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Isolation and spectroscopy study of the psoralens from *Ficus Sycomorus* sap. Its thermal and kinetic effects in collagen forms.

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Abstract – Psoralen and antioxidants were isolated for the first time from *Ficus Sycomorus* (FS) sap, localised in the trunk of the tree and are characterized by 1D and 2D NMR spectroscopy. The bioactive constituents of the sap were incorporated in the collagen gel, which is prepared further in membranes and matrices forms, using a methodology developed in our laboratories. Collagen gels with FS content were dried by different processes like freeze-drying and free drying at 25 ^{o}C in order to obtain matrices and membranes because they will be used as vehicles for topical systems with controlled release of bioactive components from FS. The thermal and structural changes caused by the presence of psoralens in membranes and matrices have been studied using Differential Scanning Calorimetry, UV and IR. In addition, *in vitro* kinetic studies revealed the possible mechanisms of the FS release and showed that FS extract has the potential to be used in these forms as a future therapeutic approach for psoriasis.

Keywords – *Ficus Sycomorus*, psoralen, psoriasis, differential scanning calorimetry, Raman spectroscopy

1. Introduction

Psoriasis is a chronic skin disease with autoimmune origin, characterized by a thickening and disorganization of the skin's protective barrier. Usually, red scaly patches are caused on the skin surface.

Several approaches including methotrexate, anthraline, immunosuppresives, vitamin D, and retinoids have been proposed for psoriasis treatment, but no effective therapy has been established yet. Novel therapeutic approaches including monoclonal antibodies such as infliximab, and efalizumab have been used but their effectiveness is associated with questionable safety profile [1]. Therapies based on compounds isolated from natural products have also been proposed and used for a long time. Such therapeutic approach is the Psoralen Ultraviolet-A (PUVA) one. PUVA is a type of treatment where the patient receives a medicine containing a psoralen before being exposed to ultraviolet light [2]. The therapeutic value of the molecules isolated from the natural sources, especially of psoralenes, with or without UV activation are under extent investigation [3]. The absence of UV-A activation in psoriasis treatment is of high value and it is a challenging strategy which minimizes the cost of therapy. *Ficus Sycomorus* sap is a well known psoralen rich natural source [4-5]. The sap of *Ficus Sycomorus* is traditionally used against psoriasis and leucoderma.

Collagen is the most predominant and important protein of the skin. There are different types of collagens characterized by a variety extend of triple helical and non-helical domains. Type I collagen is the most interesting for industrial uses. It has a basic structural unit of tropocollagen which is a molecular rod about 300 nm in length and 1.4 nm diameter and 300000 Da of molecular weight. In the extracellular matrix it forms fibrils by longitudinal and lateral staggering with a typical cross striation of 70 nm period. Fibrils are aggregated into fibril bundles originating fiber framework. Type I collagen is widely used as a raw material for transdermic and topical delivery systems owing to its low antigenicity and its ability to support cell adhesion and development [6-8]. The use of type I fibrillar collagen as biocompatible and bioresorbable biomaterial is well-known and its applications are much diversified in medical, cosmetic and pharmaceutical field [9].

Collagen sponges (matrices and membranes) can be used in drug delivery and are mentioned in the literature to be superior from the other materials for the following five reasons: (a) partial open porosity for quick release of the drug after implantation into the tissue; (b) partial close porosity for “secondary” release of the drug enclosed within pores; (c) “tertiary” release of the drug partially immobilized within the fibrillar collagen structure; (d) a three-dimensional structure which works as a “natural” distance barrier between the drug incorporated into the sponge and the surrounding environment and (e) a network which enhances cell penetration and new tissue formation

[10].

Topic drug delivery systems have been a great field of interest in the recent time with promising future applications. The main advantages of such systems are controlled release of the drug to the affected tissue. The drug is mainly delivered to the skin by means of drug delivery systems. This work presents new natural forms, for psoriasis treatments, based on collagen forms (membranes and matrices) containing FS extract which could be used as drug delivery topics.

This research activity presents new topical delivery systems based on collagen membranes and matrices containing *Ficus sycomorus* (*FS*) is a psoralen source with potential for the treatment of psoriasis and other skin diseases [1, 4]. The composition of aqueous FS extract showed the presence of gallic tannins, resinonins, reducing sugars, alkaloids and flavone aglycones [11]. Due to this complex composition the *FS* extract can be used for antisporiatic treatment. *FS* sap is able to release in a controlled way the psoralens from its extract for possible psoriasis treatment. *FS* sap was obtained by aqueous extraction and it is used for the collagen preparations. The constituents of the *FS* extract have been isolated and analyzed. Four different bioactive compounds namely, psoralen, angelicin, chlorogenic and neochlorogenic acids were isolated by HPLC and then identified by 1D and 2D COSY NMR spectroscopy.

The *in vitro* release of psoralen by a specific kinetic mechanism has been studied. In addition, spectroscopic methodologies UV, IR and thermal analysis using Differential Scanning Calorimetry to investigate the structural and thermal changes in the presence of *FS* extract have been applied.

2. Materials and Methods

2.1. Extraction and Isolation

All solvents and reagents used in the phytochemical analysis and isolation of the bioactive constituents were purchased from Riedel-de Haen and Sigma Aldrich. All the solvents were of analytical quality and used without further purification. A quantity of the *Ficus Sycomorus* sap was collected from the plant stems of five different trees planted across the southern coastline of Cyprus during the spring time of the years 2007 and 2008 and was kept in freeze. *Ficus Sycomorus* is not systematically planted in Cyprus.

For the extraction, a quantity of the sap was diluted with water and filtered under vacuum. Then, the diluted sap was extracted successively with solvents of different polarity. The solvents were used in the following order: dichloromethane, diethyl ether, ethyl acetate and n-butanol. The extracts were checked for the presence of furanocoumarins on thin layer chromatography using the solvent system AcOEt/AcOH/H_2O, 8/2/4, upper phase (TLC, Merck pre-coated silica gel plates, type G60-F254). Purifications of the extracts were performed with flush chromatography on silica gel column using AcOEt/MeOH as the eluent. Final purification was achieved by semipreparative high performance liquid chromatography (HPLC) (Waters HPLC system, Mod. 1525 equipped with a Waters dual λ absorbance detector) on normal-phase support (Waters Spherisorb@ Silica 10 μM 10X25 mm). The eluent was used as a mixture of DCM (A)/AcOEt (B) (in the presence of 2% AcOH) and the flow rate was set to 1.5 ml/min and UV detection at 365 and 254 nm. The linear gradient Scheme is presented in Table 1.

Table 1. The linear gradient scheme used in HPLC experiments.

%B	Time (min)
5	0
50	50
100	20
100	5
5	

Analytical HPLC was performed on the same system, with the same solvent system and gradient using an analytical column (Waters Spherisorb@ Silica 5 μM 10X25 mm) and 1ml/min flow. Single peaks were produced with at least 95% of the total constituent peak integrals. The final verification of the isolated structure was solved by NMR techniques as it is discussed later.

2.2. Collagen preparation

Type I fibrillar collagen gels having a concentration of 1.1% w/w were extracted from bovine hide by basic and acidic treatments at 25 ^{o}C using the current technology in INCDTP Division ICPI-Collagen Department [8]. These gels were conditioned by specific drying techniques used for collagenic biomaterials such as free drying in air at 25 ^{o}C with Venticell oven and lyophilization with Delta 2 - 24 LSC, Martin Christ, Germany (freeze-drying at –80 ^{o}C, 2x10-2 torr) in order to obtain collagen membranes (MEM) and matrices respectively (MAT). The collagen forms were named as follows: MEM-FS – collagen membrane with 5% FS extract and MAT-FS – collagen matrix with 5% FS extract. The schematic presentation of collagen with *Ficus Sycomorus* extract forms obtained is presented in Fig. 1.

2.3. NMR spectroscopy

The deuterated solvents used for the NMR experiments were CH_3OH-d4 (purity > 99.96 %) purchased by Deutero GmbH and NMR tubes by Norell (S-5-600-7). NMR spectra were recorded on a Varian 600 MHz spectrometer. Each sample was dissolved in CH_3OH-d4. The gradient selected version of the DQF-COSY experiment was used, provided in Varian libraries of pulse programs. The 1H spectral window used was 6000 Hz. The homonuclear 2D proton spectra were acquired with 1024 data points in F2 dimension, 16 scans, 256 points in F1 dimension and a relaxation delay of 1 sec.

2.4. Antioxidant Activity

Measurement of antioxidant activity: Antioxidant activity (AA%) of FS extract, collagen gel and their combination is investigated by chemiluminescence technique (Turner Design 20/20, SUA) in the presence of luminol and H_2O_2 at pH = 8.6 (in TRIS-HCl 0.2M) at λ=420-430 nm and 10-3M/L concentration. Antioxidant activity is evaluated by Equation 1.

$$AA(\%) = \frac{I_0 - I}{I_0} \cdot 100 \quad (1)$$

where I is chemiluminescence intensity measured for the samples with antioxidant (FS) and I_0 is chemiluminescence intensity for the control sample.

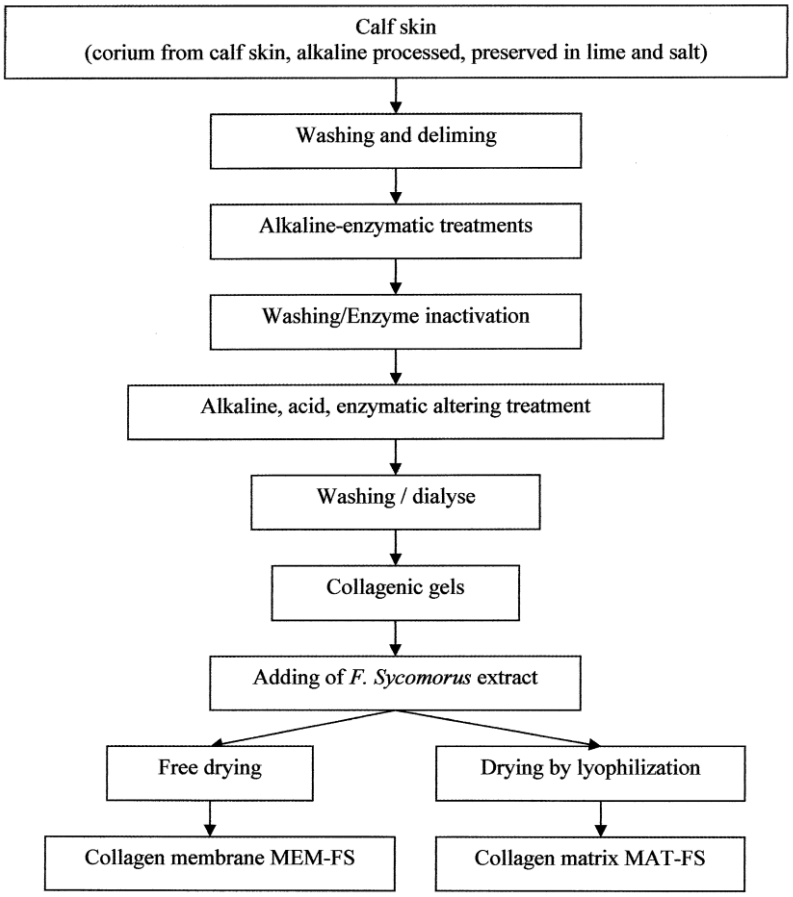


Fig. 1. Schematic presentation of collagen with *FS* extract membrane and matrix preparations.

2.5. Differential Scanning Calorimetry

Stainless steel pans were used for hermetic sealing and were purchased by Perkin Elmer (Norwalk). DSC technique was applied to study collageneous samples using a Perkin-Elmer DSC-7 calorimeter (Nowalk, Connecticut, USA). An amount of 5–10 mg of the collageneous membrane and matrix with or without Ficus Sycomorus extract was sealed into stainless steel capsules obtained from Perkin-Elmer. All samples were scanned at temperature range of 20-120 ^{o}C using a scanning rate of 10 ^{o}C/min. Thermograms were obtained on a Perkin-Elmer DSC-7 calorimeter (Nowalk, Connecticut, USA). The temperature and the enthalpy scale of the calorimeter was calibrated using indium (Tm= 156.6 ^{o}C) as the standard reference sample.

2.6. FT-IR and UV-VIS-NIR spectroscopy

Spectral characteristics were determined with FT-IR 620 (Jasco, Japan) spectrometer and FT-IR 6000 spectrophotometer with reflection ATR system MKII Golden Gate Single (Jasco) in IR (MID spectrophotometer NIR) and UV-VIS region; UV-VIS-NIR-V670 spectrometer (Jasco, Japan) with diffuse reflection (ILN – 725) and accessories for absorbance.

2.7. Water absorption

The hydrophilic properties represented by vapour water absorption were determined according with STAS 5048/1-84/A91:2008 method. The humidity of samples of membranes and matrices with *Ficus Sycomorus* were determined according with SR EN ISO 4684:2006.

2.8. In vitro kinetic and mechanism release

In vitro release of psoralen was determined in triplicate at 37±5 ^{o}C using a modified USP paddle method ("sandwich" device). The stirring paddle was rotated with a speed of 50 rpm. The distilled water was used as release/dissolution medium (200 mL). Aliquots of 5 mL were withdrawn from the medium at different times and the medium was completed with the same volume of fresh pre-heated water. Psoralen concentration was measured spectrophotometrically, at 322 nm. The cumulative amounts of psoralen released from membrane and matrix were determined using a calibration curve.

The *in vitro* release studies of *FS* from membranes and matrices took place in water at 37 ^{o}C. The amount of FS released was spectrophotometrically analyzed at 322 nm. In order to evaluate the drug release mechanism and kinetic, the in vitro release data were plotted in various kinetic models: zero order, first order, Higuchi′s kinetic and power law kinetic.

3. Results and Discussion

3.1. Extraction of psoralens

The AcOEt extract was found to be abundant in UV absorbing fouranocoumarins. Some traces of them were found to exist in the n-butanol extract. No furanocoumarin compound was detected in the extract of dichloromethane or in the etheric extract. The extraction with ethyl acetate was found to be the most efficient and, therefore, this solvent was used in all subsequent experiments for the quantitative isolation of furanocoumarins from the *Ficus Sycomorus* extract.

In the analytical HPLC of the ethylacetate extract, appeared one major peak (RT=11.655 min. 27.82%) and two minor ones (RT=15.894 min, 0.17% and RT=16.315 min. 0.29%). As it is discussed later, the main peak represents two fouranocoumarin derivatives, while the others are representing chlorogenic acid isomers.

3.2. Identification of psoralens by NMR Spectroscopy

1H and COSY NMR assignment of the first major HPLC peak revealed the presence of two furanocoumarins, namely psoralen (labelled with P) and angelicin (labelled with A) in molar ratio 3:1 respectively as it is implied by the integration of the corresponding 1H peaks. All the expected correlation peaks are observed in the COSY spectrum between the vicinal protons for both of the molecules (Fig. 2). The chemical shifts of psoralen and angelicin along with their coupling constants are presented in Table 2.

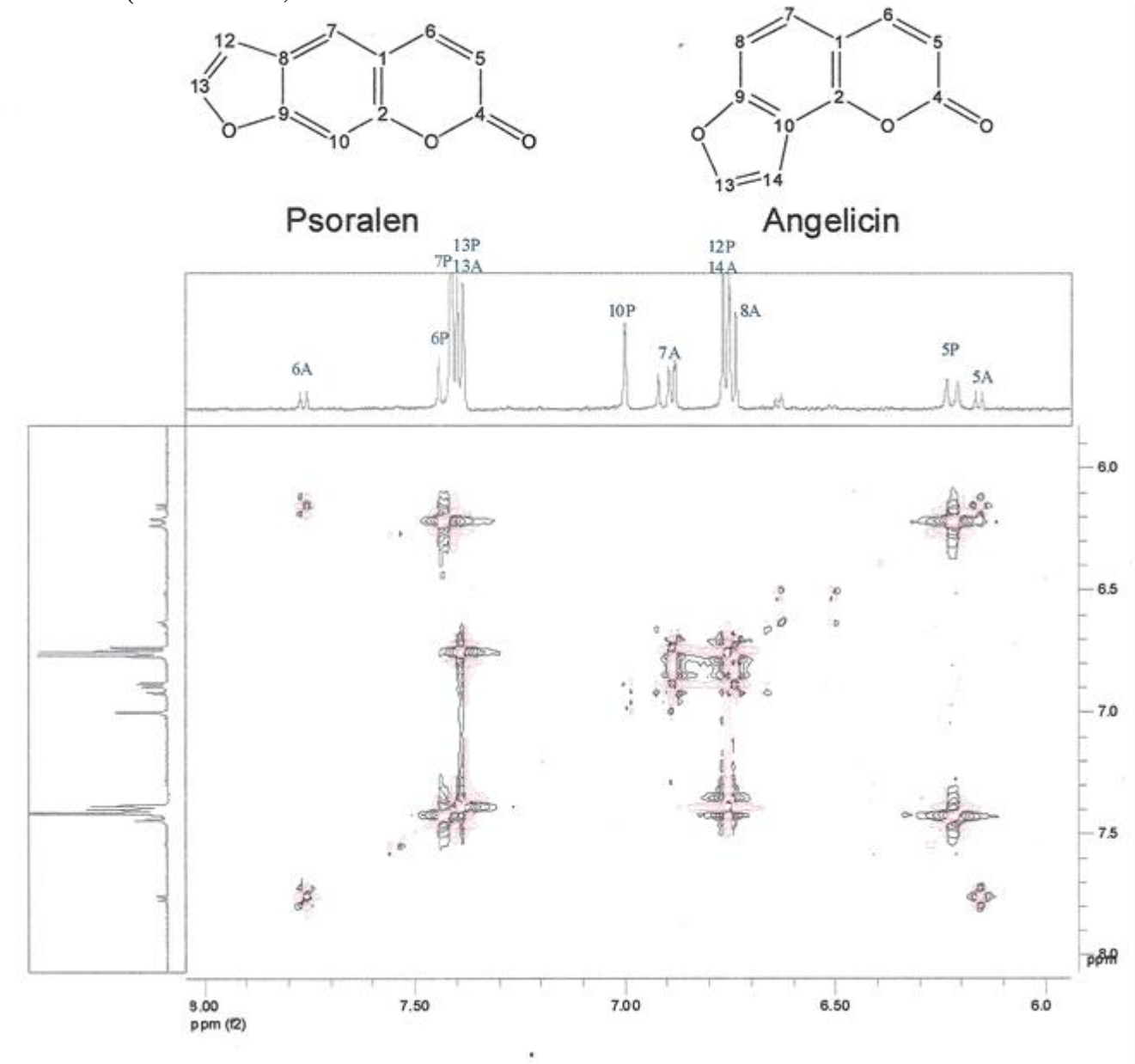


Fig. 2. 2D NMR COSY spectrum obtained at ambient temperature of psoralen and Angelicin in d4-CH_3OH using 600 MHz spectrometer. The assigned peaks labelled with P and A correspond to psoralen and angelicin respectively.

Table 2. Chemical shifts and coupling constants of psoralen and angelicin

Psoralen			Angelicin		
H	Chem. Shift (ppm)	Coupling Constants (Hz)	H	Chem. Shift. (ppm)	Coupling Constants (Hz)
5	6.22	J(5,6)=16 d	5	6.16	J(5,6)=9,5 d
6	7.44	J(6,5)=16 d	6	7.76	J(6,5)=9,5 d
7	7.42	J(7,6or12)=1.8 d	7	6.89	J(7,8)=8.2 d J(7,6)=1.9 d
10	7.00	J(10,7)=1.9 d	8	6.74	J(8,7)=8.2 d
12	6.76	J(12,13)=8.2 d	13	7.39	J(13,14)=8.2 d
13	7.39	J(13,12)=8.2 d	14	6.76	J(14,13)=8.2 d

Table 3. Chemical shifts and coupling constants of chlorogenic and neochlorogenic acid.

Chlorogenic acid			Neochlorogenic acid		
H	Chem. Shift (ppm)	Coupling Constants (Hz)	H	Chem. Shift. (ppm)	Coupling Constants (Hz)
2	6.94	J(2,3)=8.7 d J(2,6)=1.9 d	2	6.95	J(2,3)=8.7 d J(2,6)=1.9 d
3	6.77	J(3,2)=8.7 d	3	6.76	J(3,2)=8.7 d
6	7.04	J(6,2)=1.9 d	6	7.06	J(6,2)=1.9 d
9	7.60	J(9,10)=15.9 d	9	7.57	J(9,10)=15.9 d
10	6.39	J(10,9)=15.9 d	10	6.29	J(10,9)=15.9 d
14	5.54	mult.	14	5.37	mult.
15a	2.26	mult.	15a	2.08	mult.
15b	2.06	mult.	15b	2.24	mult.
17a	2.01	mult.	17a	2.49	mult.
17b	1.66	mult.	17b	1.62	mult.
18	3.53	mult.	18	3.46	mult.
19	3.88	mult.	19	3.91	mult.

NMR analysis of the second HPLC peak (RT=15.894 min.) gave evidence of the presence of two isomeric compounds, chlorogenic (labelled with C) and neochlorogenic acid (labelled with N). The correlation peaks of vicinal protons are observed in COSY spectrum for both of the molecules (Fig. 3a, 3b). The chemical shifts of chlorogenic and neochlorogenic acid along with their coupling constants are presented in Table 3. The assignment of the third HPLC peak (RT=16.315 min.) with the use of 1D and 2D COSY NMR revealed one compound, the neochlorogenic acid (Fig. 4a, 4b). The chemical shifts of neochlorogenic acid along with its coupling constants are presented in Table 4.

The presence of psoralen as a major compound, in molar ratio 3:1 relative to angelicin, at the AcOEt extract justifies the traditional use of *Ficus Sycomorus* sap for the treatment of psoriasis and leukoderma. Angelicin is inactive against these two dermatological diseases. Chlorogenic and neochlorogenic acids found in the *Ficus Sycomorus* sap are not known to be beneficial against psoriasis. However, chlorogenic acid is known to possess antioxidant, anxiolytic [12], antival [13], antibacterial [14] and antifungal [15] activity.

Chlorogenic acid and derivatives are known to absorb at the UV-B area near UV-A area with a maximum of absorption at 327 nm [16]. This is supportive to the hypothesis that UV-A activation is not necessary for the psoriasis treatment. As it was reported earlier [5], in small scale clinical trials conducted on psoriatic patients utilizing crude sap on the psoriatic plaques, the decrease of the area covered by the plaque was succeeded without any UV activation. The psoriatic plaques on the subjects of that study were not exposed directly to solar radiation.

3.3. Collagen forms preparation

In order to determine spectroscopic characteristics, the *FS* extract was dried by lyophilization. The adding of *FS* in collagen samples results in light-brown colour both for membrane and matrix. Fig. 5a shows a sample of lyophilized *FS* extract. Fig 5b presents the spongious

matrix MAT-FS and in Fig. 5c is showed the collagen membrane MEM-*FS*.

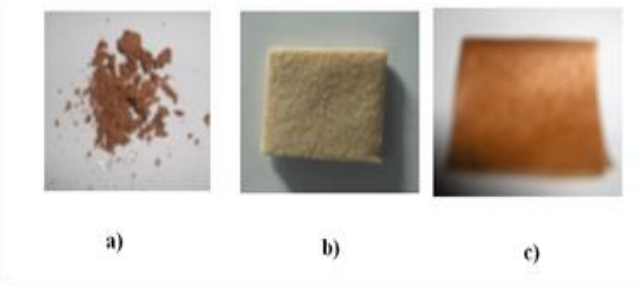
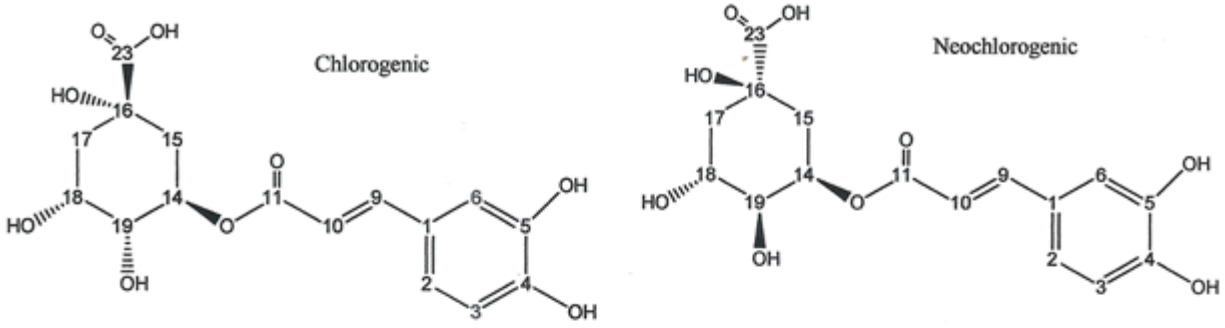


Fig. 5. Sample of (a) lyophilized FS extract, (b) MAT-FS, (c) MEM-FS.

3.4. Antioxidant Activity

The *FS* extract represents a mixture of antipsoriatic and antioxidant factors/substances. The antioxidant activity of *FS* extract measured by chemiluminescence was 99.8%. This high value is due to the presence of chlorogenic and neochlorogenic acid. Addition of *FS* extract increased antioxidant activity for collagen gel from 55.4 to 78.9%.



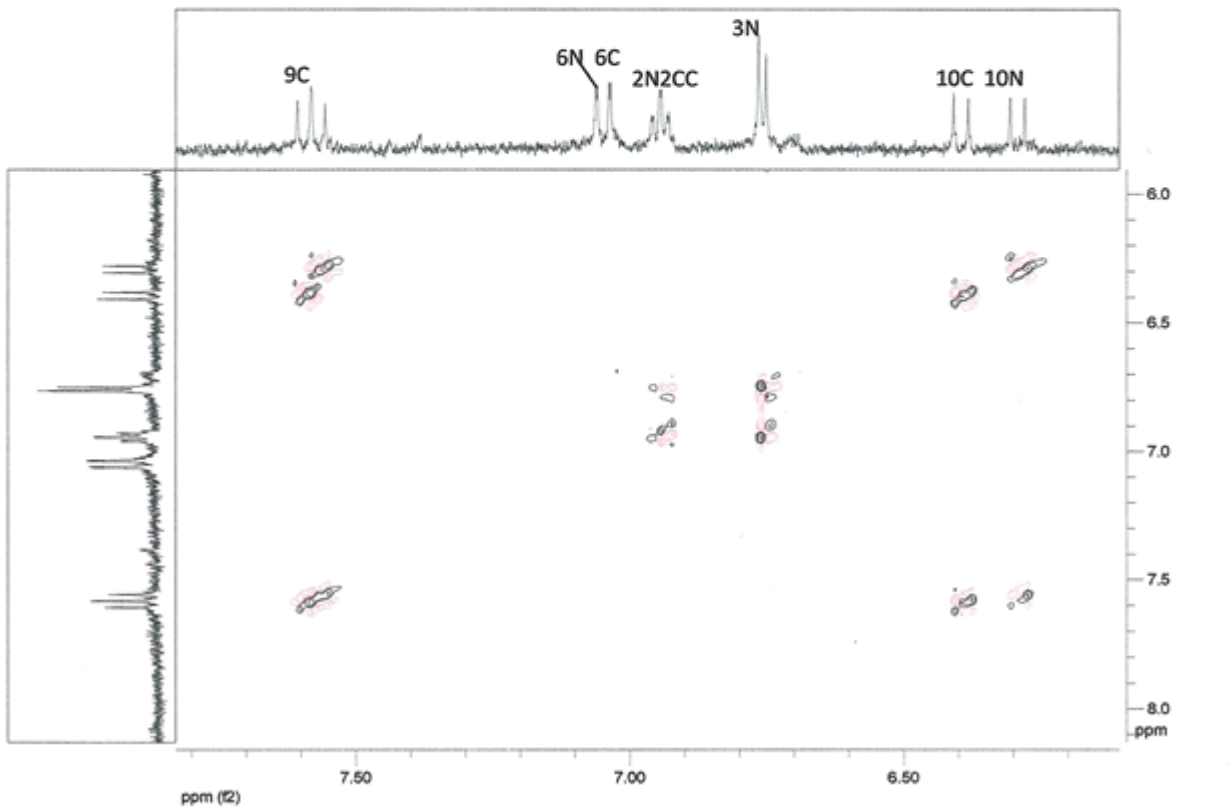


Fig. 3(a) Spectral region (6.0 – 8.0 ppm) of 2D NMR COSY spectrum of chlorogenic and neochlorogenic acid in d4-CH3OH obtained using 600 MHz s pectrometer. The assigned peaks labelled with C and N correspond to chlorogenic and neochlorogenic acid, respectively.

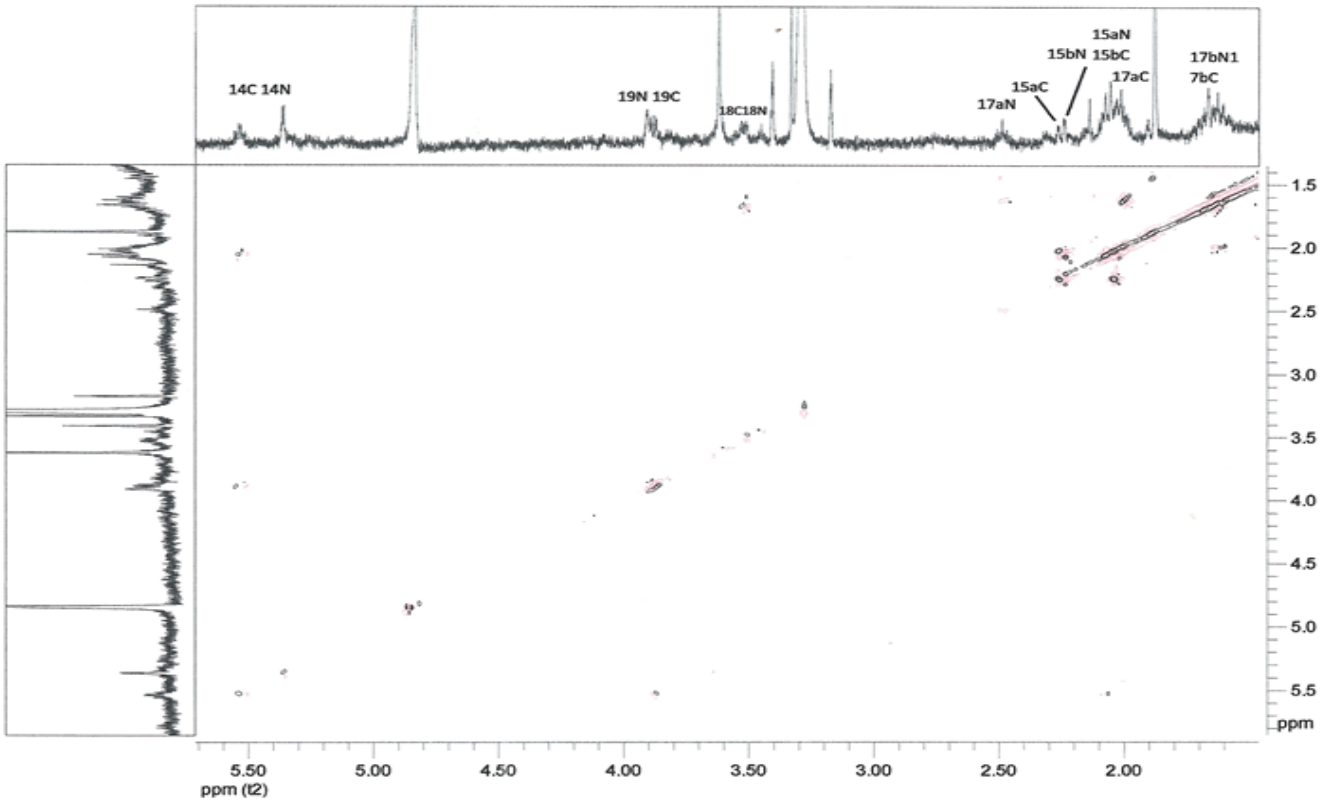
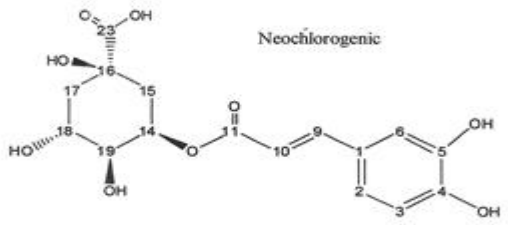


Fig. 3(b) Spectral region (1.5 – 5.5 ppm) of 2D NMR COSY spectrum of chlorogenic and neochlorogenic acid in d4-CH3OH obtained using 600 MHz spectrometer. The assigned peaks labelled with C and N correspond to chlorogenic and neochlorogenic acid, respectively.



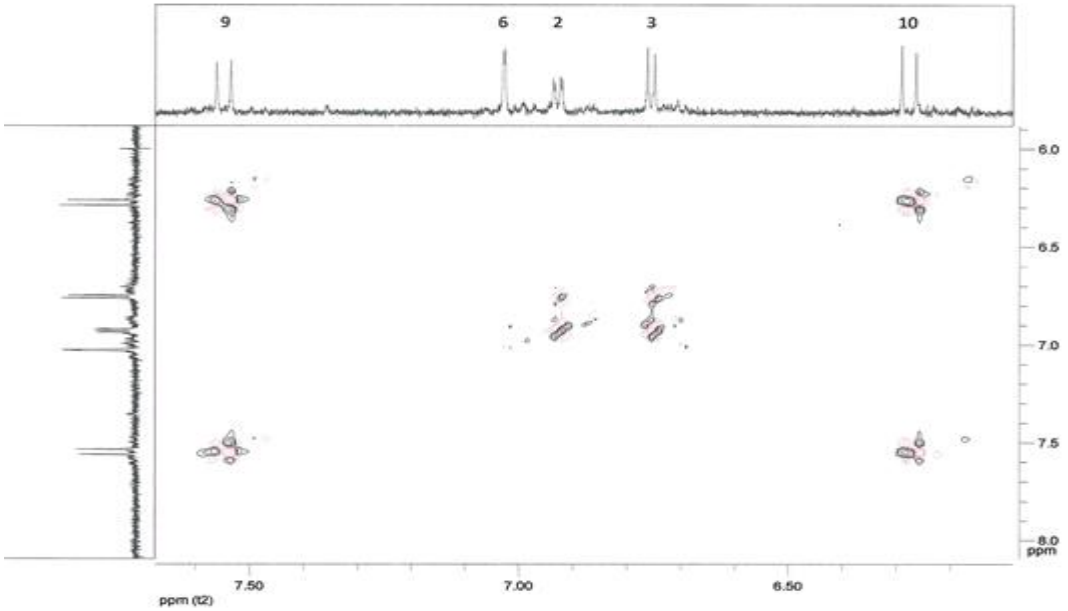


Fig. 4(a). Spectral region (6.0 – 8.0 ppm) of 2D NMR COSY spectrum of neochlorogenic acid in d4-CH3OH obtained using 600 MHz spectrometer.

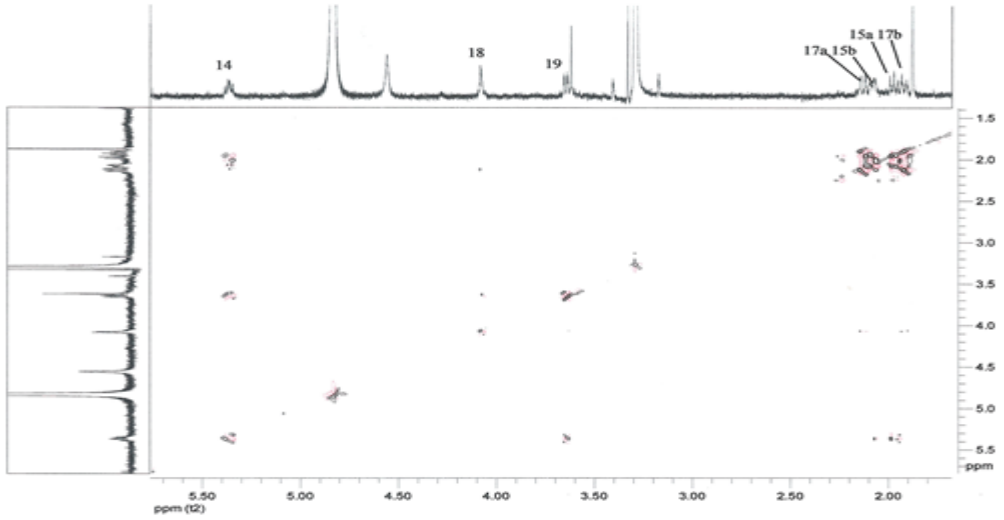


Fig. 4(b). Spectral region (1.5 – 5.5 ppm) of 2D NMR COSY spectrum of chlorogenic and neochlorogenic acid in d4-CH3OH obtained using 600 MHz spectrometer

Table 4. Chemical shifts and coupling constants of neochlorogenic acid.

Neochlorogenic		
H	Chem. Shift. (ppm)	Coupling Constants (Hz)
2	6.93	J(2,3)=8.1 d J(2,6)=2.0 d
3	6.76	J(3,2)=8.1 d
6	7.03	J(6,2)=2.0 d
9	7.55	J(9,10)=16 d
10	6.27	J(10,9)=16 d
14	5.37	mult.
15a	1.98	J(15a,15b)=12.3 m
15b	2.08	J(15b,15a)=12.3 m
17a	2.13	J(17a,17b)=14.7 m
17b	1.92	J(17b,17a)=14.7 m
18	4.08	mult.
19	3.65	J(19,14)=9.83 d J(19,18)=9.68 d

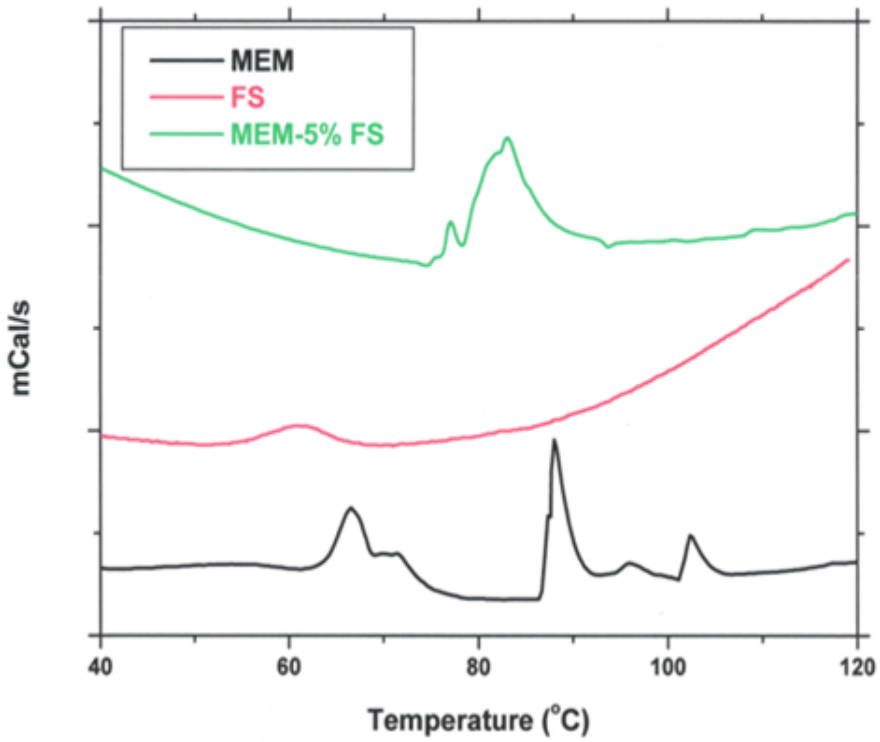


Fig. 6. DSC thermograms of collagen membrane (A) FS extract (B) and collagen matrices with 5% FS extract (C).

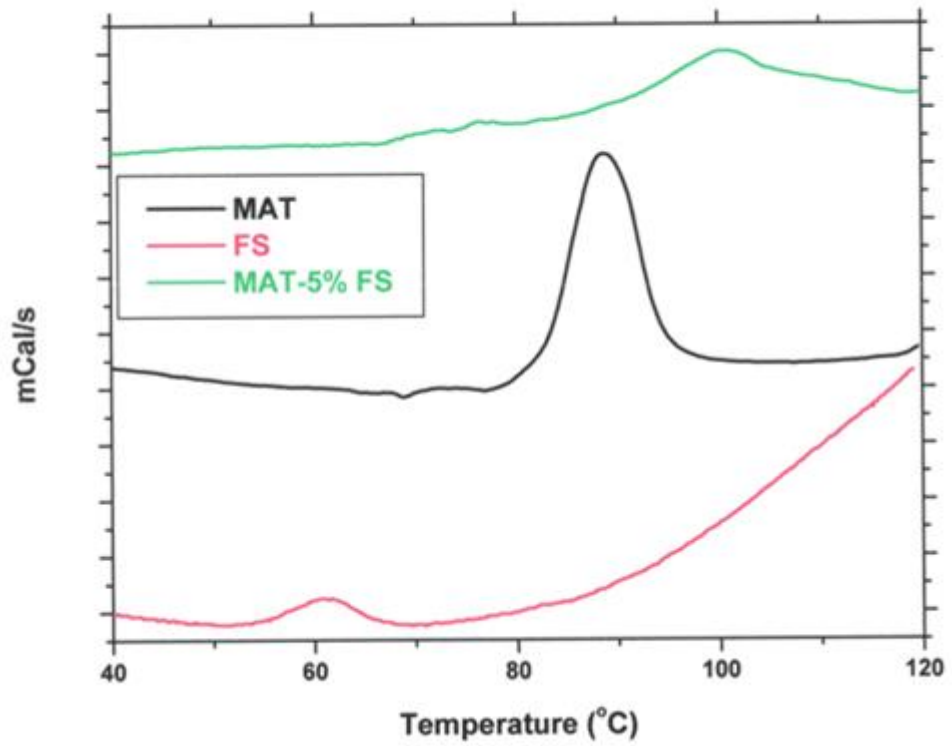


Fig. 7. DSC thermograms of collagen membrane (A) FS extract (B) and collagen membrane with 5% FS (C).

3.5. Differential Scanning Calorimetry

Figure 6 shows the effect of *FS* extract on collagen membranes [17]. The black line shows the thermal changes of collagen membranes MEM as they are depicted by increasing temperature. Two phase transitions are eminent in the temperature regions of 60-80 ^{o}C and 85-110 ^{o}C. The first multicomponent phase transition occurs at Tm of 66.37 ^{o}C and is associated with a 10.375 Jg^{-1} enthalpy change. This endothermic peak corresponds to

the denaturation phenomenon in which collagen loses its elasticity. The second region is consisted of three different endothermic peaks which are observed at 87.7, 95.7 and 102.03 ^{o}C respectively with a total ΔH of 8.68 Jg^{-1} and corresponds to the melting region of the collagen membrane. The red line in Figure 6 corresponds to the heating treatment of the freeze-dried FS extract. A broad endothermic peak at 60.67 ^{o}C is observed with ΔH= 0.876 Jg^{-1}. The green line of Figure 6 shows the effect of 5% FS extract in MEM. The presence of FS affects both the denaturation of collagen and its melting point. The peak at 66.37 ^{o}C observed with MEM preparation is shifted towards the higher temperature of 83.00 ^{o}C with higher ΔH= 12.555 Jg^{-1}. A small endothermic shoulder is observed at the left of the main peak and a small exothermic shoulder at the right of the peak.

Figure 7 presents the thermograms of collagen matrices in the absence and presence of *FS*. The control MAT (black line) shows an endothermic peak at 88.53 ^{o}C with a ΔH= 28.286 Jg-1. This peak corresponds to the dehydration of the collagen matrices. *FS* preparation (red line) shows a broad endothermic peak centered at ca 60 ^{o}C. The DSC thermal scan of MAT loaded with 5% FS sample shows an endothermic peak (green line) at 100.3 ^{o}C with a ΔH= 22.359 Jg^{-1} and corresponds to the dehydration peak of the collagen matrices. This is higher, compared to the preparation that contains only collagen matrices, indicating that the presence of *FS* causes stronger linkage with water.

3.6. *Hydrophilic properties*

The porous collagen matrix is highly hydrophilic scaffold. The absorption of water vapors is very fast and two times higher than in membranes during the first 8 hours. This behavior is mainly due to specific morphological structure as microporous sponge for freeze-dried collagen. The collagen matrices MAT and MAT-FS have a large specific surface and a very low specific density of 0.02 g/cm^3. Collagen membrane samples have a compact micro and nanoporous structure and the specific density is higher: 0.60 g/cm^3. For this reason, the amount of absorption in membranes is lower in the time period of 8 h than in matrices (Figures 8 and 9).

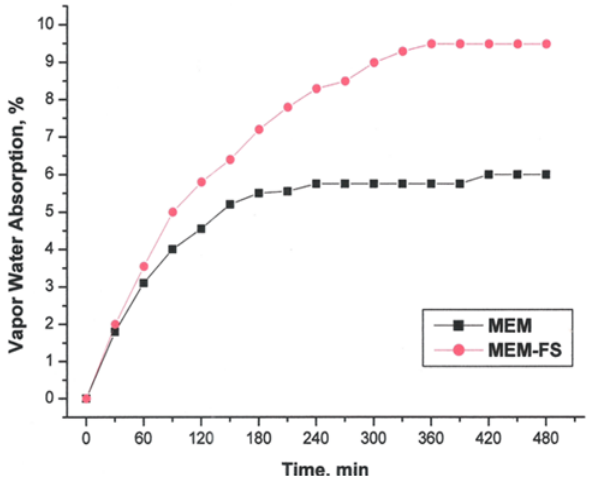


Fig. 8. Percentage vapor water absorption vs time for MEM form without and with FS incorporation.

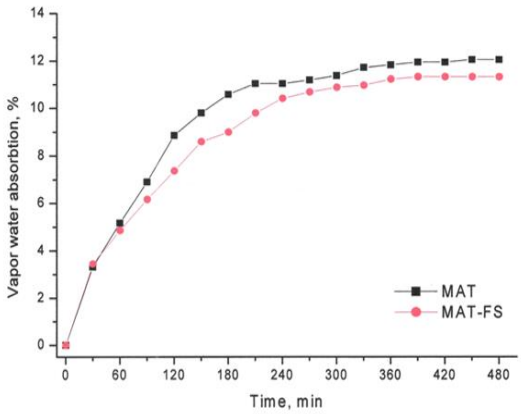


Fig. 9. Percentage vapor water absorption vs time for MAT form without and with FS incorporation.

The hydrophilic properties are in a good agreement with DSC data. Matrices in the absence and presence of FS dehydrate in a different way than membranes in the absence and presence of *FS*. Membranes give more complex thermal scan in this region, probably due to the non-homogeneous dehydration and different crosslinking with the triple helix of collagen. It is also interesting that denaturation of the matrices is not observed probably due to the higher amounts of water absorption. The increase of the cross linkage due to the presence of *FS* depicted in the DSC results as increase of the phase transition is parallel to the increase of vapor water absorption.

3.7. *UV – VIS – NIR spectroscopy*

The UV-VIS-NIR can give direct information of the presence of bioactive components of FS in the collagen membranes and matrices. For example, band at 280 nm (transition n - π^{*}) from MEM (and 250 nm from MAT) show a bathochromic shift to 350 nm due to accumulation of groups with extended conjugation (spectra not shown).

3.8. *FT-IR spectroscopy*

The FT-IR spectrum for FS extract, (SF1) presents absorption bands specifically for natural vegetal extracts formed by aromatic compounds with different structure. Thus, the large band showing maximum at 3380 cm^{-1} indicates the presence of -OH groups associated by hydrogen bonds and that at 3070 cm^{-1} shows a peak with weak intensity that derives from an aromatic structure. The bands at 2930 and 2860 cm^{-1} are due to CH_2 and CH_3 groups. The peak at 1733 cm^{-1} arises due to the present organic acids with free -OH groups. The *FS* presents absorption bands at 1243 cm^{-1} and is attributed to the aromatic -OH groups. The absorption band from range 1020 – 900 cm^{-1} is specific to aliphatic substitution.

FT-IR spectra obtained for collagen matrix (MAT-FS) and membranes (MEM-FS) show differences which can be explained by the drying process used (SF2 and SF3). The peak resonated at 1730 cm^{-1} appears only in MAT-FS spectrum preparation. The FT-IR spectra for the collagen forms show spectral characteristics of type I collagen, respectively amide I (1600 – 1740 cm^{-1}), amide II (1485 – 1590 cm^{-1}), amide III (1190 – 1300 cm^{-1}), amide A (around 3300 cm^{-1}) and amide B (around 3070 cm^{-1}).

Amide I bands are situated around the 1631 – 1642 cm^{-1} value for all samples. The shift of amide I from 1631 cm^{-1} reported for MAT to 1634 cm^{-1} in MAT-FS and from 1642 cm^{-1} in MEM and to 1641 cm-1 in MEM-FS reveals an orderly structural process through H_2O bending at carbonyl groups [18]. Also, the phenolic -OH groups of FS react with carbonylic groups of collagen. The effect consists of moderate conformational changes in secondary structure.

Other changes in spectra can be noticed in amide II band which correspond to the -NH deformation and -CN stretching modes [19]. In MEM-FS spectra the band corresponding to the triple helical structure (around 1555 cm^{-1}) is near to this value (1552 cm^{-1}). In the case of MAT-FS this band has a lower value of 1548 cm^{-1}. The bands around 1240, 1241 and 1242 cm^{-1} represent the amide III band of N-H bending vibration with significant mixing with the CH_2 wagging vibration from the glycine backbone and proline side chains in collagen.

Modification of triple helix integrity structure could be quantified by the ratio between amide III and 1450 cm^{-1} which is given by CH_3 bending vibrations. This ratio has to be higher or equal with 1. For denaturated collagen (gelatin) which hasn't triple helix conformation this ratio is 0.59 [20, 21]. The semiquantitative modifications which are correlated with conformational structure of collagen are described by the following ratios:

1) AIII/A1450 is correlated with maintaining of integrity of triple helical structure; the value has to be higher or equal with 1.
2) AI/AA ratio is correlated with crosslinkig degree: the higher the ratio, the higher the cross-linking levels.
3) $\Delta\nu = (\nu_1-\nu_2)$ cm^{-1} which represents differences between frequencies of amide I and II, gives information about denaturation process: $\Delta\nu > 100$ cm^{-1} indicates the presence of denaturated collagen.

Table 5 shows the relevant conformational modifications into collagen structure. The ratio AIII/A1450 is equal with 1 for matrices and less than one for membranes.

Table 5: Ratios AIII/A1450, AI/AA and Δν= (ν1-ν2) cm-1 for MAT and MEM collagen forms with and without the presence of FS. These indicators explore the effects of FS in the conformational structure of collagen forms.

Name of samples	A_{III}/A_{1450}	A_I/A_A	$\Delta\nu= (\nu_1-\nu_2)$ cm^{-1}
MAT	1,02	1,08	84
MAT-FS	1,03	1,21	86
MEM	0,80	0,48	91
MEM-FS	0,89	0,51	89

This decreasing is due to the obtaining process of membrane which involves before casting into shelves a slowly heating at 35 ^oC during 3-4 min necessary for desaeration. The fact that the value of this ratio is almost the same for both membranes demonstrate that FS extract does not destabilize the secondary structure. Also the Δνs for samples which contain FS, present values close to control samples and less than 100 cm^{-1}. This is indicative that there is no collagen denaturation (Table 6).

Table 6: Kinetics of FS release in various kinetic models

Collagen scaffolds with FS	Zero order (1) $\frac{m_t}{m_\infty} = k \cdot t$	First order (2) $\frac{m_t}{m_\infty} = 1 - e^{-k \cdot t}$	Higuchi's (3) $\frac{m_t}{m_\infty} = k \cdot t^{0.5}$	Power law (4) $\frac{m_t}{m_\infty} = k \cdot t^n$
	R^2 values			
A. Membrane	0.8937	0.8936	0.9730	**0.9917**
Matrix	0.9089	0.9088	0.9809	**0.9959**

The band corresponding to amide A (around 3300 cm-1) is more sensitive at structural changes of collagen than amide B band (3070 cm^{-1}). These bands are correlated with the NH stretching modes. The amide A of pure collagen presents absorption in the range of 3325-3330 cm^{-1}. When the amine groups from polypeptides are involved in hydrogen bonds these are shifted towards smaller wavelength (3300 cm-1). The shift of amide A from 3298 and 3295 cm^{-1} presented in control samples MAT and MEM towards higher wavelength, to 3301 cm^{-1} indicates interaction through hydrogen bonds of phenolic groups of FS with NH groups of collagen. This fact is confirmed by increasing of ratio AI/AA.

The absorption bands from range 900 – 1100 cm^{-1} presented in MEM and MEM-FS are not observed in matrices spectra. This fact could be explained by different types of interactions which take places between reactive groups of collagen molecules during free drying. The formation of this interaction is influenced by the presence of *FS* extract subjected to free drying. Furthermore, membranes MEM and MEM-FS show after the drying a humidity of about 30%, higher compared to matrix samples that have 15%.

3.9. In vitro kinetic and mechanism of release

The amount of *FS* was determined as having 75% psoralen. The cumulative percent of FS release from membrane and matrix is determined from the calibration

curve. Due to specific morphological structure of membranes and matrices, the release profile of *FS* is characteristic for porous and hydrophilic scaffolds with macro, micro and nanopores [22]. In Fig. 10 are presented the cumulative *FS* released (%) in 240 min. After this period of time the curves reach a plateau.

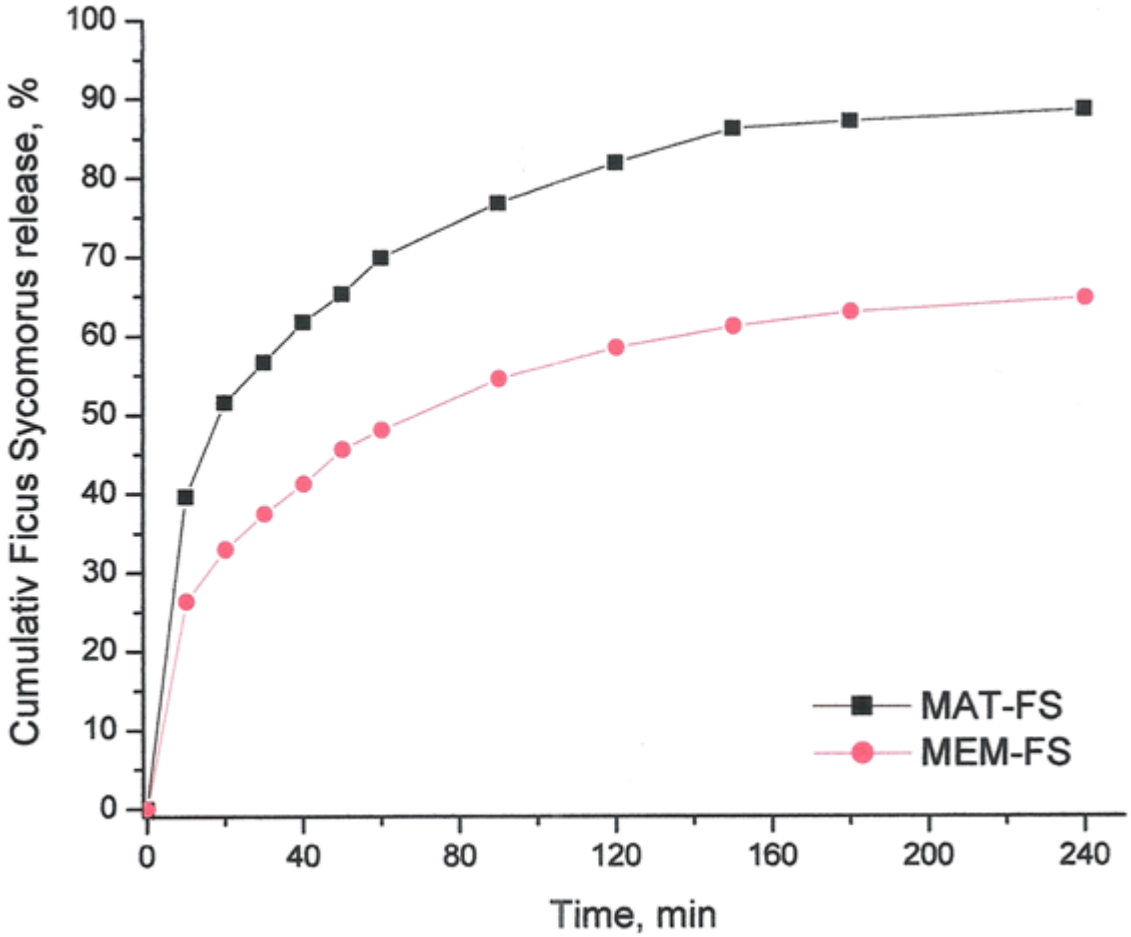
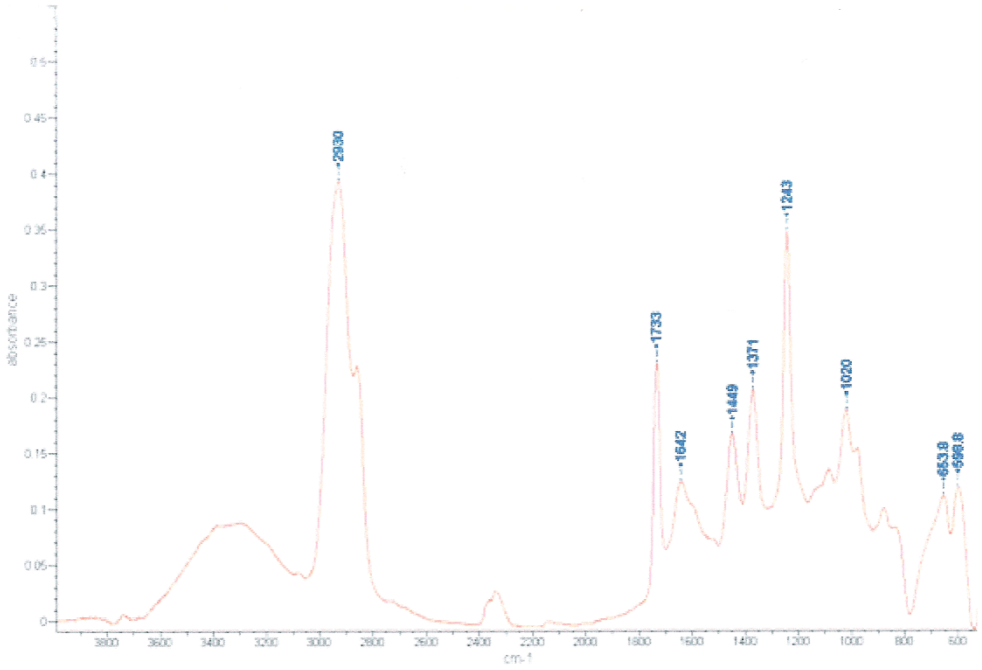
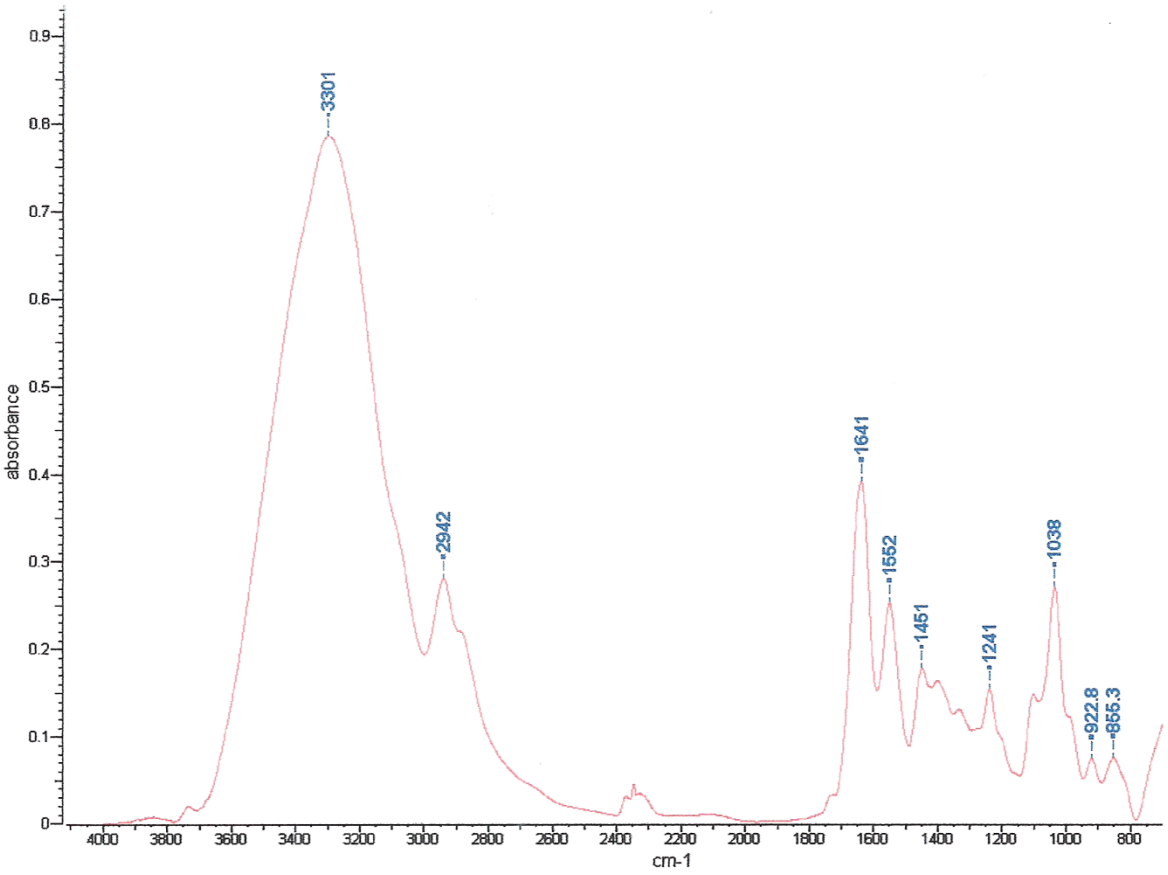


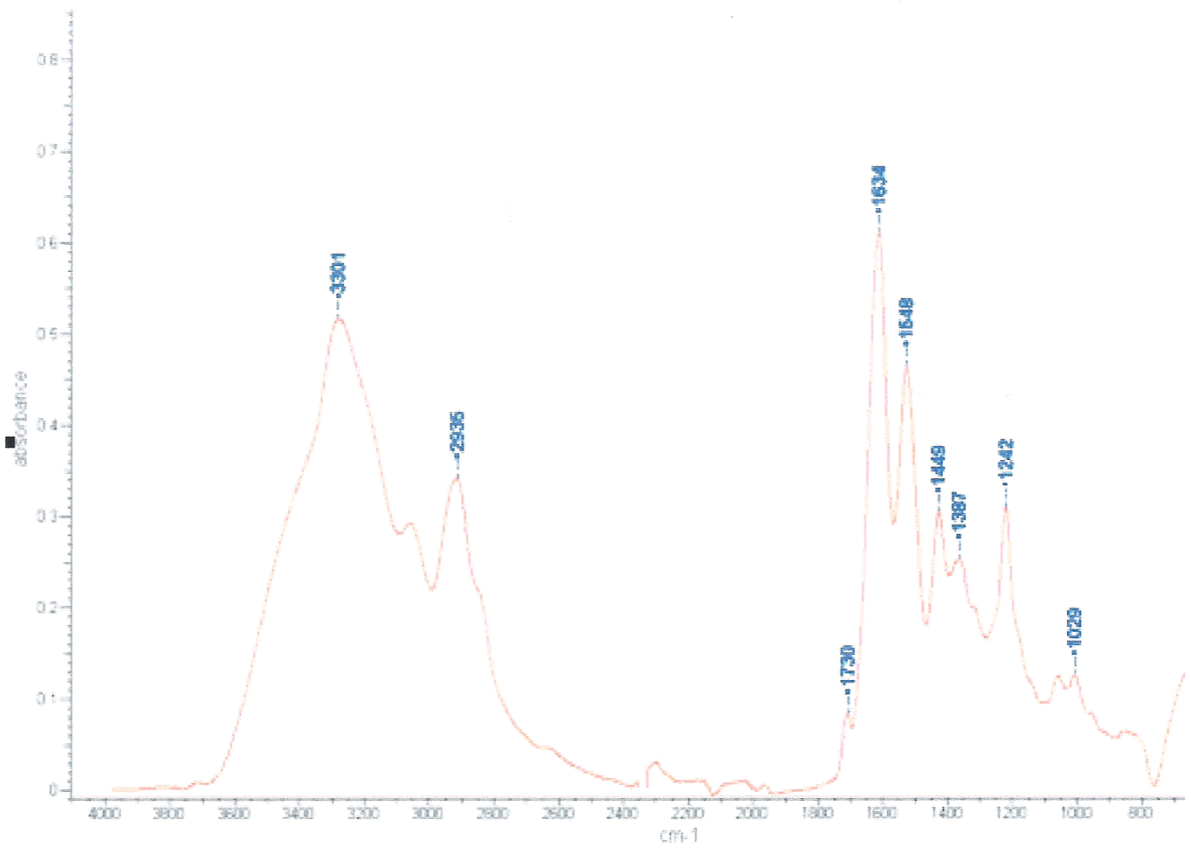
Fig. 10. Cumulative FS release versus time for MEM-FS and MAT-FS collagen forms



SF1



SF2



SF3

As it shown in Figure 10, the differences in structural morphology of membranes (transparent films) and matrices (porous sponges) determine different amount of bioactive substances released. The matrix release is maximum at 88.8% and the membrane 64.8% during 240 min. The membrane release of FS is slower than that observed in matrices.

The drug release from porous scaffolds is influenced by many factors such as hydrophilic, swelling and dissolution of scaffold; diffusion and distribution of drug into the scaffold and bioactive substance/ scaffold ratio [23]. In case of collagen scaffolds like membranes and matrices, an important role is played by hydrophilic and swelling properties that allow a fast release in the first hours followed by a slowly one until a constant value is achieved for a large period of time.

In order to evaluate the mechanism and kinetic of FS release, the data obtained from in vitro experiments are plotted in various kinetic models: zero order (Equation 1 of Table 6), first order (Equation 2 of Table 6), Higuchi's kinetic (Equation 3 of Table 6) and power law kinetic (Equation 4 of Table 6). Table 6 shows the kinetic models, their equations and correlation coefficient (R^2) for collagen membrane and matrix with FS. When the release mechanism is not known or more than one type of release phenomenon (diffusion, swelling or erosion controlled) is involved, the power law model is used to analyze the drug release.

The value of the release exponent n was calculated as the slope of the straight lines fitting the released data using the least-squares methods. The release exponent, n is 0.3194 for membrane and 0.2816 for matrix. These values indicate kinetic release mechanism that tends to be anomalous. The release data analyzed on the basis of power law equation demonstrates that the mechanism of the studied scaffolds combines characteristics of zero order, first order and Higuchi's models. The kinetic constant for matrix is 0.0681 %/sn and for membrane is 0.0344 %/sn, that proved two times faster release from matrices than membranes. In order to control the release of the *FS* extract at the local affected tissue by psoriasis is very important to know the kinetic mechanism and the rate of release.

4. Conclusion

Many interesting results are obtained which can be utilized for FS control release from collagen forms as an approach against psoriasis.

The presence of furocoumarins and antioxidants in the forms were revealed by both UV-VIS-IR. The amount of psoralen was calculated to be 75%. Low concentration of 5% *FS* causes mild structural changes in the MAT-FS and MEM-FS.

The *FS* release from the two kinds of forms shows a mechanism that tends to be anomalous. However, the *FS* release in MAT-FS is faster by a factor of two relatively to the MEM-FS. The matrix release reaches the maximum of 88.8% and the membrane 64.8% during the 240 min period.

The above properties of MAT-FS and MEM-FS forms and especially MAT make them candidate approaches for further examination for possible use as patches for antipsoriatic therapy. More work is needed *in vivo* to prove or disprove this statement.

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