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Synthesis, cytotoxicity and anti-mycobacterial activity evaluation of some newly substituted heterocyclic chalcones

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ABSTRACT

*With the aim to access the biological activity, in the present study we have synthesized a series of substituted pyridyl chalcones **(1a-1f)** by condensing different p-substituted benzaldehyde with 2-Acetyl pyridine in dilute ethanolic potassium hydroxide solution at room temperature according to Claisen- Schmidt condensation. The chemical structures of all the compounds were assigned on the basis of elemental analyses, UV-Vis., IR, ^{1}H NMR and mass spectral data. All the compounds were tested for their in vitro cytotoxic activity against human chronic myelogenous leukaemia cell line **K562** and cell viability was evaluated by 3-(4,5-dimethylthiaazol-2-yl-2,5diphenyltetrazoliumbromide(**MTT**)assay. All compounds indicated significant dose-dependent cytotoxicity in very low micro molar range **(IC_{50} 4.32-6.21μM)**. The anti-mycobacterial drug susceptibility testing (DST) performed against M.smegmatis and M.tuberculosis using microtiter plate resazurin reduction assay **(REMA)** with glycerol and acetate as two different carbon sources and results obtained were reported.*

Keywords: Pyridyl chalcones, Leukaemia cell line, Cytotoxic activity, MTT assay, Resazurin reduction assay.

INTRODUCTION

Cancer, the uncontrolled, rapid and pathological proliferation of abnormal cells, is the second leading cause of human death after cardiovascular diseases in developing as well as advanced countries.[1,2] Diseases caused by mycobacterium is one of severe problem worldwide. Despite being preventable disease, tuberculosis accounts for 2 million deaths per year. This led to development of new anticancer and anti-mycobacterial agents. Much of the research in this area is currently focussed on cancer specific mechanism and the corresponding molecular targets but the research for improved cytotoxic agents still constitutes an important part of modern anticancer drug discovery. So there is a strong need to develop new simple and potential anticancer agents.

The chemistry of heterocyclic compounds has been an interesting field of study for a long time. Chalcones constitute an important group of natural products. Chalcones either natural or synthetic are known to exhibit various biological activities. The presence of a reactive α, β-unsaturated keto function in chalcones is found to be responsible for their antimicrobial activity, [3] which may be altered depending on the type and position of substituent on the aromatic rings. The substitution of an aryl group of chalcone by a heterocyclic group would enhance their biological activity. They have been reported to possess many useful biological properties including antioxidant, antimalarial, antileishmanial, anti-inflammatory, antitumor, antiprotazoal, , antiviral, antimiotic, anticancer, anti- proliferative, antimicrobial , anti-HIV activities[4-9], inhibition of chemical mediators release, inhibition of leukotriene B_4,[10], inhibition of tyrosinase [11-12] and inhibition of aldose reductase

[13] activities. For instance, a simple chalcone-based natural product, *isoliquiritigenin*, suppresses pulmonary metastasis of mouse renal cell carcinoma [14] and effectively prevents colo-rectal tumor development at a dose of 100 ppm. [15]. In recent years a variety of chalcones have been reviewed for their cytotoxic, anticancer , mutagenic as well as antiviral, insecticidal and enzyme inhibitory properties [16].

In the present communication, we here report synthesis of a series of some new pyridyl chalcones (1a-1f) and their in-vitro biological activities. All the compounds were tested for their cytotoxic activity against myelogenous leukaemia cell line **K562** .These compounds were also screened for their anti-mycobacterial activity on *M. smegmatis mc2* and *M. tuberculosis* H37Ra culture.

MATERIALS AND METHODS

3.1. Materials and methods

Melting points were determined in an electro-thermal capillary melting point apparatus and were uncorrected. Thin layer chromatography was performed on silica gel plates and examined under UV-Vis.chamber. Elemental analysis (C, H, and N) was performed on Varian Elementar–IIIanalyser. Infra-red spectra were recorded in KBr pellets on Perkin Elmer RX-1model.^{1}H-NMR spectra of synthesized compounds were recorded in deutriated DMSO on BrukerAvance 500 spectrometer. Chemicals shifts are given on δ-scale relative to (TMS) as internal standard.UV/Visible spectra were taken on Labtronics LT-2900 spectrophotometer operating from 200-900 nm in 1.0 quartz cells in our laboratory.

Synthetic materials and reagents were purchased from Merck and Hi-media chemicals and were of analytical grade. All solvents obtained were distilled prior to use following standard procedures. RPMI-1640 medium, MB7H9 medium, fetal bovine serum was purchased from sigma chemicals.

3.2. General procedure for synthesis of compounds (1a-1e)

Chalcones were synthesized by base catalyzed Claisen-Schmidt condensation reaction, by All substituted chalcones were prepared by reaction of 2-Acetyl pyridine (10mmol,1.12ml) with different p-substituted benzaldehyde (10 mmol) in aqueous ethanolic 40%KOH. Theprogress of reaction was monitored by T.L.C. The reaction mixture was stirred for about 48h-72h and then diluted with ice cold water. This was followed by neutralization with 10% HCl. The solid obtained was collected by filtration, followed by washing with water-ethanol. These compounds were purified by recrystallisation with ethanol.

3.2.1. 3-(4-nitrophenyl)-1-(pyridin-2-yl)prop-2-en-1-one, (1a)

To a stirred mixture of 2Acetylpyridine (10mmol,1.12ml) and p-nitro benzaldehyde (10mmol,1.51g)in 95%ethanol (20ml), 40%KOH (15.0 mL) was added drop wise and treated as in the general procedure to givedark brown powder, Yield 2.20 g (81 %), mp>250^{0}C ; λ max (nm) (CH_3CN): 212,278,330 and363, IR(KBr)cm^{1} 1687υ(C=O),1594υ(C=C),1518υ(C=N),1344 υ(NO_2); Anal.Calfor$C_{14}H_{10}N_2O_3$:C,66.14; H,3.96;N,11.02. Found;C,65.83;H,4.03;N,10.89;^{1}HNMR($DMSOd_6$);δ=6.76(1H,d,COCH=),7.88(1H,d,=CHAr),8.05(2H,d,C2H,C6H) ,8.08(2H,d,C-3H,C-5H,),7.65(1H,m,C-4),7.76(1H,d,C3H),8.02(1H,m,C5H),8.77(1H,d,C6H),ESI/MS(m/z): 255.2 (M^{+}+1).

3.2.2. 3-(4-(dimethylamino)phenyl)-1-(pyridin-2-yl)prop-2-en-1-one,(1b)

To stirred mixture of 2Acetylpyridine (10mmol,1.12ml) and p-dimethylamino benzaldehye (10mml, 1.49 g) in 95% ethanol (20ml), 40%KOH (15.0 mL) was added drop wise and treated as in the general procedure to give dark orange powder, Yield 2.24 g(83 %), mp 112-114^{0},λmax (nm) (CH_3CN) 215,291, 328, and 367, IR (KBr) cm^{-1}:1651 υ(C=O), 1562 υ(C=C), 1520 υ(C=N), 1347 υ(C-N-C) Anal. Cal for $C_{16}H_{16}N_2O$: C, 76.16; H, 6.39; N, 11.10; Found; C,76.83;H,6.13;N,10.91;^{1}HNMR($DMSOd_6$);δ=6.76(1H,d,COCH=),7.88(1H,d,=CHAr), 8.05 (2H,d,C2H,C6H), 8.08(2H,d,C3H,C5H),7.76(1H,m,C4),7.78(1H,d,C3H),7.65(1H,m,C-5H),8.77(1H,d,C-6H), 2.51 (6H,s,NMe_2), ESI/MS(m/z): 253.1 (M^{+}+1).

3.2.3. 3-(4-chlorophenyl)-1-(pyridin-2-yl)prop-2-en-1-one,(1c)

To a stirred mixture of 2Acetylpyridine (10mmol,1.12ml) and p-chloro benzaldehye (10mmol,1.41g) in 95% ethanol (20ml), 40%KOH (15.0 ml) was added drop wise and treated as in the general procedure to give creamy white powder, Yield 2.25 g(86 %), mp 159-161^{0}C,λmax (nm) (CH_3CN) 214,271,337 and 365 IR (KBr) cm^{-1}: 1688 υ (C=O), 1582 υ (C=C), 1487 υ (C=N), 1088 υ (Ar-Cl) Anal. Cal for $C_{14}H_{10}ClNOC$, 69.00; H, 4.14;N,5.75;Found;C,68.57;H,4.39;N,5.91;^{1}HNMR($DMSOd_6$);δ=6.73(1H,d,COCH=),7.66(1H,d,=CHAr),8.20(2H,d, C-2H,C6-H),8.39(2H,d,C-3H,C-5H,),7.59(1H,m,C-4),7.80(1H,d,C-3H),7.88(1H,m,C-5H),8.80(1H,d,C-6H), MS(m/z): 244.01 (M^{+}+1) .

3.2.4. 3-(4-methoxyphenyl)-1-(pyridin-2-yl)prop-2-en-1-one ,(1d)
To a stirred mixture of 2Acetylpyridine (10mmol,1.12ml) and p-methoxy benzaldehye (10mmol, 1.3g) in 95% ethanol (20ml), 40%KOH (15.0 ml) was added drop wise and treated as in the general procedure to give creamy white powder, yield 2.13 g(85 %), mp: 123-125^{0}C,λmax (nm) (CH_3CN) 211, 231,269, and 358 IR (KBr) cm^{-1}: 1689 υ (C=O), 1584 υ (C=C), 1512 υ (C=N), 1178 υ (-OCH_3) Anal. Cal for $C_{15}H_{13}NO_2$: C, 75.23; H, 5.43;N,5.85;Found;C,75.81;H,5.19;N,5.97;^{1}H-NMR(DMSOd_6);δ=6.91(1H,d,CO-CH=),7.36(1H,d,=CH-Ar),8.11(2H,d,C-2H,C6-H),8.66(2H,d,C-3H,C-5H,),8.05(1H,m,C-4),8.84(1H,d,C6H),8.50(1H,m,C-5H),7.84(1H,d,C-3H),3.73(3H,s,OCH_3),ESI/MS(m/z): 240.1 (M^++1) .

3.2.5. 3-(4-(benzyloxy) phenyl)-1-(pyridin-2-yl)prop-2-en-1-one,(1e)
To a stirred mixture of 2-Acetylpyridine (10mmol,1.12ml) and p-benzyloxy benzaldehye (10mmol, 2.27g) in 95% ethanol (20ml), 40%KOH (15.0 ml) was added drop wise and treated as in the general procedure to give creamy white powder, yield 3.0 g(87 %), mp 116-118^{0}C, λmax (nm)(CH_3CN) 241,323,363 and 413,IR (KBr) cm^{-1}: 1665 υ (C=O), 1590 υ (C=C), 1507 υ (C=N), 1171υ (-OCH_2 -), Anal. Cal for $C_{21}H_{17}NO_2$: C, 79.90;H, 5.39;N, 4.43;Found; C, 78.97;H, 5.89;N, 4.16.^{1}HNMR(DMSOd_6); δ=6.95(1H,d,COCH=), 7.38(1H,d,=CHAr), 7.45(2H,d,C2H,C6H,),7.11(2H,d,C-3H,C-5H),8.03(1H,m,C-,8.10(1H,m,C-4,),8.78(1H,d,C6H,)5H,),8.15(1H,d,C-3H,),5.18(2H,s,-OCH_2),7.37(3H,m,C-3H,C-4H,C-5H,),7.41(2H,d,C-2H,C-6H,), ESI/MS(m/z):316.1 (M^++1).

3.3. Biological activity evaluation
3.3.1. Cell culture and maintenance
K562 is a human chronic myelogenous Leukemia cancer cell line (Human erythromyeoblastoic leukemia) and most commonly used cell line for screening of anticancer leukemia agents. This was obtained from NCCS Pune, India (job no 1196). The viable K562 cells, determined by trypan blue exclusion test, and 1x10^4 cells were seeded onto 96 well plates in100µL complete RPMI-1640 culture media supplemented with 4mM L-glutamine, 1.5g/lit NaHCO3, with 10% fetal bovine calf serum and allowed to grow in air in a CO_2 incubator with 5% CO_2 at 37 ^{o}C.

3.3.2. Cytotoxic assay experiment
The stock solutions of test compounds were prepared in DMSO. After 24 h incubation, different concentrations (2, 4, 6, 8 µM) of compounds, made by serial dilution in culture medium, were added in 48 h incubation. The final concentration of DMSO was 0.01% in each well. A separate well containing 0.01% DMSO only was run as DMSO-control, which was found inactive under applied conditions. The cell growth was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) reduction assay, which is based on ability of viable cells to reduce a soluble yellow tetrazolium salt to blue farmazan crystal[22,23]. Briefly, after 48 h of treatments, the10µl of MTT dye, prepared in phosphate buffered saline (PBS) were added to all wells. The plates were then incubated for 4h at 37 ^{o}C. Supernatant from each well was carefully removed, formazon crystals were dissolved in 100µL of DMSO and absorbance at 540nm wavelength was recorded (Sharma et al., 2010) and each concentration was tested in threefold.

$$\textbf{Cell toxicity (\% of control)} = [(O.D_t - O.D_b)/ (O.D_c\text{-}O.D_b)] \times 100$$

Were, $O.D_t$ –mean optical density of treated wells, $O._{Db}$- mean optical density of blank wells, O.Dc - mean optical density of control wells.The **IC_{50}** values were determined as concentration of compounds that inhibited K562 cell growth by 50%

3.3.3. Anti-mycobacterial Susceptibility Screening Assay
The antimycobacterial drug susceptibility testing (DST) was performed using micro titer plate assay (Palomino et al. 2002) with glycerol and acetate as two different carbon sources. The details are as provided: the *M. smegmatis mc2* log phase culture was diluted using MB7H9 medium to give an OD_{600} of 0.05 and 100 µl of it was taken in micro titer plate. Also, 100 µl of MB7H9 medium with either glycerol or acetate as carbon source was used. The drug concentration was initially adjusted to 50 µM. The sterility control, growth control and solvent controls were also included. All experiments were performed in duplicate. The plates were sealed properly and incubated for 48h at 37^{o}C. At the end of incubation 30 µl of resazurin stock (0.02 % w/v) was added, mixed thoroughly and incubated for o/n at 37^{o}C. The plates were visually scored after o/n incubation for colour change from blue to pink. Similarly, *M. tuberculosis* H37Ra was grown in 100 ml of Middlebrook 7H9 broth (Difco) supplemented with 0.2% (v/v) glycerol, 10% (v/v) ADC (albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween-80 at 150 rpm and 37°C, till it reached an optical density of 0.4 to 0.5 at 600 nm. The final OD was adjusted to OD_{600} of 0.05. The initial screening was performed using Middlebrook 7H9 broth (Difco) supplemented with 0.2% (v/v) glycerol, 10% (v/v) ADC (albumin, dextrose, catalase), and 0.05% (v/v) Tween 80. The compounds concentration was 50µM with a total volume of 200 µl and incubated at 37°C for 5 days. All the inhibition studies were performed in duplicate. At the end of 5th days, 30µl of freshly prepared resazurin (0.02%, w/v) was added and incubated for o/n at 37°C. The

plates were visually scored for colour change from blue to pink. The visual observation method was used to determine % growth inhibition.

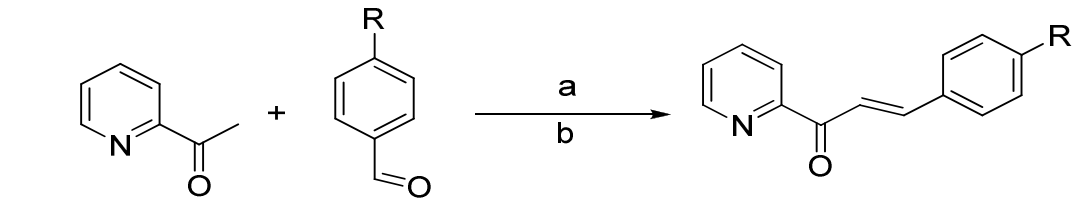
3.3.4. Statistical Analysis:

Experimental data were subjected to computer assisted statistical analysis by Graph pad prism 5 software using analysis of variance (ANOVA). The statistical significance was considered only when $p<0.05$. The IC_{50} value was determined by sigmoidal dose –response curve fitting and cell survival/viability curve was determined as log dose-response curve on this graph pad software. [24]

RESULTS AND DISCUSSION

2.1. Chemistry

Synthetic routes for chalcone derivatives are shown in scheme 1.Claisen-Schmidt condensation between 2-Acetyl pyridine and various p- substituted benzaldehyde afforded the corresponding pyridyl chalcones **(1a-1e)**.The structures of the desired compounds were determined by UV-Vis. IR, ^{1}H-NMR, mass-spectral and elemental analyses. The overall yield of all compounds exceeds 80%.



2-Acetyl pyridines p-substituted benzaldehyde Chalcone(1a-1e)

1a, R = -NO_2
1b, R = -NMe_2
1c, R = -Cl
1d, R = -OMe
1e, R = -OCH_2Ph

a: ethanolic 40% KOH,stirred
b:10% HCl, neutralised

Scheme-1

Scheme-1: Synthetic chemical pathway and reaction conditions for synthesis of compounds (1a-1e)

The I.R spectra of compounds provide valuable information about functional groups present in compounds. The IR spectrum of all substituted chalcones (1a-1e) shows a characteristic peak between 1651-1688 cm^{-1} assigned to υ(C=O) vibrations, peak between 1507-1520 cm^{-1} is due υ(C=N) vibration of pyridine ring and peak near 1582-1595 is assigned to υ(C=C) vibrations. I.R spectral data of all compounds are given in their tentative assignments.

In the ^{1}H-NMR spectra, the presence of aromatic/hetero-aromatic moiety was clearly identified by presence of in region δ 8.77-7.65 ppm. The presence of 1H, doublet in all spectra at δ 6.73-6.91 ppm is due to (-CO-CH=) proton. The presence of 1H, doublet in all spectra at δ 7.36-7.65 ppm is due to (=CH-Ar), proton. Peak near δ 5.19 (2H, singlet) in spectra of compound (1e) is due to methylene (OCH_2) group. Presence of a 3H singlet at δ 3.98 in spectra of compound (1d), a6H singlet at δ 3.05 in spectra of compound (1b) is due tois due to methoxy (-OCH_3) group and N-$(CH_3)_2$methyl group respectively. The mass spectrum of all compounds showed molecular ion peak, which is in agreement with the assigned molecular formula.

Thus, on the basis of above discussion, the structure of compounds was assigned. The ground optimized structure of chalcones 1(a) and 1(b) are shown in figure-1

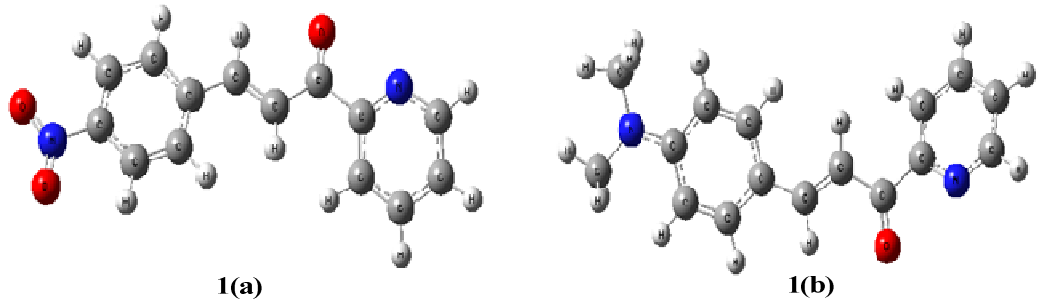
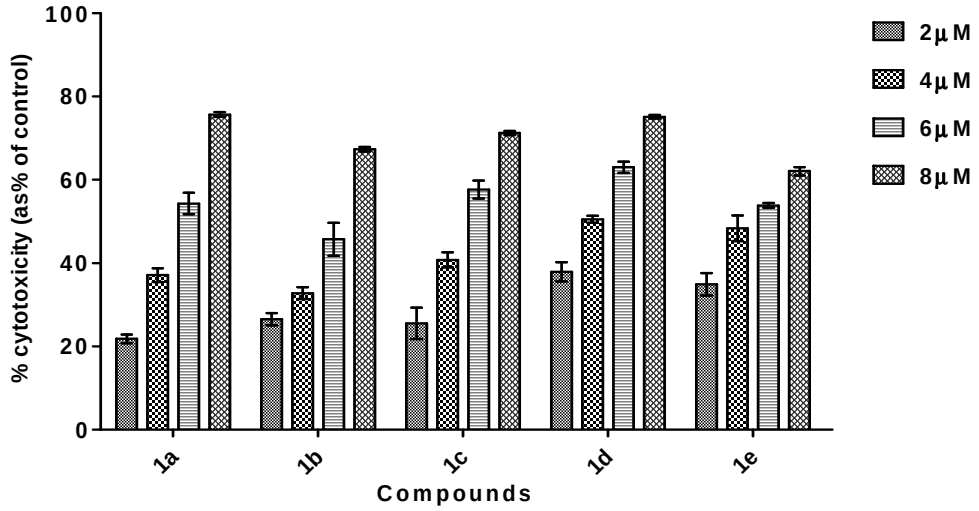
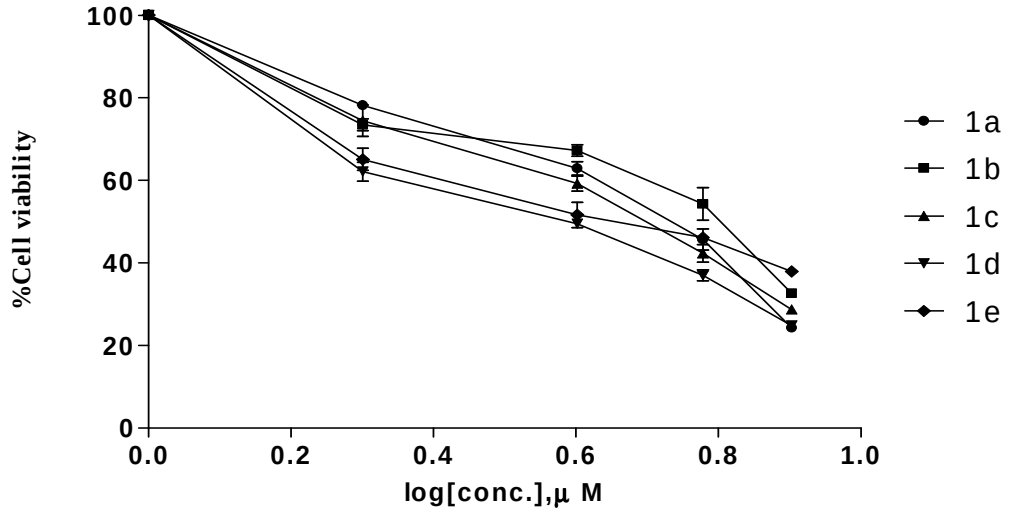


Figure-1: Geometry optimized structure of 1(a) and 1(b) using Gaussian 03(revision C0.1) at DFT/B3LYP/6-311G level



Column graph representation of % cell cytoxicity(as% of control) against lukemia K562 cell line

Figure-2: A column graph representation of % cytotoxicity(as%of control)of compounds (1a-1e) against leukemia cell line K562



log-dose vs response/survivality curve of compounds against K562 cell line

Figure-3: Log dose vs. response /viability curves of compounds (1a-1e) against leukaemia cell line K562

2.2. Cell proliferation assay

In vitro cytotoxic activity was evaluated by 3-(4,5-dimethylthiaazol-2-yl-2,5diphenyltetrazolium bromide (MTT assay), as this technique is a reliable method to determine bioactivity of compounds.[17] We also performed MTT assay in well containing 0.01% DMSO and it showed 100% cell viability suggesting no toxicity of DMSO on K562

cell lines.[18] All synthesized compounds were tested against human chronic myelogenous Leukemia cancer line K562 and shows different responses on K562 cells in culture when incubated was restricted to 48 h. The figure-2 and 3, shows effects on % cell viability (as % of control) of in vitro cultured human cancer cells incubated at 2, 4, 6 and 8µM compound concentration.

The results show that cell viability decreased with increase in concentration from 2 to 8 µM. All compounds showed significant concentration dependent cytotoxicity. These compounds are active in very low micro molar concentration with IC_{50}value ranging from **4.32-6.21µM**. Nitro substituted pyridyl chalcone 1(a) was found to have maximum cytotoxic potential with**IC_{50} 4.32 µM**, while hydroxy substituted chalcone (1e) shows minimum potential with**IC_{50} 6.21 µM.** It may be concluded from result that with increase in electron withdrawing potential of group in substitution, the cytotoxic activity of these compounds may beenhanced. The cytotoxic potencies IC_{50}data of compounds are given in table-1.

In order to know if their leukemia cancer inhibition property is exclusive of any non –specific cytotoxic activity against cell, we evaluated their cell inhibitory effect in normal epithelial kidney cell line HEK-293.All compounds were found to be almost inactive in inhibiting growth and proliferation to this normal non-cancerous cell. Results of cytotoxic activity provide only information on the restriction of the population of viable cells but not the mechanism by which restriction is observed, which is matter of further investigation.

Table-I Effect of compounds on proliferation of human chronic myelogenous cancer cell line K562 after 48 h incubation
Cytotoxic potential as [a]IC_{50}values of compounds along with their (±) standard error values.

Compounds	IC_{50} (± std. error)
1a	4.32(±0.29)
1b	6.13(±0.14)
1c	5.83(±0.32)
1d	5.94(±0.15)
1e	6.21(±0.16)

[a]50% inhibitory concentration, required to inhibit cancer cell proliferation by 50%

2.3. Anti-mycobacterial activity

The drug susceptibility studies (DST) using colorimetric methods are low cost alternatives to MGIT and BACTEC systems with good correlation (Martin et al. 2005; Martin et al. 2007; Palomino et al. 2002). [19, 20] The colorimetric indicators such as Alamar blue, MTT and resazurin have been found to have good specificity and hence resazurin was used in this study. The resazurin reduction assays using *M. smegmatis* mc^2 and *M. tuberculosis* H37Ra offers a rapid and safe screening system for antimycobacterial compounds [21].

The results from present drug susceptibility studies for inhibition of *M.smegmatis* mc^2 mainly at concentration of 50 µM, with acetate as carbon source shows inhibition up to 82% and with glycerol the growth inhibition is reduced up to about 22% only.Growth inhibition studies using against *Mycobacterium tuberculosis H37Ra*, with glycerol and acetate carbon source at concentration of 50 µM, showed inhibition only below 59 %. All the synthesized chalcones does not show much significant inhibition activities in these studies, to be considered as potential anti-mycobacterial agent.

Table –II Growth inhibition studies using Resazurin reduction assay (REMA) against *Mycobacterium smegmatis mc2*, in MB7H9 Media with glycerol and acetate as carbon source at an inhibitor concentration of 50µM
All experiments were performed in duplicates and data are reported as mean % growth inhibition

Compound	Concentration (50µM)	
	Glycerol	Acetate
1a	6.22 %	77.93 %
1b	10.01 %	77.85 %
1c	10.05 %	81.84 %
1d	21.53 %	72.91 %
1e	16.38 %	79.56 %

CONCLUSION

In the present work a series of pyridyl substituted chalcones were synthesized and their structure was confirmed by microanalysis, IR, NMR and UV/Vis. spectral studies. Obtained results clearly indicate good cytotoxic potential of these compounds are in very low micro molar range of **IC_{50} 4.32-6.21**. Antimycobacterial screening against *M.smegmatis* indicates that compounds have promising inhibition potential (up to 82%) with acetate as carbon

source in comparison to glycerol. The change in nature and position of substituted groups may enhance their biological activity up to the required level, which is a matter of further research.

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