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COLLAGEN-THUJA TINCTURE BIOMATERIALS FOR WOUND TREATMENT. 2. HYDROGELS AND POROUS MATRICES

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Collagen-based biomaterials containing *Thuja occidentalis* tincture – 0.5, 1.0 and 1.5 mL tincture/100 g 1.1% collagen hydrogel – were prepared as hydrogels having the pHs 3.8 and 7.4 and porous matrices by hydrogels' lyophilisation. FT-IR and UV-CD spectra of hydrogels show that thuja tincture does not disturb the triple helical conformation of collagen in the acid hydrogels, while rheograms, storage and loss modules suggest weak interactions with components from tincture. FT-IR spectra indicate a slight denaturation of collagen at pH 7.4 and storage and loss moduli a slight cross-linking, but the measurements were made for the hydrogels resulted by syneresis. FT-IR spectra confirm the preservation of triple helical conformation of collagen and a slight cross-linking in all the matrices. SEM shows an agglomeration of fibrils and an increase of pore size and irregularity which increase with thuja tincture amount that can be assigned to cross-linking.

INTRODUCTION

The following properties are specified for *Thuja occidentalis* tinctures:[1-3] antiviral, antifungal, anti-inflammatory and antibacterial both for gram-positive and gram-negative bacteria. That is why they are used externally to heal eczema, different wounds including the infected ones, burns, eruptions, ulcers, senile and other forms of gangrene[4] or to treat fungal infections of the skin. Applied topically they have the ability to dry the gangrenous surfaces, eliminating thus haemorrhage and suppuration, destroy fetid smell and influence granulation. Given the pronounced antibacterial properties, they are used as such or as dressings to cure a large variety of infections of different aetiologies, especially those having malodorous smells. Moreover, they seem to have effect on tissues, involving the epithelial and sub epithelial structures.[5]

Non-denatured type I collagen, due to its full biodegradability, weak antigenecity, restorability and haemostatic properties, is the primary source in biomedical application,[6,7] including wound healing.[8,9] It has as main function to provide the wound resistance and integrity,[10] but it accomplish also some other functions, interfering in all the healing stages: haemostasis – inducing the growth of fibroblasts by its three-dimensional structure, it is essential for the formation of granular tissue,[11] cell-cell and cell-matrix interactions, intervenes into the proliferation of fibroblasts and deposition of new fibres and produces reepitalization. But it is not able to promote by itself the healing process because, being a protein, it serves as a substrate for bacteria.

Collagen being the ideal support for the immobilization of active principles from plants, the combination of the anti-inflammatory, antibacterial and drying (of harmed surfaces) properties of thuja tincture with the healing capability of collagen is expected to result in efficient dressings for wound healing.

The present paper has as objective the obtaining of collagen-thuja tincture biomaterials – hydrogels and porous matrices – by incorporating thuja tincture in hydrogels, respectively by the

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lyophilisation of hydrogels. The checking of the triple helical structure of collagen molecules from the prepared 1.1% collagen hydrogels having the pHs 3.8 and 7.4 and from matrices obtained from these hydrogels, the effect of 0.5, 1.0 and 1.5 mL tincture/100 g hydrogel on this conformation by FT-IR and/or CD, depending on the collagen state, emphasizing of collagen interaction with components from thuja tincture as a function of pH and tincture content by rheological measurements for hydrogels and by morphology for porous matrices.

RESULTS

The FT-IR spectra of the collagen hydrogels containing the specified amounts of thuja tincture give for the ratios A_{III}/A_{1450} and the differences (νA_I - νA_{II}), used to evaluate the integrity of collagen triple helical conformation and the presence or absence of denatured collagen respectively, the values shown in Table 1.

The superposed UV-CD spectra of the acid collagen hydrogels that contain the indicated quantities of thuja tincture are shown in Figure 1.

Table 1

The ratios A_{III}/A_{1450} and the differences (νA_I - νA_{II}), cm^{-1}, obtained for the prepared collagen hydrogels containing the specified amounts of thuja tincture

Thuja tincture, mL/100 g hydrogel	pH			
	3.8		7.4	
	A_{III}/A_{1450}	(νA_I - νA_{II}), cm^{-1}	A_{III}/A_{1450}	(νA_I - νA_{II}), cm^{-1}
0	1.28	91	1.00	100
0.5	1.52	92	0.91	104
1.0	2.00	94	0.81	106
1.5	4.00	94	0.74	106

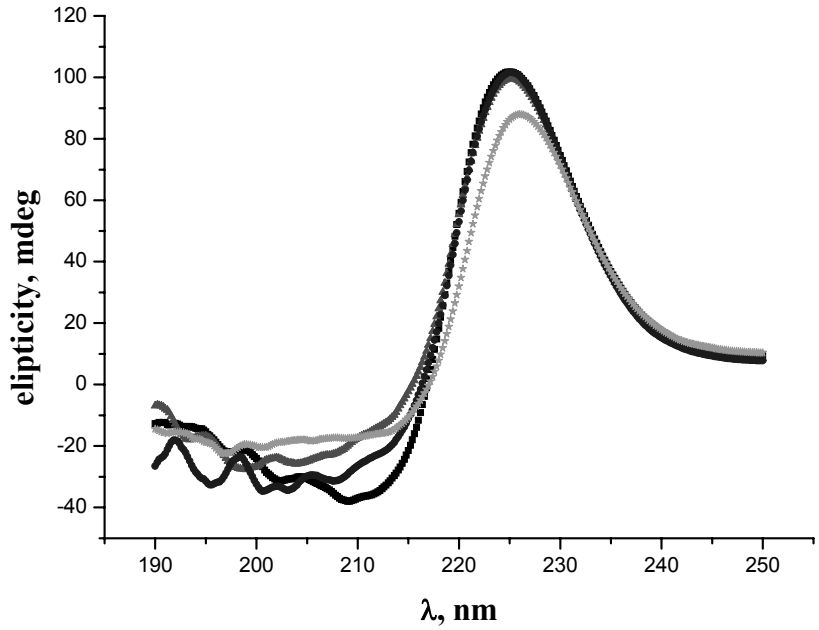

Fig. 1 – Superposed UV-CD spectra recorded for the acid hydrogels containing: ■ – 0, ▲ – 0,5, ♦ – 1,0 and ★ – 1,5 mL thuja tincture/100 g hydrogel.

The rheograms recorded for the acid collagen hydrogels with the same amounts of thuja tincture, which are homogenous, are represented in Figure 2.

The values of viscosities at zero shear rate, as well as of flowing indices, determination coefficients and Chi^2/DF calculated with Ostwald-de Waele equation for the acid hydrogels containing thuja tincture are presented in Table 2.

The dependence of storage and loss modulus, G' and G" respectively, on applied frequency for the two series of collagen hydrogels containing thuja tincture are shown in Figure 3a, b.

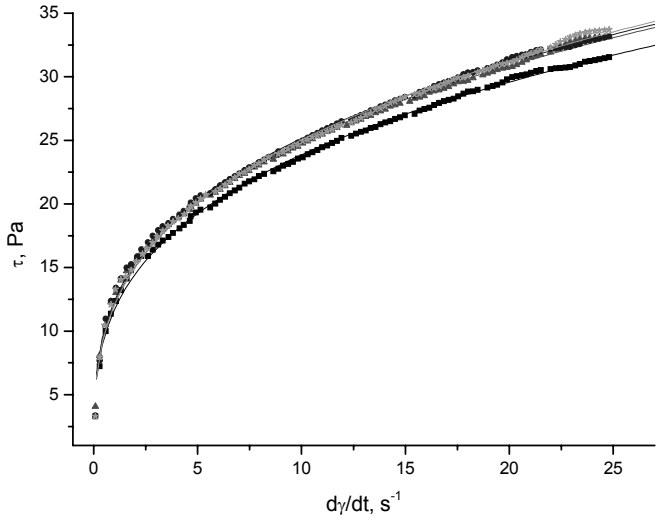

Fig. 2 – Rheograms of the acid hydrogels containing: ■ – 0, ▲ – 0,5, ♦ – 1,0 and ★ – 1,5 mL thuja tincture/100 g hydrogel.

Table 2

Viscosities at zero shear rate and rheological parameters for the acid hydrogels containing thuja tincture calculated with Ostwald-de Waele equation

Thuja tincture, mL/100g gel	η_0, Pa.s	Flowing index, n	Determination coefficient, R^2	Chi^2/DF
0	11.7±0.1	0.310±0.002	0.99706	0.1073
0.5	12.2±0.1	0.311±0.002	0.99749	0.0992
1.0	12.4±0.1	0.308±0.002	0.99678	0.1289
1.5	12.0±0.1	0.319±0.002	0.99672	0.1381

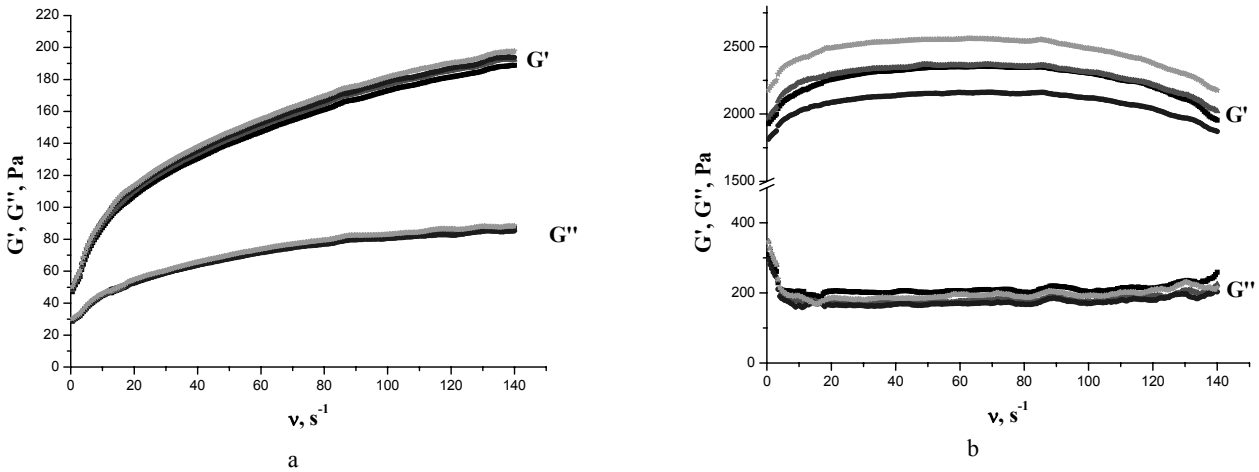

Fig. 3 – Dependence of storage, G', and loss, G", moduli on frequency for collagen hydrogels: a – pH 3.8; b – pH 7.4; thuja tincture amount: ■ – 0, ▲ – 0,5, ♦ – 1,0 and ★ – 1.5 mL/100 g hydrogel.

The superposed FT-IR spectra of collagen matrices obtained by the lyophilisation of the two series of hydrogels containing thuja tincture can be seen in Figure 4.

The values of the ratios $A_{III}/A1450$ and A_I/A_A, as well as of the differences (νA_I - νA_{II}), cm^{-1}, given by the FT-IR spectra of collagen matrices from Figure 4 are supplied in Table 3.

SEM images of the porous matrices containing the specified amounts of thuja tincture, at a magnification of 200x, are presented in the Figure 5a-h.

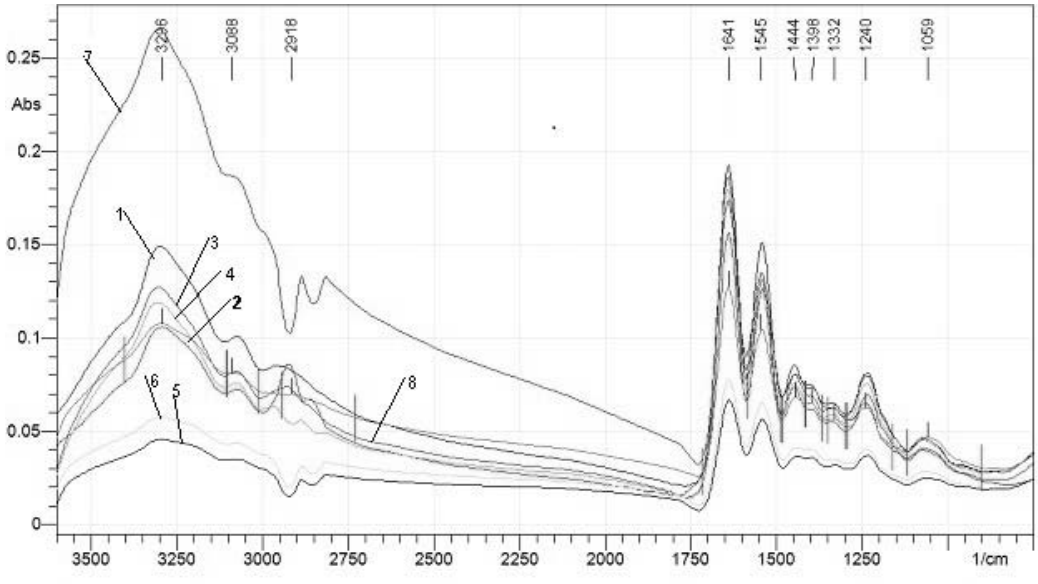

Fig. 4 – Superposed FT-IR spectra of collagen matrices obtained from hydrogels with pH 3.8 containing: **1** - 0, **2** - 0.5, **3** - 1.0 and **4** – 1.5 mL tincture/100 g hydrogel; pH 7.4 and: **5** – 0, **6** – 0.5, **7** – 1.0 and **8** – 1.5 mL tincture/100 g hydrogel.

Table 3

Ratios A_{III}/A_{1450} and A_I/A_A and differences (νA_I - νA_{II}), cm^{-1}, for the prepared collagen matrices

Thuja tincture, mL/100 g gel	pH					
	3.8			7.4		
	A_{III}/A_{1450}	A_I/A_A	νA_I - νA_{II}	A_{III}/A_{1450}	A_I/A_A	νA_I - νA_{II}
0	3.79	1,25	100	4.93	1.59	96
0.5	1.92	1,27	100	4.40	1.22	96
1.0	3.15	1,40	96	3.84	1.20	96
1.5	3.21	1,57	96	3.38	1.20	96

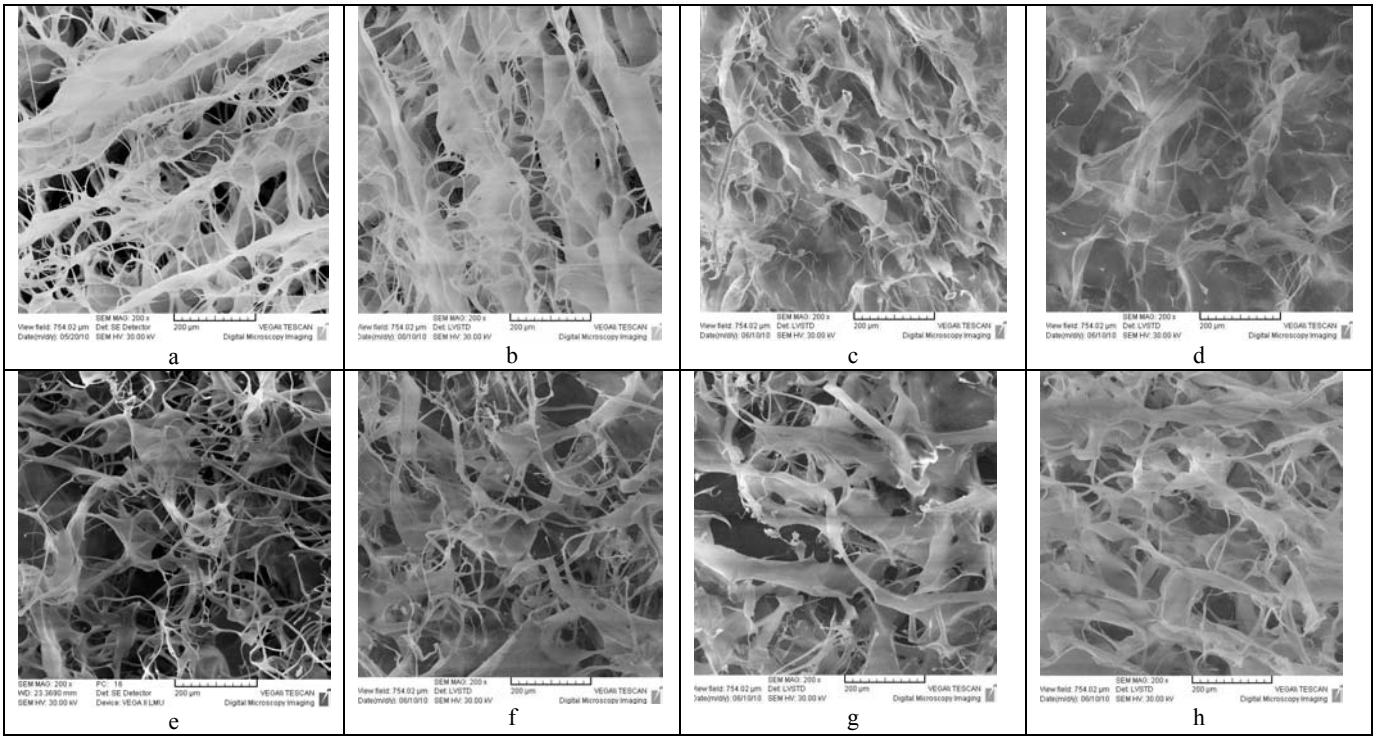

Fig. 5 – SEM images, 200x, for matrices obtained from collagen hydrogels having pH 3.8 and: a – 0, b – 0.5, c – 1.0 and d – 1.5 mL thuja tincture/100 g hydrogel; pH 7.4 and e – 0, f – 0.5, g – 1.0 and h – 1.5 mL thuja tincture/100 g hydrogel.

DISCUSSION

Combination of collagen hydrogels with plant extracts – tinctures or essential oils – is not described into literature, but a paper was found which discuses the healing properties of porous matrices containing triphala[12] – an Indian traditional ayurvedic herbal formulation having antibacterial, antifungal, antiviral and antiallergical activity – obtained by immersion of collagen matrices into triphala extract in methanol.

The alcoholic plant tinctures are usually very rich in terpenes and terpenoids, compounds with antibacterial[13-15] and antifungal[16,17] activity, as well as in tannins, which have anti-infective activity.[18]

The antibacterial activity of thuja tincture is supposed to be due to the high percentage of α- and β-thujone, terpenes known to be the main components of many extracts with antimicrobial activity similar with that of thuja tincture. The testing of antimicrobial effects of α- and β-thujone, the main components of essential oils of thuja and salvia, shown that they have a strong effect on the gram-negative bacteria *P. aeruginosa* and *K. pneumoniae* and medium one on *St. aureus*, *E. coli* and *C. albicans*.[19]

The tincture obtained from *Thuja occidentalis* var. columnaris contains not only large amounts of α- and β- thujone (47.4% and 8.3% respectively, reported to the volatile fraction), but some other more or less antimicrobial compounds:[20] totarol, pimaric acid, thymol, myrcene, fenchone, phytol, etc. as well as tannins.

The collagen hydrogels containing 0.5, 1.0 and 1.5 mL thuja tincture/100 g hydrogel remain homogenous after maturation at 4°C for 24 h only when they are acid, while those having slight basic pH undergo, starting with about 4 h, the syneresis process. The amount of dispersed phase separated and consistency of the hydrogels increase with tincture amount. The only explanation for this process is the cross-linking of collagen by some of the thuja tincture components in slight basic medium, which produces the expulsion of water. Given their inhomogeneity, they can not be characterized by CD or rheological behaviour by shearing between concentric cylinders. Moreover, the FT-IR spectra and the rheological measurements under oscillatory forces are in fact recorded for the concentrated hydrogels obtained as a result of the syneresis process. Thus, the measurements for the basic hydrogels are only approximate.

The FT-IR spectra were recorded only into the frequencies range 2000-900 cm^{-1}, in which the bands amide I-III and A and that assigned to CH_2 bending – required to establish the presence of triple helical conformation of collagen and the absence/presence of denatured collagen – are find. The ratios A_{III}/A_{1450} appreciate the triple helical conformation of collagen molecules from fibrils[21] (values higher than unity indicate the presence of triple helices) and the differences between the frequencies of bands amide I and II indicate the presence of denatured collagen (values higher than 100 cm^{-1} show the presence of denatured collagen). The frequency range higher than 2000 cm^{-1}, in which the band amide A – required for establishing of extent of cross-linking – is found was excluded, due to the low resolution, the accurate calculation of its surface being impossible.

The FT-IR bands obtained for hydrogels are pretty weak, which makes the processing of spectrum difficult and the results ambiguous. Moreover, amide I bands possess shoulders, while amide III and CH_2 bending are very weak. Thus, the results can be regarded only as informative. The only band displaced more than 4 cm^{-1}, the spectral interval between the data points, is the CH_2 banding in the hydrogel containing 1.5 mL tincture/100 g hydrogel, which is displaced with 16 cm^{-1}.

The ratios A_{III}/A_{1450} are higher than unity in acid medium and increase with thuja tincture concentration, while at slight basic pH their values are lower and decrease with tincture concentration even under unity, as can be seen from Table 1. This might suggests that some components from thuja tincture denaturise slightly the collagen at pH 7.4. The same thing is suggested by the differences (νA_I - νA_{II}), lower than 100 cm^{-1} for all the acid hydrogels containing thuja tincture and a bit higher than this value for the basic ones, the value increasing with tincture amount. Consequently, both the ratios A_{III}/A_{1450} and differences (νA_I - νA_{II}) show that thuja tincture does not disturb the triple helical conformation of collagen molecules from fibrils in acid media, while at slight basic pH produces a slight denaturation, which increases with thuja tincture amount.

Recording of UV-CD spectra was possible only for the homogenous acid hydrogels containing thuja tincture. The superposed spectra in Figure 1 show differences both as shape and height between the reference sample and the tincture containing hydrogels in the region of minima: the minimum becomes higher and displaces towards red when tincture is introduced and its amount increases. But the height and the position of maxima remain practically unmodified, excepting for the maximum amount of tincture, for which the maximum displaces with 1 nm and reduces with

14 mdeg compared with the reference. The crossover point increases slightly with thuja tincture concentration, while Rpn values are higher for all the hydrogels containing tincture than for the reference sample. These could indicate a stabilisation of the triple helix, in accordance with the high value of A_{III}/A_{1450} and (νA_I - νA_{II}) from Table 1. The high values of Rpn are mainly due to the decreasing and deformation of the negative peaks, the positive one remaining almost the same. The above results show that thuja tincture does not disturb the triple helical conformation of collagen in acid hydrogels, but it has a contrary effect, producing its strengthening.

The rheograms were recorded only for the acid hydrogels, which preserves the homogeneity in time.

The Figure 2 show that the rheograms of all the hydrogels containing thuja tincture are placed above that of the reference sample, which means that weak interactions establish between collagen and some components from thuja tincture, probably by hydrogen bonding (tincture contains tannins and tannic acid), which have as effect a slight increase of viscosity in acid medium. The rheograms' analysis demonstrates that the increasing of shear stress is perceptible for all the shear rates only when the hydrogel contains 0.5 mL tincture/100 g hydrogel. Increasing of tincture amount has an insignificant effect on shear stresses for all the shear rates, so that all the rheograms are practically superposed. This may signify that the increasing of amount of thuja tincture above 0.5 mL/100 g hydrogel has no effect on the amount of hydrogen bonding.

The viscosities at zero shear rate from Table 2 are very close for the four hydrogels, a slight increase producing when the first two amounts of thuja tincture are introduced and a slight decrease for the highest amount, due probably to the larger quantity of ethyl alcohol.

The reheological parameters from Table 2, calculated with Ostwald-de Waele equation since no hydrogel presents limiting shear stress, indicate very close values for the flowing indices, in agreement with viscosities at zero shear rate. The determination coefficients have very good values. The best correlation was obtained for the hydrogel with 0.5 mL tincture/100 g hydrogel, as the values Chi^2/DF also show.

The moduli in Figure 3a show for all the acid hydrogels storage moduli higher than the loss ones, which means that the hydrogels remain preponderant elastic. Thuja tincture produces a slight increase of elasticity, which enhances with its concentration for all the frequencies, but the viscous component of the viscoelasticity is not affected by tincture, the four curves superposing. The slight different values of the storage moduli for the acid hydrogels containing thuja tincture may indicate a weak interaction between collagen and components from tincture, emphasised also by the other methods.

The slight basic hydrogels are much more elastic than the acid ones, as Figure 3b shows (G' moduli have very high values). If the addition of 0.5 mL tincture does not modify the G' values and their variation with frequency, the curve superposing on that of the reference excepting the extremes, a double amount displace the curve down and a triple one produce an upward displacing; the curves are parallel on the whole frequency range. Regarding the effect of thuja tincture on loss moduli, a slight reduction can be observed, almost independent of tincture amount. They decrease with frequency in the range 0-10 s^{-1} and remain constant at higher frequencies. The very high values of elastic moduli suggest that in slight basic medium a cross-linking of collagen by some components from tincture is produced. However, the results obtained at pH 7.4 can not be considered very credible, they being obtained for the more concentrated hydrogels resulted by syneresis, which can explain their similitude with the behaviour of chemically cross-linked hydrogels.

The collagen porous matrices containing thuja tincture were obtained by the lyophilisation of the corresponding hydrogel and not by immersion of the previously prepared collagen porous matrices into the plant alcoholic extract as in the case of those containing triphala[12], because the last method can result in an inhomogeneous repartition of the components into the matrix and their binding only by physical forces. The two series of matrices were characterized by FT-IR – to establish the combined effect of thuja components and lyophilisation on molecule conformation from collagen fibrils, existence/absence of denatured collagen and cross-linking and by SEM – to see the tincture influence on pores' form and size. It must be remembered that the acid hydrogels were matured for 24 h at 4^oC, while the slight basic ones only 4h before lyophilisation, to avoid syneresis.

The Figure 4, in which the superposed FT-IR spectra of the two series of matrices are represented, shows that – unlike the slight basic hydrogels – the bands are more intense. Their frequencies are slightly different from those in hydrogels, due to the absence of water, which facilitate the intimacy of fibrils.

The A_{III}/A_{1450} values in the Table 3 are higher than unity for all the matrices, demonstrating that the components of thuja tincture do not affect the

conformation of molecules from collagen fibrils, but no relation was found between the amount of tincture and the value of the ratio. The ratios A_I/A_A increase a bit when the amount of tincture is increased for the matrices obtained from acid hydrogels, which might suggest a slight cross-linking of collagen, which increases with the amount of tincture. For the matrices obtained from basic hydrogels a decrease was obtained with tincture content increasing, but the value are not very accurate due to the considerable width of the amide A bands, which have also shoulders on both size. This makes the base line very difficult to be constructed. As it was no possibility to make the deconvolution, the areas may contain larger or lower contributions from other bands. The differences $\nu(A_I - A_{II})$ are all lower than 100 cm^{-1}, excepting the reference matrix and that containing 0.5 mL thuja tincture/100 g hydrogel obtained from the hydrogels with acid pH, for which it is 100 cm^{-1}. Thus it can be stated that the triple helical conformation of collagen is intact in all the matrices that contain thuja tincture, the collagen is slightly cross-linked in those obtained from acid hydrogels and the denatured collagen is absent. The effect of thuja tincture on collagen matrices resulted from slight basic hydrogels is difficult to be appreciated and explained.

The Figure 5a shows for the reference matrix obtained from acid collagen hydrogel a lattice-like lamellar structure with distances between 50 and 160μm, connected by fibrils and fibril agglomerates which form distorted ring-shaped pores, while that resulted from slight basic one – Figure 5e – contains both irregular and ring-shaped pores of different size, interconnected by larger numbers of fibrils and fibril agglomerates. The matrix obtained from the acid hydrogel containing 0.5 mL thuja tincture/100 g hydrogel retains the lattice-like lamellar structure, but the distance between lamellae increases and they are thicker (Figure 5b), which can be due to a slight cross-linking produced by components from tincture. Doubling the thuja tincture content, the aspect of matrix is modified (Figure 5c): the lamellar structure almost disappeared, the pores have very irregular forms, their distribution is very large, some agglomerates and even holes can be seen as a result of cross-linking. Large fibril agglomerates and very large pores can be seen in the matrix containing the highest amount of thuja tincture (Figure 5d). Addition of thuja tincture to the slight basic collagen hydrogel increases the fibril agglomeration, pore irregularity and size as well as the size distribution, which increase with the amount of thuja tincture that can be assigned to the collagen cross-linking. Thus it can be concluded that the components from thuja tincture have as effect the cross-linking of collagen both in acid and slight basic medium.

EXPERIMENTAL

Preparation of hydrogels. The control 1.1% collagen hydrogels having pHs 3.8 and 7.4 were obtained by diluting the 1.83% initial hydrogel with pH 2.1 with distilled water and 1M sodium hydroxide solution under mechanical stirring. Thus two series of collagen hydrogels containing 0.5, 1.0 and 1.5 mL Thuja tincture/100 g hydrogel were prepared using appropriate amounts of tincture under stirring. The acid hydrogels were matured at 4°C for 24 h, while the slight basic one for 4 h to avoid syneresis.

Preparation of matrices. The matured collagen hydrogels having the above compositions were freezed for 3½ h at (-40)°C in the already refrigerated lyophilizer and subjected to lyophilisation at 0.12 atm in the following conditions:[22] (-40)°C for 5 h, 10°C for 10 h, 20°C for 8 h, 35°C for 13 h and 40°C for 7 h using a Delta 2-24 LSC (Martin Christ, Germany) lyophiliser, the whole process lasting for 48 h.

FT-IR spectra were obtained with an ABB MB3000 MID-IR spectrometer equipped with a DTGS detector and Horizon software. Data were acquired by ATR technique using a PIKE 45 degree ZnSe trough plate with volatile cover Horizontal ATR. All the spectra were corrected for ATR effect and than transformed into absorption ones. Each spectrum is the average of 32 scans, with a spectral interval between the data points of 4 cm^{-1}.

UV-CD spectra were recorded using a Jasco J-810 spectropolarimeter equipped with a square Suprasil cuvette having a 0.02 cm path length. Working parameters: wavelength range 250-190 nm; scanning speed – 50 nm/min with 0.2 nm pitch and 2s response time, room temperature (23°C), average number of spectra accumulation – 4, continuous feeding of measure compartment with high purity nitrogen (5.5) to suppress oxygen absorption. To create a proper baseline the reference spectra of water and tincture were recorded.

Shear rheological behaviour was determined at 23 ± 0.1°C using a rotational viscometer Hake VT 550 equipped with the MV1 sensor system for medium viscosity and RheoWin 4 Thermo Fischer Scientific software. Shear rates ranging between 0.1 and 25 s^{-1}, representative for the applications on skin and releasing of drugs, were used.

Dynamic (oscillatory) rheologycal measurements were made at room temperature (23°C) with a Micro Fourier Transform Rheometer MRF 2100, GBC-Australia using the following parameters: squeezing flow, frequency range 0-140 s^{-1}, 280 discrete frequencies simultaneously analyzed in the range by a step of 0.5 s^{-1}, 30 spectra consequently acquired every tested sample, gap between the upper and bottom plates of the rheometer – 400 μm and displacement amplitude – 0.03 μm (to fall into linear viscoelastic domain). Using very

small amplitude of a pseudorandom squeezing motion exerted onto a viscoelastic sample, the force transmitted through it to the force sensor (bottom plate) is continuously monitored that both the displacement and force lead, by a Fourier transform processing, to the storage and loss modulus at every individual frequency steps within the employed frequency range.

SEM was performed with a VEGA II LMU device equipped with a LVSTD type detector using the following parameters: accelerating voltage – 30 kV, emission current – 77 μA, system pressure 10 mPa, pixel size X/Y – 1.47 μm, dwell time – 437 ms, spot size – 48 nm; magnification – 200x and 600x.

CONCLUSIONS

Collagen-based biomaterials containing *Thuja occidentalis var. columnaris* tincture were prepared both as hydrogels having the pHs 3.8 and 7.4 and containing 0.5, 1.0 and 1.5 mL tincture/100 g 1.1% collagen hydrogel and porous matrices obtained by the hydrogels' lyophilisation.

Both the ratios A_{III}/A_{1450} and differences (νA_I - νA_{II}) show that thuja tincture does not disturb the triple helical conformation of collagen molecules from fibrils in acid media, while at pH 7.4 produces a slight denaturation, which increases with thuja tincture amount.

The UV-CD spectra of the acid hydrogels show that thuja tincture does not disrupt the triple helical conformation of collagen in acid hydrogels, but has an effect of strengthening.

The rheograms of the acid hydrogels suggest a weak interactions between collagen and thuja tincture in acid medium, probably by hydrogen bonding (the tincture contains tannins and tannic acid), which have as effect a slight increase of viscosity, almost independent of its concentration, in accordance with the values of viscosities at zero shear rate and flowing indices calculates with Ostwald-de Vaele equation.

The storage and loss moduli indicate a weak interaction between collagen and components from tincture in the acid hydrogels and cross-linking in slight basic ones, but the last results were obtained for the hydrogels resulted after the syneresis process was produced.

The ratios A_{III}/A_{1450} and A_I/A_A, as well as the differences (νA_I - νA_{II}) reveal the preservation of triple helical conformation of collagen in all the matrices, a slight cross-linking of collagen in those obtained from acid hydrogels and even a more slight one for those obtained from hydrogels with pH 7.4, as well as the absence of denatured collagen in all the matrices.

SEM images exhibit the agglomeration of fibrils produced by thuja tincture both for the matrices obtained from acid and slight basic hydrogels and increasing of size and irregularity of pores, which amplify with thuja tincture amount and can be assigned to cross-linking.

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