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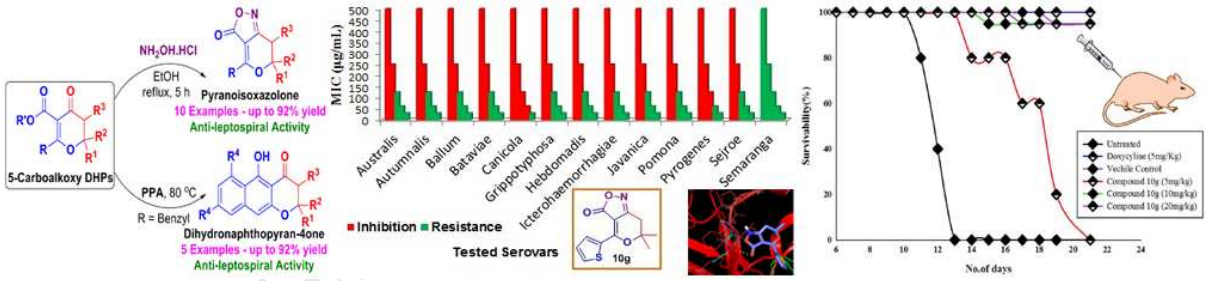
Andivelu Ilangovan,[a*] Palaniappan Sakthivel,[a] Karikalacholan Sivasankari,[b] Charles Solomon Akino Mercy,[b] Kalimuthusamy Natarajaseenivasan[b]

[a]School of Chemistry, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India

[b]Division of Medical Microbiology, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli (T.N) – 620024, India

[*]E-mail: ilangovanbdu@yahoo.com

Graphical Abstract



Discovery of 6,7-Dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones as a New Class of Pathogen Specific Anti-leptospiral Agents

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[a]School of Chemistry, Bharathidasan University, Tiruchirappalli – 620 024, Tamil Nadu, India

[b]Division of Medical Microbiology, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli (T.N) – 620024, India

[*]E-mail: ilangovanbdu@yahoo.com

Abstract

A simple and efficient method for the synthesis of a series of 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one derivatives starting from 5-carboalkoxy-2,3-dihydropyranone (5-CDHPs) has been developed. Pyranoisoxazolones **10a-j**, dihydronaphthopyran-4-one (DHNPs) class of natural product **12b** and **12c** and its analogues **12a** and **13a-c** were preliminarily screened against pathogenic leptospiral serovar Autumnalis strain N2 at various concentrations. Six pyranoisoxazolones, **10b**, **10d**, **10f**, **10g**, **10i** and **10j** which displayed very good anti-leptospiral activity was taken for secondary screening against twelve strains of pathogenic and one non-pathogenic leptospiral serovars. While all the compounds displayed significant anti-leptospiral activity against the pathogenic serovars at MIC of 62.5-500 μg/mL. Compounds **10d**, **10g** and **10j** did not show any significant effect on non-pathogenic serovar. Inhibition of leptospires at a significant level by pyranoisoxazolone **10g** was confirmed using RT-qPCR assay. *In vivo*

treatment of BALB/c mice with compound **10g** revealed that, it has 95% survivability against the pathogenic strain Canicola and also showed inhibition of renal colonization of leptospires. Compound **10g** was found to show cytotoxicity against THP-1 cells only at higher concentration (≥75 µg/mL). Effective binding of compound **10g** with leptospiral outer membrane protein LipL32 observed *via in silico* molecular docking provided a suitable explanation for pathogen specificity of compound **10g**. Antibiotics acting against leptospirosis in human are very few. The results obtained from *in vitro*, *in vivo* and *in silico* study reveals that 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones class of compounds are lead molecules for further development as pathogen specific anti-leptospiral agents.

Keywords: 5-Carboalkoxy-2,3-dihydropyranone, 6,7-Dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one, Anti-leptospiral, Pathogen-specific, LipL32.

1. Introduction

Leptospirosis is a widespread and emerging infectious disease caused by the spirochete bacteria of the genus *Leptospira* [1,2]. More than 5,00,000 cases of severe leptospirosis, with fatality rates exceeding 10-20%, are reported worldwide each year [3]. The annual morbidity of leptospirosis was estimated to be higher in developing countries of South Asia and Southeast Asia, especially in India [4]. Leptospirosis spreads either through direct and indirect contact with water or soil contaminated by the urine of infected rodents or animals [5]. Several animals such as sheep, goats, dogs, swine, horses, zebrafish, pigs, raccoons, rodents, equine, buffalo and cattle play a role as reservoir hosts [6] for leptospirosis and some of them are also affected. It is associated with various clinical symptoms such as sudden onset of febrile illness, chills, headache, myalgia, abdominal pain and conjunctival suffusion. Inappropriate treatment of acute

febrile disease leads to hepatic and renal dysfunction and hemorrhagic disorders [2,7,8]. Prevention of the infection by controlling environmental factors is difficult to implement and has considerable socio-economic implications in developing countries.

Vaccination and chemoprophylaxis are two major possible control measures for preventing the host from leptospirosis. Vaccines for serovars like Hardjo, Pomona, Canicola, Grippotyphosa, and Icterohaemorrhagiae have been developed, however they are associated with disadvantages like suboptimal protection, requirement of booster doses and failure against local serovars [1,9]. Immunization of humans with killed and attenuated whole cell vaccine (bacterins) has generally been restricted to individuals in high-risk epidemics [10]. Currently leptospirosis in human is being treated by using antibiotics such as erythromycin (**1**), amoxicillin (**2**), ceftriaxone (**3**), doxycycline (**4**), penicillin G, cefotaxime, azithromycin and ampicillin (Figure 1) which are originally developed for other bacterial infections [11-13]. Furthermore, anti-leptospiral activity of only a few other classes of organic molecules such as quinoxaline derivatives [14], xanthones [15], azomethines of aryl oxazole [16] and pseudo-peptides [17] were studied, however it did not provide useful leads. Assessment of previous clinical trials and treatment revealed unsatisfactory benefit and safety of antibiotics against leptospirosis in human [18]. Till now, there is no approved drug, specifically designed to target leptospiral bacteria life cycle in human is available. Anti-leptospiral drug used in animals leptospirosis are chlortetracycline (**5**), neomycin sulphate (**6**) and oxytetracycline. Leptospiral resistance to available antibiotics is an emerging public health threat. Therefore, the present situation emphasizes the need to develop new anti-leptospiral drugs.

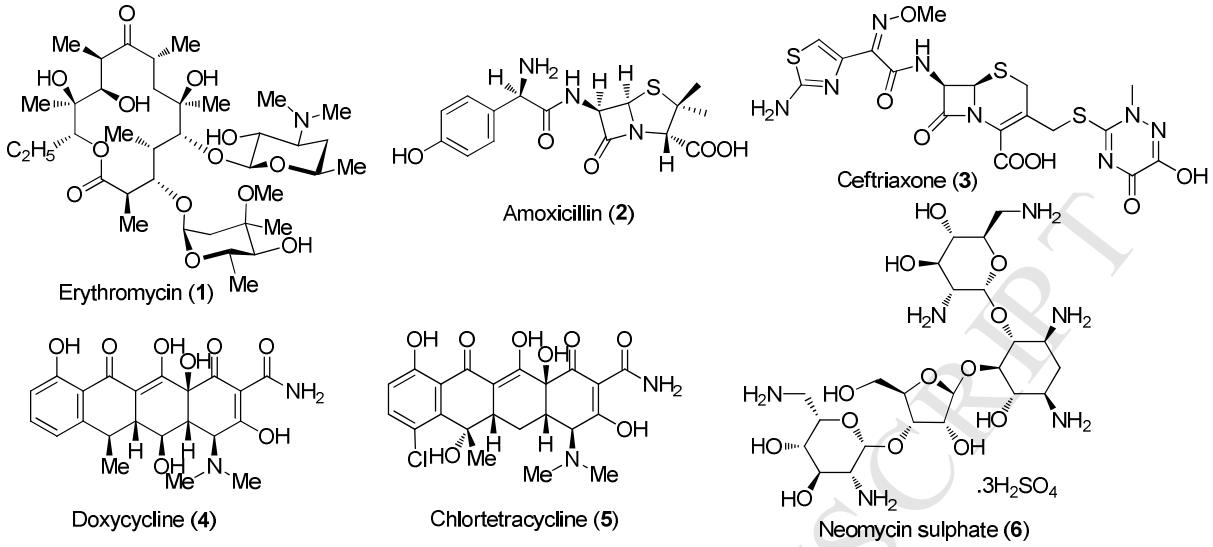


Fig. 1. Antibiotic drugs used for treatment of leptospirosis

Compounds with pyran structural motif has attracted interest of chemists owing to their anti-inflammatory [19], anti-cancer [20], anti-HIV [21], anti-Alzheimer's [22], myorelaxant [23], sex pheromones [24] and anti-microbial [25,26] activities. On the other hand, isoxazole derivatives are important pharmacophores used extensively in the field of medicinal chemistry. Isoxazole derivatives exhibit various biological activities such as, anti-bacterial, anti-convulsant, anti-cholestermic, anti-cancer, anti-inflammatory, adenosine antagonist, fungicidal, herbicidal, hypoglycemic, muscle relaxant, nematocidal, insecticidal, anti-viral and anti-microbial activities [27-29]. Although different biological activity [20,30,31], including anti-bacterial activity [32-35], of different pyrano fused hetereocycles were studied, to the best of our knowledge, biological activity of pyranoisoxazolone derivatives has not yet been examined. This literature background reveals that pyrans, isoxazoles and pyrano fused hetereocycles display very good anti-bacterial activity. In continuation of our experience on utilization of 5-carboalkoxy-2,3-dihydro-4*H*-pyran-4-one derivatives (5-CDHPs) [36], we envisaged that the compounds could be

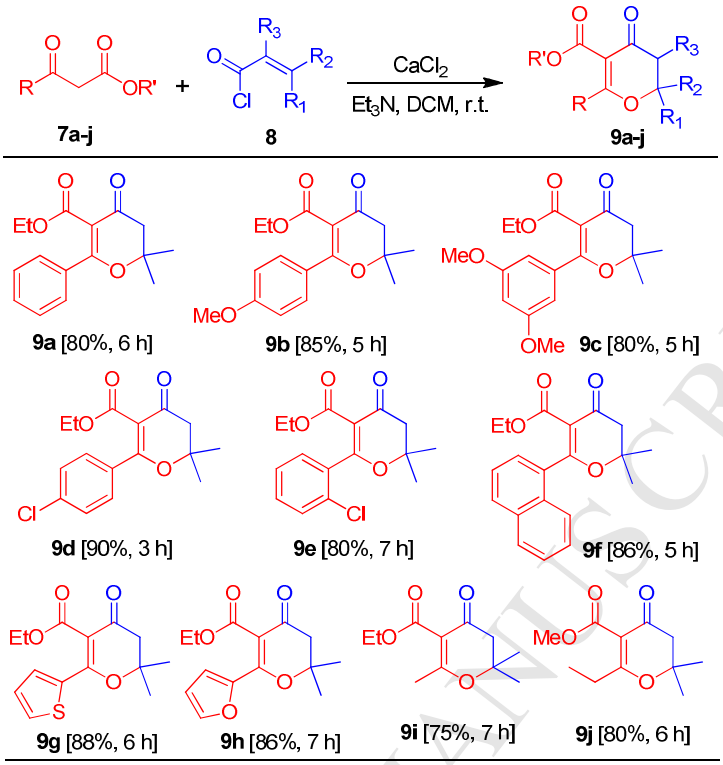
converted in to novel pyranoisoxazolone derivatives **10a-j**, which may exhibit interesting anti-bacterial *viz*., anti-leptospiral activity.

Furthermore, naphthalene fused pyran-4-one ring systems are known to exhibit different biological activities including anti-bacterial activity [37-39]. We recently reported total synthesis of phytogrowth-inhibition active dihydronaphthopyran-4-one natural products **12b** and **12c** and its analogues **12a** and **13a-c** (Scheme 3) [36]. Interestingly, compounds **12** and **13** and the drug doxycycline (**4**, Figure 1) were found to have common structural features such as the presence of ketone and phenolic functional groups, intramolecular hydrogen bonding and linear polycyclic system. This prompted us to investigate anti-leptospiral activity of compounds **12** and **13**. Thus, in continuation of our interest on study of biologically active organic molecules [40-41] herein, we report synthesis of a variety of pyranoisoxazolones **10a-j** and dihydronaphthopyran-4-one (DHNP) derivatives **12** and **13** and evaluation of their anti-leptospiral activity.

2. Results and Discussion

2.1. Chemistry

Pyranoisoxazole derivatives are usually made through dipolar cycloaddition using nitrile oxides or nitrones [42-44], multi-component reaction [45], and other methods [46-48]. Under the present study, we observed that a variety of pyranoisoxazolones **10** could be made from 5-CDHPs **9** in one pot by treating it with hydroxyl amine in ethanol. The starting materials, 5-CDHPs **9a-j** [36] and two new DHPs **9c** and **9f** were synthesised by adopting the procedure reported by us [36]. Domino C-acylation of β-ketoester **7** with α,β-unsaturated acid chlorides **8** in the presence of $CaCl_2$ (Scheme 1) followed by *in situ* 6π-oxaelectrocyclization resulted in the formation of 5-CDHPs **9**.

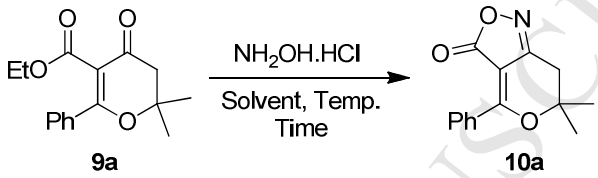


Scheme 1. Synthesis of 5-carboalkoxy-2,3-dihydro-4*H*-pyran-4-ones: *Reaction conditions*: **7** (1.0 mmol), **8** (1.0 mmol), $CaCl_2$ (0.1 mmol), triethylamine (2.0 mmol) in DCM (5 mL) at room temperature. Isolated yield.

Having synthesized variety of 5-carboalkoxy DHPs **9**, as a next step ethyl 2,2-dimethyl-4-oxo-6-phenyl-2,3-dihydro-4*H*-pyran-5-carboxylate (**9a**) was taken as model substrate, and examined its conversion to pyranoisoxazolone **10** by treatment with hydroxylamine hydrochloride in different solvents and temperature conditions. The results obtained are summarized in Table 1. With 1.0 equiv. of hydroxyl amine in ethanol (entry 1) the reaction at r.t. failed. However, after refluxing the reaction for 15 h, the desired product **10a** was obtained in 70% yield (entry 2). Pleasingly, the yield of the product **10a** increased to 90% when 2.5 equiv. of hydroxyl amine was used (entry 3). Further, when MeOH and *t*-BuOH was used as the solvent,

at reflux temperature, the product **10a** was obtained in 70% and 80% respectively (entry 4 and 5). Based on these results the optimized condition for further study was identified as treatment of 1.0 equiv. of 5-CDHP derivatives **9** with 2.5 equiv. of hydroxylamine hydrochloride in ethanol at reflux temperature.

Table 1. Optimisation of the reaction conditions[a]



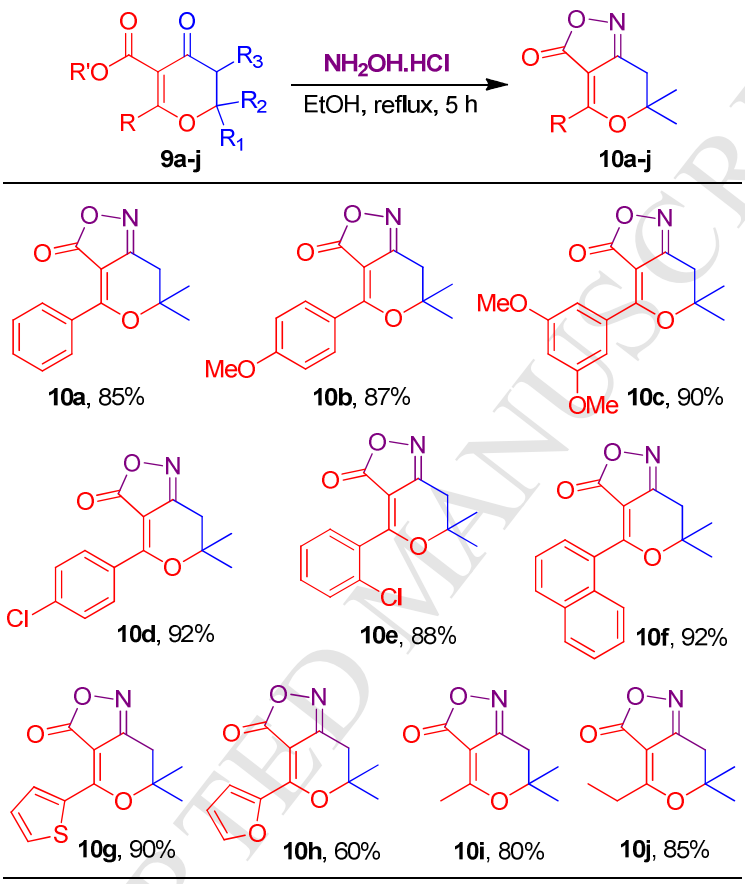
9a **10a**

Entry	$NH_2OH.HCl$ (eq.)	Solvent	Temp (^oC)	Time (h)	Yield (%)[b]
1.	1.0	EtOH	r.t.	15	NR[c]
2.	1.0	EtOH	reflux	15	70
3.	**2.5**	**EtOH**	**reflux**	**7**	**90**
4.	1.0	MeOH	reflux	10	70
5.	1.0	*t*-BuOH	reflux	7	80

[a]All reactions were performed using ethyl 2,2-dimethyl-4-oxo-6-phenyl-2,3-dihydro-4*H*-pyran-5-carboxylate (**9a**) (1.0 mmol) and hydroxylamine hydrochloride (2.5 mmol); [b]Isolated yield; [c]No Reaction.

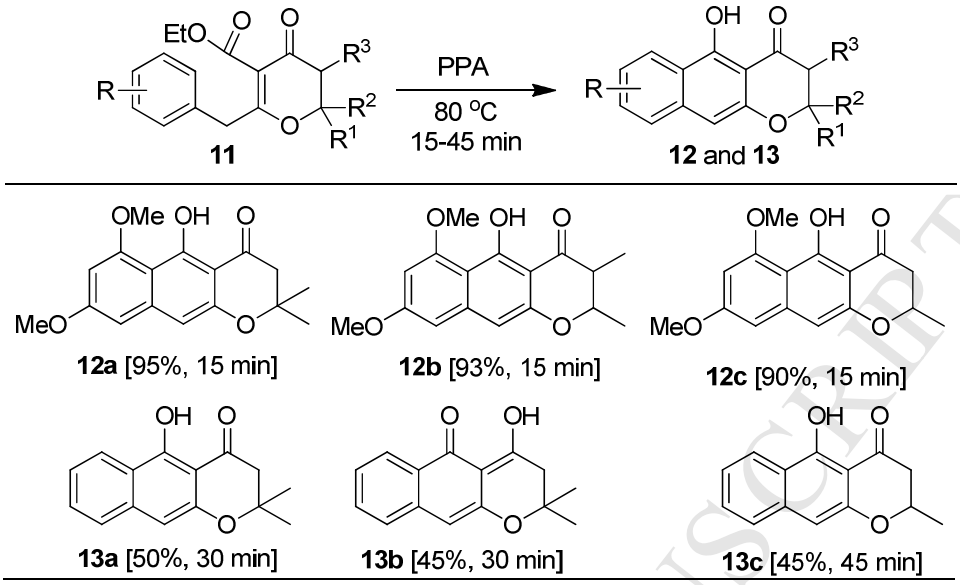
Further, different 5-CDHP **9** were treated under the optimised condition, to deliver the desired products **10** in good to excellent yield (Scheme 2). Different functional groups such as methoxy (**10b** and **10c**), chloro (**10d** and **10e**) and naphthyl (**10f**) tolerated the reaction condition well. Moreover, the easily polymerisable thienyl (**9g**) and furyl (**9h**) containing 5-CDHPs gave

corresponding pyranoisoxazolones **10g** and **10h** in good and moderate yield respectively. Aliphatic substrates **9i** and **9j** also underwent reaction to give the desired product **10i** and **10j** respectively in good yield.



Scheme 2. Synthesis of substituted 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones: *Reaction conditions*: DHPs **9** (1.0 mmol), $NH_2OH.HCl$ (1.5 mmol) in 5 mL of EtOH at 25 °C for 5 h. Isolated yield.

The DHNP analogues **12a-c** and **13a-c** required for the present study were synthesised (Scheme 3) in a concise and efficient way by making use of Friedel-Crafts acylative aromatization of 6-benzyl-5-CDHPs **11** reported by us earlier [36].



Scheme 3. Synthesis of dihydronaphthopyran-4-one natural products and its analogues

2.2. Evaluation of Biological Activity

2.2.1. Preliminary Screening of 6,7-Dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones (10a-j) and Dihydronaphthopyran-4-ones (12 and 13)

Having synthesised a series of pyrano[4,3-*c*]isoxazol-3-ones, **10a-j** and dihydronaphthopyran-4-ones **12a-c** and **13a-c**, preliminary screening against pathogenic leptospiral serovar Autumnalis strain N2 was carried out at different concentrations (500, 250, 125, 62.5 and 31.25 µg/mL, Table 2) by micro dilution assay. Doxycyline (**4**, Figure1) was used as reference. Out of the ten pyrano[4,3-*c*]isoxazol-3-ones, five compounds such as **10b**, **10d**, **10f**, **10g** and **10j** showed inhibition at lower concentration of 250 µg/mL itself whereas, **10i** showed inhibition only at 500 µg/mL. Autumnalis strain N2 was originally isolated from a human case of leptospirosis earlier. Interestingly, most of these compounds were found to show activity at concentration same as doxycyline.

Table 2. Preliminary screening of pyrano[4,3-*c*]isoxazol-3-ones **(10a-j)** and dihydronaphthopyran-4-ones (**12a-c** and **13a-c**) against *Leptospira interrogans* serovar Autumnalis strain N2

Pyranoisoxazolones	Minimum Inhibitory Concentration (MIC, µg/mL)				
	500	**250**	**125**	**62.5**	**31.25**
10a	R	R	R	R	R
10b	I	I	R	R	R
10c	R	R	R	R	R
10d	I	I	R	R	R
10e	R	R	R	R	R
10f	I	I	R	R	R
10g	I	I	R	R	R
10h	R	R	R	R	R
10i	I	R	R	R	R
10j	I	I	R	R	R
12a	R	R	R	R	R
12b	R	R	R	R	R
12c	R	R	R	R	R
13a	I	R	R	R	R
13b	I	I	R	R	R
13c	I	I	R	R	R
Doxycyline (**4**)	I	I	R	R	R

R- Resistance; I-Inhibition. All the experiments were done in triplicates.

Similarly, among naphthopyranone derivatives, compound **13a** showed activity, at a concentration of 500 µg/mL, whereas compounds **12a**, **12b** and **12c** did not show any activity. Interestingly, compound **13b** and **13c** exhibited anti-leptospiral activity at 250 µg/mL itself. This result revealed that compounds **13b** and **13c**, bearing unsubstituted naphthyl ring were potential

molecules to achieve anti-leptospiral activity compared to compounds **12a-c** substituted with OMe group. Further in order to make structure activity relationship study, six pyrano[4,3-*c*]isoxazol-3-ones such as, **10b**, **10d**, **10f**, **10g**, **10i** and **10j** (substituted at 4 position with variety of groups such as aryl, heteroaryl, and alkyl) were taken for secondary screening against locally prevalent, thirteen different serovars.

2.2.2. Secondary Screening of 6,7-Dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones

In the secondary screening, six potential pyrano[4,3-*c*]isoxazol-3-one derivatives such as **10b**, **10d**, **10f**, **10g**, **10i** and **10j** were screened at different concentrations of 500, 250, 125, 62.5, 31.25 µg/mL against twelve strains of pathogenic and one non-pathogenic serovars by micro dilution assay. The results obtained with all the concentration are shown in the figure 2. The MIC for each compound is listed out separately in table 3. The pathogenic serovars used in the study are Australis (**I**, serovar Australis, strain Ballico), Autumnalis (**II**, serovar Autumnalis, strain N2), Ballum (**III**, serovar Ballum, strain Mus 127), Bataviae (**IV**, serovar Bataviae, strain Swart), Canicola (**V**, serovar Canicola, strain Hond Utrecht IV), Grippotyphosa (**VI**, serovar Grippotyphosa, strain Moskva V), Hebdomadis (**VII**, serovar Hebdomadis, strain Hebdomadis), Icterohaemorrhagiae (**VIII**, serovar Icterohaemorrhagiae, strain RGA), Javanica (**IX**, serovar Poi, strain Poi), Pomona (**X**, serovar Pomona, strain Pomona), Pyrogenes (**XI**, serovar Pyrogenes, strain Salinem) and Sejroe (**XII**, serovar Hardjo, strain Hardjoprajitno). In addition to the pathogenic strains **I-XII**, a non-pathogenic strain Patoc I of serovar Semaranga (**XIII**) was also included.

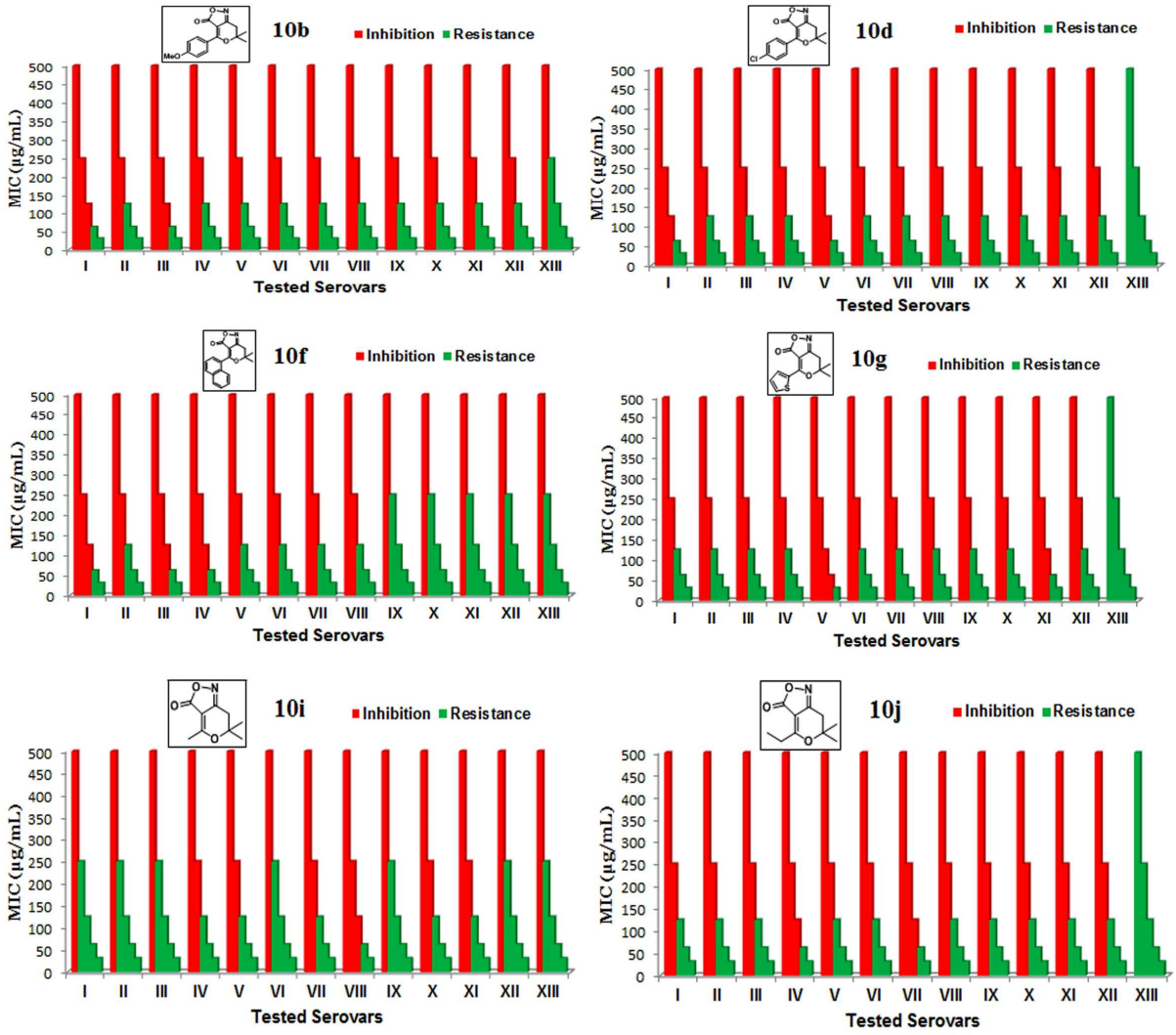


Fig. 2. Secondary screening of anti-leptospiral activity of pyranoisoxazolones **10b**, **10d**, **10f**, **10g**, **10i** and **10j** against twelve pathogenic serovar: Australis (**I**); Autumnalis (**II**); Ballum (**III**); Bataviae (**IV**); Canicola (**V**); Grippotyphosa (**VI**); Hebdomadis (**VII**); Icterohaemorrhagiae (**VIII**); Javanica (**IX**); Pomona (**X**); Pyrogenes (**XI**); Sejroe (**XII**); and one non-pathogenic serovar Semaranga (**XIII**).

Table 3. MICs of 6,7-dihydro-3*H*-pyrano[4,3-*c*]isxazol-3-ones for 13 serovars of *Leptospira*

Compound	MIC (µg/mL)												
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**
10b	125	250	125	250	250	250	250	250	250	250	250	250	500
10d	125	250	250	250	125	250	250	250	250	250	250	250	ni[a]
10f	125	250	125	125	250	250	250	250	500	500	500	500	500
10g	250	250	250	250	**62.5**	250	250	250	250	250	125	250	ni[a]
10i	500	500	500	250	250	500	250	125	500	250	250	500	500
10j	250	250	250	125	250	250	125	250	250	250	250	250	ni[a]
Doxycyline	200	200	100	100	200	200	200	200	200	100	200	100	100

[a]ni: no inhibition; Tested against serovars I-XIII, mentioned in figure 2

Very interesting trends in inhibition of serovars by six pyrano[4,3-*c*]isoxazol-3-ones **10b**, **10d**, **10f**, **10g**, **10i** and **10j** was observed (Table 3). While compound **10d**, **10g** and **10j** inhibited only the pathogenic serovars at MIC of < 250 µg/mL, the non-pathogenic serovar, semaranga (**XIII**) remained unaffected. Fascinatingly, all the six compounds inhibited the serovars Bataviae (**IV**), Canicola (**V**), Hebdomadis (**VII**), and Icterohaemorrhagiae (**VIII**) at MIC of < 250 µg/mL. However, compound **10f** with 4-naphthyl substituent and **10i** with 4-methyl substituent, showed activity against five serovars (**IX-XIII**) and seven serovars (**I-III, VI, IX, XII** and **XIII**) respectively only at highest MIC of 500 µg/mL (Table 3). The compound **10g,** with 4-thiphenyl substituent showed activity against all the serovars at MIC of < 250 µg/mL and particularly against Canicola (**V**) at a very low MIC of 62.5 µg/mL, which was much lower than the concentration observed with the reference drug doxycycline [13,49,50]. However, in case of the serovars Javanica (**IX**) and Sejroe (**XII**) all six compounds exhibited activity at only MIC > 250

μg/mL. Thus, structure activity relationship of compounds shows that substituent at 4th position of pyranoisaxazolone has an important influence on the activity against leptospiral serovars, and the compound **10g** with a thienyl substituent is a potential lead for further study. In order to establish the mechanism of action of the compound **10g**, further biological studies such as real time quantitative PCR analysis, animal survivability (*in vivo*), cytotoxicity (MTT), cell death assay (PI staining), hemocompatibility, and *in silico* molecular docking study was carried out.

2.2.3. Real Time Quantitative PCR Analysis of Leptospiral 16S rRNA Gene.

SYBR Green-based RT-qPCR assay, which is a highly efficient and widely used platform amongst available real-time PCR technologies, especially for the identification of most abundant RNA target like 16S rRNA was used under the present study to quantify the leptospiral load in the **10g** treated and untreated control [51]. The negative control was included to check the purity of the reaction mixture (Figure 3). The Ct (Threshold clycle) value is inversely proportional to the number of leptospires. The Ct value for **10g** treated and untreated leptospires was observed as 18.5 and 11.3 respectively. The primers specificity was analysed by melt curve analysis. The result shows that the leptospires growth was inhibited by pyranoisoxazolone **10g** at a significant level ($P<0.05$).

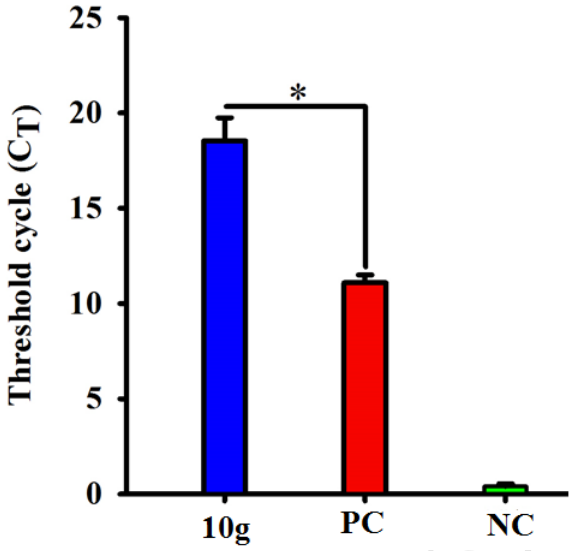


Fig. 3. Real Time quantitative PCR analysis (RT-qPCR). The propagation of leptospires was significantly less in **10g** treated samples than positive control. *P<0.05.

2.2.4. Challenge Experiments in Animal Models

Since compound **10g** showed the remarkable MIC value against pathogenic leptospiral serovars in *in vitro* experiments, it was selected for further *in vivo* studies using Cy-BALB/c mice [52,53]. At the end of the study period, 95% of surivivabilty was observed at 20 mg/kg, which was nearly equal to doxycycline treated groups and only 20% suvivability among 5 mg/kg was observed. The untreated control leads to death from day 4^{th} onwards. This has evidenced that by utilising compound **10g,** at a concentration of 20 mg/kg, the infected mice could be protected completely from leptospirosis. The antibacterial activity of compound **10g** might be related to its antibiotic activity or to the presence of metabolic toxins. This suggests that the compound **10g** provides antibacterial activity against leptospires by *in vivo.*

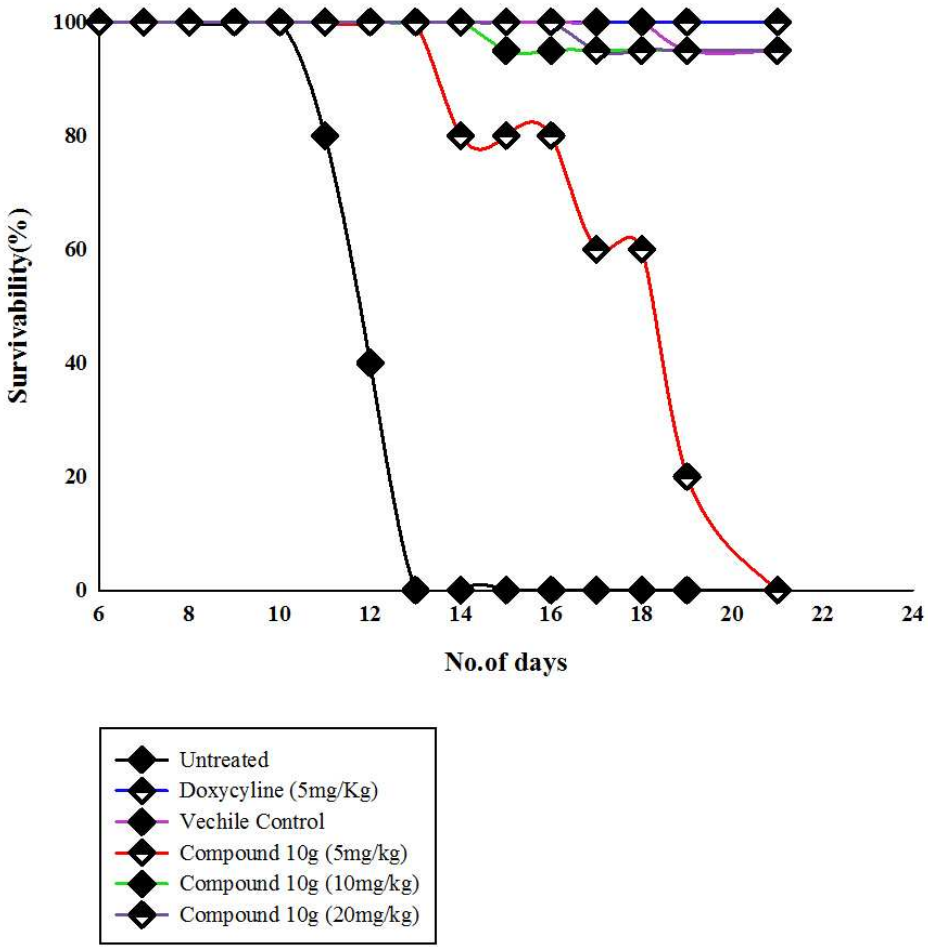


Fig. 4. Survival of mice treated for 3 days with compound **10g** (5, 10, or 20 mg/kg one time daily). Controls include untreated animals and those animals treated with doxycycline (5 mg/kg one time daily).

2.2.5. Quantitative RT-PCR for leptospiral DNA from animal tissue

The qRT-PCR analysis of inspected kidney samples revealed the presence of leptospiral renal colonization in the case of infected untreated mice (Figure 5) [51,54], and no colonization in the mice group treated with the compound **10g** at a concentration of 20 mg/kg. This finding was also supported by the re-isolation of the leptospires in EMJH from the untreated mice and among the treated mice group it was not successful with 20 mg/kg. RT-qPCR and reisolation in EMJH semi-solid medium study has shown that compound **10g** has the potential to inhibit

leptospires by clearing renal colonization of the leptospires from the system completely. This confirms that the compound **10g** shows significant protection against virulent leptospires in Cy-BALB/c mice model.

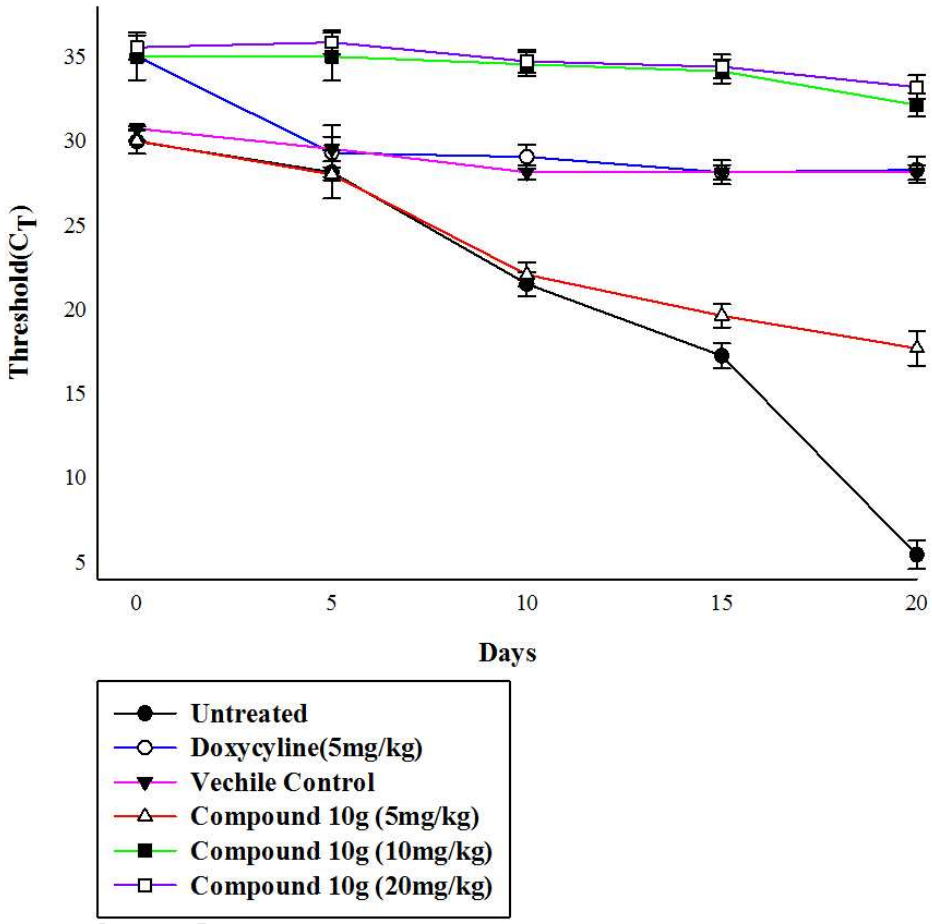


Fig. 5. The compound **10g** treated and untreated mice kidney tissue sample were analysed by RT-qPCR assay to determine the leptospiral load. Doxycycline was used as positive control.

2.2.6. MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines

mitochondrial activity. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the *in vitro* cytotoxic effects of drugs on cell lines [55]. Under the present study, *in vitro* cytotoxic effect of the compound **10g** against THP-1 cells were given in Figure 6. The results revealed that the compound **10g** exhibited dose dependent inhibition of the cell growth. At a lower concentration of ≤25 µg/mL no significant cytotoxic effect has been detected and it showed cytotoxic effect significantly only ≥75 µg/mL. This clearly indicates that compound **10g** has potential cytotoxic effect only at higher concentration, compared to 0.1% Triton X-100 treated positive controls (Amresco, USA).

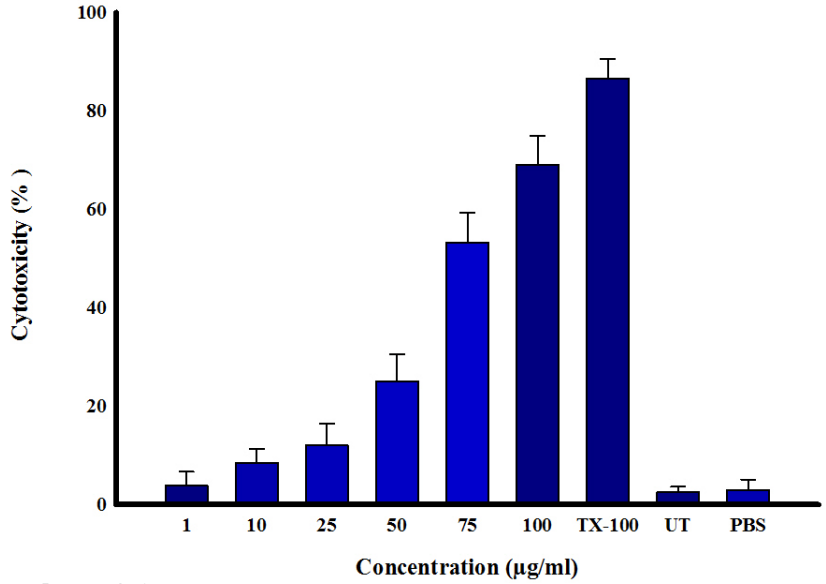


Fig. 6. Effect of compound **10g** on THP-1 cells treated at 1–100 µg/mL concentration for 24 h; TX-100 – Triton X-100; UT – Untreated cells. PBS - Phosphate-buffered saline.

2.2.7. Cell Death Assay by Propidium Iodide (PI)

Propidium lodide (PI) dye penetrates only damaged cellular membranes [56], hence used to identify the dead cells. Intercalation complexes formed by PI with double-stranded DNA

affects amplification of the fluorescence. Incubation of the total cell population with PI and subsequent fluorescence detection allowed assessment of the number of non-vital cells. The THP-1 cells treated with compound **10g**, at lower concentrations of ≤25 µg/mL showed no significant cell death, but increased cell death was observed at higher concentration ≥75 µg/mL, which is similar to the number of cells positive for PI in cells treated with tBHP (Figure 7). This reveals that compound **10g** has cytotoxicity against THP-1 cells only at higher concentration ≥75 µg/mL.

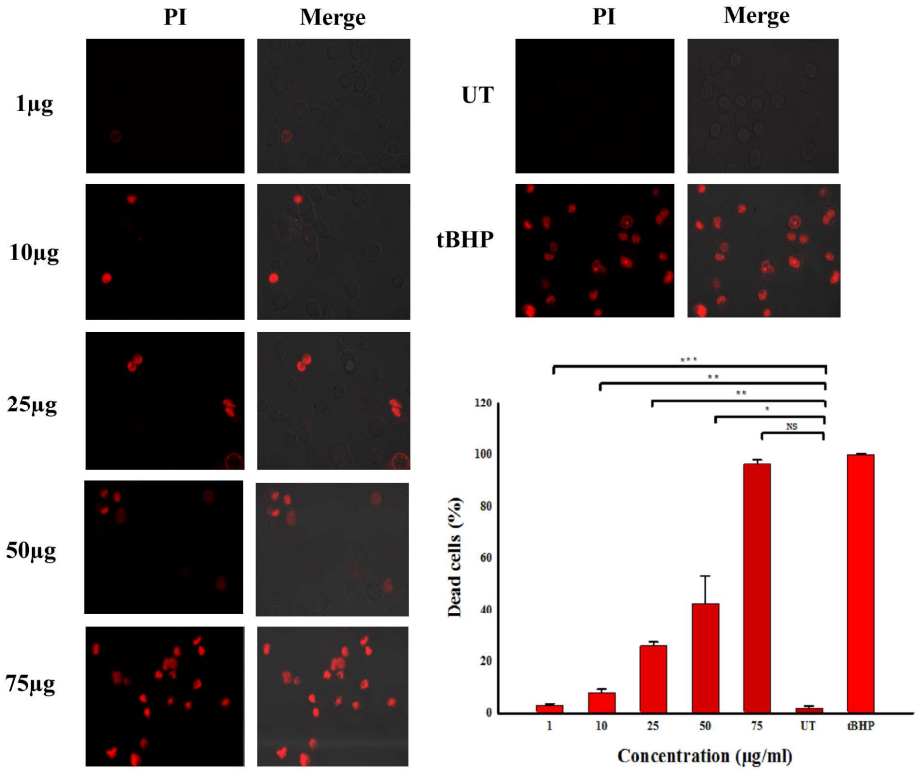


Fig. 7. PI staining of THP-1 cells at 12 h of incubations by the treatment of **10g** at different concentrations; UT – Untreated cells. tBHP – *tert*-butylhydroperoxide; The graph shows manual count of apoptotic and necrotic cells in percentage. (Data are mean percent ± SD percent of triplicate each).

2.2.8. Hemocompatibility

To be a potential anti-leptospiral agent, it is important for an organic molecule to be non-toxic to the normal human cells. In order to check toxicity, hemocompatibility of compound **10g** was examined at various concentration 500, 250, 125, 62.5, 31.25, 15.6, 7.8 µg/mL by measuring the damage to human RBCs (Figure 8). The mechanisms of direct hemolytic activity for different toxic agents were found to be non-specific. According to the International Organization for Standardization/Technical Report 7406, the admissible level of hemolysis of biological materials is 5% [57]. As shown in Figure 8, compared to the positive control (Triton X-100), compound **10g** exhibited very low hemoglobin release at various concentrations.

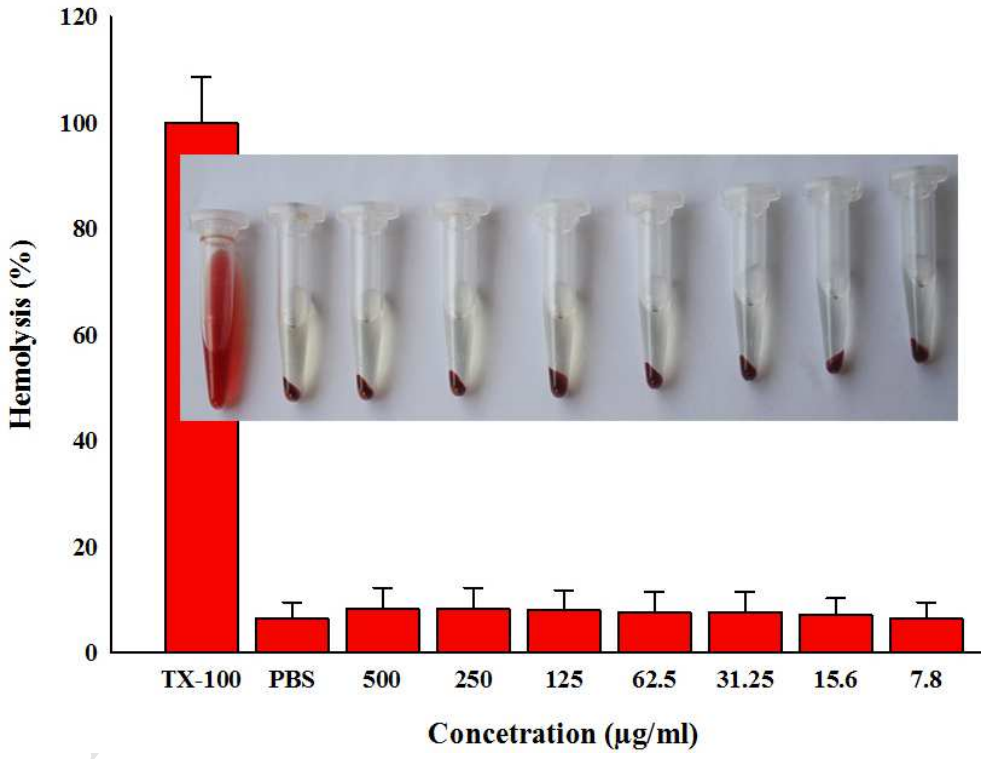


Fig. 8. *In vitro* hemocompatibility assay of pyranoisoxazolone **10g** exhibits very less hemolytic activity. No (0%) lysis was noticed in the negative control (NC-PBS buffer) whereas the positive control (PC- 1% Triton X-100) shows 100% lysis.

2.2.9. *In silico* Molecular Docking

One of the important differences among pathogenic and non-pathogenic serovars is the lack of outer membrane lipoprotein “LipL32”. It is reported to be absent in non-pathogenic avirulent *Leptospira* [58]. It is expressed during infection and highly conserved among pathogenic *Leptospira*. *In silico* comparison of amino acid sequences of LipL32 showed 97.8% average sequence identity among the pathogenic species [59]. It is important to note that while compound **10g** affected all the leptospiral strains of pathogenic serovars, it did not affect non pathogenic serovar Semaranga. Thus we were curious to know whether the lead compound **10g** could act upon leptospiral LipL32. To identify this molecular docking study was carried out for compound **10g** against LipL32 protein (PDB code: 2ZZ8) by using the Autodock4 program (Figure 9) [60].

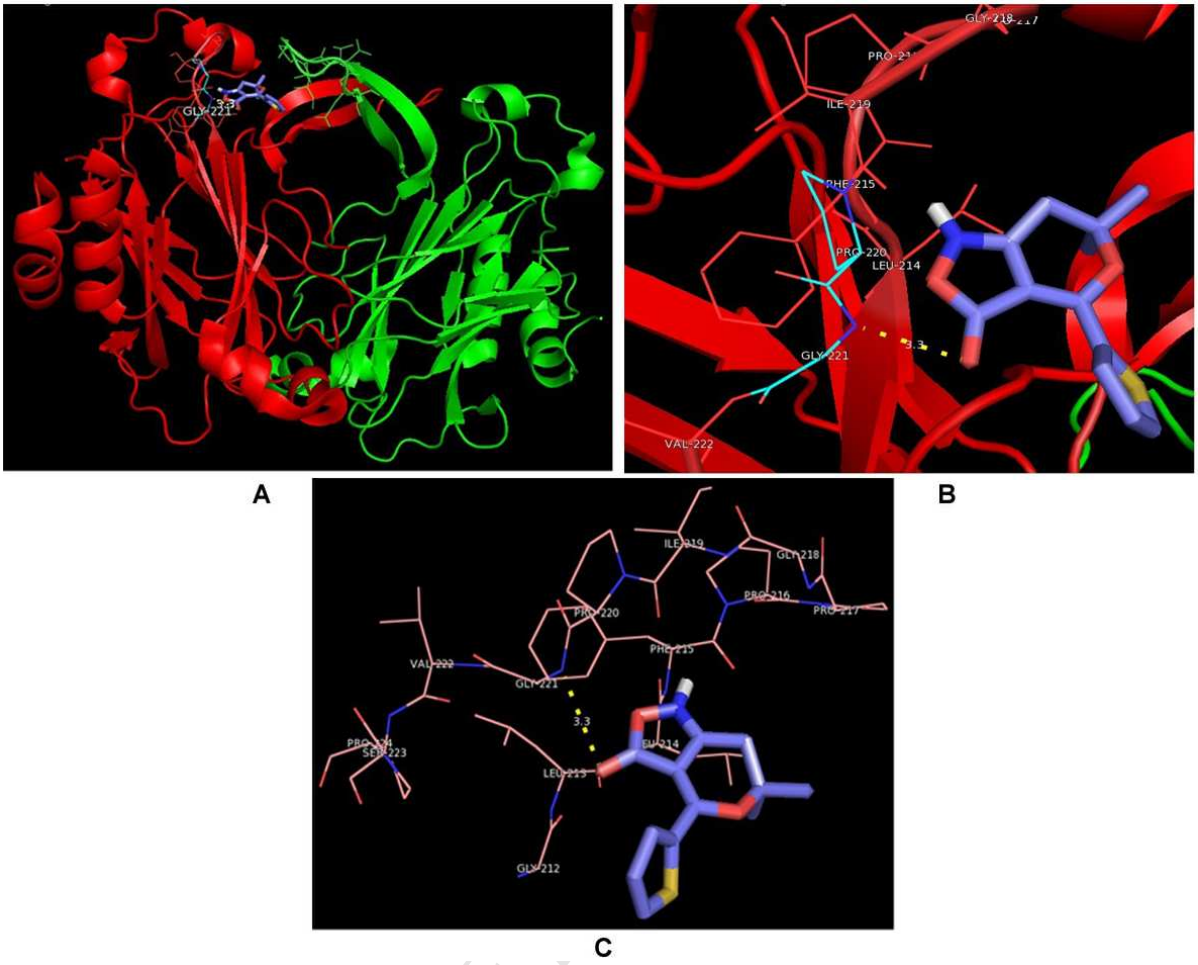


Fig. 9. (A) Docking pose of the compound **10g** with amino acid sequences of LipL32 in which chain-A shown in red color and chain-B shown in green color. (B) Docking pose of the compound **10g** with LipL32 (in zoom) (C) Line representation of selected amino acids of chain A of LipL32 interacting with compound **10g**. Yellow dotted lines indicate the hydrogen bonds. Pictures were generated by using PyMOL.

The compound **10g** upon docking was specifically surrounded by significant amino acid residues such as Leu-214, Phe-215, Pro-216, Pro-217, Gly-218, Ile-219, Pro-220, Gly-221 and Val-222 of the chain A and Thr-20, Ile-21, Pro-22, Gly-23, Thr-24, Asn-25, Glu-26 and Thr-27 of the chain B. Significant hydrogen bonding was observed between carbonyl O of the

isoxazolone ring in **10g** and HN of the Gly-221 of the chain A of LipL32 (O···HN distance = 3.3 Å). Compound **10g** binds effectively to LipL32 with a binding energy of ΔG -7.2 kcal/mol. The docking studies confirm that compound **10g** has good binding affinity towards LipL32 and explains the reason for compound **10g** being specific to leptospiral strains of pathogenic serovars.

3. Conclusions

Ten different pyrano[4,3-*c*]isoxazol-3-ones and six naphthopyranones were prepared through simple and efficient synthetic procedure. Preliminary screening of all the sixteen compounds against *Leptospira interrogans* serovar Autumnalis lead to identification of six different pyrano[4,3-*c*]isoxazol-3-ones **10** as prominent molecules for further study. Secondary screening of these molecules against twelve pathogenic and one non-pathogenic serovars lead to identification of pyrano[4,3-*c*]isoxazol-3-one **10g**, with 4-thiphenyl substituent, as the lead molecule. This compound achieved activity against all the leptospiral serovars at MIC of ≤250 μg/mL and it is particularly active against Canicola at a very low MIC of 62.5 μg/mL, which is much lower than the MIC observed with the reference drug doxycyline. Interestingly, compounds **10d**, **10g** and **10j** targeted only pathogenic serovars and did not affect the non-pathogenic serovar such as Semaranga. 16S rRNA gene based RT-qPCR study revealed inhibition of leptospires growth by compound **10g** at significant level. *In vivo* animal challenge study carried out on Cy-BALB/c mice by treating it with the compound **10g**, at a concentration of 20 mg/kg confirmed that the infected mice has 95% of survivability against leptospirosis. The RT-qPCR analysis of DNA extracted from kidney samples of mice treated with compound **10g** for leptospiral renal colonization revealed the absence of leptospiral specific DNA. This finding

was also supported by the re-isolation of the leptospires from the untreated mice. MTT assay of THP-I cells treated with compound **10g** shows potential cytotoxic effect only above 75 μg/mL which was further confirmed by PI staining. Hemolytic assay convincingly proves that compound **10g** is hemocompatible. *In silico* molecular docking studies revealed significant hydrogen bonding between carbonyl O of the isoxazolone ring in **10g** and HN of the Gly-221, of the chain A of LipL32. This substantiates the experimental result that compound **10g** is specific to pathogenic serovars. We have explored biological activity of pyranoisoxazolone class of compounds for the first time, and identified it as new class anti-leptospial active molecules. These molecules has potential for further development as pathogen specific anti-leptospiral agents. Study on anti-leptospiral active organic molecules are otherwise rare and further study on structural modification of this scaffold to achieve superior anti-leptospiral activity is under progress in our group.

4. Experimental Protocols

4.1. Chemistry

4.1.1. General remarks

Melting points were determined by the open capillary tube method using a Toshniwal melting point apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 400 (400 MHz) NMR spectrometer. Chemical shifts are reported in ppm (δ) relative to internal standard tetramethylsilane (TMS, δ 0.00 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad resonance (br)], coupling constants [Hz], integration). All the NMR spectra were acquired at ambient temperature. ESI-MS was recorded on Agilent 1100 LC/MSD (70 ev) spectrometer. High

resolution mass spectra (HRMS) were recorded on a Waters Q-Tof micro mass spectrometer. Elemental analyses were performed on a CHN analyser. Thin layer chromatography (TLC) was performed on Merck pre-coated silica gel 60F plates and visualized by exposure to UV light. ACME silica gel (100-200 mesh) was used for column chromatography.

4.1.2. Preparation of 5-carboalkoxy-2,3-dihydro-4*H*-pyran-4-one (9)

5-Carboalkoxy-2,3-dihydro-4*H*-pyran-4-one such as **9a**, **9b**, **9d**, **9e**, **9g**, **9h**, **9i** and **9j** were prepared by utilizing our previous literature procedure [36].

4.1.2.1. Ethyl 2,2-dimethyl-4-oxo-6-(3,5-dimethoxyphenyl)-2,3-dihydro-4*H*-pyran-5-carboxylate (9c):

The reaction was carried out according to reported literature method [36] using ethyl 3-(3,5-dimethoxyphenyl)-3-oxopropanoate (**7c**, 252 mg, 1.0 mmol), $CaCl_2$ (11 mg, 0.1 mmol), Et_3N (278 µL, 2.0 mmol), senecioyl chloride (**8**, 112 µL, 1.0 mmol) in DCM (4 mL) for 5 h gave **9c** (267 mg, 80%) as a yellow solid. M.p. 98-100 ^{o}C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.66 (d, *J* = 2.4 Hz, 2H), 6.54 (t, *J* = 2.2 Hz, 1H), 4.08 (q, *J* = 6.8 Hz, 2H), 3.77 (s, 6H), 2.63 (s, 2H), 1.54 (s, 6H), 1.04 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 188.9, 170.1, 165.9, 160.6, 135.5, 111.6, 106.2, 103.5, 81.9, 61.1, 55.5, 46.9, 26.0, 13.7 ppm; HRMS (ESI): $[M + Na]^+$ Calcd for $C_{18}H_{22}NaO_6$, 357.1314; found 357.1309.

4.1.2.2. Ethyl 2,2-dimethyl-4-oxo-6-(naphthalen-1-yl)-2,3-dihydro-4*H*-pyran-5-carboxylate (9f):

The reaction was carried out according to reported literature method [36] method A using ethyl 3-(naphthalen-1-yl)-3-oxopropanoate (**7f**, 242 mg, 1.0 mmol), $CaCl_2$ (11 mg, 0.1 mmol), Et_3N (278 µL, 2.0 mmol), senecioyl chloride (**8**, 112 µL, 1.0 mmol) in DCM (4 mL) for 5 h gave **9f**

(279 mg, 86%) as a yellow solid. M.p. 78-80 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 (d, *J* = 8.0 Hz, 2H), 7.88-7.86 (m, 1H), 7.56-7.45(m, 4H), 3.69 (q, *J* = 7.2 Hz, 2H), 2.77 (s, 2H), 1.65 (s, 6H), 0.46 (t, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 188.5, 172.6, 164.7, 133.3, 132.0, 130.9, 130.3, 128.4, 127.0, 126.6, 126.4, 124.7, 124.6, 113.4, 82.7, 60.4, 47.4, 26.2, 13.1 ppm; HRMS (ESI): $[M + Na]^+$ Calcd for $C_{20}H_{20}NaO_4$, 347.1259; found 347.1254.

4.1.3. General method A: Typical experimental procedure for the synthesis of 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10)

The mixture of 5-carboalkoxy-2,3-dihydro-4*H*-pyan-4-one **9** (1.0 mmol) and $NH_2OH.HCl$ (2.5 mmol) in 4 mL of ethanol was stirred at reflux for 5 h. The progress of the reaction was monitored by TLC. After the completion of reaction ethanol was removed under reduced pressure. Then the reaction mixture was quenched with water, extracted with EtOAc (3 x 8 mL) and washed with brine. The organic layer was separated, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by column chromatography (Hexane/EtOAc = 8:2) to afford pure 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one **10**.

4.1.3.1. 6,6-Dimethyl-4-phenyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10a)

The reaction was carried out according to **general method A** using ethyl 2,2-dimethyl-4-oxo-6-phenyl-2,3-dihydro-4*H*-pyran-5-carboxylate (**9a**, 274 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10a** (206 mg, 85%) as a white solid. M.p. 158-160 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.21 (dd, *J* = 8.6, 1.4 Hz, 2H), 7.64-7.60 (m, 1H), 7.53-7.49 (m, 2H), 2.92 (s, 2H), 1.60 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.9, 169.0, 157.1,

134.5, 130.1, 129.9, 128.6, 95.8, 84.2, 33.8, 26.3 ppm; HRMS (ESI): $[M + H]^+$ Calcd for $C_{14}H_{14}NO_3$, 244.0974; found 244.0967.

4.1.3.2. 4-(4-Methoxyphenyl)-6,6-dimethyl-6,7-dihydro-3H-pyrano[4,3-c]isoxazol-3-one (10b)

The reaction was carried out according to **general method A** using ethyl 6-(4-methoxyphenyl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9b**, 304 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10b** (237 mg, 87%) as a yellow solid. M.p. 147-149 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.33 (d, J = 8.8 Hz, 2H), 6.99 (d, J = 9.2 Hz, 2H), 3.89 (s, 3H), 2.89 (s, 2H), 1.58 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.3, 169.6, 164.9, 157.2, 132.7, 122.4, 114.1, 94.2, 83.5, 55.6, 33.9, 26.3 ppm; HRMS (m/z): $[M + Na]^+$ calcd. for $C_{15}H_{15}NNaO_4$, 296.0899; found 296.0894.

4.1.3.3. 4-(3,5-Dimethoxyphenyl)-6,6-dimethyl-6,7-dihydro-3H-pyrano[4,3-c]isoxazol-3-one (10c)

The reaction was carried out according to **general method A** using ethyl 6-(3,5-dimethoxyphenyl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9c**, 334 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10c** (273 mg, 90%) as a yellow solid. M.p. 140-142 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 7.49 (d, J = 2.4 Hz, 2H), 6.72 (t, J = 2.4 Hz, 1H), 3.86 (s, 6H), 2.92 (s, 2H), 1.59 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.5, 169.0, 160.6, 157.0, 131.6, 107.7, 107.2, 96.1, 84.0, 55.7, 33.8, 26.2 ppm; HRMS (m/z): $[M + H]^+$ calcd. for $C_{16}H_{18}NO_5$, 304.1185; found 304.1179.

4.1.3.4. 4-(4-Chlorophenyl)-6,6-dimethyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10d)

The reaction was carried out according to **general method A** using ethyl 6-(4-chlorophenyl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate **9d** (308 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10d** (255 mg, 92%) as a yellow solid. M.p. 156-158 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.23 (d, *J* = 8.8 Hz, 2H), 7.49 (d, *J* = 8.8 Hz, 2H), 2.94 (s, 2H), 1.61 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.3, 168.9, 156.9, 140.9, 131.4, 129.0, 128.3, 96.2, 84.4, 33.8, 26.3 ppm; HRMS (m/z): $[M + H]^+$ calcd. for $C_{14}H_{13}ClNO_3$, 278.0584; found 278.0575.

4.1.3.5. 4-(2-Chlorophenyl)-6,6-dimethyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10e)

The reaction was carried out according to **general method A** using ethyl 6-(2-chlorophenyl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9e**, 308 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10e** (244 mg, 88%) as a white solid. M.p. 140-143 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 7.54 (d, *J* = 7.6 Hz, 1H), 7.49 (d, *J* = 4.0 Hz, 2H), 7.39-7.35 (m, 1H), 2.96 (s, 2H), 1.64 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.8, 167.8, 156.3, 133.5, 133.4, 131.8, 130.9, 128.5, 126.8, 98.5, 86.0, 34.1, 26.3 ppm; HRMS (m/z): $[M + H]^+$ calcd. for $C_{14}H_{13}ClNO_3$, 278.0584; found 278.0584.

4.1.3.6. 6,6-Dimethyl-4-(naphthalen-1-yl)-6,7-dihydro-3H-pyrano[4,3-c]isoxazol-3-one (10f)

The reaction was carried out according to **general method A** using ethyl 6-(naphthalen-1-yl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9f**, 324 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10f** (269 mg, 92%) as a pale yellow solid. M.p. 177-179 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.07 (d, *J* = 8.4 Hz, 1H), 8.04-8.02 (m, 1H), 7.93-7.91 (m, 1H), 7.88 (dd, *J* = 7.2, 1.2 Hz, 1H), 7.59-7.55 (m, 3H), 3.03 (s, 2H), 1.72 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.7, 168.3, 156.5, 134.0, 133.7, 130.8, 130.3,

129.0, 127.7, 126.6, 126.3, 124.7, 124.4, 97.8, 85.3, 34.1, 26.6 ppm; HRMS (ESI): $[M + Na]^+$ calcd. for $C_{18}H_{15}NNaO_3$, 316.0950; found 316.0944.

4.1.3.7. 6,6-Dimethyl-4-(thiophen-2-yl)-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10g)

The reaction was carried out according to **general method A** using ethyl 2,2-dimethyl-4-oxo-6-(thiophen-2-yl)-2,3-dihydro-4*H*-pyran-5-carboxylate (**9g**, 280 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10g** (224 mg, 90%) as a yellow solid. M.p. 138-140 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 9.14 (dd, *J* = 4.0, 1.2 Hz, 1H), 7.82 (dd, *J* = 4.8, 0.8 Hz, 1H), 7.28 (dd, *J* = 4.8, 4.4 Hz, 1H), 2.93 (s, 2H), 1.59 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 169.3, 164.5, 156.8, 137.6, 136.0, 134.1, 129.6, 93.6, 84.2, 33.8, 26.3 ppm; HRMS (m/z): $[M + H]^+$ calcd. for $C_{12}H_{12}NO_3S$, 250.0538; found 250.0534.

4.1.3.8. 4-(Furan-2-yl)-6,6-dimethyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10h)

The reaction was carried out according to **general method A** using ethyl 6-(furan-2-yl)-2,2-dimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9h**, 264 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10h** (140 mg, 60%) as a brown solid. M.p. 170-172 ^oC; ^1H NMR (400 MHz, $CDCl_3$): δ 8.62 (dd, *J* = 3.6, 0.4 Hz, 1H), 7.78 (dd, *J* = 1.8, 0.6 Hz, 1H), 6.72 (dd, *J* = 4.0, 1.6 Hz, 1H), 2.94 (s, 2H), 1.62 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 168.7, 159.1, 156.7, 148.5, 145.0, 124.9, 113.8, 92.9, 84.4, 33.8, 26.4 ppm; MS (ESI): *m/z* 234 $(M+1)^+$. Anal. Calcd for $C_{12}H_{11}NO_4$: C, 61.80; H, 4.75; N, 6.01. Found: C, 61.98; H, 4.82; N, 6.14;

4.1.3.9. 4,6,6-Trimethyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10i)

The reaction was carried out according to **general method A** using ethyl 2,2,6-trimethyl-4-oxo-2,3-dihydro-4*H*-pyran-5-carboxylate (**9i**, 212 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol)

in ethanol (4 mL) gave the title compound **10i** (145 mg, 80%) as a pale white solid. M.p. 108-110 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.78 (s, 2H), 2.32 (s, 3H), 1.45 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.5, 169.7, 155.7, 97.4, 85.3, 33.6, 26.3, 17.9; HRMS (ESI): $[M + H]^+$ Calcd for $C_9H_{12}NO_3$, 182.0817; found 182.0826.

4.1.3.10. 4-Ethyl-6,6-dimethyl-6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-one (10j)

The reaction was carried out according to **general method A** using methyl 6-ethyl-2,2-dimethyl-4-oxo-2,3-dihydro-4H-pyran-5-carboxylate (**9j**, 212 mg, 1.0 mmol), $NH_2OH.HCl$ (174 mg, 2.5 mmol) in ethanol (4 mL) gave the title compound **10j** (166 mg, 85%) as a pale white solid. M.p. 84-86 ^{o}C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.83 (s, 2H), 2.77 (q, *J* = 7.6, 2H), 1.49 (s, 6H), 1.18 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 181.8, 169.5, 155.8, 96.5, 84.9, 33.8, 26.4, 24.6, 10.1 ppm; HRMS (ESI): $[M + H]^+$ Calcd for $C_{10}H_{14}NO_3$, 196.0974; found 196.0973.

4.1.3 Preparation of natural product dihydronaphthopyran-4-ones and its analogues 12 and 13

Dihydronaphthopyran-4-one natural products **12b** and **12c** and its analogues **12a** and **13a-c** were prepared by utilizing our previous literature procedure [36].

4.2. Biological methods

4.2.1. Anti-leptospiral assay (Preliminary and Secondary Screening)

Antileptospiral assay was performed in 96 well plates for micro dilution assay for the determination of Minimum Inhibitory Concentration (MIC). The initial stock concentration was prepared using DMSO. The initial MIC was determined for different chemical compounds against leptospiral strains of pathogenic serovars. Seven days old culture was used as inoculums

and the compounds were serially diluted at different concentration at µg/mL with appropriate controls and incubated for 2 hours and the inhibition was determined under the dark field microscopy (Optica B500 Italy) [61]. The end point was determined by 50% of reduction of leptospires compared with control. Doxycyline was used as positive control.

4.2.2. Real Time quantitative PCR analysis (RT-qPCR)

DNA from leptospires/kidneys was extracted with the DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The purified DNA was stored at -80 ^{o}C until use. The concentration of leptospires was quantified using the CFX96 Touch ^{TM} Real-Time PCR detection system (Bio-Rad, Hercules, CA) by SYBR green chemistry as reported earlier [51]. In brief, real-time quantitative PCR was performed on CFX96 Touch™ Real-Time PCR Detection System (BioRad, USA). Reactions were performed in a total volume of 20 µL consisting of 1x iQ^{TM} SYBR Green Supermix (Bio-Rad, USA) of 2x stock reagent containing 100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTP, 50 units/mL iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM fluoresein and stabilizers. Leptospiral 16S rRNA genes forward and reverse primers were added at a final concentration of 300 nM each. The compound treated and untreated leptospires were boiled and used for qPCR assay as DNA template. 10 µL of sterile water was used instead of DNA template for negative control. The amplification protocol consisted of 3 minutes at 95 ºC, followed by 40 cycles of amplification consisted 95 ºC for 10s, 55 ºC for 5s, 72ºC for 15s. Subsequently, the reaction was stopped at 95 ºC for 2 minutes, cooled at 20 ºC for 1 min and melted at 65 to 94 ºC with plate readings set at 0.5 ºC increments. The resulting data were analysed using the software CFX Manager™ version 3.1(BioRad, USA). All experiments were repeated at least thrice.

4.2.3. Challenge Experiments in Animal Model

Pathogen free BALB/c mice with a weight of 20–35 g and 4- 6 weeks old were used in the study. Animals were bought from The National Centre for Laboratory Animal Sciences, National Institute of Nutrition (NIN), Hyderabad. The mice were allowed to acclimatize to the laboratory environment at for before commencement of testing. All of the procedures were carried out in accordance with National animal ethical guidelines for animal care and use. All animal procedures reported herein were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) (BDU/IAEC/2011/29/29.03.2011) and Institutional Biosafety committee (IBC) of Bharathidasan University (BT/BS/17/29/2000 PID).

BALB/c mice were grouped in to three groups consisting of 5 animals each. Cyclophosphamide (Cy) (Sigma-Aldrich,St. Louis, MO) was injected in to animal in a single dose of 300mg/kg intraperitoneally 48h before the injection as per Adler *et al* [52,53]. The Cy-BALB/c mice were intraperitoneally challenged with 10^8 leptospires (Passaged leptospires isolated from the kidney of a cyclophosphamide treated susceptible mice inoculated with the isogenic strain of *L. interrogans* serovar Canicola strain PAI-1) in PBS intraperitoneally in experiment groups. PAI-1 originally isolated from a human urine sample of a leptospirosis case earlier [62]. On days 3–6, groups of animals were treated by daily intraperitoneal injection with compound **10g** in concentrations of 5, 10, or 20 mg/kg. A group of five BALB/c mice was left untreated as a negative control, and a group of five BALB/c mice was treated with doxycycline (5 mg/kg, IP, daily) as positive control. Remained mice were served as vehicle control. All BALB/c mice were observed multiple times a day during the 21-day of the study period, for observing significant pain or distress or characteristics of a moribund state.

4.2.4. MTT Assay

The THP-1 cells (NCCS, Pune) were cultured in 96-well tissue culture plates at a cell density of 2 X 10^3 cells/well, in RPMI containing 10% FBS and 2 mM glutamine (Sigma Aldrich, USA). After 12hrs, the cells were incubated with different concentrations (1, 10, 25, 50, 75 and 100 µg/mL) of a compound **10g** for overnight. Cytotoxicity of the compound was detected by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (HiMedia, India) [55]. After 3 hours of incubation, the medium in each well was aspirated and the formazan crystals were dissolved by adding 100µl Dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA). The optical density (OD) of each well was determined at 490 nm by using a microplate reader (Bio-Rad, USA).

4.2.5. Cell Death Assay by Propidium Iodide (PI)

THP-1 cells (1 X 10^8cells/well) in 6–well culture plates (Corning, USA) with glass cover– slips were treated with various concentration (1, 10, 25, 50 and 75µg/mL) of a compound **10g** for 12hrs. Then stained with 2µg/mL of propidium iodide (PI) (Invitrogen, USA). The number of PI positive cells (%) was measured on comparison with untreated cells by Confocal Laser Scanning Microscopy (Carl Zeiss 710, Germany)). The 0.5mM of *tert*-Butyl hydroperoxide (tBHP) (Merck, Germany) used as a positive control. Data were analysed using ZEN 2011 software.

4.2.6. Hemocompatibility Assay

Hemocompatibility assay was performed as per the earlier report [63] with slight modification. Fresh blood was collected from healthy volunteers in sterile lithium heparin vacutainers. Further, Red blood cells (RBCs) were separated by centrifugation (1500 rpm for 10 min at 4 °C) and a ficoll density gradient. After discarding the supernatant containing plasma and

platelets, the RBCs were washed thrice with sterile phosphate buffered saline (PBS). Then, the pellets (1 ml) were resuspended in 3ml of PBS. Then, 0.1 ml of the diluted RBC suspension was added to synthesized compound **10g** mixed in 0.5 ml PBS suspension at their respective IC-50 concentration (14.3, 31.2, 28.7 and 26.2 µM) and incubated at 37 °C for 4 h. After incubation, all the samples were centrifuged at 12,000 rpm at 4 °C and supernatants were transferred to a 96-well plate. The hemolytic activity was determined by measuring the absorbance at 570nm (Biorad microplate reader model 550, Japan). Control samples of 0% lysis (PBS buffer) and 100% lysis (in 1% Triton X-100) were employed in the experiment. The percent of hemolysis was calculated as follows:

% Hemolysis = sample absorbance−negative control/positive control−negative control × 100%:

This study was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (Ref no. DM/2014/101/54). A consent form with all the mandatory information was collected from the healthy volunteers for their participation in this study, and the form was submitted to IEC, Bharathidasan University.

4.2.7. *In Silico* Molecular Docking Study

The geometry of the molecules was fully optimized at the Arguslab program. We performed docking by the Autodock Vina (PyRx) program [60] in order to identify the molecules binding sites. This approach permits a scoring function evaluation during the docking process so that as many conformations as possible can be obtained. The crystal structure of the most abundant surface protein of pathogenic *Leptospira*, LipL32 (PDB Code: 2ZZ8) has been retrieved from the protein data bank. All possible poses have been considered as starting points

and the docking analysis was performed. Furthermore, both the total energy and the RMSD of each system were examined in order to ascertain whether the molecule becomes equilibrated during molecular dynamics simulation. Visualization of the docked systems has been further analyzed with PyMOL software package.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/xx.xxxx/x.xxxxx

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List of Captions

Figure Captions:

Fig. 1. Antibiotic drugs used for treatment of leptospirosis

Fig. 2. Secondary screening of anti-leptospiral activity of pyranoisoxazolones **10b**, **10d**, **10f**, **10g**, **10i** and **10j** against twelve pathogenic serovar: Australis (**I**); Autumnalis (**II**); Ballum (**III**); Bataviae (**IV**); Canicola (**V**); Grippotyphosa (**VI**); Hebdomadis (**VII**); Icterohaemorrhagiae (**VIII**); Javanica (**IX**); Pomona (**X**); Pyrogenes (**XI**); Sejroe (**XII**); and one non-pathogenic serovar Semaranga (**XIII**).

Fig. 3. Real Time quantitative PCR analysis (RT-qPCR). The propagation of leptospires was significantly less in **10g** treated samples than positive control. *$P<0.05$.

Fig. 4. Survival of mice treated for 3 days with compound **10g** (5, 10, or 20 mg/kg one time daily). Controls include untreated animals and those animals treated with doxycycline (5 mg/kg one time daily).

Fig. 5. The compound **10g** treated and untreated mice kidney tissue sample were analysed by RT-qPCR assay to determine the leptospiral load. Doxycycline was used as positive control.

Fig. 6. Effect of compound **10g** on THP-1 cells treated at 1–100 μg/mL concentration for 24 h; TX-100 – Triton X-100; UT – Untreated cells. PBS - Phosphate-buffered saline.

Fig. 7. PI staining of THP-1 cells at 12 h of incubations by the treatment of **10g** at different concentrations; UT – Untreated cells. tBHP – *tert*-butylhydroperoxide; The graph shows manual count of apoptotic and necrotic cells in percentage. (Data are mean percent ± SD percent of triplicate each).

Fig. 8. *In vitro* hemocompatibility assay of pyranoisoxazolone **10g** exhibits very less hemolytic activity. No (0%) lysis was noticed in the negative control (NC-PBS buffer) whereas the positive control (PC- 1% Triton X-100) shows 100% lysis.

Fig. 9. (A) Docking pose of the compound **10g** with amino acid sequences of LipL32 in which chain-A shown in red color and chain-B shown in green color. (B) Docking pose of the compound **10g** with LipL32 (in zoom) (C) Line representation of selected amino acids of chain A of LipL32 interacting with compound **10g**. Yellow dotted lines indicate the hydrogen bonds. Pictures were generated by using PyMOL.

Scheme Captions:

Scheme 1. Synthesis of 5-carboalkoxy-2,3-dihydro-4*H*-pyran-4-ones: *Reaction conditions*: **7** (1.0 mmol), **8** (1.0 mmol), $CaCl_2$ (0.1 mmol), triethylamine (2.0 mmol) in DCM (5 mL) at room temperature. Isolated yield.

Scheme 2. Synthesis of substituted 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones: *Reaction conditions*: DHPs **9** (1.0 mmol), $NH_2OH.HCl$ (1.5 mmol) in 5 mL of EtOH at 25 °C for 5 h. Isolated yield.

Scheme 3. Synthesis of dihydronaphthopyran-4-one natural products and its analogues

Table Captions:

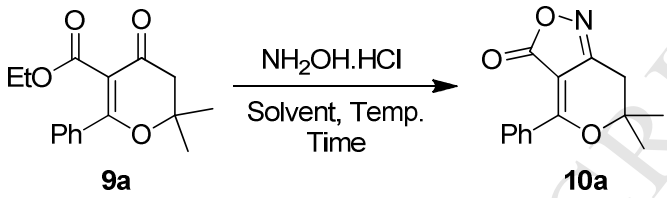
Table 1. Optimisation of the reaction conditions[a]

Table 2. Preliminary screening of pyrano[4,3-*c*]isoxazol-3-ones **(10a-j)** and dihydronaphthopyran-4-ones (**12a-c** and **13a-c**) against *Leptospira interrogans* serovar Autumnalis strain N2

Table 3. MICs of 6,7-dihydro-3*H*-pyrano[4,3-*c*]isxazol-3-ones for 13 serovars of *Leptospira*

Tables with Captions

Table 1. Optimisation of the Reaction Conditions[a]



9a **10a**

Entry	$NH_2OH.HCl$ (eq.)	Solvent	Temp (^oC)	Time (h)	Yield (%)[b]
1.	1.0	EtOH	r.t.	15	NR[c]
2.	1.0	EtOH	reflux	15	70
3.	**2.5**	**EtOH**	**reflux**	**7**	**90**
4.	1.0	MeOH	reflux	10	70
5.	1.0	*t*-BuOH	reflux	7	80

[a]All reactions were performed using ethyl 2,2-dimethyl-4-oxo-6-phenyl-2,3-dihydro-4*H*-pyran-5-carboxylate (**9a**) (1.0 mmol) and hydroxylamine hydrochloride (2.5 mmol); [b]Isolated yield; [c]No Reaction.

Table 2. Preliminary screening ofpyrano[4,3-*c*]isoxazol-3-ones **(10a-j)** and dihydronaphthopyran-4-ones (**12a-c** and **13a-c**) against *Leptospira interrogans* serovar Autumnalis strain N2

Pyranoisoxazolones	Minimum Inhibitory Concentration (MIC, µg/mL)				
	500	**250**	**125**	**62.5**	**31.25**
10a	R	R	R	R	R
10b	I	I	R	R	R
10c	R	R	R	R	R
10d	I	I	R	R	R
10e	R	R	R	R	R
10f	I	I	R	R	R
10g	I	I	R	R	R
10h	R	R	R	R	R
10i	I	R	R	R	R
10j	I	I	R	R	R
12a	R	R	R	R	R
12b	R	R	R	R	R
12c	R	R	R	R	R
13a	I	R	R	R	R
13b	I	I	R	R	R
13c	I	I	R	R	R
Doxycyline (**4**)	I	I	R	R	R

R- Resistance; I-Inhibition. All the experiments were done in triplicates.

Table 3. MICs of 6,7-dihydro-3*H*-pyrano[4,3-*c*]isxazol-3-ones for 13 serovars of *Leptospira*

Compound	MIC (µg/mL)												
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**
10b	125	250	125	250	250	250	250	250	250	250	250	250	500
10d	125	250	250	250	125	250	250	250	250	250	250	250	ni[a]
10f	125	250	125	125	250	250	250	250	500	500	500	500	500
10g	250	250	250	250	**62.5**	250	250	250	250	250	125	250	ni[a]
10i	500	500	500	250	250	500	250	125	500	250	250	500	500
10j	250	250	250	125	250	250	125	250	250	250	250	250	ni[a]
Doxycyline	200	200	100	100	200	200	200	200	200	100	200	100	100

[a]ni: no inhibition; Tested against serovars I-XIII, mentioned in figure 2

FIGURES AND SCHEMES

Figures with Captions

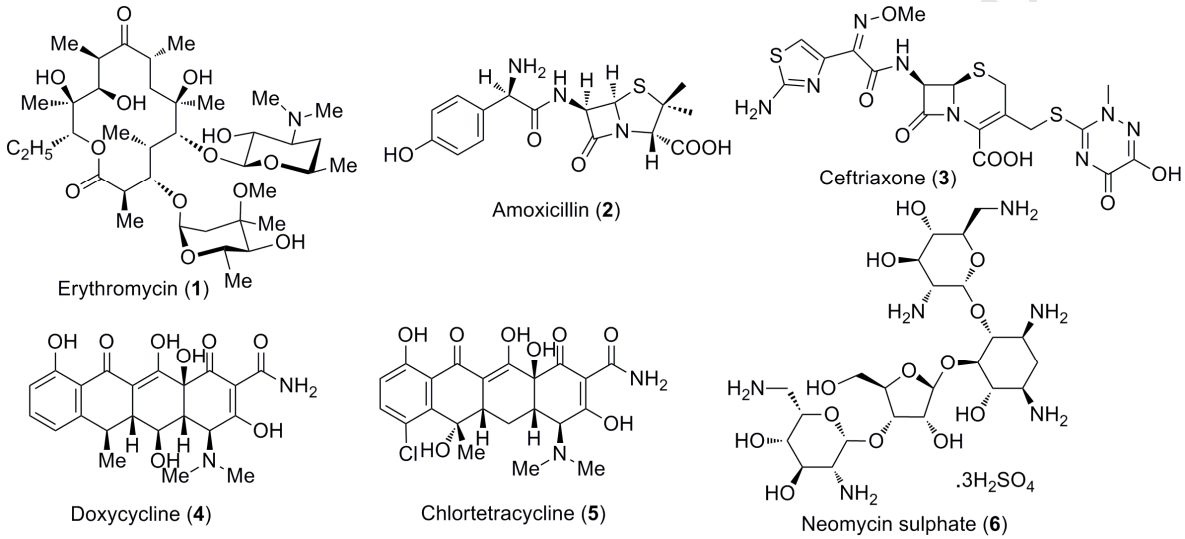


Fig. 1. Antibiotic drugs used for treatment of leptospirosis

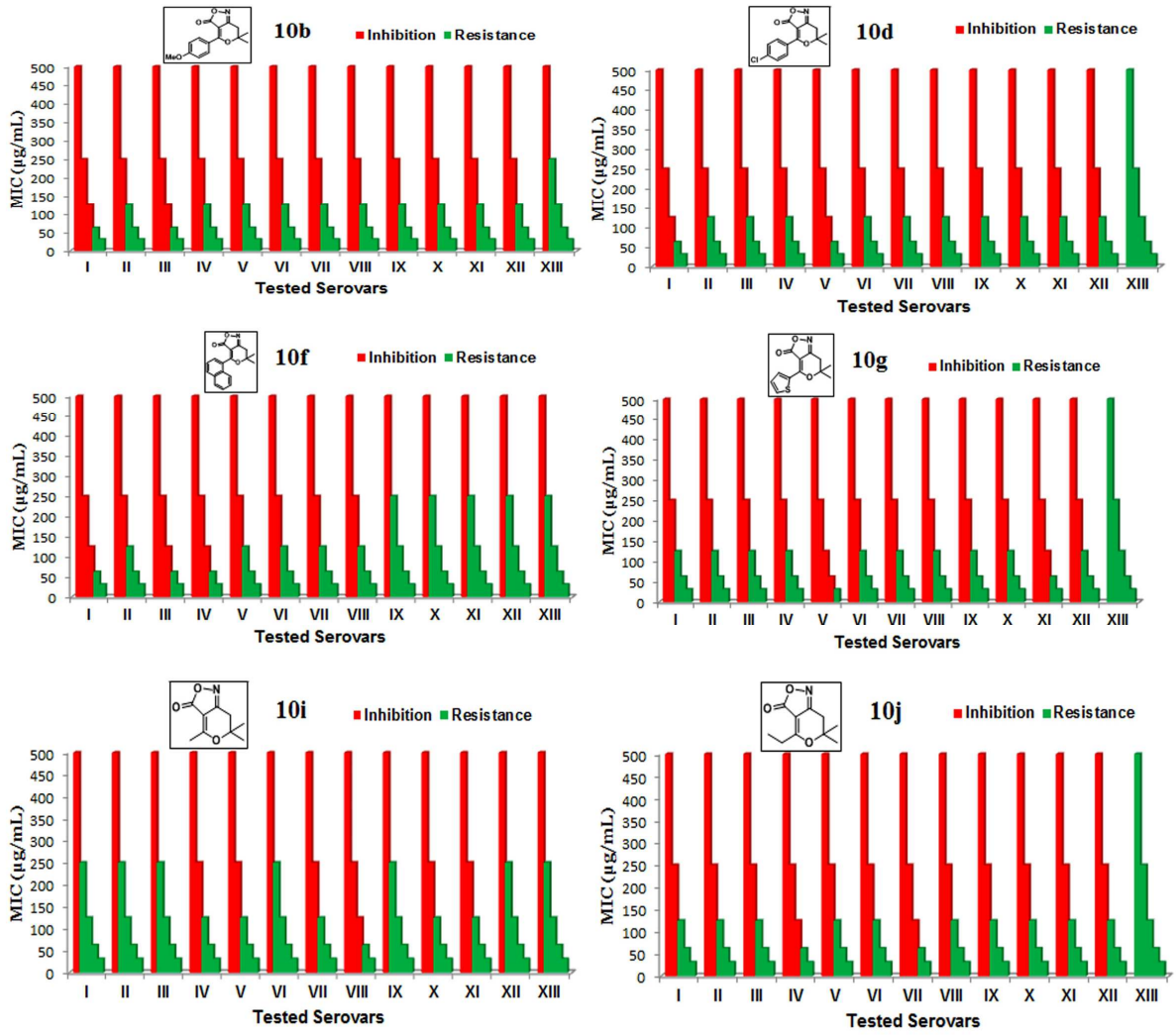


Fig. 2. Secondary screening of anti-leptospiral activity of pyranoisoxazolones **10b**, **10d**, **10f**, **10g**, **10i** and **10j** against twelve pathogenic serovar: Australis (**I**); Autumnalis (**II**); Ballum (**III**); Bataviae (**IV**); Canicola (**V**); Grippotyphosa (**VI**); Hebdomadis (**VII**); Icterohaemorrhagiae (**VIII**); Javanica (**IX**); Pomona (**X**); Pyrogenes (**XI**); Sejroe (**XII**); and one non-pathogenic serovar Semaranga (**XIII**).

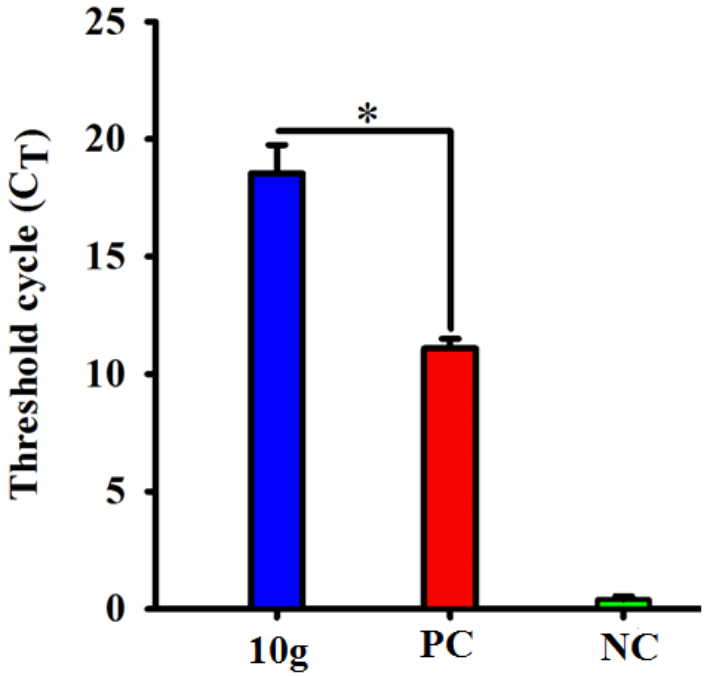


Fig. 3. Real Time quantitative PCR analysis (RT-qPCR). The propagation of leptospires was significantly less in **10g** treated samples than positive control. *P<0.05.

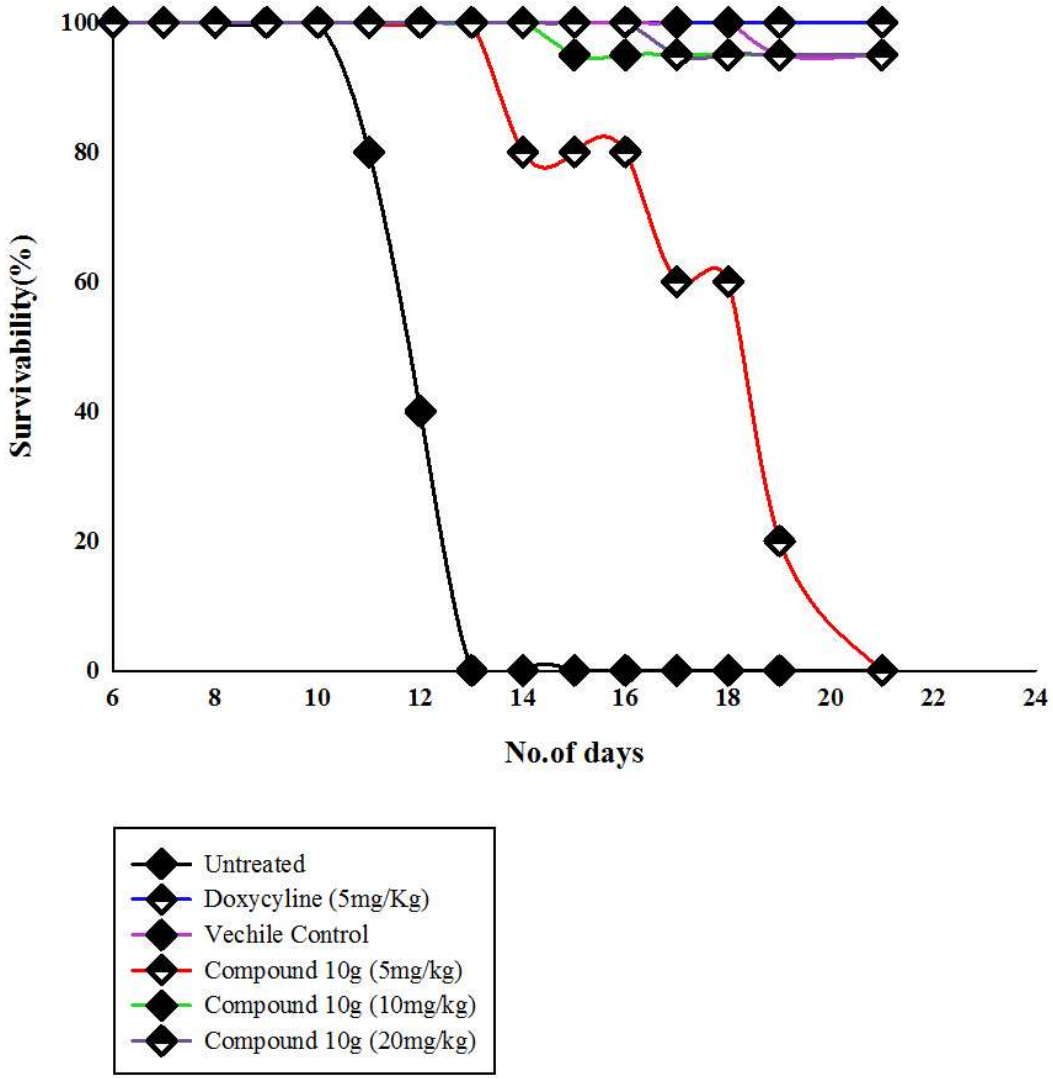


Fig. 4. Survival of mice treated for 3 days with compound **10g** (5, 10, or 20 mg/kg one time daily). Controls include untreated animals and those animals treated with doxycycline (5 mg/kg one time daily).

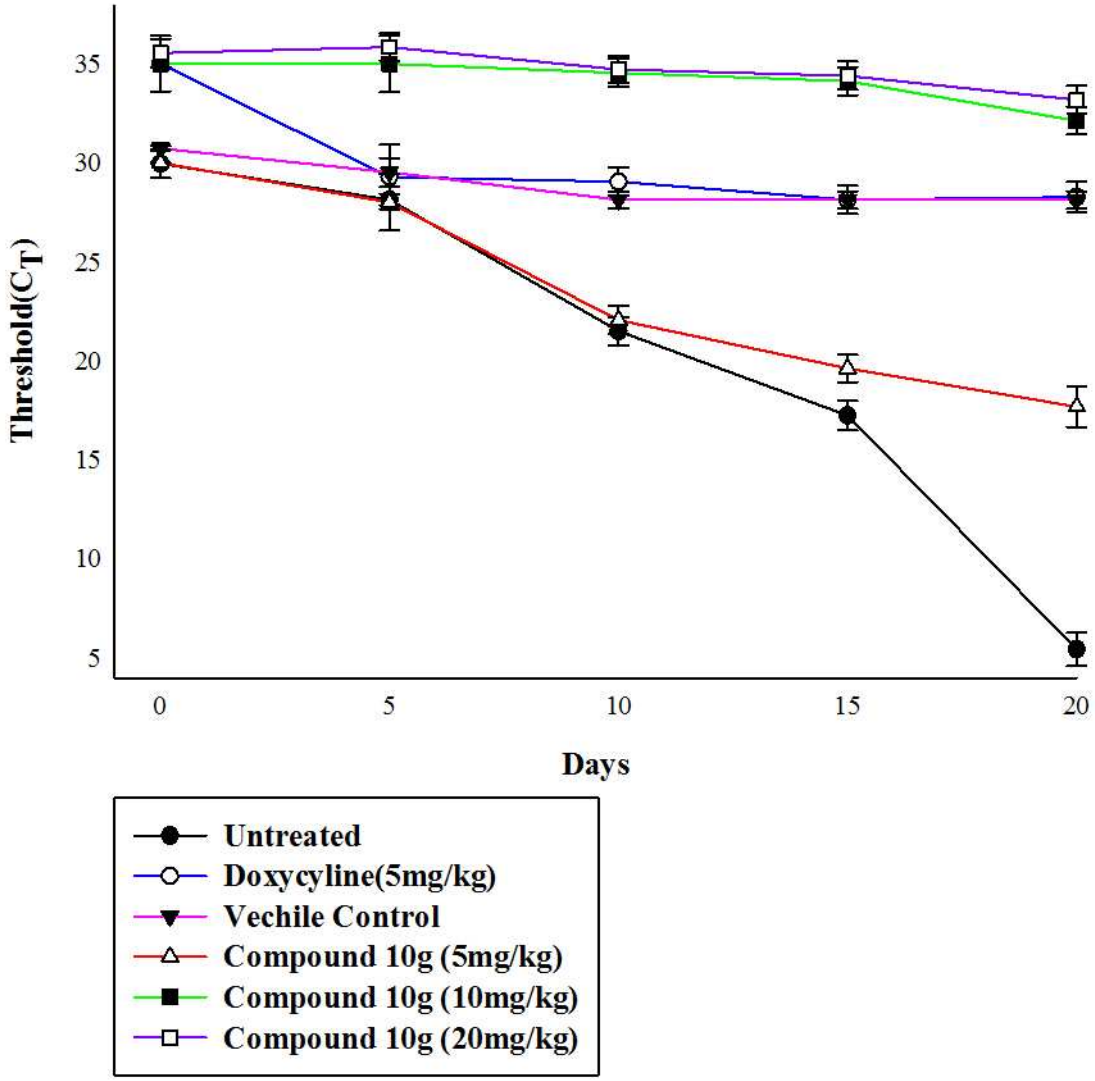


Fig. 5. The compound **10g** treated and untreated mice kidney tissue sample were analysed by RT-qPCR assay to determine the leptospiral load. Doxycycline was used as positive control.

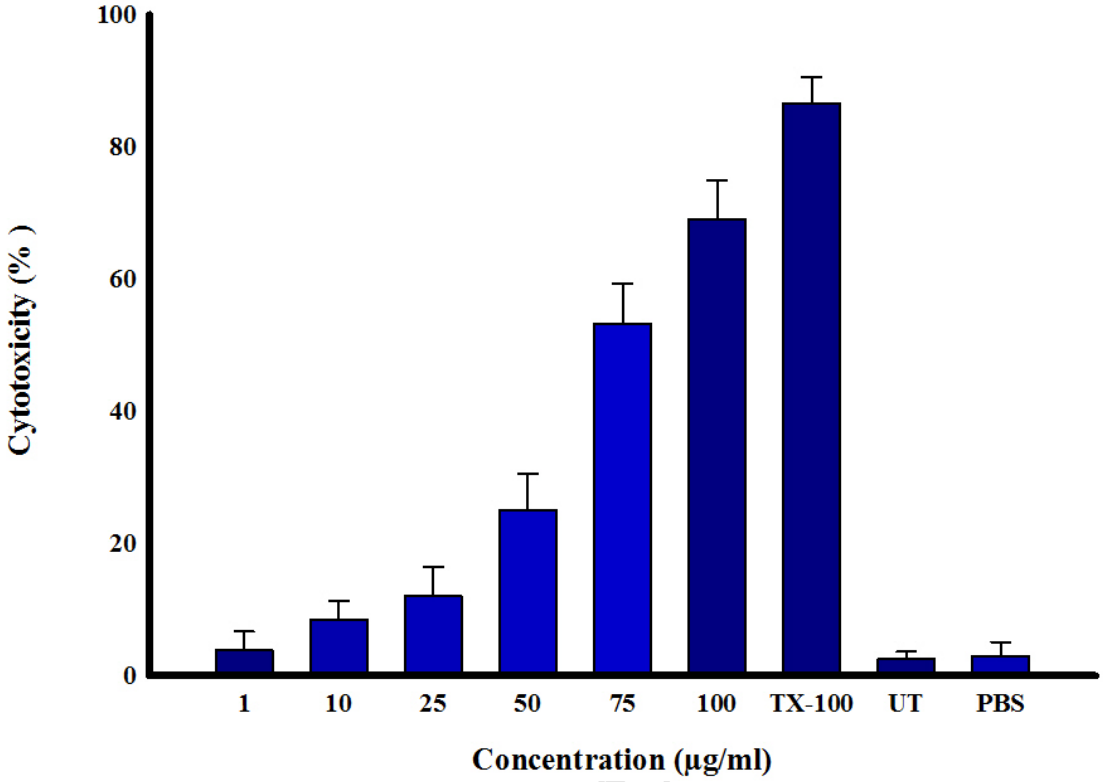


Fig. 6. Effect of compound **10g** on THP-1 cells treated at 1–100 µg/mL concentration for 24 h; TX-100 – Triton X-100; UT – Untreated cells. PBS - Phosphate-buffered saline.

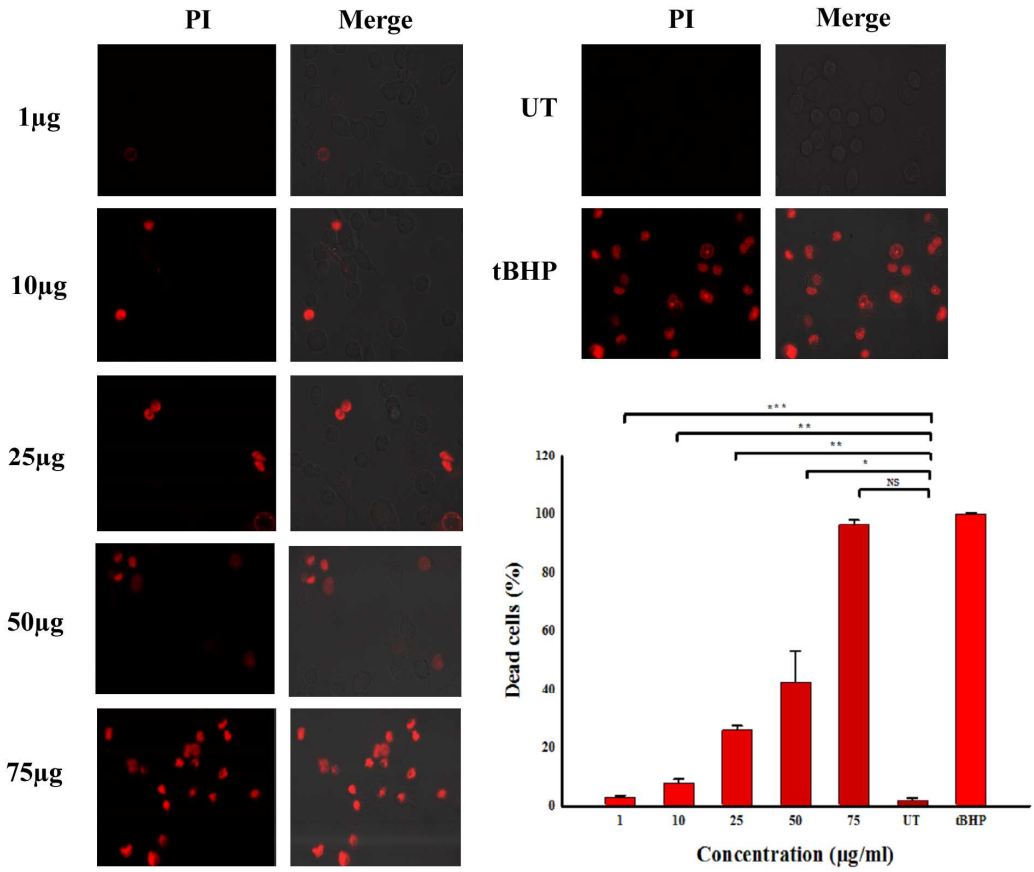


Fig. 7. PI staining of THP-1 cells at 12 h of incubations by the treatment of **10g** at different concentrations; UT – Untreated cells. tBHP – *tert*-butylhydroperoxide; The graph shows manual count of apoptotic and necrotic cells in percentage. (Data are mean percent ± SD percent of triplicate each).

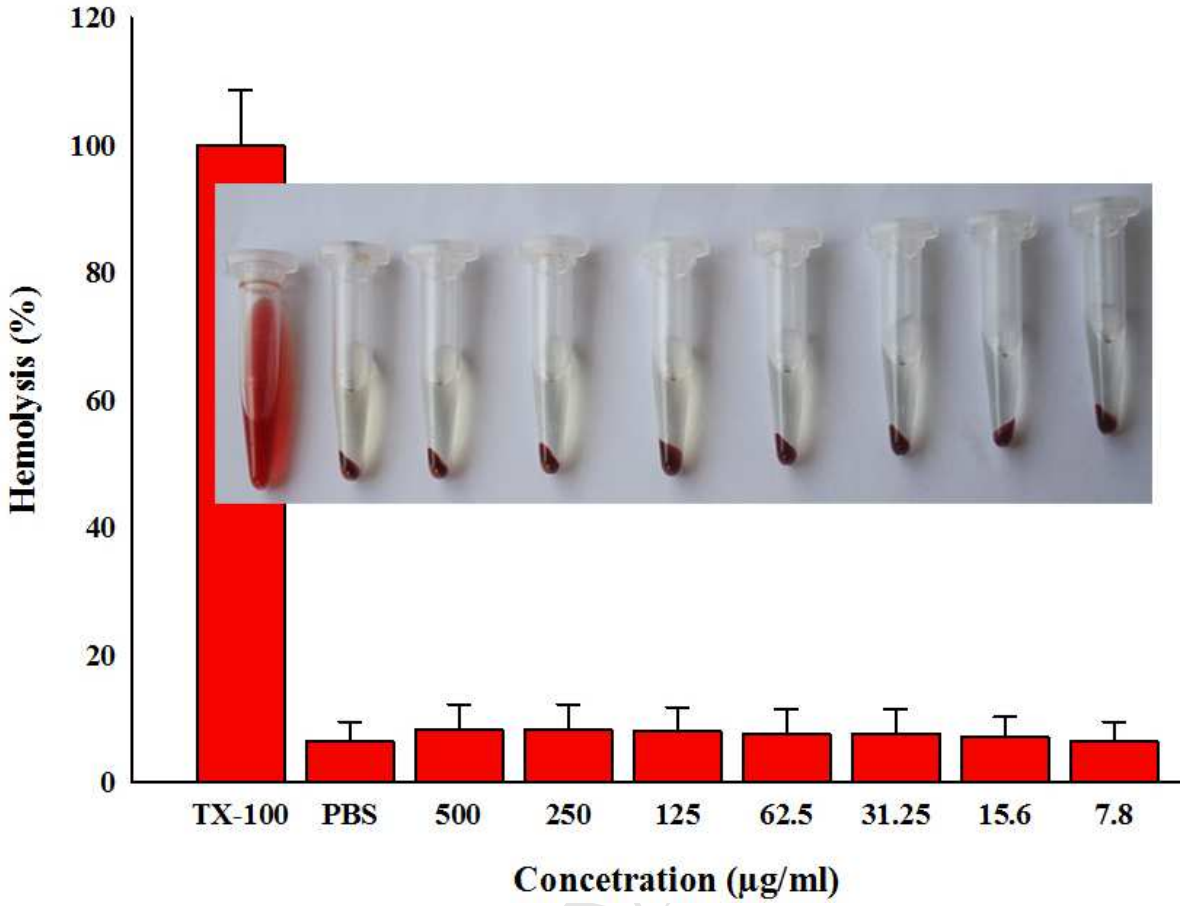


Fig. 8. *In vitro* hemocompatibility assay of pyranoisoxazolone **10g** exhibits very less hemolytic activity. No (0%) lysis was noticed in the negative control (NC-PBS buffer) whereas the positive control (PC- 1% Triton X-100) shows 100% lysis.

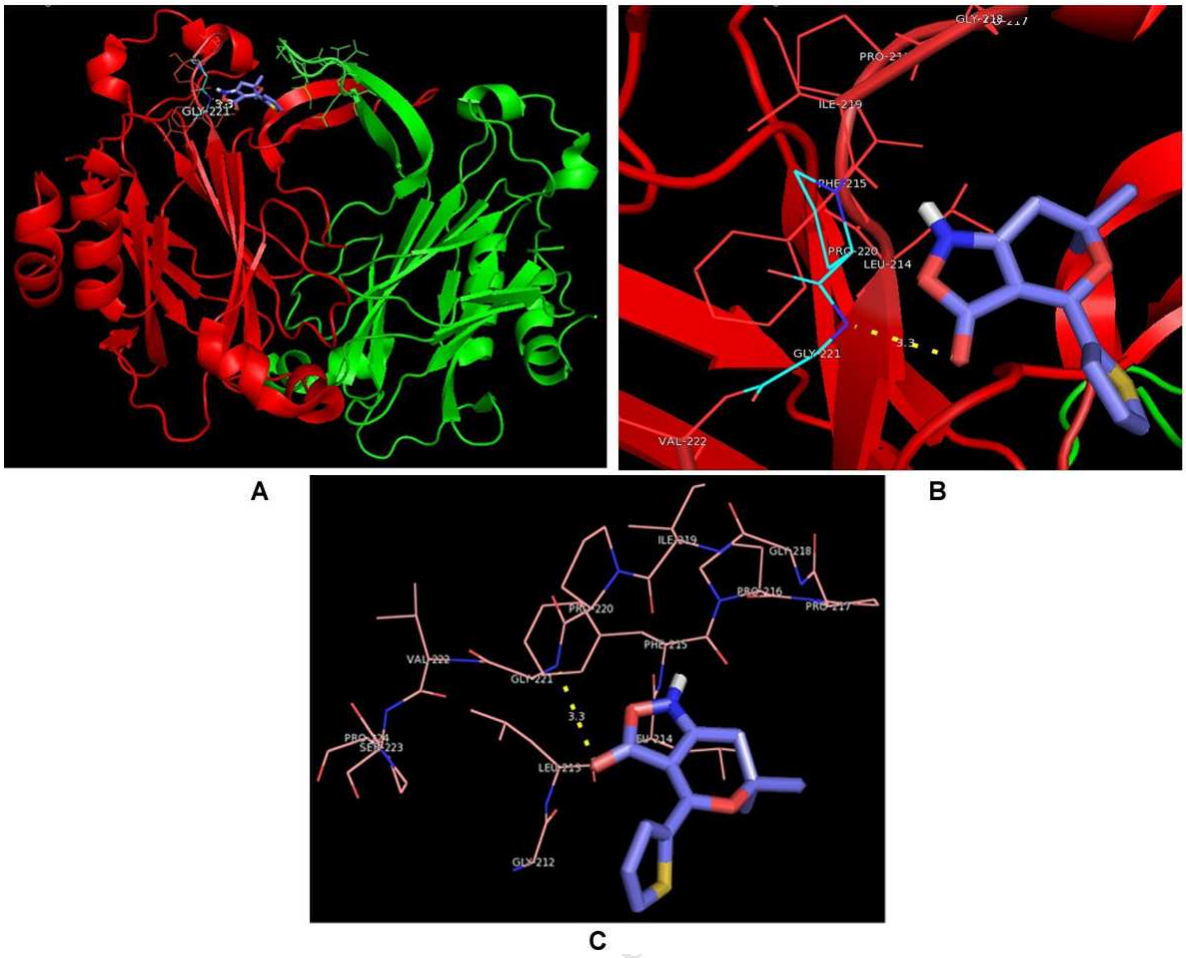
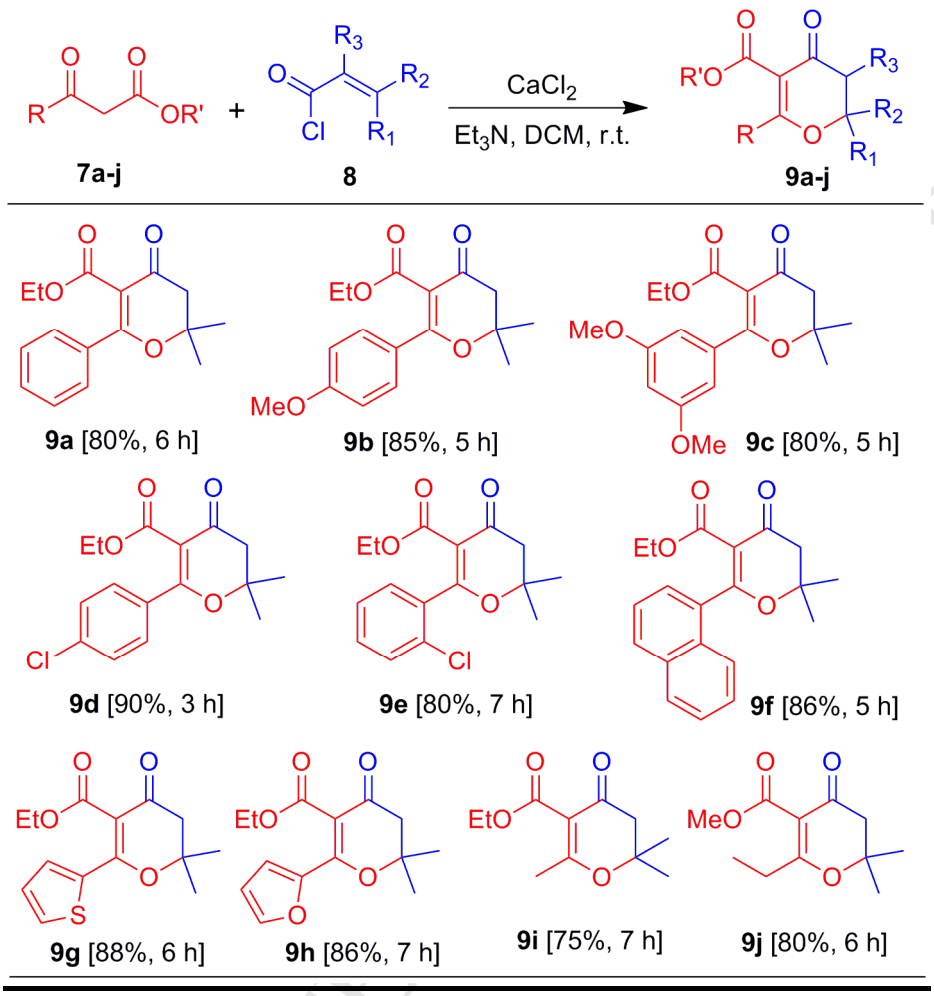
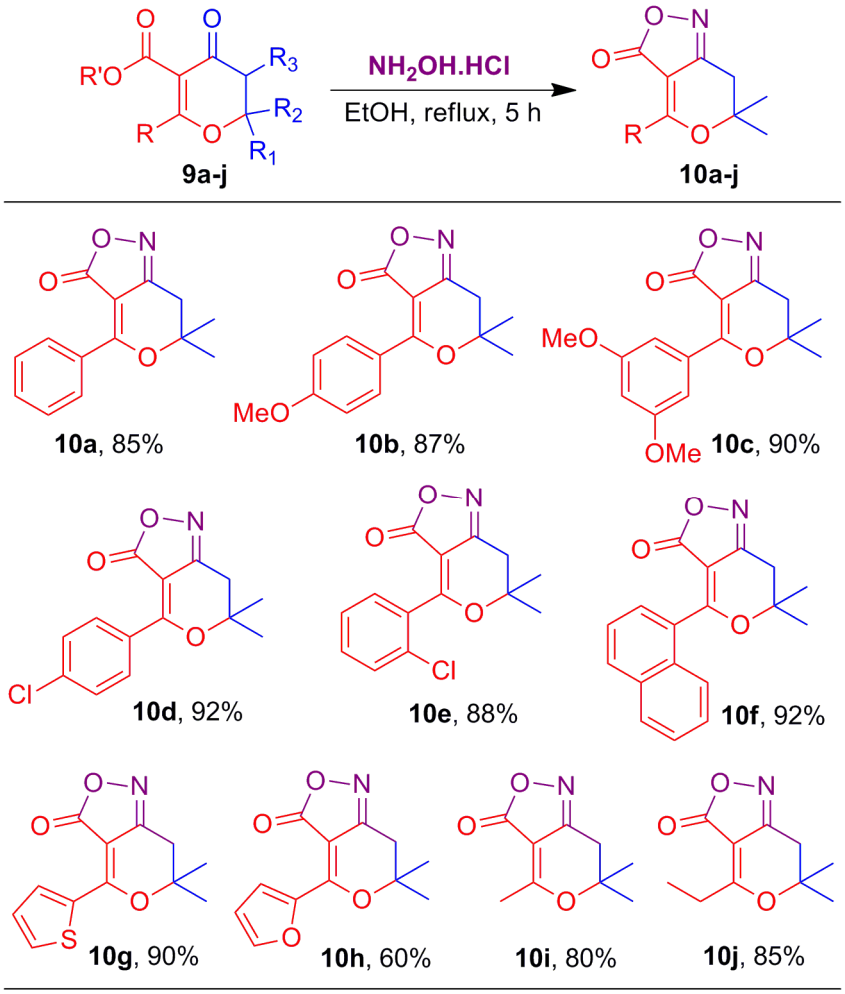


Fig. 9. (A) Docking pose of the compound **10g** with amino acid sequences of LipL32 in which chain-A shown in red color and chain-B shown in green color. (B) Docking pose of the compound **10g** with LipL32 (in zoom) (C) Line representation of selected amino acids of chain A of LipL32 interacting with compound **10g**. Yellow dotted lines indicate the hydrogen bonds. Pictures were generated by using PyMOL.

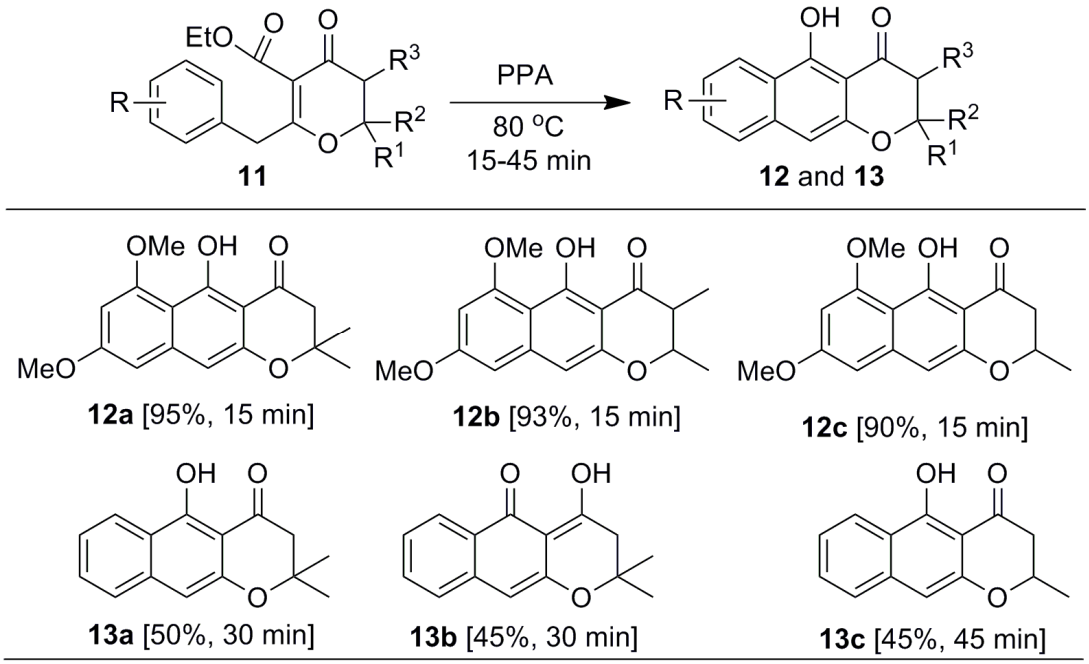
Schemes with Captions



Scheme 1. Synthesis of 5-carboalkoxy-2,3-dihydro-4*H*-pyran-4-ones: *Reaction conditions*: **7** (1.0 mmol), **8** (1.0 mmol), $CaCl_2$ (0.1 mmol), triethylamine (2.0 mmol) in DCM (5 mL) at room temperature. Isolated yield.



Scheme 2. Synthesis of substituted 6,7-dihydro-3*H*-pyrano[4,3-*c*]isoxazol-3-ones: *Reaction conditions*: DHPs **9** (1.0 mmol), $NH_2OH.HCl$ (1.5 mmol) in 5 mL of EtOH at 25 °C for 5 h. Isolated yield.



Scheme 3. Synthesis of dihydronaphthopyran-4-one natural products and its analogues

Highlights

- Pyrano[4,3-*c*]isoxazol-3-ones are found to be a new class of anti-leptospiral agents
- Compounds **10g** shows activity at concentration as low as 62.5 μg/mL
- Pathogen specific *In vitro* activity: 12 pathogens and 1 non pathogen screened
- *In vivo*: Inhibits leptospires in Cy-BALB/c mice by clearing renal colonization
- *In silico:* Compound **10g** binds with pathogen specific lipoprotein LipL32