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Simultaneous formulation of terbinafine and salvia monoterpenes into chitosan hydrogel with testing biological activity of corresponding dialysates against *C. albicans* yeast

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Abstract: This work was aimed at a progressive formulation of drugs into chitosan hydrogels. It was taken into consideration that a therapeutic effect of the drugs could be enhanced by a combination of natural compounds with chemical (synthetic) drugs. In this work, sage essential oil (SEO) bicyclic monoterpenes with antiflogistic, antiseptic, and antimycotic properties were combined with terbinafine (TB) having a strong antimycotic activity. Detail optimization of the hydrogel-drugs composition (SEO monoterpenes, TB, chitosan, and polysorbate 80 concentrations), based on permeation experiment and UV absorption/GC-MS analysis of permeated species (eucalyptol, camphor, borneol, thujone, TB) in dialysates, was made. Concerning the active drugs formulation, an optimum concentration of TB was set at the level providing maximum release of the SEO monoterpenes. In vitro activity of the dialysates from the optimized hydrogel was tested against *Candida albicans* showing that a minimum

inhibition concentration was significantly exceeded. The experimental results revealed that the chitosan hydrogel was suitable for the simultaneous formulation of the natural drugs (SEO) with chemical drug (TB) resulting in the preparation with acceptable stability, required gel properties, and significant biological activity. Such preparation should be effective in an antimycotic dermal use.

Keywords: antimycotic activity; chitosan hydrogels; permeation experiment; sage essential oil monoterpenes (*Salvia monoterpenes*); terbinafine.

1 Introduction

Hydrogels belong to semi-solid preparations for cutaneous application – Preparationes molles ad usum dermicum [1]. They are composed of macromolecular substance and dispersing hydrophilic liquid. It is known that natural polymers usually exhibit better biodegradation and biocompatibility as well as lower or zero toxicity than synthetic ones do [2, 3]. Chitosan ((1→4)-2-amino-2-deoxy-β-D-glucan) is a cationic semi-synthetic polymer obtained by partial deacetylation of chitin, suitable for the formulation of stable hydrogels [4, 5]. It is a promising biomaterial with many advantages, such as biodegradability, biocompatibility, mucoadhesivity, non-toxicity, and antimicrobial activity. Chitosan hydrogels are able to remove necrotic tissues and absorb excessive amount of exudate from wounds. A cooling effect of hydrogels, including chitosan ones, is also important; therefore, they can be used in inflammatory processes, too [2, 6, 7].

Chitosan hydrogels can be advantageously combined with various biologically active compounds. In our previous work [8], sage essential oil (SEO) (*Salvia officinalis*, L.) bicyclic monoterpenes, thujone (a mix of α- and β-thujone), camphor, borneol, and 1,8-cineol (eucalyptol), were formulated into chitosan hydrogels. The aim of the present work is to combine positive effects of the natural

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compounds of the SEO of *S. officinalis*, namely, antibacterial, local analgetic and antimutagenic (camphor), antiflogistic (borneol), and especially antifungal activity of SEO against *Candida* species [9] with the therapeutic effect of chemical drug, terbinafine (TB). TB is an allylamine antimycotic agent that blocks enzyme squalene epoxidase due to which it acts as a fungicide. It has a wide spectrum of effects on dermatofyts, some nondermatofyts (NDM), and thermal dimorph fungi. It exhibits fungi static activity against *C. albicans* and *C. tropicalis* [10, 11]. Clinical studies have shown topical and oral TB to be active in cutaneous Candidiasis and *Candida* nail infections [11–13]. Therefore, for enhancing the overall effect, it seems to be rational to combine the abovementioned natural and chemical drugs. Here, chitosan hydrogels can serve as a suitable medium for such simultaneous formulation of both groups of the biologically active compounds.

It was demonstrated in our previous work [8] that monoterpene release could be effectively controlled by both chitosan and caffeine concentration in the hydrogels. Therefore, the subject of this work was (i) to optimize the content of the SEO and TB as the main simultaneously acting active substances in the hydrogel and (ii) to evaluate the biological activity of dialysates obtained from the hydrogels after permeation experiment. To accomplish a full optimization process for a hydrogel composition, (iii) an evaluation of the influence of chitosan as well as an auxiliary substance such as polysorbate 80 (serving as an emulsifying agent) in the hydrogels on the release of both main active components (SEO, TB) was also studied.

2 Experimental

2.1 Chemicals

SEO (*Salviae aetheroleum*, *S. officinalis* L.) (SEO), serving as a natural drug, was obtained from Calendula (Nová Lubovňa, Slovak Republic). Caffeine (*Coffeinum*, SL1) (CAF) – 1,3,7-trimethyl-1,2,3,6-tetrahydro-7H-purine-2,6-dione – serving as a permeation enhancer, was obtained from Sanitas (Prague, Czech Republic). TB hydrochloride (TB) – (*E*)-*N*-(6,6-dimethyl-2-hepten-4-yl)-*N*-methyl-1-naphtylmethylamine hydrochloride – serving as an antimycotic drug, was obtained from Saneca Pharmaceuticals (Hlohovec, Slovak Republic). Chitosan (*Chitosanum*) (CHIT) – (1→4)-2-amino-2-deoxy-β-D-glucan – medium molecular weight, serving as a gelling agent, was obtained from Sigma-Aldrich (Bratislava, Slovak Republic). Degree of deacetylation of chitosan was 75%–85%. Polysorbate 80

(*Polysorbatum* 80, SL1) (P80), a nonionic surfactant, emulsifier, and solubilizing and stabilizing agent in medicinal and pharmaceutical preparations, derived from polyethoxylated sorbitan and oleic acid, was obtained from Centralchem (Bratislava, Slovak Republic). Lactic acid (*Acidum lacticum*, SL1) (LA) – 2-hydroxypropanoic acid – was obtained from Interpharm (Bratislava, Slovak Republic).

2.2 Instrumentation

The permeation experiments were carried out with the following equipments and auxiliaries: analytical scale Mettler Toledo (Greinfensee, Swiss), permeation apparatus assembled in our laboratory (Department of Galenic Pharmacy, Faculty of Pharmacy Comenius University, Bratislava, Slovak Republic). Cellophane (EKOZ, Nitra, Slovak Republic) served as a permeation membrane.

Rheological evaluations were carried out using a rotational viscometer Viscotester VT500 (Haake Mess-Technik, Karlsruhe, Germany). Thermostat U10 (Prüfgeräte-Werk Medingez, Dresden, Germany), providing constant temperature during the whole experiment, was employed for the permeation and rheological experiments.

Trace GC gas chromatography analyzer connected with Trace DSQ mass spectrometry detector, Thermo Finnigan (Waltham, MA, USA), was used for the analysis of samples from the permeation experiment.

2.3 GC-MS conditions

The amounts of particular components of the SEO were evaluated by the GC-MS analysis method employing working conditions optimized in Ref. [8]. In this work, however, the samples (dialysates) obtained from the liberation experiment were extracted, diluted, and injected into the GC-MS analyzer using different solvent, i.e. hexane (instead of acetone). This modified procedure was advantageous by its simplicity and by providing significantly enhanced recovery for the dialysate samples.

2.4 Preparation of dosage forms formulating SEO with TB and rheological properties

The hydrogels having different compositions were prepared in two parallels. The accurate amounts of the gelling agent (chitosan) and permeation enhancer (caffeine) were given into the balanced flask. Then an appropriate amount of a 1.0% (w/w) solution of lactic acid was

added into the flask (up to 50.0 g, including other substances). The system was slowly stirred until all particles were swelled and dissolved. In this way, the homogenous hydrogel was prepared.

The antimycotic drug, TB hydrochloride, was sieved by a 125- μ m sieve to form a very fine powder. Then it was suspended into the gel during continuous stirring. A specified amount of P80, as emulsifying agent for the oil/water (o/w) system, was added into the gel to keep its stability after consecutively adding the hydrophobic components (SEO) into the hydrophilic matrix (chitosan hydrogel).

The samples were prepared at the laboratory temperature $t = 25 \pm 0.2^\circ\text{C}$. The hydrogels were stored for the sake of homogenization of the inner structure of the hydrogels for at least 48 h at 5°C . Rheological properties were evaluated for each hydrogel sample. The torque was used as a directly measured parameter to calculate the following basic rheological parameters: shear stress (τ), structural viscosity, and shear rate (D). The obtained values were used for the construction of rheograms $D - \tau$. Composition of the hydrogels was systematically optimized changing amount of particular compounds in the system, i.e. SEO (monoterpenes), TB, chitosan, P80.

2.5 Liberation experiment and dialysate pretreatment

The liberation of SEO and TB from the chitosan hydrogels was carried out 48 h after their preparation using the permeation apparatus according to the procedure described in Ref. [8]. The extraction procedure, previously proposed for the isolation of the drugs from both hydrogel and saline solution, was modified in this work. The new extraction procedure was developed for saline dialysates only; however, the recoveries of the SEO drugs were considerably higher ($>70\%$) as previously. The saline dialysates were pretreated before GC-MS analysis by liquid-liquid extraction. The samples (2 ml of saline solution) were mixed with hexane (sample: hexane, 1:1). The lipophilic phase was separated from the aqueous phase after the shaking (30 min). The resulting extract (hexane sample solution) was directly injected into the GC-MS analyzer. A relative amount of the liberated component of the essential oil was obtained from GC-MS records as the ratio of peak areas measured before and after liberation.

2.6 Spectrophotometric evaluation of TB

An amount of the released drug was determined by the spectrophotometer diode array (Hewlett Packard, USA) at

the wavelength $\lambda_{\text{max}} = 224 \text{ nm}$. A solution of sodium chloride (0.9%) served as a blank, and the specific absorption coefficient at the given wavelength was $A_{1\text{cm}}^{1\%} = 204.78$. The drug concentration was evaluated according to the equation:

$$c = \frac{\bar{A}}{A_{1\text{cm}}^{1\%}} l$$

where c is the concentration of TB [10 g/1000 ml], $\bar{A}$ is the average absorption of six consecutive measurements, $A_{1\text{cm}}^{1\%}$ is the specific absorption coefficient, and l is the length of cuvette [1 cm].

3 Results and discussion

3.1 Optimization of hydrogel-drugs composition

Various concentrations of particular compounds (monoterpenes, TB, chitosan, P80) involved in the hydrogel were examined in the present permeation study using sample nos. 1–17, see Table 1. The optimum concentration pattern compromised the highest liberation of SEO with acceptable stability of hydrogel.

The liberation of TB followed the same procedure as liberation of the SEO components. The optimum TB

Table 1: Composition of hydrogels with variable amounts of particular compounds (SEO, TB, chitosan, polysorbate 80).

Composition of hydrogels (% w/w)					
No.	CHIT	SEO	TB	CAF	P80
1	2.3	3.5	1.5	2.0	0.001
2	2.3	5.0	1.5	2.0	0.001
3	2.3	6.0	1.5	2.0	0.01
4	2.3	7.5	1.5	2.0	0.01
5	2.3	10.0	1.5	2.0	0.01
6	2.3	6.0	0.5	2.0	0.01
7	2.3	6.0	1.0	2.0	0.01
8	2.3	6.0	2.0	2.0	0.01
9	2.3	6.0	2.5	2.0	0.01
10	2.0	6.0	1.5	2.0	0.01
11	2.5	6.0	1.5	2.0	0.01
12	3.0	6.0	1.5	2.0	0.01
13	3.5	6.0	1.5	2.0	0.01
14	4.0	6.0	1.5	2.0	0.01
15	2.3	6.0	1.5	2.0	0.10
16	2.3	6.0	1.5	2.0	0.15
17	2.3	6.0	1.5	2.0	0.20

concentration compromised the highest liberation of both TB itself and the SEO components.

Following subsections demonstrate the influence of the hydrogel components (including polymer, auxiliary compounds, and drugs) on the liberation of the formulated drugs (natural as well as chemical). Concentration of caffeine in the hydrogels was optimized in our previous experiment [8]; therefore, it was kept constant (optimum value 2.0%) in this work. Caffeine serves as an enhancer for liberation of SEO monoterpenes from the hydrogels.

3.2 Monoterpenes

Firstly, the concentration of SEO in the hydrogels was optimized using samples 1–5. The monitoring of released significant monoterpenes such as thujone (I), eucalyptol (II), borneol (III), and camphor (IV) was relevant for the choice of a suitable concentration of SEO in the hydrogel. The dependences of relative amounts of the liberated SEO components on the concentration of SEO in the hydrogels in Figure 1A indicated the optimum SEO amount to be 6% (sample 3). The second reason for choosing a 6.0% concentration of SEO was the stability of the gel samples. The

gel sample instability was observed as follows: initially dispersed SEO was macroscopically separated from the outer phase. Samples 1–3 (SEO 3.5–6.0%) were proved as stable, while gel stability dramatically decreased with further increase in the SEO concentration (sample 4 was decomposed a week after the preparation, sample 5 was decomposed even the same day as prepared). The SEO components such as bornylacetate, caryophyllene, α -pinene, camphene, etc. were released in negligible quantities (<1%), and therefore, they were not considered further in this study.

3.3 Terbinafine

The concentration of TB in the hydrogels was optimized using samples 3, 6–9. The dependences of relative amounts of the liberated SEO components on the concentration of TB in the hydrogels in Figure 1B indicated the optimum TB amount to be 1.5% (sample 3 providing maximum liberated SEO concentration). It is supposed that the shape of the dependences, not simply decreasing or increasing, could be affected (beside other influences) by a micellar equilibria of TB itself.

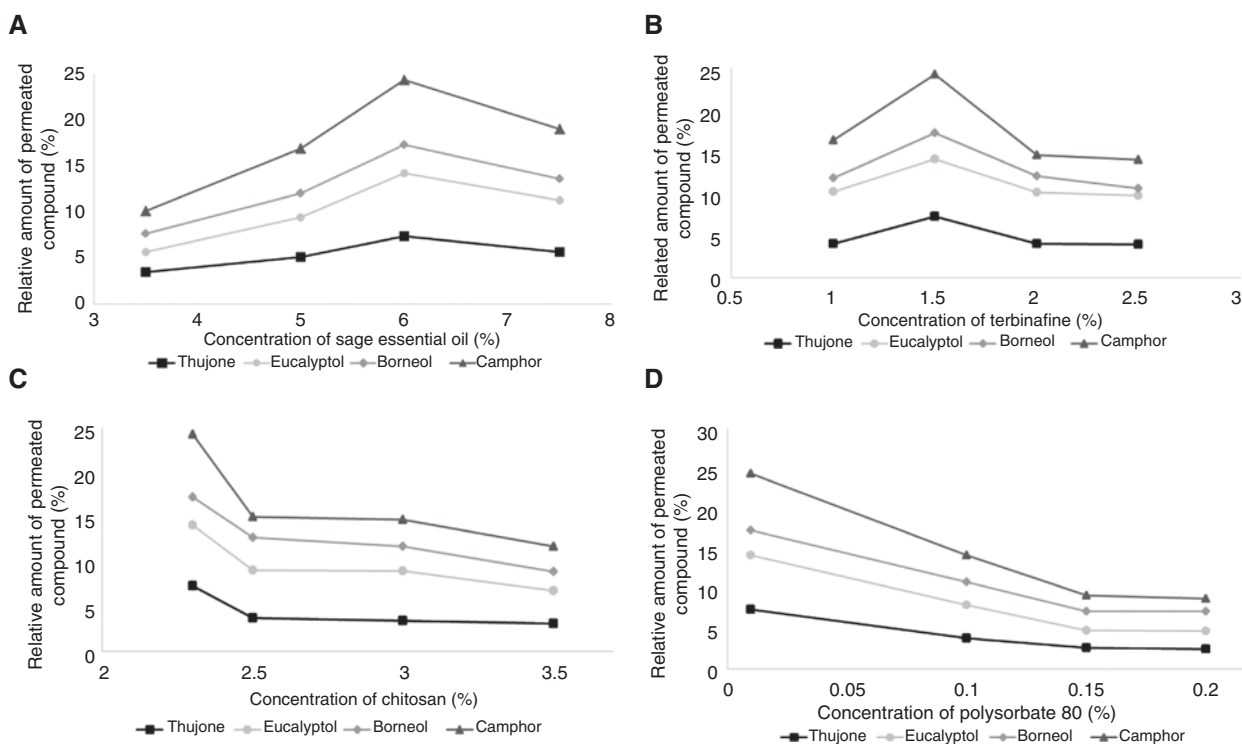


Figure 1: Effect of the concentration of particular compounds in hydrogels on the relative amounts of liberated SEO components: thujone (I), eucalyptol (II), borneol (III), camphor (IV). (A) Sample nos. 1–4 with varied SEO concentration. (B) Sample nos. 3, 6–9 with varied TB concentration. (C) Sample nos. 3, 11–13 with varied CHIT concentration. (D) Sample nos. 3, 15–17 with varied P80 concentration. List of the sample composition is in Table 1. Dialysate samples were analyzed under the conditions given in Experimental section.

The amount of TB liberated from the hydrogel increased with increasing TB concentration in the hydrogel, as it was indicated by the spectrophotometric evaluation of TB in dialysates, see Figure 2. A 1.5% concentration of TB in the hydrogel, chosen as an optimum value with respect to the highest liberated amount of SEO, represented also the maximum ratio of the released and added TB amount (at higher added TB amounts, this ratio decreased). With this composition of the drugs, a minimum inhibition concentration (MIC) of the dialysate obtained from the corresponding hydrogel should be ensured (for Biological activity testing, see Section 3.7).

3.4 Chitosan

The concentration of chitosan in the hydrogels was optimized using samples 3, 10–14. An appropriate concentration of chitosan was selected with respect to both the permeation of monoterpenes and rheological parameters.

The stability of hydrogels was determined by the measurement of rheological parameters. The resulting rheograms $\tau - D$ are shown in Figure 3. The dependences in Figure 3 demonstrate that samples 3, 11, and 12 behave as non-Newtonian systems. Such samples show the thixotropic character (thixotropy – chemical isothermal gel-sol-gel transformation [3]), which is the most preferred in hydrogels. On the other hand, samples 10 (2.0% CHIT), 13 (3.5% CHIT), and 14 (4.0% CHIT) were not stable, which was manifested by too low or too high viscosity of the hydrogel. Therefore, these unstable hydrogel samples were not used for the permeation experiment.

When the concentration of chitosan and viscosity of hydrogels increased, the amount of permeated

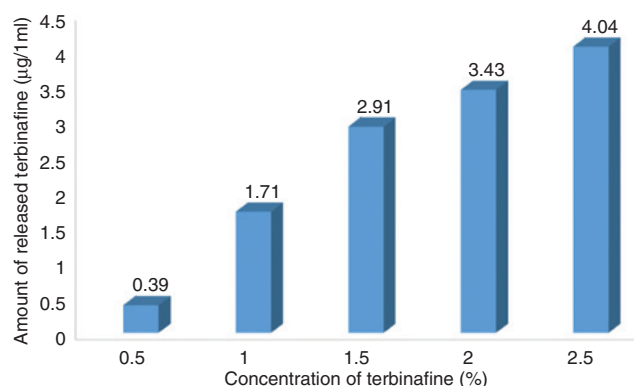


Figure 2: Effect of the concentration of TB in hydrogels on its liberated amount. Sample nos. 3, 7–10 were used in this experiment. List of the sample composition is in Table 1. Dialysate samples were analyzed under the conditions given in Experimental section.

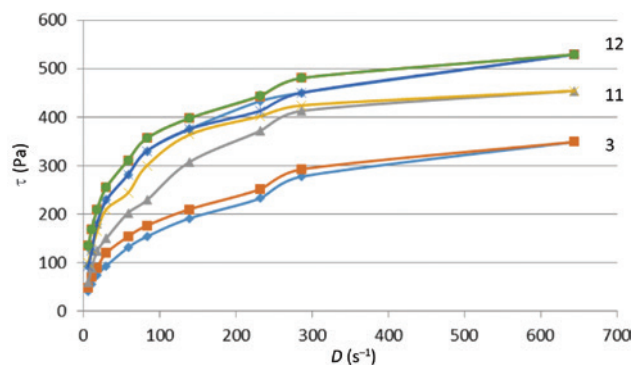


Figure 3: Rheograms $\tau - D$ of hydrogel samples. Sample nos. 3, 11, and 12 (Table 1) were used in this experiment. Rheological parameters of the hydrogel samples were measured under the conditions stated in Experimental section.

monoterpenes decreased. The dependences of relative amounts of the liberated SEO components on the concentration of chitosan in the hydrogels in Figure 1C indicated the optimum chitosan amount to be 2.3% (sample 3 providing maximum liberated SEO concentration). Hence, sample 3 with a 2.3% concentration of chitosan, providing acceptable stability as well as drugs permeation, was chosen for the next experiments.

3.5 Polysorbate 80

The last point within the process of optimization of the basic composition of hydrogel was the selection of a suitable P80 concentration. P80 was used as an emulsifying agent for the o/w system. The lowest concentration of the emulsifier, i.e. 0.001% (w/w), was used in the samples with lower concentrations of SEO (samples 1 and 2 corresponding to 3.5% and 5.0% SEO concentrations, respectively). For higher SEO concentrations, however, it was not sufficient because non-emulsified SEO caused instability of the hydrogel. Hence, the concentration of P80 in the hydrogels was further optimized using samples 3, 15–17. The dependences of relative amounts of the liberated SEO components on the concentration of P80 in the hydrogels in Figure 1D indicated the optimum P80 amount to be 0.1% (sample 3 providing maximum liberated SEO concentration).

3.6 Optimized hydrogel composition and analysis of released drugs

Systematic optimization procedure resulted in the proposal of a hydrogel enabling appropriate simultaneous

liberation of both salvia bicyclic monoterpenes and TB as well. The optimized hydrogel composition was the following: 6.0% SEO, 1.5% TB, 2.0% CAF, 2.3% CHIT, 0.01% P80. The released amount of TB was determined via ultraviolet spectral detection at a 224-nm specific wavelength, while the released SEO bicyclic monoterpenes were monitored using the GC-MS analysis. A 2.91- $\mu\text{g}/\text{ml}$ concentration of TB was determined in dialysates obtained from the hydrogel with optimized composition. Relative amounts of the permeated natural drugs (SEO monoterpenes), calculated as the ratios (w/w) of the drugs present in donor compartment of the permeation apparatus (hydrogel with optimized composition) and in acceptor compartment of the permeation apparatus (saline solution), were 24.35% (camphor), 17.28% (borneol), 14.19% (eucalyptol), and 7.35% (thujone). A GC-MS profile of the SEO bicyclic monoterpenes released from the hydrogel with the optimized composition is shown in Figure 4.

The analytical data were obtained with acceptable reliability. Student's *t*-test confirmed significant differences in the testing groups of the results in the permeation dependences (optimized versus non-optimized concentrations of the tested hydrogel constituents) and approved the optimum hydrogel composition. Relative standard deviations of the measured concentrations for all the studied drugs were < 2.5% when evaluating the intra-day precision, and they did not exceed 4.0% when evaluating the inter-day precision of the analytical method.

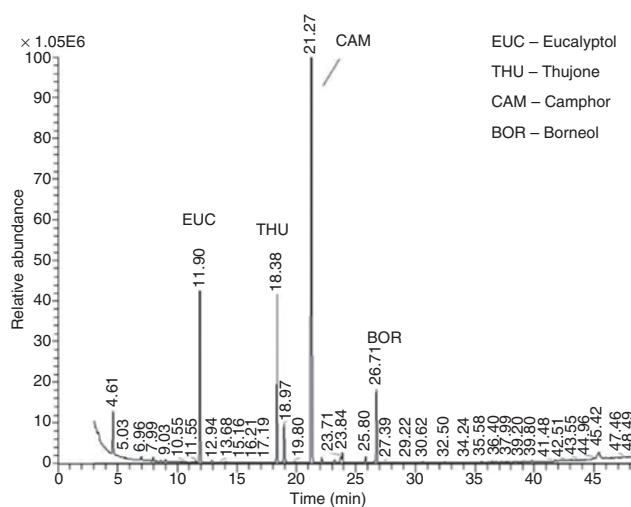


Figure 4: GC-MS profile of the SEO bicyclic monoterpenes released from the hydrogel with the optimized composition. Dialysate samples were analyzed under the conditions given in Experimental section.

3.7 Biological activity of dialysates from optimized hydrogel preparation

Before a further consideration of the optimized hydrogel preparation for a potential preclinical and clinical use, its biological activity has to be examined. The biological activity of the optimized hydrogel preparation was tested through the corresponding dialysate solution containing the permeated drugs (SEO monoterpenes, TB). *C. albicans* yeast was used as a biological medium for the in vitro experiments.

MIC is a required minimum concentration of a tested substance that stops the growth of a tested microorganism after 18–24 h of incubation. MIC of TB was evaluated for the different sorts of microscopic funguses and yeasts [11, 12]. TB was found to have an MIC of 1 $\mu\text{g}/\text{ml}$ for reference *C. albicans* strains [13]. In our experiments aimed at the determination of MIC of the studied drugs (TB, SEO), the concentration of *C. albicans* yeast was 7.5×10^5 CFU/ml. Firstly, model samples of the drugs were prepared in a 50% water solution of methanol. It was found out that MICs of TB and SEO in the model samples were 0.336 and 0.125 $\mu\text{g}/\text{ml}$, respectively. Secondly, in vitro activity of the real samples, i.e. dialyzates from the optimized hydrogel, was tested against *C. albicans* showing the positive biological effect. This observation was in a good agreement with the fact that the liberated amounts of the studied drugs (TB, SEO) from the optimized hydrogel samples were over the above-stated MIC values.

4 Conclusion

It can be summarized that critical points in the systematic optimization of the chitosan hydrogel formulating SEO monoterpenes along with TB were the following:

- (i) A 6.0% optimum concentration of SEO was chosen on the basis of two factors, which are highest amounts of permeated monoterpenes and acceptable stability of hydrogel.
- (ii) A 1.5% optimum concentration of TB was set at the level providing maximum release of the SEO monoterpenes; the concentration of caffeine (2.0%), serving as a permeation enhancer in the hydrogel, was optimized in our previous experiment [8].
- (iii) A 2.3% optimum concentration of CHIT was chosen on the basis of two factors, which are acceptable stability of the hydrogel (exhibiting a non-Newtonian system behavior with the thixotropic character, which is the

most preferred in hydrogels) and highest amounts of permeated monoterpenes.

- (iv) A 0.1% optimum concentration of P80 compromised a maximum release of the SEO monoterpenes with a sufficient emulsifying of the non-polar SEO compounds (because non-emulsified SEO caused instability of the hydrogel).

Chitosan hydrogel with the optimized composition of the natural (SEO) and chemical (TB) drugs, permeation enhancer (CAF), gelling polymer (CHIT), and emulsifier (P80) was found to be suitable for the liberation of the drugs in the concentration levels significantly exceeding their MICs against *C. albicans* yeast. This significant biological activity indicated good possibilities for a practical utilization of the proposed hydrogel preparation. After further pre-clinical and clinical testing (which will be the subject of our upcoming work), if successful, the proposed pharmaceutical preparation could be implemented into the therapeutic practice providing benefits of simultaneous therapeutical acting of antimycotic, antiflogistic, and antiseptic effects of the drugs along with favorable properties of the chitosan hydrogel in a dermal use.

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