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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of new chalcones, derived pyrazoline and cyclohexenone derivatives as potent antimicrobial, antitubercular and antileishmanial agents

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Abstract A series of chalcones (**1**–**8**) were prepared by Claisen–Schmidt condensation of 3-nitroacetophenone with various aldehydes. These chalcones on cyclization with hydrazine hydrate in the presence of glacial acetic acid, hydrazine hydrate in absolute ethanol and ethyl acetoacetate in the presence of barium hydroxide gave corresponding *N*-acetyl pyrazolines (**9**–**16**), *N*-unsubstituted pyrazolines (**17**–**19**) and cyclohexenone derivatives (**20**–**22**). All the synthesized compounds were evaluated for their in vitro antibacterial and antifungal activity by using broth microdilution method, and many compounds showed promising activity against various tested bacteria and fungi. Among various tested compounds, **16** and **19** exhibited strongest activities against *Streptococcus pyogenes* and *Pseudomonas aeruginosa*; both have MIC value of 25 μg/mL, which is fourfold greater than the standard drug. Many compounds showed good activity against *Candida albican*. Analogs **11**, **12**, **15**–**17** and **19** displayed two- to fivefold greater activity against *C. albican* as compared to the standard drug. Results of antitubercular evaluation revealed that compounds **4** and **19** displayed strong antimycobacterial activity against $H_{37}Rv$ having MIC of 25 and 62.5 μg/mL, respectively. All analogs were found to be inactive against *Leishmania braziliensis* except analogs **4** and **5** which exhibited strong leishmanicidal activity against Leishmania promastigotes with IC_{50} values of 9.3 and 8.9 μg/mL, respectively.

Keywords Chalcones · Pyrazolines · Cyclohexenones · Antimicrobial · Antitubercular · Antileishmanial

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Introduction

Diseases caused by microbial infection are a serious menace to the health of human beings and is a major cause of death in developed and developing countries (Sharma *et al.*, 2009). In recent decades, problems of drug-resistant microorganism have reached alarming level in many countries around the world (Bogatcheva *et al.*, 2006; Goldstein, 2007). A number of recent clinical reports describe the increasing occurrence of methicillin-resistant *Staphylococcus aureus* and other antibiotic-resistant human pathogenic microorganisms in United States and European countries (Moreillon, 2008; Boucher *et al.*, 2009). The high mortality rate associated with multidrug-resistant gram-negative enteric bacteria is a very serious problem (Arias, *et al.*, 2010). *Mycobacterium tuberculosis* (*M. tuberculosis*), the main causative agent for tuberculosis (TB) infecting around 32 % of the world population, is the leading cause of mortality claiming about 2 million lives annually. Further, one of the major factors contributing

toward the increasing incidence of tuberculosis is the occurrence of multidrug-resistant strains of *M. tuberculosis* (MDR-TB) (Pai *et al.*, 2006; Lawn and Zumla, 2011). Moreover, a high number of infections with non-tuberculous mycobacteria, especially in immune-compromised patients such as those infected with the human immunodeficiency virus (HIV), render the treatment even more difficult. Leishmaniasis is a group of parasitic diseases, widespread in many parts of the world, and therapy of patients with leishmaniasis still poses a serious problem due to the side effects and toxicity associated with the clinically used drugs. Besides, treatment regimen is lengthy and requires long-term parenteral administration, and resistance to these drugs has been observed in many cases (Le Pape, 2008; Kobets *et al.*, 2012). To overcome this problem of rapid development of drug resistance, one of the major challenges in drug discovery is the development of new chemical entities which should preferably consist of chemical characteristic that clearly differs from those of existing drugs.

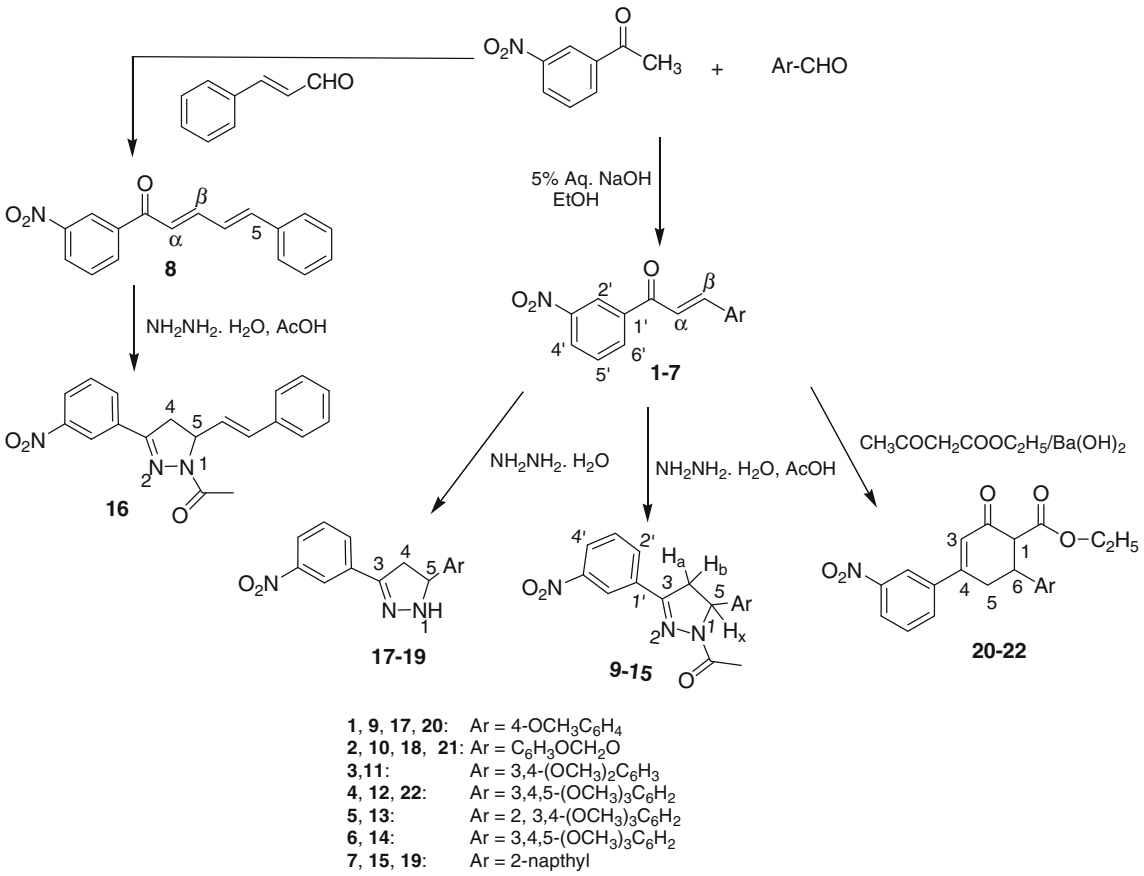
Chalcones consist of open-chain flavonoids and are well-known intermediates for synthesizing various heterocyclic compounds, which are associated with impressive array of biological activities, including anti-inflammatory, antibacterial, antioxidant, antimalarial, anticancer, antimicrobial and antileishmanial activities (Go *et al.*, 2005; Nowakowska, 2007; Romagnoli *et al.*, 2009; Kumar *et al.*, 2010; Zheng *et al.*, 2011). On the other hand, pyrazoline derivatives have attracted considerable interest due to their diverse biological properties and therapeutic applications (Palaska *et al.*, 2001; Ali *et al.*, 2007; Amir *et al.*, 2008; Kaplancikli *et al.*, 2009; Shaharyar *et al.*, 2010; Ozdemir *et al.*, 2010; Senturk *et al.*, 2012; Sharshira and Hamada 2012), namely antifungal, anti-inflammatory, antidepressant, antimicrobial, anticancer, antinociceptive, antitubercular activity and inhibitors of mammalian monoamine oxidase. Further, cyclohexenone derivatives represent another class of cyclic compounds with interesting biological activities, among which its antimicrobial activity is well documented (Mayekar *et al.*, 2010; Kanagarajan *et al.*, 2011). Prompted by these observations and in continuation of our efforts in search of new effective antimicrobial agents, it was envisaged to synthesize a series of nitrochalcones (**1–8**) that were further cyclized to *N*-acetyl-substituted and unsubstituted pyrazolines (**9–19**) and cyclohexenone (**20–22**) derivatives. All the compounds were structurally elucidated and screened for in vitro antimicrobial activity against two gram-positive bacteria, two gram-negative bacteria and three fungi. The promising results obtained prompted us to further screen them for antitubercular and antileishmanial activity. The results of antimicrobial evaluation of all the synthesized compounds are discussed in this paper.

Result and discussion

Chemistry

The synthetic route followed for the preparation of the titled compounds is illustrated in Scheme 1. The condensation of 3-nitroacetophenone with various aromatic aldehydes afforded the 1,3-diaryl-2-propen-1-one (chalcone) derivatives (**1–8**). The cyclization of chalcones with hydrazine hydrate in the presence of glacial acetic acid provided *N*-acetyl pyrazolines **9–16**, whereas cyclization with hydrazine hydrate in absolute ethanol resulted in pyrazoline derivatives **17–19.** The reaction of chalcones with ethyl acetoacetate in the presence of barium hydroxide produces cyclohexenone derivatives **20–22**. The purity of the compounds was checked by thin-layer chromatography (TLC) and elemental analyses and characterized by spectral data. The physical data of the compounds are shown in Table 1. The structures of various synthesized compounds were established by FT-IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. All derivatives showed typical absorption bands at 1,315–1,532 cm^{-1} due to their NO_2 stretching vibrations. The IR spectrum of chalcones (**1–8**) showed absorption band at 1,643–1,666 cm^{-1} due to C=O stretching. The formation of compounds **1–8** is observed in ^{1}H NMR by the disappearance of the aldehydic proton signal and the appearance of two doublets due to the olefinic protons at δ 8.16–7.72 ppm and δ 7.63–7.06 ppm, respectively, with coupling constant between them of $J = 15.6–15.9$ Hz, which agrees with a trans configuration.

The IR spectra of pyrazolines (**9–19**) showed the expected absorption bands at 1,592–1,617 cm^{-1} corresponding to C=N stretching bands because of ring closure, at 1,658–1,671 cm^{-1} due to C=O group in **9–16** and at 3,327–3,349 cm^{-1} due to NH functionality in **17–19**. In the ^{1}H NMR spectra of these compounds, the CH_2 protons of the pyrazoline ring resonated as a pair of doublets of doublets at δ 3.03–3.26 ppm (H_a) and δ 3.47–3.85 ppm (H_b), with $J_{AB} = 16.5–17.7$ Hz. The –CH(H_x) proton appeared as a doublet of doublets at δ 4.93–6.21 ppm due to vicinal coupling with the two magnetically non-equivalent protons of the methylene group at position 4 of the pyrazoline ring with $J_{AX} = 4.5–5.1$ Hz for **9–16**, $J_{AX} = 8.7–8.9$ Hz for **17–19** and $J_{BX} = 11.1–12.3$ Hz for **9–19**. The FT-IR spectrum of cyclohexenone derivatives (**20–22**) shows two strong characteristic absorptions in the range of 1,726–1,733 cm^{-1} and 1,655–1,675 cm^{-1} due to the ester carbonyl and ketone functional groups, respectively. In the ^{1}H NMR spectra, the protons of the ethyl ester group resonated as a triplet and quartet in the range of δ 1.08–1.12 ppm and 4.07–4.12 ppm, respectively, with $J = 7.2$ Hz. The signal for =CH–CO– of cyclohexenone ring appeared as singlet in the range of δ 6.60–6.62 ppm,



Scheme 1 Synthetic pathway for the compounds **1**–**22**

whereas multiplets due to the protons of two CH groups and CH_2 group of the ring resonated at δ 3.74–3.79 and 3.03–3.07 ppm. The protons belonging to the aromatic ring and phenyl substituents were observed at expected chemical shift and integral values. The spectral analyses of all the compounds are listed in the "Experimental" section and found to be in accordance with the assigned structures.

Antimicrobial screening

Antibacterial activity

All the synthesized compounds (**1**–**22**) were tested for their antibacterial activity in vitro against gram-positive bacteria (*S. aureus* MTCC-96, *Streptococcus pyogenes* MTCC-443) and gram-negative bacteria (*Escherichia coli* MTCC-442, *Pseudomonas aeruginosa* MTCC-2488). Ampicillin was used as the standard drug. The minimum inhibitory concentration (MIC) values in μg/mL are given in Table 2. A close survey of the antimicrobial data revealed that most of the tested compounds exhibited good to moderate antibacterial activity against all the tested bacterial strains with MIC values in the range of 25–200 μg/mL. Analogs **7**, **8**, **14**, **16**, **18** and **19** were found to be active against both gram-positive and gram-negative bacteria with MIC values in the range of 25–125 μg/mL. Among the various tested analogs, **16** was found to be most active against *S. pyogenes* and **19** was found to be most potent against *P. aeruginosa*, both having MIC value of 25 μg/mL, which is fourfold greater than the standard drug used. Compounds **11** and **15** exhibited very good activity against gram-positive bacteria and moderate activity against gram-negative bacteria, whereas compounds **6** and **13** displayed good antibacterial activity against gram-negative bacteria as compared to their activity against gram-positive bacteria. Analogs **2**, **5**, **9**, **10**, **12** and **22** displayed moderate activity against various tested strains of bacteria. All other derivatives exhibited moderate to weak antibacterial activity.

As regards the relationships between the structures of the various synthesized scaffolds and the detected antibacterial properties, it showed varied biological activity. However, conversion of chalcones to pyrazoles in some cases (as in case of chalcone **7** and **8** and derived pyrazolines **16** and **19**) not only improves the potency but also the spectrum of activity. Moreover, the presence of different substituents causes a certain change of activity.

Table 1 Physical data of synthesized compounds

Compound no.	Molecular formula	Molecular weight	Yield (%)	Mp (°C)	R_f^a
1	$C_{16}H_{13}NO_4$	283.28	86	132–134	0.78[a1]
2	$C_{16}H_{11}NO_5$	297.26	75	127–129	0.75[a1]
3	$C_{17}H_{15}NO_5$	313.3	62	130–132	0.52[a1]
4	$C_{18}H_{17}NO_6$	343.33	77	128–129	0.59[a1]
5	$C_{18}H_{17}NO_6$	343.33	71	115–117	0.60[a1]
6	$C_{18}H_{17}NO_6$	343.33	69	132–133	0.38[a1]
7	$C_{19}H_{13}NO_3$	303.31	68	139–141	0.75[a1]
8	$C_{17}H_{13}NO_3$	279.29	79	114–116	0.62[a1]
9	$C_{18}H_{17}N_3O_4$	339.35	65	135–137	0.32[a2]
10	$C_{18}H_{15}N_3O_5$	353.33	57	186–188	0.24[a2]
11	$C_{19}H_{19}N_3O_5$	369.37	45	138–140	0.20[a2]
12	$C_{20}H_{21}N_3O_6$	399.4	70	125–127	0.18[a2]
13	$C_{20}H_{21}N_3O_6$	399.4	49	110–111	0.28[a2]
14	$C_{20}H_{21}N_3O_6$	399.4	61	124–126	0.17[a2]
15	$C_{21}H_{17}N_3O_3$	359.38	69	131–133	0.34[a2]
16	$C_{19}H_{17}N_3O_3$	335.36	82	141–143	0.45[a2]
17	$C_{16}H_{15}N_3O_3$	297.31	60	101–102	0.50[a1]
18	$C_{16}H_{13}N_3O_4$	311.29	54	101–102	0.70[a1]
19	$C_{19}H_{15}N_3O_2$	317.34	62	98–99	0.42[a1]
20	$C_{22}H_{21}NO_6$	395.4	47	106–107	0.52[a1]
21	$C_{22}H_{19}NO_7$	409.39	54	130–131	0.54[a1]
22	$C_{24}H_{25}NO_8$	455.46	60	137–139	0.55[a1]

[a1] Solvent system—hexane/ethyl acetate (7:3)

[a2] solvent system—toluene/ethyl acetate (9.5:0.5)

Substitution with an electron-donating methoxy group at *para* position of the phenyl ring in chalcone **1**, derived pyrazoline **9** and cyclohexenone derivative **20** imparts weak to moderate activity; in case of pyrazoline **17,** it exhibits strong activity against gram-negative bacteria and weak activity against gram-positive bacteria. Analogs **2**, **10** and **21** containing methylenedioxy-substituted phenyl ring exhibited moderate antibacterial activity, whereas analog **18** displayed good antibacterial activity against various tested strains. Further, di- or tri-substitution with methoxy group improves the activity of compounds against various tested strains. Compound **4** possessing 3,4.5-trimethoxy substituent showed fivefold and compound **5** carrying 2,3,4-trimethoxy substituent showed twofold increase in potency against gram-positive bacteria as compared to chalcone **1**. Replacement of phenyl ring with napthyl ring as in case of analogs **7** and **19** and increased separation between two aromatic rings by additional double bond as in case of analogs **8** and **16** imparts good activity to the compounds. Chalcone **7** containing napthyl ring displayed twofold increase in potency against *S. aureus* and *E. coli*, whereas derived pyrazoline **19** showed two- and fourfold increased activity against *S. aureus* and *P. aeruginosa* as compared to the standard drug. Similarly, cinnamaldehyde-derived chalcone **8** exhibited almost similar pattern of activity as that of standard drug, while the derived pyrazoline derivative **16** found to posses five- and fourfold increase in activity against *S. pyogenes* and *E. coli*, respectively. The synthesized cyclohexenone derivatives **20** and **21** also showed improved antibacterial activity against both gram-positive and gram-negative bacteria as compared to the parent chalcones.

Antifungal activity

All the synthesized compounds (**1**–**22**) were tested for their antifungal activity in vitro against three fungal strains (*C. albican* MTCC-227, *Aspergillus niger* MTCC-282 and *Aspergillus clavatus* MTCC-1323). Griseofulvin was used as the standard drug. The minimum inhibitory concentration (MIC) values in μg/mL are given in Table 2. A close survey of the antifungal data revealed that some of the tested compounds exhibited good activity (100–250 μg/mL) against *C. albican* and very few compounds were found to display good activity (50–200 μg/mL) against *A. niger* and *A. clavatus.* Compounds **5**, **6** and **9** were found to have no antifungal activity against *C. albican*, *A. niger* and *A. clavatus*, respectively, even at a high concentration of 1,000 μg/mL. Chalcone derivatives **2**, **4** and **8** were found to exhibit twofold activity, whereas analogs **3** and **7** were found to exhibit similar MIC values activity against *C. albican* as that of standard. All chalcone derivatives were found to exhibit very weak or no antifungal activity against *A. niger* and *A. clavatus* except **1** which showed good

Table 2 In vitro antibacterial and antifungal activity (MIC) values of the tested compounds

No.	MIC (μg/mL)						
	Gram-positive bacteria		Gram-negative bacteria		Fungi		
	S. aureus MTCC-96	*S. pyogenes* MTCC-443	*E. coli* MTCC-442	*P. aeruginosa* MTCC-2488	*C. albican* MTCC-227	*A. niger* MTCC-282	*A. clavatus* MTCC-1323
1	500	500	200	200	1,000	200	200
2	250	250	250	250	250	1,000	1,000
3	200	200	100	200	500	>1,000	>1,000
4	100	100	250	250	250	>1,000	>1,000
5	250	250	200	200	1,000	>1,000	>1,000
6	500	500	125	125	1,000	1,000	1,000
7	100	125	62.5	200	500	1,000	1,000
8	125	125	200	100	250	1,000	1,000
9	250	250	250	250	1,000	1,000	1,000
10	250	250	250	200	500	500	500
11	125	125	500	250	200	500	500
12	250	250	250	200	250	500	500
13	250	250	100	100	1,000	250	250
14	100	125	125	125	1,000	250	250
15	100	100	250	250	250	>1,000	>1,000
16	250	25	62.5	100	500	>1,000	>1,000
17	500	500	62.5	100	250	1,000	1,000
18	100	125	100	200	250	1,000	1,000
19	50	125	250	25	100	100	250
20	250	500	100	200	500	>1,000	>1,000
21	250	200	125	200	500	1,000	1,000
22	200	200	200	200	100	>1,000	>1,000
Ampicillin	250	100	100	100	–	–	–
Griseofulvin	–	–	–	–	500	100	100

activity against both of them with a MIC value of 200 μg/mL. Napthyl-substituted pyrazoline **19** showed fivefold increased activity against *C. albican* and similar activity against *A. niger* (100 μg/mL) as compared to standard drug. Twofold increased activity was noted for **11**, **12**, **15**, **17** and **18** against *C. albican*, whereas all other pyrazoline derivatives displayed moderate to weak activity against various fungal strains. Among various tested cyclohexenone derivatives, only **22** was found to exhibit better activity (100 μg/mL) against *C. albican* and weak antifungal activity against other tested fungal strains.

Antitubercular activity

The encouraging results from the antibacterial studies impelled us to go for preliminary screening of synthesized title compounds against *M. tuberculosis*. The results of preliminary antimycobacterial screening of synthesized compounds are tabulated in Table 3. Compound **4** containing 3,4,5-trimethoxy-substituted phenyl ring and derivative **19** possessing napthyl ring attached to C-5 of the pyrazoline ring showed better antitubercular activity, having MIC values of 25 and 62.5 μg/mL, respectively. The trimethoxy-substituted *N*-acetyl pyrazoline derivative **12** turns out to be the most potent compound which exhibited a MIC value of 12.5 μg/mL and showed twofold increase in antitubercular activity as compared to the parent chalcone **4**. All other synthesized analogs displayed moderate to weak activity.

Antileishmanial activity

All the synthesized analogs (**1**–**22**) were evaluated for their inhibitory activity against *Leishmania braziliensis* promastigote forms. Amphotericin B was used as the standard drug, and the results of antileishmanial evaluation are reproduced in Table 4. Among the various tested analogs, compound **4** possessing 3,4,5-trimethoxy-substituted phenyl ring and compound **5** having 2,3,4-trimethoxy-substituted phenyl ring displayed potential inhibitory activity with IC_{50} values

Table 3 In vitro effect of the synthesized compounds on the growth of *M. tuberculosis* (MICs, μg/mL)

No.	MIC values (μg/mL) *M. tuberculosis* $H_{37}Rv$	% Inhibition
1	1,000	98
2	250	99
3	500	98
4	25	99
5	500	98
6	250	99
7	500	98
8	1,000	97
9	250	99
10	250	99
11	500	99
12	12.5	99
13	250	99
14	500	98
15	1,000	98
16	250	98
17	250	99
18	500	98
19	62.5	99
20	500	98
21	250	99
22	250	99
Rifampicin	40	98

of 9.3 and 8.9 μg/mL, respectively, whereas dimethoxyphenyl-ring-substituted chalcone derivative **3** and pyrazoline derivative **17** displayed weak activity. All other synthesized chalcones, derived pyrazoline and cyclohexenone derivatives were found to be inactive. Thus, the nature of ring may not have any effects on activity, and cyclization of chalcones resulted in the loss of activity as in case of chalcone derivatives **4** and **5**. In order to determine the toxicity/activity index, the most active compounds against promastigotes were also tested for their cytotoxicity toward mouse J774-A1 macrophages. The selectivity index (SI) was defined as the ratio of the CC_{50} values of compounds against macrophages relative to IC_{50} values obtained against *L. braziliensis* promastigotes. It has been found that compounds **4** and **5** displayed moderately good SI values of 5.26 and 10.06, respectively. Although the mechanisms of antileishmanial activity displayed by chalcones were not addressed in this work, depending upon literature reports, it can be predicted that chalcones could potentially inhibit the activity of nicotinamide adenine dinucleotide-reduced fumarate reductase (NADH-FRD), succinate dehydrogenase, NADH dehydrogenase, or succinate- and NADH-cytochrome c reductase in the parasite mitochondria (Lunardi *et al.*, 2003; Nowakowska, 2007).

Table 4 In vitro effect of the synthesized compounds on the growth of *L. braziliensis* and cytotoxicity on mouse macrophages

No.	IC_{50} values (μM) *L. braziliensis* strain H3	CC_{50} values (μM) cytotoxicity on J774-A1	SI[a]
1	NA	–	–
2	NA	–	–
3	>100	–	–
4	9.30	48.95	5.26
5	8.90	89.49	10.06
6	NA	–	–
7	NA	–	–
8	NA	–	–
9	NA	–	–
10	NA	–	–
11	NA	–	–
12	NA	–	–
13	NA	–	–
14	NA	–	–
15	NA	–	–
16	NA	–	–
17	>100	–	–
18	NA	–	–
19	NA	–	–
20	NA	–	–
21	NA	–	–
22	NA	–	–
Amphotericin B	0.3	–	–

NA no activity

[a] Assay performed at Departamento de Microbiologia e Parasitologia, UFSC, Brazil; SI = $CC_{50macrophages}/IC_{50L.\ Braziliensis}$ promastigotes

Conclusions

In conclusion, we have presented the synthesis, characterization and biological evaluation of series of chalcone, pyrazoline and cyclohexenone derivatives. Their antimicrobial activities were evaluated against *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa*, *C. albican*, *A. niger*, *A. clavatus* as well as *M. tuberculosis*. Of all the analogs screened for activity, some of the compounds were associated with higher antibacterial and antifungal activity than the standard drugs used (ampicillin and griseofulvin). Compounds **16** and **19** displayed strongest activities against tested bacteria *S. pyogenes* and *P. aeruginosa* with MIC value of 25 μg/mL, whereas **11**, **12**, **15**–**17** and **19** exhibited greater activity against *Candida albican* as compared to the standard drugs. Results of the antitubercular evaluation showed that compounds **4** and **19** exhibited potent activity against *M. tuberculosis* with MIC values of 25–62.5 μg/mL, whereas pyrazoline derivative **12**

substituted with electron-donating methoxy groups at the *meta/para* position of the phenyl rings displayed highest activity against *M. tuberculosis* with MIC value of 12.5 μg/mL. Among all the tested analogs, only trimethoxy-substituted chalcone derivatives **4** and **5** were found to be active against *L. braziliensis* with IC_{50} values of 9.3 and 8.9 μg/mL, respectively, and exhibited good therapeutic index. These results showed that cyclization of chalcones leads to improvement in their antibacterial and antifungal activity and loss of antiparasitic activity. Based on close examination of substitutions, it may be concluded that role of electron-donating groups (OCH_3) on the phenyl ring and the replacement of phenyl with napthyl ring has great influence on antimicrobial activity. The present study has generated leads with broad-spectrum activity, and further SAR studies on identified bioactive lead molecules for enhancing the antimicrobial activity are in progress.

Experimental

Chemistry

Solvents and organic reagents were purchased from Sigma-Aldrich, Merck and Loba Chemie (India) and were used without further purification. All the evaporations were carried out under reduced pressure on Buchi-rotary evaporator. Reactions were monitored by thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F_{254} silica) with UV light (256 nm), iodine and vanillin-sulfuric acid as developing agents. Melting points were determined in open capillaries and are uncorrected. IR spectra (KBr disks) were recorded on a NICOLET-380 FT-IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AM 300 (^{1}H NMR at 300 MHz and ^{13}C NMR at 75 MHz) spectrometer (Bruker Biosciences, USA) using $CDCl_3$ as solvent with TMS (tetramethylsilane) as the internal standard. Splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; and m, multiplet. Chemical shift values (δ) are given in ppm. Mass spectra were recorded on Finnigan Mat LCQ spectrometer. The results of elemental analyses (% C, H, N) for the synthesized compounds were in agreement with calculated values within ±0.4 % range.

General procedure used for the synthesis of 1,3-diaryl-2-propen-1-ones (**1**–**7**) and 1,5-diaryl-2,4-pentadien-1-one (**8**)

To a solution of substituted aromatic aldehyde (1 mmol) and *m*-nitroacetophenone (1 mmol) in ethanol (20 mL), 10 % aqueous NaOH was added. The reaction mixture was allowed to stir at room temperature for appropriate time until the starting reactants were disappeared. After completion of the reaction, the reaction mixture was quenched on ice-cold water and neutralized with dilute hydrochloric acid (5 %). The precipitated solid was filtered, washed with distilled water and dried to give the crude product that was recrystallized from ethanol. The spectral data of known compounds **2** and **7** were found to be identical with those reported in literature (Chiaradia *et al.*, 2008). The characterization data for all the compounds are given below.

*(2E)-3-(4-methoxyphenyl)-1-(3′-nitrophenyl)-2-propen-1-one (**1**)*

Light orange solid, Yield 86 %; m.p.: 132–134 °C; IR (KBr) ν (cm^{-1}): 3,041 (=C–H, aromatic), 1,655 (C=O), 1,589 (C=C), 1,526 & 1,343 (–NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.83 (s, 1H, H-2′), 8.44 (d, 1H, $J = 8.1$ Hz, H-4′), 8.35 (d, 1H, $J = 7.5$ Hz, H-6′), 7.88 (d, 1H, $J = 15.6$ Hz, H-β), 7.70 (m, 3H, H-2, 6 and 5′), 7.42 (d, 1H, $J = 15.6$ Hz, H-α), 6.97 (d, 2H, $J = 8.7$ Hz, H-3 and 5), 3.88 (s, 3H, OCH_3); δ ppm: 189.74 (C=O), 159.09 (C-4), 148.59 (C-3′), 145.98 (C-β), (133.43, 132.76, 130.84, 128.21, 127.16, 126.13, 124.62, 114.27, phenyl), 121.45 (C-α), 55.29 (OCH_3); MS (APCI) $m/z = 284$ (M + 1); Anal. Calcd. for $C_{16}H_{13}NO_4$ (283.28): C, 67.84; H, 4.63; N, 4.94, Found: C, 67.93; H, 4.61; N, 4.72.

*(2E)-1-(3′-nitrophenyl)-3-(1,3-benzodioxol-5-yl)-2-propen-1-one (**2**)*

Yellowish brown solid, Yield 75 %; m.p.: 143–145 °C; IR (KBr) ν (cm^{-1}): 3,067 (=C–H, aromatic), 1,662 (C=O), 1,566 (C=C), 1,505 & 1,347 (–NO_2), 1,248 (C–O); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.82 (s, 1H, H-2′), 8.48 (d, 1H, $J = 8.1$ Hz, H-4′), 8.34 (d, 1H, $J = 7.9$ Hz, H-6′), 7.82 (d, 1H, $J = 15.6$ Hz, H-β), 7.71 (m, 1H, H-5′), 7.36 (d, 1H, $J = 15.6$ Hz, H-α), 7.23 (s, 1H, H-2), 7.16 (d, 1H, $J = 8.0$ Hz, H-6), 6.87 (d, 2H, $J = 8.0$ Hz, H-5), 6.05 (s, 2H, –OCH_2O–); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.85 (C=O), 148.57 (C-3′), 147.24–148.16 (C-3, C-4), 146.78 (C-β), (139.55, 134.28, 131.12, 129.03, 127.19, 126.20, 118.83, 109.03, 107.02, phenyl), 123.37 (C-α), 102.1 (–OCH_2O–); MS (APCI) $m/z = 298$ (M + 1); Anal. Calcd. for $C_{16}H_{13}NO_4$ (297.26): C, 67.84; H, 4.63; N, 4.94, Found: C, 67.93; H, 4.61; N, 4.72.

*(2E)-3-(3,4-dimethoxyphenyl)-1-(3′-nitrophenyl)-2-propen-1-one (**3**)*

Light green solid, Yield 62 %; m.p.: 130–132 °C; IR (KBr) ν (cm^{-1}): 3,067 (=C–H, aromatic), 1,657 (C=O), 1,579 (C=C), 1,518 & 1,315 (–NO_2); ^{1}H NMR (300 MHz,

$CDCl_3$) δ ppm: 8.83 (s, 1H, H-2′), 8.40 (m, 2H, H-4′ and H-6′), 7.86 (d, 1H, $J = 15.59$ Hz, H-β), 7.72 (m, 1H, H-5′), 7.42 (d, 1H, $J = 15.59$ Hz, H-α), 7.28 (m, 2H, H-2 and 6), 6.93 (d, 1H, $J = 8.2$ Hz, H-5), 3.97 (s, 6H, 2 × OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.61(C=O), 155.21 (C-3), 153.93 (C-4), 148.58 (C-3′), 143.57 (C-β), (140.50, 139.78, 134.91, 127.30, 118.26, 114.51, 111.76, phenyl), 122.96 (C-α), 56.29 (OCH_3); MS (APCI) $m/z = 314$ (M + 1); Anal. Calcd. for $C_{17}H_{15}NO_5$ (313.3): C, 65.17; H, 4.83; N, 4.47, Found: C, 65.12; H, 4.72; N, 4.41.

*(2E)-1-(3′-nitrophenyl)-3-(3,4,5-trimethoxyphenyl)-2-propen-1-one (**4**)*

Yellow solid, Yield 77 %; m.p.: 128–129 °C; IR (KBr) ν (cm^{-1}): 3,092 (=C–H, aromatic), 1,666 (C=O), 1,572 (C=C), 1,514 & 1,358 (NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.84 (s, 1H, H-2′), 8.46 (d, 1H, $J = 8.1$ Hz, H-4′), 8.36 (d, 1H, $J = 7.8$ Hz, H-6′), 7.82 (d, 1H, $J = 15.6$ Hz, H-β), 7.73 (m, 1H, H-5′), 7.40 (d, 1H, $J = 15.6$ Hz, H-α), 6.90 (s, 2H, H-2 and H-6), 3.94 (s, 6H, 2 × OCH_3), 3.92 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 189.35 (C=O), 153.66 (C-3 and C-5), 148.69 (C3′), 143.78 (C-β), 140.51 (C-4), (138.21, 133.57, 131.86, 129.96, 124.73, 121.21, 102.37, phenyl), 123.23 (C-α), 60.78 (*m*-OCH_3), 56.15 (*p*-OCH_3); MS (APCI) $m/z = 344$ (M + 1); Anal. Calcd. for $C_{18}H_{17}NO_6$ (343.33): C, 62.97; H, 4.99; N, 4.08, Found: C, 62.99; H, 4.92; N, 3.98.

*(2E)-3-(2,3,4-trimethoxyphenyl)-1-(3-nitrophenyl)-2-propen-1-one (**5**)*

Light brown solid, m.p.: 115–117 °C; Yield 71 %; IR (KBr) ν (cm^{-1}): 3,109 (=C–H, aromatic), 1,646 (C=O), 1,568 (C=C), 1,533 & 1,356 (NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.82 (s, 1H, H-2′), 8.42 (d, 1H, $J = 8.1$ Hz, H-4′), 8.34 (d, 1H, $J = 7.8$ Hz, H-6′), 8.07 (d, 1H, $J = 15.9$ Hz, H-β), 7.72 (t, 1H, J = 7.8 Hz, H-5′), 7.55 (d, 1H, J = 15.9 Hz, H-α), 7.42 (d, 1H, $J = 8.7$ Hz, H-5), 6.74 (d, 1H, $J = 8.7$ Hz, H-6), 3.97 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.88 (C=O), 156.67 (C-4), 153.78 (C-2), 148.64 (C-3′), 142.18 (C-β), 140.40 (C-3), 139.50 (C-1′), (134.99, 131.02, 127.58, 124.27, 121.13, 120.17, 108.94, phenyl), 123.14 (C-α), 62.05 (OCH_3), 60.93 (OCH_3), 56.58 (OCH_3); MS (APCI) $m/z = 344$ (M + 1); Anal. Calcd. for $C_{18}H_{17}NO_6$ (343.33): C, 62.97; H, 4.99; N, 4.08, Found: C, 63.12; H, 5.05; N, 3.97.

*(2E)-1-(3′-nitrophenyl)-3-(2,4,5-trimethoxyphenyl)-2-propen-1-one (**6**)*

Yellow solid, m.p.: 132–133 °C; Yield 69 %; IR (KBr) ν (cm^{-1}): 3,094 (=C–H, aromatic), 1,643 (C=O), 1,597 (C=C), 1,522 & 1,351 (NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.82 (s, 1H, H-2′), 8.43 (m, 1H, H-4′), 8.33 (d, 1H, $J = 7.8$ Hz, H-6′), 8.16 (d, 1H, $J = 15.6$ Hz, H-β), 7.69 (t, 1H, $J = 8.1$ Hz, H-5′), 7.45 (d, 1H, $J = 15.6$ Hz, H-α), 7.13 (s, 1H, H-5), 6.51 (s, 1H, H-3), 3.96 (s, 3H, OCH_3), 3.93 (s, 6H, 2 × OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 189.82 (C=O), 153.67 (C-2), 153.68 (C-4), 148.67 (C-3′), 145.23 (C-β), 142.37 (C-5), (139.67, 133.43, 131.88, 129.55, 124.61, 118.52, 109.93, 101.02 phenyl ring), 122.86 (C-α), 56.43–56.28 (3 × OCH_3); MS (APCI) $m/z = 344$ (M + 1); Anal. Calcd. for $C_{18}H_{17}NO_6$ (343.33): C, 62.97; H, 4.99; N, 4.08, Found: C, 62.88; H, 4.79; N, 4.01.

*(2E)-1-(3′-nitrophenyl)-3-(2-naphthyl)-2-propen-1-one (**7**)*

Yellow solid, m.p.: 139–141 °C; Yield 62 %; IR (KBr) ν (cm^{-1}): 3,094 (=C–H, aromatic), 1,659 (C=O), 1,563 (C=C), 1,522 & 1,351 (NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.87 (s, 1H, H-2′), 8.45 (d, 1H, $J = 8.1$ Hz, H-4′), 8.38 (d, 1H, $J = 7.8$ Hz, H-6′), 8.07 (d, 1H, $J = 15.6$ Hz, H-β), 8.03 (s, 1H, H-1), 7.89 (d, 2H, $J = 8.4$ Hz, H-5 and 8), 7.84–7.81 (m, 2H, H-3 and 4), 7.72 (t, 1H, $J = 8.1$ Hz, H-5′), 7.63 (d, 1H, $J = 15.6$ Hz, H-α), 7.56–7.54 (m, 2H, H-6 and 7); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.97 (C=O), 148.69 (C-3′), 145.93 (C-β), 139.21 (C-1′), (134.38, 133.98, 133.21, 132.48, 131. 09, 131.12, 129.37, 129.18, 128.22, 128.01, 127.56, 125.07, 122. 76, phenyl and naphthyl), 123.43 (C-α); MS (APCI) $m/z = 304$ (M + 1); Anal. Calcd. for $C_{19}H_{13}NO_3$ (303.31): C, 75.24; H, 4.32; N, 4.62, Found: C, 75.08; H, 4.39; N, 4.86.

*(2E, 4E)-1-(3-nitrophenyl)-5-phenyl-2,4-pentadien-1-one (**8**)*

Yellow crystals; Yield 79 %; m.p.: 1,14–1,16 °C; IR (KBr) ν (cm^{-1}): 3,092 (=C–H, aromatic), 1,657 (C=O), 1,586 (C=C), 1,521 & 1,347 (NO_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.81 (s, 1H, H-2′), 8.42 (d, 1H, $J = 7.2$ Hz, H-4′), 8.31 (d, 1H, $J = 7.8$ Hz, H-6′), 7.72–7.64 (m, 2H, H-β and H-5′), 7.54–7.51 (m, 2H, H-2 and 6 of ArH), 7.42–7.35 (m, 3H, H-3, 4 and 5 of ArH), 7.13–7.06 (m, 3H, H-α, H-3 and 5 of pentadiene); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 187.91 (C=O), 148.52 (C-3′), 146.31(C-β or C-2), 143.1 (C-3), 139.27 (C-5), (136.31, 134.68, 131.06, 129.87(C-α), 129.38, 127.83, 127.56, 125.14, phenyl), 122.98 (C-4); MS (APCI) $m/z = 280$ (M + 1); Anal. Calcd. for $C_{17}H_{13}NO_3$ (279.29): C, 73.11; H, 4.69; N, 5.02, Found: C, 73.03; H, 4.63; N, 4.88.

General procedure for the synthesis of 1-acetyl-3,5-diaryl-4,5-dihydro(1*H*)pyrazoles (**9**–**16**)

To a solution of substituted chalcone (0.5 g) in glacial acetic acid (10 mL), hydrazine hydrate (0.5 mL) was

added, and the mixture was refluxed for appropriate time until the chalcone disappeared. After cooling, the mixture was poured in ice-cold water. Precipitated solid was filtered, dried and recrystallized using ethanol. The spectral data of known compounds **9–11** were found to be identical with those reported in literature (Nepali *et al.*, 2011). The characterization data for all the compounds are provided below.

*1-Acetyl-5-(4-methoxyphenyl)-3-(3′-nitrophenyl)-4,5-dihydro(1H)pyrazole (**9**)*

Pale brown solid; Yield 70 %; m.p.: 117–119 °C; IR (KBr) ν (cm^{-1}): 3,093 (=C–H, aromatic), 1,664 (C=O), 1,610 (C=N), 1,522 & 1,348 (NO_2), 1,252 (C–N); ^{1}H NMR- (300 MHz, $CDCl_3$) δ ppm: 8.53 (s, 1H, H-2′), 8.27 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.8$ Hz, H-6′), 7.62 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.15 (d, 1H, $J = 8.7$ Hz, H-2 and 6), 6.85 (d, 1H, $J = 8.7$ Hz, H-3 and 5), 5.62 [dd, 1H, $J = 4.8$, 11.7 Hz, H_x (pyrazoline ring)], 3.83–3.73 (m, 4H, OCH_3 and H_b), 3.20 [dd, 1H, $J = 4.6$, 17.7 Hz, H_a (pyrazoline ring)], 2.43 (s, 3H, C OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 168.96 (C=O), 159.17 (C-OMe), 151.38 (C-3, pyrazoline ring), 148.58 (C–NO_2), (133.55, 133.36, 131.95, 129.78, 126.80, 124.46, 121.28, 114.32, phenyl ring), 59.91 (C-5, pyrazoline ring), 55.26 (OCH_3), 42.08 (C-4, pyrazoline ring), 21.98 (CH_3); Anal. Calcd. for $C_{18}H_{17}N_3O_4$: C, 63.71; H, 5.05; N, 12.38, Found: C, 63.66; H, 5.28; N, 10.44; MS (APCI) $m/z = 340$ (M + 1); Anal. Calcd. for $C_{18}H_{17}N_3O_4$ (339.35): C, 63.71; H, 5.05; N, 12.38, Found: C, 63.66; H, 5.28; N, 10.44.

*1-(5-benzo[d][1,3]dioxol-5-yl)-4,5dihydro-3-(3′-nitrophenyl)pyrazol-1-yl)ethanone (**10**)*

Orange solid; Yield 57 %; m.p.: 196–198 °C; IR (KBr) ν (cm^{-1}): 3,083 (=C–H, aromatic), 1,660 (C=O), 1,599 (C=N), 1,529 & 1,348 (NO_2), 1,239 (C–N); ^{1}H NMR- (300 MHz, $CDCl_3$) δ ppm: 8.52 (s, 1H, H-2′), 8.26 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.6$ Hz, H-6′), 7.62 (dd, 1H, $J = 7.6$, 8.1 Hz, H-5′), 6.74–6.66 (m, 3H, H-2, 5 and 6), 5.92 (s, 2H, –OCH_2O–), 5.57 [dd, 1H, $J = 4.6$, 11.9 Hz, H_x (pyrazoline ring)], 3.77 (dd, 1H, $J = 11.9$, 17.7 Hz, H_b), 3.17 [dd, 1H, $J = 4.6$, 17.7 Hz, H_a (pyrazoline ring)], 2.44 (s, 3H, C OCH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 168.99 (C=O), 151.35 (C-3, pyrazoline ring), 148.58 (C–NO_2), (148.17, 147.20, 135.36, 133.25, 131.94, 129.79, 124.52, 121.29, 119.02, 108.55, phenyl ring), 101.15 (–OCH_2O–), 60.13 (C-5, pyrazoline ring), 42.19 (C-4, pyrazoline ring), 21.97 (CH_3); MS (APCI) $m/z = 354$ (M + 1); Anal. Calcd. for $C_{18}H_{15}N_3O_5$ (353.33): C, 61.19; H, 4.28; N, 11.89, Found: C, 61.29; H, 4.39; N, 11.78.

*1-Acetyl-5-(3,4-dimethoxyphenyl)-3-(3′-nitrophenyl)-4,5-dihydro(1H)pyrazole (**11**)*

Light orange solid; Yield 45 %; m.p.: 156–158 °C; IR (KBr) ν (cm^{-1}): 3,080 (=C–H, aromatic), 1,666 (C=O), 1,593 (C=N), 1,530 & 1,349 (NO_2), 1,258 (C–N); ^{1}H NMR- (300 MHz, $CDCl_3$) δ ppm: 8.55 (s, 1H, H-2′), 8.28 (d, 1H, $J = 8.1$ Hz, H-4′), 8.09 (d, 1H, $J = 7.8$ Hz, H-6′), 7.63 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.83–6.75 (m, 3H, H-2, 5 and 6), 5.61 [dd, 1H, $J = 4.8$, 11.7 Hz, H_x (pyrazoline ring)], 3.86–3.74 (m, 7H, 2 × OCH_3 and H_b), 3.21 [dd, 1H, $J = 4.8$, 17.7 Hz, H_a (pyrazoline ring)], 2.45 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 169.09 (C=O), 151.47 (C-3, pyrazoline ring), (149.39, 148.75, 148.64, 134.03, 133.33, 131.98, 129.84, 124.55, 121.35, 117.47, 111.65, 109.15, phenyl ring), 60.24 (C-5, pyrazoline ring), 55.98 (2 × OCH_3), 42.22 (C-4, pyrazoline ring), 22.01 (CH_3); MS (APCI) $m/z = 370$ (M + 1); Anal. Calcd. for $C_{19}H_{19}N_3O_5$ (369.37): C, 61.78; H, 5.18; N, 11.38, Found: C, 61.61; H, 5.26; N, 11.47.

*1-Acetyl-5-(3,4,5-trimethoxyphenyl)-3-(3′-nitrophenyl)-4,5-dihydro(1H)pyrazole (**12**)*

Light brown solid; Yield 70 %; m.p.: 125–127 °C; IR (KBr) ν (cm^{-1}): 3,093 (=C–H, aromatic), 1,670 (C=O), 1,595 (C=N), 1,522 & 1,348 (NO_2), 1,232 (C–N); ^{1}H NMR- (300 MHz, $CDCl_3$) δ ppm: 8.55 (s, 1H, H-2′), 8.28 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.5$ Hz, H-6′), 7.63 (dd, 1H, $J = 7.5$, 8.1 Hz, H-5′), 6.41 (s, 2H, H-2 and 6), 5.59 [dd, 1H, $J = 4.8$, 11.7 Hz, H_x (pyrazoline ring)], 3.83 (s, 6H, 2 × OCH_3), 3.79 (s, 3H, OCH_3), 3.76 [dd, 1H, $J = 11.7$ & 17.7 Hz, H_b (pyrazoline ring)], 3.20 [dd, 1H, $J = 4.5$, 17.7 Hz, H_a (pyrazoline ring)], 2.48 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 169.18 (C=O), 153.77 (2 × C-OMe), 151.59 (C-3, pyrazoline ring), 148.64 (C–NO_2), (137.54, 133.19, 131.99, 129.87, 124.62, 121.37, 102.38, phenyl ring), 60.74 (C-5, pyrazoline ring), 60.63 (OCH_3), 56.19 (2 × OCH_3), 42.37 (C-4, pyrazoline ring), 21.98 (CH_3); MS (APCI) $m/z = 400$ (M + 1); Anal. Calcd. for $C_{20}H_{21}N_3O_6$ (399.4): C, 60.14; H, 5.30; N, 10.52, Found: C, 60.03; H, 5.28; N, 10.44.

*1-Acetyl-5-(2,3,4-trimethoxyphenyl)-3-(3′-nitrophenyl)-4,5-dihydro(1H)pyrazole (**13**)*

Brown solid; Yield 49 %; m.p.: 110–111 °C; IR (KBr) ν (cm^{-1}): 3,064 (=C–H, aromatic), 1,658 (C=O), 1,592 (C=N), 1,532 & 1,349 (NO_2), 1,266 (C–N); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.54 (s, 1H, H-2′), 8.26 (d, 1H, $J = 8.1$ Hz, H-4′), 8.07 (d, 1H, $J = 7.8$ Hz, H-6′), 7.61 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.80 (d, 1H, $J = 8.7$ Hz, H-5), 6.59 (d, 1H, $J = 8.7$ Hz, H-6), 5.72 [dd, 1H, $J = 5.1$,

12.3 Hz, H_x (pyrazoline ring)], 3.90 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.73 [dd, 1H, $J = 12.3$ & 17.7 Hz, H_b (pyrazoline ring)], 3.16 [dd, 1H, $J = 5.1$ & 17.7 Hz, H_a (pyrazoline ring)], 2.44 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 169.09 (C=O), 153.57 (C-3, pyrazoline ring), (152.37, 150.79, 148.61, 142.44, 133.55, 132.00, 129.77, 127.00, 124.41, 121.36, 107.31, phenyl ring), 60.73 (C-5, pyrazoline ring), 56.91 (OCH_3), 56.91 (OCH_3), 56.01 (OCH_3), 41.58 (C-4, pyrazoline ring), 22.02 (CH_3); MS (APCI) $m/z = 400$ (M + 1); Anal. Calcd. for $C_{20}H_{21}N_3O_6$ (399.4): C, 60.14; H, 5.30; N, 10.52, Found: C, 60.18; H, 5.25; N, 10.43.

*1-Acetyl-5-(2,4,5-trimethoxyphenyl)-3-(3′-nitrophenyl)-4,5-dihydro(1H)pyrazole (**14**)*

Brown solid; Yield 61 %; m.p.: 124–126 °C; IR (KBr) ν (cm^{-1}): 3,019 (=C–H, aromatic), 1,671 (C=O), 1,611 (C=N), 1,525 & 1,348 (NO_2), 1,264 (C–N); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.54 (s, 1H, H-2′), 8.26 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.8$ Hz, H-6′), 7.61 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.61 (s, 1H, H-3), 6.53 (s, 1H, H-6), 5.77 [dd, 1H, $J = 4.8$, 11.7 Hz, H_x (pyrazoline ring)], 3.86 (s, 3H, OCH_3), 3.79 (s, 6H, 2 × OCH_3), 3.71 [dd, 1H, $J = 12.0$, 17.7 Hz, H_b (pyrazoline ring)], 3.12 [dd, 1H, $J = 5.1$, 17.7 Hz, H_a (pyrazoline ring)], 2.46 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 169.10 (C=O), 152.47 (C-3, pyrazoline ring), (150.67, 149.51, 148.59, 143.18, 133.60, 131.98, 129.75, 124.39, 121.29, 120.57, 111.31, 98.26, phenyl ring), 57.07 (C-5, pyrazoline ring), 56.61(OCH_3), 56.30 (OCH_3), 56.15 (OCH_3), 41.24 (C-4, pyrazoline ring), 21.95 (CH_3); MS (APCI) $m/z = 400$ (M + 1); Anal. Calcd. for $C_{20}H_{21}N_3O_6$ (399.4): C, 60.14; H, 5.30; N, 10.52, Found: C, 60.21; H, 5.34; N, 10.46.

*1-(5-(naphthalen-2-yl)-3-(3′-nitrophenyl)-4,5-dihydropyrazol-1-yl)ethanone (**15**)*

Brown solid; Yield 69 %; m.p.: 176–178 °C; IR (KBr) ν (cm^{-1}): 3,052 (=C–H, aromatic), 1,665 (C=O), 1,595 (C=N), 1,530 & 1,348 (NO_2), 1,270 (C–N); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.55 (s, 1H, H-2′), 8.26 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.8$ Hz, H-6′), 7.83–7.72 (m, 3H, H-4, 5 and 8), 7.68 (s, 1H, H-1), 7.61 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.46–7.41 (m, 2H, H-6 and 7), 7.31–7.28 (m, 1H, H-3), 5.82 [dd, 1H, $J = 4.8$, 11.7 Hz, H_x (pyrazoline ring)], 3.85 [dd, 1H, $J = 11.7$ & 17.7 Hz, H_b (pyrazoline ring)], 3.26 [dd, 1H, $J = 4.8$, 17.7 Hz, H_a (pyrazoline ring)], 2.48 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 164.14 (C=O), 151.50 (C-3, pyrazoline ring), 148.63 (C–NO_2), (138.53, 133.35, 133.00, 132.03, 129.85, 129.22, 127.98, 127.69, 126.43, 126.13, 124.58, 123.19, 121.38, phenyl and naphthyl), 60.64 (C-5, pyrazoline ring), 42.22 (C-4, pyrazoline ring), 20.03 (CH_3); MS (APCI) $m/z = 360$ (M + 1); Anal. Calcd. for $C_{21}H_{17}N_3O_3$ (359.38): C, 70.18; H, 4.77; N, 11.69, Found: C, 70.27; H, 4.71; N, 11.56.

*(E)-1-(3-(3′-nitrophenyl)-5-styryl-4,5-dihydropyrazol-1-yl)ethanone (**16**)*

Yellow solid, Yield 82 %; m.p.: 141–143 °C; IR (KBr) ν (cm^{-1}): 3,058 (=C–H, aromatic), 1,659 (C=O), 1,602 (C=N), 1,528 & 1,347 (NO_2), 1,248 (C–N); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.53 (s, 1H, H-2′), 8.27 (d, 1H, $J = 8.1$ Hz, H-4′), 8.08 (d, 1H, $J = 7.8$ Hz, H-6′), 7.62 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.36 (d, 2H, $J = 7.2$ Hz, H-2 and 6), 7.31–7.20 (m, 3H, H-3, 4 and 5), 6.62 (d, 1H, $J = 15.9$ Hz, –CH=C<u>H</u>–Ph), 6.21 (d, 1H, $J = 15.9$ Hz, –C<u>H</u>=CH–Ph), 5.39–5.3 [m, 1H, H_x (pyrazoline ring)], 3.60 [dd, 1H, $J = 11.9$, 17.4 Hz, H_b (pyrazoline ring)], 3.17 [dd, 1H, $J = 4.5$, 17.4 Hz, H_a (pyrazoline ring)], 2.44 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 169.29 (C=O), 151.70 (C-3, pyrazoline ring), 148.62 (C–NO_2), (136.00, 133.40, 131.95, 129.81, 128.54, 128.02, 126.65, 126.55, 124.53, 121.30, phenyl ring and styryl), 58.47 (C-5, pyrazoline ring), 39.20 (C-4, pyrazoline ring), 22.09 (CH_3); MS (APCI) $m/z = 336$ (M + 1); Anal. Calcd. for $C_{19}H_{17}N_3O_3$ (335.36): C, 68.05; H, 5.11; N, 12.53, Found: C, 67.02; H, 5.16; N, 12.41.

General procedure for the synthesis of 3,5-diaryl-4,5-dihydro-1*H*-pyrazole (**17**–**19**)

A solution of the appropriate chalcone (0.03 mol) and hydrazine hydrate (0.06 mol) in ethanol (30 mL) was refluxed for the appropriate time until the chalcone disappeared as indicated by the TLC. After completion, the reaction mixture was cooled and poured into ice-cold water and then neutralized with glacial acetic acid. The solid separated was filtered, washed with water, dried and recrystallized from ethanol to afford the titled compounds good yield and purity.

*4,5-dihydro-5-(4-methoxyphenyl)-3-(3′-nitrophenyl)-1H-pyrazole (**17**)*

Yellow solid; Yield 60 %; m.p.: 103–104 °C; IR (KBr) ν (cm^{-1}): 3,327 (N–H), 3,016 (=C–H, aromatic), 1,607 (C=N), 1,536 & 1,344 (NO_2), 1,247 (C–N); ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 8.42 (s, 1H, H-2′), 8.17 (d, 1H, $J = 8.1$ Hz, H-4′), 8.09 (d, 1H, $J = 7.8$ Hz, H-6′), 7.54 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.27 (d, 2H, $J = 8.7$ Hz, H-2 and 6), 6.88 (d, 2H, $J = 8.7$ Hz, H-3 and 5), 6.19 (bs, 1H, NH), 4.98 [dd, 1H, $J = 8.7$, 11.6 Hz, H_x (pyrazoline ring)], 3.80 (s, 3H, OCH_3), 3.48 [dd, 1H, $J = 11.1$,

16.5 Hz, H_b (pyrazoline ring)], 3.06 (dd, 1H, $J = 8.7$, 16.5 Hz, H_a (pyrazoline ring)]; ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 159.07 (C-OMe), 149.78 (C-3, pyrazoline ring), 148.56 (C–NO_2), (133.78, 133.57, 132.21, 129.97, 126.85, 124.98, 121.72, 114.62, phenyl ring), 64.69 (C-5, pyrazoline ring), 55.26 (OCH_3), 41.07 (C-4, pyrazoline ring); MS (APCI) $m/z = 298$ (M + 1); Anal. Calcd. for $C_{16}H_{15}N_3O_3$ (297.31): C, 64.64; H, 5.09; N, 14.13, Found: C, 64.51; H, 5.17; N, 14.07.

*5-(benzo[d][1,3]dioxol-5-yl)-4,5-dihydro-3-(3′-nitrophenyl)-1H-pyrazole (**18**)*

Yellow solid, Yield 54 %; m.p.: 101–102 °C; IR (KBr) ν (cm^{-1}): 3,338 (N–H), 3,024 (=C–H, aromatic), 1,611(C=N), 1,529 & 1,349 (NO_2), 1,245 (C–N); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.41 (s, 1H, H-2′), 8.16 (d, 1H, $J = 8.1$ Hz, H-4′), 8.02 (d, 1H, $J = 7.8$ Hz, H-6′), 7.54 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.86 (s, 1H, H-2), 6.81–6.75 (m, 2H, H-5 and 6), 6.23 (bs, 1H, NH), 5.95 (s, 2H, –OCH_2O–), 4.93 [m, 1H, H_x (pyrazoline ring)], 3.47 [dd, 1H, $J = 11.1$, 16.5 Hz, H_b (pyrazoline ring)], 3.03 [dd, 1H, $J = 8.7$, 16.5 Hz, H_a (pyrazoline ring)]; ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 150.22 (C-3, pyrazoline ring), 148.57 (C–NO_2), (148.49, 148.21, 147.38, 136.06, 134.75, 131.34, 129.46, 123.01, 120.63, 119.66, 108.41, 106.48, 101.19), 64.61 (C-5, pyrazoline ring), 41.02 (C-4, pyrazoline ring); MS (APCI) $m/z = 312$ (M + 1); Anal. Calcd. for $C_{16}H_{13}N_3O_4$ (311.29): C, 61.73; H, 4.21; N, 13.50, Found: C, 61.54; H, 4.23; N, 13.53.

*4,5-dihydro-5-(naphthalen-2-yl)-3-(3′-nitrophenyl)-1H-pyrazole (**19**)*

Yellow solid, Yield 62 %; m.p.: 98–99 °C; IR (KBr) ν (cm^{-1}): 3,349 (N–H), 3,013 (=C–H, aromatic), 1,617 (C=N), 1,523 & 1,348 (NO_2), 1,254 (C–N); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.45 (s, 1H, H-2′), 8.17 (d, 1H, $J = 7.2$ Hz, H-4′), 8.04 (d, 1H, $J = 7.8$ Hz, H-6′), 7.86–7.79 (m, 4H, H-1 4, 5, and 8), 7.55 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.49–7.47 (m, 3H, H-3, 6 and 7), 6.12 (bs, 1H, NH), 5.22–5.11 [m, 1H, H_x (pyrazoline ring)], 3.57 [dd, 1H, $J = 11.1$, 16.5 Hz, H_b (pyrazoline ring)], 3.16 [dd, 1H, $J = 8.9$, 16.5 Hz, H_a (pyrazoline ring)]; ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 150.02 (C-3, pyrazoline ring), 148.58 (C–NO_2), (138.96, 133.74, 133.52, 133.26, 132.37, 129.94, 129.26, 128.03, 127.72, 126.61, 126.19, 124.75, 123.21, 121.35, phenyl and naphthyl), 64.67 (C-5, pyrazoline ring), 41.04 (C-4, pyrazoline ring); MS (APCI) $m/z = 318$ (M + 1); Anal. Calcd. for $C_{19}H_{15}N_3O_2$ (317.34): C, 71.91; H, 4.76; N, 13.24, Found: C, 71.83; H, 4.87; N, 13.28.

General method for the synthesis of ethyl 6-(substituted aryl)-4-(3-nitrophenyl)-2-oxocyclohex-3-enecarboxylate (**20**–**22**)

To a solution of chalcone (0.01 mol) in the ethanol (30 mL), barium hydroxide (0.001 mol) was added followed by the addition of ethyl acetoacetate (0.01 mol). The mixture was refluxed for the appropriate time until the chalcone disappeared. After completion of reaction as indicated by the TLC, the solvent was removed under vacuum and the crude residue was extracted with chloroform (2 × 25 mL). The organic layers were combined and dried over sodium sulfate, and the chloroform layer was concentrated to get the crude product that was then recrystallized from ethanol to get pure product.

*Ethyl 6-(4-methoxyphenyl)-4-(3-nitrophenyl)-2-oxocyclohex-3-enecarboxylate (**20**)*

Yellow solid, Yield 47 %; m.p.: 106–107 °C; IR (KBr) ν (cm^{-1}): 3,076 (=C–H, aromatic), 1,731 (C=O), 1,655 (C=O), 1,609 (C=C), 1,529 & 1,349 (NO_2), 1,249 (C–O); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.38 (s, 1H, H-2′), 8.28 (d, 1H, $J = 8.1$ Hz, H-4′), 7.87 (d, 1H, $J = 7.8$ Hz, H-6′), 7.63 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 7.25 (d, 2H, $J = 8.7$ Hz, Ar–H), 6.89 (d, 2H, $J = 8.7$ Hz, Ar–H), 6.62 (s, 1H, H-3 of cyclohexenone), 4.02 (q, 2H, $J = 7.2$ Hz, –CH_2CH_3), 3.81 (s, 3H, OCH_3), 3.78–3.74 (m, 2H, H-1 and 6 of cyclohexenone), 3.06–3.02 (m, 2H, H-5 of cyclohexenone), 1.08 (t, 3H, $J = 7.2$ Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 193.72 (C-2 of cyclohexenone), 168.91 (C=O at C-1 of cyclohexenone), 159.04 (C-OMe), 155.67 (C-4 of cyclohexenone), 148.64 (C–NO_2), (139.60, 132.50, 131.82, 130.01, 128.31, 127.89, 125.90, 124.79, 121.08, 114.29, phenyl ring and C-3 of cyclohexenone), 61.08 (C-1 of cyclohexenone), 59.82 (–O–CH_2CH_3), 55.30 (OCH_3), 43.26 (C-6 of cyclohexenone), 36.22 (C-5 of cyclohexenone), 13.98 (CH_3); MS (APCI) $m/z = 396$ (M + 1); Anal. Calcd. for $C_{22}H_{21}NO_6$ (395.41): C, 66.83; H, 5.35; N, 3.54, Found: C, 66.92; H, 5.42; N, 3.68.

*Ethyl 6-(benzo[d][1,3]dioxol-5-yl)-4-(3-nitrophenyl)-2-oxocyclohex-3-enecarboxylate (**21**)*

White solid, Yield 54 %; m.p.: 152–153 °C; IR (KBr) ν (cm^{-1}): 3,091 (=C–H, aromatic), 1,726 (C=O), 1,666 (C=O), 1,597 (C=C), 1,533 & 1,347 (NO_2), 1,243 (C–O); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.37 (s, 1H, H-2′), 8.29 (d, 1H, $J = 8.4$ Hz, H-4′), 7.86 (d, 1H, $J = 7.8$ Hz, H-6′), 7.62 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.81-6.78 (m, 3H, ArH), 6.60 (s, 1H, H-3 of cyclohexenone), 5.96 (s, 2H, –OCH_2O–), 4.10 (q, 2H, $J = 7.2$ Hz, –CH_2CH_3), 3.77–3.70 (m, 2H, H-1 and 6 of cyclohexenone), 3.04 (m,

2H, H-5 of cyclohexenone), 1.12 (t, 3H, $J = 7.2$ Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 193.58 (C-2 of cyclohexenone), 168.84 (C=O at C-1 of cyclohexenone), 155.59 (C-4 of cyclohexenone), 148.65 (C–NO_2), (148.03, 147.06, 139.52, 134.29, 131.86, 130.07, 125.89, 124.88, 121.11, 120.63, 108.61, 107.50 phenyl ring and C-3 of cyclohexenone), 101.23 (OCH_2O), 61.20 (C-1 of cyclohexenone), 59.77 (–O–$\underline{C}H_2CH_3$), 43.73 (C-6 of cyclohexenone), 36.24 (C-5 of cyclohexenone), 14.06 (CH_3); MS (APCI) $m/z = 410$ (M + 1); Anal. Calcd. for $C_{22}H_{19}NO_7$ (409.39): C, 64.54; H, 4.68; N, 3.42, Found: C, 64.65; H, 4.77; N, 3.51.

*Ethyl 6-(3,4,5-methoxyphenyl)-4-(3-nitrophenyl)-2-oxocyclohex-3-enecarboxylate (**22**)*

White solid, Yield 60 %; m.p.: 162–163 °C; IR (KBr) ν (cm^{-1}): 3,084 (=C–H, aromatic), 1,733 (C=O), 1,675 (C=O), 1,591 (C=C), 1,539 & 1,349 (NO_2), 1,253 (C–O); ^1H NMR (300 MHz, $CDCl_3$) δ ppm: 8.39 (s, 1H, H-2′), 8.30 (d, 1H, $J = 8.1$ Hz, H-4′), 7.88 (d, 1H, $J = 7.8$ Hz, H-6′), 7.64 (dd, 1H, $J = 7.8$, 8.1 Hz, H-5′), 6.62 (s, 1H, H-3 of cyclohexenone), 6.53 (s, 2H, ArH), 4.12 (q, 2H, $J = 7.2$ Hz, –$C\underline{H}_2CH_3$), 3.87 (s, 6H, 2 × OCH_3), 3.84 (s, 3H, OCH_3), 3.81–3.77 (m, 2H, H-1 and 6 of cyclohexenone), 3.09–3.04 (m, 2H, H-5 of cyclohexenone), 1.11 (t, 3H, $J = 7.2$ Hz, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm: 193.48 (C-2 of cyclohexenone), 168.86 (C=O at C-1 of cyclohexenone), 155.53 (C-4 of cyclohexenone), 148.66 (C–NO_2), (153.55, 139.53, 136.15, 141.83, 125.92, 124.89, 121.11, 104.40, phenyl ring and C-3 of cyclohexenone), 61.19 (C-1 of cyclohexenone), 60.86 (OCH_3), 59.42 (-O-$\underline{C}H_2CH_3$), 56.24 (2 × OCH_3), 44.25 (C-6 of cyclohexenone), 36.30 (C-5 of cyclohexenone), 14.07 (CH_3); MS (APCI) $m/z = 456$ (M + 1); Anal. Calcd. for $C_{24}H_{25}NO_8$ (455.46): C, 63.29; H, 5.53; N, 3.08, Found: C, 63.42; H, 5.61; N, 3.14.

Microbiology

Antibacterial and antifungal activity

The MICs of synthesized compounds were carried out by broth microdilution method as described by Rattan (Rattan, 2000). DMSO was used as diluents to get desired concentration of drugs to test upon standard bacterial strains. Antibacterial activity was screened against two gram-positive (*S. aureus* MTCC 96 and *S. pyogenes* MTCC 443) and two gram-negative (*E. coli* MTCC 442 and *P. aeruginosa* MTCC 2488) bacteria by using ampicillin as a standard antibacterial agent. Antifungal activity was screened against three fungal species (*C. albican* MTCC 227, *A. niger* MTCC 282 and *A. clavatus* MTCC 1323), and griseofulvin was used as a standard antifungal agent. All MTCC cultures were collected from Institute of Microbial Technology, Chandigarh, and tested against the above-mentioned known drugs.

Mueller–Hinton broth and Sabouraud's broth were used as nutrient medium to grow bacteria and fungus, respectively. Inoculum size for the test strain was adjusted to 10^6 colony-forming unit (CFU) per milliliter by comparing the turbidity.

Serial dilutions were prepared in primary and secondary screening. The control tube containing no antibiotic was immediately subcultured (before inoculation) by spreading a loopful evenly over a quarter of plate of medium suitable for the growth of test organism and incubated at 37 °C for bacteria and 22 °C for fungi overnight. The MIC of the control organism was read to check the accuracy of the drug concentrations. The lowest concentration inhibiting growth of the organism was recorded as the MIC. Each test compound was diluted, obtaining 2,000 μg/mL concentration, as a stock solution. In primary screening, 500, 250 and 125 μg/mL concentrations of the test compounds were taken. The active synthesized compounds found in this primary screening were further tested in a second set of dilution against all organisms. The drugs found active in primary screening were similarly diluted to obtain 100, 50, 25, 12.5, 6.250, 3.125 and 1.5625 μg/mL concentrations. The highest dilution showing at least 99 % inhibition is taken as MIC. The antimicrobial screening data are shown in Table 2.

Antitubercular activity

MIC of the test compounds against *M. tuberculosis* $H_{37}Rv$ was determined by L.J. agar (MIC) method (Desai *et al.*, 1984; Shah *et al.*, 1985; Anargyros *et al.*, 1990) where primary 1,000, 500 and 250 μg/mL and secondary 200, 100, 50, 25, 12.5, 6.250 and 3.125 μg/mL dilutions of each test compound were added to liquid L.J medium and then media were sterilized by inspissation method. A culture of *M. tuberculosis* $H_{37}Rv$ growing on L.J. medium was harvested in 0.85 % saline in bijou bottles. For all test compounds, first a stock solution of 2,000 μg/mL concentration was prepared in DMSO. These tubes were then incubated at 37 °C for 24 h followed by streaking of *M. tuberculosis* $H_{37}Rv$ (5×10^4 bacilli per mL). These tubes were then incubated at 37 ± 1 °C. Growth of bacilli was seen after 12, 22 and finally 28 days of incubation. Tubes having the compounds were compared with control tubes where medium alone was incubated with *M. tuberculosis* $H_{37}Rv$. The concentration at which no development of colonies occurred or less than 20 colonies was taken as MIC of test compound. The standard strain *M. tuberculosis* $H_{37}Rv$ was tested with known drug rifampicin.

In vitro antileishmanial activity against promastigotes

Drugs All derivatives (**1**–**22**) were added into the cultures as a dimethyl sulfoxide (DMSO) solution (50 μM). The final solvent (DMSO) concentrations never exceeded 1 % (v/v) and had no effect on the parasites' proliferation or morphology.

Parasites and cell line *Leishmania braziliensis* (strain H3) promastigotes were grown at 28 °C in Schneider's medium supplemented with 5 % of heat-inactivated fetal bovine serum (FBS). The J774.A1 macrophage cell line was cultivated in RPMI 1640 (Gibco BRL) medium supplemented with 2 g/L of sodium bicarbonate and 10 % of FBS without Hepes.

Antiparasitic assay For the parasite growth inhibition assay, *Leishmania* promastigotes were harvested on the exponential phase of growth, and the concentration was adjusted to 10×10^6 parasites/mL in Schneider's medium plus 5 % FBS. The compounds solubilized in DMSO were diluted to appropriate concentrations ranging from 100 to 1.6 μM in culture medium. One hundred microliters of the parasite suspension was added to 96-well plates and incubated at 28 °C for 72 h in the presence of different compound concentrations. Amphotericin B (31.25–1,000 nM) was used as positive control, and DMSO 1 % was used as negative control. For each compound, three experiments were carried out in triplicate, and the number of surviving parasites was determined by counting in Neubauer chamber (Lunardi *et al.*, 2003).

Cytotoxicity assay Mouse peritoneal macrophages were harvested from the peritonea of healthy mice after injection of 5 mL sterile phosphate-buffered saline (PBS) containing 0.5 % EDTA. Cells (5×10^5 cells/mL) were cultivated for 72 h at 37 °C in 96-well microplates in DMEM medium supplemented with 10 % FBS in the presence of different compound concentrations. The cytotoxic effect of compounds was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Mosmann, 1983; Sieuwerts *et al.*, 1995). Thereafter, supernatant was removed, and 100 μl of DMSO was added to solublize the formazan crystals from viable cells. The samples were read at wavelength of 540 nm using ELISA plate reader. The 50 % cytotoxicity concentrations (CC_{50}) were determined by linear regression analysis (GraphPad Software, San Diego, California). All experiments were repeated three times.

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