Research Paper

2,3-Dimethylquinoxaline: A Natural Antifungal Drug with the Potential to Treat Eumycetoma, Oral Candidiasis and Other Fungal Infections

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To evaluate the pharmacokinetics of 2,3-dimethylquinoxaline using web server Swiss absorption, distribution, metabolism, and excretion, safety profile. Moreover, to assess the efficacy of 2,3-dimethylquinoxaline (a natural quinoxaline derivative) *in vitro* and *in vivo*. The absorption, distribution, metabolism, and excretion of 2,3-dimethylquinoxaline were predicted using the web servers Swiss absorption, distribution, metabolism, and excretion and ProTox-II, respectively. *In vitro* efficacy against *Madurella mycetomatis* and a wide range of other pathogenic fungi was determined by microdilution assay. *In vivo* activity was evaluated using a topical gel (1 %) in the Bagg Albino mice eumycetoma model and an oral candidiasis model. 2,3-dimethylquinoxaline showed favourable absorption, distribution, metabolism, and excretion drug-likeness features and a high safety profile. It inhibited *Madurella mycetomatis* at a minimum inhibitory concentration of 312 µg/ml and exhibited potent *in vitro* activity against a range of other fungi, including *Cryptococcus neoformans* (minimum inhibitory concentration=9 µg/ml) and *Candida tropicalis* is (minimum inhibitory concentration=1.125 µg/ml). *In vivo*, 2,3-dimethylquinoxaline gel was effective in treating both skin *Madurella mycetomatis* infection and oral candidiasis. 2,3-dimethylquinoxaline is a promising natural antifungal drug with the potential to treat eumycetoma and other fungal infections.

Key words: 2,3-dimethylquinoxaline, quinoxaline, antifungal, *Madurella mycetomatis*, *Candida tropicalis*

Fungal infections are a major global health problem, affecting millions of people each year and causing significant morbidity and mortality. They can range in severity from mild and superficial infections of the skin, hair, and nails to life-threatening invasive infections of the lungs, brain, and other organs. Invasive fungal infections can be fatal, especially in people with weakened immune systems[1].

The most common and life-threatening fungal infections are caused by *Candida, Aspergillus*, and *Cryptococcus*[2,3]. Mycetoma is a particularly challenging fungal infection that is endemic in the tropics and subtropics[4]. Limitations of current mycetoma treatment include; long duration of treatment, high risk of recurrence, side effects of medication limited efficacy expense, and lack of accessibility[5].

Most of the current antifungal drugs have one or more of the following significant limitations; severe adverse effects, parenteral administration e.g., polyenes, drug interactions[6]. Emerging resistance, relatively high cost of some antifungal formulations e.g., liposomal amphotericin[7] and limited skin permeability[8,9].

Quinoxaline derivatives are a versatile class of natural and synthetic compounds with a wide range of biological activities, including antimicrobial activity. Their ease of synthesis in the laboratory makes them a promising platform for the discovery of new antimicrobial drugs[10]. 2,3-Dimethylquinoxaline (DMQ) is a natural compound found in the *Chromolaena odorata* plant[11]. It is available commercially at a reasonable price. It has been shown

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Accepted 12 August 2024
Revised 29 December 2023
Received 02 April 2023
Indian J Pharm Sci 2024;86(4):1492-1500

to have antimicrobial activity, but its antifungal potential and pharmacological profile have not been fully explored[12].

This study aims to comprehensively study the pharmacokinetic, safety, *in vitro*, and *in vivo* antifungal potential of DMQ against different fungal infections. The results of this study could pave the way for the development of DMQ into a novel antifungal drug with a favourable pharmacological profile.

MATERIALS AND METHODS

DMQ, identified by CAS number 2379-55-7, is provided by Sigma-Aldrich in Taufkirchen, Germany (Catalog No: D184977). Hydroxypropyl Methylcellulose (HPMC) and all other components are of pharmaceutical grade quality.

Bacterial strain, growth condition and medium:

A clinical strain of the fungus *Madurella mycetomatis* (MM) was included in this study. This fungal isolate was cultured and kept on Sabouraud's Dextrose Agar (SDA) at a temperature of 37°. A total of 73 clinical isolates, featuring a broad range of pathogenic fungi, including yeasts such as *Candida albicans* (*C. albicans*), *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. auris*, *C. tropicalis*, C. *parapsilosis*, *Cryptococcus neoformans*, and molds including *Aspergillus fumigatus*, *Aspergillus niger*, and *Trichophyton mentagrophytes*, were employed in *in vitro* experiments. For both *in vitro* and *in vivo* investigations, a reference strain from the American Type Culture Collection (ATCC), *C. albicans* ATCC 10231, was utilized.

Plain and medicated DMQ hydrogel:

DMQ hydrogel refers to a hydrogel that contains a compound called 2 DMQ as one of its components. Hydrogels are three-dimensional, they can hold a significant amount of water while maintaining their structural integrity. They are commonly used in various medical and pharmaceutical applications due to their unique properties, including high water content and biocompatibility. In order to prepare plain hydrogel, 1 g of HPMC powder was added to an appropriate beaker and mixed with 49 g of purified water. The beaker was covered and left for 24 h at room temperature to allow the gel to form. To formulate DMQ hydrogel at 1 % w/w, 0.5 g of DMQ were dissolved in 4.5 g of methanol to make a final concentration of 100 mg/g. The drug solution in methanol was then gradually added to 45 g of the plain HPMC gel, with continuous gentle mixing. Both plain and medicated gels were stored in suitable umber glass jars at 8°[13].

In silico prediction of Absorption, Distribution, Metabolism and Excretion (ADME):

As they are a crucial aspect of drug discovery and development. It involves the use of computational methods and modelling techniques to predict how a potential drug compound will behave within the human body, with a particular focus on its pharmacokinetics and potential toxicological effects. Thus, the Structured Data File (SDF) for DMQ (CID16925, Simplified Molecular Input Line Entry System (SMILES) notation: CC1=NC2=CC=CC=C2N=C1C) was acquired from PubChem. SwissADME was employed for predicting its pharmacokinetic and associated parameters[14]. Toxicological data prediction was carried out using ProTox II[15].

In vitro antifungal susceptibility testing:

To assess the efficacy of DMQ *in vitro*, broth microdilution assay was used. In accordance with the guidelines set forth by the Clinical and Laboratory Standards Institute (CLSI), we conducted *in vitro* testing to assess the susceptibility of the pathogen to antifungal agents. To initiate this process, we first prepared a stock solution of the antifungal drug in Dimethyl Sulfoxide (DMSO). This concentrated solution was then meticulously diluted within Roswell Park Memorial Institute (RPMI)-1640 medium to generate a range of concentrations spanning from 0.303 to 312 µg/ml.

Concurrently, a suspension of the clinical isolate (referred to as MM) was meticulously prepared in RPMI-1640 medium and adjusted to a specific concentration of 10^4 cells/ml. Subsequently, 100 µl of this fungal suspension was dispensed into each well of a 96-well microtiter plate, followed by the addition of 100 µl of the antifungal drug solution into each respective well. The microtiter plate was carefully placed into an incubator, set to maintain a constant temperature of 37°, and left undisturbed for a duration of 24 h-36 h. After this incubation period, a visual examination of the microtiter plate was conducted to assess any signs of fungal growth. The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the antifungal drug that effectively inhibited 50 % of fungal growth[16,17].

In vivo activity against MM:

The experimental protocol was approved by the Biomedical Ethics Committee at King Abdulaziz

University (KAU) and the National Committee of Bioethics (NCBE) (Registration No: HA-02-J-008). All animal experiments were conducted in strict accordance with the ethical guidelines for animal care and use established by KAU and NCBE.

Animal model for infection by MM:

In this research, six male BALB/c mice, aged (6-8) w, were employed. Mice were purchased from Faculty of Pharmacy in KAU animal house and placed in specific-pathogen-free conditions with food and water.

A subcutaneous injection of 0.4 ml of 5×10^6 CFU MM suspension was administered into the neck of each mouse. The injection site was examined daily over a 2 w period to detect any lesion formation. Once MM infection was confirmed, a topical application of 1 % DMQ gel was provided to the lesion once a day, and the mice's lesions were subject to daily monitoring[18].

Animal model for oral candidiasis:

To assess the efficacy of the antifungal drug *in vivo*, a mouse model was used. The experimental procedure received official approval from both the Biomedical Ethics Committee at KAU and the NCBE, as evidenced by Registration No: HA-02-J-008. Every aspect of the animal experiments strictly adhered to the ethical principles and guidelines for animal care and use, as set forth by KAU and NCBE.

To assess the efficacy of DMQ gel against oral candidiasis, we employed a previously reported mouse model for the disease with minor adjustments[19]. Ten male BALB/c mice, aged 8 w, were selected for the development of oral candidiasis. Immunosuppression was induced in these mice with prednisolone (100 mg/kg subcutaneously) (Sigma-Aldrich P6004, Taufkirchen, Germany), 1 d before and 3 d after being infected with *C. albicans* ATCC 10231. Additionally, tetracycline hydrochloride solution at a concentration of 0.9 mg/ml (Sigma-Aldrich P6004, Taufkirchen, Germany) was administered orally to the mice starting 1 d before the infection.

Oral infection was initiated by swabbing the entire oral cavity of the mice with sterile cotton pads (baby cotton buds from Johnson and Johnson) saturated with a cell suspension (2×10^8 CFU/ml) of *C. albicans* ATCC 10231. The severity of the infection was assessed on a daily basis by evaluating the presence and extent of whitish, curd-like patches on the tongue surface.

After establishing the model, the mice were randomly divided into two groups, each consisting of 5 mice. One group received treatment with plain gel, while the other group was treated with a topical 1 % DMQ gel, administered daily for 7 d. The mice were closely monitored each day to determine whether they were cured or if their symptoms showed signs of improvement[18].

RESULTS AND DISCUSSION

The structure of DMQ and its predicted bioavailability was presented in fig. 1, physiochemical properties drug-likeness ADME, and bioavailability score are presented in Table 1 and Table 2.

In oral toxicity prediction; predicted Lethal Dose 50 (LD_{50}) of 500 mg/kg, predicted toxicity class was 4, (accuracy 67 %). The toxicity model report of DMQ was shown in Table 3.

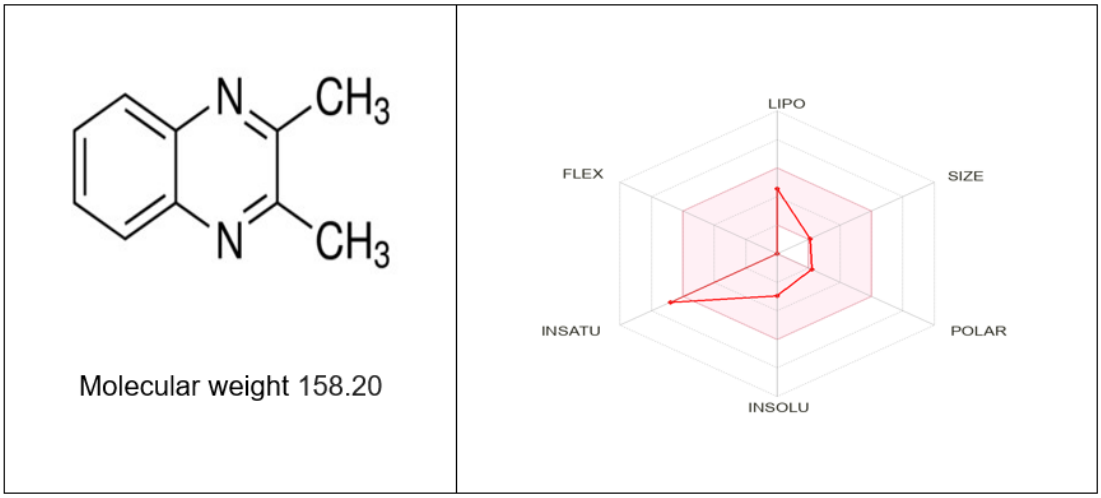

Fig. 1: The structure of DMQ and its predicated bioavailability radar, six physicochemical properties
Note: The pink represents the range of values of these six characteristics that consider a molecule as drug-like

TABLE 1: PHYSIOCHEMICAL PROPERTIES, LIPOPHILICITY AND WATER SOLUBILITY DRUG-LIKENESS OF DMQ

MW (g/mol)	H-bond donors	H-bond acceptors	Rotatable bonds	Consensus log P	TPSA (Å^2)	Water solubility	Drug likeness role		
							Lipinski	Vebers	Egan
158.20 g/mol	0	2	0	2.09	25.78 Å^2	Soluble or moderately soluble	Yes	Yes	Yes

Note: MW=Molecular Weight; consensus log P=average of all predicted log $P_{o/w}$ and TPSA=Total Polar Surface Area

TABLE 2: ADME DATA AND BIOAVAILABILITY

GI absorption	BBB permeant	Log Kp (cm/s)	CYP3A4 inhibitor	CYP2C19 inhibitor	CYP1A2 inhibitor	CYP2D6 inhibitor	CYP2C9 inhibitor	P-glycoprotein (P-gp) substrate	Bio availability score
High	Yes	-6.04	No	No	Yes	No	No	No	0.55

Note: GI=Gastrointestinal, BBB=Blood Brain Barrier and log Kp=Skin permeation. For comparison Log Kp cm/s of betamethasone=-7.32, diclofenac=-4.98 and ketoconazole=-6.46

TABLE 3: TOXICITY MODEL REPORT OF DMQ

Classification	Target	Prediction	Probability
Organ toxicity	Hepatotoxicity	Inactive	0.71
Toxicity endpoints	Carcinogenicity	Inactive	0.68
	Immunotoxicity	Inactive	0.99
	Mutagenicity	Inactive	0.5
	Cytotoxicity	Inactive	0.92
Tox21-nuclear receptor signaling pathways	Aryl hydrocarbon Receptor (AhR)	Inactive	0.8
	Androgen Receptor (AR)	Inactive	0.97
	AR Ligand Binding Domain (AR-LBD)	Inactive	0.88
	Aromatase	Inactive	0.8
	Estrogen Receptor Alpha (ERα)	Inactive	0.81
	Estrogen Receptor Ligand Binding Domain (ER-LBD)	Inactive	0.98
	Peroxisome Proliferator-Activated Receptor Gamma (PPAR-γ)	Inactive	0.79
	Nuclear factor (erythroid-derived 2)-like 2/Antioxidant Responsive Element (Nrf2/ARE)	Inactive	0.85
Tox21-stress response pathways	Heat Shock factor response Element (HSE)	Inactive	0.85
	Mitochondrial Membrane Potential (MMP)	Inactive	0.85
	Phosphoprotein (tumor suppressor) p53	Inactive	0.72
	ATPase family AAA Domain-containing protein 5 (ATAD5)	Inactive	0.96

The *in vitro* effectiveness of DMQ against MM was evaluated using the broth microdilution method. This approach involved testing various concentrations of DMQ to determine its MIC against MM. The findings revealed that the MIC of DMQ against MM was recorded at 312 µg/ml, equivalent to approximately 1.9 mM. This value signifies the concentration at which DMQ effectively inhibits the growth of MM in the test conditions.

A mice model was employed to evaluate the efficacy of DMQ in combating eumycetoma, a chronic fungal infection. The study involved two groups; one served as the control, while the other received treatment with a 1 % DMQ gel over a 14 d period.

In the initial stages, the infected mice developed distinct granulomas on their necks, which persisted despite treatment with a plain gel, indicative of the challenging nature of eumycetoma. However, in stark contrast, the mice treated with DMQ 1 % gel exhibited a remarkable outcome. After the 14 d treatment regimen, the granulomas had completely disappeared, leaving behind only a minor scar. This observation suggests that DMQ holds promise as a therapeutic agent for effectively combating eumycetoma, a condition typically known for its persistent and treatment-resistant nature (fig. 2A and fig. 2B).

DMQ demonstrated a wide-ranging and comprehensive antifungal activity, MIC across a spectrum of fungal species. The MIC values varied between 9-1125 µg/ml when tested against these microorganisms (Table 4).

An examination of the mice model revealed a noteworthy trend in infection severity 3 d post-infection. Specifically, mice subjected to treatment with DMQ 1 % gel displayed a conspicuous reduction in infection severity when compared to the control group. Intriguingly, no viable *Candida* Colony-Forming Units (CFUs) were detected in the oral cavities of the mice treated with the medicated gel, indicating a potent antifungal effect.

Furthermore, an assessment of the dorsal tongue surfaces on 5 d post-infection presented an interesting observation. These surfaces exhibited a glossy and regular appearance in the group treated with DMQ 1 % gel (fig. 3), signifying a potential therapeutic impact of DMQ in maintaining the integrity of the dorsal tongue epithelium and countering the progression of *Candida* infection.

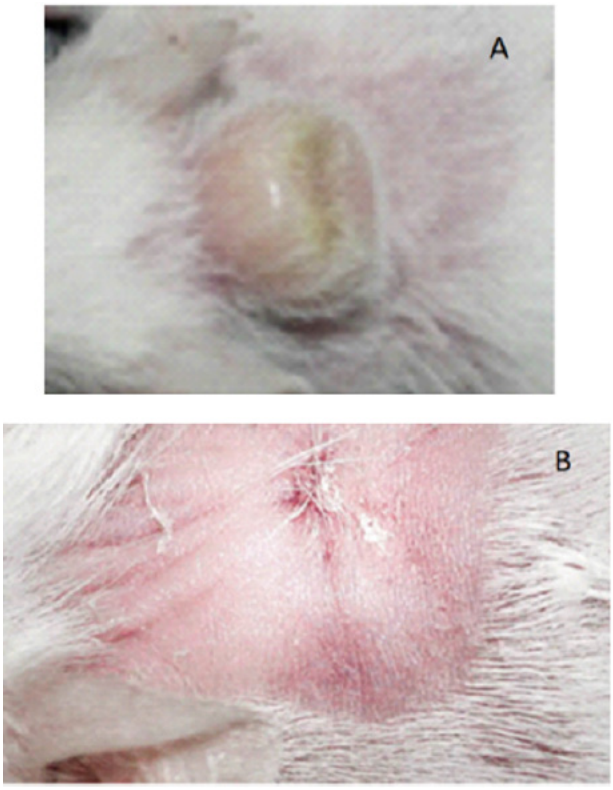

Fig. 2: A mouse model of eumycetoma, control and after DMQ 1 % gel treatment for 14 d. (A): Control mouse with granuloma (vehicle) and (B): Mouse after treatment with medicated gel-granuloma resolved left a scar

TABLE 4: *IN VITRO* ACTIVITY OF DMQ AGAINST SEVERAL FUNGAL SPECIES

Fungal species	MIC (µg/ml)	
	24 h	48 h
C. neoformans	9	9
Aspergillus fumigatus	370	370
Aspergillus niger	750	750
C. albicans ATCC 10231	190	370
C. albicans	854	935
C. auris	280	370
C. glabrata	470	560
C. krusei	370	560
C. parapsilosis	560	750
C. tropicalis	935	1125
Trichophyton mentagrophyte	750	750

Fig. 3: Effect of DMQ gel on oral candidiasis in mice
Note: Mice that were not treated with DMQ gel had thick lesions on their tongues (left panel). Mice that were treated with DMQ gel had tongues that looked almost healthy (right panel)

In silico ADME methods can help to screen compounds for drug-likeness of new compounds and optimize profile[20]. In the present study DMQ showed good Lipophilicity (LIPO) and skin permeation ability, suggesting its ability to reach target tissues at an appropriate concentration. In contrast, amphotericin B has minimal effect on MM, although it has excellent *in vitro* activity, explained by the limited drug distribution into the infected tissues[21].

In silico toxicology is essential to the evaluation of the toxicity and safety of substances as well as the process of developing new drugs. The capabilities and utility of computational techniques to predict toxicology continue to grow. These cutting-edge methodologies are used at different stages of the creation of a substance to forecast features that correlate with toxicity endpoints, construct and retrieve data from chemical databases, and model structure-activity relationships for new chemical formulations[22].

DMQ also showed a favourable toxicology profile, LD_{50} 500 mg/kg, with potential hepatoxicity, carcinogenicity, immunotoxicity, mutagenicity among other enzymes pathways. Moreover, it doesn't affect most Cytochrome P450 (CYP450) and P-gp. Good water solubility, and bioavailability factor. These features made DMQ a good candidate for oral administering.

In silico predictions in this study suggest that DMQ inhibits the CYP1A2 enzyme. A few studies have

shown that DMQ can induce the hepatic P-450 enzyme system[23,24].

It has been evaluated the mutagenicity potential of DMQ, along with 33 other quinoxaline and quinoline compounds, using the *Salmonella*/microsome assay[25]. Their results are consistent with our *in silico* predictions, which show that our tested compound has no risk of mutagenicity.

In addition to their safety profile, one of the most important characteristics of quinoxalines is their ability to reach target tissues at effective concentrations[26]. In contrast, amphotericin B has little or no value for treating deep-seated infections, despite its excellent *in vitro* activity. This is because amphotericin B does not distribute well into infected tissues[21].

The treatment of eumycetoma is challenging. It includes chronic therapy in addition to surgery. Itraconazole is usually indicated and terbinafine was considered as alternative drug with these antifungal plus surgery is low (25 %-30 %)[27]. In about 1/3rd of eumycetoma cases recurrence was documented[28]. Few studies suggested better clinical outcome with voriconazole or posaconazole, but low efficacy in case of liposomal amphotericin B[29]. Frequently patients develop extensive fibrosis, which retread the access of these antifungals to the infected tissues[30]. Regardless of variable efficacy of the aforementioned antifungals and taking into account the chronic nature of the disease, the patients likely suffer a long list of adverse effects associated with systemic use of azole antifungals or amphotericin B especially in patients with liver or renal impairment[19].

Given these issues, there is a rational need for antifungal medications with favourable pharmacokinetic and safety profiles antifungal agents *in vitro* high efficacy against MM, namely ravuconazole (MIC_{50} 0.008 mg/l) and olorofim (MIC_{50} 0.016 mg/l)[31,32]. Further studies with ravuconazole documented higher efficacy compared to itraconazole[32]. Olorofim inhibit pyrimidine biosynthesis pathway by targeting dihydroorotate dehydrogenase enzyme[33].

Extensive efforts of drug development for novel medication against MM leads to identifying 400 compounds effective at a concentration of 100 µM and 26 had an half-maximal IC (IC_{50}) <8 µM. Of these compounds 9 demonstrated *in vivo* efficacy[3]. Most studies focused on 6 compound series; aminothiazoles, phenothiazines, fenarimols, benzimidazoles ketoximes and antifolates[34]. Other promising antifungal against MM include; luliconazole and lanoconazole (median MICs 0.001-0.064 µg/ml for both) and ravuconazole (median MICs 0.008-2 µg/ml) fenbendazole and carbendazim (benzimidazole carbamates), tafenoquine (8-aminoquinolone derivative) and MMV1578570[35,36]. Few natural products were reported to showing *in vitro* activity against MM e.g., is a synthetic Cinnamon oil blend (CIN-102), with MICs ranging from <32 µg/ml to 512 µg/ml[37].

The antibacterial activity of quinoxaline derivatives were reported[38]. In 2002, researchers discovered that quinoxalines have the potential to be effective antifungal drugs against *Candida* species[39]. Other studies have shown that synthetic quinoxalines with two substituents at positions 2 and 3 of the ring have excellent antifungal activity against *Fusarium oxysporum*[40]. The inhibition of Topoisomerase II (Topo II) is one among other explanation for the mode of action of quinoxaline against eukaryotic organisms[41].

For the first time we showed that DMQ is a promising antifungal drug against mycetoma and other fungal infections *in vitro* and *in vivo*, with excellent pharmacokinetic and safety profile. Further work is needed to identify its mechanism, *in vivo* pharmacokinetics. Further work is recommended to explore the molecular pathway of DMQ antifungal activity.

Conflict of interests:

The authors declared no conflict of interests.

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