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Original article

A facile synthesis of α,α′-(*EE*)-bis(benzylidene)-cycloalkanones and their antitubercular evaluations

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ABSTRACT

An economical and facile synthesis of α,α′-(*EE*)-bis(benzylidene)-cycloalkanones was achieved by the reaction of cycloalkanones with different aromatic aldehydes using ethanolic KOH in good yields. Few of the selected compounds were reduced with $NaBH_4$ to the respective α,α′-(*EE*)-bis(benzylidene)-cycloalkanols. All these compounds and our earlier synthesized cyclohexyl phenyl methanols were evaluated for their antitubercular, antifungal and antibacterial activities. Several compounds displayed moderate antitubercular activity with MIC = 12.5–1.56 μg/mL. However, none of the compounds displayed any significant antifungal activity.

1. Introduction

Tuberculosis has re-emerged as a growing public health threat, killing young and middle-aged people faster than any other diseases. Infection of *Mycobacterium tuberculosis* remains a leading cause of death [1–3]. It is estimated that nearly 9×10^6 new cases of active TB disease occur every year [4]. A vast majority of the world's burden of tuberculosis (TB) is in developing countries, and it is estimated that only 23% of the prevalent active cases receive an appropriate antitubercular treatment [5]. An appropriate treatment of tuberculosis has led to the development of several bottlenecks in chemotherapy of tuberculosis. Emergence of MDR (multi-drug resistance) and XDR (extremely drug resistance) tuberculosis and its synergy with HIV have further aggravated the problem of chemotherapy in tuberculosis. Although, a number of new chemical entities have been discovered recently as potent antituberculars, yet no new drug has entered clinics since 1965 [6–9]. Therefore, there is an urgent need to develop new drugs that act through a novel mode of action for the chemotherapy of TB. Recently, we have designed and developed novel aryloxy cyclopropyl phenyl methanones [10] as possible inhibitors of FAS-II enzyme which is required in chain elongation of mycolic acids keeping in mind the structure of triclosan [11] and the compounds displayed potent antitubercular activity both in vitro and in vivo. Moreover, several acetophenones [12], chalcones [13] and Mannich bases of benzylidene cycloalkanones [14] have also been disclosed to possess moderate antitubercular activity and their mode of action is also postulated to be the inhibition of initial steps in fatty acid biosynthesis. Bis-benzylidene cycloalkanones have been reported to possess drug resistance reversal [15], cytotoxicity [16] and histone acetyl transferase (HAT) [17] inhibitory activities. *N*-Acetyl transferase (NAT), a subtype of HAT plays a very important role in the initial stages of mycolic acid biosynthesis in mycobacterium and it is known that human NAT inactivates [18] the antitubercular drug INH (isoniazid) in humans. The above facts prompted us to synthesize benzylidene cycloalkanone analogs in an efficient and cost effective manner, and evaluate them for their antitubercular and antifungal activities.

2. Chemistry

α,α′-(*EE*)-Bis(benzylidene)-cycloalkanones (**1**–**15**) were prepared by reacting 2 equivalents of aromatic aldehydes with 1 equivalent of cycloalkanones in the presence of solid KOH (5 mol%) in ethanol (Scheme 1). The selected aldehydes comprise furfuraldehyde, benzaldehyde, 4-bromobenzaldehyde, 4-chlorobenzaldehyde, 4-fluorobenzaldehyde, 4-nitrobenzaldehyde, 4-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde and 3,4,5-trimethoxybenzaldehyde.

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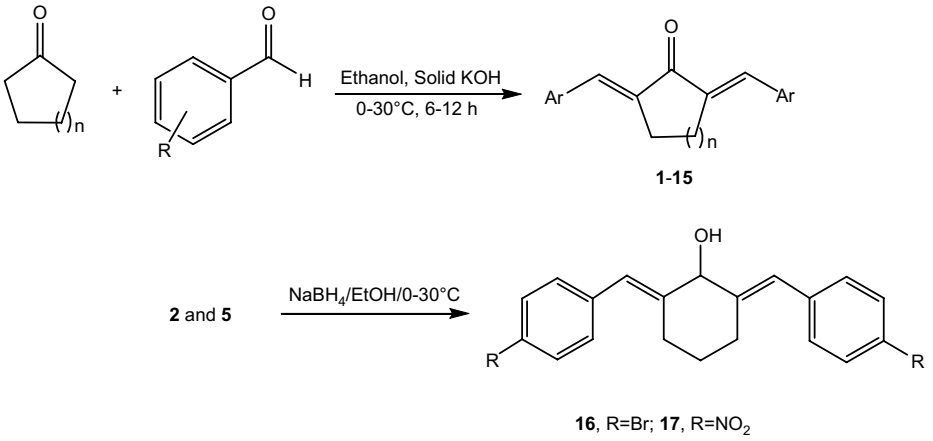


Scheme 1. Synthesis of bis-benzylidene cycloalkanones and cycloalkanols.

The selected cycloalkanones were cyclopentanone, cyclohexanone and cycloheptanone.

Two of the most active compounds **2** and **5** were reduced with $NaBH_4$ in ethanol to give the respective α,α′-bis-(benzylidene)-cycloalkanols **16** and **17** in good yields.

Cyclohexylphenyl methanols **18**, **19**, **20** and **21** were prepared by D-glucosamine catalyzed aldol reaction of cyclohexanone with 4-nitro-, 3-nitro-, 2-nitro- and 4-bromobenzaldehydes as reported by us [19] (Scheme 2).

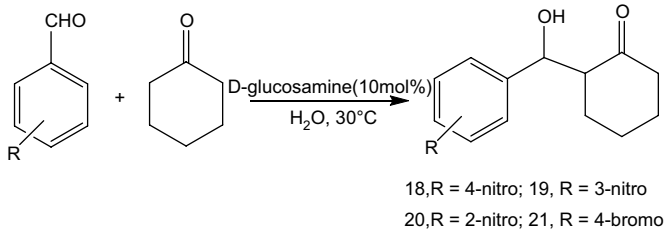
3. Biology

All of the synthesized compounds were evaluated for their antitubercular activity against *M. tuberculosis* H37Ra by MABA (Microplate Alamar Blue Assay) method [21], while agar micro-dilution method [22] was used against *M. tuberculosis* H37Rv. INH and ethambutol were used as standard drugs. All of the above compounds were also screened against different strains of bacteria and fungi viz. *Escherichia coli* (ATCC 9637), *Pseudomonasaeruginosa* (ACTT BAA-427), *Staphylococcus aureus* (ACTT-25923), *Klebsiella pneumoniae* (ACTT-27736), *Candida albicans* (ATCC-1405B), *Cryptococcus neoformans*, *Sporothrix schenckii*, *Trichophyton mentagrophytes*, *Aspergillus fumigatus* and *Candida parapsilosis* (ATCC-22019). Fungi were tested by NCCLS (National Committee for Clinical Laboratory Standards) method in RPMI 1640 medium and bacteria in Mueller Hinton Broth [34–37].

4. Results and discussion

4.1. Chemistry

Several reports exist for the synthesis [38–44] of benzylidene cycloalkanones involving the use of organic and inorganic bases, metal catalyst, different types of Friedel Craft catalysts and TCT (trichloro-1,3,5-triazine). We have used aqueous KOH/NaOH, TCT and many other catalysts for the condensation of cycloalkanones and aldehydes to get the desired α,α′-(*EE*)-bis(benzylidene)-cycloalkanones. In our work, most of the catalysts resulted in poor yields of the products as several byproducts were formed and it was difficult to isolate the desired compounds in pure form from the reaction mixture. Application of solid KOH (5 mol%) as a catalyst for the condensation of different aldehydes with cycloalkanones in minimum amount of ethanol resulted in the desired α,α′-(*EE*)-bis(benzylidene)-cycloalkanones (**1**–**15**) in good yields (Scheme 1 and Table 1) and proved to be the most convenient method. This method is economical and eco-friendly as neither any byproduct was formed nor any toxic material was used during the synthesis and the reactions were carried out at ambient temperature. Work up of the reaction mixture was very simple involving just filtration of the product and washing with cold water followed by drying and crystallization. The structures of bis-benzylidene cycloalkanones (**1**–**15**) were in accordance with their spectroscopic data and microanalyses. The IR spectrum of the compounds in general exhibited the absorption band at around 1731–1593 cm^{-1} indicating that the carbonyl group and olefinic bonds of α,α′-(*EE*)-bis(benzylidene)-cycloalkanones are in conjugation. The ESMS (mass spectra) of the compounds showed their respective $[M+H]^+$ peaks. In the ^1H NMR spectrum of the compounds the vinylic protons either occur as singlet at around δ 7.50–7.30 ppm or occur merged with the multiplet of aromatic



Scheme 2. Synthesis of hydroxy-(phenyl)-methyl cycloalkanones.

Table 1
Synthesis of bis-benzylidene cycloalkanones and cycloalkanols (**1**–**17**).

Compound no.	*n*	Ar/R	m.p. (°C)	Known m.p (°C) [Ref]	% Yield
1	2	Furyl	131–133	140–142 [20]	69
2	2	4-Bromophenyl	163–164	165–168 [26,27]	86
3	2	3,4-Dimethoxyphenyl	141–143	138–140 [23]	92
4	2	4-Fluorophenyl	154–156	156–158 [28]	85
5	2	4-Nitrophenyl	154–157	161–162 [43]	87
6	3	4-Bromophenyl	129–131	137 [24]	83
7	2	4-Benzyloxyphenyl	199–201	190–191 [25]	86
8	2	3,4,5-Trimethoxyphenyl	218–219	208 [23]	88
9	2	Phenyl	117–119	117–118 [43]	91
10	2	4-Chlorophenyl	142–145	147–148 [43]	85
11	3	4-Chlorophenyl	118–120	Not reported	85
12	3	4-Methoxyphenyl	95–97	126–127 [39]	87
13	3	3,4-Dimethoxyphenyl	155–157	164–165 [43]	90
14	1	3,4-Dimethoxyphenyl	190–192	186–188 [23]	84
15	2	2-Naphthyl	199–202	212–213 [29]	89
16	2	4-Bromophenyl	131–132	Not reported	75
17	2	4-Nitrophenyl	143–145	Not reported	65

protons ranging from δ 8.25 to 6.65 ppm, while the methylene protons of C-3 appeared as multiplet at around δ 2.96–2.36 ppm and the methylene protons of C-4 was observed as multiplet at around δ 1.97–1.67 ppm.

The *EE* geometry of the double bonds in the above compounds (**1**–**15**) was based on earlier literature reports [30–33]. It is reported that the vinylic protons are in close proximity to the carbonyl group which exerted an anisotropic effect, resulting in the downfield shifting and overlapping of the vinylic protons with the aromatic protons, and appearance of vinylic protons in the region of δ 7.15–7.95 ppm is an indication of such compounds with *E* configuration and in the region of δ 6.8 ppm indicates *Z* configuration [30,31], as for example, the olefinic protons of *Z*-2-phenyl methylenecyclohexanones and *Z*-2-phenyl methylene-6,6-diphenyl cyclohexanones are generally observed at δ 6.27 and 6.22 ppm [32,33]. In the ^{1}H NMR spectra of compounds **1**–**15** the vinylic protons appear either as singlet at around δ 7.50–7.30 ppm or observed along with the multiplets of aromatic protons ranging from δ 8.25 to 6.65 ppm. The *EE* geometry of the two double bonds was further substantiated by NOE experiment on a prototype compound (**10**). The vinylic proton at δ 7.7 ppm showed NOE with the aromatic *ortho* protons and at the same time it did not show any NOE with the methylene protons at C-3 (δ 2.8 ppm) (Fig. 1). The latter clearly shows the *EE* geometry of the olefinic bond.

The IR spectrum of the bis-benzylidene cycloalkanols (**16** and **17**) exhibited the absorption band at around 3300 and 1480 cm^{-1} indicating the presence of hydroxyl group and olefinic bonds. In the ^{1}H NMR spectrum of the compound the vinylic protons are observed to be merged with aromatic protons and appeared in the range of δ 8.21–6.54 ppm and hydroxyl proton observed around δ 4.50 ppm while the proton of C-1 occurred at δ 2.79 ppm and the methylene protons of C-3 and C-4 appeared as such.

4.2. Biology

Out of all the screened compounds **2**, **4**, **5**, **9**, **12**, **16**, **18**, **19** and **21** displayed antitubercular activity with MIC ranging from 12.5 to 1.56 μg/mL against either the avirulent strain *M. tuberculosis* H37Ra or the virulent strain *M. tuberculosis* H37Rv. As evident from Table 2, compounds **2**, **4**, **5** and **12** display antitubercular activity against the avirulent strain *M. tuberculosis* H37Ra, a surrogate of *M. tuberculosis* H37Rv. On the other hand compounds **2**, **5**, **9**, **16**, **18**, **19** and **21** show activity against *M. tuberculosis* H37Rv. Therefore, two of the above compounds **2** and **5** are active against both the avirulent and virulent strains of *M. tuberculosis*.

Among all the compounds screened against different strains of bacteria and fungi only one compound (compound **2**) showed activity against the two fungi, *S. schenckii* and *T. mentagrophytes* with MIC = 25 and 12.5 μg/mL, respectively. Other compounds displayed MIC ≥ 50 μg/mL against all the fungi which were tested for these compounds.

A closer look into the SAR (structure activity relationship) of these compounds reveals that among all the benzylidene

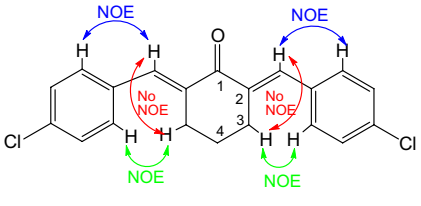


Fig. 1. NOE in *EE* geometry.

Table 2
In vitro antitubercular activity of bis-benzylidene cyclohexanones (**1**–**15**), bis-benzylidene cyclohexanols (**16** and **17**) and cyclohexyl phenyl methanols (**18**–**21**).

Compound no.	*n*	*c* Log *P*[a]	MIC (H37Ra)	MIC (H37Rv)
1	2	3.68	>12.5	>12.5
2	2	6.46	1.56	12.5
3	2	4.64	>12.5	>12.5
4	2	5.18	12.5	>12.5
5	2	7.29	3.12	12.5
6	3	6.78	>12.5	>12.5
7	2	7.59	>12.5	>12.5
8	2	4.43	>12.5	>12.5
9	2	4.64	>12.5	6.25
10	2	5.76	>12.5	>12.5
11	3	6.17	>12.5	>12.5
12	3	4.8	6.25	>12.5
13	3	4.55	>12.5	>12.5
14	1	3.72	>12.5	>12.5
15	2	7.67	>12.5	>12.5
16	2	6.13	>12.5	12.5
17	2	3.32	>12.5	>12.5
18	2	3.43	>12.5	12.5
19	2	3.53	>12.5	12.5
20	2	3.55	>12.5	>12.5
21	2	3.07	>12.5	12.5
Isoniazid	–	−0.668	–	0.75
Ethambutol	–	0.1188	–	3.25

[a] *c* Log *P* was determined by OSIRIS Property Explorer Programme which is available at http://www.organic-chemistry.org/prog/peo/.

cycloalkanones, compounds **1**, **3**, **6**, **7**, **8**, **10**, **11**, **13**, **14**, **15**, **17** and **20** do not exhibit antitubercular activity against either of the strains *M. tuberculosis* H37Ra or *M. tuberculosis* H37Rv as they have MIC > 12.5 μg/mL. Further, it is also evident that the reduction of carbonyl group in the most potent compounds (**2** and **5**) of the series to their respective bis-benzylidene cycloalkanols (**16** and **17**), results in the loss of antitubercular activity indicating that the carbonyl group is essential for their antitubercular activity. It is also interesting to note that among the bis-benzylidene cycloalkanones, only bis-benzylidene cyclohexanones display antitubercular activity while their counter parts with cyclopentanone and cycloheptanone moieties are inactive as their MIC values were >12.5 μg/mL. It is also clear that the substitution on aromatic ring at the 4th position generally leads to compounds having better activity in comparison to other compounds either with unsubstituted aromatic ring or having substitution on other aromatic positions. Compounds **16**, **18**, **19** and **21** are moderate antitubercular agents as they have MICs values < 12.5 μg/mL. However, compounds **2**, **5**, **9** and **12** are good antituberculars as they have MICs in the range of 1.56–6.25 μg/mL. Among the hydroxy-(phenyl)-methyl cycloalkanones **18**–**21**, except compound **20** with nitro substituent at 2-position of the aromatic ring, other compounds of the series **18**, **19** and **21** with substituents (–NO_2 or –Br) either at 3- or 4-positions on the aromatic rings display moderate antitubercular activity against *M. tuberculosis* H37Rv with MIC = 12.5 μg/mL while they were inactive against the avirulent strains (MIC > 12.5 μg/mL). Further as the compounds did not display any significant activity against fungi or other bacteria, they may be specific to mycobacterium.

5. Conclusion

In conclusion, we have developed a simple, economical and efficient method for the synthesis of bis-benzylidene cycloalkanones and evaluated them for their antitubercular activity. Three of the compounds displayed MIC in the range of 6.25–1.56 μg/mL, a criterion for further optimization of the series for new antitubercular agents.

6. Experimental

6.1. Chemistry

Commercially available reagent grade chemicals were used as received. All reactions were followed by TLC on E. Merck Kieselgel 60 F_{254}, with detection by UV light and/or by spraying a 20% $KMnO_4$ aq. solution. Column chromatography was performed on silica gel (60–120 mesh, E. Merck). IR spectra were recorded as thin films or in chloroform solution with a Perkin–Elmer Spectrum RX-1 (4000–450 cm^{-1}) spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Brucker DRX-300 in $CDCl_3$. Chemical shift values are reported in ppm relative to $SiMe_4$ as internal reference, unless otherwise stated; s (singlet), d (doublet), t (triplet), m (multiplet); *J* in hertz. FAB mass spectra were performed using a mass spectrometer Jeol SX-102 and ESI mass spectra with Quattro II (Micromass). Elemental analyses were performed on a Perkin–Elmer 2400 II elemental analyzer.

6.1.1. General experimental procedure for the preparation of α,α′-(EE)-bis(substituted-benzylidene)-cycloalkanones

To a stirring solution of the cycloalkanone (1 mmol) and aromatic aldehyde (2 mmol) in minimum amount of ethanol, solid KOH (5 mol%) was added. The reaction mixture was stirred at ambient temperature till the disappearance of the starting materials (TLC). The solid separated was filtered and washed with water and dried. The product, so-obtained, was crystallized with ethanol to give the desired compounds in good yields.

*6.1.1.1. 2,6-(EE)-Bis-furan-2-yl-methylene-cyclohexanone (**1**).* IR (KBr): ν_{max} cm^{-1} 3452, 1777, 1593; MS (FAB): 255 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 7.56–7.53 (d, $J = 7.1$ Hz, 4H, olefinic protons and ArH), 6.66 (d, $J = 3.18$ Hz, 2H, ArH), 6.51 (dd, $J = 1.68$ and 1.56 Hz, 2H, ArH), 3.05–2.93 (m, 4H, CH_2), 1.93–1.88 (m, 2H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 188.4, 153.2, 144.5, 133.2, 123.6, 116.1, 112.5, 31.0, 30.1, 28.4 and 22.1. Anal. Calcd for $C_{16}H_{14}O_3$: C, 75.57; H, 5.55%. Found: C, 75.54; H, 5.58%.

*6.1.1.2. 2,7-(EE)-Bis-(4-bromobenzylidene)-cycloheptanone (**6**).* IR (KBr): ν_{max} cm^{-1} 3449, 1673, 1606; MS (FAB): 447 $[M+H]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 7.61–7.11 (m, 10H, olefinic protons and ArH), 2.73–2.67 (m, 4H, CH_2), 1.98–1.81 (m, 4H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 204.0, 142.0, 134.6, 134.3, 131.7, 131.6, 130.9, 43.3, 31.3, 29.9, 28.7, 28.0, 27.7 and 25.4. Anal. Calcd for $C_{21}H_{18}O_1Br_2$: C, 56.53; H, 4.07%. Found: C, 56.54; H, 4.09%.

*6.1.1.3. 2,7-(EE)-Bis-(4-chlorobenzylidene)-cycloheptanone (**11**).* IR (KBr): ν_{max} cm^{-1} 3021, 1731, 1603; MS (FAB): 343 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.40–7.19 (m, 10H, olefinic protons and ArH), 2.65 (m, 4H, CH_2), 1.97–1.95 (m, 4H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 198.1, 142.1, 134.9, 134.6, 131.0, 129.1, 129.0, 43.6, 31.7, 29.1, 28.8 and 25.7. Anal. Calcd for $C_{21}H_{18}O_1Cl_2$: C, 70.60; H, 5.08%. Found: C, 70.59; H, 5.10%.

*6.1.1.4. 2,7-(EE)-Bis-(4-methoxybenzylidene)-cycloheptanone (**12**).* IR (KBr): ν_{max} cm^{-1} 3018, 1731, 1603; MS (FAB): 335 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.45–7.26 (m, 6H, olefinic protons and ArH), 6.93–6.87 (m, 4H, ArH), 3.81 (s, 6H, OCH_3), 2.70 (m, 4H, CH_2), 1.97 (m, 4H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 199.7, 160.0, 140.0, 135.8, 131.6, 129.0, 114.3, 55.5, 30.2, 28.8, 28.4 and 25.7. Anal. Calcd for $C_{23}H_{24}O_3$: C, 82.63; H, 7.18%. Found: C, 82.68; H, 7.09%.

*6.1.1.5. 2,7-(EE)-Bis-(3,4-dimethoxybenzylidene)-cycloheptanone (**13**).* IR (KBr): ν_{max} cm^{-1} 3010, 1695, 1596; MS (FAB): 395 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.29 (s, 2H, olefinic protons), 7.05–6.83 (m, 6H, ArH), 3.89 (s, 12H, OCH_3), 2.71 (m, 4H, CH_2), 2.00 (m, 4H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 198.1, 150.6, 149.2, 140.0, 135.9, 129.4, 122.9, 113.4, 111.4, 56.0, 29.1 and 28.6. Anal. Calcd for $C_{25}H_{28}O_5$: C, 73.51; H, 6.91%. Found: C, 73.49; H, 6.93%.

*6.1.1.6. 2,5-(EE)-Bis-(3,4-dimethoxy-benzylidene)-cyclopentanone (**14**).* IR (KBr): ν_{max} cm^{-1} 3020, 1730, 1595; MS (FAB): 367 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.50 (s, 2H, olefinic protons), 7.18–6.89 (m, 6H, ArH), 3.92 (s, 12H, OCH_3), 3.09 (m, 4H, CH_2). ^{13}C NMR (50 MHz, $CDCl_3$): δ 196.0, 150.6, 149.3, 135.6, 134.0, 129.4, 124.9, 113.9, 111.5, 56.2, and 26.8. Anal. Calcd for $C_{25}H_{28}O_5$: C, 76.61; H, 6.36%. Found: C, 76.61; H, 6.39%.

6.1.2. General experimental procedure for the preparation of α,α′-bis(benzylidene)-cycloalkanols

A solution of α,α′-(*EE*)-bis(substituted-benzylidene)-cycloalkanones (1 mmol) and ethanol (5 mL) was stirred at 0 °C for 10 min. $NaBH_4$ (1 equivalent) was slowly added and stirred at 30 °C till the disappearance of starting material (TLC). The reaction mixture was brought to 0 °C and excess of $NaBH_4$ was quenched by saturated aq. solution of NH_4Cl and solid so-obtained was filtered. The solid cake was washed with ethanol and the combined filtrate was evaporated under reduced pressure to give a crude mass. The latter was dissolved in ethylacetate, organic layer was washed with water, dried (anhy. Na_2SO_4) and concentrated in vacuum to give a gummy mass. The latter was chromatographed over SiO_2 using a gradient of hexane:EtOAc as eluent to afford the pure products.

*6.1.2.1. 2,6-Bis-(4-bromobenzylidene)-cyclohexanol (**16**).* IR (KBr): ν_{max} cm^{-1} 3021, 1724, 1596; MS (FAB): 417 $[M-OH]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 7.74–6.54 (m, 10H, olefinic protons and ArH), 4.64 (s, 1H, CHOH), 2.79–2.73 (m, 1H, CH), 2.40–2.33 (m, 4H, CH_2), 1.61 (m, 2H, CH_2). Anal. Calcd for $C_{20}H_{18}O_1Br_2$: C, 55.33; H, 4.18%. Found: C, 55.30; H, 4.20%.

*6.1.2.2. 2,6-Bis-(4-nitro-benzylidene)-cyclohexanol (**17**).* IR (KBr): ν_{max} cm^{-1} 3213, 1656 and 1513; MS (FAB): 366.9 $[M+H]^+$. ^1H NMR (200 MHz, $CDCl_3$): δ 8.21–8.16 (m, 4H, ArH), 7.37–7.33 (m, 6H, olefinic protons and ArH), 4.25 (s, 1H, CHOH), 2.79–2.72 (m, 1H, CH), 2.07–1.99 (m, 4H, CH_2), 1.67–1.58 (m, 2H, CH_2). Anal. Calcd for $C_{20}H_{18}N_2O$: C, 65.57; H, 4.95%. Found: C, 65.53; H, 4.99%.

7. Biological activity

7.1. Activity against M. tuberculosis H37Ra strain

All of the synthesized compounds were evaluated for their efficacy against *M. tuberculosis* H37Ra at active concentration ranging from 50 μg/mL to MIC using two-fold dilutions in the initial screen. Log phase culture of *M. tuberculosis* H37Ra is diluted so as to give final $OD_{550\ nm}$ of 0.05 in Sauton's medium. In 96-well white plates 190 μL of culture is dispensed in each well. A dimethyl sulfoxide (DMSO) solution of test compounds is dispensed into 96-well plates so as to make final test concentration of 25 μg/mL (5 μg test compound is dispensed into 10 μL of DMSO). Then the plate is incubated at 37 °C/5% CO_2 for 5 days. On 5th day 15 μL Alamar Blue solution is added to each well of the plate. The plate is again incubated overnight at 37 °C/5% CO_2 incubator. The fluorescence is read on BMG polar star with excitation frequency at 544 nm and emission frequency at 590 nm. The compounds, which were found to be active (>90% inhibition as compared with control) at this concentration are then tested at 6 serial dilutions starting from 50 to 1.56 μg/mL.

7.2. Activity against M. tuberculosis H37Rv strain

Drug susceptibility and determination of MIC of the test compounds/drugs against *M. tuberculosis* H37Rv were performed by agar microdilution method where two-fold dilutions of each test compound were added into 7H10 agar supplemented with OADC and organism. A culture of *M. tuberculosis* H37Rv growing on L–J medium was harvested in 0.85% saline with 0.05% Tween-80. A suspension of 1 μg/mL concentration of extracts/compounds was prepared in DMSO. This suspension was added to (in tubes) 7H10 Middle Brook's medium (containing 1.7 mL medium and 0.2 mL OADC supplement) at different concentrations of compound keeping the volume constant i.e. 0.1 mL. Medium was allowed to cool by keeping the tubes in slanting position. These tubes were then incubated at 37 °C for 24 h followed by streaking of *M. tuberculosis* H37Rv (5×10^4 bacilli per tube). These tubes were then incubated at 37 °C. Growth of bacilli was seen after 30 days of incubation. Tubes having the compounds were compared with control tubes where medium alone was incubated with H37Rv. The concentration at which complete inhibition of colonies occurred was taken as active concentration of test compound.

7.3. Antifungal and antibacterial activities

Minimum inhibitory concentration of compounds was tested according to the standard microbroth dilution technique as per NCCLS guidelines in flat bottom 96-well tissue culture plates (CELLSTAR_ Greiner bio-one GmbH, Germany) in RPMI 1640 medium buffered with MOPS (3-[*N*-morpholino]propanesulfonic acid) (Sigma Chem. Co., MO, USA) for fungal strains and in Muller Hinton Broth (Titan Biotech Ltd, India) for bacterial strains. The concentration ranges for the tested compounds were 50–0.36 and 32–0.0018 mg/mL for standard compounds. Plates were incubated at 35 °C in a moist chamber (24 h for all the bacterial strains, 48 h for *C. albicans* and *C. parapsilosis*, 72 h for *A. fumigatus*, *S. schenckii*, and *C. neoformans*, and 96 h for *T. mentagrophytes*). MICs were determined as 90% inhibition of growth with respect to the growth in control spectrophotometrically.

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Appendix. Supporting information

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2008.09.026.

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