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THIOUREA COMPOUND AS AN ALTERNATIVE TREATMENT FOR SPOROTRICHOSIS

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THIOUREA COMPOUND AS AN ALTERNATIVE TREATMENT FOR SPOROTRICHOSIS

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CERTIFICATION

It is hereby certified that I have read this project paper entitled "Thiourea compound as an alternative treatment for sporotrichosis", by Thusahtani A/P Ragu and in my opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999-Final Year Project.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek.

KOMPOUN THIOUREA SEBAGAI RAWATAN ALTERNATIVE UNTUK SPOROTRIKOSIS

Oleh

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2021

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Kompaun thiourea memainkan peranan penting dalam perubatan kimia dan pertanian disebabkan aktiviti biologi dalam kompaun tersebut seperti antibakteria, antikulat, antivirus, racun rumpai, rodentisida, inhibitor enzimatik fenoloksidase, agen anti-HIV dan anti-tumor. Oleh itu, dalam kajian ini tiga sebatian benzoiltiourea; glycinebenzoylthiourea, sarcosinebenzoylthiourea and N,N-bis(hydroxyethyl)benzoylthiourea telah disaring untuk mengenal pasti potensi aktiviti anti-kulatnya terhadap *S. schenckii.* Kaedah resapan cakera digunakan untuk menilai kehadiran zon perencatan diikuti dengan kaedah larutan mikro bagi menentukan kepekatan perencat minimum (MIC). Aktiviti anti-kular terhadap *S. schenckii* selanjutnya telah dikenal pasti melalui proses inokulasi pada agar Dekstrosa

Sabouraud (SDA) bagi menentukan kepekatan fungisidal minimum (MFC). Hasil kajian kami menunjukkan bahawa ketidakhadiran zon perencatan oleh ketiga-tiga benzoylthiourea pada kesemua kepekatan berbeza. Kompaun-kompaun benzoylthiourea yang dikaji tidak menunjukkan aktiviti anti-kulat kerana diameter ukuran zon perencatan kurang daripada zon perencatan kawalan positif. Di samping itu, MIC dan MFC untuk glycinebenzoylthiourea, sarcosinebenzoylthiourea and N,N-bis(hydroxyethyl)benzoylthiourea terhadap *S. schenckii* adalah >0.0004M yang merupakan kepekatan tertinggi digunakan dalam eksperimen ini. Justeru, kajian ini menunjukkan glycinebenzoylthiourea, sarcosinebenzoylthiourea and N,N-bis(hydroxyethyl)benzoylthiourea, dalam kepekatan 0.0004M – 0.00005M, tidak mempunyai aktiviti anti-kulat terhadap *S. schenckii.*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4999-Final Year Project.

THIOUREA COMPOUND AS AN ALTERNATIVE TREATMENT FOR SPOROTRICHOSIS

By

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2021

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Thioureas play a significant role in medicinal chemistry and agriculture due to their biological activities such as antibacterial, antifungal, antiviral, herbicides, rodenticides, phenoloxidase enzymatic inhibitors, anti-HIV and anti-tumor agents. Three types of benzoylthiourea derivatives; benzoylglycine, benzoylsarcocine and benzoyldiethanol were screened for their potential antifungal activity against *Sporothrix schenckii*.

Disc diffusion method was used to detect the presence of zone of inhibition followed by broth microdilution to determine minimum inhibitory concentration (MIC). The antifungal effects to *S. schenckii* were further assessed by inoculating onto Sabouraud Dextrose Agar (SDA) to determine the minimum fungicidal (MFC). There was no presence of zone of inhibition by all three benzoyl-thiourea. All compounds showed no antifungal activity since the diameter of inhibition zone measure was less than positive control inhibition zone. The MIC and MFC for benzoylglycine, benzoylsarcocine and benzoyldiethanol against *S. schenckii* were >0.0004M which was the highest concentration that was used in this experiment. The study demonstrated that benzoylglycine, benzoylsarcocine and benzoyldiethanol, with concentrations within 0.0004M – 0.00005M did not possess antifungal properties against *S. schenckii*.

Key words: Thiourea, antifungal activity, susceptibility testing, sporotrichosis

1.0 INTRODUCTION

Sporotrichosis is caused by *Sporothrix schenckii* which is a dimorphic fungus. Sporotrichosis can be transmitted through traumatic injury from the inoculation of contaminated soil, plants and organic matter with fungus (Barros *et al.*, 2011). It is an important zoonotic disease in which the mode of transmission to humans derived from infected cat scratches or bites (Read and Sperling, 1982). Itraconazole is a standard antifungal that has been recommended in first-line therapy. However, Ferreira *et al*. (2019) stated that failure of itraconazole in human and feline treatment in recent years might due to the fungus being resistant to it, which is a major concern in therapeutic strategy (Vandeputte *et al*., 2012). Potentially may be contributed by point mutation in either drugs target or transcription factors regulating the resistance could be the factors of apparent antifungal resistance (Vandeputte *et al.*, 2012). As a result, it has caused failure in the treatment of infectious diseases. In that respect, there is growing interest in discovery of new antifungal agents. Hence, studies have been done to find other alternative antifungal agents including thiourea compounds. Thiourea, an emerging class of compounds, were first synthesized by Neucki. According to the World Health Organization (WHO) (2003), thiourea compounds exhibit pharmaceutical values including antiviral, antibacterial, fungicidal, herbicidal and anti-HIV. A study done by Danowski and Tager in 1948 showed that thiourea compounds interfere with the growth of fungi on agar culture medium. Thus, the aim of this project is to determine the potential antifungal activity of thiourea

compounds as an alternative in antifungal treatment to fight the infection caused by *S. schenckii*.

Therefore, the main objective of this study is to identify antifungal effects of thiourea compounds against Sporothrix schenckii through minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) broth dilution technique.

2.0 LITERATURE REVIEW

2.1 Sporotrichosis

Sporotrichosis is an infection caused by dimorphic fungi, *Sporothrix spp* which may lead to subacute or chronic infection and the most prevalent and known species affecting animals and humans in Malaysia to date is *S. schenckii* (Tang *et al.,* 2012). According to Barros *et al.,* (2011), *Sporothrix schenckii* is a eukaryotic organism that belongs to kingdom Fungi. *S. schenckii* can be found in both filamentous as well as yeast form. Filamentous form is composed of hyaline and septate hyphae and the colonies can be observed as smooth to wrinkled, white to creamy at first and later turning to brown to black after a few days within 7 days. Apart from that, this fungus can also be found in yeast form in both human and animal tissues. Microscopically, it appears as round to oval and usually has elongated buds. It is known that traumatic cutaneous inoculation by contacting the fungus from an infected cat or contaminated environment are the common route of transmission. Edmund and Robert (1990) stated sporotrichosis can be divided into three clinical forms; cutaneolymphatic, cutaneous and disseminated.

2.2 Treatment for Sporotrichosis

Potassium iodide and itraconazole are the usual drug used for treating sporotrichosis (Mathias *et al*., 2020). There was a report describing a combination of treatment approach comprising of surgical excision, application of amphotericin and oral administration of sodium iodide which showed the importance of treating the infection. Besides, itraconazole is a broad spectrum triazole antifungal agent, that is also the drug of

choice for cutaneous and lymphangitic sporotrichosis. In general, patients can tolerate well to itraconazole with doses of up to 400 mg/day without adverse effects. However, there is possibility for the drug interaction, mediated through the cytochrome P450 enzyme 3A4 system, which should be used as part of a multi-drug regimen. (Piegard *et al*., 2000).

2.3 Antifungal susceptibility testing

According to European Committee of Antimicrobial Susceptibility Test (EUCAST), (2002), antifungal susceptibility tests were performed on pathogenic fungi that cause disease especially when infections are invasive, relapsing or failing therapy, when acquired resistance is possible or when susceptibility cannot be predicted merely from species identification. Disc diffusion method is commonly used to determine the susceptibility of fungus to various anti-fungal agents. Empty discs preloaded with anti-fungal drugs were preliminarily used to determine the optimal concentration that produced inhibition zones (Nweze *et al*., 2010). Broth dilution methods were used to determine the minimum inhibitory concentrations (MICs) of anti-fungal agents. Minimum inhibition concentration (MIC) is defined as the lowest concentration of antimicrobial agents that can inhibit the growth of microorganism. EUCAST (2002) proposed that in this susceptibility test, the fungal will be tested to determine if it can grow in micro-titration plate wells of broth that contain serial dilution of anti-fungal agents.

2.4 Antifungal resistance

Over the past three decades, the incidence of fungal infections has increased persistently and was simultaneously accompanied by increasing acquired and innate

resistance to antifungal drugs (Vandeputte *et al*., 2012). However, occurrence of antifungal resistance is independent for each fungal genus as well as each antifungal class.

Based on CLSI MICs data of antifungal agent available worldwide, Espinel-Ingroff *et al.,* (2017) characterized the epidemiological cutoff values (ECVs). ECVs allows identifying the non-wild type (non-WT) of *Sporothrix sp.* with reduced susceptibility to antifungal agent due to acquired mutational resistance or resistance mechanism from wild-type (WT) isolates, which are isolates with no acquired resistance mechanism.

Table 1: Epidemiological cutoff value (ECVs) of different antifungal agents for identifying the wild-type (WT) and non-wild-type (non-WT) strains of *Sporothrix schenckii* and *Sporothrix brasiliensis,* (Adopted from research done by Espinel-Ingroff *et al.,* 2017)

Sporothrix species	Antifungal agents†	ECVs*	
		WT (µg/mL)	Non-WT (µg/mL) §
S. schenckii	AMB	≤ 4	>4
	ITR	≤ 2	>2
	POS	≤ 2	>2
	VOR	≤ 64	> 64
S. brasiliensis	AMB	≤ 4	>4
	ITR	≤ 2	>2
	POS	≤ 2	>2
	VOR	≤ 32	> 32
	KET	≤ 2	>2
	TERB	≤ 0.12	>0.12

AMB, amphotericin B; ITR, itraconazole; POS, posaconazole; VOR,voriconazole; KTZ, ketoconazole; TRB, terbinafine

Non-WT strains are recognized as Sporothrix spp. strains with reduced susceptibility to antifungals and, therefore, less likely to respond to the antifungal therapy.

According to a study done by Vettorato *et al.*, (2017), 9.8 % of clinical isolates of *S. schenckii* senso stricto and 6.5% of Brazilian isolates had MIC equal to or greater than 4 µg/ml for itraconazole. Based on ECV of 2 µg/ml for itraconazole indicating non-wild type, this can be considered as reduced susceptibility to antifungals. Apart from that, a study conducted by Gutierez-Galhardo *et al.,* (2010) where 91 Brazilian and Spanish *S. schenckii* strains using microdilution tests by the EUCAST, showed the emergence of antifungal-resistant isolates of *S. schenckii* to fluconazole, itraconazole, voriconazole, posaconazole and terbinafine.

Although the mechanism of antifungals resistance is not fully elucidated, the resistance development on *Sporothrix spp.* is related to the melanin production capacity (DHN-melanin, L-DOPA-melanin, and pyomelanin). According to Almeida-Paes *et al.* (2012), DHN-melanin, L-DOPA-melanin, or pyomelanin is associated with lower susceptibility to amphotericin B. Besides, melanin can also protect *Sporothrix spp.* from the antifungal effect of terbinafine. Next, genetic diversity plays an important role in acquiring resistances against antifungals. Prolonged exposure to antifungals and host immunity against pathogenic fungi cause selective pressure. This can eventually lead to

chromosomal polymorphism in *Sporothrix spp* for the acquisition of variability of antifungal susceptibility profiles (Sasaki *et al.,* 2014). Lastly, mutations in cytochrome P450 that involve in ergosterol biosynthesis can cause development of antifungal resistances. Azole antifungal agents act by inhibiting cytochrome P450 monooxygenases (Kelly *et al.,* 1995). In a study conducted by Matowane *et al.* (2018), showed that mutations at the itraconazole binding site associates with increased resistance against azoles.

2.5 Thiourea

Thiourea was first synthesized by Neuki in 1873 (Normark and Normark, 2002). According to the World Health Organization (WHO) (2003), thiourea derivatives represent a well-known important application in fields of medicine, agriculture and analytical chemistry. These compounds also hold a wide range of biological activities such as antiviral, antibacterial, fungicidal, analgesics, herbicidal, plant growth regulating, anti-aggregating, antiarrhythmic, local anesthetic and anti-hyperlipidemic activities. Thiourea is an analogue compound to urea as it is synthesized by replacing the oxygen atom in urea by a sulfur atom (Alkan *et al*., 2011). The properties of thiourea and urea differ significantly in electronegativity between sulfur and oxygen. Recently, substituted thiourea gained much interest as it may have significant attribution in preparation of biologically active compounds. Alkherraz *et al*., (2014) suggested that one of the important thiourea derivatives is benzoyl-thiourea compounds as the exhibit a comprehensive range of biological activities including antiviral, antibacterial, antifungal, anti-tubercular, herbicidal, insecticidal and pharmacological properties and also acting as chelating agents.

3.0 MATERIALS AND METHODS

3.1 Preparation molar concentration of Thiourea compound

Stock solution with 0.0004M was prepared by diluting 2mg of benzoylglycine, benzoylsarcosine, and benzoyldiethanol in 0.1ml of dimethyl sulfoxide (DMSO) and 19.9ml of distilled water. Then, four different concentrations were prepared aseptically from stock solution in order to be tested.

3.2 Fungal isolation

Clinical isolates of *Sporothrix schenckii* from stock culture were sub-cultured onto Sabouraud Dextrose Agar (SDA) where the fungus was able to sporulate sufficiently and kept them at room temperature for 5-7 days.

3.3 Inoculum suspension

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2020) suggested that inoculum suspensions are prepared from fresh, mature (2-5 day-old) cultures. However, in some cases, an extended incubation period is needed for proper sporulation of isolates. The conidia from pure culture was transferred aseptically using sterile swab into 3-4ml of sterile distilled water in a test tube and mixed thoroughly. Using 0.5 McFarland, the turbidity of the inoculum was compared.

3.4 Disc diffusion method

Inoculum of *Sporothrix schenckii* were then streaked evenly on Sabouraud dextrose agar (SDA) using sterile swab. Ten microliter of benzoylglycine with concentration of 0.0004M, 0.0002, 0.0001M, 0.00005M were dropped onto the empty discs using

micropipette. Discs were then adhered onto the Sabouraud dextrose agar (SDA) media containing *S. schenckii* aseptically using sterile forceps. Each disc was adhered to each individual inoculated SDA plate. The steps were then repeated for benzoylsarcosine and benzoyldiethanol. The culture plates were then kept at room temperature for 7 days. Itraconazole (32mg/ml) was used as a control. The whole procedure was repeated thrice to ensure reproducibility. Antimicrobial activity was evaluated by observing and measuring the diameter of zones of inhibition (ZOI) using a digital caliper.

3.5 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

A sterile plastic, disposable, 96-well microdilution plates with flat-bottom with normal capacity of approximately 300µl were used. Two-fold dilution factor with nine serial thiourea dilutions in RPMI medium from stock solution were carried out using a multichannel pipette was done systematically. Wells No. 11 containing 100µl of inoculum and RPMI medium, served as positive control while wells No. 12 that contained RPMI medium and titrated solubilizing vehicle (DMSO) served as negative control. All assays were performed in triplicate. Quality control (QC) strains *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 were tested in parallel using the same concentration dilution of itraconazole (Kohler *et al.* 2006). Microplates were then incubated at 25˚C for 72 hours (Waller *et al*., 2018). In order to determine the minimum inhibitory concentration (MIC), the turbidity of well plates was observed and compared with positive and negative controls. MIC was designed as the lowest concentration that produced no visible fungal growth. Meanwhile, to determine the minimum fungicidal concentration (MFC), 10µL of

aliquots from each well was inoculated on Sabouraud Dextrose agar (SDA) and incubated at 25˚C for 5 days to allow the growth of fungi.

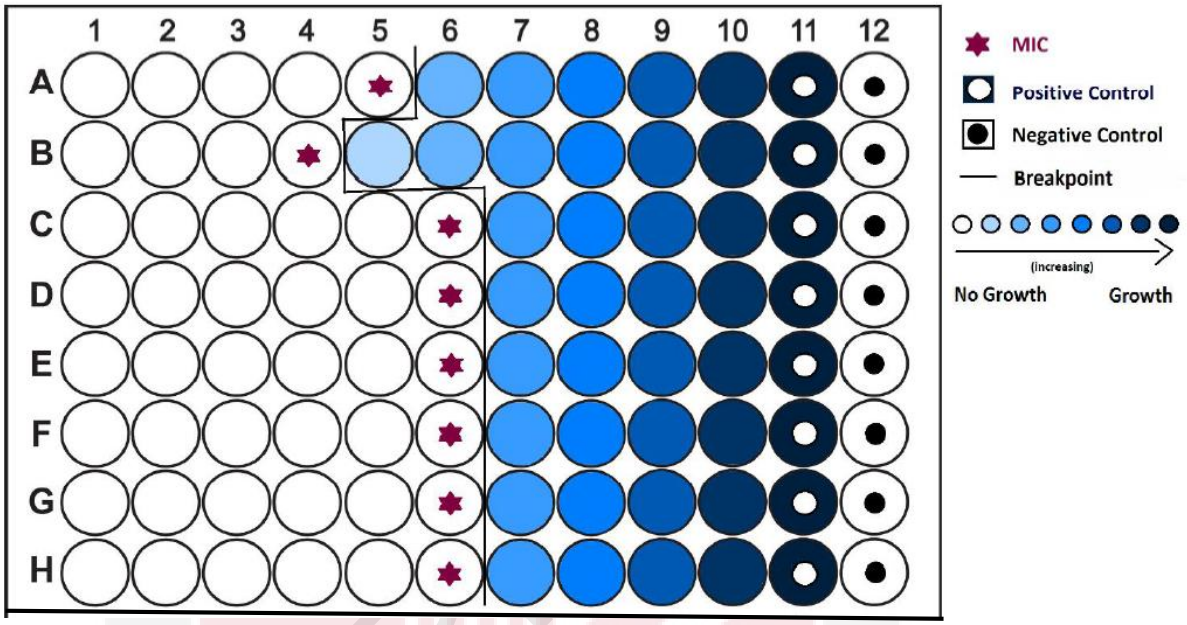

Figure 1: Example MIC microtiter plate. (Adopted from Emery Pharma, 2018)

4.0 RESULTS

Based on disc diffusion experiment, it was observed that there was no presence of ZOI seen on Sabouraud Dextrose Agar (SDA) plate streaked with *Sporothrix schenckii* against benzoylglycine, benzoylsarcocine, and benzoyldiethanol at concentrations of 0.0004 M, 0.0002 M, 0.0001 M, 0.00005M. As for itraconazole 32mg/ml, there was zone of inhibition produced on the agar streaked with *S. schenckii.*

Minimum Inhibitory Concentration (MIC) was determined based on the turbidity of the well plate. Meanwhile, Minimum Fungicidal Concentration (MFC) was determined by taking a loop of each suspension from each well including positive and negative

controls and plated onto SDA medium and incubate at 25˚C for 4-5 days. The MIC and MFC of benzoylglycine, benzoylsarcocine, and benzoyldiethanol against *S. schenckii* at concentrations from 0.0004 M to 7.813×10^{-6} M, was predicted to be > 0.0004 M. Meanwhile, itraconazole showed turbidity starting from 4 µg/ml, indicating its MIC is 8 µg/ml. *S. schenckii* started to show growth at a concentration of 4 µg/ml, indicating the MFC is 8 µg/ml. The MIC and MFC for itraconazole on both yeasts, *C. krusei* and *C. parapsilosis* were determined to be 16 µg/ml and 32 µg/ml, respectively.

5.0 DISCUSSION

The antifungal susceptibility tests showed that benzoylglycine, benzoyldiethanol and benzoylsarcosine did not show any antifungal effects on *Sporothrix schenckii* when tested within 0.0004 M - 0.00005 M. Based on antifungal susceptibility tests for the tested compounds using disk diffusion method, there were absence of zone of inhibition (ZOI) values. It was known that thiourea compounds exhibit good antifungal properties (Wang *et al*., 2006). However, our findings revealed the contrary. Meanwhile, benzoylglycine, benzoylsarcocine, and benzoyldiethanol, with concentrations of 0.0004 M to 7.813×10^{-6} M showed no growth inhibition on *S. schenckii*. Higher concentrations are required to ensure the studied compounds could be potent alternatives to existing antifungal agents. Apart from that, the fungus that was used in this study was a clinical isolate of *S. schenckii*, which possesses a stronger fungi properties and may have already acquired resistance. A study conducted by Kurt *et al*., (2009), using new and different benzoyl thiourea derivatives; (E)-N-[(2 benzamidomethyleneamino)ethylcarbamothioyl] benzamide, N-(1-

(3 benzoylthioureido)propan-2-ylcarbamothioyl) benzamide, (E)-N-[4 (benzamidomethyleneamino)phenylcarbamothioyl]benzamide, revealed that no antifungal properties were shown when screened against *Candida albicans*. Meanwhile, Rodriguez-Fernandez *et al.*, (1999), studied the antifungal effects of different thiourea derivatives, namely α-chlorobenzylidene thiourea derivatives and their complexes with Ni(II) against *Botrytis cinerea*. The studied thiourea derivatives and their complexes with Ni(II) effectively inhibits the growth of Botrytis cinerea. Therefore, it is important to understand that different compounds consist of variable chemical composition and structure, thus will be responsible for the inhibitory effect in different organisms.

According to the results obtained from the experiment, the range of MIC values of itraconazole against *S. schenckii* was ≥8 µg/ml. Studies have classified the antifungal susceptibility of *S. schenckii,* in which the MIC values equal to or higher than 8 µg/ml may be considered resistance. The Clinical and Laboratory Standard Institute published M38-A2 guideline 2017, proposed that the MIC value which is 8-16 µg/ml is considered intermediately susceptible.

In this experiment, mycelial form of *S. schenckii* has been used. Due to the low nutrient requirement and the facility to grow mycelia at room temperature, most studies that have been carried out to determine the susceptibility profile of *S. schenckii* to antifungal drugs have used the mycelial form of *S. schenckii* (McGinnis *et al.*, 1997; Espinel-Ingroff 1998*a*, 1998*b*; Noguchi *et al*., 1999; McGinnis *et al*., 2001; Odabasi *et al*., 2004). The conversion of mycelial to yeast forms requires different conditions including temperature of 35˚C occasionally, an atmosphere of 5% of carbon dioxide (Casali and

Hamdan, 1997) and several passages through rich BHI media supplemented with 0.5% glucose (Kohler *et al*., 2004). The conversion will not be always rapid and completely done, hence shows how difficult it is to use the yeast form of *S. schenckii*. Recently, CLSI approved a protocol document M38-A for susceptibility testing for filamentous fungus that cause invasive infection including *S. schenckii* against antifungal drugs. Thus, it can be concluded that the antifungal sensitivity findings derived from mycelial form of *S. schenckii* can be used to predict the activity of antifungal against the fungus.

6.0 CONCLUSION

There was no presence of antifungal properties by benzoylglycine, benzoylsarcocine and benzoyldiethanol with concentration range of $\leq$0.0004M against *S. schenckii* observed.

7.0 RECOMMENDATION

Subsequent of our findings, a higher range of concentration of the studied compounds can be tested in future. Since these are new compounds, the exact effective anti-fungal concentration is still uncertain. It is also recommended that the test should be tested on other types of fungi as it is known that the *S. schenckii* is resistance towards itraconazole, thus testing new compounds with resistant *S. schenckii* may not give reliable susceptibility. Moreover, in order to understand the mode of action by the subject, microscopy evaluation should be included in the future. It is also recommended that this

susceptibility test should be done with *S. schenckii* which is in yeast form. This is because the yeast form is pathogenic to the organism.

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9.0 APPENDICES

Preparation of four different molar concentrations of Benzoyl-glycine thiourea, Benzoyl-sarcocine thiourea and Benzoyl-diethanol thiourea

Molarity (M)	Benzoyl-glycine thiourea, Benzoyl-sarcocine thiourea, Benzoyl-diethanol thiourea		Final volume (mL)
	Mass (mg)	DMSO + Distilled water (mL)	
0.004	2	1ml + 19.9ml	20
0.002	1	1ml + 19.9ml	20
0.001	0.5	1ml + 19.9ml	20
0.0005	0.25	1ml + 19.9ml	20

List of compounds, inoculums and serial dilution initial concentration to be tested.

Compounds	Inoculum suspension	Serial dilution initial concentration (M)
Benzoyl-glycine thiourea	*S. schenckii*	0.004
Benzoyl-sarcocine thiourea	*S. schenckii*	0.004
Benzoyl-diethanol thiourea	*S. schenckii*	0.004

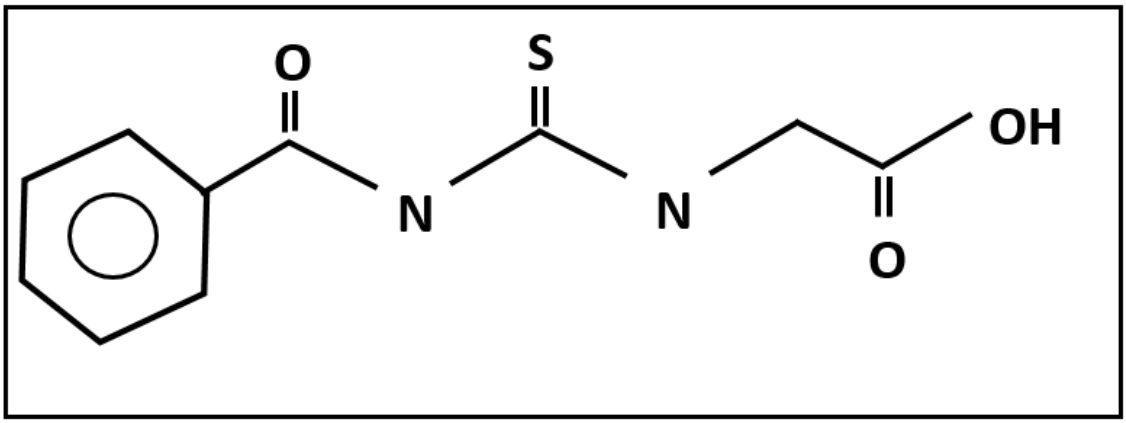
Volume of compound suspension and diluent with molar concentration.

Well no.	Benzoyl-glycine, Benzoyl-sarcocine, Benzoyl-diethanol (µL)	RPMI solution (µL)	Final volume (µL)	Molar concentration (M)
1	100 from stock	-	100	0.004
2	100 from stock	100	100	0.002
3	100 from 2	100	100	0.001
4	100 from 3	100	100	5×10^{-4}
5	100 from 4	100	100	2.5×10^{-4}
6	100 from 5	100	100	1.25×10^{-4}
7	100 from 6	100	100	6.25×10^{-5}
8	100 from 7	100	100	3.125×10^{-5}
9	100 from 8	100	100	1.563×10^{-5}
10	100 from 9	100	100	7.813×10^{-6}

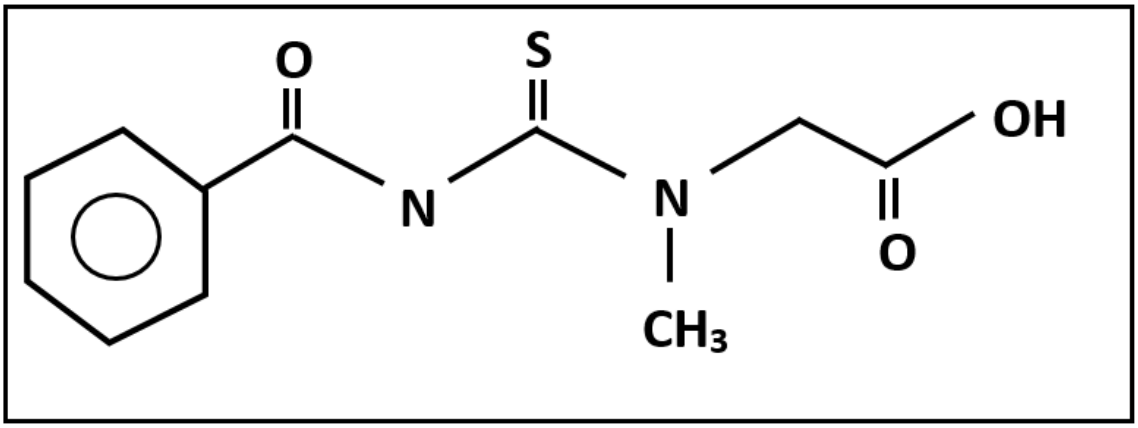
Volume of itraconazole suspension and diluent with concentration (µg/ml)

Well no.	Itraconazole (µL)	RPMI solution (µL)	Final volume (µL)	Concentration (µg/ml)
1	100 from stock	-	100	32
2	100 from stock	100	100	16
3	100 from 2	100	100	8
4	100 from 3	100	100	4
5	100 from 4	100	100	2
6	100 from 5	100	100	1
7	100 from 6	100	100	0.5
8	100 from 7	100	100	0.25
9	100 from 8	100	100	0.125
10	100 from 9	100	100	6.25×10^{-2}

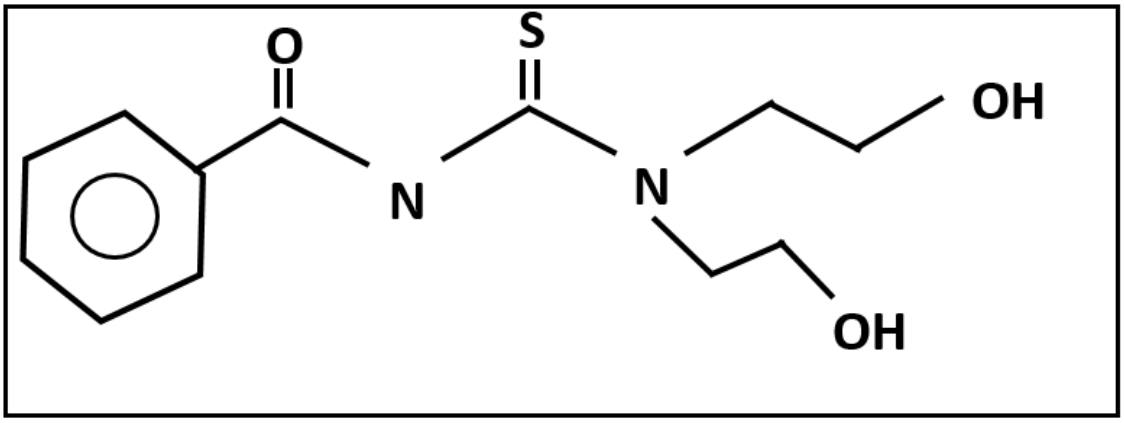
a)



b)



c)



Molecular structure of a) Benzoyl-glycine, b) Benzoyl-sarcocine, c) Benzoyl-diethanol