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Synthesis and in vitro evaluation of substituted 3-Cinnamoyl-4-hydroxy-pyran-2-one (CHP) in pursuit of new potential antituberculosis agents

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Running title: Pyrones against Tuberculosis

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Abstract

Tuberculosis is an ever evolving infectious disease that demands urgent need for new drugs. In search for new antituberculosis agents, a library of *3-Cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-ones* (CHPs) (**2a-2y**) was synthesised and evaluated against standard virulent laboratory strain of *Mycobacterium tuberculosis* H37Rv. Out of 25 compounds 11, 5, 7 and 2 (**2a** and **2u)** showed least, moderate, good and appreciable activities based on minimum inhibitory concentrations (MICs). Both **2a** and **2u** exhibited an MIC value of 4μg/ml that was close to those of standard antituberculosis drugs ethambutol, streptomycin and levofloxacin. Neither **2a** nor **2u** showed any activity either against gram positive or gram negative bacteria and even against non tuberculous mycobacterium i.e. *Mycobacterium smegmatis*; thus like antituberculosis drugs rifampicin, isoniazid and pretomanid they are highly TB specific. All the pyrone based chalcones showed no recognizable level of cytotoxicity against normal human kidney cell line (HEK-293) up to 80 μM concentration and 11 exhibited an IC50 ≤ 100 μM (highest tested concentration). On further investigation, both **2a** and **2u** proved to be non toxic against four human cell lines but **2a** proved to be better choice as it did not reach IC50 even at 100 μM (highest tested concentration) while IC50 of **2u** was around 80 μM. In conclusion our results demonstrate that **2a** is specifically against *M.tuberculosis* with no appreciable toxicity and its activity matches with few clinically approved antituberculosis drugs and therefore it merits further evaluation.

Key Words: Pyrone, Chalcones, Cinnamoyl pyrone, *Mycobacterium tuberculosis*, Tuberculosis drug discovery, Structure activity relationship

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Tuberculosis (TB) is an old but ever evolving infectious disease that still poses a huge threat to the human global population[1]. About one third of the world's population is harbouring the causative agent *Mycobacterium tuberculosis (M.tuberculosis)* as asymptomatic latent TB infection (LTI)[2]. The chances of reactivation to active TB disease are higher in HIV infected and other immune compromised patients[3]. HIV-TB co-infection is a deadly setback as multitude of complications makes the concurrent treatment of HIV-TB more complicated. These include drug-drug interactions[4], overlapping toxicity[5], non-adherence[6] and TB-associated immune reconstitution inflammatory syndrome (IRIS)[5]. Another major challenge is the drug resistance. Although current first-line anti-TB drug regimen can achieve more than 95% efficacy, this is often reduced owing to the emergence of drug resistant strains of *M.tuberculosis*[7]. This demands urgent need for new drugs[8]. Considering the failure rates during discovery phase and clinical trials, the search of new compounds with low cytotoxicity and better efficacy is a daunting scientific challenge[9]. Therefore it seems intellectually wise to take forward the core scaffolds that have already acclaimed some clinical relevance in humans.

In this direction, *pyran-2-one* (2P) represents the core structural subunit of a number of biologically significant natural and synthetic compounds[10]. On one hand, *pyran-2-one* based scaffolds have received FDA approval against HIV and on the other hand have displayed appreciable level of anti-TB activity. (+)*Calanolide A* isolated from *Calophyllum lanigerum*[11] is a first *pyran-2-one* based anti-HIV agent that significantly inhibited the growth of *M.tuberculosis* H37Rv[12]. In particular, *4-hydroxy-pyran-2-one* (4-HP) represents the active component of naturally isolated anti-TB agents like (*Pseudopyronine B*[13] and *Myxopyronin*[14]) and clinically approved anti-HIV agent like *Tipranavir*[15] (**Fig. 1**).

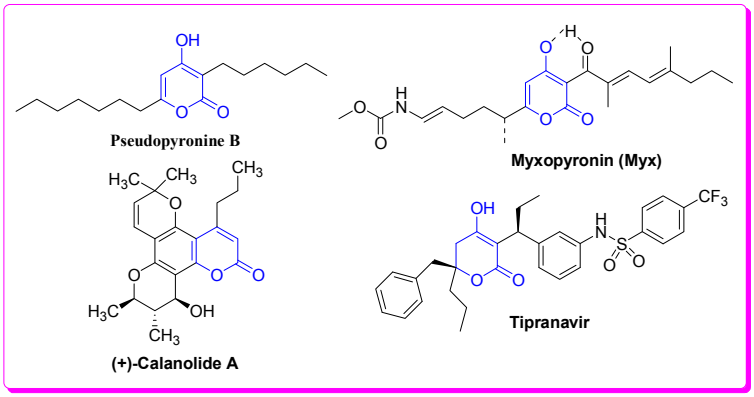

Fig. 1: **2-Pyrone based anti-tuberculosis and anti-HIV agents.**

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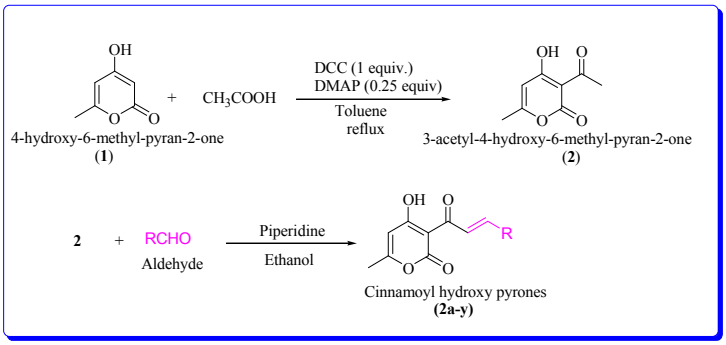
Another class of compounds that are of great interest to medicinal chemists are *chalcones (1,3-diaryl-2-propene-1-ones)* due to their simple chemistry, ease of synthesis and broad spectrum of pharmacological activities against major human diseases like cancer[16], inflammation[17], malaria[18], HIV[19–21] and TB[22–28].The anti-TB activity of chalcones is attributed to their lipophilic nature that allows them to easily penetrate the mycobacterial cell wall [29,30].

Interestingly, a class of *Cinnamoyl pyrone* (CP) based compounds exists that contains both pyrone moiety (4-HP scaffold) as aromatic ring A and cinnamoyl moiety as ring B, referred to as *3-Cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-ones* (CHPs). Till date, the anti-HIV[31] and anticancer[32] potential of CHPs is documented but no report about their anti-tuberculosis potential exists so far. Considering the fact that both pyrones and chalcones have shown some anti-TB potential and have attained some clinical relevance on one hand and the need for new antituberculosis drug on the other hand this study was aimed at synthesising CHPs and exploiting them for TB drug discovery. To the best of our knowledge the results of this study show for the first time promising antituberculosis potential of CHPs against *M.tuberculosis*.

A general synthesis of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one derivatives **2a-2y** is exhibited in **Scheme 1**.



Scheme 1: Synthesis of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one derivatives.

3-acetyl-4-hydroxy-6-methyl-pyran-2-one (**2**) was synthesized from 4-hydroxy-6-methyl-pyran-2-one **(1)** using acetic acid, DCC and DMAP in toluene under reflux in almost quantitative yield. The targeted cinnamoyl pyrones **(2a-y)** were synthesized by reacting 3-acetyl-4-hydroxy-6-methyl-pyran-2-one (**2**) with specific benzaldehyde in ethanol using catalytic piperidine under reflux in good to excellent yield (**Table 1**). Structure of all the synthesized compounds was

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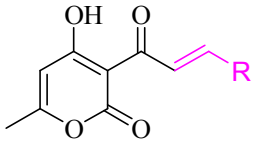
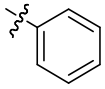
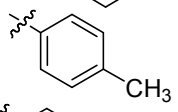
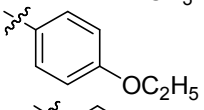
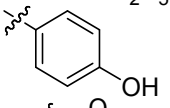
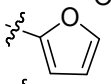
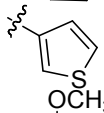
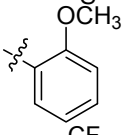
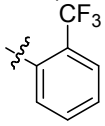
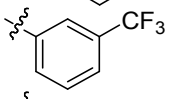
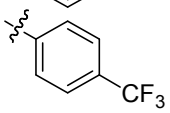
characterized by using ^{1}H and ^{13}C NMR. For example the ^{1}H NMR spectrum of **2a** exhibited two doublet signals at 8.30 ppm (d, J = 15.7 Hz, 1H) and 7.94 ppm (d, J = 15.8 Hz, 1H), corresponding to β and α-H of enone C=C respectively. The peaks of 7.67 ppm (dd, J = 6.5, 2.8 Hz, 2H), 7.39 ppm (dd, J = 11.3, 7.7 Hz, 3H) are attributed to five aromatic benzene C-H. The proton of C5-H of pyrone ring appeared as singlet at 5.94 ppm. Similarly C6-methyl group showed a broad singlet at 2.26 ppm.

Table 1: Library α-pyrone based substituted 3-cinnamoyl-4-hydroxy-6-methyl-2-pyrone derivatives (2a-2y) and their MIC values against *Mycobacterium tuberculosis* H37Rv and *M.smegmatis*.



Cinnamoyl hydroxy pyrones
(2a-y)

S.No	Code	%Yield	Structure (R)	*M.tuberculosis** MIC (µg/ml)	*M.Smegmatis*** MIC (µg/ml)
1	**2a**	85		**4**	>128
2	2b	81		8	>128
3	2c	79		256	>128
4	2d	80		64	>128
5	2e	78		16-32	>128
6	2f	82		32-64	>128
7	2g	77		32-64	>128
8	2h	80		8	>128
9	2i	81		256	>128
10	2j	83		256	>128

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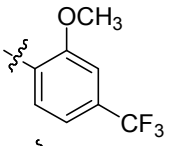
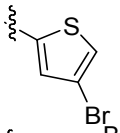
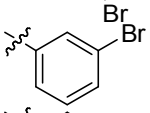
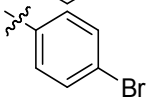
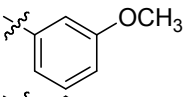
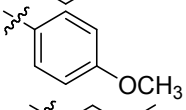
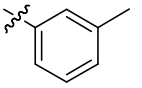
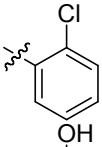
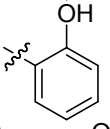
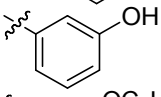
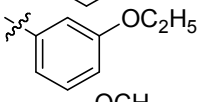
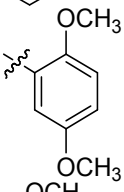
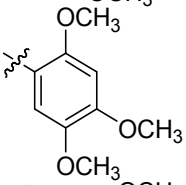
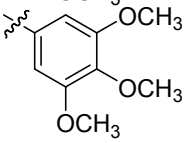
11	2k	79		256	>128
12	2l	78		4-8	>128
13	2m	80		4-8	>128
14	2n	77		8-16	>128

S.No	Code	%Yield	Structure (R)	*M.tuberculosis** MIC (µg/ml)	*M.Smegmatis*** MIC (µg/ml)
15	2o	81		8-16	>128
16	2p	82		32	>128
17	2q	79		32	>128
18	2r	82		16	>128
19	2s	82		8-16	>128
20	2t	78		8-16	>128
21	**2u**	83		**4**	>128
22	2v	80		4-8	>128
23	2w	81		8	>128
24	2x	83		8	>128

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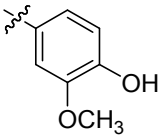
25	2y	80		32	>128

*Antituberculosis drugs RIF, INH, EMB, STR and LVX showed MICs values of 0.078, 0.312, 2.5, 1.25 and 2.5µg/mL µg/ml respectively against *M. tuberculosis*. ** LVX served as positive control against *M. smegmatis* as RIF and INH have been reported to show 500 and 1000 fold higher MIC values against *M. smegmatis* (100 and 25 mg/ml) compared to those of *M. tuberculosis* (0.2 and 0.02µg/mL) respectively and therefore can't act as controls. LVX exhibited an MIC of 2.5 µg/ml against *M. smegmatis*.

Antimicrobial susceptibility testing (AST) was done by determining minimum inhibitory concentration (MIC) of antimicrobial agents and test compounds by 7H9 broth microdilution assay as discussed previously with slight modifications[33]. Briefly, Middlebrook 7H9 broth with 10% ADC (albumin, dextrose and catalase) containing the CHP based test compounds in concentration range (0.25-128.0 µg/ml) were added in duplicate in 96 well titer plates (Nest Biotech, China). Each well was inoculated with 50 µl of appropriately diluted mid log phase *M.tuberculosis* H37Rv culture corresponding to ~1×10^5 CFU/ml. Isoniazid (INH), Rifampicin (RIF), ethambutol (EMB), streptomycin (STR) and levofloxacin (LVX) were used as positive controls and drug free broth served as negative control respectively.

Original CFU were verified by plating serial 10 fold dilutions of the inoculum onto Middlebrook 7H11 agar plates that were incubated at 37°C and read after four weeks. The minimum inhibitory concentration (MIC) was taken as the lowest concentration (µg/ml) of an antibiotic that inhibits the visible growth after two weeks of incubation. MIC against *M. smegmatis* was done as above but reading was taken only after 72 hrs owing to its fast growing nature.

MICs of standard antituberculosis drugs RIF, INH, EMB, STR and LVX were found to be 0.078, 0.312, 2.5, 1.25 and 2.5µg/mL respectively against *M. tuberculosis* H37Rv. MIC results of all the 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-one (CHP's) derivatives (**2a-2y)** against *M. tuberculosis* are presented in **Table 1**. Among 25 derivatives 11 did not show any attractive activity i.e. their MIC values were ≥ 32 µg/mL. Five compounds (**2n**, **2o**, **2r**, **2s** and **2t**) showed moderate antituberculosis activity with MIC values ranging from 8-16 µg/ml (Table 1**)**. Seven compounds (**2b**, **2h**, **2l**, **2m**, **2v, 2w** and **2x**) showed good anti-TB potential reflected by their MIC values ranging from 4-8 µg/ml (Table 1**)**. Two compounds (**2a** and **2u**) displayed excellent antituberculosis activity against *M.tuberculosis* with an MIC value of 4 µg/ml (Table 1**)**. This

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MIC value is close to those of standard antituberculosis drugs EMB, STR and LVX, thereby reflecting promising antituberculosis potential of these two compounds that strongly merits further evaluation. This significant antituberculosis potential can be due to 2-pyrone based polyketides which represent a diverse class of secondary metabolites with crucial roles in *M.tuberculosis*. Indeed a putative chalcone synthase (PKS11) and a tri-ketide as well as tetra-ketide pyrone synthase (PKS18) in *M.tuberculosis* have been characterized that synthesize a unique cyclic 5-methyl-6-alkyl-4-hydroxy-2-pyrone (MAHP) via various intermediates. These small polyketide molecules are finally incorporated in *M.tuberculosis* cell wall where they are perceived to regulate permeability [34-35]. It is one of the ways by which pyrone based molecules attack *M. tuberculosis*. Additionally pyrones have been shown to inhibit *M. tuberculosis* translation like rifampicin but even in rifampicin resistant strains also. So there are experimental indications how these molecules might work against *M. tuberculosis*. Further reasons for this anti-TB activity include their chalcones side which owing to its lipophilic nature easily penetrates mycobacterial cell wall and hence causes the damage [29-30]. It is to be noted none of the molecule exhibit appreciable activity against *M. smegmatis* (Table 1).

Based on our experimental results, the structure-activity relationship (SAR) of this class of anti-TB agents with respect to benzene ring can be summarised as follows. **2a** being the most potent molecule, it can be concluded that substitution of any sort in the benzene ring leads to decrease in activity. Though $–OC_2H_5$ group is well tolerated but the position of the same is very important since its presence at para position proved to be detrimental for activity. Though the presence of halogens and hydroxyl groups is tolerated, electron withdrawing groups like $-CF_3$ totally proved unfavourable for activity. In general presence of alkyl and alkoxy groups also decrease the potency of the molecules. Additionally replacement of phenyl ring with heterocyclic rings like furan and thiophene also decreased antituberculosis activity.

Since first line antituberculosis drugs are specifically active against *M.tuberculosis* only and exhibit very poor activity against other microbes, we therefore evaluated the most two potant CHPs (**2a** and **2u**) against four Gram positive bacteria (*Staphylococcus aureus* (*ATCC 25923*), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 10240), *and Bacillus subtilis* (ATCC 11774)) and four Gram negative (*Escherichia coli* (ATCC 10536), *Enterococcus faecalis* (ATCC 51299), *Pseudomonas aeruginosa* (ATCC 10145), *Klebsilla pneumonia* (ATCC BAA-2146)). Strains were revived according to written instructions, platted onto Muller Hinton

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Agar (MHA) and incubated at 37 °C for 24 h. The MIC was determined by previously described method with minor modification using MH broth and reading was taken after 24 h of incubation at 37 °C[36]. The second line anti-TB drug levofloxacin (LVX) served as positive control owing to its broad spectrum of activity. LVX exhibited MICs values of 0.078-0.156, 0.078, 0.039-0.078, 0.39, 0.078, 0.312-0.625, 0.156 and 0.625-1.25µg/ml against *S. aureus*, *B. subtilis*, *M. luteus*, *S. epidermidis*, *E. coli*, *P. Aeruginosa*, *E. faecalis* and *K. Pneumonia* respectively; these MIC values of LVX are well in agreement with reported ones (Table 2). MIC values of 2a and 2u observed in this study are presented in Table 2. None of them exhibited any significant activity against gram positive bacteria: *S. aureus* and *B. subtilis*, *S. epidermidis* and *M. luteus* gram negative bacteria: *E. coli*, *E. faecalis*, *K. pneumonia* and *P. Aeruginosa* (Table 2). Full visual turbidity in 96-well plate was observed even up to the highest tested concentration i.e. 128 µg/ml for all compounds against each bacteria. Our results clearly show that 2a and 2u exhibit unique activity specifically against *M.tuberculosis*. This observation is of great significance for developing CHP based antituberculosis drug as it is less likely to interfere with normal human flora.

Table 2: Activity of lead compounds (2a and 2u) against Gram negative and Gram positive bacteria

Type of bacteria	Bacterial strain	MIC (µg/ml)		
		2a	2u	LVX [a]
Gram positive	*S. aureus*	>128	>128	0.078-0.156
	S. epidermidis	>128	>128	0.039-0.078
	M. luteus	>128	>128	0.078
	B. subtilis	>128	>128	0.39
Gram negative	*E.coli*	>128	>128	0.078
	E. faecalis	>128	>128	0.156
	P. aeruginosa	>128	>128	0.312-0.625
	K. pneumonia	128	128	0.625-1.25

[a] LVX means standard broad spectrum antibacterial as well as anti-TB drug that served as positive control

Among the critical factors that allow any molecule to proceed successfully during drug discovery process is its selective toxicity to words pathogenic microbes and non/less toxicity on mammalian cells as it's by this character activity and toxicity get separated and therapeutic index becomes acceptable. We therefore evaluated cytotoxicity of CHP based derivatives in concentrations ranging from 1 to 80 µM (1µM, 20µM, 40µM and 80µM) against normal human

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kidney (HEK-293) cell line which was purchased from National Centre for Cell Science (*NCCS*), Pune, India. This was done on the basis of percentage growth inhibition assessed by MTT assay. Over all compounds showed lesser growth inhibition of HEK-293 cells up to 80 μM concentration and thus CHPs were found to be non toxic at concentrations that are usually high end concentrations for such studies. We therefore evaluated all compounds at much higher concentration i.e. at 100 μM. 11 compounds (**2x, 2w, 2v, 2u, 2q, 2p, 2n, 2j, 2k, 2l, and 2m**) inhibited HEK cell growth by 50 or >50 % at this highest tested concentration and thus exhibited an IC50 value of 100 μM. Five compounds (**2b, 2i, 2r, 2t and 2y**) showed 45- <50% inhibition at 100 μM, thus though their IC50 is > 100 μM but will be close to this value. Nine compounds did not even inhibit 45% growth at highest experimental concentration and thus their 1C50 is clearly > 100 μM, demonstrating them to be safe for human cells.

Toxicity of the two most potent compounds with respect to antituberculosis activity **2a** and **2u** (MIC = 4 μg/ml) was further evaluated at a concentration range (0.0- 100 μM) against four human cell lines those include HEK-293, breast cancer (MCF-7), colon cancer (HCT-116) and prostate cancer (PC-3) by double dilution method using MTT cell viability assay. MCF-7, HCT-116 and PC3 cells were obtained from National Cancer Institute (NCI), USA. The results of this study as mean± standard deviation of percentage growth inhibition are presented in **Figure 2 i-iv**. Both these compounds **2a** and **2u** showed some dose dependent growth inhibition response against all cell lines (Figure 2 i-iv). **2a** at the highest experimental concentration inhibited < 50% (38-44%) growth of three cell lines HEK-293, MCF-7 and PC3; inhibited 50% of growth of HCT-116 cell line at the same concentration (Figure 2 i-iv). In contrast **2u** inhibited 67-70% growth of all the cell lines tested at the same concentration and its IC_{50} appeared to be 80 μM against all cell lines (Figure 2 i-iv). Therefore **2a** displayed crystal clear advantage over **2u** with respect to toxicity profile. Further for **2a** MIC is 4 μg/ml against *M.tuberculosis* and only 40-50% inhibition was observed at 100 μM (25.6μg/ml); this demonstrates its broad therapeutic window. From antituberculosis activity to cytotoxic studies, it is crystal clear that compound **2a** is the choice for future studies.

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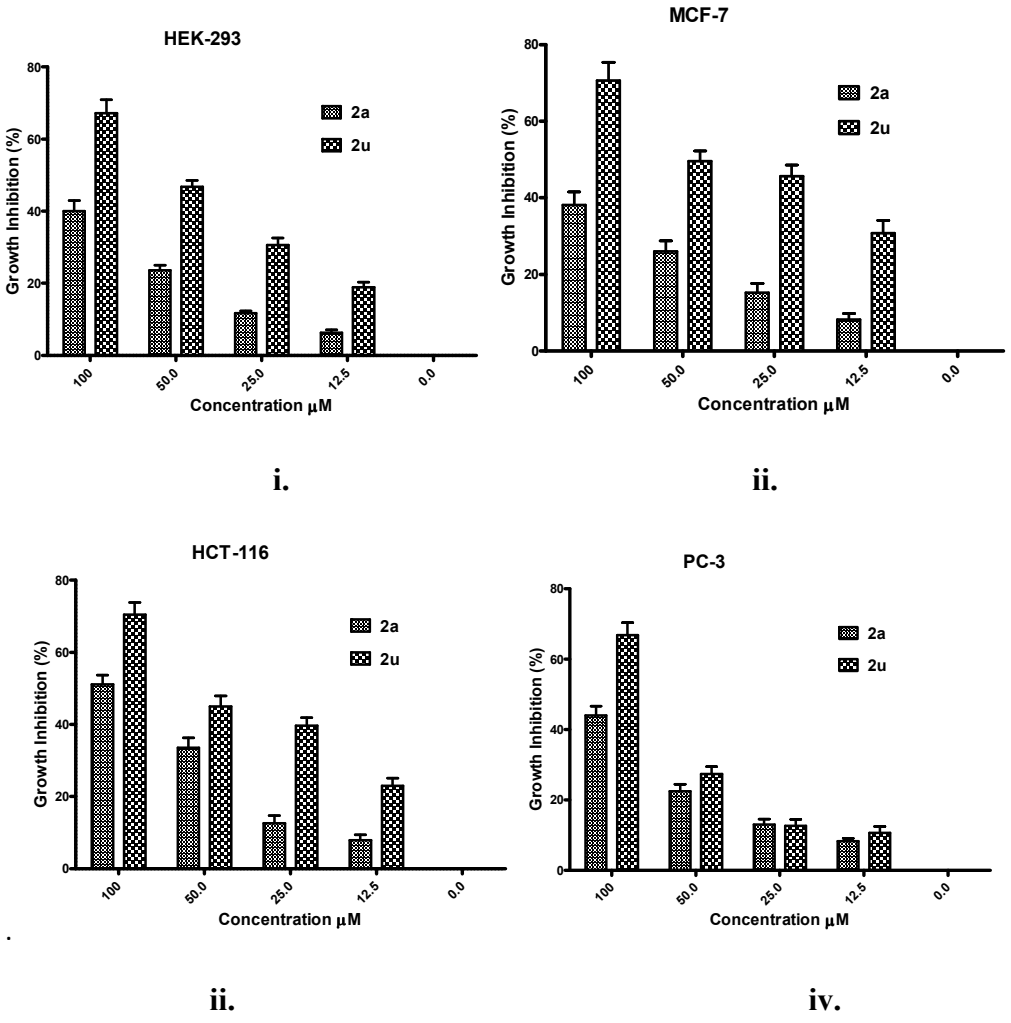
Figure 2: Cytotoxicity profile of 3-cinnamoyl-4-hydroxy-6-methyl-2H-pyran-2-ones (2a and 2u) expressed as percentage growth inhibition.



i) normal human embryonic kidney (HEK-293); **ii)** breast cancer (MFC-17); **iii)** colon cancer (HCT-116) and **iv)** prostate cancer (PC3) cell. Values are mean ± SD of three sets

Conclusion

Out of 25 compounds subjected to antimycobacterial susceptibility testing, two compounds displayed potent anti-TB activity (**2a** and **2u**) that matches to those of few known antituberculosis drugs. **2a** and **2u** were found to be ineffective against a panel of gram-positive, gram-negative and even against *M. smegmatis*, reflecting thereby their specific antituberculosis activity as reported for some antituberculosis drugs. Further cytotoxicity data favoured significantly **2a** over **2u**. The structure activity relationship reveals that substitution of any sort in

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the benzene ring leads to decrease in activity.Undoubtly therefore **2a** merits further evaluation with respect to its anti-TB potential.

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Conflict of Interest: All authors declare that there is no conflict of interest.

Ethical approval: This article does not contain any study involving animals

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