**In vitro** anticandidal evaluation of novel highly functionalized bis cyclohexenone ethyl carboxylates

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**Abstract.** - OBJECTIVES: Novel highly functionalized bis cyclohexenone ethyl carboxylates 7-12 were designed, synthesized and their structures were elucidated by their elemental analysis, MS, FT-IR, one-dimensional 1H, and 13C NMR spectroscopic data.

MATERIALS AND METHODS: All the synthesized compounds 7-12 were tested for their in vitro antifungal activities against Candida sp. namely Candida albicans, Candida tropicalis, Candida glabrata, Candida parapsilosis, Candida dubliiniensis and Candida krusei.

RESULTS: A close inspection of the in vitro antifungal activity profile in differently withdrawing (-F, -Cl, and -Br) functional group and electron donating (CH3 and OCH3) substituted phenyl rings of novel highly functionalized bis cyclohexenone ethyl carboxylates 7-12 exerted strong antifungal activity against all the tested Candida species. All the synthesized compounds 7-12 exhibited MIC value in the range of 6.25-200 µg/mL against all the tested Candida (C.) species.

CONCLUSIONS: Compound 8 against C. albicans, 9,11 against C. glabrata, 8,10 against C. parapsilosis, 7,9 against C. dubliiniensis, 8,10 against C. krusei exhibited excellent antifungal activity at a MIC value of 6.25 µg/mL. Likewise compound 7, 9-11 against C. albicans, 8, 9,11 against C. tropicalis, 8 against C. glabrata, 9 against C. parapsilosis, 10 against C. dubliiniensis, 9 against C. krusei revealed superior activity at a MIC value of 12.5 µg/mL.

Key Words: Functionalized bis cyclohexenones, Cyclocondensation, Michael addition, Synthesis, Candida sp.

**Introduction**

Infections due to Candida (C.) species are the most common of the fungal infections1. Candida species produce a broad range of infections, ranging from non-life-threatening mucocutaneous illnesses to invasive process that may involve virtually any organ. Such a broad range of infections requires an equally broad range of diagnostic and therapeutic strategies. In general, both amphotericin B and the azoles have a role to play in treatment. Choice of therapy is guided by weighing the greater activity of amphotericin B for some non-albicans species (e.g., Candida krusei) against the lesser toxicity and ease of administration of theazole antifungal agents. Fluconazole has activity against many isolates of Candida but is not often used. Vaginal candidiasis2 is an infection caused by Candida albicans (80-90%) or related fungi such as C. glabrata and C. tropicalis (10%). Fluconazole, a bis-triazole antifungal agent has the potential for reducing episodes of vaginal candidiasis3. In animal models, fluconazole has been shown to be more potent than Ketoconazole against Candida infections4. Clotrimazole is effective against dermatophyte and other fungal infections5, which has been used for local treatment.

A growth of interest is growing now-a-days in exploiting more than one proximal functional pharmacophoric groups for designing novel structures capable of performing a variety of functions6,7. One of the essential components of the search for new leads in drug designing programme is to synthesis molecules, which are novel still resembling known biologically active molecules by virtue of the presence of some critical pharmacophoric structural features8. The motive for the preparation of highly functionalized cyclohexenone ethyl carboxylates is due to the fact that they are excellent carriers of different types of biological activity.9-12. Cyclohexenoic long chain fatty alcohols are used in the treatment of neurological disorders13. Ambucic acid, a highly functionalized cyclohexenone exhibits antifungal activity14. Jesterone and hydroxyl jesterone are highly functionalized cyclohexenylester derivatives with potent antifungal activity15.

In view of the above mentioned biological properties and as part of our research program aimed at
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the synthesis of biologically active small yet novel structurally diverse compounds, herein is reported the highly functionalized cyclohexenone ethyl carboxylates has been designed and synthesized and to study their in vitro anticanidal activity against clinically isolated fungal strains namely C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. dubliniensis and C. krusei and their structure-activity relationship results are discussed.

Materials and Methods

Chemistry

The progress of the reaction is monitored by thin layer chromatography (TLC) analysis. All the reported melting points are taken in open capillaries and are uncorrected. IR spectra are recorded in KBr (pellet forms) on a Nicolet-Avatar–330 FT-IR spectrophotometer (Fisher Scientific Inc, Waltham, MA, US) and note worthy absorption values (cm⁻¹) alone are the reported melting points are taken in open thin layer chromatography (TLC) analysis. All analyses are obtained on Carlo Erba 1106 CHN analyzer (Thermo Fisher Scientific Inc, Waltham, MA, US). By adopting the previous literature, bis chalcones 1-6 are prepared.

Typical Experimental Procedure for the Synthesis of Highly Functionalized bis Cyclohexenone Ethyl Carboxylates 7-12

To a solution of sodium ethoxide (0.001 mol) in 30 mL of absolute ethanol, freshly distilled ethyl acetocetate (0.01 mol) and respective bis chalcones 1-6 (0.01 mol) in absolute ethanol (40 mL) is mixed and it is refluxed in a water bath for 3-6 h by maintaining the temperature around (70-80)°C. The reaction mixture is allowed to cool and filtered. Then the crude product is recrystallized from absolute ethanol to afford bis cyclohexenone ethyl carboxylates 7-12.

Compound 7

Reflux time: 5 h, Yield 82%, m.p. 62°C, m.f. C₃₆H₃₄Br₂O₆, 562 M⁺, C 76.71, found 76.82 cal H 5.99, found 6.09, cal IR (KBr) ν (cm⁻¹): 3052, 2980, 2924, 2854, 1663, 1738, 1607, 757, 694; ¹H NMR (δ ppm), (J Hz): 0.93 (6H, t, CH₃CH₂ at C-1, J=5.2), 2.99-2.95 (2H, H₃p, m), 3.14-3.00 (2H, H₅p, m), 3.68-3.61 (1H, H₃, m), 3.95-3.87 (4H, m, CH₂CH₂ at C-1), 4.11 (1H, H₂, d, J=13.6), 6.54 (1H, d, H₂p, J=2.0), 7.72-7.37 (14H, m, H₅pm); ¹³C NMR (δ ppm): 13.79 CH₂, 69.20 CH₃, 71.63 CH₂CH₂, 123.21 C-1, 122.84 C-2, 157.78 C-4, 168.64 C=O at C-1, 194.21 C-2, 129.81-127.61 -C₆, 130.14 C-3, 139.86, 138.00, 137.32 -C₅, 128.91-115.6 -C₄, 129.18 C-5.

Compound 8

Reflux time: 3 h, Yield 86%, m.p. 90°C, m.f. C₃₆H₃₂Cl₂O₆, 598 M⁺, C 72.14, found 72.23 cal H 5.31, found 5.39 cal IR (KBr) ν (cm⁻¹): 3063, 2986, 2925, 1664, 1738, 1600, 832, 756; ¹H NMR (δ ppm), (J Hz): 0.92 (6H, t, CH₂CH₂ at C-1, J=7.2), 2.99-2.94 (2H, H₃p, m), 3.12-3.05 (2H, H₅p, m), 3.67-3.59 (1H, H₁, m), 3.95-3.85 (4H, m, CH₂CH₂ at C-1), 4.09 (1H, H₂, d, J=14.3), 6.52 (1H, s, H₃), 7.81-7.18 (12H, m, H₅pm); ¹³C NMR (δ ppm): 13.79 CH₂, 69.20 CH₃, 71.63 CH₂CH₂, 123.21 C-1, 122.84 C-2, 157.78 C-4, 168.64 C=O at C-1, 194.19 C-2, 129.81-115.64 -C₆, 130.14 C-3, 139.86, 138.00, 137.32 -C₅, 128.91-115.6 -C₄, 129.18 C-5.

Compound 9

Reflux time: 4 h, Yield 80 %, m.p. 72°C, m.f. C₃₆H₃₂F₂O₆, 630 M⁺, C 68.31, found 68.47 cal H 4.98, found 5.11 cal IR (KBr) ν (cm⁻¹): 3052, 2980, 2927, 1665, 1738, 1609, 825, 677; ¹H NMR (δ ppm), (J Hz): 0.92 (6H, t, CH₂CH₂ at C-1, J=7.0), 2.97-2.93 (2H, H₃p, m), 3.07-3.00 (2H, H₅p, m), 3.66-3.63 (1H, H₁, m), 3.93-3.87 (4H, m, CH₂CH₂ at C-1), 4.11 (1H, H₂, d, J=15.6), 6.56 (1H, d, H₂p, J=2.0), 7.76-7.38 (12H, m, H₅pm); ¹³C NMR (δ ppm): 13.79 CH₂CH₂ at C-1, 35.20 C-5, 43.35 C-6, 59.89 CH₂CH₂ at C-1, 59.89 C-5, 122.84 C-3, 158.07 C-4, 169.15 C=O at C-1, 194.19 C-2, 129.81-115.6 -C₆, 130.14 C-3, 139.86, 138.00, 137.32 -C₅, 128.91-115.6 -C₄, 129.18 C-5.

Compound 10

Reflux time: 3 h, Yield 78 %, m.p. 128°C, m.f. C₃₆H₃₂Br₂O₆, 718 M⁺, C 59.87, found 60.02 cal H 4.37, found 4.48 cal IR (KBr) ν (cm⁻¹): 3063, 2974, 2923, 2849, 1664, 1737, 1607, 825, 756; ¹H NMR (δ ppm), (J Hz): 0.85-0.97 (6H, m, CH₂CH₂ at C-1), 2.99-2.92 (2H, H₃p, m), 3.08-3.00 (2H, H₅p, m), 3.68-3.56 (1H, H₁, m), 3.94-3.86 (4H, m, CH₂CH₂ at C-1), 4.03 (1H, H₂, d, J=14.5), 6.58 (1H, d, H₂p, J=3.0), 7.67-7.28 (12H, m, H₅pm); ¹³C NMR (δ ppm): 14.31 CH₂CH₂ at C-1, 35.44 C-5, 43.90 C-6, 60.51 CH₂CH₂ at C-
1, 59.22 C-1, 123.78 C-3, 158.41 C-4, 169.82 C=O at C-1, 194.59 C-2, 128.23-124.46 -C arom., 137.04, 132.22, 129.07 ipso C’s.

**Compound 11**

Reflux time: 6 h, Yield 85 %, m.p. 186°C, m.f. C₅H₇O₅ 622 M⁺, C 73.11found 73.29 cal H 6.03found 6.15cal; IR (KBr) ν (cm⁻¹): 3030, 2958, 2924, 2850, 1653, 1737, 1601, 831, 756; ¹H NMR (δ ppm), (J Hz): 0.94 (6H, m, CH₂CH₃ at C-1, J=7.2), 3.02-2.94 (2H, H₅a, m), 3.09-3.03 (2H, (1H, d, H₃, J=1.5), 7.71-6.99 (12H, m, H arom.); ¹³C NMR (δ ppm), (δ ppm): 14.30 CH₂ at phenyl rings, 2.99-2.95 (2H, H₅a, m), 3.13-3.04 (2H, H₅a, m), 3.68-3.57 (1H, H₆, m), 3.92-3.90 (4H, m, CH₂CH₃ at C-1), 4.09 (1H, H₆, d, J=14.3), 6.52 (1H, s, H₆), 7.63-7.24 (12H, m, H arom.); ¹³C NMR (δ ppm): 14.30 CH₂CH₃ at C-1, 21.32, 21.34 CH₃ at phenyl rings, 35.66,35.56 C-5, 43.96, 44.08 C-6, 60.40, 60.37 CH₂CH₃ at C-1, 59.17 C-1, 123.88, 122.55 C-3, 159.63, 158.93 C-4, 169.85, 169.73 C=O at C-1, 194.71 C-2, 129.93-126.78 -C arom., 143.02, 141.00, 140.83, 140.53, 135.54, 134.87 ipso C’s.

**Microbiology**

**Materials**

All the clinically isolated fungal strains namely C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. dubliniensis and C. krusei are obtained from Faculty of Medicine, Annamalai University, Annamalainagar-608002, Tamil Nadu, India.

**In vitro Anticandidal Activity By Two Fold Serial Dilution Method**

Minimum inhibitory concentration (MIC) in µg/mL values is carried out by two-fold serial dilution method₁. The respective test compounds (15-21) are dissolved in dimethyl sulphoxide (DMSO) to obtain 1 mg mL⁻¹ stock solution. Seeded broth (broth containing microbial fungal spores) is prepared at 37 ± 1°C from 1 to 7 days old Sabouraud’s agar (Hi-media, Mumbai, India) slant cultures were suspended in seeded broth (SDB). The colony forming units (CFU) of the seeded broth are determined by plating technique and adjusted in the range of 10⁴-10⁵ CFU/mL. The final inoculum’s size was 1.1-1.5 Ξ 10⁵ CFU/mL for antifungal assay. Testing is performed at a pH 5.6 for fungi (SDB). Exactly 0.4 mL of the solution of test compound was added to 1.6 mL of seeded broth to form the first dilution. One milliliter of this is diluted with a further 1 mL of seeded broth to give the second dilution and so on till six such dilutions are obtained. A set of assay tubes containing only seeded broth is kept as control. The tubes are incubated in BOD incubators (Sigma Instruments, Chennai, India) at 28±1°C for fungi. The minimum inhibitory concentrations (MICs) are recorded by visual observations after 72-96 h (for fungi) of incubation. Fluconazole is used as a standard drug for Candida species.

**Results**

The straight forward approach for the synthesis of highly functionalized cyclohexenone ethyl carboxylates 7-12 is as follows: Novel bis chalcones 1-6 are synthesized by the Claisen-Schmidt condensation of terephthaldehyde with substituted acetophenones in the presence of alcoholic sodium hydroxide base catalyst. Treatment of bis chalcones 1-6 with ethyl acetoacetate in the presence of sodium ethoxide in refluxing ethanol (Figure 1) afford highly functionalized cyclohexenone ethyl carboxylates 7-12. The reaction mechanism (Figure 2) involves the formation of Michael addition product by ethyl acetoacetate with bis chalcones 1-6 in the presence of base, sodium ethoxide. Afterwards the Michael addition product undergoes intramolecular aldol reaction in the presence of sodium ethoxide base to yield the title compounds 7-12. The structures of all the synthesized compounds 7-12 are confirmed by m.p.’s, FT-IR, MS, ¹H NMR, ¹³C NMR spectra and elemental analysis. In vitro anticandidal activity of highly functionalized cyclohexenone ethyl carboxylates 7-12 is studied against the Candida species viz., C. albicans, C. tropicalis, C. glabrata, C. parapsilosis, C. dubliniensis and C. krusei. Fluconazole is used as a standard drug. Minimum inhibitory
**Figure 1.** Synthetic route for the formation of highly functionalized cyclohexanone ethyl carboxylates.

**Figure 2.** Mechanistic pathway for the formation of title compounds 7-12.
concentration (MIC) in µg/mL values is reproduced in Table I and their pictorial representation is shown in Figure 3.

**Discussion**

**Structural Elucidation of bis Cyclohexenone Ethyl Carboxylate 7**

In order to discuss the spectral data of the synthesized compounds 7-12, compound 7 is chosen as the representative compound.

**Analysis of FT-IR Spectrum of bis Cyclohexenone Ethyl Carboxylate 7**

FT-IR spectrum of compound 7 shows two strong characteristic absorptions at 1738 and 1663 cm⁻¹ due to ester carbonyl and ketone functional groups respectively. The band at 1607 cm⁻¹ is due to the presence of C=C stretching frequency. The absorption frequency at 3052, 2980 cm⁻¹ is assigned to aromatic C-H stretching vibration and the absorption frequencies at 2924 and 2854 cm⁻¹ is assigned to aliphatic C-H stretching vibration. The observed ester carbonyl, ketone and C=C stretching vibrational bands are supporting evidence for the formation of synthesized compound 7.

**Analysis of ¹H NMR spectrum of bis Cyclohexenone Ethyl Carboxylate 7**

In the ¹H NMR spectrum of 7, a triplet observed at 0.93 ppm (J=5.2 Hz) corresponding to six protons and this signal is due to ester methyl protons at C-1. A multiplet observed at 3.95-3.87 ppm corresponding to four protons and this signal is due to ester methylene protons at C-1. Three multiplets are obtained in the range 2.99-2.95, 3.14-3.00 and 3.68-3.61 ppm and they are due to H-5a, H-5e and H-6 protons. The doublet at 4.11 ppm (J=13.6 Hz) has been assigned to H-
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Analysis of $^{13}$C NMR Spectrum of bis Cyclohexenone Ethyl Carboxylate 7
The $^{13}$C resonances at 194.27 ppm is assigned to C-2 carbonyl carbon whereas carbon resonances observed at 169.31 ppm are assigned to ester carbonyl carbons. The $^{13}$C resonances at 35.28 and 43.46 ppm are due to the C-5 and C-6 carbons respectively. The $^{13}$C resonance observed at 59.89 and 13.79 ppm are assigned to ester methylene and methyl carbons at C-1 respectively. The signal observed at 58.67 ppm is assigned to C-1 carbon, whereas the signal at 122.89 ppm is assigned to C-3 carbon. The aromatic carbons are observed in the range of 130.10-124.16 ppm. C-4 carbon resonates at 159.29 ppm. The remaining $^{13}$C signals at 140.28, 139.86, 138.00 and 137.32 are due to ipso carbons.

In vitro Anticandidal Evaluation of Highly Functionalized bis Cyclohexenone Ethyl Carboxylates 7-12
A close survey of the MIC values indicates that all the tested derivatives 7-12 exhibited a varied range (6.25-200 µg/mL) of anticandidal activity against all the tested Candida species. Compound 7, having no substitution at the phenyl rings attached to C-4 carbon of cyclohexenone moiety exhibits excellent to moderate activity against all the tested Candida species and show MIC value in the range of 6.25-200 µg/mL. Compound 7 shows four fold increases in activity (MIC value = 6.25 µg/mL) against C. dubliniensis when compared to standard drug, Fluconazole which show MIC value of 25 µg/mL. But compound 7 shows potent equal activity like that of drug Fluconazole against C. albicans and shows MIC value of 12.5 µg/mL.

Electron withdrawing functional groups like fluoro, chloro and bromo groups in compounds 8, 9 and 10 at the phenyl rings attached to C-4 carbon of cyclohexenone moiety all exert excellent anticandidal activity against all the tested Candida species. All these compounds 8, 9 and 10 exhibit MIC value in the range of 6.25-25 µg/mL against all the tested strains. Compound 8 which have fluoro functional group exhibits excellent activity against C. albicans, C. parapsilosis and C. krusei at a MIC value of 6.25 µg/mL whereas against C. tropicalis and C. glabrata it shows activity at a MIC value of 12.5 µg/mL. Two fold increase in activity is noticed by compound 8 (MIC value = 6.25 µg/mL) against C. albicans, C. parapsilosis and C. krusei than the standard drug Fluconazole (MIC value = 12.5 µg/mL). Chloro substituted compound 10 exhibits superior activities against C. glabrata and C. dubliniensis at a MIC value of 6.25 µg/mL whereas against C. albicans, C. tropicalis, C. parapsilosis and C. krusei it shows activity at a MIC value of 12.5 µg/mL. Four fold increase in activity is noticed by compound 10 (MIC value = 6.25 µg/mL) against C. glabrata and C. dubliniensis than the standard drug Fluconazole (MIC value = 25 µg/mL). Bulky bromo substituted compound reveals excellent activity against C. parapsilosis and C. krusei at a MIC value of 6.25 µg/mL whereas against C. albicans and C. dubliniensis it shows activity at a MIC value of 12.5 µg/mL. Two fold increase in activity is noticed by compound 10 (MIC value = 6.25 µg/mL) against C. parapsilosis and C. krusei than the standard drug Fluconazole (MIC value = 12.5 µg/mL). Replacement of electron withdrawing functional groups like fluoro, chloro and bromo groups in compounds 8, 9 and 10 by electron donating methoxy or methyl functional groups at the phenyl rings attached to C-4 carbon of cyclohexenone moiety for compounds 11 and 12 exert intermediate to good activity against all the tested Candida species which all show MIC in the range of 6.25-200 µg/mL. Methoxy substituted compound 11 against C. albicans and C. tropicalis show potent equal activity like that of standard drug Fluconazole and all of them show MIC value of 12.5 µg/mL. But four fold increase in anticandidal activity is noticed than drug Fluconazole by compound 11 against C. glabrata and shows MIC value of 6.25 µg/mL. Methyl substituted compound 12 shows moderate activity against all the tested Candida sp., except against C. parapsilosis which shows activity only at a higher concentration of 200 µg/mL.

Conclusions
In crunch, a series of novel highly functionalized cyclohexenone ethyl carboxylates 7-12 are designed and synthesized from bis chalcones 1-6 and their structures are elucidated by their by their physical and analytical data. This reaction may have wide applicability in building a variety of heterocycles by choosing highly functionalized cyclohexenone ethyl carboxylates 7-12 as synthon, which has three versatile functional groups i.e., ketone, olefin and ester for the synthesis of structurally diverse organic compounds. Compound 8 against C. albicans, 9,11 against C. glabrata, 8,10 against C. parapsilosis, 7,9
against C. dubliniensis, 8, 10 against C. krusei exhibited excellent antican didal activity at a MIC value of 6.25 μg/mL. Likewise compound 7, 9-11 against C. albicans, 8, 9, 11 against C. tropicalis, 8 against C. glabrata, 9 against C. parapsilosis, 10 against C. dubliniensis, 9 against C. krusei revealed superior activity at a MIC value of 12.5 μg/mL. Results of the biological activity show that electron withdrawing substituents like fluoro, chloro and bromo substituted derivatives exerted excellent antifungal activities, since electron withdrawing substituent increases the lipophilicity due to the strong electron withdrawing capability. Moreover, electron withdrawing substituents namely fluorine substitution was commonly used in contemporary medicinal chemistry to improve metabolic stability, bioavailability and protein ligand interactions. These observations may promote a further development of our research in this field. Furthermore, the observed marked antican didal activity of this group of highly functionalized cyclohexenone ethyl carboxylate derivatives may be considered as key steps for the building of novel chemical entities with comparable pharmacological profiles to that of the standard drugs.

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