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Research Article

Determination of *In Vitro* Antifungal Activity of Dihydroisoxazole Dimethanol Compound Against Dermatophytes Obtained From Clinical Isolates and Design of Some New Dihydroisoxazole Dimethanol Compounds

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ABSTRACT

In this study, the *in vitro* antifungal activity of (3-(4-chlorophenyl)-4,5-dihydroisoxazole-4,5-diyl) dimethanol compound (1) was investigated against two pathogenic fungi species (*Trichophyton mentagrophytes* and *Microsporum canis*) and important results were obtained. The difficulty of treating fungal diseases induced us to assess the antifungal properties of dihydroisoxazole dimethanol compound. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC). The compound (1) showed an antifungal effect with MIC=12.5 µg ml^{-1} on *M. canis* and MIC=50 µg ml-1 on *T. mentagrophytes*, respectively. To prepare potentially more effective antifungal agents, new dihydroisoxazole derivatives (2-32) were theoretically designed. The geometrical optimization of all compounds (1-32) was performed using the semi-empirical AM-1 level. Some structural parameters of the optimized compounds (1-32) were also calculated.

Keywords: Antifungal activity, dermatophyte, dihydroisoxazole, MIC, *Microsporum canis*, structural parameters, *Trichophyton mentagrophytes*.

1. INTRODUCTION

Infections of the skin, hair, and nails caused by dermatophytes, which are one of the most common agents of superficial fungal infections, are called dermatophytosis.[1-3] Dermatophytosis infection is seen in a significant part of the patients who apply to the dermatology outpatient clinic. The frequency and the type of etiological agent of dermatophytosis vary depending on many factors (socioeconomic level of the community as well as geographical area, lifestyle of the population, migrations, time of study, climate changes, age of persons, and pet feeding habits). Dermatophytes need keratin tissue to reproduce, so they settle in hair, nails, and superficial skin areas and reproduce in hot, humid conditions and unhygienic environments.[4-11] Dermatophytes are classified into three anamorphic species, *Trichophyton*, *Epidermophyton*, and *Microsporum*. It can also be classified as anthropophilic (transmitted from human to human), zoophilic (transmitted from animal to human), and geophilic (transmitted from soil to human or animal).[4-8] *Trichophyton* is the most common genus causing superficial fungal infections among dermatophytes.[10-13] *M. canis* and *T. mentagrophytes* are the most important factors causing dermatophytosis in dogs and cats.[14-19] Infection by dermatophytes is generally cutaneous and limited to non-viable cornified tissues.[14,20] They form typical lesions called tinea, which cause dandruff, vesicle formation and inflammation on the skin, breakage and shedding in the hair, deformity of the nails.[5]

The insufficiency of antifungal agents in the treatment of dermatophytes leads to the search for different therapeutic agents. Antibiotic resistance, which

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threatens human and animal health, is becoming a bigger problem day by day, causing difficulties in treatment. Conventional treatment of fungal diseases is limited compared to antibiotic therapy used for bacterial infections. Treatment of dermatophyte infection primarily includes oral and/or topical formulations of azoles, allylamines, itraconazole, and terbinafine. In recent years, the increasing problems of antifungal drug resistance of dermatophytic fungus have been described.[21-24] Thus, drug resistance in pathogenic fungi, including dermatophytes, is gaining importance. With this requirement, there is a need for new drugs especially new drug classes to combat this clinical resistance with new compound derivatives and various chemical agents.

Antifungal studies on dermatophytes in the literature are boric acid and ozonated olive oil[25], ozone[26], oxaborole-6-benzene sulphonamide[27], some medical plants[28], essential oil and extracts[29-31], silver nanoparticles[32], gelatin-stabilized selenium oxide nanoparticles[33], lichen compounds[34]. The synthesis and antimicrobial activity results of some 4,5-dihydroisoxazole derivatives have been reported in the literature.[35-37] However, there are no studies on (3-(4-chlorophenyl)-4,5-dihydroisoxazole-4,5-diyl) dimethanol compound (**1**) that we synthesized in our previous study.[38]

We examined the antifungal activity of the (3-(4-chlorophenyl)-4,5-dihydroisoxazole-4,5-diyl) dimethanol compound (**1**) (Figure 1) against *T. mentagrophytes* and *M. canis*. Because of these facts and as a continuation of our research on the biological properties of dihydroisoxazole derivatives, we have designed many dihydroisoxazole dimethanol derivatives (**2-32**), as potential antifungal agents (Figure 1). The theoretically geometric optimization of compounds (**1-32**) was made using the semi-empirical AM-1 level. Then, QSAR parameters, quantum parameters, and derived parameters of compounds (**1-32**) were calculated.

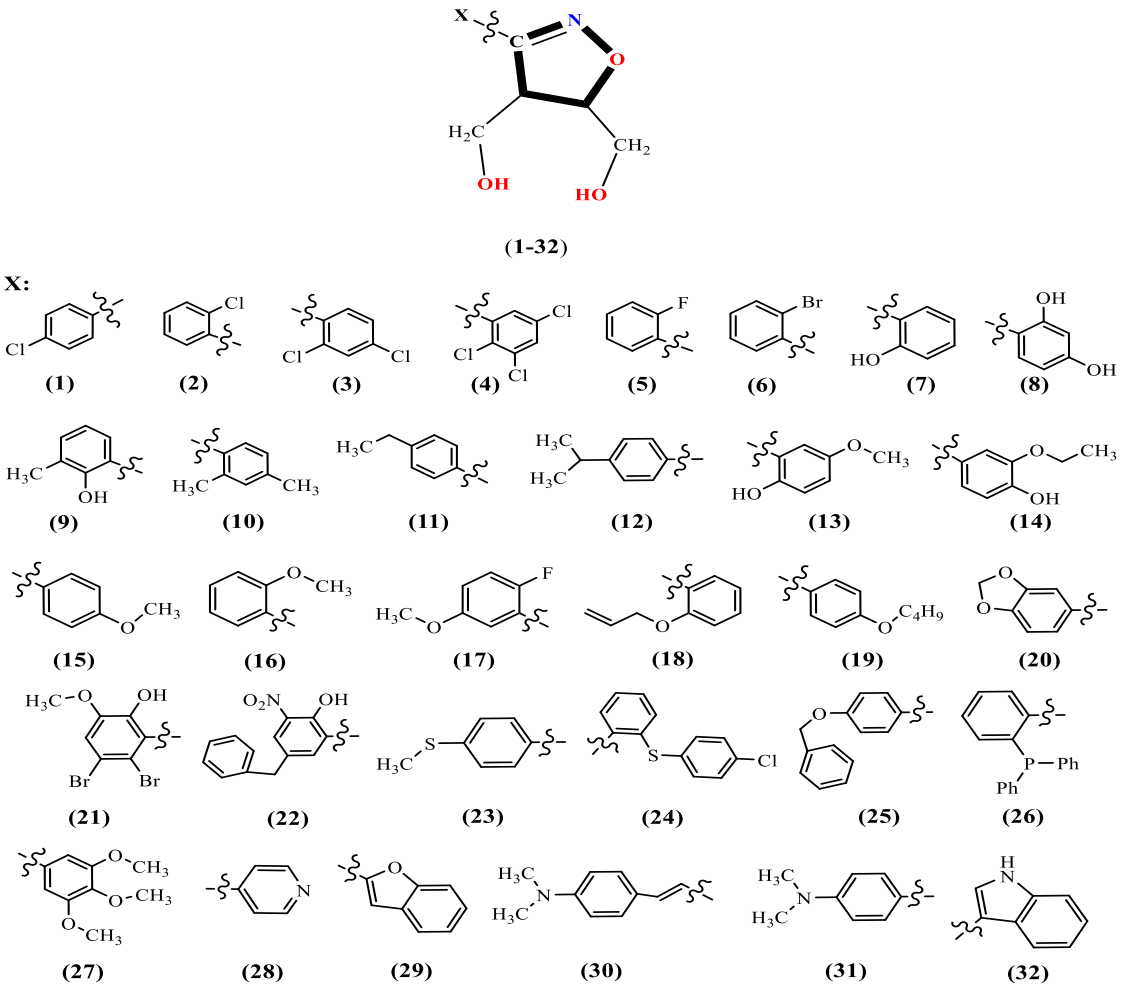

Figure 1. Chemical structures of 4,5-dihydroisoxazole-4,5-diyl dimethanol compounds (**1-32**).

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2. EXPERIMENTAL

2.1. Materials

The synthesis, analytical, and spectroscopic data of compound (**1**) (Figure 1), which we synthesized in our previous study[38], are available in the literature.

2.2. Antifungal Agent

Compound (**1**) was dissolved in sterile 100% ethanol (Sigma) at the stock solution of 2000 µg ml^{-1}. The final desired concentration (1600 µg ml^{-1}) was prepared with RPMI 1640 medium. The concentration ranges of compound (**1**) was 1600 to 0.04 µg ml^{-1}. In the studied concentrations, ethanol did not affect the microorganisms.

2.3. Antifungal Strains

The *T. mentagrophytes* and *M. canis* strains used in antifungal activity were obtained from the Faculty of Veterinary Medicine, Department of Microbiology Culture Collection, Istanbul University-Cerrahpasa. Isolation and identification were done by conventional methods. At the time of testing, the isolates were subcultured twice to achieve exponential growth and to ensure purity.

2.4. Antifungal Activity for *T. mentagrophytes* and *M. canis*

The broth microdilution method was used according to a standard protocol by the Clinical and Laboratory Standards Institute (CLSI) recommendation.[39] RPMI 1640 broth with L-glutamine without sodium bicarbonate was used. The medium was buffered to pH=7.0 at 25 °C with 0.165 M morpholinepropanesulfonic acid (MOPS). The preparation of inoculum suspensions of dermatophytes was based mainly on the CLSI guidelines[39] and the procedure in the literature.[40] The microdilution assay was performed in multiwell microdilution plates (sterile, disposable 96 U-shaped wells). Fluconazole (Sigma) was used as the control antimycotic (MIC=2 µg ml^{-1}), which was also obtained from the manufacturer.

Before testing, *T. mentagrophytes* and *M. canis* were subcultured onto Potato dextrose agar (PDA) plates were incubated at a temperature of 25±1 °C for 7-14 days (Figure 2). All tested strains grew well during this period. The fungal colonies were covered with ca. 10 ml of distilled sterile water, and suspensions were prepared by gently probing the surface with the tip of a Pasteur pipette. The resulting mixtures of conidia and hyphal fragments were withdrawn and transferred to a sterile tube. They were incubated at room temperature for 30 minutes to precipitate heavy particles and the upper homogeneous suspensions were collected and mixed with a vortex mixer for 15 seconds (Figure 3). The density of these suspensions was adjusted with a spectrophotometer at a wavelength of 530 nm to obtain inoculum quantification (65 to 75% T). These stock suspensions were diluted 1:50 in RPMI medium to obtain the final inoculum size, which ranges from $0.4x10^4$ to $5x10^4$ CFU ml^{-1} (colony forming unit). Microdilution plates were incubated at 25±1 °C for 7-14 days and were examined daily for the presence of fungal colonies. The lowest concentration that completely inhibits reproduction and can be determined with the naked eye was established as the MIC value (Table 1).

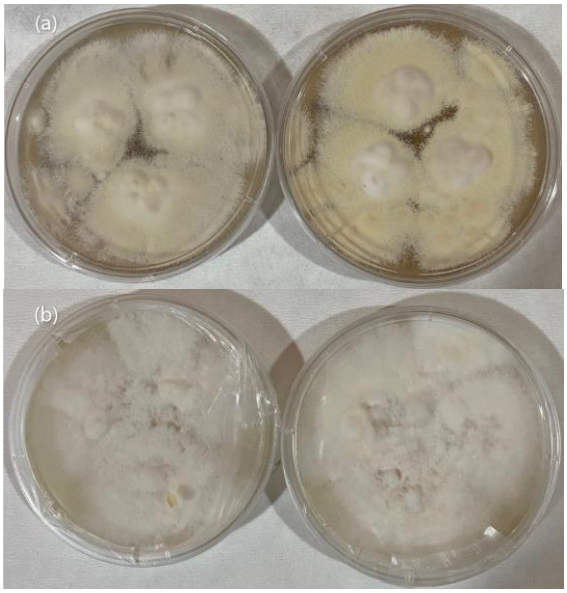

Figure 2. Images of (a) *T. mentagrophytes* and (b) *M. canis* strains used in the antifungal activity.

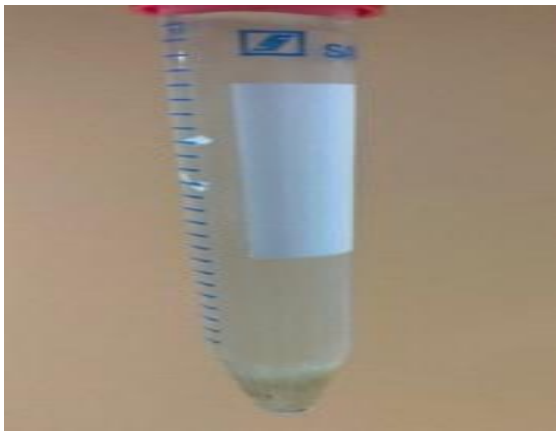

Figure 3. Image of mixture of conidia and hyphal fragments transferred into a sterile tube.

2.5. Structural and Theoretical Parameters

We also have studied the structural parameters of compound (**1**). The geometrical optimization of compounds (**1-32**) has been carried out by semi-empirical AM-1 parameterization in the Hyperchem package for calculations.[41] Some structural parameters [calculated log of octanol-water partition coefficient (*c*logP), Van der Waals Surface Area (Approx) (SAA), Van der Waals Molecular Volume (MV), Molar Refractivity (MR), Polarizability (polar), E_{HOMO}

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(energies of the highest occupied molecular orbital), E_{LUMO} (energies of the lowest unoccupied molecular orbital) and Mass] of the optimized compounds (**1-32**) were calculated by Hyperchem software AM-1 level (Table 2). Several derived parameters[42] were also calculated as below;

$$O\ (ovality) = \frac{SAA}{4\pi\left(\frac{3MV}{4\pi}\right)^{2/3}}$$

$$\chi\ (electronegativity) = \frac{E_{HOMO} - E_{LUMO}}{2}$$

$$\eta\ (hardness) = \frac{E_{LUMO} - E_{HOMO}}{2}$$

$$\omega\ (electrophilicity) = \frac{\chi^2}{2\eta}$$

$$\eta^{-1}\ (softness) = \frac{1}{\eta}$$

This parameter (*c*log P), the oldest and most widely used in quantitative structure-effect relationships analysis, is the logarithmic value of P, which defines the partition coefficient of the chemical compound in the organic (lipid) phase and the aqueous phase. For this reason, the *c*log P constant, which is used as a physicochemical parameter in quantitative structure-effect relationships analysis, enables the observation of linear relationships between the lipophilic properties and biological effects of the compounds in the investigated sequence. E_{HOMO} and E_{LUMO} values as various quantum mechanical parameters, which are calculated theoretically through semi-empirical or ab initio quantum chemical methods, are used in quantitative structure-effect relations analyzes.[43]

3.RESULTS and DISCUSSION

The microplates were incubated at 25±1 °C and were read at 7-14 days of incubation. The MICs were determined by visual inspection of the growth inhibition of each well compared with that of the growth control (drug-free) well. The MIC endpoints were determined for each isolate in Table 1; **(i)** *T. mentagrophytes*, MIC=50 µg ml^{-1}; **(ii)** *M. canis*, MIC=12.5 µg ml^{-1}, respectively. The obtained MIC values (Table 1) for compound (**1**) and calculated various structural parameters (Table 2) showed the relationship between the structure and the activity. In addition to computational results, future QSAR studies with theoretically designed dihydroisoxazole derivatives (**2-32**) will also give us important info concerning the organic-structural typicals of the dihydroisoxazole compounds.

Table 1. In vitro antifungal activity data of compound (1).

Compound and Reference	Microorganisms / MIC in µg ml^{-1}	
	T. mentagrophytes	*M. canis*
1	50	12.5
Fluconazole	2	2

Also, in structural calculations for *c*logP, the largest positive value (*c*logP=2.18) was found for molecule (**12**), which is containing isopropyl group, and the smallest negative value (*c*logP= –1.68) was found for molecule (**27**), which is containing three methoxy groups, respectively.

The increasing number of reports of fungal infections among immunocompromised patients and additional problems associated with toxicity and resistance to standard antifungal drugs cause an urgent need for the development of novel safe and effective antifungals.[44] As a result, the limited number of antifungal drugs used in the treatment of dermatophyte infections and the failure of these treatments in some cases has led to the search for new therapeutic resources in recent years. This situation leads researchers to find new antifungal drugs with more effective and broad-spectrum antifungal effect. Therefore, we believe that compound (**1**), which is an easily synthesizable molecule with broad-spectrum antimicrobial activity[38,45,46], and its new designed derivatives (**2-32**) may have an important place among anti-infectious agents in the coming years.

Table 2. Calculated and derived structural parameters of compounds (1-32).

Comp.	clogP	Mass (amu)	E_{HOMO} (eV)	E_{LUMO} (eV)	SAA (Å^2)	MV (Å^3)	MR (Å^3)	Polar (Å^3)	ω (eV)	χ (eV)	η (eV)	η^{-1} (eV^{-1})	O
1	1.07	241.67	-9.27	-0.54	229.8	202.7	63.9	23.61	2.18	-4.36	4.36	0.23	1.377
2	1.07	241.67	-9.70	-0.45	232.6	202.4	63.9	23.61	2.32	-4.63	4.63	0.22	1.395
3	0.85	276.12	-9.42	-0.67	246.7	216.6	68.7	25.54	2.19	-4.38	4.38	0.23	1.414
4	0.63	310.56	-9.61	-0.77	263.3	230.7	73.4	27.46	2.21	-4.42	4.42	0.23	1.447
5	0.70	225.22	-9.33	-0.44	219.5	191.1	59.4	21.59	2.23	-4.45	4.45	0.22	1.368
6	1.35	286.13	-9.73	-0.48	237.8	209.6	66.8	24.31	2.32	-4.63	4.63	0.22	1.393
7	0.27	223.23	-9.23	0.04	223.2	194.4	60.9	22.32	2.32	-4.64	4.64	0.22	1.375
8	-0.75	239.23	-9.37	-0.33	233.9	201.2	62.5	22.95	2.26	-4.52	4.52	0.22	1.408
9	0.43	237.26	-9.20	-0.00	244.5	211.3	65.2	24.15	2.30	-4.60	4.60	0.22	1.425
10	1.60	235.28	-9.25	-0.29	260.5	221.5	67.8	25.35	2.24	-4.48	4.48	0.22	1.471
11	1.85	235.28	-9.22	-0.43	257.7	221.9	68.2	25.35	2.20	-4.40	4.40	0.23	1.454
12	2.18	249.31	-9.27	-0.38	278.4	238.7	72.7	27.18	2.23	-4.45	4.45	0.22	1.496
13	-0.72	253.25	-8.75	-0.39	258.3	219.3	67.2	24.79	2.09	-4.18	4.18	0.24	1.468

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14	-0.38	267.28	-8.89	-0.46	280.3	236.4	71.9	26.62	2.11	-4.22	4.22	0.24	1.516
15	0.30	237.26	-8.88	-0.22	248.9	212.9	65.6	24.15	2.17	-4.33	4.33	0.23	1.443
16	0.30	237.26	-9.63	-0.21	251.9	213.2	65.6	24.15	2.36	-4.71	4.71	0.21	1.459
17	-0.30	255.25	-9.32	-0.45	257.6	215.6	65.8	24.06	2.22	-4.44	4.44	0.23	1.481
18	1.04	263.29	-9.70	-0.26	282.2	241.9	74.8	27.63	2.36	-4.72	4.72	0.21	1.503
19	1.51	279.34	-8.84	-0.20	311.2	263.5	79.5	29.66	2.16	-4.32	4.32	0.23	1.565
20	-0.50	251.24	-8.95	-0.47	236.5	210.8	64.9	24.01	2.12	-4.24	4.24	0.24	1.380
21	-0.62	411.05	-9.33	-0.59	300.5	261.4	82.3	30.04	2.19	-4.37	4.37	0.23	1.520
22	0.46	358.35	-9.71	-1.10	349.1	304.6	100.2	35.53	1.66	-3.31	3.31	0.30	1.594
23	0.65	253.32	-8.25	-0.39	257.4	222.8	72.1	26.51	1.97	-3.93	3.93	0.25	1.448
24	0.92	349.83	-8.51	-0.86	286.4	291.9	101.4	36.27	1.92	-3.83	3.83	0.26	1.346
25	1.32	313.35	-8.98	-0.31	323.0	287.4	94.4	33.81	2.17	-4.34	4.34	0.23	1.533
26	0.94	391.41	-7.97	-0.42	382.8	354.3	129.2	41.39	1.89	-3.78	3.78	0.26	1.581
27	-1.68	297.31	-8.87	-0.52	320.7	261.9	78.4	29.10	2.09	-4.18	4.18	0.24	1.620
28	-0.18	208.22	-9.73	-0.53	206.8	183.6	55.7	20.97	2.30	-4.60	4.60	0.22	1.324
29	-0.27	247.25	-9.01	-0.83	237.9	214.7	69.6	25.02	2.05	-4.09	4.09	0.25	1.372
30	0.76	276.34	-8.20	-0.29	313.3	262.7	83.1	30.18	1.98	-3.96	3.96	0.25	1.579
31	0.35	250.30	-8.41	-0.23	278.1	233.9	72.9	26.70	2.05	-4.09	4.09	0.25	1.515
32	-0.54	246.27	-8.56	-0.31	243.3	218.3	70.6	25.73	2.07	-4.13	4.13	0.24	1.387

4.CONCLUSION

It has become mandatory to search for better therapeutic agents with antifungal effects and to find new solutions. Our results demonstrate that dihydroisoxazole dimethanol compound showed in vitro antifungal activity against *T. mentagrophytes* and *M. canis*. Therefore, further studies should be carried out to confirm the usefulness of these dihydroisoxazole dimethanol compounds *in vivo*.

Conflict of Interest

The authors declare that they have no conflict of interest.

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