Study on antibacterial activity of newly synthesized derivatives of pyranopyrazole, pyrazolo[1,2-b]phtalazine and bis-pyrazole

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Summary

In recent years, due to a sharp increase of antibiotic resistance, synthesized derivative compounds have been considered as a superseded source for new drugs. With regard to the high therapeutic behavior of isatin derivatives from many aspects of drug discovery, in this study, the antibacterial effects of newly synthesized derivatives of pyranopyrazole, pyrazolo[1,2-b]phtalazine and bis-pyrazole against *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Enterococcus faecalis* were examined.

Twelve individual compounds were weighted and prepared at a final concentration of 1 mg/mL in dimethyl sulfoxide (DMSO). Bacterial test organisms were maintained in nutrient agar slants at 4°C and subcultured in Petri plates prior to use. The tested compounds are randomly added into the wells with a 100 µl volume on the plate under sterile condition and then were incubated at 37°C for 24 hours. All experiments were repeated three times and the mean values

are reported as the results. The antibacterial effects of the compounds that produced ≥ 8mm zone of inhibition were tested quantitatively by Micro broth dilution method for determination of minimal inhibitory concentration (MIC) value. After incubation overnight, the first tube with clean appearance determined the MIC.
According to the results, the MIC of the compounds defined as **c** and **d** against *S. aureus* is 64 (µg/ml). It is found that the synthesized compounds are only effective against *S. aureus*.
The comparison of the maximum zone of inhibition (22±0.4) and MICs between the present study and those in literature, shows the privilege of using compound **c** and **d** against *S. aureus*.

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Antibacterial activity; Antibiotic resistance; Maximum zone of inhibition; Minimum inhibitory concentration (MIC); Isatin

Introduction

Each year many people die because of Gram-positive and Gram-negative bacterial infections. These bacteria mostly result in rheumatic diseases, salmonellosis, food poisoning, and diarrhea.[1] Regardless of the kind of microbial infections, antibiotics are considered as the main basis for treatment, although overuse of antibiotics induced a problematic antibiotic resistance situation in recent years.[2,3] This problem will become more pronounced if the alarming number of life-threatening infectious disease caused by multiple drug resistant pathogen bacteria in recent years are taken into account.[4]

Lipopeptides, oxazolidinones, and streptogramins are the only three classes of antibiotics which have been discovered in the past four decades.[5] Because older antibiotics are losing their effectivenes, second or third line drugs are being used even in spite of possible side effects.[2] Hence, considering the mentioned facts about the resistant clinical isolates, it can be seen that the discovery of new antimicrobial agents will pave a bright future for treating infectious diseases caused by drug resistant pathogen bacteria.

The metabolically versatile bacterium, *Pseudomonas aeruginosa*, normally inhabits the soil and surfaces in aqueous environments,[6] infections caused are often nosocomial and frequently are associated with compromised host defenses.[7] It is among the most common hospital pathogens and is the second most common pathogen isolated from patients with ventilator-associated pneumonia.[8] *Staphylococcus aureus* which can be transmitted among hospital personnel and hospitalized patients, is one of the most common nosocomial bacteria of infectious diseases[9] possessing many mechanisms of pathogenicity.[10] *Bacillus cereus* is a Gram-positive bacterium which can cause diarrhea, emesis, fatal meningitis, and spoilage of different food products.[11] This bacterium can transmit through processed, pasteurized, sterilized, and heat-treated food products.[12] *Escherichia coli* is commonly found in the digestive system, particularly intestine of humans and animals. Drug resistant *E. coli* can be transferred from animals to people.[13] As normal human commensals which are adapted to the nutrient-enriched, oxygen-depleted and ecologically complex environments, *Enterococci* can cause a wide variety of diseases in humans, infecting the urinary tract, bloodstream, endocardium and indwelling foreign devices.[14] *Enterococcus faecalis* causes 80 to 90% of human enterococcal infections.[15]

Isatin (2,3-dioxindole) is an important class of he-

terocyclic compounds[16] and a core component of numerous natural products and drugs. In recent years, isatin derivatives have attracted interest in organic and medicinal chemistry because of their potent biological and pharmacological activities, like antitumor, anti-inflammatory and analgesic, antimicrobial, antimycobacterial, anticonvulsant, antiviral, anthelmintic, anti-HIV, antioxidant, CNS depressant ones.[16] Therefore, many research teams focused on the synthesis and study of isatin compounds, like pyranopyrazole, pyrazolo[1,2-b]phtalazine and bis-pyrazole.

In that respect, we have attempted to evaluate the antibacterial activity of some newly synthesized derivatives of pyranopyrazole, pyrazolo[1,2-b]phtalazine[17] and bis-pyrazole[18] against five bacteria, *Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Enterococcus faecalis* is investigated by disk diffusion and minimum inhibitory concentration (MIC) methods.

Materials and Methods

Synthesis and characterization of the compounds

Synthesis and characterization of the compounds used in this study has been reported in previous works.[17,18] Twelve individual compounds were weighted and prepared at a final concentration of 1 mg/mL in Dimethyl sulfoxide (DMSO).

Bacterial culture

Bacterial strains used in the present study were taken from Bahar Afshan Company, Tehran, Iran, and included *B. cereus* ATCC11778, *S. aureus* ATCC25923, *E. faecalis* ATCC29212, *E. coli* ATCC25922 and *P. aeruginosa* ATCC27853. Bacterial test organisms were maintained in nutrient agar (Merck, Germany) slants at 4°C and subcultured in Petri plates prior to use. From the petri plates, bacterial suspension was prepared at a final concentration of 0.5 McFarland standards (1.5 x 10^8 cfu/mL).

Diffusion disc agar and minimal inhibitory concentration (MIC) methods

Antimicrobial sensitivity was tested by disk diffusion method and minimum inhibitory concentration (MIC) according to Clinical and Laboratory Standards Institute (CLSI) guideline.[19]

Briefly, the 0.5 McF suspension of bacteria was spread over Muller-Hinton Agar plates, and a 100 μl volume of the tested compounds were randomly added into 9.6 mm paper disks on the plate under sterile condition and then were incubated at 37°C for 24 hours. DMSO was used as a negative control, which has no antimicrobial activity. All experiments were repeated in triplicate and the mean values are reported at the results.

The antibacterial effects of the compounds that produced a zone of inhibition larger than 8mm were further tested quantitatively by broth microdilution method for determination of MIC value (that was defined as the lowest concentration of the compound inhibiting the growth of tested bacteria). Overall compounds concentration of 512, 256, 128, 64, 32, 16, 8 μg/mL were prepared and tested under the same incubation conditions. One tube without inoculation was used as a negative control, while one that contained gentamicin antibiotic was used as a positive control. After incubation overnight, the first tube without any growth determined the MIC.

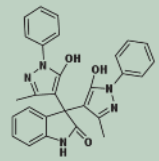
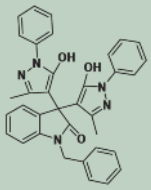
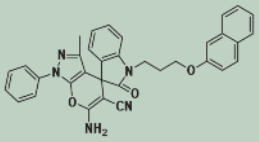
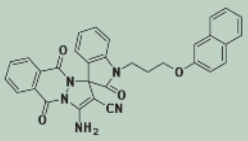
Results and discussion

In total four compounds (designated a, b, c and d) were tested in the present work. Their chemical characteristics and the disk diffusion results are presented in Table 1. It can be observed that activity was detected only against *S. aureus*. More specifically, compounds **a** and **b** demonstrated low activity (zones of 11 and 16 mm) whereas compounds **c** and **d** demonstrated higher activity against *S. aureus*. In that respect, only *S. aureus* was tested using the broth microdilution method. The MIC of the four compounds against *S. aureus* is presented in Table 2, where it is shown that compounds a and b exhibited high MIC values (512 and 128 μg/ml, respectively), whereas compounds c and d demonstrate lower MIC values (64 μg/ml), thus indicating a possible antibacterial activity against *S. aureus*.

Pyrazole derivatives which are extensively studied and used as antimicrobial agents,[20,21] possess wide spectrum of biological activities, such as antifungal,[22] herbicidal,[23] anti-inflammatory,[24] cytotoxic[25] and A3 adenosine receptor antagonists.[26] In some pharmaceutical drugs such as celecoxib[27] and rimonabant[28] pyrazole is the core molecular entity.[28,29] In addition, 5-chloropyrazole derivatives show antimicrobial[30] and anti-inflammatory activities.[31]

In the present study we showed that new synthesized pyrazole derivatives exhibited activity against S. aureus, but not against *B. cereus, E. coli, P. aeruginosa,* or *E. faecalis*. This has been shown in other studies of similar comounds with comparable chemistry as well, as indicated in Table 3. Nevertheless, inhibition zones against *S. aureus* in the present study (compounds c and d) are higher than those reported in the comparable studies,[32-34] although the actual difference is mi-

Table 1 Antibacterial activity of chemical compounds; zone of inhibition in mm

Compound		Disk diameters (mm)				
	Name	B. cereus	E. coli	P. aeroginosa	S. aureus	E. faecalis
a 	3,3-bis(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)indolin-2-oney	No activity	No activity	No activity	11±0.6	No activity
b 	1-benzyl-3,3-bis(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)indolin-2-one	No activity	No activity	No activity	16±0.4	No activity
c 	6'-amino-3'-methyl-1-(3-(naphthalen-2-yloxy)propyl)-2-oxo-1'-phenyl-1'H-spiro[indoline-3,4'-pyrano[2,3-c]pyrazole]-5'-carbonitrile	No activity	No activity	No activity	22±0.1	No activity
d 	3'-amino-1-(3-(naphthalen-2-yloxy)propyl)-2,5',10'-trioxo-5',10'-dihydrospiro[indoline-3,1'-pyrazolo[1,2-b]phthalazine]-2'-carbonitrile	No activity	No activity	No activity	22±0.4	No activity
Gentamicin (Positive Control)		18±0.6	16±0.5	18±0.6	18±0.4	17±0.7
DMSO		No activity	No activity	No activity	–	–

DMSO: Dimethyl sulfoxide (negative control)

Minimum inhibitory concentration of the chosen chemical compounds against *S. aureus ATCC25923* (µg/ml)

Table 2

Compound	S. aureus MIC (mg/L)
a	512
b	128
c	64
d	64
DMSO	No activity

DMSO: Dimethyl sulfoxide (negative control)

Table 3

A quantitative comparison of antibacterial activity against *S. aureus*; maximum zone of inhibition and minimum inhibition concentration (MIC).

Max. zone of inhibition (mm)	MIC (μg/ml)	Reference
22±0.4[a]	64	Present study
–	200	(32)
21±0.17	–	(33)
21.3	32	(34)

[a] *Values are means of three replicates*

nimal[34] and MIC values are either lower or within one dilution difference. Finally based on comparison between our results and other similar studies working on synthesis of newly derivatives of pyranopyrazole, pyrazolo [1,2-b]phtalazine and bis-pyrazole, isatin presence in our compounds can increase antibacterial activity. A possible explanation for this might be that isatin and its derivatives have a good antimicrobial and antibacterial activity.[16,35]

Conclusions

In conclusion, the comparison of the maximum zone of inhibition and MICs between the present study and those in literature, shows the privilege of using two compound against *S. aureus*. Further studies for the application of these compounds *in vivo* should be performed.

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Declaration of competing interests

The authors declare that they have no conflict of interest.

Περίληψη

Μελέτη της αντιβακτηριακής δράσης νέων παραγώγων πυρανοπυραζόλης: pyrazolo[1,2-b]phtalazine και bis-pyrazole

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Τα τελευταία χρόνια, λόγω των αυξανόμενων αντοχών στα αντιβιοτικά, συνθετικά παραγόμενες νέες ουσίες μελετώνται ως υποψήφια νέα αντιμικροβιακά φάρμακα. Στην παρούσα μελέτη εξετάζεται η δράση νέων παραγώγων πυρανοπυραζόλης, συγκεκριμένα pyra-zolo[1,2-b]phtalazine και bis-pyrazole, έναντι στελεχών *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* και *Enterococcus faecalis*. Συνολικά μελετήθηκαν 12 ουσίες. Τέσσερεις από αυτές, a, b, c και d* σε τελική συγκέντρωση 1 mg/mL σε dimethyl sulfoxide (DMSO) με τη μέθοδο διάχυσης δίσκων και μέθοδο αραιώσεων σε ζωμό (σύμφωνα με το CLSI), βρέθηκαν δραστικές. Συγκεκριμένα, εναιώρημα 0,5 McF εκάστου μικροβιακού στελέχους επιστρώθηκε σε Muller Hinton άγαρ και προστέθηκε δισκίο χαρτιού 9.6 mm εμποτισμένο με 100μl της υπό εξέταση ουσίας. Μετά από επώαση 24 ωρών σε 37°C έγινε μέτρηση των διαμέτρων αναστολής ανάπτυξης των υπό μελέτη μικροβιακών στελεχών. Ως αρνητικός μάρτυρας χρησιμοποιήθηκε δισκίο εμποτισμένο με DMSO και ως θετικός δισκίο γενταμικίνης. Όλα τα πειράματα έγιναν τρεις φορές και αξιολογήθηκαν οι μέσες τιμές. Αποτελέσματα με διάμετρο αναστολής ανάπτυξης μικροβίου ≥ 8mm μελετήθηκαν περαιτέρω ποσοτικά με μακρομέθοδο αραιώσεων σε ζωμό για καθορισμό της Ελάχιστης Ανασταλτικής Συγκέντρωσης (MIC). Ως τιμή MIC καθορίστηκε η ποσότητα ουσίας στο σωληνάριο χωρίς ορατή μικροβιακή ανάπτυξη. Σύμφωνα με τα αποτελέσματα, η MIC για τις ουσίες c και d έναντι *S. aureus* ήταν 64 μg/ml. Οι μελετούμενες ουσίες ήταν δραστικές μόνο έναντι του *S. aureus*. Σύγκριση των τιμών ζωνών αναστολής (22±0.4) και MIC μεταξύ της παρούσας μελέτης και αυτών από τη βιβλιογραφία έδειξε την αξία των ουσιών c και d έναντι στελεχών *S. aureus*, αλλά όχι έναντι των λοιπών υπό μελέτη στελεχών *B. cereus, E. coli, P. aeruginosa,* και *E. faecalis*.

***a:** 3,3-bis(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)indolin-2-oney

b: 1-benzyl-3,3-bis(5-hydroxy-3-methyl-1-phenyl-1H-pyrazol-4-yl)indolin-2-one

c: 6'-amino-3'-methyl-1-(3-(naphthalen-2-yloxy)propyl)-2-oxo-1'-phenyl-1'H-spiro[indoline-3,4'-pyrano[2,3-c]pyrazole]-5'-carbonitrile

d: 3'-amino-1-(3-(naphthalen-2-yloxy)propyl)-2,5',10'-trioxo-5',10'-dihydrospiro[indoline-3,1'-pyrazolo[1,2-b]phthalazine]-2'-carbonitrile

Λέξεις κλειδιά

Αντιβακτηριακή δράση, αντιβιοτική αντοχή, μέγιστη ζώνη αναστολής, ελάχιστη ανασταλτική συγκέντρωση, ισατίνη

References

1. Khan SA, Kumar P, Joshi R, Iqbal PF, Saleem K. Synthesis and in vitro antibacterial activity of new steroidal thiosemicarbazone derivatives. *Eur J Med Chem.* 2008;43:2029-34.
2. Moyo B, Mukanganyama S. Antibacterial Effects of Cissus welwitschii and Triumfetta welwitschii Extracts against *Escherichia coli* and *Bacillus cereus*. *Int J Bacteriol*. 2015;2015:10.
3. Willey JM, Sherwood L, Prescott LM, Woolverton CJ. *Prescott, Harley, and Klein's Microbiology:* McGraw-Hill Higher Education; 2008.
4. Berber I, Cokmus C, Atalan E. Characterization of Staphylococcus species by SDS–PAGE of Whole-Cell and Extracellular Proteins. *Microbiology.* 2003;72:42-7.
5. Parisien A, Allain B, Zhang J, Mandeville R, Lan CQ. Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *J Appl Microbiol*. 2008;104:1-13.
6. Gellatly SL, Hancock REW. Pseudomonas aeruginosa: new insights into pathogenesis and host defenses. *Pathog Dis*. 2013;67:159-73.
7. Lyczak JB, Cannon CL, Pier GB. Establishment of Pseudomonas aeruginosa infection: lessons from a versatile opportunist. *Microb Infect.* 2000;2:1051-60.
8. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, et al. Antimicrobial-Resistant Pathogens Associated With Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. *Infect Control Hosp Epidemiol.* 2016;30:1-14.
9. Motevasel M, Okhovat MA, Zomorodian K, Farshad S. A Study of the Effect of *Zataria multiflora* Extract on Methicillin Resistant *Staphylococcus aureus. Jundishapur J Microbiol.* 2013;6:e5453.
10. Win Jr. W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, et al. *Koneman's Color Atlas and Textbook of Diagnostic Microbiology.* 6 ed: Lippincott Williams & Wilkins; 2006.
11. Evreux F, Delaporte B, Leret N, Buffet-Janvresse C, Morel A. [A case of fatal neonatal Bacillus cereus meningitis]. *Arch Pediatr.* 2007;14:365-8.
12. Rahimi E, Abdos F, Momtaz H, Torki Baghbadorani Z, Jalali M. *Bacillus cereus* in Infant Foods: Prevalence Study and Distribution of Enterotoxigenic Virulence Factors in Isfahan Province, Iran. *Scientific World J.* 2013;2013:5.
13. Guardabassi L, Schwarz S, Lloyd DH. Pet animals as reservoirs of antimicrobial-resistant bacteria. *J Antimicrob Chemother.* 2004;54:321-32.
14. Kayaoglu G, Orstavik D. Virulence factors of *Enterococcus faecalis:* relationship to endodontic disease. *Crit Rev Oral Biol Med.* 2004;15:308-20.
15. Jett BD, Huycke MM, Gilmore MS. Virulence of enterococci. *Clin Microbiol Rev*. 1994;7:462-78.
16. Grewal AS. Isatin derivatives with several biological activities. *Int J Pharm Res.* 2014;6:4.
17. Khazaei A, Zolfigol MA, Karimitabar F, Nikokar I, Moosavi-Zare AR. N, 2-Dibromo-6-chloro-3, 4-dihydro-2 H-benzo [e][1, 2, 4] thiadiazine-7-sulfonamide 1, 1-dioxide: an efficient and homogeneous catalyst for one-pot synthesis of 4 H-pyran, pyranopyrazole and pyrazolo [1, 2-b] phthalazine derivatives under aqueous media. *RSC Adv*. 2015;5:71402-12.
18. Zolfigol AM, Khazaei A, Karimitabar F, Hamidi M. Alum as a Catalyst for the Synthesis of Bispyrazole Derivatives. *Appl Sci.* 2016;6.
19. Standards NCfCL. *Performance Standards for Antimicrobial Disk Susceptibility Tests: Approved Standards:* National Committee for Clinical Laboratory Standards; 2006.
20. Aggarwal R, Kumar V, Tyagi P, Singh SP. Synthesis and antibacterial activity of some new 1-heteroaryl-5-amino-3H/methyl-4-phenylpyrazoles. *Bioorg Med Chem.* 2006;14:1785-91.
21. Akbas E, Berber I. Antibacterial and antifungal activities of new pyrazolo[3,4-d]pyridazin derivatives. *Eur J Med Chem.* 2005;40:401-5.
22. Prakash O, Kumar R, Parkash V. Synthesis and antifungal activity of some new 3-hydroxy-2-(1-phenyl-3-aryl-4-pyrazolyl) chromones. *Eur J Med Chem.* 2008;43:435-40.
23. Ohno R, Watanabe A, Matsukawa T, Ueda T, Sakurai H, Hori M, et al. Synthesis and Herbicidal Activity of New Pyrazole-4-carboxamide Derivatives. *J Pest Sci.* 2004;29:15-26.
24. Bekhit AA, Fahmy HTY, Rostom SAF, Baraka AM. Design and synthesis of some substituted 1H-pyrazolyl-thiazolo[4,5-d]pyrimidines as anti-inflammatory–antimicrobial Agents. *Eur J Med Chem.* 2003; 38:27-36.
25. Vera-Divaio MA, Freitas AC, Castro HC, de Albuquerque S, Cabral LM, Rodrigues CR, et al. Synthesis, antichagasic in vitro evaluation, cytotoxicity assays, molecular modeling and SAR/QSAR studies of a 2-phenyl-3-(1-phenyl-1H-pyrazol-4-yl)-acrylic acid benzylidene-carbohydrazide series. *Bioorg Med Chem.* 2009; 17:295-302.
26. Baraldi PG, Bovero A, Fruttarolo F, Romagnoli R, Tabrizi MA, Preti D, et al. New strategies for the synthesis of A3 adenosine receptor antagonists. *Bioorg Med Chem.* 2003;11:4161-9.
27. Penning TD, Talley JJ, Bertenshaw SR, Carter JS, Collins PW, Docter S, et al. Synthesis and Biological

Evaluation of the 1,5-Diarylpyrazole Class of Cyclooxygenase-2 Inhibitors: Identification of 4-[5-(4-Methylphenyl)-3- (trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (SC-58635, Celecoxib). *J Med Chem.* 1997;40:1347-65.

28. Deng X, Mani NS. Base-Mediated Reaction of Hydrazones and Nitroolefins with a Reversed Regioselectivity: A Novel Synthesis of 1,3,4-Trisubstituted Pyrazoles. *Org Lett*. 2008;10:1307-10.
29. Katritzky AR, Wang M, Zhang S, Voronkov MV, Steel PJ. Regioselective Synthesis of Polysubstituted Pyrazoles and Isoxazoles. *J Org Chem.* 2001;66:6787-91.
30. Kumar R, Malik S, Chandra R. Synthesis and antimicrobial activity of 4-[5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl]-dihydropyridines and 4-[5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl]-3,4-dihydropyrimidin-2-ones. *Indian J Chem.* 2009;48B:718-24.
31. Girisha KS, Kalluraya B, Narayana V, Padmashree. Synthesis and pharmacological study of 1-acetyl/propyl-3-aryl-5-(5-chloro-3-methyl-1-phenyl-1*H*-pyrazol-4-yl)-2-pyrazoline. *Eur J Med Chem.* 2010;45:4640-4.
32. Ranmal A. Varu, Pancholi KS, Karia DC. Synthesis of diverse pyrano[2,3-c]pyrazoles derivatives as potential antimicrobial agents. *Der Pharmacia Sinica*. 2013;4:1-5.
33. Khoraamabadi-zad A, Azadmanesh M, Karamian R, Asadbegy M, Akbari M. Triethanolamine as an inexpensive and efficient catalyst for the green synthesis of novel 1 H-pyrazolo [1, 2-a] pyridazine-5, 8-diones under ultrasound irradiation in water and their antibacterial activity. *RSC Adv.* 2014;4:47721-5.
34. Pundeer R, Kiran V, Sharma C, Aneja K, Prakash O. Synthesis and evaluation of antibacterial and antifungal activities of new (Z)-3-bromo-4-(1, 3-diaryl-1*H*-pyrazol-4-yl) but-3-en-2-ones and 4-(3-methyl-1-phenyl-1*H*-pyrazol-5-yl)-1, 3-diaryl-1*H*-pyrazoles. *Med Chem Res.* 2013;22:4715-26.
35. Sridhar SK, Saravanan M, Ramesh A. Synthesis and antibacterial screening of hydrazones, Schiff and Mannich bases of isatin derivatives. *Eur J Med Chem.* 2001;36:615-25