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SYNTHESIS, ANTITUBERCULAR AND ANTIBACTERIAL ACTIVITIES OF NOVEL *N*'-(SUBSTITUTED)-2-(2,5-DIMETHYL-1*H*-PYRROL-1-YL)PHENYL)BENZAMIDE DERIVATIVES

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ABSTRACT

Novel series of pyrrolylbenzamide derivatives were synthesized with an aim to combat the increasing anti-tubercular resistance and to develop more potent anti-tubercular agents with reasonably less side effect. Herein, we synthesized a series of substituted 2-(2,5-dimethyl-1*H*-pyrrol-1-yl)phenyl)benzamides (3a-f) by reacting different substituted aromatic acids with 2-(2,5-dimethyl-1*H*-pyrrol-1-yl)aniline (2) by using HBTU as a coupling agent, DIEA as a catalyst and DMF as a solvent. Structures of all the newly synthesized compounds were established by spectral analysis viz., IR, ^{1}H NMR, ^{13}C NMR and Mass. Further they were tested for their anti-tubercular and antibacterial activities and compounds showed moderate to good activity.

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INTRODUCTION

Mycobacterium tuberculosis is the major causative microbe to develop Tuberculosis (TB) in living beings, which is stands one among the top ten diseases to cause highest death worldwide. *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti* and *Mycobacterium canetti*are also likely cause TB but to limited extent [1, 2] and TB is characterized by tubercle lesions in the lungs [3]. It has infected man since the birth of civilization and in spite of the introduction of TB chemotherapy in the 1950s, it has made a dramatic resurgence in the past decades and it still remains a leading infectious disease worldwide [4]. The World Health Organization (WHO) estimated nearly 1.3 million deaths in 2017 are due to TB,which included 300,000 TB, associated HIV infected deaths [5-**7]** and india alone accounts for the total 27% deaths of worldwide figure, also India and China alone accounted for 40% emergence of multidrug-resistant (MDR-TB) and extensive drug resistance (XDR-TB) of the global gap that has become a main threat to human kind [8].

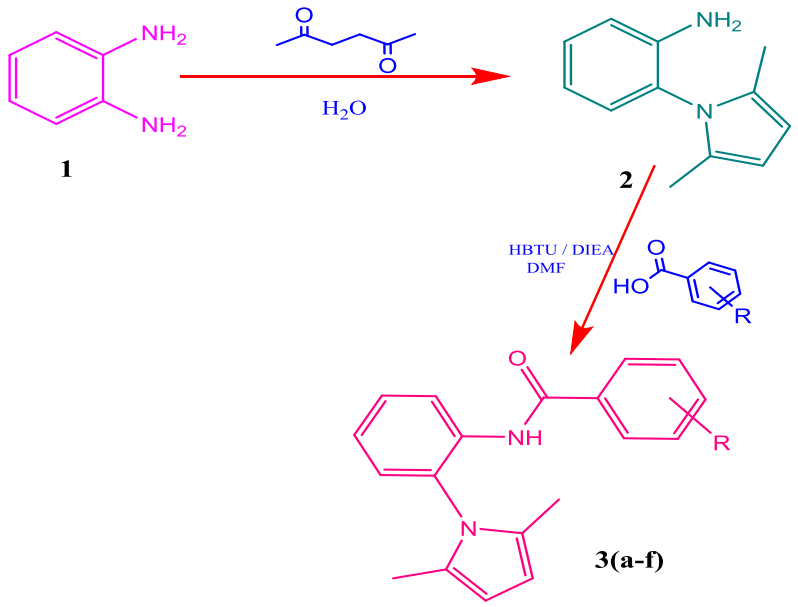
Pyrrole and its derivatives have well reported for their biological activities such as antibacterial, antitumor, analgesics, antitubercular, anti-inflammatory, and anti-allergic [9-14]. Several macromolecular antibiotics containing pyrrole structure were isolated from biological sources and their activities were defined [15, 16]. It is an important heterocycle in plant and animal kingdom because of its participation as a subunit of chlorophyll in plant cells and hemin and vitamin B_{12} in animal cells. Joshi and co-workers have reported some pyrrolebenzamide derivatives and they have shown significant antimycobacterial activities [17.18]. Literature shows the biological and medicinal importance of pyrroles and the potentiality of peptide linkage in tuberculosis studies, with above references we have carried out the synthesis of such molecules that contain both pyrrole and peptide linkages to treat alongside of the resistant TB.

In this work, we have synthesized some dimethylpyrrolylbenzamide derivatives by reacting different aromatic acids with 2-(2,5-dimethyl-1*H*-pyrrol-1-yl)aniline (**2**) by using HBTU as a coupling agent and DIEA as a catalyst and DMF as a solvent to get the titled products.

Experimental

All the chemicals used in the synthetic experiment were purchased from Sigma-Aldrich, S. D. Fine-Chem Limited and Spectrochem Pvt. Ltd. Solvents used were of reagent grade and they were purified and dried by the standard methods. Melting points were determined using the Shital-digital programmable melting point apparatus and are uncorrected. FTIR spectra in KBr pellets were recorded on a Bruker FTIR spectrophotometer. The ^{1}H NMR spectra were recorded on a Bruker AVANCE II at 400 MHz, chemical shifts are expressed in parts per million (*ppm*) relative to TMS. The abbreviations used to describe the peak patterns are: (*s*) singlet, (*d*) doublet, (*t*) tripletand (*m*) multiplet. Mass spectra (MS) were recorded in a JEOL GCMATE II GC-Mass spectrometer and SchimadzuQP 20105 GC-Mass spectrometer. Analytical thin-layer chromatography (TLC) was performed on precoated TLC sheets of silica gel 60 F254 (Merck, Darmstadt, Germany) visualized by long-(365nm) and short- (253nm) wavelength ultraviolet (UV) lamps.

SCHEME I:



R = a) H, b) 4-Cl, c) 4-Br, d) 4-F, e) NO_2, f) 3-Cl.

Procedure for the synthesis of 2-(2,5-dimethyl-1H-pyrrol-1-yl)aniline (2)

To a solution of O-phenylenediamine (10 mmol) in 20 ml water was added slowly 2,5-hexanedione (15 mmol) at room temperature and the mixture was refluxed for 45 min. The reaction mixture was poured onto crushed ice and neutralized by adding saturated solution of $NaHCO_3$. The separated solid was collected, washed with water, dried and recrystallized from chloroform. [11]

General procedure for the synthesis of N'-substituted-2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)benzamides (3a-f):

A mixture of 0.0018 mol of substituted aromatic acids, 0.0019 mol of *2-(2,5-dimethyl-1H-pyrrol-1-yl)aniline* (**2**) were dissolved in dry DMF (20ml), HBTU (0.0023 mol) and DIEA (0.93ml) were added and stirred for 25 h at room temperature and the reaction was monitored by TLC after the completion of reaction, the mixture was quenched by brine and then it is extracted with ethyl acetate (3 x 50 ml), then the ethyl acetate layer was washed with 1N HCl and saturated $NaHCO_3$ solution followed by brine, then the organic layer was evaporated using rotary flash evaporator to obtain compounds (**3a-f)** and further compounds were purified by column chromatography using chloroform: methanol (9:1) as mobile phase and visualized by long-(365nm) and short- (253nm) wavelength ultraviolet (UV) lamps.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)benzamide(3a)

IR (KBr) υ_{max}, cm^{-1}: 3397.03 (-NH), 2920.09, 2852.80 (CH-Ar), 1672.68 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.91 (s, 6H, pyrrole-diCH_3-H), 6.09 (d, 2H, pyrrole-C_3, C_4-H), 7.21-7.51 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.90-7.92 (d, 2H, phenyl-C_2, C_6-H), 7.96-7.98 (d, 2H, phenyl-C_3, C_5-H),8.05 (t, 1H, phenyl-C_4-H),8.90 (s, 1H, s, -NH).
MS (EI): m/z = Found =291.48 [M+1], Calcd. = 290.37.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-4-chloro-benzamide (3b)

IR (KBr) υ_{max}, cm^{-1}: 3359.47 (-NH), 2919.67, 2852.14 (CH-Ar), 1680.92 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.92 (s, 6H, pyrrole-diCH_3-H), 6.09 (d, 2H, pyrrole-C_3, C_4-H), 7.28-7.58 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.90-7.92 (d, 2H, phenyl-C_2, C_6-H), 7.95-7.96 (d, 2H, phenyl-C_3, C_5-H),8.68 (s, 1H, s, -NH).^{13}C NMR (100MHz, δ ppm CDCl3): 12.90, 106.78, 128.21, 128.36, 128.89, 128.92, 130.31, 132.43, 137.80, 139.62, 141.20, 166.78; MS (EI): m/z = Found =326.48 [M+2], Calcd. = 324.81.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-4-bromo-benzamide (3c)

IR (KBr) υ_{max}, cm^{-1}: 3358.73 (-NH), 2919.77, 2853.58 (CH-Ar), 1679.66 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.91 (s, 6H, pyrrole-diCH_3-H), 6.10 (d, 2H, pyrrole-C_3, C_4-H), 7.18-7.55 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.89-7.90 (d, 2H, phenyl-C_2, C_6-H), 7.96-7.98 (d, 2H, phenyl-C_3, C_5-H),8.71 (s, 1H, s, -NH).^{13}C NMR (100MHz, δ ppm CDCl3): 13.10, 106.88, 128.11, 128.22, 128.62, 128.89, 130.01, 133.43, 136.20, 139.62, 142.20, 166.88; MS (EI): m/z = Found =368.99 [M+1], 370.05 [M+2], Calcd. = 369.26.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-4-fluorobenzamide (3d)

IR (KBr) υ_{max}, cm^{-1}: 3201.47 (-NH), 2919.59, 2853.80 (CH-Ar), 1678.18 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.93 (s, 6H, pyrrole-diCH_3-H), 6.09 (d, 2H, pyrrole-C_3, C_4-H), 7.20-7.49 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.52-7.54 (d, 2H, phenyl-C_2, C_6-H), 7.67-7.69 (d, 2H, phenyl-C_3, C_5-H),8.65 (s, 1H, s, -NH).
MS (EI): m/z = Found =307.99 [M+], 309.88 [M+2], Calcd. = 308.36.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-4-nitrobenzamide (3e)

IR (KBr) υ_{max}, cm^{-1}: 3378.80 (-NH), 2974.25, 2921.07 (CH-Ar), 1640.93 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.90 (s, 6H, pyrrole-diCH_3-H), 6.10 (d, 2H, pyrrole-C_3, C_4-H), 7.18-7.44 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.60-7.62 (d, 2H, phenyl-C_2, C_6-H), 7.84-7.86 (d, 2H, phenyl-C_3, C_5-H),8.67 (s, 1H, s, -NH).^{13}C NMR (100MHz, δ ppm CDCl3): 12.96, 106.08, 127.21, 128.16, 128.69, 128.98, 130.61, 132.33, 136.80, 139.22, 140.55, 165.78; MS (EI): m/z = Found =335.48 [M+], Calcd. = 335.36.

N-(2-(2,5-dimethyl-1H-pyrrol-1-yl)phenyl)-3-chloro-benzamide (3f)

IR (KBr) υ_{max}, cm^{-1}: 3382.35 (-NH), 2919.97, 2852.99 (CH-Ar), 1679.44 (C=O); ^{1}H NMR (400 MHz, δ ppm $CDCl_3$): 1.95 (s, 6H, pyrrole-diCH_3-H), 6.09 (d, 2H, pyrrole-C_3, C_4-H), 7.29-7.53 (m, 4H, bridging ph-C_3, C_4, C_5, C_6-H), 7.93-7.94 (d, 2H, phenyl-C_2, C_6-H), 7.96-8.05 (m, 2H, phenyl-C_4, C_5-H), 8.68 (s, 1H, s, -NH). ^{13}C NMR (100MHz, δ ppm CDCl3): 12.90, 106.78, 128.21, 128.36, 128.89, 128.92, 130.31, 132.43, 137.80, 139.62, 141.20, 166.78; MS (EI): m/z = Found =324.03 [M+], 326.48 [M+2], Calcd. = 324.81.

Biological activity:

Antitubercular activity

MIC values were determined for *N*'-substituted-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)phenyl)benzamides **(3a-f)** against *M. tuberculosis* H37Rv strain by Micro plate Alamar Blue assay (MABA) [19] using INH as the standard drug. The 96 wells plate received 100 µL of Middle brook 7H9 broth and serial dilution of compounds were made directly on the plate with drug concentrations of 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 µg/mL. Plates were covered, sealed with parafilm and incubated at 37 ^{o}C for 5 days. Then, 25 µL of freshly prepared 1:1 mixture of almar blue reagent and 10% Tween 80 were added to the plate and incubated for 24 h. A blue color in the well was interpreted as no bacterial growth and pink color was scored as the growth. The MIC was defined as the lowest drug concentration, which prevented the color change from blue to pink. Table **2** reveals antitubercular activity (MIC) data.

Antibacterial activity

MIC determination of the tested compounds was investigated by a side-by-side comparison with norfloxacin and ciprofloxacin against Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) by the broth microdilution method [20, 21]. Serial dilutions of the test compounds and reference drugs were prepared in Mueller-Hinton agar. Drugs (10 mg) were dissolved in dimethylsulfoxide (DMSO, 1 mL). Further progressive dilutions with the molten Mueller-Hinton agar were performed to obtain the required concentrations of 0.2, 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 and 100 mg/mL. The tubes were inoculated with 105cfu/ mL (colony forming unit/mL) and incubated at 37 ^{o}C for 18 h. MIC was the lowest concentration of the tested compound that yielded no visible growth on the plate. To ensure that solvent had no effect on bacterial growth, a control was performed with the test medium supplemented with DMSO at the same dilutions as used in the experiments and DMSO had no effect on the microorganisms in the concentrations studied. Table **2** reveals the antibacterial activity (MIC values) data

RESULTS AND DISCUSSION:

Synthesis

Synthetic route adopted to obtain the target compounds are depicted in Scheme **I**. FTIR, ^{1}H NMR,^{13}C NMR and mass spectral data are in agreement with the proposed structures of all the synthesized compounds. The physicochemical properties of the newly synthesized compounds are depicted in Table 1.

Table 1. Physical data of the synthesized compounds:

Comp.	R	Yield (%)	Mobile phase for Column Chromatography	M.P (^{0}C)	Molecular Formula
3a	-H	75	Chloroform: Methanol	88-88	$C_{19}H_{18}N_2O$
3b	4-Cl	61	Chloroform: Methanol	100-102	$C_{19}H_{17}N_2OCl$
3c	4- Br	63	Chloroform: Methanol	110-112	$C_{19}H_{17}N_2OBr$
3d	4-F	66	Chloroform: Methanol	94-96	$C_{19}H_{17}N_2OF$
3e	4-NO_2	70	Chloroform: Methanol	96-98	$C_{19}H_{17}N_3O_2$
3f	3-Cl	74	Chloroform: Methanol	92-94	$C_{19}H_{17}N_2OCl$

All the synthesized compounds of pyrrolylbenzamide derivatives (3a-f) were obtained in a good yield and their structures were established by NMR spectral analysis, FTIR spectra of compound 3b showed the presence of C=O peak at 1680 cm^{-1} and -NH's peak at 3359 cm^{-1}, further the structure of compound **3b** was confirmed by ^{1}H NMR spectra which showed the presence of two -NH as singlet at 8.68 ppm, doublet peak at 6.02 corresponds to pyrrole-H and a peak at 1.90 ppm was attributed to the presence of -CH_3 . ^{13}C NMR spectra showed a peak at 166.78, 106.78, 12.90 ppm which was attributed to the presence of carbonyl group, pyrrole and CH_3.

Biological activity

Antitubercular Activity

All the synthesized compounds (**3a-f**) were screened against *M. tuberculosis* H37Rv strain which showed MIC value ranging from 3.12-25 µg/ml. Among all the screened compounds, compounds **3b, 3c** and **3f** inhibited microorganism growth efficiently compared to other synthesized compounds in the series with a MIC value of 0.4 µg/ml, and the compounds **3a, 3d** and **3e** showed a good activity with MIC value of 3.12 and 6.25 µg/ml respectively (Table **2**).

Antibacterial Activity

The results of antimicrobial activities (expressed in MIC) of the compounds against selected Gram-positive and Gram-negative bacteria are illustrated in Tables 2. The activity of ciprofloxacin and norfloxacin are used for comparison. All the compounds showed moderate to significant microbial inhibition. Pyrrole derivatives have shown antibacterial activity between MIC of 6.25-100 µg/ml. Compounds 3b, 3c and 3f have showed highest activity against *E. coli* at MIC value of 6.25 µg/ml than the other tested compounds. (Table 2).

Table 2: *Invitro* antitubercular and antibacterial activity results of newly synthesized compounds.

Comp.	*In vitro antitubercular activity* MIC value (µg/ml)	*In vitro antitubercular activity* MIC value (µg/ml)	
		Gram +ve (*S. aureus*)	Gram -ve (*E. coli*)
3a	3.12	50	12.5
3b	0.4	100	6.25
3c	0.4	100	6.25
3d	3.12	100	25
3e	6.25	100	25
3f	0.4	100	6.25
Pyrazinamide	3.125	--	--
Streptomycin	6.25	--	--
Ciprofloxacin	--	2	2
Norfloxcin	--	3	12

CONCLUSION

Novel series of *N'*-substituted-2-(2,5-dimethyl-1*H*-pyrrol-1-yl)phenyl)benzamides **(3a-f)** were synthesized and identified as potent antitubercular and antibacterial agents. Among all the compounds **3b**, **3c** and **3f** have displayed significant activity against *M. tuberculosis* with MIC value of 0.4 µg/ml. These compounds will be useful as the lead compounds for developing antitubercular and antibacterial agents.

LIST OF ABBREVIATIONS

C	Degree centigrade
$CDCl_3$	Deuterated chloroform
FTIR	Fourier Transfer Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
Ppm	Parts per million
MP	Melting point
R_f	Retention factor
Min	Minutes
H	Hours
Mol	Mole
TLC	Thin Layer Chromatography
MIC	Minimum Inhibitory Concentration

Conflictof Interests:

All the authors have no conflict of interests.

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REFERENCES

1. WHO report, Global Tuberculosis Report, 2018
2. Raviglione M, Marais B,Floyd K, Lonnroth K, Getahun H, Migliori GB, et al. Scaling up interventions to achieve global tuberculosis control: progress and new developments. Lancet.2012; 379: 1902-13.
3. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications.Int J Tuberc Lung Dis. 1999; 3: S231-79.
4. Biava M, Poretta GC, Poce G, Battilocchio C. Identification of a novel pyrrole derivatives endowed with anti-mycobacterial activity and protection index comparable to that of the current antitubercular drugs streptomycin and rifampin. Bioorg Med Chem. 2010; 18: 8076-84.
5. Joshi SD, Dixit SR, More UA, Aminabhavi TM, Kulkarni VH, Gadad AK.Enoylacpreductase as effective target for the synthesized novel antitubercular drugs: A-state-of-the-art.Mini Rev Med Chem.2014; 14: 678-93.
6. Heath RJ, Li J, Roland GE, Rock CO, Inhibition of the *Staphylococcus aureus*NADPH-dependent enoyl-acyl carrier protein reductase by triclosan and hexachlorophene. J Biol Chem. 2000; 275: 4654-59.

7. Heath RJ, Rock CO. Enoyl-acyl carrier protein reductase (*fabI*) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli.* J Biol Chem. 1995; 270: 26538-42.
8. Banerjee A, Dubnau E,Quemard A, Balasubramanian V, Um KS, Wilson T, et al.InhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. Science.1994; 263: 227-30.
9. Estevez V, Villacampa M, Menendez JC. Multicomponent reactions for the synthesis of pyrroles. ChemSoc Rev.2010; 39: 4402-21.
10. More UA, Joshi SD, Aminabhavi TM, Kulkarni VH, Badiger AM, Lherbet C, Discovery of target based novel pyrrolylphenoxy derivatives as antimycobacterial agents: An in silico approach. Eur J Med Chem. 2015; 94: 317-39.
11. Joshi SD, Dixit SR, Kirankumar MN, Aminabhavi TM, Raju KVSN, Narayan R. Synthesis, antimycobacterial screening and ligand-based molecular docking studies on novel pyrrole derivatives bearing pyrazoline, isoxazole and phenyl thiourea moieties. Eur J Med Chem. 2016; 107: 133-52.
12. Joshi SD, Vagdevi HM, Vaidya VP, Gadaginamath GS. Synthesis of new 4-pyrrol-1-yl benzoic acid hydrazide analogs and some derived oxadiazole, triazole and pyrrole ring systems: A novel class of potential antibacterial and antitubercular agents. Eur J Med Chem. 2008; 43: 1989-96.
13. Halazy S, Mangus P. Synthesis on the antitumor agent CC-1065: 1-Phenylsulfonyl-1,3-butadiene. An electrophilic equivalent to 1,3-butadiene for the synthesis of 3,31 bipyrroles. Tetrahedron Lett. 1984;25:1421-24.
14. Debashish B, Sanghamitra M, Jose C, Grandos JD, Short BK, et al. Ultrasound- assisted bismuth nitrate- induced green synthesis of novel pyrrole derivatives and their biological evaluation as anticancer agents. Eur J Med Chem. 2012; 50: 209-15.
15. Jones RA, Bean GP. The Chemistry of Pyrroles, Academics Press, London, 1997.
16. Jones RA. Pyrrole, Part II: The Chemistry of Heterocyclic Compounds, Wiley, New York, 1992.
17. Joshi SD, More UA, Dixit SR, Balmi SV, Kulkarni BG et al. Chemical synthesis and in *silico* molecular modeling of pyrollylbenzohydrazide derivatives: Their biological evaluation against enoyl ACP reductase (InhA) and *mycobacterium tuberculosis.* Bio Org Chem. 2017; 75:181-200.
18. Joshi SD, Dixit SR, Kulkarni VH, Lherbet C, Nadagouda MN, Aminabhavi TM. Synthesis, biological evaluation and in silico molecular modeling of pyrrolylbenzohydrazides derivatives as enoyl ACP reductase inhibitors. Eur J Med Chem. 2017; 126:286-297.
19. Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, et al.Rapid, low-technology MIC determination with clinical *Mycobacteriumtuberculosis* isolates by using the microplateAlamar Blue assay. J ClinMicrobiol. 1998; 36: 362-66.
20. Goto S, Jo K, Kawakita T, Mitsuhashi S, Nishino T, Ohsawa N. Determination method of minimum inhibitory concentrations. Chemotherapy. 1981; 29: 76-9.
21. A. Villanova, National Committee for Clinical Laboratory Standards, Methods for Dilution Antimicrobial Susceptibility for Bacteria Grown Aerobically, Approved Standard, National Committee for Clinical Laboratory Standards, 1985.

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