Preparation, characterization and *in vitro* efficacy studies of antifungal topical formulations

Mehmet Tayfur, Nurseli Saylam, Sibel Döşler, Büşra Örenli, Gülgün Yener
Adresler
Orcid No:0000 0002 7234 0034

Corresponding author Nurseli Saylam

Name SURNAME [1] *, Name SURNAME [2]

ABSTRACT: Fungal skin infections are among common and serious global health issues. In recent years, there has been an increase in fungal infections due to frequent use and wrong consumption of antibiotics and antifungals that suppress the immune system. Current treatment options have limitations such as the high toxicity of antifungal agents, the effects of pharmacodynamic-pharmacokinetic mechanisms, and the occurrence of antifungal resistance. The side effects of synthetic drugs used in the treatment of fungal infections necessitated the development of alternative formulations. Essential oils from various plants are natural resources that have been used in modern and traditional medicine for centuries as antifungal and antimicrobial agents.

In this study planned in the light of this information, antifungal efficacy tests were carried out on fungi causing superficial fungal infections by preparing gel formulations of tea tree oil, thyme oil, clove oil and hackberry oil, which are natural sources that have been frequently used in both modern and traditional medicine for centuries. the most appropriate formulation has been investigated. Gel formulations of tea tree oil, thyme oil, clove essential oils and hackberry oil were prepared and their antifungal activities on T. rubrum, M. canis, and C. albicans strains causing skin fungal infections were investigated comparatively. The MIC values of the gels prepared with essential oils were evaluated according to the CLSI accuracy range. The results have revealed that it could be predicted this gel formulations with natural components may be effective and alternative in the treatment of skin fungal infections in humans.

KEYWORDS: Topical; antifungal; gel; tea tree oil, thyme oil, clove oil, hackberry oil

1. INTRODUCTION

Fungal diseases are a global health problem; it is estimated that more than one billion people suffer from serious fungal diseases and more than 1.6 million deaths occur annually [1]. Fungal infections have increased in recent years due to the irregular use of antibiotics and antifungal drugs that weaken the immune system [2]. This fact has accelerated the development of new antifungals of plant origin with no or acceptable side effects [3].

The main agents of fungal infections on the skin are dermatophytes, yeasts and saprophytic fungi, although not very common [4]. In the treatment of fungal diseases on the skin, topical preparations are used in addition to systemic treatment. Since these preparations contain corticosteroids, they may cause the treatment of fungal infection, which requires a long treatment period, to be interrupted due to side effects [5].

Approximately two hundred and fifty thousand different fungal species have been investigated in nature and about one hundred and fifty of them may be pathogenic for animals and humans [6]. There are three main types of superficial fungal infections caused by dermatophytes, Candida species and Pityrosporum organisms. Dermatophyte fungi spread to keratin-rich areas outside the skin and can also affect nails and hair, which contain keratin. Most of these infections are transmitted from person to person and the most common known pathogen is Trichophyton rubrum [7]. Candida species infections of the skin are most commonly caused by the opportunistic skin pathogen Candida albicans. Skin infections, which are usually not severe, are treated locally with superficial imidazole-containing preparations or with allylamine and terbinafine. Two weeks of treatment is necessary in most patients. If the infected area is much larger, systemic antifungal drugs are recommended.

Medicinal plants are used extensively in traditional medicine to treat many diseases. Aromatherapy is one of the methods used to cure and prevent diseases. The method of action is based on triggering the sensory centers and heat receptors in the limbic system and brain, and destroying germs and fungi. In aromatherapy, aromatic essential oils obtained from different parts of the plant such as roots and woody parts, flowers, bark, leaves, balsam, seed resin are used [8]. Tea tree oil is a volatile aromatic oil extracted by water vapor distillation from the leaf parts of Melaleuca alternifolia, which grows endemically in Australia, and has been used as a surface germicide for more than eighty years in Australia [9]. Tea tree oil is recognized as a suitable disinfectant for topical use due to its antimicrobial action against a wide range of microorganisms, its easy absorption by the skin and its non-irritating properties. Tea tree oil is included in many pharmaceutical and cosmetic preparations at different concentrations or undiluted. Studies prove that tea tree oil has a wide range of antimicrobial activity thought to be due to its high content of terpinen-4-ol [10]. Thyme plant is known to have antioxidant, antispasmodic, antimicrobial and digestive stimulant properties. In particular, thymol and carvacrol in thyme essential oil are known to have antimicrobial properties [11]. In addition, phenolic compounds in thyme oil activate the phospholipid layer in the cell membrane of microorganisms, increase the permeability of intracellular vital structures or disrupt the enzyme systems of microorganisms [12]. This ethereal essential oil is known to contain around 50% Thymol, Carvacrol, Borneol, Cymol, Pimen, Tannin and other flavones [13]. The seeds, fruits and leaves of hackberry, which have many different names in different regions, are used by the people in that region to cure many different ailments in traditional medicine [14]. The fruits that grow on the hackberry tree are slightly larger than lentils and have a bitter peanut flavor. E and B group vitamins are abundant in it. Its composition contains many minerals such as sodium, potassium, zinc, calcium, phosphorus and manganese. Prevents foot sweating. It has diuretic and wound healing properties. It relieves chronic pain in the stomach and cough. Leaf parts are applied to the hair to make it black [14] . It is thought to be very effective for medicinal purposes due to its many effects such as being analgesic, relieving chronic stomach pains, healing wounds with its antiseptic properties and being useful in stone reduction [15]. In addition, antimicrobial and especially antifungal activities of extracts prepared with hackberry leaves and ethyl alcohol against Candida Albicans and the emerging yeast-like fungi Candida Parapsilosis and Rhodotorula mucilaginosa were determined [16]. In addition to the use of many parts of the plant in folk medicine and medicine for the treatment of various health problems, the use of chitlembic in cosmetics has started to increase day by day due to the increasing demand for pleasant odors in recent years [14]. Clove oil is a volatile aromatic oil extracted from the stems, leaves and buds of the Syzygium aromaticum plant. In alternative medicine, clove oil has been used to treat cuts and burns and even to relieve toothache and infection. Clove has been shown to be effective against bacteria associated with dental caries and periodontal disease, as well as many other bacteria. Research has also reported antifungal, antiallergic, anticarcinogenic and antimutagenic activity of this plant [17]. The essential oil extracted from clove buds is widely used for medicinal purposes, especially in oral and dental care. Clove oil is effective against Escherichia coli, Listeria monocytogenes, Salmonella enterica, Staphylococcus aureus, Campylobacter jejuni and other bacteria [18]. The main chemical components of clove essential oil are caryophyllene, iso-eugenol, eugenol acetate and eugenol. Eugenol, a phenylpropanoid compound, is the main component (85.3%) of clove essential oil with antioxidant properties. Eugenol has demonstrated antifungal activity against Trichophyton mentagrophyte and Candida albicans [19].

Antifungals consumed after the emergence of fungal diseases have also revealed the problem of resistance as a result of the formation of immunity in molds or yeasts that cause disease. For this reason, new antifungal studies that do not have such problems are accelerating. In this study, formulations developed with aromatherapy oils, which are intended to be an alternative or support to the chemical topical antifungal drugs used in the market, were prepared and their effectiveness was evaluated comparatively.

2. RESULTS

2.1. Preparation of Formulations

There were six distinct formulations prepared. Since there are no active ingredients in the F1 formulation, it is a placebo formulation. F2 formulation contains 0.5% tea tree oil. F3 formulation contains 0.5% hackberry oil. F4 formulation contains 0.5% clove oil. F5 formulation contains 0.5% thyme oil. The F6 formulation is a mix formulation because it contains 0.5% each of tea tree, hackberry, clove, and thyme oils.

Table 1. Constituents of formulations (w/v)

Ingredients	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)	F6 (%)
Distilled Water	89.8	88.8	88.8	88.8	88.8	88.8
EDTA	0.2	0.2	0.2	0.2	0.2	0.2
Carbomer	1	1	1	1	1	1
Polysorbate 20	-	0.5	0.5	0.5	0.5	2
Dimethicone	1	1	1	1	1	1
Vitamin E	3	3	3	3	3	3
Tea Tree Oil	-	0.5	-	-	-	0.5
Thyme Oil	-	-	-	-	0.5	0.5
Clove Oil	-	-	-	0.5	-	0.5
Hackberry Oil	-	-	0.5	-	-	0.5
Glycerin	2	2	2	2	2	2
D-Panthenol	2	2	2	2	2	2
Triethanol Amine	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Sensiva	1	1	1	1	1	1

2.2. Physical Stability Test

The organoleptic properties of the formulations were analyzed for 3 months. There was no change in color, odor and appearance, they were stable. pH and viscosity changes are as given below.

Table 2 . 3-month pH measurements of formulations stored under 3 different conditions

pH	Temp.	F1	F2	F3	F4	F5	F6
Initial	25°C	5.35	4.78	5.47	4.995	4.935	5.23
1st Month	5°C	5.42	5.155	5.435	5.14	5.02	5.455
	25°C	5.495	5.73	5.56	5.29	5.906	5.375
	40°C	5.69	5.193	5.716	5.243	5.663	5.57
2nd Month	5°C	5.415	5.11	5.42	5.15	5	5.42
	25°C	5.555	5.77	5.96	5.415	5.945	5.41
	40°C	5.765	5.265	5.825	5.33	5.72	5.625
3rd Month	5°C	5.325	5.025	5.31	5.035	4.91	5.325
	25°C	5.51	5.675	5.56	5.365	5.885	5.37
	40°C	5.705	5.26	5.735	5.37	5.645	5.53

Table 3 . 3-month viscosity measurements of F1, F2 and F3 formulations stored under 3 different conditions

Speed (rpm)	Temp.	Viscosity (cP)											
		F1				F2				F3			
		Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
20	5°C		4916.35	3859.66	3358.595		3408.21	3150.235	3060.935		5253.7	3824.935	2599.565
	25°C	7079.35	5154.475	4975.885	5218.975	2217.565	5491.83	4742.715	4767.52	4722.875	4871.705	4534.355	4003.525

	40°C		6940.435	5938.315	5918.475		4598.845	3671.14	3552.08		6166.525	5442.22	5189.205
	5°C		3707.52	2943.53	2616.1		2635.945	2457.35	2463.965		3889.425	2946.835	2136.535
30	25°C	5080.065	3955.57	3800.125	3932.42	1696.66	4048.175	3657.91	3674.45	3568.61	3720.75	3476.01	3141.965
	40°C		4980.845	4501.28	4372.295		3456.165	2834.385	2682.245		4574.04	4147.395	3866.27
	5°C		3046.05	2401.12	2155.555		2170.435	2006.725	2041.45		3155.195	2386.24	1810.765
40	25°C	3941.515	3197.365	3117.99	3180	1413.885	3284.185	3033.65	3021.25	2884.82	3108.07	2872.42	2599.565
	40°C		3998.565	3614.09	3465.26		2817.845	2334.155	2175.4		3693.465	3361.08	3113.025
	5°C		2583.69	2051.87	1843.51		1861.365	1742.305	1740.32		2708.705	2047.9	1591.49
50	25°C	3313.95	2686.875	2672.985	2678.94	1234.295	2819.835	2617.425	2549.955	2464.625	2665.05	2474.545	2196.735
	40°C		3367.525	3022.245	2875.395		2397.155	2016.15	1851.445		3123.445	2859.52	2597.58
	5°C		2235.76	1799.19	1602.4		1647.055	1552.79	1539.565		2358.13	1810.765	1410.575
60	25°C	2882.34	2384.585	2348.205	2275.445	1101.345	2462.31	2293.635	2217.565	2179.53	2331.67	2164.65	1929.825
	40°C		2892.26	2583.03	2460.655		2106.77	1769.42	1607.365		2712.015	2455.695	2244.025
	5°C		2009.91	1618.7	1417.43		1484.05	1404.67	1380.575		2107.72	1635.71	1272.85
70	25°C	2554.205	2127.56	2093.545	1987.235	1012.045	2174.335	2034.01	1965.97	1965.975	2066.61	1924.865	1726.43
	40°C		2534.36	2270.72	2161.58		1890.85	1584.685	1431.6		2396.87	2167.245	1971.645
	5°C		1803.325	1478.38	1276.22		1355.59	1292.34	1250.175		1916.19	1488.3	1162.115
80	25°C	2274.62	1898.82	1881.46	1754.955	936.39	1963.315	1836.81	1761.155	1788.445	1847.975	1736.35	1551.555
	40°C		2268.42	2017.89	1921.145		1710.305	1439.93	1303.5		2158.035	1957.115	1767.355
	5°C		1636.03	1364.83	1157.565		1251.275	1195.05	1153.155		1746.27	1373.645	1073.78
90	25°C	2060.47	1757.295	1734.145	1602.955	870.93	1787.06	1677.92	1599.65	1649.255	1692.25	1586.415	1423.255
	40°C		2052.75	1855.415	1719.815		1570.98	1319.625	1189.54		1972.27	1780.445	1616.18
	5°C		1485.325	1266.05	1061.65		1156.905	1117.22	1065.625		1608.36	1275.97	998.15
100	25°C	1915.94	1605.38	1598.435	1477.385	817.57	1644.08	1545.845	1470.44	1532.95	1559.74	1461.51	1308.71
	40°C		1884.185	1710.555	1572.635		1448.61	1221.4	1096.38		1820.685	1638.125	1479.37

Table 4 . 3-month viscosity measurements of F4, F5 and F6 formulations stored under 3 different conditions

Speed (rpm)	Temp.	Viscosity (cP)											
		F4				F5				F6			
		Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month	Initial	1st Month	2nd Month	3rd Month
20	5°C		3507.425	2376.32	2103.465		2956.755	2162.995	1850.45		4072.985	3140.315	2991.485

	25°C	3929.115	3869.58	3145.275	3184.96	2272.14	6082.185	4861.78	5164.4	3631.45	3547.115	3041.095	3492.545
	40°C		4375.6	4321.03	4579		5665.46	5199.125	5085.025		4330.95	4296.23	3889.425
	5°C		2635.945	1848.8	1676.82		2285.365	1620.59	1432.075		3089.05	2480.5	2401.125
30	25°C	2966.68	2950.14	2437.5	2454.04	1699.965	4468.21	3760.44	3968.8	2741.78	2725.24	2424.275	2791.39
	40°C		3350.33	3247.805	3380.095		4296.225	3968.8	3743.905		3307.33	3300.72	3012.98
	5°C		2145.63	1532.95	1396.52		1890.14	1339.47	1208.005		2537.555	2031.53	2036.49
40	25°C	2443.29	2403.605	2024.09	2078.66	1406.44	3626.49	3120.47	3207.29	2234.93	2254.775	2004.245	2341.59
	40°C		2735.99	2614.445	2698.785		3465.26	3214.73	2993.96		2743.43	2740.955	2468.1
	5°C		1843.505	1335.5	1206.515		1633.16	1168.815	1041.81		2216.575	1762.145	1815.725
50	25°C	2099.495	2069.73	1736.35	1776.035	1220.405	3077.805	2686.88	2712.675	1956.615	1960.585	1764.13	2057.82
	40°C		2353.495	2220.54	2276.105		2909.135	2722.6	2516.22		2381.28	2371.36	2119.34
	5°C		1620.59	1187.33	1081.5		1440.345	1043.465	937.63		1962.9	1567.675	1609.02
60	25°C	1860.375	1815.725	1539.56	1559.405	1091.42	2669.015	2343.245	2399.47	1736.35	1746.27	1567.68	1807.455
	40°C		2043.93	1939.75	1962.9		2540.03	2366.395	2169.61		2123.31	2090.235	1838.88
	5°C		1462.79	1075.83	978.03		1301.195	958.18	849.04		1774.62	1425.935	1458.53
70	25°C	1678.24	1627.21	1390.5	1401.84	995.03	2357.185	2092.125	2180.005	1581.85	1593.19	1430.185	1624.37
	40°C		1824.23	1723.595	1734.935		2248.04	2087.875	1920.615		1903.605	1865.335	1631.46
	5°C		1341.95	982.28	891.74		1201.8	883.06	776.4		1621.005	1314.665	1345.67
80	25°C	1535.43	1478.375	1271.255	1273.735	911.585	2102.225	1913.705	1953.395	1469.695	1464.735	1320.865	1487.06
	40°C		1660.695	1560.235	1567.675		2019.13	1872.78	1721.47		1726.43	1687.98	1477.14
	5°C		1239.15	909.515	831.24		1106.85	819.115	722.105		1488.3	1217.1	1230.33
90	25°C	1424.36	1354.905	1174.105	1168.59	853.29	1902.82	1750.68	1776.035	1363.725	1362.62	1225.92	1357.11
	40°C		1505.935	1433.175	1424.36		1839.975	1704.38	1573.19		1589.725	1540.115	1343.88
	5°C		1149.96	848.33	774.91		1035.855	764.99	673.705		1379.16	1135.075	1145
100	25°C	1323.595	1256.125	1093.405	1076.54	799.71	1740.32	1616.295	1637.13	1278.945	1272	1144.01	1256.125
	40°C		1392.055	1326.575	1314.665		1695.67	1569.665	1453.575		1468.455	1418.845	1239.255

2.3. Textural analysis

Table 5. Values of texture profile analysis of formulations

Formulation code	Hardness (N)	Compressibility (mm)	Adhesiveness (mJ)	Cohesiveness
F1	0,05 ± 0	4,49 ± 0	0,50 ± 0	-0,66 ± 0
F2	0,05 ± 0	4,49 ± 0	0,50 ± 0	-0,66 ± 0
F3	0,05 ± 0	4,625 ± 0,005	0,25 ± 0,05	-0,205 ± 0,005
F4	0,06 ± 0	4,015 ± 0,125	0,2 ± 0,2	1,965 ± 3,515
F5	0,05 ± 0	4,49 ± 0	0,5± 0	-0,66 ± 0
F6	0,04 ± 0	3,375 ± 1,315	0,15 ± 0,15	-0,61 ± 0,61

Data represent mean ±SD; n=3

2.4. Microbiological Test

The MIC values of the selected mold and yeast strains against the prepared formulations are given in Table-1. The MIC values of the fluconazole was within the accuracy range in CLSI throughout the study [20].

According to the results, the MIC values against the yeast C. albicans std strain were 1-0.06 µg/ml, while hose against clinical C. albicans isolate were 0.03-0.007 µg/ml. Against dermatophyte T. rubrum std strain the MICs were 0.015-0.003 µg/ml, while those against clinical isolate were 0.125 -0.03 µg/ml. Against mold A. fumigatus std strain, the MIC values were 0.5-0.015 µg/ml, and the clinical M. canis isolate were found as 0.007-0.001 µg/ml. These values seem to be comparable to standard commercial product Fuggy cream in the market.

Table 6 . Antimicrobial effects of formulations against placebo

	C.albicans ATCC 10231	*C.albicans* clinical Isolate	*T. rubrum* ATCC 28188	*T. rubrum* clinical Isolate	*A.fumigatus* ATCC 26934	*M. canis* clinical Isolate
Placebo (F1)	0,25	0,015	0,007	0,06	0,03	0,001
Tea tree (F2)	0,5	0,015	0,015	0,06	0,125	0,007
Hackberry (F3)	1	0,015	0,015	0,125	0,125	0,003
Clove (F4)	0,25	0,007	0,007	0,06	0,03	0,003
Thyme (F5)	0,06	0,007	0,007	0,03	0,015	0,001
Mix (F6)	0,25	0,015	0,003	0,06	0,015	0,001

Table 7 . Antimicrobial effects of formulations against control Fuggy Cream (%1 Isoconazole)

	C.albicans ATCC 10231	*C.albicans* clinical Isolate	*T. rubrum* ATCC 28188	*T. rubrum* clinical Isolate	*A.fumigatus* ATCC 26934	*M. canis* clinical Isolate
Fuggy Cream: %1 Isoconazole	1	0,03	0,015	0,125	0,5	0,003
Tea tree (F2)	0,25	0,015	0,003	0,06	0,015	0,007
Hackberry (F3)	1	0,03	0,015	0,125	0,03	0,003
Clove (F4)	0,25	0,015	0,003	0,03	0,015	0,003
Thyme (F5)	0,125	0,015	0,003	0,03	0,015	0,003
Mix (F6)	0,06	0,015	0,003	0,03	0,015	0,003

3. DISCUSSION

The color, odor and appearance of the formulations are compatible with gel formulations. pH values hover around 5.5 in line with skin pH and remain close to this value throughout the 3-month stability test. In the viscosity evaluation of the formulations, it is seen that the viscosity decreases as the shear rate increases, all formulations show pseudoplastic flow as expected from gels and maintain this throughout the stability tests.

Texture profile analysis of the gels was performed at 25 ± 2°C. The results of texture profile analysis are shown in Table 5. In this study, all formulations showed similar hardness. The formulation with the highest active ingredient concentration F6, i.e. the formulation containing a mixture of oils, showed the lowest compressibility. F6 showed the lowest adhesiveness while F1, F2 and F5 showed the highest adhesiveness. F4, the formulation containing clove essential oil, had the highest cohesiveness value.

When the results obtained for T. rubrum ATCC strains were examined, it was observed that the preparations gave an MIC value equal to or 4 times lower than the Fuggy Cream accepted as control, especially the mixture, and Thyme formulations were highly effective. When the experiments were performed on clinical T. rubrum isolate, the most effective formulations were observed with thyme and clove, and the results were lower than the MICs against standard strain. Similarly, the results on C.

albicans standard strain showed that the MIC values of thyme, clove, tea, and mix formulations were at least two times lower than the control, similarly against clinical C. albicans isolate, the mix, thyme, and clove formulations MIC values were two times lower than the control. No significant difference was found with the formulations against M. canis strains, except the two times decrease thyme and mix formulations MIV values. The greatest difference in results were observed against A. fumigatus standard strain as a five-fold decrease in the MIC values of the mix, thyme, tea and clove formulations than control.

4. CONCLUSION

The aim of this study was to prepare herbal antifungal gels that can be an alternative or supplement to the synthetic topical antifungal drugs used in the market. Depending on the results obtained, it was concluded that the prepared formulations showed good texture properties and pseudoplastic flow and were compatible with skin pH. During the 3-month stability tests, they maintained their organoleptic and other general properties in all 3 environmental conditions. When the antimicrobial activity of the formulations was analyzed, all formulations showed equal or better antimicrobial activity compared to the market product. This indicates that the formulations containing essential oils may support the existing treatment and may be an alternative to treatment to break antifungal resistance in accordance with the aim of the study.

5. MATERIALS AND METHODS

5.1. Materials

The oils selected for the preparation of the formulation: tea tree oil, clove oil, thyme oil were obtained by NU-KA Defne Essencia (Turkey), and hackberry oil was supplied by Mecitefendi (Turkey). Vitamin E and D-Panthenol were purchased from BASF (Germany). Glycerin was provided from EVYAP (Malaysia). Dimethicone and Polysorbate 20 were obtained from Innospec (UK). Carbomer was purchased from Spectrum Chem. (USA). EDTA (Ethylene Diamine Tetra Acetic Acid) and TEA (Triethanol Amine) were supplied from MERCK (Germany). Sensiva® PA 30 was obtained from Schülke & Mayr (Germany). The other substances and solvents that were utilized were all pure analytical grade.

5.2. Preparation of Formulations

The carbomer was weighed and 1/3 of the total distilled water was added. It was left to swell on a magnetic stirrer (Daihan Smhs-6, Korea) at 500 rpm for 1 night. Vitamin E, D-Panthenol, glycerin, dimethicone, polysorbate 20, EDTA, sensiva and the active ingredient (tea tree oil, clove oil, thyme oil, hackberry oil) are weighed in determined ratio. The swollen carbomer and other weighed substances are mixed. A sufficient amount of triethanolamine is added to this mixture.

5.3. Characterization of Formulations

5.3.1. Rheological Characteristics

The viscosity of the formulations at different shear rates was measured using a Brookfield cone and plate rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA) at 25 ± 0.5 °C. The rheological properties of the formulations were evaluated according to the correlation between viscosity and shear rate.

5.3.2. pH Measurement

After diluting the formulations, pH measurements were performed with a portable pH meter (Hanna Edge, USA) at 25 ± 2 °C.

5.3.3. Textural analysis

Texture profile analyses of prepared gels were evaluated using CT3 Texture Analyser (Brookfield, UK) to determine the mechanical parameters like hardness, compressibility, adhesiveness, and cohesiveness. An analytical probe name of TA5 was immersed twice into each sample to a defined depth (5 mm), at a defined rate (2 mm/s), with a defined recovery period (15 s), between the end of the first

compression and the beginning of the second. A trigger force of 0.01 N was applied. Three replicate analyses of each sample were performed at 25 ± 1°C. Data collection and calculation were done by TexturePro CT V1.6 Build. From the resulting force-time plots, hardness (the force required to attain a given deformation), compressibility (the work required to deform the product during the first pass of the probe), adhesiveness (the work necessary to overcome the attractive forces between the surface of the sample and the surface of the probe), and cohesiveness (the ratio of the area under the force-time curve produced on the second compression cycle to that produced on the first compression cycle) were obtained.

5.3.4. Physical Stability Test

Formulations were prepared for physical stability evaluation. pH and viscosity measurements of the prepared formulations were performed and their organoleptic control was assessed. The formulations were stored in closed glass bottles at different conditions: 25 ± 2 °C and 60% RH, 40 ± 2 °C and 75% RH and 5 ± 3 °C. These formulations were evaluated for pH, viscosity and appearance for 3 months (1st, 2nd and 3rd months).

5.3.5. Microbiological Test

T. rubrum, M. canis, and C. albicans clinically isolates which were obtained from specimens submitted to the Clinical Microbiology Laboratories of Istanbul University, Istanbul Faculty of Medicine were tested. Mold samples were incubated on Saboraud Dextrose Agar at 27°C for one week. After incubation period, 5 ml of sterile water was added to the formed mold colonies, then samples were collected with a sterile swab which added 1% Tween80.

Media: RPMI-1640 broth (Sigma), which was added to morpholine propane sulfonic acid (MOPS, Sigma) and adjusted to pH 7.0, was used to calculate the minimum inhibitory concentration (MIC) values of the fungi. Saboraud Dextrose Agar was used for colony counts.

Indicator called resasurin was used to distinguish the turbidity of growth in microplates from the turbidity formed as a result of mixing of the substance with the medium. For resasurin staining method, 5 mg resasurin dye was weighed and dissolved in 10 ml of distilled water as a stock solution and diluted 1:5 for the experiments.

Determination of minimum inhibitory concentration (MIC): The antifungal activities of the prepared topical preparations were determined against the above-mentioned molds and yeast samples by microdilution method based on the Clinical Laboratory Standards Institute [21] criteria. Serial two-fold dilutions of formulations were prepared in RPMI-1640 medium (Sigma) in a 96 well microplates. The prepared inoculum of the mold and yeast samples were adjusted to a turbidity equivalent to a 0.5 McFarland standard, and added to the wells to give a final concentration of 0.5x103 to 2.5x103 cfu/ml. The trays were covered and placed in plastic bags to prevent evaporations and incubated for 5-7 days at 25°C for molds and 24 hours at 37°C for C. albicans. At the end of the incubation, 25 µl of resasurin dye was added to the wells, after one-hour incubation, purple colored wells were evaluated as growth negative and pink colored wells were evaluated as growth positive. The MICs were defined as the lowest concentration of compound giving complete inhibition of growth. Fluconazole was used as reference antifungal for yeast, and commercial antifungal cream “Fuggy Cream”, and placebo which does not contain active substance, were also used as the controls for experiments.

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