3.96	3.608 ± 2.448	593.151
p-Coumaric acid	4.25	65498.024 ± 2.648	163.040
Rutin	4.61	159.968 ± 2.456	609.146
Hyperoside	4.71	339.928 ± 2.768	463.088
Dihydrokaempferol	4.96	136.904 ± 2.288	287.056
Quercitrin	5.25	101.712 ± 3.024	447.093
Ellagic acid	5.27	104.448 ± 3.360	300.999
Myricetin	5.31	1.504 ± 0.044	317.030
Quercetin	5.81	471.296 ± 2.361	301.035
Naringenin	6.00	12955.712 ± 3.344	271.061
Kaempferol	6.21	504.017 ± 2.848	285.040
Caffeic asit phenethyl ester	6.88	825.264 ± 2.504	283.097
Chrysin	7.17	764.672 ± 2.592	253.050
Acacetin	7.41	1002.000 ± 3.184	283.061
Genkwanin	7.41	112.096 ± 3.552	283.061
Chrysoeriol	6.42	259.224 ± 1.664	299.056
Apigenin 7-metilat	7.41	143.528± 2.352	283.061

[a] The values represent the mean ± S.E.M. of three parallel measurements ($p < 0.05$); [b] RT: Retention time; [c] Values in mg/L (w/v) of extract

Physico-chemical and morphological characterization

The results of pH, conductivity and viscosity measurements of the electrospinning solutions are given in Table V. It can be observed the pH values were between 5.5 - 5.6 for all of the solutions, while the conductivity of the polymeric blend ranges between 17.06 ± 0.11 µS/cm and 68.63 ± 0.17 µS/cm. It can be observed that the viscosity of the polymeric blends decreased with the addition of either 10% or 20% (v/v), while 30% (v/v) propolis addition increased the viscosity of the mixture. Nevertheless, the viscosity values of all electrospinning solutions were acceptable for good electrospinning.

Table V

pH, conductivity and viscosity values of the electrospinning solutions

Solutions	pH	Conductivity (µS/cm)	Viscosity (cP)
HPC/PVP	5.69 ± 0.02	17.06 ± 0.11	226.29 ± 21.36
10-PE/HPC/PVP	5.63 ± 0.03	31.43 ± 0.10	184.88 ± 14.88
20-PE/HPC/PVP	5.54 ± 0.06	47.78 ± 0.09	199.61 ± 36.52
30-PE/HPC/PVP	5.51 ± 0.01	68.63 ± 0.17	271.39 ± 5.81

The surface morphology of the nanofiber formulations is presented in Figure 1; the micrographs being recorded with different magnifications (2000x, and respectively, 10000x). It can be observed the uniform, long, continuous and non-beaded feature of the fibres, in all the samples. Moreover, when high propolis concentrations (20% and 30% (v/v)) were incorporated into the nanofibers, and their appearance seems to be more homogenous. The average fibre diameters were calculated using Image J software (National Institute of Health, USA) by randomly measuring the diameters of 60 separate fibres from each sample, the results being displayed in Figure 2, along with the size distribution. The average fibre diameter of HPC/PVP formulation was found to be 528.764 ± 197.263 nm, while the average size of propolis nano-fibers ranges between 197.261 nm and 268.483 nm (Figure 2).

The structural characterization of the nanofiber formulations performed by FTIR spectroscopy is presented in Figure 3, displaying the vibrational features of raw polymers (HPC and PVP) and the nanofiber formulations HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP. The vibrational features of PVP at 2950 cm^{-1} (asymmetric CH2 pyrrole ring), at 1647 cm^{-1} (C=O stretching), at 570 cm^{-1} (N-C=O) and of HPC at 3378 cm^{-1} (OH vibrational stretching), 2920 - 2874 cm^{-1} (symmetric C-H stretching of methyl and hydroxypropyl groups), 1646 cm^{-1} (stretching vibration of C–O for six-membered cyclic rings), 1076 cm^{-1} (ethereal C–O-C stretching) and 837 cm^{-1} (rocking mode of CH_2) are clearly observed. In the spectra of HPC/PVP nanofibers, besides of these characteristic peaks of PVP and HPC, there is a broad band at 3406 cm^{-1} and the sharp peak at 1079 cm^{-1} due to the hydrogen bonding between the carbonyl groups of PVP and the hydroxyl groups of HPC. In the case of propolis nanofibers, especially at the highest concentration of propolis, the peak at 3406 cm^{-1} shifted to 3419 cm^{-1} due to the phenolic compounds and amino acids in propolis. In addition, the peaks at 1084 cm^{-1} and 1439 cm^{-1} correspond to the stretching

vibration of aromatic ether C-O groups and stretching of C=C groups, respectively, being more prominent in 30-PE/HPC/PVP formulation. Moreover, the peaks around 1660 cm^{-1} are related to stretching of C=O, C=C and asymmetric bending of N-H groups of flavonoids and amino acids in propolis [25-27].

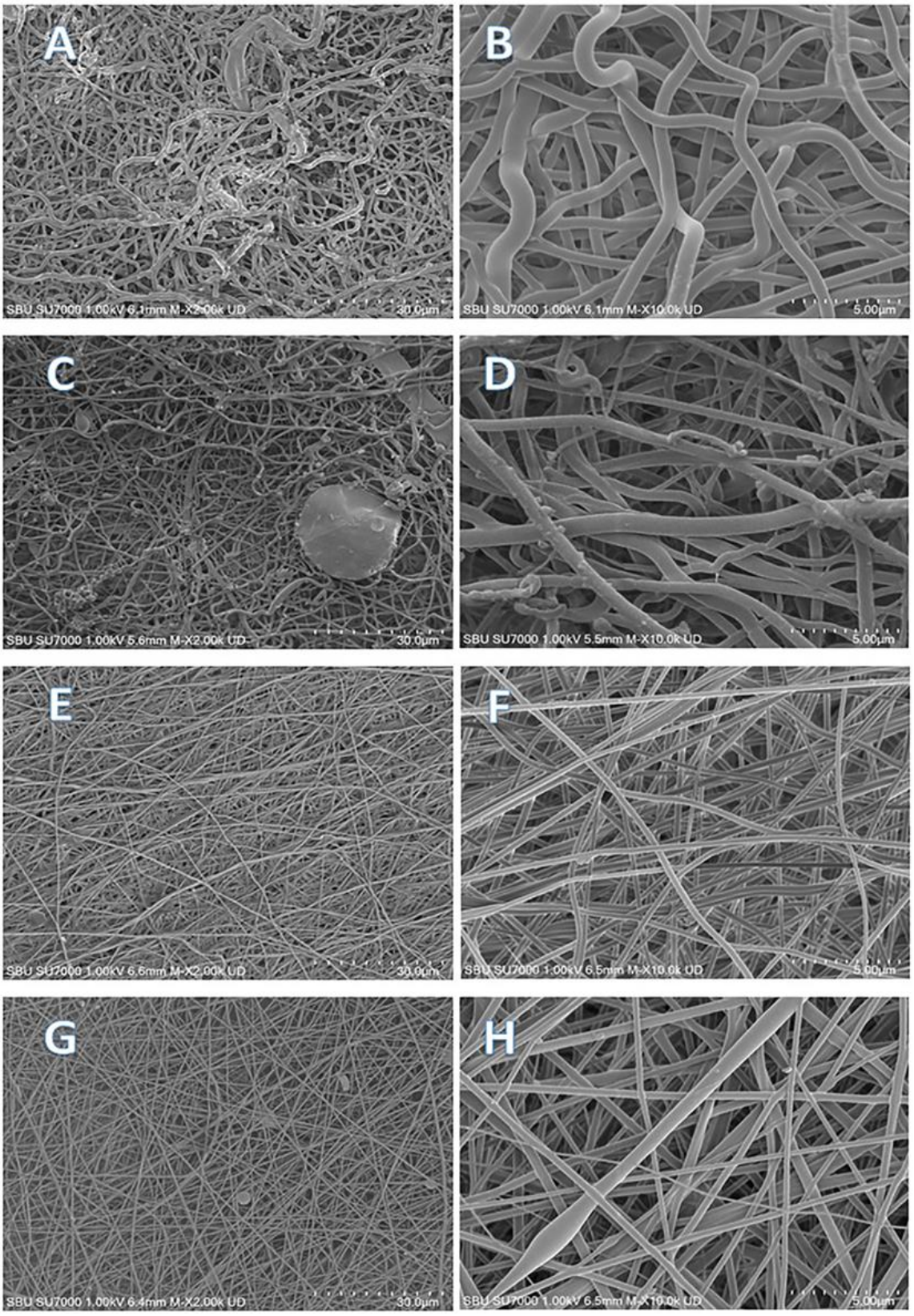


Figure 1.
SEM images of the nanofiber formulations: HPC/PVP (A, B); 10-PE/HPC/PVP (C, D); 20-PE/HPC/PVP (E, F) and 30-PE/HPC/PVP (G, H), recorded with different details and magnifications (2000x, respectively 10000x)

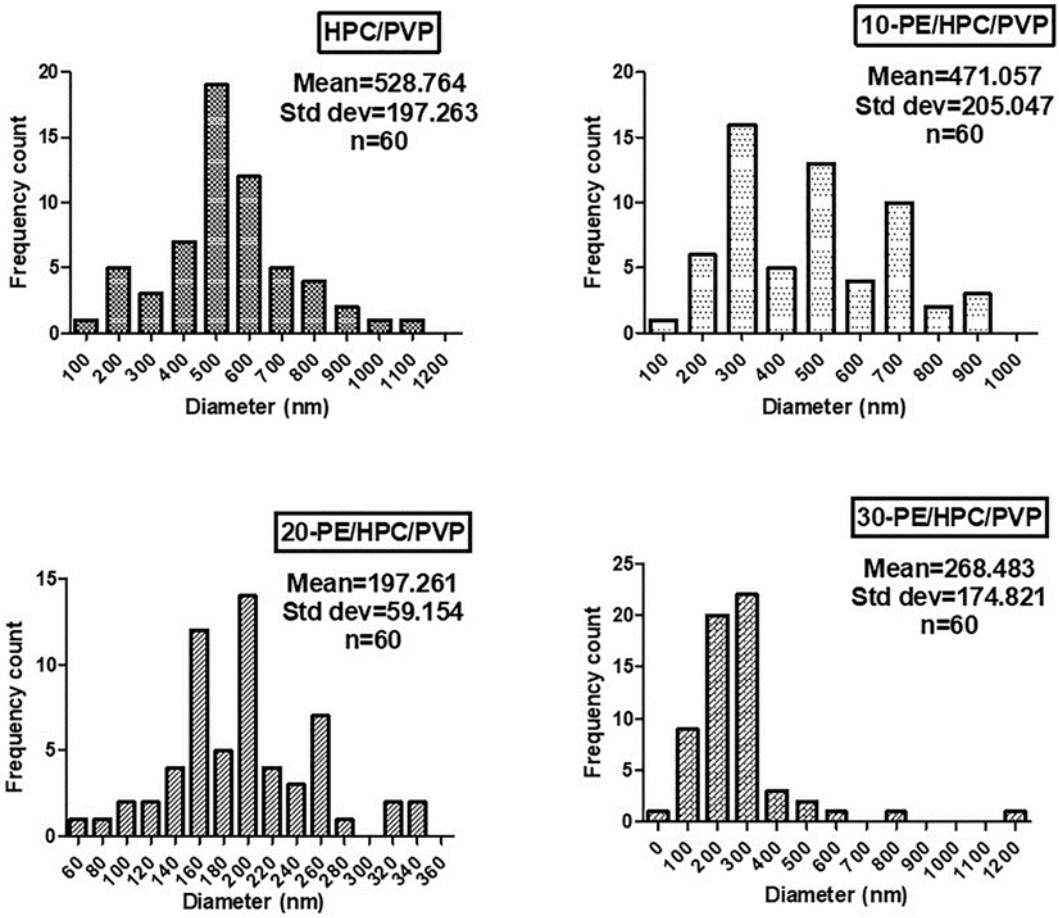


Figure 2.
Graphical representation of the size distribution (diameter) of the nanofiber formulations: HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP

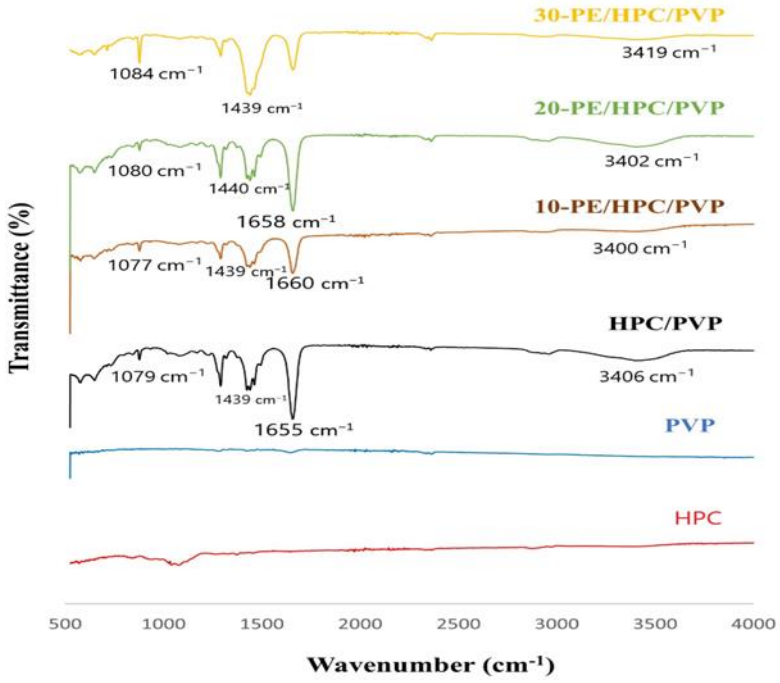


Figure 3.
FTIR spectra of the raw polymers (HPC and PVP) and the nanofiber formulations: HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP

Wettability is an important parameter in nanofiber mats, especially for effective wound healing purpose [28]. In order to prevent excessive dehydration and exudate accumulation, a suitable biomaterial should be able to maintain a moist environment for wound healing. Contact angle measurements were carried out to evaluate the wettability of the nanofiber mats. The contact angle values of the nanofiber formulations are listed in Table VI, while the photographic details are presented in Figure 4.

Table VI
Mean contact angle values for nanofiber formulations

Formulation	Mean contact angle value (°)
HPC/PVP	26.33
10-PE/HPC/PVP	38.04
20-PE/HPC/PVP	52.57
30-PE/HPC/PVP	45.62

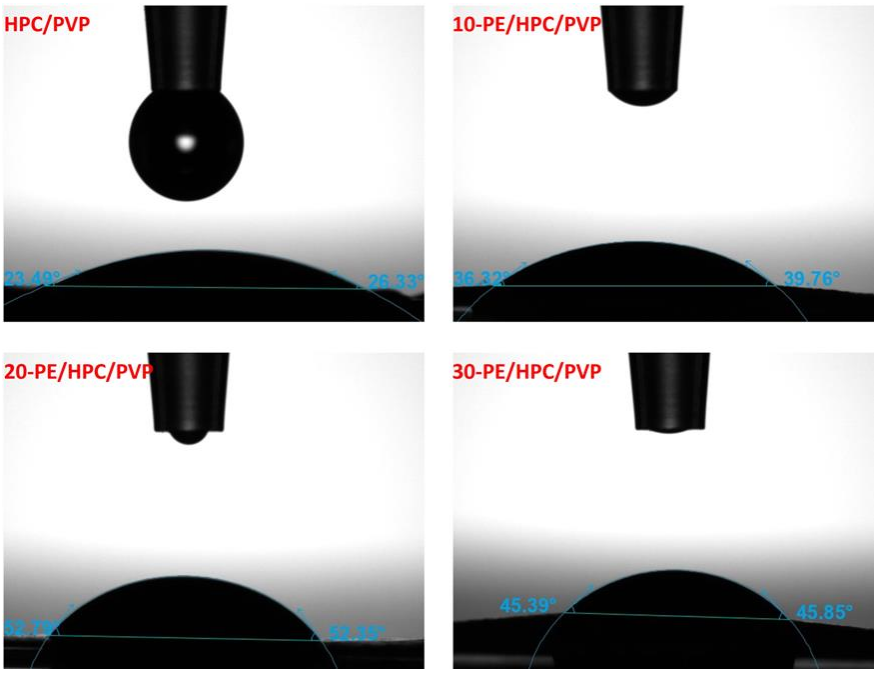


Figure 4.
Contact angle measurements on the surface of the nanofiber materials

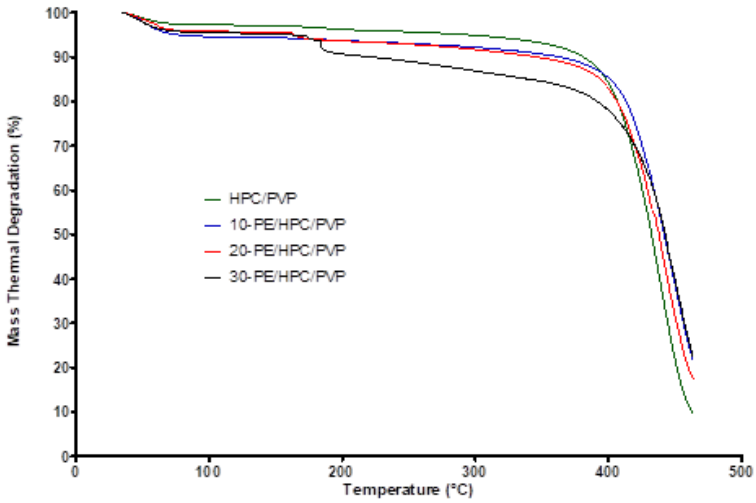


Figure 5.
TGA thermograms of the nanofiber formulations of HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP

The thermal degradation pattern of the nanofiber formulations is shown in Figure 5. Except HPC/PVP formulation, all the samples shown good thermal stability, with degradation and mass loss beginning around the 400°C. It can be inferred that inclusion

of propolis in the polymeric mixture resulted in improved thermal stability of the nanofibers.

In vitro assay: evaluation of antioxidant activity, cytotoxicity and biocompatibility of the nanofiber formulations

The antioxidant capacity of the nanospun materials evaluated by DPPH assay (Figure 6) revealed that the incorporation of propolis extract in HPC/PVP formulations significantly increased the antioxidant activity of the nanofibers ($p < 0.05$).

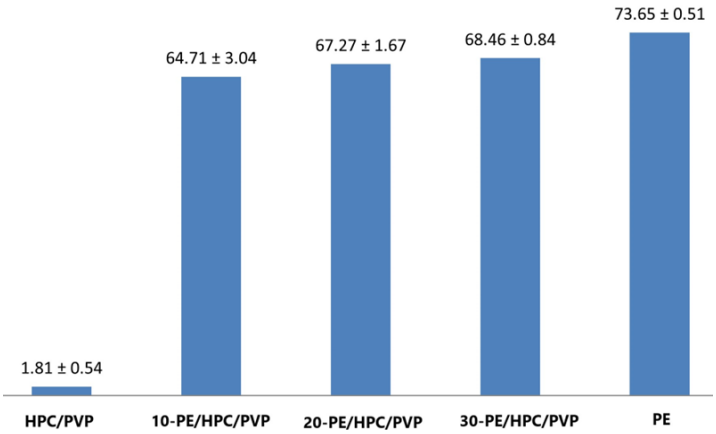


Figure 6.

Antioxidant activity (DPPH assay) of PE and nanofiber formulations: HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP

The results are expressed as the half-maximum inhibitory concentration (IC50) of DPPH radicals in µg/mL

In vitro cytotoxicity results are presented in Figure 7 and Figure 8. According to ISO10993-5 standards, if the cell survival rate is higher than 70%, the material does not present cytotoxicity. At the end of the 1st and 3rd day, for all the tested concentrations (100 µg/mL, 200 µg/mL, 500 µg/mL and 1 mg/mL), the formulations presented almost 100 % viability of the fibroblast cells, showing lack of toxicity. By comparison, it can be noticed that propolis extract alone showed an average of 50% viability. In addition, all the nanofiber formulations showed lack of toxicity, when compared to positive control.

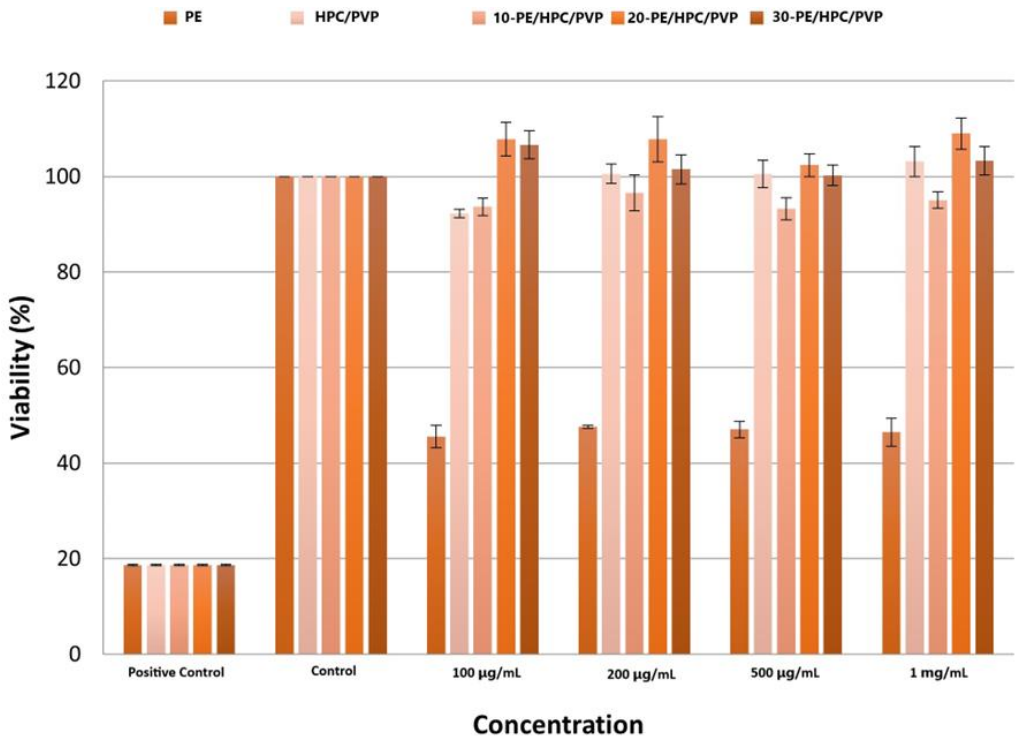


Figure 7.

Viability of NIH/3T3 cells with respect to PE and nanofiber formulations: HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP at different concentrations: 100 µg/mL, 200 µg/mL, 500 µg/mL and 1 mg/mL, at the end of 1st day

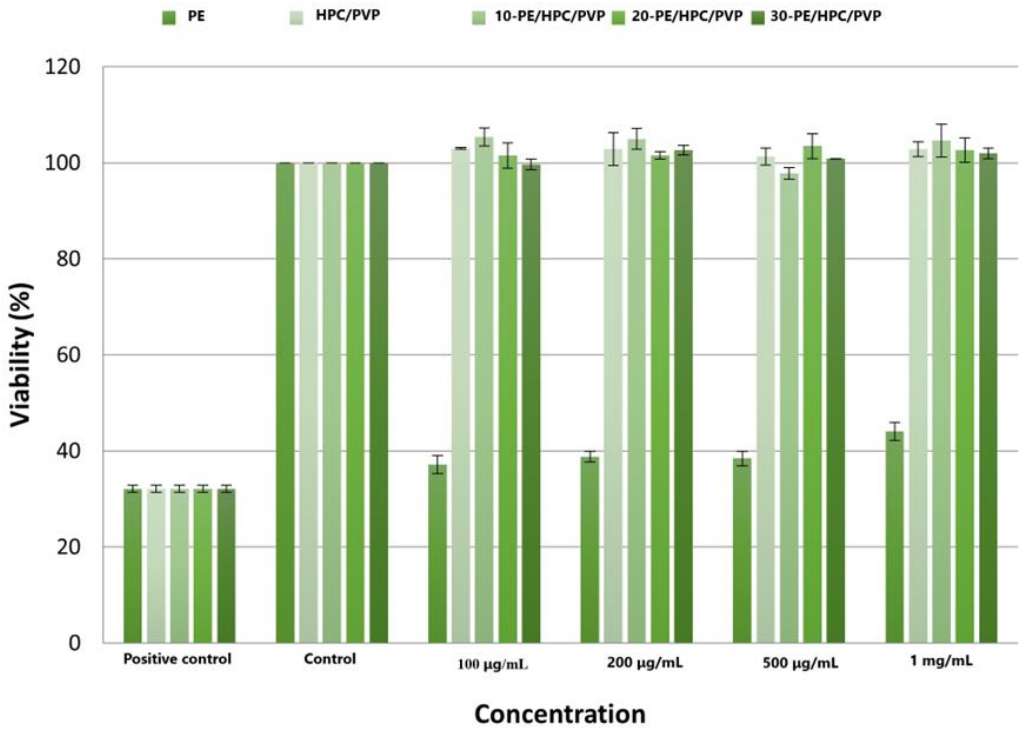


Figure 8.

Viability of NIH/3T3 cells with respect to PE and nanofiber formulations: HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP at different concentrations: 100 µg/mL, 200 µg/mL, 500 µg/mL and 1 mg/mL at the end of 3rd day

In vitro biocompatibility results are shown in Figure 9; while 90% viability determined at the end of the first day for the propolis-free formulation (HPC/PVP), at the end of the 7th day, the viability decreased to 70%. With the increasing concentrations of propolis extract, the cell viability increased to 100% and remained unchanged for 7 days. These results showed that the bioactive compounds released from the nanofiber formulations over time ensure the growth and proliferation of NIH/3T3 cells.

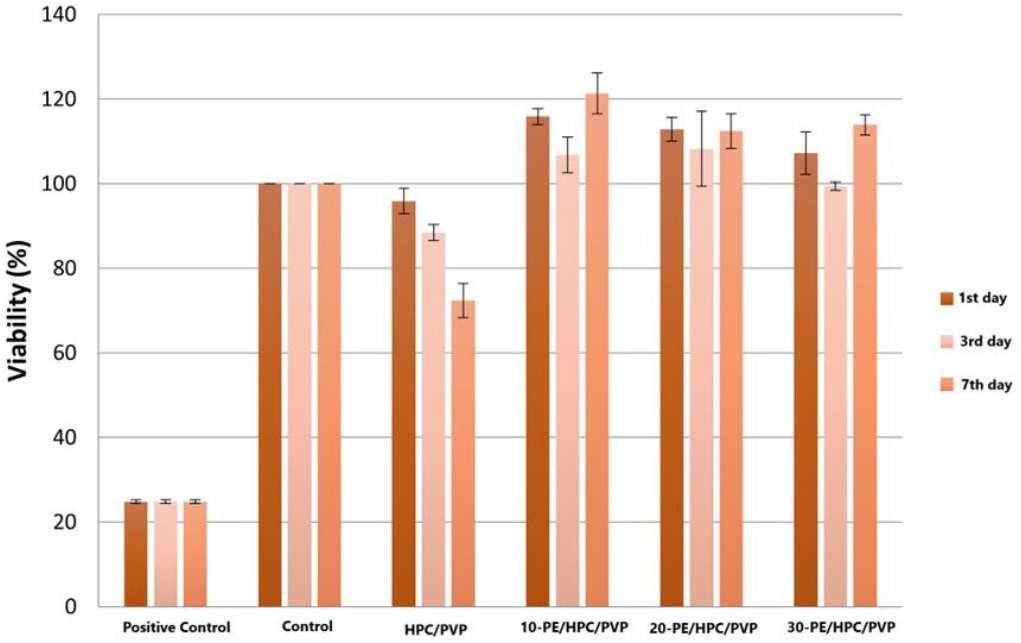


Figure 9.

In vitro biocompatibility of nanofiber formulations of HPC/PVP, 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP at the end of the 1st, 3rd and 7th days

In this study, a total of 23 bio-compounds were identified in propolis extract was obtained from Sivas, Türkiye. It has been demonstrated its rich content in polyphenols: p-coumaric acid, naringenin, caffeic acid, acacetin and caffeic acid phenethyl ester were detected as the major compounds along with chrysin, kaempferol, quercetin, hyperoside, rutin and ellagic acid. The incorporation of propolis extract into the nanofiber mats resulted in improved antioxidant capacity of the 10-PE/HPC/PVP, 20-PE/HPC/PVP and 30-PE/HPC/PVP formulation toward the propolis-free HPC/PVP nanofibers. It is well known that polyphenolic compounds possess high antioxidant, antimicrobial and anti-inflammatory activities, as previously demonstrated by numerous *in vivo* and *in vitro* studies [29, 30].

Cellulose derivatives are one of the most important classes of biomolecules possessing optimal physicochemical, mechanical and biological properties, suitable for cutaneous applications such as wound healing, revitalizing and antiaging. Moreover, these polymers are generally preferred in drug delivery systems due to high stability, controlled drug release, non-toxicity, biodegradability and bioavailability properties [31]. Propolis has been incorporated into nanofibers composed of cellulose-derived polymers, according to several studies. Sharaf *et al*. [32] prepared cellulose acetate (CA) electrospun nanofiber mats and loaded with honeybee propolis extract. CA was used as a carrier for honeybee propolis extract and investigated for scaling-up potential. It was found that prepared electrospun mats might be employed as both wound healing dressing and antibacterial drugs due to their sustained release of propolis from the nanofibers. In another study, CA and polycaprolactone (PCL) nanofibers were impregnated with a propolis-extracted solution to obtain an antibacterial and antioxidant material for wound healing applications. The resulted propolis/nanofiber mats exhibited a high antioxidant potential, determined by DPPH assay, and antibacterial activity against both the Gram-positive and Gram-negative bacteria, thus accepted as a suitable system for wound healing [33].

Abdi *et al*. [34] loaded propolis into pH-sensitive nanofiber mats composed of Eudragit® L-100 and hydroxypropyl methyl cellulose for wound healing purpose. Propolis was loaded at the concentrations of 10 - 30% (v/v) and according to their SEM images, the average diameter of nanofibers increased from 589.82 nm to 676.01 nm, with increasing propolis content. In our study, propolis was also successfully incorporated into nanofiber in concentrations of 10 - 30% (v/v), but in we noticed a decrease of the average fibre diameter due to increasing conductivity of PE/HPC/PVP solutions. The thinnest fibre obtained in our experiment was 20-PE/HPC/PVP formulation, with average fibre diameters of 197.261 ± 59.154 nm, followed by 30-PE/HPC/PVP with average diameter 268.483 ± 174.821 nm. The higher conductivity of electrospinning solution was associated to the thinner fibres, while higher viscosity resulted in increasing fibre diameters [10]. Also, the addition of increasing propolis concentration (10%, 20% and 30%) into the polymer blend, resulted in higher conductivity, promoted electro spinnability. The successful incorporation of propolis into the nanofibers was confirmed by FTIR analysis and the results were consistent with the literature [25-27].

The hydrophilicity of the novel formulations, when topically applied, might affect the interactions between the extracellular matrix and the skin cells [35]. As a general rule, if the contact angle < 90°, the material is considered to be hydrophilic, while a contact angle > 90°, means hydrophobicity [36]. Our propolis-free formulation HPC/PVP had a mean contact angle value of 26.33°, indicating high hydrophilic feature. When propolis was incorporated, hydrophilicity decreased slightly in all the formulations. This might be due to the absence of low soluble compounds in propolis extract. Decreased hydrophilicity of the nanofiber mats after incorporation of high amounts of propolis was also reported in other studies [37, 38]. Nevertheless, all the propolis-containing nanofiber formulations could be accepted as moderate hydrophilic, which supports cell interaction and growth [39]. In our study, all of the formulations were non-cytotoxic to skin fibroblast cells; the moderate hydrophilicity of the propolis incorporated nanofibers resulted in increased cell viability during 7 days. These findings are consistent with the research of Mirbagheri *et al*. [40] on propolis-loaded nanofiber scaffolds based on polyvinyl alcohol and polycaprolactone and Eskandarinia *et al*. [41] on propolis enriched polyurethane-hyaluronic acid nanofibrous wound dressings. Overall, owing their highly antioxidant, antimicrobial and anti-inflammatory properties, it is accepted that polyphenolic compounds can be effective in dermatological conditions such as acne, alopecia, fungal infections, photoaged skin and hyperpigmentation, as well as chronic diseases including cardiovascular disorders and cancer [42, 43]. A cream formulation based on p-coumaric acid - which is the most abundant compound of the propolis extract - was demonstrated to effectively reduce the UV-induced erythema and subsequent pigmentation in human skin, according to a clinical trial conducted by Seo *et al*. [44]. Naringenin-based topical formulation's potential to alleviate ultraviolet-induced inflammation and the effect of reducing oxidative stress was demonstrated by *in vivo* study conducted by Martinez *et al*. [45]. A lotion formulation rich in polyphenolic compounds including 2% green tea extract (*Camellia sinensis*) was found to decrease the effect on inflammatory acne lesions, as revealed by Sharquie *et al*. [46]. Moreover, chrysin was found to diminish the effect of imiquimod-induced psoriasis-like skin lesions in mice,

suggesting its potential use against inflammatory skin diseases, according to Li *et al*. [47]. In another study, the effect of caffeic acid was demonstrated *in vivo*, by reducing the acute and chronic cutaneous inflammations in mice, as well *in vitro* towards human keratinocytes, according to Zhang *et al*. [48]. In our study, high antioxidant potential of all propolis incorporating formulations was proven, and the results were consistent with other studies [33, 49]. Di Capua *et al.* [50] conducted a study with supercritical assisted atomization using hydroxypropyl-β-cyclodextrin and polyvinylpyrrolidone carriers to protect propolis against oxidation and to increase its bioavailability. Similar to our study, the authors found a direct correlation between the amount of propolis extract incorporated into polymeric carriers and their anti-oxidant capacity.

Conclusions

As a conclusion, efficacious, safe and easy to apply propolis nanofiber mats were successfully produced, possessing suitable morphological, physiochemical and biological properties. According to LC-HRMS analyses, propolis extract collected from Sivas, Türkiye, is very rich in polyphenolic compounds such as chrysin, kaempferol, quercetin, hyperoside, rutin and ellagic acid. All of the nanospun formulation prepared by propolis incorporation into HPC/PVP possess beadles, nanofibrous shape, moderate hydrophilicity, thermo-stability, high antioxidant activity, biocompatibility and no cytotoxicity. We suggest that topical application of propolis *via* nanofiber type carriers may localize a high concentration of the bioactive compounds in the superficial skin layers, to treat cutaneous disorders, by increasing their solubility, stability and penetration, while minimizing systemic exposure. Particularly, with the highest propolis concentration, 30-PE/HPC/PVP nanofiber formulation is a promising alternative to conventional treatments for cutaneous applications.

Acknowledgement

The APC for this manuscript was supported by University of Oradea, Romania.

Conflict of interest

The authors declare no conflict of interest.

References

1. López BGC, Schmidt EM, Eberlin MN, Sawayaa ACHF, Phytochemical markers of different types of red propolis. *Food Chem.*, 2014; 146: 174-180.
2. Kurek-Górecka A, Keskin Ş, Bobis O, Felitti R, Górecki M, Otręba M, Stojko J, Olczyk P, Kolayli S, Rzepecka-Stojko A, Comparison of the antioxidant activity of propolis samples from different geographical regions. *Plants (Basel)*, 2022; 11(9): 1203.
3. Bankova V, Popova M, Trusheva B, Propolis volatile compounds: chemical diversity and biological activity: a review. *Chem Cent J.*, 2014; 8(1): 1-8.
4. Kapare HS, Sathiyanarayanan L, Nutritional and therapeutic potential of propolis: A review. *Res J Pharm Technol.*, 2020; 13(7): 3545.
5. Bobiş O, Berretta AA, Vilas-Boas M, De Jong D, Editorial: Therapeutic potential of propolis—from *in vitro* studies to clinical trials. *Front Pharmacol.*, 2023; 14: 1192045.
6. Pasca PM, Cavalu S, The influence of propolis nanoparticles on dermal fibroblasts migration: premises for development of propolis-based collagen dermal patches. *Dig J Nanomater Biostructures*, 2021; 16(3): 929-938.
7. Kapare HS, Giram PS, Raut SS, Gaikwad HK, Paiva-Santos AC, Formulation development and evaluation of Indian propolis hydrogel for wound healing. *Gels*, 2023; 9(5): 375.
8. Abdellatif MM, Elakkad YE, Elwakeel AA, Allam RM, Mousa MR, Formulation and characterization of propolis and tea tree oil nanoemulsion loaded with clindamycin hydrochloride for wound healing: *In-vitro* and *in-vivo* wound healing assessment. *Saudi Pharm J.*, 2021; 29(11): 1238-1249.
9. Hozzein WN, Badr G, Al Ghamdi AA, Sayed A, Al-Waili NS, Garraud O, Topical application of propolis enhances cutaneous wound healing by promoting TGF-beta/Smad-mediated collagen production in a streptozotocin-induced type I diabetic mouse model. *Cell Physiol Biochem.*, 2015; 37(3): 940-954.
10. Esentürk İ, Erdal MS, Güngör S, Electrospinning method to produce drug-loaded nanofibers for topical/ transdermal drug delivery applications. *Istanbul J Pharm.*, 2016; 46(1): 49-69.
11. Esentürk Güzel I, Güngör S, Erdal MS, Improved skin penetration and deposition of naftifine from transethosomes and transethosomal gel formulations. *Farmacia*, 2022; 70(3): 514-521.
12. Pelipenko J, Kocbek P, Kristl J, Critical attributes of nanofibers: Preparation, drug loading, and tissue regeneration. *Int J Pharm.*, 2015; 484: 57-74.
13. Elsadek NE, Nagah A, Ibrahim TM, Chopra H, Ghonaim GA, Emam SE, Cavalu S, Attia MS, Electrospun nanofibers revisited: An update on the emerging applications in nanomedicine. *Materials*, 2022; 15: 1934.
14. El-Newehy MH, El-Naggar ME, Alotaiby S, El-Hamshary H, Moydeen M, Al-Deyab S, Green electrospining of hydroxypropyl cellulose nanofibres for drug delivery applications. *J Nanosci Nanotechnol.*, 2018; 18(2): 805-814.
15. Gencturk A, Kahraman E, Güngör S, Özhan G, Özsoy Y, Sarac AS, Polyurethane/hydroxypropyl cellulose electrospun nanofiber mats as potential transdermal drug delivery system: characterization studies and *in vitro* assays. *Artif Cells Nanomed Biotechnol.*, 2017; 45(3): 655-664.
16. Wang Y, Shi L, Wu H, Li Q, Hu W, Zhang Z, Huang L, Zhang J, Chen D, Deng S, Tan S, Jiang Z, Graphene Oxide–IPDI–Ag/ZnO@Hydroxypropyl Cellulose nanocomposite films for biological wound-dressing applications. *ACS Omega*, 2019; 4(13): 15373-15381.

17. Ali EA, Abo-Salem HM, Arafa AA, Nada AA, Chitosan Schiff base electrospun fabrication and molecular docking assessment for nonleaching antibacterial nanocomposite production. *Cellulose*, 2023; 30(6): 3505-3522.
18. Franco P, De Marco I, The use of poly (N-vinyl pyrrolidone) in the delivery of drugs: A review. *Polymers*, 2020; 12(5): 1114.
19. Kayabaşı S, Comparison of phytochemical properties and antioxidant capacities of propolis extracts prepared using new generation techniques. Bayburt University Graduate School of Education Food Engineering Department Master Thesis, Bayburt, Türkiye, 2019, 45.
20. Yıldız O, Evaluation of solvents (menstruums) used in consumable propolis extracts. *U Bee J.*, 2020; 20: 24-37.
21. Voicu ME, Drăgănescu D, Anuța V, Stoian AB, Ioniță D, Demetrescu I, The interrelationship between the drug embedded TiZr surface properties and antibacterial effect. *Farmacia*, 2022; 70(6): 1064-1071.
22. Singh G, Maurya S, DeLampasona MP, Catalan CA, A comparison of chemical, antioxidant and antimicrobial studies of cinnamon leaf and bark volatile oils, oleoresins and their constituents. *Food Chem Toxicol.*, 2007: 45(9); 1650-1661.
23. Karavelioglu Z, Cakir-Koc R, Preparation of chitosan nanoparticles as Ginkgo Biloba extract carrier: *In vitro* neuroprotective effect on oxidative stress-induced human neuroblastoma cells (SH-SY5Y). *Int J Biol Macromol.*, 2021; 192: 675-683.
24. Chogan F, Mirmajidi T, Rezayan AH, Sharifi AM, Ghahary A, Nourmohammadi J, Kamali A, Rahaie M, Design, fabrication, and optimization of a dual function three-layer scaffold for controlled release of metformin hydrochloride to alleviate fibrosis and accelerate wound healing. *Acta Biomater.*, 2020; 113: 144-163.
25. El-Guendouz S, Aazza S, Lyoussi B, Bankova V, Lourenço JP, Costa AMR, Mariano JF, Miguel MG, Faleiro ML, Impact of biohybrid magnetite nanoparticles and Moroccan propolis on adherence of methicillin resistant strains of *Staphylococcus aureus*. *Molecules*, 2016; 21(9): 1208.
26. Bilginer R, Ozkendir-Inanc D, Yildiz UH, Arslan-Yildiz A, Biocomposite scaffolds for 3D cell culture: Propolis enriched polyvinyl alcohol nanofibers favoring cell adhesion. *J Appl Polym Sci.*, 2021; 138(17): 50287.
27. Sutjarittangtham K, Sanpa S, Tunkasiri T, Chantawannakul P, Intatha U, Eitssayeam S, Bactericidal effects of propolis/polylactic acid (PLA) nanofibres obtained *via* electrospinning. *J Apic Res.*, 2014; 53(1): 109-115.
28. Zahedi P, Rezaeian I, Ranaei-Siadat SO, Jafari SH, Supaphol P, A review on wound dressings with an emphasis on electrospun nanofibrous polymeric bandages. *Polym Adv Technol.*, 2010; 21(2): 77-95.
29. Uzel E, Durgun ME, Esentürk-Güzel İ, Güngör S, Özsoy Y. Nanofibers in Ocular Drug Targeting and Tissue Engineering: Their Importance, Advantages, Advances, and Future Perspectives. Pharmaceutics. 2023; 15(4):1062.
30. Tuong W, Walker L, Sivamani RK, Polyphenols as novel treatment options for dermatological diseases: A systematic review of clinical trials. *J Dermatol Treat.*, 2015; 26(4): 381-388.
31. Albuquerque PBS, de Oliveira WF, dos Santos Silva PM, dos Santos Correia MT, Kennedy JF, Coelho LCBB, Skincare application of medicinal plant polysaccharides—A review. *Carbohydr Polym.*, 2022; 277: 118824.
32. Sharaf S, El-Naggar ME, Eco-friendly technology for preparation, characterization and promotion of honey bee propolis extract loaded cellulose acetate nanofibers in medical domains. *Cellulose*, 2018; 25: 5195-5204.
33. Khoshnevisan K, Maleki H, Samadian H, Doostan M, Khorramizadeh MR, Antibacterial and antioxidant assessment of cellulose acetate/polycaprolactone nanofibrous mats impregnated with propolis. *Int J Biol Macromol.*, 2019; 140: 1260-1268.
34. Abdi M, Zakeri-Milani P, Ghorbani M, Designing and evaluating pH-responsive electrospun Eudragit® L-100/Hydroxypropyl methyl cellulose composite mats for release of propolis as a novel wound dressing. *J Polym Environ.*, 2023; 13: 1-15.
35. Unal S, Arslan S, Yilmaz BK, Kazan D, Oktar FN, Gunduz O, Glioblastoma cell adhesion properties through bacterial cellulose nanocrystals in polycaprolactone/ gelatin electrospun nanofibers. *Carbohydr Polym.*, 2020; 233: 115820.
36. Ma Y, Xin L, Tan H, Fan M, Li J, Jia Y, Ling Z, Chen Y, Hu X, Chitosan membrane dressings toughened by glycerol to load antibacterial drugs for wound healing. *Mater Sci Eng.*, 2017; 81: 522-531.
37. Ulag S, Ilhan E, Demirhan R, Sahin A, Yilmaz BK, Aksu B, Sengor M, Ficai D, Titu AM, Ficai A, Gunduz O, Propolis-based nanofiber patches to repair corneal microbial keratitis. *Molecules*, 2021; 26(9): 2577.
38. Bilginer R, Yildiz AA, A facile method to fabricate propolis enriched biomimetic PVA architectures by co-electrospinning. *Mater Lett.*, 2020; 276: 128191.
39. Bilginer R, Ozkendir-Inanc D, Yildiz UH, Arslan-Yildiz A, Biocomposite scaffolds for 3D cell culture: Propolis enriched polyvinyl alcohol nanofibers favoring cell adhesion. *J Appl Polym Sci.*, 2021; 138(17): 50287.
40. Mirbagheri MS, Akhavan-Mahdavi S, Hasan A, Kharazmi MS, Jafari SM, Propolis-loaded nanofiber scaffolds based on polyvinyl alcohol and polycaprolactone. *Int J Pharm.*, 2023; 642: 123186.
41. Eskandarinia A, Kefayat A, Gharakhloo M, Agheb M, Khodabakhshi D, Khorshidi M, Sheikhmoradi V, Rafienia M, Salehi H, A propolis enriched polyurethane-hyaluronic acid nanofibrous wound dressing with remarkable antibacterial and wound healing activities. *Int J Biol Macromol.*, 2020; 149: 467-476.
42. Sun M, Deng Y, Cao X, Xiao L, Ding Q, Luo F, Huang P, Gao Y, Liu M, Zhao H, Effects of natural polyphenols on skin and hair health: A review. *Molecules*, 2022; 27(22): 7832.
43. Matran IM, Martin-Hadmaș RM, Niculaș C, Muntean DL, Tarcea M, Dietary and pharmaco-therapy in skin diseases. *Farmacia*, 2022; 70(2): 177-183.
44. Seo YK, Kim SJ, Boo YC, Baek JH, Lee SH, Koh JS, Effects of p-coumaric acid on erythema and

pigmentation of human skin exposed to ultraviolet radiation. *Clin Exp Dermatol*., 2011; 36(3): 260-266.

45. Martinez RM, Pinho-Ribeiro FA, Steffen VS, Silva TC, Caviglione CV, Bottura C, Fonseca MJV, Vicentini FTMC, Vignoli JA, Baracat MM, Georgetti SR, Verri WA, Casagrande R, Topical formulation containing naringenin: efficacy against ultraviolet B irradiation-induced skin inflammation and oxidative stress in mice. *PLoS One*, 2016; 11(1): e0146296.
46. Sharquie KE, Noaimi AA, Al-Salih MM, Topical therapy of acne vulgaris using 2% tea lotion in comparison with 5% zinc sulphate solution. *Saudi Med J*., 2008; 29(12): 1757-1761.
47. Li HJ, Wu NL, Pu CM, Hsiao CY, Chang DC, Hung CF, Chrysin alleviates imiquimod-induced psoriasis-like skin inflammation and reduces the release of CCL20 and antimicrobial peptides. *Sci Rep*., 2020; 10(1): 2932.
48. Zhang M, Zhou J, Wang L, Li B, Guo J, Guan X, Han Q, Zhang H, Caffeic acid reduces cutaneous tumor necrosis factor alpha (TNF-α), IL-6 and IL-1β levels and ameliorates skin edema in acute and chronic model of cutaneous inflammation in mice. *Biol Pharm Bull*., 2014; 37(3): 347-354.
49. Maroufi LY, Norouzi R, Ramezani S, Ghorbani M, Novel electrospun nanofibers based on gelatin/oxidized xanthan gum containing propolis reinforced by Schiff base cross-linking for food packaging. *Food Chem*., 2023; 416: 135806.
50. Di Capua A, Bejarano A, Adami R, Reverchon E, Preparation and characterization of Chilean propolis coprecipitates using Supercritical Assisted Atomization. *Chem Eng Res Des*., 2018; 136: 776-785.