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Short communication

Proton-pumping-ATPase-targeted antifungal activity of cinnamaldehyde based sulfonyl tetrazoles

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ABSTRACT

Azoles are generally fungistatic, and resistance to fluconazole is emerging in several fungal pathogens. We designed a series of cinnamaldehyde based sulfonyl tetrazole derivatives. To further explore the antifungal activity, *in vitro* studies were conducted against 60 clinical isolates and 6 standard laboratory strains of *Candida*. The rapid irreversible action of these compounds on fungal cells suggested a membrane-located target for their action. Results obtained indicate plasma membrane H^+-ATPase as site of action of the synthesized compounds. Inhibition of H^+-ATPase leads to intracellular acidification and cell death. Presence of chloro and nitro groups on the sulfonyl pendant has been demonstrated to be a key structural element of antifungal potency. SEM micrographs of treated *Candida* cells showed severe cell breakage and alterations in morphology.

1. Introduction

Candida albicans is an emerging cause of infection in both adults and infants, and its resistance to azole antifungals is an increasing problem [1,2]. Amphotericin B and azoles, basically fluconazole are the currently available treatment options, which suffer from many serious drawbacks [3–5]. Such serious global health problem demands a renewed effort seeking the development of new antimicrobial agents effective against pathogenic microorganisms resistant to currently available treatments. To overcome this problem we designed a series of spice oil cinnamaldehyde based sulfonyl tetrazole derivatives. Sulfonamide derivatives possess significant antimicrobial activity [6,7]. Some of them are HIV protease inhibitors [8], carbonic anhydrase inhibitors [9], and anticonvulsant agents [10,11]. Tetrazoles on the other extreme are regarded as biologically equivalent to the carboxylic acid group with strong growth inhibitory activity against different *Candida* species [12]. It is reported that tetrazole derivatives were the most active against *C. albicans* in comparison to standard antifungal drug [13]. However, a concrete mechanism for their antimicrobial action has yet to be explained.

The yeast plasma membrane H^+ ATPase is a promising new antifungal target [14,15]. This enzyme plays a crucial role in fungal cell physiology, intracellular pH regulation, cell growth and has been implicated in the pathogenicity of fungi through its effects on dimorphism, nutrient uptake and medium acidification [14,16]. Previously we have reported proton translocating ATPase mediated fungicidal activity of various essential oil components [17–19]. The objective of this study was to further elucidate the antimicrobial mechanism of action of newly synthesized cinnamaldehyde based compounds by determining their effect on the activity of H^+ ATPase located in the membranes of pathogenic *Candida* species. Importantly, we also illustrate that these synthesized compounds are effective *in vitro* against *Candida* species which were demonstrated to be largely insensitive to azoles.

2. Results and discussion

2.1. Chemistry

Present study was undertaken to synthesize some novel sulfonyl tetrazole derivatives and study their probable antifungal behaviour.

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Target compounds were obtained in a two step reaction procedure as outlined in Scheme 1. First of all, 5-[(*E*)-2-phenylethenyl]-2*H*-tetrazole (**1**) was synthesized from cinnamaldehyde by following a reported procedure [20]. 5-[(*E*)-2-phenylethenyl]-2*H*-tetrazole was treated with different arylsulfonylchlorides (substituted/unsubstituted), in presence of triethylamine using dry CH_2Cl_2 as a solvent to get the target compounds. All the synthesized compounds were characterized by elemental analysis, IR, ^1H, ^{13}C NMR and ESI-MS studies and their data is presented in experimental section.

2.2. Pharmacology

2.2.1. Minimal inhibitory concentration

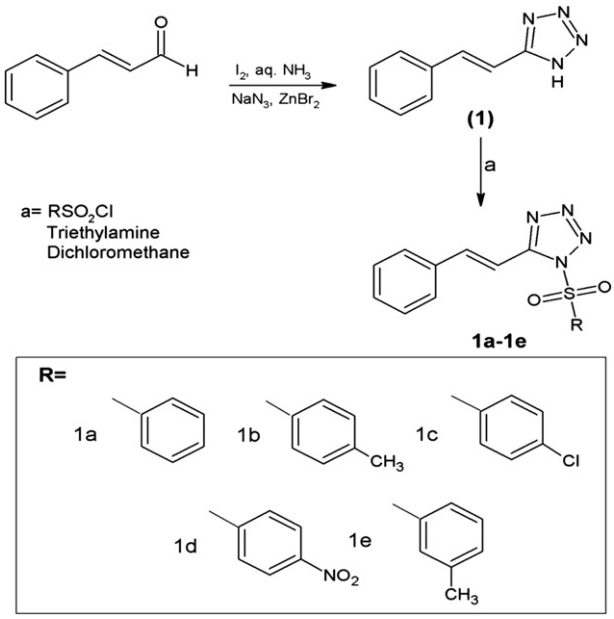
Table 2 summarizes the *in vitro* susceptibilities of 46 *Candida* isolates of fluconazole-susceptible and 20 *Candida* isolates of fluconazole-resistant category (Table 1). The data are reported as MICs required to inhibit 90% growth of the *Candida* cell population. Species exhibiting fluconazole MIC $\geq$ 64 μg/ml were considered as resistant.

2.2.2. Disc diffusion

The results summarized in Table 3 give the sensitivity assay, using standard discs of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**), compound (**1e**) and fluconazole (100 μg/ml). Both the standard and clinical types of *Candida* isolates showed high degree of sensitivity as is evident from the zone of clearance in each case. Index of sensitivity defined as

$$\sum \text{Zone diameter(mm)/concentration(mg)} = \text{clearing(mm)/mg}$$

is greater (3.47 ± 0.53) for resistant isolates when treated with compound (**1d**) and least (0.73 ± 0.15) for clinical isolates when treated with compound (**1a**). Results obtained demonstrated that the ability to kill *Candida* species is dependent on the concentration of the test compound. The discs impregnated with DMSO (negative control) showed no zone of inhibition and hence 10% DMSO had no effect on the strains tested in the present study. Our results show that the growth was inhibited by synthesized compounds, and the halo was completely clear. Fig. S1 in the supplementary information indicates potential fungicidal activity in fluconazole-sensitive strains, whereas in case of the resistant strains fluconazole showed a turbid halo and in most of the cases the zone was absent an indication of its fungistatic nature.



Scheme 1. Synthesis of 1-(phenyl/substituted phenyl) sulfonyl]-5-[(*E*)-2-phenyl ethenyl]-1*H*-tetrazoles.

Table 1
Isolates used in the present study.

Classification of isolates	Type of isolate
Sensitive (Standard, $n = 6$)	
ATCC 10261	*C. albicans*
ATCC 44829	*C. albicans*
ATCC 90028	*C. albicans*
ATCC 750	*C. tropicalis*
ATCC 90030	*C. glabrata*
ATCC 6258	*C. krusei*
Sensitive (Clinical, $n = 40$)	
Invasive ($n = 8$)	*C. albicans*(3), *C. tropicalis*(1), *C. glabrata*(3), *C. krusei* (1)
Cutaneous ($n = 10$)	*C. albicans*(6), *C. glabrata*(3), *C. krusei* (1)
Respiratory:	
Bronchioalveolar ($n = 7$)	*C. albicans* (3), *C. glabrata*(2), *C. krusei* (2)
Oropharyngeal ($n = 9$)	*C. albicans*(3), *C. tropicalis*(1), *C. glabrata*(3), *C. guilliermondii*(1), *C. krusei* (1)
Resistant[a] (Clinical, $n = 20$)	
Invasive ($n = 7$)	*C. albicans*(4), *C. tropicalis*(1), *C. glabrata*(1), *C. krusei* (1)
Cutaneous ($n = 7$)	*C. tropicalis*(2), *C. glabrata*(2), *C. guilliermondii*(2), *C. krusei* (1)
Respiratory ($n = 6$)	*C. albicans*(3), *C. tropicalis*(2), *C. glabrata*(1)

[a] Fluconazole MIC $\geq$ 64 μg/ml considered as resistant.

2.2.3. Growth studies (Turbidometric measurement)

Growth pattern of *Candida* species was investigated at different concentrations of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**). Fig. 1 (A)–(C) shows the effect of different concentrations of compound **1c**, compound **1d** and compound **1e** on growth pattern of *C. albicans* ATCC 10261. Significant and pronounced effect is observed for other tested species (data not shown). Control cells showed a normal pattern of growth with lag phase of 4 h, active exponential phase of 8–10 h before attaining stationary phase. Increase in concentration of test agents leads to significant decrease in growth with suppressed and delayed exponential phases with respect to control. At MIC_{90} values almost complete cessation of growth was observed for all the yeast species. Effectiveness of growth inhibition was measured by comparing optical densities on full growth (24 h) at half MIC of respective compounds. Average decrease in optical densities of four *Candida* isolates of fluconazole-susceptible and three *Candida* isolates of fluconazole-resistant category with respect to control was 60.64% for compound (**1a**), 71.06% for compound (**1b**), 82.92% for compound (**1c**), 91.53% for compound (**1d**) and 77.31% for compound (**1e**) respectively.

Table 2
Minimum inhibitory concentrations (90%) of the synthesized compounds against *Candida* isolates.

Bioactive compound	Mean MIC_{90} (μg/ml)	
	Fluconazole-sensitive ($n = 46$)	Fluconazole-resistant ($n = 20$)
Compound (**1a**)	250–450	200–300
Compound (**1b**)	150–250	150–250
Compound (**1c**)	80–110	70–90
Compound (**1d**)	60–90	50–70
Compound (**1e**)	150–200	100–200

Table 3
In vitro sensitivity of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**) against *Candida* isolates as determined by disc diffusion assay.

Candida isolates	Sensitivity index (mm/mg)				
	Compound (**1a**)	Compound (**1b**)	Compound (**1c**)	Compound (**1d**)	Compound (**1e**)
Standard ($n = 6$)	0.77 ± 0.18	0.81 ± 0.41	2.77 ± 0.33	3.16 ± 0.41	1.42 ± 0.60
Clinical ($n = 10$)	0.73 ± 0.15	1.05 ± 0.09	2.31 ± 0.51	2.71 ± 0.37	1.18 ± 0.19
Resistant ($n = 10$)	0.98 ± 0.21	1.27 ± 0.23	3.29 ± 0.47	3.47 ± 0.53	1.71 ± 0.28

Fluconazole (100 μg/ml) showed inhibitory zone (mm) of 14.33 (±2.58), 15.8 (±1.61) and 02 (±1.24) for standard, clinical and resistant isolates. Sensitivity index is expressed as mean ± SD and was calculated as diameter of zone of inhibition (mm)/concentration of drug (mg/ml). *n* is number of isolates. Each isolate was tested in duplicate.

2.2.4. Proton efflux measurements

H^+-efflux is an immediate event associated with H^+ ATPase activity. Proton-pumping ability of fungi mediated by the H^+ ATPase at the expense of energy is crucial for the regulation of internal pH and growth regulation of fungal cell. Fungal cells depleted of carbon-source when exposed to glucose, rapidly acidify medium to generate proton motive force for nutrient uptake. *Candida* cells susceptible to the synthesized compounds were examined for the ability to pump intracellular protons to the external medium (as measured by the alteration of pH of the external medium) in the presence of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**). Table 4 gives relative rates of H^+-efflux by *Candida* sp. in presence of synthesized compounds and fluconazole (5 μg/ml). H^+-extrusion inhibition in standard isolates was 32.37%, 50.22%, 89.90%, 96.05% and 62.88% when treated with compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**), respectively. H^+-extrusion rate was also decreased to 21.00%, 38.91%, 80.75%, 88.89% and 56.82% for compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**), in clinical isolates. In case of resistant isolates the decrease was 36.81%, 54.54%, 93.56%, 97.21% and 66.35% when cells were treated with compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**), respectively. Glucose (5 mM) stimulated H^+-efflux in all the strains by 4–5 folds. Glucose-stimulated H^+-efflux was also inhibited by the synthesized compounds. Glucose-stimulated H^+-efflux rates in standard, resistant and clinical isolates with respect to control, were 16.68%, 16.81% and 11.17% in the presence of compound (**1a**), 36.85%, 36.37% and 26.29% in the presence of compound (**1b**), 60.53%, 61.90% and 49.57% in presence of compound (**1c**), 64.50%, 69.86% and 59.42% in presence of compound (**1d**) and 45.35%, 45.91% and 38.57% in presence of compound (**1e**), respectively. Detailed studies of test compounds on the same can give us more insight into the possible mechanisms of action.

2.2.5. Measurement of intracellular pH

Intracellular pH and the H^+ pump are thought to play an important role in yeast growth. Many cellular processes are regulated by the internal pH, and many transport processes depend on the H^+ cycle. Generally internal pH of yeast cells is maintained between 6.0 and 7.5 by H^+ ATPase activity. We tried to investigate whether cells with normal H^+ ATPase activity maintain the constant internal pH as compared to cells with low activity. Fig. 2 shows changes in the pattern of pHi with control and treated cells. Only yeast control cells with normal H^+ ATPase activity maintain the pHi (6.52), while the treated cells show increase in internal acidification. The decrease in pHi was more in cells exposed to compound (**1d**) than rest of the four compounds.

2.2.6. Scanning electron microscopy

Finally SEM observations were used to study the anticandidal activity of the synthesized compound. Interaction between compound (**1d**) and *Candida* cells was demonstrated by SEM after a 14 h exposure. Fig. 3 results clearly showed differences in morphology between untreated and compound **1d** treated *Candida* cells. On the basis of present microscopic analyses of the *Candida* cells, it can be concluded that compound (**1d**) causes irreparable damage to *Candida* cells. The SEM micrographs for untreated *C. albicans* Seq No 1138 shows well defined shape with normal smooth surfaces Fig. 3 (A). There was complete damage to the cell of compound **1d** treated fungi, indicated by black arrow in Fig. 3 (B). In addition to this multiple breaks were also noticed in the membrane of the treated cell which is indicated by white arrow in Fig. 3 (B). Interesting finding is that compound **1d** causes complete cellular damage at MIC_{90} concentrations.

2.2.7. Hemolytic activity

To evaluate the toxicity of synthesized compounds, they were tested against human red blood cells. Effects of test agents on human red blood cells are shown in Fig. 4. Compound (**1a**),

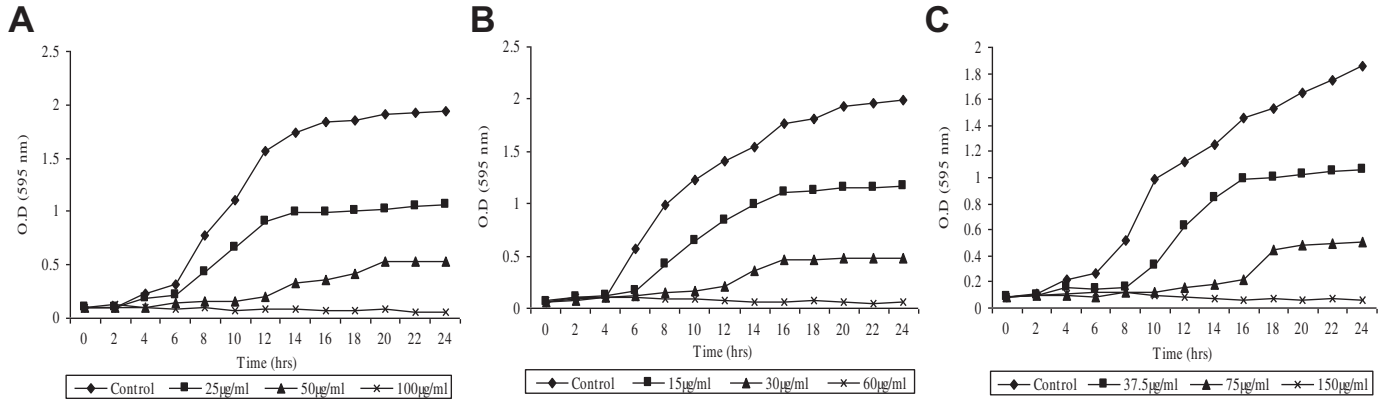


Fig. 1. Effect of synthesized compounds on growth of *C. albicans* ATCC 10261. Growth curve plotted against absorbance at 595 nm and time (h) shows complete inhibition of growth at MIC_{90} values. (A) In presence of compound **1c**. (B) In presence of compound **1d**. (C) In presence of compound **1e**.

Table 4
Effect of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**) on the rate of H^+-efflux by various *Candida* isolates at pH 7. Cells were suspended in 0.1 mM $CaCl_2$ and 0.1 M KCl at 25 °C.

Incubation with	Range of relative H^+-efflux rate		
	Standard	Clinical	Resistant
Control	1	1*	1**
Compound **1a** (MIC_{90})	0.67 ± 0.02 (32.37)	0.78 ± 0.03 (21.00)	0.62 ± 0.02 (36.81)
Compound **1b** (MIC_{90})	0.49 ± 0.03 (50.22)	0.60 ± 0.04 (38.99)	0.44 ± 0.04 (54.45)
Compound **1c** (MIC_{90})	0.09 ± 0.01 (89.90)	0.18 ± 0.03 (80.75)	0.05 ± 0.02 (93.56)
Compound **1d** (MIC_{90})	0.03 ± 0.02 (96.05)	0.10 ± 0.02 (88.89)	0.02 ± 0.01 (97.21)
Compound **1e** (MIC_{90})	0.36 ± 0.01 (62.88)	0.42 ± 0.02 (56.82)	0.33 ± 0.01 (66.35)
Glucose (5 mM)	4.19	3.80	3.71
Glucose + Compound **1a**	3.48 ± 0.51 (16.68)	3.37 ± 0.48 (11.17)	3.09 ± 0.24 (16.81)
Glucose + Compound **1b**	2.65 ± 0.60 (36.85)	2.78 ± 0.40 (26.29)	2.35 ± 0.26 (36.70)
Glucose + Compound **1c**	1.64 ± 0.63 (60.53)	1.90 ± 0.26 (49.57)	1.41 ± 0.17 (61.90)
Glucose + Compound **1d**	1.48 ± 0.63 (64.50)	1.53 ± 0.22 (59.42)	1.12 ± 0.18 (69.86)
Glucose + Compound **1e**	2.29 ± 0.63 (45.35)	2.32 ± 0.33 (38.57)	1.99 ± 0.25 (45.91)

Control had an average (of 4 independent recordings) H^+-efflux rate of 5.58 nmol/min/mg cells in standard isolates (1); 5.51 nmol/min/mg yeast cells in clinical isolates (1*) and 5.73 nmol/min/mg yeast cells in resistant isolates (1**). Values in parentheses give the %-age inhibition of H^+-efflux w.r.t. control.

compound (**1b**), compound (**1c**), compound (**1d**), compound (**1e**) and the reference compound fluconazole showed a viability of 98%, 93%, 91%, 97%, 90%, 89% at the concentration range of 10 μg/ml. At 50 μg/ml and 100 μg/ml the viability was 95%, 90%, 84%, 89%, 85%, 71% and 93%, 87%, 79%, 86%, 78%, 45%. At much higher concentrations 300 μg/ml and 600 μg/ml compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**), compound (**1e**) showed 10%, 17%, 24%, 16%, 26% and 11%, 20%, 25%, 18%, 28% hemolysis while as conventional antifungal therapeutic agent showed 79% and 82% hemolysis. At the MIC_{90} concentration of synthesized compounds, which had profound effect on growth, H^+ ATPase activity and ultrastructure of *Candida*, the hemolysis observed was very little. This indicates that the synthesized compounds have low cytotoxic activity.

The fungal cell wall may be considered to be a prime target for selectively toxic antifungal agents because of its chitin structure, absent from human cells. In the present study we demonstrated that the synthesized compounds at MIC_{90} values exhibit fungicidal and not fungistatic activity, by halo assay and growth curve studies against all the *Candida* isolates *in vitro*. In addition to our six laboratory wild-type *Candida* strains, forty (susceptible) and twenty (resistant), recently obtained clinical isolates were also tested for evidence of antifungal activity of these compounds. Synthesized derivatives exhibit varying degrees of antifungal activity. Generally, all the fluconazole-susceptible as well as-resistant *Candida* isolates investigated were found to be sensitive to the synthesized compounds. The use of total mean MICs obtained gave a good indication of the overall antimicrobial effectiveness of each synthesized compound. This may indicate that the yeast physiology may not be better equipped to counteract the antifungal properties of these synthesized compounds.

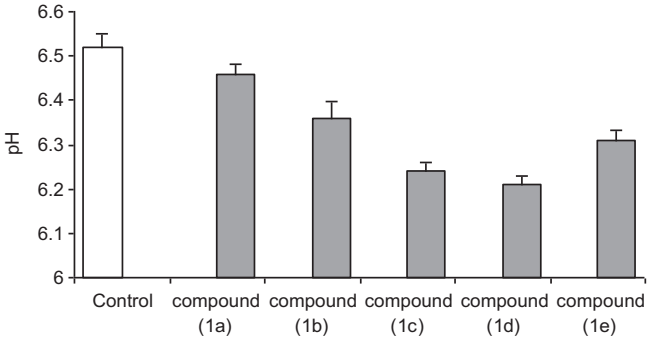


Fig. 2. Intracellular pH in presence of synthesized compounds in *Candida* cells. Mid logarithmic cells were incubated with MIC_{90} of compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**) and compound (**1e**). Remarkable decline in pH as shown in figure is indicative of induced acidity.

In liquid medium on full growth (24 h) at half MIC, decrease in optical densities of 7 randomly selected *Candida* isolates with respect to control was 60.64% for compound **1a**, 71.06% for compound **1b**, 82.92% for compound **1c**, 91.53% for compound **1d** and 77.31% for compound **1e** respectively. Control cells showed normal growth pattern. At lower concentration of the synthesized compounds slight decline in curve was observed as compared to control, whereas at MIC_{90} values of the test agents, normal S shaped curve declined to flat line showing almost complete arrest of cell growth (Fig. 1). Anticandidal activity order of the compounds on solid medium leads to similar conclusion, compound (**1d**) > compound (**1c**) > compound (**1e**) > compound (**1b**) > compound (**1a**). The conclusion that compound **1d** has higher antifungal activity than rest of the compounds is based on the differences in compound concentrations needed to inhibit yeast growth (Table 2). These results suggest that presence of nitro and chloro groups in the structure plays a key role in the antifungal activity. A total of two of the five compounds feature a nitro group (compound **1d**) and chloro group (compound **1c**) in their structure, these represented the most active antifungal compounds. However, in this study, the less effective compounds were shown to be compound (**1a**), compound (**1b**) and compound (**1e**), Compound **1a** bears an unsubstituted phenyl ring while as the phenyl ring is substituted at para and meta positions by a methyl group in compounds **1b** and **1e** respectively. Furthermore, it was also observed that in less effective compounds the presence and position of methyl group has a marked effect on the antifungal potential of the compound. These findings indicate that the presence of electron withdrawing groups on the phenyl ring of the sulfonyl pendant increases the antifungal activity of the compounds under investigation than the compounds bearing electron donating groups. Based upon the results it will also be necessary to optimize the led compound by substitution in C-4 position of phenyl ring of the sulfonyl fragment by electron withdrawing groups, which seem to be very important for antifungal effect, besides the position of the substituents seems to be an important factor behind the antifungal potential of the tested compounds.

The *in vitro* hemolytic assay is a feasible screening tool for gauging *in vivo* toxicity to host cells [21,22]. The comparative study of synthesized compounds indicates that the test compounds were significantly less cytotoxic than conventional antifungals.

Low MIC values and low cytotoxicity obtained against *Candida* with synthesized compounds encouraged us to study their mode of action. Although tetrazole derivatives showed higher anticandidal activity than that of comparable membrane-targeting antifungal

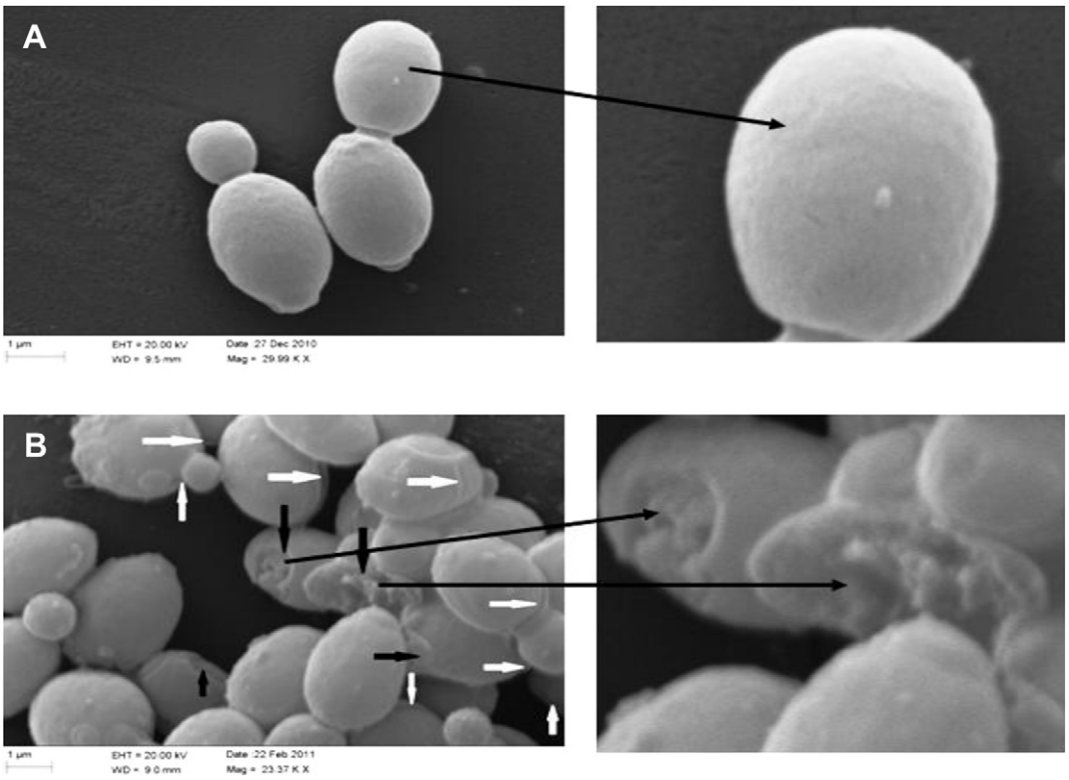


Fig. 3. Scanning electron micrographs of *C. albicans* Seq No 1138 (A) Untreated control and (B) Treated with compound **1d**. The black arrows indicate damage to the cell while as the white arrows indicate breakage of the membrane.

drugs [13], however, specific mechanisms involved in the antimicrobial action of tetrazoles remain poorly characterised. The rapid irreversible action of these compounds suggests that there may be a cellular target(s) accessible to the compound externally. *Candida* isolates showing susceptibility to the test compounds also showed inhibition of H^+-ATPase-mediated proton pumping suggesting that the two events are linked. It is to be noted that the inhibition of H^+-ATPase function was achieved at the MIC_{90} concentrations of the compounds. The decrease in H^+-extrusion being less when cells were exposed to the test compounds in presence of glucose. It is well established that plasma membrane ATPase undergoes modification in glucose medium [23]. Glucose induced acidification of the external medium by yeast cells are a convenient measure of H^+-ATPase-mediated proton pumping [24]. The enzyme may exist in a different conformational state in the two situations. It is thus possible that the synthesized compounds may be directly interacting with the enzyme, which serves as the primary reason for their antifungal activity. Thus, it would be useful to further investigate the interaction of these compounds with the purified PM-ATPase enzyme and study its activity in both steady state and pre-steady state. Regulation of pHi, appears to be a fundamental prerequisite for growth of *Candida* and activation of plasma membrane ATPase as it is involved in maintenance of pHi [16]. We therefore studied the role of plasma membrane ATPase activation in the regulation of pHi, in control as well as treated cells. The pHi was near neutrality in absence of test compounds while as in presence of the MIC_{90} of the synthesized compounds pHi declined to 6.46 (compound **1a**), 6.36 (compound **1b**), 6.24 (compound **1c**), 6.21 (compound **1d**) and 6.31 (compound **1e**) respectively. Surface features of cells after 14 h treatment with compound **1d** revealed extensive cell damage and significant breakage of cell membrane of compound **1d** treated cells Fig. 3 (B). In addition to this significant morphological change in cell shape was also observed. Taken together our results emphasize that multiple drug susceptibility assays therefore should be used for exact assessment. The excellence of these compounds demand more insight studies into all of the possible mechanisms of these compounds.

3. Conclusion

Present study has achieved the excellent synthesis of cinnamaldehyde based sulfonyl tetrazoles. The results of the study also

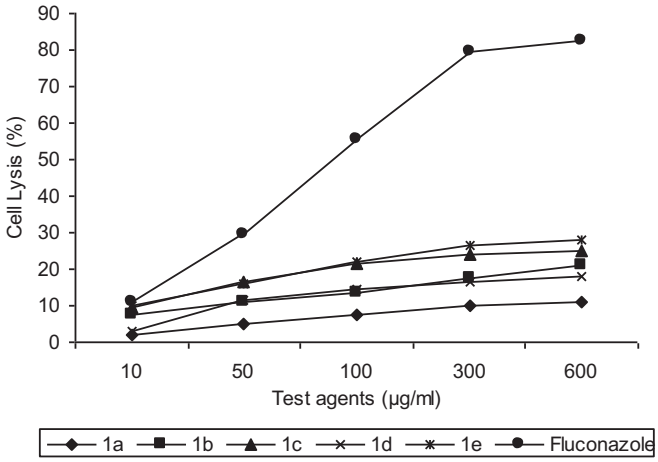


Fig. 4. Hemolysis caused by different agents: compound (**1a**), compound (**1b**), compound (**1c**), compound (**1d**), compound (**1e**) and fluconazole. Hemolysis was determined by an absorbance reading at 450 nm and compared to hemolysis achieved with 1% Triton X-100 (reference for 100% hemolysis). The data are means of triplicate experiments.

present a concrete mechanism for their antimicrobial action. The synthesized compounds show significant anticandidal activity both in liquid and solid medium. These compounds have insignificant toxicity at MIC_{90}. Tentative mechanism of action appears to originate from inhibition of plasma membrane ATPase activity. These synthesized compounds could be promising drugs after improved formulations and also advocates the determination of optimal concentrations for clinical applications.

4. Experimental Protocol

All the chemicals were purchased from Aldrich (USA) and Merck (Germany). Precoated aluminum sheets (silica gel 60 F_{254}), Merck (Germany) were used for thin-layer chromatography (TLC) and spots were visualized under UV light. Elemental analyses were performed on Heraeus Vario EL III analyzer at Central Drug Research Institute, Lucknow, India. The results were within ±0.4% of the theoretical values. IR spectra were recorded on Perkin–Elmer model 1600 FT-IR RX1 spectrophotometer as KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AVANCE 300 spectrometer using DMSO-d_6 as solvent with TMS as internal standard. Splitting patterns are designated as follows; s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. Chemical shift values are given in ppm. ESI-MS was recorded on a MICROMASS QUATTRO II triple quadrupole mass spectrometer.

4.1. General procedure for the transformation of cinnamaldehyde into 5-[(E)-2-phenylethenyl]-2H-tetrazole (**1**)

A solution of (2*E*)-3-phenylprop-2-enal (cinnamaldehyde) (5 mmol) and iodine (5.5 mmol) in ammonia water (50 ml of 30% solution) and THF (5 ml) was stirred at room temperature for 1 h. The dark solution became colorless at the end of reaction. A mixture of NaN_3 (6.0 mmol) and $ZnBr_2$ (7.5 mmol) was then added. The reaction mixture was heated at reflux for 12–15 h with vigorous stirring. HCl (40 ml of 3 M solution) and EtOAc (100 ml) were added, and vigorous stirring was continued until no solid was present and the aqueous layer had a pH of 1. The organic phase was concentrated in vacuo, and the remaining solids were rinsed with EtOAc (50 ml) to give a pure tetrazole product.

4.2. General procedure for the synthesis of 5-[(E)-2-phenylethenyl]-2-(phenyl/substituted phenyl sulfonyl)-2H-tetrazoles (**1a**–**e**)

To a solution of 5-[(*E*)-2-phenylethenyl]-2*H*-tetrazole **1** (1 eq.) and triethylamine (3 eq.) in dry dichloromethane at 0 °C was added aryl sulfonyl chlorides (1.2 eq.). The reaction mixture was stirred at 0 °C for about 2 h and stirring was continued at room temperature for about 4–5 h (completion of reaction was monitored by TLC). After the completion of reaction the reaction mass was quenched with distilled water and extracted with dichloromethane. Finally the combined organic layer was washed with distilled water again and dried over anhydrous Na_2SO_4. After removal of the solvent in vacuum, the residue was purified by recrystallization.

4.2.1. 5-(2-Phenylethenyl)-2H-tetrazole (**1**)

Solid; yield: 84%; mp 152–155 °C; Anal. Calc. for $C_9H_8N_4$: C 62.78, H 4.68, N 32.54%, found: C 62.73, H 4.72, N 32.58%; IR ν_{max}cm^{-1}: 3280 (N–H br stretch), 2864 (C–H), 1632 (C=N); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.79 (2H, m), 7.66 (1H, d, J = 15.2 Hz), 7.46–7.36 (3H, m), 7.32 (1H, d, J = 15.0 Hz), 7.09 (1H, NH); ^{13}C NMR (DMSO-d_6) δ(ppm): 147.50 (C=N), 132.12 (C=C); ESI-MS m/z: [M$^+$+1] 173.

4.2.2. 5-[(E)-2-Phenylethenyl]-1-(phenylsulfonyl)-1H-tetrazole (**1a**)

Yield 72%; White crystals; mp 182–185 °C; Anal. Calc. for $C_{15}H_{12}N_4O_2S$: C 57.68, H 3.87, N 17.94%, found: 57.70, H, 3.84, N, 18.05%; IR ν_{max}cm^{-1}: 3072 (Ar–H), 3045 (C–H), 1650 (C=N) 1150 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.80–7.72 (2H, m) 7.64 (2H, m), 7.53 (1H, d, J = 15.2 Hz), 7.48–7.38 (3H, m), 7.33–7.22 (3H, m), 7.10 (1H, d, J = 15.1); ^{13}C NMR (DMSO-d_6) δ(ppm): 167.5 (C=N), 145.6, 137.2, 123.4, 115.7, 113.0, (Ar–C), 128.5, 108.0; ESI-MS m/z: [M$^+$+1] 313.

4.2.3. 1-[(4-Methylphenyl)sulfonyl]-5-[(E)-2-phenylethenyl]-1H-tetrazole (**1b**)

Yield 68%; Yellow crystals; mp 175–178 °C; Anal. Calc. for $C_{16}H_{14}N_4O_2S$: C 58.88, H 4.32, N 17.17%, found: C 59.03, H 4.30, N 17.22%; IR ν_{max}cm^{-1}: 3068 (Ar–H), 3038 (C–H), 1660 (C=N) 1158 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.89–7.80 (2H, m) 7.75 (2H, m), 7.60 (1H, d, J = 15.2 Hz), 7.55–7.48 (2H, m), 7.46–7.36 (3H, m), 7.33 (1H, d, J = 15.1) 2.35 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=N), 147.5, 135.0, 125.4, 112.5, 113.0, (Ar–C), 132.0, 104.5, 28.0; ESI-MS m/z: [M$^+$+1] 327.

4.2.4. 1-[(4-Chlorophenyl)sulfonyl]-5-[(E)-2-phenylethenyl]-1H-tetrazole (**1c**)

Yield 75%; White crystals; mp 170–173 °C; Anal. Calc. for $C_{15}H_{11}ClN_4O_2S$: C 51.95, H 3.20, N 16.16%, found: C 52.01, H 3.24, N 16.27%; IR ν_{max}cm^{-1}: 3080 (Ar–H), 3055 (C–H), 1650 (C=N) 1165 (S=O) 735 (C–Cl stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.85–7.86 (2H, m) 7.70 (2H, m), 7.65 (1H, d, J = 15.2 Hz), 7.52–7.46 (2H, m), 7.40–7.32 (3H, m), 7.26 (1H, d, J = 15.1); ^{13}C NMR (DMSO-d_6) δ(ppm): 165.5 (C=N), 148.0, 130.0, 125.5, 115.0, 110.4, (Ar–C),129.5, 110.5; ESI-MS m/z: [M$^+$+1] 348.

4.2.5. 1-[(4-Nitrophenyl)sulfonyl]-5-[(E)-2-phenylethenyl]-1H-tetrazole (**1d**)

Yield 80%; White crystals; mp 165–168 °C; Anal. Calc. for $C_{15}H_{11}N_5O_4S$: C 50.42, H 3.10, N 19.60%, found: C 50.45, H 3.14, N 19.67%; IR ν_{max}cm^{-1}: 3067 (Ar–H), 3045 (C–H), 1660 (C=N), 1380 (NO_2 stretch), 1152 (S=O stretch); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.82–7.75 (2H, m) 7.73 (2H, m), 7.68 (1H, d, J = 15.0 Hz), 7.50–7.43 (2H, m), 7.38–7.30 (3H, m), 7.25 (1H, d, J = 14.3); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=N), 145.0, 133.5, 127.0, 113.7, 110.5, (Ar–C) 130.0, 110.5; ESI-MS m/z: [M$^+$+1] 358.

4.2.6. 1-[(3-Methylphenyl)sulfonyl]-5-[(E)-2-phenylethenyl]-1H-tetrazole (**1e**)

Yield 70%; Light Yellow crystals; mp 165–168 °C; Anal. Calc. for $C_{16}H_{14}N_4O_2S$: C 58.86, H 4.33, N 17.17%, found: C 58.98, H 4.34, N 17.20%; IR ν_{max}cm^{-1}: 3074 (Ar–H), 3046 (C–H), 1648 (C=N) 1175 (S=O); ^{1}H NMR (DMSO-d_6) δ(ppm): 7.76–7.68 (2H, m) 7.75 (2H, m), 7.63 (1H, d, J = 14.8 Hz), 7.50–7.42 (2H, m), 7.45–7.34 (3H, m), 7.28 (1H, d, J = 15.1) 2.25 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ(ppm): 168.0 (C=N), 148.9, 137.6, 126.8, 115.5, 110.5, (Ar–C), 127.8, 112.5, 28.0; ESI-MS m/z: [M$^+$+1] 327.

4.3. Determination of MIC_{90}

Microtiter assay: Cells were grown for 48 h at 30 °C to obtain single colonies, which were resuspended in a 0.9% normal saline solution to give an optical density at 600 nm (OD_{600}) of 0.1. The cells were then diluted 100-fold in YNB medium containing 2% glucose. The diluted cell suspensions were added to the wells of round-bottomed 96-well microtiter plates (100 μl/well) containing equal volumes of medium and different concentrations of synthesized compounds [25]. A drug-free control was also included. The plates were incubated at 30 °C for 24 h. The MIC test end point was

evaluated both visually and by observing the OD_{620} in a microplate reader (BIO-RAD, iMark, US) and is defined as the lowest compound concentration that gave ≥90% inhibition of growth compared to the controls.

4.4. Disc diffusion halo assays

Strains were inoculated into liquid YPD medium and grown overnight at 37 °C. The cells were then pelleted and washed three times with distilled water. Approximately 10^5cells/ml were inoculated in molten agar media at 40 °C and poured into 100-mm-diameter petri plates. The synthesized compounds initially dissolved in 10% DMSO were further diluted in distilled water to concentration ranges of 10 fold of their respective MICs. 4-mm-diameter sterile filter discs were impregnated with the test compounds as described earlier [18]. 10% DMSO (solvent) and 100 μg/ml of fluconazole were also applied on the discs to serve as negative and positive controls, respectively. The diameter of zone of inhibition was recorded in millimeters after 48 h and was compared with that of control. This experiment was performed on fluconazole-sensitive [standard ($n = 6$), clinical ($n = 10$)], and fluconazole-resistant ($n = 10$) isolates selected randomly. Results are reported as mean ± standard error of mean (SEM) of all three respective categories.

4.5. Growth curve studies

For growth studies, 10^6 cells/ml (optical density $A_{600} = 0.1$) culture of *Candida* cells were inoculated and grown aerobically in YEPD broth for control along with varied concentrations of the synthesized compounds in individual flasks. Growth was recorded turbidometrically at 595 nm using LaboMed Inc. Spectrophotometer (USA) as described previously [18,19]. The growth rate study of different *Candida* species in absence as well as in presence of inhibitor was performed for each concentration in triplicate, average of which was taken into consideration.

4.6. Proton efflux measurements

The proton pumping activity of *Candida* species was determined by monitoring acidification of external medium by measuring the pH as described previously [17,26]. Briefly, mid-log phase cells harvested from YEPD medium were washed twice with distilled water and routinely 0.1 g cells were suspended in 5 ml solution containing 0.1 M KCl, 0.1 mM $CaCl_2$ in distilled water. Suspension was kept in a double-jacketed glass container with constant stirring. The container was connected to a water circulator at 25 °C. Initial pH was adjusted to 7.0 using 0.01 M HCl/NaOH. Synthesized compounds were added to achieve the desired concentrations (MIC_{90}) in 5 ml solution. For glucose stimulation experiments, 100 μl of glucose was added to achieve a final concentration of 5 mM in total volume of suspension. H^+-extrusion rate was calculated from the volume of 0.01 N NaOH consumed.

4.7. Measurement of intracellular pH (pHi)

Intracellular pH was measured as done earlier [16] with modifications. Mid-log phase cells grown in YEPD medium were harvested and washed twice with distilled water. Cells (0.1 g) were suspended in 5 ml solution containing 0.1 M KCl and 0.1 mM $CaCl_2$. Desired concentrations of synthesized compounds (their respective MIC_{90} values) were added to the suspension and pH was adjusted to 7.0 in each case. Following incubation for 30 min at 37 °C with constant shaking at 200 rpm, pH was again adjusted to 7.0. Nystatin (20 μM) dissolved in DMSO was added to the unbuffered cell suspension and incubated at 37 °C for 1 h. The change in pH of suspension was followed on pH meter with constant stirring. The value of external pH at which nystatin permeabilization induced no further shift was taken as an estimate of pHi.

4.8. Scanning electron microscopy (SEM)

Candida cell suspensions from overnight cultures were prepared in YEPD medium. Test compound at equivalent to MIC_{90} concentration was added to the cell ($\sim 1 \times 10^6$) and incubated for 14 h at 30 °C and prepared for an electron microscopy. All *Candida* cells were fixed with 2% glutaraldehyde in 0.1% phosphate buffer for 1 h at room temperature (20 °C) [27,28]. Washed with 0.1 M phosphate buffer (pH 7.2) and post fixed 1% OsO_4 in 0.1 M phosphate buffer for 1 h at 4 °C. For SEM, samples were dehydrated in acetone and dropped on round glass cover slip with HMDS and dried at room temperature then sputter coating with gold and observed under the SEM (Zeiss EV040).

4.9. Hemolytic activity

Human erythrocytes from healthy individuals were collected in tubes containing EDTA as anti-coagulant. The erythrocytes were harvested by centrifugation for 10 m at 2000 rpm at 20 °C, and washed three times in PBS. To the pellet, PBS was added to yield a 10% (v/v) erythrocytes/PBS suspension. The 10% suspension was then diluted 1:10 in PBS. From each suspension, 100 μl was added in triplicate to 100 μL of a different dilution series of synthesized compounds (or fluconazole) in the same buffer in eppendorf tubes. Total hemolysis was achieved with 1% Triton X-100. The tubes were incubated for 1 h at 37 °C and then centrifuged for 10 m at 2000 rpm at 20 °C. From the supernatant fluid, 150 μl was transferred to a flat-bottomed microtiter plate (BIO-RAD, iMark, US), and the absorbance was measured spectrophotometrically at 450 nm. The hemolysis percentage was calculated by following equation:

$$\% \text{ hemolysis} = [(A_{450} \text{ of test compound treated sample} - A_{450} \text{ of buffer treated sample})/(A_{450} \text{ of 1\% Triton X} - A_{450} \text{ of buffer treated sample})] \times 100\%.$$

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Appendix. Supplementary Data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.12.007.

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