Indian Journal of Biotechnology
Vol 9, July 2010, pp 265-270

Anti-HIV, anti-tubercular and mutagenic activities of borrelidin

D V R N Bhikshapathi, D R Krishna and V Kishan*
University College of Pharmaceutical Sciences, Kakatiya University, Warangal 506 009 India

Received 2 January 2009; revised 5 October 2009; accepted 20 December 2009

Borrelidin, an antibiotic found to be an angiogenesis inhibitor, is a potential drug candidate due to its antiangiogenic activity and other biological activities. Keeping in view the scope of the antibiotic, we have performed some of the biological activities like effect of borrelidin on *Mycobacterium tuberculosis* $H_{37}Rv$ *(*ATCC 27294*)* by BACTEC 460 TB and multidrug resistant *M. tuberculosis*. In these studies, the minimum inhibitory concentration (MIC) of borrelidin against *M. tuberculosis* $H_{37}Rv$ was found to be 3.12 µg/mL. When compared with known antituberculous drugs like rifampicin, isoniazid, ethambutol and streptomycin, borrelidin showed higher MIC on *M. tuberculosis* $H_{37}Rv$ (ATCC 27294). The MIC was, however, lower than that of pyrazinamide. The inhibitory activity on multidrug resistant *M. tuberculosis* was, observed at 256 µg/mL for borrelidin, rifampicin and isoniazid. The anti-HIV reverse transcriptase activity of borrelidin was tested by ELISA test, and mutagenic effect of borrelidin was performed by Ames test. Borrelidin showed moderate activity against anti-HIV reverse transcriptase enzyme. Results of Ames test indicated that borrelidin was non-mutagenic up to 500 µg/mL concentration.

Keywords: Borrelidin, *Mycobacterium tuberculosis,* mutagenicity, anti-HIV reverse transcriptase enzyme

Introduction

Borrelidin, a non-glycosidic 18 membered macrolide antibiotic, was isolated for the first time from *Streptomyces rochei* in 1949 by Berger and co-workers[1]. Borrelidin, which showed anti-borrelia activity, was later isolated from *Streptomyces* C2989[2], *S. griseus* BS 1325[3], *S. parvulus Tü* 4055[4], *S. candidus* (ATCC 202148)[5] and from an Indian soil isolate, *S. californicus*[6].

Borrelidin was demonstrated to have various biological activities like antibacterial, antimitotic[7], antiviral, herbicidal, insecticidal and antitumour activities. The activities were attributed to the inhibition of threonyl tRNA synthetase and the activation of caspase-3 and caspase-8. Borrelidin exhibited excellent antimalarial activity against both chloroquine-sensitive and -resistant strains in mice[8]. Further, recent reports indicated that borrelidin had potent antiangiogenesis activity and induced apoptosis of the capillary tube forming cells in a dose dependent manner[9]. In our search for antibiotics active against drug resistant bacteria, *S. californicus* (MTCC 4401) was isolated from Indian soil, which also produced borrelidin[6]. Interest was regenerated on borrelidin, once again, due to recent reports of its antiangiogenic activity. Keeping in view the potential antiangiogenesis, antimalarial and other activities reported for borrelidin, it is speculated to be a possible drug candidate. Therefore, we performed anti-HIV, -tubercular and other studies on borrelidin.

Borrelidin presents a unique structural feature, not described for any other known macrolide, a nitrile moiety present at C12 of macrolide ring containing natural products produced by microorganisms are relatively rare[10]. The structural novelty and relevant biological activity of borrelidin presented an exciting challenge for its chemical synthesis[11]. Several workers[12] contributed towards the total synthesis of borrelidin.

In this paper, we describe the antimycobacterial effects of borrelidin on *Mycobacterium tuberculosis* and its multidrug resistant strain in comparison to standard drugs, along with anti-HIV activity and mutagenic activity by Ames test.

Materials and Methods

Effect of Borrelidin on *M. tuberculosis* $H_{37}Rv$ (ATCC 27294)

Radio labeled palmitic acid containing 12B medium and Bactec 460 TB obtained from Becton Dickinson, Maryland, USA, were used to carryout the study. The protocol followed in brief was:

*Author for correspondence:
Tel: 91-870-2446259, 2432699; Fax: 91-870-2438844, 2453508
E-mail: vbkishan@yahoo.com

M. tuberculosis $H_{37}Rv$ (ATCC 27294) strain was routinely maintained on LJ (Lowenstein Jensen medium) slants[13]. Broth cultures were incubated at 37°C until the turbidity exceeded that of a 0.5 McFarland standard, when the growth reached a growth index reading 400-500, 0.1 mL of this culture was used to inoculate each test vial. Borrelidin solutions as well as other standard drug solutions were freshly prepared using a solvent mixture (0.1 mL DMSO+0.9 mL sterile distilled water), in the concentration range of 0.8 to 100 µg/mL and passed through millipore filter. The effect of borrelidin was compared with that of known antimycobacterial drugs like streptomycin, rifampicin, ethambutol, pyrazinamide and isoniazid (described in literature). Positive and negative controls were included in the study. The vials were read daily and the radio active CO_2 released was recorded in terms of a growth index on a scale of 0 to 999[14]. Minimum inhibitory concentration (MIC) of borrelidin on *M. tuberculosis* was determined using Becton Dickinson BACTEC system.

Effect of Borrelidin on Multidrug Resistant *M. tuberculosis*

Rifampicin and isoniazid were purchased from Sigma Chemicals and borrelidin was isolated in our laboratory. The LJ medium[13] was prepared as per the formula. The multidrug resistant *Mycobacterium strain* C_{77}/08 was isolated from a patient's sputum with a known history of tuberculosis (Multidrug resistant), by Dr Chitra Chandrasekhar in Dr Iravatham Clinical Laboratory, Hyderabad. Four mL of LJ medium was poured into a sterile McCartney bottle, containing different concentrations of drug solutions, tightly capped and kept in a slanting position over night. The culture was streaked on LJ slants and incubated at 37°C for 3-4 wks[15]. Antibiotic concentrations of rifampicin, isoniazid and borrelidin were prepared geometrically in the range of 2-256 µg/mL. A positive control and solvent control (0.1 mL DMSO and 0.9 mL sterile distilled water) were included in the study.

Anti-HIV RT Activity of Borrelidin

Reverse Transcriptase (RT) Assay kit, (Gmbh Roche Diagnostics, Germany, Cat. No. 11 468 120 910) was used to carryout the activity. The protocol followed in brief was: 20 µL of diluted lysis buffer containing recombinant HIV-1-RT, 4-6 ng was taken into an Eppendorff tube initially. 20 µL of RT inhibitors (Zidovudine) or test compound (borrelidin) diluted in lysis buffer was added along with 20 µL reaction mixture per reaction tube and incubated for 1 h at 37°C. Lysis buffer without recombinant HIV-1-RT were used as a negative control. Total 60 µL of the solution from each of the Eppendorff tubes was transferred into the wells of the ready to use MP modules, covered and incubated for 1 h at 37°C. The solution was discarded completely. Anti DIG-POD (Digoxigenin-peroxidase), working dilution (200 mU/mL solution, 5a, of protocol), 200 µL per well was added. MP modules were covered and incubated further for 1 h at 37°C. Finally, the solution was completely discarded and rinsed 5 times with 250 µL of washing buffer. To each well, 200 µL of ABTS (sodium perborate and citric acid/phosphate buffer) substrate solution was added and incubated for 10-15 min at 15-25°C for the development of green colour. Using a micro plate (ELISA; Anthos 2010, Germany) reader, the absorbance of the samples was measured at 405 nm[16].

Ames Mutagenicity

Ames mutagenicity of borrelidin was conducted at different concentrations (10-1000 µg/mL) and with a positive control of sodium azide (10 µg/mL) on 24 h culture of *Salmonella typhimurium* TA-1538 (ATCC e 29631). Davis minimal agar[17] plates were prepared. Molten top agar tubes at 45°C, containing 0.2 mL of sterile biotin-histidine solution and 0.1 mL of the *S. typhimurium* test culture were prepared and the contents of test tubes were mixed by rotating them between the palms of the hands. The top agar cultures were poured on to the minimal agar plates and allowed to solidify. Sterile filter paper discs were dipped in the test chemical solutions i.e., borrelidin and that of sodium azide, a positive known control, with sterile forceps and placed the discs after draining off the extra chemical solution in the centre of the minimal agar plates. A sterile disc without any chemical i.e., with sterile water was placed on the plate labeled as negative control. All the plates in triplicate were incubated in an inverted position for 48 h at 37°C[18]. The number of revertant colonies were enumerated on each plate and average was found in each case and compared with positive and negative controls.

Results and Discussion

Although borrelidin was reported as an antibiotic for a long time, due to toxicity of the molecule not much work was conducted subsequently. During the

search for new antibiotics, by several groups, this compound was reported to have been produced from many species of *Streptomyces*[2-5]. The information on toxicity of borrelidin was limited to the determination of LD_{50} values in mice (39.0 mg/kg by I.V & 74-7 mg/kg body wt by subcutaneous route)[19]. It is known that there are many drugs in the current therapy with much lower LD_{50} values, e.g. Chlorambucil 20 mg/kg[20], Doxorubicin 26 mg/kg[21]. Cytotoxic activity of borrelidin (purified by HPLC) was tested at 10 µg/ml against HBL 100 breast cancer cell lines and the percentage of inhibition was found to be 10.23, whereas doxorubicin (standard) at 200 n *M*showed 87.35% of inhibition[6]. Accordingly, we performed experiments to know the histological changes in liver of albino rats after treating for 5 d by oral route at 400 µg/kg body wt/day, which resulted in histopathological changes as described in our earlier paper[22]. When the oral dose was increased to 1 mg/kg body wt/day of rats, the rats died on the 4th d (Unpublished result).

Further, the recent findings like antiangiogenic[9], anticancer, antimalarial, antimitotic[7], herbicidal and other activities stimulated various groups to concentrate once again on this molecule. Studies related to biosynthesis, chemistry and structure analogues were also performed[12]. Mechanism of action was also studied[8]. The structure of this molecule is unique in having a nitrile group containing macrolide antibiotic. It is known that macrolides like clarithromycin are having anti mycobacterial activities and ansa macrolides like rifampicin are having antitubercular activities. Due to this structural uniqueness and no information on the antitubercular activities of the borrelidin, the compound was tested initially for activity on *M. tuberculosis.* Further, anti-HIV activity of borrelidin was also not reported. Hence, the studies were conducted to find out the anti-HIV properties of the molecule. In addition, the scope of carcinogenicity or mutagenicity was also explored.

BACTEC 460 TB test is a very commonly used test for studying the sensitivity of *M. tuberculosis* against different drugs. Consequently, in this study BACTEC 460 TB was used to assess the effectiveness of borrelidin against *M. tuberculosis*. This study revealed that borrelidin inhibited the growth of *M. tuberculosis* $H_{37}Rv$. The MIC's of the tested drugs are given in Table 1. In these serial dilution experiments, the MIC of borrelidin against *M. tuberculosis* $H_{37}Rv$ was found to be 3.12 µg/mL. When compared with known drugs like rifampicin, isoniazid, ethambutol and streptomycin, borrelidin exhibited higher MIC on *M. tuberculosis* $H_{37}Rv$ (ATCC 27294)[14]. But, when compared with pyrazinamide it was having lower MIC, i.e., 10-fold more active[23]. These results clearly indicated that borrelidin could be an alternative drug candidate for treating tuberculosis. The results of the present study are in accordance with the reported activities of many macrolide antibiotics on *M. tuberculosis*.

Further, the effect of this drug on multidrug resistant *M. tuberculosis* was also tested. In this study, a clinical isolate was used to grow on LJ medium. Various concentrations of drugs were geometrically prepared and tested. The incubation period was for 3 wks. The effects on control, solvent control (DMSO+Water (1:9) and on multidrug resistant *M. tuberculosis* were observed after 3 wks of incubation (Table 2). The tested concentrations varied geometrically from 2 µg/mL to 256 µg/mL for all the drugs and borrelidin, respectively. The inhibitory activity was observed at 256 µg/mL for borrelidin, rifampicin and isoniazid, whereas for other and lower concentrations no activity was observed.

Borrelidin and reference standards like rifampicin and isoniazid showed inhibitory activity against

Table 1—Comparative MICs of standard drugs and borrelidin on *M. tuberculosis* ($H_{37}Rv$)

No.	Antibiotic	MIC (µg/mL)
1	Isoniazid	0.03[14]
2	Rifampicin	0.25[14]
3	Streptomycin	0.5[14]
4	Ethambutol	1[14]
5	Pyrazinamide	32[23]
6	Borrelidin	3.12

Table 2—Inhibitory activity of rifampicin, isoniazid and borrelidin on multidrug resistant *M. tuberculosis* (incubated for 3 wks in L J medium)

Antibiotic	Conc. range of drug (2-128 µg/mL)	Conc. of drug (256 µg/mL)
Rifampicin	3/3 growth	No growth (3/3)
Isoniazid	2/3 growth*	No growth (3/3)
Borrelidin	3/3 growth	No growth (3/3)
Control	Growth	Growth
Solvent control	Growth	Growth

*Note: 2/3-2 out of 3 culture tubes showed growth in case of a conc. 128 µg/ml only.
Lower doses showed growth. However, this conc. is not considered as inhibitory conc.

multidrug resistant *M. tuberculosis* at 256 µg/mL. These results, therefore, indicated that borrelidin was also effective against the used strain of multidrug resistant *M. tuberculosis* (C_{77}/08) when compared with other drugs tested. The reported mechanisms of action of the two known drugs are different when compared to that of borrelidin. Thus, this finding indicated the possibility of an additional mechanism for the inhibition of *M. tuberculosis.*

Table 3—Inhibitory activity of borrelidin on recombinant-HIV reverse transcriptase enzyme

No.	Drugs	(µg/mL)	% Inhibition of RT enzyme activity
1	Zidovudine (Standard)	10	50.2
2	Zidovudine (Standard)	100	64.0
3	Borrelidin (Test)	10	12.57
4	Borrelidin (Test)	100	37.58

Anti-HIV reverse transcriptase activity was used as marker for the anti-HIV effect of borrelidin. The anti-HIV reverse transcriptase activity of borrelidin

Table 4—Effect of borrelidin on mutagenicity of *S. typhimurium his*$^-$ (ATCC e 29631)

Test chemical	Av. no. of revertant colonies	No. of induced mutations	Degree of Mutagenicity
Negative control	04	—	(-)
Positive control (Sodium azide,10µg/ml)	206	202	(2+)
Borrelidin(10µg/ml)	01	—	(-)
Borrelidin(50µg/ml)	01	—	(-)
Borrelidin(500µg/ml)	03	—	(-)
Borrelidin(1000µg/ml)	18	14	(1+)

Note: Degree of mutagenicity is based on the no. of revertant colonies obtained - Below 10(-), If more than 10 (1+), more than 100 (2+), more than 500 (3+)[18].

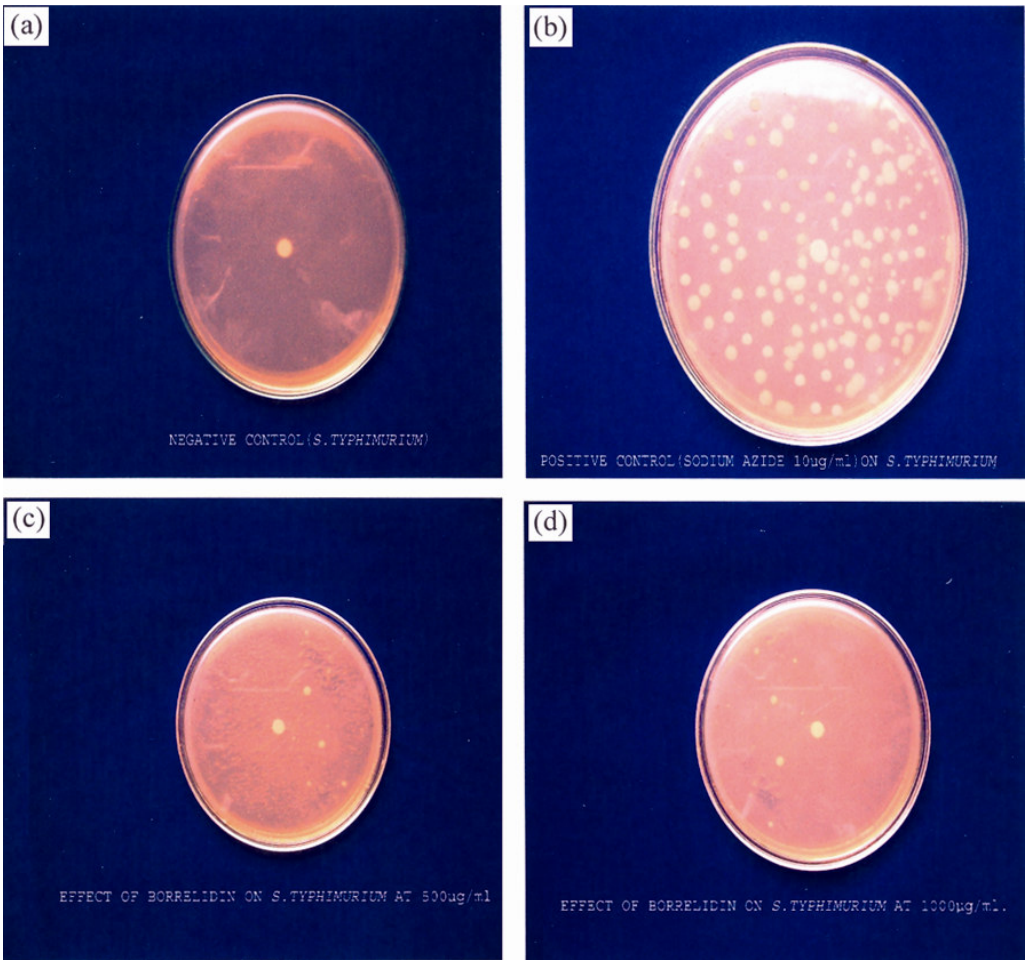

Fig. 1—The mutagenic effect of borrelidin on *S. typhimurium* (TA-1538) tested by Ames method at different concentrations: a. Negative control showing four spontaneous revertant colonies in the absence of any drugs, which were very small and not to be seen in the photograph. The central disc was wet with sterile distilled water; b. Sodium azide (10 µg/mL)-A positive control showing large number of revertant colonies on a minimal medium plate; c & d. Borrelidin at 500 µg/mL, 1000 µg/mL, respectively show very few number of revertant colonies.

was tested by ELISA test and results are shown in Table 3. Zidovudine was used as standard antiviral drug. In the test, the absorbance of the sample was measured at 405 nm. The maximum percentage of enzyme inhibition by borrelidin was found to be 37.58, at 100 µg/mL. Whereas, Zidovudine resulted in 64.08% inhibition activity. From these results, it was concluded that borrelidin showed moderate activity against recombinant HIV reverse transcriptase enzyme. Following this, borrelidin is expected to be another lead structure for further anti-HIV drug development. Further, it is clinically known that HIV infected patients are prone for tuberculosis, due to lack of immunity. Borrelidin, being active against *M. tuberculosis* and with moderate anti-HIV activity will become a potential drug candidate for further investigations in developing suitable drug for treating HIV +ve and tuberculosis infected cases.

The purpose of testing the mutagenic effect of borrelidin by Ames test was to find out its possibility as a potential carcinogen/mutagen. Different concentrations (10, 50, 500 and 1000 µg/mL) of borrelidin were used and the *S. typhimurium his*$^-$ mutant (ATCC e 29631) was used as sensitive culture. Sodium azide, a known mutagen (10 µg/ml), was used as positive control. Number of colonies observed on the plates was counted for negative and positive control and test sample i.e., borrelidin at different concentrations. The results observed are shown in Table 4 and Figs 1a-d. The number of mutants or revertant colonies induced by mutations was very nominal for borrelidin in comparison with positive control (sodium azide). Sodium azide produced 202 colonies on the plates i.e., highly mutagenic at 10 µg/ml, whereas for borrelidin at 1000 µg/ml only few colonies were observed. Below 10 revertant colonies, the degree of mutagenicity is (-), if more than 10 (1+), more than 100 (2+), more than 500 (3+)[18]. The degree of mutagenicity is quantitatively expressed as 1+, 2+ and 3+ based on the number of revertant colonies. Borrelidin showed less than 10 revertant colonies up to 500µg/mL conc. These results clearly indicated that borrelidin is non-mutagenic up to 500 µg/mL conc.

Acknowledgement

The authors would like to thank the AICTE, New Delhi for providing research funds under MODROB scheme vide File No. 8020/RID/MODROB-5/2001-02. DVRNB is also thankful to AICTE for providing scholarship under Quality Improvement Programme for teachers. We thank Prof. Axel Zeeck of Goettingen University, Germany for the gift samples of borrelidin. In addition, we thank the DAAD for equipment grant, Germany. Further, the authors are also thankful to Dr Deepak More (Vimta Labs, Hyderabad) and Mrs Dr Chitra Chandrasekhar (Dr Iravatham Clinical Laboratory, Hyderabad), for providing the facilities for testing on *M. tuberculosis* $H_{37}Rv$ and multidrug resistant *M. tuberculosis,* respectively and to Dr A Sadanandam, Biotechnology Department, Kakatiya University for the help extended to carry out the anti-HIV RT activity test.

References

1 Berger J, Jampolsky L M & Goldberg M W, Borrelidin a new antibiotic with anti-borrelia activity and penicillin enhancement properties, *Arch Biochem*, 22 (1949) 476-478.

2 Lumb M, Macey P E, Spyvee J, Whitmarsh J M & Wright R D, Isolation of vivamycin and borrelidin, two antibiotics with anti-viral activity from a species of *Streptomyces* (C2989), *Nature (Lond)*, 206 (1965) 263-265.

3 Dorgerloh M & Kret S A, Borrelidin insecticide and herbicide and its preparation by fermentation, *Ger Offen,* DE 3607287, 11, 1988.

4 Olano C, Steven J M, Alfredo F B, Rose M S, Math V *et al,* Biosynthesis of the angiogenesis inhibitor borrelidin by *Streptomyces parvulus Tü* 4055: Insights into nitrile formation, *Mol Microbiol*, 52 (2004) 1745-1756.

5 Shiang M & Kuo M Y, New strain of *Streptomyces* and its applications, *Ger Offen,* DE 19835669, 22, 1999.

6 Saisivam S, Bhikshapathi D V R N, Krishnaveni J & Kishan V, Isolation of borrelidin from *Streptomyces californicus*- An Indian soil isolate, *Indian J Biotechnol,* 7 (2008) 349-355.

7 Vamsee C K, Maitra S, Mukkanti K & Iqbal J, Studies towards the total synthesis of (-) borrelidin; Strategies for the C11-C15 fragment construction and the utility of RCM reaction for macrolide cyclization using model systems, 231st ACS National Meeting, Atlanta, United States, 2006, 26-30.

8 Kazuro S & Omura S, Antiparasitic agents produced by microorganisms, *Proc Jpn Acad*, 81 (2004) 245-258.

9 Wakabayashi T, Kageyama R, Naruse N, Tsukahara N, Funahashi Y *et al,* Borrelidin is an angiogenesis inhibitor: Disruption of angiogenic capillary vessels in a rat aorta matrix culture model, *J Antibiot*, 50 (1997) 671-676.

10 Flemming F F, Nitrile containing natural products, *Nat Prod Rep*, 16 (1999) 597-606.

11 Duffey M O, Arnaud L T & Morken J P, Enantio selective total synthesis of borrelidin, *J Am Chem Soc*, 125 (2003) 1458-1459.

12 Nagamitsu T, Harigaya Y & Omura S, Total synthesis of borrelidin, *Proc Jpn Acad*, *Ser B*, 81(b) (2005) 244-256.

13 Ronald M A & James W S (eds), *Hand book of media for clinical microbiology*, 2nd edn (CRC Press, Boca Raton, USA) 1988, 291.

14 Chung G A C, Aktar Z, Jackson S & Duncan K, High-throughput screening for detecting antimycobacterial agents, *Antimicrob Chemother*, 39 (1995) 2235-2238.

15 Alessandro D L, Valentina O, Barbara S, Cenzo C, Maria L *et al,* Activity of a new class of isonicotinoylhydrazones used

alone and in combination with isoniazid, rifampicin, ethambutol, para-aminosalicylic acid and clofazimine against *Mycobacterium tuberculosis, J Antimicrob Chemother,* 49 (2002) 275-282.

16 Kiran U V, Radhika K, Mahendra D, Sadanandam A & Ramakrishna D, Anti HIV-Reverse transcriptase activity of some selected bio flavonoids, *Adv Pharmacol Toxicol,* 9 (2008) 71-76.

17 Davis B D & Elizabeth S M, Mutants of *Escherichia coli* requiring methionine or Vitamin B12, *J Bacteriol,* 60 (1950) 17-28.

18 Cappuccino J G & Sherman N (eds), The Ames test: A bacterial test system for chemical carcinogenicity, in *Microbiology, A laboratory manual* (The Benjamin/Cummings Publishing Company Inc., California) 1987, 345-350.

19 Glasby J S (ed), *Encyclopedia of antibiotics*, 2nd edn (Wiley-Interscience Publication) 1979, 145.

20 Morabito F, Callae I & Irrera G, *In vitro* improvement of chlorambucil induced cytotoxicity by deflazacort and 6-methylprednisolone in B-cell chronic lymphocytic leukaemia, *Eur J Haematol*, 58 (1997) 301-306.

21 Cevc G (ed), Medical applications of phospholipids*, in Phospholipids hand book*, 2nd edn (CRC Press Boca Raton, Taylor & Francis Group) 1993, 883-885.

22 Bhikshapathi D V R N, Shravan Kumar Y, Madhusudan Rao Y & Kishan V, Borrelidin: A prospective drug, *Indian J Biotechnol*, 9 (2010) 18-23.

23 Speirs R J, Welch J T & Cynamon M H, Activity of n-propyl pyrazinamate against pyrazinamide resistant *M. tuberculosis*, *Antimicrob Chemother*, 39 (1995) 1269-1271.