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Original Article

Nano-$SnCl_4.SiO_2$, an efficient catalyst for synthesis of benzimidazole drivatives as antifungal and cytotoxic agents

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Abstract

The concept of green chemistry has made significant impact on many frontages including the use of green solvents or sustainable catalyst materials. Benzimidazole ring is an important nitrogen-containing heterocyclic, which exhibits a broad spectrum of bioactivities and are widely utilized by the medicinal chemists for drug discovery. A simple and efficient method was developed for the synthesis of some benzimidazole derivatives *via* reaction of *o*-phenylenediamine and substituted aldehydes in the presence of nano-$SnCl_4/SiO_2$ as a mild catalyst. Ten 2-substituted benzimidazole compounds (***J*$_1$-*J*$_{10}$**) were synthesized. All compounds were evaluated against different species of yeasts and filament fungi using broth micro dilution method as recommended by clinical and laboratory standard institute. Among these compounds, the active ones were chosen for their cytotoxic activities evaluation against MCF-7 and A549 cell lines using MTT method. Compound ***J*$_2$** showed the best antifungal activity against all tested species. Compounds ***J*$_5$-*J*$_7$** had also desirable antifungal activities. Our cytotoxic results were also similar to the antifungal activities except for ***J*$_7$** which had no cytotoxic activity.

Keywords: Antifungal; Benzimidazole; Cytotoxic; MTT; Nano-$SnCl_4/SiO_2$

INTRODUCTION

Systemic fungal infections have enlarged in the past few decades, especially in immune-compromised individuals and in organ transplant recipients (1,2). The expansion of azoles has updated the treatment of many fungal infections, but still treatment of many of them necessitates application of the highly toxic drugs. Emergence of new resistant species of fungi in addition to the poor safety and pharmacokinetics profile, challenges the clinicians in their way to handle the fungal infections (3). Therefore, an urgent need for new antifungal chemical structures as alternative agents is required. On the other hand, cancer is a major public health problem worldwide (4). Many publications have shown an increase in the incidence of different type of cancer in the last 40 years (5). Several chemotherapeutic agents are now being used to treat human cancers but they have limited success in clinical trials. The main limitations in straight cancer chemotherapy rise from the absence of drug-specific affinity to tumor cells. Thus, there is a critical need to discover novel anticancer agents as well (6). Benzimidazole is a heterocyclic aromatic molecule with electron rich nitrogen atoms. Its derivatives have many biological applications such as antitumor (7), antimicrobial (8), anthelmintic (9), anti-inflammatory (10) and anti-diabetic (11). Different synthetic methods including solid phase synthesis (12), thermal or acid catalyzed cyclization (13), ceric ammonium nitrate catalysed (14) and using of samarium triflate (15) were reported for synthesis of benzimidazole compounds.

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However, some of the above cited methods suffer from several weaknesses like harsh reaction conditions, high catalyst cost, and existence of side reactions or tedious work-up procedure. We also described the synthesis of some benzothiazole (16), benzoxazine (17), benzimidazole (18), tetrazole (19,20), and benztriazole (21) derivatives as antifungal agents in our previous studies. Our previous results showed that benzimidazole compounds exhibited favorite biological activities and drug like properties (22). We also evaluated different type of catalysts for synthesis of azole compounds in our earlier works (23). Now in continuation of our work here we aimed to use nano-$SnCl_4.SiO_2$ as a reusable heterogeneous catalyst for synthesis of 2-substituted benzimidazole compounds with possible antifungal and cytotoxic activities. We also planned to evaluate the antifungal and cytotoxic activities of synthesized compounds. Different species of fungi were chosen for this purpose. In order to identify the potential antitumor candidates, the cytotoxic activities of the potent antifungal compounds were determined against MCF-7 and A549 cell lines.

MATERIAL AND METHODS

All chemicals were obtained from Merck and Fluka companies (Germany). Fourier-transform infrared attenuated total reflection (FT-IR, ATR) spectra were acquired on a Bruker, Equinox 55 spectrometer (Germany). The proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were run on a DRX-400 Avane (Germany). Melting points were recorded on a *Büchi B 545* apparatus (Germany) in open capillary tubes and all are uncorrected.

General procedure for synthesis of benzimidazole derivatives

A mixture of aldehyde (1 mmol), *o*-phenylenediamine (1 mmol), and nano-$SnCl_4/SiO_2$ (0.06 g) were placed in a round bottom flask and heated at 90 °C for 1 h under solvent free condition. Progress of the reaction was followed by thin-layer choromatography (TLC) using EtOAc/n-hexane. After completion of the reaction, the product was dissolved in chloroform and filtered to recover the catalyst. Then the solvent was evaporated, and the crude mixture was solidified from a mixture of ethanol and water. The pure product was obtained by re-crystallization in ethanol (Scheme 1).

Spectroscopy data

2-Phenyl-1H-benzimidazole ***(J_1)***

Yellow solid, FT-IR: ν_{max} (ATR, neat, cm^{-1}): 1462 (C=C stretch), 1277 (C-N bend), 743 and 703 (C-H bend); ^{1}H-NMR (400 MHz, DMSO-d_6): 7.29 (m, $2H_{6,5}$), 7.5 (m, $3H_{3',4',5'}$), 7.66 (brs, $2H_{4,7}$), 8.075 (dd, J = 7.4, 2 Hz, $2H_{2',6'}$), 10 (brs, 1H, N-H1) ppm.

2-(4'-Fluorophenyl)-1H-benzimidazole ***(J_2)***

Yellow crystal, FT-IR: ν_{max} (ATR, neat, cm^{-1}) = 1429 (C=C stretch), 1273 (C-N bend), 746 (=C-H), cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): 7.32 (brs, $2H_{6,5}$), 7.42 (m,$3H_{3',5',4}$), 7.52 (d, J = 7.6 Hz, $1H_7$), 7.8 (brs, $1H_{2'}$), 8.83 (d, J = 7.6 Hz, $1H_{6'}$), 10.3 (brs, 1H, N-H_1) ppm.

2-(4'-Chlorophenyl)-1H-benzimidazole ***(J_3)***

Orange solid, FT-IR: ν_{max} (ATR, neat, cm^{-1}): 1429 (C=C stretch), 1273 (C-N bend), 746 (=C-H); ^{1}H-NMR (400 MHz, $CDCl_3$): 7.32 (brs, $2H_{6,5}$), 7.42 (m, $3H_{3',5',4}$), 7.52 (d, J = 7.6 Hz, $1H_7$), 7.83 (brs, $1H_{2'}$), 8.47 (d, J = 7.6 Hz, $1H_{6'}$), 10.3 (brs, 1H, N-H_1) ppm.

2-(4'-Bromophenyl)-1H-benzimidazole ***(J_4)***

Yellow solid, FT-IR: ν_{max} (ATR, neat, cm^{-1}): 1599 (C=N stretch), 1458 (C=C stretch), 1231 (C-N bend), 747 (C-H bend); ^{1}H-NMR (400 MHz, DMSO-d_6): 7.20 (brs, $2H_{6,5}$), 7.6 (brs, $2H_{3',5'}$), 7.8 (d, J = 7.2 Hz, $2H_{4,7}$), 8.1 (d, J = 7.2 Hz, $2H_{2',6'}$), 13.1 (s, 1H, N-H_1) ppm.

2 - (2', 3'- Dihydroxyphenyl) - 1 H - benz-imidazole ***(J_5)***

Red crystal; FT-IR: ν_{max} (KBR, cm^{-1}) = 3453 (O-H), 1618 (C=N stretch), 1580 (C=C stretch), 1543 (N-H bend), 1460 (C=C stretch), 1210 (C-N bend), 1032 (C-H bend), 763 (C-H bend); ^{1}H-NMR (400 MHz, DMSO-d_6): 6.77 (t, J = 7.6 Hz, $1H_{5'}$), 6.92 (d, J = 6.4 Hz, $1H_{4'}$), 7.095 (d, J = 6.4 Hz, $1H_{6'}$), 7.4 (brs, $4H_{4,5,6,7}$), 8.86 (s, O-$H_{2'}$), 9.27 (s, O-$H_{3'}$), 12.91 (s, 1H, N-H_1) ppm.

J_1: R_1=R_2=H
J_2: R_1=H, R_2=4-F
J_3: R_1=H, R_2=4-Cl
J_4: R_1=H, R_2=4-Br
J_5: R_1=H, R_2=2,3-di(OH)
J_6: R_1=CH_3, R_2=4-NO_2
J_7: R_1=NO_2, R_2=4-NO_2
J_8: R_1=H, R_2=4-CH_2CH_2Ph
J_9: R_1=NO_2, R_2=2,3-di(OH)
J_{10}: R_1=NO_2, R_2=4-OH

Scheme 1. Synthesis of benzimidazole derivatives.

2 - (4 '- Nitrophenyl)- 1 H - 5 – methyl - benz-imidazole ***(J_6)***

Yellow crystal; FT-IR: ν_{max} (KBR, cm^{-1}) = 1603 (C=N stretch), 1520 (NO_2 stretch), 1344 (NO_2 stretch), 1109 (C-N bend) cm^{-1}, ^{1}H NMR (400 MHz, DMSO-d_6): 2.43 (s, $3H_{methyl}$), 7.1 (s, 1H7), 7.4-7.6 (m, 2H4,6), 8.4 (m, $4H_{2',3',5',6'}$), 13.3 (s, 1H, NH_1) ppm.

2 - (4' - Nitrophenyl) – 1 H – 5 – nitro - ben z-imidazole ***(J_7)***

Orange crystal; FT-IR: ν_{max} (KBr, cm^{-1}) = 1605 (C=N stretch), 1522 (NO_2 stretch), 1418 (C=C stretch), 1344 (NO_2 stretch), 759 (C-H bend) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): 7.80 (brs, $1H_7$), 8.12 (brs, $1H_4$), 8.41 (brs, $4H_{6,2',6',3'}$), 8.49 (brs, $1H_{5'}$), 13.9 (s, 1H, N-H_1) ppm.

2-(2-Ethylephenyl)-benzimidazole ***(J_8)***

Red solid; FT-IR: ν_{max} (KBr, cm^{-1}) = 3160 (=C-H stretch), 1600 (C=N stretch), 1575 (C-N bend) cm^{-1}; ^{1}H NMR (400 MHz, DMSO-d_6): 3.15-3.55 (m, $4H_{ethyl}$), 6.5-7.8 (m, $9H_{2',3',4',5',6',4,5,6,7}$, and 1H, N-$H_1$) ppm.

2-(2',3'-Dihydroxyphenyl)-1H-5-nitro-benz-imidazole ***(J_9)***

Red solid, FT-IR: ν_{max} (ATR, neat, cm^{-1}): 3391 (O-H), 1603(C=N stretch), 1459 (C=C stretch), 1338 (NO_2 stretch), 1264 (C-N bend); ^{1}H-NMR (400 MHz, $(CD_3)_2CO$): 6.9 (t, J = 7.6 Hz, $1H_{4'}$), 6.98 (d, J = 8.8 Hz, $1H_{5'}$), 7.04 (d, J = 7.6 Hz, $1H_{6'}$), 7.2 (d, J = 7.6 Hz, $1H_7$), 7.8 (s, $1H_4$), 7.9 (d, J = 8.4, $1H_6$), 8.04 (s, O-H), 8.9 (s, O-H), 12.5 (s, 1H,N-H_1) ppm.

2 - (4' - Hydroxyphenyl) - 1 H5 – nitro - benz-imidazole ***(J_{10})***

Yellow solid, FT-IR: ν_{max} (ATR, neat, cm^{-1}): 3401 (O-H stretch), 1609 (C=N stretch), 1425 (C=C stretch), 1126 (C-N bend), 758 (C-H bend); ^{1}H NMR (DMSO-d_6): δ 3.5 (br s, O-H), 6.9 (d, J = 8.4 Hz, $2H_{3',5'}$), 7.38(m, $2H_{4,7}$), 7.51(m, $2H_{4,7}$), 7.98 (d, J = 8.4 Hz, $2H_{2',6'}$), 9.98 (s, 1H, N-H_1) ppm.

Antifungal activity

Microorganisms

The antifungal activities of the synthesized compounds were examined against some American type culture collection (ATCC) strains of fungi including *Candida albicans (C. albicans, ATCC 10261), C. dubliniensis (CBS 8501), C. parapsilosis (ATCC 4344), C. krusei (ATCC 6258), C. tropicalis (ATCC 750), Cryptococcus neoformanse (H99), Aspergillus flavus (ATCC 64025), Aspergillus clavatus (CBS 514.65), Alternaria alternate, Microsporum canis,* and *Trichophyton mentagrophytes* . The susceptibility of all clinical isolates of fungi against selected antibiotics was examined by microdilution and disk diffusion methods.

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) were determined using the broth micro dilution method recommended by the clinical and laboratory standard institute. Briefly, for determination of antimicrobial activities

against fungi, serial dilutions of the synthesized compounds (***J1-J10***) were prepared in 96-well micro titer plates using RPMI-1640 media buffered with 3-(N-morpholino)propanesulfonic acid (MOPS). Stock inoculums were prepared by suspending three colonies of the examined yeast in 5 mL sterile 0.85% NaCl, and adjusting the turbidity of the inoculums to 0.5 McFarland standards at 530 nm wavelengths. For molds, conidia were recovered from the 7-day old cultures grown on potato dextrose agar by a wetting loop with Tween20. The collected conidia were transferred in sterile saline and their turbidity was adjusted to 0.09-0.11 optical density. Working suspension was prepared by making a 1:50 and 1:1000 dilution with RPMI of the stock suspension for molds and yeasts, respectively. Working inoculums were added to the micro titer plates and incubated in a humid atmosphere at 30 °C for 24-48 h. Inoculated medium was included as a sterile control. In addition, growth controls (medium with inoculums but without antibiotics or the synthetic compounds) were also included. The growth in each well was compared with that of the growth in the control well. Fluconazole was used as positive control.

Cytotoxic activity

Cell lines and cell culture

Two human cancer cell lines, MCF-7 and A549, were purchased from the National Cell Bank of Pasteur Institute of Iran. Using aseptic techniques, the cells were cultured in RPMI 1640 medium, containing 10% fetal bovine serum, 100 units, penicillin, streptomycin (100 µg/mL), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. Following enough confluence, the cells were treated with 25% trypsin-EDTA and sub-cultured. The cells were then washed, counted, and prepared for cytotoxic MTT assay as previously described (24-26).

MTT assay

More active benzimidazole compounds (***J2, J5-J7***) according to antifungal studies were chosen for cytotoxic evaluation. Briefly 10^4 cells in the complete culture medium were seeded per well in a 96-well cell culture microplate. After attachment the cells were treated with different concentrations of each compound (1-1000 µM). Three wells were left without treatment as cell-based negative controls, and three wells containing cell culture medium alone were considered as blanks. Cisplatin was used as positive control. After 48 h incubation, the culture media were removed and 100 µL of MTT solution (0.5 mg/mL) were added to the wells including controls. The plate was incubated for 3-4 h at 37 °C and checked periodically for the appearance of purple precipitate. After complete removing of MTT solution, 150 µL of DMSO was added to the wells and left in the 37 °C incubator for more 30 min. The absorbance of all wells including the blanks, were measured at 570 nm. The optical density (OD) of individual well was obtained at 570 nm using a microplate ELISA reader (Biotek, USA). Data are presented as mean ± SD of three separate experiments.

Data analysis

The average values from triplicate readings were determined and subtracted the average value for the blank. The inhibitory concentration (IC) of each compound was calculated and reported using following equation:

$$\text{IC} = 100 - \frac{\text{OD of tteated well} - \text{OD of blank}}{\text{OD of negative control}} \times 100$$

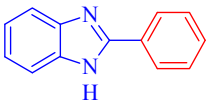
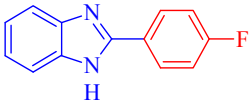
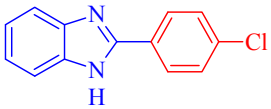
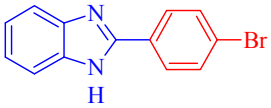
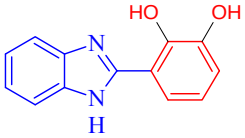
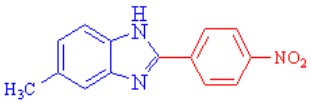
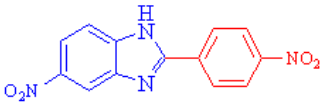
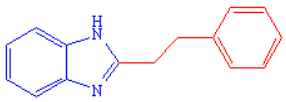
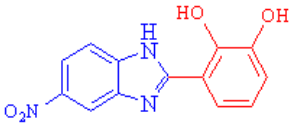
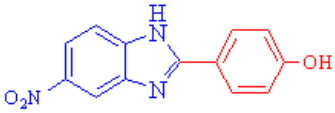
For each chemical a plot of the IC versus concentration was depicted using Curve Expert 1.4 software and IC_{50} was obtained for each compound. *P* values less than 0.05 were considered statistically significant.

RESULTS

Chemistry

Ten 2-substituted benzimidazole analogs were synthesized in the presence of nano-$SnCl_4.SiO_2$ under solvent free conditions. All synthesized compounds and their characteristics are shown in Table 1.

Table 1. Different characteristics of the synthesized compounds.

Entry	Chemical structure	Molecular weight (g/mol)	Melting point (°C)	Yield (%)
***J*1**		194.08	287-288	95
***J*2**		212.22	290-292	97
***J*3**		228.67	287-289	95
***J*4**		273.12	248-252	93
***J*5**		226.23	201-202	89
***J*6**		253.25	234-238	95
***J*7**		284.22	255-256	97
***J*8**		298.38	246-247	92
***J*9**		271.22	201-204	84
***J*10**		249-252	211-213	86

Antifungal activities of the synthetic compounds

Table 2 summarizes the inhibitory activities of the synthetic compounds against the tested fungi. MIC values of the synthetic compounds indicated that ***J*2** exhibited strong inhibitory activities against most of the tested fungi. Compounds ***J*5** and ***J*6** showed their greatest inhibitory activities against *C. krusei* and *C. dubliniensis* at 32 and 64 µg/mL, respectively. Compounds ***J*7** showed moderate inhibitory activity against *C. dubliniensis, C. krusei,* and *C. neoformance*.

Cytotoxic activities

The cytotoxic activities of ***J*2, *J*5-*J*7** were investigated against MCF-7 and A549 cells. The IC_{50} values of tested compounds are presented in Table 3. ***J*2** was found as the most active compounds which exhibited higher toxicities with IC_{50} of 127.2 ± 4.6 and 157.55 ± 5.3 µM against MCF-7 and A549 cell lines respectively. ***J*5** and ***J*6** with IC_{50} values of 656.4 ± 10.8 and 590.12 ± 2.2 µM for MCF-7 and 254.2 ± 9.6 and 635.65 ± 1.9 µM for A549 cell lines showed moderate to weak cancer cell toxicity. ***J*7**, however, showed no cytotoxic effects against investigated cell lines.

Table 2. Minimum inhibitory and fungicidal concentrations (μg/mL) of the synthesized compounds against examined fungi.

Microorganisms		synthetic compounds										
		J1	***J2***	***J3***	***J4***	***J5***	***J6***	***J7***	***J8***	***J9***	***J10***	**Fluconazole**
Candida albicans	MIC_{50}		64									2
	MIC_{90}		128									8
Candida dubliniensis	MIC_{50}		16			128	64	128				0.5
	MIC_{90}		32			256	256	256				1
Candida parapsilosis	MIC_{50}		128									1
	MIC_{90}		256									2
Candida krusei	MIC_{50}		16			32	128	128				32
	MIC_{90}		32			128	256	256				128
Candida tropicalis	MIC_{50}		128									16
	MIC_{90}		256									32
Cryptococcus neoformans	MIC_{50}		8				128	128				8
	MIC_{90}		32				256	256				32
Aspergillus flavus	MIC_{50}											8
	MIC_{90}											32
Aspergillus clavatus	MIC_{50}											4
	MIC_{90}											8
Alternaria	MIC_{50}											8
	MIC_{90}											16
Microsporum canis	MIC_{50}		32			128						0.5
	MIC_{90}		64			256						1
Trichophyton mentagrophytes	MIC_{50}		64			128						1
	MIC_{90}		128			256						2

$MIC_{50,}$ minimal concentration of an antimicrobial necessary to inhibit the growth of 50% of a target micro-organism; $MIC_{90,}$ minimal concentration of an antimicrobial necessary to inhibit the growth of 90% of a target micro-organism.

Table 3. *In vitro* cytotoxicity (IC_{50}, μM) of four investigated benzimidazole on two different cancer cell lines.

Entry	**IC_{50} (μM)**	
	MCF-7	**A549**
J2	127.2 ± 4.6	157.55 ± 5.3
J5	656.4 ± 10.8	254.2 ± 9.6
J6	590.12 ± 2.2	635.65 ± 1.9
J7	> 1000	> 1000
Cisplatin	61.56 ± 0.98	50.81 ± 3.10

DISCUSSION

Solid acids do less harm to the environment and have many advantages over liquid acids (27). We demonstrated a simple method for synthesis of azole compounds as possible biologically active compounds. Benzimidazoles are important class of heterocyclic compounds which have a wide range of therapeutic effects such as antimicrobial, antiviral, antifungal, antihypertension, and anticancer activities (28). Synthesized benzimidazoles exhibited wide range of antifungal activities. Substitution of chloro, trifluoromethyl, methoxy, and ethoxy groups at 5 position were found to be more potent substituents (29). A highly efficient method for the synthesis of benzimidazole analogues was developed by treatment of *o*-phenylenediamine with aldehyde moiety. We used nano-$SnCl_4.SiO_2$ as eco-friendly and efficient catalyst in a one-pot procedure. Short reaction time, high yield, clean and simple procedure, easy work-up, and green condition are advantages of this protocol. The lower cost of the catalyst and the utilization of environmental protection are the other benefits of this method. Ten derivatives were synthesized and characterized by different spectroscopic methods. Different aldehydes were used to create the benzimidazole ring and also put the desired substitution

at the 2 position of the ring. Compounds ***J₂*** as the most potent compound, showed antifungal activity against *C. neoformans, C. krusei, C. dubliniensis, Microsporum canis, C. albicans,* at 8-64 µg/mL. The cytotoxic activities of four derivatives (***J₂, J₅-J₇***) were also assessed against two cancerous cell lines. Similar to antifungal results, ***J₂*** had satisfactory anti-tumour activities especially on breast cancer cell line (MCF-7) compared to carcinoma cell line (A549). Compounds ***J₅*** and ***J₆*** had also moderate activities while ***J₇*** showed no toxicities. This activity may be related to chemical structure of the compounds. As it is observed in Table 1, the presence of small electronegative halogen group (fluorine) in ***J₂*** structure at para position of the benzene ring increased the cytotoxic activities.

CONCLUSION

Recently, the emphasis on green chemical principles introduced some significant advances in organic synthesis. In this regard, heterogeneous catalysts have found considerable interest in organic reactions. Reactions with these catalysts are generally clean and selective and give high yields of products (29). Our results collectively indicated that nano-$SnCl_4.SiO_2$ was a mild and cost-effectiveness catalyst for synthesis of benzimidazole compounds. Among our synthesized compounds, ***J₂*** showed antifungal activity against different fungi species at lower concentration. Based on the cytotoxic effects of potent antifungal derivatives, ***J₂*** also had reasonable antitumor activities on both examined cancerous cell lines. Our data suggest that among these analogues of benzimidazoles, ***J₂,*** with the highest potency on both antifungal and cytotoxic tests, could be introduced as a proper candidate for further *in vitro* and *in vivo* studies.

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