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SYNTHESIS AND BIOLOGICAL EVALUATION OF HALOGEN SUBSTITUTED 1,4-NAPHTHOQUINONES AS POTENT ANTIFUNGAL AGENTS

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ABSTRACT

A series of halogen containing 1,4-naphthoquinon derivatives (**1-4,5a-f**) were synthesized and studied for their antifungal activities against *C. albicans* ATCC10231, *C. albicans* 955, *T. mentagrophytes* and *M. gypseum*.

The results indicate that compound 2-hydroxy-3-chloro-1,4-naphthoquinone (**2),** 2-(N-acetyl)-acetamido-3-chloro- 1,4-napthoquinone (**3**) and 2-(*N*-acetyl)-acetamido-3-chloro- 1,4-napthoquinone (**4**) have potent antifungal activity. Among these promising antifungal candidates, **2** and **4** showed better activity than that of clinically antifungal drug clotrimazole (MIC = 8 µg/ml) with MIC = 1 µg/ml and 4 µg/ml, respectively against *C.albicans* ATCC10231. Compound **2** also exhibited an extremely potent activity (MIC = 0.25 µg/ml) against *C.albicans* 955 strain compared with clotrimazole (MIC = 16 µg/ml).

Structure and activity relationship (SAR) study demonstrated that replacing of 3-position in 1,4-naphthoquinon by a Cl group is essential for antifungal activity. Meanwhile, antifungal activity was decreased considerably when the hydrogen atom at position-2 in naphthoquinone structure were replaced by a bulky group (e.g. diacetyl of phenyl group).

KEY WORDS: 1,4-naphthoquinon, antifungal activity, *Candida albicans*, dermatophytes.

BACKGROUND

The increase in the incidence of fungal infections may be attributed primarily to increased numbers of critically ill and immunocompromised patients, including those with AIDS, cancer patients undergoing chemotherapy, and organ transplant recipients taking immunosuppressive drugs.[1] However azole derivatives including fluconazole and itraconazole are widely used in clinical settings but there are major weaknesses in their spectra, potency, safety and pharmacokinetic properties. In addition, the emergence of fungal strains resistant to existing antifungal drugs is becoming a significant problem. Thus the development of new effective antifungal agents is strongly needed in medicine.[2-3]

Quinones, in particular benzoquinone and naphthoquinone derivatives, have been repeatedly isolated from lower as well as higher species of plants, and are found frequently in animals.[4] In addition to quinones possessing a biological function in cell metabolism as electron carriers, other compounds of this class have been found active against bacteria and fungi.[5-10] Recently, many studies have demonstrated that naphthoquinone derivatives substituted with a halogen atom show a particularly marked activity against fungi.[11-14]

The present study was carried out to design and synthesis a series of halogen containing 1,4-naphthoquinone derivatives and test against several fungal pathogens.

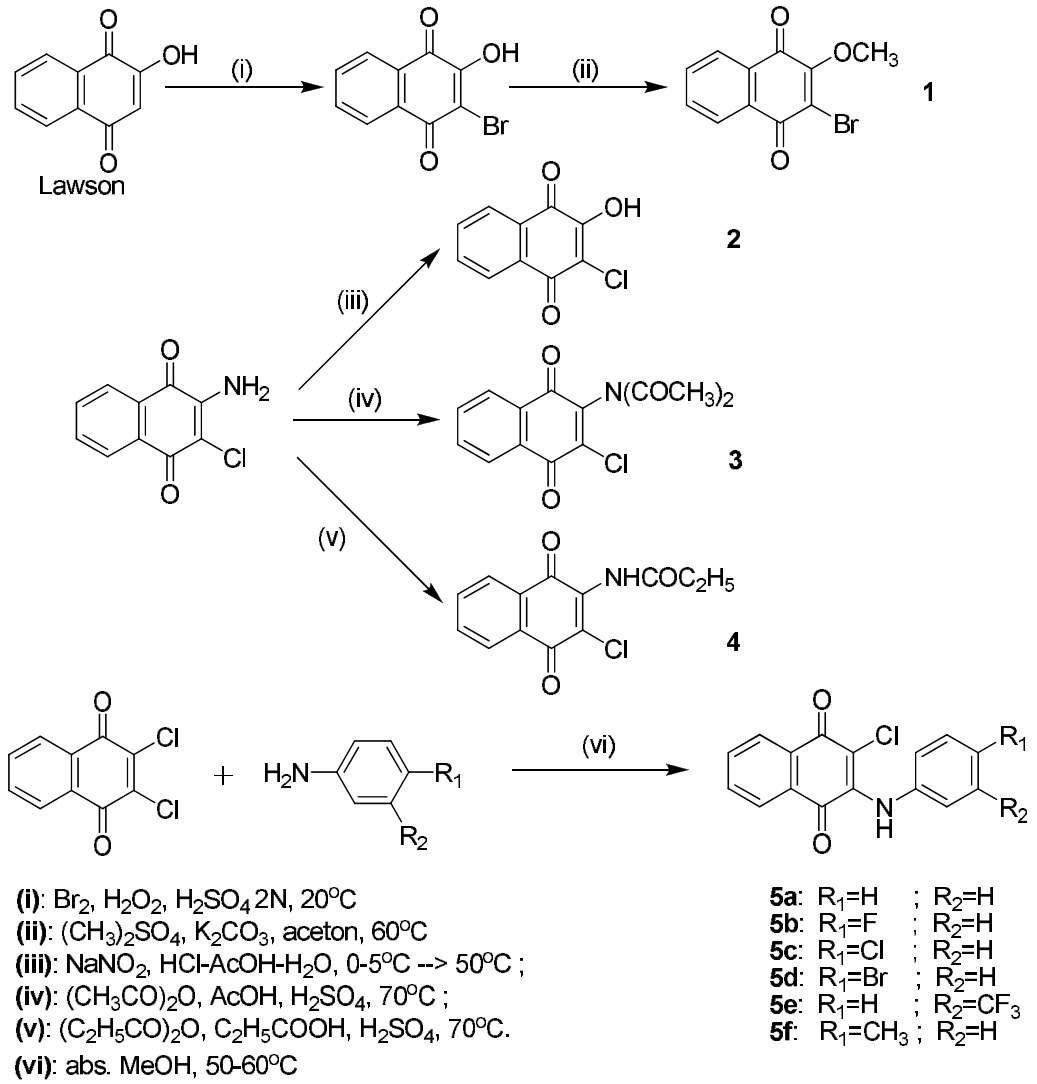
MATERIALS AND METHODS

Chemistry

The reagents and the solvents used in this study were of analytical grade were used without further purification. Lawsone was synthesized according to the previous publication.[15] 2-amino-3-chloro-1,4-naphthoquinone and 2,3-dichloro-1,4-naphthoquinone were purchased

form Acros. Progress of reactions and purity of compounds were monitored by thin layer chromatography (TLC), which was performed on silica gel 60F254 and compounds were detected with UV Chamber at 254nm and 365nm (where required). Column chromatography was performed on silica gel G60 (230-400 mesh, ASTM, Merck). All melting points were measured in open capillary tubes using Galenkampt melting point and were uncorrected. UV-Vis spectra were taken with a Hitachi U-2010 spectrophotometer. IR spectra were recorded on Schimadzu FTIR 8201 PC Spectrophotometers. Proton Nuclear Magnetic Resonance (^{1}H NMR) spectra were recorded on Bruker Ultrashield 500 spectrometers using tetramethylsilan (TMS) as an internal reference. Chemical shifts were given in ppm with TMS as a standard. ESI mass spectra (ESI-MS) were obtained on Waters Quattro micro API mass spectrometer.

In order to study the structure-activivity relationship (SAR) of halogen substituted 1,4-naphthoquinone derivatives, several 1,4-naphthoquinon were synthesized (see **Scheme 1**).



Scheme 1. Synthesis of novel halogen substituted 1,4-naphthoquinones

2-Methoxy-3-bromo-1,4-naphthoquinone (**1**) was obtained by two-step-reaction. Fistly, bromination of lawsone with bromine and hydroperoxide in acidic medium provide 2-hydroxy-3-bromo-1,4-naphthoquinone in good yield (92%). This compound was then

reacted with dimethylsulfate in acetone to obtain **1** as a yellow solid. The methylation reaction was catalysted by K_2CO_3.

Form 2-amino-3-chloro-1,4-naphthoquinone, we have successfully carried out the synthesis of several chloro-1,4-naphthoquinone analogs (**2-4**). 2-hydroxy-3-chloro-1,4-naphthoquinone (**2**) was obtained by the diazoniation of 2-amino-3-chloro-1,4-naphthoquinone with sodium nitrite as reagent, the mixture was then hydrolyzed at 50 ^{o}C providing **2** as an orange solid. On the other hand, reaction of 2-amino-3-chloro-1,4-naphthoquinone with several acid anhydride producing two amino substituted derivatives (**3-4**) in average yields, concentrated H_2SO_4 was used as catalyst.[13]

The reaction of 2,3-dichloro-1,4-naphthoquinone with arylamine was carried out in absolute methanol at 50-60 ^{o}C. Only one chlorine atom of 2,3-dichloro-1,4-naphthoquinone was substituted by the nucleophile arylamines due to electronic enrichment of the quinone structure. [10,14] We have studied this reaction with six different aryl amine (shown in Scheme 1) to obtain six different arylamine analogs of naphthoquinone (**5a-f**).

In vitro antifungal activities

The compounds **1-4** and **5a-f** were evaluated for their *in vitro* antifungal activity against *C. albicans* ATCC10231, *C. albicans* 955, *T. mentagrophytes* and *M. gypseum* by diffusion technique and minimum inhibitory concentration (MIC) assay. [16]

Diffusion technique

All fungi were grown in Sabouraud Dextrose Agar (SDA) medium. Lorian disks were soaked with 2.5µL solution 10mg/mL of substances (**1-4, 5a-f**) in DMSO. Disks were put on an exponentially growing plated culture with appropriate dilution to 10^{-6} colony forming unit (CFU mL^{-1}). The plates were then incubated for 2 days (*C. albicans*) and 7 days (dermatophytes) at 37 ^{o}C. The results were recorded by measuring the zones surrounding the disk. Control disk containing DMSO and clotrimazole was used as reference in the assay.

MIC assay

In this process MIC of compounds **1-4** and **5a-f** were tested according to standard micro-broth dilution. Briefly, testing was performed in flat-bottomed 96-well tissue culture plate in SDA medium. The tested compounds was dissolved in DMSO and the concentration range was 64 – 0.5 µg/mL. Initial inocula of fungal strains were maintained at 10^{-3} $CFU.mL^{-1}$ (*C. albicans*) and 10^{-4} $CFU.mL^{-1}$ (dermatophytes). These plates were incubated in a moist chamber at 37 ^{o}C for 2 days (*C. albicans*) or 7 days (dermatophytes), before being read. MIC was defined as the lowest compound concentration preventing visible fungal growth. Clotrimazole was used as antifungal standard substance.

RESULTS AND DISCUSSION

Chemistry

Procedure for the synthesis of 2-methoxy-3-bromo-1,4-naphthoquinone (1)

Synthesis of 2-hydroxy-3-bromo-1,4-naphthoquinone from lawsone

A stirred solution of lawsone (1g, 5.7 mmol) in chloroform (100mL) was cooled to 20 ^{o}C and then 11.5ml H_2SO_4 2N was added. The mixture was then slowly added bromine (1.17ml; 3 mmol), and 0.7ml of solution H_2O_2 30%. The reaction was monitored by TCL until complete consumption of lawsone. The reaction was allowed to warm to room temperature and then a solution of $Na_2S_2O_3$ 10% was added to eliminate any bromine

remaining. The reaction mixture was extracted with chloroform. The combined organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure yielding (0.92g, 92%) as a brown yellow solid; mp 192 ^oC ; UV (1%, methanol, λ_{max} nm): 259.4 ; IR (KBr, cm^{-1}): 3186 (OH), 1674 (C=O), 1651, 1635 (C=C); ^{1}H-NMR (500MHz, DMSO-d_6): 8.04-8.02 (m, 2H, H6 and H7), 7.87-7.81 (m, 2H, H5 and H8).

Synthesis of 2-methoxy-3-bromo-1,4-naphthoquinone (1)

To a solution of 2-hydroxy-3-bromo-1,4-naphthoquinone (0.3g, 1.2 mmol) in acetone was slowly added solid K_2CO_3 (0.1794g, 1.3 mmol) and dimethylsulfat (0.124ml, 1.3 mmol). The mixture was refluxed in 6 hours and then was filtered to eliminate the solid K_2CO_3. The solution was then concentrated under reduced pressure producing **1** as a yellow solid (0.27g, 80%) ; mp 187 ^oC ; UV (λ_{max}, nm): 277.2 ; 247.4; 205 ; IR (ν_{max}/cm^{-1}): 1676, 1635, 1591 ; ^{1}H-NMR (DMSO-d_6, 500 MHz): 8.03-8.01 (m, 2H, H6 and H7); 7.87-7.85 (m, 2H, H5 and H8); 4.22 (s, 3H, OCH_3).

Synthesis of 2-hydroxy-3-chloro-1,4-naphthoquinone (2)

Dissolve 2-amino-3-chloro-1,4-napthoquinone (0.5g) in 20 ml mixture of glacial acetic acid – water – HCl (7:2:1). To above solution cooling at 10 - 15^oC, a solution of $NaNO_2$ 1% (10.7 mL) was slowly added. The solution color turned form red to orange yellow. The mixture was warmed to 50 ^oC in 30 minutes to hydrolyze the diazonium salt and then extracted with dichloromethane (3 x 20mL). The combined organic phase was dried over anhydrous Na_2SO_4 and then concentrated under reduced pressure to obtain an orange solid. Recrystallization from $CHCl_3$ - methanol (3 : 1) provided **2** as bright-orange crystals ; mp 299 ^oC ; UV (methanol, λ max, nm): 332; 275.5; 249.5 ; 243.5 ; IR (KBr, cm^{-1}): 3186 (OH); 1674 (C=O); 719(Cl) ; ^{1}H-NMR (MeOH-d_4, 500 MHz): 8.25 (m, 2H, H6 and H7); 7.96-7.87 (m, 2H, H5 and H8).

Synthesis of 2-(N-acetyl)-acetamido-3-chloro- 1,4-napthoquinone (3)

To a suspension of 2-amino-3-chloro-1,4-naphthoquinone (6.2 g, 0.03 mol) in acetic acid (10mL), an amount of 20g anhydride acetic (0.2 mol) was added. Then one drop of concentrated sulfuric acid was added. The mixture was refluxed at 70 ^oC in 6 hours. The reaction was cooled to 0 ^oC in 2 hours to form a yellow precipitate. The solid material was collected by vacuum filtration. Recrystallization from $CHCl_3$ - methanol (3 : 1) provided **3** as yellow crystals (3.9g, 45%) ; mp 206 ^oC ; UV (methanol, λmax, nm): 314; 288; 253.5; 248; IR (KBr, cm^{-1}): 1699 (C=O); 1637 (C=C); 1363 (CH3); ^{1}H-NMR (MeOH-d_4, 500 MHz): 8.18-8.13 (m, 2H, H6 and H7); 7.87-7.85 (m, 2H, H5 and H8); 2.24(s, 3H, CH3), 2.17 (s, 3H, CH3).

Synthesis of 2-ethylcarboxamido-3-chloro-1,4-naphthoquinone (4)

To a suspension of 2-amino-3-chloro-1,4-naphthoquinone (6.2 g, 0.03 mol) in propionic acid (10mL), 6.7g (0.05 mol) of anhydride propionic was added. Then one drop of concentrated sulfuric acid was added. The stirred mixture was refluxed at 70 ^oC in 6 hours. The reaction was cooled to 0 ^oC in 2 hours to form a pale yellow precipitate. The solid material was collected by vacuum filtration. Recrystallization from $CHCl_3$ - methanol (3 : 1) provided **4** as pale yellow crystals (3.5g, 45%) ; mp 166 ^oC ; UV (methanol, λ max, nm): 272.5; 252; 215.5 ; IR (KBr, cm^{-1}): 3068 (NH); 1660 (C=O); 1608 (C=C); ^{1}H-NMR (MeOH-d_4, 500 MHz): 8.15-8.09 (m, 2H, H6 and H7); 7.85-7.83 (m, 2H, H5 and H8); 1.31 (s, 2H, CH2), 1.23 (s, 3H, CH3).

General procedure for the synthesis of 2-arylamino-3-chloro-1,4-naphthoquinones (5a-f)

To a solution of 2,3-dichloro-1,4-naphthoquinone (1mmol) in absolute methanol was

added arylamine (1mmol). The reaction mixture was warmed to 50-60 ^{o}C and monitored by TCL. The solution was then allowed to cool to 0 ^{o}C. The solid material was collected by vacuum filtration. Recrystallization from $CHCl_3$ - methanol (3 : 1) provided **5a-f**

2-Chloro-3-(phenylamino)naphthalen-1,4-dione (**5a**) (100mg, 35%) ; red crystal solid ; mp 218-221 ^{o}C ; UV (methanol, λmax, nm): 476 và 274 ; IR (KBr, cm^{-1}): 3238 (N-H), 1676, 1637 (C=O), 1597, 1562, 1508 (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz,): 8.20 (d, *J* =7.5Hz, 1H, H5/H8); 8.13 (d, *J* = 7.5Hz, 1H, H8/H5); 7.77 (dt, *J* = 7.5Hz ; 1Hz, 1H, H6/H7); 7.69 (dt, *J* = 7,5Hz ; 1,0Hz, 1H, H7/H6); 7.68 (bs, 1H, NH), 7.37-7.34 (m, 2H, H3' and H5'); 7.22 (t, *J* = 7.5Hz, 1H, H4'); 7.09 (d, *J* = 8Hz, 2H, H2' and H6') ; ESI-MS (m/z): $[M+H]^+$ (284.054), $[M+Na]^+$ (306.035).

2-Chloro-3-(4-fluorophenylamino)naphthalen-1,4-dione (**5b**) (100mg, 33%) ; red crystals solid; mp 237-239 ^{o}C ; UV (methanol, λmax, nm): 274 ; IR (KBr, cm^{-1}): 3233 (N-H), 1674, 1639 (C=O), 1593, 1566, 1512 (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz,): 8.19 (dd, *J* =7.5Hz ; 1Hz, 1H, H5/H8); 8.12 (dd, *J* = 7.5Hz ; 1Hz, 1H, H8/H5); 7.77 (dt, *J* = 7.5Hz ; 1.5 Hz, 1H, H6/H7); 7.70 (dt, *J* = 7.5Hz ; 1.5Hz, 1H, H7/H6); 7.59 (bs, 1H, NH), 7.10-7.03 (m, 4H, H3', H4', H5', H6) ; ESI-MS (m/z): $[M+H]^+$ (302.064), $[M+Na]^+$ (324.045).

2-Chloro-3-(4-chlorophenylamino)naphthalen-1,4-dione (**5c**) (200mg, 55%) ; red crystals solid ; mp 263-265^{o}C; UV (methanol, λmax, nm): 361, 273, 215 ; IR (KBr, cm^{-1}): 3263 (N-H), 1672 , 1636 (C=O), 1603, 1566, 1512, (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz,): 8.20 (d, *J* =7.5Hz, 1H, H5/H8); 8.13 (d, *J* =7.5Hz, 1H, H8/H5); 7.78 (t, *J* = 7Hz, 1H, H6/H7); 7.70 (t, *J* = 7Hz, 1H, H7/H6); 7.59 (bs, 1H, NH), 7.32 (d, *J* = 8.5Hz, 2H, H3' and H5'); 7.01 (d, *J* = 8.5Hz, 2H, H2' and H6') ; ESI-MS (m/z): $[M+H]^+$ (317.998).

2-Chloro-3-(4-bromophenylamino)naphthalen-1,4-dione (**5d**): (190mg, 53%) ; red crystals solid ; mp 237-239 ^{o}C ; UV (methanol, λmax, nm): 476, 277; IR (KBr, cm^{-1}): 3244 (N-H), 1676, 1638 (C=O), 1600, 1566, 1504 (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz): 8.20 (d, *J* = 8Hz, 1H, H5/H8); 8.12 (d, *J* = 8Hz, 1H, H8/H5); 7.78 (dt, *J* = 7.5Hz ; 1.5 Hz, 1H, H6/H7); 7.70 (dt, *J* = 7.5Hz ; 1.5 Hz, 1H, H7/H6); 7.57 (bs, 1H, NH), 7.47 (d, *J* = 8.5Hz, 2H, H3' and H5'); 6.95 (d, *J* = 8.5Hz, 2H, H2' and H6') ; ESI-MS (m/z): $[M+H]^+$ (364.011).

2-Chloro-3-(3-(trifluoromethyl)phenylamino)naphthalen-1,4-dione (**5e**) (170mg, 48%) ; red crystals solid ; mp 195-198 ^{o}C ; UV (methanol, λmax, nm): 360, 275 ; IR (KBr, cm^{-1}): 3234 (N-H), 1674; 1645 (C=O), 1598, 1576, 1516 (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz) 8.21 (d, *J* = 8Hz, 1H, H5/H8); 8.18 (dd, *J* = 8Hz ; 1Hz, 1H, H8/H5); 7.79 (dt, *J* = 7.5Hz ; 1Hz, 1H, H6/H7); 7.72 (dt, *J* = 7.5Hz ; 1Hz, 1H, H7/H6); 7.66 (bs, 1H, NH), 7.48-7.46 (m, 2H, H3' and H4'); 7.32 (s, 1H, H6'); 7.24 (d, *J* = 7Hz, 1H, H2') ; ESI-MS (m/z): $[M+H]^+$ (352.057), $[M+Na]^+$ (374.046).

2-Chloro-3-(p-tolylamino)naphthalen-1,4-dione **(5f)** (200mg, 67%) ; red crystals solid ; mp 184-186 ^{o}C ; UV (methanol, λmax, nm): 484, 275 ; IR (KBr, cm^{-1}): 3227 (N-H), 1676, 1636 (C=O), 1599, 1562, 1518 (C=C) ; ^{1}H-NMR ($CDCl_3$, 500MHz,): 8.20 (d, *J* =7.5Hz, 1H, H5/H8); 8.11 (d, *J* = 7.5Hz, 1H, H8/H5); 7.79 (t, *J* = 7.5Hz, 1H, H6/H7); 7.67 (t, *J* = 7.5Hz, 1H, H7/H6); 7.64 (bs, 1H, NH), 7.15 (d, *J* = 8.25Hz, 2H, H3' and H5'); 6.99 (d, *J* = 8.25Hz, 2H, H2' and H6') ; ESI-MS (m/z): $[M+H]^+$ (298.095), $[M+Na]^+$ (320.007).

Antifungal activity

These 1,4-naphthoquinone derivatives (**1-4, 5a-f**) subjected to the *in vitro* antifungal test.

Results (Table 1) reported the inhibition zones (mm) of tested compounds determined for several fungal strains including *C. albicans* ATCC10231, *C. albicans* 955, *T. mentagrophytes* and *M. gypseum.* Compounds **2, 3** and **4** have significant inhibitory activity on *C. albicans* ATCC10231 with inhibitory zone similar to that of clotrimazole (30

mm). On the drug-resistant-*C. albicans* 955 strain, compounds **2** and **4** demonstrated an potential activity with inhibitory zone range between 36 and 15 mm, respectively compared to that of clotrimazole (12 mm). On dermophytes, all compounds have a low to average activity with inhibition zone range between 13-48 mm lower than that of the reference clotrimazole (60 mm).

Table 1. Antifungal activity of **1-4** and **5a-f** determined by diffusion technique

Compounds	Inhibitory zone (mm)			
	C .albicans ATCC10231	*C. albicans* 955	*T. mentagrophytes*	*M. gypseum*
Lawsone	0	0	0	0
1	22	0	30	34
2	40	36	24	40
3	30	0	48	38
4	30	15	43	30
5a	0	0	0	0
5b	0	0	0	0
5c	0	0	0	0
5d	10	0	14	16
5e	8	0	16	13
5f	10	0	20	20
Clotrimazole	30	12	60	60

The MIC of compounds **1-4** and **5a-f** were compared with that of clotrimazole (shown in Table 2). The results showed that compound **2** (MIC = 1 μg/ml), 4 (MIC = 4 μg/ml) had better activity than and compound **3** (MIC = 8 μg/ml) had same antifungal profile with clotrimazole against *C. albicans* ATCC10231. Compounds **2** and **4** (MIC = 0.25 and 32 μg/ml, respectively) also exhibited a promising antifungal activity on comparison with antifungal drug such as clotrimazole.

Table 2. Minimum inhibitory concentration (MIC) for **1-4** and **5a-f**

Compounds	MIC (μg/ml)			
	C. albicans ATCC10231	*C. albicans* 955	*T. mentagrophytes*	*M. gypseum*
Lawsone	-	-	-	-
1	8	-	8	8
2	1	0.25	4	8
3	8	-	1	2
4	4	32	1	0.5
5a	-	-	-	-
5b	-	-	-	-
5c	-	-	-	-
5d	32	-	64	32
5e	64	-	64	32
5f	32	-	8	2
Clotrimazole	8	16	1	1

- : not determined

The results revealed that substituted 1,4-naphthoquinone posses potent antifungal activity against *C. albicans* when 3-position is replaced by Cl group (**2** and **4**). The MIC values of tested compounds also demonstrated that antifungal activity was decreased considerably when the hydrogen atom at position-2 in naphthoquinone structure were replaced by a bulky group such as diacetyl (**3**) of phenyl group (**5a-f**).

CONCLUSION

In conclusion, we have synthesized a series of novel halogen substituted 1,4-naphthoquinone. The antifungal profile of these compounds indicated that compounds **2, 3** and **4** have potent antifungal activity. Among these promising antifungal candidates, 2-hydroxy-3-chloro-1,4-naphthoquinone (**2**) and 2-ethylcarboxamido-3-chloro-1,4-naphthoquinone (**4**) showed better antifungal activity than that of the clinically prevalent antifungal drug clotrimazole against *C. albicans* ATCC10231 and the drug-resistant *C. albicans* 955.

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