Salvia Officinalis Essential Oil Loaded Gelatin Hydrogel as Potential Antibacterial Wound Dressing Materials

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Salvia officinalis essential oil loaded gelatin hydrogels with improved antibacterial activity and enhanced stability was prepared by microwave-assisted polymerization method. FT-IR spectra indicated no chemical interaction between the hydrogel matrix and the essential oil functional groups. According to the swelling studies, enhanced stability in all pH media was obtained. Studying two kinetic models: Fickian transport and Schott second order kinetic model, it was demonstrated that the swelling process of the prepared hydrogels occurs after a second order kinetics. Antibacterial activity, investigated by the agar diffusion method, regarding S. aureus and E. coli is comparable to that of silver nanoparticles and twice more efficient compared to cinnamon essential oil.

Keywords : polymer gels, Salvia officinalis, antibacterial activity, swelling kinetic

Hydrogels, three-dimensional cross-linked water-swollen polymeric materials, have potential biomedical and pharmaceutical application due to their biocompatibility, hydrophilic and non irritating nature. Hydrogels are suitable for wound dressing, because they can absorb moisture up to thousand times of its dry polymer weight, maintain wet environment preventing wound desiccation and maceration, provide cool sensation which reduces pain, and ensure bacteria entrapment and bacterial penetration protecting lesion from bacterial infections [1-4]. Due to these areas of application, in order to improve, especially the antibacterial activity of hydrogels, metal ions, in the form of micro- or nanoparticles, such as gold [5], silver [6-8], TiO_2 [9], ZnO [10], zinc carbonate [11], CuO [12] and graphene oxide [13] were tested. Because these nanoparticles show potential toxic properties when are in contact with human tissue, limiting their applicability, plant essential oils (EO) represent a new interest and a long term friendly alternative, replacing conventionally used preservatives and antibacterial agent. Many EO, such as thyme oil, lavender, peppermint, cinnamon, tea tree, rosemary, chamomile blue, eucalyptus, lemongrass [14,15], citronella and cedarwood [16], sage [17-19] and few others have been demonstrated to have a wide spectrum of antimicrobial activity. For most of these oil extracts have also been reported to have antifungal, antitermite, anti-inflammatory, antioxidant, anticancer or other therapeutic effects for some skin disorders and not only [20]. Among bioactive and antimicrobial properties, plant EO incorporated into wound dressing biofilms act as skin permeation enhancers promoting the diffusivity of drugs, by modification the skin barrier but withouth any change of their structure [21]. Considering the high volatility of EO, the incorporation into polymeric matrices seems to be an effective strategy for their usability. S. T. Khalili et al. [22], produced chitosan-benzoic acid nanogel with encapsulated thyme EO for enhanced antimicrobial activity. The minimum inhibitory concentration of the encapsulated EO was recorded at 300 mg/L while the free thyme extract could only completely prevent the growth of *Aspergillus flavus* at an elevated concentration of 400 mg/l. J. Liakos et al. [14], prepared natural polymeric composite films with remarkable anti-microbial and anti-fungal properties, by dispersing some EO (chamomile blue, cinnamon, lavender, tea tree, peppermint, eucalyptus, lemongrass and lemon oils) in sodium alginate. Extracts from the leaves of *Salvia officinalis* tested on mice have shown anti-inflammatory activity twice stronger than indomethacin which was used as a reference [18]. A study realised on human tissue, demonstrated a better diffusivity of *p*-aminobenzoic acid when sage oil was used. Recent research results demonstrate the benefits of essential oils compared to metal oxides and nanoparticles on getting improved wound dressing biofilms.

According to our knowledge and research, there are no previous data regarding gelatin based hydrogels loaded with *Salvia officinalis* EO. The aim of this study is to obtain *Salvia officinalis* EO loaded gelatin hydrogels as potential wound dressing material, with enhanced antibacterial activity.

Experimental part

Materials

Gelatin from porcine skin (gel strength 300, type A) and glycerol (≥ 99.5%) used as plasticizer were purchased from Sigma-Aldrich. Glucose (≥99.9%) purchased from S.C. HIPOCRATE 2000 SRL was used for the preparation of the cross-linking solution. *Salvia officinalis* (sage) EO were separated by *Stejarul* Biological Research Centre, Piatra Neam, Romania, using dry plant material from *Salvia officinalis* leaves according to the method indicated in the Romanian Pharmacopoeia, the X edition (RF X) [23,24].

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Escherichia coli ATCC 25922 (Gram negative) and *Staphylococcus aureus* ATCC 25923 (Gram positive) were obtained from Microbiology Laboratory (Faculty of Biology and Geology of Babes-Bolyai University, Cluj-Napoca, Romania) where the bactericidal activity of the prepared hydrogels was investigated. Both bacterial strains were grown on Nutrient Broth (Oxoid) and spread on Petri dishes with Nutrient Agar (Oxoid) for inhibition assay.

Preparation of hydrogel

Gelatin-based biopolymer films plasticized with glycerol and cross-linked with 10% glucose solution were prepared by microwave-assisted polymerization method from a mixture of gelatin and glycerol, mixed in a weight ratio 1.25:1, using the following procedure: gelatin, glycerol and *Salvia officinalis* EO (0.2 % w/w) were added to 6 mL of distilled water. The mixture was irradiated by microwave in household microwave system at 480 W for different time periods following 5 second heating and 1-2 s mixing steps, to prepare two different sheets: blank sample (BS) - without EO and sample loaded with *Salvia officinalis* EO (OLS). After complete dissolution the mixture was poured into polystyrene Petri dishes and air dried at ambient temperature for 5 to 6 days. The dried biodegradable gelatin films were wrapped in wax paper and kept at temperatures between 3-5°C.

Characterization

Fourier Transmission Infra-Red Spectroscopy (FT-IR)

The structure of the EO loaded gelatin hydrogel and blank sample were confirmed by using a Spectrum BX FT-IR spectrometer with an attenuated reflectance accessory. The FT-IR spectra of air dried films were recorded with one scan at a resolution of 4 cm^{-1}. Scanning was carried out in the range 4000-400 cm^{-1} for each sample.

Swelling studies

Swelling behavior of the prepared hydrogels were studied by dipping a 2 x 2 cm large sample of each dried films in three different media (neutral, acidic and basic) at room temperature until constant weight was obtained. At predetermined time intervals, the swollen samples were gently blotted and weighed. The swelling ratio (SW(%)) was determined by equation (1):

$$SW = \frac{W_t - W_i}{W_i} \times 100 \quad (1)$$

where: Wt is the weight of the swollen samples and Wi is the weight of the dry samples.

Bacterial resistance test

The bactericidal activity was investigated by the agar diffusion [25,26] (disc diffusion) method using a Gram positive bacterium, *Staphylococcus aureus* (*S. aureus*) and a Gram negative bacterium, *Escherichia coli* (*E.coli.)* [26-28]. The hydrogels were sterilized using butan gas flame. About 1 µL of colony forming units of the bacterial cell was spread on agar-agar plates and small disks of gelatin based hydrogels (2x2 cm) were placed on them aseptically. The disks were incubated at 28°C for 24h after incubation at 37°C for 1h. Inhibition zone was the area around the disc where bacteria would not survive as they were susceptible to the antibacterial agent that diffused from the sample to the surrounding medium.

Results and discussions

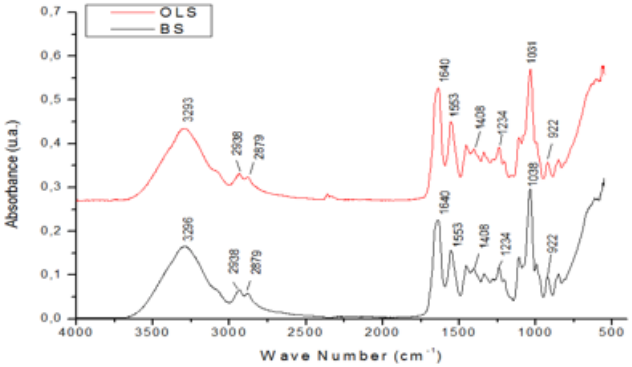


Fig. 1. FT-IR spectra of Salvia officinalis essential oil loaded and blank gelatin based hydrogels

FT-IR spectroscopy was used as a tool to investigate the structure and the bonding of the hydrogel samples. Figure 1 shows the FT-IR spectra of blank and sage EO loaded gelatin hydrogels.

The characteristic absorption bands of the hydrogel matrix constituents were identified as presented: absorption bands of gelatin at 1640 cm^{-1}, 1553 cm^{-1} and 1234 cm^{-1} were attributed to the amide I (C=O and C-N stretching vibration) [29-32], amide II [29, 31-34] and amide III (mainly N-H bending vibration and C-N stretching vibration) [33,35], respectively. The wide absorption band around 3296 cm^{-1} was assigned to the stretching vibration of O-H bonded to N-H [29,31,32,34]. The absorption bands at 1038 cm^{-1} respectively at 1031 cm^{-1} in the spectra of blank and sage oil loaded sample for C-O streching vibration, indicate the presence of alcoholic groups of glycerol [31]. As it can observed from figure 1, the characteristic absorption band of amide I appears at the same wave number in the spectra of the blank film and EO loaded film, suggesting that there was no change in the secondary structure of the gelatin molecules after converting them into hydrogels. The spectra of the EO loaded hydrogel show no new bands formed which means that there are no chemical interactions happened between the hydrogel matrix and the tested oil. This is a very important aspect because; antibacterial activity can be inhibited if essential oils interact chemically by phenolic groups [36]. There are notable differences in the region of 1031-1640 cm^{-1} regarding the intensities of the characteristic absorption bands. For comparing the intensities, the absorption peak at 1640 cm^{-1} was considered as internal standard, due to the stability and inactive nature of C=O groups of gelatin molecule in the process of hydrogel preparation. The ratio between the intensity of the band at 1038 cm^{-1} reported to the internal standard decreased from 1.20 in the spectra of blank sample, to 1.06 in the spectra of essential oil loaded sample. After sage oil incorporation the intensity of the peak at 1038 cm^{-1} decreased compared to the intensity of the same absorption band in the blank sample spectra. This behavior suggest that after oil incorporation the vibration of C-OH group of glycerol is blocked or can no longer vibrate with the same intensity, because of some steric impediments, whether because of certain physical interactions with the essential oil functional groups. These interactions are basically hydrogen bonds, which stabilize the hydrogel by generating a secondary network.

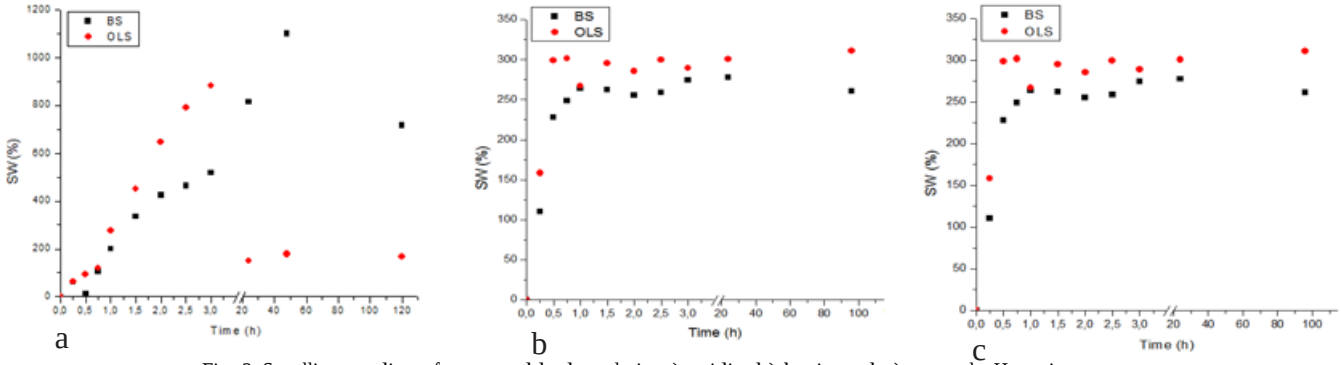


Fig. 2. Swelling studies of prepared hydrogels in a) acidic, b) basic and c) neutral *p*H environment.

Swelling studies

The swelling properties of the prepared hydrogels were evaluated from their water uptake value in different pH media. The results are shown in figure 2.

Even if essential oils, due to their hydrophobicity, tend to minimize their interactions with any hydrophilic phase, according to the data presented in figure 2, the tested sage EO increased the swelling degree of gelatin hydrogels in all pH media, compared to blank sample. This behavior of EO loaded sample can be attributed to a possible physical interaction by intermolecular hydrogen bond between phenolic groups of EO and -NH_2 respectively -OH groups of hydrogel matrix. The interactions between hydrogel forming groups lead to the shifting of the absorption band from 3296 cm^{-1}, corresponding to blank sample to 3293 cm^{-1} for EO loaded hydrogel sample. These intermolecular hydrogen bonds stabilize the macromolecule by forming a secondary network but does not decrease swelling behavior.

Increased SW of hydrogel samples under acidic conditions can be explained by the protonation of a primary amino group on gelatin, which induced repulsion between polymeric chains [36]. In acidic conditions the degradation process of gelatin based hydrogels is faster and more sharply suggesting that the decrease of weight is due to the interchains bonds breakage. In basic medium, after reaching the maximum swelling capacity, the hydrogels mass decreases slightly due to decomposition by breaking of low molecular weight fractions which were not linked by cross-linking. In neutral medium the presence of two inflection points in the swelling profile can be due to the breakage of the internal polymeric architecture represented by hydrogen bonds between gelatin and EO, with the increase of the water molecules uptake [33]. After secondary network destruction, SW increased because the crosslinking density is continuously decreased.

Swelling kinetics

Swelling is a continuous process of reorientation of polymer molecules during the process of transition from unsolvated glassy state to a relaxed rubbery region [37]. Transport of solvent molecules through a gel under constrained swelling shrinkage may be described by Fickian transport [37-39], according to the equation (2):

$$SW = k \times t^n \quad (2)$$

where: SW is the swelling degree (%) at time t, k and n (≤ 0.5) are diffusion coefficient and diffusional exponent, respectively.

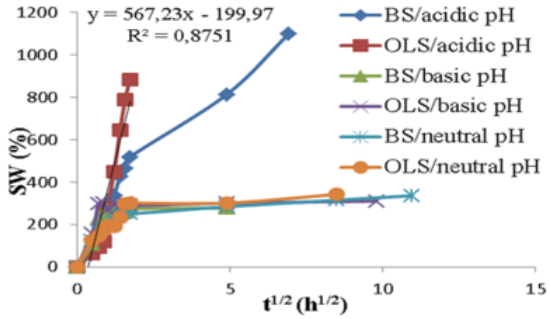


Fig. 3. SW - $t^{1/2}$ relation curve of hydrogels at different pH environments

The fitting curves $SW_t = f(t^{1/2})$ for the prepared hydrogels are presented in figure 3 at different *p*H environment. For all the samples, except OLS in acidic condition, the entire swelling process did not exhibit a Fickian behavior, because there are no linear relationships between SW_t - $t^{1/2}$.

In the case of OLS in acidic *p*H, first order swelling kinetic can be assigned to the fast reorientation or relaxation of polymer molecules due to the electrostatic repulsion forces, which distances polymer chains and promotes the diffusion of water [36].

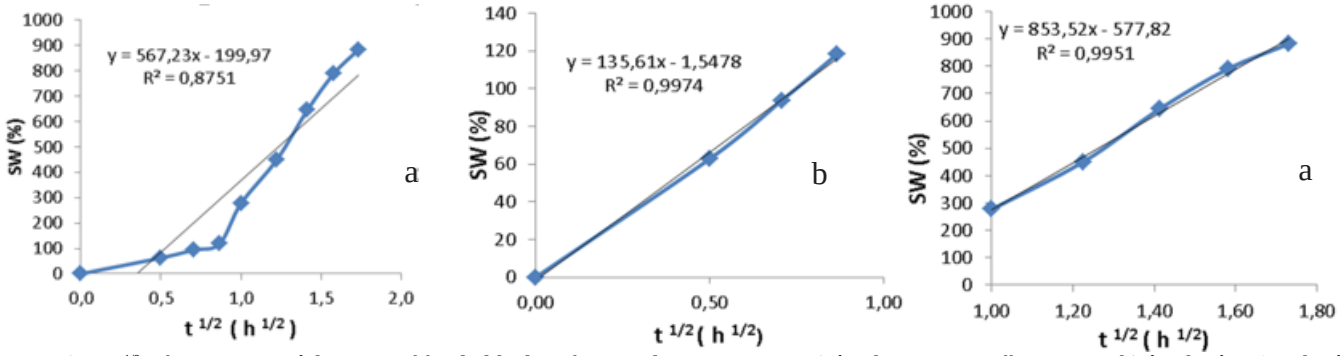


Fig. 4. SW - $t^{1/2}$ relation curve of the sage oil loaded hydrogels in acidic environment a) for the entire swelling proces b) for the first 0.75 h of swelling c) from 0.75 h of swelling until equilibrium

According to figure 4, the entire swelling process of sage oil loaded sample exhibited a first order kinetic with case particularity to have two different diffusion coefficients for the extensive swelling system. For the first 0.75 h of swelling the diffusion coefficient, k = 135.61, from this point until reaching equilibrium the diffusion coefficient increased significantly to k = 853.52. These behavior suggest that, in acidic condition the OLS swelling occurs at a higher velocity after 0.75h of immersion. Probably, in these experimental conditions, this is the time needed for relaxation of the material, so as to favor the diffusion of water throught the polymer chains of the hydrogel. Considering the two specific swelling domains, characterized by different diffusion coefficients, there are linear relationship between SW_t -$t^{1/2}$ [37,38].

Another approach to describe the swelling process of hydrogels is presented by Schott-second order kinetics [40,41]. The dynamic equation is expressed according to the equation (3):

$$\frac{dSW_t}{dt} = k \times (SW_{eq} - SW_t)^2 \quad (3)\ (5)$$

where: dSW_t/dt is the swelling velocity, k is the rate constant (1/h), SW_{eq} is the maximum swelling degree. (6)
Equation (3) is reorganized as follows:

(7)

$$\frac{dSW_t}{k \times (SW_{eq} - SW_t)^2} = dt \quad (4)$$

The integral form in scope [0,t] and [0, SW_t] is represented by the equation (5):

$$\frac{1}{(SW_{eq} - SW_t)} - \frac{1}{SW_{eq}} = k \times t$$

$$SW_{eq} = k \times SW_{eq}^2 \times t - k \times SW_{eq} \times SW_t$$

$$\frac{t}{SW_t} = A + B \times t$$

where: $A = \frac{1}{k \times SW_{eq}^2}$ and $B = \frac{1}{SW_{eq}}$.

The fitting curves of $t/SW = f(t)$ at different *p*H environments are shown in figure 5.

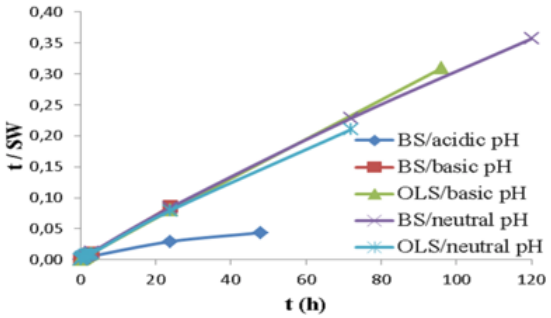


Fig. 5. t/SW - t relation curve of hydrogels at different *p*H environments

The slope B and intercept A can be defined from the regression equationand the theoretical maximum swelling degree (SW_{eq}) and the rate constant k (1/h) were calculated and presented in table 1. The theoretical analysis data of SW_{eq} were well consistent with the experimental data, therefore, Schott second order dynamic equation can be employed to discuss the swelling process in different *p*H conditions of OLS. Table 1 also illustrated that, for a constant composition, the swelling process can be influenced by the *p*H of the solvent solution. SW of sage EO loaded gels increased along with the decreasing of the pH of the environmental solution, while the rate constant decreased, suggesting that it takes more time for the tested hydrogels to reach maximum SW at higher *p*H value, due to the slower reorientation or relaxation of polymer molecules.

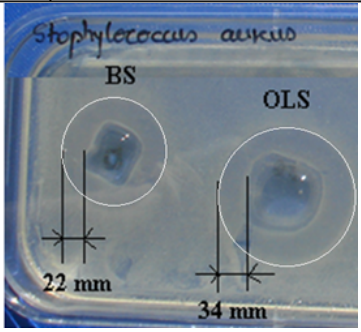
Bacterial resistace test

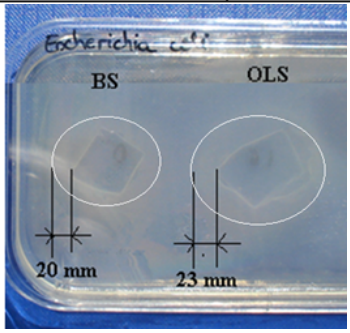
After 24h of incubation at room temperature the EO loaded hydrogels showed an inhibition towards *E. coli* and *S. aureus* of the tested microorganisms. Based on the diameter of inhibition zone (fig. 6.), the bacterial killing ability of sage EO is comparable to that of silver nanoparticles (12 mm for *S. aureus* and 14 mm for *E. coli*) [8,26,27], a widely used antibacterial agent in cosmetics and pharmaceutics. Compared to the activity of other essential oils used in order to obtain antibacterial materials, sage loaded hydrogel is twice more efficient towards *E. coli* compared to cinnamon essential oil (12 mm diameter of inhibition zone of cinnamon loaded sodium alginate hydrogel) [14].

Sample	Linearization equation/ Correlation coefficient	SW_{eq} (%) – experimental data	SW_{eq} (%) – calculated data	k (1/h)
BS/acidic pH	y = 0.0009x + 0.0039/ R^2 = 0.9706	1098	1111.11	0.0002
BS/basic pH	y = 0.0036x + 0.0005/ R^2 = 0.9998	277	277.77	0.0259
OLS/basic pH	y = 0.0032x + 0.0025/ R^2 = 0.9999	310	312.50	0.0205
BS/neutral pH	y = 0.003x + 0.0029/ R^2 = 0.9984	336	333.33	0.0031
OLS/neutral pH	y = 0.0029x + 0.0023/ R^2 = 0.9983	342	344.82	0.0036

Table 1
FITTING KINETIC PARAMETERS OF DIFFERENT *p*H ENVIRONMENT

a





b

Fig. 6. Sensitivity of a) Staphylococcus aureus and b) Eschericia coli bacteria on Salvia officinalis essential oil

Enhanced antibacterial activity of sage EO is related to their chemical composition. According to A. Porte et al. [42] and G. Bernotiené et al. [43] the major constituents of the *Salvia officinalis* oil are: α-thujone activity of sage EO is related to their chemical composition. According to A. Porte et al. [42] and G. Bernotiene et al.[43] the major constituents of the *Salvia officinalis* oil are: α-thujone (40.90%), camphor (26.12%), α-pinene (5.85%) and β-thujone (5.62%) [44].

Conclusions

Water-absorbent EO loaded hydrogels were prepared by microwave-assisted polymerization method. The structure of the biodegradable films was confirmed by FT-IR spectra. The results of FT-IR analysis suggested that there are no chemical interactions between the gel matrix and the incorporated essential oil. According to the data obtained from swelling studies there are possible physical interactions by intermolecular hydrogen bond between phenolic groups of sage EO and $–NH_2$ respectively –OH groups of hydrogel matrix, which stabilize the macromolecule by forming a secondary network. Antibacterial activity of sage EO regarding *S. aureus* and *E. coli* bacteria was demonstrated by agar diffusion method. Based on the diameter of inhibition zone, the tested EO antibacterial activity is comparable to that of most commonly used silver nanoparticles, and twice stronger than that of other EO used for this purpose. The results of this work can help to design new antibacterial wound dressing materials with enhanced stability and swelling properties, replacing toxic and expensive antibacterial agents.

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