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ORIGINAL ARTICLE

Design, Synthesis and Evaluation of Antifungal activity of some maleimide derivatives

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ABSTRACT

*In recent years, the use of antifungal drugs in human medicine has increased, especially with the advent of AIDS epidemic. The purpose of this study was to synthesize and perform activities of N-phenylalkyl-3, 4-dichloromaleimide (**L**) but not those of N-phenyl alkyl-maleimide derivatives showed to be dependent on the length of the alkyl chain. The compound **L** with three methylenes chain length showed the broadest spectrum of action and lower MIC (1.56 < MICs < 6.25µg/mL) in all of the fungi tested, being active against standardized as well as clinical strains of Candida albicans (MIC= 6.25 µg/mL). Compound **L** strongly inhibits chitin synthase with 89% of inhibition at 20 µg/assay (IC_{50} =0.032 µg/µL). The presence of a flexible connecting chain in N-phenylalkyl maleimides appears not to be essential for antifungal activity, although its length shows a correlation with the antifungal behavior, displaying maleimides with alkyl chains of n = 3 and n = 4 the best antifungal activities in most fungi.*

Keywords: *Antifungal; maleimide; chitin synthase.*

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INTRODUCTION

Several *N*-phenyl, *N*-aryl, *N*-phenyl alkyl-maleimide and 3, 4-dichloromaleimide derivatives have been reported to have good antifungal activity against the human pathogenic fungi.[1, 2] Also, the *para*-substituted anilines have been part of the structure of the compounds possessing antifungal properties (**Fig. 1**).[3-7]

The activities of *N*-phenylalkyl-3, 4-dichloromaleimide (**L)** but not those of *N*-phenyl alkyl-maleimide derivatives showed to be dependent on the length of the alkyl chain. The compound **L** with three methylenes chain length showed the broadest spectrum of action and lower MIC (1.56 < MICs < 6.25µg/mL) in all of the fungi tested, being active against standardized as well as clinical strains of *Candida albicans*(MIC= 6.25 µg/mL).Compound **L** strongly inhibits chitin synthase with 89% of inhibition at 20 µg/assay (IC_{50} =0.032 µg/µL).[8]The presence of a flexible connecting chain in *N*-phenylalkyl maleimides appears not to be essential for antifungal activity, although its length shows a correlation with the antifungal behavior, displaying maleimides with alkyl chains of n = 3 and n = 4 the best antifungal activities in most fungi.[1]

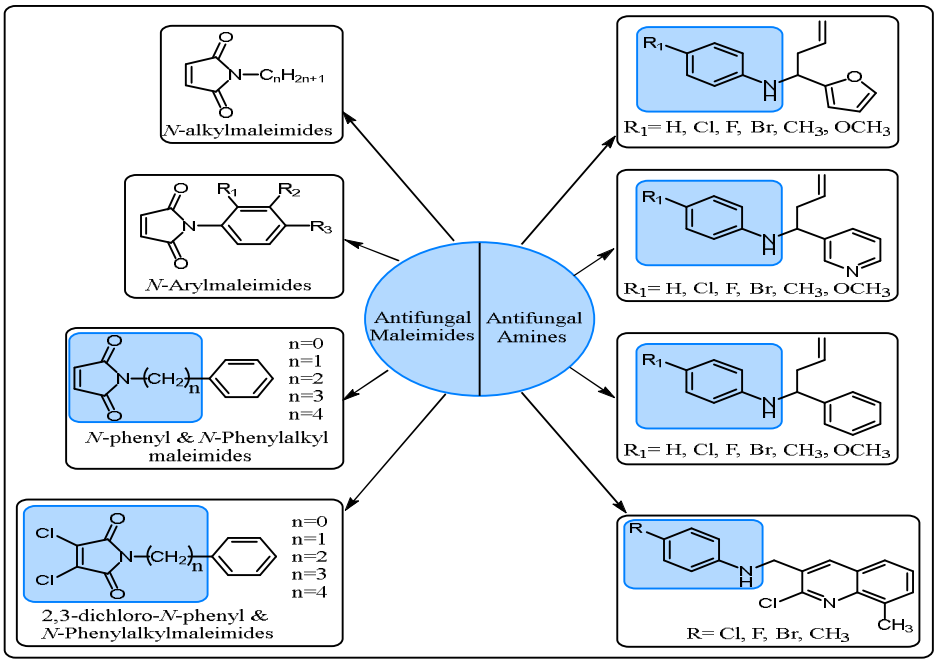

Figure 1. Antifungal maleimide and aniline derivatives.

MATERIAL AND METHODS

Synthesis of maleimide and 3, 4-dichloromaleimide derivatives.

The *N*-(3-anilinopropyl) maleimide (**M1-M6)** and *N*-(3-anilinopropyl)-3, 4- dichloromaleimide (**M7-M12)** derivatives were synthesized by following scheme 1 (**Fig. 1**), as per literature methods.[1, 8, 13, 14]

Preparation of 3-chloropropylamine solution[9] (3)

To a solution of sodium hydroxide (0.92, 23.00mmol) in 10mL water, was added 3-chloropropan-1-amine hydrochloride (3.0gm, 23.00mmol) and toluene (20mL) at cold temperature. The mixture was stirred for 10-15min and the organic layer was separated. Resulting aqueous layer was extracted with toluene (20mL x 2) and the combined organic layers were dried over anhydrous $MgSO_4$ and used for next step.

Synthesis of *N*-(3-chloropropyl) maleamic acid (4)

Equimolar solution of maleic anhydride (**1)** and 3-chloropropylamine (3**)** in toluene were mixed with stirring under cold condition. The mixture was further stirred for 1h and filtered at pump. The residue was washed with water and dried at 100^{o}C in a hot air oven. The synthesized compound gave a positive test for the presence of carboxylic acid, elements like nitrogen and chlorine. The structure of the synthesized maleamic acid was confirmed by FTIR

IR (KBr) (νcm^{-1}): 3481**,** 3354, 3073, 3031, 2965, 2933, 2838, 1663, 1609, 1585, 1502

Synthesis of *N*-(3-chloropropyl) 3, 4-dichloromaleamic acid (5)

Equimolar solution of dichloromaleic anhydride (**2)** and 3-chloropropylamine (**3)** in toluene were mixed with stirring under cold condition. The mixture was further stirred for 1h and filtered at pump. The residue was washed with water and dried at 60^{o}C in hot air oven.

The synthesized compound gave positive test for the presence of carboxylic acid, elements like nitrogen and chlorine. The structure of the synthesized maleamic acid was confirmed by FTIR IR (KBr) (νcm^{-1}): 3184**,** 3118, 2934, 2840, 1691, 1601, 1511, 1451

Synthesis of *N*-(3-chloropropyl) maleimide (6)

A reaction mixture containing *N*-(3-chloropropyl) maleamic acid (**4)** (1.0gm, 5.2mmol) and sodium acetate (0.246gm, 3.00mmol) in 5.0mL acetic anhydridewas heated at 90^{o}C for 2 h. The mixture was cooled and added to 200mL of ice cold water and stirred for 2 h. The aqueous solution was extracted with chloroform (50mL x 3).The combined organic extracts were dried over sodium sulfate and filtered. The residue on solvent evaporation was purified by column chromatography using ethyl acetate: n-Hexane (10:90) as mobile phase.

The structure of the synthesized compound was confirmed by FTIR, ^{1}HNMR, ^{13}CNMR, and GC-MS.

***N*-(3-chloropropyl) maleimide**: IR(KBr) (ν cm^{-1}):3169**,** 3101, 2961, 2948 , 2872, 1768, 1708, 1702, 1585, 1442; ^{1}H NMR ($CDCl_3$)**:** δ 2.09 (quin,*J* = 5.50Hz, 2H), 3.56 (t,*J* = 6.60Hz, 2H), 3.69 (t, *J* = 6.69Hz, 2H), 6.73 (s, 2H) ; ^{13}C NMR ($CDCl_3$)**:**δ 30.27, 36.83, 41.94, 134.24, 169.95; MS m/z (EI): 173, 174(M+1), 139, 110, 82.

Synthesis of *N*-(3-chloropropyl) 3, 4-dichloromaleimide[2] (7)

A reaction mixture containing *N*-(3-chloropropyl) 3, 4-dichloromaleamic acid (**5)** (1.0gm, 4.1mmol) and sodium acetate (0.246gm, 3.00mmol) in 20.0mL glacial acetic acidwas refluxed gently for 1 h. The mixture was cooled and added to 100mL of ice cold water and stirred for 30 min. The solid obtained was filter at pump and allowed to dry at room temperature. The residue was purified by column chromatography using ethyl acetate: n-Hexane (10:90) as mobile phase.

The structure of the synthesized compound was confirmed by FTIR, ^{1}HNMR, ^{13}CNMR, and GC-MS.

***N*-(3-chloropropyl) 3, 4-dichloromaleimide**: IR (KBr)(ν cm^{-1}):2957, 2921, 2870, 1728, 1707, 1627, 1452 ; ^{1}H NMR ($CDCl_3$): δ 2.14 (quin, *J*=6.42 Hz, 2 H), 3.55 (t, *J*=6.51 Hz, 2 H), 3.78 (t, *J*=6.83 Hz, 2 H); ^{13}C NMR ($CDCl_3$): δ 31.05, 37.08, 41.66, 133.43, 162.96; MS m/z (EI): 243, 244(M+1), 179, 121, 86.

Synthesis of *N*-[3-(4-arylamino) propyl] maleimide (M1-M6)

General procedure[15]

To asolution of aniline (0.279gm, 3.0mmol) in acetonitrile (3mL) in a microwave Discover LabMate vial *N*-(3-chloropropyl) maleimide (4**)**(0.173gm, 1.0mmol)and KI (0.166gm, 0.1mmol)were added. The vial was sealed and heated in a Discover LabMate microwave at 110^{o}C for 10min. The cooled reaction mixture was diluted with 20mL Chloroform and washed successively with $NaHCO_3$ (aqueous saturated solution) and brine. The organic layer was dried over anhydrous sodium sulphate and distilled off at reduced pressure. The residue was purified by column chomatography using ethyl acetate: n-Hexane (20:90) mobile phase to obtain *N*-[3-(anilino) propyl] maleimide (**M1).** Similarly other maleimide derivatives (**M2-M6)** were synthesized. The structures of the synthesized compounds were confirmed by FTIR, ^{1}HNMR, ^{13}CNMR, and GC-MS.

***N*-[3-(*p*-tolylamino) propyl] maleimide (M5)**:IR (KBr)(ν cm^{-1}):3389, 3361, 3051, 3024, 2989, 2951, 2917, 2851, 2815, 1770, 1692, 1681, 1522; ^{1}H NMR ($CDCl_3$) : δ2.11 (quin, *J*=6.51 Hz, 2 H), 2.26 (s, 3 H), 3.56 (t, *J*=6.28 Hz, 2 H), 3.74 (t, *J*=6.64 Hz, 2 H), 4.23 (s, 1 H), 6.56 (d, *J*=8.10 Hz, 2 H), 7.03 (d, *J*=7.8 Hz, 2 H), 7.26 (s, 1 H); ^{13}C NMR ($CDCl_3$):δ 20.32, 26.62, 38.08, 41.21, 113, 129, 143, 174; MS m/z (EI): 244, 245(M+1), 138, 120, 106, 91

Similarly, the structures of other maleimide derivatives were confirmed by spectral studies.

Synthesis of *N*-[3-(4-arylamino) propyl] 3, 4-dichloromaleimide (M7-M12):

General procedure[15]

To asolution of aniline (0.279gm, 3.0mmol) in acetonitrile (3mL) in a microwave Discover LabMate vial *N*-(3-chloropropyl) 3, 4-dicloromaleimide (**5)**(0.242gm, 1.0mmol) and KI (0.166gm, 0.1mmol)were added. The vial was sealed and heated in a Discover LabMate microwave at 110^{o}C for 10min. The cooled reaction mixture was diluted with 20mLchloroform and washed successively with $NaHCO_3$ (aqueous saturated solution) and brine.The organic layer was dried over anhydrous sodium sulphate and distilled off at reduced pressure. The residue was purified by column chomatography using ethyl acetate: n-Hexane (20:90) mobile phase to obtain *N*-[3-(anilino) propyl] 3, 4- dichloromaleimide (**M7).** Similarly other 3, 4-dichloromaleimide derivatives (**M8-M12)** were synthesized.

The structures of the synthesized compounds were confirmed by FTIR, ^{1}HNMR, ^{13}CNMR, and GC-MS.

***N*-{3-[(4-chlorophenyl) amino] propyl}-3, 4-dichloromaleimide** (**M8**): IR (KBr)(ν cm^{-1}):3248, 3105, 3016, 2962, 2937, 2866, 1759, 1703, 1641, 1587; ^{1}H NMR ($CDCl_3$) : δ 2.13 (quin, *J*=6.60 Hz, 2 H), 3.49 (s, 1 H), (3.56 (t, *J*=6.42 Hz, 2 H), 3.75 (t, *J*=6.74 Hz, 2 H), 7.13 (t, *J*=8.75 Hz, 2 H), 7.37 (t, *J*=8.84 Hz, 2 H); ^{13}C NMR ($CDCl_3$): δ 31, 37, 51, 113, 129, 134, 164; MS m/z (EI):335, 336(M+1), 300 212, 126, 91.

Similarly, the structures of other 3, 4-dichloromaleimide derivatives were confirmed by spectral studies.

Antifungal Screening (*in vitro*)

Microorganisms and media

The microorganisms used for the antifungal evaluation were obtained from National Collection of Industrial Microorganism (Pune): *Candida albicans 3471, Saccharomyces cerevisiae 3046, Aspergillus niger 545*and*Aspergillus flavus 524.* Yeast and Fungi strains were grown on the Malt extract glucose yeast extract peptone and Potato dextrose agar slants for 48 h at 30°C, respectively. Cell suspensions in sterile distilled water were adjusted to give a final concentration of 10^{8} viable yeast cells and 10^{6} viable fungi spores per mL.

Agar diffusion assay (Zone of inhibition)

All the synthesized compounds were screened for their *in vitro* antifungal activities. For preliminary screening the antifungal tests were carried out by disc-diffusion method.[16]The preweighed benzenamine and maleimide derivatives were dissolved in DMSO to get final concentrations 100µg/mL. The test microorganisms were seeded into respective medium by gently mixing 0.5 mL (containing 10^{8} viable yeast cells and 10^{6} viable fungi spores per mL) of the 48 h. fresh cultures with 20 mL sterile melted agar cooled to about 45^{o}C in sterile Petri plates. After hardening, the 6 mm diameter disc (Disc size,

6mm) containing compounds (100 µg/disc) or solvent blanks were blanks. The plates were kept at 4°C in refrigerator for diffusion of the respective liquid in the plate. The petri plates containing antifungal assay assembly were incubated at 30°C for 48 h. The standard discs of Nyastatin (100U/mL) served as a positive antifungal control. DMSO (50% v/v) was used as a control. The diameter of zone of inhibition around each of the disc was taken as measure of the antifungal activity. Each experiment was carried out in triplicate and mean diameter of inhibition zone was recorded (**Table 4**).

Broth dilution assay (Minimum inhibitory concentration)

The antifungal activity of benzenamine and maleimide derivatives was determined by the broth dilution method according to reported procedures.[16] The minimum inhibitory concentration (MIC) values were determined for microorganisms, which were sensitive to the compounds in the agar disc diffusion assay. The compounds were prepared at the highest concentration (1.5mg/mL).The two rows of 12 sterile 7.5 x1.3 cm capped tubes were arranged in the rack. Various dilutions of compounds were achieved by serial double dilution method. Compounds were diluted in sequential range- 1025, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, and 0.125µg/mL. In a capped tube 5mL of each solution was taken and to it added 5mL double strength media. Tubes were inoculated with 0.2mL (containing 10^8 viable yeast cells and 10^6 viable fungi spores per mL) suspension of yeast and fungi spores. A blank 2mL of compound free broth was added to the last tube in each row. The tubes were incubated for 48 h at 37°C. A tube containing 2mL broth was inoculated with the organism and kept at +4°C in a refrigerator overnight to be used as standard for the determination of complete inhibition.MIC was defined as the lowest compound concentration, showing no visible fungal growth after incubation time (**Table 5**).

Chitin synthase assay

The chitin synthase inhibition assays were performed using yeast cell extracts, which was performed according to a modified procedure described by *Lucero* and *Bulik*.[12]The yeast *Saccharomyces cerevisiae* were cultured overnight with shaking in YPD (1% yeast extract, 2% peptone, and 2% glucose) at 30°C, and then the cells were harvested by centrifugation at 1500 x g for 10 min at 4°C. Cells were disrupted by grinding method with glass beads under liquid nitrogen condition.

The WGA-coated 96-well microtiter plates stored at -20°C in blocking buffer were thawed at room temperature and emptied by shaking. Then the pretreated whole cell extracts, tested samples and reaction mixtures were added to the appropriate wells, respectively, to a final volume of 100µL. For each complete assay mixture the corresponding incomplete reaction mixture (without UDP-GlcNAc) was used to assess the assay background. Immediately after the addition of reaction mixtures, plates were shaken slowly ona vortex shaker for 60 s, and then incubated on the shaking table at room temperature for about 90min. Then 20 µL of 50 mM EDTA was added and plates were gently shaken for 30s ona vortex shaker. After this, plates should be emptied and washed eight times with amount of double distilled water, followed by the addition of 100 µL WGA-HP (1 µg/mL, 20 mg/mL BSA, 50 mM Tris-HCl, pH 7.5), and incubated for 15 min at room temperature. Plates were then emptied by vigorous shaking of their content and washed six times by double distilled water. Then 100 µL TMB reaction reagent was added and plates were immediately placed on the enzyme-linked analyzer for detection of the optical density (OD) at 600 nm by enzyme kinetic method. Each reaction was carried out in triplicate.

RESULTS AND DISCUSSION

By connecting maleimide/2, 3-dichloromaleimide ring through a spacer of three carbon chain to *para*-substituted anilines may give potent antifungal agent with chitin synthase inhibitory activity. Based on this conclusion we have designed the following maleimide/2, 3-dichloromaleimide derivatives (**Table 1**).

Table 1. Designed maleimide derivatives

Compound	R_1	R_2	R_3
M1	H	H	H
M2	H	H	Cl
M3	H	H	F
M4	H	H	Br
M5	H	H	CH_3
M6	H	H	OCH_3
M7	Cl	Cl	H
M8	Cl	Cl	Cl
M9	Cl	Cl	F
M10	Cl	Cl	Br
M11	Cl	Cl	CH_3
M12	Cl	Cl	OCH_3

Maleimide derivatives were synthesized starting with maleic anhydride and 3-chloropropylamine. The 3-chloropropylamine (3-CPA) free base diluted in a nonpolar aprotic solvent toluene ameliorated the self-polymerization reaction at 25-30°C for about 72 hr.[9] Hence, the 3-CPA free base was extracted in toluene at cold condition and this solution was directly used for further reaction. Then, the ring opening occurs through the nucleophilic attack of the amine nitrogen atom on carbonyl carbon and the formation of amic acids. The amic acids were cyclized by dehydration using acetic anhydride/ glacial acetic acid to give *N*-(3-chloropropyl) maleimides. The maleimides were then mono *N*-alkylated with *p*-substituted anilines in the presence of potassium iodide under microwave irradiation to yield the desired compounds.

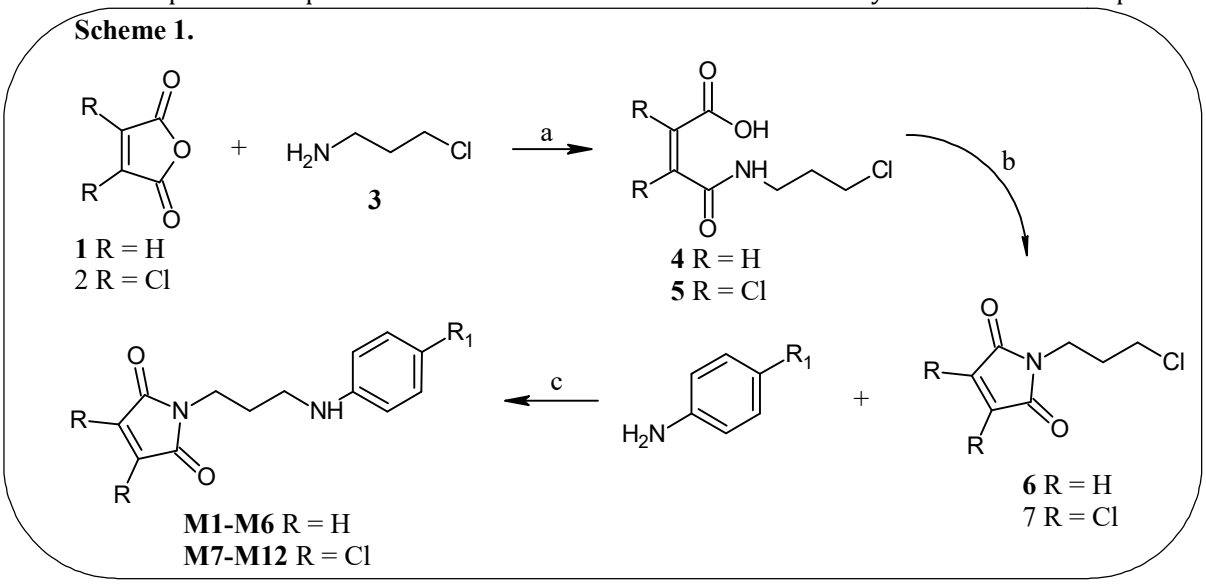

Figure 2. Synthesis of maleimide and 3, 4- dichloromaleimide derivatives. Reagent and condition: **a-** Toluene; **b** - Acetic anhydride (**4**), Glacial acetic acid (**5**), Sodium acetate, reflux.; **c** - Acetonitrile, KI, MW for 10min (**M1-M6**) and Acetonitrile, KI, reflux for 1.0h (**M7-M12**); R_1- H, Cl, F, Br, CH_3, OCH_3.

Their structure was established using IR, NMR spectroscopy and mass spectrometry. Their IR spectra showed the bands of the –NH stretching vibrations in the region of 3200-3400 cm^{-1},and-NH bending vibration band in the region of 1502-1560 cm^{-1}. The retention of carbonyl stretching vibrations in the region 1680-1707 cm^{-1} supports the formation of desired maleimide derivatives. In the ^1H NMR spectra of these compounds the broad singlet of NH appeared in region 4.00 ppm, two triplets signal of H_2C- appeared in a region 3.5 ppm and 3.7ppm, and one multiplet of CH_2-group appeared in the region 2-3 ppm. Molecular ion peaks and fragmentation pattern of the synthesized maleimide derivatives obtained on the mass spectrum adequately corresponds with their structures. This spectral data satisfactorily supports the formation of maleimide derivatives.

For preliminary screening the synthesized maleimide **M1-M6** /3, 4-dichloromaleimide **M7-M12**were subjected to antifungal tests by disc-diffusion method at 100µg/mL. Nyastatin (100U/mL) was used as a positive control in the disc diffusion method. The yeast cell suspension for *Candida albicans* and *Saccharomyces cerevisiae*, fungal suspension for *Aspergillus niger and Aspergillus flavus* were spread on sterile YPG (yeast extract,0.3%, peptone, 0.5%, and glucose, 1%) and PDA (potato,20% dextrose, 2%) agar plates separately. The entire synthesized compounds were found to be active against the tested fungi.

The synthesized maleimide M1-M6 /3, 4-dichloromaleimide M7-M12 were evaluated for antifungal activity (MIC) using the broth dilution method against *Candida albicans 3471, Saccharomyces cerevisiae 3046*, *Aspergillus niger 545 and Aspergillus flavus 524.* Amphotericin B and ketoconazole was taken as positive control because antifungal efficacy of nikkomycin Z is low.[10] Concentrations up to 1000µg/mL of each compound were incorporated into growth media according to reported procedures.

All synthesized maleimide/3 ,4-dichloromaleimide derivatives were found to be active against the panel of human pathogenic fungi. Compounds M2-M4, M8-M10 containing *p*-haloaniline as a part of its structure showed good antifungal activities. The substitution of -chloro group at 3 & 4 position on maleimide ring increases its activity. Compounds M8-M10 too showed excellent antifungal activities (MICs 1.0 – 4.0 µg/mL).

Table 4. *In vitro* antifungal activity detection by using disc-diffusion method

Compound	Zone of inhibition (mm) (mean±SD)			
	S. cerevisiae	***C. albicans***	***A. niger***	***A. flavus***
M1	8.18±0.24	9.58±0.34	8.14±0.51	8.24±0.33
M2	9.63±0.31	8.20±0.56	8.54±0.38	8.74±0.44
M3	9.35±0.42	8.42±0.39	8.92±0.47	9.12±0.31
M4	9.02±0.38	8.86±0.20	9.79±0.21	9.91±0.41
M5	8.32±0.29	7.20±0.19	8.64±0.39	8.44±0.37
M6	8.20±0.67	8.13±0.29	8.23±0.54	8.03±0.61
M7	10.25±0.72	10.63±0.45	10.43±0.32	10.34±0.21
M8	13.36±0.36	12.51±0.35	12.86±0.19	12.56±0.41
M9	11.27±0.55	10.32±0.38	10.07±0.23	9.97±0.5
M10	10.34±0.57	11.89±0.41	12.35±0.65	12.55±0.37
M11	8.23±0.48	9.14±0.32	9.26±0.63	9.16±0.21
M12	9.20±0.23	11.32±0.34	9.12±0.21	9.53±0.3
Nyastatin (100 U/mL)	9.53±0.41	9.53±0.32	9.53±0.26	9.55±0.45

Table 5. Minimum inhibitory concentration values of the compounds against the fungal species using broth dilution method

Compound	Minimum Inhibitory Concentration (µg/mL)			
	S. cerevisiae	***C. albicans***	***A. niger***	***A. flavus***
M1	128	128	128	128
M2	64	64	32	32
M3	32	64	32	64
M4	64	64	32	32
M5	64	128	128	128
M6	256	256	128	128
M7	64	32	16	32
M8	2	1	2	4
M9	1	2	4	4
M10	8	32	64	32
M11	64	64	16	32
M12	64	32	64	64
Amphotericin B	0.25	0.25	0.5	0.25
Ketoconazole	0.125	0.25	0.25	0.25
Nikkomycin Z[10]	--	<0.5–32	>64	--

To gain insight into the mode of action of the most active antifungal compounds, their capacity to inhibit *in vitro* chitin synthase activities were tested. Results of the *in vitro* assays are listed in Table 6.

Table 6. Chitin synthases inhibition activity of compounds.

Compound	Percent inhibition(mean±SD)
M3	74±0.27
M8	91±0.16
M9	93±0.29
Nikkomycin Z	100

Nikkomycin Z (Chitin synthase inhibitor) was used as a positive control. Chitin synthase inhibition activity from *S. cerevisiae* was estimated with compounds (4 µg/mL), and without also, using a nonradioactive chitin synthase assay according to *Lucero et al.*[12] It was found that compound **M8** and **M9** inhibited 91% and 93% chitin synthase activity respectively. The rest of the compounds were showed the considerable percentage of inhibition in the range of 71-84 %

CONCLUSION

In the present study, **12** maleimide derivatives have been designed and synthesized. The 3,4-dichloromaleimide derivatives showed better antifungal properties compared to maleimide derivatives.

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CONFLICT OF INTEREST

Authors declared that there is no conflict of interest.

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