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COLLAGEN-THUJA TINCTURE BIOMATERIALS FOR WOUND TREATMENT. 3. HYDROGELS CONTAINING THUJA TINCTURE AND CHLORHEXIDINE DIGLUCONATE

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Collagen-based biomaterials containing *Thuja occidentalis var.* columnaris tincture and chlorhexidine digluconate (CHDG) were prepared as hydrogels having pHs 3.8 or 7.4 and containing all the combinations between 0.5, 1.0 and 1.5 mL tincture/100 g hydrogel and 0.02, 0.05 and 0.10 % CHDG. The acid hydrogels are homogenous, while the slight basic ones undergo syneresis. FT-IR spectra of the acid hydrogels show very weak bands, with A_{III}/A_{1450} ratios and (νA_I - νA_{II}) diferences proving the integrity of the native conformation of collagen, excepting 0.10% CHDG which produces a slight perturbation that increases gradually with tincture amount. UV-CD spectra show the same thing. The rheograms – placed above that of the collagen hydrogel – show slight interactions between collagen, CHDG and coponents from thuja tincture, independent on tincture amount for 0.02 and 0.10% CHDG and increasing with tincture amount for 0.05%. Tthuja tincture enhances the amount of CHDG delivered, which increases with concentration of both materials.

INTRODUCTION

Excess using of antimicrobials leads to the appearance of some antibiotics-resistant microorganism species. Methicillin-resistant *Staphylococcus aureus*, the main agent producing intra-hospital infections worldwide, can be given as example. With the increase in the prevalence of microbial resistance to conventional antibiotics and antiseptics, attention has been turning to natural antimicrobial compounds,[1] especially phytochemicals extracted from a range of botanic origins, most of which used traditionally.[2]

The plant extract containing compounds with antimicrobial properties are essential oils[3] and tinctures extracted using concentrated ethanol solutions. They contain high amounts of terpenes and terpenoids, recognized as antibacterials[4-6] and antifungals,[7,8] and tannins, having anti-infecting activity.[9] Terpenes act also as penetration enhancers, increasing permeation and improving retention of drugs within the skin.[10,11]

CHDG, one of the most widely used antimicrobials in clinical practice for skin antisepsis,[12] is recommended within the Evidence-Based Practice in Infection Control (EPIC)[13] and Healthcare Infection Control Practices Advisory Committee (HICPAC)[14] guidelines. It is potent also against mutant streptococci,[15,16] but its efficacy is reduced by factors as pH and organic matter.[17] That is why its activity must be improved.

It was demonstrated[18] that the combination of antibiotics or antiseptics with terpenes may result in synergistic effects, the obtained mixtures having the property to eliminate both the microorganism becoming resistant to antibiotics and the natural resistant ones. Some essential oils, as cinamon, manuka and *Leptospernum morrisonii*, decrease at

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least ten times the concentration of CHDG required to produce an equivalent inhibition of *Lactobacillus plantarum* and *Streptococcus mutants* biofilms, transforming thus the CHDG *in vivo* from bacteriostat into bactericid.[2,19]

Thuja occidentalis var. columnaris tincture containing large amounts of thujone[20] – 55.7% reported to volatile compounds, from which 47.4 % is α- and 8.3% β-thujone – and some other more or less antimicrobial compounds: totarol, pimaric acid, thymol, myrcene, fenchone, phytol, etc., is expected to have as effect the increase of CHDG antibacterial activity. Considering the beneficial effect of type I collagen in wound healing, its combination with CHDG and thuja tincture may result in biomaterials with high efficiency in wound healing.

The objective of the present paper is to present the preparation and characterization of 1.1% collagen hydrogels containing all the combinations between 0.02, 0.05 and 0.10% CHDG and 0.5, 1.0 and 1.5 mL thuja tincture/100 g hydrogel, as well as the delivery of CHDG from the respective hydrogels.

RESULTS

The superposed FT-IR spectra of the acid collagen hydrogels containing the specified amounts of thuja tincture and CHDG recorded into the frequencies range 2000-900 cm^{-1} are presented in Fig. 1.

The UV-CD spectra of the acid collagen hydrogels containing the above combinations between CHDG and thuja tincture are given in Fig. 2a-c.

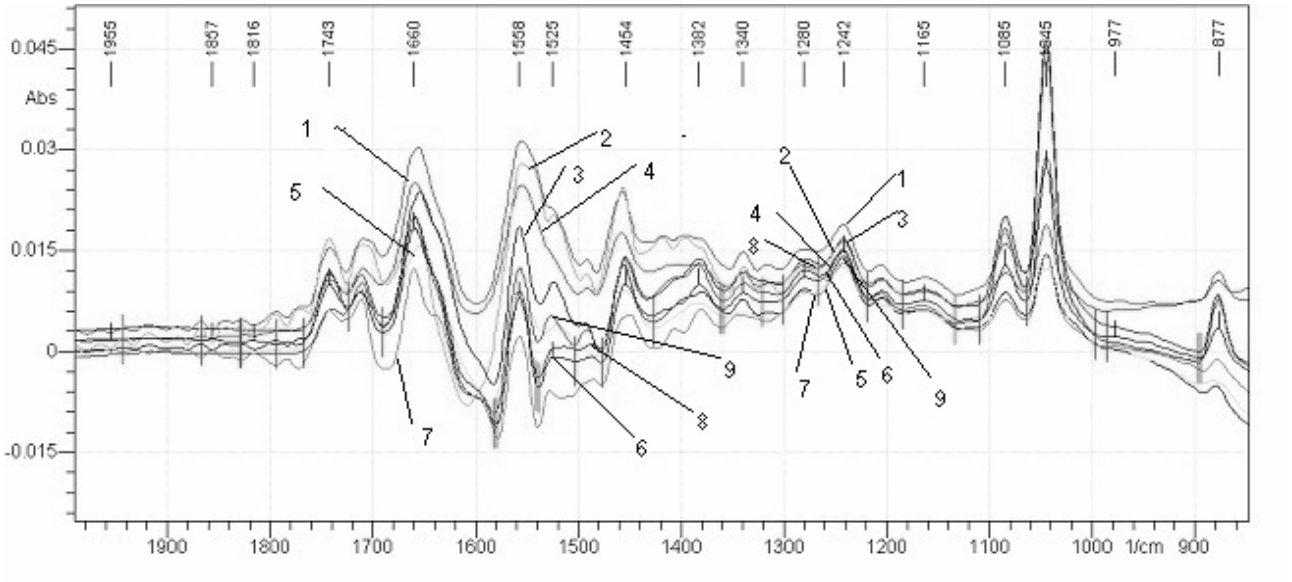


Fig. 1 – Superposed FT-IR spectra of acid collagen hydrogels containing: 0.02% CHDG and 1 – 0.5, 2 – 1.0 and 3 – 1.5 mL thuja tincture/100 g hydrogel; 0.05% CHDG and 4 – 0.5, 5 – 1.0 and 6 – 1.5 mL; 0.10% CHDG and 7 – 0.5, 8 – 1.0 and 9 – 1.5 mL.

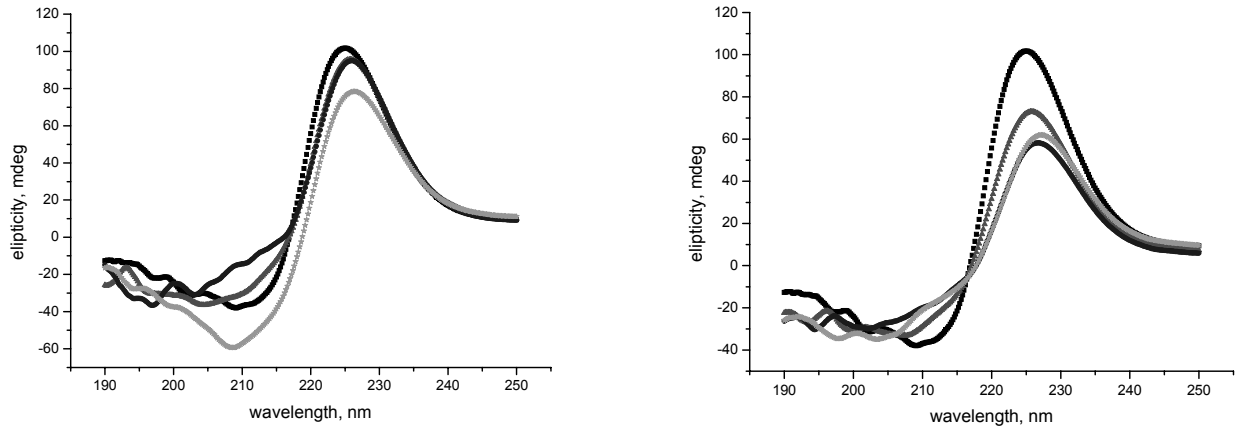
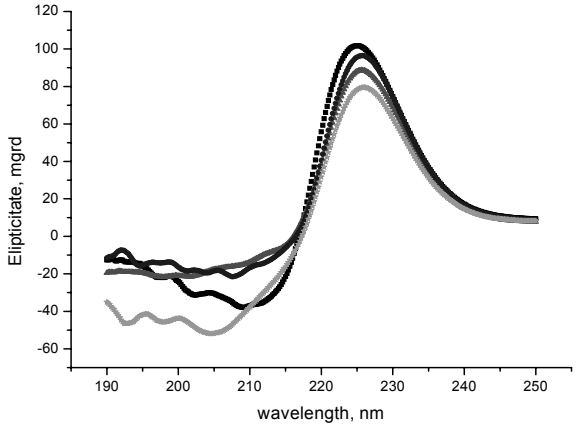


Fig. 2 – UV-CD spectra of acid hydrogels containing: a – 0.02%, b – 0.05% and:
▲ – 0.5 ♦ – 1.0 and ★ – 1.5 mL thuja tincture/100 g hydrogel; ■ – reference collagen hydrogel.

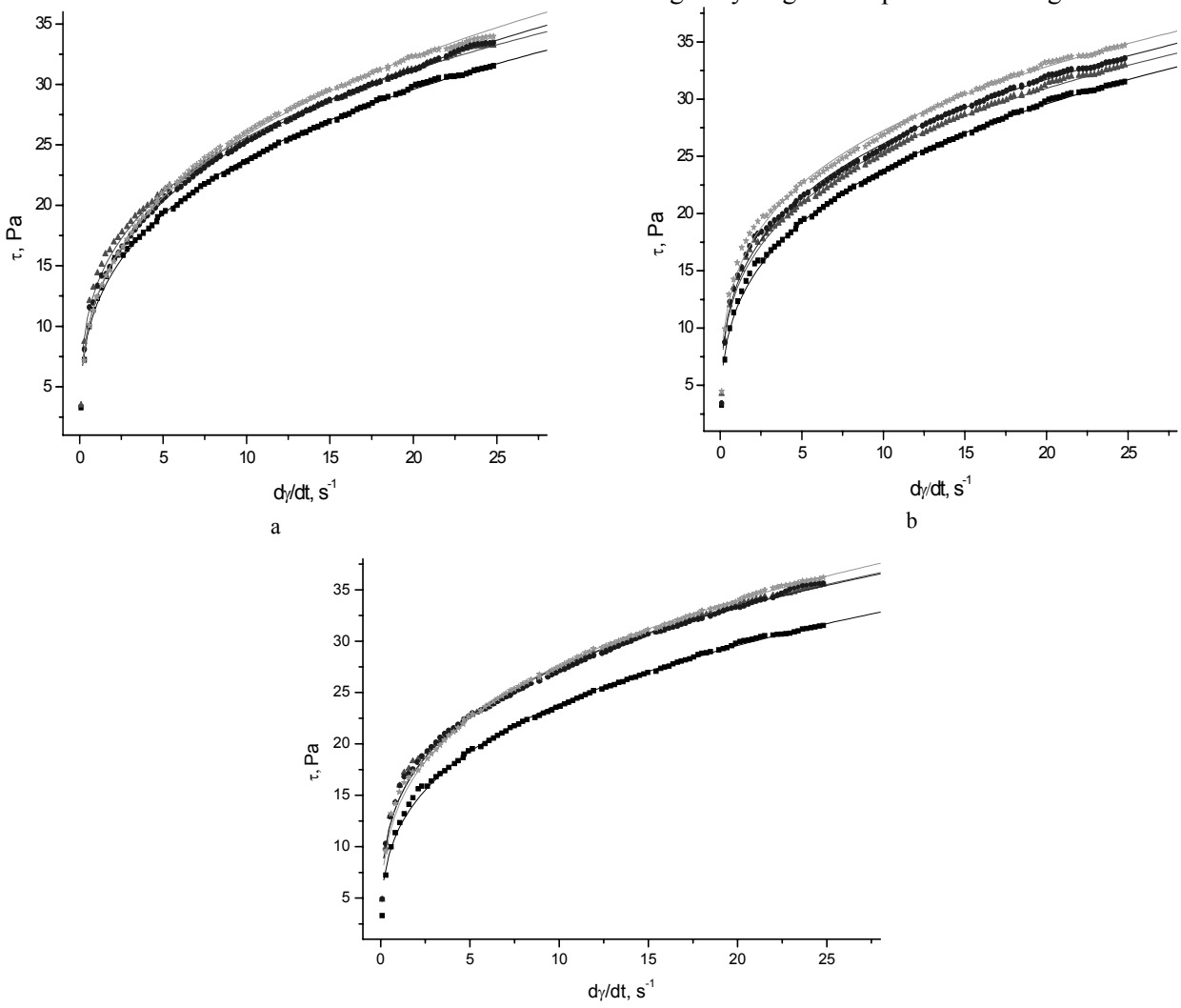


c

Fig. 2 – UV-CD spectra of acid hydrogels containing: c – 0.10% CHDG and: ▲ – 0.5 ♦ – 1.0 and ★ – 1.5 mL thuja tincture/100 g hydrogel; ■ – reference collagen hydrogel.

The rheograms of the acid hydrogels having the same concentrations of CHDG and amounts of thuja tincture are represented in Fig. 3.

The cumulative release of CHXD from all the collagen hydrogels is represented in Fig. 4.



a b c

Fig. 3 – Rheograms of acid hydrogels containing: a – 0.02, b – 0.05, c – 0.10% CHDG and: ▲ – 0.5, ♦ – 1.0 and ★ – 1.5 mL thuja tincture/100 g hydrogel; ■ – reference collagen hydrogel.

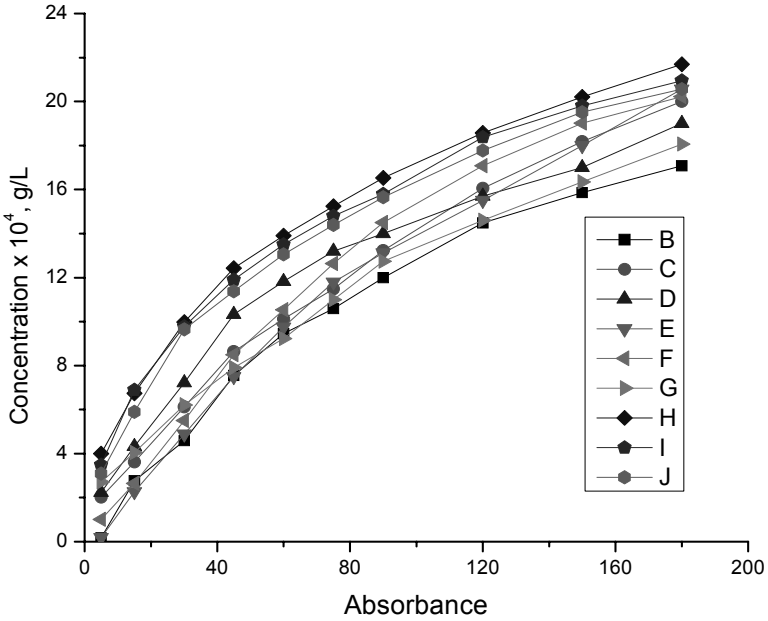


Fig. 4 – CHXD cumulative release from collagen hydrogels containing: $2x10^{-2}$% DGCH and B – 0.5, C – 1.0 and 1.5 mL tincture/100 g hydrogel; $5x10^{-2}$% DGCH and the same amounts of tincture (E, F, G); 10^{-1}% DGCH and the above tincture amounts (H, I, J).

DISCUSSION

The antibacterial activity of CHDG can be increased by its mixing with terpenes[18]. Indeed the mixing of CHDG with essential oils of cinnamon, manuka and Leptospernum morrisonii[2], containing large amounts of terpens, resulted in a drastic reduction of CHDG concentration required to produce an equivalent inhibition for increasing of *Lactobacillus plantarum* and *Streptococcus mutants* biofilms: it is at least ten times lower. Thus, the CHDG was transformed, *in vivo*, from bacteriostat into bactericid.[2,19]

Mixtures of CHDG with plant tinctures were not found in literature.

It is supposed that the antibacterial properties of essential oils and tinctures are due to the lipophilic compounds contained, such as terpenes. Although the mechanism of action of terpens at molecular level is not completely known, the high lipophilic nature of the majority of terpens suggests that they act by disrupting the cell membranes.[21,22] However, the essential oils contain a lot of compounds, so that the main components responsible for their antimicrobial activity and the way they act are not well understood yet.[1]

The antibacterial activity of *Tuia occidentalis* tincture is supposed to be due to the high content of α- and β-thujone. Testing of their antibacterial properties has shown a powerful effect on gram-negative bacteria *P. Aeruginosa* and *K. Pneumoniae* and medium on *St. aureus*, *E. coli* and *C. albicans.*[23] But, besides the high content of α- and β-thujone – 55.7%, (47.4 and 8.3% respectively), the prepared thuja tincture contains some other compounds with antibacterial activity, such as totarol, pimaric acid, thymol, myrcene, fenchone, phytol, etc.,[20] as well as tannins, that have anti-infecting activity,[9] acting by proteins complexing by non-specific forces (hydrogen bonds and hydrophobic interactions) and covalent bonds,[9,24] as well as by the direct inactivation of microorganisms.[25] At the same time they have the property to cross-link the collagen.

The three series of acid collagen hydrogels containing the used combinations between the amounts of CHDG and thuja tincture remain homogenous after maturation for 24 h at 4°C, while the slight basic ones (pH 7.4) undergo, starting with about 4 h, the syneresis phenomenon, as in the case of those containing thuja tincture,[26] excepting the hydrogels with 0.1% CHDG, which are pretty homogenous. This can be due to the cross-linking of collagen by components from tincture, which expels a part of water, but higher amounts of CHDG have as effect the reducing of cross-linking, probably by its reaction with these components.

The FT-IR spectra of the acid hydrogels, recorded into the spectral range 2000-900 cm^{-1} in which the bands amide I-III and that assigned to

CH_2 bending – required to establish the presence of triple helical conformation of collagen and of denatured collagen – can be found (Fig. 1), show pretty weak absorption bands and look more similar with those obtained for collagen hydrogels containing thuja tincture[26] than with those containing CHDG.[27,28] This can signify that some components from thuja tincture interact more strongly with collagen than with CHDG. Moreover, the bands amide I and II have shoulders, making the calculation of surfaces difficult and the results uncertain. The difference of 4 cm^{-1} (experimental error) between the collagen hydrogel bands and those of the hydrogels containing the used mixtures between CHDG and thuja tincture is not surpassed, which demonstrates that no significant interactions between these mixtures and collagen take place.

The ratios A_{III}/A_{1450}, given in Table 1, higher than unity, indicate intact triple helical conformation of collagen[29] in the hydrogels containing 0.02 and 0.05% CHDG and 0.5 and 1.0 mL thuja tincture/100 g hydrogel; they are very close to 1 for those containing the same amounts of CHDG but 1.5 mL tincture, and lower than 1 for 0.1% CHDG regardless of tincture amount. In the limits of the experimental errors, it can be said that the collagen conformation is not affected in the first two series, but desturbed a bit in the third one.

The ratios, so not very accurate the bands being very weak and large, are in agreement with the differences (νA_I - νA_{II}) from the column 3 of Table 1, which are more precise: lower or equal with 100 cm^{-1} for the first five hydrogels and slightly higher than 100 cm^{-1} for the last four, which shows that the last contain very low amounts of denatured collagen. Consequently high amounts of CHDG combined with thuja tincture alter to a certain extent the triple helical conformation of collagen in acid medium, which increase with tincture amount.

The UV-CD spectra of the acid hydrogels given in Fig. 2a-c and, for a better following of data, in Table 2 show important changes only into the minima region when the hydrogels contain 0.02% CHDG (Fig. 2a): their wavelengths decrease with increasing amount of tincture for 0.5 and 1.0 mL tincture/100 g hydrogel but is very close to reference for 1.5 mL; the heights are the same for the first two hydrogels and over 21 mdeg lower for the third. The maxima remain almost unchanged, excepting the hydrogel containing 1.5 mL tincture which is with about 17 mdeg lower. The crossover points, very close to that of the reference, increases with 2 mdeg only for the last hydrogel. Rpn values decrease with increasing amount of tincture, which may suggest a slight denaturation of collagen, in agreement with the sub-unitary value of the A_{III}/A_{1450} ratios from Table 1.

Increasing of CHDG concentration to 0.05% brings nearer the heights of the minima, but their wavelengths vary as above (Fig. 2b). All the hydrogels containing tincture have the maxima under that of the reference and the wavelength increase slightly with tincture amount. The same variation has the crossover points. Rpn values decrease with increasing amount of tincture and are lower than those of the previous hydrogels, excepting the one containing the maximum amount of tincture. This may signify either a lower denaturation, suggesting that CHDG protect collagen, or a reducing of collagen molecule flexibility as a result of cross-linking. Corroborating with FT-IR results, it seems that the last hypothesis is real.

Table 1

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

Thuja tincture, mL/100 g hydrogel	A_{III}/A_{1450}	(νA_I - νA_{II}), cm^{-1}
	0.02% CHD	
0.5	1,00	96
1.0	1,00	100
1.5	0,97	100
	0.05% CHD	
0.5	1,08	96
1.0	1,04	96
1.5	0,95	102
	0.10% CHD	
0.5	0,92	102
1.0	0,86	102
1.5	0,84	102

Table 2

Wavelength location of minima, maxima and crossover points and Rpn values for specified concentrations of CHDG and thuja tincture in collagen hydrogels

Thuja tincture, mL/100 g hydrogel	Elipticity (mdeg) and wavelength location (nm)			Rpn
	Minimum	Maximum	Crossover point	
Reference	-38.0/209	101.7/225	216.8	2.68
	0.02% CHDG			
0.5	-36.5/204.4	95.6/226	216.8	2.62
1.0	-36.7/196.8	95.0/226	216.2	2.59
1.5	-59.4/208.6	78.4/226.4	218.8	1.32
	0.05 % CHDG			
0.5	-33.2/207.6	73.0/225.8	217.2	2.20
1.0	-30.3/194.4	58.2/226.8	217.8	1.92
1.5	-34.96/203.4	62.9/227.2	217.8	1.80
	0.10% CHDG			
0.5	-21.8/197.8	88.6/225.6	216	4.06
1.0	-21.5/207.6	96.4/225.8	216.4	4.48
1.5	-52.0/204.6	79.5/226	217.2	1.53

When the hydrogels contain 0.10% CHDG thuja tincture changes considerable the position and location of the minima: they are 17 mdeg above that of collagen for 0.5 and 1.0 mL tincture/100 g hydrogel and 14 mdeg below for 1.5 mL, whilst the wavelength are lower, as Fig. 2c shows. All the maxima are placed under that of collagen, but the wavelength are almost identical. The same is valid for the crossover points. Rpn has the highest value for the hydrogels containing 0.5 and 1.0 mL tincture and decreases drastically for 1.5 mL, indicating denaturation.

To avoid the superpositions, the rheograms of the acid hydrogels were represented at constant CHDG concentrations (Fig. 3a-c). They reveal that the relative positions depend on CHDG concentration: they superpose practically for 0.5 and 1.0 mL tincture/100 g hydrogel and is a little above for 1.5 mL when the CHDG concentration is 0.02%, displace towards higher values with tincture amount when it is 0.05% and superpose again for 0.10. This may indicate slight interactions between collagen, CHDG and components from thuja tincture, dependent on their relative concentrations, and that 0.1% CHDG is high enough to make the interaction independent of thuja tincture amount.

The release of CHDG from the hydrogels containing the mentioned amounts of CHDG and thuja tincture, shown in Fig. 4, demonstrates that the released amounts increases generally with CHDG and thuja tincture amounts, all the hydrogels have high delivery ratios within the first 20 min with slopes that increse with CHDG and tincture concentration, as in the case of hydrogels containing CHDG,[27,28] excepting the hydrogel containing 0.05% and 1 mL tincture (curve F) which has the lowest delivery rates. The delivery ratios decrease significantly between 30 and 45 min, particularly when the amount of CHDG is 0.1% (curves H-J). After 45 min they are very small or even zero, as in the case of curves H-J, excepting the same hydrogel. Thus, the highest amount of CHDG is released by the hydrogel containing maximum amount od CHDG and thuja tincture.

Compared with the amounts released by the acid hydrogels containing the same amounts of CHDG,[27,28] it can be seen that thuja tincture intensify – between 1.77 and 6.67 times – the delivery of CHDG, depending on concentration of both materials. The intensifying effect of thuja tincture can be explained by the lower viscosities of hydrogels containing thuja tincture and antiseptic, which make the difusion coefficeints higher.

EXPERIMENTAL

Preparation of hydrogels. The collagen hydrogels having the concentration of 1.1%, pH 3.8 and containing the amounts of CHDG and thuja tincture from Table 1 were prepared by diluting the 1.83% (w/w) collagen hydrogel having the pH 2.39 with proper amounts of distilled water, 1M sodium hydroxide, 4% CHDG solution and thuja tincture under stirring. CHDG was supplied by FAGRON, Germany, as 20% aqueous solution (w/w) and thuja tincture was prepared in our laboratory from fresh ground leaves and twigs harvested from Rădaia – Cluj, Roumania in the middle of April 2009.[20] All the hydrogels were matured at 4°C for 24 h. The slight basic hydrogels undergo the syneresis phenomenon.

FT-IR and **UV-CD spectra** as well as **the rheological behaviour** were measured using the same equipment and conditions as in the previous papers.[20,27,28]

The measurement of ***in vitro*** **release of chlorhexidine digluconate** was described in a previous paper.[27]

CONCLUSIONS

The acid hydrogels containing all the combinations between 0.5, 1.0 and 1.5 mL tincture/100 g hydrogel and 0.02, 0.05 and 0.10 % CHDG are homogenous after maturation, while the slight basic ones present the syneresis phenomenon, excepting those containing 0.1% CHDG, which continue to be homogenous.

The FT-IR spectra show very weak bands, with A_{III}/A_{1450} ratios and (νA_I - νA_{II}) diferences proving the preservation of integrity of collagen conformation for 0.02 and 0.05% CHDG and all the tincture amounts and slight denaturation for hydrogels containing 0.10%, which increases with tincture amount.

The UV-CD spectra of all the hydrogels confirm the findings obtained by FT-IR.

The rheograms of all the hydrogels containing CHDG and thuja tincture are placed above that of collagen hydrogel, but their relative positions depend on CHDG/thuja tincture ratio, especially when CHDG concentration is 0.05%, showing slight interaction between collagen, CHDG and compounds from thuja tincture.

The presence of thuja tincture in hydrogels containing CHDG enhances the amount of antiseptic delivered, which increases with concentration of both materials, due to the lower viscosity of hydrogels containing thuja tincture and CHDG compared with the corresponding one containing only CHDG.

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