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FORMULATION AND EVALUATION OF ANTIFUNGAL MICONAZOLE NITRATE MEDICATED SOAP

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ABSTRACT. Miconazole nitrate containing soaps were formulated using one commercially available soap base and three cold process soap bases with various lipids. The aim of our work was to investigate the antimicrobial activity and physicochemical properties of the obtained formulations. The water loss and hardness of the soaps was determined 1, 2, 4 and 9 weeks after preparation. The pH and foamability were determined after 9 weeks of curing time. Two Gram-positive, four Gram-negative bacteria and two yeast (Candida spp.) strains were used in the microbial study. The miconazole content was determined with HPLC-UV analysis. Antimicrobial assay showed that all medicated soaps were effective against Candida albicans. The API had minimal influence on the physicochemical properties (water loss, pH, foamability and consistency) of the soaps. All soaps possessed

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good lathering properties and an alkaline pH (9.2-11.3). The miconazole content of the soaps varied from 2.72 % to 5.12 % as revealed by HPLC-UV analysis.

Keywords: *miconazole, medicated soap, Candida albicans, olive oil.*

INTRODUCTION

The aim of our work was to formulate and obtain miconazole containing medicated soaps using different soap bases (containing various natural oils), and to investigate the antimicrobial activity and physicochemical properties of the obtained formulations, such as water loss, consistency, pH and foamability. To determine the active substance content, HPLC method was applied.

Superficial mycoses

Superficial fungal infections, involving cutaneous stratum corneum, hair and nails are estimated to affect billions of people worldwide, thus being among the most frequent forms of infections. Due to the lack of regular and national surveillance, poor diagnostic and no reporting obligations, incidence rates are usually underestimated and determined by extrapolating local and literature data [1]. The fungal pathogens causing skin mycoses are dermatophytes (*Epidermophyton spp., Microsporum spp., Trichophyton spp.*), yeasts (*Candida spp., Malessezia spp*.) and molds (*Aspergillus spp.).* The most common clinical manifestations of these infections are dermatophytosis, onychomycosis, superficial candidiasis and pityriasis versicolor [2].

Their prevalence depends on socio-economic conditions, geographical location and environmental and cultural habits [3]. Several factors predispose to superficial fungal infections, including physiological factors (infancy, pregnancy, aging, menses), dermatoses, trauma, endocrine diseases (diabetes mellitus, hypothyroidism), immunological dysfunctions (HIV), chemotherapy, antibiotics and immunosuppressant therapy [2,4].

In order to invade healthy human tissue, fungi have to adapt to various environmental factors. Hence, fungal pathogens can respond to host-derived stresses, grow at temperatures of 37°C, take up and metabolize nutrients from their host [5]. In addition, fungal pathogenicity also depends on virulence factors, such as dimorphism, biofilm formation, the expression of adhesins, invasins and virulence enzymes (keratinase, cellulase, protease) [6]. Fungal pathogens adapt to changing environmental pH and modulate the skin surface pH from acidic (4.7) to alkaline through various mechanisms [7]. Most fungi take up amino acids and alkalinize their environment by releasing urea

or ammonia; as an additional mechanism, C. albicans, seems to neutralize the pH of its environment through the utilization of carboxylic acids (such as pyruvate, α-ketoglutarate and lactate) [8]. The resulting neutral-alkaline pH disturbs the regulation of the keratinization enzymes required for the renewal of the stratum corneum, leading to host tissue damage and increased virulence [9].

Candida strains such as *C. parapsilosis, C. krusei, C. tropicalis, C. albicans* and *C. glabrata* have also been found on healthy skin, *C. parapsilosis* being the most prevalent species [10]. *C. albicans* is seldom an inhabitant of healthy skin, yet it was identified as the most common infectious agent of this genus, followed by *C. parapsilosis* [11,12]. When examining the skin microbiome of patients with atopic dermatitis or primary immunodeficiencies, *Malassezia spp*. remained the predominant species, however the abundance of *Candida* genus increased [13,14]. In the case of diabetic patients, a high number of *Cladosporium herbarum* and *C. albicans* was found in chronic non healing wounds, *C. parasilopsis* and *C. tropicalis* were also identified [15].

The most frequent forms of superficial candidiasis are intertrigo, interdigital candidiasis, diaper dermatitis, perianal dermatitis, paronychia and onychomycosis. Intertrigo or inflammatory dermatosis is the result of friction and irritation created by opposing skin surfaces, which often occur in infancy, obesity, diabetes mellitus, hyperhidrosis and in those who are bedridden. Due to the damaged epidermal tissue, warm and moist medium, secondary fungal infections with *Candida* are frequent [4,16]. Diapers also ensure favorable conditions for *C. albicans* due to moisture, elevated pH and the presence of irritating urine and stool enzymes. As a result, diaper candidiasis often complicates non-infectious diaper dermatitis in infants, small children and elderly people [17].

To treat superficial fungal infections, antifungal drugs can be administered topically and systematically. Topical treatment of fungal skin infections is preferred to systemic delivery due to direct drug administration onto the infection site, high local drug concentration, reduced systemic adverse effects, ease of administration and improved patient compliance [18].

Pharmacological use of miconazole nitrate

Miconazole nitrate (1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)-methoxy]-ethyl]-1H-imidazole mononitrate) is a lipophilic imidazole derivative with a broad-spectrum of fungistatic activity [19, 20]. Imidazole derivatives target the ergosterol biosynthesis in yeast through lanosterol 14-α-demethylase (CYP51).

Ergosterol, a 5,7-diene oxysterol is an essential fungal cell membrane sterol synthesized in the endoplasmic reticulum, which maintains the fluidity, permeability and thickness of plasma membranes and protects the phospholipid bilayer against mechanical and oxidative stress [21, 22]. Besides plasma membranes, ergosterol has been found in the membranes of intracellular organelles such as endoplasmic reticulum (ER), mitochondria, peroxisomes and vacuoles, where it regulates the localization and activity of enzymes within the membrane (Cdr1 efflux pump, H+-ATPase, V-ATPase) [23]. To form ergosterol, CYP51 catalyzes the three-step C14-demethylation of lanosterol [24]. This step of the ergosterol synthesis is inhibited by imidazole derivatives, which bind to the haem group of CYP51 enzyme through the free nitrogen atom in the imidazole ring [25]. Takahashi et al. used miconazole soaps for cleaning diaper-covered sites of elderly patients, which significantly reduced the pseudohyphae/blastoconidia of *Candida spp*. when compared to control [26]. Jagdale et al. demonstrated the effect of medicated soap strips prepared by dipping paper in miconazole soap solutions [27].

Miconazole also exerts antibacterial effect against gram-positive aerobic bacteria including *Enterococcus spp., Streptococcus spp., Staphylococcus aureus*, methicillin-susceptible, methicillin-resistant and fusidic acid-resistant S. aureus [28, 29]. The antibacterial activity of miconazole is attributed to its ability to bind to the haem group of flavohemoglobin, thus inhibiting its NO dioxygenase and alkyl hydroperoxide reductase activity and increasing the intracellular oxidative stress [30]. Miconazole has rapid clearance and low oral bioavailability due to its poor aqueous solubility and low gastrointestinal absorption [31], therefore topical administration is preferred. Topical pharmaceutical dosage forms such as cream, gel, ointment, powder, spray and tincture usually contain 2% miconazole, which are generally well tolerated and effective in the treatment of superficial fungal infections [32].

Soap obtaining: saponification process, lipids and alkali

Natural soaps are anionic surfactants defined as the alkali salts of fatty acids obtained from plant oils and animal fats [33]. The triglycerides, esters and fatty acids contained in these lipids are saponified using a warm aqueous base, typically potassium hydroxide (KOH) or sodium hydroxide (NaOH). Triglycerides (triesters of fatty acids with glycerin) and fatty acid esters are converted to fatty acids, which are then neutralized to produce the salts [34]. Glycerin is a by-product of the saponification process that is left in or sometimes added to soaps for skin conditioning, enhancing product quality (softness, appearance), and as a processing aid [35].

Soaps can be manufactured via cold, hot (semi-boiled) or fully boiled process considering the temperature whereas the saponification reaction takes place. In the cold process, an exact amount of lye is used in order to avoid excess unreacted lye in the finished soap. The saponification index of the lipids is used to ensure the correct ratios of water, oils and lye, as the various fat and oil sources contain different types and amounts of triglycerides, esters and fatty acids [36]. Once the lye and lipids are mixed, saponification takes 18 to 24 hours to complete, and no additional heat is required [37]. Soaps are often formulated to contain an excess of fatty acids (approximately 5%) in order to ensure the absence of unneutralized lye and to reduce the harshness of the soap [38]. Superfatted soaps with 2-5% excess fats or oils are harder, less prone to cracking, and show good leathering and moisturizing ability [39, 40]. In the hot process, the mixture is heated to 80-100 °C (near boiling temperature) in order to accelerate the reaction. In the fully boiled process, the reactants are boiled, and the mixture is washed to remove any impurities, spent lye and glycerin [41, 42].

Besides the manufacturing process, the chemical properties of soaps depend on the composition and the nature of the lipids, and the alkali used in the saponification process. Unsaturated fats are susceptible to oxidation, slowly become rancid, reduce soap hardness, but also provide moisturizing and skin nourishing properties [43,44]. To balance out the soap formula, saturated fatty acids are used to produce harder soaps [39]. Short chain fatty acids (C10-C14) produce more soluble and harder soaps with good foamability. Longer chain fatty acids (C16-C18) contribute to the cleansing property of soap and provide a longer lasting soap [34, 45]. Very long-chain fatty acids (C22 and higher) provide bar integrity [46].

Animal fats (lard, tallow) tend to be richer in long chain saturated fats (C16:0, C18:0) and monounsaturated fats (C18:1), hence these are often combined with coconut oil which contains shorter chain length (C8:0–C14:0) saturated fatty acids. The ratio of tallow/coconut oil used for soap manufacture generally ranges from 85:15 to 75:25. Lather quickness improves, while foam stability decreases with the increase of the coconut oil content [37, 47]. Beeswax contains approximately 12% free fatty acid and 40-45% monoesters of C24-C34 alcohols [48, 49]; it produces harder soaps with high foamability, which have lower foam stability at higher concentrations (35%) [50]. High beeswax content causes the soap to solidify quickly during preparation [36].

The most used oil sources in natural soaps are coconut, palm kernel, palm, olive, rice bran and sunflower seed [51]. Nut oils such as coconut and palm kernel oil are found in a variety of commercial soaps due to their low cost. Short chain saturated fatty acids from these oils produce harder soaps which lather readily but not stably; however, saturated fatty acids with 10 or fewer

carbons confer objectionable odors, irritate and dry out the skin [44]. Palm oil is often used as an alternative for tallow as it also contains C16-C18 fatty acids, which produce slow-lathering soaps with more resistant foam [52]. Kuntom et al. found that the foamability and hardness of soaps derived from palm and palm kernel oil blends is reduced as the palm oil content increases [45].

Olive oil is composed mainly of triacylglycerols of unsaturated free fatty acids, yet it still produces hard soaps with stable lather and skin conditioning properties. Cocoa butter is used for its moisturizing effect, and it also helps to produce harder soaps with good lather [53].

The aqueous solutions of natural soaps neutralized with NaOH have an alkaline pH of 10-11 due to the quantity of alkali released after the dissociation of fatty acid salts. The pH can be further increased when residual amount of alkali is retained in the soap during manufacture [49]. Most commercial soaps have a pH ranging between 9 and 11 with a few soaps having higher pH levels (up to 11.5) [54]. The physiological skin pH is normally acidic (pH 4.1-5.8); elevated values have been detected in infant and elderly skin due to reduced buffer capacity [55]. The skin surface pH also increases after a single washing procedure with alkaline soaps; however, these increases are recovered gradually in 6 hours [56]. Anionic surfactants interact with lipids and keratin filaments of the skin causing reversible expansion of the cell membranes and enhancing the permeability of the skin for active pharmaceutical ingredients [57].

Soap monographs are official in Austrian Pharmacopoeia (ÖAB 2008), British Pharmacopoeia (BP 2019), Korean Pharmacopoeia (KP 10), Japanese Pharmacopoeia (JP XVII), Swiss Pharmacopoeia (Ph. Helv. 11) and US Pharmacopoeia (USP 42-NF 37). Medicinal soap mentioned in both Korean Pharmacopoeia and Japanese Pharmacopoeia is defined as the sodium salts of fatty acids. The British Pharmacopoeia includes a monograph for soft soap, which is “made by the interaction of potassium hydroxide or sodium hydroxide with a suitable vegetable oil or oils or with fatty acids derived there from. It yields not less than 44.0% of fatty acids”. Hard soap (*Sapo durus*) in Austrian Pharmacopoeia is prepared by melting together lard and olive oil, adding a mixture of sodium hydroxide solution and ethanol. US Pharmacopoeia also mentions soaps and shampoos as a dosage form with surface-active properties that facilitate the topical administration of the active pharmaceutical ingredient (usually antimicrobial agents).

RESULTS AND DISCUSSION

Weight loss observed during soap curing can be attributed to the evaporation of water. The soaps were kept at room temperature at a relative humidity of 40-60%. Water loss was followed by comparing the weekly weight decrease to the initial weight measured one week after preparation (**Table 1**). Soaps prepared using the melt-and-pour method do not require curing time as they are ready to use after they harden [58]. Cold process soaps are generally cured for 4 to 6 weeks [59]. The maturation process for castile soap (made from olive oil) is longer, sometimes up to 6 months [60]. At 2 weeks MIX-1-M soaps had the smallest decrease in weight. At 4 weeks soap MIX-2 lost the most amount of water. After the 9-week period the commercial soap had the highest weight loss. OLI and OLI-M soaps present the same weight loss, and the lowest one from all compositions.

Table 1. Weekly weight loss during a 9-week period, and physicochemical and active substance measurements at the end of the curing time.

Soap type	***Weekly weight loss (%) compared to initial measurements***			***pH***	***Foam volume ml***	***Miconazole content %±SD***
	Week 2 AVG±SD	Week 4 AVG±SD	Week 9 AVG±SD			
COM	5.2%±0.003	6.1%±0.004	13.3%±0.007	9.2	80	
COM-M	5.1%±0.001	6.3%±0.003	13.9%±0.003	9.3	66	2.72%± 0.5
OLI	5.0%±0.006	6.3%±0.008	8.1%±0.009	10.2	50	
OLI-M	4.6%±0.002	7.1%±0.003	8.1%±0.005	10.2	57	2.85%± 1.69
MIX-1	3.0%±0.002	6.4%±0.001	10.9%±0.011	11.3	59	
MIX-1-M	3.0%±0.006	6.5%±0.014	9.2%±0.015	10.1	65	4.92%± 1.68
MIX-2	4.9%±0.001	8.7%±0.002	12.4%±0.007	11.1	56	
MIX-2-M	4.6%±0.006	8.2%±0.005	10.7%±0.008	9.2	80	5.12%± 0.65

Penetration depth value reflects the hardness of the product. Low penetration value indicates harder consistency, high penetration value indicates softer soap. Initial penetration values (**Figure 1**) indicate that soaps were softer immediately after production.

The hardness of medicated soaps was different from control soap during the first period of curing. At 9 weeks each soap had similar consistency, the commercial soaps being softer when compared to cold process soaps. The two commercial soaps and OLI control showed mild changes in their consistency during the maturation process. MIX-1-M medicated soaps had

the highest penetration values from 1 to 4 weeks, suggesting soft consistency despite it losing the most amount of moisture out of the cold process soaps. Medicated cold process soaps were initially softer than control, this tendency remained the same in the case of MIX-1-M and MIX-2-M blends at the end of the maturation process. In the case of the OLI soaps, control had higher penetration values, indicating softness compared to medicated OLI soap.

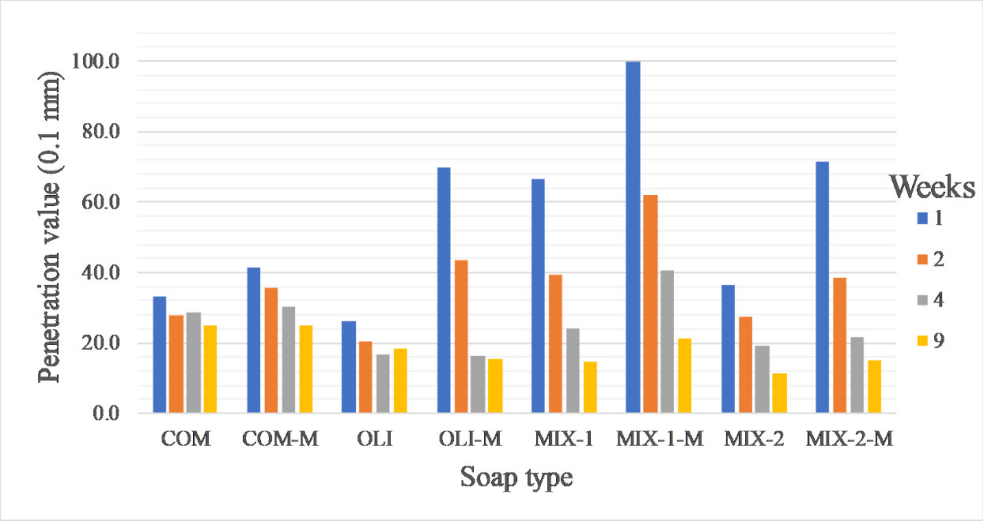

Figure 1. Initial penetration values measured at 1, 2, 4 and 9 weeks

The overall homogeneity of the soap was evaluated at the end of the maturation process (9 weeks) by comparing the difference between the penetration values measured at 5, 10, 15, 20, 25 and 30 s. As the needle entered deeper in the soap, the penetration value increased but with a different amount. The difference between the penetration values of two timestamps (5-10, 10-15, 15-20, 20-25 and 25-30, respectively) decreases with the depth of penetration, suggesting that the soaps were harder in the centre. Soaps with similar penetration value differences dried more homogenously. OLI soaps showed almost no difference between the penetration values of two different timestamps further suggesting the hardness of the soap. The penetration values of medicated MIX-1-M blend indicate the largest variation in the soap consistency compared to other soaps.

Penetration values probably depend on the composition of the soaps. Olive oil containing soaps are known for their unique properties that may be explained by the components of the oil (fatty acids present in olive oil are palmitic, palmitoleic, stearic, oleic, linoleic, and linolenic acids) [61]. Beeswax

and cocoa butter are solid soap components modifying the consistency of the soaps MIX, MIX-1-M, MIX-2 and MIX-2-M (**Figure 2**). The foamability and pH of the various soap samples are represented in **Table 1**. Each soap showed great lathering properties. Cold process soaps with active pharmaceutical ingredient had better foamability than control, while the contrary was observed in the case of commercial soap. The commercial control soap exhibited the highest foamability followed by the medicated MIX-2-M blend. High foamability does not mean high cleansing, however, most consumers think that way, therefore it is important for product desirability [46].

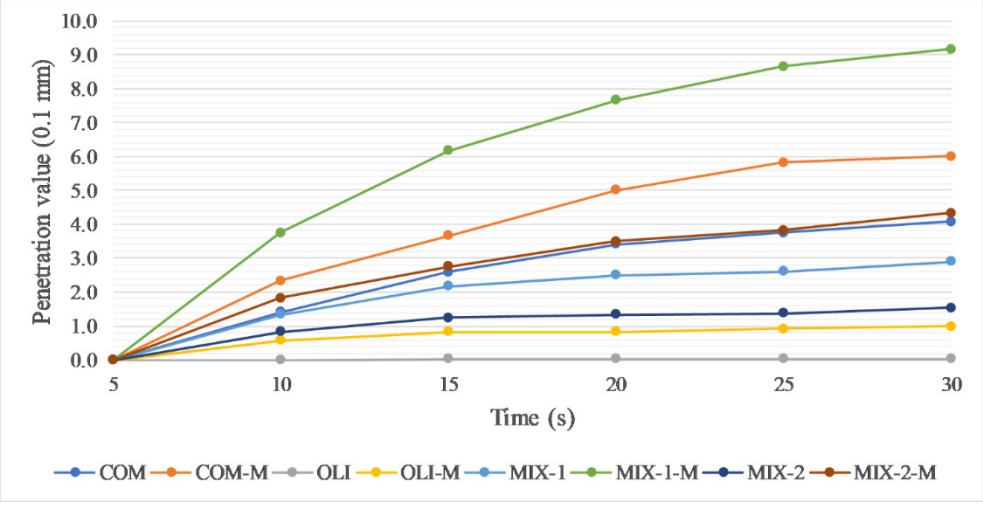

Figure 2. Penetration values measured at 9 weeks

The pH of the medicated soaps was within the normal range for commercial soaps (9-11). Two control soaps had higher pH values (MIX-1-M and MIX-2-M); these were still under 11.5. The pH of the commercial soap was the lowest. Among the prepared soaps OLI and OLI-M have the lowest pH.

Table 2 shows antimicrobial activity of the soap samples. Each medicated soap was effective against C. albicans, commercial and OLI soap having the largest inhibition zones (**Figure 3**).

Table 2. Microbial test results

Microorganism	Diameter of zone of inhibition (mm)							
	COM	COM-M	OLI	OLI-M	MIX-1	MIX-1-M	MIX-2	MIX-2-M
Staphylococcus aureus	R	11[a]	R	R	R	R	R	R
Enterococcus sp.	R	R	11	11	R	R	R	R
Escherichia coli	R	R	R	R	R	R	R	R
Klebsiella sp.	R	R	R	R	R	R	R	R
Salmonella typhimurium	R	R	R	R	R	R	R	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R
Candida albicans	R	20	R	20	R	19	R	15[a]
Candida parapsilosis	R	11	R	R	R	R	R	R

The diameters of the soap solution drops were 10 mm, R – resistance, a – drop with a 9 mm diameter

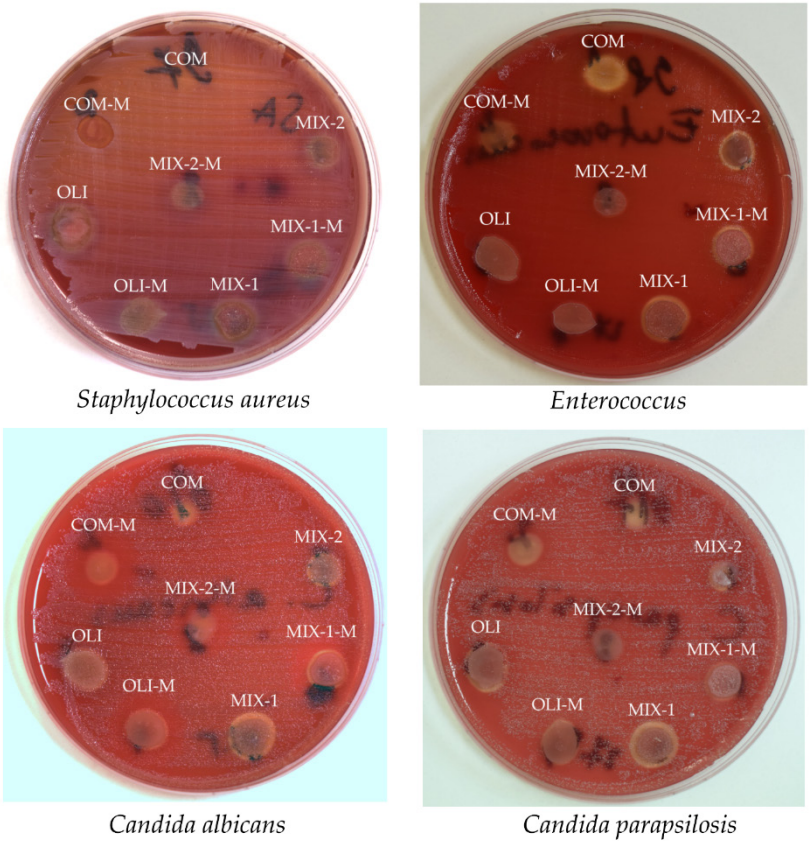

Figure 3. Plates showing zone of inhibition

HPLC method validation

Miconazole shows a linear response between 5-100 µg/ml. The accuracy was tested at three concentration level by adding known amount of miconazole to the soap matrix: then dissolving it in 10% methanol aqueous solution to obtain solutions with 20 µg/ml, 40 µg/ml and 60 µg/ml concentration, respectively. The precision of the method tested at 40 µg/ml expressed in CV% is 0.76. The LOD and LOQ concentrations were 1.25 µg/ml and 4.16 µg/ml, respectively.

The validation parameters of the method are shown in **Table 3**.

Table 3. The validation parameters of the method

Parameter	Result	Statistical analysis	Comment
Linearity	5-100 µg/ml	R^2=0.9990	
		W=0.918 (p=0.51)	Shapiro-Wilk test accepted range 0.787-1.0
		F=5017.80	p<0.05
Accuracy (%) **20 µg/ml** **40 µg/ml** **60 µg/ml**	 101.7 - 102.3 101.5 - 102.6 100.2 – 101.3	R^2=0.9991	-
Precision (CV%)	0.76%	-	-
LOD	1.25 µg/ml	-	-
LOQ	4.16 µg/ml	-	-

Figure 4 shows a typical chromatogram of a soap sample (tr miconazole 7.14 ± 0.10 min), a spiked sample and the matrix solution, no interference from the matrix can be observed on the retention time of miconazole.

The miconazole content of the soaps shows a content of 2.72 % to 5.12 %. Uniformity content is influenced by the preparation method and the conservation of the soaps. The uniformity had been determined after more than 9 weeks of preparation. In the meantime, the soaps lost water, presented weight loss, which leads to the increase of miconazole content. Miconazole had been suspended in the soap base and the turn into moulds, then allowed to cool. At sampling from the soaps, probes were collected from different places, heights of the soaps. Miconazole might have sedimented during the cooling phase of preparation.

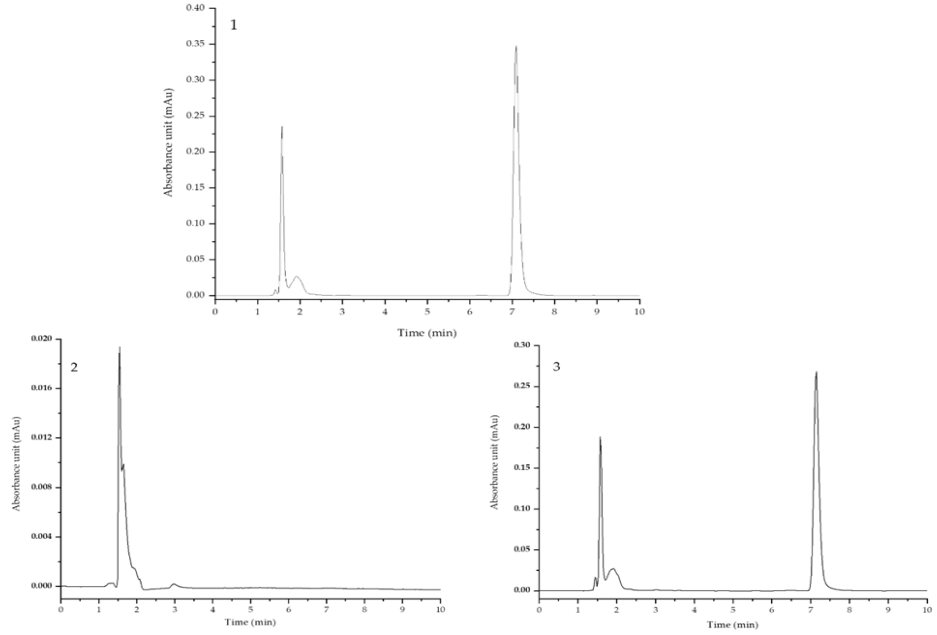

Figure 4. Chromatogram of a soap sample (1 – 60 µg/ml spiked matrix solution; 2 – matrix; 3 – sample OLI-M)

CONCLUSIONS

Medicated, miconazole nitrate containing soaps may be used by medical staff for handwashing and for cleaning skin zones of patients predisposed to fungal infections, the active pharmaceutical ingredient inhibiting the growth of *Candida albicans* in microbiological study. The influence of miconazole nitrate seems to be minor on the properties of the soaps. Olive oil containing soaps presented the lowest weight loss, the lowest pH, and proper consistency during conservation.

EXPERIMENTAL SECTION

Lipids and alkali used for soap preparation

Medicated and control soaps were prepared using four soap bases, three of which were formulated using various lipids and a 25% sodium hydroxide (NaOH) solution. Solid NaOH was weighed and dissolved in distilled

water to prepare the 25% NaOH solution. Each formulation contained olive oil, which was combined with beeswax, cocoa butter and sunflower oil in some of the mixtures (**Table 4**). A commercial ly4e calculator X was used to select the composition of the different formulations considering their saponification index [62]. An approximately 1:2 ratio of lipid and alkaline solution was used with a 5% excess lipid. A commercial soap base was used for comparison, containing coconut oil, palm oil, safflower oil, glycerin, water, sodium hydroxide, sorbitol, propylene glycol, sorbitan oleate, oat protein.

Table 4. Composition of various soap samples

	Soap type							
Ingredient (g)	COM	COM-M	OLI	OLI-M	MIX-1	MIX-1-M	MIX-2	MIX-2-M
Miconazole nitrate	-	0.67	-	0.89	-	0.89	-	0.89
Commercial soap base	33	30	-	-	-	-	-	-
Olive oil	-	-	30	29.1	12	11.1	9	8.11
25% NaOH solution	-	-	15.4	15.4	14.3	14.3	14.5	14.5
Sunflower oil	-	-	-	-	12	12	12	12
Beeswax	-	-	-	-	6	6	3	3
Cocoa butter	-	-	-	-	-	-	6	6

Cold process soap formulation

The oils and butters were mixed in an evaporating dish and heated to 70 °C on an electronic water bath. In the case of the beeswax containing mixtures, the lipids were melted at 85°C to get a homogenous liquid. The olive oil content of medicated soap bases was reduced and replaced with the active pharmaceutical ingredient (2% miconazole nitrate) which was added after melting the lipids. The required amount of alkali solution with the same temperature as the lipid mixtures was slowly poured into the melted lipids, while stirring vigorously until the trace stage, which corresponds to the mixture thickening to the consistency of pudding. Then the mixture was poured gradually into molds and dried for 48 h before unmolding. The soap bars were cured at room temperature for several weeks and the maturation process was followed at 1, 2, 4 and 9 weeks.

To prepare the medicated soap from commercial soap base the melt-and-pour method was used, during which the soap base was melted at 50°C, then mixed with miconazole nitrate and poured into molds.

Following water loss

To measure water loss from soap overtime, the weight of each soap sample was determined 1, 2, 4 and 9 weeks after soap preparation with Kern ABJ 220-4NM analytical balance at room temperature. Three parallel samples were measured.

Hardness measurement by penetrometry

The hardness of the soaps was determined using an automatic penetrometer (VEB Feinmess Dresden) equipped with a penetration needle with a 1 mm diameter and 40 mm exposed needle length. The penetration rate was recorded in penetration units (0,1 mm). The penetration depth was measured at room temperature every 5 seconds during a 30 second period. The measurements were repeated at 1, 2, 4 and 9 weeks after soap preparation with three samples.

pH measurement

50 ml solutions were prepared by dissolving 2.0 g of each soap sample in distilled water. The pH value of each sample was measured after 30 minutes at 25°C using a pH meter (Consort C831 multi-parameter analyzer).

Foamability test

The foamability of the soap samples was determined by dissolving 2.0 g of soap in distilled water for 30 minutes to prepare 50 ml solutions in a graduated cylinder. Later, the solutions were shaken vigorously for 1 minute, allowed to settle for 10 minutes; then the foam volume was measured in ml.

Antimicrobial assay

Two Gram-positive (*Staphylococcus aureus, Enterococcus*), four Gram-negative (*Escherichia coli, Klebsiella sp., Salmonella typhimurium, Pseudomonas aeruginosa*) and two yeast (*Candida albicans, C. parapsilosis*) strains were used in microbial study. Each strain was inoculated in a sterile saline solution to make a suspension equal to 0.5 McFarland (1,5x108 CFU/ml) and plated on Mueller Hinton Agar supplemented with 10% defibrinated sheep blood. 1:10 soap solutions were prepared by dissolving the soaps in distilled water for 24 h. 10 µl of each soap solution was placed on the plate at 2.5 cm distance from each other. The inoculated plates were kept at room temperature for 30

minutes to allow diffusion of the agent into the agar; this was followed by 24 h incubation at 37 °C. After incubation, the zone of inhibition and the diameter of the 10 µl soap solution were measured in millimeters.

HPLC measurement of drug content

The HPLC-UV (Merck Hitachi HPLC system: D-7000 interface, L-7100 quaternary pump, L-7612 solvent degasser, L-7200 autosampler, L-7455 DAD detector and HSM 4.0 software) determination of miconazole nitrate content of the soap samples was performed isocratically on EC HPLC column 150 x 4.6 mm NUCLEODUR® PolarTec, 3 µm (Macherey Nagel, Germany) and 5 mM phosphate buffer (pH=8.73±0.02) and acetonitrile (25:75 w/w%) as mobile phase, with a flow rate of 0.75 ml/min. Detection and best chromatogram extraction were carried out at 222 nm. The column was maintained at an ambient temperature and an injection volume of 30 µl was used.

Analytical validation parameters such as linearity, precision, accuracy, specificity, LOD and LOQ were tested. For these parameters a stock solution of miconazole nitrate in 1 mg/ml concentration was prepared by dissolving the active ingredient in methanol, and this was diluted with blank solution (suitable amount of soap, without active ingredient, dissolved in 10% methanol solution in purified water) to obtain standard solutions. The linearity of the method was tested in 5-100 µg/ml concentration range. The accuracy was verified at three concentration levels by adding known amount of miconazole to the soap matrix: then dissolving it in 10% methanol aqueous solution to obtain solutions with 20 µg/ml, 40 µg/ml and 60 µg/ml concentrations, respectively. The precision of the method, tested at 40 µg/ml was expressed in CV%. The LOD and LOQ values were evaluated at a 3:1 and 10:1 signal-to-noise ratio.

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