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Synthesis and evaluation of novelquinuclidinonederivatives as potential anti-proliferative agents

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ABSTRACT

In this study a new series of substituted (Z)-4-(3-oxoquinuclidin-2-ylidene) benzamide and substituted (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate has been designed and synthesised as potential anti-cancer agents. These set of compounds were prepared by using common intermediate (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoic acid. They were well characterized by various spectroscopic techniques as well as crystallographic study and screened for anti-cancer activity. Cell viability assay using MTT was performed on A549 & L132 cell lines and IC_{50} values were determined. Analogues **4c** and **5e** exhibited most potent anti-cancer activity among all the analogues synthesized in this present study. Haemolytic assay using normal human erythrocyte was performed to study the blood compatibility of the compounds. Acridine orange/ethidium bromide (AO/EB) staining also showed cell death. To get better insight into the mechanism of cell death DAPI (4',6-diamido-2-phenylindol nuclear staining) and DNA fragmentation studies were performed. A Structure Activity Relationship (SAR) was explored to facilitate further development of this new class of compounds.

Keywords: 3-quinuclidinone hydrochloride, anticancer activity, MTT assay, DNA ladder assay,Hemolytic assay.

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1. Introduction

Cancer, a serious health problem, is one of the main causes of mortality in the developing as well as developed countries.[1]Different theories have been proposed for the cause of cancer and several strategies have been formulated and examined for combating the disease. Survival rates for five year of some cancers have significantly improved in the past two decades while those of other cancers, such as lung cancer and pancreatic remain low.[2]The major form of cancer treatment is Chemotherapy, alone or combined with radiation.However, the majority of cancers developed resistant to chemotherapy during treatment. As a result, the design and discovery of non-traditional, efficient and safe classes of chemical agents are the prime targets in contemporary medicinal chemistry.[3]

The molecule 3-quinuclidinone hydrochloride possesses variety of biological spectrum and is a part of many existing drugs such as azasetron, palonosetron, solifenacin, cevimeline, quinupramine (**Fig.1**). Literaturesurvey revealed that several derivatives of quinuclidines have been reported to show wide range of biological activitysuch as Alzheimer's disease[4]chronic obstructive pulmonary disease,[5]Antihistamine-Bronchodilating Agents,[6] α7 and α4β2 nicotinic receptors inhibitory activity.[7]According toMalki et al analogs of quinuclidinone can provide an excellent scaffoldfor novel anti-cancer agents with improved safety profile.[8]They used lung carcinoma cells for study and observed that in more potent derivatives, the carbonyl group of quinuclidinone was intact.[8]Further in another study they observed that quinuclidinone derivatives induce apoptosis in human breast cancer cells via reduced expression level of Bcl-2, Bcl-XL and increased mitochondrial apoptotic pathways by activating the release of cytochrome C.[9]The derivativesof this moleculemayhave a selective mode of action as they are structurally unique, and yet have a great deal of known chemistry upon which to prepare analogs. In search of novel more potent anti-cancer compoundswith greater affinity for cancer cells than healthy normal cells,we decided to

explore the anti-cancer activity of some novel quinuclidinone derivatives.We have recently reported the synthesis of quinuclidinone hydrochloride from isonepecotic acid.[10]In the present article we report the synthesis of some quinuclidinone based ester and amide derivatives with cytotoxicity and apoptosis-inducing property in lung cancer cells.

2 Result and discussion

2.1 Synthesis

In the first step 3-quinuclidinone hydrochloride**(1)**was refluxed with 4-formyl benzoic acid**(2)** in the presence of sodium hydroxide, using absolute ethanol as solvent to give 4-(3-oxo-1-aza-bicyclo[2.2.2]oct-2-ylidenemethyl)-benzoic acid**(3)(Scheme-1).**Formation of this intermediateacid (**3**) was confirmed fromthe broad absorption bandat around 3200 cm^{-1} in the infrared spectrum(IR) and downfield peak at 13.1 δ ppmin ^{1}H NMR. The acid derivative **(3)** was converted to the acid chloride with thionyl chloride in the presence of triethylamine. The acid chloride was further converted in to the corresponding ester or amide derivative by reaction with appropriate alcohol/ amine.The structure of synthesized compounds established on the basis of elemental analysis and spectral data.In case of amide derivatives (**4a to 4f**), the NH stretching frequency appeared around 3300 to 3450 cm^{-1}.The formation of ester (**5a to 5e**)was confirmed from the disappearance of the broadband of carboxyl in the IR spectrumand appearance of absorption band in the region 1728-1750 cm^{-1}. ^{13}C NMR downfield peakappeared around165-169δppm, whichfurther confirms the formation of ester derivatives.Single crystal of compound **4c** and **5c (Fig.6 and fig.7)**were obtained by slow evaporation method using methanol as a solvent. The crystal structures of compound **4c** and **5c** shows the presence of double bond with Z geometry. This is well in agreement with previousreport.[11]

2.2Biological assay and Structure Activity Relationship (SAR)

Thesynthesized compounds were screened for MTT assay. All compounds were screened at 1, 5, 10 and 20μMconcentrations. The results obtained in a cytotoxicity assay for quinuclidinone and its ester and amide analogues accounted for decreased cell viability in a dose dependent manner. The results also suggest that the synthesized compounds showed specificity for cancer cells over normal lung cells.

The half inhibitory concentration IC_{50} valuesin μM concentrationwere determinedand presented in Table 1. The results indicate that compared to esters all amide derivative exhibited reasonably good activity. Among amides the derivative having unsubstituted phenyl ring **(4a)** was found to be less active among all. The introduction of either electron donating group (methyl) or electron withdrawing group (bromo) increases the activity.Amide derivative having *para*bromophenyl group **(4c)** was found to be most potent among all aromatic amides. In order to determine if aromatic ring is essential for activity, derivatives with cyclohexyl**(4d)** and heterocyclic ring such as morpholine**(4e)** and pyrrolidine**(4f)** were synthesized. In all these cases an increase in the activity was observed.

In ester series thederivatives in which the oxygen of ester group was attached to a secondary carbon exhibited better activity. Thus esters of secondary alcohols such as isopropyl alcohol, *sec*-butanol, and cyclopropyl alcohol were found to be more active. The compound **(5c)**bearing isopropyl group was found to be most active followed by (**5d)**and**(5e)** which were moderately active. Lowering of activity was observed when methyl (**5a**) and ethyl groups (**5b**) were introduced.

Synthesis of new chemotherapeutic agents with selective cytotoxicity towards cancer cells is always a major challenge for chemists and biotechnologists. Based upon the results obtained

in the cytotoxicity assaythe derivatives **4c, 4e,5c** and**5d** were selected for a detailed scrutiny to assess the mode of cell death.

Cancer cells were stained with fluorescent stains to gain a deeper insight into the mechanism of cell death. Induction of apoptosis is a key event and a preferred pathway for induction of cell death by a test compound.[12]Hence, a fluorescent probe (AO/EB) was used to gather qualitative evidence on apoptosis. It has been reported that the viable cells show green fluorescence and late apoptotic cells show orange to red fluorescence with condensed chromatin.[13]We could observe more orange to red fluorescent cells **4c, 4e** and**5c**as compared to **5d** and quinuclidinoneHCl**1** treated cells suggesting induction of apoptosis(fig.2).

Nuclear condensation resulting due to any test compound is assessed using DAPI staining.[14]In our study, condensation/fragmentation/distortion of nuclei was evident in all treatment groups (fig.3). Further confirmation on apoptosis was obtained from DNA ladder assay, wherein apoptosis induced DNA damage is accredited to possible induction of apoptotic pathway.[15]In the present study, all the test compounds showed moderate to heavy ladder formation (fig.4) suggesting induction of apoptosis.

Novel compounds that may be potent in destruction of cancer cells often cause damage to the red blood corpuscles (RBC) and hence, haemolytic assay provides a clue on its merit in not destroying the RBC.[16] Hence, haemolytic assay is a popular tool to assess the relative impact of test compounds on RBCs.[16]In our study 4e**,5e**, **5f**, and the quinuclidinoneHCl**1**accounted for moderate haemolysis indicating at their relative safety for *in vivo* use as a possible therapeutant(fig.5).

3 Conclusions

In conclusiona series of novel quinuclidinone based amides and esters (**4a to4f** and **5a** to **5f**) were synthesized. The structuresoftitlecompounds were well supported by spectroscopic data

and elemental analysis. Test compounds were able to induce apoptosis of A549 lung carcinoma cells with minimal damage to the L132 normal lung cells. The most potent compounds (**4c**, **4e 5c**and 5**d**,) weresubjected to further investigations. DNA fragmentation suggests that the cytotoxic effect of the compound is selectively mediated through the induction of apoptosis.Additional experiments are required to determine the mechanism of action andfor better elucidation of structure activity relationships of this class of molecules.

4Experimental

4.1 Materials and methods

Commercial grade solvents and reagents (alcohols and amines) were purchased from Sigma Aldrich or Alfa aeser or SpectrochemMumbai India and used without further purification. Quinuclidinone Hydrochloride was prepared as described in literature.[10]Melting points were measured using a (Buchi B-545) melting point apparatus and were uncorrected. Infrared spectra were recorded on a Perkin-Elmer RX 1 spectrometer. Elemental analyses were recorded on Thermo finnigan Flash 11-12 series EA.^{1}H and ^{13}C NMR spectra were recorded on an Advance Bruker (400 MHz) spectrometer in suitable deuterated solvents. ^{1}HNMR data were recorded as follows: chemical shift measured in parts per million (ppm) downfield from TMS multiplicity, observed coupling constant (*J*) in Hertz (Hz), proton count. Multiplicities are reported as singlet (s), broad singlet (br s), doublet (d), triplet (t), quartet (q) and multiplet (m). ^{13}C NMR chemical shifts are reported in ppm downfield from TMS. Solvents and reagents were purified by literature methods. Mass spectra were determined by LC/MS, using Shimadzu LCMS 2020 and AB Sciex 3200 QTRAP. The reaction progress was monitored by TLCin ultraviolet light as well as with iodine vapor.

4.2 Biological assay

4.2.1 Cell line and culture

The A549 and L132 cell lines were obtained from the National Center for Cell Sciences, Pune whereas Dubecoos Modified Essential Medium (DMEM), Fetus Bovine Serum (FBS) and antimycotic–antibiotic solution were obtained from HiMedia. The human cell lines A549 and L132 were seeded in T-25 flask with DMEM, 10% FBS and 1% antimycotic-antibody solution in a humidified atmosphere supplied with 5% CO_2 at 37^0C. Cells were subsequently sub-cultured every third day by trypsinization with 0.25% Trypsin versus glucose solution. Both the cell lines were utilized to examine the antitumor activity of testing compound at varying concentration.

4.2.2 Cell viability assay

The IC_{50}values of cell proliferation were determined using MTT assay. Quinuclidinone and its derivatives were dissolved in 0.5% dimethyl sulfoxide and subsequent doses were prepared in the media. A549 cells were seeded in 96 well culture plates and were treated with different concentrations of the compoundsfor 24 h. The positive control cells were treated with quinuclidinone in culture medium at subsequent doses. At the end of the incubation period 100µl of 3-(4, 5-dimethylthiazol-2-yl) -2, 5-diphenyl tetrazolium bromide (MTT; $1mgml^{-1}$) was added to the wells and plates were incubated at 37^0C for 4 h. Later, culture medium was discarded and 150µl DMSO was added. Absorbance was read at 540nm in ELX800 Universal Microplate Reader.

4.2.3. Nuclear morphology assessment (DAPI staining)

A549 cells were seeded in 6 well plate (5×10^5) and were allowed to achieve 80% confluence. Cells were treated with IC_{50} values of the compounds for 24 h at 37^0C. Cells were washed with PBS and fixed with 1% paraformaldehyde, rewashed with PBS and incubated with DAPI for 5 min. Treated cells were examined for condensed and fragmented nuclei and photographed under Leica DMRB fluorescence microscope.

4.2.4 Assessment of apoptosis AO/EtBr staining

Cells were grown on glass cover slip (5 x 10^6) and were incubated in a CO_2 incubator at 37^0C. Cells were dosed with IC_{50} concentration of compounds. After 24 h incubation, cells were washed with PBS and stained with 5µl of AO-EtBr mixture. The coverslip was placed on clean microscopic slides and photograph was taken under confocalmicroscope(Carl Zeiss LSM-710.)

4.2.5 Haemolytic assay

Whole blood was collected for haemolytic assay from a healthy human volunteer after taking a written consent. Protocol was approved and experiments were performed in compliance with the relevant laws and guidelines of Indian medical association for research on human subjects at Blue cross pathology lab (IMA-BMWMC No. 1093), Vadodara, India. The blood samples were placed in vacutainer tubes coated with ethylenediaminetetra-acetic acid (EDTA) and were gently mixed and treated with IC_{50} concentration of the compounds. Control was untreated (with 0% haemolysis) and positive control was sample treated with 3% hydrogen peroxide (with 100% haemolysis). After incubation for 3 h (an adjustment of the standard ASTM F-756[17]) the tubes containing blood sampleswere centrifuged at 1500 rpm for 10 min to collect the plasma. The supernatant was analysed for the presence of the haemoglobin at 540 nm and percentagehaemolysis calculated according to the procedure described by Shiny et al.[18]

4.2.6. DNA ladder assay

A549 cells (3 x 10^6) were exposed to the IC_{50} concentration of the compounds. Cells were centrifuged and then washed with PBS, and the pellet was lysed with 400 µL hypotonic buffer solution (containing 10 mM of tris (pH 7.5), 1 mM of EDTA and 0.2% triton X-100) for 15 min at room temperature, and then centrifuged at 13000 rpm for 15 min. 350 µL of the supernatant was again lysed in 106 µL of second lysis buffer (150 mMNaCl, 10 mMTris–HCl (pH 8.0), 40 mM EDTA, 1% SDS and 0.2 $mgmL^{-1}$ of proteinase K, at final concentration) for

4 h at 37^0C. The DNA was extracted with phenol/chloroform/isoamyl alcohol (25:25:1 v/v/v), and the pellet thus obtained was washed with ethanol and re-suspended for RNAase digestion in 15 µL of 10 mMTris, 1 mM of EDTA (pH 8.5), and 50 μgmL^{-1} of RNAase for 1 h at 37^0C. The fragmented DNA was quantified on 2% agarose gel electrophoresis.[19]

4.3 Synthesis of (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoic acid (3)

Compound 1 and formyl benzoic acid were taken in absolute ethanol and the solution was refluxed with catalytic amount of sodium hydroxide for about 3-4 h. The reaction progress was monitored by TLC. After completion the reaction mixture was acidified with glacial acetic acid. Crude product precipitated was filtered and washed with water. It was dried under vacuum and recrystallized using IPA-water.

4.4 General procedure for the preparation of compounds amides (4)

The product of step one (1 mmol) was taken in 20 ml dichloromethane andtriethyl amine (3 mmol) was added. The resulting mixture was stirred at 0 $^{\circ}$C andthionyl chloride (1.1 mmol) was added carefully. It was then warmed to room temperature and refluxed for 30 min. After solvent evaporation the unreacted thionyl chloride was removed under vacuum. The acid chloride thus obtained was taken in 20 ml acetonitrile and appropriate amine (1.12 mol) was added followed by K_2CO_3 (3 mmol).The reaction mixture was refluxed andthe progress of the reaction was monitored by TLC. After completion, the alcohol was evaporated under vacuum. The residue was dissolved in ethyl acetate (50 ml) and washed with water and sat. $NaHCO_3$ solution. The organic layer was dried with sodium sulfate and solvent evaporated to give the ester as solid product.

4.5 General procedure for the preparation of esters (5)

To the acid chloride obtained above, appropriate alcohol was added in excess and the mixture was refluxed. The progress of the reaction was monitored by TLC. After completion, the

alcohol was evaporated under vacuum. The residue was dissolved in ethyl acetate (50 ml) and washed with water and sat. $NaHCO_3$ solution. The organic layer was dried with sodium sulfate and solvent evaporated to give the ester as solid product.

4.3.1(Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoic acid (3)

Yellow solid, mp: >250 ºC; yield 78.6%.^{1}H NMR (400 MHz, DMSO): δ ^{1}H NMR (400 MHz, DMSO): δ 1.88-1.94 (2H, m, -CH_2); 1.99-2.02 (2H, m, -CH_2); 2.49-2.50 (1H, m, -CH);2.82-2.92 (2H, m, -CH_2); 3.12-3.25 (2H, m, -CH_2); 6.95 (1H, s, vinylic proton);7.93-7.95 (2H, d, *J*=8.0, -ArH); 8.14-8.16 (2H, d, *J*=8.0, -ArH); 13.15 (1H, b, Acid proton). ^{13}C NMR (100 MHz, DMSO): δ 25.3, 40, 47.3, 122.9, 129.6, 132.1, 137.7, 146.7, 167.5, 205.4.DEPT- 135 (100 MHz, DMSO): δ 25.3, 40.0, 47.3, 122.8, 129.6, 132.1.IR (KBr, cm^{-1}): 3396, 2971, 2956, 2941, 2869, 1706, 1689, 1624, 1290, 805. ESI/MS 258.1 $[M+1]^+$ calculated for $C_{15}H_{15}NO_3$.Anal.Calcd. for: C,70.02; H ,5.88; N,5.44; O,18.65; found C, 70.42; H, 5.90; N,5.38.

4.4.1 (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)-N-phenylbenzamide (4a)

Yellow solid, mp: 130-133 ºC; yield 60%.^{1}H NMR (400 MHz, DMSO): δ 2.00-2.03 (4H, m, 2-CH_2); 2.50-2.52 (1H, m, -CH); 2.86-2.91 (2H, m, -CH_2); 3.13-3.91 (2H, m, -CH_2); 6.98 (1H, s, vinylic proton); 7.08-7.12 (1H, t, *J*=7.2, 14.2 Hz, -ArH), 7.33-7.37 (2H, t, *J*=7.6, 15.6 Hz, -ArH); 7.77-7.79 (2H, d, *J*=8 Hz, -ArH), 7.94-7.96 (2H, d, *J*=8.4 Hz, -ArH); 8.17-8.19 (2H, d, *J*=8.0 Hz, -ArH), 10.33 (1H, s , Amide proton). ^{13}C NMR (100 MHz, DMSO): δ 25.3, 47.3, 114.3, 120.7, 123.0, 124.2, 128.1, 129.1, 129.2, 129.4, 131.5, 132.0, 135.8, 136.9, 139.5, 146.6, 165.5, 205.5.IR (KBr, cm^{-1}): 3388, 2960, 2943, 2869, 1699, 1656, 1597, 685.ESI/MS 333.2 $[M+1]^+$ calculated for $C_{21}H_{20}N_2O_2$.Anal.Calcd. For: C, 75.88; H, 6.06; N, 8.43; O, 9.63; found C,75.71; H,6.27; N,8.51.

4.4.2. (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)-N-(p-tolyl)benzamide (4b)

Yellow solid, mp: >250ºC; yield 58%.^{1}H NMR (400 MHz, $CDCl_3$): δ 2.06-2.08 (4H, m, 2-CH_2); 2.36 (3H, s, -CH_3); 2.67-2.68 (1H, m, -CH), 2.99-3.05 (2H, m, -CH_2); 3.17-3.22 (2H, m, -CH_2); 7.04 (1H, s, vinylic proton); 7.18-7.02 (2H, d, *J*=8.4 Hz, -ArH); 7.53-7.55 (2H, d, *J*=8.0 Hz, -ArH); 7.85-7.87 (2H, d, *J*=8.0 Hz, -ArH), 8.13-8.15 (2H, d, *J*=8.4 Hz, -ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 20.9, 25.7, 40.1, 47.4, 120.2, 123.6, 127.0, 129.6, 132.2, 134.3, 135.3, 137.1, 146.1, 165.1, 206.2.IR (KBr, cm^{-1}): 3295, 3130, 3042, 2950, 2869, 1703, 1644, 1607, 808.ESI/MS 347.2 $[M+1]^+$ calculated for $C_{22}H_{22}N_2O_2$.Anal.Calcd.for: C, 76.28; H, 6.40; N, 8.09; O, 9.24; found C, 76.51; H, 6.26; N, 8.27.

4.4.3. (Z)-N-(4bromophenyl)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzamide (4c)

Yellow solid, mp: 207-208ºC; yield 69%.^{1}H NMR (400 MHz, DMSO): δ 1.91-2.07 (4H, m, 2-CH_2); 2.85-2.92 (2H, m, -CH_2); 3.13-3.20 (2H, m, -CH_2); 6.98 (1H, s, vinylic proton); 7.53-7.55 (2H, d, *J*=9.4 Hz, -ArH); 7.76-7.79 (2H, d, *J*=9.2 Hz, -ArH); 7.94-7.96 (2H, d, *J*=8.4 Hz, -ArH); 8.17-8.20 (2H, d, *J*=8.4 Hz, -ArH); 10.50 (1H, s, Amide). ^{13}C NMR (100 MHz, DMSO): δ 25.3, 47.3, 115.8, 122.6, 122.9, 128.1, 129.3, 131.9, 132.0, 135.5, 137.1, 139.0, 146.6, 165.6, 205.4.IR (KBr, cm^{-1}): 3373, 3302, 3086, 2942, 2866, 1698, 1677, 1620, 1588, 1466, 1067, 1036, 854.ESI/MS 410.2 $[M]^+$ calculated for $C_{21}H_{19}N_2BrO_2$.Anal.Calcd. for: C,61.33; H,4.66; N,6.81; O,7.78; Br, 19.43;found C,61.47; H,4.45; N,6.97.

4.4.4. (Z)-N-cyclohexyl-4-((3-oxoquinuclidin-2-ylidene)methyl) benzamide (4d)

Yellow solid, mp: >225ºC; yield 73%.^{1}H NMR (400 MHz, DMSO): δ 1.11-1.13 (1H, m, -CH_2); 1.24-1.35 (4H, m, 2-CH_2); 1.59-1.62 (1H, m, -CH_2); 1.72-1.73 (2H, m, -CH_2); 1.73-1.72 (2H, m, -CH_2) ;1.86-1.92 (2H, m, -CH_2);1.98-2.03 (2H, m, -CH_2); 2.83-2.90 (2H, m, -

CH_2); 3.12-3.19 (2H, m, -CH_2); 3.73-3.75 (1H, m, -CH); 6.94 (1H, s, - vinylic proton); 7.81-7.83 (2H, d, *J*=8.4 Hz, -ArH); 8.09-8.11 (2H, d, *J*=8.4 Hz, -ArH); 8.22-8.24 (1H, d, Amide).

^{13}C NMR (100 MHz, DMSO): δ 25.3, 32.8, 47.3, 48.8, 123.1, 127.7, 131.9, 135.7, 136.4, 146.35, 165.3, 205.4.IR (KBr, cm^{-1}): 3341, 2926, 2853, 1745, 1703, 1653, 1534, 1500, 800.ESI/MS 338.3 $[M+1]^+$ calculated for $C_{21}H_{26}N_2O_2$.Anal.Calcd. for: : C,74.53; H,7.74; N,8.28; O, 9.45;found C,74.78; H,7.51; N,8.37.

4.4.5. (Z)-2-(4-(morpholine-4-carbonyl)benzylidene)quinuclidin-3-one (4e)

Yellow solid, mp: 155-156 ºC; yield 55%.^{1}H NMR (400 MHz, DMSO): δ 2.02-2.07 (4H, m, 2-CH_2); 2.65-2.66 (1H, m, -CH); 2.96-3.03(2H, m, -CH_2); 3.15-3.22 (2H, m, -CH_2); 3.47-3.79 (8H, m, -4CH_2); 7.00 (1H, s,-vinylic proton); 7.40-7.42 (2H, d, *J*=8.4 Hz, -ArH); 8.07-8.07 (2H, d, *J*=8 Hz, -ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 25.3, 40.1, 47.4, 51.6, 66.9, 123.8, 127.2, 132.1, 135.5, 135.8, 145.7, 169.9, 206.2.IR (KBr, cm^{-1}): 3444, 3027, 2962, 2869, 1707, 1683, 1635, 1438, 1110.ESI/MS 327.3 $[M+1]^+$ calculated for $C_{15}H_{17}NO_2$.Anal.Calcd. for: C,69.92; H,6.79; N,8.58; O,14.71.; C,70.14; H,6.51; N,8.42.

4.4.6. (Z)-2-(4-(pyrrolidine-1-carbonyl)benzylidene)quinuclidin-3-one (4f)

Yellow solid, mp: 170-172 ºC; yield 49%.^{1}H NMR (400 MHz,$CDCl_3$): δ 1.90-1.96 (2H, m, -CH_2); 1.97-1.99 (2H, m, -CH_2); 2.01-2.07 (4H, m, 2-CH_2); 2.64-2.66 (1H, m, -CH); 2.96-3.03 (2H, m, -CH_2); 3.14-3.21 (2H, m, -CH_2); 3.42-3.46 (2H, t, -CH_2); 3.63-3.67 (2H, m, -CH_2); 7.00 (1H, s,-vinylic proton); 7.50-7.52 (2H, d, *J*=8.4 Hz, -ArH); 8.05-8.07 (2H, d, *J*=8.4 Hz, -ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 24.4, 25.7, 26.4, 40.2, 46.2, 47.4, 49.5, 124.1, 127.1, 131.9, 135.3, 137.8, 145.5, 169.1, 206.3.IR (KBr, cm^{-1}): 3444, 2970, 2944, 2872, 1703, 1623, 1431, 1097.ESI/MS 311.4 $[M+1]^+$ calculated for $C_{19}H_{22}N_2O_2$.Anal.Calcd. for: C,73.52; H,7.14; N,9.03; O, 10.31; found C,73.37; H,7.29; N,9.25.

4.5.1. Methyl (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate (5a)

Yellow solid, mp: 142-143ºC; yield 70%.^{1}H NMR (400 MHz, $CDCl_3$): δ2.02-2.07 (4H, m, CH_2); 2.64-2.66 (1H, m, CH); 2.98-3.05 (2H, m, CH_2); 3.15-3.22 (2H, m, CH2); 3.84 (3H, s, -CH_3);6.90-6.93 (1H, dd, J= 4.0, 0.8 Hz, ArH) 7.00 (1H, s, vinylic proton); 7.28-7.32 (1H, t, J= 8.0, 7.6 Hz, ArH); 7.53-7.55 (1H, d, *J*= 7.2 Hz, ArH); 7.81 (1H, s, ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 25.8, 40.2, 47.4, 51.2, 115.3, 117.0, 124.9, 125.0, 129.3, 135.2, 169.3, 206.5.IR (KBr, cm^{-1}): 3380, 3049, 2290, 2220, 2869, 1759, 1728, 1700,1620.ESI/MS 272.2 $[M+1]^+$ calculated for $C_{16}H_{17}NO_3$ .Anal.Calcd.for $C_{15}H_{17}NO_2$: C, 70.83; H,6.32; N,5.16; O, 17.69 Found: C, 70.92; H,6.28; N,5.20.

4.5.2.Ethyl(Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate (5b)

Yellow solid, mp: 112-115ºC; yield 68%.^{1}H NMR (400 MHz, $CDCl_3$): δ 1.39-1.42 (3H, t,-CH_2); 2.04-2.08 (4H, m, CH_2); 2.65-2.68 (1H, m, CH); 2.99-3.04 (2H, m, CH_2); 3.14-3.23 (2H, m, CH_2); 4.46-4.42 (1H, q, CH_2); 7.04 (1H, s, vinylic proton); 8.02-8.10 (4H, m, ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 8.60, 14.3, 25.7, 40.1, 45.6, 47.4, 61.1, 123.8, 129.4, 130.0, 130.7, 131.8, 131.9, 138.1, 146.2, 166.2, 206.Hz. IR (KBr, cm^{-1}): 3387, 3051, 2994, 2963, 2943, 2922, 2871, 1754, 1701, 1679, 1623,806. ESI/MS 286.2 $[M+1]^+$ calculated for $C_{17}H_{19}NO_3$ .Anal.Calcd.for: C, 71.56 ; H,6.71: N,4.91; O,16.82.Found: C, 71.87; H, 6.50; N,5.13.

4.5.3. Isopropyl (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate (5c)

Yellow solid, mp: 96-97ºC; yield 56%.^{1}H NMR (400 MHz, $CDCl_3$): δ 1.37-1.39 (6H, d, CH_3); 2.04-2.08 (4H, m, CH_2); 2.66-2.68 (1H, m, CH); 2.99-3.02 (2H, m, CH_2); 3.16-3.21 (2H, m, CH_2); 5.23-5.29 (1H, m, CH_2); 7.04 (1H, s, vinylic proton); 8.02-8.04 (2H, m, ArH). 8.08-8.10 (2H, d, *J*=8.4 Hz, ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 21.0, 25.7, 40.2, 47.4,

68.5, 123.8, 129.4, 131.8, 138.0 146.2, 166.2, 206.2.IR (KBr, cm^{-1}): 3057, 2984, 2944, 2964, 2875, 1758, 1724, 1707, 1606, 1458, 810.ESI/MS 300.1$[M+1]^+$ calculated for $C_{18}H_{21}NO_3$ .Anal.Calcd. for: C, 72.22 ; H,7.07: N,4.68; O,16.03.Found: C,72.45; H,7.00: N,4.93

4.5.4 Sec-butyl (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate (5d)

Yellow solid, mp: 100-102 ºC; yield 72%.^{1}H NMR (400 MHz, $CDCl_3$): δ 0.96-1.06 (3H, t,-CH_3);1.34-1.35 (3H, d, - CH_2); 1.68-1.74 (2H, m, -CH_2); 2.04-2.90 (4H, m,-CH_2); 2.67-2.68 (1H, m, CH); 3.00-3.05 (2H, m, CH_2); 3.17-3.23 (2H, m, CH_2); 7.05 (1H, s, vinylic proton); 8.03-8.05 (2H, m, -ArH). 8.08-8.10 (2H, d, *J*=8.66 Hz, -ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 9.7, 19.5, 25.7, 28.9, 40.1, 47.4, 123.9, 129.4, 131.8, 165.8, 206.2. ESI/MS 313.3 $[M+1]^+$ calculated for $C_{19}H_{23}NO_2$.Anal.Calcd. for: C, 72.22 ; H,7.07; N,4.68; O,16.03.Found: C,72.45 ; H,7.00; N,4.93.

4.5.5Cyclopentyl (Z)-4-((3-oxoquinuclidin-2-ylidene)methyl)benzoate (5e)

Yellow solid, mp: 140-143ºC; yield 43%.^{1}H NMR (400 MHz, $CDCl_3$): δ 1.47-1.50 (2H, m, -CH_2); 1.56-1.64 (2H, m, -CH_2); 1.78-1.81 (2H, m, -CH_2); 1.92-1.95 (2H, m, -CH_2); 2.04-2.08 (4H, m, 2-CH_2); 2.66-2.68 (1H, m, -CH); 2.97-3.04 (2H, m, -CH_2); 3.15-3.22 (2H, m, -CH_2); 5.02-5.06 (1H, m, -CH); 7.04-7.05 (1H, s, vinylic proton); 8.03-8.13 (4H, m, -ArH).^{13}C NMR (100 MHz, $CDCl_3$): δ 23.6, 25.4, 25.7, 31.5, 40.1, 47.9, 123.8, 129.4, 130.0, 131.2, 131.7, 131.8, 138.0, 146.2, 165.6, 206.2.IR (KBr, cm^{-1}): 3392, 3056, 2942, 2869, 1755, 1708, 1675, 1624, 805.ESI/MS 325.2 $[M+1]^+$ calculated for $C_{20}H_{23}NO_3$.Anal.Calcd. for: C, 73.37 ; H,7.70; N,4.28; O,14.66. C, 73.08; H,7.91; N,4.45.

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Supporting information

CCDC No.1025491 and 1051487 contains the crystallographic data for the compounds**4c** and **5c**respectively. These data can be obtained free of charge via http://www.ccdc.cam.ac.uk/conts/retrieving.html,or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (+44) 1223 336 033; or e-mail: deposit@ccdc.cam.ac.uk

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Captions to the Figures

Scheme 1 Synthetic route for compounds (4) and (5);(Reagents and conditions: (a) NaOH, EtOH, reflux; (b) (1) MDC, TEA, $SOCl_2$, reflux; (2) R'-NH_2, ACN, K_2CO_3, Reflux; (c) (1) MDC, TEA, $SOCl_2$, reflux; (2) R-OH reflux.)

Fig. 1 Structures of some quinuclidine based drugs.

Fig. 2 Photomicrographs ofAO/EB staining for apoptosis,treated with (a) control,compounds (b) 4c, (c) 4d, (d) 5c, (e) 5d and (f) 1 respectively at their IC_{50} values for 24h. (Green fluorescence indicated live cells whereas reddish orange stained cells indicate late apoptosis).

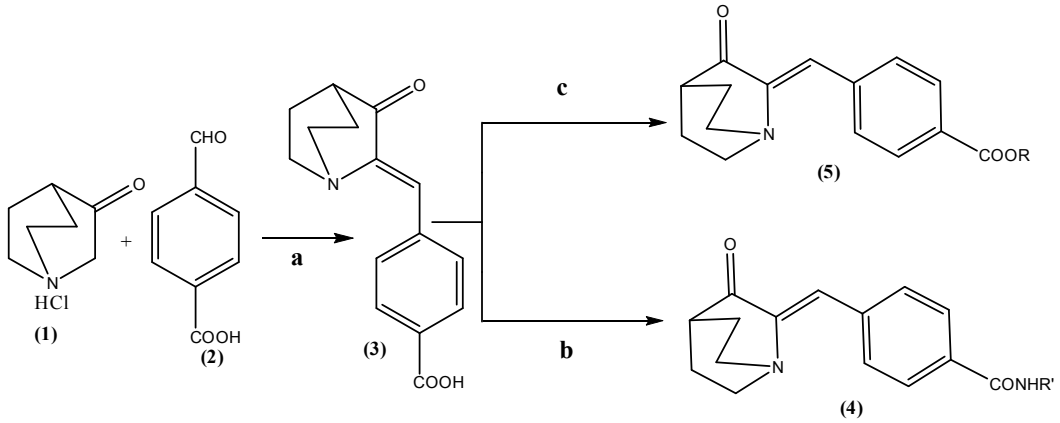
Fig. 3 Nuclear condensation test with DAPI for treated with (a) control, compounds (a) 4c, (b) 4d, (c) 5c, (d) 5d and (f)1 respectively at their IC_{50} values for 24h. (Arrows indicate condensation/fragmentation/distortion of nuclei as compared to the control)

Fig. 4 DNA ladder test for apoptosis: Ladder formation suggests induction of apoptosis (lane 1: Ladder, lane 2: control, lane 3: 5d, lane 4: 5c, lane 5: 4c, lane 6: 4e, lane 7: quinuclidinoneHCl 1).

Fig.5 Haemolysis of RBC

Fig. 6ORTEP diagram of compound 4c with atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability.

Fig.7ORTEP diagram of compound 5c with atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability.



Scheme 1

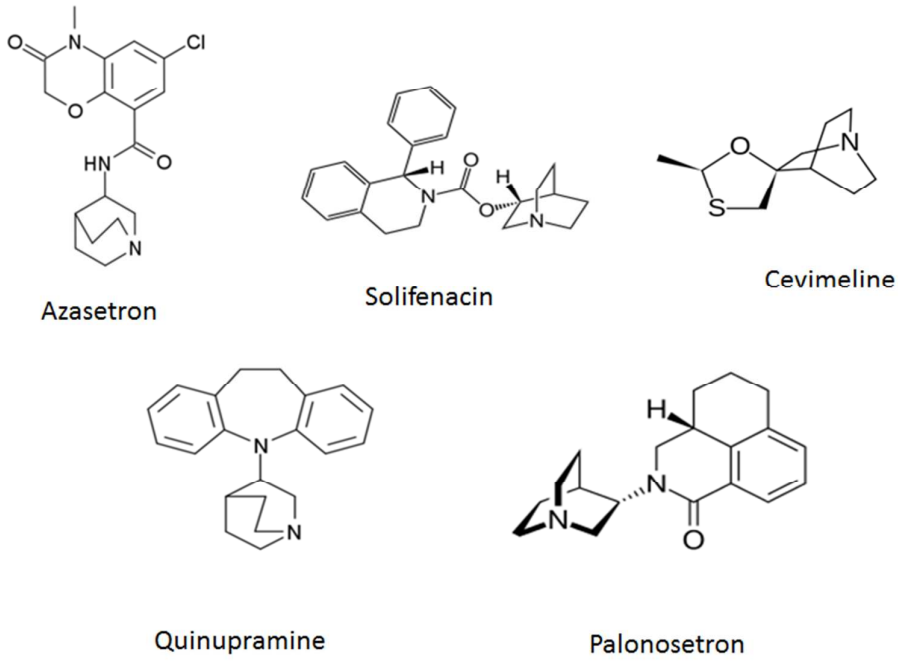


Fig.1

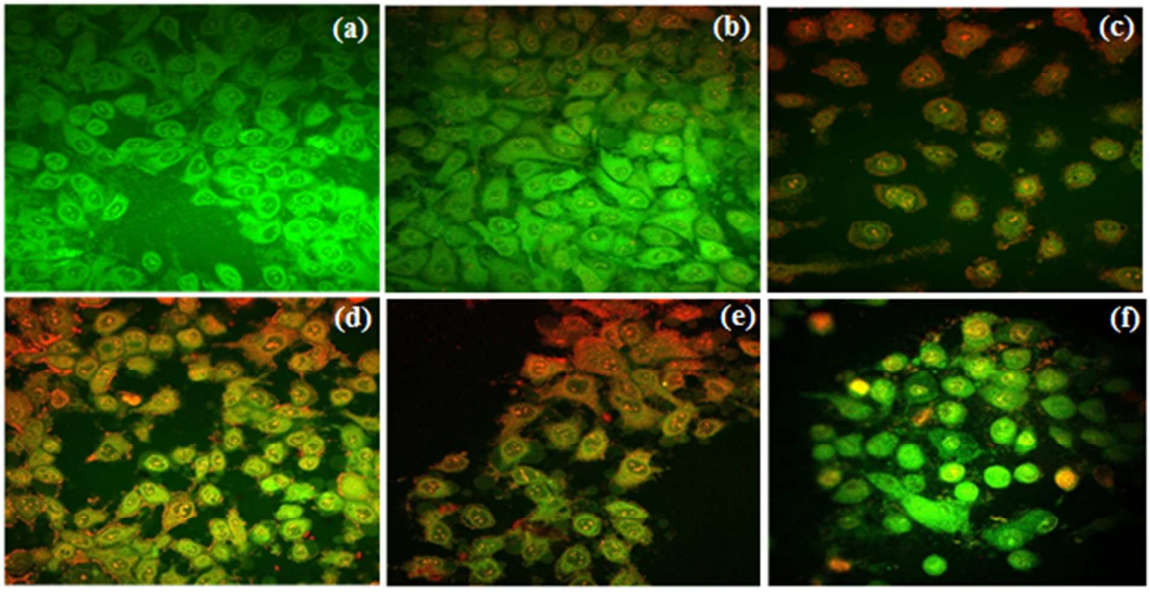


Fig.2

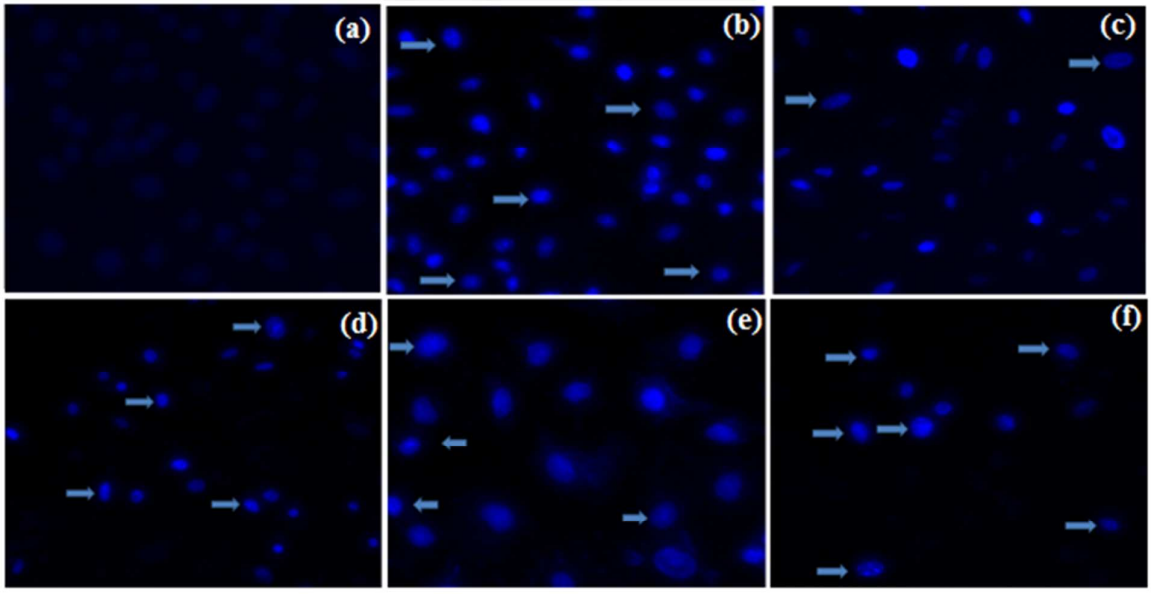


Fig.3

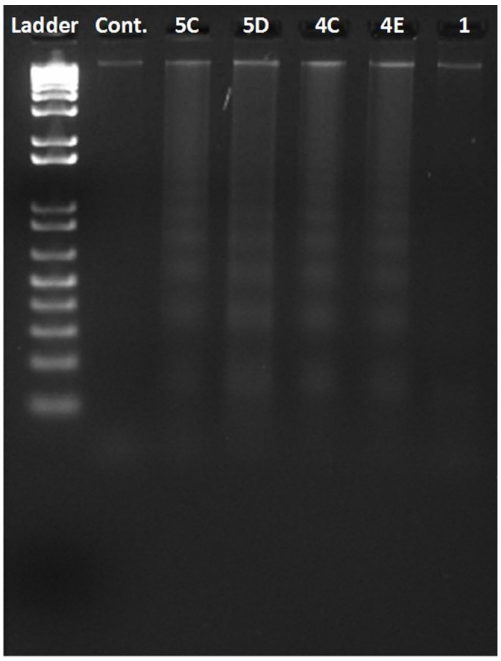


Fig.4

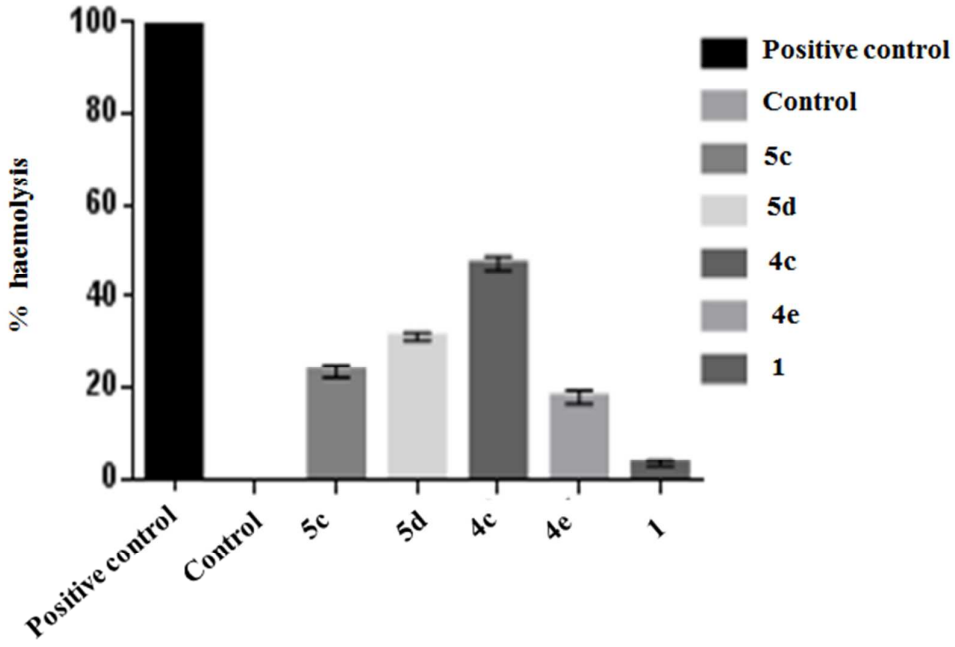


Fig. 5

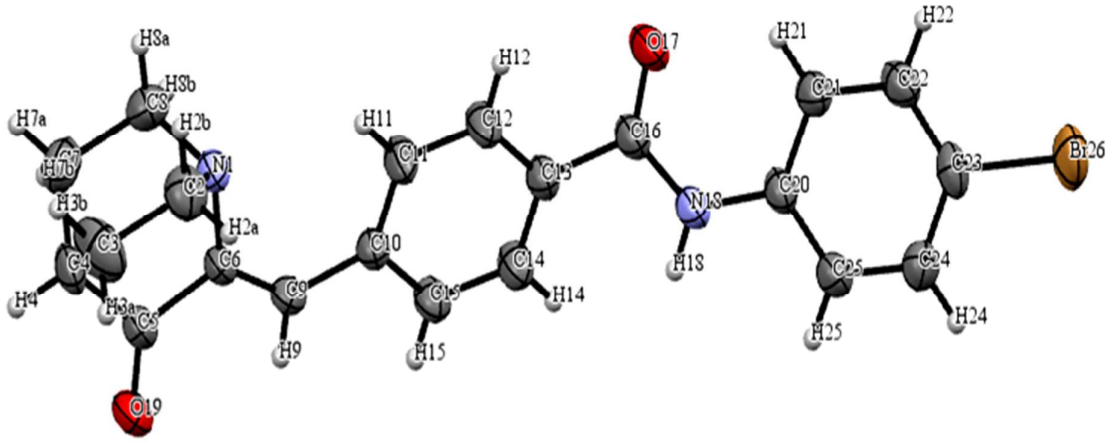


Fig.6

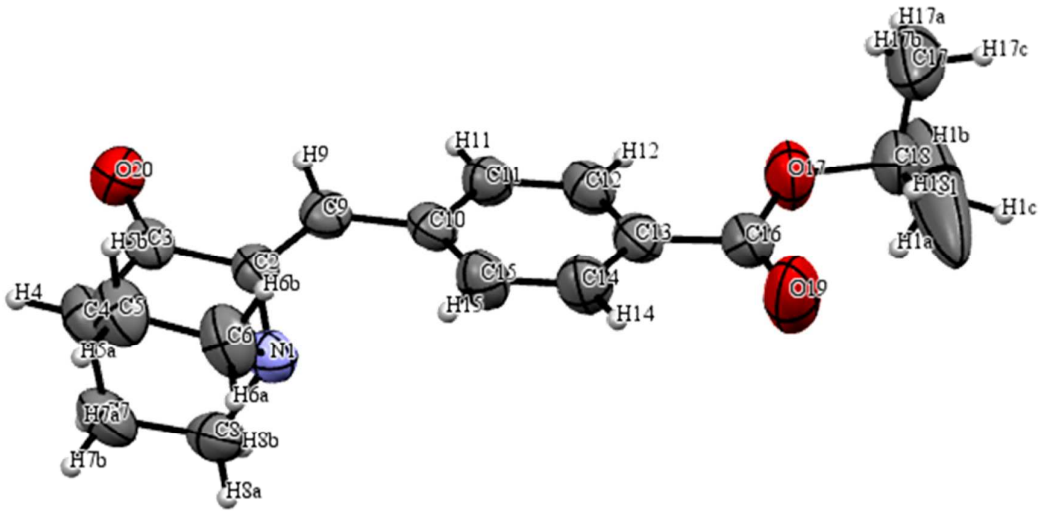
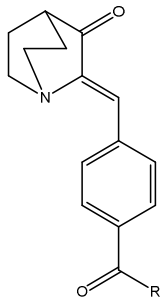
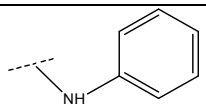
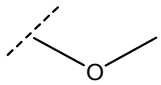
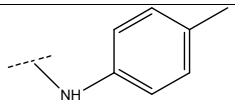
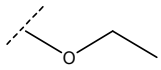
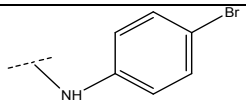
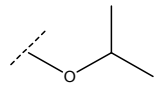
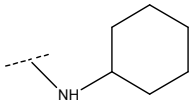
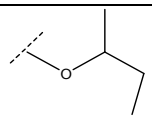
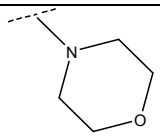
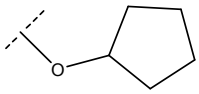
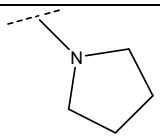


Fig.7

Table-1.IC_{50} values of the compounds 4 (a-f) and 5 (a-e)



Compound	Structure	IC_{50} (µM)	Compound	Structure	IC_{50} (µM)
4a		3.26 ± 1.4	**5a**		9.22 ± 1.2
4b		1.24 ± 0.5	**5b**		8.77 ± 0.6
4c		0.225 ± 0.7	**5c**		1.5 ± 0.9
4d		1.301 ± 0.4	**5d**		5.74 ± 0.8
4e		0.665 ± 1.4	**5e**		6.15 ± 1.0
4f		1.24 ± 0.9			