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Design, synthesis and pharmacological evaluation of substituted benzeneacetic acid ester derivatives

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ABSTRACT

Following the principles of bioisosterism we envisioned to synthesize novel analogs of lead compound α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester with planned structural modifications. Compounds synthesized were characterized by spectroscopic techniques to establish their structures and evaluated for their anti-spasmodic, anti-cholinergic and antimicrobial potency. The anti-spasmodic and anti-cholinergic testing was carried out by muscle relaxation studies on isolated Wistar rat's ileum, contracted with acetylcholine and acetylcholine antagonist property on frog rectus abdominal muscle preparation respectively. The sedative potency of the compounds was evaluated following one-way ANOVA followed by Scheffe's post hoc analysis to find out the significance. The compounds were evaluated in vitro, for their antibacterial activity using acetone as a solvent and MIC was done by broth dilution method. The fungal susceptibility testing on different fungi was done by cup-diffusion method using Clotrimazole (100mcg/mL) as standard using acetone as a solvent. The compounds have shown promising results and definite structure-activity relationship could be established.

Key Words: anti-spasmodic, anti-cholinergic, anti-microbial, Wistar Rats

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INTRODUCTION

Antispasmodic drugs relieve cramps or spasms of the stomach, intestines, and bladder. [1] Anti-cholinergic agent is a substance that blocks the neurotransmitter acetylcholine in the central and the peripheral nervous system. Anti-cholinergic are administered to reduce the effects mediated by acetylcholine on acetylcholine receptors in neurons through competitive inhibition [2]. These drugs are always associated with unavoidable side effects reported in the literature like dizziness, drowsiness, lightheadedness, nausea, nervousness, blurred vision, dry mouth [3]. Therefore, the discovery of new, safer drugs represents a challenging goal for such a research area [4]. Recently, Barreiro et al. reviewed that bioisosterism is a useful strategy for the lead optimization process and molecular modification for rational drug design [5]. The present work is an extension of our ongoing efforts towards the development

and identification of new drug candidates; by the bioisotere concept. We designed some diverse analogs of lead compound, α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride.

Synthesis of lead compound involves condensation of key process intermediates; 4-diethylamino-2-butynyl acetate and α-cyclohexyl-α-hydroxybenzeneacetic acid methyl ester**.** The desired structural diversities in the basic framework of lead compound were achieved by suitable modifications in above process intermediates using respective raw materials. These modifications led us to the structurally diverse derivatives of lead compound and were characterized by structure elucidation techniques like NMR, IR and Mass spectra.
The compounds were screened for their antispasmodic and anti-cholinergic potency against Papaverine and Dicyclomine as standard drugs by muscle relaxation studies on isolated Wistar rat's ileum, contracted with acetylcholine and acetylcholine antagonist property on frog rectus abdominal muscle preparation respectively. All compounds exhibited significant antispasmodic and anti-cholinergic activity.

It is evident from the literature reports that the compounds with two benzene rings and secondary or tertiary nitrogen in its core molecule structure are expected to exhibit antimicrobial potency [6]. Different studies on search of newer antimicrobials and antibacterial have revealed that moderate to remarkable antimicrobial or antibacterial action is present in several compounds, belonging to various pharmacological categories, such as antihistamines [7-9], tranquilizers [10], antihypertensive [11], anti-psychotics [12-16] anti-spasmodic [6] and anti-inflammatory agents [6]. Such compounds, having antibacterial properties in addition to their predesignated pharmacological actions, are termed as non-antibiotics [6]. Based on this rationale present study is extended towards evaluating sedative, antibacterial and antifungal potency of our compounds.

Based on various potency levels exhibited and their planned structural diversity, a definite correlation on structure-activity could be established.

MATERIALS AND METHODS

Chemistry

Synthesis of lead molecule i.e. α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride involves simple condensation of 4-diethylamino-2-butynyl acetate and α-cyclohexyl-α-hydroxybenzeneacetic acid methyl ester **(**Scheme 1). The standard laboratory synthetic process is presented in following chapters. The diverse analogs of intermediate 4-diethylamino-2-butynyl acetate were synthesized in a two step process by synthesis of different propargyl acetate homologs in the first step and condensation with various substituted amines followed by reaction with different α-cyclohexyl-α-hydroxybenzeneacetic acid methyl ester analogs synthesized by Grignard process involving preparation of cyclohexyl magnesium bromide and in situ condensation with respective homologs of α-oxo-benzeneacetic acid methyl ester.

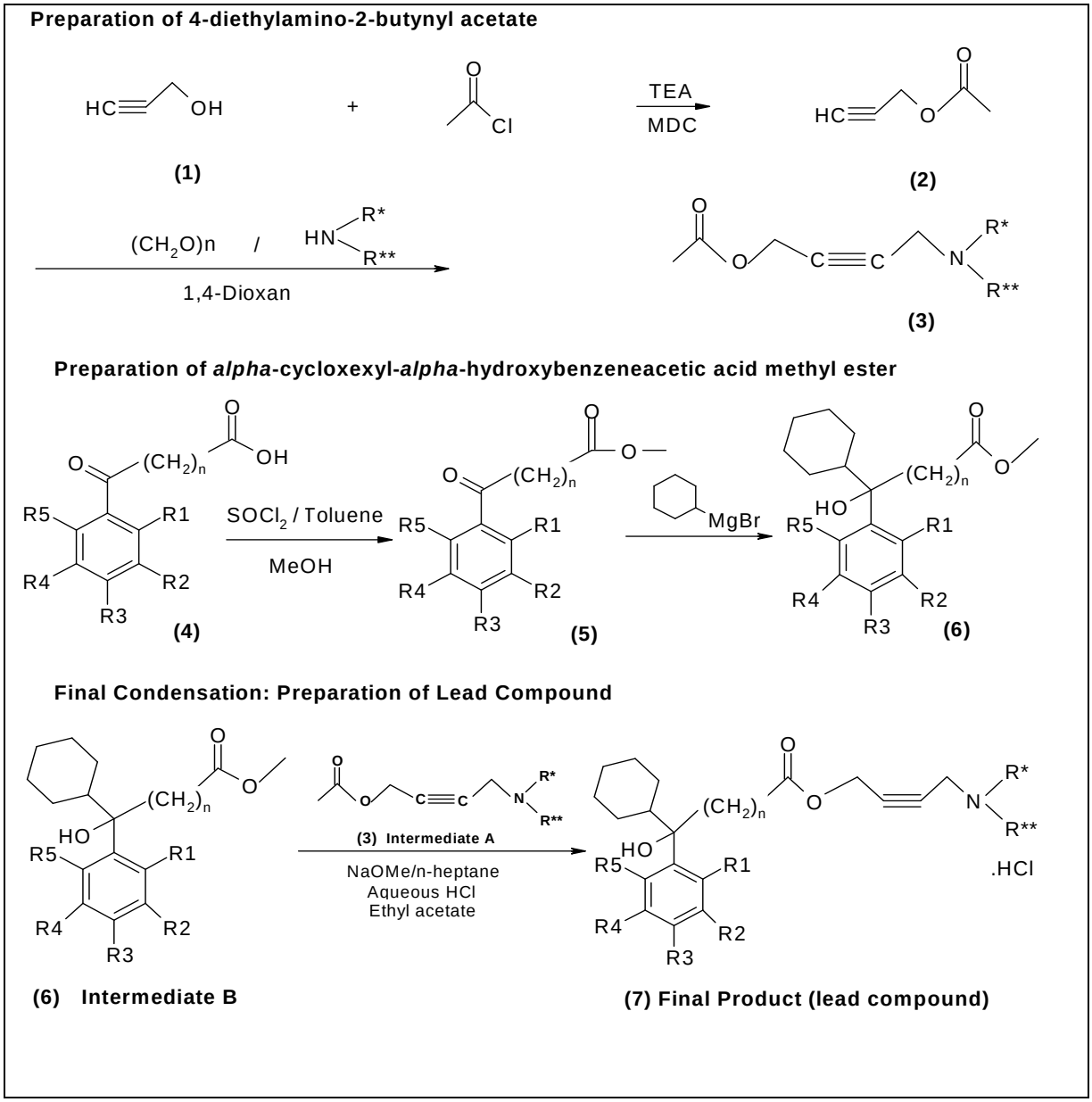
Melting points of the synthesized compounds were determined on Thomas Hoover capillary apparatus and are uncorrected. IR spectra of the synthesized compounds were acquired on Perkin Elmer FTIR Spectrum. ^{1}H NMR, spectra were acquired on Bruker 300MHz NMR spectrometer and mass spectra on Shimadzu Qp-2010 mass spectrometer. Perkin Elmer Clarus 500 GC system and Agilent 1100 HPLC system was used to monitor progress of the reaction and to evaluate purity of compounds. Chemicals and solvents used were procured from Sigma-Aldrich or from Rankem Ltd.

Synthesis of Lead Compound (7) and its analogs (7a-7j)

Standard process for preparation of propargyl acetate (2) and its analogs.

To a precooled solution (<10°C) of propargyl alcohol (200 g, 3.57 M) and triethylamine (430.5 g, 4.26 M) in dichloromethane (1.0 L), was added acetyl chloride (311.0 g, 3.96 M) maintaining temperature less than 15°C. The reaction mass was slowly warmed to 20-25°C and stirred for 0.5 h. Completion of reaction was confirmed by GC and water was added to the reaction mass. The reaction mass was then stirred for 15 min, layers settled and organic layer containing product was separated. The product was isolated by distillation after recovery of dichloromethane solvent. Product Chemical Yield: 318.5 g, 91 %.

Scheme 1: Synthetic Pathway for preparation of compounds (7a-7j)



Standard process for the preparation of 4-diethylamino-2-butynyl acetate (3) and its analogs.
To the mixture of *p*-formaldehyde (90 g, 3.0 M), diethylamine (210 g, 2.87 M) and cuprous chloride (5.0 g) in 1,4-dioxane (1.050 L) was added propargyl acetate (250 g, 2.55 M) under stirring at 30-35^{o}C. The reaction mass was then heated using an oil bath to 90-95°C and maintained for 1 h. Completion of reaction was confirmed by GC, cooled to 15^{o}C and reaction mass was filtered. On recovery of solvent 1, 4-dioxane (70 %) crude product was isolated (415.5 g, 89 %) which was purified by distillation under reduced pressure (1mmHg, 75-80^{o}C) to get pure product as brownish yellow oil. Product Chemical Yield: 373.5 g, 80.2 %.

Standard process for the preparation of α-oxo-benzeneacetic acid methyl ester (5) and its analogs.
To the solution of α-oxo-benzeneacetic acid (150 g, 1.0 M) and dimethylformamide (1.0 mL) in toluene (0.450 L) was added thionyl chloride (178.5g, 1.5 M). The solution was heated to 45-50^{o}C and continued for 1 h. The reaction mass was then subjected to distillation when toluene was recovered (~0.390 L) followed by distillation of product under reduced pressure (1 mmHg, 60^{o}C). The isolated acid chloride product (155 g, 92 %) was poured in methanol (0.300 L) and methyl ester of was isolated by distillation under reduced pressure (1 mmHg, 98^{o}C) after initial recovery of methanol. Product Chemical Yield: 143.5 g, 87 %.

Standard process for the preparation of α-cyclohexyl-α-hydroxybenzeneacetic acid methyl ester (6) and its analogs.
Magnesium turnings (15.8 g) and iodine (0.02 g) were added to tetrahydrofuran (73.0 g) under nitrogen atmosphere and the mixture was stirred at about 25^{o}C for 0.5 h. Initially cycloxexyl bromide (4.1 g, 0.025 M) was added drop wise followed by tetrahydrofuran (292.0 g) was added and remaining cyclohexyl bromide (86 g, 0.53 M) was added drop wise at 60-70^{o}C. The mixture was stirred at 60-70^{o}C and reaction completion was confirmed by GC. The mixture was cooled to 20-30^{o}C and this Grignard solution was added drop wise to a mixture of α-oxo-benzeneacetic acid methyl ester (82.1 g, 0.5 M) and tetrahydrofurane (82.0 ml) at 5-15^{o}C. The reaction mass further stirred for 1 hour and reaction completion was confirmed by HPLC for absence of α-oxo-benzeneacetic acid methyl ester. Tetrahydrofurane was evaporated under reduced pressure at 65-80^{o}C and toluene (0.150 L) was added. This mixture was then added drop wise at < 35^{o}C to 7N hydrochloric acid (0.215 L) and allowed to stir for 0.5 h. The layers were settled, organic layer containing product was separated, cooled to 0-5^{o}C and product isolated by filtration. The product was purified by crystallization from ethyl acetate. Product Chemical Yield: 80.7 gm, 65 %.

Standard process for the preparation of α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride (7) and its analogs (7a-7j).
To the solution of α-cyclohexyl-α-hydroxybenzeneacetic acid methyl ester (160 g, 0.65 M) in heptane (0.800 L) was added solution of 4-diethylamino-2-butynyl acetate (130 g, 0.71 M) in heptane (0.800 L) followed by sodium methoxide (8.0 g) at 25-30^{o}C. Heat the reaction mass to 90-95^{o}C when distillation of methyl acetate and n-heptane mixture starts. This is continued further for 3 hours. Reaction completion was confirmed by HPLC and added *n*-heptane (0.400 L) followed by water (0.400 L). The reaction mass stirred for 10 min, layers settled and organic layer containing product was separated and washed further with water (0.100 L). The organic layer was then extracted with 10 % HCl solution (4 x 0.100 L) and combined acidic aqueous layer was subjected to chilling (0 ± 5^{o}C) when product is precipitated as hydrochloride salt. The product slurry stirred for 1 hour at same temperature, filtered. The crude product is purified by crystallization from ethyl acetate to isolate pure product as off white crystalline powder. Product Chemical Yield 218.5 gm, 86.4 %

Pharmacology

All the pharmacological testing was performed following prescribed protocols on approval of Institutional animal ethics committee constituted for the purpose.

Test Protocol: Anti-spasmodic activity

The work includes muscle relaxation studies on isolated Wistar rat's ileum, contracted with acetylcholine [17, 18]. Wistar rats (n=6) of both sexes (300-500 g) were used for the study. The animals were sacrificed by using ether as anesthetic agent, until death. The ileum was removed immediately and placed in aerated Krebs saline at 37^0C. The saline contained (in mM): NaCl, 120.7; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 15.5; and glucose, 11.5 at pH 7.3.For tension recording 2 cm ileal strips were mounted in a 10 ml organ bath and were connected to physiograph (Polyrite, Recorders and Medicare systems) through force tension transducer. In the concentration range 10 µM - 150 µM, papaverine, the lead compound and all its derivatives caused relaxation of spontaneous rhythmic contractions of both Wistar rat ileum accompanied by a fall in resting tension.

The inhibition contraction was measured simply as percentage reduction in the height of spontaneous contractions. The percentage relaxation of all derivatives is compared in Table 4. The results are expressed as mean ± S.E. The statistical significance was treated with the paired student`s t-test. P value < 0.01 was considered to be significant. Increase or decrease in tension was expressed as percent of maximal response to Papaverine and lead compound. The results are reported in Table 4.

Test Protocol: Anti-cholinergic activity

The compounds were assayed for their acetylcholine antagonistic property on frog rectus abdominis muscle preparation. The results were compared with "dicyclomine' which is used as reference standards and also with their parent compound.

Solution of the drugs: The weighed quantity of each drug was dissolved in a minimal known amount of warm ethanol and the volume was made with warm distilled water so that the solution in no case contained more than 5% ethanol. The solution was cooled to room temperature before use and the studies were conducted with freshly prepared solution of each drug. Since the control studies with 5% ethanol alone did not exhibit any anti-acetylcholine activity, only the activity of the compounds is reported in Table 5.

Rectus abdominis muscle of frog was mounted in an organ bath as described by [19-20]. Acetylcholine induced spasm was recorded with 0.1 mg/ml concentration. After the controls were obtained, the test drug was added and was allowed to remain in contact with the tissue for 3 min. Again the same amount of acetylcholine was added and the tracing was recorded. The percentage inhibition was calculated and compared with that of the parent drug. The preparation was tested at intervals of 5 min, for 30 min, with acetylcholine to observe the recovery period.

Test Protocol: Sedative activity

Male Wister rats weighing in the range of 200-400 gm were selected from an inbred strain colony. They were maintained at constant temperature and relative humidity. Acute toxicity was done by following the proposed test method from literature [21]. Thiopental sodium (Thiosol ®) was used as standard drug, 2% CMC suspension was used as control and suspensions of the synthesized compounds were used. The mean sleeping times of compounds were compared with the standard, using one-way ANOVA followed by Scheffe's post hoc analysis to find out the significance. The results are presented in Table 6.

Test Protocol: Antibacterial activity

Antibacterial activity of the synthesized compounds (7, 7a-7j) was evaluated using acetone as a solvent and MIC was done by broth dilution method [22]. The bacterial stains used for the assay include, against

gram-positive organisms B.Subtilis (MTCC 441), B.sphaericus (MTCC 511) & S.aureus (MTCC 96) and Gram-negative organisms P.aeruginosa (MTCC 741) K.aerogenes (MTCC 39) & C.violaceum (MTCC 2656) at 100µg/ml concentration. Standard antibacterial drugs were also screened under similar conditions for comparison. Penicillin (100mcg/mL) from stock solution of 1000mg/mL was used as standard for B.Subtilis, B.sphaericus & S.aureus and Gentamycin (100mcg/mL) from stock solution of 1000mg/mL was used as a standard for other organisms. The results are presented in Table 7.

Test Protocol: Antifungal activity

The antifungal activity of compounds (7,7a-7j) was evaluated against A. niger (MTCC 282), C. tropicum (MTCC 2821), R.oryzae (MTCC 262), F.moliliforme (MTCC 1848) and C. lunata (MTCC 2030) using Acetone as a solvent by cup diffusion method [22] at 100mcg/mL concentrations. The fungal susceptibility testing was done by cup-diffusion method using Clotrimazole (100mcg/mL) from stock solution of 1000mg/mL as standard. The results are presented in Table 8. The zone of inhibition was measured after 24 hr of incubation at 37^{o}C. The zone of inhibition developed if any, was then accurately measured and recorded.

RESULTS AND DISCUSSION

Chemistry

The structurally diverse derivatives of lead compound α-cyclohexyl-α-hydroxy-benzeneacetic acid-4-(diethylamino)-2-butynyl ester hydrochloride as presented in Table 1. The chemical yields of synthesized compounds were in the range of 60-86 % and purity levels were in expected range of not less than ≥ 98 %.

Table 1: Analogs of Lead Compound (7-7j)

Product	R1	R2	R3	R4	R5	n	R*	R**	% Yield
7	H	H	H	H	H	0	$-C_2H_5$	$-C_2H_5$	86.4 %
7a	H	H	H	H	H	2	$-C_2H_5$	$-C_2H_5$	62.3 %
7b	H	H	H	H	H	3	$-C_2H_5$	$-C_2H_5$	71.9 %
7c	H	CH_3	H	H	H	2	$-C_2H_5$	$-C_2H_5$	76.8 %
7d	CH_3	CH_3	H	H	H	2	$-C_2H_5$	$-C_2H_5$	84.3 %
7e	H	H	H	H	H	0	$-CH(CH_3)_2$	$-CH(CH_3)_2$	79.5 %
7f	H	H	H	H	H	0	$-(CH_2)_3-CH_3$	$-(CH_2)_3-CH_3$	80.5 %
7g	H	H	H	H	H	2	$-CH(CH_3)_2$	$-CH(CH_3)_2$	69.4 %
7h	H	H	H	H	H	3	$-CH(CH_3)_2$	$-CH(CH_3)_2$	65.4 %
7i	H	H	H	H	H	2	$-CH_3$	$-CH_3$	71.5 %
7j	H	H	H	H	H	3	$-CH_3$	$-CH_3$	80.3 %

Characterization data of the synthesized compounds by M. P., MS (M/Z), FTIR and NMR spectroscopy are presented in Table 2 and Table 3 respectively.

Table 2: M. P., Mass (M/Z) and FTIR spectral data of synthesized compound (7-7j)

Comp.	M.P. ^{0}C	Mass (M/Z)	IR Data
7a	143-144.5	386 (M+H)	-OH stretching (3512 cm^{-1}), aromatic C-H stretching (3095 cm^{-1}), aliphatic C-H stretching (2928 cm^{-1}),-C≡C- stretching (2071 cm^{-1}), -C=O stretching (1743 cm^{-1}), symmetric -C=O stretching (1467 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1246-1171 cm^{-1}), monosubstituted benzene stretching (two bands at770 cm^{-1} and 698 cm^{-1})
7b	161-162	400 (M+H)	-OH stretching (3318 cm^{-1}), aromatic C-H stretching (3096 cm^{-1}), aliphatic C-H stretching (2992-2845 cm^{-1}), -C≡C- stretching (2069 cm^{-1}), -C=O stretching (1756 cm^{-1}), asymmetrical -C-O-C and --C-O stretching

			(1246-1133 cm^{-1}), monosubstituted benzene stretching (two bands at 770 cm^{-1} and 695 cm^{-1})
7c	157-158.5	400 (M+H)	-OH stretching (3514 cm^{-1}), -NH stretching (3317 cm^{-1}), aromatic C-H stretching (3096 cm^{-1}), aliphatic C-H stretching (2992-2845 cm^{-1}), -C≡C- stretching (2070 cm^{-1}), -C=O stretching (1745 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1273-1161 cm^{-1}), monosubstituted benzene stretching (two bands at (770 cm^{-1} and 695 cm^{-1}), mata substituted benzene stretching (two bands at704 cm^{-1} and 781 cm^{-1})
7d	177-178.6	413 (M)	-OH stretching (3510 cm^{-1}), aromatic C-H stretching (3095 cm^{-1}), aliphatic C-H stretching (2991-2859 cm^{-1}), -C≡C- stretching (2071 cm^{-1}), -C=O stretching (1744 cm^{-1}), symmetric -C=O stretching (1467 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1246-1162 cm^{-1}), monosubstituted benzene stretching (two bands at 770 cm^{-1} and 697 cm^{-1}),mata disubstituted benzene stretching(bands at 697 $cm^{-1}$and 809cm^{-1})
7e	121-123	386 (M+H)	-OH stretching (3316 cm^{-1}), aromatic C-H stretching (3096 cm^{-1}), aliphatic C-H stretching (2992-2861 cm^{-1}), -C≡C- stretching (2069 cm^{-1}), -C=O stretching (1745 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1274-1161 cm^{-1}), monosubstituted benzene stretching (two bands at770 cm^{-1} and 695 cm^{-1})
7f	183-184.7	414 (M+H)	-OH stretching (3510 cm^{-1}), aromatic C-H stretching (3095 cm^{-1}), aliphatic C-H stretching (2928-2860 cm^{-1}), -C≡C- stretching (2069 cm^{-1}), -C=O stretching (1743 cm^{-1}), symmetric -C=O stretching (1464 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1246-1141 cm^{-1}), monosubstituted benzene stretching (two bands at 771 cm^{-1} and 703 cm^{-1})
7g	191-193	414 (M+H)	-OH stretching (3316 cm^{-1}), aromatic C-H stretching (3096 cm^{-1}), aliphatic C-H stretching (2992-2861cm^{-1}), -C≡C- stretching (2142 cm^{-1}), -C=O stretching (1746 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1274-1161 cm^{-1}), monosubstituted benzene stretching (two bands at 770 cm^{-1} and 695 cm^{-1})
7h	173-175	427 (M)	-OH stretching (3316 cm^{-1}), aromatic C-H stretching (3096 cm^{-1}), aliphatic C-H stretching (2992-2861cm^{-1}), -C≡C- stretching (2070 cm^{-1}), -C=O stretching (1744 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1274-1161 cm^{-1}), monosubstituted benzene stretching (two bands at 770 cm^{-1} and 695 cm^{-1})
7i	163-165.3	358 (M+H)	-OH stretching (3512 cm^{-1}), aromatic C-H stretching (3095 cm^{-1}), aliphatic C-H stretching (2928 cm^{-1}),-C≡C- stretching (2071 cm^{-1}), -C=O stretching (1743 cm^{-1}), symmetric -C=O stretching (1467 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1246-1171 cm^{-1}), monosubstituted benzene stretching (two bands at770 cm^{-1} and 698 cm^{-1})
7j	153-153.4	372 (M+H)	- OH stretching (3317 cm^{-1}), aromatic C-H stretching (3095 cm^{-1}), aliphatic C-H stretching (2992-2860cm^{-1}), -C≡C- stretching (2069 cm^{-1}), -C=O stretching (1744 cm^{-1}), asymmetrical -C-O-C and --C-O stretching (1274-1161 cm^{-1}), monosubstituted benzene stretching (two bands at 770 cm^{-1} and 695 cm^{-1})

Table 3: ^{1}H NMR spectral data of synthesized compound (7-7j)

Comp.	^{1}H NMR (ð)
7	Chemical Shift ð, 1.12-0.90 ppm (m, 6H, -CH_3), 1.41-1.37 ppm (m, 6H, -CH of cyclohexyl),1.57-1.48 (m, 4H, -CH of cyclohexyl),1.75-1.71 (m, 1H, -CH of cyclohexyl), 3.05-2.51 (q, 4H, -CH_2), 3.36 (s, 2H, -CH_2), 4.88 (s, 2H, -CH_2), 5.78 (s, 1H, -OH), 7.38-7.24 (m, 3H, CH-Ar), 7.57-7.55 (m, 2H, CH-Ar)
7a	Chemical Shift ð, 0.98-1.20 ppm (t, 6H, -CH_3), 1.32-1.43 (m, 2H, -CH of cyclohexyl), 1.44-1.52 (m, 8H, -CH of cycloxexyl), 2.10-2.00 (m, 1H, -CH of cyclohexyl), 2.18-2.12 (t, 2H, -CH_2), 2.31-2.25 (t, 2H, -CH_2), 2.60-2.48 (q, 4H, -CH_2), 3.05 (s, 2H, -CH_2), 4.77 (s, 2H, -CH_2), 5.52 (bs, 1H, -OH), 7.35-7.40 (d,

	3H, CH-Ar), 7.73-7.52 (m, 2H, CH-Ar)
7b	Chemical Shift ð,1.24-1.10 ppm (t, 6H, -CH_3), 1.36-1.32 (m, 2H, -CH of cyclohexyl), 1.56-1.45 (m, 8H, -CH of cycloxexyl), 1.68-1.73 (m, 4H, -CH_2), 2.02-2.00 (m, 1H, -CH of cyclohexyl), 2.50 (s, 2H, -CH_2), 3.02-2.80 (q, 4H, -CH_2), 4.15 (s, 2H, -CH_2), 4.90 (s, 2H, -CH_2), 5.89 (s, 1H, -OH), 7.25-7.34 (d, 3H, CH-Ar), 7.65-7.55 (m, 2H, CH-Ar)
7c	Chemical Shift ð,1.24-1.19 ppm (t, 6H, -CH_3),1.40-1.36 ppm (m, 2H, -CH of cyclohexyl), 1.56-1.47 (m, 6H, -CH of cyclohexyl),1.66-1.80 ppm (m, 2H, -CH of cyclohexyl), 2.00-1.90 (m, 1H, -CH of cyclohexyl), 2.32-2.31 (t, 2H, -CH_2), 2.55-2.45 (t, 2H, -CH_2), 2.70 (s, 3H, -CH_3-Ar), 2.90-2.75 (m, 4H, -CH_2), 4.10 (s, 2H, -CH_2), 4.87 (s, 2H, -CH_2), 5.90 (s, 1H, -OH), 7.37-7.23 (m, 3H, CH-Ar), 7.56-7.54 (t, 1H, CH-Ar)
7d	Chemical Shift ð, 0.95-1.10 ppm (t, 6H, -CH_3), 1.36-1.23 ppm (m, 2H, -CH of cyclohexyl), 1.36-1.51 (m, 8H, -CH of cyclohexyl), 1.90-1.95 (m, 1H, -CH of cycloxexyl), 2.17-2.10 (t, 2H, -CH_2), 1.19-2.27 (t, 2H, -CH_2), 2.39 (s, 6H, -CH_3-Ar), 2.45-2.57 (q, 4H, -CH_2), 3.10 (s, 2H, -CH_2), 4.73 (s, 2H, -CH_2), 5.85 (bs, 1H, -OH), 6.83-6.95 (m, 3H, -CH-Ar),
7e	Chemical Shift ð, 1.05 (s, 12H, -CH_3), 1.19 (s, 2H, -CH of cycloxexyl), 1.40-1.61 (m, 8H, -CH of cyclohexyl), 2.35-2.12 (m, 1H, -CH of cycloxexyl), 3.00-2.91 (m, 2H, -CH), 3.15 (s, 2H, -CH_2), 4.71 (s, 2H, -CH_2), 6.75 (bs, 1H, -OH), 7.40-7.18 (bd, 5H, CH-Ar)
7f	Chemical Shift ð, 0.96 ppm (s, 6H, -CH_3), 1.25 (s, 2H, -CH of cycloxexyl), 1.36-1.70 (m, 16H, -CH of cyclohexyl, CH_2), 2.38-2.21 (m, 4H, -CH_2), 2.55-2.38 (m, 1H, -CH of cyclohexyl), 3.05 (s, 2H, -CH_2), 4.73 (s, 2H, -CH_2), 6.81 (bs 1H, -OH), 7.30-7.21 (m, 4H, CH-Ar), 7.57-7.22 (m, 1H, CH-Ar)
7g	Chemical Shift ð,1.08 (s, 12H, -CH_3),1.24-1.22 (m, 2H, -CH of cycloxexyl),1.31-1.49 (m, 8H, -CH of cyclohexyl),1.90 (s, 1H, -CH of cycloxexyl), 2.13-2.10 (t, 2H, -CH_2), 2.45-2.30 (t, 2H, -CH_2), 3.01-2.90 (m, 2H, -CH), 3.10 (s, 2H, -CH_2), 4.72 (s, 2H, -CH_2), 5.90 (s, 1H, -OH), 7.40-7.37 (m, 3H, CH-Ar), 7.62-7.57 (t, 2H, CH-Ar)
7h	Chemical Shift ð, 1.15-1.00 (d, 12H, -CH_3), 1.16-1.26 (m, 2H, -CH of cycloxexyl), 1.42-1.58 (m, 2H, -CH of cycloxexyl), 1.60-1.89 (m, 7H, -CH_2, -CH of cyclohexyl), 2.31-2.25 (t, 2H, -CH_2), 2.97 (s, 2H, -CH), 3.10 (s, 2H, -CH_2), 4.75 (s, 2H,-CH_2), 6.91 (bs, 1H, -OH), 7.50-7.25 (m, 3H, CH-Ar), 7.72-7.51 (m, 2H, CH-Ar)
7i	Chemical Shift ð, 1.27-0.90 ppm (m, 2H, -CH of cyclohexyl), 1.45-1.27 (m, 8H, -CH of cycloxexyl),1.90-1.80 (m, 1H, -CH of cyclohexyl), 2.00 (s, 6H, -CH_3), 2.30-2.12 (m, 2H, -CH_2), 2.45-2.40 (t, 2H, --CH_2), 3.05 (s, 2H, -CH_2), 4.77 (s, 2H, -CH_2), 5.50 (s, 1H, -OH), 7.29-7.26 (m, 2H, CH-Ar), 7.38-7.29 (m, 2H, -CH-Ar), 7.67-7.65 (m, 1H, CH-Ar)
7j	Chemical Shift ð, 1.24-1.10 (m, 3H, -CH of cyclohexyl), 1.50-1.25 (m, 7H, -CH of cycloxexyl), 1.54-1.75 (m, 4H, -CH_2), 1.93-1.82 (m, 1H, -CH of cyclohexyl), 2.30-2.20 (t, 2H, -CH_2), 2.27 ppm (s, 6H, -CH_3), 3.05 (s, 2H, -CH_2), 4.71 (s, 2H, -CH_2), 6.81 (bs, 1H, -OH), 7.41-7.22 (m, 3H, -CH-Ar), 7.61-7.72 (m, 2H, -CH-Ar)

Anti-spasmodic activity

Table 4: Test results of anti-spasmodic activity of the synthesized compounds

Compound	% Relaxation at various concentrations (µM, 500µL)			
	150	**100**	**50**	**10**
Papaverine	68.46 ± 3.49	43.90 ± 2.72	26.70 ± 0.32	7.80 ± 0.27
7	85.46 ± 3.25	65.29 ± 2.15	44.86 ± 3.90	19.89 ± 1.13
7a	80.35 ± 1.35	61.12 ± 1.55	39.36 ± 2.33	15.67 ± 0.45
7b	79.26 ± 2.05	60.44 ± 1.87	38.76 ± 2.42	14.89 ± 1.16
7c	77.36 ± 1.75	58.35 ± 2.15	38.58 ± 2.32	14.79 ± 0.23
7d	75.11 ± 2.75	56.15 ± 1.33	37.27 ± 1.78	13.51 ± 1.19
7e	65.73 ± 1.89	40.52 ± 2.75	24.83 ± 0.64	6.51 ± 0.24
7f	67.59 ± 1.39	42.12 ± 1.13	25.10 ± 0.23	6.95 ± 0.84
7g	60.29 ± 2.39	52.92 ± 2.12	31.70 ± 0.58	10.71 ± 0.34
7h	59.18 ± 1.29	50.22 ± 1.14	28.40 ± 1.23	09.41 ± 1.21
7i	86.51 ± 2.19	65.12 ± 1.32	45.70 ± 0.18	20.36 ± 0.29
7j	86.39 ± 1.57	66.22 ± 1.57	45.39 ± 0.38	21.71 ± 0.54

Pharmacology

Anti-spasmodic activity testing was performed using healthy Wistar rats of average weight 300-500 g of either sex. The results compared with lead compound and Papaverine as standard drug. Compounds were tested for their acetylcholine antagonistic property on frog rectus abdominal muscle preparation method and test results were compared with lead compound and Dicyclomine as reference standard. Samples of reference standards were obtained from "Biocon Laboratories", Bangalore, India.

Structure-activity relationship

The results of antispasmodic activity described in Table 4, indicated that compounds "7a" and "7b" where an ethyl and propyl linker is incorporated prior to carbonyl carbon, (n = 2 and 3 respectively) have exhibited slightly reduced antispasmodic activity compare to the lead compound "7". The activity is further reduced with placement of electron donating alkyl groups on the phenyl ring as in the compound "7c" and "7d". While potency of these compounds is lowered compare to the lead molecule "7", it is found higher than reference standard Papaverine.

Replacing diethyl amine with diisopropyl amine and dibutyl amine in the synthesis of intermediate, 4-diethylamino-2-butynyl acetate provided respective modification in compounds "7e" and "7f". Introduction of these groups on amino nitrogen showed no benefits over antispasmodic potency. These compounds exhibited extremely low antispasmodic activity compare to lead molecule but surprisingly equipotent to reference standard Papaverine. The compounds "7g' and "7h" with two isopropyl groups on amino nitrogen and respective linker (n=2 and 3) incorporated have also shown diminished activity compare to lead compound and reference standard Papaverine. Results indicated that replacing ethyl groups on amino nitrogen with higher alkanes reduce the antispasmodic potency and incorporation of linker (n=2 or 3) provides no benefit either. These four compounds "7e", "7f", "7g" and "7h" can be claimed inactive and are not recommended for further studies. It's interesting that compounds "7i" and "7j" where two ethyl are replaced with two methyl on amino nitrogen and with respective linker (n= 2 and 3) incorporated have exhibited excellent antispasmodic activity higher than lead compound "7" and can be claimed as promising candidates. The results are in line with our earlier conclusion on compounds"7e", "7f", "7g' and "7h" that replacing two ethyl groups with higher alkanes diminish the potency while with lower alkanes led compounds potent candidates.

Anticholinergic potency

Structure-activity relationship

Results of anti-cholinergic activity presented in Table 5, indicated that the compound "7a" where ethyl linker is incorporated prior to carbonyl carbon (n = 2) has shown enhanced anti-cholinergic activity compared to lead compound and reference compound dicyclomine and can be claimed as promising candidate. Surprisingly the anti-cholinergic potency is reduced with propyl linker (n = 3) incorporation as in compound "7b". Similar reduction in activity is observed in compound "7c" with aromatic proton substitution by electron donating alkyl group on the phenyl ring (C-R2). The potency is further decreased in compound "7d" with two alkyl group substitutions on C-R1 and C-R2. But interestingly potency of all these compounds was well comparable with reference standard Dicyclomine. In compound "7e" and "7f", two ethyl groups on amino nitrogen are replaced with two isopropyl and butyl groups respectively. Respective modifications in these compounds have diminished their anti-cholinergic potency against the lead molecule and reference standard dicyclomine. They can be claimed as inactive and are not recommended for further work. Interesting fact is potency is improved when similar substitutions on amino nitrogen with ethyl or propyl linker incorporation (n=2 and 3) as in compound "7g' and "7h". These compounds have exhibited anti-cholinergic potency equivalent to reference standard Dicyclomine while slightly lower than lead compound.

In the series of novel analogs, an excellent activity results were obtained in compounds "7i" and "7j" where two ethyl groups on amino nitrogen are replaced with two methyl groups along with respective linker introduced prior to carbonyl carbon (n = 2 and 3 respectively). Both these compounds have exhibited anti-cholinergic activity much higher than the lead compound and reference standard Dicyclomine and can be claimed as promising candidates. The recovery period of these compounds was observed to be also higher compare to standards and other analogs. Results of anti-cholinergic activity indicated similar facts of structure-activity relationship as stated earlier for antispasmodic potency. The structural modifications like different substitutions on amino nitrogen and incorporation of appropriate linker prior to carbonyl carbon has pronounced effect on anti-cholinergic potency. In conclusion most of the newly designed analogs of lead molecule with various structural modifications have exhibited higher or equivalent anti-cholinergic activity against reference standard Dicyclomine and lead molecule.

Table 5: Test results of anti-cholinergic activity of the synthesized compounds

Compound	Conc. µg/l 0 ml	% Inhibition	Compound	Conc. µg/l 0 ml	% Inhibition
Dicyclomine (Reff. Std.)	1	31.2 (10)	7e	1	25.2 (05)
	2	41.1 (10)		2	36.5 (05)
	10	52.9 (30)		10	47.7 (10)
7 (Lead Comp.)	1	34.5 (10)	7f	1	22.2 (05)
	2	43.6 (10)		2	32.5 (05)
	10	54.7 (30)		10	43.7 (10)
7a	1	35.2 (10)	7g	1	32.2 (10)
	2	46.5 (10)		2	43.7 (10)
	10	57.7 (>30)		10	53.4 (30)
7b	1	30.2 (10)	7h	1	30.2 (10)
	2	39.8 (10)		2	39.8 (10)
	10	50.8 (30)		10	50.8 (30)
7c	1	31.9 (10)	7i	1	39.1 (15)
	2	41.5 (10)		2	64.5 (20)
	10	52.7 (>30)		10	85.7 (>50)
7d	1	30.2 (10)	7j	1	41.9 (15)
	2	38.5 (10)		2	70.5 (30)
	10	50.7 (30)		10	94.7 (>50)

Note:

- The values in parenthesis denote recovery period in minutes.

Sedative activity

The four compounds viz. "7a", "7b", "7g", "7h"; with n=2 or 3 were found to be statistically significant against standard Thiopental sodium at $P<0.05$ by applying Scheffe's Post Hoc method at 100 mg/kg (Table 6). The lead compound "7" and its analogs "7e" and "7f" were not significant where n=0. This indicated structural effect of presence of linker (n=2 or 3) prior to carbonyl carbon in the molecular framework. Results obtained for compound "7i" and "7j" are contradicting this fact and can be correlated to replacement of two ethyl groups by lower alkyl. Also the compounds "7c" and "7d" with methyl and dimethyl substitution on phenyl ring were not significant in-spite of presence of ethyl linker where n=2 which is correlated to substitutions on phenyl ring.

Table 6: Sedative Activity of the Synthesized Compounds (7, 7-7j)

Compound	Mean Sleeping Time min ± S.E.	Compound	Mean Sleeping Time min ± S.E.
Control	-	7d	13.26 ± 0.65
Standard Thiopental sodium	13.16 ± 1.66	7e	12.25 ± 0.37
		7f	12.05 ± 0.65
7	11.36 ± 0.76	7g	21.10 ± 0.66
7a	22.10 ± 0.55	7h	21.53 ± 0.57
7b	21.33 ± 0.37	7i	11.45 ± 0.61
7c	10.36 ± 0.76	7j	13.10 ± 0.43

Antibacterial Activity

From Table 7, it is clearly evident that the compounds are active against the bacterial stains. Among eleven compounds (7-7j), the compounds "7", "7i" and "7j" has activity comparable to that of reference standard Penicillin.

Table 7: Test results of Antibacterial Activity of the Compounds (7-7j)

Test Comp.	Microorganisms					
	Gram-positive			Gram-negative		
	B. subtilis	B. sphaericus	S. aureus	P. aeruginosa	K. aerogenes	C. violaceum
Penicillin	28	26	22	20	15	15
7	25	25	15	18	12	13
7a	14	15	10	10	09	07
7b	16	14	06	06	04	07
7c	19	17	11	11	09	08
7d	20	16	10	12	10	08
7e	13	12	07	05	04	05
7f	12	13	06	07	05	04
7g	11	11	05	08	05	07
7h	13	17	04	09	08	09
7i	26	18	10	12	12	09
7j	24	22	14	16	10	11

The compounds "7a" and "7b" exhibited reduced antibacterial potential than the lead compound "7", where respective linker is introduced in the structural framework prior to carbonyl carbon with n=2 or 3. The activity is slightly enhanced with introduction of methyl and dimethyl group on phenyl ring as in compounds "7c" and "7d" with n=2. Similarly reduced potency was shown by compounds 7g and 7h where diethyl amine is replaced with di-isopropyl amine. Similar results were observed in compounds "7e" and "7f" where two ethyl groups are replaced with isopropyl and butyl respectively which has shown extremely low antibacterial potential. The data indicated that in compounds "7i" and "7j" with methyl replacements against ethyl on amino nitrogen and n= 2 and 3 respectively has shown promising antibacterial activity which is correlated to presence of lower alkyl on amino nitrogen.

Antifungal Activity

Table 8: Test results of Antifungal Activity of compounds (7-7j)

Compound	Zone of inhibition in mm				
	A. niger	**C. tropicum**	**R. oryzae**	**F. moniliforme**	**C. lunata**
Clotrimazole (Reference Standard)	26	29	23	27	28
7	24	26	21	24	25
7a	19	18	19	17	17
7b	18	16	19	16	18
7c	17	17	18	16	17
7d	17	19	17	16	15
7e	12	13	12	12	10
7f	13	12	15	11	10
7g	10	09	10	08	09
7h	10	07	09	06	08
7i	23	26	23	22	27
7j	25	25	22	24	21

None of the compounds tested have exhibited MIC more than that of the reference standard Penicillin. The data presented in Table 8 indicated that the compounds “7a”, “7b”, “7c” and “7d” have shown moderate activity (<20), whereas compounds “7”, “7i” and “7j” exhibited an excellent antifungal activity (21-27) close to reference standard Clotrimazole (23-29) and can be exploited for formulation of fungicide. The compounds “7e” and “7f” with respective structural substitutions have shown reduced antifungal activity (≤13) and similar results are observed for compounds “7g” and “7h” that can be claimed as inactive (<15).

It is evident from the test results that incorporating linker prior to carbonyl carbon and replacing two ethyl groups on nitrogen with higher alkanes in the structural framework of lead compound, led to diminished antifungal potential of respective compounds except in compounds “7i” and “7j” with lower alkyl substitution on amino nitrogen that has given promising potency. Similar results are observed during antibacterial testing.

In conclusion, the tested novel compounds have moderate to excellent activity towards bacteria and fungi stains under investigation. The lead compound “7” and analogs “7i” and “7j” can be exploited for evaluating their formulation potential as bactericidal and fungicide.

CONCLUSION

The compound “7a” has exhibited excellent anti-cholinergic activity but no promising results of antispasmodic potency. Within the limits of our study described in this paper, compounds “7i” and “7j” are claimed as most potent and promising candidates with desired anti-spasmodic and anti-cholinergic potency and are recommended.

In evaluation of sedative activity compounds “7a”, “7b”, “7g” and “7h” have demonstrated significant sedative activity and are recommended for further detailed evaluation. Testing of antibacterial activity indicated that the compounds, “7”, “7i” and “7j” showed promising results and can be evaluated further for next phase studies. In evaluation of antifungal activity the compounds “7”, “7i” and “7j” were promising candidates and can be further exploited for formulations.

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